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Eight new mitogenomes for exploring the phylogeny and classification of Vetigastropoda

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ABSTRACT

Vetigastropoda, comprising marine gastropods of both snail-like and limpet-like form, were common during the Palaeozoic and remain so in modern marine environments. The most resolved molecular phylogenetic study to date at the family level in Vetigastropoda was based on molecular data of complete mitochondrial genomes, but only 15 mitochondrial genomes are available and the taxonomic coverage remains insufficient to resolve many systematic questions. Notably, among the vetigastropod superfamilies, ‘Trochoidea’ is the most diverse, but no mitogenome has been yet published for its representative family Trochidae. We here provided eight newly reconstructed mitogenomes from the following vetigastropods: *Angaria delphinus*, *Phasianella australis*, *Astraliium haematragum*, *Lunella granulata*, *Chlorostoma argyrostomum*, *Omphalius nigerrimus*, *Stomatella planulata* and *Variagemarginula punctata*. *Stomatella planulata* is the first available mitogenome for Trochidae. Our analyses of the extended mitogenome dataset show that the two trochoid families Turbinidae and Tegulidae group together, while their relationship to Trochidae (represented by *S. planulata*) is uncertain. Within the Tegulidae, monophyly of the genus *Tegula* is not recovered. The analysis with additional fissurelloid mitogenome confirms that within Vetigastropoda this superfamily is a distinct clade. Except for *V. punctata* the mitogenomes reconstructed show the ancestral gene order for Vetigastropoda. The additional fissurelloid mitogenome reveals that gene order in Fissurelloidea is variable, which might suggest a faster rate of mitochondrial evolution that in turn may cause artefacts in phylogenetic analyses.

INTRODUCTION

Vetigastropoda (including abalones, slit snails, true limpets, key-hole limpets, seguenzid snails and turban snails) are a large and morphologically diverse group of marine gastropods with approximately 3,700 described living species (Knight *et al.*, 1960; Ponder & Lindberg, 1997). They originated at the end of the Cambrian and have been common in marine faunas since the Palaeozoic (Frýda, Nutzelt & Wagner, 2008). Currently, the Vetigastropoda are classified in 38 families and 10 superfamilies (Angarioidea, Fissurelloidea, Halioidea, Lepetelloidea, Lepetodriolea, Phasianelloidea, Pleurotomarioidea, Scissurelloidea, Seguenzioidea and Trochoidea) (Bouchet *et al.*, 2005).

Contemporary studies on the phylogeny of Vetigastropoda were initially based on morphology (Salvini-Plawen & Haszprunar, 1987; Haszprunar, 1987a, b, c, 1988; Ponder & Lindberg, 1996, 1997; Hedegaard, 1997) and have since been complemented by molecular analyses (Harasewych *et al.*, 1997; Colgan *et al.*, 2003; McArthur & Harasewych, 2003; Geiger & Thacker, 2005; Yoon & Kim, 2005; Williams & Ozawa, 2006; Kano, 2008; Williams,

Karube & Ozawa, 2008; Aktipis & Giribet, 2010, 2012; Aktipis, Boehm & Giribet, 2011; Williams, 2012; Uribe *et al.*, 2016). However, the proposed hypotheses are insufficiently consistent for establishing a stable classification system.

The most recent molecular phylogenetic studies differ in using either a large taxonomic sampling (e.g. Aktipis & Giribet, 2012) or more informative markers (e.g. Uribe *et al.*, 2016). Aktipis & Giribet (2012) provided the most complete study of vetigastropod phylogeny to date. This included 69 terminal taxa of Vetigastropoda, covering all the vetigastropod superfamilies and 22 of its 38 families. Using three nuclear and two mitochondrial markers, these authors recovered Vetigastropoda as nonmonophyletic and they therefore excluded Pleurotomarioidea and Lepetelloidea from Vetigastropoda *s. s.* However, the relationships within Vetigastropoda *s. s.* were still unresolved due to weak support for the inferred relationships among superfamilies and families. Consequently, more data are still needed, especially in the phylogenomic field, to resolve the conflicting phylogenetic issues within Vetigastropoda *s. s.*

Most recently, Uribe *et al.* (2016) investigated vetigastropod phylogeny using data from whole mitochondrial genomes (mitogenomes). Indeed, mitogenomes are now widely used for resolving the deep branching lineages at the level of phylum, class or order (Smith *et al.*, 1993; Boore & Brown, 1994, 2000; Boore, Lavrov & Brown, 1998; Saccone *et al.*, 1999; Stöger & Schrödl, 2013; Bernt *et al.*, 2013a). Compared with the commonly used molecular markers for gastropod systematic studies (*cox1*, *16S*, *18S*, *28S* and *histone H3*, etc.), mitogenomes contain many more informative sites than shorter sequences and also provides genome-level characters (Boore, Macey & Medina, 2005). In some studies, mitogenomes have been successfully used to reconstruct the phylogenetic relationships of molluscan groups, e.g. in bivalves (Doucet-Beaupré *et al.*, 2010), cephalopods (Allcock, Cooke & Strugnell, 2011), Heterobranchia (Medina *et al.*, 2011; White *et al.*, 2011; Kocot, Halanych & Krug, 2013) and Caenogastropoda (Cunha, Grande & Zardoya, 2009; Osca, Templado & Zardoya, 2015). However, in comparison with these other molluscan groups, mitogenomic data for Vetigastropoda are still scarce. Uribe *et al.* (2016) reconstructed seven new vetigastropod mitogenomes, allowing them to reconstruct phylogenetic relationships based on 12 mitogenomes covering seven of the ten superfamilies and eight of the 38 families of Vetigastropoda *s. s.* The mitogenomic dataset of Uribe *et al.* (2016) included only two trochoid families, Tegulidae and Turbinidae, which formed a clade together with Liotiidae, *Tectus*, *Rochia* and *Cittarium* in previous studies (Williams *et al.*, 2008; Williams, 2012). The taxonomic sampling by Uribe *et al.* (2016), although the best to date, is insufficient to reveal the phylogenetic relationships within the Trochoidea. For example, in order to examine further the interrelationships among Angarioidea, Phasianelloidea and Trochoidea, and to assess the superfamily rank of Angarioidea and Phasianelloidea, more trochoid taxa should be included in the analysis.

In this context we sequenced eight new mitogenomes in Vetigastropoda (Table 1), to provide a second species in Angarioidea (*Angaria delphinus*) and in Phasianelloidea (*Phasianella australis*), two additional genera in Tegulidae (*Chlorostoma argyrostomum* and *Omphalius nigerrimus*), one new genus (*Astralium haematragum*) and an additional species (*Lunella granulata*) in Turbinidae, the first mitogenome for Trochidae (*Stomatella planulata*) and a new genus in Fissurelloidea (*Variemarginula punctata*). These newly reconstructed gastropod mitogenomes, combined with all other available ones, were used in phylogenetic analyses to provide new insights into the evolutionary relationships of the Vetigastropoda and in particular to test the monophyly of the Trochoidea.

MATERIAL AND METHODS

Sample collection and genomic DNA extraction

Seven species were sampled from Taiwan and *Phasianella australis* from Esperance, Western Australia (Table 1). The specimens were

fixed in 95% ethanol and, in order to maintain the quality of genomic DNA, all except *P. australis* were stored at -20°C . Genomic DNA was extracted by using a phenol–chloroform extraction protocol (Sambrook & Russell, 2001) for *Astralium haematragum*, *Chlorostoma argyrostomum*, *Lunella granulata* and *Omphalius nigerrimus* and by using the DNeasy Blood & Tissue Kit (Qiagen, Hilden) for *Angaria delphinus*, *P. australis*, *Stomatella planulata* and *Variemarginula punctata*. The genomic DNA was treated in one of two ways to reconstruct the mitogenomes, as described below.

PCR amplification and Sanger sequencing

The complete mitogenomes of the seven vetigastropods from Taiwan were amplified by long-range polymerase chain reaction (long PCR), then sequenced by traditional Sanger sequencing. Several PCR primers of 40 bp were designed from two mitochondrial genes, cytochrome *c* oxidase I (*cox1*) and 16S rRNA (*16S*) from each species. DNA fragments were amplified and sequenced using the following two sets of universal primers: LCO-1490 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3' and HCO-2198 5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3' (Folmer *et al.*, 1994) for *cox1* sequence, 16SaL 5'-CGC CTG TTT ATC AAA AAC AT-3' and 16SbH 5'-CCG GTC TGA ACT CAG ATC ACG T-3' (Palumbi, 1996) for *16S*. An aliquot of DNA (10 ng) was added to 48 μl of PCR mix containing 0.4 μM of each primer, 10 \times LA Taq Buffer (Mg²⁺ plus, TaKaRa Bio.), 5 mM of each dNTP (TaKaRa Bio.) and 2.5 units of LA Taq polymerase (TaKaRa Bio.). Long PCR was then carried out in a thermocycler (PTC-200, MJ Research) with one cycle of 94 $^{\circ}\text{C}$ for 2 min, 32 cycles of 96 $^{\circ}\text{C}$ for 20 s, 62–70 $^{\circ}\text{C}$ for 5–9 min and one cycle of 72 $^{\circ}\text{C}$ for 15 min. Nested PCRs were done in some cases using the DNA templates above. The sequences of each PCR product were obtained by direct sequencing with an ABI 3730 XL sequencer by primer walking.

Next-generation sequencing and data analysis

The mitogenome of *P. australis* was reconstructed by next-generation sequencing (NGS) in the Illumina platform, without mitochondrion enrichment. The NGS library was prepared and run at the Center of Genomic Medicine, National Taiwan University. Two lanes of an Illumina HiScan SQ flow cell were used for sequencing *P. australis* together with seven additional gastropod samples and eight additional fish samples for another parallel study to reduce the costs of the sequencing. Gastropods and fishes were combined into eight pairs. Eight libraries, each built on the mixed genomic DNAs of one fish-gastropod pair, were built for the analysis.

Mitogenomes were processed and assembled using MIRA (Chevreux *et al.*, 1999) and then MITObim (Hahn *et al.*, 2013) for mitogenome baiting and mapping after shotgun sequencing. The mitogenome of *L. granulata* obtained from Sanger sequencing was

Table 1. New vetigastropod mitogenomes in this study.

| Superfamily | Family | Species | Accession number | Locality and voucher material |
|-----------------|----------------|---|------------------|--|
| Angarioidea | Angariidae | <i>Angaria delphinus</i> (Linnaeus, 1758) | KX298893 | South coast of Taiwan, intertidal |
| Fissurelloidea | Fissurellidae | <i>Variemarginula punctata</i> (A. Adams, 1852) | KX298889 | Wanlilong, south coast of Taiwan, intertidal |
| Phasianelloidea | Phasianellidae | <i>Phasianella australis</i> (Gmelin, 1791) | KX298888 | Esperance, Western Australia, intertidal; voucher MNHN IM-2009-31771 |
| Trochoidea | Tegulidae | <i>Chlorostoma argyrostomum</i> (Gmelin, 1791) | KX298892 | Yanliao, north coast of Taiwan, intertidal |
| | | <i>Omphalius nigerrimus</i> (Gmelin, 1791) | KX298895 | Yanliao, north coast of Taiwan, intertidal |
| | Turbinidae | <i>Astralium haematragum</i> (Menke, 1829) | KX298891 | Yanliao, north coast of Taiwan, intertidal |
| | | <i>Lunella granulata</i> (Gmelin, 1791) | KX298890 | Penghu I., Taiwan, intertidal |
| | Trochidae | <i>Stomatella planulata</i> (Lamarck, 1816) | KX298894 | South coast of Taiwan, intertidal |

Abbreviation: MNHN, Muséum National d'Histoire Naturelle, Paris.

used as reference to map the *P. australis* mitochondrial reads from the genomic readpool prepared by Illumina HiScan SQ. In the MITObim workflow, reads from the readpool were mapped to the original reference and used iteratively to rebuild new references from the target genome based on the conserved region. Then these new references were used as baits to ‘fish’ those reads from the readpool that overlapped with the references. The target mitogenome was thus constructed after several iterations. After many iterations, several contigs were formed based on vetigastropod reference mitogenomes that overlapped with each other.

Mitochondrial gene annotation

The DNA sequences of each mitogenome (from Sanger sequencing and NGS) were assembled with CodonCode Aligner v. 3.7.2.2 (Codoncode Corporation, Dedham, MA, USA), then managed using Se-AL v. 2.0a11 (Rambaut, 1996). Gene annotation was carried out using MITOS Webserver (Bernt et al., 2013b) using the invertebrate genetic code and default settings. The obtained sequences were also aligned with known mitochondrial protein-coding genes, rRNAs and tRNAs from the *Haliois rubra* mitogenome available from GenBank (Maynard et al., 2005) to define the gene boundaries. In addition Nucleotide and Open Reading Frame BLAST were used to detect gene positions in each mitogenome.

Phylogenetic analysis

The eight new mitogenomes were included in phylogenetic analyses with other vetigastropod mitogenomes available from GenBank. Based on the work of Uribe et al. (2016), who found that the combined group of Caenogastropoda and Neritimorpha was sister to Vetigastropoda, we used four caenogastropods and three nerites as outgroups.

The nucleotide sequences of all 13 protein-coding genes and the two rRNA genes (*12S* and *16S*), and also the amino acid sequences of the protein-coding genes, were used to reconstruct the phylogenetic tree in two separate analyses. Nucleotide sequences were aligned using the automatic multiple-alignment program MUSCLE (Edgar, 2004). For *12S* and *16S* this was based on the nucleotide sequences themselves. To avoid the creation of gaps that would lead to shifts in the open reading frame, for aligning the protein-coding genes we first translated the nucleotide sequences to amino acids. The resulting multiple sequence alignments were adjusted manually. Gblocks v. 0.91b (Castresana, 2000) was used gene-by-gene to remove the ambiguous alignments, with default settings but including the parameter ‘gap positions within the final blocks were allowed’. The final alignment consisted of 11,930 bp. PartitionFinder and PartitionFinderProtein v. 1.1.0 (Lanfear et al., 2012) were used to test the best partition schemes and the best-fit models for nucleotide and amino acid sequence alignments, respectively. Due to the limitations of included models in RAxML and MrBayes, we ran PartitionFinder and PartitionFinderProtein twice, restricting the analysing model to that implemented in RAxML and MrBayes, respectively.

Phylogenetic analysis was performed using both maximum likelihood (ML) and Bayesian inference (BI) approaches. A ML tree was reconstructed with 27 taxa and the nucleotide dataset of the 15 concatenated genes as implemented in RAxML v. 8.0 (Stamatakis, 2014) with the GTR+G+I model and 11 partitions (see Supplementary material). The robustness of the nodes was assessed by bootstrapping (Felsenstein, 1985) with 1,000 pseudoreplicates. The BI tree was reconstructed with the same dataset using MrBayes v. 3.2.6 (Ronquist et al., 2012) on the CIPRES Science Gateway (Miller, Pfeiffer & Schwartz, 2010) with 12 partitions and with different best-fit models for each partition (Supplementary material). In the BI analysis four Markov chains

were run for 30,000,000 generations in each of two parallel runs, with a sampling frequency of one tree per thousand generations and with the heating temperature 0.02. The convergence was further examined using Tracer v. 1.6 (Rambaut et al., 2014) to make sure all the ESS values were over 200.

The ML and BI analyses were also carried out using the alignment of predicted amino acid sequences for the 13 protein-coding genes, to avoid reconstruction artefacts resulting from different biases in synonymous codon-usage (as reported in Arthropoda by Rota-Stabelli et al., 2013). For this amino acid alignment, five and two partitions were used in ML and BI approaches, respectively, based on PartitionFinderProtein. ML and BI trees were also reconstructed based on the amino acid alignment of the 13 concatenated protein-coding genes and with the same parameters of nucleotide analysis and different substitution models (Supplementary material). Following Williams, Foster & Littlewood (2014) and Uribe et al. (2016), the MtZoa model (Rota-Stabelli, Yang & Telford, 2009) was also tested manually to examine if it was a better fit than the models given by PartitionFinderProtein (Supplementary material). Based on the likelihood values of models given by PartitionFinderProtein and of MtZoa, we used the MtZoa model for all the partitions in the ML analysis.

RESULTS

Long PCR, primer walking and Sanger sequencing for mitogenomes

The size of the seven mitochondrial genomes sequenced by Sanger Sequencing ranged from 14,440 bp (*Variemarginula punctata*) to 19,554 bp (*Angaria delphinus*) (Table 2). All but one (*V. punctata*) of these mitogenomes contained 13 protein-coding genes, two rRNAs and 21–23 tRNAs. However, several segments were missing in three mitogenomes. The *trnE* sequence, which is common in other gastropods, was not found in *Astraliium haematragum*, *Chlorostoma argyrostomum* or *Stomatella planulata*. In *V. punctata* a segment containing the protein-coding genes *atp6*, *trnF*, *trnS* and part of *nad5* could not be sequenced. The main structure of the newly reconstructed mitogenomes is shown in Table 2.

NGS for *Phasianella australis*

The sample from *P. australis* yielded 5.2 Gb from the Illumina HiScan SQ. A total of 51,596,536 reads were acquired, with average length of 101 bp, for which Q30 values were greater than 85%. Reads from *P. australis* and from fish were not separated and all reads were used in the MITObim pipeline.

The mitogenome of *P. australis* could not be completed by MITObim. The elongation sequences stopped at the putative control regions during the process and the contigs could not be overlapped between the two ends. The cause of this break might be the structure of the control region, which has a large number of duplicates of AT that confused MITObim. Nevertheless, we successfully acquired all the 13 protein-coding genes, two rRNAs and 22 tRNAs and their arrangement is shown in Table 2.

Phylogenetic analysis

The tree topology obtained from the different analyses was similar, except for the interrelationships among the trochoidean families, the Angarioidea and the Phasianelloidea (Fig. 1; see below). Three major clades were resolved with strong nodal support within the Vetigastropoda: (1) Fissurelloidea (full support in all analyses); (2) a clade including Lepetodriloidae, Seguenzioidea and Halioidea (nucleotide alignment: bootstrap value [BP] = 89, posterior probability [PP] = 1.00; amino acid alignment: BP = 98, PP = 1.00) and (3) a clade including three trochoidean families, Angarioidea and Phasianelloidea (nucleotide alignment: BP = 99, PP = 1.00; amino

Table 2. Summary of features of the new vetigastropod mitogenomes, including total size of the genome (bp), percentage of A and T (AT %) and sizes of protein-coding genes (bp).

| | <i>Angaria delphinus</i> | <i>Astralium haematragum</i> | <i>Chlorostoma argyrostomum</i> | <i>Lunella granulata</i> | <i>Omphalius nigerrimus</i> | <i>Phasianella australis*</i> | <i>Stomatella planulata</i> | <i>Variemarginula punctata*</i> |
|--------------|--------------------------|------------------------------|---------------------------------|--------------------------|-----------------------------|-------------------------------|-----------------------------|---------------------------------|
| Total size | 19,554 | 16,310 | 17,780 | 17,190 | 17,755 | 18,397 | 17,151 | 14,440 |
| AT% | 64.3 | 67.0 | 66.3 | 66.4 | 67.0 | 65.8 | 67.5 | 69.6 |
| <i>cox1</i> | 1,536 | 1,536 | 1,536 | 1,536 | 1,536 | 1,545 | 1,542 | 1,554 |
| <i>cox2</i> | 693 | 690 | 696 | 693 | 696 | 690 | 693 | 699 |
| <i>cox3</i> | 780 | 780 | 780 | 780 | 780 | 780 | 780 | 780 |
| <i>atp6</i> | 693 | 696 | 699 | 696 | 699 | 705 | 702 | – ** |
| <i>atp8</i> | 201 | 165 | 177 | 189 | 177 | 192 | 168 | 213 |
| <i>cytb</i> | 1,140 | 1,140 | 1,140 | 1,140 | 1,140 | 1,140 | 1,140 | 1,038 |
| <i>nad1</i> | 945 | 960 | 954 | 942 | 951 | 945 | 945 | 984 |
| <i>nad2</i> | 1,173 | 1,128 | 1,152 | 1,155 | 1,152 | 1,131 | 1,122 | 1,110 |
| <i>nad3</i> | 354 | 354 | 354 | 354 | 354 | 354 | 354 | 345 |
| <i>nad4</i> | 1,392 | 1,305 | 1,392 | 1,302 | 1,392 | 1,377 | 1,365 | 1,407 |
| <i>nad4l</i> | 300 | 339 | 300 | 297 | 300 | 306 | 300 | 297 |
| <i>nad5</i> | 1,743 | 1,740 | 1,743 | 1,740 | 1,743 | 1,737 | 1,737 | 378*** |
| <i>nad6</i> | 507 | 507 | 507 | 507 | 507 | 504 | 507 | 465 |

*incomplete genome; **gene could not be sequenced; ***incomplete sequence.

acid alignment: BP = 98, PP = 1.00). Concerning the first two major clades, Fissurelloidea were resolved as the sister clade of the remaining vetigastropods (nucleotide alignment: BP = 96, PP = 1.00; amino acid alignment: BP = 98, PP = 1.00); *Lepetodrilus* (Lepetodriolea) was sister to *Granata* (Seguenzioidea) and *Haliotis* (Haliotoidea) (nucleotide alignment: BP = 97, PP = 1.00; amino acid alignment: BP = 100, PP = 1.00). Within the remaining major clade, the sister-group relationships remained unstable. With the nucleotide alignment, ML and BI gave the same topology: *Stomatella* (Trochidae) was sister to all other taxa (supported only in BI: PP = 0.99) and *Angaria* (Angarioidea) was grouped with Tegulidae and Turbinidae (BP = 81, PP = 0.99) (Fig. 1A). In the ML tree based on amino acid alignment, *Phasianella* (Phasianelloidea) was sister to Trochoidea and Angarioidea, but the clade formed by Trochoidea and Angarioidea was without significant support (BP = 68) (Fig. 1B). Trochidae, Tegulidae and Turbinidae were grouped together also without support (BP = 51) (Fig. 1B). In the BI tree based on amino acid alignment, *Phasianella* (Phasianelloidea) was sister to *Angaria* (Angarioidea) without significant support (PP = 0.6) (Fig. 1C). Using the amino acid alignment, the two turbinid genera *Astralium* and *Bolma* were grouped together, again without significant support (BP = 52, PP = 0.91).

DISCUSSION

Phylogenetic relationships of the vetigastropod superfamilies

The three major clades retrieved within the Vetigastropoda: (1) Fissurelloidea, (2) Lepetodriolea, Seguenzioidea and Haliotoidea, and (3) three trochoidean families (Trochidae, Tegulidae, Turbinidae), Angarioidea and Phasianelloidea, were consistent with those demonstrated by Uribe *et al.* (2016). Our increased taxonomic sampling yielded a stronger resolution of the basal relationships than in the tree of Uribe *et al.* (2016): Fissurelloidea were sister to all other analysed vetigastropods, while Lepetodriolea were sister to Seguenzioidea and Haliotoidea. The additional family, Trochidae, examined in our analysis, was recovered as sister to all other trochoideans, Angarioidea and Phasianelloidea (see below).

To undertake a comprehensive phylogenetic analysis of Vetigastropoda based on mitogenomes, several important groups are still lacking. These groups are rather small (Scissurelloidea, Lepetelloidea: Marshall, 1985; McLean, 1992; Geiger, 2012) and/

or inhabit deep water (Lepetelloidea, Pleurotomarioidea and some scissurelloidean families: Warén & Bouchet, 2001; Harasewych, 2002; Geiger, 2012), which increases the difficulty of sampling.

Gene arrangements of vetigastropods

Vetigastropod mitochondrial gene arrangements are mostly conserved. In the eight newly reconstructed mitogenomes, most showed the inferred ancestral gastropod mitochondrial gene order (Stöger & Schrödl, 2013), with a few tRNA translocations (Fig. 2). Based on the ancestral gastropod gene order, *tmE* was expected to be located with the tRNA series MYCWQG. In our analysis of trochoid species *tmE* was found in this location only for *Lunella granulata*. For other species even by checking manually *tmE* could not be found at this position. This problem was also found in Uribe *et al.* (2016) who suggested that *tmE* was missing in *Tegula lividomaculata* and *Tegula brunnea*. Interestingly, in *Omphalius nigerrimus* the *tmE* was identified between *atp8* and *atp6*, which was not similar to other vetigastropod mitogenomes.

Fissurelloidea and Lepetodriolea presented distinct gene arrangements (Fig. 2). This result confirms that of Nakajima *et al.* (2016), who recently provided an additional *Lepetodrilus* mitogenome. In the fissurellid *Variemarginula punctata* we found a very different mitochondrial gene arrangement compared with other vetigastropods and even with other fissurellids. We could not find *atp6* but, based on other vetigastropod gene orders, we hypothesize that the position of *atp6* is between *tmV* and *cytb*. These data suggest that there is at least one inversion between *16S* – *nad1* – *nad6* – *cytb* – (*atp6*) in the fissurellid mitogenome.

Phylogenetic relationships of trochoidean gastropods

Trochoidea are the largest, most diverse and best-studied group in Vetigastropoda, containing more than 2,000 species (Hickman & McLean, 1990; Williams & Ozawa, 2006; Geiger, Nutzel & Sasaki, 2008; Williams *et al.*, 2008, 2010; Williams, 2012). Besides Trochoidea, the two superfamilies Angarioidea and Phasianelloidea are also ‘trochiform’ gastropods and the interrelationships among these superfamilies remain unsettled. We provided data for two additional tegulids, two turbinids and one trochid. Notably, we provided the first mitogenome from Trochidae, which is the largest family in the Trochoidea. We found a sister relationship between the Trochidae and the other trochiform gastropods in

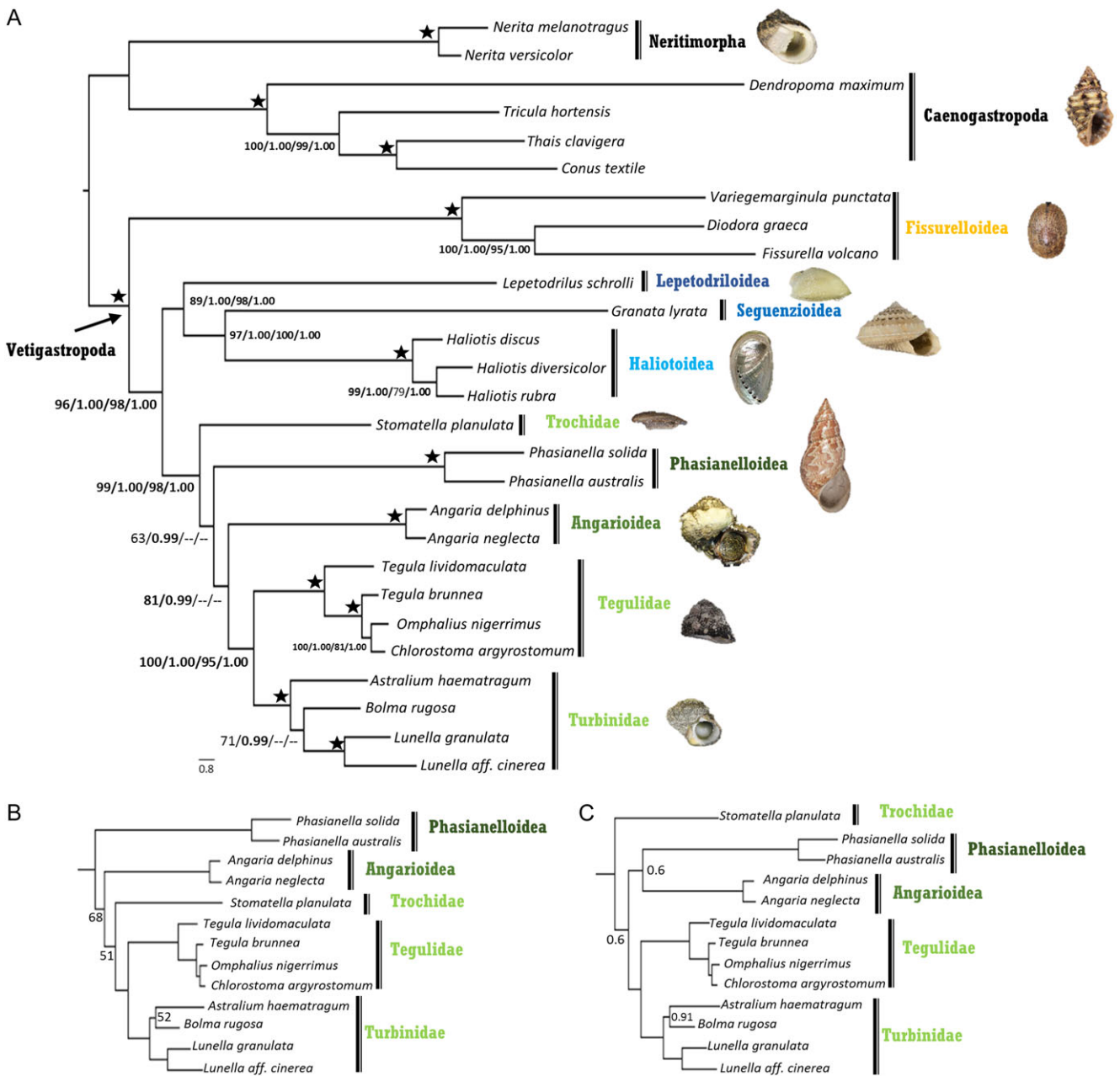


Figure 1. Phylogenetic relationships within Vetigastropoda inferred using partitioned maximum likelihood (ML) and Bayesian (BI) methods. **A.** ML tree based on 11,930 bp nucleotide sequences combined from 13 protein-coding genes and two rRNA genes. Nodal support is shown as nucleotide BP/nucleotide PP/amino acid BP/amino acid PP. **B.** ML tree based on 3,461 amino acid sequences from 13 protein-coding genes. Nodal support is shown as BP. **C.** BI tree based on the same amino acid dataset as in B. Nodal support is shown as PP. Values higher than BP = 80 and PP = 0.95 are considered significant and shown in bold. Absence of nodal support is shown as a dash and full support (100/1.00/100/1.00) as an asterisk. Superfamilies are shown in different colours.

the phylogenetic tree based on the nucleotide alignment, with strong support in the BI analysis (Fig. 1A). According to this topology, Angarioidea and Phasianelloidea belong in the Trochoidea clade. However, not all nodes are supported in the ML analysis and the topology is different in the tree based on the amino acid alignments, although with low nodal support (Fig. 1B, C). This unstable topology could be caused by the long branch of Fissurelloidea (as suggested by Uribe *et al.*, 2016), but also probably by the limited taxon sampling within Trochoidea. The superfamily Trochoidea contains eight living families, Calliostomatidae, Liotiidae, Margaritidae, Skeneidae, Solariellidae, Tegulidae, Trochidae and Turbinidae (Williams, 2012), of which we included only three. Thus, more mitogenomes of other

trochoidean families are required to resolve the phylogeny of this large superfamily. Nevertheless, our results suggest that Trochidae, Tegulidae and Turbinidae do not form a monophyletic group, and also question the currently accepted superfamily status of Angarioidea and Phasianelloidea.

Tegulidae were first considered as a subfamily within either Trochidae or Turbinidae, based on morphological characters (Hickman & McLean, 1990). Bouchet *et al.* (2005) and Williams *et al.* (2008) placed this subfamily within Turbinidae, based on morphological and molecular data, respectively. Williams (2012) included four of the six genera previously placed in Teguliniae and found that *Tegula* and *Norissia* formed a clade (recognized as Tegulidae), while *Tectus* and *Cittarium* formed another

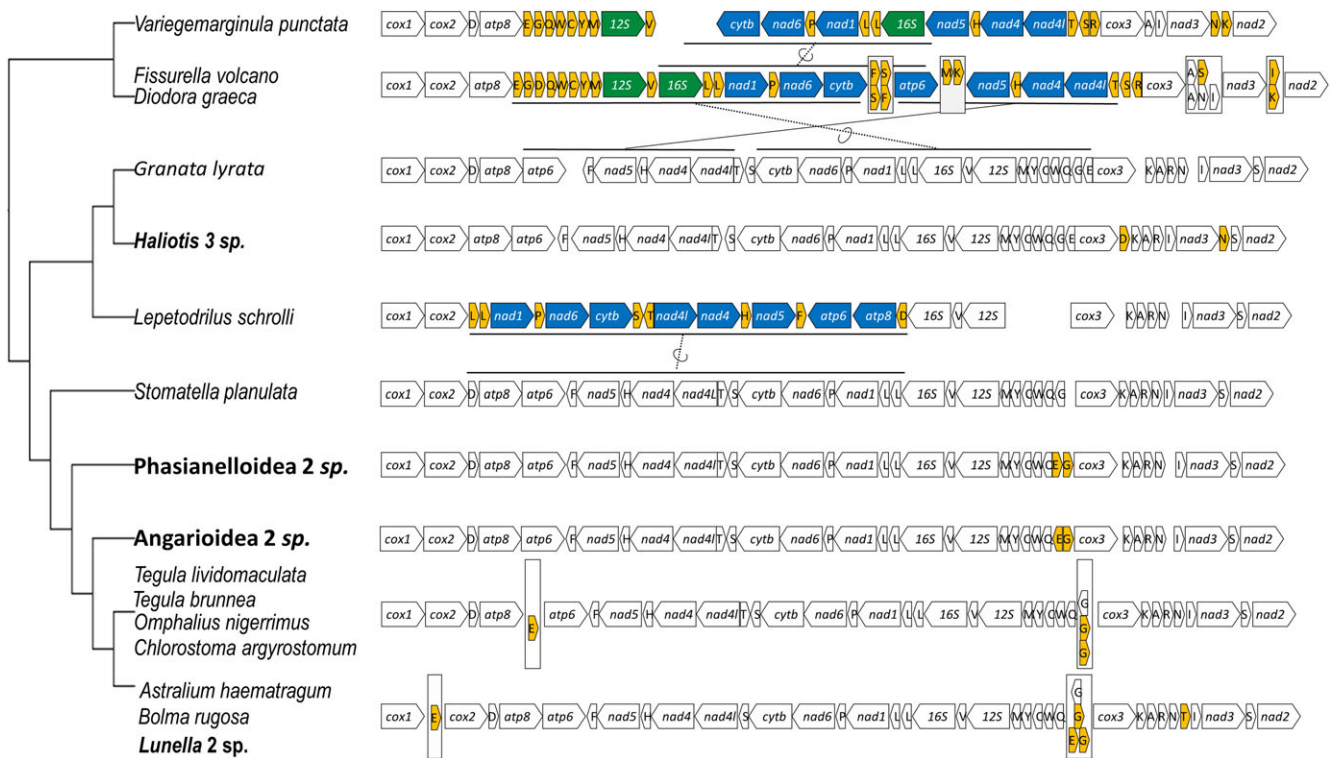


Figure 2. Gene arrangements of 21 vetigastropod mitogenomes. Genes without colour correspond to the hypothetical ancestral gastropod arrangement. Coloured genes show the differences from this ancestral arrangement. The four names in bold correspond to taxa (nodes) for which there are two or more species with exactly same gene order (three species for *Haliotis*, two for Phasianelloidea, Angarioidea and *Lunella*). For nodes at which there is some variability in gene order, the whole list of species names is given and boxes indicate the alternative gene orders (e.g. four boxes show differences between *Fissurella volcano* and *Diodora graeca*).

monophyletic group (still waiting for additional taxa before formal naming as a new taxon). Güeler & Zelaya (2014) described a new genus *Carolesia* in Tegulidae, but no molecular data are yet available to confirm its taxonomic placement. Here, we present two new mitogenomes for *Chlorostoma* and *Omphalius*, which in the study of Williams *et al.* (2008) formed a monophyletic group with *Tegula*. Our analysis confirms that these two genera belong to Tegulidae, but shows that *Tegula* is not monophyletic. This result is congruent with that of Hellberg (1998), who showed that *T. brunnea* from California was closer to *Chlorostoma argyrostomum* and *O. nigerrimus* from East Asia, rather than to a supposedly congeneric species *T. lividomaculata* from the Gulf of Mexico.

Fast mitochondrial evolution in Fissurelloidea

Uribe *et al.* (2016) proposed that Fissurelloidea were the first lineage to diverge within the analysed set of vetigastropod taxa. However, because of a long branch and an unstable position in their analysis they cautioned that its position could be a reconstruction artefact. They also showed a unique gene order for Fissurelloidea, with a large inversion compared with the inferred ancestral gene order of gastropods. The two species they sampled belonged to two fissurellid subfamilies, Diodorinae and Fissurellinae, which had been shown to be sister groups in a previous analysis (Aktipis *et al.*, 2011). To limit the long-branch artefact and to give a better resolution to the tree, we added one more species from another fissurellid subfamily, Hemitominae. We still found a long branch for the fissurelloid clade, but its position was stable and well supported as sister to the other Vetigastropoda *s. s.* Interestingly, the added mitogenome of *Variemarginula* showed a new gene arrangement. We thus revealed (1) that Fissurelloidea displays the most variable gene order among vetigastropod clades and (2) that gene inversion and relocation have happened not only

among superfamilies, but also within the family Fissurellidae. This fast rate of gene inversions and relocations may reflect an overall increased rate of evolution of the mitogenome in comparison with other vetigastropods, potentially affecting phylogenetic analyses.

SUPPLEMENTARY MATERIAL

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

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