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Into the deep: A phylogenetic approach to the bivalve subclass Protobranchia

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ABSTRACT

A molecular phylogeny of Protobranchia, the subclass of bivalve mollusks sister to the remaining Bivalvia, has long proven elusive, because many constituent lineages are deep-sea endemics, which creates methodological challenges for collecting and preserving genetic material. We obtained 74 representatives of all 12 extant protobranch families and investigated the internal phylogeny of this group using sequence data from five molecular loci (16S rRNA, 18S rRNA, 28S rRNA, cytochrome *c* oxidase subunit I, and histone H3). Model-based and dynamic homology parsimony approaches to phylogenetic reconstruction unanimously supported four major clades of Protobranchia, irrespective of treatment of hypervariable regions in the nuclear ribosomal genes 18S rRNA and 28S rRNA. These four clades correspond to the superfamilies Nuculoidea (excluding Sareptidae), Nuculanoidea (including Sareptidae), Solemyoidea, and Manzanelloidea. Salient aspects of the phylogeny include (1) support for the placement of the family Sareptidae with Nuculanoidea; (2) the non-monophyly of the order Solemyida (Solemyidae + Nucinellidae); (3) and the non-monophyly of most nuculoid and nuculanoid genera and families. In light of this first family-level phylogeny of Protobranchia, we present a revised classification of the group. Estimation of divergence times in concert with analyses of diversification rates demonstrate the signature of the end-Permian mass extinction in the phylogeny of extant protobranchs.

1. Introduction

Among the poorest known molluscan groups is the subclass Protobranchia, a bivalve lineage that has diversified and colonized the deepest oceans, with numerous cosmopolitan species at abyssal depths (Allen and Sanders, 1996; Etter et al., 2011; Zardus et al., 2006). Of the ca. 750 protobranch species (Table 1; Zardus, 2002), most are deposit feeders in soft sediments, but two lineages host chemoautotrophic, sulfide-oxidizing bacteria, with concomitant reductions of the hosts' alimentary system (Cavanaugh, 1983; Gustafson and Reid, 1988; Yamanaka et al., 2008; Oliver et al., 2011; Oliver and Taylor, 2012). The incidence of doubly uniparental inheritance (i.e., mitochondrial heteroplasmy), once thought to occur only in Autobranchia, has been discovered very recently in a protobranch species, suggesting an earlier origin of this exceptional mode of mitochondrial transmission in bivalves (Doucet-Beaupré et al., 2010; Boyle and Etter, 2013). Protobranchs have a probable Cambrian origin (Cope, 1996, 1997; but see Carter et al., 2000), with several lineages radiating thereafter in the deep sea, where they constitute the dominant group of bivalves (Allen, 1978, 1979).

Early studies on bivalve phylogenetics based on nucleotide sequence data frequently recovered non-monophyly of Protobranchia and suggested an early split into Opponobranchia (the clade Nuculida + Solemyida) and Foliobranchia (Nuculanida + Autobranchia) (e.g., Giribet and Wheeler, 2002; Giribet and Distel, 2003; Giribet, 2008; Wilson et al., 2010). More recently, the monophyly of Protobranchia has become well established on the basis of larger molecular analyses (Kocot et al., 2011; Smith et al., 2011; Sharma et al., 2012), consistent with a compelling number of morphological characters that have traditionally united the protobranch bivalves. These characters include the eponymous protobranch gill, which resembles the putatively plesiomorphic gill of patellogastropods; the palp proboscides (absent in the solemyoids, likely a consequence of obligate chemosymbiosis, as with reductions of the alimentary system); and characteristic taxodont dentition, consisting of a series of identical or very similar vertical teeth (Coan et al., 2000). Additionally, protobranchs are distinguished from other Bivalvia in having a pericalymma larva (e.g., Drew, 1899; Gustafson

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Table 1
Diversity and sampling of extant protobranch families.

Family	Described species	Sampled species
Solemyidae	29	6
Nucinellidae	20	2
Nuculidae	167	10
Sareptidae	7	3
Bathyspinulidae	19	5
Malletiidae	60	3
Neilonellidae	39	3
Nuculanidae	214	17
Phaseolidae	3	1
Siliculidae	6	3
Tindariidae	30	2
Yoldiidae	158	9

and Reid, 1986; Zardus and Morse, 1998). By contrast, the autobranch bivalves bear a typical veliger larva, comparable to gastropod counterparts (Jablonski and Lutz, 1983).

Several classifications of Protobranchia have been proposed, but most agree on division into three orders, Nuculida Dall, 1889, Solemyida Dall, 1889 (divided into Solemyoidea Gray, 1840 and Manzanelloidea Chronic, 1952), and Nuculanida Carter, Campbell and Campbell, 2000 (Bieler et al., 2010). However, the monophyly of Manzanelloidea has been questioned (Oliver and Taylor, 2012) and the number of families and their constituent genera remains in flux (Table 1). Protobranch phylogenetic study is still in its infancy, as little morphological and molecular work has focused on this basal clade of bivalves. Due to the increasing predominance of protobranchs with depth, this group of bivalves has figured prominently in studies on speciation in the deep sea (Allen, 1971; Etter et al., 2005), with recent efforts highlighting discovery of species from extreme environments (e.g., Oliver et al., 2011; Oliver and Taylor, 2012) or the nature of endosymbiosis with sulfide-oxidizing bacteria (e.g., Taylor and Glover, 2010; Oliver and Taylor, 2012). The presence of chemosymbiosis in Nucinellidae has been inferred (Reid, 1990, 1998; Taylor and Glover, 2010), and corroborated by both anatomical and molecular data (Oliver and Taylor, 2012).

Resolution within Protobranchia has been analysis-dependent, but previous studies have supported the sister relationship of Solemyidae to the clade (Nuculida + Nuculanida), albeit without sampling Manzanelloidea (Smith et al., 2011; Sharma et al., 2012). The relationships of Nucinellidae and Solemyidae were reviewed by Oliver and Taylor (2012; see also Pojeta, 1988), and a small analysis of Solemyidae was recently published (Taylor et al., 2008). Although with limited taxon sampling, Taylor et al.'s (2008) study addresses the taxonomy of Solemyidae and considerably advances our knowledge of these bivalves, supporting the reciprocal monophyly of *Acharax* and *Solemya*, and the monophyly of the subgenus *Solemyarina*. Analysis of an 18S rRNA dataset of the solemyid genus *Acharax* has similarly revealed aspects of diversification among Indo-Pacific species (Neulinger et al., 2006). Barring these few advances, protobranch internal phylogeny remains largely unknown, because few families have been included in previous sampling efforts. For example, Manzanelloidea has heretofore not been represented in a molecular phylogenetic analysis.

The state of protobranchiate phylogenetics is in marked contrast to that of major groups within Autobranchia, many of which have been investigated using molecular data and have demonstrably stable phylogenies (e.g., pterioids: Tëmkin, 2010; palaeoheterodonts: Graf and Cummings, 2006; anomalodesmatans: Harper et al., 2006; veneroids: Mikkelsen et al., 2006; heterodonts: Taylor et al., 2007). In part, the recalcitrance to include Protobranchia in molecular phylogenetic datasets is attributable to operational challenges stemming from their habitat; protobranch tissues suitable for molecular techniques are notoriously difficult to obtain for some groups because of the great depths that these bivalves inhabit. Inherent to the task is the difficulty of identifying living (or recently expired) and minute (often less than 3 mm) specimens that require several hours to raise from the deep sea via dredging (Boyle et al., 2004). Moreover, the solubility of calcium carbonate at great depths is such that for some specimens, only the periostracum remains by the time the specimen is recovered. The mainly deep-sea solemyid genus *Acharax* (see Yamanaka et al., 2008 for a shallow example) is particularly susceptible to this phenomenon, hence is rarely obtained alive (Coan et al., 2000; Neulinger et al., 2006). Many *Acharax* also burrow deeply and are capable of swimming when disturbed, hampering collecting efforts.

To redress this long-standing lacuna in bivalve phylogeny, we assembled a multilocus dataset to infer a protobranch phylogeny, which required multiple collecting campaigns extending over a decade. Our taxon sampling encompasses for the first time all extant families of Protobranchia described heretofore, including the enigmatic Sareptidae. On the basis of this phylogeny, we present an updated classification of the protobranchs.

2. Materials and methods

2.1. Species sampling

Specimens of Protobranchia were collected by the authors and multiple other individuals over several collecting campaigns. Rare species were largely obtained by deep-sea dredging. Data collected in previous studies (e.g., Giribet and Wheeler, 2002; Giribet and Distel, 2003; Passamaneck et al., 2004) were additionally accessed from GenBank. Collected specimens were stored in 96% EtOH.

Sequenced specimens consisted of seven Solemyidae, two Nucinellidae, 48 Nuculanoidea, and 17 Nuculoidea (including Sareptidae). These spanned all 12 recognized families of extant Protobranchia *sensu* Bieler et al. (2010). Outgroup taxa for the study consisted of three Gastropoda, three Pteriomorphia, and nine Heterodonta. However, we have previously observed that ribosomal-dominated datasets consistently result in non-monophyly of Protobranchia (Giribet and Wheeler, 2002; Giribet and Distel, 2003; Wilson et al., 2010; reviewed in Sharma et al., 2012) (Supplementary Fig. 1). Given that the monophyly of protobranch bivalves and the sister relationship of Solemyidae to the remaining Protobranchia have been demonstrated recently using nuclear genes and phylogenomic approaches (Smith et al., 2011; Sharma et al., 2012), and that this study is concerned only with internal relationships, we limited outgroup sampling to the subset of gastropods for principal analyses. The full list of specimens included in our study is found in Table 2; collecting data are provided in Supplementary Table 1.

2.2. Molecular methods

Total DNA was extracted from dissected tissues or whole animals using Qiagen's DNeasy® tissue kit (Valencia, CA, USA). Formalin-fixed tissues were extracted following the protocol of Boyle et al. (2004). Purified genomic DNA was used as a template for PCR amplification. Molecular markers consisted of two mitochondrial genes (16S rRNA and cytochrome *c* oxidase subunit I), two nuclear ribosomal genes (18S rRNA and 28S rRNA), and one nuclear protein-encoding gene (histone H3). Primer sequences and obtained fragment lengths are indicated in Supplementary Table 2.

Polymerase chain reactions (PCR), visualization by agarose gel electrophoresis, and direct sequencing were conducted for most specimens as described by Sharma and Giribet (2009). For rare specimens, PCR was conducted using illustra™ Ready-To-Go™ PCR Beads (GE Healthcare, Little Chalfont, UK). Chromatograms obtained from the automatic sequencer were read and sequences

Table 2
List of species and gene fragments included in phylogenetic analyses.

	Family	Source	18S rRNA	28S rRNA	COI	16S rRNA	histone H3
MANZANELLOIDEA							
Huxley munita (Dall, 1898)	Manzanellidae	BivAToL-137	KC429323	KC429412-13			KC429157
Nucinella sp.	Manzanellidae	MNHN; MCZ MAL-379095/DNA101571	KC429324	KC429414	KC429089		KC429158
SOLEMYOIDEA							
Acharax bartschii (Dall, 1908)	Solemyidae	MCZ DNA106839 / CASIZ 188907	KC984714	KC984828		KC984671	KC984781
Acharax gadirae Oliver, Rodrigues & Cunha, 2011	Solemyidae	MCZDNA106719	KC984715	KC984793		KC984672	
Solemya elarraichensis Oliver, Rodrigues & Cunha, 2011	Solemyidae	MCZ MAL-379147/DNA106718	KC984719	KC984795	KC984743	KC984673	KC984779
Solemya pervernicosa Kuroda, 1948	Solemyidae	GenBank	AF117737				
Solemya velesiana Iredale, 1931	Solemyidae	BivAToL-73	KC984717	KC984794	KC984744	KC984674	KC984780
Solemya velum Say, 1822	Solemyidae	MCZ MAL-379150	KC984718	KC984796	KC984745	KC984675	KC984778
Solemya velum Say, 1822	Solemyidae	BivAToL-17	AF120524	KC429415	U56852	JQ728447	AY070146
NUCULANOIDEA							
Bathyspinula calcar (Dall, 1908)	Bathyspinulidae	AMNH PRTB001	KC993875			KC993870	
Bathyspinula filatovae (Knudsen, 1967)	Bathyspinulidae	AMNH PRTB002	KC993876	KC984841		KC993871	KC993889
Bathyspinula hilleri (Allen & Sanders, 1982)	Bathyspinulidae	UMASS 3D534.2	KC984712	KC984806	KC984733	KC993874	KC984773
Tindariopsis agatheda (Dall, 1890)	Bathyspinulidae	AMNH PRTB003	KC993877			KC993869	
Tindariopsis sulcata (Gould, 1852)	Bathyspinulidae	AMNH PRTB004	KC993878			KC993868	
Clencharia abyssorum (Verrill & Bush, 1898)	Maletiidae	BivAToL-217	KC429320	KC429409			KC429154
Malletia cuneata (A) Jeffreys, 1876	Maletiidae	UMASS Ice142.5	KC984697	KC984809		KC984669	KC984759
Malletia cuneata (B) Jeffreys, 1876	Maletiidae	UMASS 3D534.6	KC984698	KC984810			KC984758
Malletia johnsoni Clarke, 1961	Maletiidae	AMNH PRTB005	KC993879	KC984837		KC993872	KC993888
Neilonella salicensis (Seguenza, 1877)	Neilonellidae	AMNH PRTB006	KC993881	KC984838			KC993887
Neilonella subovata (Verrill & Bush, 1897)	Neilonellidae	GenBank	AF207645	AF207652	AF207656		
Neilonella whoii Allen & Sanders, 1996	Neilonellidae	BivAToL-218	KC984695	KC984822	KC984732	KC984659	KC984756
Adrana scaphoides Rehder, 1939	Nuculanidae	MCZ DNA100657	KC984691	KC984819			KC984753
Jupiteria sematensis (Suzuki & Ishizuka, 1943)	Nuculanidae	GenBank		AB103131			
Jupiteria sp.	Nuculanidae	MNHN; MCZ DNA105568		KC984825			KC993885
Jupiteria sp.	Nuculanidae	MNHN; MCZ DNA105566		KC984824			KC993884
Jupiteria sp.	Nuculanidae	MNHN; MCZ DNA105567		KC984821			KC993886
Ledella ecaudata (Pelseneer, 1903)	Nuculanidae	AMNH PRTB007	KC984701	KC984843		KC984666	KC984792
Ledella jamesi Allan & Hannah, 1989	Nuculanidae	UMASS 3D534.3	KC984700	KC984839	KC984739		KC984770
Ledella pustulosa (Jeffreys, 1876)	Nuculanidae	UMASS Ice142.3	KC984710	KC984804		KC993873	KC984771
Ledella sp.	Nuculanidae	MCZ DNA105564	KC984711	KC984805	KC984738		KC984772
Ledella ultima (Smith, 1885)	Nuculanidae	DIVA2; MCZ DNA104865	KC984685	KC984820	KC984740	KC984667	KC984769
Nuculana conceptionis (Dall, 1896)	Nuculanidae	AMNH PRTB008	KC984688	KC984800			KC984763
Nuculana minuta (Müller, 1776)	Nuculanidae	GenBank	DQ279938	DQ279961	DQ280018	DQ280030	DQ280002
Nuculana minuta (Müller, 1776)	Nuculanidae	Protostome AToL	AF120529	AF120586	AF120643	KC984664	KC984765
Nuculana pella (Linnaeus, 1767)	Nuculanidae	MCZ MAL-379011/DNA100065	AY070111	AY070124	AY070138		AY070148
Nuculana pernula (Müller, 1779)	Nuculanidae	MCZ MAL-379111/DNA100121	AF207644	AF207651			KC984764
Nuculana pernula (Müller, 1779)	Nuculanidae	GenBank	AY145385	AY145419			
Nuculana pernula (Müller, 1779)	Nuculanidae	BivAToL-134.1a	KC984693	KC984801	KC984737		KC984766
Propeleda cf. *carpenteri*	Nuculanidae	UMASS 3D534.2	KC984687	KC984799	KC984735		KC984761
Propeleda cf. *longicaudata*	Nuculanidae	AMNH PRTB009	KC984692	KC984802	KC984736	KC984665	KC984785
Scaeoleda caloundra (Iredale, 1929)	Nuculanidae	BivAToL-100	KC429321	KC429410			KC429155
Lametila abyssorum Allen & Sanders, 1973	Phaseolidae	UMASS EN_10UC1	KC984705	KC984798		KC984661	KC984783
Silicula rouchi Lamy, 1911	Siliculidae	AMNH PRTB010	KC984686	KC984836		KC984663	KC984767
Silicula sp.	Siliculidae	MNHN; MCZ DNA105569	KC984703	KC984840	KC984734		KC984762
Silicula sp.	Siliculidae	UMASS 3D561.13	KC984694	KC984803			KC984760
Tindaria kennerlyi (Dall, 1897)	Tindariidae	AMNH PRTB011	KC984702	KC984812	KC984731		KC984755
Tindaria sp.	Tindariidae	MNHN;MCZ DNA105565	KC993882	KC984823			
Megayoldia sp.	Yoldiidae	MCZ MAL-378912/DNA104864	KC984699	KC984811			KC984757
Yoldia eightsi (Jay, 1839)	Yoldiidae	MCZ MAL-379181/DNA101624	KC984696	KC984808	KC984730		KC984754
Yoldia limatula (Say, 1831)	Yoldiidae	MCZ MAL-379182/DNA100119/BivAToL-19	KC429322	KC429411	KC429088		KC429156
Yoldia myalis (Couthouy, 1838)	Yoldiidae	MCZ MAL-379185/DNA100120	AF207643	AF207650	AF207655		

Yoldia scissurata Dall, 1897	Yoldiidae	AMNH PRTB012	KC984706	KC984797	KC984729		KC984790
Yoldiella americana Allen, Sanders & Hannah, 1995	Yoldiidae	UMASS 3D8369.2	KC984707	KC984842	KC984726	KC984662	KC984787
Yoldiella inconspicua inconspicua Verrill & Bush, 1898	Yoldiidae	UMASS Icel42.7.1	KC984689	KC984807	KC984727	KC984668	KC984788
Yoldiella orcia (Dall, 1916)	Yoldiidae	AMNH PRTB013	KC984690	KC984832	KC984728		KC984789
Yoldiella cf. valleri	Yoldiidae	AMNH PRTB014	KC993880	KC984831			KC993883
NUCULOIDEA							
Acila castrensis (Hinds, 1843)	Nuculidae	GenBank	AF120527	AF120584			
Acila castrensis (Hinds, 1843)	Nuculidae	BivAToL-205	KC429319	KC429408	KC429087	KC429241	
Brevinucula verrilli (Dall, 1886)	Nuculidae	UMASSEN 14CC1	KC984722	KC984814		KC984680	KC984782
Ennucula cf. *cardara*	Nuculidae	AMNHPRTB015	KC984716	KC984829	KC984748	KC984681	KC984751
Ennucula granulosa (Verrill, 1884)	Nuculidae	UMASS EN_3aAC8	KC984721	KC984817	KC984749	KC984678	KC984774
Ennucula tenuis expansa (Montagu, 1808)	Nuculidae	MCZ MAL-379107/DNA105848	KC984684	KC984826	KC984747	KC984682	KC984775
Leionucula cf. *cumingi*	Nuculidae	MCZ MAL-379010/DNA103781	KC984724	KC984813	KC984750	KC984683	KC984752
Nucula atacellana Schenck, 1939	Nuculidae	BivAToL-215/MCZ DNA101159	KC984723	KC984818	KC984742	KC984676	KC984768
Nucula profundorum Smith, 1885	Nuculidae	AMNH PRTB016	KC984720	KC984830	KC984741	KC984677	
Nucula proxima Say, 1822	Nuculidae	GenBank	AF120526	AF120583	AF120641	AY377617	
Nucula sulcata Bronn, 1831	Nuculidae	MCZ MAL-379108/DNA100067	KC984713	KC984816		KC984679	KC984776
Nucula sulcata Bronn, 1831	Nuculidae	MCZ MAL-379109/DNA100104		KC984827			
Nucula sulcata Bronn, 1831	Nuculidae	MCZ MAL-379098/DNA100117	AF120525	AF120582			AY070147
Nucula sulcata Bronn, 1831	Nuculidae	MCZ MAL-379099/DNA100118	KC984725	KC984815	KC984746		KC984777
Nucula sulcata Bronn, 1831	Nuculidae	BivAToL-189/Protostome AToL T68	AF207642	DQ279960	DQ280017	DQ280029	DQ280001
SAREPTOIDEA							
Pristigloma cf. *alba*	Sareptidae	UMASS EN_18aLC1	KC984704	KC984834			KC984784
Pristigloma cf. *nitens*	Sareptidae	UMASS EN_10RC1	KC984708	KC984833		KC984670	KC984786
Pristigloma sp.	Sareptidae	UMASS EN_18aXC1	KC984709	KC984835			KC984791
OUTGROUPS							
AUTOBRANCHIA							
Arcopsis adamsi (Dall, 1886)	Noetiidae	GenBank	KC429327	KC429419-20	KC429092	KC429245	KC429162
Lima lima (Linnaeus, 1758)	Limidae	GenBank	KC429339	KC429434	KC429101	KC429257	KC429174
Pinna carnea Gmelin, 1791	Pinnidae	GenBank	KC429337	KC429431-32	KC429099	KC429255	KC429172
Eucrassatella cumingii (Adams, 1854)	Crassatellidae	GenBank	KC429350	KC429448	KC429110	KC429267	KC429187
Neotrigonia lamarckii (Gray, 1838)	Trigoniidae	GenBank	KC429345	KC429443	KC429105	KC429262	KC429182
Unio pictorum (Linnaeus, 1758)	Unionidae	GenBank	KC429349	KC429447	KC429109	KC429266	KC429186
Cardiomya sp.	Cuspidariidae	GenBank	KC429362	KC429463-64	KC429118	KC429276	KC429198
Lyonsia floridana Conrad, 1849	Lyonsiidae	GenBank	KC429353	KC429451	AF120654	KC429268	KC429191
Thracia sp.	Thraciidae	GenBank	KC429356	KC429454-56	KC429115	KC429271	KC429194
Dreissena polymorpha (Pallas, 1771)	Dreissenidae	GenBank	AF120552	KC429513-14	KC429149	DQ280038	KC429234
Solen vaginoides Lamarck, 1818	Solenidae	GenBank	KC429399	KC429507		KC429308	KC429230
Thyasira flexuosa (Montagu, 1803)	Thyasiridae	GenBank	KC429367	KC429469	KC429122		KC429200
GASTROPODA							
Crepidula fornicata (Linnaeus, 1758)	Calyptraeidae	GenBank	AY377660	AY145406	AF353154	AY377625	AY377778
Haliotis tuberculata Linnaeus, 1758	Haliotidae	GenBank	AY145418	AY145418	AY377729	AY377622	AY377775
Siphonaria pectinata (Linnaeus, 1758)	Siphonariidae	GenBank	X91973	DQ256744	AF120638	AY377627	AY377780

assembled using the sequence editing software Sequencher™ (Gene Codes Corporation, Ann Arbor, MI, USA). Sequence data were edited in Se-Al v. 2.0a11 (Rambaut, 1996).

2.3. Phylogenetic Analyses

Maximum likelihood (ML) and Bayesian inference (BI) analyses were conducted on static alignments, which were inferred as follows. Sequences of ribosomal genes were aligned using MUSCLE v. 3.6 (Edgar, 2004) with default parameters, and subsequently treated with GBlocks v. 0.91b (Castresana, 2000) to cull positions of ambiguous homology. Sequences of protein encoding genes were aligned using MUSCLE v. 3.6 with default parameters as well, but alignments were additionally confirmed using protein sequence translations prior to treatment with GBlocks v.0.91b. The size of data matrices for each gene prior and subsequent to treatment with GBlocks v. 0.91b is provided in Supplementary Table 3.

ML analyses were conducted using RAxML ver. 7.2.7 (Stamatakis, 2006). For the maximum likelihood searches, a unique General Time Reversible (GTR) model of sequence evolution with corrections for a discrete gamma distribution (GTR + Γ) was specified for each data partition, and 100 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.

BI analysis was performed using MrBayes v. 3.1.2 (Huelsenbeck and Ronquist, 2005) with a unique GTR model of sequence evolution, corrections for a discrete gamma distribution and a proportion of invariant sites (GTR + Γ + I) specified for each partition, as selected in jModeltest v. 0.1.1 (Guindon and Gascuel, 2003; Posada, 2008) under the Akaike information criterion (AIC) (Posada and Buckley, 2004) (Supplementary Table 3). Default priors were used starting with random trees. Two runs, each with three hot and one cold Markov chains, were executed until the average deviation of split frequencies reached <0.01 (10^7 generations). Convergence diagnostics were assessed using Tracer ver. 1.5 (Rambaut and Drummond, 2009).

Parsimony analyses were based on a direct optimization (DO) approach (Wheeler, 1996) using POY v. 4.1.2 (Varón et al., 2010). Tree searches were performed using the timed search function in POY, i.e., multiple cycles of (a) building Wagner trees, (b) subtree pruning and regrafting (SPR) and (c) tree bisection and reconnection (TBR), (d) ratcheting (Nixon, 1999), and (e) tree-fusing (Goloboff, 1999, 2002). Timed searches of 24 h were run on 24 processors under a mixed parameter set, such that ribosomal genes were weighted using the parameter set 3221 (indel opening cost = 3; transversions = transitions = 2; indel extension cost = 1) and protein-encoding genes were weighted using the parameter set 121 (indel cost = 2; transversion cost = 2; transition cost = 1). The design of this parameter set follows previous exploration of invertebrate datasets (Sharma et al., 2011). Nodal support for the optimal parameter set was estimated via jackknifing (250 replicates) with a probability of deletion of e^{-1} (Farris et al., 1996).

2.4. Likelihood-based tests of topology

To assess the strength of phylogenetic evidence for the placement of key taxa, Shimodaira–Hasegawa (SH) tests were conducted using RAxML v. 7.2.7 (Shimodaira and Hasegawa, 1999). We enforced topological constraints consistent with alternative hypotheses of taxon placement for three scenarios: (1) the monophyly of Solemyida (=Solemyidae + Nucinellidae), (2) the monophyly of Opponobranchia (=Solemyida + Nuculida), and (3) the monophyly of Nuculida, including Sareptidae (i.e., the traditional definition of this order). For each evaluation, we compared the resulting sub-optimal likelihood trees to the unconstrained ML topology obtained using the same dataset. To generate the null distribution, 500 resampling replicates were conducted.

2.5. Estimation of divergence times

Ages of clades were inferred using BEAST v. 1.7.4 (Drummond et al., 2006; Drummond and Rambaut, 2007). We specified a unique GTR model of sequence evolution with corrections for a discrete gamma distribution and a proportion of invariant sites (GTR + Γ + I) for each partition (as with BI analysis). An uncorrelated lognormal clock model was inferred for each partition, and a Yule speciation process was assumed for the tree prior. We selected the uncorrelated lognormal model because its accuracy is comparable to an uncorrelated exponential model, but it has narrower 95% highest posterior density intervals. Additionally, the variance of the uncorrelated lognormal model can better accommodate data that are already clock-like (Drummond et al., 2006). Priors were sequentially optimized in a series of iterative test runs; the command files are available upon request from the authors. Two Markov chains were run for 5×10^7 generations, sampling every 5000 generations. Convergence diagnostics were assessed using Tracer ver. 1.5 (Rambaut and Drummond, 2009).

Fossil taxa were used for divergence time calibration, as follows: We constrained the diversification of Nucinellidae using the early Jurassic (Hettangian stage, 201.6–197 Ma) fossil *Nucinella liasina* (Bistram, 1903), which is inferred to be the earliest crown nucinellid (Conti, 1954). To account for uncertainty in estimation of the fossil age we applied a normal distribution prior to this node with a mean of 197 Ma and standard deviation of 5 Myr. While the Permian species *Manzanella cryptodonta*Chronic, 1952 is the type of the superfamily, our examination of the holotype suggests that this specimen may not be a protobranch and thus was not used in the estimation of divergence times. The systematics of *Manzanella* have been addressed elsewhere (Oliver and Taylor, 2012). Diversification of Solemyoidea was constrained using the Ordovician fossil *Ovatoconcha* (Cope, 1999); a normal distribution prior with a mean of 475 Ma and standard deviation of 10 Myr was applied to this node. Nuculida was constrained using the same prior distribution as for Solemyoidea, on the basis of several Arenig fossils (Cope, 2004). Finally, the age of the ingroup was constrained using a uniform prior bounding the maximum age of Protobranchia at 545 Ma, based on the age of the earliest crown-group bivalve, *Fordilla troyensis* (Pojeta et al., 1973; Pojeta and Runnegar, 1974; Pojeta, 2000; Parkhaev, 2008).

2.6. Diversification through time

Likelihood analyses of speciation and extinction were conducted using Laser v. 2.3 (Rabosky, 2006) on the dated tree topology. To overcome the effect of incomplete sampling of extant lineages and the associated rate slowdown artifact (Cusimano and Renner, 2010), branching times younger than 65 Myr were culled. Six models were fitted to the truncated protobranch chronogram: a pure-birth (Yule) model, a birth-death model, a logistic density dependence model (DDL), an exponential density dependence model (DDX), a two-rate Yule model, and a three-rate Yule model. The ΔAICrc was calculated as the difference in AIC scores of the best-fit rate-constant and best-fit rate-variable model.

To compare the empirical net diversification curve to a scenario corresponding to the end-Permian mass extinction, we simulated 500 phylogenies with 700 extant taxa using TreeSim v. 1.6 (Stadler, 2012), under a constant-rate birth-death model with parameters equal to the initial model inferred by LASER v. 2.3 at the base of the protobranch radiation. Seven hundred taxa reflect an approximation of the number of valid extant protobranch species

described, which we treated as a proxy for total extant diversity. To simulate the effect of mass extinction, we induced a single non-selective cull of 99% of lineages at time $t = 250$, with no subsequent modification of evolutionary rates. To simulate the effect of sampling error, we randomly sampled subtrees of 60 taxa from the 700-taxon trees. Sixty taxa were subsampled to enable comparison to the empirical chronogram.

3. Results

3.1. Maximum likelihood

ML analysis of the five-gene, 77-ingroup taxon dataset using RAxML v. 7.2.7 resulted in a tree topology with ln $L = -39026.88$ (Supplementary Fig. 2). However, 12 of the ingroup taxa were demonstrably unstable in their phylogenetic placement due to large amounts of missing data. We therefore constructed a smaller and denser dataset excluding these 12 terminals, using the same methods for inferring tree topology. Analysis of the 65-taxon dataset resulted in topologies with ln $L = -38412.02$ (Fig. 2).

Major aspects of the ML topology include the mutual monophyly of the solemyoid genera *Solemya* and *Acharax*; the non-monophyly of Solemyida (=Solemyidae + Nucinellidae) (BS = 88); and the monophyly of the sister clades Nuculida (excluding Sareptidae) and Nuculanida (including Sareptidae). These relationships received significant nodal support (bootstrap resampling frequency [BS] > 70), irrespective of inclusion of taxa with missing data. The ML topology also weakly supported the monophyly of Nuculida + Nuculanida (BS = 75).

All exemplars of Sareptidae (traditionally placed in Nuculida) were recovered as nested within Nuculanida, and closely related to *Yoldia eightsi* (BS = 71). Henceforth we refer to Nuculida *sensu stricto* as the clade that does not include Sareptidae, i.e., the traditionally defined superfamily Nuculoidea.

Except Solemyidae and Sareptidae, few of the families or genera represented by multiple specimens were recovered as monophyletic. Among nuculids, both *Nucula* and *Ennucula* were polyphyletic. Among nuculanids, *Nuculana* was rendered paraphyletic due to the inclusion of a *Jupiteria* (BS = 99); *Propeleda* and *Silicula* formed a grade (without significant support) sister to *Nuculana* + *Jupiteria*; and *Yoldiidae* was recovered as a polyphyletic assemblage of at least three lineages. The inclusion of taxa that had significant amounts of missing data mostly resulted in the recovery of generic non-monophyly (e.g., *Jupiteria*, *Tindaria*, *Bathyspinula*) and/or significantly depressed nodal support, with the exception of the genus *Neilonella* (BS = 95) (Supplementary Fig. 2).

3.2. Bayesian inference

Runs of MrBayes v.3.1.2 reached stationarity in $<10^6$ generations; 2.5×10^6 generations (25%) runs were discarded as burnin.

Fig. 1. Exemplars of Protobranchia. (A) *Solemya velesiana* (Solemyidae); (B) *Huxleyia munita* (Nucinellidae); (C) same as (B), detail of hinge dentition; (D) *Yoldiella orcia* (Yoldiidae); (E) *Pristigloma* cf. *nitens* (Sareptidae); (F) *Scaeoleda caloundra* (Nuculanidae); (G) *Leionucula cumingi* (Nuculidae); (H) *Nucula tenuis expansa* (Nuculidae); (I) *Ledella ultima* (Nuculanidae).

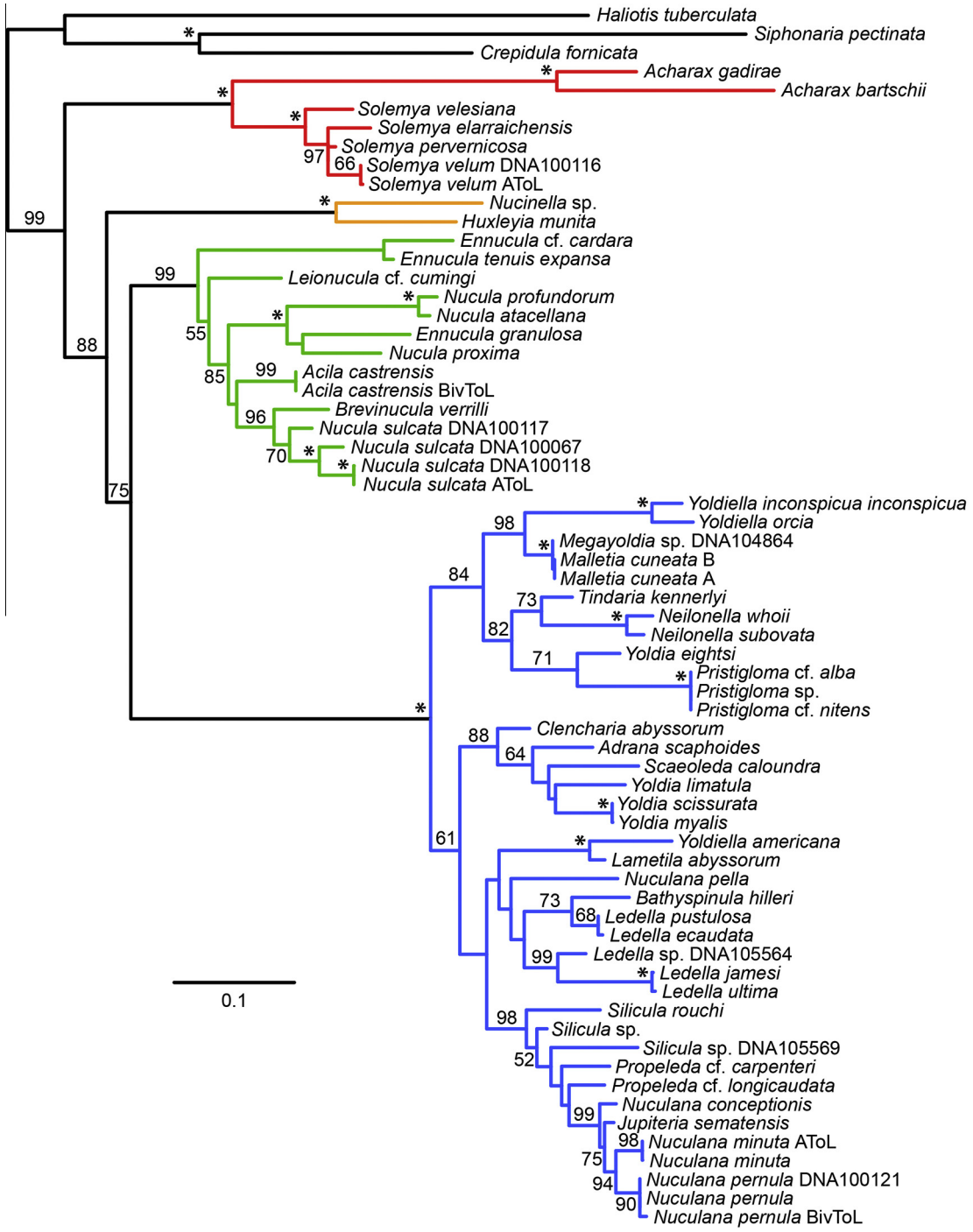

Fig. 2. Phylogenetic relationships of Protobranchia based on maximum likelihood analysis of five genes (ln $L = -38412.02$). Numbers on nodes indicate bootstrap resampling frequencies. Colors in tree topology correspond to major lineages (red: Solemyidae; orange: Nucinellidae; green: Nuculoidea *sensu stricto*; blue: Nuculanoidea + Sareptoidea). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

BI analysis recovered a topology highly congruent with respect to the monophyly of Solemyoidea (posterior probability [PP] = 0.98), Nucinellidae (PP = 1.00), Nuculida *sensu stricto* (PP = 1.00) and Nuculanida (PP = 1.00) (Fig. 3). Both the non-monophyly of Solemyida and the monophyly of Nuculida + Nuculanida were weakly supported (PP = 0.94 and PP = 0.91, respectively). Minor topological differences exist between the ML and BI topologies (e.g., the placement of *Scaeoleda caloundra* and *Nuculana pella*), but these differences were unsupported in both topologies.

3.3. Direct optimization

Iterative rounds of tree-fusing and driven searches using direct optimization in POY v. 4.1.2 resulted in a single most parsimonious tree with a length of 18,827 weighted steps. In general, the parsimony DO tree was very similar to the topologies based on static alignments, particularly with respect to derived relationships (Fig. 4). Two major topological differences are (1) the placement of Nucinellidae, which was recovered as sister to Sareptidae + Nuculanida

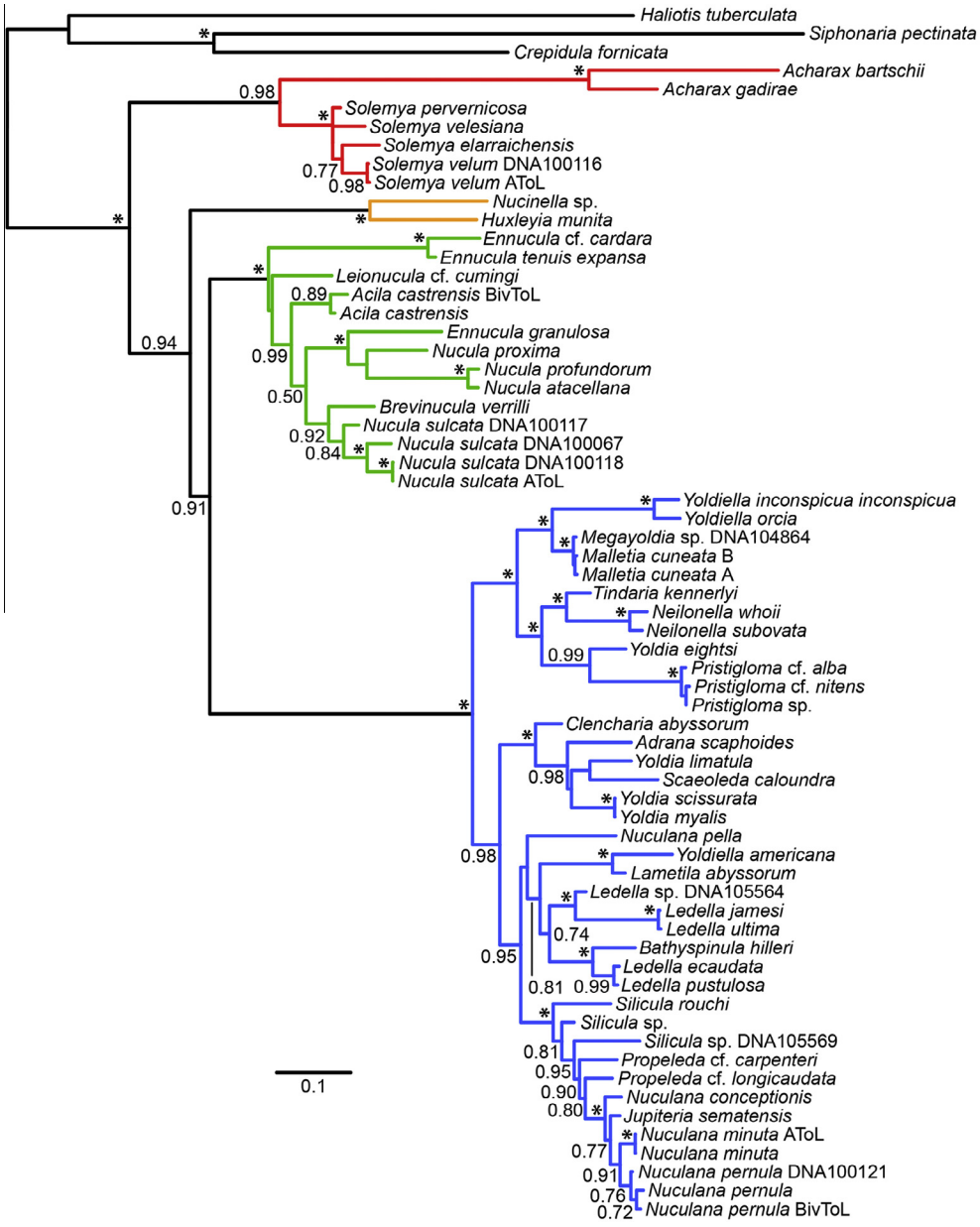

Fig. 3. Phylogenetic relationships of Protobranchia based on Bayesian inference analysis of five genes. Numbers on nodes indicate posterior probabilities. Colors in tree correspond to major lineages (as in Fig. 2).

(jackknife resampling frequency [JF] = 67), and (2) the placement of Sareptidae, which was recovered sister to the remaining Nuculanida (JF < 50). Nodal support values for other relationships (e.g., monophyly of the four major clades; mutual monophyly of *Solemya* and *Acharax*) were generally comparable to those based on model-based approaches.

3.4. Shimodaira–Hasegawa tests

The unconstrained ML topology of 65-ingroup taxa (ln $L = -38412.02$) was not significantly better than a topology consistent with the Solemyida hypothesis (ln $L = -38421.95$ for best suboptimal tree) at $\alpha = 0.05$ (Fig. 5). However, the unconstrained ML topology was found to be significantly better than topologies consistent with either Opponobranchia (ln $L = -38430.90$ for best suboptimal tree) or Nuculida *sensu lato*., i.e., including Sareptidae (ln $L = -38631.41$ for best suboptimal tree), at $\alpha = 0.05$ and $\alpha = 0.01$, respectively (Fig. 5).

3.5. Estimation of divergence times

Diversification of major lineages using BEAST for the 147-taxon dataset is estimated as follows: Protobranchia, 522.7 Ma (95% highest posterior density interval [HPD] 507.1–539.1 Ma); Solemyoidea, 465.8 Ma (95% HPD 448.1–482.9 Ma); Nucinellidae, 196.7 Ma (95% HPD 187.1–206.6 Ma); Nuculida *sensu stricto*, 259.4 Ma (95% HPD 181.3–332.0 Ma); Sareptidae + Nuculanida, 456 Ma (95% HPD 438.6–473.2 Ma); Nuculanida, 282.3 Ma (95% HPD 201.9–368.8 Ma) (Fig. 6). Most aspects of the dated topology are comparable to ML and BI results, particularly the placement of Nucinellidae. However, as in the DO topology, Sareptidae is recovered sister to the remaining Nuculanida with high support ($PP_{BEAST} = 1.00$).

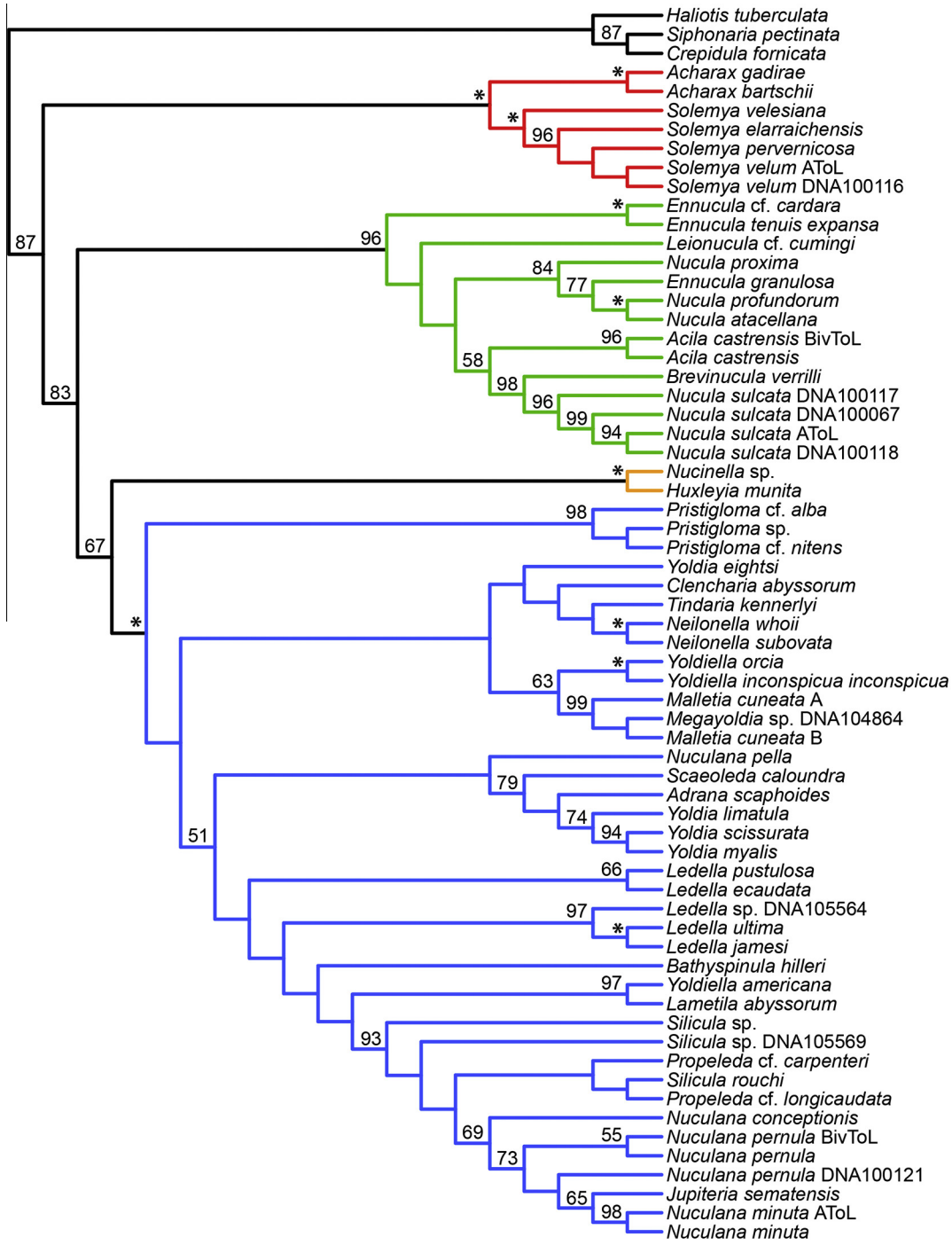

Fig. 4. Phylogenetic relationships of Protobranchia based on parsimony under direct optimization of five genes. Numbers on nodes indicate jackknife resampling frequencies. Colors in tree correspond to major lineages (as in Fig. 2). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.6. Analyses of diversification rates

The log-lineage through time plot obtained from the chronogram corresponded to an anti-sigmoidal curve (Fig. 8A), consistent with either an upturn in diversification after a period of low cladogenetic potential, or a cryptic mass extinction (Crisp and Cook, 2009). The timing of the upturn in diversification corresponded to ca. 250 Ma, the timing of the end-Permian extinction. Of the six competing diversification models, the optimal was a Yule-three-rate model ($\ln L = -102.95$; AIC = 215.89), incorporating a diversification rate slowdown at 456 Ma and rate acceleration at 260 Ma (Table 3). With respect to the Yule-three-rate model, all suboptimal models differed by ΔAIC > 5.

Comparison of the empirical log-lineage through time plot of Protobranchia to trees simulated under (1) constant net diversification with 700 extant species, (2) a single cull of 99% at time $t = 250$ Ma, and (3) taxon sampling of 60 extant species indicated that the empirical chronogram is not significantly different from the simulated null distribution ($p > 0.05$) (Fig. 8B).

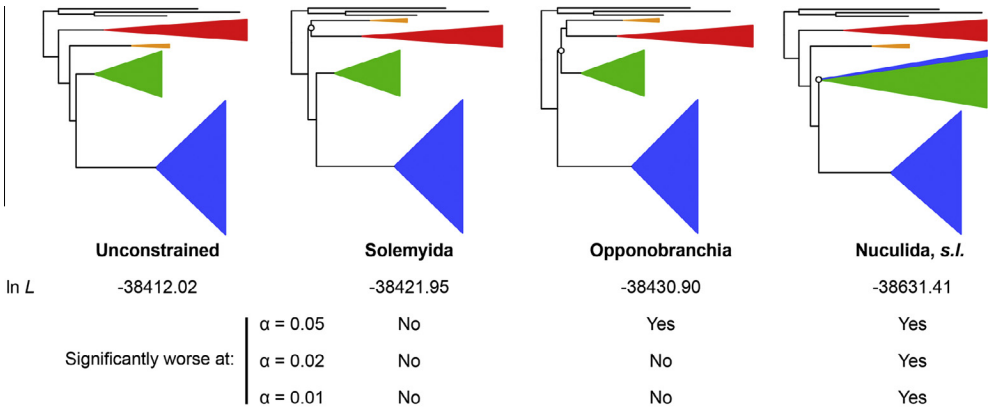

Fig. 5. Comparisons of tree topologies using Shimodaira-Hasegawa tests. Colors in tree correspond to major lineages (as in Fig. 2). Open circles indicate constrained nodes.

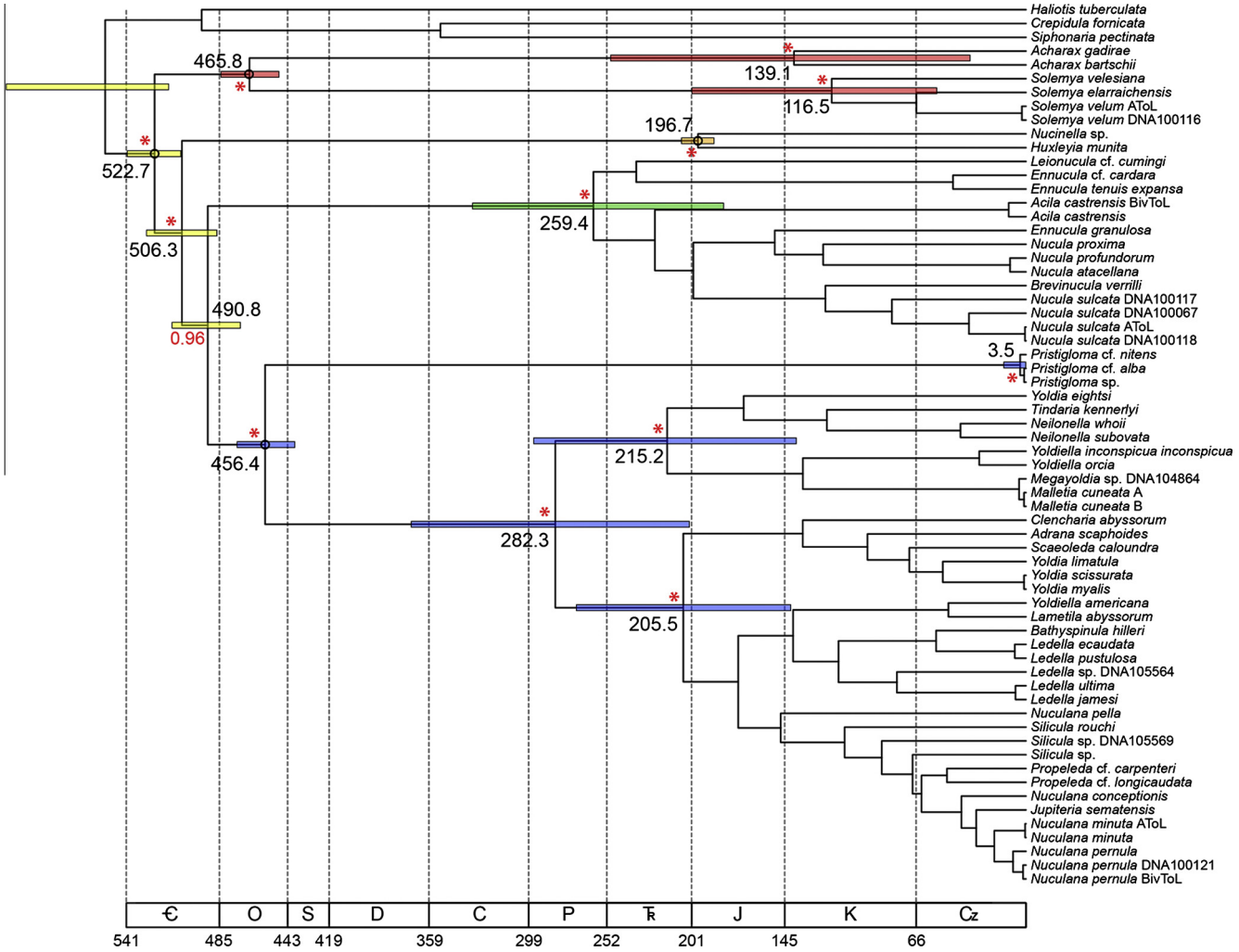

Fig. 6. Evolutionary timetree of Protobranchia inferred from BEAST analysis of all molecular data. Colored bars indicate 95% highest posterior density intervals for nodes of interest. Black text adjacent to selected nodes indicates median ages; red text indicates posterior probabilities (for selected nodes). Asterisks indicate posterior probability of 1.00. Open circles indicate calibrated nodes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

The use of various algorithmic approaches for investigating protobranch phylogeny was prompted by the challenging nature of this multilocus dataset. For example, the genus *Acharax* is known to have hypervariable regions within the small ribosomal subunit (18S rRNA; Neulinger et al., 2006). In the present study, we observed that both sampled species of *Acharax* bear highly variable regions in the large ribosomal subunit (28S rRNA) as well, adding hundreds of nucleotide characters to static alignments. Similarly, many species were distinguished in available sequence data only within length variable regions of ribosomal genes (e.g., the three

Table 3
Fit of models to the protobranch log-lineage through time curve, truncated at 65 Ma. Boldface text indicates optimal model; parameters of Yule-3-rate model indicate speciation rates (λ) and shift points in time.

Model	Parameters	ln*L*	AIC
Pure birth	1	−109.66	221.31
Birth–death	2	−109.14	222.28
DDL	2	−109.66	223.31
DDX	2	−109.53	223.06
Yule-2-rate	3	−107.82	221.63
Yule-3-rate	**5**	**−102.95**	**215.89**

Yule-3-rate model parameters:
$\lambda_1 = 0.01734$; $\lambda_2 = 0.00083$; $\lambda_3 = 0.00799$.
shift_1 = 455.66 Ma; shift_2 = 259.60 Ma.

Pristigloma, discussed below). Indel characters are inherently informative for analyses under direct optimization, inasmuch as POY v.4.1.2 can incorporate both indel opening and extension parameters. Both RAxML v.7.2.7 and MrBayes v.3.1.2 incorporate rapid heuristic algorithms and sophisticated modeling of substitution events, although neither can distinguish indels from missing data (but see Simmons and Ochoterrena, 2000 for a common workaround). Consequently, model-based approaches may constitute an unsatisfactory compensation for the loss of information that transpires when phylogenetic signal resides exclusively in length-variable regions (e.g., Lindgren and Daly, 2007). The protobranch dataset we generated thus presented an opportune case where both static and dynamic homology approaches could elucidate different aspects of protobranch phylogeny.

4.1. Higher-level relationships of Protobranchia

All phylogenetic analyses based on molecular sequence data unambiguously recover with significant support the division of Protobranchia into four clades, corresponding to Solemyidae, Nucinellidae, Nuculida, and Nuculanida (Figs. 2–4 and 6, Supplementary Fig. 2). Barring the placement of Sareptidae within or sister to Nuculanida, and the non-monophyly of Solemyida (=Solemyidae + Nucinellidae)—both results insensitive to algorithmic treatment—the constituent families and genera of these four clades are consistent with the traditional classification of the protobranchs.

Part of the discordant phylogenetic signal for a monophyletic Solemyida appears to stem from the nuclear ribosomal genes; analyzed on their own for the present species sampled, these will recover Solemyida, albeit without significant nodal support (Supplementary Fig. 3). Although model-based algorithmic approaches to the entire molecular dataset support the paraphyly of Solemyida (Figs. 2 and 3), the resulting topology is not significantly better than a suboptimal Solemyida topology (Fig. 5). Morphologically, a sister relationship of Solemyidae and Nucinellidae is consistent with the large, bipinnate protobranch gill and the reduced (or absent) palp proboscides in these two superfamilies. However, these characters may be attributable to habitat and/or the incidence of chemosymbiosis in both lineages, and thus constitute either convergence or protobranch symplesiomorphies (Oliver and Taylor, 2012). Moreover, the ultrastructure of the nucinellid shell suggests affinity to Nuculanida (Coan et al., 2000), an observation consistent with the direct optimization topology (Fig. 4).

Nucinellidae therefore constitutes a curious lineage with ambiguous affinities to other protobranchs. The uniqueness of this clade, whose fossil record extends to the Permian (Chronic, 1952), is evident in its hinge structure, which is strong, short, and consists of one to two prominent lateral teeth and several cardinal taxodont teeth (Fig. 1C). By contrast, the hinge plate of true Solemyoidea is weak and edentate. Given (1) the lack of unambiguous morphological synapomorphies uniting solemyids and nucinellids, (2) the absence of significant nodal support uniting these families in analyses of molecular data, and (3) the apomorphic hinge structure of nucinellids, we reaffirm the validity of Nucinellidae as a fourth and distinct clade of protobranchs.

All model-based inferences of tree topology recover a clade comprised of Nuculida and Nuculanida (the traditional Palaeotaxodonta sensu Newell, 1969; Nuculoida sensu Sanders and Allen, 1973; Beesley et al., 1998), which bear prominent hinge plates and a characteristic taxodont dentition (Figs. 2, 3 and 6). This clade is additionally supported by the presence of small gills that are situated posteriorly, and large labial palps and palp proboscides. Previous efforts toward higher-level bivalve phylogeny supported a clade of Solemyida + Nuculida (Waller, 1998; Carter et al., 2000; Giribet and Wheeler, 2002; Giribet and Distel, 2003), a hypothesis formalized by the name Opponobranchia (Giribet, 2008). Comparative assessment of the strength of the Opponobranchia hypothesis indicates that the Palaeotaxodonta topology is significantly better than a topology consistent with Opponobranchia, albeit only at $\alpha = 0.05$ (Fig. 5). The sister group relationship of Solemyoidea to the remaining protobranchs is also supported by recent phylogenetic and phylogenomic datasets (Kocot et al., 2011; Smith et al., 2011; Sharma et al., 2012), though Nucinellidae was not sampled in those studies. As the present study constitutes the most comprehensive sampling of protobranch bivalves for phylogenetic analysis, we tentatively favor the palaeotaxodont hypothesis, but advocate reassessment of this topology by sampling of nucinellids in future phylogenomic analysis and/or re-evaluation using a suite of non-overlapping molecular markers (e.g., Sharma et al., 2012).

4.2. Sareptidae is a lineage of Nuculanida

One of the smaller and more curious families of protobranchs is Sareptidae Stoliczka, 1870, one of the two families of Nuculoidea, with ca. 10 known species (Huber, 2010). As currently understood by Bieler et al. (2010), Sareptidae includes the nominal genus *Sarepta* Adams, 1860 and the genera *Pristigloma* Dall, 1900 and *Setigloma* Schileyko, 1983, and therefore the new Sareptidae concept includes Pristiglomidae and Setiglomidae as junior synonyms. However, this taxonomic assignment is not without controversy and is not based on any recent phylogenetic analysis. For example, Coan et al. (2000) used the superfamily Pristiglomoidea Sanders and Allen, 1973 to include the single family Pristiglomidae Sanders and Allen, 1973, with the genera *Pristigloma*, *Setigloma*, and *Pseudoglomus* Dall, 1898, the latter now in Malletiidae, although it probably does not belong there. They treated Pristiglomoidea as a rank comparable to Nuculoidea and Nuculanoidea (in their Nuculoidea). Sanders and Allen (1973), when they proposed Pristiglomidae, also included the genus *Microgloma* Sanders and Allen, 1973 in this family, and removed *Pristigloma* from 'Nuculanacea' to be placed in 'Nuculacea', formalizing the transfer of the new family to the current Nuculida. Allen and Hannah (1986) also included *Pseudoglomus* in Pristiglomidae, but treated *Sarepta* as a member of Yoldiidae Allen and Hannah, 1986, in their Nuculanacea. Ockelmann and Warén (1998) transferred *Microgloma* to Nuculanidae and discuss the possible synonymy of Pristiglomidae with Sareptidae, as well as cast doubts on the position of Pristiglomidae. The most recent bivalve compendium uses Sareptoidea as a superfamily, comparable to Nuculoidea, Solemyoidea, Manzanelloidea and Nuculanoidea, without arranging them (Huber, 2010).

Sareptidae are distinguished from both Nuculoidea and Nuculanoidea in having few hinge teeth, often of chevron shape, and being greatly miniaturized. The miniaturization of sareptids appears to be achieved by smaller cell size and lowered reproductive output (Sanders and Allen, 1973). A relationship between part of Sareptidae (*Pristigloma*) and Nuculoidea has thus often been suggested, largely on the basis of the shell shape (a rounded posterior end, resulting in antero-posterior [AP] symmetry), disposition of major

organs, and the absence of a pallial sinus. It is therefore surprising that we obtain all sareptid species (genus *Pristigloma*) as nested within or sister to Nuculanoidea across all topologies (Figs. 2–4 and 6), and with significant support in SH tests (Fig. 5). Moreover, Sareptidae is recovered sister to *Yoldia eightsi*, a genus of Yoldiidae that includes species with rounded posterior ends and chevron teeth, in the probabilistic analyses (BS = 71; PP = 0.99), as suggested by the placement of *Sarepta* by Allen and Hannah (1986), but sister group to Nuculanida in the direct optimization and BEAST analyses (JF < 50; PP_{BEAST} = 1.00). The three *Pristigloma* species are sufficiently closely related that their sequences are rendered identical upon removal of length-variable regions. However, analysis of divergence time suggests that this lineage, if indeed sister to true Nuculanida, is ancient, with an origin dating to the Ordovician and/or Early Silurian (Fig. 6).

We therefore re-examined the morphological characters supporting the placement of Sareptidae within Nuculida and found these unconvincing. Sanders and Allen (1973) contend that *Pristigloma* shared many characters with true Nuculida, such as the anterior (rather than posterior) inhalant current, the lack of mantle fusion, anterior mucus glands of the mantle, absence of siphons, transversely oriented ctenidia, and the large, broad palp. However, many of these features may result from the dramatic miniaturization of sareptids and the atypical AP symmetry of the shell in this cryptic family. Reduction of hinge tooth number and the pallial sinus may also represent further effects of miniaturization in Sareptidae. Many species of Nuculanoidea (e.g., *Yoldiella capsa*, *Yoldiella subcircularis*, *Neilonella mexicana*) also do not have the marked AP asymmetry characteristic of most nuculanids. The absence of the pallial sinus, mantle fusion, and siphons in *Pristigloma* and true nuculoids is also unsatisfactory, insofar as absence of a character may not constitute a sound basis for diagnosis given prevalence of homoplasy. Moreover, the placement of *Pristigloma* in all of our topologies is consistent with their lack of nacreous shell layers, which occur only in modern Nuculida (Carter, 1990). Given the number of morphological and molecular sequence characters shared by Sareptidae and various clades of Nuculanida, we consider Sareptidae to constitute a lineage of Nuculanida (Table 4). As a conservative measure, we maintain this lineage in the superfamily Sareptoidea for the present, due to the basal (i.e., non-nested) placement of Sareptidae in the direct optimization and dated tree topologies (Fig. 4), but recognize that additional molecular data should be gathered, specifically from the rare genus *Sarepta*.

4.3. Systematic validity of protobranch families and genera

Under either approach to alignment, few of the genera and families within the four major clades of protobranchs were recovered as monophyletic. A notable exception is the monophyly of Solemyidae and the mutual monophyly of the genera *Solemya* and *Acharax*, which were invariably supported in all phylogenetic analyses, including under parametric treatment of hypervariable regions in POY v.4.1.2 (Figs. 2–4). The deep-sea genus *Acharax* is known to form at least two clusters of species, as inferred from 18S rRNA sequences (Neulinger et al., 2006). Both species of *Acharax* sampled here correspond to the JAC clade defined by Neulinger et al. (2006), as inferred from multiple sequence alignments (data not shown). The 28S rRNA sequences of *Acharax bartschii* and *A. gadirae* bear numerous hypervariable regions as well. The significance of the elongated insertions in the ribosomal array of *Acharax* is not known, but obtaining more 28S rRNA sequences from other species of this genus may prove useful for corroborating the clusters delimited by Neulinger et al. (2006).

Within the palaeotaxodont genera, only *Neilonella* was monophyletic (*Acila* and *Malletia* were represented by multiple conspecifics, and thus cannot test generic monophyly) (Figs. 2–4).

Table 4
Proposed classification of Protobranchia.

Subclass Protobranchia Pelseneer, 1889

Order Solemyida Dall, 1889
- **Superfamily** Solemyoidea Gray, 1840
  - **Family** Solemyidae Gray, 1840
- **Superfamily** Manzanelloidea Chronic, 1952
  - **Family** Manzanellidae Chronic, 1952
  - **Family** Nucinellidae Vokes, 1956

Order Nuculida Dall, 1889
- **Superfamily** Nuculoidea Gray, 1824
  - **Family** Nuculidae Gray, 1824

Order Nuculanida Carter, Campbell & Campbell, 2000
- **Superfamily** Nuculanoidea H. Adams & A. Adams, 1858
  - **Family** Bathyspinulidae Coan and Scott, 1997
  - **Family** Malletiidae H. Adams & A. Adams, 1858
  - **Family** Neilonellidae Schileyko, 1989
  - **Family** Nuculanidae H. Adams & A. Adams, 1858
  - **Family** Phaseolidae Scarlato & Starobogatov, 1971
  - **Family** Siliculidae Allen & Sanders, 1973
  - **Family** Tindariidae Verrill & Bush, 1897
  - **Family** Yoldiidae Dall, 1908
- **Superfamily** Sareptoidea Stocliczka, 1870
  - **Family** Sareptidae Stocliczka, 1870

Among nuculoids, *Nucula* is a triphyletic assemblage, owing to the placement of *Acila*, *Ennucula*, and *Leionucula* (Figs. 2–4). The systematic validity of *Ennucula* has been in question for some time, as this genus is distinguished from *Nucula* only by the absence of crenulations on the ventral interior surface of the shell (Maxwell, 1988; Kilburn, 1999). The arrangement of clades in the nuculoid phylogeny suggests that absence of ventral crenulations is a symplesiomorphy within this subfamily (these do not occur in *Ennucula* or *Brevinucula*) and/or has been lost repeatedly in unrelated lineages. These results herald future revision of nuculoid genera.

Among nuculanoids (Fig. 7), the genus *Nuculana* is represented by three species and is a somewhat coherent entity, save for the inclusion of *Jupiteria*, an erstwhile subgenus of *Nuculana* (Allen and Hannah, 1986) (BS = 99; PP = 1.00; JF = 69; Figs. 2–4). Our results therefore suggest that *Jupiteria* should once again be synonymized with *Nuculana*. Similarly, the genus *Ledella* (Nuculanidae; Ledellinae in Allen and Hannah, 1986) is largely coherent in ML and BI topologies, but for the inclusion of *Bathyspinula hilleri* (Bathyspinulidae)—heretofore a subfamily of Nuculanidae (Coan and Scott, 1997; Coan et al., 2000; Spinulinae in Allen and Hannah, 1986) (Figs. 2 and 3). The placement of *Bathyspinula* within *Ledella* is supported by multiple nuclear gene tree topologies (Boyle, 2011). Marked morphological similarities between *Bathyspinula* and many *Ledella* that also bear an attenuate rostrum (e.g., *Ledella robusta*, *Ledella ultima*) is dissuasive of a distinction between the two families, much less the genera *Ledella* and *Bathyspinula* (Boyle, 2011).

Another pair of genera with greatly asymmetrical shells and/or recurved rostra are *Propeleda* (Nuculanidae) and *Silicula* (Siliculidae), which form a grade sister to the *Nuculana* + *Jupiteria* clade (Figs. 2–4). As nodal support for the non-monophyly of these genera is not significant, we cannot dismiss the possibility that they are systematically valid. However, support for the inclusion of *Silicula* with a clade of *Propeleda* + *Nuculana* + *Jupiteria* is strong (BS = 98; PP = 1.00; JF = 93), and this clade appears nested within other nuculanids, disputing the validity of the family Siliculidae as an entity separate from Nuculanidae.

Of all the nuculanoid families, Yoldiidae (represented here by the genera *Yoldia*, *Yoldiella*, and *Megayoldia*) appears to be in direst need of dissolution, having been recovered as a polyphyletic assemblage across all topologies (Figs. 2–4). At least two *Yoldiella* are supported as members of a clade with *Malletia* and *Megayoldia*. In model-based analyses, *Yoldia eightsi* is sister to *Pristigloma* (BS = 71; PP = 0.99), whereas another three species of *Yoldia* form

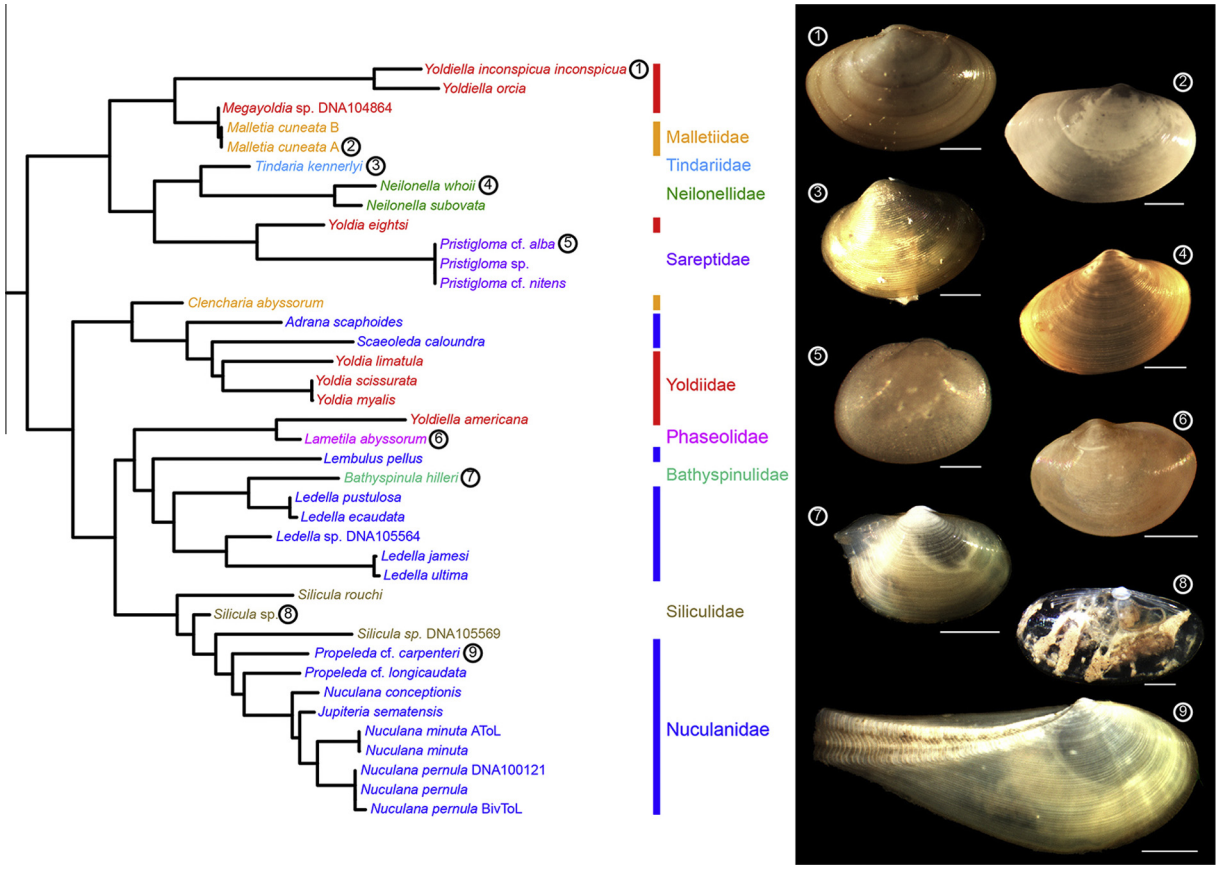

Fig. 7. Interfamilial relationships within Nuculanoidea based on ML analysis.

a clade with the malletiid *Clencharia abyssorum* and the nuculanids (BS = 88; PP = 1.00; Figs. 2 and 3). Clades of Yoldiidae are somewhat more coherent in the direct optimization topology, but are still recovered as triphyletic (Fig. 4). The type genus *Yoldia* is not monophyletic in any of the analyses (Figs. 2–4). The great variation in yoldiid shell morphology accords with the incoherent position of yoldiids among malletiids and nuculanids across the phylogeny. Yoldiidae may have a rounded or truncated posterior shell margin, and the resilifer may be large or small (Coan et al., 2000; Huber, 2010). Consistent with the placement of *Malletia* as derived Yoldiidae, both yoldiids and malletiids have large labial palps with narrow palp proboscides. Only the absence of the resilifer in Malletiidae distinguishes this lineage from nuculanids and yoldiids. But as with the absence of AP asymmetry in Sareptidae or the lack of ventral crenulations in *Ennucula* (discussed above), the absence of a character is a demonstrably poor justification for defining derived clades in Protobranchia. The only character system that reasonably distinguishes Nuculanidae, Malletiidae and Yoldiidae is the alimentary system (e.g., Sanders and Allen, 1985; Allen, 1992; Allen et al., 1995), but this is also in need of re-examination at the family and genus-level.

The placement of Neilonellidae, Phaseolidae and Tindariidae as derived lineages within the Nuculanidae-complex (nested among Nuculanidae, Malletiidae and Yoldiidae) further highlights inconsistencies in the present classification of Protobranchia. The addition of terminals with significant missing data does lend support to some groups (e.g., *Neilonella* is still recovered as monophyletic; Fig. 7), but mostly casts additional doubt upon the validity of several genera (e.g., *Tindaria*, *Jupiteria*, *Malletia*). Meanwhile, many protobranch genera remain to be sampled for testing familial and generic relationships. Forthcoming efforts are therefore anticipated to redefine and reestablish a classification of Nuculida and Nuculanida.

4.4. Protobranch phylogeny retains the signature of the end-Permian mass extinction

One of the ideas that has dominated paleontological and evolutionary thinking for several decades is the Sepkoski Curve, the outcome of detailed tabulation of fossil lineages through the stratigraphic record (Sepkoski, 1981; Sepkoski and Bambach, 1981). Observing the phenomenon of early bursts in radiation, tandem plateaus of stability, and abrupt declines, Sepkoski quantitatively described three "evolutionary faunas"—the Cambrian, the Paleozoic, and the Modern—comprising distinct assemblages of taxa associated with particular geological periods (Sepkoski, 1978, 1979, 1981). An important component of transitions from one fauna to the next are mass extinctions, many of which both define certain geological periods and precede rapid diversification of the ensuing faunal assemblage (Raup and Sepkoski, 1982, 1984). The single greatest episode of these is the end-Permian mass extinction ca. 254 Ma. Estimated to have extinguished 95–99% of marine species and approximately 75% of families of terrestrial vertebrates, the end-Permian event radically altered the composition of Earth's biota. The marine realm, theretofore dominated by such Palaeozoic lineages as crinoids, bryozoans, brachiopods, belemnites, ammonites, and trilobites, subsequently bore spectacular radiations of bivalves, gastropods, and echinoids—constituents of the Modern fauna.

Mass extinctions are measured by the persistence and decline of fossil lineages, but their effects on the phylogenies of extant taxa are largely inferred through theory and simulations (Rabosky and Lovette, 2008; Crisp and Cook, 2009). One of the characteristic features of a simulated mass extinction on evolutionary history is to engender long branches in a tree topology. This is observed as a log-lineage through time (LTT) plot—a visualization of net

diversification rate through time—of anti-sigmoidal shape (Crisp and Cook, 2009). Generally, the greater the extinction event, the greater will be the curvature of the LTT plot (Crisp and Cook, 2009). The intermittent plateau of net diversification rate is a consequence of extinction, and subsequent upturn in diversification rate corresponds to recovery from the extinction event. The amplitude of lineage loss during the end-Permian extinction is therefore expected to give rise to a characteristic net diversification rate curve for lineages that originated prior to, and survived, this event.

However, an anti-sigmoidal curve is infrequently observed in empirical studies, principally because a large number of extant lineages that diversified before the extinction event is necessary to

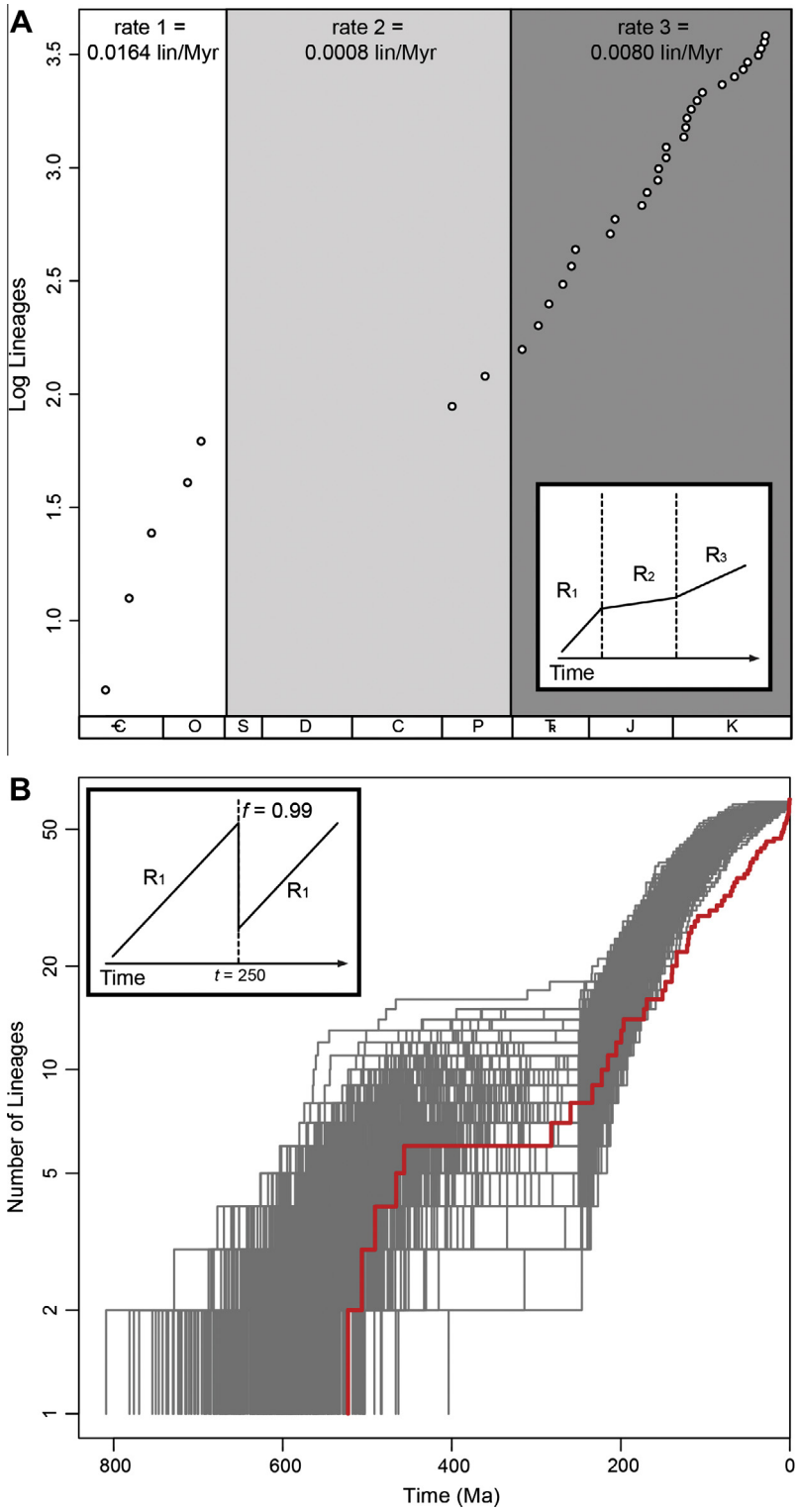

Fig. 8. (A) Log-lineage through time (LTT) plot inferred from molecular dating of Protobranchia. Shading and rates indicate parameters of optimal model; note truncation of post-Cretaceous branching times. Inset: Schematic of Yule-three-rate model fitted to data by Laser v. 2.3. (B) Simulated LTT plots (in gray) corresponding to a constant speciation process interrupted by a 99% cull at time $t = 250$ (as shown in inset schematic). Apparent downturn in net diversification rate as time $t \rightarrow 0$ is caused by simulation of sampling limitations. The observed LTT plot of Protobranchia is shown in red.

observe such a curve. Many taxa with ancient origins have too few extant species to infer diversification through time (e.g., nautiloids, Lindgren et al., 2004; horseshoe crabs, Obst et al., 2012), too few fossils to calibrate dated phylogenies reliably (e.g., most soft-bodied invertebrates; Kawauchi et al., 2012), or are survived by a single lineage that diversifies in the wake of the mass extinction (revenant clades, *sensu* Sharma and Wheeler, 2013). As an example, all crown-group Crinoidea are inferred to have diversified immediately after the end-Permian, engendering a characteristic long branch subtending a clade of ca. 620 extant species (Rouse et al., 2013). Additionally, simulation studies have demonstrated that prolonged extinction events can cause shifts in root ages, causing diversifications of extant taxa to appear younger, even with complete taxon sampling; this effect is especially pronounced for small clades (Yedid et al., 2012; Sharma and Wheeler, 2013).

In the case of protobranch bivalves, early diversification gave rise to all extant superfamilial lineages prior to the Silurian, but diversification of most of their constituent clades occurred in the Mesozoic (Fig. 6). The long branches subtending crown-group superfamilies engender an LTT plot with a characteristic anti-sigmoidal curve, with upturn in diversification toward the end of the Permian (260 Ma; Table 3; Fig. 8A). The diversification rate estimated for the middle portion (the "saddle" of the anti-sigmoidal curve) of protobranch evolutionary history is remarkably low under the optimal model (0.0008 lin/Myr; Fig. 8A). However, although recovered as optimal, the Yule-three-rate model is only designed to infer three phases of pure speciation, with no mechanism for parameterizing either intrinsic extinction (μ) or extrinsic diversity culls, such as mass extinction events. To test whether protobranch evolutionary history is discernible from a constant diversification process experiencing mass extinction, we employed simulations of an end-Permian event-like process to generate a null distribution for comparison. The empirical protobranch LTT is indistinguishable from such a null distribution (Fig. 8B). Some deviation from the simulated evolutionary histories occurs in the Recent, likely stemming from assumptions made for the purpose of generating a tractable null distribution (e.g., actual clade diversity approximately equal to described number of extant species; equal pre- and post-extinction diversification rates).

Taken together with the fossil record of the group, these analyses indicate that the phylogeny of extant Protobranchia retains the signature of the end-Permian mass extinction, consistent with predictions from theory and simulations (Crisp and Cook, 2009). Protobranchia provide a compelling contrast in this regard to such groups as crinoids, which similarly arose in the Cambrian and have a comparable number of extant species, yet were survived by a single lineage through the end-Permian (Rouse et al., 2013). In concert with denser sampling of the protobranch tree of life, future investigation of this extinction signature should incorporate direct measurements of speciation and extinction rates from the protobranch fossil record, particularly for gauging post-extinction recovery in the Recent and improving inference of evolutionary history through modelling approaches.

5. Conclusion

We comprehensively sampled the families of Protobranchia, and generated a molecular phylogeny of this bivalve subclass based on a multilocus dataset that is largely insensitive to algorithmic approaches. All tree topologies obtained distinguish Nucinellidae from Solemyidae with support and indicated that Sareptidae is more closely allied to Nuculanida than to Nuculida, either as a derived, miniaturized family (probabilistic approaches) or as a basal lineage (direct optimization and BEAST analyses). Forthcoming systematic revisions of Nuculida and Nuculanida are imperative for establishing a new classification of these orders based on natural, monophyletic groups. Estimation of divergence times and analysis of diversification rates reveal characteristic hallmarks of mass extinction in the evolutionary history of protobranchs.

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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.2013.05.018.

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