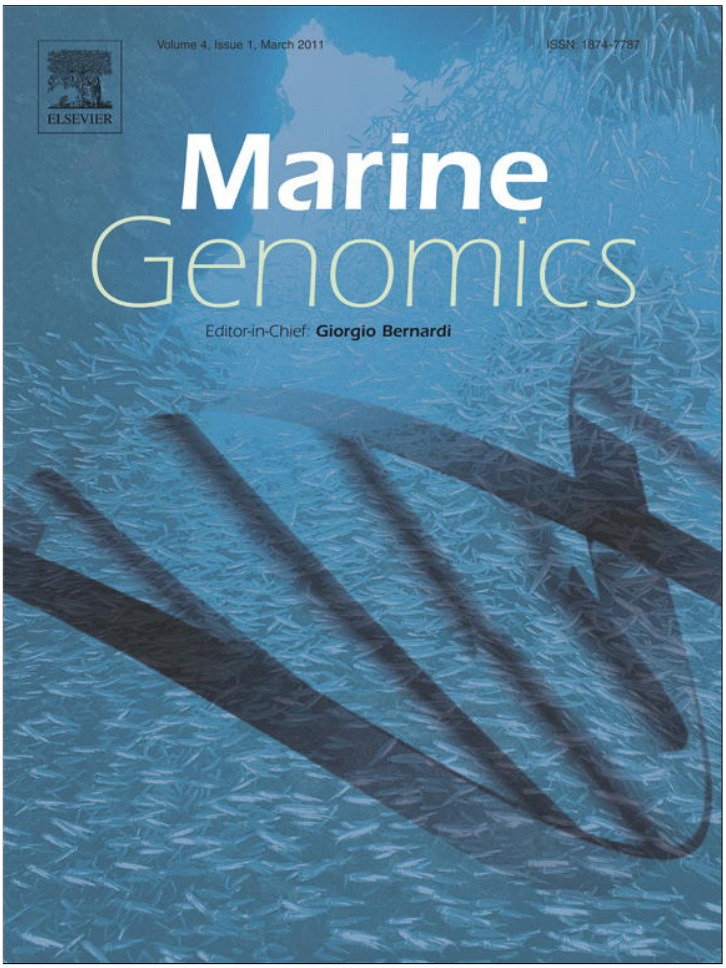

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# Crawling through time: Transition of snails to slugs dating back to the Paleozoic, based on mitochondrial phylogenomics

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ABSTRACT

Sea slugs (Gastropoda: Opisthobranchia) are characterized by extensive morphological homoplasy. In particular, reduced or absent shells are predominant throughout the group. This trend towards shell loss has resulted in a poor fossil record. DNA-based phylogenies have been helpful in improving our understanding of the evolution of this group and major clades are emerging. We report 13 new complete opisthobranch mitochondrial genomes that provide robust support for some of these emerging nodes. We name three new clades within the Opisthobranchia, the Actopleura (Acteonoidea plus Nudipleura), Placoesophaga (Cephalaspidea plus Anaspidea), and Siphoglossa (Sacoglossa plus the *Siphonaria*). Finally we use molecular clock dating that suggests an earlier opisthobranch divergence than previously reported. The implications of this evolutionary scenario are discussed.



## 1. Introduction

Sea slugs (Gastropoda: Opisthobranchia) are a diverse group of gastropods characterized by reduced or absent shells. The evolutionary trend toward shell reduction has led to unique ecological adaptations such as chemical defense mechanisms (elaborate toxic compounds sequestered from their diet or synthesized de novo) (Avila, 1995; Cimino and Ghiselin, 2009; Ghiselin, 1966), as well as widespread Mullerian and Batesian mimicry (Gosliner, 1991, 1994; Gosliner and Ghiselin, 1984). In addition to the evolution of shell loss, this group has undergone extensive morphological homoplasy of other features such as detorsion, cephalization and division of genital ducts, in some cases arising independently in various lineages (Ghiselin, 1966; Gosliner, 1991, 1994; Gosliner and Ghiselin, 1984; Mikkelsen, 1996; Valdés et al., 2010). Because most opisthobranchs are soft-bodied and often lack a strong adult shell structure, there is no good fossil record for this group. Consequently, little is known about the time and conditions when most major groups diverged. Parallel evolution and convergence of anatomical traits have limited the resolution of morphology-based phylogenies (Gosliner and Ghiselin, 1984; Mikkelsen, 1996). Yet these early morphological phylogenies already suggested a great deal of ecological diversification reflected in the evolution of distinct feeding strategies. In the past decade, partial single-gene and multi-gene molecular phylogenies have steadily improved our understanding of opisthobranch evolution (Grande et al., 2004a; Grande et al., 2004b; Klussmann-Kolb et al., 2006; Klussmann-Kolb et al., 2008; Malaquias et al., 2009a; Martynov and Schrodl, 2008; Vonnemann et al., 2005; Wägele and Klussmann-Kolb, 2003; Wägele et al., 2005). Also, mitochondrial phylogenomics has recently become a fruitful approach to address unresolved relationships within euthyneuran gastropods (pulmonates and opisthobranchs) (Kurabayashi and Ueshima, 2000; Grande et al., 2002, 2008; Knudsen et al., 2006). In particular, mitogenomic data have provided more robust topologies than those based on gene fragments. However, because the taxon sampling for sea slugs is still scarce, the current mitogenomic-based phylogenies provide limited insight into how opisthobranch radiations took place.

### 1.1. Ecological diversification

The adaptive radiation of sea slugs invokes several intriguing questions. Did opisthobranch lineages radiate in response to major geological and biological historical events such as mass extinctions?

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Did the loss of the shell precede the evolution of chemical defenses? How many times has the shell been lost in different opisthobranch lineages? Was the evolution of feeding specificity a consequence of shell loss? In terms of prey specificity, we here focus on how specializations to various diets (*e.g.* carnivorous versus herbivorous clades of cephalaspideans) arose. Are there multiple evolutionary events or do specific clades reflect a single specialization of diet? Did detorsion precede shell loss or vice versa? Have aposematic color patterns evolved through common ancestry or convergence or both? The answers to all of these questions require mapping of characters on a robust phylogeny and some understanding of the ages of the various lineages.

### 1.2. Mitochondrial genomics

Both sequences and gene arrangements of mitochondrial genomes have been compared to successfully address phylogenetic relationships among various branches of the animal tree (Cameron et al., 2007; Medina et al., 2006; Miya et al., 2003; Muller, 2006; Yokobori et al., 2004). Mollusca is the second largest animal phylum after Arthropoda, so the 77 complete molluscan mitochondrial genome sequences available still constitute scarce taxon sampling, and robust class-level phylogenies remain elusive [reviewed in Simison and Boore (2008)]. However, these data have been useful in genome evolution research since molluscan mitochondrial genomes show evidence of major gene reshuffling throughout the evolution of the phylum. Some bivalves and gastropods, in particular, exhibit highly rearranged genomes relative to the ancestral bilaterian genome [reviewed in Medina and Collins, 2003]. Thus, reconstructing the evolutionary history of molluscan mitochondrial genomes has been particularly obscured by the fast rate of rearrangements coupled with deep divergences within each class.

The majority of gastropod lineages (Vetigastropoda and Caenogastropoda) show relatively minor gene order shuffling (Simison and Boore, 2008) from that of chitons, while the Patellogastropoda and Heterobranchia exhibit major rearrangements. Some of the more recently diverged branches within the gastropods such as the euthyneurans have been well supported from mitogenomic data and comparing the patterns of gene arrangements within this group may serve to clarify how gene order changes evolve. These euthyneuran mitochondrial genomes show two striking features: (1) a reduction in size to approximately 14 Kb on average, and (2) a highly rearranged genome relative to other known gastropod outgroups. The robust results of mitochondrial phylogenomics for euthyneuran taxa (Grande et al., 2002, 2008) and the success of large mitogenomic datasets in other taxa (Medina et al., 2006; Miya et al., 2003; Muller, 2006; Simison and Boore, 2008; Yokobori et al., 2004), suggest that this approach may improve our understanding of the evolution within the Opisthobranchia.

In this manuscript we present a phylogenomic analysis with 13 new complete mitochondrial genomes (including a pulmonate outgroup). We use this reconstruction to examine the evolution of some noteworthy morphological and ecological traits. Finally, we attempt to date the appearance of several opisthobranch lineages using molecular evidence with the ultimate goal to shed light into the environmental circumstances that led to evolutionary innovations within major opisthobranch lineages.

## 2. Materials and methods

### 2.1. Sample collection and amplification

We included complete mitochondrial genome data for 13 species from six different opisthobranch orders and one outgroup (Table 1). We extracted DNA by either a standard SDS/Proteinase K digestion protocol as described in Medina and Walsh (2000), or with the QIAquick tissue extraction kit (Qiagen). We performed long PCR (Takara) to amplify large mitochondrial fragments. The PCR conditions used were denaturation at 94 °C for 2 min, followed by 30 cycles of a 30 s denaturation step at 94 °C, a 30 s annealing step at 48 °C, with an extension step of 10 min at 70 °C, with a final extension step of an additional 10 min at 72 °C. In a few instances annealing temperatures were modified as necessary for successful amplification. In several cases, we obtained 50–70% of the mitochondrial genome with universal primers for *rrnL, rrnS, cob* and *cox3*. Once we sequenced these portions, we amplified the remainder of each genome with individual-specific primers. The list of primer combinations per species is presented in Appendix A. Primer sequences are reported in Appendix B. We amplified two of the mitochondrial genomes (*Aplysia dactylomela* and *Aplysia vaccaria*) by rolling circle amplification (RCA) instead of PCR using random hexamers on templates enriched for mtDNA by CsCl centrifugation (Boore et al., 2005).

**Table 1**
Collection location, genome size and GC content of new opisthobranch mitochondrial genomes reported in this publication.

| Classification | Species name | Collection locality | Genome size | GC content |
|---|---|---|---|---|
| Cephalaspidea | *Smaragdinella calyculata* | Lifou, New Caledonia | 13,855 | 38 |
| | *Bulla* sp. | Lifou, New Caledonia | 13,889 | 38 |
| | *Odontoglaja guamensis* | Papua New Guinea | 15,038 | 32 |
| | *Sagaminopteron nigropunctatus* | Palau | 13,889 | 30 |
| Anaspidea | *Aplysia vaccaria* | San Diego, CA | 13,130 | 35 |
| | *Aplysia dactylomela* | Florida Bay, FL | 14,128 | 34 |
| Aplustridae | *Hydatina physis* | Papua New Guinea | 14,153 | 34 |
| | *Micromelo undata* | Papua New Guinea | 14,160 | 34 |
| Sacoglossa | *Thuridilla gracilis* | Palau | 14,259 | 29 |
| Nudibranchia | *Aegires gardineri* | Papua New Guinea | 14,424 | 34 |
| | *Chromodoris magnifica* | Palau | 14,446 | 33 |
| Notaspidea | *Berthellina ilisima* | Pacific, Costa Rica | 15,688 | 32 |
| Pulmonate outgroup | *Onchidella borealis* | Pillar Point, CA | 14,510 | 34 |

### 2.2. Cloning and sequencing

We randomly sheared the products of long PCR or RCA in a HydroShear (Gene Machines), blunt end repaired them enzymatically, size selected them from an agarose gel (1.5 kb), and ligated them into pUC vector. We transformed the ligated DNA into *E. coli* DH10b to create plasmid libraries, then plated the clones, for overnight growth. We randomly selected individual clones for sequencing using RCA with TempliPhi (Epicentre), sequencing reactions with standard primers for forward and reverse reactions, purification of the reaction products using SPRI (solid phase reversible immobilization), and sequencing using capillary electrophoresis on an ABI 3730xl instrument.

### 2.3. Genome assembly and annotation

We made base calls using Phred (Ewing and Green, 1998; Ewing et al., 1998), generated assemblies with Phrap (Gordon et al., 2001), and called the consensus sequence in Consed (Gordon et al., 1998). Sequencher (Gene Codes Corporation) was used to proofread the final consensus sequences and assemblies. We used DOGMA (Wyman et al., 2004) along with tRNAscanSE (Lowe and Eddy, 1997) and further manual examination to identify genes using the genetic code for invertebrate mitochondria. We identified the tRNA genes by their potential to form tRNA-like secondary structures, with specific identifications made according to anticodon sequence. DNA sequences are available at NCBI (GenBank accession numbers DQ991927–DQ991939). Putative tRNA structures are available upon request.

### 2.4. Phylogenetic analysis

We generated amino acid alignments using Clustal W in the GCG Wisconsin package (Accelrys) for all protein encoding genes. *Ilyannassa*

*obsoleta, Lophiotoma cerithiformis* and *Conus textile* were used as outgroups to Euthyneura. Only *Biomphalaria glabrata* and *Onchidella borealis* were used as pulmonate sister taxa to Opisthobranchia. We initially considered all available pulmonate sequences, but preliminary analyses indicated that some taxa have particularly long branches, likely due to both poor sequence quality and annotation, so these were eliminated from consideration. We determined regions of unambiguous alignment for 12 of the 13 protein coding genes (*atp8* was excluded), using Gblocks (Castresana, 2000) by implementing the most stringent settings. Those regions were excluded from further analysis. We subsequently concatenated the resulting alignments into a single file that comprised 2622 amino acids for phylogenetic analysis using two methods: (1) RAxML (Randomized Axelerated Maximum Likelihood High Performance Computer) estimates phylogenetic trees from sequence data with Maximum Likelihood (ML) methods (Stamatakis et al., 2005). The mtREV model of substitution was implemented, as it was specifically developed to model mitochondrial amino acid substitution rates with a gamma distribution. Support values were determined by executing one thousand bootstrap replicates in RAxML V. 7.2.7. (2). (2) The BEAST (Drummond and Rambaut, 2007) program uses a Bayesian MCMC based algorithm for estimating phylogenies from molecular data. As for the ML analysis, we implemented the mtREV model of substitution with the site heterogeneity model set to gamma + invariant sites (gamma categories set to 8), for 35,000,000 generations. All trees were viewed and edited in Dendroscope (Huson et al., 2007).

### 2.5. Molecular dating

We estimated divergence times using both BEAST and r8s (Sanderson, 2003). For both methods we fixed the age of three nodes – Acteonoidea to 196 mya, Bullidae to 167 mya and the clade comprising *Hydatina* and *Micromelo* to 171 mya as calibrating points (Tracey et al., 1993; Wenz and Zilch, 1959–1960) – as calibrating points. In BEAST we implemented the relaxed uncorrelated lognormal clock model, with prior set to a normal distribution with a standard deviation of 2. In r8s we used the ML tree and branch lengths estimated by RAxML. The Penalized Likelihood method was used to estimate the divergence times with the Truncated Newton (TN) algorithm and a smoothing value of 7. The latter was first estimated by implementing the cross-validation procedure.

## 3. Results

### 3.1. Mitochondrial genomes

The opisthobranch mitochondrial genomes reported here range in size from 13,130 to 15,038 nucleotides (Table 1), similar in size to the previously known euthyneuran genomes, with a compact arrangement of genes and few non-coding nucleotides and GC content ranging from 29 to 38%. In all 13 new genomes, we have identified the same putative control region within the *cox3-trnI* junction as previously reported for related genomes (Kurabayashi and Ueshima, 2000; Grande et al., 2002). This region ranges in size from 31 to 915 nucleotides. In three genomes (*Onchidella borealis, Sagaminopteron nigropunctatus, Thuridilla gracilis*), a second non-coding intergenic region is present within the *nad6–nad5* junction, ranging in size from 33 to 145 nucleotides in length. All 13 genomes have the typical metazoan mitochondrial gene content with 13 encoding proteins, 22 encoding tRNAs and two encoding ribosomal RNAs. Protein genes are similar in length to those of published mtDNAs, and have a similar variety of initiation (ATG, ATA, ATT, TTG, TTA, GTG, CTT, and ATT) and termination (TAA, TAG, and abbreviated terminations) codons. Some of the tRNAs are relatively short and characterized by unpaired D or T arms, in particular *trnS1* and *trnS2*, as has commonly been found. As observed in other opisthobranch gastropods, most of the new genomes have genes overlapping by several nucleotides. These overlaps occur mainly between tRNA genes, but overlaps between protein and tRNA genes are also common. The least common overlap is observed between protein encoding genes. Some of the common overlaps across species are consistent with phylogenetic relationships.

### 3.2. Phylogenetic relationships

The phylogenomic reconstruction with the new mitogenomic data provides stronger support than previously reported for several of the opisthobranch nodes (Fig. 1). In the Bayesian analysis all nodes are supported by 100% posterior probability values. The Maximum Likelihood reconstruction resulted in high bootstrap supports for most nodes as well (77% or higher), with the exception of the pulmonate outgroup clade and the monophyly of opisthobranchs, with the inclusion of *Siphonaria pectinata*. The main clades within the Opisthobranchia supported are: 1) a clade including the Sacoglossa (*Thuridilla gracilis, Ascobulla fragilis* and *Elysia chlorotica*) and the supposedly-pulmonate *Siphonaria pectinata*; this first clade is here named Siphoglossa new name. 2) A clade with the Cephalaspidea (*Smaragdinella calyculata, Bulla* sp., *Sagaminopteron nigropunctatus,* and *Odontoglaja guamensis*) as sister taxon of the genus *Aplysia* (Anaspidea); this second clade is called Placoesophaga new name. 3) A clade including the Nudipleura (*Aegires gardineri, Chromodoris magnifica, Roboastra europea,* and *Berthellina illisima*) and the Acteonacea (*Micromelo undata, Hydatina physis,* and *Pupa strigosa*) as sister taxa; this last taxon is here named Acteopleura new name.

### 3.3. Gene order

The gene order of some of the genomes is identical to published genomes (Fig. 2): 1) *A. vaccaria, A. dactylomela, M. undata* and *H. physis* have the same gene order as *P. strigosa* (Kurabayashi and Ueshima, 2000); and 2) *B. illisima, A. gardineri* and *C. magnifica* share the same gene order as *Roboastra europea* (Grande et al., 2002). Several genomes have novel gene orders with respect to published data. Some of the rearrangements involve as few as two adjacent tRNAs while others involve larger portions of the genome containing multiple genes. In particular, the four genomes of the Cephalaspidea (*Bulla* sp., *S. calyculata, S. nigropunctatus,* and *O. guamensis*) exhibit the most divergent gene order relative to the ancestral state within opisthobranchs.

### 3.4. Molecular dating

After 35,000,000 generations the BEAST analysis never reached convergence, and therefore only the r8s divergence time estimates are reported here. Dates for all nodes are depicted below branches in Fig. 1, and also included the dates of the oldest know fossils for each taxon (Table 2). Discussion of the criteria for considering oldest fossils and their significance is considered below.

## 4. Discussion

### 4.1. Opisthobranch phylogeny and systematics

Although taxon sampling is still limited, the phylogeny presented in this study substantiates the use of mitochondrial genomics as a reliable approach to tackle difficult nodes within opisthobranch gastropods. We report strong support values for lineages that have been suggested in the recent literature with either fewer taxa or partial gene trees. A robust mitochondrial genome-based phylogeny provides the basis for dating the divergence of multiple branches of the opisthobranch tree, offering the opportunity to examine different aspects of their anatomical and ecological adaptations in a geological framework. Recent molecular phylogenetic studies of opisthobranchs (Dayrat, 2001; Dayrat and Tillier, 2002; Dinapoli and Klussmann-Kolb, 2010; Grande et al., 2004a,b;

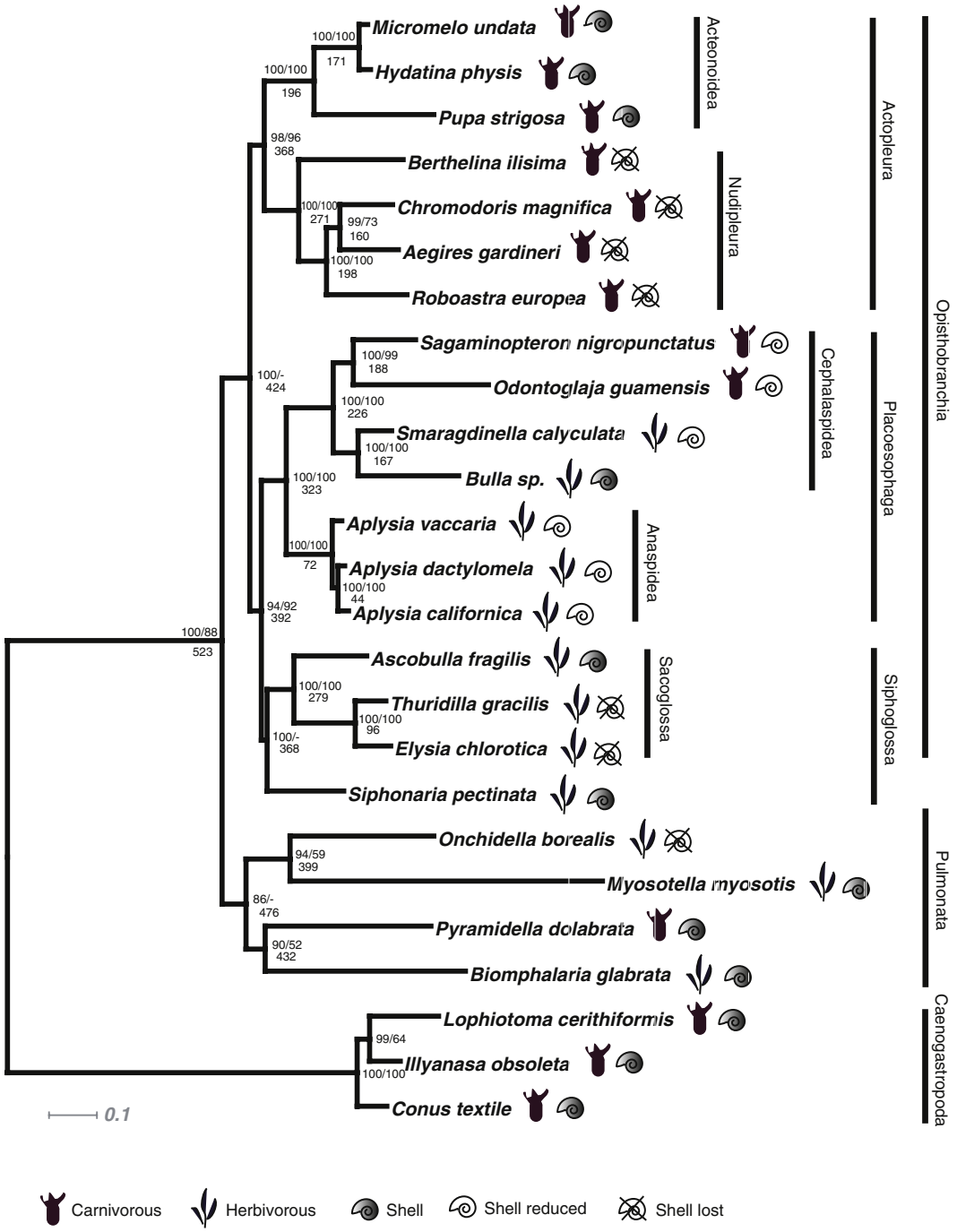


**Fig. 1.** Phylogenetic reconstruction of opisthobranch gastropods based on complete mitochondrial genomes. Bayesian posterior probabilities (left) and Maximum Likelihood boostrap (right) values are depicted above each branch. Divergence time estimates are depicted below each branch in millions of years. Evolution of both shell reduction/loss and feeding strategies are mapped onto the tree.

Klussmann-Kolb et al., 2008; Thollesson, 1999; Wagele and Klussmann-Kolb, 2005) have yielded trees with a paraphyletic or polyphyletic Opisthobranchia and a paraphyletic or polyphyletic Pulmonata. In the present study, Opisthobranchia is monophyletic, as long as the genus *Siphonaria*, traditionally regarded as a pulmonate, is included. *Siphonaria* has been classified within Pulmonata because of the presence of the so-called pulmonary cavity. However, the pulmonary cavity of *Siphonaria* is quite distinct from that of all pulmonates because its pneumostome (cavity opening) is not contractile (Dayrat and Tillier, 2002, 2003). Also, the roof of the "pulmonary" cavity of *Siphonaria* bears a gill which is anatomically very similar to that of opisthobranchs such as the shelled sacoglossans and cephalaspideans (Dayrat and Tillier, 2002, 2003). Therefore, the inclusion of *Siphonaria* within Opisthobranchia allows to reinterpret the "pulmonary" cavity of *Siphonaria* as a pallial cavity serving to exchange gases in water and, independently from the pulmonates, also evolved to serve as a lung. *Siphonaria* was in many ways an unusual pulmonate lineage but it now also is an unusual opisthobranch lineage: it is the only lineage of Opisthobranchia known to survive when not fully submerged being able to breathe air. Pulmonata also is monophyletic if *Siphonaria* is excluded. In our analysis, Opisthobranchia and Pulmonata are sister taxa to each other. Within the Opisthobranchia two major clades are recovered: the Acteonoidea

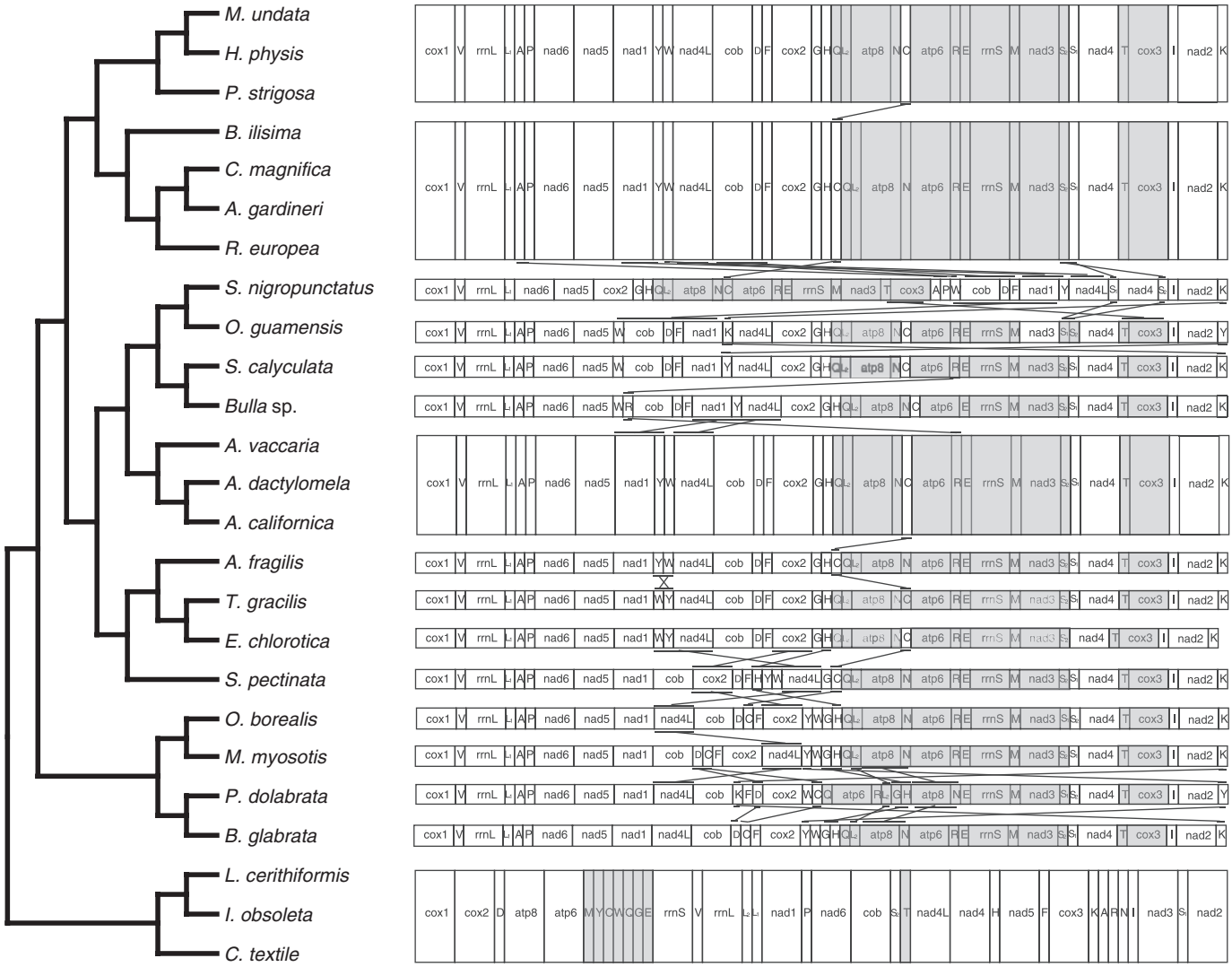


**Fig. 2.** Linearized mitochondrial gene order comparisons mapped onto the cladogram obtained after phylogenetic reconstruction. Shaded areas depict rearranged regions across genomes. Wide boxes depict protein encoding and ribosomal genes, thin boxes depict tRNAs. tRNA translocations are highlighted by lines in the interconnected genomes.

**Table 2**
Dates of divergence (in millions of years) from the Dinapoli and Klussmann-Kolb (Dinapoli and Klussmann-Kolb, 2010) and estimated in this study [Fossil dates are from Tracey et al. (1993), and Wenz and Zilch (1959–1960)].

| Taxon | Dinapoli and Klussmann-Kolb | This study | Oldest known fossil |
|---|---|---|---|
| Nudipleura | 131 | 271 | 26 |
| Acteonoidea | 210 | 196 | 196 |
| Nudibranchia | 66 | 160 | None |
| Actopleura | | 368 | 360 |
| Cephalaspidea | 49 | 226 | 208 |
| Aplustridae | | 167 | 208 |
| Anaspidea | | 72 | 55 |
| Sacoglossa | 85 | 279 | 22 |
| Siphonariidae | 22 | 282 | 161 |
| Siphoglossa | 106 | 368 | 161 |
| Euthyneura | 231 | 523 | 360 |
| Pulmonata | | 476 | 325 |
| Hydatinidae | 210 | 171 | 171 |
| Bullidae | | 167 | 167 |
| Heterobranchia | 390 | | 390 |
| Opisthobranchia | | 424 | 360 |
| Philinoidea | | 179 | 360 |
| Omalogyridae | 88 | | 88 |

plus Nudipleura (here called the Acteopleura) and the remainder of the opisthobranchs. The Acteopleura has been recovered in previous studies (Grande et al., 2004a; Klussmann-Kolb et al., 2008; Vonnemann et al., 2005). The remainder of opisthobrachs includes two major sister clades: the Placoesophaga (the gizzard-bearing opisthobranchs) and the Siphoglossa (Sacoglossa plus Siphonariidae). The Placoesophaga was also recovered (Dinapoli and Klussmann-Kolb, 2010) while the Siphoglossa has been previously documented (Klussmann-Kolb et al., 2008) but were not named as such. Rampant morphological homoplasy and highly derived mitochondrial genome features make opisthobranchs a suitable system to simultaneously examine evolutionary processes both at the anatomical and genomic levels.

### 4.2. Mitochondrial genome evolution

Although molluscs are known to have experienced major rearrangements throughout the evolution of their mitochondrial genome, this study is among the first to attempt examining this phenomenon in a large number of closely-related taxa. While most gastropods are quite stable in terms of mitochondrial gene order (Simison and Boore, 2008; Ponder and Lindberg, 2008), heterobranch

gastropods show significant rearrangements. We have been able to determine that gene rearrangements can be frequent in recently diverged taxa (Fig. 2). Within the Acteopleura, there is only one rearrangement in gene order found between the Acteonoidea and the Nudipleura. Within these clades, these taxa show great consistency in arrangement of mitochondrial genes in the taxa studied here. In striking contrast, the two species of carnivorous Cephalaspidea show multiple rearrangements, while the two herbivorous members exhibit only a single change. The rearrangements observed in these two genomes seem to have been caused by independent events. These differences in closely related taxa are suggestive of rapid evolutionary changes in some clades while others demonstrate remarkable stability. These differences do not appear to be related to clade age or to trophic diversification, as Nudipleura exhibit large differences in trophic diversification but show consistency in gene order. Other explanations need to be sought to determine the evolutionary basis for these disparate differences in gene order. We are still far from being able to understand and postulate all the possible molecular mechanisms that lead to such changes in the mitochondrial genome. Nevertheless, as shown in this study, we were able to identify the lineages that are more prone to suffer rearrangements. As new complete mitochondrial genomes become available we will find that even for slow evolving taxa, changes in gene order are not as rare as they initially appeared.

### 4.3. Divergence dates

A previous study by Dinapoli and Klussmann-Kolb (2010) estimated divergence dates for various euthyneuran and heterobranch lineages based on molecular clock data. These authors utilized three points to calibrate their data: the oldest known heterobranch (390 mya), the oldest known Acteonoidea (210 mya) and the oldest known Omalogyridae (88 mya). There is considerable debate as to what is the oldest fossil heterobranch. Some authors (Bandel and Heidelberger, 2002; Fryda and Blodgett, 2004) maintain that the earliest definitive heterobranchs are Devonian, while others (Hua-Zhang et al., 2003) suggest that some Early Triassic heterobranchs are intermediate between Paleozoic streptacidid heterobranchs and Mesozoic opisthobranchs. Presence of a heterostrophic protoconch is generally used to determine whether a gastropod is a member of the Heterobranchia or not. Unfortunately, the protoconch is often not well preserved or entirely eroded. Similarly, Dinapoli and Klussmann-Kolb (2010) use Omalogyridae as one of the nodes for calibration. Omalogryids are minute gastropods, generally around 1 mm in diameter when fully mature. While the oldest known fossil omalogyrids only extend back into the Santonian of the Late Cretaceous (Tracey et al., 1993), they are not the best candidates for good fossilization, owing to their minute size and thin calcification. If one is trying to find the oldest representative of a particular lineage and the diagnostic feature is often not preserved or the shells are minute and poorly calcified, it might be more prudent to use different taxa to calibrate the nodes of the phylogenetic tree. For this reason we chose to calibrate nodes for our clock data using Acteonidae, Aplustridae and Bullidae, that are larger, more heavily calcified and likely to be fossilized and be recognizable as having diagnostic features of these taxa.

There has also been considerable controversy in the paleontological literature as to the age of the oldest opisthobranch fossil. Some authors (Hua-Zhang et al., 2003) suggest that the oldest generally accepted opisthobranch fossil is *Cylindrobullina convexa* (Batten and Stokes, 1986) from the Moekopi formation in the early Triassic with an age of about 240 mya. However, others (Tracey et al., 1993) point to the Cydrobullinidae extending into the Tournaisian section of the Carboniferous at about 360 mya. Wenz and Zilch (1959–1960) also cite *Acteonina carbonaria* (de Koninck, 1843) from the Tournaisian as the oldest opisthobranch. Hua-Zhang et al. (2003) contended that *A. carbonaria* has an orthostrophic rather than heterostrophic protoconch and as such it is not an opisthobranch. Others (Fryda and Blodgett, 2004; Hua-Zhang et al., 2003; Hanger and Strong, 1998) have suggested Paleozoic taxa regarded as opisthobranchs and pulmonates should be regarded as caenogastropods, based on the form of the protoconch. Hua-Zhang et al. (2003) also stated that protoconch of *Acteonina permiana* (Hanger and Strong, 1998) from the early Permian (Wolfcampain–Leonardina) of Oregon is badly eroded and is ambiguous as to whether it is heterostrophic. However, Hanger and Strong (1998) described the protoconch as heterostrophic and a clearly heterostrophic protoconch is evident in their figures of the shell. The shell, with flat whorls and a narrow, elongate aperture clearly resembles modern specimens of the cephalaspidean *Acteocina* (Gosliner et al., 2008). *A. carbonaria*, as illustrated by several authors (Wenz and Zilch, 1959–1960; Knight, 1941), clearly resembles modern cephalaspideans, as well. This establishes the presence of Cephalaspidea into the Paleozoic in either the early Permian or likely into the early Carboniferous. These dates are also highly consistent with the molecular clock data presented here.

Dinapoli and Klussmann-Kolb (2010) provided molecular clock data divergent from ours and that of the oldest known euthyneuran fossils (Table 2). For example, their clock data (Dinapoli and Klussmann-Kolb, 2010) suggest that Siphonariidae originated 22 mya, although there are fossil siphonariids dating back to the Jurassic (161 mya), and that Cephalaspidea originated 49 mya. The present study suggests an age of 226 mya for the Cephalaspidea more in congruence with the oldest certain cephalaspidean fossil of 208 mya. As mentioned above, some possible reasons for these differences were (1) the choice of taxa that have poorer fossil records for calibration, and (2) the taxon sampling within clades not reflecting sufficient divergence.

The present molecular clock data were based on the presence of three fixed dates with relatively reliable fossil records (Acteonidae, Aplustridae and Bullidae). When we ground truth these data with other oldest known fossils there is fairly a good agreement with the clock data. For example, the oldest known fossil Acteopleuran (*A. carbonaria*) is 360 mya and our clock data suggest an age of 362 mya.

Our divergence estimate for the radiation of euthyneurans goes back to the Cambrian (523 mya) with the pulmonates diverging earlier (476 mya) than opisthobranchs (424 mya). These early dates are considerably earlier than the oldest known opisthobranch fossil, which dates back to the Carboniferous (360 mya). We also found earlier divergence estimates for recent branches (Table 2). A particularly interesting case is that of the Nudipleura since the oldest known fossil dates back only 26 mya yet the clock estimates place the origin of this clade back to the Paleozoic (late Carboniferous or early Permian, 271 mya). Nudibranchs, which entirely lack shells and have no known fossil record, likely extend back to the Jurassic (160 mya). Similarly, the Sacoglossa, which has a few shelled representatives and many shell-less representatives, has its oldest fossil in the Miocene (22 mya). Based on our clock estimates, however, this lineage extends back into the Permian (279 mya). Thus taxa with reduced shells or those that entirely lack shells appear to be much older than previously indicated by the fossil record.

Others (Hua-Zhang et al., 2003) have noted that the opisthobranchs may have been present, but rare in the late Paleozoic, but were certainly common in the early Triassic. Fryda et al. (2008) state that opisthobranchs and pulmonates are not known from the Paleozoic. Dinapoli and Klussmann-Kolb (2010) suggest that euthyneurans originated between the mid-Carboniferous and early Triassic based on their clock data, while our data suggest an even earlier origin. The clock estimates presented in this study, together with the strong conchological similarity of Paleozoic fossils to modern opisthobranchs, strengthen the likely Paleozoic origins of opisthobranchs and pulmonates.

Based on our molecular estimates (Fig. 1; Table 2) it appears that pulmonates and opisthobranchs, as well as other heterobranchs, were present in the Paleozoic and survived the Permian-Triassic extinction (251.4 mya). Taxa such as the Nudipleura and Sacoglossa may have also

originated prior to this mass extinction, and some taxa such as the Cephalaspidea and other Placoesophaga began radiating following the extinction. As a result this extinction, which may have eliminated half of the major groups of animals, must have created new opportunities for euthyneuran diversification. Under this scenario, this event would likely account for the period when most modern opisthobranchs were able to embark on the trophic diversification now evident in all modern lineages.

### 4.4. Evolution of shell loss

As suggested by morphological and partial gene phylogenies, we find strong support for multiple homoplastic events leading to the reduction, internalization or loss of shell in different lineages of adult opisthobranchs (Fig. 1). Calculation of the number of independent evolutionary events of shell reduction and loss and is highly dependent on taxon sampling. In the present study, shell reduction occurs independently at least four times, while shell loss occurs at least three times. Due to the limited taxon sampling in our study these numbers represent only a conservative approximation of these events in the Opisthobranchia and Pulmonata. The addition of other taxa with reduced shells or those that lack adult shells will provide a better approximation of the number of independent evolutionary events. Wagele and Klussmann-Kolb (2005), with a larger taxon sampling, and based on 18S and 28S nuclear sequences, provided evidence for at least three instances of shell reduction and six instances of shell loss in the Euthyneura. However, the full extent of this evolutionary trend in euthyneurans still requires additional molecular phylogenetic study.

### 4.5. Evolution of feeding strategies

The present taxon sampling does not allow us to say much about the plesiomorphic feeding state of the Euthyneura as only carnivorous caenogastropod outgroup taxa were included in the present analysis. To better answer the question of the plesiomorphic feeding arrangement in euthyneurans, a broad sampling of both carnivorous and herbivorous caenogastropod and heterobranch gastropods needs to be undertaken.

Regardless of the plesiomorphic arrangement within the Euthyneura, several major trophic patterns are present within the group. The members of the Actopleura are exclusively carnivorous. Within this group, broader taxon sampling is required to further examine the evolution of trophic specialization with the Nudipleura, in particular, which are known for a great deal of trophic diversity within and between subclades. The Siphoglossa and Anaspidea are exclusively herbivorous, but as in the case of the Nudipleura, the sacoglossans exhibit a great deal of trophic specialization and are worthy of in depth study. The Cephalaspidea are most interesting in that there are two distinct subclades, one of which is herbivorous and the other that is carnivorous and recent analyses suggest that herbivory was the plesiomorphic state within this lineage (Malaquias et al., 2009b). Within the Pulmonata, the parasitic pyramidellids are shown to be carnivorous. There are other representatives of the Pulmonata that are predatory and their inclusion in future analyses will shed light on whether carnivory in pulmonates represents a single evolutionary event or represents more than one event. The preliminary results shown here suggest distinct patterns of trophic radiation within clades and demonstrate that future analyses directed to explore trophic specializations within clades would be a fruitful focus for future research.

## 5. Conclusion

Mitogenomic approaches are greatly enhancing our ability to reconstruct phylogenies and provide robust patterns of relationship within taxa that have remained difficult to study phylogenetically. We can now proceed to address elusive questions in opisthobranch evolutionary biology. As mitochondrial genome sequencing costs continue to drop through next-gen technologies, it will be easier and more cost-effective to increase taxon sampling and therefore strength to our current phylogenies. We will soon be able to tackle more precisely the link between morphological homoplasy and evolutionary history within not only Opisthobranchia but also within any specific internal lineage of interest. More exciting is the fact that there is an increase in new tools of study for euthyneuran species (i.e. *Aplysia californica,* and *Biomphalaria glabrata*) such as whole genomes, which will allow us to examine the molecular underpinnings of the phenotypic and genomic traits that have made sea slugs both fascinating and difficult to study.

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## Appendix A. Universal primers used in this study

| Gene | Primer | Sequence | Reference |
|---|---|---|---|
| *rrnS* | 12s380H | RAGAGTGACGGGCGATTTGTRCAC | This publication |
| | 12s424H | TTCARBTACYYCTACTWTGTTACGACTTATC | |
| | 12s62L | YTMAAACTYAAANARCTAGGCGG | |
| | 12saiL | AAACTAGGATTAGATACCCTATTAT | Palumbi et al. (1991) |
| *rrnL* | 16s1148H | ATTAYGCTACCTTWGCACRGTCARRRTACYGCGG | Boore et al. (2005) |
| | 16s1148L | CCGCRGTAYYYTGACYGTGCBAAGGTAGCRTAAT | |
| | 16s1620H | TACATGATCTGAGTTCAGACCGGCGYRAGCCAGG | This publication |
| | 16s1620L | TACATGATCTGAGTTCAGACCGGCGYRAGCCAGG | |
| *cob* | CobF | GGWTAYGTWYTWCCWTGRGGWCARAT | Boore and Brown (2000) |
| | CobR | GCRTAWGCRAAWARRAARTAYCAYTCWGG | |
| *cox3* | Cox3F | TGGTGGCGAGATGTKKTNCGNGA | |
| | Cox3R | ACWACGTCKACGAAGTGTCARTATCA | |

## Appendix B. Species specific primers

| | |
|---|---|
| Berth2947F | AGAAAATACGACAAACTAATCCTGCTGAG |
| Berth4568R | CCCAAGACACAACCCGACTAATACCACTG |
| Bulla1038F | TAAACGGACTAAATCAAAGAAGAAAGC |
| Bulla1880R | AGTGGAAAGCAAAATAAAGAAAACAG |
| Bulla656F | ATAGAAACTGACCTGGCTTGC |
| Bulla831R | GAGTTGAATTGGGCTGTAGAG |
| Chrom3021F | TGTCCATACCGTTTCTAATCGTTGAGC |
| Chrom4728R | TTTTGGAGAAGATAATGTGTAGATGAG |
| Chrom4097R | TAATTTTCCCACCTAAGCACTATGACT AA |
| Hyda417F | CCCTTTTGTTGGTTTTCTCTGTTGGTAG |
| Hyda1685R | AGTTTGTTTCTTCTTCGCTTTCTTTTGA |
| OnchB4155R | CTCAAAATCTACAAATACAAATACAACC |
| OnchB2871F | AAATGAATCAAGGAACGAAAAGTCTACA |
| Odonto289F | AAAAACCCAACCAGATACTAATACCAAGAG |
| Odonto2103R | AAA ACTAAAAAACACATACAACAAGCCAGAT |
| Smarg823F | TGTTACAACGAGAGTGACGGGCGATTT |
| Smarg1305R | CGGACAACAACCACTATTATCACCAGT |
| Thu1929F | ACCCTAGGGATAACAGCGTAAT |
| Thur2707R | TCATAACCCAACAATAGAGGAACAA |

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