Hemichordate Molecular Phylogeny Reveals a Novel Cold-Water Clade of Harrimaniid Acorn Worms

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Abstract. Hemichordates are instrumental to understanding early deuterostome and chordate evolution, yet diversity and relationships within the group have been understudied. Recently, there has been renewed interest in hemichordate diversity and taxonomy, although current findings suggest that much hemichordate diversity remains to be discovered. Herein, we present a molecular phylogenetic study based on nuclear 18S rDNA sequence data, which includes 35 previously unsampled taxa and represents all recognized hemichordate families. We include mitochondrial 16S rDNA data from 66 enteropneust taxa and three pterobranch *Rhabdopleura* species, and recover colonial pterobranchs and solitary enteropneusts as reciprocally monophyletic taxa. Our phylogenetic results also reveal a previously unknown clade of at least four species of harrimaniid enteropneusts from cold waters, including Antarctica, the North Atlantic around Iceland and Norway, and the deep sea off Oregon. These small worms (1–5 mm in length), occur from 130 to 2950 m and are not closely related to other deep-sea harrimaniids, indicating that diversity of enteropneusts within the deep sea is broader than previously described in the literature. Discovery of this clade, as well as larger torquaratorids from Antarctica, strengthens hypotheses of close associations between Antarctic and deep-sea fauna.

Introduction

Hemichordates, a small marine phylum integral to hypotheses on deuterostome and chordate evolution, occur throughout the world's oceans. At present, 135 hemichordate species are described: 121 are solitary Enteropneusta, or acorn worms; and 24 are colonial, tube-dwelling Pterobranchia (Appeltans *et al.*, 2012; Halanych and Cannon, unpubl. data). In the last decade, there has been revived interest in hemichordate taxonomy. The number of enteropneust genera has increased by 40% since 2005, with several papers on the deep-sea family Torquaratoridae (Holland *et al.*, 2005, 2009, 2012a, b; Smith *et al.*, 2005; Osborn *et al.*, 2011; Priede *et al.*, 2012), as well as recent evaluations of Ritter's and Bullock's historical collections leading to the description of 16 new species and three new genera (Deland *et al.*, 2010; Cameron *et al.*, 2010; Cameron and Ostiguy, 2013; Cameron and Perez, 2012). Another novel genus of enteropneust, *Meioglossus*, has been described on the basis of meiofaunal samples collected in Bermuda and Belize (Worsaae *et al.*, 2012). Evidently, much of the diversity within this group remains undiscovered (Appeltans *et al.*, 2012, supplement).

There are four currently recognized families of enteropneusts—Harrimaniidae (39 species in 10 genera), Ptychoderidae (38 species in 3 genera), Spengelidae (19 species in 4 genera), and Torquaratoridae (6 species in 4 genera). Molecular phylogenetic work has indicated that Harrimaniidae, Ptychoderidae, and Torquaratoridae are likely to be valid families (Cannon *et al.*, 2009; Osborn *et al.*, 2011; Worsaae *et al.*, 2012). Spengelids, on the other hand, have thus far been represented in phylogenetic analyses by a single species, *Glandiceps hacksi*, which is recovered as sister to Ptychoderidae + Torquaratoridae (Osborn *et al.*, 2011; Worsaae *et al.*, 2012).

Extant pterobranchs comprise two groups, Cephalodiscida (18 species in 1 genus) and Rhabdopleurida (6 species in 1 genus). Recent analyses of morphology from fossil grap-
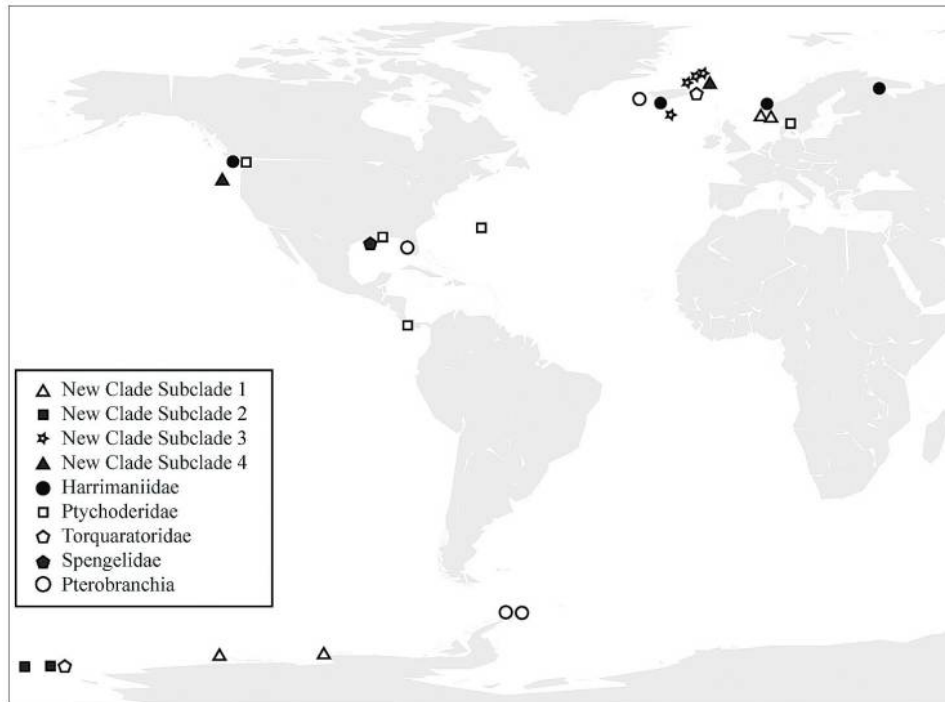
Figure 1.   Collection localities for hemichordates collected in this study. Taxonomic groups indicated by filled circles.

tolites and extant pterobranchs place Cephalodiscida as sister to Graptolithina, including Rhabdopleurida (Mitchell *et al.,* 2012). Interrelationships between Enteropneusta and Pterobranchia have been controversial. Several 18S rDNA studies have recovered pterobranchs within Enteropneusta sister to Harrimaniidae (Halanych, 1995; Cameron *et al.*, 2000; Bourlat *et al.*, 2003; Cannon *et al.*, 2009; Worsaae *et al.*, 2012), but other 18S analyses do not recover this relationship (Osborn *et al.,* 2011). By contrast, recent microRNA data (Peterson *et al.*, 2013), morphological cladistic analysis (Cameron, 2005), and 28S rDNA (Winchell *et al.*, 2002) suggest that Enteropneusta and Pterobranchia are reciprocally monophyletic taxa, although the latter two data matrixes have been criticized for containing few informative characters (Cannon *et al.*, 2009), and the microRNA analysis included only three hemichordate lineages.

In 1959, Libbie Hyman observed that "nearly all enteropneusts of the cold waters of both hemispheres belong to Harrimaniidae" (Hyman, 1959). Today, this observation remains largely correct, particularly within higher latitudes. Deep-sea torquaratorids, which were unknown in her time, are the obvious exception. Three enteropneusts are known from Arctic seas, *Harrimania kupfferi*, *Harrimania maculosa*, and *Saccoglossus mereschkowskii*, all harrimaniids. Ptychoderidae and Spengelidae each contain one deep-sea member and are otherwise predominantly tropical or subtropical. Most pterobranch species, on the other hand, occur in deep cold water, many in Antarctica.

Here we present a molecular phylogeny of Hemichordata that includes novel sequence data from 35 taxa representing all hemichordate families, at least 12 of which are distinct genetic lineages not attributable to known species. Our phylogenetic results reveal a previously unknown clade of harrimaniid enteropneusts from cold waters and provide an updated molecular phylogenetic hypothesis for hemichordates with broader taxon sampling across all lineages.

Materials and Methods

Organismal collection

Figure 1 shows a world map with collection localities for samples in this study, and Table 1 provides more detailed locality information and sampling method used. Eleven specimens sequenced in this study were collected in September 2011 during Senckenberg's German Center for Marine Biodiversity Research (DZMB) IceAGE expedition led by Dr. Saskia Brix aboard the R/V *Meteor*, which circled Iceland, crossing the Mid-Atlantic and Greenland-Scotland ridges. *Cephalodiscus* specimens were collected during two research expeditions to the Antarctic Peninsula aboard the R/V *Lawrence M. Gould* in 2001 and 2004. Antarctic enteropneusts were collected in January–February 2013 in the Amundsen and Ross Seas by the R/V *Nathaniel B. Palmer*. Enteropneusts from Norway were collected on the R/V *Håkon Mosby* or R/V *Aurelia* with the aid of Dr. Christiane

Table 1

Collection and locality information by taxon

Family	Taxon	Locality	Latitude	Longitude	Depth (m)	Collection method
Cephalodiscidae	*Cephalodiscus hodgsoni* H11.3	Elephant Island, Antarctica	62°44.74′S	56°44.88′W	207	Dredge
	Cephalodiscus fumosus H5.3	South Shetlands, Antarctica	62°45.03′S	56°46.26′W	220	Dredge
	Cephalodiscus nigrescens H12.3	Elephant Island, Antarctica	62°44.74′S	56°44.88′W	207	Dredge
Rhabdopleuridae	*Rhabdopleura* sp. 1 H71.2	Iceland	63°56.07′N	25°56.53′W	209	Agassiz Trawl
	Rhabdopleura sp. 2	Gulf of Mexico	27°36.94′N	83°28.02′W	36.5	Hourglass Dredge
Harrimaniidae	Harrimaniidae H42.1	Norway	60°18.37′N	5°12.07′E	130	Epibenthic Sled
	Harrimaniidae H61.1	Norway	60°16.58′N	05°11.09′E	183	Epibenthic Sled
	Harrimaniidae H72.1	Iceland	69°6.51′N	9°55.09′W	2177	Epibenthic Sled
	Harrimaniidae H72.2	Iceland	69°6.51′N	9°55.09′W	2177	Epibenthic Sled
	Harrimaniidae H72.3	Iceland	69°6.51′N	9°55.09′W	2177	Epibenthic Sled
	Harrimaniidae H69.1	Iceland	60°20.87′N	18°8.52′W	2569	Epibenthic Sled
	Harrimaniidae H75.1	Iceland	69°6.51′N	9°55.09′W	2177	Epibenthic Sled
	Harrimaniidae H76.1	Iceland	67°5.79′N	13°0.42′W	1612	Agassiz Trawl
	Harrimaniidae H77.1	Iceland	67°37.39′N	12°4.06′W	1781	Agassiz Trawl
	Harrimaniidae H86.2	Wright's Gulf, Antarctica	73°15.30′S	129°12.94′W	481	SmithMac Grab
	Harrimaniidae H83.1	Amundsen Sea, Antarctica	72°10.64′S	103°30.84′W	341	Blake Trawl
	Harrimaniidae H93.1	Ross Shelf, Antarctica	75°19.80′S	176°59.26′W	567	MegaCore
	Harrimaniidae H94.1	Ross Shelf, Antarctica	75°19.80′S	176°59.26′W	567	MegaCore
	Harrimaniidae H98.2	Ross Shelf, Antarctica	76°20.47′S	170°51.02′W	531	MegaCore
	Harrimaniidae H103.1	Oregon, USA	43°50.60′N	127°34.03′W	2950	MultiCore
	Saccoglossus mereschkowskii H53.1	White Sea, Russia	66°33.19′N	33°6.35′E	<20	Dredge
	Saccoglossus sp. H44.2	Friday Harbor, Washington, USA	48°32.61′N	123°0.68′W	10–5	Dredge
	Harrimaniidae H62.1	Norway	60°27.85′N	05°05.96′E	130	Epibenthic Sled
	Harrimaniidae H74.1	Iceland	62°56.46′N	20°44.06′W	916	Epibenthic Sled
Ptychoderidae	*Balanoglossus* sp. 1 H50.4	Bocas del Toro, Panama	9°24.3′N	82°19.45′W	<1	Shovel
	Balanoglossus cf. *aurantiaca* H51.1	Ship Island, Mississippi, USA	30°14.8′N	88°32.4′W	1	Yabby Pump
	Balanoglossus cf. *aurantiaca* H52.1	Horn Island, Mississippi, USA	30°14.75′N	88°46.51′W	1	Yabby Pump
	Glossobalanus marginatus H48.2	Norway	58°55.00′N	10°33.16′W	184	Agassiz Trawl
	Glossobalanus berkeleyi H68.2	False Bay, Washington, USA	48°28.93′N	123°4.23′W	<1	Shovel
	Ptychodera bahamensis H54.2	Shelley Bay, Bermuda	32°19.88′N	64°44.36′W	1	Snorkel
Spengelidae	*Schizocardium* cf. *braziliense* H47.5	Bay St. Louis, Mississippi, USA	30°14.09′N	89°20.09′W	12.5	Box Core
Torquaratoridae	Torquaratoridae H78.1	Iceland	66°18.06′N	12°22.40′W	732	Box Core
	Torquaratoridae H89.3	Ross Shelf, Antarctica	76°20.47′S	170°51.02′W	531	Blake Trawl
	Torquaratoridae H90.3	Ross Shelf, Antarctica	76°20.47′S	170°51.02′W	531	Blake Trawl

Todt and the late Dr. Christoffer Schander. *Balanoglossus* sp. specimens from Mississippi were collected with the assistance of Dr. Richard Heard. Dr. Jon Norenburg and Dr. Darryl Felder provided material from *Rhabdopleura* sp. collected in the Gulf of Mexico on the R/V *Pelican*.

Specimens were collected at depths ranging from the intertidal to over 2500 m, using diverse sampling methods (Table 1). Enteropneusts from Iceland were collected by decanting sediment through a 1–1.5-mm sieve, and were retained on either a 500- or 300-μm sieve; enteropneusts from Antarctica and off Oregon were decanted from sediment directly onto a 250-μm sieve. Freshly collected worms were preserved in 95%–100% ethanol, and when multiple specimens were available, voucher specimens were relaxed in 7.5% magnesium chloride and fixed in 4%–10% formalin for morphological studies.

Fragile and soft-bodied enteropneusts are easily damaged by dredging or sorting of sediment. Interestingly, all novel taxa reported here were collected using standard sampling techniques, including epibenthic sleds, which are typically used to recover more robust animals. Minuscule acorn worms may be present in samples collected elsewhere using similar methods, but may have been overlooked due to their minimal and nondescript external characters (Fig. 2). During collection, many enteropneusts were passed through a 1-mm mesh, and therefore could be classified as meiofaunal (Higgins and Thiel, 1988). However, most worms were broken during sieving, so their intact length is unknown. Additionally, because worms fragment easily, they are less amenable to detailed morphological analyses. Whereas even very small fragments can be used for DNA or RNA extraction, worms with intact proboscis, collar, and even a small part of the trunk are much more difficult to find (Fig. 2C). This simple fact may have hindered previous discovery of these worms and has delayed morphological characterization of novel worms reported in this study. Complete morphological species descriptions are forthcoming, but herein we focused on molecular data in order to provide a more comprehensive overview of unknown hemichordate diversity.

Molecular methods

Molecular laboratory methods followed Cannon *et al.* (2009). In addition to in-house sequencing on a CEQ8000 (Beckman-Coulter, Inc., Brea, CA), some PCR products were sent to Genewiz (South Plainfield, NJ). In the case of *Rhabdopleura* cf. *normani* from Bermuda, we were able to generate a full-length 18S rDNA sequence using publically available partial sequences (Genbank Accession numbers: U15664, JF900483, and JF900484) and 454 transcriptome data (Cannon and Halanych, unpubl.) prepared following the methods of Kocot *et al.* (2011). Assembled 454 contigs were formatted into a BLAST (Altschul *et al.*, 1997) data-
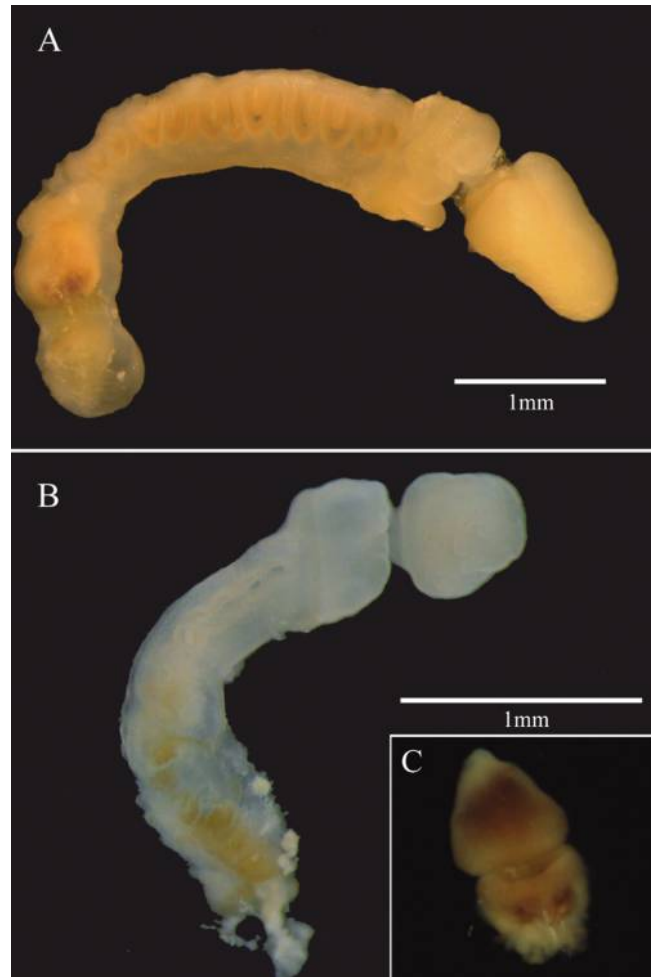

Figure 2. Cold-water harrimaniids. (A) Harrimaniidae Antarctica H83.1 (Subclade 1). Proboscis, collar, mouth, trunk, and gill slits are visible. (B) Harrimaniidae Antarctica H94.1 (Subclade 2). (C) Harrimaniidae Iceland H72.2 (Subclade 4), showing damaged condition in which many specimens were collected, with only proboscis and collar. Mouth is visible between proboscis and collar. Lower scale bar applies to B and C.

base, which was searched with a query composed of diverse hemichordate 18S sequences. Fragments with E-values below 1×10^{-30} were aligned to the partial 18S sequences as a backbone, and then assembled into a full-length 18S rDNA contig using the software package CLC Genomics Workbench ver. 5 (Aarhus, Denmark). As reported previously (Cannon *et al.* 2009), *Stereobalanus canadensis* (Enteropneusta, Harrimaniidae) has a highly divergent 18S rDNA sequence. Previously we had sequenced this acorn worm multiple times, but to further verify this sequence, genomic DNA was extracted from *S. canadensis* and sequenced *via* an Illumina MiSeq (San Diego, CA) at Auburn University. This work employed a 2 × 150-bp paired end run and the Nextera DNA sample kit. Scaffolds generated using the genome assembler Ray (Boisvert *et al.*, 2010) were formatted into a BLAST database, and queried as

above. A single 18S rDNA sequence was recovered, with 99.6% identity to our previously reported sequence; thus we have retained *Stereobalanus canadensis* in our analyses.

Taxon sampling and NCBI accession numbers for sequences used in phylogenetic analyses are given in Table 2. Representatives of each echinoderm class were used as outgroup taxa (Table 2). Sequences were aligned with MAFFT ver. 6.09b (Katoh *et al.*, 2005) using the L-INS-i method, and uninformative sites were trimmed using the perl scripts Aliscore and Alicut (Misof and Misof, 2009). Models of evolution were selected under the Akaike Information Criterion (AIC) implemented by MrModelTest (Nylander, 2004). The best fitting model for both 16S and 18S rDNA, GTR+I+G, was used in maximum likelihood (ML) and Bayesian inference analyses (BI) of concatenated alignments. ML analyses were performed with RaxML version 7.3.9 (Stamatakis, 2006) using 5000 bootstrap replicates. Bayesian analyses were conducted using MrBayes ver. 3.2.0 (Ronquist and Huelsenbeck, 2003). Four independent BI analyses were run for each dataset for 5,000,000 generations with trees sampled every 100 generations using three heated and one cold chain. Plotting likelihood values *versus* generation number revealed that stationarity was reached after approximately 1,000,000 generations, and thus the first 25% of sampled trees was discarded as burn-in. Competing hypotheses of hemichordate phylogeny were evaluated using the SH-test (Shimodaira, 2002) as implemented in RAxML ver. 7.3.8 with the GTR+I+G model.

Results

The final combined 18S rDNA + 16S rDNA alignment was 2776 nucleotides in length (18S = 2053 nucleotides, 16S = 723 nucleotides), with 88 included taxa and 965 parsimony informative sites. Several lengthy indel regions in the 16S of rhabdopleurid pterobranchs account for the long 16S alignment. Table 2 reports GenBank accession numbers for all data used, and aligned data have been deposited to TreeBase (http://www.treebase.org). Maximum likelihood and Bayesian inference analyses yielded topologies with an identical branching pattern (Fig. 3) in which Hemichordata and all currently recognized families were recovered as monophyletic. Notably, unlike previous 18S results (Halanych, 1995; Cameron *et al.*, 2000; Bourlat *et al.*, 2003; Cannon *et al.*, 2009; Worsaae *et al.*, 2012), Enteropneusta and Pterobranchia were recovered as reciprocally monophyletic, with strong support (bootstrap/posterior probability = 98/1.00), although the alternative hypothesis (Pterobranchia + Harrimaniidae) was not rejected by Shimodaira-Hasegawa tests (*P* value > 0.05).

Within Harrimaniidae, we recovered a well-supported (98/1.00), but hitherto unknown, clade of small (1–3 mm in length) undescribed cold-water harrimanid worms from Antarctica, Norway, Iceland, and Oregon. This clade consists of four distinct and strongly supported subclades (Fig. 3) that likely represent at least four distinct species. Subclade 1 is composed of small worms from Norway and the Amundsen Sea in Antarctica, subclade 2 of Antarctic worms from the Ross Sea, subclade 3 of Icelandic worms, and subclade 4 of Icelandic worms and a single specimen from deep waters off Oregon. Pairwise genetic distances from 16S rDNA sequences were calculated using the K2P model as implemented by MEGA 5 (Tamura *et al.*, 2011). Nucleotide substitution values within these subclades were 0.0%–12.0%, while distances between subclades were 12.6%–26.9%. For comparison, species within the harrimaniid genus *Saccoglossus* showed distances of 0.1%–17.9%, whereas genetic distances between recognized harrimaniid genera were 18.2%–40.0% (not including the highly divergent *Stereobalanus canadensis*). This places the four subclades at the higher end of the range for species distinction, and taken together they may represent a new harrimaniid genus. The morphological work needed to confirm their taxonomic status relative to current generic diagnosis will be part of a subsequent study. Other novel harrimanids we sequenced include an unidentified species of *Saccoglossus* from Norway and a single specimen collected at 916 m off Iceland that is most closely related to the deep-sea genus *Saxipendium*. *Saccoglossus* was recovered as monophyletic (100/1.0), whereas *Harrimania* was non-monophyletic, with *H. planktophilus* sister to *Protoglossus* and *H. kupfferi* (Worsaae *et al.,* 2012) recovered within *Saxipendium*. At present, whether *Harrimania planktophilus* or *Harrimania kupfferi* were misidentified cannot be determined. Alternatively, *Harrimania* may be in need of revision. Additionally, *Protoglossus* deserves further attention, as Cedhagen and Hansson (2013) reported a new species, *Protoglossus bocki*, which they placed close to *Saxipendium*. (As their sequences are not publicly available, *P. bocki* is not included here.) Interestingly, the late Cyril Burton-Jones, an accomplished hemichordate biologist, confirmed the *Protoglossus* designation of both the *P. bocki* and the *Protoglossus* used in Cannon *et al.* (2009).

Within Ptychoderidae, *Ptychodera* and *Balanoglossus* were monophyletic genera (100/1.0, 97/1.0, respectively). *Glossobalanus* was rendered non-monophyletic because *Balanoglossus* was nested with *Glossobalanus* (95/100). Two spengelid species, *Schizocardium braziliense* and *Glandiceps hacksi,* formed a clade with strong support (100/1.0). Spengelidae was sister to Ptychoderidae + Torquaratoridae, as in Osborn *et al.,* 2011. Interestingly, even with increased representation of all known families that reproduce *via* tornaria larvae, a tornaria larva collected in the Gulf Stream (Cannon *et al.*, 2009) was still distinct from all other sequences, and fell sister to Spengelidae + Ptychoderidae + Torquaratoridae.

Within Torquaratoridae, two specimens, one from Iceland and one from Antarctica, both with distinctly torquaratorid

Table 2

Complete taxon sampling and NCBI accession numbers for sequences used in phylogenetic analyses; new sequences for this study indicated in bold

Taxa	18S rDNA	16S rDNA
HEMICHORDATA		
Cephalodiscidae		
Cephalodiscus hodgsoni H11.3	**KF683576**	—
Cephalodiscus hodgsoni	EU728441	—
Cephalodiscus fumosus H5.3	**KF683575**	—
Cephalodiscus nigrescens H12.3	**KF683574**	—
Cephalodiscus gracilis	AF236798	—
Cephalodiscus densus	EU728439	—
Cephalodiscus nigrescens	EU728440	—
Rhabdopleuridae		
Rhabdopleura sp. 1 H71.2	**KF683598**	**KF683562**
Rhabdopleura sp. 2	**KF683597**	**KF683563**
Rhabdopleura normani	**KF683596**	—
Rhabdopleura compacta	—	FN908482
Harrimaniidae		
Harrimaniidae H42.1	**KF683595**	**KF683548**
Harrimaniidae H61.1	**KF683581**	**KF683547**
Harrimaniidae H72.1	**KF683591**	**KF683534**
Harrimaniidae H72.2	**KF683580**	**KF683533**
Harrimaniidae H69.1	**KF683594**	**KF683537**
Harrimaniidae H75.1A	**KF683578**	**KF683536**
Harrimaniidae H75.1B	**KF683590**	**KF683540**
Harrimaniidae H75.1C	**KF683579**	**KF683535**
Harrimaniidae H76.1	**KF683592**	**KF683539**
Harrimaniidae H77.2	**KF683593**	**KF683538**
Harrimaniidae H83.1	**KF683582**	**KF683549**
Harrimaniidae H86.2	**KF683583**	**KF683550**
Harrimaniidae H93.1	**KF683585**	**KF683541**
Harrimaniidae H94.1	**KF683586**	**KF683543**
Harrimaniidae H98.2	**KF683584**	**KF683542**
Harrimaniidae H103.1	—	**KF683532**
Harrimania kupfferi MCZ	JF900487	JX855286
Harrimania planktophilus	AF236799	EU728421
Protoglossus sp.	EU728432	EU728420
Meioglossus psammophilus	JF900488	JX855287
Saccoglossus mereschkowskii H53.1	**KF683588**	**KF683545**
Saccoglossus sp. H44.2	**KF683577**	**KF683544**
Harrimaniidae H62.1	**KF683587**	**KF683546**
Saccoglossus bromophenolosus	AF236801	L26348
Saccoglossus kowalevskii	L28054	NC_007438
Saccoglossus pusillus	AF236800	EU728422
Saxipendium coronatum A	EU728433	EU728423
Saxipendium coronatum B	EU520505	EU520493
Saxpendium implicatum	JN886774	JN886756
Harrimaniidae H74.1	**KF683589**	**KF683551**
Stereobalanus canadensis	EU728434	EU728424
Ptychoderidae		
Balanoglossus sp. 1 H50.4	**KF683570**	**KF683557**
Balanoglossus cf. aurantiaca H51.1	**KF683569**	**KF683555**
Balanoglossus cf. aurantiaca H52.1	**KF683568**	**KF683556**
Balanoglossus clavigerus	—	EU728425
Balanoglossus carnosus MCZ	JF900489	—
Balanoglossus carnosus	D14359	AF051097
Tampa Ptychoderid	AF278685	EU728427
Glossobalanus marginatus H48.2	**KF683566**	**KF683559**
Glossobalanus berkeleyi H68.2	**KF683567**	**KF683554**
Glossobalanus berkeleyi	EU728435	EU728426
Glossobalanus minutus	AF119089	—
Ptychodera bahamensis H54.1	**KF683571**	**KF683560**
Ptychodera bahamensis 101774	JF900485	JX855285
Ptychodera bahamensis 103686	JF900486	—
Ptychodera flava	EU728436	EU728429
Ptychodera flava	AF278681	EU728428

Table 2

(Continued)

	Taxa	18S rDNA	16S rDNA
Spengelidae	**Schizocardium cf. braziliense H47.5**	**KF683572**	**KF683561**
	Glandiceps hacksi	JN886773	JN886755
Incertae sedis	Gulf Stream Tornaria	EU728437	EU728430
	Enteropneusta incertae sedis H89.3	**KF683573**	**KF683558**
Torquaratoridae	**Torquaratoridae H78.1**	**KF683565**	**KF683553**
	Torquaratoridae H90.3	**KF683564**	**KF683552**
	Tergivelum baldwinae T1076-1	EU520506	EU520494
	Tergivelum baldwinae T1094	EU520509	EU520497
	Tergivelum baldwinae T1078-1	JN866772	EU520495
	Tergivelum cinnabarinum I165-24	JN886770	JN886753
	Genus C T886-A4	EU520511	EU520499
	Genus C D80-A2	JN886768	JN886751
	Allapasus aurantiacus	JN886767	JN886750
	Allapasus isidis	JN886766	JN886749
	Genus B sp 1 T176-A1	JN886761	JN886744
	Genus B sp 1 t879-A8	JN886760	EU520500
	Genus B sp2 T1011	EU520515	EU520503
	Yoda purpurata I171-36a	JN886757	JN886740
	Yoda purpurata I171-36b	JN886758	JN886741
	IFREMER Enteropneust	EU728438	EU728431
ECHINODERMATA			
Crinoidea	*Metacrinus rotundus*	AY275898	AY275905
Ophiuroidea	*Gorgonocephalus eucnemis*	DQ060790	DQ297092
	Amphipholis squamata	X97156	NC_013876
Asteroidea	*Solaster stimpsoni*	DQ060819	DQ297113
	Odontaster validus	DQ060801	GQ294457
Holothuroidea	*Psychropotes longicauda*	Z80956	DQ777099
	Apostichopus japonicas	AB595140	NC_012616
Echinoidea	*Paracentrotus lividus*	AY428816	NC_001572

morphotypes, were most closely related to undescribed Genus C from Osborn *et al.*, 2012. A single specimen collected in the Ross Sea, Antarctica, was recovered as sister to Ptychoderidae + Torquaratoridae with poor bootstrap support (41/0.98).

Discussion

Cold-water enteropneusts

Our results greatly increase our knowledge of deep-sea, Arctic, and Antarctic enteropneusts. Within the novel harrimaniid clade, subclades 3 and 4 comprise specimens collected at depths greater than 1500 m, yet this group is phylogenetically distinct from *Saxipendium*, the only known genus of deep-sea Harrimaniidae. This finding suggests that diversity of enteropneust taxa in the deep sea is underestimated, despite the recent spate of papers on large-bodied deep-sea species (Holland *et al.*, 2005, 2009, 2012a, b; Osborn *et al.,* 2011, Priede *et al.,* 2012). These smaller worms were collected in cold waters either within or just south of the Arctic Circle, in Antarctica, or in the deep sea off the Oregon coast. Connections between deep-sea fauna and polar fauna have been hypothesized for other taxa (*e.g.,*

Strugnell *et al.*, 2008), and this clade may be yet another example of this phenomenon.

Within our molecular phylogeny, this clade is distinct from other genera, yet three harrimaniid genera, *Horstia, Mesoglossus,* and *Ritteria*, have recently been described solely on the basis of morphology (Deland *et al.,* 2010). The new clades' position in our phylogeny is consistent with the placement of *Horstia* in the phylogenetic hypothesis of harrimaniid genera by Deland *et al.* (2010). However, the present specimens do not demonstrate the extremely narrow trunk or conspicuous gonads arranged in a series of protruding nodules indicated in the generic description of *Horstia* (Deland *et al.*, 2010) (Fig. 2A, B). Therefore, we suggest that the new clade represents a novel genus, although internal morphological characterization will be needed to fully assess its relationship to other harrimaniid taxa. In terms of morphology and apparent feeding strategy, these novel harrimaniids appeared similar to larger enteropneusts rather than to the considerably smaller (<0.6-mm long) *Meioglossus psammophilus* (Worsaae *et al.*, 2012).

We also collected Arctic and Antarctic members of Torquaratoridae. Two torquaratorids were recovered sister to Genus C from Osborn *et al.* (2012)—but see Osborn *et*

Figure 3. Combined nuclear 18S rDNA and mitochondrial 16S rDNA phylogeny. Maximum likelihood tree is shown with bootstrap values (BS) and Bayesian posterior probabilities (PP) indicated at the nodes. BS/PP values of 100/100 are indicated by filled circles, and BS/PP values of 95–100/99–100 are indicated by filled squares. BS/PP values <60/<0.80 are not shown. Maximum likelihood and Bayesian branching patterns were identical.

al. (2013). These specimens were collected from 732 m in the Norwegian Sea and 531 m in the Ross Sea, far shallower than previously reported depths for Torquaratoridae (1600–4000). Another specimen from Antarctica, "Enteropneusta Antarctica H89.3R" in Figure 3, is recovered sister to Ptychoderidae + Torquaratoridae with moderate support. This is an intriguing result, as this specimen may represent a basal member of Torquaratoridae, or possibly a distinct lineage. Enteropneusts have been essentially unknown from Antarctica, demonstrating gaps in our knowledge of Southern Ocean biodiversity. Sea floor imaging from the Ross Sea reveals large numbers of torquaratorids of multiple morphotypes (Halanych *et al.*, 2013). Similarly to the harrimaniids discussed above, Antarctic continental shelf representatives of a predominantly deep-sea group strengthen hypotheses connecting deep-sea and Antarctic fauna (Gage, 2004; Strugnell *et al.*, 2008). With these discoveries, we provide genetic evidence of at least four enteropneust species in Antarctic seas.

Revised molecular hypothesis of hemichordate evolution

Holland *et al.* (2012b) and Osborn *et al.* (2011) have hypothesized a shallow-water origin for enteropneusts on the basis of the comparatively few deep-sea species in Harrimaniidae, Ptychoderidae, and Spengelidae. Our results indicate broader diversity of harrimaniids in the deep sea and a close relationship between deep-sea *Saxipendium* and shallower species (*Harrimania kupfferi* and our specimen H74.1, collected at 916 m); they also demonstrate the presence of torquaratorids at <1000 m (Halanych *et al.,* 2013; Osborn *et al.,* 2013). In view of these findings, we suggest that statements on biogeographic patterns within this group may be premature. Continued discovery of unknown species and broader taxonomic categories, such as the novel clade of cold-water harrimaniids reported here, indicate that today's view of hemichordate diversity is highly incomplete, as recently described (Appeltans *et al.,* 2012, supplement).

In the context of understanding the origins and diversity of hemichordate clades, the age and diversification times of various lineages are of interest. Fossils of rhabdopleurid and cephalodiscid pterobranchs are known from the Early and Middle Cambrian (Maletz *et al.,* 2005; Rickards and Durman, 2006; Hou *et al.,* 2011; Mitchell *et al.,* 2012). In general, however, soft-bodied enteropneusts have a poor fossil record. The recently described *Spartobranchus tenius* (Caron *et al.,* 2013) appears similar to modern torquaratorids (Halanych *et al.*, 2013), and *Mesobalanoglossus buergeri* Bechly and Frickhinger, 1999 (in Frickhinger, 1999) from the Upper Jurassic may be within Ptychoderidae, but most other body fossils are outlines only, precluding family-level identification (Maletz, 2013). Thus, although several lineages do appear to be hundreds of millions of years old, rendering time calibration of lineages on a molecular topology is not possible. Similarly, discerning clear trends in evolution for lineages associated with particular habitats (*e.g.,* deep sea, polar seas) is still difficult. Future efforts that uncover additional novel hemichordate taxa will help resolve such issues.

Understanding hemichordate interrelationships is critical for inferring hemichordate ancestral states, and thus, further questions of deuterostome evolution. In particular, whether pterobranchs evolved from within enteropneusts or the two groups are reciprocally monophyletic has major bearing on character polarization within Hemichordata. Prior studies using 18S rDNA have recovered a Pterobranchia + Harrimaniidae clade (Halanych, 1995; Cameron *et al.,* 2000; Bourlat *et al.,* 2003; Cannon *et al.,* 2009; Worsaae *et al.,* 2012). This result suggests that early hemichordates were enteropneust-like, with pterobranchs arising from acorn worm ancestors (Rychel and Swalla, 2007; Brown *et al.*, 2008; Cannon *et al.*, 2009; Peterson *et al.*, 2013). In contrast, a recent microRNA study (Peterson *et al*., 2013) found that *Saccoglossus kowalevskii* and *Ptychodera flava* share 12 microRNAs not found in *Cephalodiscus hodgsoni*. While these authors suggested that enteropneusts are monophyletic to the exclusion of pterobranchs, they examined only the three hemichordate taxa mentioned above, and thus taxon sampling is a major concern. Unfortunately, determination of early hemichordate character states cannot be conducted without further studies of individual homologous characters (Cannon *et al.,* 2009; Peterson *et al.,* 2013).

We were able to include three 16S rDNA sequences from the pterobranch genus *Rhabdopleura* in our analyses, as well as 18S rDNA data from two additional *Rhabdopleura* and three additional *Cephalodiscus* taxa. In individual analyses of 16S rDNA alone (not shown), *Rhabdopleura* sequences were recovered within the echinoderm outgroup as sister to ophiuroids. This result has previously been observed in analyses using the complete mitochondrial genome of *Rhabdopleura compacta* (Perseke *et al.*, 2011). However, mitochondrial sequences of *Rhabdopleura* are extremely AT rich (Perseke *et al*., 2011; present study) and may produce artifacts due to long-branch attraction. In combined analyses, however, Pterobranchia and Enteropneusta are reciprocally monophyletic sister taxa with good support (98/1.00). Notably, support for enteropneust monophyly is moderate (65/0.99), and SH testing did not reject the alternative hypothesis (enteropneust paraphyly). If enteropneusts and pterobranchs are indeed monophyletic, studies on pterobranch morphology, evolution, and development must advance to the forefront, so that assessments of character evolution across Hemichordata can be made. To further validate these results, additional data, particularly genome-scale information, will be required.

Acknowledgments

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