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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

Table 1
Specimens used for this study, with sampling locality and taxonomy following Millard (2001). Only species whose sequences were obtained in our laboratory are shown.

Subclass	Order	Suborder	Superfamily	Family	Subfamily	Species	Provenience
Anomalodesmata	Pholadomyoida	Cuspidariina		Cuspidariidae		*Cuspidaria rostrata*	Malta
		Pholadomyina	Pandoroidea	Pandoridae		*Pandora pinna*	Trieste, Italy
				Thraciidae		*Thracia distorta*	Secche di Tor Paterno, Italy
Heterodonta	Chamida		Astartoidea	Astartidae	Astartinae	*Astarte* cfr. *castanea*	Woods Hole, MA, USA
			Mactroidea	Mactridae	Mactrinae	*Mactra corallina*	Cesenatico, Italy
						Mactra lignaria	Cesenatico, Italy
			Tellinoidea	Pharidae	Cultellinae	*Ensis directus*	Woods Hole, MA, USA
			Tridacnoidea	Tridacnidae		*Tridacna derasa*	Commercially purchased
						Tridacna squamosa	Commercially purchased
	Myida	Myina	Myoidea	Myidae	Myinae	*Mya arenaria*	Woods Hole, MA, USA
	Veneroida		Carditoidea	Carditidae	Carditinae	*Cardita variegata*	Nosi Bè, Madagascar
			Veneroidea	Veneridae	Gafrarinae	*Gafrarium alfredense*	Nosi Bè, Madagascar
					Gemminae	*Gemma gemma*	Woods Hole, MA, USA
Palaeheterodonta	Unionida		Unionoidea	Unionidae	Anodontinae	*Anodonta woodiana*	Po River delta, Italy
Protobranchia	Nuculoida		Nuculanoidea	Nuculanidae	Nuculaninae	*Nuculana commutata*	Malta
			Nuculoidea	Nuculidae		*Nucula nucleus*	Goro, Italy
Pteriomorphia	Arcida	Arcina	Arcoidea	Arcidae	Anadarinae	*Anadara ovalis*	Woods Hole, MA, USA
					Arcinae	*Barbatia parva*	Nosi Bè, Madagascar
						Barbatia reeveana	Galápagos Islands, Ecuador
						Barbatia cfr. *setigera*	Nosi Bè, Madagascar
	Limida		Limoidea	Limidae		*Lima pacifica galapagensis*	Galápagos Islands, Ecuador
	Ostreoida	Ostreina	Ostreoidea	Ostreidae	Pycnodonteinae	*Hyotissa hyotis*	Nosi Bè, Madagascar
		Pectinina	Anomioidea	Anomiidae		*Anomia sp.*	Woods Hole, MA, USA
			Pectinoidea	Pectinidae	Chlamydinae	*Argopecten irradians*	Woods Hole, MA, USA
						Chlamys livida	Nosi Bè, Madagascar
						Chlamys multistriata	Krk, Croatia
					Pectininae	*Pecten jacobaeus*	Montecristo Island, Italy
	Pteriida	Pinnina	Pinnoidea	Pinnidae		*Pinna muricata*	Nosi Bè, Madagascar

Gel and PCR Clean-Up System (ProMega, Madison, WI, USA), following manufacturer's instructions.

Sometimes, amplicons were not suitable for direct sequencing; thus, PCR products were inserted into a pGEM®-T Easy Vector (ProMega, Madison, WI, USA) and transformed into Max Efficiency® DH5α™ Competent Cells (Invitrogen, Carlsbad, CA, USA). Positive clones were PCR-screened with M13 primers (see Appendix A2) and visualized onto a 1–2% electrophoresis agarose gel. However, as far as possible, we only cloned whenever it was strictly necessary; actually, as in DUI species some "leakage" of M mitotype may occur in somatic tissues of males, sensible cloning procedures could sometimes amplify such rare variants. Suitable amplicons and amplified clones were sequenced through either GeneLab (ENEA-Casaccia, Rome, Italy) or Macrogen (World Meridian Center, Seoul, South Korea) facilities.

2.3. Sequence alignment

Electropherograms were visualized by Sequence Navigator (Parker, 1997) and MEGA4 (Tamura et al., 2007) softwares. Sequences were compared to those available in GenBank through BLAST 2.2.19+ search tool (Altschul et al., 1997). Four outgroups were used for this study: the polyplacophoran *Katharina tunicata*, the scaphopod *Graptacme eborea* and two gastropods, *Haliotis rubra* and *Thais clavigera*. Appendix A3 lists all DNA sequences used for this study, along with their GenBank accession number.

Alignments were edited by MEGA4 and a concatenated data set was produced; whenever only three sequences out of four were known, the fourth was coded as a stretch of missing data, since the presence of missing data does not lead to an incorrect phylogeny by itself, given a correct phylogenetic approach (as long as sufficient data are available for the analysis; see Hartmann and Vision, 2008; and reference therein). In other cases, there were not sufficient published sequences for a given species to be included in our concatenated alignment; nevertheless, we could add the genus itself by concatenating DNA sequences from different co-generic species, as this approach was already taken in other phylogenetic studies (see, f.i., Li et al., 2009). This was the case for *Donax*, *Solemya*, *Spisula*, and *Spondylus* (see Appendix A3 for details). Given the broad range of the analysis, which targets whole class phylogeny above the genus level, we do not think that such an approximation significantly biased our results. In any case, phylogenetic positions of such genera were taken with extreme care.

Sequences were aligned with ClustalW (Thompson et al., 1994) implemented in MEGA4. Gap opening and extension costs were set to 50/10 and 20/4 for protein- and ribosomal-coding genes, respectively. Because of the high evolutionary distance of the analyzed taxa, sequences showed high variability, and the problem was especially evident for ribosomal genes, where different selective pressures are active on different regions. These genes showed a lot of indels, which were strikingly unstable across alignment parameters; thus, we could not resolve alignment ambiguities in an objective way. The method proposed by Lutzoni et al. (2000), though very appealing, is problematic for big data sets with high variability, as shown by the authors themselves. On the other side, likelihood analyses are also problematic with the fixed character state method proposed by Wheeler (1999). Elision, as introduced by Wheeler et al. (1995), is a possibility that does not involve particular methods of phylogenetic analyses, but only a "grand alignment". However, variability in our ribosomal data set was so high that alignments with different parameters were almost completely different; thus, elision generated only more phylogenetic noise, whereas the original method by Gatesy et al. (1993) was not conceivable because alignment-invariant positions were less than twenty. All that considered, we preferred to use a user-assisted standard alignment method (i.e., ClustalW) since we think this is yet the best alignment strategy for such a complex dataset. Alignment was also visually inspected searching for misaligned sites and ambiguities, and where manual optimization was not possible, alignment-ambiguous regions were excluded from the analysis. Indels were treated as a whole and converted to presence/absence data to avoid many theoretical concerns on alignments (simple indel coding; see Simmons and Ochoterena, 2000, for more details). In fact, ambiguities in alignments are mainly due to indel

insertions; therefore, this technique also eliminates a large part of phylogenetic noise. We then coded indels following the rules given by Simmons and Ochoterena (2000), as implemented by the software GapCoder (Young and Healy, 2003), which considers each indel as a whole, and codes it at the end of the nucleotide matrix as presence/absence (i.e. 1/0). Possibly, a longer indel may completely overlap another across two sequences; in such cases, it is impossible to decide whether the shorter indel is present or not in the sequence presenting the longer one. Therefore, the shorter indel is coded among missing data in that sequence. Data set was then analyzed treating gaps as missing data and presence/absence data of indel events as normal binary data.

2.4. Phylogenetic analyses

A preliminary test was made on saturation: transition and transversion uncorrected *p*-distances were plotted on global pairwise *p*-distances, as computed with PAUP* 4.0b10 (pairwise deletion of gaps; Swofford, 1999); the test was repeated on third positions only for protein-coding genes. Linear regression and its significance were tested with PaSt 1.90 (Hammer et al., 2001).

Partitioning schemes used in this study are 10, based on 26 different partitions (Supplementary Materials Fig. 1), although they are not all the conceivable ones; we describe our 10 partitioning patterns in Table 2. The Bayesian Information Criterion (BIC) implemented in ModelTest 3.7 (Posada and Crandall, 1998) was used to select the best-fitting models; the graphical interface provided by MrMTgui was used (Nuin, 2008). As MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) currently implements only models with 1, 2 or 6 substitutions, a GTR + I + Γ model (Tavaré, 1986) was chosen for all partitions. ModelTest rejected the presence of a significant proportion of invariable sites in three cases only; GTR + Γ were selected for *cox1* third positions and for *cytb* second and third positions.

Maximum Likelihood was carried out with PAUP* software at the University of Oslo BioPortal (<http://www.bioportal.uio.no>). Gap characters were treated as missing data and the concatenated alignment was not partitioned. Nucleotides frequencies, substitution rates, gamma shape parameter and proportion of invariable sites were set according to ModelTest results on global alignment. Outgroups were set to be paraphyletic to the monophyletic ingroup. Bootstrap with 100 replicates, using full heuristic ML searches with stepwise additions and TBR branch swapping, was performed to assess nodal support.

Machine time is a key issue in Maximum Likelihood, and, unfortunately, a parallel version of PAUP* has not been published yet. To speed up the process, we used a slightly restricted dataset and set up the analysis to simulate a parallel computation, therefore taking higher advantage of the large computational power of the BioPortal. We run 10 independent bootstrap resamplings with 10 replicates each, starting with different random seeds generated by Microsoft Excel® 2007 following PAUP* recommendations. Trees found in each run were then merged and final consensus was computed with PAUP*. A comparative analysis on a smaller but still representative dataset showed, as expected, that this strategy does not affect the topology of the tree, nor significantly changes bootstrap values (data not shown).

Although less intuitive than in the case of parsimony (Baker and DeSalle, 1997), a Partitioned Likelihood Support (PLS) can be computed for likelihood analyses (Lee and Hugall, 2003). We chose this kind of analysis because other methods (Templeton, 1983; Larson, 1994; Farris et al., 1995a, 1995b) measure overall levels of agreement between partitions in the data set, but they cannot show which parts of a tree are in conflict among partitions (Wiens, 1998; Lambkin et al., 2002). A positive PLS indicates that a partition supports a given clade, and a negative PLS indicates that the partition contradicts the clade itself. Parametric bootstrapping (Huelsenbeck et al., 1996a; Huelsenbeck et al., 1996b) and Shimodaira–Hasegawa test (Shimodaira and Hasegawa, 1999) can assess the statistical significance of PLS results (Goldman et al., 2000; Lee and Hugall, 2003; and reference therein). However, PLS analyses are currently difficult because no widely available phylogenetic software implement such an algorithm. Therefore, Partitioned Likelihood Support (PLS) was evaluated following the manual procedure described in Lee and Hugall (2003). TreeRot 3.0 (Sorenson and Franzosa, 2007) was used to produce PAUP* command file, whereas individual-site log-likelihood scores were analyzed by Microsoft Excel® 2007. Shimodaira–Hasegawa test was employed to assess confidence in PLS, following Shimodaira and Hasegawa (1999). VBA macros implemented in Microsoft Excel® 2007 to perform PLS and Shimodaira–Hasegawa analyses are available from F. P.

MrBayes 3.1.2 software was used for Bayesian analyses, which were carried out at the BioPortal (see above). We performed a Bayesian analysis for each partitioning scheme. Except as stated elsewhere, two MC^3 algorithm runs with four chains were run for 10,000,000 generations; convergence was estimated through PSRF (Gelman and Rubin, 1992) and by plotting standard deviation of average split frequencies sampled every 1000 generations. The four outgroups were constrained, trees found at convergence were retained after the burnin, and a majority-rule consensus tree was computed with the command **sumt**. Via the command **sump printtofile = yes** we could obtain the harmonic mean of the Estimated Marginal Likelihood (EML). EML was used to address model selection and partition choice.

Since there is no obvious way to define partitions in ribosomal-encoding genes and secondary structure-based alignments did not result in correct phylogenetic trees (data not shown; see also Steiner and Hammer, 2000), we first decided to test data partitioning schemes on protein-coding genes only. Therefore, after a global analysis merging all markers within the same set, we tested six different partitioning schemes for protein-coding genes, taking

Table 2
Partitioning schemes. See Supplementary Materials Fig. 1 for details on partitions.

Partitioning scheme	Number of partitions	Partitions (see fig. 1)
t01	2	all, all_indel
t02[a]	4	rib, rib_indel, prot, prot_indel
t03	5	rib, rib_indel, prot_12, prot_3, prot_indel
t04	6	rib, rib_indel, prot_1, prot_2, prot_3, prot_indel
t05	6	rib, rib_indel, cox1, cox1_indel, cytb, cytb_indel
t06	8	rib, rib_indel, cox1_12, cox1_3, cox1_indel, cytb_12, cytb_3, cytb_indel
t07	10	rib, rib_indel, cox1_1, cox1_2, cox1_3, cox1_indel, cytb, cytb_1, cytb_2, cytb_3, cytb_indel
t08	8	12s, 12s_indel, 16s, 16s_indel, prot_1, prot_2, prot_3, prot_indel
t09	12	12s, 12s_indel, 16s, 16s_indel, cox1_1, cox1_2, cox1_3, cox1_indel, cytb_1, cytb_2, cytb_3, cytb_indel
t10	4	cox1 (amminoacids), cox1_indel, cytb (amminoacids), cytb_indel

[a] *tNy98* and *tM3* were also based on this partitioning scheme.

ribosomal ones together (Table 2; *t02–t07*). As *t04* and *t07* were selected as the most suitable ones (see Section 3.5), we designed two more schemes splitting *12s* and *16s* based on these datasets only (Table 2; *t08–t09*). Finally, we tested some strategies to further remove phylogenetic noise: we first constructed an amminoacid dataset (Table 2; *t10*; we were forced to completely remove ribosomal genes, as MC^3 runs could not converge in this case). However, the use of amminoacids is not directly comparable with other datasets by AIC and BF, because it not only implies a different model, but also different starting data: as a consequence, we implemented the codon model (Goldman and Yang, 1994; Muse and Gaut, 1994) on the *prot* partition. This allowed us to start from an identical dataset, which makes results statistically comparable. As *t04* scheme turned out to be essentially comparable with *t09* (see Section 3.5), we did not implement codon model also on separate *cox1* and *cytb* genes, because codon model is computationally extremely demanding. Two separate analyses were performed under such a codon model: in both cases, metazoan mitochondrial genetic code table was used; in one case Ny98 model was enforced (*tNy98*; Nielsen and Yang, 1998), whereas in the other case M3 model was used (*tM3*). Only one run of 5000,000 generations was performed for codon models, sampling a tree every 125. Dealing with one-run analyses, codon models trees were also analytically tested for convergence via AWTY analyses (<http://king2.scs.fsu.edu/CEBProjects/awty/awty_start.php>; Nylander et al., 2008). Moreover, our analysis on codon models allowed us to test for positive selection on protein-coding genes (see Ballard and Whitlock, 2004): MrBayes estimates the ratio of the non-synonymous to the synonymous substitution rate (ω) and implements models to accommodate variation of ω across sites using three discrete categories (Ronquist et al., 2005).

Finally, to test for the best partitioning scheme and evolutionary model, we applied Akaike Information Criterion (AIC; Akaike, 1973) and Bayes Factors (BF; Kass and Raftery, 1995). AIC was calculated, following Huelsenbeck et al. (2004), Posada and Buckley (2004), and Strugnell et al. (2005), as

$$AIC = -2EML + 2K$$

The number of free parameters K was computed taking into account branch number, character (nucleotide, presence/absence of an indel, amminoacid, or codon and codon-related parameters) frequencies, substitution rates, gamma shape parameter and proportion of invariable sites for each partition.

Bayes Factors were calculated, following Brandley et al. (2005), as

$$B_{ij} = \frac{EML_i}{EML_j}$$

and, doubling and turning to natural logarithms

$$2\ln B_{ij} = 2(\ln EML_i - \ln EML_j)$$

where B_{ij} is the Bayes Factor measuring the strength of the ith hypothesis on the jth hypothesis. Bayes Factors were interpreted according to Kass and Raftery (1995) and Brandley et al. (2005).

All trees were graphically edited by PhyloWidget (Jordan and Piel, 2008) and Dendroscope (Huson et al., 2007) softwares. Published Maximum Likelihood and Bayesian trees, along with source data matrices, were deposited in TreeBASE under SN4787 and SN4789 Submission ID Numbers, respectively.

2.5. Taxon sampling

Taxon sampling is a crucial step in any phylogenetic analysis, and this is certainly true for bivalves (Giribet and Carranza, 1999; Puslednik and Serb, 2008). Actually, many authors claim for a bias in taxon sampling to explain some unexpected or unlikely results (Adamkewicz et al., 1997; Canapa et al., 1999; Campbell, 2000; Kappner and Bieler, 2006). As we want to find the best performing methodological pipeline for reconstructing bivalve phylogeny, we assessed taxon sampling following rigorous criteria, in order to avoid misleading results due to incorrect taxon choice. We approached this with both *a priori* and *a posteriori* perspectives, following two different (and complementary) rationales.

Quite often, taxa that are included in a phylogenetic analysis are not chosen following a formal criterion of representativeness: they are rather selected on accessibility and/or analyzer's personal choice. To avoid this, we developed a method to quantify sample representativeness with respect to the whole class. The method is based on Average Taxonomic Distinctness (AvTD) of Clarke and Warwick (1998). The mathematics of this method has been proposed in a different paper (Plazzi et al., 2010), but here we would like to mention the rationale behind it: estimating *a priori* the *phylogenetic representativeness* of a sample is not conceptually different from estimating its *taxonomic representativeness*, i.e. testing whether our taxon sampling is representative of a given master taxonomic list, which may eventually be retrieved from bibliography. This approach does not require any specific knowledge, other than the established taxonomy of the sampled taxa; neither sequence data, nor any kind of measure are used here, which means the AvTD approach comes *before* seeing the data. Our source of reference taxonomy (master list) was obtained from Millard (2001). The AvTD was then computed for our sample and confidence limits were computed on 1000 random resamplings of the same size from bivalve master list. If the taxon sample value is above the 95% lower confidence limit, then we can say that our dataset is representative of the whole group. We developed a software to compute this, which is available for download at <www.mozoolab.net>.

On the other hand, *after* seeing the data, we were interested in answering whether they were sufficient or not to accurately estimate phylogeny. For this purpose, we used the method proposed by Sullivan et al. (1999). The starting point is the tree obtained as the result of our analysis, given the correct model choice (see below). Several subtrees are obtained by pruning it without affecting branch lengths; each parameter is then estimated again from each subtree under the same model: if estimates, as size increases, converge to the values computed from the complete tree, then taxon sampling is sufficiently large to unveil optimal values of molecular parameters, such as evolutionary rates, proportion of invariable sites, and so on (Townsend, 2007). At first, we checked whether MC^3 Bayesian estimates of best model were comparable to Maximum Likelihood ones computed through ModelTest. We took into consideration all 6 mutations rates and, where present, nucleotide frequencies, invariable sites proportion and gamma-shaping parameter (which are not used into M3 codon model). In most cases (see Supplementary Materials Table 1) the Maximum Likelihood estimate fell within the 95% confidence interval as computed following Bayesian Analysis and, if not, the difference was always (except in one case) of 10^{-2} or less order of magnitude. Therefore, we used Bayesian estimates of mean and confidence interval limits instead of bootstrapping Maximum Likelihood, as in the original method of Sullivan et al. (1999). Fifty subtrees were manually generated from best tree by pruning a number of branches ranging from 1 to 50. Following Authors' suggestions, we used different pruning strategies: in some cases, we left only species very close in the original tree, whereas in others we left species encompassing the whole biodiversity of the class (Appendix A4). Model parameters were then estimated from each subtree for each partition (*rib* and *prot*) using original sequence data and the best model chosen by ModelTest as above. The paupblock of ModelTest was used into PAUP* to implement such specific Maximum Likelihood analyses for each partition, model, and subtree.

2.6. Dating

The r8s 1.71 (Sanderson, 2003) software was used to date the best tree we obtained. Fossil collections of bivalves are very abundant, so we could test several calibration points in our tree, but in all cases the origin of Bivalvia was constrained between 530 and 520 million years ago (Mya; Brasier and Hewitt, 1978), and no other deep node was used for calibration, as we were interested in molecular dating of ancient splits. Data from several taxa were downloaded from the Paleobiology Database on 4 November, 2009, using group names given in Table 3 and leaving all parameters as default. Some nodes were fixed or constrained to the given age, whereas others were left free. After the analysis, we checked whether the software was able to predict correct ages or not, i.e. whether the calibration set was reliable. The tree was re-rooted with the sole *Katharina tunicata*; for this reason, two nodes "*Katharina tunicata*" and "other outgroups" are given in Table 3. Rates and times were estimated following both PL and NPRS methods, which yielded very similar results. In both cases we implemented the Powell's algorithm. Several rounds of fossil-based cross-validation analysis were used to determine the best-performing smoothing value for PL method and the penalty function was set to log. Four perturbations of the solutions and five multiple starts were invoked to optimize searching in both cases. Solutions were checked through the **checkGradient** command. NPRS method was also used to test variability among results. 150 bootstrap replicates of original dataset were generated by the SEQBOOT program in PHYLIP (Felsenstein, 1993) and branch lengths were computed with PAUP* through r8s-bootkit scripts of Torsten Eriksson (2007). A complete NPRS analysis was performed on each bootstrap replicate tree and results were finally profiled across all replicates through the r8s command **profile**.

3. Results

3.1. Obtained sequences

Mitochondrial sequences from partial ribosomal small (*12s*) and large (*16s*) subunit, cytochrome b (*cytb*) and cytochrome oxidase

Table 3
r8s datation of *tM3* tree. If a fossil datation is shown, the clade was used for calibrating the tree using Paleobiology Database data; in bold are shown the eight calibrations point of the best-performing set, whereas the others were used as controls. Constraints enforced are shown in the fourth and fifth column; if they are identical, that node was fixed. Ages are in millions of years (Myr); rates are in substitutions per year per site and refer to the branch leading to a given node. PL, Penalized Likelihood; NPRS, Non Parametric Rate Smoothing; StDev, Standard Deviation.

	Fossil datation	Reference[a]	Constraints		PL		NPRS			
			Min	Max	Age	Local rate	Age	Local rate	Mean	StDev
Katharina tunicata					627.58		625.44			
Other outgroups					561.45	1.65E−03	560.05	1.67E−03	533.95	2.67
Bivalvia	**530.0–520.0**	**5**	**520.00**	**530.00**	**529.99**	**3.46E−03**	**530.00**	**3.63E−03**	**530.00**	**0.00**
Autolamellibranchiata					520.32	2.01E−02	520.31	2.01E−02	517.04	1.70
Pteriomorphia + Heterodonta					513.59	2.26E−02	513.59	2.26E−02	508.51	1.74
Pteriomorphia					505.74	1.81E−02	505.82	1.83E−02	501.13	2.29
Heterodonta					497.83	1.51E−02	498.20	1.55E−02	490.24	3.11
Traditional Pteriomorphia					496.63	1.26E−02	496.13	1.19E−02	488.88	2.38
Hiatella + Cardiidae					481.34	1.10E−02	481.61	1.09E−02	476.05	3.65
Limidae + Pectinina					474.51	1.71E−02	474.82	1.78E−02	468.49	3.49
Veneroida sensu lato					471.38	3.80E−03	471.87	3.82E−03	471.22	6.63
Anomioidea + Pectinoidea					464.44	1.19E−02	464.92	1.21E−02	459.25	4.26
Protobranchia					454.28	1.34E−03	455.67	1.37E−03	482.02	14.61
Arcidae	**457.5–449.5**	**29**	**449.50**	**457.50**	**449.51**	**2.35E−02**	**449.50**	**2.38E−02**	**449.50**	**0.00**
Pectinoidea	428.2–426.2	21, 27, 30			431.77	1.27E−02	433.44	1.32E−02	417.82	4.20
Anomalodesmata					431.45	3.29E−03	434.04	3.40E−03	461.87	9.59
Cardiidae	**428.2–426.2**	**18**	**427.20**	**427.20**	**427.20**	**1.18E−02**	**427.20**	**1.18E−02**	**427.20**	**0.00**
Cuspidaria clade					418.58	4.87E−03	421.63	5.04E−03	477.22	9.28
Veneroida 2					407.08	3.58E−03	407.42	3.58E−03	410.56	9.26
Ostreoida + Pteriida					393.59	3.48E−03	395.13	3.55E−03	435.47	10.95
Pectinidae	**388.1–383.7**	**2, 6, 14, 22, 26**	**385.90**	**385.90**	**385.90**	**5.18E−03**	**385.90**	**5.00E−03**	**385.90**	**0.00**
Limidae	**376.1–360.7**	**1**	**360.70**	**376.10**	**360.74**	**4.66E−03**	**360.71**	**4.65E−03**	**370.13**	**6.31**
Veneridae	**360.7–345.3**	**19, 30**	**345.30**	**360.70**	**345.33**	**3.30E−03**	**345.31**	**3.28E−03**	**347.28**	**4.57**
Pectininae					324.88	1.57E−03	327.18	1.63E−03	342.84	7.76
Unionidae	245.0–228.0	8			293.93	3.68E−03	298.00	3.74E−03	347.74	20.25
Gafrarium + Gemma					282.57	2.24E−03	283.03	2.25E−03	280.55	22.38
Ostreoida	251.0–249.7	28			264.75	3.00E−03	266.21	3.00E−03	333.04	16.09
Mactrinae	196.5–189.6	25			243.80	2.27E−03	244.76	2.28E−03	261.16	21.60
Argopecten + Pecten					220.05	1.22E−03	222.43	1.22E−03	256.84	14.94
Unioninae	**228.0–216.5**	**9, 13, 16, 20, 23**	**216.50**	**228.00**	**216.53**	**1.71E−03**	**216.51**	**1.62E−03**	**227.86**	**0.93**
Chlamys livida + Mimachlamys					190.34	1.24E−03	194.24	1.27E−03	336.20	8.12
Ensis + Sinonovacula					189.33	1.16E−03	189.83	1.16E−03	305.30	18.57
Astarte + Cardita					188.86	3.26E−03	191.12	3.25E−03	274.37	23.58
Dreissena + Mya					185.03	2.62E−03	185.82	2.62E−03	224.89	19.55
Barbatia	**167.7–164.7**	**4, 10, 24**	**166.20**	**166.20**	**166.20**	**6.93E−04**	**166.20**	**6.93E−04**	**166.20**	**0.00**
Tridacna	23.0–16.0	17			147.15	1.26E−03	149.69	1.27E−03	383.21	11.43
Setigera + Reeveana					77.29	2.20E−03	75.19	2.15E−03	92.77	12.17
Crassostrea	145.5–130.0	15			63.17	3.08E−03	63.52	3.07E−03	92.38	10.04
Gigas + Hongkongensis					23.47	2.72E−03	23.65	2.71E−03	36.93	9.36
Mactra	196.5–189.6	25			21.63	1.50E−03	21.80	1.49E−03	31.48	6.91
Mytilus	418.7–418.1	3, 7, 11, 12			1.88	2.92E−03	1.77	2.92E−03	1.79	0.60

[a] References as follows: (1) Amler et al. (1990); (2) Baird and Brett (1983); (3) Berry and Boucot (1973); (4) Bigot (1935); (5) Brasier and Hewitt (1978); (6) Brett et al. (1991); (7) Cai et al. (1993); (8) Campbell et al. (2003); (9) Chatterjee (1986); (10) Cox (1965); (11) Dou and Sun (1983); (12) Dou and Sun (1985); (13) Elder (1987); (14) Grasso (1986); (15) Hayami (1975); (16) Heckert (2004); (17) Kemp (1976); (18) Kříž (1999); (19) Laudon (1931); (20) Lehman and Chatterjee (2005); (21) Manten (1971); (22) Mergl and Massa (1992); (23) Murry (1989); (24) Palmer (1979); (25) Poulton (1991); (26) Rode and Lieberman (2004); (27) Samtleben et al. (1996); (28) Spath (1930); (29) Suarez Soruco (1976); (30) Wagner (2008).

subunit I (*cox1*) were obtained; GenBank accession numbers are reported in Appendix A3. A total of 179 sequences from 57 bivalve species were used for this study: 80 sequences from 28 species were obtained in our laboratory, whereas the others were retrieved from GenBank (see Appendix A3 for details). Alignment was made by 55 taxa and 2501 sites, 592 of which, all within *12s* and *16s* genes, were excluded because they were alignment-ambiguous. After removal, 1623 sites were variable and 1480 were parsimony-informative. It is clearly impossible to show here a complete *p*-distance table, but the overall average value was 0.43 (computed by MEGA4, with pairwise deletion of gaps).

Quite interestingly, we found few anomalies in some of the sequences: for instance, a single-base deletion was present in *cytb* of *Hyotissa hyotis* and *Barbatia* cfr. *setigera* at position 2317 and 2450, respectively. This can suggest three possibilities: (i) we could have amplified a mitochondrial pseudogene (NUMT); (ii) we could have faced a real frameshift mutation, which may eventually end with a compensatory one-base insertion shortly downstream (not visible, since our sequence ends quite soon after deletion); (iii) an error in base calling was done by the sequencer. At present no NUMTs have been observed in bivalves (Bensasson et al., 2001; Zbawicka et al., 2007) and the remaining DNA sequences are perfectly aligned with the others, which is unusual for a NUMT; therefore, we think that the second or the third hypotheses are more sound. In all subsequent analyses, we inserted missing data both in nucleotide and in amminoacid alignments. Moreover, several stop codons were found in *Anomia sp.* sequences (within *cox1*, starting at position 1796 and 1913; within *cytb*, starting at 2154, 2226, 2370, 2472 and 2484). Again, we could have amplified two pseudogenes; however, all these stop codons are TAA and the alignment is otherwise good. A possible explanation is an exception to the mitochondrial code of this species, which surely demands further analysis, but this is beyond the scope of this paper. In any case, we kept both sequences and placed missing data in protein and codon model alignments in order to perform subsequent analyses. Of course, phylogenetic positions of all the abovementioned species have been considered with extreme care, taking into account their sequence anomalies.

3.2. Sequence analyses

No saturation signal was observed by plotting uncorrected p-distances as described above (see Supplementary Materials Fig. 2), since all linear interpolations were highly significant as computed with PaSt 1.90. Moreover, deleting third codon positions we obtained a completely unresolved Bayesian tree, confirming that these sites carry some phylogenetic signal (data not shown).

Selective pressures on protein-coding genes were tested through ω. In the Ny98 model (Nielsen and Yang, 1998), there are three classes with different potential ω values: $0 < \omega_1 < 1$, $\omega_2 = 1$, and $\omega_3 > 1$. The M3 model also has three classes of ω values, but these values are less constrained, in that they only have to be ordered $\omega_1 < \omega_2 < \omega_3$ (Ronquist et al., 2005). As M3 was chosen as the best model for our analysis (see below), we only considered M3 estimates about ω and its heterogeneity. Boundaries estimates for *tM3* are very far from one (Supplementary Materials Table 2) and more than 75% of codon sites fell into the first two categories. Moreover, all codon sites scored 0 as the probability of being positively selected. Therefore, we conclude that only a stabilizing pressure may be at work on these markers, which may enhance their phylogenetic relevance. This also allows to analyze protein-coding genes together.

3.3. Taxon sampling

Supplementary Materials Fig. 3 shows results from Average Taxonomic Distinctness test. Our sample plotted almost exactly on the mean of 1000 same-size random subsamples from the master list of bivalve genera, thus confirming that our sample is a statistically representative subsample of the bivalves' systematics.

Supplementary Materials Fig. 4 shows results from *a posteriori* testing of parameter accurateness. Analysis was carried out for all main parameters describing the models, but, for clarity, only gamma-shaping parameters (alpha) and invariable sites proportions (pinv) for *rib* partition are shown. In any case, all parameters behaved the same way: specifically, estimates became very close to "true" ones starting from subtrees made by 30–32 taxa. Therefore, at this size a dataset is informative about evolutionary estimates, given our approach. As we sampled nearly twice this size, this strengthens once again the representativeness of our taxon choice – this time from a molecular evolution point of view.

3.4. Maximum Likelihood

Maximum Likelihood analysis gave the tree depicted in Fig. 1. The method could not resolve completely the phylogeny: bivalves appear to be polyphyletic, as the group corresponding to Protobranchia (*Nucula* + *Solemya*) is clustered among non-bivalve species, although with low support (BP = 68). A first node (BP = 100) separates Palaeoheterodonta (*Inversidens* + *Lampsilis*) from the other groups. A second weak node (BP = 51) leads to two clades, one corresponding to Pteriomorphia + *Thracia* (BP = 68) and the other, more supported, to Heterodonta (BP = 83). A wide polytomy is evident among Pteriomorphia, with some supported groups in it, such as *Thracia*, *Mytilus*, Arcidae (all BP = 100), Limidae + Pectinina (BP = 87), and Pteriida + Ostreina (BP = 85). Heterodonta subclass is also not well resolved, with *Astarte* + *Cardita* (BP = 100) as sister group of a large polytomy (BP = 73) that includes *Donax*, *Ensis*, *Hiatella* + (*Acanthocardia* + *Tridacna*), and an heterogeneous group with Veneridae, *Spisula*, *Dreissena* and *Mya* (BP = 66).

PLS tests turned out to be largely significant (Supplementary Materials Fig. 5). High likelihood support values were always connected with highly supported nodes, whereas the opposite is not always true (see node 11). High positive PLS values are generally showed by the *cytb* partition; good values can also be noted for *cox1* and *16s* genes, even if *16s* is sometimes notably against a given node (see nodes 23 and 24). *12s* has generally low PLS absolute values, with some notable exceptions (see nodes 15 and 16). Globally, deeper splits (see nodes 6, 13, 14, 22, 23, 24, 29) have a low likelihood support absolute value, and generally a low bootstrap score too.

3.5. Bayesian analyses

Table 4 shows results of model-decision statistical tests. Among classical 4by4 models (i.e., not codon models) AIC favored *t04* as best trade-off between partitions number and free parameters. However, if considered, *tM3* (a codon model) was clearly favored. As BF does not take into account the number of free parameters, *t04* is not clearly the best classical 4by4 model in this case. More complex models (with the notable exception of *t05*) turned out to be slightly favored: *t09*, the most complex model we implemented, has positive (albeit small) BF values against each simpler partition scheme. Again, when considered, *tM3* is straightforwardly the best model, with the highest BF scores in the matrix (see Table 4). It is notable that *tNy98*, even not the worst, has instead very low BF scores. Therefore, using *tM3* we obtained the best phylogenetic tree, which is shown in Fig. 2. In this tree, several clusters agreeing with the established taxonomy are present: the first corresponds to Protobranchia (*sensu* Giribet and Wheeler, 2002) and it is basal to all the remaining bivalves (Autolamellibranchiata *sensu* Bieler and Mikkelsen, 2006; PP = 1.00). A second group, which is basal to the rest of the tree, is composed by Palaeoheterodonta (PP = 1.00). Sister group to Palaeoheterodonta a major clade is found (PP = 1.00), in which three

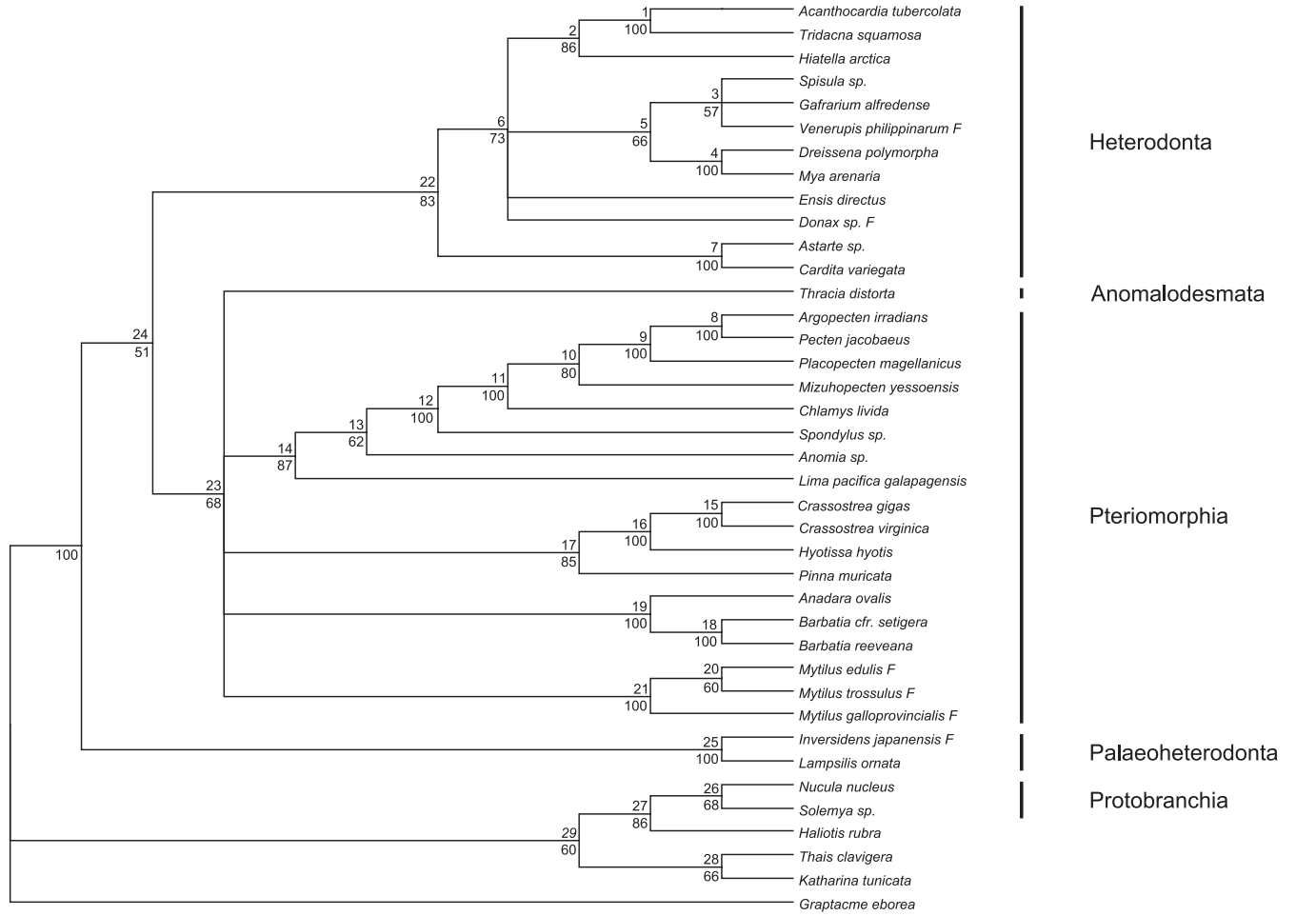

Fig. 1. Majority-rule consensus tree of 100 Maximum Likelihood bootstrap replicates: node have been numbered (above branches), and numbers below the nodes are bootstrap proportions.

Table 4
Results from Akaike Information Criterion (AIC) and Bayes Factors (BF) tests. EML, Estimated Marginal Likelihood; *p*, number of partitions in the partitioning scheme; FP, Free Parameters. Partitioning schemes as in Table 2.

Tree	EML	*p*	FP	AIC	*t02*	*t03*	*t04*	*t05*	*t06*	*t07*	*t08*	*t09*	*t10*	*tNy98*	*tM3*
t01	−64,914.04	2	225	130,278.08	479.76	1870.00	2203.28	494.92	1950.86	2290.48	2326.90	2424.26	N/A	884.14	3721.44
t02	−64,674.16	4	450	130,248.32		1390.24	1723.52	15.16	1471.10	1810.72	1847.14	1944.50	N/A	404.38	3241.68
t03	−63,979.04	5	567	129,092.08			333.28	−1375.08	80.86	420.48	456.90	554.26	N/A	−985.86	1851.44
t04	−63,812.40	6	684	128,992.80				−1708.36	−252.42	87.20	123.62	220.98	N/A	−1319.14	1518.16
t05	−64,666.58	6	675	130,683.16					1455.94	1795.56	1831.98	1929.34	N/A	389.22	3226.52
t06	−63,938.61	8	907	129,691.22						339.62	376.04	473.40	N/A	−1066.72	1770.58
t07	−63,768.80	10	1140	129,817.60							36.42	133.78	N/A	−1406.34	1430.96
t08	−63,750.59	8	909	129,319.18								97.36	N/A	−1442.76	1394.54
t09	−63,701.91	12	1365	130,133.82									N/A	−1540.12	1297.18
t10	−13,725.38	4	450	28,350.76										N/A	N/A
tNy98	−64,471.97	4	512	129,967.94											2837.30
tM3	−63,053.32	4	513	127,132.64											

main groups do separate. Heterodonta constitute a cluster (PP = 1.00), with two branches: *Hiatella* + Cardiidae (PP = 1.00) and other heterodonts (PP = 0.98). Within them, only one node remains unresolved, leading to a Veneridae + Mactridae + (*Dreissena* + *Mya*) polytomy. Another cluster (PP = 0.96) is made by *Pandora* + *Thracia*, as sister group of all Pteriomorphia + *Nuculana* (both PP = 1.00). A wide polytomy is evident within Pteriomorphia, with *Mytilus* species, Limidae + Pectinina, Pteriida + Ostreina, Arcidae and *Nuculana* itself as branches, all with PP = 1.00. Another cluster (PP = 1.00) is made by *Cuspidaria* + (*Astarte* + *Cardita*). All families have PP = 1.00: Cardiidae (genera *Acanthocardia* and *Tridacna;* see Section 4.2.4), Mactridae (genera *Mactra* and *Spisula*), Veneridae (genera *Gafrarium*, *Gemma* and *Venerupis*), Unionidae (genera *Hyriopsis*, *Inversidens*, *Anodonta* and *Lampsilis*), Arcidae (genera *Anadara* and *Barbatia*), Limidae (genera *Acesta* and *Lima*), Ostreidae (genera *Crassostrea* and *Hyotissa*) and Pectinidae (genera *Mizuhopecten*, *Chlamys*, *Mimachlamys*, *Argopecten*, *Pecten* and *Placopecten*).

3.6. Dating the tree

Results from r8s software are shown in Table 3. The relative ultrametric tree is shown in Fig. 3 along with the geological timescale. The best-performing smoothing value for PL analysis was set to 7.26 after a fossil-based cross-validation with an increment of

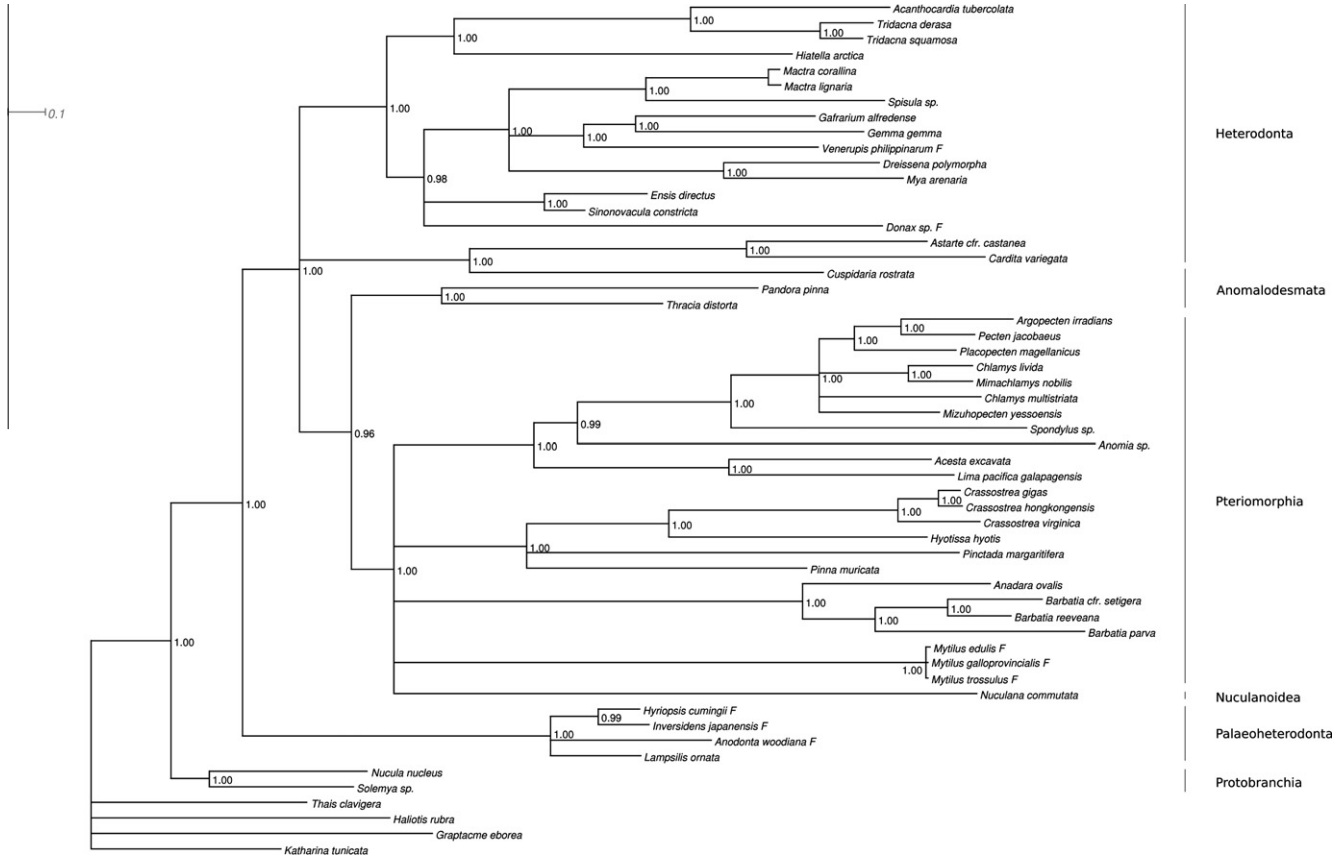

Fig. 2. Majority-rule *tM3* consensus tree from the Bayesian multigene partitioned analysis. Numbers at the nodes are PP values. Nodes under 0.95 were collapsed. Bar units in expected changes per site.

0.01. The best calibration set comprises genus *Barbatia*, subfamily Unioninae, families Veneridae, Limidae, Pectinidae, Cardiidae, Arcidae, and Bivalvia; all constraints were respected. Age for many other taxa were correctly predicted with an error of always less than 50 million years (Myr), as shown in Table 3. This was not the case for genera *Mytilus*, *Mactra*, *Crassostrea*, and *Tridacna*: with the notable exception of *Tridacna*, they were predicted to be much more recent than they appeared in fossil records. This is easily explained by the fact that in all cases (except *Tridacna*) strictly related species were represented in our tree, which diverged well after the first appearance of the genus. Results from PL and NPRS were substantially identical: as in four cases NPRS analysis did not pass the **checkGradient** control, we will present and discuss PL results only.

Deep nodes were all dated between 530 and 450 million years ago (see Fig. 3): the origin of the class was dated 530 Mya, Autolamellibranchiata 520 Mya and their sister group Protobranchia 454 Mya. Within Autolamellibranchiata, the big group comprehending Heterodonta and Pteriomorphia would have arisen about 514 Mya; the radiation of Palaeoheterodonta was not computed as only specimens from Unionidae (293.93 Mya) were present. Pteriomorphia and Heterodonta originated very close in time, about 506 and 498 Mya, respectively. Within Pteriomorphia, the basal clade of Anomalodesmata is more recent (431 Mya) than the main group of traditional Pteriomorphia (497 Mya). On the other hand, the main split within Heterodonta gave rise to *Hiatella* + Cardiidae about 481 Mya, and to Veneroida *sensu lato* 471 Mya. Evolutionary rates (expressed as mutations per year per site) varied consistently, ranging from 0.000693 of branch leading to genus *Barbatia* to 0.011 of the *Hiatella* + Cardiidae group. Table 3 also lists the mean value of NPRS dating across 150 bootstrap replicates and its standard deviation, and it is worth noting that deeper nodes do have very little standard deviation.

4. Discussion

4.1. The methodological pipeline

As the correct selection of suitable molecular markers was (and still is) a major concern in bivalves' phylogenetic analysis, we tested for different ways of treating the data. Our best-performing approach is based on four different mitochondrial genes, and because we obtained robust and reliable phylogenies in our analysis, we can now confirm that this choice is particularly appropriate in addressing deep phylogeny of Bivalvia, given a robust analytical apparatus.

As mentioned, our mitochondrial markers were highly informative, especially protein-coding ones and our results from model selection were straightforward. The phylogenetic signal we recovered in our dataset is complex, as different genes and different positions must have experienced different histories and selective pressures. Moreover, performed single-gene analyses yielded controversial and poorly informative trees (data not shown).

Specifically, both AIC and BF separated ribosomal and protein-coding genes for traditional 4by4 models. AIC tends to avoid overparametrization, as it presents a penalty computed on free parameters, and selected a simpler model; conversely, BF selected the most complex partitioning scheme. BF has been proposed to be generally preferable to AIC (Kass and Raftery, 1995; Alfaro and Huelsenbeck, 2006), but Nylander et al. (2004) pointed out that BF is generally consistent with other model selection methods, like AIC. Indeed, trees obtained under models *t04*, *t07*,

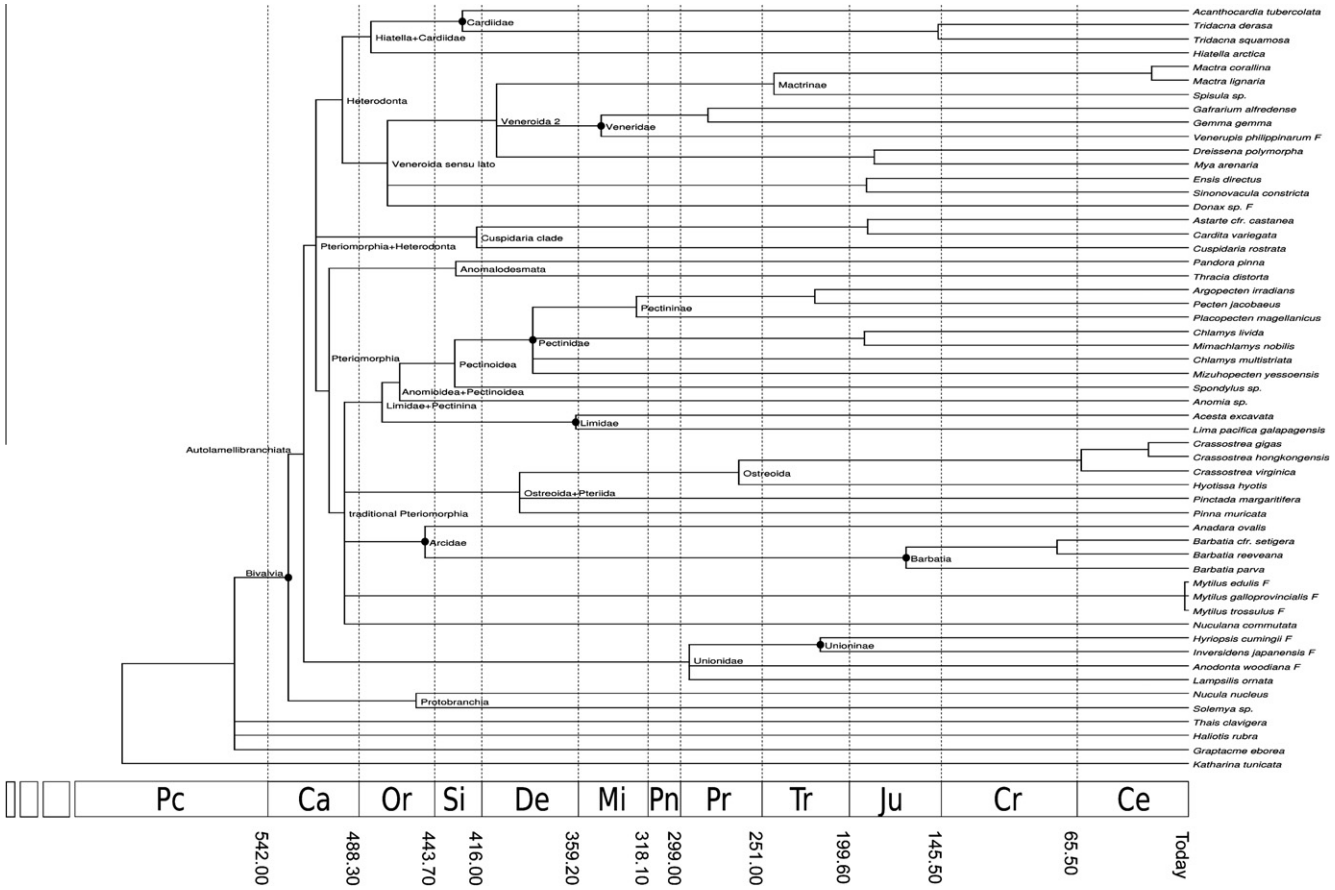

Fig. 3. Results from time calibration of *tM3* tree. The ultrametric *tM3* tree computed by r8s (under Penalized Likelihood method, see text for further details) is shown along with geological time scale and major interval boundaries (ages in million years). Only deep nodes are named: for a complete survey of node datations, see Table 3. Geological data taken from Gradstein et al. (2004) and Ogg et al. (2008). Pc, Precambrian (partial); Ca, Cambrian; Or, Ordovician; Si, Silurian; De, Devonian; Mi, Mississippian; Pn, Pennsylvanian; Pr, Permian; Tr, Triassic; Ju, Jurassic; Cr, Cretaceous; Ce, Cenozoic.

t08, and *t09* are very similar (data not shown). Anyway, the *tM3* model clearly outperformed all alternatives, following both AIC and BF criteria (see Table 4). Furthermore, this was not the case for models *tNy98* and *t10*, which we used to reduce possible misleading phylogenetic noise, albeit in different ways (by a Ny98 codon model or by amminoacids, respectively). *t10* tree was similar to *tM3* one, but significantly less resolved on many nodes, thus indicating a loss of informative signal (data not shown). M3 codon model allows lower ω categories than Ny98; on the other hand, it does not completely eliminate nucleotide information level, as amminoacid models do. All this considered, we propose that M3 codon model is the best way for investigating bivalve phylogeny.

Finally, it is quite evident that Bayesian analysis yielded the most resolved trees, when compared to Maximum Likelihood and this was especially evident for ancient nodes. The tendency of Bayesian algorithms to higher nodal support has been repeatedly demonstrated (Leaché and Reeder, 2002; Suzuki et al., 2002; Whittingham et al., 2002; Cummings et al., 2003; Douady et al., 2003; Erixon et al., 2003; Simmons et al., 2004; Cameron et al., 2007), though Alfaro et al. (2003) found that PP is usually a less biased predictor of phylogenetic accuracy than bootstrap. Anyway, it has to be noted that most of our recovered nodes are strongly supported by both methods; we therefore think that the higher support of Bayesian analysis is rather due to a great affordability of the method in shaping and partitioning models, which is nowadays impossible with Maximum Likelihood algorithms. All that considered, we suggest that a suitable methodological pipeline for bivalves' future phylogenetic reconstructions should be as such: (i) sequence analyses for saturation and selection; (ii) rigorous evaluation of taxon coverage; (iii) tests for best data partitioning; (iv) appropriate model decision statistics; (v) Bayesian analysis; (vi) eventual dating by cross-validation with fossil records.

4.2. The phylogeny of Bivalvia

4.2.1. Protobranchia Pelseneer

Our study confirms most of the recent findings (Giribet and Wheeler, 2002; Giribet and Distel, 2003; Kappner and Bieler, 2006): Nuculoidea and Solemyoidea do maintain their basal position, thus representing Protobranchia *sensu stricto*, which is a sister group to all Autolamellibranchiata. On the contrary, Nuculanoidea, although formerly placed in Nuculoida, is better considered within Pteriomorphia, placed in its own order Nuculanoida. The split separating *Nucula* and *Solemya* lineages is dated around the late Ordovician (454.28 Mya); since the first species of the subclass must have evolved earlier (about 500 Mya), this is a clear signal of the antiquity of this clade. In fact, based on paleontological records, the first appearance of Protobranchia is estimated around 520 Mya (early Cambrian) (He et al., 1984; Parkhaev, 2004), and our datation is only slightly different (482.02 Mya, with a standard deviation of 14.61).

4.2.2. Palaeoheterodonta Newell

Freshwater mussels are basal to all the remaining Autolamellibranchiata (Heterodonta + Pteriomorphia), as supposed by Cope (1996). Therefore, there is no evidence for Heteroconchia *sensu*

Bieler and Mikkelsen (2006) in our analysis. The monophyletic status of the subclass was never challenged in our Bayesian analyses, nor in traditional Maximum Likelihood ones. Finally, since we obtained sequences only from specimens from Unionoidea: Unionidae, a clear dating of the whole subclass is not sound, as shown by a relatively high difference between PL values and mean across bootstrap replicates (294 and 348 Mya, respectively). Therefore, the origin of the subclass must date back to before than 350 Mya, which is comparable to paleontological data (Morton, 1996).

4.2.3. Pteriomorphia Newell

Here we obtained a Pteriomorphia *sensu novo* subclass comprising all pteriomorphians *sensu* Millard (2001), as well as Nuculanoidea and anomalodesmatans. This diverse taxon arose about 506 Mya, which makes it the first bivalve radiation in our tree, dated in the middle Cambrian, which is perfectly in agreement with paleontological data. Moreover, our results proved to be stable also with bootstrap resampling, with a standard deviation of slightly more than 2 million of years (Table 3). A wide polytomy is present within the subclass; as this polytomy is constantly present in all the analyses, and it has been found also by many other authors (see Campbell, 2000; Steiner and Hammer, 2000; Matsumoto, 2003), we consider it as a "hard polytomy", reflecting a true rapid radiation dated about 490 Mya (Cambrian/Ordovician boundary). Sister group to this wide polytomy is the former anomalodesmatan suborder Pholadomyina. In our estimate, the clade *Pandora* + *Thracia* seems to have originated something like 431.45 Mya, as several pteriomorphian groups, like Pectinoidea (431.77 Mya) or Arcidae (449.51 Mya). On the other hand, we failed in retrieving *Cuspidaria* within the pteriomorphian clade, while this genus is strictly associated with *Astarte* + *Cardita*. Not only the nodal support is strong, this relationship is also present across almost all trees and models. It has to be noted that the association between *Cuspidaria* and (*Astarte* + *Cardita*) has been evidenced already (Giribet and Distel, 2003). On the other side, suborder Pholadomyina is always basal to pteriomorphians (data not shown). Maybe it is worth noting that *Cuspidaria* branch is the longest among anomalodesmatans and that *Astarte* and *Cardita* branches are the longest among heterodonts (see Fig. 2). Moreover, this clade is somewhat unstable across bootstrap replicates (see Table 3). Maybe the large amount of mutations may overwhelm the true phylogenetic signal for such deep nodes, as also expected by their relatively high mutation rates. Hence, we see three possible alternatives: (i) an artifact due to long-branch-attraction – all anomalodesmatans belong to Pteriomorphia, whereas *Astarte* and *Cardita* belong to Heterodonta; (ii) anomalodesmatans do belong to Heterodonta, whose deeper nodes are not so good resolved, whereas a strong signal is present for Pteriomorphia monophyly, thus leading to some shuffling into basal positions; (iii) anomalodesmatans are polyphyletic, and the two present-date suborders do not share a common ancestor. The two last possibilities seem unlikely to us, given our data and a considerable body of knowledge on the monophyletic status of Heterodonta and Anomalodesmata (Canapa et al., 2001; Dreyer et al., 2003; Harper et al., 2006; Taylor et al., 2007). We therefore prefer the first hypothesis, albeit an anomalodesmatan clade nested within heterodonts has also been appraised by some authors (Giribet and Wheeler, 2002; Giribet and Distel, 2003; Bieler and Mikkelsen, 2006; Harper et al., 2006). Interestingly, in *t10* tree the whole group *Cuspidaria* + (*Astarte* + *Cardita*) nested within pteriomorphians species; a similar result was also yielded by a wider single-gene *cox1* dataset (data not shown). This would also account for the great difference found in *Astarte* + *Cardita* split across bootstrap replicates. A major taxonomical revision is needed for basal pteriomorphians, including also anomalodesmatans, as well as for superfamilies Astartoidea and Carditoidea.

As mentioned above, the main groups of pteriomorphians, arising in the late Cambrian, comprehend the genus *Nuculana* also. This placement was first proposed by Giribet and Wheeler (2002) on molecular bases and our data strongly support it. Its clade must have diverged from other main pteriomorphian groups at the very beginning of this large radiation. Among the main groups of Pteriomorphia, it is also worth noting the breakdown of the orders Pterioida *sensu* Vokes (1980) and Ostreoida *sensu* Millard (2001): the suborder Ostreina constitutes a net polyphyly with suborder Pectinina. The former is better related with order Pteriida *sensu* Millard (2001) (*Pinna*, *Pinctada*), whereas the latter is better related with superfamilies Limoidea (*Lima* + *Acesta*) and Anomioidea (*Anomia*). This is in agreement with most recent scientific literature about Pteriomorphia (Steiner and Hammer, 2000; Matsumoto, 2003).

4.2.4. Heterodonta Newell

The subclass seems to have originated almost 500 Mya (late Cambrian) and its monophyletic status is strongly confirmed by our analysis, but a major revision of its main subdivisions is also required. The placement of *Astarte* and *Cardita* has already been discussed. At the same time, the orders Myoida and Veneroida, as well as the Chamida *sensu* Millard (2001), are no longer sustainable. A first main split separates (*Hiatella* + Cardiidae) from all remaining heterodonts. This split may correspond to two main orders in the subclass. As we sampled only 15 specimens of Heterodonta, we could only coarsely assess their phylogenetic taxonomy. However, we could precisely demonstrate the monophyly of families Veneridae and Mactridae and their sister group status. This could correspond together with *Dreissena* + *Mya* to a superfamily Veneroidea *sensu novo*, which is stably dated around the early Devonian; however, further analyses are requested towards an affordable taxonomical revision, which is beyond the aims of this paper. Finally, recent findings about Tridacninae subfamily within Cardiidae family (Maruyama et al., 1998) are confirmed against old taxonomy based on Cardioidea and Tridacnoidea superfamilies (Millard, 2001).

Concluding, our work evidenced that all main deep events in bivalve radiation took place in a relatively short 70 Myr time during late Cambrian/early Ordovician (Fig. 3). Dates are stable across bootstrap replicates, especially those of deeper nodes, which were one of the main goals of this work (Table 3): most NPRS bootstrap means are indeed very close to PL estimates and standard deviations are generally low. Notable exceptions are some more recent splits on long branches (*Chlamys livida* + *Mimachlamys*, *Ensis* + *Sinonovacula*, *Astarte* + *Cardita*, *Tridacna*), which clearly are all artifacts of low taxon sampling for that specific branch, and Unionidae and Ostreoida. Unionidae are the only palaeoheterodonts we sampled and this could account for this anomaly; anyway, it is worth taking

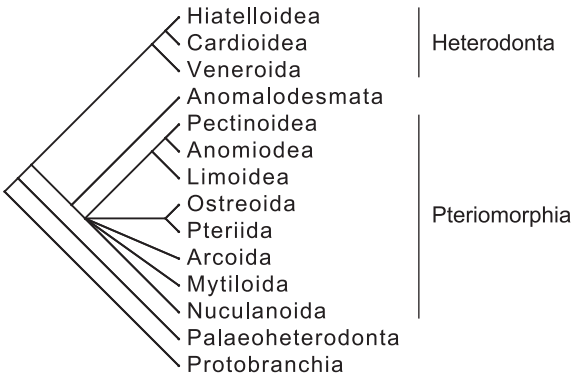

Fig. 4. Global survey of the bivalve phylogeny.

Table A1
PCR conditions.

		12s		*16s*		*cox1*		*cytb*	
		Annealing	Primers	Annealing	Primers	Annealing	Primers	Annealing	Primers
1	*Anadara ovalis*	50 °C 30″	SR-J14197 ÷ SR-N14745			56 °C 20″	coIF ÷ coIR	48 °C 30″	cobF ÷ cobR
2	*Anodonta woodiana*					48 °C 1′	LCO ÷ HCO	48 °C 1′	cobF ÷ cobR
3	*Anomia sp.*			48 °C 1′	16SbrH(32) ÷ 16Sar(34)	56–46 °C 30″–1′	coIF ÷ coIR	48 °C 30″	cobF ÷ cobR
4	*Argopecten irradians*	50 °C 30″	SR-J14197 ÷ SR-N14745	48 °C 1′	16SbrH(32) ÷ 16Sar(34)	56–46 °C 30″–1′	coIF ÷ coIR	55–45 °C 30″–1′	cobF ÷ cobR
5	*Astarte* cfr. *castanea*	50 °C 30″	SR-J14197 ÷ SR-N14745					48 °C 30″	cobF ÷ cobR
6	*Barbatia parva*	50 °C 30″	SR-J14197 ÷ SR-N14745			48 °C 1′	LCO ÷ HCO	48 °C 1′	cobF ÷ cobR
7	*Barbatia reeveana*	50 °C 30″	SR-J14197 ÷ SR-N14745			52 °C 20″	coIF ÷ coIR	53–43 °C 30″–1′	cobF ÷ cobR
8	*Barbatia* cfr. *setigera*	50 °C 30″	SR-J14197 ÷ SR-N14745			54 °C 20″	coIF ÷ coIR	48 °C 1′	cobF ÷ cobR
9	*Cardita variegata*	50 °C 30″	SR-J14197 ÷ SR-N14745			48 °C 1′	LCO ÷ HCO	48 °C 1′	cobF ÷ cobR
10	*Chlamys livida*	50 °C 30″	SR-J14197 ÷ SR-N14745	48 °C 1′	16SbrH(32) ÷ 16Sar(34)	52 °C 20″	coIF ÷ coIR	48 °C 1′	cobF ÷ cobR
11	*Chlamys multistriata*			54 °C 2′	16SbrH(32) ÷ 16SDon			48 °C 1′	cobF ÷ cobR
12	*Cuspidaria rostrata*	50 °C 30″	SR-J14197 ÷ SR-N14745			48 °C 1′	LCO ÷ HCO	58–48 °C 1′	cobF ÷ cobR
13	*Ensis directus*	46 °C 30″	SR-J14197 ÷ SR-N14745	54 °C 2′	16SbrH(32) ÷ 16SDon	56–46 °C 30″–1′	coIF ÷ coIR	53–43 °C 1′	cobF ÷ cobR
14	*Gafrarium alfredense*	50 °C 30″	SR-J14197 ÷ SR-N14745	48 °C 1′	16SbrH(32) ÷ 16Sar(34)			48 °C 1′	cobF ÷ cobR
15	*Gemma gemma*			48 °C 1′	16SbrH(32) ÷ 16Sar(34)	52 °C 20″	coIF ÷ coIR	58–48 °C 1′	cobF ÷ cobR
16	*Hyotissa hyotis*	50 °C 30″	SR-J14197 ÷ SR-N14745	48 °C 1′	16SbrH(32) ÷ 16Sar(34)	52 °C 20″	coIF ÷ coIR	58–48 °C 1′	cobF ÷ cobR
17	*Lima pacifica galapagensis*	50 °C 30″	SR-J14197 ÷ SR-N14745	48 °C 45″[a]	16SbrH(32) ÷ 16SarL[a]	52 °C 20″	coIF ÷ coIR	53–43 °C 30″–1′	cobF ÷ cobR
18	*Mactra corallina*	48 °C 1′	SR-J14197 ÷ SR-N14745	56 °C 1′	16SbrH(32) ÷ 16Sar(34)	48 °C 1′	LCO ÷ HCO	48 °C 1′	cobF ÷ cobR
19	*Mactra lignaria*	48 °C 1′	SR-J14197 ÷ SR-N14745	56 °C 1′	16SbrH(32) ÷ 16Sar(34)	48 °C 1′	LCO ÷ HCO		
20	*Mya arenaria*							48 °C 1′	cobF ÷ cobR
21	*Nucula nucleus*	50 °C 30″	SR-J14197 ÷ SR-N14745	54 °C 2′	16SbrH(32) ÷ 16SDon				
22	*Nuculana commutata*	50 °C 30″	SR-J14197 ÷ SR-N14745			48 °C 1′	LCO ÷ HCO	48 °C 1′	cobF ÷ cobR
23	*Pandora pinna*	50 °C 30″	SR-J14197 ÷ SR-N14745	53–43 °C 1′20″	16SbrH(32) ÷ 16SarL	48 °C 1′	LCO ÷ HCO	53–43 °C 1′20″	UCYTBF144F ÷ UCYTB272R
24	*Pecten jacobaeus*							58 °C.48 °C 1′	cobF ÷ cobR
25	*Pinna muricata*	50 °C 30″	SR-J14197 ÷ SR-N14745	48 °C 1′	16SbrH(32) ÷ 16Sar(34)	52 °C 20″	coIF ÷ coIR	48 °C 1′	cobF ÷ cobR
26	*Thracia distorta*	50 °C 30″	SR-J14197 ÷ SR-N14745			48 °C 1′	LCO ÷ HCO	48°C 1′	cobF ÷ cobR
27	*Tridacna derasa*					48 °C 1′	LCO ÷ HCO	48 °C 1′	cobF ÷ cobR
28	*Tridacna squamosa*							48 °C 1′	cobF ÷ cobR
	Transformed inserts	55 °C 30″	M13F ÷ M13R	55 °C 30″	M13F ÷ M13R	55 °C 30″	M13F ÷ M13R	55 °C 30″	M13F ÷ M13R

[a] This amplification was carried out with Herculase reaction kit (Stratagene, Cedar Creek, TX, USA), following manufacturer's instructions.

Table A2
Primer used in this study.

	5′–3′ Sequence	Reference
SR-J14197	GTACAYCTACTATGTTACGACTT	Simon et al. (2006)
SR-N14745	GTGCCAGCAGYYGCGGTTANAC	Simon et al. (2006)
16SbrH(32)	CCGGTCTGAACTCAGATCACGT	Palumbi et al. (1996)
16Sar(34)	CGCCTGTTTAACAAAAACAT	Modified from Palumbi et al. (1996)
16SarL	CGCCTGTTTATCAAAACAT	Palumbi et al. (1996)
16SDon	CGCCTGTTTATCAAAAACAT	Kocher et al. (1989)
LCO1490	GGTCAACAAATCATAAAGATATTGG	Folmer et al. 1994)
HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. (1994)
COIF	ATYGGNGGNTTYGGNAAYTG	Matsumoto (2003)
COIR	ATNGCRAANACNGCNCCYAT	Matsumoto (2003)
CobF	GGWTAYGTWYTWCCWTGRGGWCARAT	Passamonti (2007)
CobR	GCRTAWGCRAAWARRAARTAYCAYTCWGG	Passamonti (2007)
UCYTB144F	TGAGSNCARATGTCNTWYTG	Merritt et al. (1998)
UCYTB272R	GCRAANAGRAARTACCAYTC	Merritt et al. (1998)
M13F	GTAAAACGACGGCCAGT	
M13R	CAGGAAACAGCTATGAC	

into account that the r8s-bootkit follows a slightly different method than *tout court* PL, therefore the results are not expected to perfectly coincide. When this happens, however, i.e. for most nodes in Fig. 3, it accounts for a substantial stability in timing estimates.

We show in Fig. 4 the survey on bivalve taxonomy which we described above. Given the still limited, but statistically representative, taxon sampling available, it is nowadays inconceivable to propose a rigorous taxonomy at order and superfamily level; therefore, we used in Fig. 4 the nomenclature of Millard (2001) and Vokes (1980). More taxa and genes to be included will sharp resolution and increase knowledge on bivalves' evolutionary history.

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Table A3
GenBank accession numbers of sequences used in this study. Bold sequences were obtained for this work.

	12s	*16s*	*cox1*	*cytb*
Acanthocardia tubercolata	DQ632743	DQ632743	DQ632743	DQ632743
Acesta excavata	AM494885	AM494899	AM494909	AM494922
Anadara ovalis	**GQ166533**		**GQ166571**	**GQ166592**
Anodonta woodiana F		DQ073815	EF440349	**GQ166594**
Anomia sp.		**GQ166557**	**GQ166573**	**GQ166595**
Argopecten irradians	**GQ166535**	**GQ166558**	**GQ166574**	**GQ166596**
Astarte castanea			AF120662	
Astarte cfr. castanea	**GQ166536**			**GQ166597**
Barbatia parva	**GQ166537**		**GQ166575**	**GQ166599**
Barbatia reeveana	**GQ166538**		**GQ166576**	**GQ166600**
Barbatia cfr. setigera	**GQ166539**		**GQ166577**	**GQ166601**
Cardita variegata	**GQ166540**		**GQ166578**	**GQ166605**
Chlamys livida	**GQ166541**	**GQ166559**	**GQ166579**	**GQ166606**
Chlamys multi striata	AJ571604	**GQ166560**		**GQ166607**
Crassostrea gigas	AF177226	AF177226	AF177226	AF177226
Crassostrea hongkongensis F	EU266073	EU266073	EU266073	EU266073
Crassostrea viriginica	AY905542	AY905542	AY905542	AY905542
Cuspidaria rostrata	**GQ166542**		**GQ166580**	**GQ166608**
Donax faba F			AB040844	
Donax trunculus F		EF417549		EF417548
Dreissena polymorpha		DQ280038	AF120663	DQ072117
Ensis directus	**GQ166543**	**GQ166561**	**GQ166581**	**GQ166610**
Gafrarium alfredense	**GQ166544**	**GQ166562**		**GQ166611**
Gemma gemma		**GQ166563**	**GQ166582**	**GQ166612**
Graptacme eborea	AY484748	AY484748	AY484748	AY484748
Haliotis rubra	AY588938	AY588938	AY588938	AY588938
Hiatella arctica	DQ632742	DQ632742	DQ632742	DQ632742
Hyotissa hyotis	**GQ166545**	**GQ166564**	**GQ166583**	**GQ166613**
Hyriopsis cumini	FJ529186	FJ529186	FJ529186	FJ529186
Inversidens japanensis F	AB055625	AB055625	AB055625	AB055625
Katharina tunicata	U09810	U09810	U09810	U09810
Lampsilis ornata	AY365193	AY365193	AY365193	AY365193
Lima pacifica galapagensis	**GQ166548**	**GQ166565**	**GQ166584**	**GQ166616**
Mactra corallina	**GQ166550**	**GQ166566**	**GQ166585**	**GQ166617**
Mactra lignaria	**GQ166551**	**GQ166567**	**GQ166586**	
Mimachlamys nobilis	FJ415225	FJ415225	FJ415225	FJ415225
Mizuhopecten yessoensis	AB271769	AB271769	AB271769	AB271769
Mya arenaria		AY377618	AF120668	**GQ166619**
Mytilus edulis F	AY484747	AY484747	AY484747	AY484747
Mytilus galloprovincialis F	AY497292	AY497292	AY497292	AY497292
Mytilus trossulus F	DQ198231	DQ198231	DQ198231	DQ198231
Nucula nucleus	**GQ166552**	**GQ166568**	AM696252	
Nuculana commutata	**GQ166553**		**GQ166587**	**GQ166622**
Pandora pinna	**GQ166554**	**GQ166569**	**GQ166588**	**GQ166623**
Pecten jacobaeus	AJ571596	AJ245394	AY377728	**GQ166624**
Pinctada margariti fera	AB250256	AB214436	AB259166	
Pinna muricata	**GQ166555**	**GQ166570**	**GQ166589**	**GQ166625**
Placopecten magellanicus	DQ088274	DQ088274	DQ088274	DQ088274
Sinonovacula constricta	EU880278	EU880278	EU880278	EU880278
Solemya velesiana				AM293670
Solemya velum		DQ280028	U56852	
Spisula solidissima				AF205083
Spisula solidissima solidissima			AY707795	
Spisula subtruncata		AJ548774		
Spondylus gaederopus	AJ571607	AJ571621		
Spondylus varius			AB076909	
Thais clavigera	DQ159954	DQ159954	DQ159954	DQ159954
Thracia distorta	**GQ166556**		**GQ166590**	**GQ166626**
Tridacna derasa		AF122976	**GQ166591**	**GQ166627**
Tridacna squamosa		AF122978	EU346361	**GQ166628**
Venerupis philippinarum F	AB065375	AB065375	AB065375	AB065375

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Appendix A

See Tables A1–A4.

Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2010.08.032.

Table A4
Subtrees used for assessing parameter estimate accurateness.

Taxon labels:							
1	*Acanthocardia tubercolata*	15	*Crassostrea hongkongensis*	29	*Katharina tunicata*	43	*Pecten jacobaeus*
2	*Acesta excavata*	16	*Crassostrea virginica*	30	*Lampsilis ornata*	44	*Pinctada margaritifera*
3	*Anadara ovalis*	17	*Cuspidaria rostrata*	31	*Lima pacifica galapagensis*	45	*Pinna muricata*
4	*Anodonta woodiana* F	18	*Donax* sp. F	32	*Mactra corallina*	46	*Placopecten magellanicus*
5	*Anomia* sp.	19	*Dreissena polymorpha*	33	*Mactra lignaria*	47	*Sinonovacula constricta*
6	*Argopecten irradians*	20	*Ensis directus*	34	*Mimachlamys nobilis*	48	*Solemya* sp.
7	*Astarte* cfr. *castanea*	21	*Gafrarium alfredense*	35	*Mizuhopecten yessoensis*	49	*Spisula* sp.
8	*Barbatia parva*	22	*Gemma gemma*	36	*Mya arenaria*	50	*Spondylus* sp.
9	*Barbatia reeveana*	23	*Graptacme eborea*	37	*Mytilus edulis* F	51	*Thais clavigera*
10	*Barbatia* cfr. *setigera*	24	*Haliotis rubra*	38	*Mytilus galloprovincialis* F	52	*Thracia distorta*
11	*Cardita variegata*	25	*Hiatella arctica*	39	*Mytilus trossulus* F	53	*Tridacna derasa*
12	*Chlamys livida*	26	*Hyotissa hyotis*	40	*Nucula nucleus*	54	*Tridacna squamosa*
13	*Chlamys multistriata*	27	*Hyriopsis cumingii* F	41	*Nuculana commutata*	55	*Venerupis philippinarum* F
14	*Crassostrea gigas*	28	*Inversidens japanensis* F	42	*Pandora pinna*		

Tree tM3:

(51,29,24,23,(((((7,11),17),(((1,(53,54)),25),((20,47),(((32,33),49),((21,22),55),(19,36)),18)),(((37,38,39),((2,31),(5,((35,13,(12,34),((6,43),46)),50))),((((14,15),16),26),44,45),(3,((10,9),8)),41),(42,52))),((27,28),4,30)),(40,48)));

Subtrees:

1 (51,29,24,23,((((17),(((1,(53,54)),25),((20,47),(((32,33),49),((21,22),55),(19,36)),18)),(((37,38,39),((2,31),(5,((35,13,(12,34),((6,43),46)),50))),((((14,15),16),26),44,45),(3,((10,9),8)),41),(42,52))),((27,28),4,30)),(40,48)));
2 (51,29,24,23,((((((1,(53,54)),25),((20,47),(((32,33),49),((21,22),55),(19,36)),18)),(((37,38,39),((2,31),(5,((35,13,(12,34),((6,43),46)),50))),((((14,15),16),26),44,45),(3,((10,9),8)),41),(42,52))),((27,28),4,30)),(40,48)));
3 (51,29,24,23,(((((7,11),17),(((1,(53,54)),25),((20,47),(((32,33),49),((21,22),55),(19,36)),18)),(((37,38,39),((2,31),(5,((35,13,(12,34),((6,43),46)),50))),((((14,15),16),26),44,45),(3,((10,9),8)),41),(42,52)))),(40,48)));
4 (51,29,24,23,(((((7,11),17),(((1,(53,54)),25),((20,47),(((32,33),49),((21,22),55),(19,36)),18)),(((37,38,39),((2,31),(5)),((((14,15),16),26),44,45),(3,((10,9),8)),41),(42,52))),((27,28),4,30)),(40,48)));
5 (51,29,23,(((((7,11)),(((1,(53,54)),25),((20,47),(((32,33),49),((21,22),55),(19,36)))),(((37,38,39),((2,31),(5,((35,13,(12,34),((6,43),46)),50))),((((14,15),16),26),44,45),(3,((10,9),8)),41))),((27,28)))));
6 (51,29,24,23,(((((7,11),17),(((1),25),((20,47),((49),((21,22),55),(19,36)),18)),((((2,31),(5,((35,13,(12,34),((6,43),46)),50))),((((14,15),16),26),44,45),(3),41),(42,52))),((27,28),4,30)),(40,48)));
7 (51,24,23,(((((7,11),17),((((53,54)),25),((20),(((32,33),49),((21,22)),(19,36)),18)),(((38,39),((2,31),(5,((35,13,(12),((6,43),46)),50))),((((14),16),26),44,45),(3,((9),8))),(52))),((27),4,30)),(40)));
8 (23,(((((7,11),17),(((((32,33),49),((21,22),55),(19,36)),18)),(((37,38,39),((2,31),(5,((35,13,(12,34),((6,43),46)),50))),((((14,15),16),26)),(3,((10,9),8)),41),(42,52))),((28),4)),(48)));
9 (51,29,24,23,(((((7,11),17),((41,(37,38,39),((2,31),(5,((35,13,(12,34),((6,43),46)),50))),((((14,15),16),26),44,45),(3,((10,9),8))),(42,52))),((27,28),4,30)),(40,48)));
10 (51,29,(((((7),17),(((1,(54)),25),((20),(((32)),((21,22)),(19,36)))),(((37,38),((2,31),(((35,(12,34),((6,43),46)),50))),((((14,15)),26),44),(3,((10),8)),41),(52))),((27,28),30)),(40)));
11 ((((7,11),17),(((1,(53,54))),((((32,33),49),((21,22),55)))),(((37,38,39),((5,((35,13,(12,34),((6,43))),50))),((((14,15),16)),44,45),(((10,9),8))))),((27,28),4,30));
12 (51,29,24,23,(((((7,11),17),(((1,(53,54)),25),((20,47),(((32,33),49),((21,22),55),(19,36)),18)),(((((10,9),8))))),((27,28),4,30)),(40,48)));
13 (29,23,(((((11)),(((1,(54))),((20),(((32),49),((22)),(19)),18)),(((38),((2),(5,((13,(34),((43))),50))),((((15)),26),45),(((10),8))),(42))),((27),4)),(40)));
14 (23,((((17),((((54))),((20,47))),((((2,31),(5,((13,(34),((6),46))))),((((14,15),16)),44,45),41),(42))),((27),4,30)),(40,48)));
15 (29,24,23,((((((1,(53,54))),(20),(((32,33),49),((22)),(19)))),(((38,39),((5,((13,(34),(46))))),((((14))),44)))),((27)))));
16 (((((7,11),17),((25),(((36)),18)),(((37),((5)),(((16))),41),(42,52))),((27,28),4,30)),(40,48));
17 (((((53,54))),((((32,33)),(55)))),(((37,38,39),(((((12,34)))))),((((14,15),16))),(((10,9),8)))));
18 (51,24,(((((7),17),(((((33)),(19)))),((((2),(((35)))),((26))),(52))),((28),30)),(40,48)));
19 ((2,31),(5,((35,13,(12,34),((6,43),46)),50)));
20 (((1,(53,54)),25),((20,47),(((32,33),49),((21,22),55),(19,36)),18));
21 (29,(((((11)),((((49)))),((((5,(50))),((8)),41),(42))),((27))),(48)));
22 (51,(((((7)),(((20))),(((37,38,39),((((14))))))),(40)));
23 (((((((21)))))),(((45)),(52))),(4)),(48));
24 (51,29,24,23,(((((7,11),17),(((1,(53,54)),25),(18,(20,47),(((32,33),49),((21,22),55),(19,36)))),((41,(37,38,39),((2,31),(5,((35,13,(12,34),((6,43),46)),50))),((((14,15),26),44,45),(3,((10,9),8))),(42,52))),((27,28),4,30)),(40,48)));
25 (51,29,24,23,(((((7,11),17),(((1,(53,54)),25),(18,(20,47),(((32,33),49),((21,22),55),(19,36)))),((41,(37,38,39),((2,31),(5,((35,13,(12,34),((6,43),46)),50))),(44,45),(3,((10,9),8))),(42,52))),((27,28),4,30)),(40,48)));
26 (51,29,23,(((((7,11),17),((1,25),(18,(20,47),(((32,33),49),((21,22),55),(19,36)))),((41,((2,31),(5,((35,13,(12,34),((6,43),46)),50))),((((14,15),16),26),44,45),(3,((10,9),8))),(42,52))),((27,28),4,30)),(40,48)));
27 (51,29,24,23,(((((7,11),17),(((1,(53,54)),25),(18,(20,47))),((41,(37,38,39),((2,31),(5,((35,13,(12,34),((6,43),46)),50))),((((14,15),16),26),44,45),(3,((10,9),8))),(42,52))),((27,28),4,30)),(40,48)));
28 (51,24,23,((((11,17),(((53,1),25),(18,(20,47),((32,49),((21,22),55),(19,36)))),((3,(37,39),((2,31),(5,((46,35,13,(12,34)),50))),((((14,15),16),26),45)),(42,52))),((27,28),4)),(40,48)));
29 (51,29,24,23,((((((1,(53,54)),25),(18,(20,47),(((32,33),49),(19,36)))),((41,(37,38,39),((2,31),(5,((35,13,(12,34),((6,43),46)),50))),((((14,15),16),26),44,45)),(42,52))),((27,28),4,30)),(40,48)));
30 (51,29,24,23,((((7,17),(((1,(53,54)),25),((20,47),(36,((32,33),49),((21,22),55))))),((41,38,(31,(5,((43,35,13,(12,34)),50))),((14,26),44),(8,3)),(42,52))),(4,28,30)),(40,48)));
31 (51,((((7,17),(((1,(53,54)),25),((20,47),(36,((32,33),49),((21,22),55))))),((41,38,(31,(5,((43,35,13,(12,34)),50))),((14,26),44),(8,3)),(42,52))),(4,28,30)),(40,48)));
32 (51,29,24,23,(((18,(41,(37,38,39),((2,31),(5,((35,13,(12,34),((6,43),46)),50))),((((14,15),16),26),44,45),(3,((10,9),8))),((7,11),17)),((27,28),4,30)),(40,48)));
33 (51,29,23,(40,((7,(((53,1),25),((20,47),((21,22),(32,49),(19,36)))),(42,(41,((2,31),(5,((46,35,(12,34)),50))),((15,16),44,45),(9,3)))),(27,28))));
34 (29,23,((((7,11),(((53,54),25),((20,47),((32,33),(21,22)))),((41,(10,9),(38,39),((13,(6,43),(12,34)),(2,31)),(((14,15),26),45)),(42,52))),(27,28)),(40,48)));
35 (51,29,24,23,((7,(((1,(53,54)),25),(18,(((32,33),49),((21,22),55)))),(((2,31),(5,((35,13,(12,34),((6,43),46)),50))),(42,52))),((27,28),4,30)));
36 (40,((((7,11),17),((1,(53,54)),(18,(20,47),(36,32,(22,55))))),(42,(41,39,((15,16),26),(2,(5,((35,34,(6,46)),50))),(8,3)))),(4,27)));
37 (51,24,(((((1,(53,54)),25),(18,(20,47))),(((37,38,39),(26,44,45),(3,((10,9),8)),(5,((35,((6,43),46)),50))),(42,52))),(40,48)));

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38  ((((2,31),(5,((35,13,(12,34),((6,43),46)),50))),(((((14,15),16),26),44,45)),((20,47),(((32,33),49),((21,22),55),(19,36)))));
39  (48,((17,((25,54),(47,(22,19,(33,49)))),((41,39,(14,26),(31,((13,12),50)),(3,(8,9))),(42))),(4,27)));
40  (51,29,23,(40,(28,((7,17),(1,(47,18,(36,(21,55)))),(52,(41,(38,39),(31,(5,((34,13,6),50))),(26,45)))))));
41  ((40,48),(((27,28),4,30),((41,(37,39),(31,(5,((34,(6,46)),50))),((14,26),45),(9,3)),(42,52))));
42  (51,29,24,23,(((1,(53,54)),25),(18,(20,47)),(((32,33),49),((21,22),55),(19,36)))));
43  ((40,48),((41,((2,31),(5,((35,13),50))),(((((14,15),16),26),44,45)),(42,52)));
44  (51,(40,((11,((32,22),(25,54)),(52,(41,39,8,6,(26,45))))),(4,27))));
45  (29,24,23,((((42,52),((7,11),17)),((27,28),30)),(40,48)));
46  (((27,28),4,30),(41,3,(2,31),(26,44,45)));
47  (23,(40,(30,(18,((7,11),17),(42,31))))));
48  (51,(((27,28),4,30),(40,48)));
49  (((6,43),46),(12,34));
50  (24,(55,(37,(10,9))));
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