**Running head:** mitogenomes Nacella limpets

# Antarctic and sub-Antarctic Nacella limpets reveal novel evolutionary characteristics of mitochondrial genomes in Patellogastropoda

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**Abstract**

Mitochondrial genomes (mitogenomes) provide valuable phylogenetic information and genome-level characters that are useful in resolving evolutionary relationships within major lineages of gastropods. However, for more than one decade, these relationships and the phylogenetic position of Patellogastropoda have been inferred based on the genomic architecture as well as the nucleotide and protein sequences of a single representative, the limpet *Lottia digitalis*. This mitogenome exhibits extensive rearrangements and several repetitive units that may not represent universal features for Patellogastropoda. Here, we sequenced the complete mitogenomes of three *Nacella* limpets, providing new insights into the dynamics of gene order and phylogenetic relationships of Patellogastropoda. Comparative analyses revealed novel gene rearrangements in Gastropoda, characterised by two main translocations that affect the KARNI and the MYCWQ clusters in *Nacella* limpets. Our phylogenetic reconstructions using combined sequence datasets of 13 mitochondrial protein-coding genes and two rRNAs, recovered Patellogastropoda, and Gastropoda in general, as non-monophyletic. These findings could be related to the long-branch attraction tendency of these groups, and/or taxon sampling bias. In our novel mitogenome-based phylogenetic hypothesis, *L. digitalis* is placed in a sister position to Bivalvia and Heterobranchia, whereas *Nacella* limpets are placed sister to a clade containing Caenogastropoda + Neritimorpha and Vetigastropoda + Neomphalina.

## 1. Introduction

Patellogastropods or true limpets, are abundant and conspicuous keystone inhabitants of intertidal rocky shores throughout the world's oceans from the polar regions to the tropics (Lindberg, 2008). Due to their particular morphological characteristics, these molluscs have been considered as the most external branch of living gastropods (Ponder and Lindberg, 1997). However, the phylogenetic position of the group is highly controversial. For example molecular analyses have placed patellogastropods as sister group of Gastropoda, Vetigastropoda, Noemphalina, Heterobranchia or within Caenogastropoda (Colgan et al., 2000; Harasewych and McArthur, 2000; Aktipis et al., 2008; Nakano and Sasaki, 2011; Zapata et al., 2014).

The evolutionary relationships among families of true limpets are also controversial (Harasewych and McArthur, 2000; Nakano and Ozawa, 2007; Lindberg, 2008; Nakano and Sasaki, 2011). Molecular studies based on nuclear genes (*18S* and *28S* rRNAs) have recovered the family Nacellidae as paraphyletic at the base of Patellogastropoda (Harasewych and McArthur, 2000; Nakano and Ozawa, 2007), whereas analyses based on mitochondrial genes (*rrnS*, *rrnL* and *cox1*) recovered the monophyly of this group in a close relationship with Patellidae and clearly distinguished from Lottiidae (Nakano and Ozawa, 2007; González-Wevar et al., 2017b). The general consensus is that mitochondrial genes are more informative for relationships within Patellogastropoda due to their faster and more uniform evolutionary rates among species of this order, compared to nuclear genes (Harasewych and McArthur, 2000; Nakano and Ozawa, 2007; Nakano and Sasaki, 2011). Nevertheless, although more than seven thousand mitochondrial sequences of 253 species of patellogastropods have been deposited in GenBank (30/03/2018), most of these are partial sequences of *rrnS*, *rrnL, cox1* and *cob*, and only one of them represents a

complete mitochondrial genome (mitogenome) of the limpet *Lottia digitalis*. The genomic architecture as well as the nucleotide and protein sequences of this mitogenome have been widely used as references of Patellogastropoda to reconstruct the evolutionary history of different molluscan lineages (e.g. Grande et al., 2008; Stöger and Schrödl, 2013; Uribe et al., 2016b). Nonetheless, *L. digitalis* mitogenome exhibits extensive rearrangements and several repeating units (Simison et al., 2006; Grande et al., 2008) that may not represent universal features for Patellogastropoda. The aims of this study were thus: (a) to sequence the complete mitogenome of three Antarctic and sub-Antarctic *Nacella* limpets, a genus that is distantly related to *Lottia* (Nakano and Sasaki, 2011); (b) to investigate the dynamics of gene order arrangements within patellogastropods and in comparison with other gastropods; and (c) to understand the contribution of these newly sequenced mitogenomes to the phylogenetic relationships of Patellogastropoda.

## 2. Materials and Methods

### *2.1. Species collection and mitogenome sequencing*

Adult limpets of *Nacella concinna, N. magellanica* and *N. clypeater* were collected from the intertidal and subtidal zones in the King George Island (62°10′S, 58°51′W), the Strait of Magellan (53° 37′S, 70° 54′W) and La Misión, Chile (39° 46′ S, 73° 23′W), respectively. For each species, pieces of foot muscle were isolated from 15 individuals for RNA and DNA sequencing. Total RNA was isolated by homogenization of each sample in a TRIzol (Invitrogen, Carlsbad, CA)/chloroform mixture, followed by a DNase treatment and cleaning with the RNeasy mini kit (Qiagen, Chatsworth, CA). Purified mRNA samples were pooled for cDNA library preparation (paired-end) and sequenced. DNA was extracted by the isolation of intact mitochondria from

approximately 150 mg of tissue from single individuals using the Mitochondrial Isolation Kit (Thermo Scientific). The isolated mitochondrial pellet of each limpet was used for the mtDNA extraction by mean of the Mitochondrial-DNA Isolation kit (BioVision). RNA and DNA quality and quantity were analysed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). RNA and DNA library preparations and sequencing were done in the AUSTRAL-omics Core Facility (www.australomics.cl) on an Illumina Hiseq 2500 and a 454 GS Junior Titanium platform (Roche), for RNA and DNA respectively.

*2.2. Assembly and annotation*

Quality control of the raw data was performed using the CLC Genomics Workbench software v.8.5 (CLC bio, Denmark) by removing adapters and reads containing more than 5% of ambiguous nucleotides ('N'). Additionally, sequences were trimmed with a Phred quality score ≤ 40; and reads shorter than 30 bp were removed after trimming. For the 454 raw reads we also used the condensation tool found in NextGENe v2.3.3 (Softgenetics, USA) for correction of homopolymer errors and other base call errors produced by the pyrosequencing process. Then, DNA reads were assembled using the software Geneious Pro 8.1(Biomatters), resulting in a single contig with uniform coverage distribution for each species. Assembly of high quality reads from Illumina sequencing (RNA-cDNA) was carried out using the Trinity software (Grabherr et al., 2013) with default settings and a minimum contig length of 200 nt.

Mitochondrial DNA sequences were identified by local BLAST searches (BLASTn and BLASTx) using Geneious Pro 8.1(Biomatters). In addition, protein-coding genes (PCGs) were identified with the ORF Finder at NCBI using the invertebrate mitochondrial genetic code. The limits of both protein-coding and ribosomal RNA genes were adjusted

118 manually based on the location of adjacent genes and the presence of start and stop codons.
119 Transfer RNA genes were located and folded by their proposed cloverleaf to confirm their
120 secondary structures using ARWEN v.1.2 (Laslett and Canbäck, 2008) following the
121 generalized invertebrate mitochondrial tRNA settings. Finally, our annotations were
122 double-checked using MITOS standalone version (Bernt et al., 2013b) under the
123 invertebrate mitochondrial code. Mitogenome sequences obtained in this work were
124 deposited in GenBank under the accession numbers KT990124 (*N. clypeater*), KT990125
125 (*N. magellanica*) and KT990126 (*N. concinna*).
126
127 *2.3. Concatenated alignments*
128 Nucleotide sequences of the 13 mitochondrial protein-coding genes (PCGs) and two
129 rRNAs from gastropods used in this study (Table 1) were aligned separately using the
130 MAFFT platform implemented in TranslatorX (Abascal et al., 2010). Alignments were
131 done using the L-INS-i option (accurate for alignment of ≤ 200 sequences) and default
132 settings. For the PCGs, the alignments were translated into amino acid sequences using the
133 invertebrate mitochondrial genetic code. Ambiguously-aligned sites were removed using
134 Gblocks v.0.19b implemented in TranslatorX (Abascal et al., 2010) with default settings.
135 Phylogenetic reconstructions were conducted based on the concatenation of the deduced
136 amino acid sequences of the 13 PCGs and the nucleotide sequences of the two rRNAs.
137
138 *2.4. Phylogenetic reconstructions*
139 Best Partition Scheme (BPS) analyses were conducted with the program
140 PartitionFinder2 and PartitionFinder2 Protein (Lanfear et al., 2016), using the Akaike
141 information criterion (AIC) and a heuristic search algorithm. Following Uribe et al.

142 (2016b), the MtZoa model (Rota-Stabelli et al., 2009) was also tested to examine if it could
143 provide a better fit than the models selected by PartitionFinder2. Based on the likelihood
144 values (Supplementary Information S1), we selected the models of molecular evolution
145 used for both Bayesian (BI) and Maximum Likelihood (ML) analyses. ML inference were
146 performed with RAXML v.7.2.6 (Stamatakis, 2006), invoking the GTRGAMMA (less than
147 50 taxa) and the rapid bootstrap option with 10000 replicates. In addition, a Bayesian
148 inference (BI) MCMC analysis was conducted using MrBayes v.3.2 (Ronquist and
149 Huelsenbeck, 2003), running six Markov chains in two simultaneous runs of 10 million
150 generations. Trees were sampled every 1000 generations and those inferred prior to
151 stationarity were discarded as burn-in (first 25% of the sampled trees), and the remaining
152 trees were used to construct a 50% majority-rule consensus tree.
153 Finally, an additional BI analysis was conducted in PhyloBayes MPI v.1.5a.
154 (Lartillot et al., 2013) using the CAT-GTR model of evolution. This method is described as
155 a good strategy to alleviate the long-branch attraction biases that exist between
156 Heterobranchia and Patellogastropoda (Uribe et al., 2016b). The performance of the CAT-
157 GTR+G model was assessed using a 10-fold cross-validation performed on subsamples of
158 6000 non-constant positions randomly drawn from the original matrices. Convergence was
159 assessed using tools implemented in PhyloBayes. Posterior probabilities provided branch
160 support for BI analyses.
161
162 **3. Results and discussion**
163 *3.1. Mitogenomic architectures*
164 Combined DNA and RNA sequencing approaches were able to recover the
165 complete mitogenomes of Antarctic (*N. concinna*) and sub-Antarctic (*N. magellanica* and

166 *N. clypeater*) limpets. The size of these mitogenomes (~16 kb) falls within the range of
167 other gastropods but is considerably smaller than in *L. digitalis* (Table 1), for which
168 several repeated units are distributed across two regions of the genome (*rrnS-cob* and
169 *nad1-nad6*) (Simison et al., 2006). As for most molluscs, *Nacella* mitogenomes consist of
170 a single circular chromosome with the typical set of 13 protein-coding genes (PCGs), two
171 ribosomal RNA genes (rRNA), 22 transfer RNA genes (tRNA), and a large AT-rich non-
172 coding region (control region; CR) (Bernt et al., 2013a) that is implicated in the initiation
173 of transcription and replication processes (Gaitán-Espitia et al., 2013). In these
174 mitogenomes, genes are divided between the two strands about evenly, with the heavy
175 strand (H) having 10 tRNAs and seven PCGs, and the light (L) strand having 12 tRNAs,
176 six PCGs and the two rRNAs (Fig. 1). Although both strands are AT rich (65-68%), there
177 is a compositional asymmetry favouring GT in the H-strand and AT in the L-strand
178 (Supplementary Information S2). The cause of this asymmetry is accounted to an
179 asymmetric mutation process that favours transitions over transversions on the H-strand,
180 which is prone to mutations when it is single stranded (i.e., during replication and
181 transcription) (Bernt et al., 2013a).
182 In the 13 PCGs, three types of start codons (ATG, ATT and ATA) were registered,
183 of which ATG started 8 genes while ATA and ATT initiated two and three genes
184 respectively. Nine open-reading frames ended with TAA whereas four ended with TAG.
185 The highest AT content was observed in *nad4L* (71.3%), whilst the lowest one was
186 detected in *cox3* (61.9%) (Supplementary Information S2). For the tRNAs, our findings
187 are consistent with those documented for other metazoans (Bernt et al., 2013a), in which
188 most of the tRNAs have the potential to fold into the typically cloverleaf secondary

189 structure, with the exception of *tRNA-$S_1$* that lacks the dihydrouracil (DHU) arm
190 (Supplementary Information S3).
191 Gene arrangements in Antarctic and sub-Antarctic *Nacella* mitogenomes were
192 highly conserved within the genus but exhibited clearly distinctive genomic architectures
193 compared to *L. digitalis* (Fig. 1). Contrasting to this patellogastropod, as well as to other
194 gastropods, no duplications and/or translocations+inversions were detected in *Nacella*
195 limpets (Fig. 1). However, our newly sequenced mitogenomes showed similar
196 arrangements to the hypothetical ancestral gene order of Gastropoda (Uribe et al., 2016b),
197 and to some Neritomorpha and Vetigastropoda species (Fig. 1). Major differences with
198 these groups could be explained by two main translocations that affect the KARNI (H-
199 strand) and the MYCWQ (L-strand) clusters (Fig. 1). These translocations represent novel
200 gene rearrangements in Gastropoda (Grande et al., 2008). With the exception of *nad3*,
201 these findings are consistent with the idea that in Gastropoda, tRNA genes are more prone
202 to switch their position than larger PCGs and rRNAs (Grande et al., 2008; Gaitán-Espitia
203 et al., 2013).
204
205 *3.2. Phylogenetic analysis*
206 Best Partition Scheme (BPS) analyses for combined amino acid sequences of 13 PCGs
207 and nucleotide sequences of the two rRNAs, revealed slightly better fit using the MtZoa
208 model than the models selected by PartitionFinder2 (Supplementary Information S1).
209 Similar results has been documented in phylogenetic studies using mitochondrial genomes
210 in other gastropod lineages (Stöger and Schrödl, 2013; Lee et al., 2016; Uribe et al.,
211 2016b). The MtZoa BPS was used for both Bayesian (BI) and Maximum Likelihood (ML)
212 analyses, which produced nearly identical topologies with similar branch lengths and strong

support of bootstraps (ML analysis) and posterior probabilities (Bayesian inference) for most of the nodes (Fig. 2 and Supplementary Information S4). Both, the ML (-LnL = -143247.86) and the BI (-LnL = -137981.4) reconstructions, recovered with high statistical support the monophyly of the genus *Nacella* (Fig. 2). The evolutionary relationships within this group of Antarctic and sub-Antarctic limpets were consistent with previous molecular studies using partial sequences of mtDNA (*cox1* and *cob*) and nDNA (28S rRNA) genes, supporting the division of *Nacella* according to the biogeographic regions described for the Southern Ocean (González-Wevar et al., 2010, 2017a). Here, our results suggest that the Antarctic limpet *N. concinna* branched off early in this monophyletic lineage (Fig. 2). This split is estimated to have occurred during late Miocene (~8 – 5.5 Mya), parallel to climatic transitions and intense thermal zonations between Antarctic and sub-Antarctic provinces (González-Wevar et al., 2010, 2017a). Additionally, our results suggest that the sub-Antarctic limpet *N. magellanica* shares a most recent common ancestor with *N. clypeater* (Fig. 2), in which their divergence time is estimated to be approximately 6-5 Mya in the transition between late Miocene and early Pliocene (González-Wevar et al., 2010).

In our study, the evolutionary relationships among gastropods and the phylogenetic position of Patellogastropoda, were based on a taxon sampling that included representative species of Cephalopoda, Scaphopoda, Polyplacophora Aplacophora, Bivalvia and Monoplacophora as outgroups. Our results recovered Gastropoda and Patellogastropoda as non-monophyletic (Fig. 2), probably as a result of the long-branch attraction tendency of these groups (Stöger and Schrödl, 2013; Osca et al., 2014), and/or taxon sampling bias. The general topology for Mollusca was highly consistent to other studies based on mitochondrial genome architectures (Stöger et al., 2016) and mitogenome sequences (Stöger and Schrödl, 2013; Osca et al., 2014; Li et al., 2018). Within each Gastropoda

order, our results are highly aligned to previous mitogenomic phylogenies of Vetigastropoda (Uribe et al., 2016b) and Neritimorpha (Uribe et al., 2016a), with some discrepancies regarding the position of Patellogastropoda, which is based only on the mitogenome of *L. digitalis*. Previous phylogenetic reconstructions of evolutionary relationships among gastropods have used a variety of different datasets, ranging from morphology (e.g., shell structure) to few molecular sequences (e.g., partial genes *18S*, *28S*, *rrnS*, *rrnL* and *cox1*), supermatrices of genes derived from transcriptomes, and the gene order of mitogenomes (Ponder and Lindberg, 1997; Colgan et al., 2000; Harasewych and McArthur, 2000; Aktipis et al., 2008; Grande et al., 2008; Lindberg, 2008; Zapata et al., 2014). These studies have shown contrasting results placing patellogastropods as the most external branch of Gastropoda or in a sister group relationship with Vetigastropoda, Noemphalina, Heterobranchia or within Caenogastropoda (Colgan et al., 2000; Harasewych and McArthur, 2000; Aktipis et al., 2008; Nakano and Sasaki, 2011; Zapata et al., 2014). In this study, patellogastropods were split in two groups with *L. digitalis* placed in a sister position to Bivalvia and Heterobranchia, whereas *Nacella* limpets were placed sister to a clade containing Caenogastropoda + Neritimorpha and Vetigastropoda + Neomphalina (Fig. 2).

Our novel phylogenetic hypothesis highlights the importance of increasing taxon sampling in patellogastropods in order to ameliorate the impact of long-branch attraction artefacts and to improve our understanding of their phylogenetic relationships with other molluscan lineages. Moreover, considering that Patellogastropoda contains at least seven families (Nakano and Ozawa, 2007), a denser taxon sampling of mitogenomes of representative species from other groups such as Lepetidae, Acmaeidae, Patellidae and Nacellidae (e.g., *Cellana*, a sister genus of *Nacella*), will provide a better framework of the

evolutionary relationships within patellogastropods and the dynamics of genome-level characters (e.g., rearranged structures) for this order.

## Conflict of interest

The authors declare no conflicts of interest.


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## Figure Legends

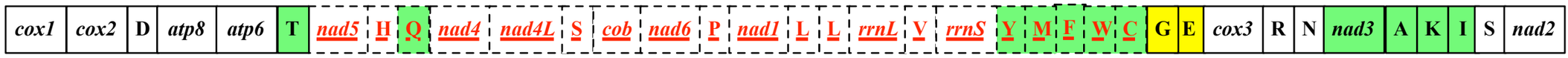
**Figure 1.** Mitochondrial gene order of patellogastropods in comparison with other gastropod lineages. Protein coding and ribosomal genes are labelled with gene names and tRNAs are denoted by single-letter codes according to the amino acid they represent: A, Ala; G, Gly; P, Pro; T, Thr; V, Val; S, Ser; R, Arg; L, Leu; F, Phe; N, Asn; K, Lys; D, Asp; E, Glu; H, His; Q, Gln; I, Ile; M, Met; Y, Tyr; C, Cys; W, Trp. Transcripts in the light strand (reverse) are represented in red and dashed boxes.

**Figure 2.** Maximum likelihood (ML) tree of concatenated amino acid sequences of the 13 PCGs and the nucleotide sequences of the two rRNAs describing phylogenetic relationships among Gastropoda. The ML bootstrap and Bayesian posterior probability values for each node are indicated (black circles: bootstrap value ≥ 90% and posterior probability of 1; grey circles: bootstrap value < 90% and posterior probability of 1; white circles: bootstrap value < 90% and posterior probability < 1). The scale bar represents the number of nucleotide substitutions per site.

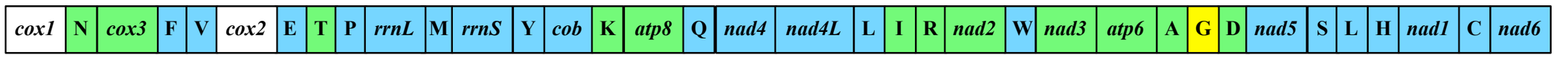
## Hypothetical Ancestral Gastropoda


## Patellogastropoda

*Nacella*

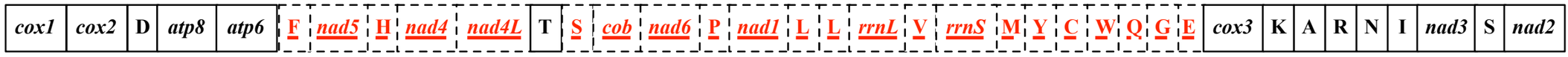



*Lottia*




## Neritomorpha

*Nerita, Georissa*



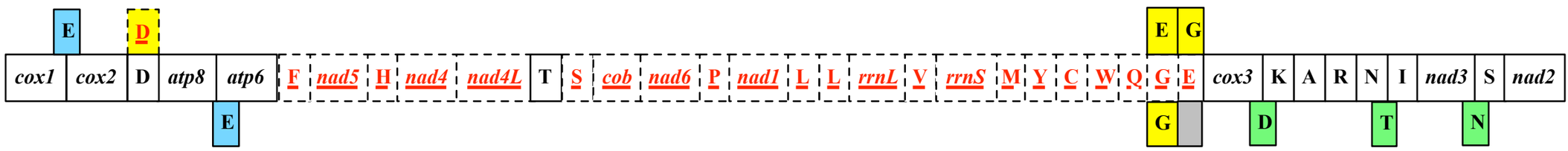

*Pleuropoma*


## Neomphalina

*Chrysomallon*


## Vetigastropoda

*Granata, Tegula, Stomatella, Chlorostoma, Phasieanella, Angaria, Lunella, Astralium, Omphalius, Bolma, Haliotis*



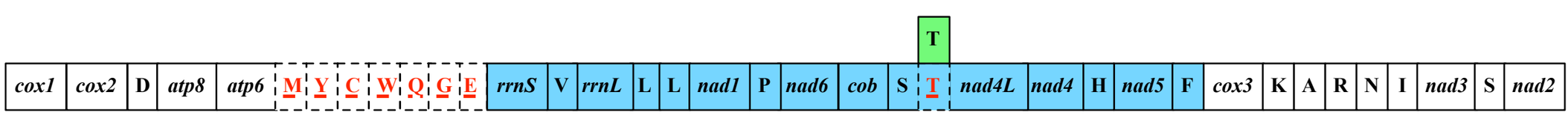

*Diodora, Fisurela*


*Variegemarginula*


*Lepetodrilus*

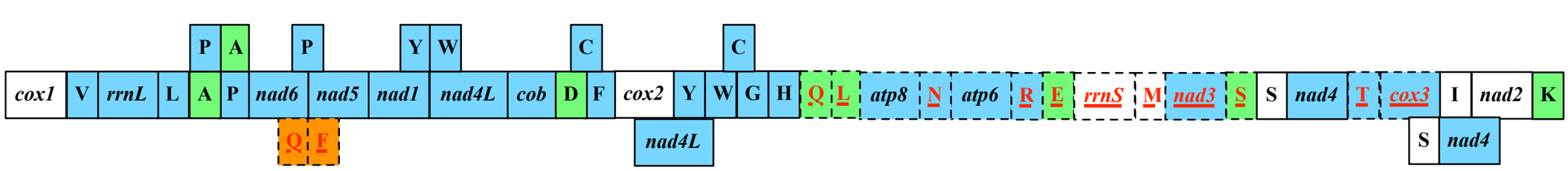

## Caenogastropoda

*Rapana, Tricula, Thais, Oncomelania, Ilyanassa, Conus*

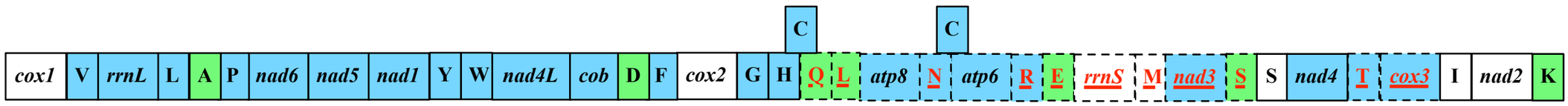



## Heterobranchia

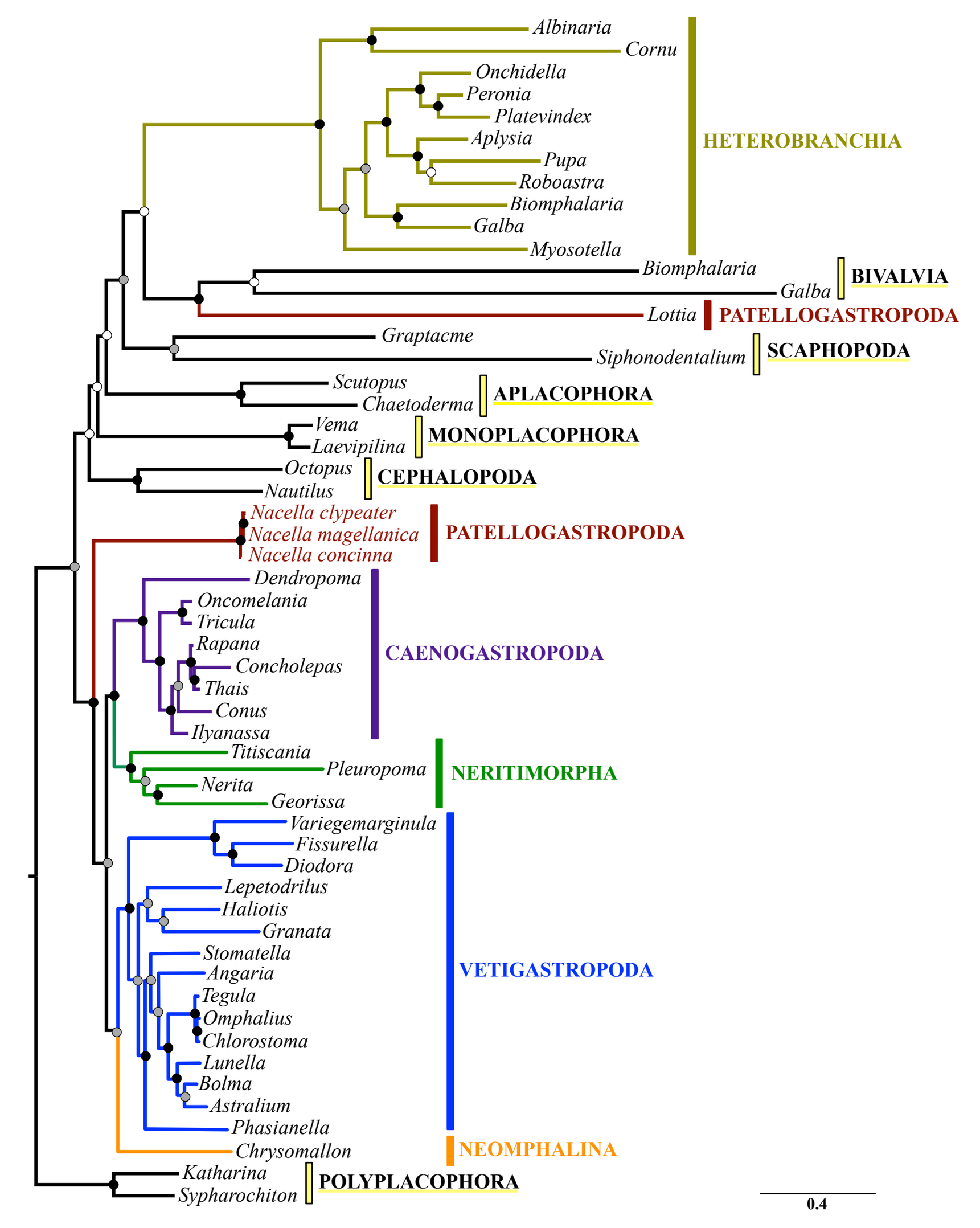
*Peronia, Galba, Albinaria, Biomphalaria, Onchidella, Myosotella, Cornu*




*Roboastra, Aplysia, Pupa*




Translocation | Inversion | Translocation and inversion | Duplication | No information


Albinaria
Cornu
Onchidella
Peronia
Platevindex
Aplysia
Pupa
Roboastra
Biomphalaria
Galba
Myosotella
HETEROBRANCHIA
Biomphalaria
Galba
BIVALVIA
Lottia
PATELLOGASTROPODA
Graptacme
Siphonodentalium
SCAPHOPODA
Scutopus
Chaetoderma
APLACOPHORA
Vema
Laevipilina
MONOPLACOPHORA
Octopus
Nautilus
CEPHALOPODA
Nacella clypeater
Nacella magellanica
Nacella concinna
PATELLOGASTROPODA
Dendropoma
Oncomelania
Tricula
Rapana
Concholepas
Thais
Conus
Ilyanassa
CAENOGASTROPODA
Titiscania
Pleuropoma
Nerita
Georissa
NERITIMORPHA
Variegemarginula
Fissurella
Diodora
Lepetodrilus
Haliotis
Granata
Stomatella
Angaria
Tegula
Omphalius
Chlorostoma
Lunella
Bolma
Astralium
Phasianella
VETIGASTROPODA
Chrysomallon
NEOMPHALINA
Katharina
Sypharochiton
POLYPLACOPHORA
0.4