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Phylogenetic analysis of four nuclear protein-encoding genes largely corroborates the traditional classification of Bivalvia (Mollusca)

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ABSTRACT

Revived interest in molluscan phylogeny has resulted in a torrent of molecular sequence data from phylogenetic, mitogenomic, and phylogenomic studies. Despite recent progress, basal relationships of the class Bivalvia remain contentious, owing to conflicting morphological and molecular hypotheses. Marked incongruity of phylogenetic signal in datasets heavily represented by nuclear ribosomal genes versus mitochondrial genes has also impeded consensus on the type of molecular data best suited for investigating bivalve relationships. To arbitrate conflicting phylogenetic hypotheses, we evaluated the utility of four nuclear protein-encoding genes—ATP synthase β, elongation factor-1α, myosin heavy chain type II, and RNA polymerase II—for resolving the basal relationships of Bivalvia. We sampled all five major lineages of bivalves (Archiheterodonta, Euheterodonta [including Anomalodesmata], Palaeoheterodonta, Protobranchia, and Pteriomorphia) and inferred relationships using maximum likelihood and Bayesian approaches. To investigate the robustness of the phylogenetic signal embedded in the data, we implemented additional datasets wherein length variability and/or third codon positions were eliminated. Results obtained include (a) the clade (Nuculanida + Opponobranchia), i.e., the traditionally defined Protobranchia; (b) the monophyly of Pteriomorphia; (c) the clade (Archiheterodonta + Palaeoheterodonta); (d) the monophyly of the traditionally defined Euheterodonta (including Anomalodesmata); and (e) the monophyly of Heteroconchia, i.e., (Palaeoheterodonta + Archiheterodonta + Euheterodonta). The stability of the basal tree topology to dataset manipulation is indicative of signal robustness in these four genes. The inferred tree topology corresponds closely to those obtained by datasets dominated by nuclear ribosomal genes (18S rRNA and 28S rRNA), controverting recent taxonomic actions based solely upon mitochondrial gene phylogenies.

1. Introduction

Bivalvia is the second largest class of mollusks after Gastropoda and is comprised of aquatic (predominantly marine), bilaterally symmetrical animals characterized by a laterally compressed body enclosed in a bivalved shell and the lack of a radular apparatus. Extant bivalves are abundantly represented from intertidal to hadal marine environments and many species have significant commercial importance. Five distinct groups of bivalves are often recognized: the protobranchs (solemyoids, nuculoids, and nuculanoids); the pteriomorphians (mussels, scallops, oysters, and arks); the palaeoheterodonts (most freshwater mussels and trigoniids); the archiheterodonts (which include members with hemoglobin); and the euheterodonts, including Anomalodesmata (the most species-rich and widely distributed group of bivalves) (Bieler and Mikkelsen, 2006; Giribet, 2008).

The monophyly of Bivalvia is supported by numerous morphological apomorphies, but has historically proven elusive to demonstrate based on molecular sequence data, owing to early limitations in sampling of molecular loci and/or taxa (e.g., Steiner and Müller,

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1996; Adamkewicz et al., 1997; Campbell et al., 1998; Giribet and Carranza, 1999; Giribet and Wheeler, 2002; Giribet and Distel, 2003; Giribet et al., 2006; but see Wilson et al., 2010). Recent application of second-generation sequencing techniques to outstanding questions of molluscan systematics has corroborated the monophyly of bivalves and their sister relationship to either Gastropoda (Kocot et al., 2011; based on 308 genes) or the clade (Gastropoda + Scaphopoda) (Smith et al., 2011; based on 1185 genes). Similarly, morphological cladistic analyses in concert with molecular phylogenies have elucidated relationships of many internal bivalve clades, such as within Pteriomorphia (Canapa et al., 2000; Matsumoto and Hayami, 2000; Steiner and Hammer, 2000; Matsumoto, 2003; Tëmkin, 2006, 2010; Waller, 2006), Unionida (Hoeh et al., 1999; Graf, 2000; Graf and Ó Foighil, 2000; Huff et al., 2004; Graf and Cummings, 2006), Anomalodesmata (Dreyer et al., 2003; Harper et al., 2006), and other Heterodonta (Canapa et al., 1999, 2001, 2003; Park Ó Foighil, 2000; Campbell et al., 2004; Williams et al., 2004; Taylor et al., 2005, 2007, 2009, 2011; Mikkelsen et al., 2006; Taylor and Glover, 2006).

Following decades of dispute concerning the position of major bivalve lineages (Fig. 1; reviewed by Giribet and Wheeler, 2002; Giribet, 2008), certain aspects of basal bivalve phylogeny have begun to stabilize. For example, although protobranch monophyly has long been contentious (Campbell et al., 1998; Giribet and Wheeler, 2002; Giribet and Distel, 2003; Wilson et al., 2010), the mutual monophyly of Protobranchia (Nuculanida + Opponobranchia *sensu* Giribet, 2008) and Autobranchia—the group comprised of all bivalves with ctenidia modified for filter-feeding—was obtained in a recent phylogenomic analysis sampling all three protobranch superfamilies (Smith et al., 2011; although Archiheterodonta and Anomalodesmata were not sampled). Similarly, the monophyly of Heteroconchia (Palaeoheterodonta + Archiheterodonta + Euheterodonta [including Anomalodesmata]) was obtained previously (Giribet and Wheeler, 2002; Wilson et al., 2010), with phylogenetic studies corroborating either a sister relationship of Archiheterodonta and Euheterodonta (Giribet and Wheeler, 2002; Taylor et al., 2007), or of Archiheterodonta and Palaeoheterodonta (Wilson et al., 2010). A noteworthy commonality of these phylogenetic studies is the use of datasets heavily or exclusively represented by nuclear ribosomal genes (18S rRNA and 28S rRNA).

A notable exception to this accruing consensus was a molecular phylogeny based on four genes (18S rRNA, 28S rRNA, histone H3, and cytochrome *c* oxidase subunit I) that recovered a sister relationship of Nuculanida and Archiheterodonta, and a more basal divergence of Palaeoheterodonta (Giribet and Distel, 2003). Part of the discrepancy may have been attributable to the analytical treatment of the gene histone H3, which was newly sequenced for Bivalvia at the time. In that study, the optimal parameter set selected for parsimony analysis assigned all substitutions the same weight. Reexamination of histone H3 performance across various invertebrate taxa has demonstrated the suppression of phylogenetic signal of this gene given equal weighting of transversion and transition events—typically caused by the disproportionate influence of the nuclear ribosomal genes—and ensuing false topological incongruity (Sharma et al., 2011). Nevertheless, certain aspects of the topology of Giribet and Distel (2003) were corroborated by subsequent analyses (e.g., Wilson et al., 2010), specifically with respect to derived relationships and the monophyly of Euheterodonta (including Anomalodesmata).

In spite of these advances toward a stable bivalve phylogeny, basal relationships have once again come into question owing to topologies obtained using mitochondrial genes and genomes. One such study, examining doubly uniparental inheritance (DUI) of the mitochondrial genome (which has heretofore been documented in Mytilida, Unionida, and Venerida; reviewed by Breton et al., 2007), constructed a 12-mitochondrial gene phylogeny sampling only unionoids, veneroids, and three groups of pteriomorphians (Pectinida, Ostreida, and Mytilida) (Doucet-Beaupré et al., 2010). Although this sampling was deemed sufficient for the purposes of that study (i.e., mapping gains and losses of DUI in Autobranchia), the omission of several major groups could have engendered the unusual sister relationship of Pteriomorphia and Euheterodonta (not including Anomalodesmata) to the exclusion of Palaeoheterodonta. Furthermore, two recent phylogenetic analyses, both based exclusively on four mitochondrial genes (12S rRNA, 16S rRNA, cytochrome *c* oxidase subunit I, and cytochrome *b*), recovered highly counterintuitive topologies. In the first case, bivalves were recovered as polyphyletic owing to the placement of Solemyida and Nuculida, and the remaining relationships obtained included polyphyly of Anomalodesmata, and Nuculanida clustering with Pteriomorphia (Fig. 1I; Plazzi and Passamonti, 2010). A subsequent rendition with increased taxonomic sampling obtained the monophyly of Bivalvia, but a markedly different topology (Fig. 1L; Plazzi et al., 2011). The authors proposed the name "Amarsipobranchia" for all bivalves except Nuculida, Solemyida, and Unionida, based upon a gill character that does not in fact occur in all Archiheterodonta, Euheterodonta, or Pteriomorphia—but "has most probably to be considered as a symplesiomorphy of this group" (Plazzi et al., 2011, p. e27147). The authors concluded that mitochondrial genes should not be discarded *a priori* from phylogenetic analysis and that sophisticated analytical treatment, particularly with respect to third codon positions in protein-encoding genes, can reveal underlying phylogenetic signal (Plazzi et al., 2011).

We concur with Plazzi et al. (2011) that mitochondrial genes can be highly informative markers, particularly with respect to shallow nodes. Empirical phylogenetic studies of bivalves already actively sample mitochondrial genes (both ribosomal and protein-encoding) in addition to their nuclear counterparts, and for resolving various taxonomic ranks (e.g., Giribet and Wheeler, 2002; Giribet and Distel, 2003; Campbell et al., 2005; Giribet et al., 2006; Kappner and Bieler, 2006; Mikkelsen et al., 2006; Wood et al., 2007; Tëmkin, 2010; Wilson et al., 2010). However, the second claim, that sophisticated analytical treatment of mitochondrial genes can unearth phylogenetic signal among deep divergences, is dubious for two reasons. First, the approach taken by Plazzi et al. (2011) with respect to evaluating phylogenetic utility is to test complex models and an assortment of partitioning schemes for describing their dataset, and to obtain an optimal set of model parameters. But they do not evaluate phylogenetic utility in the context of previous phylogenetic studies, particularly those that have implemented both nuclear and mitochondrial genes together. Consequently, it is clarified neither why the mitochondrial data are so markedly discordant with respect to other multilocus data, nor how reconciliation is possible. As a corollary, it is also not clarified why exclusive use of mitochondrial data should be preferred over nuclear data, given the significant topological discord that they engender.

Second, a number of studies has previously demonstrated topological incongruence between mitochondrial and nuclear datasets (e.g., Degnan, 1993; Slade et al., 1994; reviewed by Ballard and Whitlock, 2004). Although algorithmic approaches can elucidate phylogenetic signal, over-parameterization of nucleotide and codon models can reduce their predictive power, or even engender problems of non-identifiability, in addition to artificially inflating nodal support (Chang, 1996; Steel, 2005). This could partially explain why the approach of analyzing the same four mitochondrial genes yielded radically different topologies upon the addition of taxa, with almost flawless nodal support values in the more recent study (Plazzi and Passamonti, 2010, *contra* Plazzi et al., 2011).

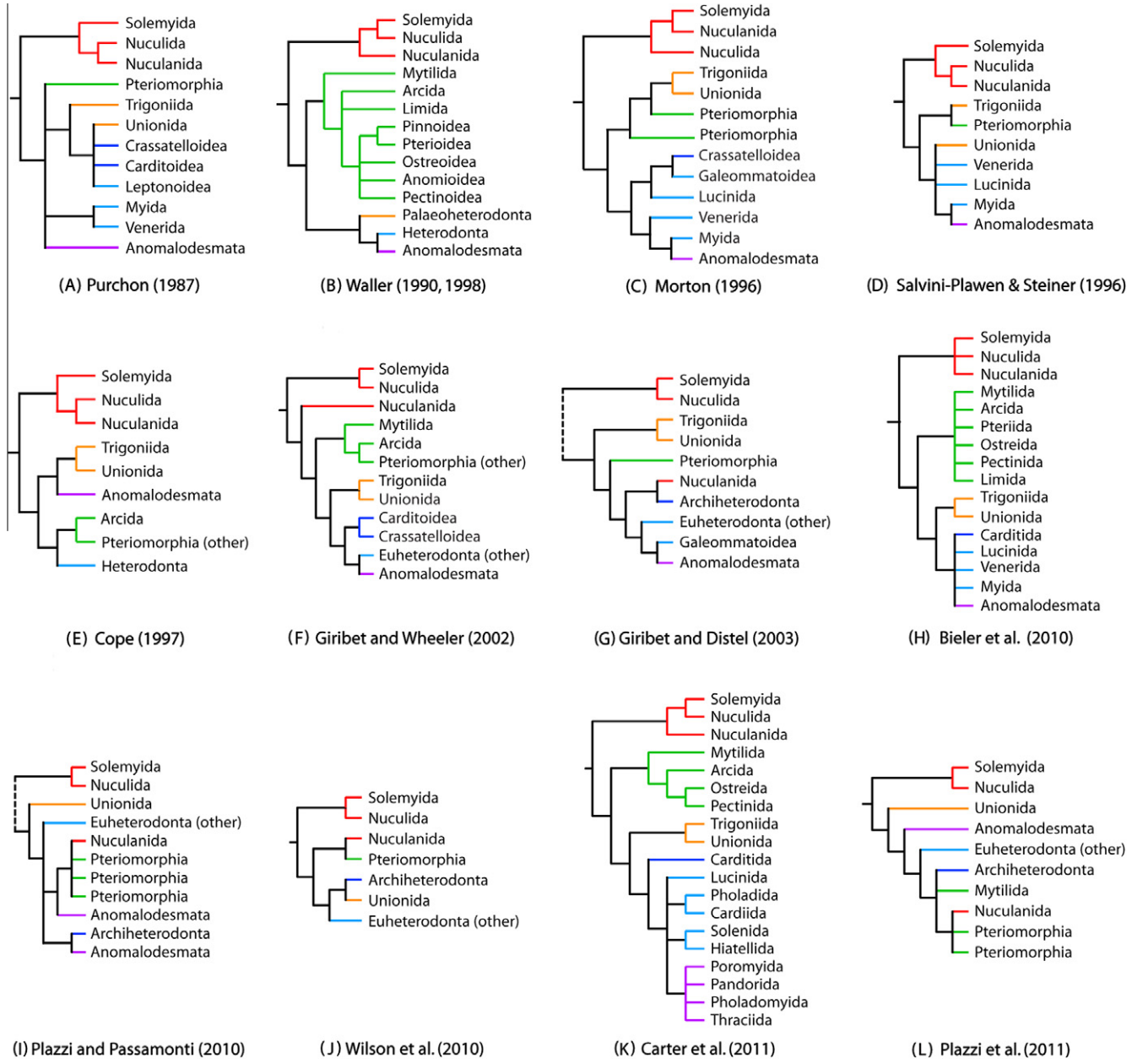

Fig. 1. Phylogenetic hypotheses of higher bivalve relationships proposed by different authors. (A) Purchon (1987) based on phenetic analysis of morphological data. (B) Waller (1990, 1998) based on non-numerical cladistic analyses of morphology. (C) Proposed evolutionary tree of Morton (1996). (D) Salvini-Plawen and Steiner (1996), parsimony analysis of morphological data. (E) Suggested evolutionary tree of Cope (1997). (F) Giribet and Wheeler (2002), based on the parsimony analysis of morphology and three molecular markers (18S rRNA, 28S rRNA, COI). (G) Giribet and Distel (2003), based on parsimony analysis of four molecular markers (18S rRNA, 28S rRNA, COI, histone H3). (H) Synoptic classification of Bieler et al. (2010). (I) Plazzi and Passamonti (2010), based on Bayesian analysis of four molecular markers (12S rRNA, 16S rRNA, COI, CytB). (J) Wilson et al. (2010), based on Bayesian analysis of five molecular markers (16S rRNA,18S rRNA, 28S rRNA, COI, histone H3). (K) Synoptic classification of Carter et al. (2011). (L) Plazzi et al. (2011), based on Bayesian analysis of four molecular markers (12S rRNA, 16S rRNA, COI, CytB). Colors in tree topology correspond to major lineages (red: Protobranchia; green: Pteriomorphia; orange: Palaeoheterodonta; indigo: Archiheterodonta; purple: Anomalodesmata; blue: remaining Euheterodonta). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Phylogenomic assessments of molluscan relationships shed little further light on basal relationships, beyond favoring the mutual monophyly of Protobranchia and Autobranchia. This is a consequence of limitations in sampling of Autobranchia, whose internal relationships were not the focus of these studies. For example, Kocot et al. (2011) did not include representatives of Nuculanida, Archiheterodonta, or Anomalodesmata. Similarly, Smith et al. (2011) did not sample Archiheterodonta or Anomalodesmata, and both studies used a previously published small EST data set for a single exemplar of Palaeoheterodonta. This may in part explain the counterintuitive sister relationship of Pteriomorphia and Euheterodonta (to the exclusion of Palaeoheterodonta) obtained by Smith et al. (2011), although this result was not supported under some algorithmic treatments.

To redress the topological conflict caused by the use of exclusively mitochondrial datasets versus datasets that include nuclear genes, as well as to test hypotheses of basal relationships, we investigated the phylogeny of bivalves using four nuclear, protein-encoding loci: ATP synthase β, elongation factor-1α, myosin heavy chain type II, and RNA polymerase II. The study included representatives of all major bivalve lineages. We performed a phylogenetic analysis of nucleotide sequence data, manipulating the

treatment of length variability and/or third codon positions to investigate phylogenetic robustness. From the topologies obtained, we reexamined hypotheses of basal relationships and evaluated these four molecular markers as predictors of bivalve phylogeny.

2. Materials and methods

2.1. Taxon sampling

Specimens for the study were obtained as part of the Bivalve Tree of Life (BivAToL) project (where most will also serve as exemplar species in separate analyses of other molecular loci, as well as morphological characters); additional sequence data were obtained from the Protostome Tree of Life project (see project information in Acknowledgments), or accessed from GenBank. The 45 ingroup taxa sampled consisted of 5 Protobranchia, 14 Pteriomorphia, 3 Palaeoheterodonta, 2 Archiheterodonta, 3 Anomalodesmata, and 18 other Euheterodonta. Outgroup taxa for the study consisted of 7 gastropods, 1 chiton, 1 scaphopod, and 2 cephalopods. However, we observed that the highly divergent sequences of all outgroups except Gastropoda resulted in non-monophyly of the ingroup (Supplementary Fig. 1). Given that bivalve monophyly has been demonstrated recently using phylogenomic approaches (Smith et al., 2011) and this study is concerned only with internal relationships, we limited the outgroup sampling to a subset of gastropods for our principal analyses. The full list of taxa included in our study is provided in Supplementary Table 1.

2.2. Molecular methods

Total RNA was isolated from tissues preserved in RNA*later*® (Ambion) or frozen at −80 °C, using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA synthesis was performed with 1–2 mg of total RNA using the RETROscript™ kit (Ambion). Sequence data were obtained using degenerate PCR primers, whose sequences and original references are provided in Table 1.

Fragments of the head portion of myosin heavy chain type II and elongation factor-1α were amplified from template cDNA following nested PCR reaction protocols, described by Aktipis and Giribet (2010). Fragments of RNA polymerase II were amplified following protocols described by Regier and Shultz (2000).

ATP synthase β fragments were amplified from cDNA following touchdown PCR reaction protocols modified from Sperling et al. (2007). Initial touchdown reactions amplified fragments using the external primers ATPβF and ATPβR, in a 25 μL reaction (1 μL cDNA template, 20.5 μL ddH2O, 2.5 μL AmpliTaq™ 10x PCR buffer, 0.5 μL dNTPs [10 μmol L^{-1}], 0.25 μL of each primer [100 μmol L^{-1}], and 0.625 U AmpliTaq™ enzyme) with the following parameters: 1 min of denaturation at 94 °C, 45 s of annealing at 52 °C for two cycles, then annealing temperature was lowered one degree every two cycles, terminating at 40 °C for a total of 26 cycles, followed by 45 s of annealing at 52 °C for 10 cycles, and a 2 min extension at 72 °C, with a final 7 min extension at 72 °C. This initial PCR product was then amplified in a second PCR reaction using the internal primers ATPβ200F and ATPβ1088R, in a 50 μL reaction (2 μL touchdown template, 41 μL ddH2O, 5.0 μL AmpliTaq™ 10x PCR buffer, 1 μL dNTPs [10 μmol L^{-1}], 0.5 μL of each primer [100 μmol L^{-1}], and 1.25 U AmpliTaq™enzyme, Applied Biosystems, Carlsbad, CA, USA) with the following parameters: initial 2 min denaturation at 94 °C, then 35 cycles of 30 s of denaturation at 94 °C, 45 s of annealing at 52 °C, and 2 min extension at 72 °C, with a final 7 min extension at 72 °C. Annealing temperatures for ATP β ranged between 46 °C and 55 °C.

All amplified samples were purified using an Eppendorf vacuum (Hamburg, Germany) and Millipore Multiscreen® $PCR_{\mu 96}$ cleanup filter plates (Billerica, MA, USA) following the manufacturers' instructions. Sequencing was performed in a GeneAmp® PCR system 9700 (Perkin Elmer, Waltham, MA, USA) using ABI PRISM™ BigDye™ v.3 Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) and following the manufacturer's protocol. The BigDye-labeled PCR products were cleaned with Performa® DTR v3 96-well short plates (Edge BioSystems, Gaithersburg, MD, USA) and directly sequenced using an automated ABI Prism® 3730 Genetic Analyzer.

Chromatograms obtained from the automatic sequencer were analyzed using the sequence editing software Sequencher™4.8 (Gene Codes Corporation, Ann Arbor, MI, USA). External and internal primer regions were removed from these edited sequences. The lengths of the amplicons for each gene are provided in Supplementary Table 2.

2.3. Phylogenetic analysis

Bayesian inference (BI) and maximum likelihood (ML) analyses were conducted on static alignments, which were inferred as follows. Length variable sequences (elongation factor-1α and myosin) were converted to amino acid sequences, which were aligned using MUSCLE ver. 3.6 (Edgar, 2004) with default parameters. The resulting amino acid alignments were used to guide the alignment of the corresponding nucleotide sequences. Length invariable sequences (ATP synthase β and RNA polymerase II) were confirmed using amino acid sequence translations to ensure that no treatment with MUSCLE ver. 3.6 was required. These four data partitions constituted the full dataset (henceforth Dataset 1).

Length variable data partitions (elongation factor-1α and myosin) were subsequently treated with GBlocks v. 0.91b (Castresana, 2000) to cull positions of ambiguous homology from amino acid sequence alignments. We allowed less strict flanking positions for both genes, as use of this feature retains the conserved positions at the beginnings and ends of each dataset (disuse of this feature affected only the termini of the amino acid alignments). Together with the length invariable data partitions, these formed a second dataset free of length variability entirely (henceforth Dataset 2).

We removed the third codon positions of the full dataset to form a third dataset, wherein only first and second codon positions were retained in addition to some length variability (henceforth Dataset 3). Finally, we also removed the third codon positions of Dataset 2 to form the smallest dataset, wherein only 1st and 2nd codon positions, but no length variable regions, were retained (henceforth Dataset 4). The lengths of the aligned datasets in each treatment are indicated in Supplementary Table 2. Aligned datasets are available upon request from the authors.

BI analyses were performed using MrBayes ver. 3.1.2 (Huelsenbeck and Ronquist, 2005) on 12 CPUs of a cluster at Harvard University, FAS Research Computing (odyssey.fas.harvard.edu), with a unique model of sequence evolution with corrections for a discrete gamma distribution and/or a proportion of invariant sites specified for each partition, as selected in jModeltest ver. 0.1.1 (Posada, 2008; Guindon and Gascuel, 2003) under the Akaike Information Criterion (Posada and Buckley, 2004). Model recommendations for each dataset are indicated in Supplementary Table 2. Default priors were used starting with random trees, and three runs, each with three hot and one cold Markov chains, were performed for all four datasets until the average deviation of split frequencies reached <0.01 (10^7 generations). Stationarity was checked using Tracer ver. 1.5 (Rambaut and Drummond, 2009). After burn-in samples were discarded, sampled trees were combined in a single majority-rule consensus topology, and the

Table 1
List of primer sequences used for amplification and sequencing with original references.

Myosin heavy chain		
mio3	5′-GGN GTN YTN GAY ATH GC-3′	Ruiz-Trillo et al. (2002)
mio4	5′-GGR AAN CCY TTN CKR CAD AT-3′	Ruiz-Trillo et al. (2002)
mio6	5′-CCY TCM ARY ACA CCR TTR CA-3′	Ruiz-Trillo et al. (2002)
mio7	5′-TGY ATC AAY TWY ACY AAY GAG-3′	Ruiz-Trillo et al. (2002)
ATP synthase subunit B		
ATPβF	5′-GTN GAY GTN CAR TTY GAY GA-3′	Sperling et al. (2007)
ATPβR	5′-NCC NAC CAT RTA RAA NGC-3′	Sperling et al. (2007)
ATPβ200F	5′-RTW GGD GAM CCA ATT GAY GA-3′	This study
ATPβ1088R	5′-CYA TYT TGG GTA TGG ATG AA-3′	This study
Elongation factor-1α		
RS2F (52.4F)	5′- TCN TTY AAR TAY GCN TGG GT-3′	Regier and Shultz (1997)
RS4R (52RC)	5′- CCD ATY TTR TAN ACR TCY TG-3′	Regier and Shultz (1997)
RS3F (45.71F)	5′- GTN GSN GTI AAY AAR ATG GA-3′	Regier and Shultz (1997)
RS6R (53.5RC)	5′- ATR TGV GMI GTR TGR CAR TC-3′	Regier and Shultz (1997)
RNA polymerase II		
15F	5′-ACW GCH GAR ACH GGK TAY ATY CA-3′	Shultz and Regier (2000)
14F	5′-YTK ATH AAR GCT ATG GA-3′	Shultz and Regier (2000)
17R	5′-TTY TGN GCR TTC CAD ATC AT-3′	Shultz and Regier (2000)

percentage of trees in which a node was recovered was taken as the posterior probability for that node.

ML analyses were conducted using RAxML ver. 7.2.7 (Stamatakis, 2006) on 24 CPUs of a cluster at Harvard University, FAS Research Computing (odyssey.fas.harvard.edu). For the maximum likelihood searches, a unique GTR model of sequence evolution with corrections for a discrete gamma distribution (GTR + Γ) was specified for each data partition, and 250 independent searches were conducted. Nodal support was estimated via the rapid bootstrap algorithm (250 replicates) using the GTR-CAT model (Stamatakis et al., 2008). Bootstrap resampling frequencies were thereafter mapped onto the optimal tree from the independent searches.

A Shimodaira–Hasegawa (SH) test was conducted using RAxML ver. 7.2.7. We enforced an ingroup topology consistent with the Amarsipobranchia hypothesis (*sensu* Plazzi et al., 2011) and compared it to the ML topology obtained using Dataset 1. To generate the null distribution, 500 resampling replicates were conducted.

3. Results

Runs of MrBayes ver. 3.1.2 generally reached stationarity in ca. 10^6 generations; 2×10^6 generations (20%) were hence discarded as burn-in. One of the three runs for Dataset 4 became trapped on a local optimum and was therefore abandoned after the remaining two runs converged. BI analysis of all datasets recovered the monophyly of all six major lineages except Palaeoheterodonta, and basal relationships as follows: (Protobranchia (Pteriomorphia ((Archiheterodonta + Palaeoheterodonta) (Euheterodonta)))) (Fig. 2). Datasets 1–3 also recovered a basal dichotomy between Anomalodesmata and the remaining Euheterodonta, but Dataset 4 recovered Anomalodesmata nested within Euheterodonta (with a lineage of Cardioidea as sister to the remaining Euheterodonta). Derived relationships within Pteriomorphia and Euheterodonta were generally unstable, with numerous superfamilies recovered as non-monophyletic (Fig. 3).

ML analysis using RAxML ver. 7.2.7 resulted in tree topologies with $\ln L = -44419.380$, $\ln L = -42102.014$, $\ln L = -15746.721$, and $\ln L = -14510.373$ for Datasets 1–4, respectively. ML topologies of each dataset were almost identical to those obtained by corresponding BI analyses. However, Dataset 1 recovered the monophyly of all six major lineages, whereas Datasets 2–4 favored the paraphyly of Palaeoheterodonta with respect to Archiheterodonta. All analyses recovered a sister group relationship of Palaeoheterodonta and Archiheterodonta, and therefore non-monophyly of Heterodonta. As in the BI topologies, the sister relationship of Anomalodesmata to the remaining Euheterodonta was obtained by all datasets except Dataset 4 (Fig. 2).

Removal of the variable third codon positions is expected to limit phylogenetic inference among shallow nodes. Accordingly, Datasets 3 and 4 resulted in non-monophyly of one or more congeneric species pairs (*Modiolus* and *Mytilus*) in both BI and ML analyses. There is also a trend toward declining posterior probabilities among BI topologies upon removal of both third codon positions and length variability. Bootstrap resampling frequencies across all four ML topologies were limited. A strict consensus of all tree topologies obtained, underscoring those nodes consistently found across the analytical space, is shown in Fig. 4. Salient points are the monophyly of Protobranchia, Autobranchia, Pteriomorphia, Heteroconchia, Palaeoheterodonta + Archiheterodonta, Euheterodonta and Anomalodesmata.

The SH test comparing the ML topology (obtained with Dataset 1; $\ln L = -44419.380$) to the topology consistent with the Amarsipobranchia hypothesis ($\ln L = -44653.593$) recovered a difference in log likelihood of 234.20 (standard deviation of 30.19), and rejected the null hypothesis of equal likelihood of the two topologies.

4. Discussion

Exclusive use of phylogenetic data from one source—nuclear, mitochondrial, plastid, or any other—can engender biases in tree topology. These biases can only be tested by comparing topological congruence from other data sources. Topological discordance between mitochondrial and nuclear datasets was observed early in the history of molecular phylogenetics (e.g., Degnan, 1993; Slade et al., 1994) and is attributable to multiple idiosyncrasies of the mitochondrial genome. For example, the length of the mitochondrial genome constitutes only a minuscule fraction of the length of the nuclear haploid genome—approximately 0.0073% in the case of the gastropod *Lottia gigantea* G.B. Sowerby, I, 1834 (~26,400 bp in the mitochondrial genome, compared to ca. 359.5 Mbp in the nuclear genome). Mitochondrial and nuclear genomes also differ in degree of recombination, modes of inheritance, and incidence of introns—differences that can affect inferences of evolutionary history. Additionally, mutation rates of mitochondrial DNA are generally higher than those of nuclear DNA, with typical estimates ranging from two- to over tenfold with respect to nuclear genes

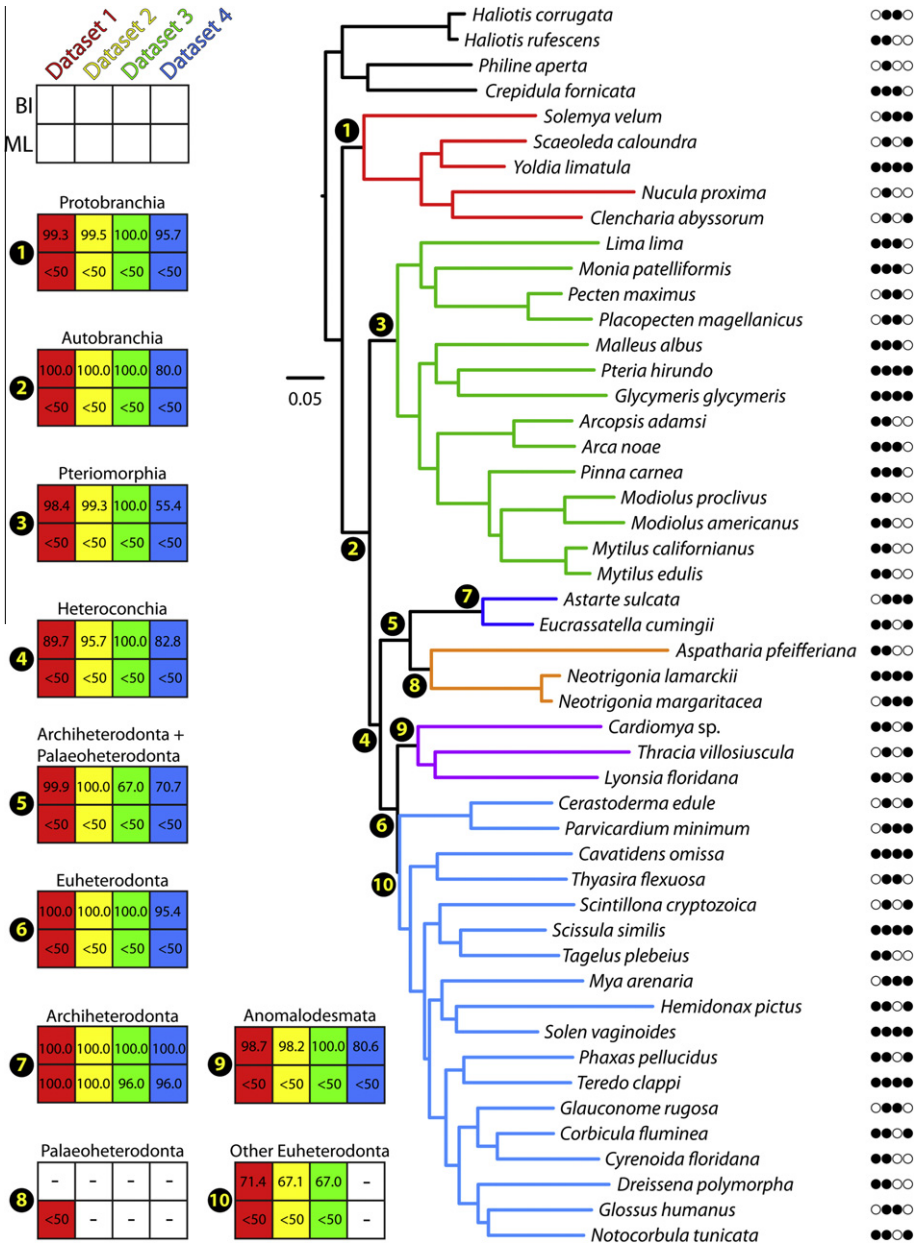

Fig. 2. Phylogenetic relationships of Bivalvia based on maximum likelihood analysis of four nuclear protein-encoding genes (ln $L = -44419.380$). Colors in tree topology correspond to major lineages (as in Fig. 1). Navajo rugs correspond to 10 nodes of interest. Colors in Navajo rugs correspond to each dataset; numbers in Navajo rugs indicate posterior probabilities from Bayesian analysis (top row) or bootstrap resampling frequency (bottom row). Failure to retrieve a node is indicated as a white entry (without a number indicating nodal support). Filled circles at the right of each taxon indicate representation by the gene of interest, from left to right: ATP synthase β, elongation factor-1α, myosin heavy chain type II, and RNA polymerase II. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Brown et al., 1979; Moriyama and Powell, 1997; Denver et al., 2000; Lambert et al., 2002; Howell et al., 2003), limiting inference of deep phylogenetic events, namely, the origin and early diversification of bivalves. Strand-specific substitution biases have also been shown to occur in mitochondrial genomes (Ballard, 2000; Haag-Liautard et al., 2008). Consequently, a number of reviews have critiqued the role of mitochondrial DNA in phylogenetics, suggesting caution in their use, simultaneous deployment with nuclear genes, and/or omission from phylogenetic study altogether (e.g., Ballard and Whitlock, 2004; Rubinoff and Holland, 2005; Fisher-Reid and Wiens, 2011).

These concerns weigh heavily upon recent inferences of bivalve basal relationships based solely on mitochondrial genes (Plazzi and Passamonti, 2010; Plazzi et al., 2011). As proponents of the total evidence approach, we do not countenance in principle or in practice the omission of mitochondrial genes from assessment of phylogenetic relationships. Previous studies have demonstrated the utility of mitochondrial genes for resolving shallow nodes in bivalve phylogenies, a property stemming from the variability and increased mutation rate of the mitochondrial genome (e.g., Giribet and Wheeler, 2002; Giribet and Distel, 2003; Wilson et al., 2010)—the very property that discourages their use for resolving deep nodes. Moreover, as algorithms and models are improved, especially for analyzing mitochondrial gene order and amino acid sequence data, the utility of mitochondrial genes is anticipated to increase with improved taxonomic sampling. For this reason, we

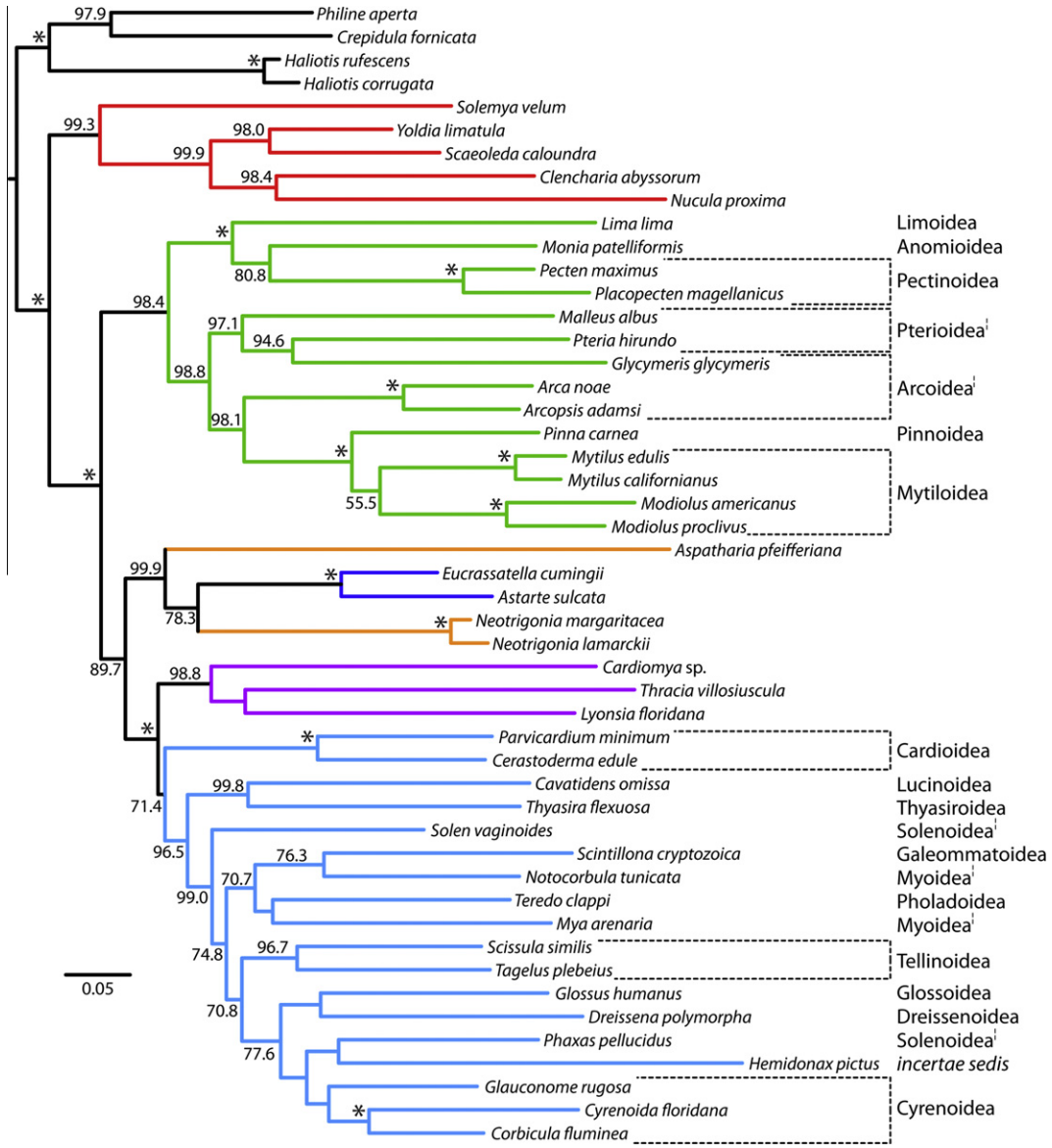

Fig. 3. Phylogenetic relationships of Bivalvia based on Bayesian inference analysis of four nuclear protein-encoding genes. Colors in tree topology correspond to major lineages (as in Fig. 1). Numbers on nodes indicate posterior probabilities, with asterisks indicating a value of 100%. Superfamilies of Pteriomorphia and Euheterodonta (not including Anomalodesmata) are as indicated, with a broken line symbol indicating non-monophyletic groups. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

continue to advocate the inclusion of mitochondrial genes in concert with nuclear ones in order to resolve relationships of various phylogenetic depths.

Accordingly, we regard with skepticism a topology of basal bivalve relationships that is derived exclusively from a particular genomic source with a high mutation rate and concomitant complex algorithmic treatment that yields uniformly high nodal support. The topology supporting Amarsipobranchia (*sensu* Plazzi et al., 2011) is based on four mitochondrial genes and suggests radical reorganization of bivalve higher-level systematics. In addition, the use of multiple protein-encoding sequences with single nucleotide indels among the datasets of Plazzi et al. (2011) is particularly suspect; the authors admitted to being unable to rule out sequencing errors or numts as potential explanations, but nevertheless favored the functionality of their sequenced amplicons.

Separately, we observe that the contending hypotheses of basal bivalve relationships (Giribet and Wheeler, 2002; Wilson et al., 2010) are based on multilocus datasets, but those dominated by nuclear ribosomal genes. These datasets have previously employed the "workhorses" of molecular phylogenetics: the mitochondrial genes 16S rRNA and cytochrome *c* oxidase subunit I; the nuclear ribosomal genes 18S rRNA and 28S rRNA; and a small (327-bp) fragment of the nuclear protein-encoding gene histone H3. This combination of nuclear and mitochondrial genes was anticipated to be capable of resolving relationships of various depths, by sampling both slow (e.g., 18S rRNA) and fast evolving sites (e.g., 16S rRNA). However, the amplicons of these genes are not uniformly distributed in length; the two nuclear ribosomal genes alone typically constitute ca. 70% of the entire dataset (e.g., Wilson et al., 2010). Therefore, evidence of misleading signal stemming from disproportionate representation of nuclear ribosomal genes must also be tested independently of mitochondrial gene signal.

Here we have reevaluated basal bivalve relationships using a separate set of genes altogether to redress the topological conflicts in early bivalve phylogenies. Our four molecular loci are not inherited as a linked or structurally dependent unit, as are the

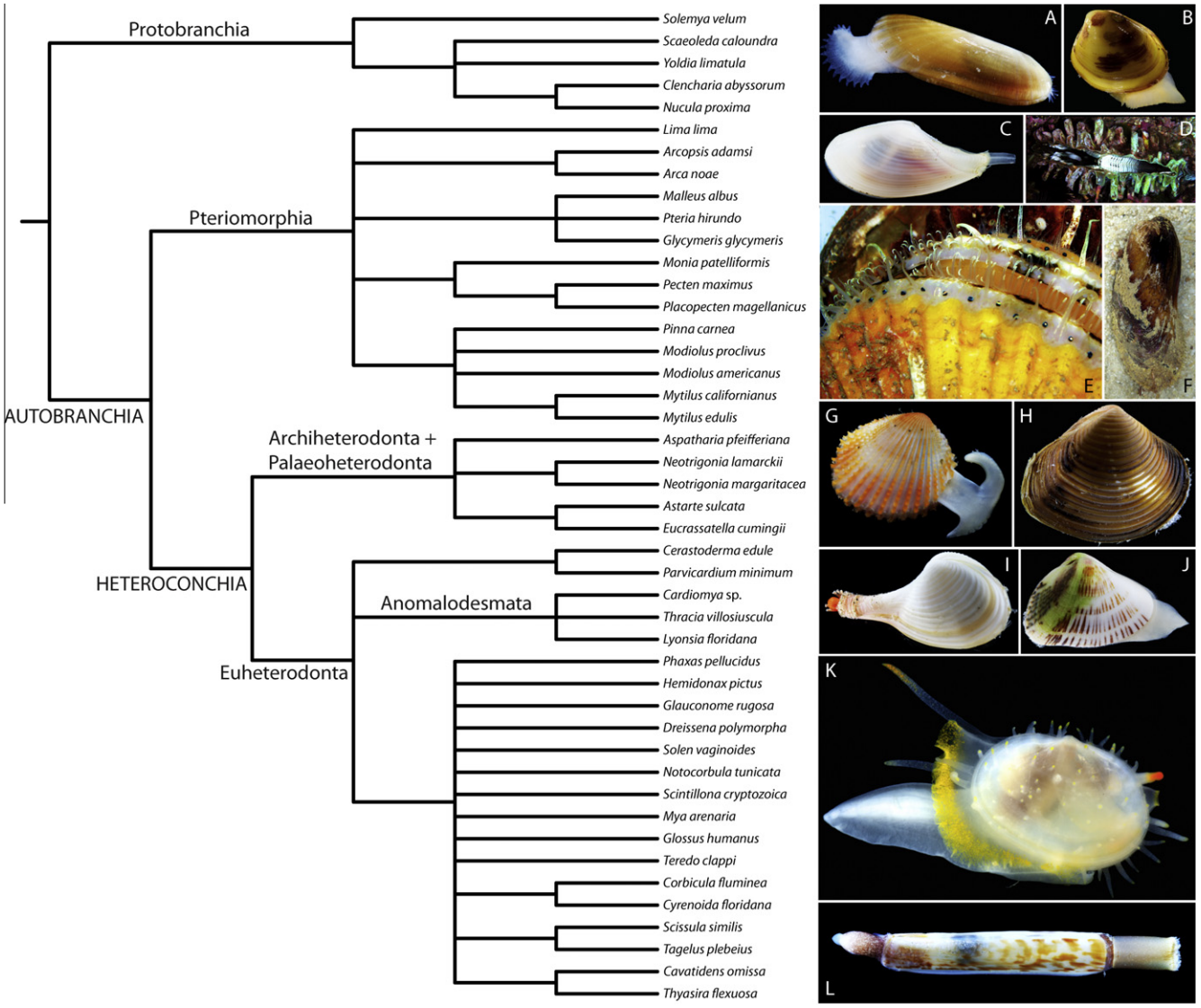

Fig. 4. Strict consensus of all eight topologies obtained (two from each dataset). Bivalve exemplars photographed are: (A) *Solemya velum* (Protobranchia); (B) *Nucula expansa* Reeve, 1855 (Protobranchia); (C) *Scaeoleda caloundra* (Protobranchia); (D) *Pinna rudis* Linnaeus, 1758 (Pteriomorphia); (E) *Aequipecten opercularis* (Linnaeus, 1758) (Pteriomorphia), detail of simple eyes; (F) *Modiolus proclivus* (Pteriomorphia); (G) *Neotrigonia lamarckii* (Palaeoheterodonta); (H) *Eucrassatella cumingi* (Archiheterodonta); (I) *Cuspidaria latesulcata* (Tenison-Woods, 1878) (Anomalodesmata); (J) *Hemidonax pictus* (Euheterodonta); (K) *Scintillona cryptozoica* (Euheterodonta); (L) *Solen vaginoides* (Euheterodonta).

mitochondrial genes. The tree topologies that we obtained are remarkably congruent with the traditional classification of bivalves (*sensu* Bieler and Mikkelsen, 2006). Although bootstrap resampling frequencies were low in ML analyses, all topologies examined recovered the monophyly of Protobranchia, Autobranchia, Heteroconchia, Pteriomorphia, Archiheterodonta, and Euheterodonta (including a monophyletic Anomalodesmata) (Figs. 2–4). Within Euheterodonta, we obtained a sister relationship of Anomalodesmata to the remaining Euheterodonta in the majority of topologies examined. A basal position of Anomalodesmata among Euheterodonta accords with previous hypotheses based on morphological and molecular characters (e.g., Giribet and Wheeler, 2002; Harper et al., 2006; Taylor et al., 2007).

Among derived Euheterodonta, we observe with interest the clade consisting of *Corbicula fluminea*, *Cyrenoida floridana*, and *Glauconome rugosa* (Figs. 2 and 3), insofar as exemplars of the same three genera were found to cluster in a separate phylogenetic analysis using nuclear ribosomal markers (Taylor et al., 2009). This clade, comprising the superfamilies Cyrenoidea and Cyrenoidoidea, was used to justify the further dismantling of Lucinoidea by Taylor et al. (2009), wherein Cyrenoididae had been placed previously. Our analyses thus corroborate the exclusion of cyrenoidids from Lucinoidea, with support (PP = 99.0%, 98.1%, 95.4%, and 97.0% in Datasets 1–4, respectively), but places Cyrenoididae as sister group to Cyrenidae (formerly Corbiculidae), with Glauconomidae as their sister family, therefore making Cyrenoidea paraphyletic with respect to Cyrenoidoidea.

Palaeoheterodonta was obtained as monophyletic in only one of eight topologies examined. The recovery of a paraphyletic Palaeoheterodonta with respect to Archiheterodonta appears to stem from missing data for the taxon *Aspatharia pfeifferiana*, which is represented here by only two gene partitions (Fig. 2, Supplementary Table 1). We observe similar sensitivity to analytical treatment among other taxa that are represented by fewer sequence data, visualized as little structure within Pteriomorphia and Euheterodonta subsequent to strict consensus across all topologies examined (Fig. 4). The monophyly of Palaeoheterodonta is contentious (Purchon, 1987; Morton, 1996; Salvini-Plawen and Steiner,

1996; Waller, 1998; Cope, 2000) and we are as yet unable to test it, given our present sampling of only two of the 175 extant palaeoheterodont genera (Roe and Hoeh, 2003). Similarly, we observe extensive non-monophyly among the constituent superfamilies of Pteriomorphia and Euheterodonta that were sampled in this study (Fig. 3), but the internal relationships of these diverse subclasses is beyond the scope of the present study.

We also uniformly obtain the monophyly of the curious clade (Archiheterodonta + Palaeoheterodonta), a result obtained by a previous phylogenetic analysis (using the aforementioned "workhorses"; Wilson et al., 2010) (Figs. 2–4). In addition to phylogenetic support and stability based on molecular data, this sister relationship is potentially supported by morphological synapomorphies, namely the hind end of the ctenidia unattached to the mantle (Purchon, 1990), or the presence of Atkin's type D ciliary currents, although both characters require further scrutiny. Members of Carditida (i.e., *Cyclocardia ventricosa* and *Astarte sulcata*—see Yonge, 1969; but see Saleuddin, 1965, for a different view on *Astarte*) and Unionida show Atkin's type D ciliary currents (see Atkins, 1937). Tevesz (1975) also considered the ctenidial ciliation of *Neotrigonia* to be of type D, as in Unionida, but this was disputed by Morton (1987). Further phylogenetic study is anticipated to test the monophyly of this clade using separate molecular loci, in addition to morphological characters.

In general, the removal of length variable regions and/or third codon positions from the alignment had no effect on the relationships obtained (nodes 1–7, 9 in Fig. 2). Bootstrap resampling frequencies were very low across all topologies, possibly as a consequence of the degree of character conflict, missing data, the short length of the combined dataset, or some combination of these. In any case, we cannot assess the effects of analytical treatment based on the bootstrap values. However, posterior probabilities in Bayesian analyses marginally increased upon removal of length variable regions and tended to decrease for some nodes upon removal of both length variability and third codon positions. These data suggest that complexity of algorithmic treatment is not required to elucidate phylogenetic signal among these nuclear protein-encoding genes, particularly with respect to treatment of third codon positions, as suggested for mitochondrial genes (Plazzi et al., 2011).

Comparison of the consensus topology obtained in the present study to previous hypotheses of bivalve relationships indicates significant congruence with a topology using five genes (Wilson et al., 2010) with respect to relationships among Autobranchia, and with the topology recovered by second-generation sequencing techniques (Smith et al., 2011) with respect to the monophyly of Protobranchia. These results favor the traditional classification of Bivalvia (Bieler and Mikkelsen, 2006), albeit with the emended relationship of (Archiheterodonta + Palaeoheterodonta). None of these results is consistent with a group uniting Nuculanida, Pteriomorphia, Archiheterodonta, Anomalodesmata, and the other Euheterodonta, i.e., Amarsipobranchia (*sensu* Plazzi et al., 2011). We therefore reject the putative homology of the gill character that supposedly unites this clade, given (a) the homoplastic mapping engendered by this character upon superimposition on either our consensus topology or the topology obtained by Wilson et al. (2010)—congruent with ours but derived from a completely non-overlapping set of genes; (b) the enforced homoplasy of other morphological characters traditionally considered synapomorphies for various groups of bivalves, if the topology of Plazzi et al. (2011) were accepted; and (c) the results of the SH test, which indicates that the Amarsipobranchia hypothesis is significantly worse than the maximum likelihood topology obtained using either the "workhorses" or the four genes analyzed in this study.

The robustness of the phylogenetic signal embedded in these four protein-encoding genes, in addition to the marked congruence observed between these markers and the "workhorses" of molecular phylogenetics (see Aktipis and Giribet, 2010, 2012, for a similar case in gastropods), strongly favors their continued use in the study of molluscan phylogenetics specifically, and invertebrate systematics generally. Regrettably, we were unable to sample more broadly the diversity of both Euheterodonta and Pteriomorphia, which would have enabled an assessment of the utility of these markers for elucidating relationships within diverse subclasses (Fig. 3), but consistent amplification across taxa can prove difficult, even when using freshly collected tissue. The utility of these markers for elucidating shallow relationships is beyond the scope of this study. However, comparable use of one or more of these loci in studies of other invertebrate taxa (e.g., Regier and Shultz, 1997, 2000; Sperling et al., 2007; Aktipis and Giribet, 2010) offers promising prospects for the applicability of these markers in multilocus datasets, albeit with the added challenge of traditional RT-PCR techniques. We additionally observe that widespread proliferation of phylogenomic data for non-model invertebrate taxa (e.g., Dunn et al., 2008; Hejnol et al., 2009; Meusemann et al., 2010; Kocot et al., 2011; Smith et al., 2011) heralds access to an unprecedented stockpile of efficacious molecular loci for phylogenetic study of diverse invertebrate groups.

5. Conclusion

Concordance between topologies based on the "workhorses"—the five traditionally used molecular loci that include nuclear and ribosomal genes (e.g., Wilson et al., 2010)—and the four nuclear protein-encoding markers employed here suggest that robust assessment of phylogenetic relationships of various depths is best achieved by sampling markers spanning a spectrum of evolutionary rates.

Author contributions

PPS: Designed, conducted phylogenetic analyses; wrote manuscript; made figures.

VLG: Collected fresh tissues for RNA extraction; collected nucleotide sequence data; designed bivalve-specific primers for ATP Synthase beta (Table 1); generated and checked alignments; accessioned sequences in GenBank; wrote Molecular Methods section; edited manuscript.

GYK: Generated and checked alignments; conducted likelihood ratio tests using jModeltest; edited manuscript.

SCSA: Collected nucleotide sequence data; approved manuscript.

AG: Collected fresh tissues for RNA extraction; collected nucleotide sequence data.

TMC, EAG, EMH, JMH, PMM, JDT: Collected fresh tissues for RNA extraction; read and approved manuscript.

RB: Conceived and supervised Bivalve Tree of Life project; collected fresh tissues for RNA extraction; wrote and checked discussion on most recent systematics of bivalves; edited manuscript.

GG: Conceived and supervised Bivalve Tree of Life project; collected fresh tissues for RNA extraction; supervised molecular work; edited manuscript.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2012.05.025.

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