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# Towards a molecular phylogeny of Mollusks: Bivalves' early evolution as revealed by mitochondrial genes

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ABSTRACT

Despite huge fossil, morphological and molecular data, bivalves' early evolutionary history is still a matter of debate: recently, established phylogeny has been mostly challenged by DNA studies, and little agreement has been reached in literature, because of a substantial lack of widely-accepted methodological approaches to retrieve and analyze bivalves' molecular data. Here we present a molecular phylogeny of the class based on four mitochondrial genes (*12s*, *16s*, *cox1*, *cytb*) and a methodological pipeline that proved to be useful to obtain robust results. Actually, best-performing taxon sampling and alignment strategies were tested, and several data partitioning and molecular evolution models were analyzed, thus demonstrating the utility of Bayesian inference and the importance of molding and implementing non-trivial evolutionary models. Therefore, our analysis allowed to target many taxonomic questions of Bivalvia, and to obtain a complete time calibration of the tree depicting bivalves' earlier natural history main events, which mostly dated in the late Cambrian.



## 1. Introduction

Bivalves are among the most common organisms in marine and freshwater environments, summing up to about 8000 species (Morton, 1996). They are characterized by a bivalve shell, filtrating gills called ctenidia, and no differentiated head and radula. Most bivalves are filter-feeders and burrowers or rock-borers, but swimming or even active predation are also found (Dreyer et al., 2003). Most commonly, they breed by releasing gametes into the water column, but some exceptions are known, including brooding (Ó Foighil and Taylor, 2000). Free-swimming planktonic larvae (veligers), contributing to species dispersion, are typically found, which eventually metamorphose to benthonic sub-adults.

Bivalve taxonomy and phylogeny are long-debated issues, and a complete agreement has not been reached yet, even if this class is well known and huge fossil records are available. In fact, bivalves' considerable morphological dataset has neither led to a stable phylogeny, nor to a truly widely accepted higher-level taxonomy. As soon as they became available, molecular data gave significant contributions to bivalve taxonomy and phylogenetics, but little consensus has been reached in literature because of a substantial lack of shared methodological approaches to retrieve and analyze bivalves' molecular data. Moreover, to improve bivalves' phylogenetics, several attempts to join morphology and molecules have also been proposed (Giribet and Wheeler, 2002; Giribet and Distel, 2003; Harper et al., 2006; Mikkelsen et al., 2006; Olu-Le Roy et al., 2007), since, according to Giribet and Distel (2003), morphology resolves deeper nodes better than molecules, whereas sequence data are more adequate for recent splits.

Bivalves are generally divided into five extant subclasses, which were mainly established on body and shell morphology, namely Protobranchia, Palaeoheterodonta, Pteriomorphia, Heterodonta and Anomalodesmata (Millard, 2001; but see e.g., Vokes, 1980, for a slightly different taxonomy). In more detail, there is a general agreement that Protobranchia is the first emerging lineage of Bivalvia. All feasible relationships among Protobranchia superfamilies (Solemyoidea, Nuculoidea and Nuculanoidea) have been proposed on morphological approaches (Purchon, 1987b; Waller, 1990; Morton, 1996; Salvini-Plawen and Steiner, 1996; Cope, 1997; Waller, 1998), albeit some recent molecular findings eventually led to reject the monophyly of the whole subclass: while Solemyoidea and Nuculoidea do maintain their basal position, thus representing Protobranchia *sensu stricto*, Nuculanoidea are better considered closer to Pteriomorphia, placed in their own order Nuculanoida (Giribet and Wheeler, 2002; Giribet and Distel, 2003; Kappner and Bieler, 2006).

The second subclass, Palaeoheterodonta (freshwater mussels), has been considered either among the most basal (Cope, 1996) or the most derived groups (Morton, 1996). Recent molecular analyses confirm its monophyly (Giribet and Wheeler, 2002) and tend to support it as basal to other Autolamellibranchiata bivalves (Graf and Ó Foighil, 2000; Giribet and Distel, 2003).

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Mussels, scallops, oysters and arks are representatives of the species-rich subclass Pteriomorphia. In literature, this subclass has been resolved as a clade within all Eulamellibranchiata (Purchon, 1987b), as a sister group of Trigonioidea (Salvini-Plawen and Steiner, 1996), of Heterodonta (Cope, 1997), of (Heterodonta + Palaeoheterodonta) (Waller, 1990, 1998), or as a paraphyletic group to Palaeoheterodonta (Morton, 1996). Moreover, some authors hypothesize its polyphyly (Carter, 1990; Starobogatov, 1992), while others claimed that a general agreement on Pteriomorphia monophyly is emerging from molecular studies (Giribet and Distel, 2003). Such an evident lack of agreement appears to be largely due to an ancient polytomy often recovered for this group, especially in molecular analyses, which is probably the result of a rapid radiation event in its early evolution (Campbell, 2000; Steiner and Hammer, 2000; Matsumoto, 2003).

Heterodonta is the widest and most biodiversity-rich subclass, including some economically important bivalves (f.i., venerid clams). This subclass has been proposed as monophyletic (Purchon, 1987b; Carter, 1990; Starobogatov, 1992; Cope, 1996, 1997; Waller, 1990, 1998), or paraphyletic (Morton, 1996; Salvini-Plawen and Steiner, 1996), but it seems there is a growing agreement on its monophyly. At a lower taxonomic level, doubts on the taxonomic validity of its major orders, such as Myoida and Veneroida, are fully legitimate, and, in many cases, recent molecular analyses led to throughout taxonomic revisions (Maruyama et al., 1998; Williams et al., 2004; Taylor et al., 2007a).

Little agreement has been reached in literature on Anomalodesmata: this subclass shows a highly derived body plan, as they are septibranchiate and some of them are also carnivore, features that possibly evolved many times (Dreyer et al., 2003). Anomalodesmata were considered as sister group of Myoida (Morton, 1996; Salvini-Plawen and Steiner, 1996), Mytiloidea (Carter, 1990), Palaeoheterodonta (Cope, 1997), or Heterodonta (Waller, 1990, 1998); alternatively, Purchon (1987b) states that they represent a monophyletic clade nested in a wide polytomy of all Bivalvia. Anomalodesmata were also considered as basal to all Autolamellibranchiata (e.g., Starobogatov, 1992). Whereas the monophyletic status of Anomalodesmata seems unquestionable on molecular data (Dreyer et al., 2003), some authors proposed that this clade should be nested within heterodonts (Giribet and Wheeler, 2002; Giribet and Distel, 2003; Bieler and Mikkelsen, 2006; Harper et al., 2006).

Molecular analyses gave clearer results at lower taxonomic levels, so that this kind of literature is more abundant: for instance, key papers have been published on Ostreidae (Littlewood, 1994; Jozefowicz and Ó Foighil, 1998; Ó Foighil and Taylor, 2000; Kirkendale et al., 2004; Shilts et al., 2007), Pectinidae (Puslednik and Serb, 2008), Cardiidae (Maruyama et al., 1998; Schneider and Ó Foighil, 1999) or former Lucinoidea group (Williams et al., 2004; Taylor et al., 2007b).

In this study, we especially address bivalves' ancient phylogenetic events by using mitochondrial molecular markers, namely the *12s*, *16s*, cytochrome b (*cytb*) and cytochrome oxidase subunit 1 (*cox1*) genes. We chose mitochondrial markers since they have the great advantage to avoid problems related to multiple-copy nuclear genes (i.e. concerted evolution, Plohl et al., 2008), they have been proved to be useful at various phylogenetic levels, and, although this is not always true for bivalves, they largely experience Strict Maternal Inheritance (SMI; Gillham, 1994; Birky, 2001).

Actually, some bivalve species show an unusual mtDNA inheritance known as Doubly Uniparental Inheritance (DUI; see Breton et al., 2007; Passamonti and Ghiselli, 2009; for reviews): DUI species do have two mitochondrial DNAs, one called F as it is transmitted through eggs, the other called M, transmitted through sperm and found almost only in males' gonads. The F mtDNA is passed from mothers to complete offspring, whereas the M mtDNA is passed from fathers to sons only. Obviously, DUI sex-linked mtDNAs may result in incorrect clustering, so their possible presence must be properly taken into account. DUI has a scattered occurrence among bivalves and, until today, it has been found in species from seven families of three subclasses: palaeoheterodonts (Unionidae, Hyriidae, and Margaritiferidae), pteriomorphians (Mytilidae), and heterodonts (Donacidae, Solenidae, and Veneridae) (Theologidis et al., 2008; Fig. 2 and reference therein). In some cases, co-specific F and M mtDNAs do cluster together, and this will not significantly affect phylogeny at the level of this study: this happens, among others, for *Donax trunculus* (Theologidis et al., 2008) and *Venerupis philippinarum* (Passamonti et al., 2003). In others cases, however, F and M mtDNAs cluster separately, and this might possibly result in an incorrect topology: f.i. this happens for the family of Unionidae and for *Mytilus* (Theologidis et al., 2008). All that considered, bivalves' mtDNA sequences should not be compared unless they are surely homolog, and the possible presence of two organelle genomes is an issue to be carefully evaluated (see Section 2.1, for further details). On the other hand, we still decided to avoid nuclear markers for two main reasons: (i) largely used nuclear genes, like 18S rDNA, are not single-copy genes and have been seriously questioned for inferences about bivalve evolution (Littlewood, 1994; Steiner and Müller, 1996; Winnepenninckx et al., 1996; Adamkewicz et al., 1997; Steiner, 1999; Distel, 2000; Passamaneck et al., 2004); (ii) data on putative single-copy nuclear markers, like β-actin or *hsp70*, lack for the class, essentially because primers often fail to amplify target sequences in Bivalvia (pers. obs.).

## 2. Materials and methods

### 2.1. Specimens' collection and DNA extraction

Species name and sampling locality are given in Table 1. Animals were either frozen or ethanol-preserved until extraction. Total genomic DNA was extracted by DNeasy® Blood and Tissue Kit (Qiagen, Valencia, CA, USA), following manufacturer's instructions. Samples were incubated overnight at 56 °C to improve tissues' lysis. Total genomic DNA was stored at −20 °C in 200 μL AE Buffer, provided with the kit.

DUI species are still being discovered among bivalves; nevertheless, as mentioned, a phylogenetic analysis needs comparisons between orthologous sequences, and M- or F-type genes under DUI are not. On the other hand, F-type mtDNA for DUI species and mtDNA of non-DUI species are orthologous sequences. As M-type is present mainly in sperm, we avoided sexually-mature individuals and, when possible (i.e., when the specimen was not too tiny), we did not extract DNA from gonads. If possible, DNA was obtained from foot muscle, which, among somatic tissues, carries very little M-type mtDNA in DUI species (Garrido-Ramos et al., 1998), thus reducing the possibility of spurious amplifications of the M genome. Moreover, when downloading sequences from GenBank, we paid attention in retrieving female specimen data only, whenever this information was available.

### 2.2. PCR Amplification, cloning, and sequencing

PCR amplifications were carried out in a 50 μL volume, as follows: 5 or 10 μL reaction buffer, 150 nmol $MgCl_2$, 10 nmol each dNTP, 25 pmol each primer, 1–5 μL genomic DNA, 1.25 units of DNA Polymerase (Invitrogen, Carlsbad, CA, USA or ProMega, Madison, WI, USA), water up to 50 μL. PCR conditions and cycles are listed in Appendix A1; primers used for this study are listed in Appendix A2. PCR results were visualized onto a 1–2% electrophoresis agarose gel stained with ethidium bromide and purified through Wizard® SV