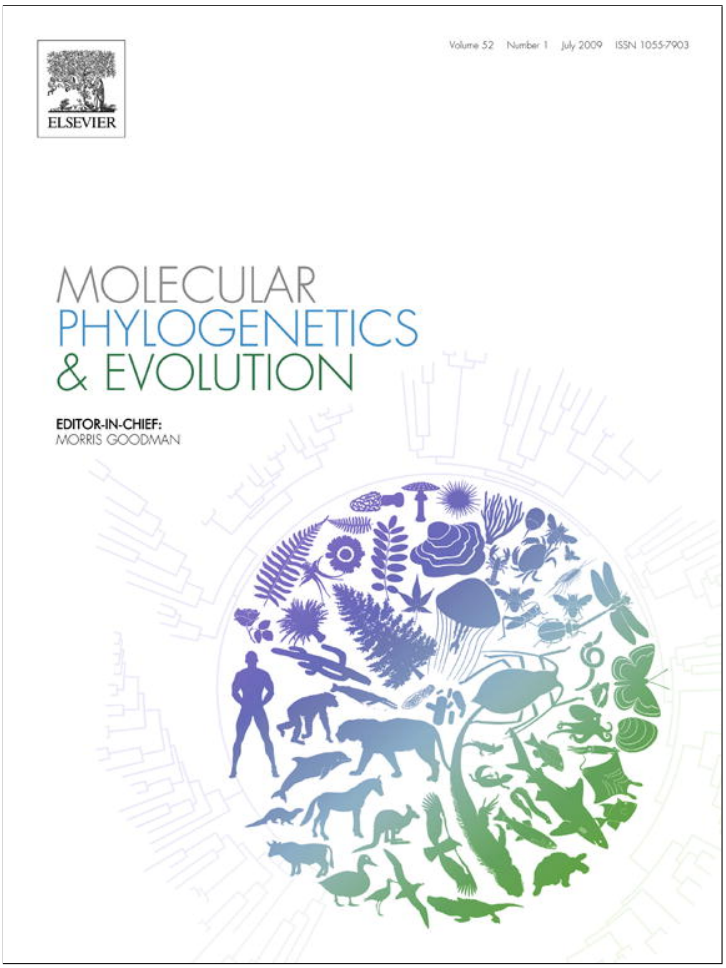

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Molecular phylogeny of hemichordata, with updated status of deep-sea enteropneusts

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ABSTRACT

Hemichordates have occupied a central role in hypotheses of deuterostome and early chordate evolution. However, surprisingly little is understood about evolution within hemichordates, including hemichordate ancestral characters that may relate to other deuterostome taxa. Previous phylogenetic studies suggested that enteropneust worms are either monophyletic (based on 28S rDNA) or paraphyletic (based on 18S rDNA). Here, we expand the number of hemichordate taxa used in phylogenetic analyses for 18S rDNA data and employ more quickly evolving mitochondrial gene sequences. Novel data from an undescribed deep-sea enteropneust species similar to *Torquarator bullocki* and a Gulf Stream tornaria larva suggest that these taxa are closely allied to or possibly within Ptychoderidae. *Saxipendium coronatum*, another deep-sea species commonly called the spaghetti worm, is shown to be a member of Harrimaniidae. Recognition of these deep-sea lineages as distinct families calls into question features used in hemichordate taxonomy. In the new analyses, enteropneusts fall into two distinct monophyletic clades, with the colonial pterobranchs sister to Harrimaniidae, similar to earlier published 18S results. These results indicate that colonial pterobranchs may have evolved from a solitary acorn worm-like hemichordate ancestor. If true, pterobranchs would be unlikely to represent the deuterostome ancestral form as has been suggested by many traditional theories of deuterostome evolution.

1. Introduction

Hemichordata are deuterostome animals with a tripartite body plan, and separate coeloms for each body region: proboscis (prosome), collar (mesosome), and trunk (metasome) (Fig. 1) (Barrington, 1965; Hyman, 1959; Ruppert and Barnes, 1994). The name Hemichordata was erected to reflect the idea that hemichordates shared morphological characteristics with chordates (Bateson, 1885). Gill slits, for example, have been shown to be homologous between hemichordates and chordates based on morphology (Schaeffer, 1987), developmental expression of the transcription factor *Pax1/9* (Lowe et al., 2003; Ogasawara et al., 1999; Rychel and Swalla, 2007), and the anterior–posterior positioning of gill slits along the body axis (Aronowicz and Lowe, 2006; Lowe et al., 2003). Additionally, expression of *Hox11/13* genes in a post-anal region of developing *Saccoglossus kowalevskii* embryos is similar to *Hox10-13* expression in the post-anal tail of developing chordates, suggesting possible homology between these structures (Aronowicz and Lowe, 2006; Lowe et al., 2003). However, molecular data have shown that hemichordates and echinoderms are sister groups, together comprising the Ambulacraria (Bourlat et al., 2006; Cameron et al., 2000; Halanych, 1995, 2004; Peterson, 2004; Swalla and Smith, 2008). This relationship is well supported by developmental, morphological, larval, and molecular characteristics (Barrington, 1965; Bromham and Degnan, 1999; Cameron, 2005; Cameron et al., 2000; Castresana et al., 1998; Furlong and Holland, 2002; Hyman, 1959; Lowe et al., 2003; Peterson, 2004; Ruppert and Barnes, 1994; Rychel and Swalla, 2007; Winchell et al., 2002; Zeng and Swalla, 2005). Because hemichordates are more closely related to echinoderms than chordates, any homologous features shared between hemichordates and chordates must have been present in the last common ancestor of deuterostomes (Lowe et al., 2006; Swalla, 2007; Brown et al., 2008).

At present, Hemichordata are divided into two classes, the solitary, free-living Enteropneusta (Fig. 1), and the colonial,

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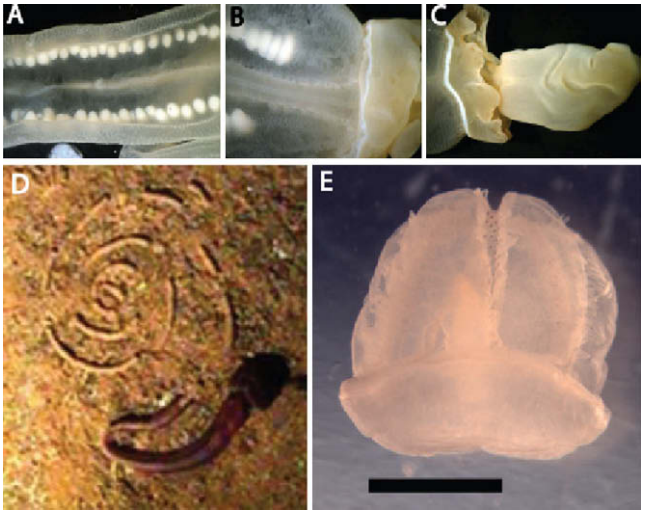

Fig. 1. Deep-sea Hemichordates and Gulf Stream Tornaria Larva. (A) Deep-sea "spaghetti worm" hemichordate *Saxipendium coronatum* trunk with gonads. (B) Close-up of *S. coronatum* trunk showing gill pore openings. (C) Proboscis and short collar of *S. coronatum*. Photos (A, B, and C) courtesy of Greg Rouse. (D) Photo of the deep-sea enteropneust similar to *Torquarator* (Holland et al., 2005) used in this study. Photo (D) was taken at 13° N on the EPR, in the Central Valley. Copyright IFREMER-99. (E) Photo of a fixed tornaria larva found in the Gulf Stream near New Providence Island, Bahamas and sequenced in this study. Scale bar = 2 mm (applies only to photo (E)).

tube-dwelling Pterobranchia (Fig. 2). There are estimated to be approximately 100 recognized species of hemichordates, of which about 70 are enteropneusts. Enteropneusts, or acorn worms, are characterized by numerous gill slits in the trunk region and a straight gut with a terminal anus in the adult, although harrimaniid enteropneusts possess a post-anal tail as juveniles (Aronowicz and Lowe, 2006). Acorn worms grow from moderate to considerable lengths (2 cm to 2.5 m) and typically live in benthic habitats, buried in sand or mud. In contrast, all pterobranchs are colonial, minute moss-like animals that live in secreted tubes called coenecia (Fig. 2). As noted by Halanych (1996), *Atubaria* is likely a *Cephalodiscus* species, and the report of its solitary nature erroneous. The pterobranch gut is U-shaped, and individual zooids suspension feed with ciliated tentacles. Members of the genus *Cephalodiscus* possess a single pair of gill slits, whereas *Rhabdopleura* has no gill slits. Pterobranchs reproduce sexually or asexually by budding. Sexual reproduction produces non-feeding larvae that are brooded

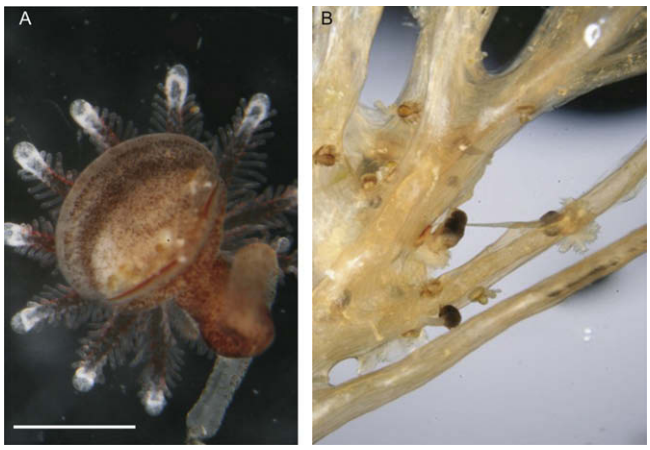

Fig. 2. Antarctic Pterobranchs. (A) Individual zooid of *Cephalodiscus hodgsoni* viewed from underside of cephalic shield showing the tentacular arms, trunk, and stolon. Scale bar = approximately 0.5 mm. (B) *Cephalodiscus hodgsoni* colony, showing coenecium structure and several zooids.

in the colony and are likely to have limited dispersal potential (Sato et al., 2008a). The extinct group Graptolithina (graptolites) was a primarily pelagic group of colonial animals that is alternately considered a separate class of hemichordates (Bulman, 1955) or occasionally with extant pterobranchs as a single class called Graptolitoidea (Sato et al., 2008b; Urbanek, 1994).

Solitary enteropneust hemichordates are split into five groups (Dawydoff, 1948): Harrimaniidae Spengel, 1901, Ptychoderidae Spengel, 1893, Spengelidae Willey, 1898, Saxipendiidae Woodwick and Sensenbaugh, 1985, and Torquaratoridae Holland et al., 2005. Ptychoderidae is defined by the presence of gill slit synapticles and pronounced regions of the trunk including hepatic sacculations, whereas Harrimaniidae lacks these features. Spengelidae is defined by the very long horns of the proboscis skeleton, and a vermiform process extending anteriorly from the stomochord (Hyman, 1959). Saxipendiidae is defined by weak proboscis muscles and a crown-shaped (in cross section) proboscis skeleton (Woodwick and Sensenbaugh, 1985). Torquaratoridae lacks synapticles, and has an exceptionally broad collar and proboscis (Holland et al., 2005). Members of the rarely collected group Planctosphaeroidea are only known as large, modified tornaria larvae found in plankton tows in the open ocean (Hart et al., 1994; Scheltema, 1970; Van der Horst, 1936). Larvae assigned to this group probably belong to adults of a solitary enteropneust group (Hadfield and Young, 1983). Saxipendiidae contains a single described deep-sea species, *Saxipendium coronatum*, which was first found on rocky outcroppings near deep-sea hydrothermal vents (Fig. 1A–C) (Woodwick and Sensenbaugh, 1985). These long, thin worms were first discovered tangled up together, leading to the common name "spaghetti worms". Torquaratoridae is a recently described family of deep-sea enteropneusts, typified by a broad proboscis and collar. There is currently one described species, *Torquarator bullocki*, although many other individuals with similar features have been photographed (Fig. 1D) (Holland et al., 2005).

Previous molecular systematic work on hemichordates sampled several taxa within Harrimaniidae and Ptychoderidae as well as colonial pterobranchs (Cameron et al., 2000; Halanych, 1995). Based on 18S rDNA sequence data, the solitary enteropneust worms were found to be paraphyletic, with pterobranchs sister to the harrimaniids (Bourlat et al., 2003; Cameron et al., 2000; Halanych, 1995). A more limited dataset of a conserved portion of 28S found enteropneusts to be monophyletic, with a single sequenced pterobranch as sister to the solitary worms (Winchell et al., 2002). A morphological study using parsimony also found pterobranchs to be the basal-most member of Hemichordata, and recovered a monophyletic Hemichordata, Enteropneusta, Ptychoderidae, and Ptychoderidae + Spengelidae (Cameron, 2005).

Here, we present an updated hemichordate phylogeny with expanded taxon sampling, including two previously unsampled deep-sea solitary enteropneusts (Fig. 1A–D), and a Gulf Stream tornaria larva (Fig. 1E). Novel 18S ribosomal DNA sequences from Antarctic cephalodiscid pterobranchs (Fig. 2) are also presented. We show that the deep-sea species *Saxipendium coronatum* is a member of Harrimaniidae, and a Gulf Stream tornaria larva and deep-sea *Torquarator*-like worm are allied with Ptychoderidae. Enteropneusts are paraphyletic in our expanded analyses using 18S rDNA, with pterobranchs allied with harrimaniids, suggesting that the hemichordate ancestor may have been a solitary enteropneust-like worm.

2. Materials and methods

2.1. Taxonomic sampling

Table 1 lists sample locality information and GenBank Accession numbers for taxa analyzed in this study. Shallow water enteropneusts

Table 1
Collection localities and GenBank Accession numbers for taxa used in phylogenetic analyses.

Taxon		Locality[a]	GenBank Accession No.[b]		
			18S	16S	Cytochrome *b*
Asteroid	*Asterina pectinifera*		AB084551	NC_001627	NC_001627
Ophiuroid	*Ophiopholis aculeata*		DQ060806	NC_005334	NC_005334
Echinoid	*Strongylocentrotus purpuratus*		L28056	NC_001453	NC_001453
Holothuroid	*Cucumaria miniata*		DQ777082	NC_005929	NC_005929
Crinoid	*Gymnocrinus richeri*		AY275895	NC_007689	NC_007689
Xenoturbellida	*Xenoturbella bocki*		AY291292	NC_008556	NC_008556
Cephalochordate	*Branchiostoma lanceolatum*		AY428817	NC_001912	NC_001912
Harrimaniid	*Saccoglossus bromophenolosus*	Padilla Bay, WA	AF236801	L26348	**EU728444**
	Saccoglossus kowalevskii		L28054	NC_007438	NC_007438
	Saccoglossus pusillus	Cape Beal, B.C. Can	AF236800	**EU728422**	
	Saccoglossus cambrensis		X59119		
	Harrimania planktophilus	Cape Beal, B.C. Can	AF236799	**EU728421**	**EU728443**
	Stereobalanus canadensis	Maine	**EU728434**	**EU728424**	**EU728446**
	Protoglossus sp.	South Australia	**EU728432**	**EU728420**	**EU728442**
	Saxipendium coronatum	37° 47.5 S on SEPR—Alvin dive # 4090	**EU728433**	**EU728423**	**EU728445**
Ptychoderid	*Ptychoderid* sp. Tampa	Tampa, FL	AF278685	**EU728427**	**EU728448**
	Balanoglossus carnosus		D14359	AF051097	AF051097
	Balanoglossus clavigerus	La Baule, France		**EU728425**	
	Glossobalanus berkeleyi	Hood Canal, WA	**EU728435**	**EU728426**	**EU728447**
	Glossobalanus minutus		AF119089		
	Torquarator-like Enteropneust	13°N on the EPR	**EU728438**	**EU728431**	**EU728449**
	Ptychodera flava	Honolulu, HI	AF278681	**EU728428**	
	Ptychodera flava	Moorea	**EU728436**	**EU728429**	
	Tornaria larva	Gulf Stream, Bahamas	**EU728437**	**EU728431**	
Pterobranch	*Cephalodiscus gracilis*		AF236798		
	Cephalodiscus nigrescens	Antarctica	**EU728440**		
	Cephalodiscus hodgsoni	Antarctica	**EU728441**		
	Cephalodiscus densus	Antarctica	**EU728439**		
	Rhabdopleura normani		U15664.1		

[a] Locality information provided only for samples that are new from this study.
[b] New sequences from this study indicated in bold.

were dug with shovels at low tide at specified locations, stored in individual tubes with seawater and carried back to the lab for processing. *Stereobalanus canadensis* was collected by dredging from a small boat with the help of Darling Marine Center. *Protoglossus* sp. was collected using SCUBA at 5 m depth in sediment under Edithburg Jetty in South Australia, and was generously provided by Greg Rouse. Tornaria larvae were collected in a plankton tow in the Gulf Stream off Fort Pierce, Florida and in the Bahamas on the Southwest reef, New Providence Island (provided by Will Jaeckle) and stored in 100% ethanol. Tissue samples obtained by submersible (ALVIN dive 4090) from *Saxipendium coronatum* were kindly provided by Bob Vrijenhoek and Greg Rouse. Broad collared deep-sea worms similar to *Torquarator bullocki* (Fig. 1D) were obtained by submersible by IFREMER (French Research Institute for Exploitation of the Sea), stored in ethanol and provided by Stéphane Hourdez. Antarctic pterobranchs were collected during two cruises to the southern tip of South America and the Antarctic Peninsula aboard the *R/V Laurence M. Gould*. The first cruise took place from 23 November–22 December 2004 and the second from 12 May–13 June 2006. Samples intended for DNA analysis were either frozen at −80 °C or preserved in ~85% ethanol upon collection.

2.2. Data collection

Genomic DNA was extracted from enteropneust proboscis muscle or gonad tissue using a phenol/chloroform protocol (Swalla et al., 2000), GenElute mammalian genomic DNA miniprep kit (Sigma, St. Louis, MO), or DNeasy Tissue extraction kits (Qiagen, Valencia, CA). DNA extractions from pterobranch zooids were performed using the DNeasy Tissue kit (Qiagen). Table 2 shows the 18S, 16S, and cytochrome *b* (cyt *b*) primers as well as PCR programs used in this study. PCR was performed in 25 μl reactions with the following components: 0.75 units GoTaq DNA Polymerase, 1x Green GoTaq Reaction Buffer (Promega, Madison, WI), 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.5 μM each primer, and 50–200 ng total template DNA. Alternatively, PCRs contained 0.15 μl Taq DNA Polymerase (Eppendorf, Westbury, NY), 2.5 μl 10x Eppendorf PCR Buffer, 2.5 μl of 25 mM $MgCl_2$, 2.5 μl of 2 mM dNTPs, 1–2 μl of 10 μM primer, 1 μl DNA template, and water to a final volume of 25 μl. PCR products were run on a 1% TAE agarose gel and the bands were excised and purified using Sephaglas (GE Healthcare, Piscataway, NJ), EZNA micoelute gel extraction kit (Omega Bio-tek, Doraville, GA), or QIAquick Gel Extraction Kit (Qiagen). Purified PCR products were ethanol precipitated and bidirectionally sequenced on an ABI 3100 sequencer (Foster City, CA) in the UW Biology Comparative Genomic Center or a Beckman Coulter CEQ 8000 (Fullerton, CA) at Auburn University. Sequences from additional taxa, including outgroups, were obtained from GenBank. A representative from each echinoderm class, as well as *Xenoturbella bocki* and the cephalochordate *Branchiostoma lanceolatum* were used as outgroups.

Sequences were aligned using Clustal X (Thompson et al., 1997), edited manually using MacClade 4.08 (Maddison and Maddison, 2000), and regions of questionable alignment were excluded. Cyt *b* sequences were checked against the amino acid translation to ensure proper alignment. The individual gene alignments have been deposited in TreeBASE (http://www.treebase.org) under the submission ID SN3905. We analyzed datasets separately and in

Table 2
Primer sequences and thermocycler parameters.

Primer pair	Primer sequence (5′ → 3′)	PCR parameters	References
16S ar	CGCCTGTTTATCAAAAACAT	94° 45 s, 50° 45 s, 72° 1 min	Palumbi (1996)
16S br	CCGGTCTGAACTCAGATCACGT		Palumbi (1996)
cyt *b* F	CAAATGTCRTTYTGGGGWGC	94° 45 s, 40° 45 s, 72° 1 min	Designed by Rychel
cyt *b* R	GGRAANARRAARTAYCAYTC		Yokobori et al. (2005)
18S PH	CTGGTTGATCCTGCCAG	94° 45 s, 55° 45 s, 72° 1 min	Swalla et al. (2000)
18S D	CGATCAGATACCGTCCTAGT		Swalla et al. (2000)
18S BS	TAATGATCCATCTGCAGGTTCACCT	94° 45 s, 55° 45 s, 72° 1 min	Swalla et al. (2000)
18S F	GCCTGCTTTGAACACTCTAA		Swalla et al. (2000)
18e	CTGGTTGATCCTGCCAGT	94° 3 min, add polymerase, 94° 3 min, 94° 1 min, 40° 1 min 30 s, 72° 2 min 30 s	Hillis and Dixon (1991)
18L	GAATTACCGCGGCTGCTGGCACC		Halanych et al. (1998)
18H	AGGGTTCGATTCCGGAGAGGGAGC	94° 3 min, add polymerase, 94° 3 min, 94° 1 min, 50° 1 min 30 s, 72° 2 min 30 s	Hillis and Dixon (1991)
18 M	GAACCCAAAGACTTTGGTTTC		Halanych et al. (1998)
18F997	TTCGAAGACGATCAGATACCG	94° 3 min, add polymerase, 94° 3 min, 94° 1 min, 40° 1 min 30 s, 72° 2 min 30 s	Burnette et al. (2005)
18P	TAATGATCCTTCCGCAGGTTCACCT		Hillis and Dixon (1991)

combination, using maximum likelihood (ML), and Bayesian inference methods (BI). Likelihood trees were generated using PAUP* 4.0b10 (Swofford, 1999) with appropriate models of evolution chosen using Modeltest (Posada and Crandall, 1998) under the Akaike Information Criterion (Posada and Buckley, 2004). Heuristic analyses with Tree-Bisection-Reconnection (TBR) branch swapping were used with 100 iterations started with random starting trees. Nodal support was assessed with 500 bootstrap iterations (heuristic search using the fast-step option). Bayesian inference topologies were generated using MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003) with models chosen using MrModeltest (Nylander, 2004). Four independent Bayesian analyses on separate datasets were run for 10,000,000 generations with tree samples every 100 generations using 3 heated and 1 cold chain. To assess 'burn-in', likelihood values were plotted against generation number to determine when likelihood values reached stationarity. Combined Bayesian analyses modeled parameters for each partition separately, and partitions were run as unlinked. Four independent Bayesian analyses on the combined data set were run for 5,000,000 generations with trees sampled every 100 generations using 3 heated and 1 cold chain.

Likelihood values in BI analyses reached stationarity after 5000 generations, which were discarded as burn-in. The general time reversible (GTR) model of nucleotide substitution with proportion of invariable sites (18S $I = 0.3357$; 16S $I = 0.2163$) and gamma distribution (18S $\Gamma = 0.6144$; 16S $\Gamma = 0.6158$) was selected for the 18S and 16S data sets, and the Hasegawa, Kishino, Yano 85 (HKY) model with proportion of invariable sites ($I = 0.2584$) and gamma distribution ($\Gamma = 0.7564$) was selected for cytochrome *b*. These models were used in individual analyses of each gene as well as the combined partitioned BI analyses. For the complete alignment, the symmetrical model (SYM) with proportion of invariable sites ($I = 0.3327$) and gamma distribution ($\Gamma = 0.5065$) was selected under the Akaike Information Criterion (AIC) in ModelTest, and this model was used in ML analyses of the total data set.

Alternative phylogenetic hypotheses were compared using the Shimodaira and Hasegawa (1999) test as implemented in PAUP* 4.0b10 using RELL with 1,000 bootstrap replicates. Two times natural log Bayes factors were calculated using the harmonic mean estimated marginal likelihood of stationary phase samples generated by the sump command in MrBayes, and were interpreted according to the criteria of Kass and Raftery (1995) to assess alternative phylogenetic hypotheses.

3. Results

In the combined data set there were 3252 positions, 2550 of which could be unambiguously aligned. Of these, 1166 characters were variable, and 805 were parsimony-informative across all operational taxonomic units (OTUs). There were 1678 included characters in the 18S rDNA data set, 379 were parsimony-informative. For mitochondrial genes, there were 468 included characters from 16S rDNA, including 190 parsimony-informative characters, and 404 included characters for cyt *b*, with 236 parsimony-informative characters. Nucleotide frequencies across taxa were stationary for 18S and 16S, but not for cytB (Chi-square test of homogeneity of base frequencies across taxa, $P = 0.000000$). A harrimaniid enteropneust, *Stereobalanus canadensis*, was found to have highly divergent 18S and 16S sequences, and was extracted and sequenced twice to ensure the accuracy of the data.

ML and BI analyses of the combined 18S, 16S and cyt *b* data yielded topologies with identical relationships (Fig. 3). Hemichordates formed a monophyletic clade in all analyses (BI posterior probability = 1.00, ML bootstrap = 100). The Harrimaniidae + *Saxipendium coronatum* clade is well-supported, with Bayesian pp = 1.00, and ML bootstrap = 96. A Ptychoderidae/Torquaratoridae/tornaria clade is supported by a posterior probability value of 1.00 and ML bootstrap value of 73. Polymerase chain reactions on pterobranch DNA using conserved mitochondrial primers (Table 2) as well as specifically designed primers for 16S rDNA and cytochrome *b* in hemichordates (not shown) have failed to produce products despite extensive optimization and the use of several extraction techniques for genomic DNA. Therefore, pterobranchs as a whole were excluded from combined analyses due to unavailability of mitochondrial data.

Results from 18S rDNA analyses (Fig. 4) supported a paraphyletic Enteropneusta, with pterobranchs as sister to Harrimaniidae. In the ML tree, the harrimaniid + pterobranch clade is poorly supported (bootstrap value = 48), whereas the BI analysis showed strong support (0.99 posterior probability). Removal of the long-branched harrimaniid *Stereobalanus canadensis* and the partial *Rhabdopleura normani* increases the maximum likelihood bootstrap value for Harrimaniidae + Pterobranchia to 68 in a separate 18S rDNA analysis (data not shown). The *Cephalodiscus* clade within pterobranchs is very well supported (pp = 1.00, bootstrap = 100), however, support for pterobranchs as a whole (*Cephalodiscus* + *Rhabdopleura*) is low. The Harrimaniidae + *Saxipendium coronatum* clade has limited support in 18S rDNA analyses (pp = 0.90, bootstrap = 70). However, these marginal values may be due to the long-branched *Stereobalanus canadensis*; all the other harrimaniids + *Saxipendium* group together with high support (pp = 1.00, bootstrap = 87).

Table 3 presents Shimodaira-Hasegawa (S-H) test and Bayes Factor results for alternative phylogenetic hypotheses tested. We tested alternative hypotheses using 18S rDNA sequence data

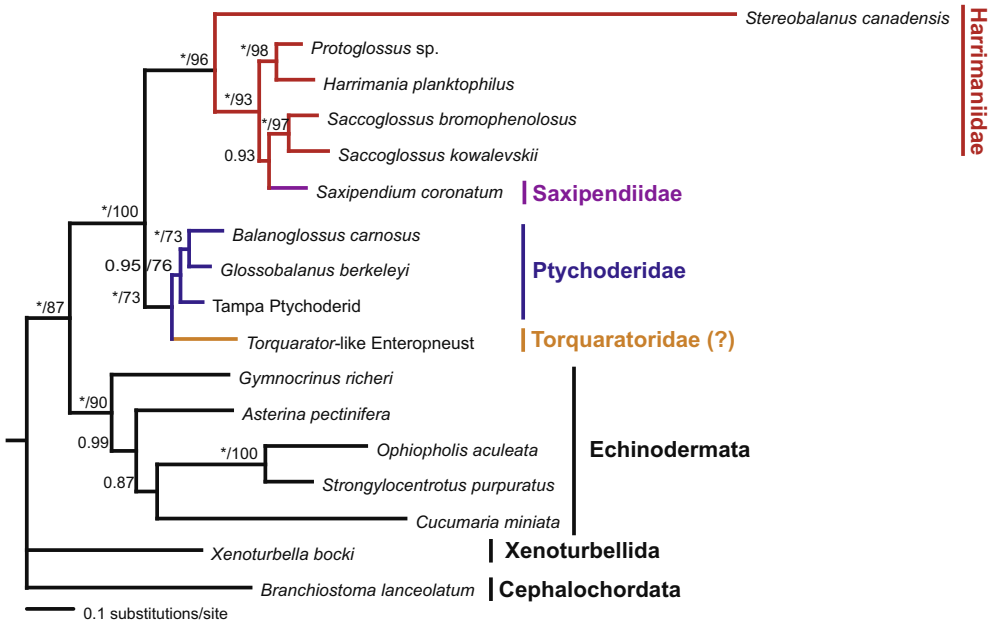

Fig. 3. Combined 18S and mitochondrial phylogeny. Bayesian tree is shown with posterior probabilities greater than 0.60 and maximum likelihood bootstrap values above 60 indicated above the nodes. Bayesian posterior probabilities of 1.00 are indicated by asterisks. Methodological details given in the text.

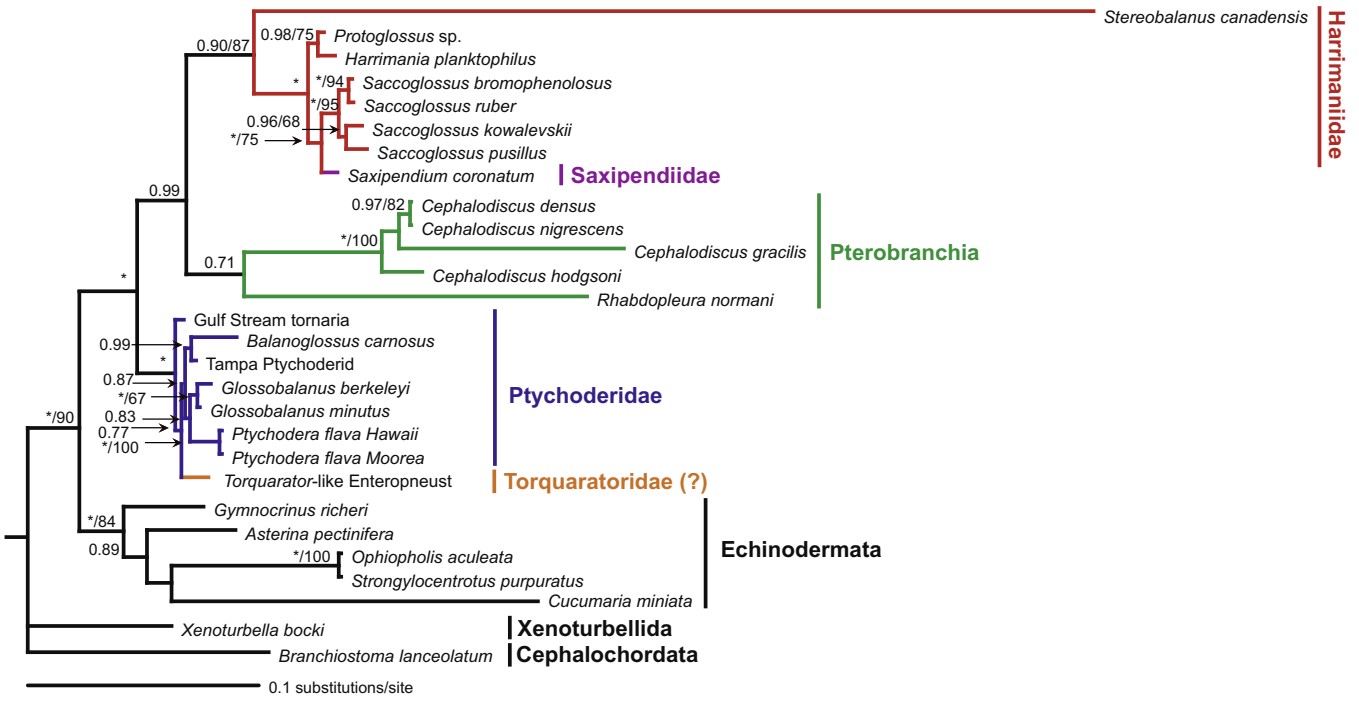

Fig. 4. 18S rDNA topology. Bayesian tree is shown with posterior probabilities greater than 0.60 and maximum likelihood bootstrap values above 60 indicated above the nodes. Bayesian posterior probabilities of 1.00 are indicated by asterisks. Methodological details are given in the text.

alone, as well as the combined data set described above. The alternative hypothesis that *Saxipendium coronatum* falls outside Harrimaniidae was not significantly worse than the best tree in S-H tests, although *P*-values were close to the cut-off point for significance. Bayes Factor comparisons demonstrate strong support against *Saxipendium coronatum* outside Harrimaniidae. Trees in which the broad-collared deep-sea enteropneust was constrained outside Ptychoderidae were not rejected by either S-H tests or Bayes Factor comparisons. The hypothesis that *Protoglossus* sp. is not a member of Harrimaniidae (as proposed by Caullery and Mesnil, 1904) was rejected by both S-H tests using the combined data set and Bayes Factor comparisons. A Shimodaira-Hasegawa test using 18S rDNA did not reject the hypothesis that Enteropneusta is monophyletic. Bayes Factor comparisons, on the other hand, showed evidence in favor of the Bayesian consensus tree (Fig. 3) over a constrained tree in which enteropneusts were monophyletic, as assessed by the criteria outlined in Kass and Raftery (1995) (2ln Bayes factor = 10.94). Interestingly, S-H

Table 3
Shimodaira-Hasegawa test and Bayes factor results comparing alternative hypotheses.[a]

Alternative hypotheses tested	Ln likelihood score	*P*-value	Total harmonic mean	2ln Bayes factor	Ln likelihood score	*P*-value	Total harmonic mean	2ln Bayes factor
	18S rDNA				Combined data set			
Best tree	−8794.103		−8862.59		−17074.777		−16568.37	
Enteropneusta monophyletic	−8802.364	0.198	−8868.06	10.94	n/a	n/a	n/a	n/a
Saxipendium coronatum outside Harrimaniidae	−8807.850	0.053	−8873.54	20.82	−17086.304	0.095	−16584.35	31.96
Torquarator-like enteropneust outside Ptychoderidae	−8795.948	0.307	−8862.44	0.3	−17074.777	1.00	−16567.34	2.06
Protoglossus sp. outside Harrimaniidae	−8803.958	0.064	−8873.54	17.28	−17099.537	0.001*	−16592.11	47.48

[a] Alternative hypotheses are constrained contrary to what is observed in the best tree. *P*-values for S-H tests represent significance of support for the relationships seen in the best tree versus the alternative hypotheses. 2ln Bayes factors > 10 are considered strong support in favor of the best tree over alternative hypotheses, as outlined in Kass and Raftery (1995).
* $P < 0.05$.

tests were less sensitive than Bayes Factor comparisons in rejecting alternative hypotheses.

4. Discussion

4.1. Phylogenetic relationships and taxonomic changes

Hemichordate taxonomy is in need of revision. Data presented herein raise concerns as to the status of the two families of deep-sea enteropneusts that have been described within the last 25 years (Holland et al., 2005; Woodwick and Sensenbaugh, 1985). Each family is monospecific, and in both cases, worms were described based on morphological analysis of either one or a few specimens. Due to the fragility of their tissue, deep-sea enteropneusts are difficult to collect (Holland et al., 2005). Saxipendiidae examined here is shown to be a member of Harrimaniidae, not a separate family. Similarly, the broad-collared Torquaratoridae-like enteropneust is very closely allied to Ptychoderidae and may be nested within the taxon. Unfortunately, although the tornaria larvae we examined looked ptychoderid-like, we cannot confirm its taxonomic position based on morphology until the adult is obtained. These results underscore the importance of molecular data when morphology may be highly derived in deep-sea taxa when compared to more familiar intertidal species.

The deep-sea Torquaratoridae is characterized by an exceptionally broad proboscis and collar. The presence of hepatic caecae and genital wings (enlarged in *Torquarator*) unite the Torquaratoridae with ptychoderids (Holland et al., 2005). However, *Torquarator* lacks gill bar synapticulae, an apomorphy of Ptychoderidae (Spengel, 1893). Our molecular sequence data from a broad-collared deep-sea worm similar to *Torquarator* indicate that this species is allied to Ptychoderidae. Unfortunately, the organism did not remain intact upon surfacing, precluding morphological analyses by means other than photographs. Based upon its broad collar (Fig. 1D), we provisionally ally this species with Torquaratoridae, and suggest that *Torquarator* and similar species may belong within the enteropneust family Ptychoderidae. DNA sequence data from a specimen verified by morphology to be *Torquarator* will be required to confirm this result.

Similarly, *Saxipendium coronatum* was originally placed in its own separate family (Saxipendiidae) due to a few autapomorphic characters coupled with several losses (Woodwick and Sensenbaugh, 1985). The authors noted that Saxipendiidae is most similar to Harrimaniidae, although *Saxipendium* does not possess the long muscular proboscis and large yolky eggs that are characteristic of harrimaniids. Morphological analysis of *S. coronatum* showed that they have small eggs, a short collar, and a proboscis skeleton that is crown-shaped in cross section (Woodwick and Sensenbaugh, 1985). Both 18S and mitochondrial sequence data place *Saxipendium coronatum* within harrimaniids with strong support, suggesting that the long muscular proboscis is not a reliable taxonomic character for the Harrimaniidae.

Molecular data can help classify taxa that may have unusual morphological characters related to their unique habitat. The broad proboscis and collar of *Torquarator* and similar species, as well as the weakly developed proboscis muscles in *Saxipendium*, are likely to be such adaptations, and may not be useful diagnostic features. Additionally, our results highlight the utility of molecular data in classifying species for which collection of intact specimens is difficult.

We were also able to generate 18S rDNA and 16S rDNA sequence data from single tornaria larvae from the Gulf Stream off New Providence Island in the Bahamas, as well as 16S rDNA from a single tornaria larva from the Gulf Stream off the Florida coast. Sequence divergence levels of 16S between individuals from the two locations were very low (0.00000–0.00736 between three Bahaman individuals and 0.00000–0.00553 between a Florida individual and three Bahaman individuals), suggesting that these larvae belong to the same species. Sequence data from a single Bahaman individual are presented here, termed Gulf Stream tornaria in our analyses. In 18S and combined analyses, the tornaria clusters with, but is basal to, known ptychoderid adults, while in 16S analyses it pairs weakly with the *Torquarator*-like deep-sea enteropneust (Supplementary Fig. 2). Although we were unable to link these tornaria larvae with specific adult species, additional sequence data from widely distributed ptychoderids should facilitate future identification. Similar success has already been achieved with matching echinoderm larvae to adults using molecular data (Janosik et al., 2008; Knott et al., 2003).

Protoglossus, a harrimaniid considered to have such simple morphology that it was originally placed in its own family as a basal enteropneust (Caullery and Mesnil, 1904), is shown here to be nested within the harrimaniids. The species presented here as Tampa pychoderid was originally identified as a member of the genus *Ptychodera*, species unknown (Winchell et al., 2002). In our analyses, this species pairs with either *Balanoglossus* (18S rDNA) or as sister to *Glossobalanus* + *Balanoglossus* (combined dataset), raising the possibility that this species may have been misidentified, and we have remained conservative with naming herein until the identification can be confirmed by morphology.

Finally, results shown here are consistent with previous findings based on 18S data that Pterobranchia originated within Enteropneusta as sister to Harrimaniidae (Cameron et al., 2000; Halanych, 1995). Ptychoderids have been historically thought of as the most derived hemichordates due to the apparently complex features of the trunk, including genital ridges or wings, lateral septa, and gill bar synapticules (Hyman, 1959), indicating to some

phylogeneticists that ptychoderids are not a basal group within hemichordates. However, they have the most complex adult dorsal neural structures and ptychoderid worms are the only hemichordate group with feeding tornaria larvae (Brown et al., 2008), suggesting that they are basal hemichordates. Our analyses also indicate that ptychoderids may be the earliest branching lineage within the hemichordates. Nodal support for this arrangement is not high in our ML analyses, and although Bayes factor comparisons reject the alternative hypothesis that Enteropneusta is monophyletic, S-H tests do not. The addition of three Antarctic *Cephalodiscus* species strengthens former results, but data from additional genes will be required to definitively resolve this issue.

4.2. Implications for chordate origins

Earlier hypotheses of deuterostome evolution in which pterobranchs represented an ancestral deuterostome form typically included lophophorates (phoronids and brachiopods) within deuterostomes (Jefferies, 1986; Jefferies et al., 1987; Romer, 1967). Molecular and morphological data have since shown that the lophophore is not homologous to pterobranch feeding structures, because lophophorates are protostomes (Halanych, 1993, 1996; Halanych et al., 1995). With lophophorates no longer within deuterostomes, the strongest support for pterobranchs as basal hemichordates comes from similarities in the mesocoel-derived branched tentacular system possessed by pterobranchs and echinoderms (Swalla and Smith, 2008). There are two recent phylogenetic analyses supporting the hypothesis that pterobranchs are basal within Hemichordata. In Cameron's (2005) morphological cladistic study, pterobranchs were found to be the earliest lineage within hemichordates, as sister to a monophyletic Enteropneusta. However, the analysis included many characters that were too general to be informative within hemichordates (e.g., segmentation, U-shaped gut), or that were specific to non-hemichordate taxa (e.g., pentaradial symmetry, madreporite, ossicles in tube feet). Due to the drastic changes that occur with the evolution of coloniality, solitary forms within a clade are likely to be more morphologically similar to each other than to colonial forms in the same clade, which can confound morphological analyses (Zeng and Swalla, 2005).

Using 28S rDNA, Winchell et al. (2002) recovered a monophyletic Enteropneusta, using a more limited taxonomic sample of hemichordates. A potential problem with this data set is lack of phylogenetic signal. There were just 109 parsimony-informative characters from the conserved stem regions out of 2642 total characters for the in-group, and 91 of these 109 are informative just between the solitary hemichordates in 28S rDNA alignments. Between the harrimaniid enteropneusts (three species) and *Cephalodiscus gracilis* there are only 15 parsimony-informative 28S characters, suggesting that this conserved portion of 28S rDNA has little phylogenetic signal within Hemichordata.

An alternate view, that the ancestral hemichordate was enteropneust-like, has been proposed on the basis of homologies between enteropneust and cephalochordate gill slits (Peterson and Eernisse, 2001; Rychel and Swalla, 2007), as well as similarities in axis specification genes in enteropneusts and chordates (Cameron et al., 2000; Swalla, 2007; Zeng and Swalla, 2005). Additionally, pychoderid tornaria and echinoderm larvae have very similar ciliary band patterning, digestive structures, and tri-coelomic organization (Hyman, 1959; Brown et al., 2008). Harrimaniids and pterobranchs, on the other hand, are direct developers with a short-lived larval stage that bear little resemblance to tornaria larvae (Hyman, 1959; Lester, 1988; Sato et al., 2008a). Similarities between ptychoderid larvae and echinoderm larvae support a basal position for Ptychoderidae, while harrimaniids and pterobranchs have lost this complex larval form (Brown et al., 2008). Our results are consistent with this hypothesis, although additional data are necessary. In the future, comparison of the 18S dataset presented here with one constructed from genomic information—single copy nuclear genes, introns, and entire mitochondria—should provide us with a better picture of the evolutionary relationships among the solitary and colonial hemichordates.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2009.03.027.

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