

Comparative Large-Scale Mitogenomics Evidences Clade-Specific Evolutionary Trends in Mitochondrial DNAs of Bivalvia

Federico Plazzi*, Guglielmo Puccio, and Marco Passamonti

Department of Biological, Geological and Environmental Sciences, University of Bologna, via Selmi, 3 – 40126 Bologna, Italy

*Corresponding author: E-mail: federico.plazzi@unibo.it.

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Abstract

Despite the figure of complete bivalve mitochondrial genomes keeps growing, an assessment of the general features of these genomes in a phylogenetic framework is still lacking, despite the fact that bivalve mitochondrial genomes are unusual under different aspects. In this work, we constructed a dataset of one hundred mitochondrial genomes of bivalves to perform the first systematic comparative mitogenomic analysis, developing a phylogenetic background to scaffold the evolutionary history of the class' mitochondrial genomes. Highly conserved domains were identified in all protein coding genes; however, four genes (namely, *atp6*, *nad2*, *nad4L*, and *nad6*) were found to be very divergent for many respects, notwithstanding the overall purifying selection working on those genomes. Moreover, the *atp8* gene was newly annotated in 20 mitochondrial genomes, where it was previously declared as lacking or only signaled. Supernumerary mitochondrial proteins were compared, but it was possible to find homologies only among strictly related species. The rearrangement rate on the molecule is too high to be used as a phylogenetic marker, but here we demonstrate for the first time in mollusks that there is correlation between rearrangement rates and evolutionary rates. We also developed a new index (HERMES) to estimate the amount of mitochondrial evolution. Many genomic features are phylogenetically congruent and this allowed us to highlight three main phases in bivalve history: the origin, the branching of palaeoheterodonts, and the second radiation leading to the present-day biodiversity.

Key words: mitochondrial genomics, comparative mitogenomics, bivalves, phylogeny, HERMES.

Introduction

Mitochondria are key eukaryotic organelles involved not only in the well-known synthesis of ATP through oxidative phosphorylation (OXPHOS; Mitchell 1961; Wallace 2013), but also in many other biological functions, such as intracellular signaling, cell differentiation, programmed cellular death, fertilization, and aging (Scheffler 2008; Tait and Green 2010; Van Blerkom 2011; Lane and Martin 2012; López-Otín et al. 2013; Sousa et al. 2013; Chandel 2014). Currently, it is widely accepted that mitochondria originated from a single endosymbiotic event that took place over a billion of years ago (Gray et al. 1999, 2001; Sicheritz-Ponten and Andersson 2001; Gray 2012). It has been proposed that the putative ancestor of all mitochondria was an alpha-proteobacterium (Müller and Martin 1999; Gray et al. 2001; Fitzpatrick et al. 2006; Williams et al. 2007; Atteia et al. 2009; Abhishek et al. 2011; Thrash et al. 2011; Degli Esposti et al. 2014).

The following evolution of mitochondria is still unclear and a matter of debate. In animals, mitochondrial DNA (mtDNA) is a small (~16 kb) and compact circular molecule that typically contains 13 OXPHOS-related genes, 2 rRNAs encoding for the two subunits of mitochondrial ribosomes, and an array of tRNAs used for translation within the organelle (Boore 1999; Breton et al. 2014). Most genes of the original endosymbiont were therefore lost or have been transferred to the nucleus during a process called Genome Reductive Evolution (GRE) (Andersson and Kurland 1998; Khachane et al. 2007; Ghiselli et al. 2013; Kannan et al. 2014). As claimed by Meisinger et al. (2008), in mammals no less than 1,500 proteins are needed to keep mitochondria alive and working. Why then only a small cluster of protein coding genes (PCGs) was spared by GRE—the typical 13 in metazoans, and even down to 3 in apicomplexans (Feagin 1994; Rehkopf et al. 2000)?

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Many tentative answers were proposed to this question. It is well known, for example, that mitochondria employ a genetic code slightly different from that of the nucleus, which would possibly lead to erroneous translations of some genes if transferred to nucleus (Adams and Palmer 2003); however, this is true for animals, but not for plants (Jukes and Osawa 1990), which also underwent mitochondrial GRE.

The high hydrophobicity of some of these proteins would hamper the import from the cytosol as well (von Heijne 1986; Popot and de Vitry 1990; Claros et al. 1995; Pérez-Martínez et al. 2000, 2001; Daley et al. 2002; Funes et al. 2002; Adams and Palmer 2003). Martin and Schnarrenberger (1997) also claimed that certain gene products are toxic in the cytoplasm. Finally, it has been proposed that the genes still encoded by mtDNAs must be efficiently and directly regulated by mitochondrial redox conditions, so they need mandatory colocalization for redox regulation (CoRR) (Race et al. 1999; Allen 2003a, 2003b; Lane 2007).

The sequencing and characterization of several complete mitochondrial genomes is a key to address GRE of mitochondria. Pioneering comparative works of large mtDNA datasets could recently, e.g., shed light on the reduction and ultimate loss in animals' mitochondria of ribosomal protein genes (Maier et al. 2013) and establish that the mitochondrion-encoded genes of almost all eukaryotes are different subsets of the mitochondrial gene complement of jakobids, a group of free-living, heterotrophic flagellates (Kannan et al. 2014).

At the same time, mitochondrial genomes of single metazoan groups often exhibit peculiar evolution that deserves a careful examination. In this regard, bivalves (Mollusca, Bivalvia) are among the most notable animal taxa, because of several interesting features. Most strikingly, some bivalves follow a non-canonical way of mitochondrial inheritance, the Doubly Uniparental Inheritance (DUI; Skibinski et al. 1994a, 1994b; Zouros et al. 1994a, 1994b). In these species, two separate sex-linked mitochondrial lineages (and their respective mtDNAs) are present, namely, the F and the M (Breton et al. 2007; Passamonti and Ghiselli 2009; Zouros 2013; Breton et al. 2014). F mitochondria are passed from the mother to the complete offspring, and they are typically found in the soma and in the female germline (eggs); the M mitochondria are passed from the father to the male offspring, and they are typically found in the male germline (sperms). However, these general DUI rules may hold imperfectly, thus resulting in a leakage of the M mitochondrial lineage in somatic cells of either sex (Chakrabarti et al. 2006; Batista et al. 2010; Kyriakou et al. 2010; Ghiselli et al. 2011; Obata et al. 2011). DUI represents a case of a triple genomic conflict (nucleus vs. F mtDNA; nucleus vs. M mtDNA; F vs. M mtDNAs) (Passamonti and Ghiselli 2009; Breton et al. 2014).

There are more outstanding features of bivalves mtDNA that are generally shared across the entire class. In fact, bivalve mitochondrial genomes are often very large, up to the 46,985 bp of *Scapharca broughtonii* (Liu et al. 2013); they

present many putative unassigned regions (URs) (Ghiselli et al. 2013); they may encode for supernumerary open reading frames (ORFans) (Breton et al. 2009; Milani et al. 2013); they show an unpaired degree of gene rearrangement (Vallès and Boore 2006; Simison and Boore 2008; Plazzi et al. 2013); they exhibit strong differences in strand usage (see Additional file 6 in Plazzi et al. 2013).

All this considered, the interest of bivalves in the wider picture of the evolution of animal mitochondria is self-evident. In this work, we present the first meta-analysis of 100 bivalve mtDNAs, corresponding to all the species whose mtDNA was available in GenBank in August, 2014.

The polyphyly of bivalves in molecular phylogenetic reconstructions is a well-known artifact (Giribet and Wheeler 2002; Giribet and Distel 2003; Passamaneck et al. 2004; Giribet et al. 2006; Sharma et al. 2012; Plazzi et al. 2013; Stöger and Schrödl 2013; Bieler et al. 2014) and it is probably related to the fact that a small group of bivalves (the protobranchs) separated very early and, regarding mitochondrial markers, before the burst of genomic novelties described above (Plazzi et al. 2013). In the present work, we do not address the issue of bivalve monophyly: therefore, the only protobranch bivalve whose complete mitochondrial genome is available in GenBank in August, 2014 is used as outgroup for phylogenetic analyses.

The comparative mitogenomics of bivalves is critically explored to (i) achieve a deeper knowledge about factors that shaped mitochondrial evolution in these metazoans, and (ii) focus on the specific features of bivalve mitochondrial PCGs.

Materials and Methods

The Database

Hundred bivalve complete mitochondrial genomes (supplementary file S1, Supplementary Material online) were downloaded from GenBank in August, 2014 using CLC Sequence Viewer 7.5 (Qiagen A/S, Aarhus). Annotations and sequences were imported and managed in Microsoft Excel® 2007 through custom functions and VBA macros that were also used to output files in the correct format for downstream analyses. In August, 2014 the annotation of *Tegillarca granosa* (Sun et al. 2015) was already published, but the sequence was not available yet; the sequence of *Nucula nucleus* (GenBank accession number EF211991) is still incomplete and unpublished: as a consequence, these species were included in the overall analysis, but not in alignments presented in this paper.

Alignment Algorithms

The first step of most analyses was a structural alignment and data masking: to this purpose, a custom tool was written in **bash** and R (R Development Core Team 2008) environments, loading the package *seqinr* (Charif and Lobry 2007). The *atp8*

gene and tRNAs were excluded from alignments because of many uncertainties in annotations.

This tool was called `masking_package` and is available with a brief tutorial from the GitHub repository https://github.com/mozoo/masking_package.git. It performs:

- (i) structural alignment using T-Coffee (Notredame et al. 2000) and nested packages PSI-BLAST (Altschul et al. 1997), Muscle (Edgar 2004), ProbconsRNA (Do et al. 2005), RNAplfold (Lorenz et al. 2011), and MAFFT (Katoh and Standley 2013), using the option series `PSI-Coffee > Espresso > accurate` for Protein Coding Gene (PCG) amino acids and the MR-Coffee mode for rRNA nucleotides;
- (ii) alignment masking in order to eliminate phylogenetic noise using the four softwares Aliscore 2.0 (Misof and Misof 2009), BMGE 1.1 (Criscuolo and Gribaldo 2010), Gblocks 0.91b (Castresana 2000), and Noisy (Dress et al. 2008), setting adequate options for distantly related sequences;
- (iii) comparison of outputs from different masking strategies in terms of number of sites and percentage of removed sites and output of a final concatenated alignment where only sites kept by at least *k* softwares are present (where *k* is up to the user and was set to 2, 3, or 4);
- (iv) computation of the number of sites selected by all the possible combinations of the four softwares.

All the `masking_package` scripts adapted for amino acid (PCGs) and nucleotide (rRNAs) alignments of the present study are available as [supplementary files S2 and S3](#), [Supplementary Material](#) online, respectively.

Split *rrnL* genes of ostreids were concatenated prior to alignment. Moreover, all *Crassostrea* species (with the exception of *C. virginica*) have a duplicated *rrnS* gene. In these cases, the two copies were aligned with MR-Coffee, a consensus sequence was computed using `seqinr` and the result was inserted into the final alignment.

Genomic Features

Five large-scale features of bivalve mtDNAs were extracted: length, percentage of URs, number of genes (including newly annotated ones; see below), and A-T and G-C skew following Reyes et al. (1998). Instead of a typical ANOVA, because of non-normality and non-homoscedasticity of data, differences between subclasses had to be tested using a non-parametric alternative: we used the Kruskal–Wallis test (Kruskal and Wallis 1952), while relative pairwise comparisons were carried out using the Dunn's test (Dunn 1964). Given the poor sample size of Anomalodesmata (*N* = 1) and Opponobranchia (*N* = 2), these subclasses were excluded from these preliminary analyses.

Afterwards, single alignments were used as input for several custom `bash` and R scripts that were used to:

- (i) subsample the original dataset, producing subsets corresponding to largest families (Mytilidae, Ostreidae, Pectinidae, Unionidae, Veneridae) and subclasses (Heterodonta, Palaeoheterodonta, Pteriomorphia) to be aligned and masked;
- (ii) compute the percentage of cases in which each site of each gene was kept after the masking phase, using the agreement of at least 3 out of 4 masking softwares (across the five families, the three subclasses, and in the complete-dataset analysis);
- (iii) comparing masking results using a one-sided *t*-test;
- (iv) back-translate the original, unmasked amino acid alignment into nucleotides;
- (v) use EMBOSS (Rice et al. 2000) to compute different metrics of pairwise and overall distances, both on total gene length (using `distmat`) and over a sliding window (using `plotcon`);
- (vi) compare nucleotide and amino acid distances through the Kolmogorov–Smirnov test;
- (vii) test alignments for saturation by plotting uncorrected *p*-distance over the Jin–Nei distance (for nucleotides; Jin and Nei 1990) or the Kimura distance (for amino acids; Kimura 1983);
- (viii) perform a Principal Component Analysis (PCA) on concatenated distance matrices, using the FactoMineR (Lê et al. 2008) package for computations and `ggplot2` (Wickham 2010) for graphics.

The ratio of non-synonymous vs. synonymous substitutions (d_N/d_S) was computed for back-translated alignments using PAML 4.8a (Yang 1997, 2007); d_N/d_S was computed both for each pair of sequences and along the phylogenetic tree (see below). In the latter case, we used the Likelihood Ratio Test (LRT) to compare the fitting of single d_N/d_S for the complete tree with the use of 12 different d_N/d_S , allowing different d_N/d_S ratios for different clades. All LRTs were carried out in the R environment; to be conservative, we used a chi-square distribution with 11 degrees of freedom (Wong et al. 2004).

Phylogenetic Analyses

Using the original annotations available in GenBank, phylogenetic analysis was carried out on PCGs and rRNAs; given many uncertainties in annotation and orthology, the *atp8* gene and tRNAs were excluded from the analysis. The best-fitting partitioning scheme and molecular evolution models were estimated using PartitionFinderProtein and PartitionFinder 1.1.0 (Lanfear et al. 2012) under the Bayesian Information Criterion and a greedy approach. Gaps were coded following the simple indel method of Simmons and Ochoterena (2000) as implemented in GapCoder (Young and Healy 2003). As a result, the final alignment spanned over 14 genes and three types of data: amino acids for PCGs, nucleotides for rRNAs, and binary data for coded gaps.

The software RAXML 8.2.0 (Stamatakis 2014) was used to infer Maximum Likelihood (ML) phylogeny. The strand usage is variable across different bivalve mtDNAs: while in most cases

all genes locate to the same strand, genes are evenly distributed on both strand in *N. nucleus*, *S. velum*, and Unionidae. As a strand usage bias can be associated with a compositional bias, which may in turn lead to phylogenetic artifacts, preliminary analyses with 500 bootstrap replicates were separately conducted on those genes mapping on the “+” and on the “-” strand of unionids. If a significant compositional bias is working, we expect to detect possible phylogenetic artifacts by recovering different topologies from the two analyses.

The final tree was computed as follows; explicit RAxML commands are listed in [supplementary file S4, Supplementary Material](#) online.

- (i) five randomized maximum parsimony (MP) starting trees were generated;
- (ii) a maximum likelihood (ML) tree was inferred from each MP starting tree with a fixed rearrangement radius of 10 and with an automatically determined one, choosing the setting yielding the best results;
- (iii) a ML tree was inferred from each MP starting tree with the selected initial rearrangement option under different number of rate categories (10, 25, 40, or 55) for the CAT model accounting for evolutionary rate heterogeneity (Stamatakis 2006), choosing the setting yielding the best results;
- (iv) the best-known likelihood tree was inferred from the original alignment under the selected rearrangement and rate categories options calling 10 runs from 10 randomized MP starting trees;
- (v) 500 bootstrap replicates were run; and
- (vi) confidence values were computed from bootstrap replicates and annotated on the best-known likelihood tree.

The software MrBayes 3.2.1 (Ronquist et al. 2012) was used to carry out Bayesian Inference (BI) using 2 separate runs, 4 chains, and 10,000,000 generations of MC³, sampling every 100 trees. Convergence between runs and burn-in were estimated looking to standard deviation of average split frequencies sampled every 1,000 generation and to Potential Scale Reduction Factor (PSRF) (Gelman and Rubin 1992).

The best-known likelihood tree computed by RAxML was used to obtain a time-scaled chronogram using r8s 1.70 (Sanderson 2003) and the r8s bootstrap kit by Torsten Eriksson (downloaded from <https://github.com/TorstenEriksson/r8s-bootstrap> in February, 2015). First appearance data for six calibration point were downloaded from the Paleobiology Database (<http://fossilworks.org>) in February, 2015: the root of all bivalves, Mytilidae, Ostreidae, Pectinidae, Unionidae, and Veneridae (M'Coy 1847; Tillyard and Dunstan 1916; Crompton and Parrington 1955; Drysdall and Kitching 1963; Nakazawa and Newell 1968; Brasier and Hewitt 1978; Baird and Brett 1983; Grasso 1986; Brett et al. 1991; Mergl and Massa 1992; Kříž 2008; Nesbit et al. 2010). We used the Penalized Likelihood method and the truncated Newton algorithm; the cross-validation approach allowed us to estimate the best smoothing parameter up to the sixth

decimal digit and five restarts and five guesses were used each round.

Following the r8s bootstrap kit procedure, 163 bootstrap replicates of the original alignment were generated and the original tree was optimized on each of them; node ages were estimated under the same r8s setting, estimating the best smoothing parameter up to the second decimal digit. The 99% confidence intervals of each node age were computed using custom R script loading the package ape (Paradis et al. 2004) and following methodological recommendations of Hyndman and Fan (1996), i.e., type = 8. Trees were graphically edited using the software PhyloWidget (Jordan and Piel 2008) and Dendroscope 3.3.2 (Huson and Scornavacca 2012).

Phylogenetic Informativeness (PI) was investigated on the amino acid dataset using the PhyDesign portal (López-Giráldez and Townsend 2011) and Rate4Site (Pupko et al. 2002) to estimate site-specific evolutionary rates. Best-fitting amino acid evolutionary models were selected using ProtTest 3.4 (Darriba et al. 2011) and PhyML (Guindon and Gascuel 2003). PI was computed over five different epochs, whose boundaries were taken from the International Stratigraphic Chart v2015/01 (Cohen et al. 2013): Quaternary, “Cenozoic” (Paleogene + Neogene), Mesozoic, “Paleozoic” (from Ordovician to Permian), and “Cambrian” (from 520 to 485.4 Mya). Following Townsend et al. (2012) and Simmons et al. (2004), the phylogenetic signal and noise analysis was carried out in a state space of five.

Finally, we investigated the correlation between gene rearrangements and substitution rates, which has been demonstrated for insects by Shao et al. (2003) and hypothesized for mollusks by Stöger and Schrödl (2013). However, the RGR test of Downton (2004) and its modified version of Xu et al. (2006) cannot be useful in the case of bivalves to describe gene arrangement variability because it is a relative rate test that involves a comparison with the ancestral gene arrangement. As detailed in Plazzi et al. (2013), this is most likely that of the chiton *Katharina tunicata*, but, with the notable exception of *Solemya velum* and *Nucula nucleus* (Plazzi et al. 2013), most bivalve gene orders are not comparable with each other and seem to be equally very different from that. Therefore, while there is no remarkable difference in terms of distance from the ancestral state, higher rates of mitochondrial gene rearrangement are straightforward in some clusters with respect to others, but this would not be detected by RGR: for this reason, we quantified rates of mitochondrial rearrangement in the clades obtained in the best-known likelihood tree using the architecture rate (AR) as introduced by Gissi et al. (2008). This is given by

$$AR = \frac{N_{GA} - 1}{N_{OTU} - 1}$$

where N_{GA} is the number of different gene arrangements found in a clade and N_{OTU} is its number of OTUs. In fact, this index conservatively estimates the number of gene

rearrangement events along a clade's evolutionary history, as it must be at least as large as the number of different gene arrangements found in extant taxa, and normalizes it with the number of species, so that clades of different sizes are comparable. AR rate ranges from 0 (the whole clade shares the same gene arrangement) to 1 (each OTU shows a different gene arrangement). The sum of branch lengths for a given clade was computed with the software Phylocom 4.2 (Webb et al. 2008), thus estimating the total number of expected substitution per site in a given cluster; this was divided by the root age in millions of years (see above). The correlation of AR rate and substitution rate was assessed with the Spearman's rho and Kendall's tau tests using R.

Annotation of the *atp8* Gene and of ORFans

The EMBOSS suite was used to find all the possible Open Reading Frames (ORFs) in all the complete mitochondrial genomes, using all the six possible frames. A Hidden Markov Model (HMM) was constructed for each ORF using HHblits 2.0 (Remmert et al. 2012) and the latest PDB70 release. All the HMMs were merged in a custom database, which was called Biv_mtDNA_ORFs.

All the annotated *atp8* genes were aligned using the T-Coffee (again following the PSI-Coffee > Expresso > accurate option series). A Hidden Markov Model (HMM) was constructed using HHblits 2.0 and the latest Uniprot release and a HMM-HMM alignment was run against the Biv_mtDNA_ORFs database to check for homology with *atp8*. Positive results were manually screened and original annotations were consequently updated.

Bivalve supernumerary mitochondrial ORFs do not have known homologs and are therefore considered as ORFans (Fischer and Eisenberg 1999). However, it is possible to find homologies at least within Bivalvia. All the published supernumerary ORFs (Breton et al. 2009; Milani et al. 2013; Plazzi et al. 2013; Bettinazzi et al. 2016; GenBank accession numbers HM856636, HQ283344, KC848655, KF030963, NC_015310, NC_015476, NC_015477, NC_015479, NC_015481, NC_015483, NC_018763, NC_022803, NC_023250, NC_023942) were annotated; a HHblits database of all the known ORFans was created as above using Biv_mtDNA_ORFs and was called Biv_mtDNA_ORFans; finally, HHblits was used for each ORFan against this custom database to investigate putative relationships between known ORFans.

Summarizing Data

A PCA was carried out as above using many different parameters (supplementary file S5, Supplementary Material online): nucleotide composition; AT content; A-T and G-C skews; use of between-genes overlapping nucleotides; length; percentage of Unassigned Regions (URs); total number of genes; the strand usage skew (SU skew); number of truncated T→TA-stop codons in the genome; the Amount of Mitochondrial

Identical Gene Arrangements (AMIGA). SU skew and AMIGA were defined as follows.

$$\text{SUskew} = \frac{H - L}{H + L}$$

where H is the number of genes on the H strand and L is the number of genes on the L strand. If the strand usage is perfectly balanced (i.e., $H=L$), the SU skew is equal to 0; a negative SU skew indicates a bias towards the L strand, while a positive SU skew indicates a bias towards the H strand.

$$\text{AMIGA} = \frac{N_{\text{IGA}} - 1}{N - 1}$$

where N_{IGA} is the number of taxa in the analyzed sample that share an Identical Gene Arrangement and N is the total number of taxa. As a consequence, unique gene arrangements will have an AMIGA score equal to 0. Conversely, when all the taxa in a given sample share the same gene arrangement, each AMIGA score will be equal to 1. An intermediate value expresses an intermediate value of conservation. Given many uncertainties in annotations, rRNAs and tRNAs were excluded from the analysis, therefore the AMIGA index relies solely on PCGs.

The HERMES Index

A widespread method of quantifying molecular evolution of mitochondrial genomes in different species and clusters is still lacking for mitogenomic analyses. We developed a new index in this regard, which relies on maximum likelihood factor analysis to summarize different measures that are typically found to be linked with evolutionary rates; it is intended to be computed *a posteriori*, i.e. after the phylogenetic and genomic analysis. As different empirical measures are merged together in a single score, this is a "hyper-empirical" index. Moreover, a taxon retaining most genomic plesiomorphies of the group (at least following state-of-art knowledge) is selected as a benchmark: thus, it is a relative measure, and that taxon was in our case *N. nucleus*. All this considered, the index was called Hyper-Empirical Relative Mitochondrial Evolutionary Speed (HERMES) index.

We explored the use of different subsets of the following variables to compute the HERMES index:

- (i) the AT content;
- (ii) the genome length;
- (iii) the number of (annotated) genes;
- (iv) the percentage of URs;
- (v) the absolute value of SU skew;
- (vi) AMIGA;
- (vii) the root-to-tip distance computed on the best-known likelihood tree using Phylocom 4.2;
- (viii) the ML distance from *N. nucleus* computed with RAxML specifying the model as above.

The factor analysis was carried out using the psych (Revelle 2014) package of R; the plot was prepared using the ggplot2 package. Normalization and varimax rotation were used, factor scores were found using correlation preserving, and correlations were found using the Pearson method; given the possible presence of a missing value (as *T. granosa* was not included in the phylogeny and we were therefore unable to compute either the root-to-tip and the ML distance), missing data were set to be imputed using the median. All the variables were pooled together for each species into the value of a single loading: we define this score as the HERMES score of a given species.

The best-performing variable set and the goodness-of-fit of the analysis was assessed following the recommendations of Hu and Bentler (1999): Tucker–Lewis Index (TLI) (Tucker and Lewis 1973) > 0.95; root mean square of the residuals (SRMR) < 0.08; root mean squared error of approximation (RMSEA) < 0.06; moreover, the Kaiser–Meyer–Olkin index (KMO) (Kaiser 1970) was taken into account on this regard.

A Python script was written to compute the HERMES scores of a given assemblage of species, providing the GenBank annotation, gene alignments and a phylogenetic tree; this software can be downloaded from the GitHub repository at the URL <https://github.com/mozoo/HERMES.git>, along with sample data and a tutorial.

Results

Overall Genomic Features

The 100 mitochondrial genomes (mtDNAs) of the present study range from 14,622 (*Lanternula elliptica*) to 46,985 (*Scapharca broughtonii*) bp in length. Excluding the abnormally large genomes of Arcidae, the longest mtDNA is that of *Placopecten magellanicus* (32,115), followed by the female genome of *Venerupis philippinarum* (22,676 bp). Conversely, the proportion of putatively Untranslated Regions (URs) span from 1.44% (*L. elliptica*) and 2.13% (*S. velum*) to the high scores of Pectinidae (29.86–51.04%) and Arcidae (52.30–70.76%). The number of annotated genes on the molecule is much more stable: exception made for *Scapharca kagoshimensis* and *S. broughtonii* (55 and 54, respectively), this number span from 30 (*Mizuhopecten yessoensis*) to 46 (*P. magellanicus*), with a mean value of 38.11 ± 3.20 .

As summarized in figure 1, mtDNA length and %UR increases in the order Palaeoheterodonta < Pteriomorphia < Heterodonta. Palaeoheterodonta are in both cases significantly different from either Pteriomorphia or Heterodonta ($P < 0.001^{***}$; [supplementary file S6, Supplementary Material](#) online). The Kruskal–Wallis test detected a significant location difference also for the number of annotated genes, which is due to the Pteriomorphia/Heterodonta comparison only ($P < 0.05^*$; [supplementary file S6, Supplementary Material](#) online). Finally, each subclass was significantly

different from each other when considering A-T and G-C skews—again, comparisons involving Palaeoheterodonta showed the highest significance values ($P < 0.001^{***}$; [supplementary file S6, Supplementary Material](#) online). Single-species data used in this study are extensively listed in [supplementary file S5, Supplementary Material](#) online.

Notwithstanding the large variability, especially in length and %UR, the genome content is quite stable: if we do not take tRNAs into account, all the mtDNAs share the same gene content and few duplication events are present. The most evident duplication is that of the *rnrS* gene in all species of the genus *Crassostrea* (with the exception of *C. virginica*), whose genes show a divergence between 0.11% (*C. iredalei*) and 5.05% (*C. ariakensis*), as estimated using uncorrected *p*-distance (mean = 2.92%). Moreover, as already signaled (Milbury and Gaffney 2005; Ren et al. 2010), in all Ostreidae species the *rnrL* gene is split in two separate fragments. Finally, a duplication of *cox2* was already signaled in *Musculista senhousia*, male type (Passamonti et al. 2011), and *V. philippinarum*, female type (GenBank Accession Number AB065375).

Alignments

Protein Coding Genes (PCGs) alignment lengths range from 186 amino acids of *nad3* to 1,313 amino acids of *cox2* (before masking). The amount and percentage of sites selected by at least 2, 3, or 4 softwares is detailed in [supplementary file S7, Supplementary Material](#) online. The use of three masking softwares out of four is a good compromise between the elimination of noise and the elimination of apparently noisy useful sites, therefore this was taken as our preferred setting. All alignments are available from FP upon request.

The masking step affected different genes differently: *cox1* and *cytb* are the genes that were least affected during masking phase (68.63% and 60.13% of their sites were kept, respectively), while *cox2*, *nad4L*, and *nad6* alignments were heavily reduced after this phase (to the 16.45%, 18.32%, and 21.73% of the original size, respectively). As expected, rRNA alignments are much longer (4,210 and 2,891 bp for *rnrL* and *rnrS*) and much more shortened after the masking phase (to the 14.06% and 11.45% of the original size, respectively).

The amount of shortening was not reduced when smaller, more-related subsets were analyzed ([supplementary file S8, Supplementary Material](#) online). Differences in the percentages of kept sites among different subsets were never significantly higher when moving towards a smaller, nested taxon, but rather in some cases they were significantly lower (Bivalvia > Pteriomorphia, $P < 0.001^{***}$; Palaeoheterodonta > Unionidae, $P < 0.05^*$; Pteriomorphia > Pectinidae, $P < 0.01^{**}$).

Actually, in all the cases, phylogenetically-informative sites are almost the same, being the dataset a single family, a subclass, or the whole class (fig. 2). The longest conserved

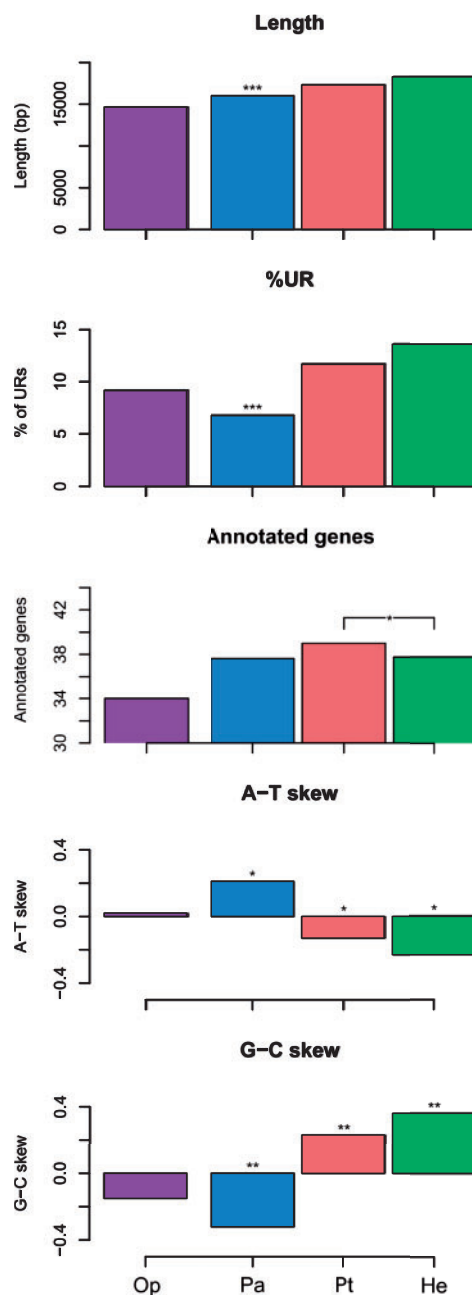


Fig. 1.—Main features of mitochondrial genomes. Five large-scale mitochondrial features are compared across four bivalvian subclasses (the fifth subclass, Anomalodesmata, was excluded because only one mitogenome was available): Op, Opponobranchia (purple); Pa, Palaeoheterodonta (blue); Pt, Pteriomorphia (red); He, Heterodonta (green). Length, median length of the genome; %UR, median percentage of Untranslated Regions; Annotated genes, mean number of annotated genes; A-T skew, median A-T skew; G-C skew, median G-C skew. Differences between subclasses were tested using the Kruskal–Wallis and the Dunn’s test; Anomalodesmata and Opponobranchia were excluded because of low sample size ($N=1$ and $N=2$, respectively). Asterisks above columns refer to levels of significance in both pairwise comparisons or in a single one (bracketed); * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$.

domains are those of *cox1* and *cytb*, even if also *nad* genes (with the exception of *nad4L* and *nad6*) show extended well-aligned regions. Interestingly, *nad4* is the only case of a conserved domain that appears with reduced and less variable alignments: about 120 amino acids at the N-terminus of the protein are not conserved when the complete dataset is considered, but are increasingly more conserved at the subclass and family level (fig. 2). The similarity measure of *plotcon* over a sliding window show highest values for the most conserved regions, as expected (supplementary files S9 and S10, Supplementary Material online).

Uncorrected (p -) distances are slightly, but significantly ($P=0$), higher for amino acids than for nucleotides; however, when a more complex model is used to account for multiple substitution events at a single site, the situation is the opposite, and nucleotide distances are significantly ($P=0$) higher than amino acid ones (fig. 3). Moreover, with the exception of the low-scoring *cox1*, all uncorrected distances are comparable among genes, while using the Jin–Nei/Kimura method (for nucleotides/amino acids, respectively), some genes (namely, *atp6*, *nad2*, *nad4L*, and *nad6*) have higher distance values than others (fig. 3). A similar scenario is retrieved through saturation plots. The uncorrected distance was plotted on Jin–Nei/Kimura distance: while in many cases uncorrected distances tend to increase with the other model, the same four genes show a plateau, indicating that p -distance is not able to uncover multiple hits and that a significant degree of saturation is present. As an example, *cytb* and *nad6* plots are shown in figure 4, while all genes are detailed in supplementary file S11, Supplementary Material online. Expectedly, amino acid (aa) plot is farther from the plateau than nucleotide (nt) plot in all cases.

A PCA was carried out using pairwise nucleotide and amino acids distances as variables for each gene; the result is shown in figure 5. Taken together, the first two principal components explain the 85.64% of the variance, but it has to be noted that the first component alone explains the 77.62%. The PCA (and specifically the first component) clearly separates *atp6*, *nad2*, *nad4L*, and *nad6* from other genes.

Finally, the d_N/d_S ratios for each gene are given in table 1. In all cases, the null hypothesis that a single d_N/d_S applies to all the tree branches was not rejected by the LRT ($P=1$); the d_N/d_S value computed along the entire tree is one or two orders of magnitude higher than the median of pairwise comparisons (table 1). The highest values are shown by *nad6* ($d_N/d_S = 0.344$; median of pairwise comparisons = 0.045), while the lowest are shown by *cox1* ($d_N/d_S = 0.080$; median of pairwise comparisons = 0.004). However, namely in *atp6*, *nad4L*, and *nad6*, a quite large number of high (i.e., greater than 10) pairwise d_N/d_S values were computed (table 1). In the vast majority of cases, these values expectedly come from pairs of distantly related species.

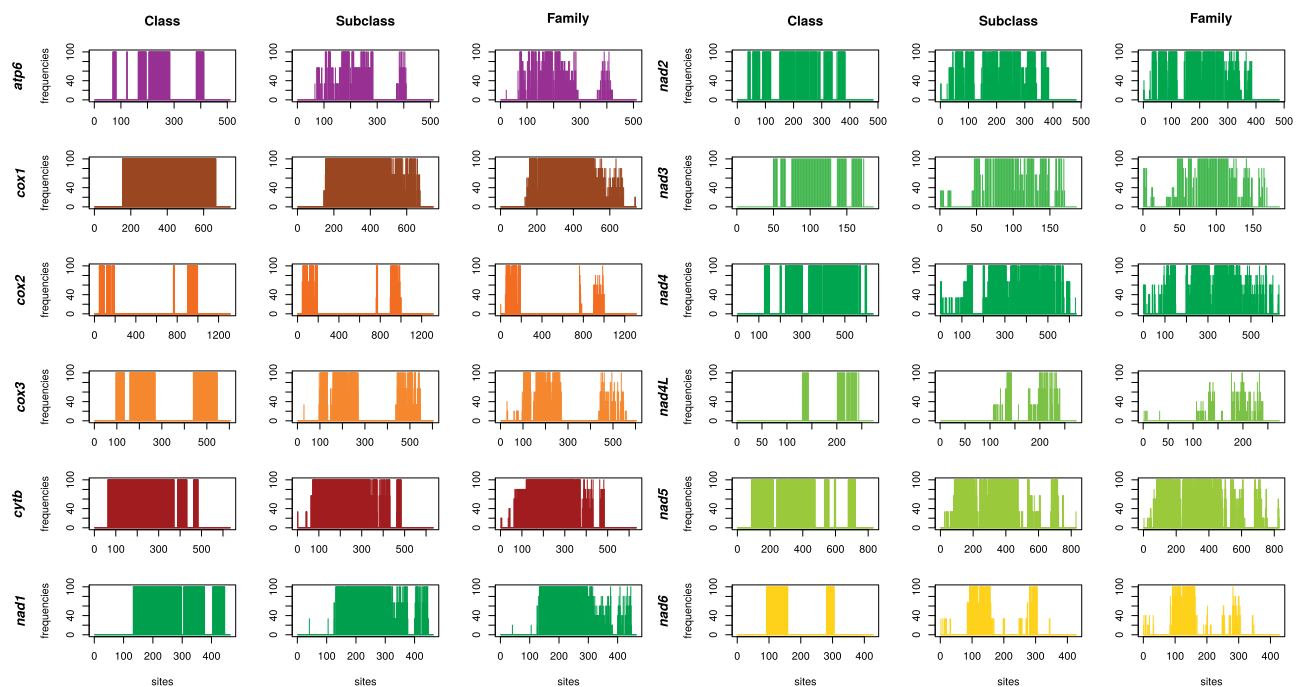


Fig. 2.—Conserved sites of protein coding genes. Single genes were aligned, masked using four different approaches, and finally only sites selected as phylogenetically informative by at least three softwares were kept. Whenever possible in terms of sample size, the same procedure was carried out for reduced datasets, corresponding to single subclasses (3 datasets) and single families (5 families). Each chart shows the proportion of datasets keeping single sites: this is 0 (discarded site) or 100 (kept site) at the class level, and ranges from 0 to 100 at the subclass and family level, because more datasets are available.

Phylogenetic Analysis

The two subsets of markers (i.e., genes that are on the “+” strand and genes that are on the “-” strand of Unionioidea) yielded the same topology in the preliminary analyses, therefore the final tree was computed on the complete set of genes. The best partitioning scheme selected by PartitionFinder separated *atp6* + *nad* genes, cytochrome subunit genes, and rRNAs (which were taken together; see [supplementary file S12, Supplementary Material](#) online, for details).

The best-known likelihood tree annotated with support values is shown in figure 6 over a geological timescale, while original phylograms are shown in [supplementary files S13, Supplementary Material](#) online (best-known likelihood tree with bootstrap proportions) and 14 (Bayesian consensus tree). Both Bootstrap Proportions (BPs) and Bayesian Posterior Probabilities (PPs) are generally high: as BP is typically lower than PP, we conservatively show the consensus tree after collapsing nodes with $BP > 70$. After the separation of the outgroup *S. velum*, the tree is divided into two major branches: Palaeoheterodonta ($BP = 100$, $PP = 1.00$) and Amarsipobranchia *sensu* Plazzi et al. (2011) ($BP = 97$, $PP = 1.00$). Within Palaeoheterodonta, M and F genomes (if non-DUI species genomes are considered as F ones) cluster separately, both with $BP = 100/PP = 1.00$; the branching order

is also the same. Within Amarsipobranchia, relationships between *L. elliptica* (Anomalodesmata), Pteriomorpha ($BP = 91$, $PP = 1.00$), and Heterodonta ($BP = 73$, $PP = 1.00$) are not resolved. Within Pteriomorpha, Mytilidae ($BP = 100$, $PP = 1.00$) are the sister group of all remaining pteriomorphians ($BP = 79$, $PP = 1.00$). With the exception of *M. californianus* M, DUI genomes of *Mytilus* spp. cluster by sex, as in Palaeoheterodonta, while *M. senhousia* genomes cluster by species. Within Heterodonta, Lucinindae (*Loripes lacteus* + *Lucinella divaricata*, $BP = 100$, $PP = 1.00$) are the sister group of all remaining heterodonts ($BP = 100$, $PP = 1.00$). All heterodont DUI genomes (i.e., *M. lamarckii* and *V. philippinarum*) cluster by species.

The main split of the class (Palaeoheterodonta, one side, and Amarsipobranchia, the other side) took place about 500 million years ago (Mya), with a confidence interval (CI) of about 4.5 million years (My). The origin of pteriomorphians is dated at 484 mya ($CI = 6$ My), while the origin of heterodonts is dated slightly later, 454 mya ($CI = 16$ My). Oldest families are those of mytilids (421 Mya), pectinids (384 Mya), and venerids (336 Mya). All details about time calibration, including node names, mean estimates across bootstrap replicates, and CIs are shown in [supplementary files S15 and S16, Supplementary Material](#) online.

Different genes have different Phylogenetic Informativeness (PI) scores for different periods (fig. 6). Most

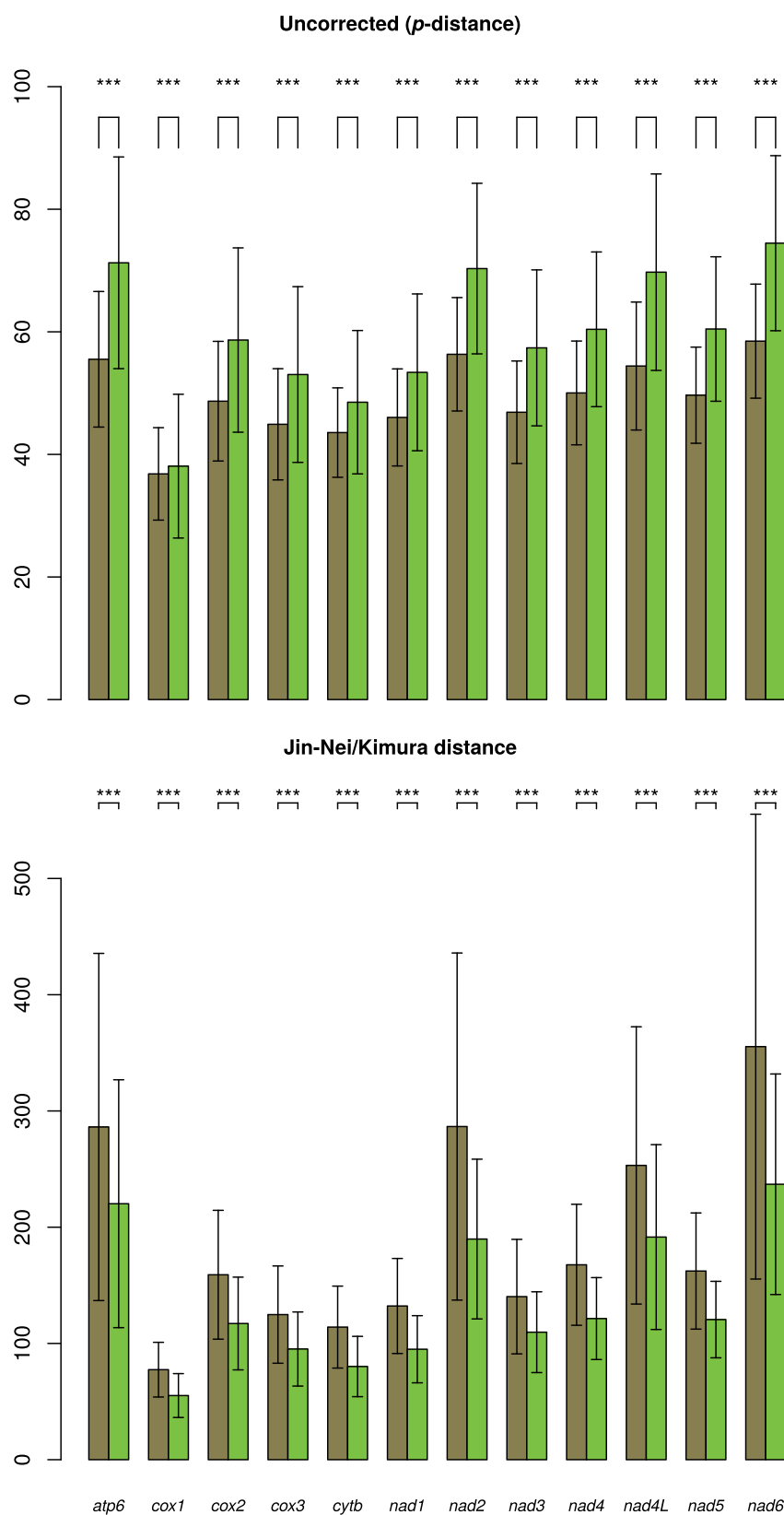


FIG. 3.—Nucleotide and amino acid genetic distances. The mean genetic distance across the complete dataset \pm one standard deviation is shown for each PCG; brown, nucleotides; green, amino acids. Differences in distance distributions were tested using the Kolmogorov–Smirnov test; *** $P < 0.001$.

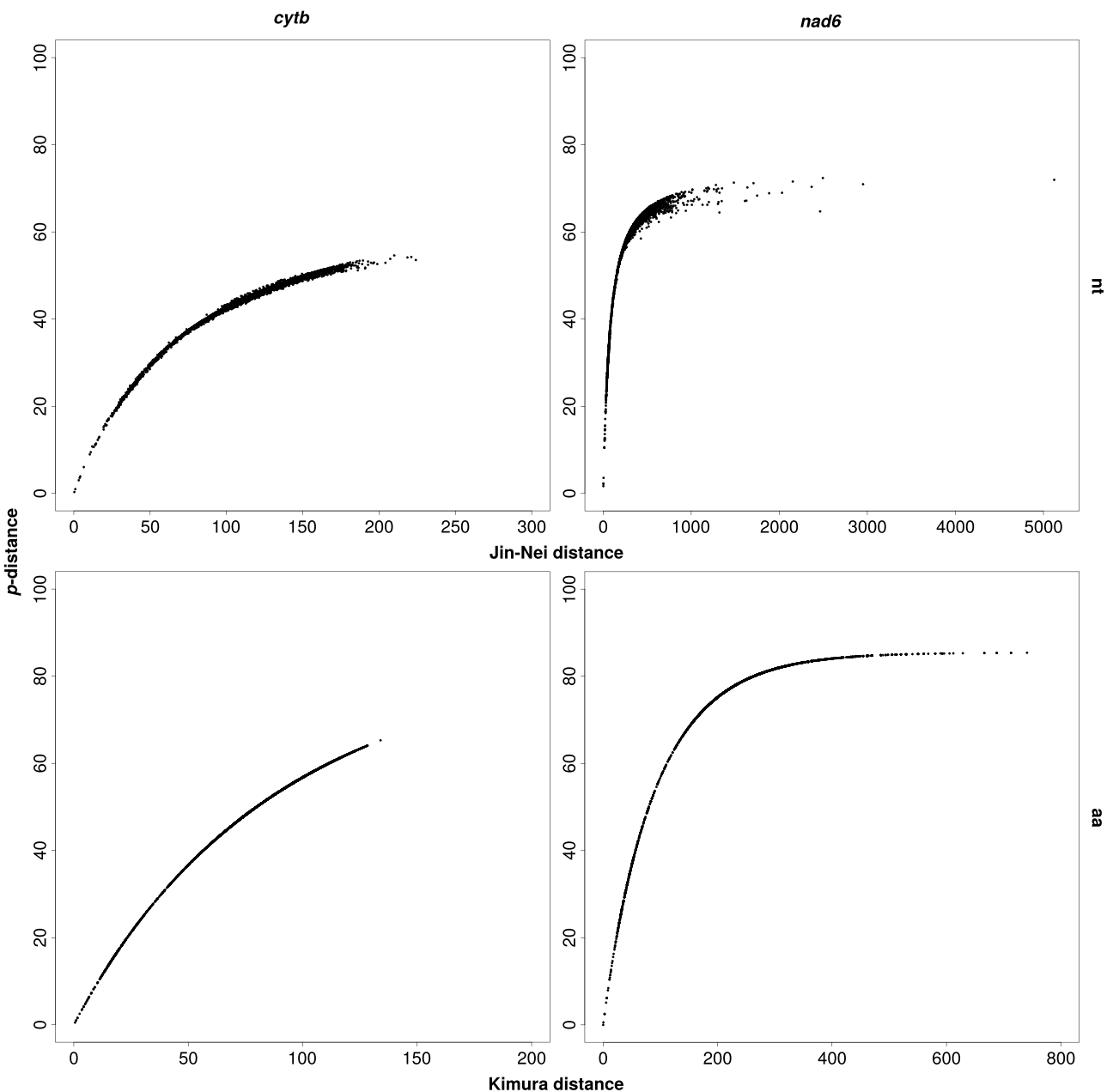


FIG. 4.—Example of four saturation plots. Pairwise p -distance is plotted over Jin–Nei/Kimura distance for nucleotides (above)/amino acids (below), respectively; nt, nucleotides; aa, amino acids. All saturation plots are available as [supplementary file S11, Supplementary Material](#) online.

nad genes and the *cytb* gene reach their peak in the Cenozoic, while *cox* genes and *atp6* reach it in the Cretaceous. Notably, the peak of *cox1* informativeness is shifted back to the Jurassic–Cretaceous limit and decreases slowly towards the deeper past. In noise vs. signal analysis ([supplementary file S17, Supplementary Material](#) online) *cox1*, *cytb*, *nad1*, and *nad5* typically outperformed all the other markers, although the complete dataset always shows much higher values than single genes.

The AR and substitution rates ([supplementary file S18, Supplementary Material](#) online) showed a good correlation

when six clades were used, corresponding to the major clades of the tree (Palaeoheterodonta F, Palaeoheterodonta M, Mytilidae, other Pteriomorphia, Lucinidae, other Heterodonta). The value of Spearman's rho was 0.90 ($P < 0.01^{**}$) and that of Kendall's tau was 0.83 ($P < 0.05^*$).

Annotation of the *atp8* Gene and of ORFans

Twenty *atp8* genes were annotated using the HHblits approach; they are listed in table 2. All the newly annotated *atp8* had a homology probability $> 95\%$, E -value < 0.01 ,

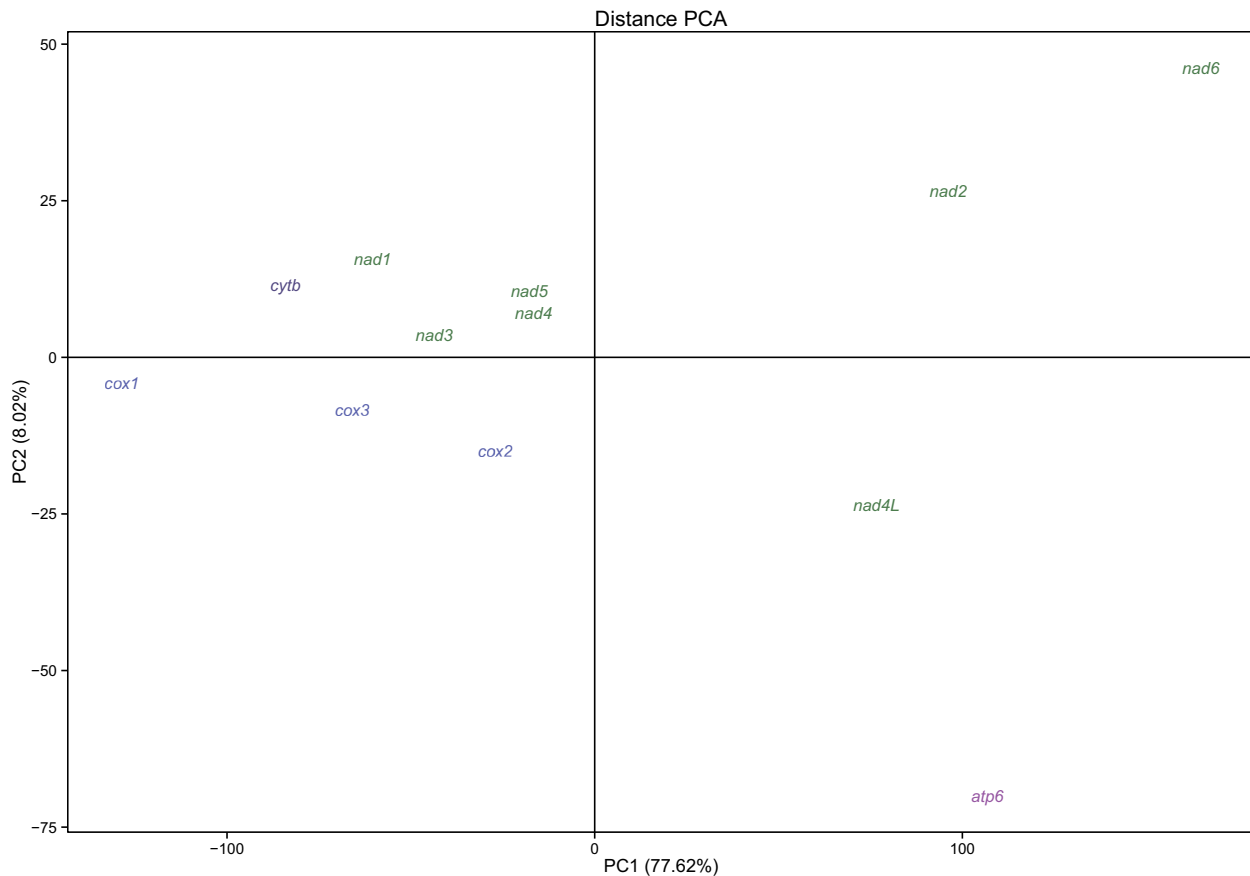


Fig. 5.—Distance PCA. Single-gene pairwise distance matrices were used as input data in order to investigate differences between PCGs.

Table 1

Values of d_N/d_S

Genes	d_N/d_S	Pairwise median	$d_N/d_S > 10$
<i>atp6</i>	0.255	0.023 ± 6.030	41
<i>cox1</i>	0.080	0.004 ± 1.436	1
<i>cox2</i>	0.222	0.008 ± 1.436	1
<i>cox3</i>	0.155	0.009 ± 0.027	0
<i>cytb</i>	0.164	0.008 ± 0.017	0
<i>nad1</i>	0.162	0.009 ± 0.026	0
<i>nad2</i>	0.310	0.019 ± 2.949	9
<i>nad3</i>	0.216	0.009 ± 1.461	2
<i>nad4</i>	0.230	0.010 ± 0.022	0
<i>nad4L</i>	0.312	0.032 ± 8.441	67
<i>nad5</i>	0.252	0.008 ± 0.020	0
<i>nad6</i>	0.344	0.045 ± 11.223	103

d_N/d_S , value of d_N/d_S computed by PAML using the best-known likelihood tree (see text for details) as the given phylogeny; pairwise median, median of all pairwise comparisons ± standard deviation; $d_N/d_S > 10$, number of pairwise d_N/d_S ratios greater than 10.

and P -value $< 1 \times 10^{-6}$. The only exceptions to these are the *atp8s* in *Paphia euglypta* (Prob. = 92.7%, E -value = 0.071, P -value = 4.8×10^{-6}), *Acanthocardia tuberculata*

(Prob. = 86.6%, E -value = 0.46, P -value = 3.1×10^{-5}), and *Musculista senhousia* M (Prob. = 80.6%, E -value = 0.74, P -value = 5.0×10^{-5}): as the *atp8* gene was not annotated in these species, it is highly probable that the homology is correct, but they anyway should be regarded as only tentatively annotated; the complete list of HHblits scores is available as [supplementary file S19, Supplementary Material](#) online. The annotation of *atp8* led us to make small changes to the original GenBank annotations; these, along with other corrections of minor flaws that we detected during the analyses, are listed in [supplementary file S20, Supplementary Material](#) online, where the complete, updated annotations of all the present mtDNAs are given.

Conversely, ORFans seem to be poorly connectible across the entire class: the complete list of HHblits clusters of homologs shows that any given ORFan shows sharp similarities only with ORFans of strictly related species ([supplementary file S21, Supplementary Material](#) online). Indeed, ORFans from Unionidae are phylogenetically clumped in the same clusters that appear in the tree (fig. 6) and, notably, F and M ORFans are never intermingled. The same apply for F ORFans of mytilids, but not for M ORFans, whose homology scores are always

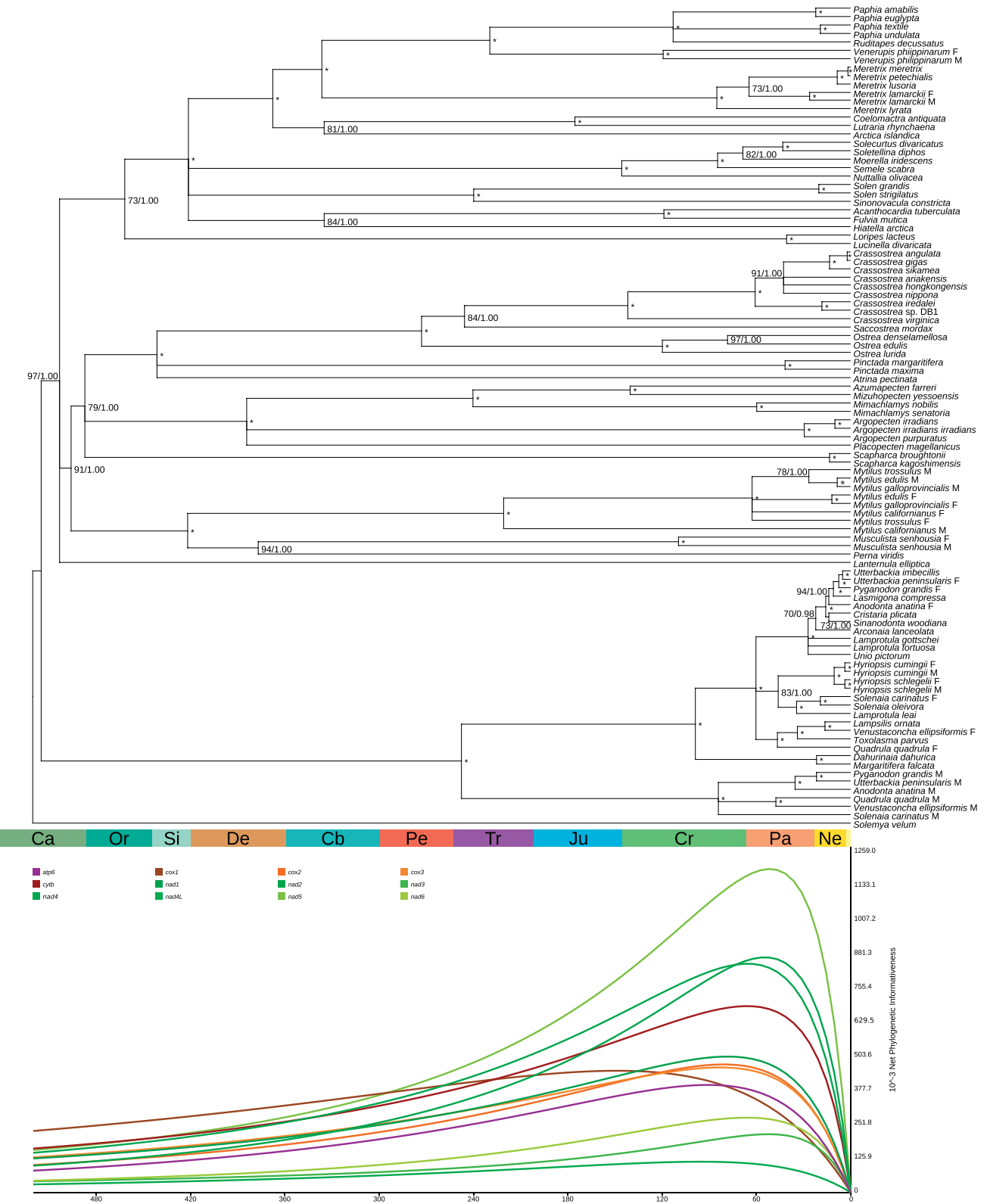


Fig. 6.—The mitochondrial phylogeny of bivalves. Ultrametric tree of 98 bivalve species computed using r8s starting from the best-known likelihood tree computed with RAxML and the consensus Bayesian tree. RAxML bootstrap values/MrBayes posterior probabilities are printed at each node (an asterisk means a bootstrap of 100 and a posterior probability of 1.00). Below the tree the phylogenetic informativeness for each PCG is shown. The geological timescale in Mya follows Cohen et al. (2013).

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Table 2Newly annotated *atp8* genes

Species	Start	Stop	Length	aa
<i>Mytilus galloprovincialis</i> M	8,530	8,871	345	115
<i>Mytilus edulis</i> M	9,789	10,133	345	115
<i>Mytilus californianus</i> F	8,735	9,037	303	101
<i>Mytilus galloprovincialis</i> F	8,802	9,062	264	88
<i>Mytilus edulis</i> F	10,396	10,656	264	88
<i>Mytilus californianus</i> M	8,262	8,570	312	104
<i>Ruditapes philippinarum</i> M	4,630	4,752	126	42
<i>Arctica islandica</i>	10,343	10,493	151	50
<i>Semele scabra</i>	11,969	12,097	129	43
<i>Solecurtus divaricata</i>	11,321	11,452	135	45
<i>Nuttallia olivacea</i>	12,930	13,058	132	44
<i>Soletellina diphos</i>	11,214	11,342	132	44
<i>Mimachlamys nobilis</i>	7,937	8,086	153	51
<i>Moerella iredescens</i>	11,625	11,753	132	44
<i>Meretrix petechialis</i>	8,532	8,669	141	47
<i>Meretrix meretrix</i>	8,532	8,669	141	47
<i>Ruditapes philippinarum</i> F	5,968	6,084	120	40
<i>Paphia euglypta</i>	12,994	13,107	117	39
<i>Acanthocardia tuberculata</i>	12,546	12,648	103	34
<i>Musculista senhousia</i> M	7,403	7,591	192	64

Start and stop refer to the complete mitochondrial genome as referenced in [supplementary file S1, Supplementary Material](#) online; length, nucleotide length without stop codon; aa, amino acid length. All newly annotated *atp8* are on the "+" strand; ORFs are listed in order of hit, as in [supplementary file S19, Supplementary Material](#) online.

unsignificant for all entries of the database. The only exception to this is given by *Musculista senhousia*. Three ORFans were described on the F mtDNA of this species (Guerra et al. 2014): while no significant hits were retrieved for one of these, the other two and the single M ORFan are clearly homolog ([supplementary file S21, Supplementary Material](#) online; see also Guerra et al. 2014). Finally, *S. velum* ORFans did not show significant homologies with other ORFans.

Summarizing Data: PCA and HERMES Score

All bivalve mitogenomic features are listed in [supplementary file S5, Supplementary Material](#) online. The resulting PCA is shown in figure 7; the first two principal components account for the 66.61% of the dataset variability. In the PCA plot, larger taxonomic assemblages are easily identified: Palaeoheterodonta on one side and the wide cluster of Amarsipobranchia on the other side. Most families create also a cluster, like Pectinidae, Ostreidae, and Margaritiferidae. Contrastingly, some points are sharply separated from others: *N. nucleus*, *S. velum*, *Scapharca* spp., *P. magellanicus*, and *L. elliptica*.

The best-performing variable set to compute the HERMES score was the following:

- (i) percentage of URs;
- (ii) absolute value of SU skew;

- (iii) AMIGA;
- (iv) root-to-tip distance; and
- (v) ML distance from *N. nucleus*.

For example, when inserting also the AT content, the goodness-of-fit parameters become only slightly better, but AT content was given a communality of 0.25%; therefore, we may conclude that this variable is not highly linked with the other five and thus it not significant in quantifying molecular evolution of mitochondrial genomes.

The HERMES factor analysis index (fig. 8) shows good levels of correlation between the selected variables: TLI = 0.965, SRMR = 0.061, RMSEA 95% CI = 0.025–0.233, KMO = 0.764, which are all within boundaries suggested by Hu and Bentler (1999). The lowest communality was scored by %UR (13.20%), while the highest was scored by the ML distance from *N. nucleus* (98.66%); the mean communality of the model was 60.62%, meaning that the HERMES index accounts for the 60.62% of the total variability of the source matrix.

Discussion

To the best of our knowledge, the present study is the first overall and detailed appraisal to the mitogenomics of bivalve mollusks. Many similar studies have been published in the past on similar topics, but generally they focused on a single family, like Mytilidae (Breton et al. 2006), Unionidae (Breton et al. 2009), Pectinidae (Wu et al. 2009), or on the DUI phenomenon (Doucet-Beaupré et al. 2010). As stated in the introduction, however, large comparative studies are essential to understand the main pathways followed by the evolution of mitochondrial genomes.

At the class level, we decided to especially concentrate on PCGs, as annotations are more trustworthy, homology is certain, and the same set of genes is present in all genomes under study. Actually, we find only two duplication events (*cox2* in *M. senhousia* M and *V. philippinarum* F) and we were able to detect the *atp8* gene in 20 species where this gene was originally described as missing (table 2): some of these *atp8* genes were already signaled (Stöger and Schrödl 2013), namely those of *A. tuberculata*, *H. arctica*, and *V. philippinarum* (Dreyer and Steiner 2006); *Mytilus* spp. (Breton et al. 2010; Smietanka et al. 2010); Unionidae (Doucet-Beaupré et al. 2010). After our analysis, 71 species out of 100 have an annotated *atp8*. The nucleotide/amino acid sequence of this gene is scarcely conserved, and this probably led to annotation flaws, as already stated (Plazzi et al. 2013; Zouros 2013; Bettinazzi et al. 2016; Gaitán-Espitia et al. 2016). Structural analyses are needed to recover some homology with known *atp8*, and we may suggest the importance of such analyses in other groups where *atp8* is reported as missing, like nematodes (Okimoto et al. 1992), platyhelminths (Le et al. 2000), and chaetognaths (Boore et al. 2004).

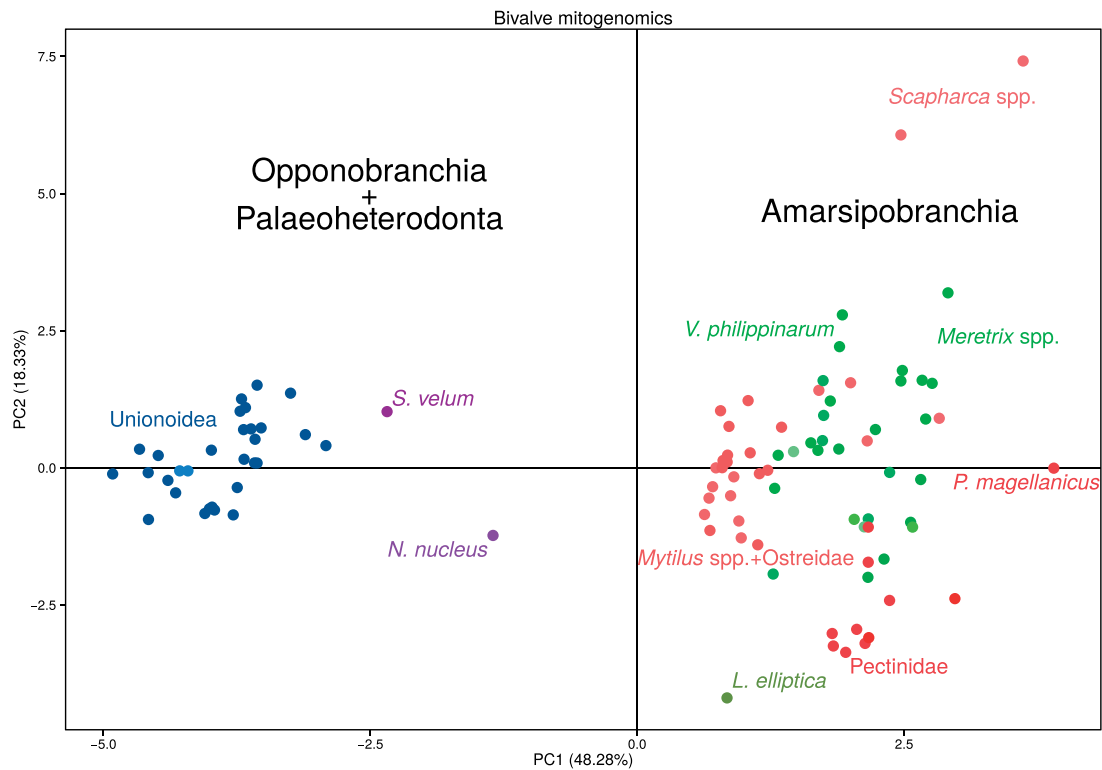


Fig. 7.—Bivalve mitogenomics PCA. Input data are shown in [supplementary file S5, Supplementary Material](#) online (see text for details). Different subclasses are indicated with different shades: shades of blue, Palaeoheterodonta; shades of violet, Opponobranchia; shades of red, Pteriomorphia; shades of green, Heterodonta + Anomalodesmata; species of the same family are indicated with the same color.

Contrastingly, the GenBank annotation of rRNAs is still commonly obtained by the boundaries of the upstream and downstream genes, despite accurate ongoing work of re-annotation (Bernt et al. 2013; Stöger and Schrödl 2013). The use of these genes is therefore problematic for analyses like nucleotide composition, skews, or biases. However, phylogenetic methods should be robust enough to overcome this issue (but a masking phase is required): for this reason, we decided to insert rRNAs in the phylogenetic analysis anyway.

Finally, tRNAs are extremely prone to gene rearrangement (Boore 1999; Vallès and Boore 2006; Xu et al. 2006; Gissi et al. 2008) and recruitment (Lavrov and Lang 2005; Wu, Li, Li, Xu, et al. 2012; Wu, Li, Li, Yu, et al. 2012), and therefore they have to be assessed at a lower taxonomical level (Wu et al. 2014).

PCGs in Bivalve Mitochondrial Genomes

The distance analyses reveal a high degree of divergence for single genes. p -Distance, which is an underestimation of the true distance in that it does not account for multiple substitution events, is generally comprised between 40% and 60% (fig. 3). However, when the correct model of molecular evolution is used, it is clear that most of this variability is found in

synonymous mutations (fig. 3), which is clearly demonstrated by the low values of d_N/d_S (table 1).

Pairwise d_N/d_S have very low medians, but a very high standard deviations, probably due to high d_N/d_S computed for very distantly related species; therefore, we consider as the best estimation of d_N/d_S in our dataset the value computed along the phylogenetic tree, which is comprised between 0.080 (*cox1*) and 0.344 (*nad6*; table 1). Such a pattern of overall negative selection was already detected for the species of genus *Mytilus* (Zbawicka et al. 2014; Gaitán-Espitia et al. 2016).

However, conservation and variability should not be assessed at the general gene level, because our results clearly indicate a sharp contrast between strongly conserved domains and highly variable regions. Those domains are shown in figure 2 (see also [supplementary files S9 and S10, Supplementary Material](#) online). Concluding, some domains of the mitochondrial PCGs are currently under severe purifying selection in bivalves, while, in most cases, variability is due to indel events typical of some species (see, e.g., the *cox2* gene).

If the general pattern is the conservation of specific domains in each PCG, some genes seem to follow different evolutionary pathways. In most analyses, *atp6*, *nad2*, *nad4L*, and

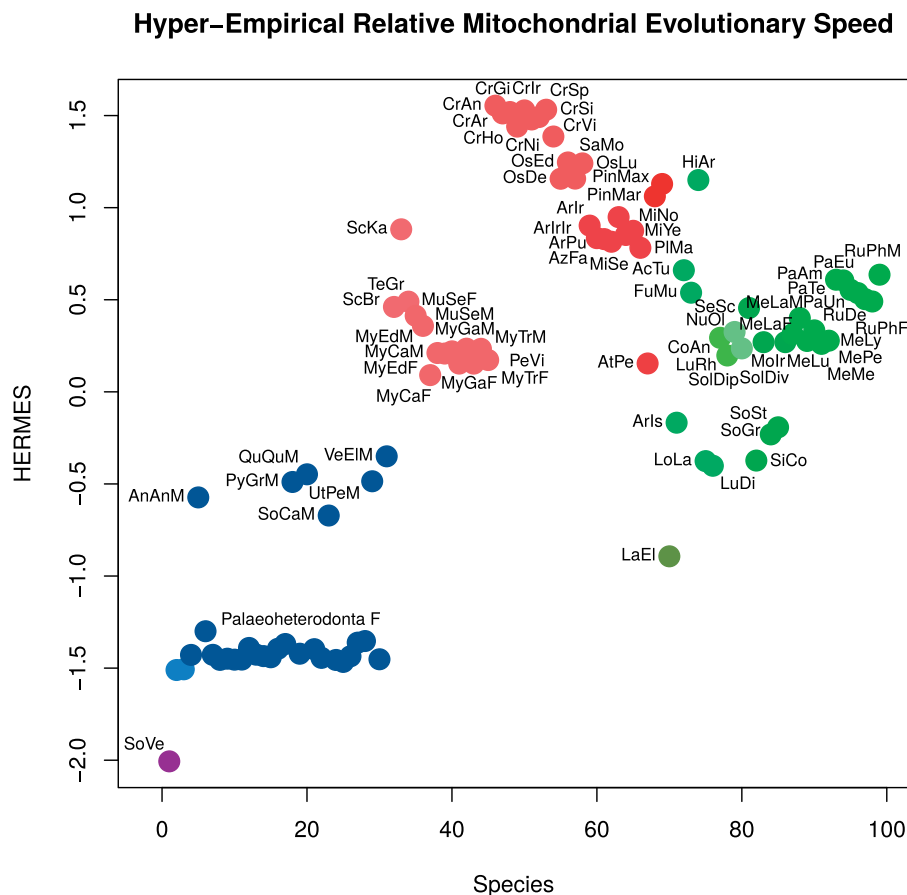


Fig. 8.—The HERMES index. To highlight HERMES differences between subclasses, species are horizontally listed by subclass and then in alphabetical order.

nad6 behave differently from other PCGs: they are more heavily affected by masking phase (supplementary file S7, Supplementary Material online), show higher d_N/d_S values (table 1), are heavily saturated (fig. 4 and supplementary file S11, Supplementary Material online), and are much more variable (fig. 3). In a nutshell, these genes are driven by different evolutionary constraints with respect to the others (fig. 5); although the second principal component accounts for only the 8.02% of the variability, this may indicate that, furthermore, each of these genes follows its own evolutionary pathway.

History of Bivalve Mitochondria

The present phylogeny (fig. 6) corroborates a view of bivalve evolution where most extant families were essentially already present in the Lower Ordovician (Mondal and Harries 2016b). Expectedly, the mitogenomic differentiation of the main clades of extant bivalves predates the paleontological evidences of the well-known Ordovician bivalve radiation (Cope 1996; Fang 2006; Sánchez 2008; Fang and Sánchez 2012; Polechová 2015; Mondal and Harries 2016b).

Conversely, the root of most families is placed after the Ordovician, probably because of limited taxon sampling: the same would hold for Palaeoheterodonta, but they appear to be much more recent (Early Triassic) since only members of the superfamily Unionioidea were actually inserted in this phylogenetic analysis.

The topology of our phylogenetic tree is in perfect agreement with our previous results (Plazzi and Passamonti 2010; Plazzi et al. 2011). It is particularly noteworthy the presence of the Amarsipobranchia clade, i.e., Pteriomorphia + Heterodonta. Our previous analyses were based on four mitochondrial genes (*cox1*, *cytb*, *rrnL*, and *rrnS*), and the use of the complete mitochondrial gene array led to the same result, even with a larger sample. Furthermore, the signal vs. noise analysis indicates that the phylogenetic signal of all genes is suitable (supplementary file S17, Supplementary Material online), and it becomes even stronger for the complete, concatenated dataset. Indeed, the same Amarsipobranchia clade was also retrieved by other studies using mitochondrial markers (Giribet and Distel 2003; Doucet-Beaupré et al. 2010; Stöger and Schrödl 2013).

On the other side, the use of morphological characters leads generally to the Heteroconchia *sensu* Waller (1998) clade (i.e., Palaeoheterodonta + Heterodonta; Giribet and Distel 2003; Bieler et al. 2014)—however, some morphological analyses retrieved instead the Amarsipobranchia clade (Cope 1996). Recent analyses based on nuclear genes and transcriptomes (Kocot et al. 2011; Smith et al. 2011; Sharma et al. 2012; González et al. 2015) also recovered the Heteroconchia clade.

Thus, the discrepancy between phylogenies based on morphology/nuclear genes vs. mitochondrial genes still holds. The amount of data in mitochondrial genomes is clearly restricted with respect to nuclear genomes. It is also possible that specific mitochondrial features may lead to a wrong relationship between heterodonts and pteriomorphians, thus disrupting Heteroconchia. Nucleotide composition may be one of these features: Amarsipobranchia have all negative A-T and positive G-C skews, while the situation is reversed for Palaeoheterodonta (supplementary file S5, Supplementary Material online). Another feature which is correlated with nucleotide composition is the substitution rate (Kowalczyk et al. 2001; Siepel and Haussler 2004; Gowri-Shankar and Rattray 2006; Hobolth et al. 2006), which we show is linked with AR rate (supplementary file S18, Supplementary Material online), as already hypothesized by Stöger and Schrödl (2013).

However, our HERMES factor analysis demonstrates that the AT content practically does not explain other phylogenetic parameters like root-to-tip distance and ML distance from *N. nucleus*, and the protobranch *Solemya velum* show the same situation of Palaeoheterodonta, corroborating the idea that this is the plesiomorphic condition of bivalves. The same can be seen from the general PCA (fig. 7): Amarsipobranchia and Palaeoheterodonta are separated, many features are considered (not only nucleotide composition), and Opponobranchia are shifted towards Palaeoheterodonta.

Conclusions

The HERMES Index: Tempo and Mode of Mitochondrial Evolution

The use of a single score to quantify mitochondrial evolution is a complex task, as many different parameters (that, furthermore, are linked together to different extents) can be considered and have to be summarized into a single number. High goodness-of-fit test results (given the complexity of the dataset) show that HERMES (fig. 8) is indeed a suitable measure of this evolution. We are planning to apply the HERMES index in other taxa, and this will lead us to the identification of taxon-specific mitochondrial features that are suitable to measure molecular evolution; a Python script was written and made publicly available for this purpose (available at <https://github.com/mozoo/HERMES.git>).

Concerning bivalves, there is consistence between all the aforementioned data and the HERMES measure: *S. velum* is the slowest-evolving mitogenome, followed by those of Palaeoheterodonta. Amarsipobranchia have similar HERMES scores, typically higher than Palaeoheterodonta; finally, the position of *L. elliptica* is intriguing and deserves further investigation. Within Palaeoheterodonta, F genomes have HERMES scores that are sharply lower than the M counterparts. The very early onset of DUI in Palaeoheterodonta led to the highest inter-sex distance values for DUI species (Bettinazzi et al. 2016) and to the well-known gender-joining pattern in phylogenetic trees (Curole and Kocher 2005; Doucet-Beaupré et al. 2010; Zouros 2013; fig. 6). The divergence of the two lineages started at least from the origin of Unionidae, ~250 Mya (Tillyard and Dunstan 1916; Cromptok and Parrington 1955; Drysdall and Kitching 1963; Nesbitt et al. 2010; fig. 6; supplementary file S16, Supplementary Material online): F and M genomes are separately evolving since then. Recall that the organization of the ancestral bivalve mitochondrial genome should have resembled that of *Solemya* and *Nucula* (Plazzi et al. 2013), we can conclude that, while somehow the F mtDNAs of unionids retain much of this original condition, the M mtDNAs seem to have diverged more quickly on their own. DUI in other bivalve lineages appears to be a much more recent phenomenon (fig. 6), or masked by masculinization role reversals (Hoeh et al. 1997; Quesada et al. 1999; Zouros 2013), therefore the gender-joining pattern of unionids remains essentially unique across the phylogeny of the entire class.

It is tempting to explore links between the molecular evolution of bivalve mitochondrial genomes as depicted by the HERMES score (and the mitogenomic feature-based PCA as well; fig. 7) and the fossil evidences of the class. First known bivalves originated in the Early-Middle Cambrian and slowly faded away during the Late Cambrian; no Cambrian species are known from the Ordovician (Fang 2006; Sánchez 2008; Fang and Sánchez 2012; Cope and Kříž 2013; Polechová 2015; Mondal and Harries 2016a).

However, some of them gave rise to extant bivalve clades in the Ordovician period (Cope 2002; Sánchez 2008; Polechová 2015). Protobranch forms were the first branching clade (Morton 1996; Cope and Babin 1999; Kocot et al. 2011; Plazzi et al. 2011; Smith et al. 2011; Fang and Sánchez 2012; Sharma et al. 2012; Bieler et al. 2014; González et al. 2015; and reference therein) before the evolution of the true feeding gill of all remaining autobranchs. The strict similarity of the mitochondrial genome of *Solemya* (and *Nucula* as well) with some gastropods (Plazzi et al. 2013) strengthens this hypothesis and allows to set a direction in the evolution of bivalve mtDNAs after the original appearance of extant bivalves; the HERMES index detects two main phases of this evolution (fig. 8).

According to the HERMES pattern, a first phase was the split of palaeoheterodonts from Amarsipobranchia. Again,

fossil data may shed light on this issue. The most ancient families of Ordovician bivalves (dating to the Lower Ordovician, upper Tremadoc, ~480 Mya) are Ucumariidae, Modiolopsidae (Goniophorinidae), and Lipanellidae (Sánchez 2006; Fang and Sánchez 2012): following current revised bivalve systematics, they should be classified within Heterodonta, Pteriomorpha, and Heterodonta, respectively (Carter et al. 2011).

However, the state-of-art phylogenetic reconstruction is Lipanellidae + (Modiolopsidae + Ucumariidae) (Sánchez 2006, p. 117; Fang and Sánchez 2012, p. 12), and Heterodonta should be therefore considered as polyphyletic at their root. Moreover, Ucumariidae are interpreted as connected to extant anomalodesmatans (Sánchez 2006; Fang and Sánchez 2012), which are currently considered as nested within heterodonts (Giribet and Wheeler 2002; Dreyer et al. 2003; Giribet and Distel 2003; Harper et al. 2006; Taylor et al. 2007, 2009; Sharma et al. 2012; Bieler et al. 2014; González et al. 2015).

Indeed, our phylogenetic reconstruction and the Amarsipobranchia hypothesis retrieve the same clade; namely, in the present phylogenetic tree it exhibits a basal tritomy (Heterodonta + Pteriomorpha + Anomalodesmata; fig. 6). Furthermore, it is worth recalling the fossil record of the family Thoraliidae, which is also known from the Lower Ordovician (Morris 1980; Sánchez and Babin 2003; Cope and Kříž 2013) and it is currently classified within Palaeoheterodontia (Carter et al. 2011). Thus, in the Lower Ordovician a monophyletic Amarsipobranchia-like clade is hypothesized, while the first palaeoheterodonts have been found from the same epoch: concluding, phylogenetic paleontological reconstructions are not discordant with our molecular phylogenetic tree or with the HERMES pattern.

Eventually, the second phase of bivalve mitochondrial evolution as depicted by the HERMES score is the diversification of Amarsipobranchia, which definitely lost most plesiomorphic mitogenomic features (fig. 7): all genes, with rarest exceptions, migrated on the same coding strand; an increase in length was coupled to an increase of the genomic regions not assigned to canonical genes; there was the inversion in A-T and G-C skews (fig. 1; [supplementary file S5, Supplementary Material](#) online); most strikingly, the gene rearrangement rate was given an unprecedented boost ([supplementary file S18, Supplementary Material](#) online).

Supplementary Material

[Supplementary file S1–S21](#) are available at *Genome Biology and Evolution* online (<http://www.gbe.oxfordjournals.org/>).

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