Hemichordate Phylogeny: A molecular, and genomic approach

by

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Abstract

The phylogenetic relationships within Hemichordata are significant for understanding the evolution of the deuterostomes. Hemichordates possess several important morphological structures in common with chordates, and they have been fixtures in hypotheses on chordate origins for over 100 years. However, current evidence points to a sister relationship between echinoderms and hemichordates, indicating that these chordate-like features were likely present in the last common ancestor of these groups. Therefore, Hemichordata should be highly informative for studying deuterostome character evolution. Despite their importance for understanding the evolution of chordate-like morphological and developmental features, relationships within hemichordates have been poorly studied. At present, Hemichordata is divided into two classes, the solitary, free-living enteropneust worms, and the colonial, tube-dwelling Pterobranchia. The objective of this dissertation is to elucidate the evolutionary relationships of Hemichordata using multiple datasets. Chapter 1 provides an introduction to Hemichordata and outlines the objectives for the dissertation research. Chapter 2 presents a molecular phylogeny of hemichordates based on nuclear ribosomal 18S rDNA and two mitochondrial genes. In this chapter, we suggest that deep-sea family Saxipendiidae is nested within Harrimaniidae, and Torquaratoridae is affiliated with Ptychoderidae. Next, in Chapter 3, using a gene genealogy approach, we 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. Chapter 4 presents a phylogenomics approach to relationships within Ambulacraria (Hemichordata + Enteropneusta). Our results strongly support reciprocal monophyly of Pterobranchia and Enteropneusta, and Asterozoa (Asteroidea + Ophiuroidea). Lastly, Chapter 5 provides conclusions, synthesis of the preceding chapters, and future directions.

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List of Abbreviations

BI	Bayesian inference
cDNA	complimentary DNA
DNA	deoxyribonucleic acid
EST	expressed sequenced tag
FHL	Friday Harbor Laboratories
LCA	last common ancestor
ML	maximum likelihood
MYA	million years ago
OG	orthology group
OTU	operational taxonomic unit
PCR	polymerase chain reaction
PE	paired-end
rDNA	ribosomal DNA
RNA	ribonucleic acid
rRNA	ribosomal RNA
SH test	Shaimodaira-Hasegawa test
UCEs	Ultraconserved genomic elements
UW	University of Washington

Chapter 1. Introduction to dissertation

1.1 General Introduction and Background

Hemichordates are a small phylum of marine organisms that have been integral to hypotheses of early deuterostome and chordate evolution for over 150 years. There are two major body plans within extant hemichordata; solitary, vermiform enteropneusts, or acorn worms, and colonial, tube-building pterobranchs (Figure 1). Both enteropneusts and pterobranchs display a tripartite body plan, with a proboscis (protosome), collar (mesosome), and trunk (metasome). Hemichordates share some important morphological characters with chordates, most notably pharyngeal gill slits (Bateson, 1885, 1886; Lowe et al., 2003; Ogasawara et al., 1999) and a post-anal tail in some species (Aronowicz and Lowe, 2006; Lowe et al., 2003; but see Stach and Kaul 2011 for an alternative interpretation). However, hemichordates are sister taxon to echinoderms (Figure 2, see discussion below), which suggests that these “chordate” characters are instead sympleisiomorphies for the last common ancestor (LCA) of Deuterostomia.

Understanding early deuterostome evolution has far-reaching implications in fields such as neurobiology, developmental biology, and physiology. Elucidating the early evolution of chordates, including chordate sympleisiomorphies, has been a major question since the 1800’s (Geoffroy Saint-Hilaire, 1822; Garstang, 1894, 1928). Curently, Chordata, Hemichordata, Echinodermata and Xenoturbellida comprise Deuterostomia (Table 1, see section 1.1.4 for further discussion). Comparative studies between chordates and these taxa, including phylogenetic studies, are required in order to trace early deuterostome and chordate character evolution. Echinodermata have a highly modified pentaradial symmetry that makes them relatively uninformative for reconstructing ancestral deuterostome characters. Xenoturbellida's simplified body plan contains very few informative characters for morphological comparisons. Hemichordates, on the other hand, retain apparent sympleisomorphic characters of the deuterostome LCA, and are therefore instrumental to comparative studies on deuterostome character evolution. Indeed, numerous authors have hypothesized that the deuterostome ancestor shared morphological similarities with either enteropneusts (Brown et al. 2008; Peterson and Eernisse, 2001; Rychel and Swalla, 2007) or pterobranchs (Jeffries, 1986; Romer, 1967). The following sections provide a synopsis of hemichordate biology, including history, taxonomy, and evolutionary relationships.

1.1.1 Historical Overview

In 1825, *Ptychodera flava* was the first hemichordate species to be described, although it was identified as an aberrant holothurian (Eschscholtz 1825). *Balanoglossus clavigerus* was next described in 1829, named for the local fishermen's common name of "ox tongue" for the worm (Greek, *glossa,* tongue), and an observed resemblance by the author to the barnacle *Balanus* (Greek, *balanos,* acorn) (Delle Chiaje, 1829; Hyman, 1959). Over the next 60 years, acorn worms were placed into *Balanoglossus*, until Spengel (1891), who revived *Ptychodera*, and added *Schizocardium* and *Glandiceps*. Enteropneusti (Greek, *enteron*, intestine; *pneumon*, lung) was proposed for *Balanoglossus* animals by Gegenbaur in 1870, which is today modified to Enteropneusta. Bateson, recognizing similarities between acorn worms and chordates, proposed the name Hemichordata (1885), and also proposed that hemichordates should included within

Chordata. Although this idea was rejected by Spengel (1893), and others (Dawydoff, 1948; Hyman, 1959; Van der Horst, 1939), most traditional views maintained that Hemichordata was the sister group of Chordata until the late 20th century (Holland et al., 1991; Maisey, 1986; Nielsen et al., 1996; Romer, 1967; Schaeffer, 1987; Schram, 1991; 1997).

In 1869, Metschnikoff tracked the development of tornaria larvae, which were thought to be echinoderm larvae at the time, and recognized that they were instead larval enteropneusts. He later suggested that similarities between coelomic organizations of echinoderm larvae and tornaria larvae warranted that echinoderms and hemichordates be united in a clade called Ambulacraria (Metschnikoff 1881). In 1995, Halanych recovered Hemichordata sister to Echinodermata using 18S rDNA data, and formalized Ambulacraria as the node-based name for this clade.

In 1866, G.O. Sars discovered an unusual colonial organism dredged off the coast of Northern Norway. Sars did not publish descriptions of this animal until 1873 and 1874, by which time Allman had described it as *Rhabdopleura normani* from samples taken off the Shetland Islands (Allman, 1869). Both authors considered these animals to be allied to Bryozoans (Polyzoa). Pterobranchia was erected by Lankester in 1877 as the group name for *Rhabdopleura* within Bryozoa. When the equally unusual colonial animal *Cephalodiscus dodecalophus* was described by M'Intosh in 1882, he recognized similarities to *Rhabdopleura*, and considered both genera to be bryozoans. However, in his appendix to M'Intosh's report, Harmer (1887) suggested similarities between *Cephalodiscus* and *Balanoglossus*, and proposed that *Cephalodiscus* be placed in Hemichordata. Fowler added *Rhabdopleura* to Hemichordata in 1892. The present organization of Hemichordata comprised of Enteropneusta and Pterobranchia has been in place since 1899 (Willey), although Neilson (2012) splits the group into two phyla.

1.1.2 Enteropneusta

Enteropneusts, or acorn worms, are the largest group of hemichordates, with 110 species divided into four families. Despite renewed interest in enteropneust taxonomy (see discussion below), diversity within the group is dramatically underestimated, with some authors estimating species numbers closer to 500-1,000 (Appeltans et al., 2012 Supplement). These benthic animals often form U-shaped burrows in sand or mud, although some species have a more epibenthic lifestyle (Halanych *et al*, 2013; Smith et al., 2005; Woodwick and Sensenbaugh, 1985). They are found from the intertidal zone to the deep sea, and range from less than 1 mm (*Meioglossus psammophilus* Worsaae et al., 2012) to over 2 meters in length (*Balanoglossus gigas* Müller in Spengel 1893). Enteropneusts are deposit feeders, consuming sediment particles in a mucous stream, digesting organic material, and in some species, excreting a distinctive fecal cast (Barrington 1965).

The four currently recognized enteropneust families are Ptychoderidae Spengel 1893, Torquaratoridae Holland et al. 2005, Spengelidae Willey 1898 and Harrimaniidae Spengel 1901. Ptychoderidae includes 44 species in three recognized genera, *Ptychodera* (see Figure 1C)*, Glossobalanus,* and *Balanoglossus*. Ptychoderids have been described as the “most highly evolved” enteropneusts, due to structures such as synapticles in the gill slits, pronounced regionalization of the trunk, and lateral septa (Hyman, 1959). Ptychoderids develop indirectly via tornaria larvae. Most studies on indirect development in enteropneusts are based on ptychoderids, specifically *Ptychodera flava* (e.g. Henry, 2001; Nakajima et al., 2004; Tagawa et al. 1998). More recently, *Balanoglossus misakiensis* and *Balanoglossus simodensis* have been proposed as models for hemichordate indirect development, in part due to the ease with which these species can be reared in the lab (Ikuta et al., 2009; Miyamoto et al., 2010; Miyamoto and Saito, 2007; Miyamoto and Saito, 2010; Urata and Yamaguchi, 2004).

Torquaratoridae was described in 2005 based on morphological analysis of a few specimens collected in the deep northeastern Pacific (Holland et al. 2005). This family now contains six species in four genera, *Torquarator, Tergivelum, Allapasus,* and *Yoda*, all found in the deep sea. Torquaratorids are characterized by a broad proboscis and collar, but are otherwise similar to ptychoderids, although synapticles are absent. Broad-collared acorn worms have been photographed in the deep sea since the 1960's (Bourne and Heezen, 1965; Ewing and Davis, 1967), but due to the extreme fragility of these animals, collecting intact specimens for species description has not been possible until recent advances in deep sea remote-operated vehicles (ROVs) (Osborn *et al.* 2011). Molecular phylogenetic results have indicated that Torquaratoridae is sister to Ptychoderidae (Cannon et al., 2009; Holland et al., 2009; Osborn et al., 2011; Worsaae et al. 2012). Torquaratorid life history is still poorly understood, although some species demonstrate unusual characteristics. For example, videos taken by ROV have shown *Allapasus aurantiacus* both burrowing and drifting above the benthos, indicating a possible benthopelagic life history (Holland et al., 2012).

Schizocardium, Willeyia, *Glandiceps*, and *Spengelia* comprise Spengelidae (20 species), which is defined by the presence of a digit-like projection at the anterior end of the stomochord and very long horns of the proboscis skeleton. Spengelids are described as having a combination of ptychoderid and harrimaniid features. For example, *Spengelia* and *Schizocardium* possess gill slit synapticles, whereas *Glandiceps* and *Willeyia* do not (Hyman 1959). In species where development is known, spengelids develop via tornaria larvae (e.g., *Glandiceps*, Rao, 1953). In general, this group contains many rare and poorly-studied species, thus there are few studies on spengelids, and no representative species from this group has been studied extensively. Illustrating this, a specimen of *Glandiceps abyssicola*, not seen since its discovery on the Challenger expedition in 1873, was recently rediscovered (Holland *et al.* 2013).

Much better known, Harrimaniidae (40 species) is comprised of members of the genera *Saccoglossus* (see Figure 1A)*, Harrimania, Stereobalanus, Protoglossus, Mesoglossus, Ritteria, Saxipendium, Horstia, Meioglossus,* and *Xenopleura*. Harrimaniids are defined by the absence of many features; including circular muscles in the trunk, lateral septa, gill slit synapticles, and hepatic caeca (Deland et al. 2010). In 2010, Deland et al. presented a revised taxonomy of Harrimaniidae based on historical collections, started over 100 years prior, from Ritter, Bullock and Rao. These collections, comprised of complete specimens, sections, notes, and micrographs, had been passed from Bullock to Burdon-Jones, who deposited the material at the Smithsonian Institution upon his retirement. Bullock contacted Cameron to complete the work, and after Bullock's death in 2005, several papers have now been published posthumously based on this material (Cameron *et al.*, 2010; Cameron and Ostiguy, 2013; Cameron & Perez, 2012; Deland *et al.*, 2010). In addition to genera established in these revisions (*Mesoglossus, Horstia, Ritteria*), Deland *et al*. (2010) formally synonymized the hydrothermal vent dwelling Saxipendiidae Woodwick and Sensenbaugh 1985 as a member of Harrimaniidae after molecular phylogenetic analyses demonstrated that *Saxipendium coronatum* belonged in this group (Cannon et al., 2009, see Chapter 2). Lastly, a new genus of meiofaunal harrimaniid acorn worm, *Meioglossus*, has been described from material found in Bermuda and Belize (Worsaae *et al.*, 2012).

Harrimaniids have direct development, with a non-descript larval stage bearing little resemblance to tornaria. *Saccoglossus kowalevskii* has become a well-known study organism for developmental work (Colwin and Colwin, 1950; 1953; 1963; Kaul-Strehlow and Stach, 2013; Stach and Kaul, 2011) and developmental gene expression work (Aronowicz and Lowe, 2006; Darras et al. 2011; Green et al., 2013; Lowe et al., 2006, 2003; Lowe, 2008). These studies have yielded important insights by comparing hemichordate and chordate development. Additionally, a draft genome for *Saccoglossus kowalevskii* is currently available (Freeman et al., 2008; Baylor College of Medicine Human Genome Sequencing Center).

In addition to the four recognized enteropneust families, Planctosphaeroidea is a rarely collected monotypic group known only as large, modified tornaria larvae of *Planctosphaera pelagica*. These organisms have previously been considered a separate class of hemichordate (Hyman, 1959; Van der Horst, 1936), or members of Enteropneusta (Hadfield and Young, 1983). These planktonic organisms can measure up to 25 mm, and are likely the larval form of an unknown adult enteropneust. They have been collected in the Atlantic and the Pacific, from depths of 75 to ~1000m, but only about 30 individuals have been collected since their discovery in 1910 (Hart et al., 1994).

Due to their uncertain affinities and taxonomic status, we have not included them in species counts herein.

Largely due to their fragile, soft bodies, there are few definitive enteropneust fossils. Body fossils of enteropneusts are known from the Lower Jurassic of Italy (Arduini *et al*., 1981), and trace fossils of the genus *Schaubcylindrichnus* of the Miocene and Pleistocene of Japan may have been produced by an enteropneust (Nara 2006). Recently, Caron *et al.* (2013) described *Spartobranchus tenuis*, a fossil enteropneust from the Burgess Shale. Several specimens of *S. tenuis* were observed within fibrous tubes, which are interpreted as the precursor to the pterobranch coenecium (Caron *et al.,* 2013). However, Halanych *et al.* (2013) demonstrate that modern-day torquaratorids in Antarctica produce mucous sheaths that strongly resemble *Spartobranchus tenuis* tubes, and a connection between pterobranch coenecia and these enteropneust tubes is unlikely.

1.1.3 Pterobranchia

The other group of hemichordates, Pterobranchia, is morphologically very distinct from enteropneusts. These animals are tiny (2-5 mm), tube-dwelling organisms that form colonies of zooids. They suspension feed with ciliated tentacles, and secrete a proteinaceous tube system structure called the coenecium. Their body is divided into the cephalic shield, which is used for locomotion and tube secretion, the metasome, bearing tentacular arms, and the trunk, with a U-shaped gut. The trunk is connected to other zooids via the contractible stolon, which has been hypothesized to be a homologous structure to the harrimaniid post-anal tail. Pterobranchs have direct development via a short-lived lecithotrophic larval stage (Hyman, 1959; Lester 1988). There are 24 extant species of pterobranch, divided into two genera, *Rhabdopleura* (6 species) and *Cephalodiscus* (18 species). *Rhabdopleura* colonies consist of a branching or creeping tube that attaches to the substratum, and shorter, erect tubes that branch off the creeping tube that house individual zooids. All zooids are attached to each other via a common stolon that extends throughout the length of the colony. *Rhabdopleura* has a single pair of tentacular feeding arms, and lacks pharyngeal gill slits.

Cephalodiscus zooids, on the other hand, are attached to each other in clusters of mature zooids with young buds all branching from a common attachment point, and these clusters move about the colony freely (Figure 1B). *Cephalodiscus* zooids have multiple pairs of arms, and have a single pair of pharyngeal gill openings. *Cephalodiscus* coenecium structures have several different organizations, and a system of sub-genera has been developed to reflect the distinct coenecium types (Andersson, 1907; John, 1931; Ridewood, 1907). A third genus, *Atubaria*, named due to the lack of a coenecium, has been found only once in Japan (Sato, 1936). These specimens were likely *Cephalodiscus* zooids that became dislodged from their tubes (Halanych, 1995).

Pterobranchs have a long fossil record, with mid-Cambrian specimens showing both clear fusellar structure and internal stolons (Maletz *et al.,* 2005; Rickards and Durman, 2006). Both cephalodiscids and rhabdopleurids are believed to have been present by the end of the Middle Cambrian (Swalla and Smith, 2008). Fossil graptolites are a large group of benthic and pelagic animals closely related to pterobranchs (see discussion in Mitchell et al., 2012; Sato et al., 2008). Graptolites are found from the Cambrian until the Carboniferous, having reached their peak of diversity in the Ordovician and Silurian (Clarkson, 1998; Rickards and Durman, 2006). Pelagic graptolites, also known as Eugraptoloidea Maletz et al. 2005 or graptoloids, appear to have detached from the seafloor around 490 million years ago (MYA), subsequently diversifying into approximately 2,000 species before their extinction ~418 MYA (Bapst et al., 2012; Clarkson, 1998). Graptoloids and benthic graptolites have both been used in studies of ancient speciation and extinction events (Bapst et al., 2012; Bulman, 1970; Melchin and Mitchell, 1991), and have been used extensively as stratigraphic markers, even by gold prospectors in Australia (Clarkson, 1998; Cooper and Lindholm, 1990; Svitil 1993).

Some authors place pterobranchs and graptolites within the same group, Graptolithoidea (Urbanek, 1994), while others have treated graptolites as a separate class of hemichordates, Graptolithina (Bulman, 1970). Although earlier authors denied the relationship between graptolites and pterobranchs (Decker, 1956; Decker and Gold, 1957; Hyman, 1959), the discovery of extant species *Cephalodiscus graptolitoides* Dilly 1993 with long spires of the coenecium structured similarly to graptolite spines called nema essentially ended this debate (Dilly, 1993; 1994). While the association between graptolites and pterobranchs is now widely accepted, the line between the two groups has been difficult to discern (Maletz et al., 2005). Fossils such as *Melanostrophus* (Mierzejewski and Urbanek, 2004), remarkable zooid body fossil *Galeaplumosus abilis* (Hou et al., 2011), and extant species like *Cephalodiscus graptolitoides* (Dilly, 1993; 1994) show what have been interpreted as mixtures of pterobranch and graptolite features. The most recent morphological cladistic analysis placed *Rhabdopleura* within Graptolithina, with *Cephalodiscus* sister to the extinct graptolites + *Rhabdopleura* (Mitchell et al., 2012). Based on their analysis, they postulate that *Rhabdopleura* may be useful for inferring graptolite zooidal characters (Mitchell et al., 2012). Their proposed taxonomic scheme defines Pterobranchia as the least inclusive clade including both *Rhabdopleura normani* and *Cephalodiscus dodecalophus*, with Graptolithina including *Rhabdopleura* as a subclass of Pterobranchia (Mitchell *et al*., 2012).

1.1.4 Phylogenomics and deuterostome relationships

Since the advent of molecular phylogenetics, there has been a general consensus on relationships between the three major deuterostome phyla, Chordata, Echinodermata, and Hemichordata (see discussion in Halanych, 2004; Kocot et al., 2010; Swalla and Smith 2008). Echinodermata + Hemichordata forming Ambulacraria, sister to Chordata, is now broadly accepted. However, since 2005, phylogenomic studies have been responsible for several major shifts in deuterostome phylogenetic hypotheses. In phylogenomic studies, randomly sequenced cDNA provides snapshot of the transcribed mRNA, or transcriptome, of a given sample. Many of the transcribed genes will be constitutively expressed ‘housekeeping’ genes, or other genes that are vital to cell function. Due to their integral nature, sequences of these genes are often highly conserved, and as such, are useful for inferring deep evolutionary relationships.

A series of high-profile phylogenomics studies have addressed relationships within deuterostomes. Delsuc et al. (2006) recovered tunicates and craniates as sister taxa in a non-traditional clade of chordates called Olfactores, while cephalochordates (the traditional sister group to craniates) clustered with the sole echinoderm in their analyses, the purple urchin *Strongylocentrotus purpuratus*. The urchin + cephalochordate result was soon shown to be due to poor taxon sampling (Bourlat et al. 2006), although Bourlat et al. (2006) and others (Delsuc et al., 2008; Dunn et al., 2008; Lartillot and Philippe, 2008; Philippe et al., 2009) have also recovered Olfactores. Two groups that were previously thought to be Platyhelminthes have recently been placed within deuterostomes based primarily upon phylogenomic results, Xenoturbellida (Bourlat et al., 2006; Bourlat et al, 2008; Dunn et al., 2008; Lartillot and Philippe, 2008) and Acoelomorpha (Philippe et al., 2011), although this is an area of active debate (see Chapter 5, future directions).

Phylogenomics has strongly upheld Ambulacraria (Bourlat et al., 2006; Dunn et al., 2008; Lartillot and Philippe, 2008; Philippe et al. 2009), and there is additional support for this clade from morphology (Cameron, 2005; Ruppert, 2005), microRNAs (Wheeler et al., 2009), and a combined analysis of ribosomal, mitochondrial and nuclear protein-coding genes (Bourlat et al., 2008). The Ambulacraria hypothesis suggests that shared morphological features between hemichordates and chordates, such as pharyngeal gill slits and the post-anal tail, were likely to have been present in the deuterostome ancestor. However, in order to trace evolutionary history of character traits throughout deuterostomes, an understanding of the relationships within the two Ambulacrarian groups is required. Relationships within these two taxa have not been addressed using a phylogenomics approach (see Chapter 4), and relationships within hemichordates in particular have received little attention (see discussion below).

1.1.5 Hemichordate interrelationships

Although there is currently general consensus about the position of hemichordates within deuterostomes, relationships within this group are poorly resolved. Historically, there were few explicit phylogenetic hypotheses, although traditional wisdom assumed pterobranchs and enteropneusts were reciprocally monophyletic taxa based on their disparate morphology and life history (Dawydoff, 1948; Hyman, 1959). Within enteropneusts, phylogenetic hypotheses have been lacking, although Hyman (1959) noted that harrimaniids were the least complex of the enteropneusts, followed by spengelids and ptychoderids. Aside from this type of subjective assessment, the earliest objective studies addressing hemichordate relationships included Halanych (1995), with three hemichordate taxa in his 18S rDNA, and Winchell et al. (2002), who included six hemichordates in a broader study on deuterostome relationships using 18S and 28S rDNA data. Cameron (2005) conducted a morphological cladisitic analysis including all known hemichordate genera at the time, although several of the morphological characters used were too general, or specific to non-hemichordate taxa included in the analyses (e.g., pentaradial symmetry, prototroch), and resolution within enteropneusta was poor.

Cannon et al. (2009) specifically addressed hemichordate relationships (see Chapter 2), and several subsequent studies have built upon the Cannon et al. (2009) dataset (Cedhagen and Hansson, 2013; Osborn et al. 2011; Worsaee et al. 2012; Holland et al. 2013). Generally, these studies have recovered monophyletic Harrimaniidae, Ptychoderidae, Torquaratoridae, and have suggested that Ptychoderidae is sister to Torquaratoridae, while Spengelidae is sister to Torquaratoridae + Ptychoderidae. Spengelids are only represented by a single taxon in the above studies.

Beginning with Halanych (1995), studies using 18S rDNA sequence data have recovered Enteropneusta as paraphyletic, with pterobranchs originating within the acorn worm lineage sister to Harrimaniidae (Bourlat et al., 2003; Cameron et al., 2000; Cannon et al., 2009; Worsaae et al., 2012). In their 28S rDNA analyses, however, Winchell et al.

(2002) found pterobranchs as sister to a monophyletic Enteropneusta, a result that has also been recovered by Cameron’s (2005) morphological cladistic analysis and recent microRNA analyses (Peterson et al., 2013). However, the first two studies have been criticized for a small number of informative characters (Cannon *et al.,* 2009), and the microRNA study included only three hemichordate taxa.

The question of whether pterobranchs are basal hemichordates or are derived from within Enteropneusta has important consequences for reconstructing the last common ancestor (LCA) of the hemichordates. If enteropneusts are paraphyletic, polarization of characters for the hemichordate LCA becomes fairly straightforward, and the hemichordate ancestor can be inferred to have been a solitary vermiform animal with some enteropneust characters. If however, enteropneusts and pterobranchs are reciprocally monophyletic, this question becomes more difficult to resolve, necessitating more complex studies assessing homology of key structures, because the characters cannot be polarized by the tree topology. Due to hemichordates’ unique phylogenetic position and shared characters with chordates, early deuterostome animals likely shared certain morphological characters with hemichordates (Brown et al., 2008). Therefore, understanding phylogenetic relationships within Hemichordata will provide context for further studies of early deuterostome evolution.

1.2 Objectives

As discussed above, there are two current hypotheses regarding deep hemichordate phylogeny: 1) pterobranchs are sister to a monophyletic Enteropnuesta; and 2) enteropneusts are paraphyletic, with pterobranchs originating from within

Enteropneusta. Resolving whether pterobranchs are derived from within or sister to enteropneusts has broad implications for our understanding of early deuterostome and chordate evolution. The following chapters address these two hypotheses using three different datasets, and test additional hypotheses surrounding hemichordate and Ambulacrarian relationships. The broad aims of this dissertation are: 1) to provide a robust evolutionary framework for Hemichordata; and 2) to generate testable phylogenetic hypotheses within Enteropneusta and Pterobranchia. In addition to the these two broad aims, specific objectives of the research chapters are as follows:

1. To test the validity of deep-sea families Saxipendiidae and Torquaratoridae using a gene genealogy approach.
2. To place undescribed cold-water enteropneusts in acorn worm phylogeny, and test their relationship to known deep-sea taxa using a gene genealogy approach.
3. To place pterobranchs within Hemichordata and to investigate Ambulacrarian phylogeny using a phylogenomics approach.

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Table 1. Current classification of Deuterostomia.

Taxon	Class
Chordata	
	Cephalochordata
	Tunicata
	Craniata
Xenoturbellida*	
	Xenoturbella
Ambulacraria	
Hemichordata	
	Pterobranchia
	Enteropneusta
Echinodermata	
Crinozoa	
	Crinoidea
Eleutherozoa	
	Asteroidea
	Ophiuroidae
Echinozoa	
	Echinoidea
	Holothuroidea

* monogeneric taxon

Table 2. Hemichordate species and authority information. Family and genus authorities are given in the text.

Family	**Species**	**Authority**
Harrimaniidae	*Harrimania borealis*	Okuda & Yamada, 1955
	Harrimania kupfferi	von Willemoes-Suhm, 1871
	Harrimania maculosa	Ritter, 1900
	Harrimania planktophilus	Cameron 2002
	Horstia kincaidi	Deland, Cameron, Rao, Ritter & Bullock, 2010
	Meioglossus psammophilus	Worsaae, Sterrer, Kaul-Strehlow, Hay-Schmidt & Giribet, 2012
	Mesoglossus bournei	(Menon, 1904)
	Mesoglossus caraibicus	(van der Horst, 1924)
	Mesoglossus gurneyi	(Robinson, 1927)
	Mesoglossus intermedius	Deland, Cameron, Rao, Ritter & Bullock, 2010
	Mesoglossus macginitiei	Deland, Cameron, Rao, Ritter & Bullock, 2010
	Mesoglossus pygmaeus	(Hinrichs & Jacobi, 1938)
	Protoglossus bocki	Cadhagen & Hansson, 2012
	Protoglossus koehleri	Caullery & Mesnil, 1900
	Protoglossus mackiei	Deland, Cameron, Rao, Ritter & Bullock, 2010
	Protoglossus graveolens	Giray & King, 1996
	Ritteria ambigua	Deland, Cameron, Rao, Ritter & Bullock, 2010
	Saccoglossus apantesis	Thomas, 1955
	Saccoglossus aulakoeis	Thomas, 1968
	Saccoglossus bromophenolosus	King, Giray & Kornfield, 1994
	Saccoglossus horsti	Brambell, Rogers & Goodhart, 1941
	Saccoglossus hwangtauensis	Tchang & Koo, 1935
	Saccoglossus inhacensis	Kapelus, 1936

	Saccoglossus kowalevskii	Agassiz, 1873
	Saccoglossus madrasensis	Rao, 1957
	Saccoglossus mereschkowskii	Wagner, 1885
	Saccoglossus otagoensis	Benham, 1899
	Saccoglossus palmeri	Cameron, Deland & Bullock, 2010
	Saccoglossus porochordus	Cameron, Deland & Bullock, 2010
	Saccoglossus pusillus	Ritter, 1902
	Saccoglossus rhabdorhyncus	Cameron, Deland & Bullock, 2010
	Saccoglossus ruber	Tattersall, 1905
	Saccoglossus shumaginensi	Cameron, Deland & Bullock, 2010
	Saccoglossus sonorensis	Cameron, Deland & Bullock, 2010
	Saccoglossus sulcatus	Spengel, 1893
	Saxipendium coronatum	Woodwick & Sensenbaugh, 1985
	Saxipendium implicatum	Holland, Osborn, & Kuhnz, 2012
	Stereobalanus canadensis	Spengel, 1893
	Stereobalanus willeyi	Ritter, 1904 / Deland, Cameron, Rao, Ritter & Bullock, 2010
	Xenopleura vivipara	Gilchrist, 1925
Spengelidae	*Spengelia alba*	Willey, 1899
	Spengelia amboinensis	Spengel, 1907
	Spengelia discors	Spengel, 1907
	Spengelia maldivensis	Punnett, 1903
	Spengelia porosa	Willey, 1898
	Spengelia sibogae	Spengel, 1907
	Glandiceps abyssicola	Spengel, 1893
	Glandiceps bengalensis	Rao, 1955
	Glandiceps coromandelicus	Spengel, 1907
	Glandiceps eximius	Spengel, 1907

	Glandiceps hacksi	Marion, 1885
	Glandiceps malayanus	Spengel, 1907
	Glandiceps qingdaoensis	Jianmei, 2005
	Glandiceps talaboti	Marion, 1876
	Schizocardium californicum	Cameron & Perez, 2012
	Schizocardium peruvianum	Spengel, 1893
	Schizocardium braziliense	Spengel, 1893
	Willeyia bisulcata	Punnett, 1906
	Willeyia delagoensis	Horst, 1940
	Willeyia loya	Petersen, 1965
Ptychoderidae	*Balanoglossus apertus*	Spengel, 1893
	Balanoglossus aurantiaca	Girard, 1853
	Balanoglossus australiensis	Hill, 1894
	Balanoglossus borealis	Willey, 1899
	Balanoglossus capensis	Gilchrist, 1908
	Balanoglossus carnosus	Müller in Spengel, 1893
	Balanoglossus catharensis	Sawaya & Forneris, 1953
	Balanoglossus clavigerus	Delle Chiaje, 1829
	Balanoglossus eufrosinoi	Sawaya & Forneris
	Balanoglossus gigas	Müller in Spengel, 1893
	Balanoglossus hydrocephalus	van der Horst, 1940
	Balanoglossus jamaicensis	Willey, 1899
	Balanoglossus misakiensis	Kuwano, 1902
	Balanoglossus natalensis	Gilchrist, 1908
	Balanoglossus nonatoi	Sawaya & Forneris, 1953
	Balanoglossus occidentalis	Ritter, 1902
	Balanoglossus parvulus	Punnett, 1903
	Balanoglossus robinii	Giard, 1882

	Balanoglossus proterogonius	Belichov, 1928
	Balanoglossus salmoneus	Giard, 1882
	Balanoglossus stephensoni	van der Horst, 1937
	Balanoglossus simodensis	Miyamoto & Saito, 2007
	Balanoglossus studiosorum	Horst, 1940
	Glossobalanus alatus	Horst, 1940
	Glossobalanus barnharti	Cameron & Ostiguy, 2013
	Glossobalanus berkeleyi	Willey, 1931
	Glossobalanus crozieri	van der Horst, 1924
	Glossobalanus elongatus	Spengel, 1904
	Glossobalanus hartmani	Cameron & Ostiguy, 2013
	Glossobalanus hedleyi	Hill, 1897
	Glossobalanus marginatus	Meek, 1922
	Glossobalanus minutus	Kowalevsky, 1866
	Glossobalanus mortenseni	van der Horst, 1932
	Glossobalanus parvulus	Punnett, 1906
	Glossobalanus polybranchioporus	Tchang & Liang, 1965
	Glossobalanus tuscarorae	Belichov, 1971
	Glossobalanus ruficollis	Willey, 1899
	Glossobalanus sarniensis	Koehler, 1886
	Glossobalanus williami	Cameron & Ostiguy, 2013
	Ptychodera bahamensis	Spengel, 1893
	Ptychodera asymmetrica	Punnett, 1903
	Ptychodera flava	Eschscholtz, 1825
	Ptychodera pelsarti	Dakin, 1916
	Ptychodera viridis	Punnett, 1903
Torquaratoridae	*Allapasus aurantiacus*	Holland, Kuhnz, Osborn, 2012

	Allapasus isidis	Priede, Osborn, Gebruk, Jones, Shale, Rogacheva, Holland, 2012
	Torquarator bullocki	Holland, Clague, Gordon, Gebruk, Pawson & Vecchione, 2005
	Tergivelum baldwinae	Holland, Jones, Ellena, Ruhl & Smith, 2009
	Tergivelum cinnabarinum	Priede, Osborn, Gebruk, Jones, Shale, Rogacheva, Holland, 2012
	Yoda purpurata	Priede, Osborn, Gebruk, Jones, Shale, Rogacheva, Holland, 2012
Cephalodiscidae	*Cephalodiscus agglutinns*	Harmer & Ridewood, 1913
	Cephalodiscus atlanticus	Bayer, 1962
	Cephalodiscus australiensis	Johnston & Muirhead, 1951
	Cephalodiscus calciformis	Emig, 1977
	Cephalodiscus densus	Andersson, 1907
	Cephalodiscus dodecocephalus	M'Intosh 1887
	Cephalodiscus evansi	Ridewood, 1918
	Cephalodiscus fumosus	John, 1931
	Cephalodiscus gilchristi	Ridewood, 1906
	Cephalodiscus gracilis	Harmer, 1905
	Cephalodiscus graptolitoides	Dilly, 1993
	Cephalodiscus hodgsoni	Ridewood, 1906
	Cephalodiscus indicus	Schepotieff, 1909
	Cephalodiscus kempi	John, 1932
	Cephalodiscus levinseni	Harmer, 1905
	Cephalodiscus nigrescens	Lankester, 1905
	Cephalodiscus sibogae	Harmer, 1905
	Cephalodiscus solidus	Andersson, 1907
Rhabdopleuridae	*Rhabdopleura annulata*	Norman, 1910

	Rhabdopleura compacta	Hincks, 1880
	Rhabdopleura grimaldi	Jullien, 1890
	Rhabdopleura manubialis	Jullien & Calvet, 1903
	Rhabdopleura normani	Allman, 1869
	Rhabdopleura striata	Schepotieff, 1909
Incertae sedis	*Atubaria heterolopha*	Sato, 1935
	Planctosphaera pelagica	Spengel, 1932

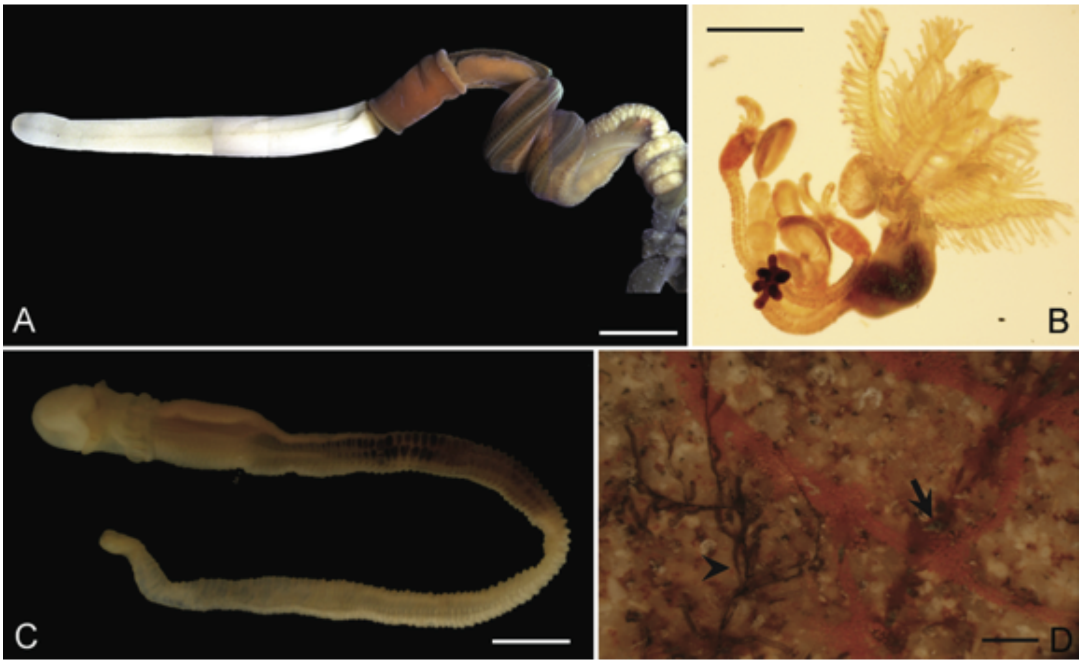

Figure 1. Representatives of enteropneust and pterobranch hemichordates.

A. *Saccoglossus bromophenolosus*, Padilla Bay, Washington. Scale bar = 5mm. (Photo by Billie J. Swalla). **B.** *Cephalodiscus gracilis*, Causeway, Bermuda. Scale bar = 0.5mm. **C.** *Ptychodera flava*, Snug Harbor, Bermuda. Scale bar = 5mm. **D.** Coenecia of sympatric pterobranch colonies on a rock from Bermuda. Left (arrowhead), *Rhabdopleura normani*; Right (arrow), *Cephalodiscus gracilis.* Arrow points to body of adult *Cephalodiscus* zooid within transparent coenecium. Scale bar = 2mm.

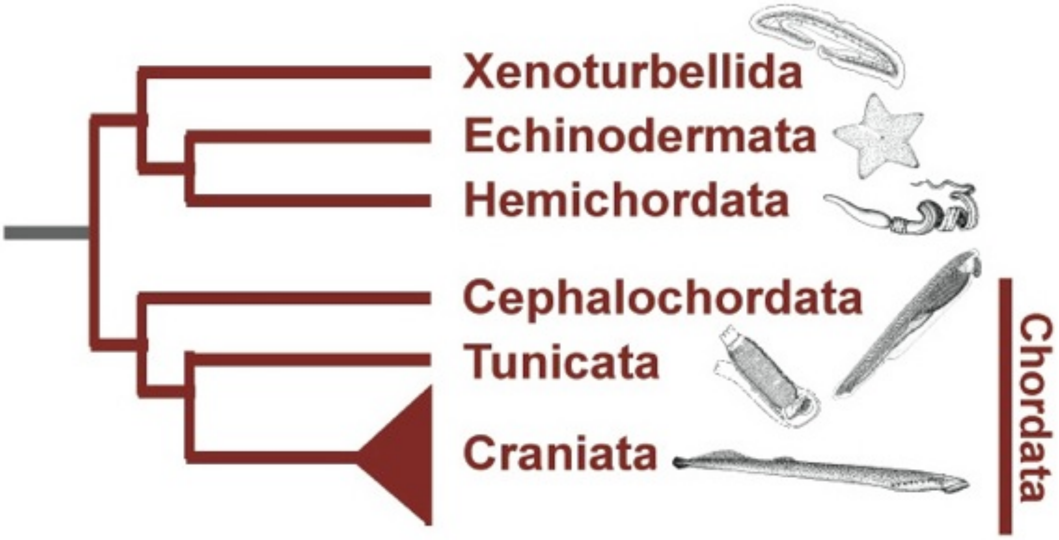

Figure 2. Current understanding of deuterostome relationships. Modified from Kocot, Cannon, and Halanych (2010).

Chapter 2. Molecular Phylogeny of Hemichordata, with Updated Status of Deep-Sea Enteropneusts

2.1 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.

2.2 Introduction

Hemichordata are deuterostome animals with a tripartite body plan, and separate coeloms for each body region: proboscis (prosome), collar (mesosome) and trunk (metasome) (Figure 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; Halanych, 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 (Figure 1), and the colonial, tube-dwelling Pterobranchia (Figure 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 (Figure 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 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.

2.3 Materials and Methods

2.3.1 Taxonomic sampling

Table 1 lists sample locality information and GenBank Accession numbers for taxa analyzed in this study. Shallow water enteropneusts 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 5m 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 *Saxipedium 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 -80ºC or preserved in ~85% ethanol upon collection.

2.3.2 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 $MgCl_2$, 0.2mM each dNTP, 0.5 μM each primer, and 50-200 ng total template DNA. Alternatively, PCRs contained 0.15μl Taq DNA Polymerase (Eppendorf, Westbury, NY), 2.5μl 10x Eppendorf PCR Buffer, 2.5μl of 25mM $MgCl_2$, 2.5μl of 2 mM dNTPs, 1-2μl of 10μM primer, 1 μl DNA template, and water to a final volume of 25μl. 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, 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 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 trees 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 Γ=0.6144; 16S Γ=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 (Γ=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 (Γ=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-

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.

2.4 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 of these were parsimony informative. For mitochondrial genes, there were 468 included characters from 16S rDNA, including 190 parsimony-informative characters, and 404 included characters for cytB, 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 cytB data yielded topologies with identical relationships (Figure 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 (Figure 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 test and Bayes Factor results for alternative phylogenetic hypotheses tested. We tested alternative hypotheses using 18S rDNA sequence data 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 (Figure 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 tests were less sensitive than Bayes Factor comparisons in rejecting alternative hypotheses.

2.5 Discussion

2.5.1 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 (Figure 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 larvae 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 (Figure 5). 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, SH 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.

2.5.2 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.

2.6 Acknowledgements

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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 aculeate*		DQ060806	NC_005334	NC_005334
Echinoid	*Strongylocentrotus purpuratus*		L28056	NC_001453	NC_001453
Holothuroid	*Cucumaria miniata*		DQ777083	NC_005929	NC_005929
Crinoid	*Gymnocrinus richeri*		AY275895	NC_007689	NC_007689
Xenoturbellid	*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 ruber		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

Table 2. Primer sequences and thermocycler parameters

Primer Pair	Primer Sequence (5’ → 3’)	PCR Parameters	Reference
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	
cyt b R	GGRAANARRAARTAYCAYTC		
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
18M	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

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).

* $P < 0.05$

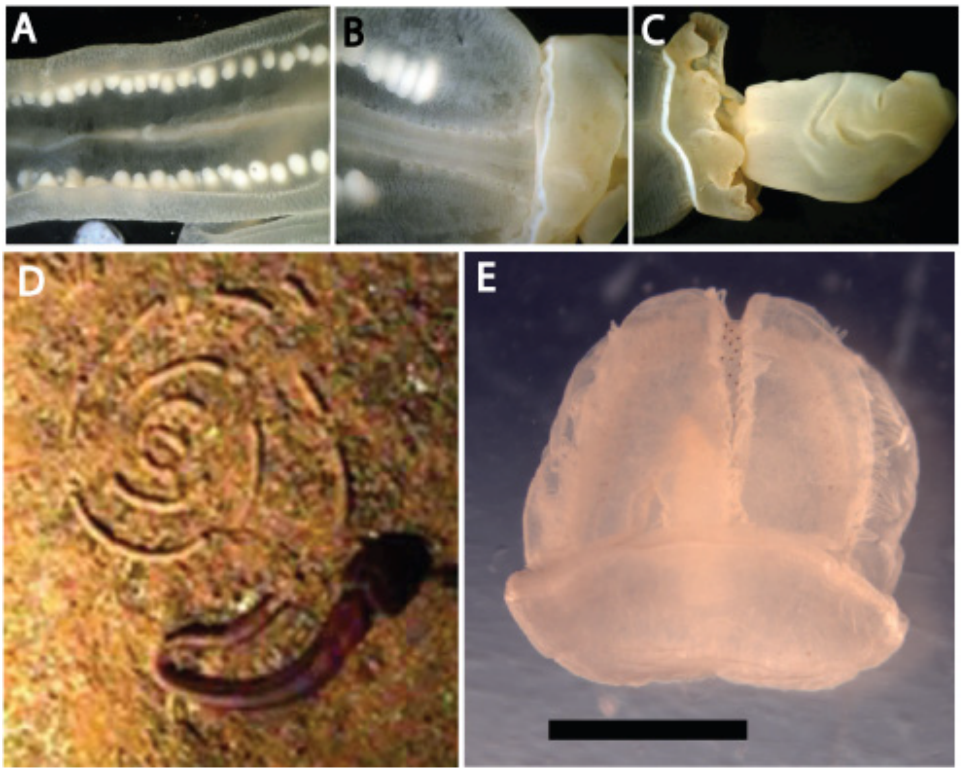

Figure 1. Deep-sea Hemichordates and Gulf Stream Tornaria larva. A. Deep-sea “spaghetti worm” hemichordate *Saxipendium coronatum* trunk with gonads. B. Close-up of *S. coronatum* trunk showing gill pore openings. C. Proboscis and short collar of *S. coronatum*. Photos A., B., C. courtesy of Greg Rouse. 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 = 2mm (applies only to photo E).

Figure 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 0.5mm. B. *Cephalodiscus hodgsoni* colony, showing coenecium structure and several zooids.

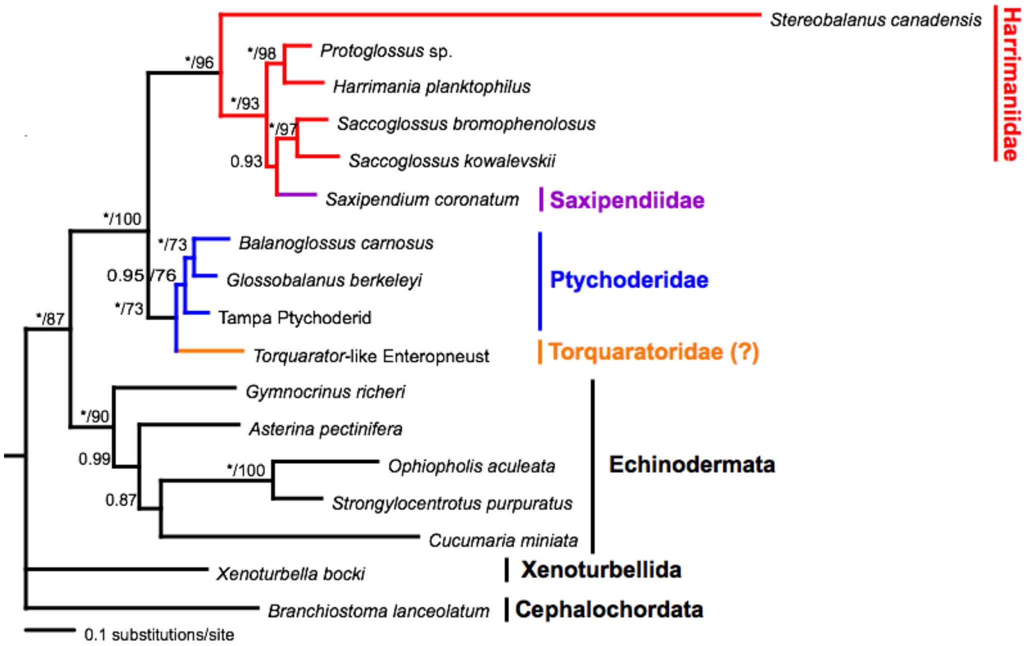

Figure 3. Combined 18S and mitochondrial phylogeny. Bayesian tree is shown with posterior probabilities greater than 0.60 and maximum likelihood bootstrap values above 60 indicated above the nodes. Bayesian posterior probabilities of 1.00 are indicated by asterisks. Methodological details given in the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

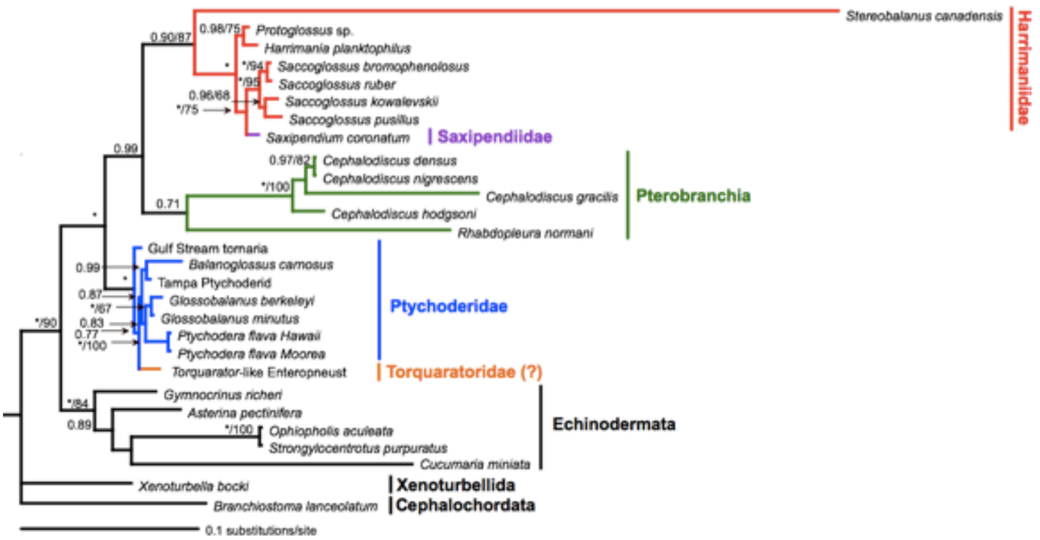

Figure 4. 18S rDNA topology. Bayesian tree is shown with posterior probabilities greater than 0.60 and maximum likelihood bootstrap values above 60 indicated above the nodes. Bayesian posterior probabilities of 1.00 are indicated by asterisks. Methodological details are given in the text.

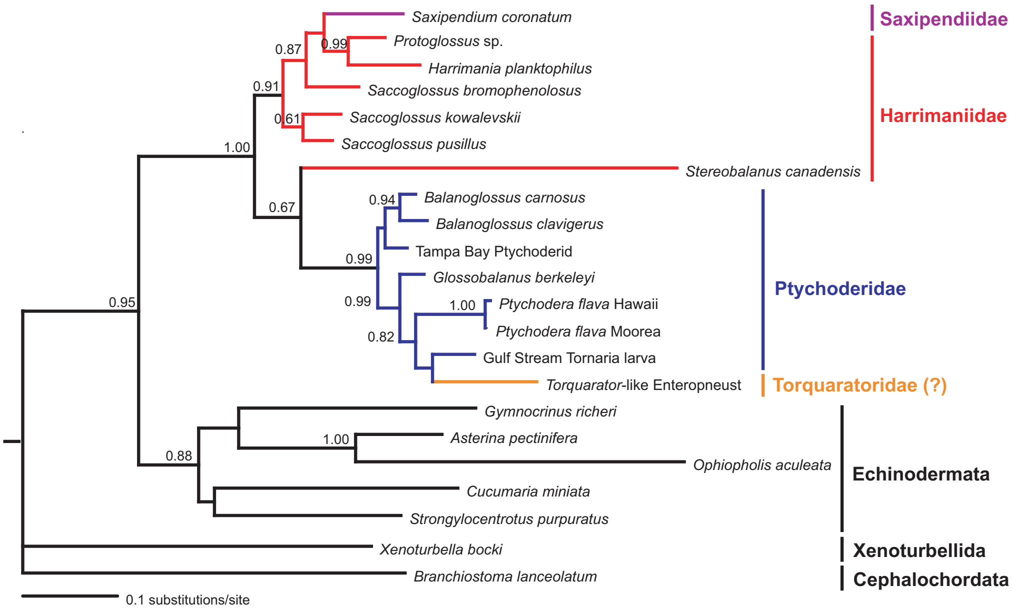

Figure 5. 16S rDNA topology. Bayesian tree is shown with posterior probabilities greater than 0.60 indicated above the nodes. Methodological details are given in the text.

Chapter 3. Hemichordate molecular phylogeny reveals a novel cold-water clade of harrimaniid acorn worms

3.1 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 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 meters, 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. The discovery of this clade, as well as larger torquaratorids from Antarctica, strengthen hypotheses of close associations between Antarctic and deep sea fauna.

3.2 Introduction

Hemichordates, a small marine phylum integral to hypotheses on deuterostome and chordate evolution, occur throughout the world's oceans. At present, 125 hemichordate species are described; 101 are solitary Enteropneusta, or acorn worms, and 24 are colonial, tube-dwelling Pterobranchia (Appeltans *et al.*, 2012). 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, 2013; Cameron & Perez, 2012). Another novel genus of enteropneust, *Meioglossus*, has been described based on 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 hacski*, which is recovered 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 graptolites 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 only included 3 hemichordate lineages (Peterson *et al.,* 2013).

In 1959, Libbie Hyman observed, "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 are the obvious exception, which were unknown at the time. Three enteropneusts are known from Arctic seas, *Harrimania kupferri*, *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 including 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.

3.3 Materials and Methods

3.3.1 Organismal collection

Figure 1 shows a world map with collection localities for samples collected for 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 lead 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 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 meters, 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. Miniscule acorn worms may be present in samples collected elsewhere using similar methods, but may have been overlooked due to their minimal and non-descript external characters. During collection, many enteropneusts were passed through a 1 mm mesh, and therefore could be classified as meiofaunal (Higgins & 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 (Figure 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.

3.3.2 Molecular methods

Molecular laboratory methods followed Cannon *et al.* (2009). In addition to in-house sequencing on a CEQ8000 (Beckman-Coulter, Inc., Brea, California), some PCR products were sent to Genewiz (South Plainfield, NJ). In the case of *Rhabdopleura* c.f. *normani* from Bermuda, we were able to generate a full-length 18S rDNA sequence using a publically available partial sequences (Genbank Accession #s: U15664, JF900483 and JF900484) and 454 transcriptome data (Cannon and Halanych, unpublished) prepared following Kocot *et al.*'s (2011) methods. Assembled 454 contigs were formatted into a BLAST (Altschul *et al.*, 1997) database, which was searched with a query comprised 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 (Aarhus, Denmark). As reported previously, *Stereobalanus canadensis* (Enteropneusta, Harrimaniidae) has a highly divergent 18S rDNA sequence (Cannon *et al.* 2009). Previously we had sequenced this acorn worm multiple times, but in order to further verify this sequence, genomic DNA extracted from *S. canadensis* and sequenced via Illumina MiSeq (San Diego, California) at Auburn University. 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 version 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 & 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 5,000 bootstrap replicates. Bayesian analyses were conducted using MrBayes version 3.2.0 (Ronquist & 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 with the GTR+I+G model.

3.3.3 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 has been deposited to TreeBase (http://www.treebase.org). Maximum likelihood and Bayesian inference analyses yielded topologies with an identical branching pattern (Figure 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 are 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 recover 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 (Figure 3) that likely represent at least 4 distinct species. Subclade 1 is comprised of small worms from Norway and the Amundsen Sea in Antarctica, subclade 2 is comprised of Antarctic worms from the Ross Sea, subclade 3 is comprised of Icelandic worms, and subclade 4 is comprised 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 4 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 meters off Iceland that is most closely related to deep-sea genus *Saxipendium*. *Saccoglossus* is recovered as monophyletic (100/1.0), whereas *Harrimania* is non-monophyletic, with *H. planktophilus* is sister to *Protoglossus* and *H. kupferri* (Worsaae *et al.* 2012) recovered within *Saxipendium*. At present, whether *Harrimania planktophilus* or *Harrimania kupferri* may have been misidentified cannot be determined. Alternatively, *Harrimania* may be in need of revision.

Within Ptychoderidae, *Ptychodera* and *Balanoglossus* are monophyletic genera (100/1.0, 97/1.0, respectively). *Glossobalanus* is rendered non-monophyletic because *Balanoglossus* is nested with *Glossobalanus* (95/100). Two spengelid species, *Schizocardium braziliense* and *Glandiceps hacksi* form a clade with strong support (100/1.0). Spengelidae is 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) is still distinct from all other sequences, and falls sister to Spengelidae + Ptychoderidae + Torquaratoridae.

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

3.4 Discussion

3.4.1 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 suggests 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, Holland *et al.* 2009, Holland *et al.* 2012[a], Holland *et al.* 2012[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 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 based solely on morphology (Deland *et al.* 2010). The new clades' position in our phylogeny is consistent with the placement of *Horstia* in Deland *et al.*'s (2010) phylogenetic hypothesis of harrimaniid genera. 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) (Figure 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 the considerably smaller (<0.6 mm long) *Meioglossus psammophilus* (Worsaae *et al*. 2012).

We also collected Arctic and Antarctic members of Torquaratoridae. Two toquaratorids were recovered sister to Genus C from Osborne *et al*. 2012, which is as-yet undescribed. 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 reveal large numbers of torquaratorids of multiple morphotypes (Halanych *et al*., in revision). 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.

3.4.2 Revised molecular hypothesis of hemichordate evolution

Holland *et al.* 2012 and Osborn *et al.* 2011 have hypothesized a shallow water origin for enteropneusts, based on the comparatively few deep-sea species in Harrimaniidae, Ptychoderidae, and Spengelidae. Our results indicate broader diversity of harrimaniids in the deep sea, a close relationship between deep-sea *Saxipendium* and shallower species (*Harrimania kupferri* and our specimen H74.1, collected at 916 m), and demonstrate the presence of torquaratorids at <1000m. Based on these factors, 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).

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 if 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 & Swalla, 2007; Brown *et al.*, 2008; Cannon *et al.*, 2009; Peterson *et al.*, 2013). In contrast, recent a microRNA study (Peterson *et al*. 2013) found that *Saccoglossus kowalevskii* and *Ptychodera flava* share 12 microRNAs not found in *Cephalodiscus hodgsoni*. While this manuscript suggested that enteropneusts are monophyletic to the exclusion of pterobranchs, they only examined 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 are recovered within the echinoderm outgroup as sister to ophiuroids. This result has been previously 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. In order to further validate these results, additional data, particularly genome-scale information, will be required.

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Table 1.

Collection and locality information by taxon.

Family	Taxon	Locality	Latitude	Longitude	Depth	Collection method
Cephalodiscidae	*Cephalodiscus hodgsoni* H11.3	Elephant Island, Antarctica	62° 44.748' S	56° 44.881' W	207m	Dredge
	Cephalodiscus fumosus H5.3	South Shetlands, Antarctica	62º 45.034' S	56º 46.26' W	220m	Dredge
	Cephalodiscus nigrescens H12.3	Elephant Island, Antarctica	62° 44.748' S	56° 44.881' W	207m	Dredge
Rhabdopleuridae	*Rhabdopleura* sp. 1 H71.2	Iceland	63° 56.07' N	25° 56.53' W	209m	Agassiz Trawl
	Rhabdopleura sp. 2	Gulf of Mexico	Not available	Not available	N/A	Not available
Harrimaniidae	Harrimaniidae H42.1	Norway	60° 18.37' N	5° 12.077' E	130m	Epibenthic Sled
	Harrimaniidae H61.1	Norway	60° 16.584' N	05° 11.092' E	183m	Epibenthic Sled
	Harrimaniidae H72.1	Iceland	69° 6.51' N	9° 55.09' W	2177m	Epibenthic Sled
	Harrimaniidae H72.2	Iceland	69° 6.51' N	9° 55.09' W	2177m	Epibenthic Sled
	Harrimaniidae H72.3	Iceland	69° 6.51' N	9° 55.09' W	2177m	Epibenthic Sled
	Harrimaniidae H69.1	Iceland	60° 20.87' N	18° 8.52' W	2569m	Epibenthic Sled
	Harrimaniidae H75.1	Iceland	69° 6.51' N	9° 55.09' W	2177m	Epibenthic Sled
	Harrimaniidae H76.1	Iceland	67° 5.79' N	13° 0.42' W	1612m	Agassiz Trawl
	Harrimaniidae H77.1	Iceland	67° 37.39' N	12° 4.06' W	1781m	Agassiz Trawl
	Harrimaniidae H86.2	Wright's Gulf, Antarctica	73° 15.3017' S	129° 12.9483' W	481m	SmithMac Grab
	Harrimaniidae H83.1	Amundsen Sea, Antarctica	72° 10.6480' S	103° 30.8478' W	341m	Blake Trawl
	Harrimaniidae H93.1	Ross Shelf, Antarctica	75° 19.8083' S	176° 59.2676' W	567m	MegaCore
	Harrimaniidae H94.1	Ross Shelf, Antarctica	75° 19.8083' S	176° 59.2676' W	567m	MegaCore
	Harrimaniidae H98.2	Ross Shelf, Antarctica	76° 20.4730' S	170° 51.0297' W	531m	MegaCore

	Harrimaniidae H103.1	Oregon, USA	43° 50.604' N	127° 34.037' W	2950m	MultiCore
	Saccoglossus mereschkowskii H53.1	White Sea, Russia	66° 33.192' N	33° 6.35' E	<20m	Dredge
	Saccoglossus sp. H44.2	Friday Harbor, Washington, USA	48° 32.6167′ N	123° 0.6833′ W	10-15m	Dredge
	Harrimaniidae H62.1	Norway	60° 27.858' N	05° 05.967' E	130m	Epibenthic Sled
	Harrimaniidae H74.1	Iceland	62° 56.46' N	20° 44.06' W	916m	Epibenthic Sled
Ptychoderidae	*Balanoglossus* sp. 1 H50.4	Bocas del Toro, Panama	9°24.3' N	82°19.45' W	<1m	Shovel
	Balanoglossus c.f. *aurantiaca* H51.1	Ship Island, Mississippi, USA	30°14.8' N	88°32.4' W	1m	Yabby Pump
	Balanoglossus c.f. *aurantiaca* H52.1	Horn Island, Mississippi, USA	30°14.75' N	88°46.5167' W	1m	Yabby Pump
	Glossobalanus marginatus H48.2	Norway	58° 55.002' N	10° 33.165' W	184m	Agassiz Trawl
	Glossobalanus berkeleyi H68.2	False Bay, Washington, USA	48°28.9333' N	123°4.2333' W	<1m	Shovel
	Ptychodera bahamensis H54.2	Shelley Bay, Bermuda	32°19.8833' N	64°44.3667' W	1m	Snorkel
Spengelidae	*Schizocardium* c.f. *braziliense* H47.5	Bay St. Louis, Mississippi, USA	30° 14.09' N	89° 20.09' W	12.5 m	Box Core
Torquaratoridae	Torquaratoridae H78.1	Iceland	66° 18.06' N	12° 22.40' W	732m	Box Core
	Torquaratoridae H89.3	Ross Shelf, Antarctica	76° 20.4730' S	170° 51.0297' W	531m	Blake Trawl
	Torquaratoridae H90.3	Ross Shelf, Antarctica	76° 20.4730' S	170° 51.0297' W	531m	Blake Trawl

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* c.f. *aurantiaca* H51.1**	**KF683569**	**KF683555**
	***Balanoglossus* c.f. *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
Spengelidae	***Schizocardium* c.f. *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

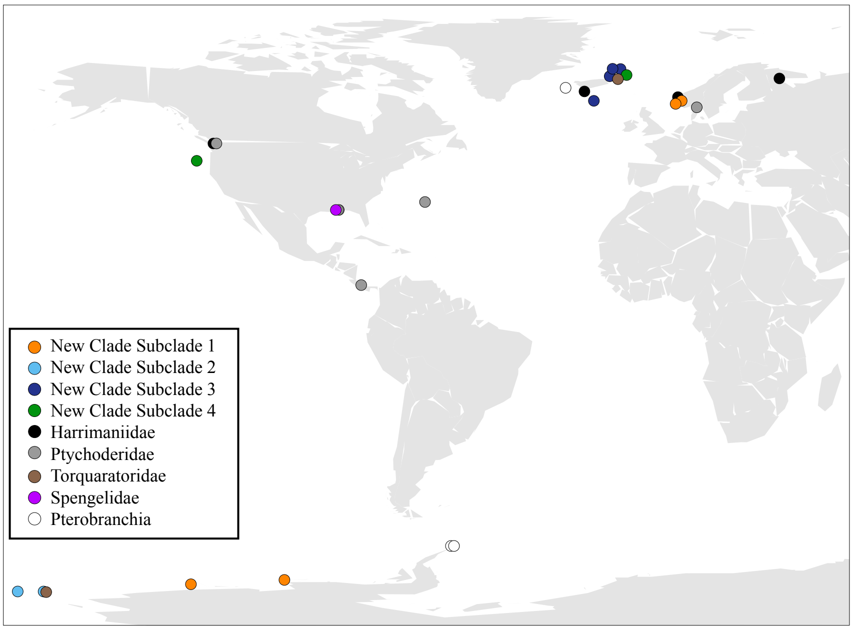

Figure 1. Map showing collection localities for hemichordates collected in this study. Taxonomic groups indicated by filled circles.

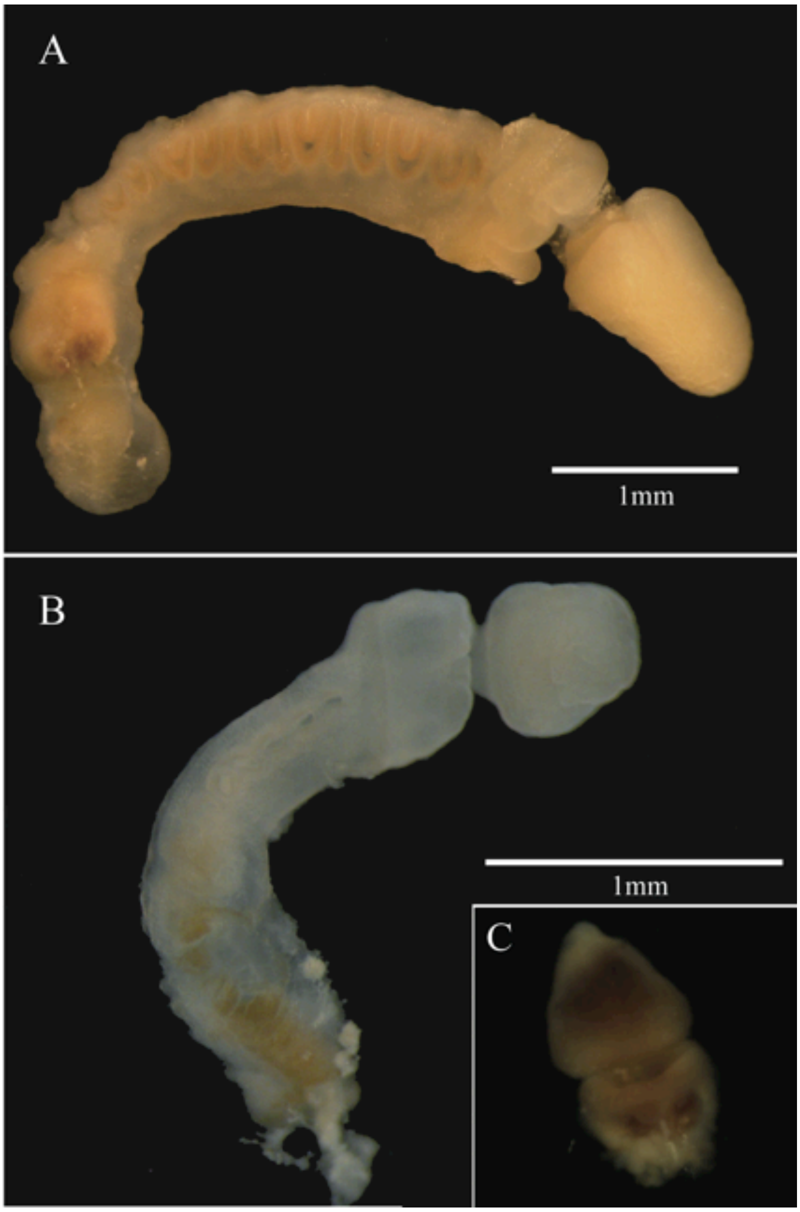

Figure 2. Cold-water harrimaniids. A. Harrimaniidae Antarctica H83.1 (Sublcade 1). Proboscis, collar, mouth, trunk, and gill slits are visible. B. Harrimaniidae Antarctica H94.1 (Subclade 2). C. Harrimaniidae Iceland H72.2 (Subclade 4), showing damaged condition in which many specimens were collected,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

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.

Chapter 4. Phylogenomics reveals relationships within Ambulacraria (Hemichordata + Echinodermata)

4.1 Abstract

As the sister group to chordates, Ambulacraria (Hemichordata + Echinodermata) plays a central role in understanding of chordate origins, as well as ancestral deuterostome character states. Despite their evolutionary significance, relationships within Hemichordata and Echinodermata remain unresolved. There are two major hemichordate body plans, wormlike Enteropneusta commonly called acorn worms, and colonial, tube-dwelling Pterobranchia. Previous studies have placed enteropneusts and pterobranchs as sister taxa, or alternatively, enteropneusts as paraphyletic, with pterobranchs derived from within acorn worms. Each hypothesis has unique implications for interpretation of ancestral forms. Likewise, echinoderm interrelationships have posed considerable challenges. Crinoidea is recognized as sister to Eleutherozoa (= Asteroidea + Ophiuroidea + Holothuroidea + Echinoidea), and there is strong evidence for a sister relationship between echinoids and holothuroids, but asteroids and ophiuroids have not been reliably placed. In order to directly address relationships within Ambulacraria, we generated transcriptome data from fourteen hemichordate species (five pterobranchs and nine enteropneusts) and eight echinoderms using next generation sequencing. These data were combined with publically available ambulacrarian sequences to generate a data matrix of 185 orthologous genes and 33 ambulacrarian taxa. Our analyses provide strong support for Asterozoa (Asteroidea + Ophiuroidea), and place pterobranchs as sister group to monophyletic enteropneusts.

4.2 Introduction

Ambulacraria, composed of Hemichordata + Echinodermata is recognized as the sister clade to Chordata (Bourlat et al., 2006; Dunn et al., 2008; Halanych, 1995; Turbeville et al., 1994; Wada & Satoh, 1994; Cameron et al., 2000). Phylogenetic relationships of these three taxa suggests that homologous traits shared between chordates and hemichordates, such as pharyngeal gill slits (Lowe et al., 2003; Ogasawara et al., 1999; Rychel and Swalla, 2007) and a post-anal tail observed in some species (Aronowicz and Lowe, 2006; Lowe et al., 2003; but see Stach and Kaul 2011) are likely to be deuterostome sympleisiomorphies (Brown et al., 2008; Swalla and Smith, 2008). Hemichordates also share remarkable similarities to chordates in transcriptional regulatory architecture related to early body patterning (Aronowicz and Lowe, 2006; Darras et al., 2011; Gillis et al., 2012; Lowe et al., 2003), and nervous system development (Luttrell et al., 2012; Miyamoto and Wada, 2013; Nomaksteinsky et al., 2006; Pani et al., 2012). As the sistergroup to chordates, Ambulacraria is key to understanding chordate origins, as well as early deuterostome evolution. However, relationships within the two ambulacrarian phyla have remained unresolved.

Within Hemichordata, there are two major body plans: solitary, vermiform enteropneusts (Figure 1), and colonial, tube-dwelling pterobranchs (Figure 2). Due to morphological and life history differences between these organisms, they have traditionally been divided into two classes, Enteropneusta and Pterobranchia. However, multiple molecular phylogenetic studies based on 18S rDNA have suggested that enteropneusts are paraphyletic, with Pterobranchia nested within them (Halanych, 1995; Cameron et al., 2000; Cannon et al., 2009; Worsaae et al., 2012). The more traditional hypothesis of pterobranchs and enteropneusts as sister taxa has been recovered by morphological cladistic analysis (Cameron, 2005), a short segment of 28S rDNA (Winchell et al., 2002), and microRNAs (Peterson et al., 2013), although the first two studies are limited in number of informative characters (Cannon et al., 2009), and the third included only three hemichordate taxa. Mitochondrial sequences from the pterobranch *Rhabdopleura compacta* are highly AT rich and phylogenetically uninformative (Perseke et al., 2011).

Relationships within echinoderms have posed long-standing questions in metazoan molecular phylogeny (Field et al., 1988; Janies, 2001; Janies et al., 2011; Littlewood et al., 1997; Pisani et al., 2012; Raff et al., 1988; Smith et al., 2004). Figure 3 shows representatives of the five extant classes of echinoderms: Crinoidea, Echinoidea, Holothuroidea, Asteroidea, and Ophiuroidea. Molecular and morphological analyses generally agree that Crinoidea is sister to the remaining four classes (= Eleutherozoa). Within Eleutherozoa, however, relationships are unclear. Most molecular phylogenetic and morphological studies place Echinoidea and Holothuroidea as sister taxa (= Echinozoa), but differ as to placement of Asteroidea and Ophiuroidea. Two major hypotheses have emerged: first, the Cryptosyringida hypothesis, placing ophiuroids sister to Echinozoa (Heinzeller and Welsh, 2001; Littlewood et al., 1997; Pisani et al., 2012; Smith, 1984); and second, the Asterozoa hypothesis, placing ophiuroids sister to asteroids, together sister to Echinozoa (Janies, 2001; Janies et al., 2011; Mallatt and Winchell, 2007; Mooi and David, 2000; Sumrall, 1997). In many of the studies listed above, the authors point out that there was little statistical difference between these two hypotheses, or multiple analyses within individual studies yielded different results.

Mitochondrial genomes have yielded conflicting topologies (Perseke et al. 2008, 2010; Scouras et al., 2004), and microRNAs lack phylogenetically informative characters within echinoderms (Pisani et al., 2011). Janies et al. (2011) and Pisani et al. (2012) demonstrated that echinoderm class-level relationships are particularly susceptible to parameter changes in phylogenetic analyses. This is likely due to long stem group lineages leading to these taxa (Janies et al., 2011; Pisani et al., 2012).

At present, there are differing degrees of phylogenetic resolution within each of the five living echinoderm classes. Crinoid interrelationships have recently been addressed by Rouse et al. (2013), who find several robust relationships (i.e., Cyrtocrinida, Hyocrinida, and Isocrinida as monophyletic groups, and two stalked crinoid groups nesting within comatulids) despite low support at basal nodes. Echinoids are fairly well resolved, with Cidaroidea as the earliest branching lineage (Littlewood and Smith, 1995; Smith et al., 2004). Holothuroids have received some attention, with more recent analyses agreeing on the basal position of Apodida and paraphyly of some of the major historical lineages (Kerr, 2001; Lacey et al., 2005). Ophiuroids are the most diverse extant class in terms of species numbers, but their interrelationships have received little attention (Smith et al., 1995). Interrelationships within Asteroidea, on the other hand, are well studied, but unclear. Numerous authors have addressed asteroid interrelationships with quite variable results (Hrincevick et al, 2004; Janies, 2001; Knott and Wray, 2000; Lafay et al., 1995; Mah, 2007; Mah and Foltz, 2011; O'Loughlin and Waters, 2004; Smith, 1997; Wada et al., 1996).

Despite difficulties in resolving hemichordate and echinoderm relationships, understanding evolutionary relationships within these two phyla has major implications for our understanding of deuterostome evolution. A clear phylogenetic framework is fundamental to meaningful comparisons within hemichordates and echinoderms, between these two phyla, and significantly, between Ambulacraria and Chordata. Within the last decade, phylogenomic studies have yielded important insights into deuterostome relationships. Within chordates, tunicates and craniates have been regularly recovered as sister taxa in phylogenomic analyses (Bourlat et al., 2006; Delsuc et al., 2006; Dunn et al., 2008; Lartillot and Philippe, 2008; Philippe et al., 2009), a grouping that has rapidly gained acceptance. Additional support has been shown from morphology (Ruppert 2005), cadherin gene structure (Oda et al. 2004), and chordate fibrillar collagen genes (Wada et al. 2006). Additionally, Xenourbellida (Bourlat et al., 2006; Bourlat et al, 2008; Dunn et al., 2008; Lartillot and Philippe, 2008), and more recently Acoelomorpha (Philippe et al., 2011) have been proposed as members of Deuterostomia based on phylogenomics results, although their placement remains controversial. In particular, evidence for acoelomorphs within deuterostomes is questionable. Philippe et al. (2011) suggest that microRNA distribution supports acoelomorphs within deuterostomes, yet their own calculations of steps required to explain this distribution indicate that this in fact the least parsimonious topology, and support values at the relevant nodes in their phylogenomic analysis are quite low. Ambulacraria, however, has been consistently supported in phylogenomics studies, although the number of sampled echinoderm and hemichordata taxa has been limited.

Herein, we directly address Ambulacrarian relationships using a phylogenomics approach. To further clarify the position of these groups, we sequenced transcriptome data for 14 hemichordate species (9 enteropneusts and 5 pterobranchs) and 8 echinoderms and combined them with existing data for a total of 33 ambulacarian operational taxonomic units (OTUs) . Contrary to previous results using 18S rDNA, enteropneusts form a monophyletic clade sister to monophyletic pterobranchia, which is more consistent with morphology-based hypotheses. Additionally, we recover Asteroidea + Echinoidea, refuting Cryptosyringida.

4.3 Methods

4.3.1 Taxon selection and sample collection

We sampled representatives from all recognized hemichordate families and all echinoderm classes except Echinoidea, for which genomic data and several transcriptomes were publically available. At least two species of each taxonomic group were collected, except in the case of Spengelidae. Table 1 provides detailed collection information for all taxa sequenced in this study. Enteropneusts from Norway were collected from *R/V Hakön Mosby* or *R/V Aurelia* with the aid of Dr. Christiane Todt and the late Dr. Christoffer Schander. Icelandic specimens were collected during Senckenberg's German Center for Marine Biodiversity Research (DZMB) IceAGE expedition aboard *R/V Meteor* led by Dr. Saskia Brix. University of Washington's Friday Harbor Laboratories provided assistance with collection of holothuroid taxa. *Rhabdopleura normani, Cephalodiscus gracilis*, and *Ptychodera bahamensis* were collected with the aid of Bermuda Institute of Ocean Sciences. Antarctic *Cephalodiscus* species were collected in 2001, and *Labidiaster annulatus*, *Odontaster validus*, *Ophionotus victoriae* and *Promachocrinus kerguelensis* were collected in 2006 during two cruises along the Antarctic Peninsula aboard the *R/V L.M. Gould*. The stalked crinoid, *Dumetocrinus* sp., was collected aboard the *R/V N.B. Palmer* in January 2013. *Saccoglossus mereschowskii* was collected near the White Sea Marine Station, Moscow University, Russia with the aid of Dr. Alexander Tzetlin. Enteropneusts from Mississippi were collected with the aid of Dr. Richard Heard and the staff and crew of *R/V Kit Jones*. *Stereobalanus canadensis* was collected at Darling Marine Center from *R/V Ira C.* Samples were transported live to the laboratory, transferred to RNAlater, flash frozen at -80°C, or kept in ethanol at -20 °C (see Table 1).

4.3.2 Molecular Techniques

Total RNA was extracted from enteropneust proboscis tissue or from intact anteriors (including proboscis, collar, and trunk). Prior to preservation or extraction, enteropneust gut contents were cleared. For pterobranch species, multiple zooids from the same colony were extracted together. Echinoderm RNA extractions were taken from arm tissue (Asteriodea, Ophiuroidea, and Crinoidea), or internal body muscle tissue (Holothuroidea). Complementary DNA (cDNA) preparation methods varied somewhat between samples that were subsequently sequenced using Roche 454 (GS-FLX or Titanium) or Illumina (see Table 2). For all taxa, total RNA was prepared by TRIzol (Invitrogen) extractions followed by purification using RNeasy (Qiagen) spin columns with on-column DNase treatment (Qiagen). First-strand cDNA was prepared using the SMART cDNA library construction kit (Clontech). For taxa sequenced using Illumina, the provided 3’ oligo in the SMART cDNA kit was replaced with the Cap-Trsa-CV oligo (5’-AAGCAGTGGTATCAACGCAGAGTCGCAGTCGGTACTTTTTTCTTTTTTV-3’) as per Meyer et al. (2009). Double-stranded cDNA libraries were amplified using the

Advantage 2 PCR system (Clontech). For libraries sequenced via 454 FLX or Titanium, double-stranded full-length cDNA was normalized using the Trimmer-Direct kit (Evrogen) following manufacturers recommendations. These normalized libraries were sequenced at the former University of South Carolina Environmental Genomics Core Facility, now Selah Genomics (Columbia, South Carolina). Non-normalized libraries were sent to HudsonAlpha Institute for Biotechnology (Huntsville, Alabama) for Illumina TrueSeq paired-end (PE) library preparation and Illumina sequencing (2x100). Each library was sequenced using approximately one-sixth of an Illumina HiSeq 2000 lane (see Table 2 for number of reads for each library).

4.3.3 Bioinformatics pipeline

Sequence data generated above were augmented with publically available data (Table 2). Because we sought to directly address relationships within Ambulacraria and because monophyly for extant hemichordates and echinoderms is well established (reviewed in Halancyh, 2004; Kocot et al., 2010; Swalla and Smith, 2008), we rooted the tree so that the phyla were reciprocally monophyetic. Data from taxa sequenced by 454 or Sanger sequencing were assembled and processed using the EST2uni pipeline (Forment et al., 2008). This software pipeline integrates lucy (Chou and Holmes, 2001) to remove low-quality regions and vector sequences, RepeatMasker to mask low complexity repetitive sequence (www.repeatmasker.org), and CAP3 (Huang and Madan, 1999) for contig assembly using quality data when available. For taxa sequenced by Illumina, raw PE Illumina reads were digitally normalized using khmer (normalize-by-median.py -C 30 -k 20 -N 4 -x 2.5e9; Brown et al., 2012). A custom bioinformatic pipeline including scripts provided with khmer was used to restore read pairing. The remaining PE reads were assembled on the Auburn University Molette Lab SkyNet server using the October 2012 version of Trinity for all taxa except *Schizocardium* c.f. *braziliense*, *Dumetocrinus* sp., and the torquaratorid from Antarctica, for which the February 2013 version was used (Grabherr et al., 2011) with the default settings. Contigs (for Illumina libraries) or contigs + high quality singletons (for 454 and Sanger libraries) were translated using Transdecoder (https://sourceforge.net/p/transdecoder/). Table 2 provides the number of unigenes (contigs for Illumina libraries, and contigs + singletons for 454 and Sanger libraries).

Identification of putatively orthologous groups (OGs) was conducted with HaMStR (Hidden Markov Model based Search for Orthologs using Reciprocity) local, version 9 (Ebersberger et al., 2009). This program generates profile hidden Markov models (pHMMs), each one representing a set of orthologous genes for selected reference taxa from the InParanoid database (Ostlund et al., 2009) for which whole genomes are available. Ambulacrarian sequences were searched against the “model organisms” reference taxon set, which includes 1,032 orthologous groups with sequences from *Homo sapiens, Ciona intestinalis, Drosophila melanogaster, Caenorhabditis elegans*, and *Saccharomyces cerevisiae*. Translated unigenes were scanned for significant hits to each OG’s pHMM. Matching sequences were then compared to the proteome of a selected primer taxon (*Homo sapiens* in our analyses) using BLASTP (-strict option). If the *Homo sapiens* amino acid sequence that contributed to the pHMM was the best BLASTP hit, then the sequence was assigned to that OG. If this reciprocity criterion was not met, the sequence was discarded. Table 2 provides the number of HaMStR orthology groups identified for each of the taxa included in our analyses.

After orthology determination, sequences from two 454 libaries for *Ophionotus victoriae* and four small libraries from *Rhabdopleura* species were combined into chimerical OTUs in order to reduce the amount of missing data per taxon. Orthology groups were then sorted according to the number of taxa contained in each, and only OGs with ≥15 ambulacrarian taxa were retained for further analyses. Also, because one of our primary questions was pterobranch placement, only OGs containing at least 1 pterobranch sequence were retained. Translated sequences shorter than 100 amino acids (AAs) were deleted for all taxa. Sequence ends were sometimes obviously mistranslated, so amino acid sequences were scanned for presence of X codons (corresponding to a stop codon, or a codon with an ambiguity, gap, or missing data) in either the first or last 20 characters. Where these X codons were found, indicating mistranslation, missing data, or ambiguities, the X and all characters between that character and the sequence end were deleted. Each OG was aligned with MAFFT (mafft --auto --localpair --maxiterate 1000; Katoh et al., 2005), and then trimmed with the perl scripts Aliscore and Alicut (Misof and Misof, 2009) to remove columns with ambiguous alignment or little phylogenetic signal.

Next, individual alignments were manually evaluated for partially mistranslated sequences and presence of two or more incomplete non-overlapping sequences from a single OTU that could be combined into a single more complete chimeric sequence. Mistranslated sequences were deleted or trimmed as appropriate. Single-OG trees were then constructed for each OG using RAxML version 7.3.8 with the PROTGAMMALGF model. Model organism taxa from HaMStR (*Homo sapiens, Ciona intestinalis, Drosophila melanogaster, Caenorhabditis elegans*, and *Saccharomyces cerevisiae*) were retained in alignments until this step to provide a means of screening for sequence contamination as follows. Individual OG trees were rooted on *Saccharomyces.* Sequences forming sister relationships with model organism taxa in clades outside of Ambulacraria were taken to be contamination and deleted (randomly selected BLAST searches of these sequences validated this approach). When ingroup taxa formed sister relationships with model organism sequences within Ambulacraria, these sequences were searched against the NCBI non-redundant (nr) protein database using blastp and were deleted if the results did not return a hemichordate (for enteropneust taxa) or an echinoderm (for echinoderm taxa) as the top BLAST hit. For pterobranch sequences, if the top BLAST hit was either a hemichordate or an echinoderm, the sequence was retained (pterobranch sequences tend to be more divergent). In cases where model organism taxa were dispersed throughout the tree, the maximally inclusive subtree containing no model organism taxa was retained when possible, or the entire OG was deleted due to paralogy concerns. OGs were also discarded at this step if they no longer retained any pterobranch sequences after evaluation. After manual screening of alignments and individual OG trees, 299 alignments remained.

Subsequent to individual gene tree screening, model organism taxa were removed from alignments, and amino acid sequences shorter than 100 codons were discarded. The utility PhyloTreePruner (Kocot and Citarella et al., 2013) was used to screen for potential paralogs. Individual OG trees were generated using FastTreeMP (-slow -gamma; Price et al., 2010), and nodes supported by ≤0.50 support were collapsed into polytomies. Resulting Newick files were provided to PhyloTreePruner, which screened trees for instances where multiple sequences from the same OTU did not form monophyletic clades. Suspected paralogs were trimmed from the data matrix, leaving the maximally inclusive subtree in which sequences from each OTU formed monophyletic clades or were part of the same polytomy (Kocot and Citarella et al., 2013). The program was set to generate new alignments for OGs with ≥15 ambulacrarian OTUs remaining after pruning. If an OG still possessed more than one sequence for an OTU (inparalogs), PhyloTreePruner selected the longest sequence for inclusion in the final concatenated alignment. A concatenated alignment of all remaining OGs was generated using FASconCAT (Kück and Meusemann, 2010).

4.3.4 Phylogenetic analyses

Maximum likelihood phylogenetic analyses were conducted using RAxML 7.7.6 (Released by Alexandros Stamatakis, August 2013) using a PROTGAMMALGF model for each individual OG partition. Nodal support was assessed with 100 replicates of nonparametric bootstrapping. Bootstrapped trees were used to calculate leaf stability and taxonomic instability indices of each OTU using the Roguenarok server (http://193.197.73.70:8080/rnr/roguenarok/about). Competing hypotheses of ambulacrarian phylogeny were evaluated using the SH-test (Shimodaira, 2002) as implemented in RAxML with the PROTGAMMALGF model for each OG partition. All maximum likelihood analyses were conducted on the Auburn University CASIC HPC supercomputer.

4.4 Results

Following paralogy screening by PhylotreePruner, a final alignment including 185 orthology groups and 70,498 total amino acids was retained. Harrimaniid enteropneust *Stereobalanus canadensis* and chimaeric OTU *Rhabdopleura* spp. had poor leaf stability and taxonomic instability index values (lsi/tii = 0.90/613.9 and lsi/tii = 0.83/828.2, respectively, Table 3). Therefore, we performed analyses including all taxa, or excluding one or both of these unstable OTUs (Figures 5-7). Unless otherwise noted, bootstrap values given below refer to analyses in which *Stereobalanus canadensis* was excluded (Figure 4).

Echinodermata and Hemichordata were supported as monophyletic clades with bootstrap support (BS) of 100 in all analyses. Within Hemichordata, pterobranchs and enteropneusts were recovered as reciprocally monophyletic (BS = 67). When unstable taxa *Rhabdopleura* spp. and *Stereobalanus canadensis* were removed, this value increased to BS = 100 (Figure 7). Additionally, for the dataset excluding *S. canadensis* but including *Rhabdopleura* spp., Shamodaira-Hasegawa tests strongly rejected the alternative hypothesis of paraphyletic Enteropneusta, or Pterobranchia + Harrimaniidae (Table 4). Although our maximum likelihood tree under the best model recovers pterobranchs as monophyletic, paraphyletic pterobranchia (e.g., Cephalodiscus sister to Rhabdopleura + Enteropneusta) could not be ruled out in our analyses. Interestingly, using models with poorer fit to our data, or a less thorough approximately-ML analysis performed by the program FastTree 2.1 (Price et al., 2001) we recover paraphyletic pterobranchs. Unfortunately, despite combining three 454 libraries (although notably, *Rhabdopleura compacta* from dbEST is particularly small) and an Illumina library into a chimeric OTU, *Rhabdopleura* spp. was still only represented by 8% of genes in our data matrix.

Within enteropneusta, Harrimaniidae (excluding *Stereobalanus*) was recovered as monophyletic (BS = 100) sister to remaining enteropneusts. When *Stereobalanus* was included, it was the earliest branching lineage of Enteropneusta, albeit with poor support (all taxa, BS = 51; excluding *Rhabdopleura* spp., BS = 63). Spengelidae, represented singly by *Schizocardium* c.f. *braziliense*, was sister to a clade consisting of Ptychoderidae + Torquaratoridae. In contrast to results based on 18S rDNA analyses, we found Torquaratoridae nested within Ptychoderidae (BS = 78) sister to the two *Balanoglossus* species. In a taxon-restricted analysis including only enteropneust taxa, this value increased to BS = 92 (Figure 8). An alternative tree topology in which Ptychoderidae was constrained as monophyletic to the exclusion of torquaratorid taxa was significantly rejected by SH tests (Table 4).

Each of the 5 recognized echinoderm classes was recovered as monophyletic with 100% bootstrap support. Crinoidea is sister to Eleutherozoa (BS = 100). Within Eleutherozoa, we find strong support for Asterozoa + Echinozoa (BS = 100). Cryptosyringida is rejected by SH tests (Table 4). Relationships within echinoderm classes were consistent with current taxonomy. Within sea stars, *Labidiaster annulatus* (Forcipulata) is sister to the remaining three asteroids (all Valvatacea). The cidaroid urchin *Eucidaris tribuloides* is sister to two euechinoids, and *Leptosynapta clarki* (Apodida) is sister to Aspidochirotida (Holothuriidae and Stichopodidae).

4.5 Discussion

Enteropneust monophyly and monophyly of Asterozoa (Ophiuroidea + Asteroidea) were strongly supported. Taken together, the phylogeny presented here is consistent with earlier perspectives on pleisiomorphies of Ambulacraria, including pharyngeal gill slits, a single axocoel, and paired hydrocoels and somatocoels (Smith, 2008; Swalla and Smith, 2008). The axial complex, which filters fluid from the blood vascular system via contractions of the heart vesicle (hemichordates) or madreporic vesicle (echinoderms) across podocytes in the glomerulus (hemichordates) or the axial gland (echinoderms) into a coelomic cavity, and finally through a hydropore out of the body, was also likely present (Balser and Ruppert, 1990; Ruppert and Balser, 1986; Smith et al., 2004). Although pterobranchs and crinoids have non-feeding larval forms, shared characters of eleutherozoan and enteropneust feeding larvae (ciliated band patterning, subdivision of the digestive tract, and coelomic organization) are unlikely to be due to convergence (Brown et al., 2008; Peterson et al., 1997; Swalla and Smith, 2008). Thus, a planktotrophic larval stage was likely present in the Ambulacrarian LCA.

The ancestral hemichordate has been hypothesized to be a solitary, ptychoderid-like worm that filter-feeds via gill slits, and has indirect development (Brown et al., 2008; Cameron et al., 2000; Swalla, 2001, 2007). Our results suggest that ptychoderids have a more derived placement, but as gill slits and feeding larval forms are shared with chordates and echinoderms, respectively, these characters are likely ancestral within hemichordates. However, neither a strictly acorn worm-like or a strictly pterobranch-like hemichordate ancestor can be assumed based on reciprocal monophyly the two groups. Morphological, developmental, and paleontological evidence, particularly from data-limited pterobranchs, will be needed to further address these questions (Cannon et al., 2009; Peterson et al, 2013).

Within Enteropneusta, recently described (Holland et al., 2005) and predominantly deep-sea Torquaratoridae appears to be derived from within ptychoderids. Some torquaratorid taxa have shown unusual reproductive structures or strategies, such as externalized ovaries in *Allapasus aurantiacus* (Holland et al, 2012), or brooding in *Coleodesmium karaensis* (Osborn et al, in press). The position of Torquaratoridae within Ptychoderidae suggests that these features, as well as the defining characters of the group (reduced or absent proboscis skeleton, absent stomochord or stomochord separated from buccal cavity of collar) are derived from ptychoderid-like forms. Our results also indirectly support the interpretation of Halanych et al. (2013) that fossil enteropneust *Spartobranchus tenuis* is allied to torquaratorids, rather than harrimaniids, as suggested by Caron et al. (2013). Harrimaniid affinities of this fossil were informed by the presumed relationship between pterobranchs and harrimaniids, which is refuted here. Thus, tubes of *Spartobranchus* are unlikely to represent the precursor to the pterobranch periderm, as suggested by Caron et al. (2013).

The harrimaniid enteropneust *Stereobalanus canadensis* is a highly unstable taxon in our analyses. This species has a remarkably long-branched 18S rDNA sequence (Cannon et al., 2009; Cannon et al., accepted), and is morphologically unusual among enteropneusts, possessing four short gonad regions directly poster to the collar, and gill pores fused into a common slit. Two independently generated *S. canadensis* cDNA libraries were sequenced via Illumina, the equivalent of 1/3 of an Illumina HiSeq 2000 2x100 paired end lane. A combined assembly of these two libraries contributed to only 5% of the 185 genes in our analyses, in stark contrast to other enteropneust taxa sequenced at 1/3 of a lane, which contributed up to 95% OGs. Causes of these poor sequencing results are unclear. When *S. canadensis* is included in our analyses, it is recovered sister to all other enteropneust taxa, whereas in 18S + 16S rDNA analyses, it is recovered sister to all other harrimaniids (Cannon et al., 2009; Cannon et al., accepted). Additional sequence data will likely be required to place this unusual worm with confidence.

Our results concerning echinoderm class-level relationships are marked by near-perfect bootstrap values. Support for Asterozoa + Echinozoa suggests that features uniting Cryptosyringida [enclosed radial elements of water vascular system in adults, pluteus larval stages in ophiuroids and echinoids, jaw apparatus construction (Smith, 1984), and neurulation of radial nerve (Heinzeller and Welsh, 2001)] are convergence or secondary losses in Asteroidea. Morphological synapomorphies of Asterozoa include saccate gut and adambulacral ossicles (Janies, 2001). Pisani et al (2012) used site stripping methods to suggest that Asterozoa was a result of artifacts such as LBA using a dataset comprised of seven nuclear housekeeping genes and three ribosomal genes. For our analyses, in order to minimize artifacts and phylogenetic noise, we have employed perl scripts Aliscore and Alicut (Misof and Misof, 2009) to remove uninformative or ambiguously aligned sites, manually evaluated individual alignments and single-gene trees, and removed paralogs with Phylotreepruner (Kocot and Citarella et al., 2013). Additionally, parameter changes (i.e., model selection, use of rapid approximately-ML analyses) do not affect support for Asterozoa.

In this study we have greatly expanded the amount of data available for non-model organisms within Ambulacraria by producing transcriptomes for 22 taxa. Our phylogenetic results indicate pterobranchs, enteropneusts, and Asterozoa are monophyletic clades. Monophyly of acorn worms and pterobranchs makes determining ancestral character states for Hemichordata and Ambulacraria a greater challenge. However, we can infer an ambulacrarian ancestor with a tricoelomic planktotrophic larva, an axial complex, and pharyngeal gill slits.

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Table 1. Collection information for species sequenced in this study.

Species	**Locality**	**Depth**	**Collection Date**	**Collection method**	**Preservation**	**Tissue extracted**
Astrotomma agassizii	Antarctica 54° 22.936' S, 61° 52.968' W	274 m	May, 2006	Dredge	Cryo (-80° C)	arm
Balanoglossus c.f. *aurantiacus*	Mississippi, USA 30°14.8' N, 88° 32.4' W	1 m	June, 2011	yabby pump	Live	whole anterior
Cephalodiscus gracilis	Bermuda 32° 21.467' N, 64° 42.35' W	1 m	November, 2009	Snorkel	RNAlater	multiple zooids
Cephalodiscus hodgsoni	Antarctica 62° 45.034' S, 56° 46.26' W	220 m	December, 2001	Dredge	Cryo (-80° C)	multiple zooids
Cephalodiscus nigrescens	Antarctica 62° 45.034' S, 56° 46.26' W	220 m	December, 2001	Dredge	Cryo (-80° C)	multiple zooids
Glossobalanus marginatus	Norway 58° 55.002' N, 10° 33.165' W	184 m	June, 2009	Dredge	RNAlater	proboscis
Harrimaniidae sp. Iceland	Iceland 69° 6.51' N, 9° 55.09' W	2177 m	September, 2011	epibenthic sled	Ethanol (-20° C)	whole anterior
Harrimaniidae sp. Norway	Norway 60° 18.37' N, 5° 12.077' E	130 m	June, 2008	epibenthic sled	RNAlater	whole anterior

Labidiaster annulatus lib. 1	Antarctica 63° 08.838' S, 57° 07.441' W	192 m	May, 2006	dredge	Cryo (-80° C)	arm
Labidiaster annulatus lib. 2	Antarctica 63° 40.145' S, 61° 10.047' W	126 m	May, 2006	dredge	Cryo (-80° C)	arm
Leptosynapta clarki	Washington, USA 48° 32.6167′ N, 123° 0.6833′ W	1 m	August, 2011	shovel	RNAlater	muscle tissue
Odontaster validus	Antarctica 62° 18.834' S, 61° 45.086' W	334 m	May, 2006	dredge	Cryo (-80° C)	arm
Ophionotus victoria	Antarctica 62° 18.834' S, 61° 45.086' W	334 m	May, 2006	dredge	Cryo (-80° C)	arm
Parastichopus californicus	Washington, USA 48° 32.6167′ N, 123° 0.6833′ W	1 m	August, 2011	dredge	RNAlater	muscle tissue
Promachocrinus kerguelensis	Antarctica 62° 44.75' S, 56° 44.883' W	207 m	December, 2004	dredge	Cryo (-80° C)	arm
Ptychodera bahamensis	Bermuda 32° 19.8833' N, 64° 44.3667' W	1 m	November, 2009	snorkel	RNAlater	proboscis
Rhabdopleura normani	Bermuda	1 m	November,	snorkel	RNAlater	multiple zooids

	32° 21.467' N, 64° 42.35' W		2009			
Rhabdopleura sp. Iceland	Iceland 63° 56.07' N, 25° 56.53' W	209 m	September, 2011	Dredge	RNAlater	multiple zooids
Saccoglossus mereschkowskii	Russia 66° 33.192' N, 33° 6.35' E	<20 m	September, 2011	Dredge	RNAlater	proboscis
Schizocardium c.f. *braziliense*	Mississippi, USA 30° 14.09' N, 89° 20.09' W	12.5 m	February, 2009	box core	Live	whole anterior
Dumetocrinus sp.	Antarctica 70° 48.713’ S, 92° 31.304' W	430 m	January, 2013	Dredge	RNAlater	arm, stalk
Stereobalanus canadensis	Maine, USA 43°55.48' N, 69°34.243' W	15-20 m	June, 2011	box core	RNAlater	proboscis, gonad
Torquaratoridae sp. Iceland	Iceland 66° 18.06' N, 12° 22.40' W	732 m	September, 2011	box core	RNAlater	proboscis
Torquaratoridae sp. Antarctica	Antarctica 76° 20.4730' S, 170° 51.0297' W	531 m	February, 2013	Dredge	RNAlater	proboscis

Table 2. Taxon sampling and dataset assembly information for all species included in this study

Taxon	Species	Source	# Reads	# unigenes	HaMStR OGs	Accession #s
Ptychoderidae	*Ptychodera flava*	NCBI Trace Archive (Sanger)	3360	1420	97	downloaded 2009 (no updates)
	Ptychodera bahamensis	**HudsonAlpha (Illumina)**	41174126	115310	948	
	Glossobalanus marginatus	**Engencore (454 Titanium)**	230141	101125	319	
	Balanoglossus c.f. aurantiacus	**HudsonAlpha (Illumina)**	52013666	143815	972	
	Balanoglossus clavigerus	dbEST (454)	4118	1675	80	FN985644-FN989759
Harrimaniidae	*Saccoglossus kowalevskii*	NCBI Trace Archive (Sanger)	202190	37266	842	downloaded 2009
	Saccoglossus mereschkowskii	**HudsonAlpha (Illumina)**	50630972	145937	979	
	Harrimaniidae sp. Iceland	**HudsonAlpha (Illumina)**	66151572	230054	983	
	Harrimaniidae sp. Norway	**HudsonAlpha (Illumina)**	69266416	274434	964	
	Stereobalanus canadensis	**HudsonAlpha (Illumina)**	19290646,	18843	170	

			50206164			
Spengelidae	***Schizocardium c.f. braziliense***	**HudsonAlpha (Illumina), Engencore(454 GS-FLX)**	40083244, 98989	101457	744	
Torquaratoridae	**Torquaratorid H78 Iceland**	**HudsonAlpha (Illumina)**	73725698	102971	727	
	Torquaratorid H89 Antarctica	**HudsonAlpha (Illumina)**	45751208	145544	944	
Cephalodiscida	***Cephalodiscus hodgsoni***	**HudsonAlpha (Illumina), Engencore(454 GS-FLX)**	17183978, 85028	14441	113	
	Cephalodiscus nigrescens	**HudsonAlpha (Illumina)**	23413166	23130	100	
	Cephalodiscus gracilis	**HudsonAlpha (Illumina)**	47746306	57139	538	
Rhabdopleurida	***Rhabdopleura normani***	**Engencore (454 GS-FLX, Titanium)**	82283, 104634	66988	78	
	***Rhabdopleura sp.* Iceland**	**HudsonAlpha (Illumina)**	13703832	4790	43	
	Rhabdopleura compacta	dbEST (454)	4115	308	6	FN989760-FN993914
Echinoidea	*Strongylocentrotus purpuratus*	UnigeneDB (Sanger)	n/a	28867	964	downloaded 2009
	Paracentrotus lividus	dbEST (Sanger)	140897	52232	865	AM184308-AM228715, AM504150-AM600638
	Eucidaris tribuloides	SRA (454 Titanium)	2309861	55933	433	SRR101490

Holothuroidea	*Apostichopus japonicus*	SRA (Illumina)	91860733	139677	979	SRR414930
	Parastichopus parvimensis	SRA (Illumina)	130417338, 118404785	252996	994	SRR496203, SRR496204
	Parastichopus californicus	**HudsonAlpha (Illumina)**	52003372	134640	898	
	Holothuria glaberrima	SRA (Illumina)	63760530	98449	749	SRR490864
	Leptosynapta clarki	**HudsonAlpha (Illumina)**	56022502	242126	891	
Asteroidea	*Patiria pectinifera*	dbEST (Sanger)	56831	14054	354	DB384948-DB441778
	Patiria miniata	Hart and Foster, 2013 (Illumina)	n/a	5501	464	Hart and Foster, 2013
	Odontaster validus	**Engencore (454 Titanium)**	171417	39461	48	
	Labidiaster annulatus	**HudsonAlpha (Illumina)**	71361592, 53268480	210764	823	
Crinoidea	***Promachocrinus kerguelensis***	**Engencore (454 Titanium)**	190565	35867	140	
	***Dumetocrinus* sp. Antarctica**	**HudsonAlpha (Illumina)**	49481384	127039	931	
Ophiuroidea	***Ophionotus victoriae***	**Engencore (454 Titanium)**	157023	34062	71	
	Ophionotus victoriae	SRA (454 GS-FLX)	358243	16420	215	SRR500294
	Astrotoma agssizii	**HudsonAlpha (Illumina)**	54375996	156062	680	

Table 3. Leaf stability and taxonomic stability information by taxon.

Operational Taxonic Unit	**Leaf Stability Index**	**Taxonomic Instability Index**	**Percent coverage OGs**
Apostichopus japonicus	0.985986	112.092595	84%
Astrotoma agassizii	0.985986	192.766409	69%
Balanoglossus c.f. aurantiacus	0.970867	325.252716	95%
Balanoglossus clavigerus	0.970867	336.627512	3%
Cephalodiscus gracilis	0.936871	539.829811	89%
Cephalodiscus hodgoni	0.936871	591.56933	10%
Cephalodiscus nigrescens	0.936871	608.799203	7%
Eucidaris tribuloides	0.985986	166.905759	29%
Glossobalanus marginatus	0.965125	401.550233	16%
Harrimaniidae Iceland	0.981992	245.178821	96%
Harrimaniidae Norway	0.981992	245.178821	94%
Holothuria glaberrima	0.985986	135.283678	82%
Labidiaster annulatus	0.981018	368.123334	74%

Leptosynapta clarki	0.985986	166.905759	84%
Odontaster validus	0.980667	288.744566	3%
Ophionotus victoriae	0.985986	192.766409	22%
Paracentrotus lividus	0.985986	135.283678	77%
Parastichopus californicus	0.985986	94.523085	72%
Parastichopus parvimensis	0.985986	94.523085	93%
Patiria miniata	0.98324	271.962195	46%
Patiria pectinifera	0.98324	277.5042	37%
Promachocrinus kerguelensis	0.985986	253.735047	5%
Ptychodera bahamensis	0.973085	325.413803	93%
Ptychodera flava	0.973085	325.413803	6%
Rhabdopleura spp.	0.83304	828.203014	8%
Saccoglossus kowalevskii	0.981992	245.178821	72%
Saccoglossus mereschkowskii	0.981992	245.178821	96%
Schizocardium c.f. *braziliense*	0.981992	388.634574	64%

Stalked Crinoid	0.985986	253.735047	86%
Stereobalanus canadensis	0.90504	613.894647	5%
Strongylocentrotus purpuratus	0.985986	135.283678	89%
Torquaratoridae Antarctica	0.972242	242.010832	92%
Torquaratoridae Iceland	0.972242	242.010832	70%

Table 4. Results of hypothesis testing by Shaimodaira-Hasegawa tests. Likelihood value for the best tree was -783118.9207.

Hypothesis	**Likelihood**	**D(LH)**	**Standard Deviation**	**Significantly Worse (5%)**
Pterobranchia + Harrimaniidae	-784505.2477	-1386.326992	70.188218	Yes
Paraphyletic Pterobranchia	-783120.9067	-1.986053	3.390795	No
Cryptosyringida	-783198.5781	-79.657465	23.045378	Yes
Monophyletic Ptychoderidae (excluding Torquaratoridae)	-783383.8421	-264.921379	40.085201	Yes

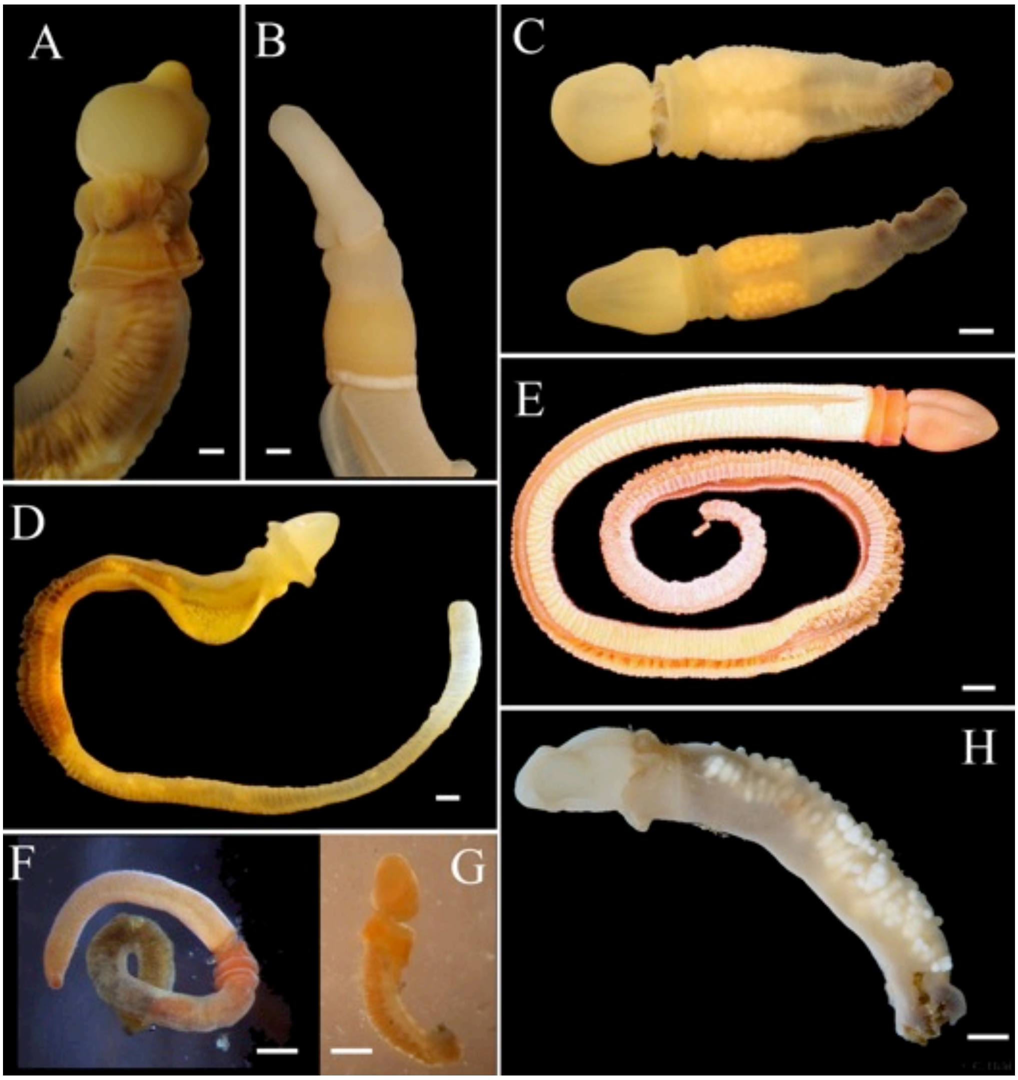

Figure 1. Representative enteropneusts. A. *Glossobalanus marginatus*, scale bar = 2 mm, B. *Balanoglossus* c.f. *aurantiacus*, scale bar = 2 mm, C. *Stereobalanus canadensis*, scale bar = 5 mm, D. *Ptychodera bahamensis*, scale bar = 2 mm, E. *Schizocardium* c.f. *braziliense*, scale bar = 5 mm, F. *Saccoglossus mereschkowskii*, scale bar = 1 mm, G. Harrimaniidae n. sp. Norway, scale bar = 1 mm, H. Torquaratoridae Antarctica, scale bar = 5 mm.

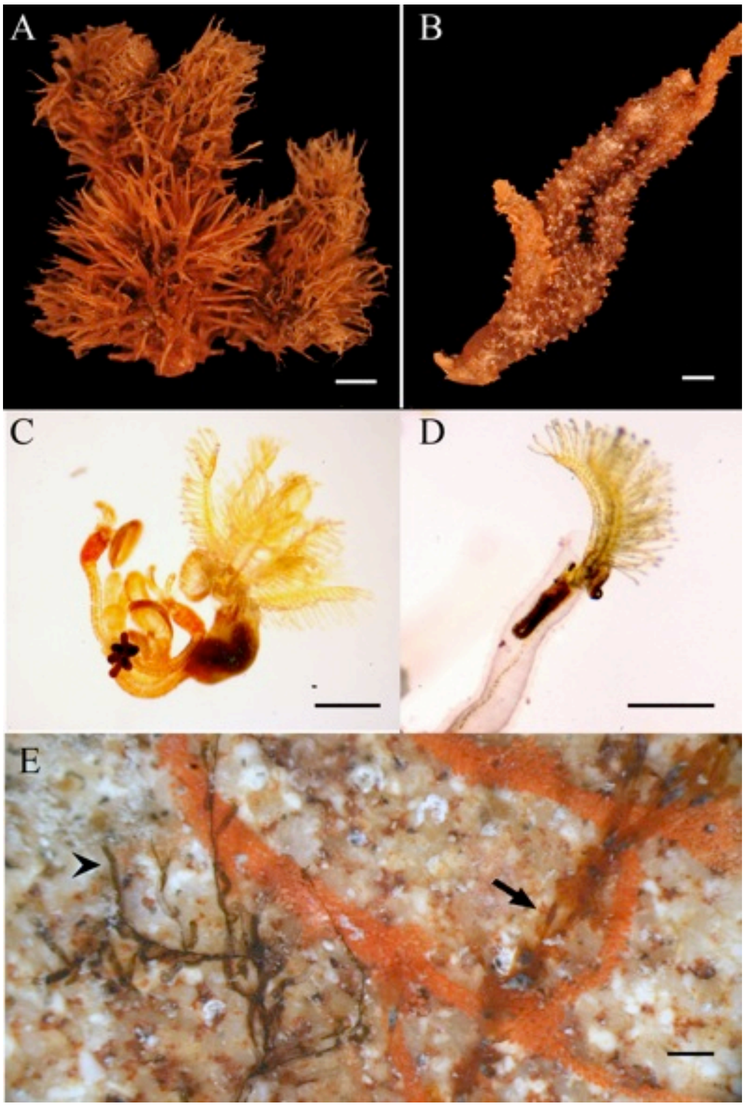

Figure 2. Representative pterobranchs. A. Coenecium of *Cephalodiscus hodgsoni*, scale bar = 2 cm, B. Coenecium of *Cephalodiscus nigrescens*, scale bar = 2 cm, C. Zooid of *Cephalodiscus gracilis*, scale bar = 0.5 mm, D. Zooid of *Rhabdopleura* c.f. *normani*, scale bar = 0.5 mm, E. Coenecia of sympatric pterobranch colonies on a rock from Bermuda. Left (arrowhead), *Rhabdopleura normani*; Right (arrow), *Cephalodiscus*

gracilis. Arrow points to body of adult *Cephalodiscus* zooid within transparent coenecium. Scale bar = 2mm.

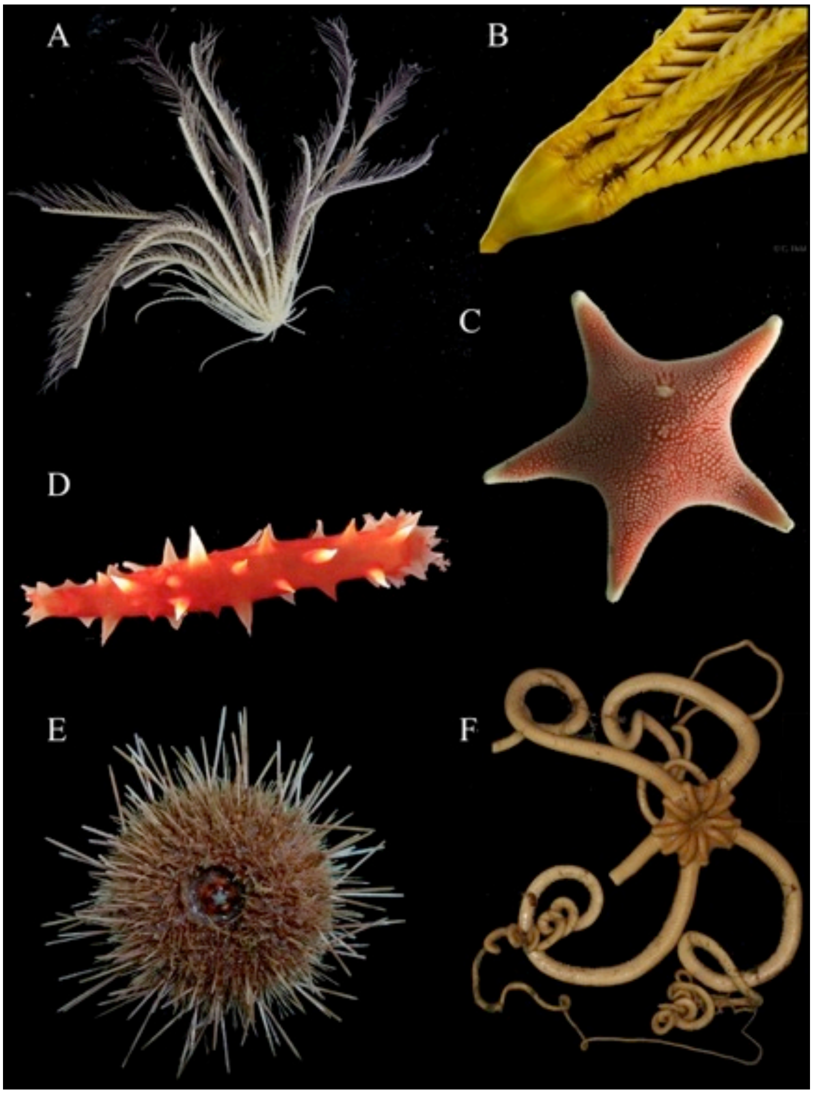

Figure 3. Representative echinoderms. A. Crinoid *Promachocrinus kerguelensis*, B. Stalked crinoid, *Dumetocrinus* sp., C. Asteroid *Odontaster validus*, D. Holothuroid *Parastichopus californicus*, E. Echinoid *Sterechinus neumayeri*, F. Ophiuroid *Astrotoma agassizi.*

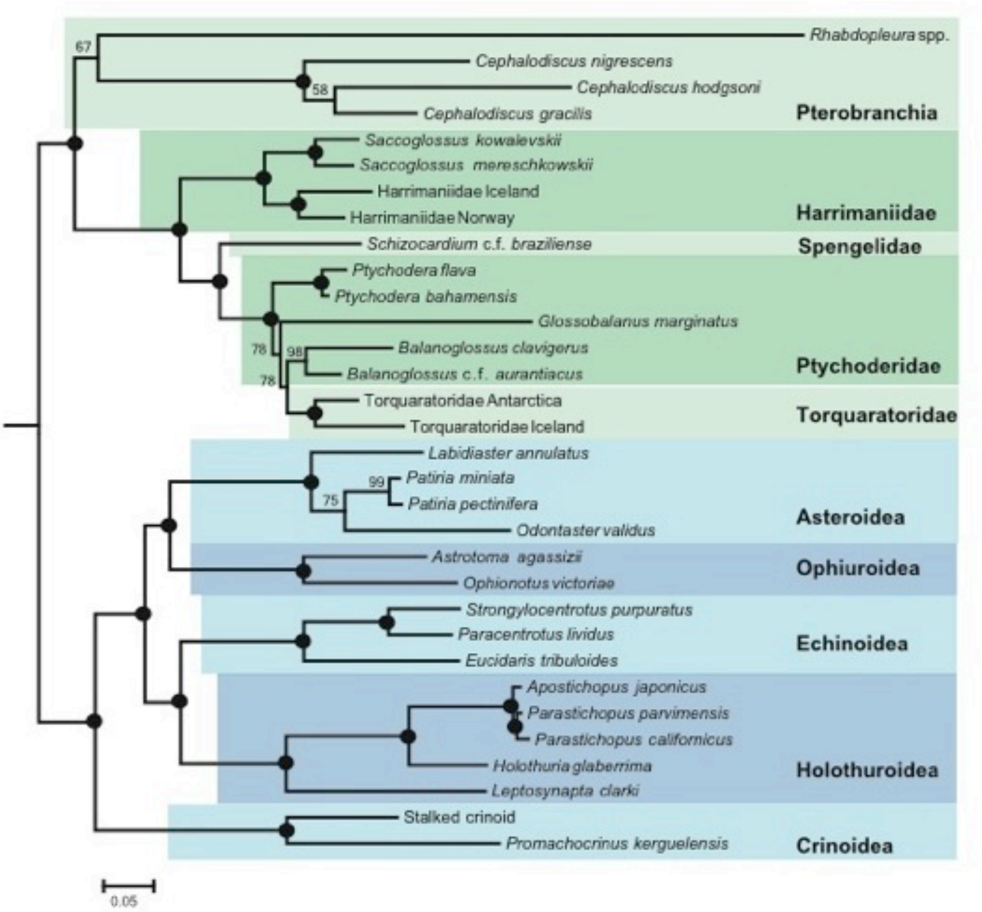

Figure 4. Ambulacrarian phylogeny based on 185 genes. Maximum likelihood tree shown with *Stereobalanus canadensis* excluded, with bootstrap values are indicated at nodes. BS values of 100 are indicated by filled circles.

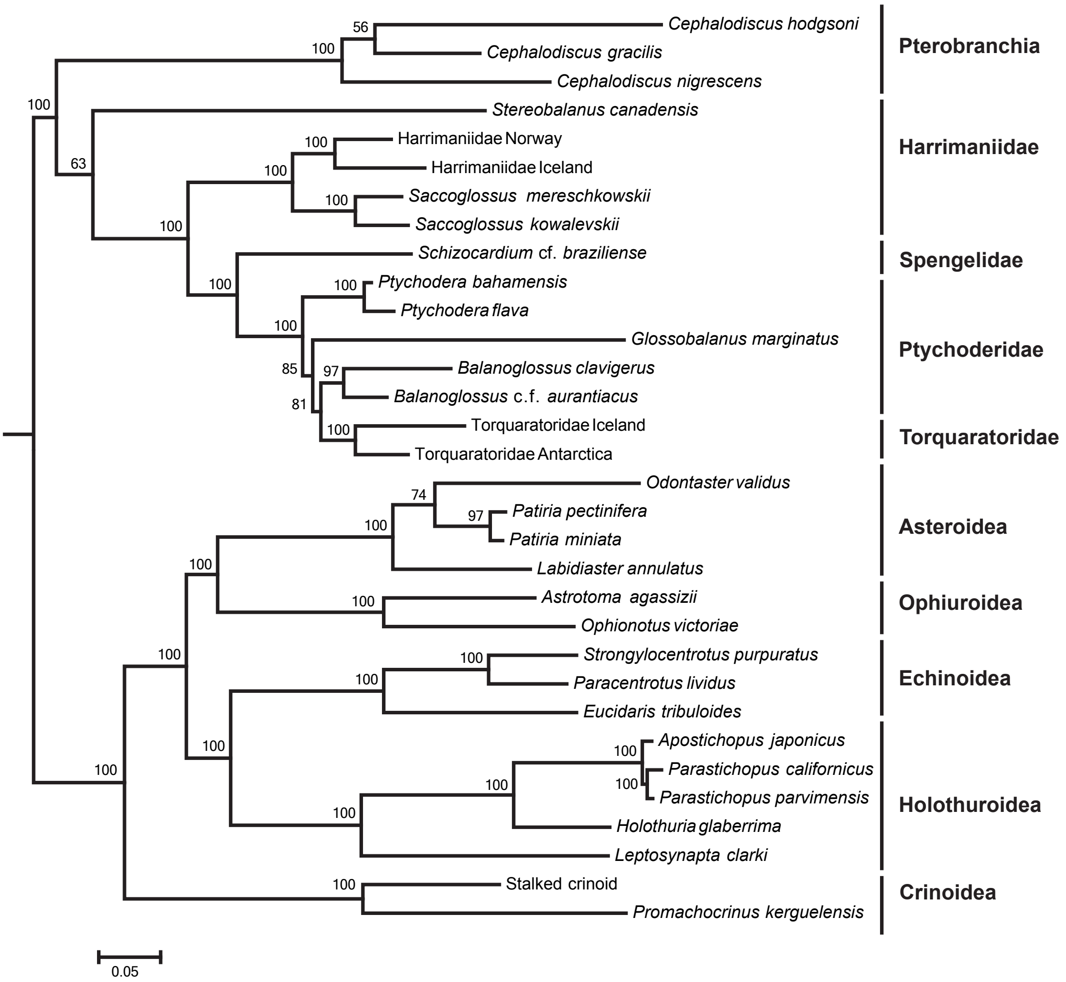

Figure 5. Ambulacrarian phylogeny based on 185 genes, excluding *Rhabdopleura* spp. Maximum likelihood tree shown, with bootstrap values indicated at nodes.

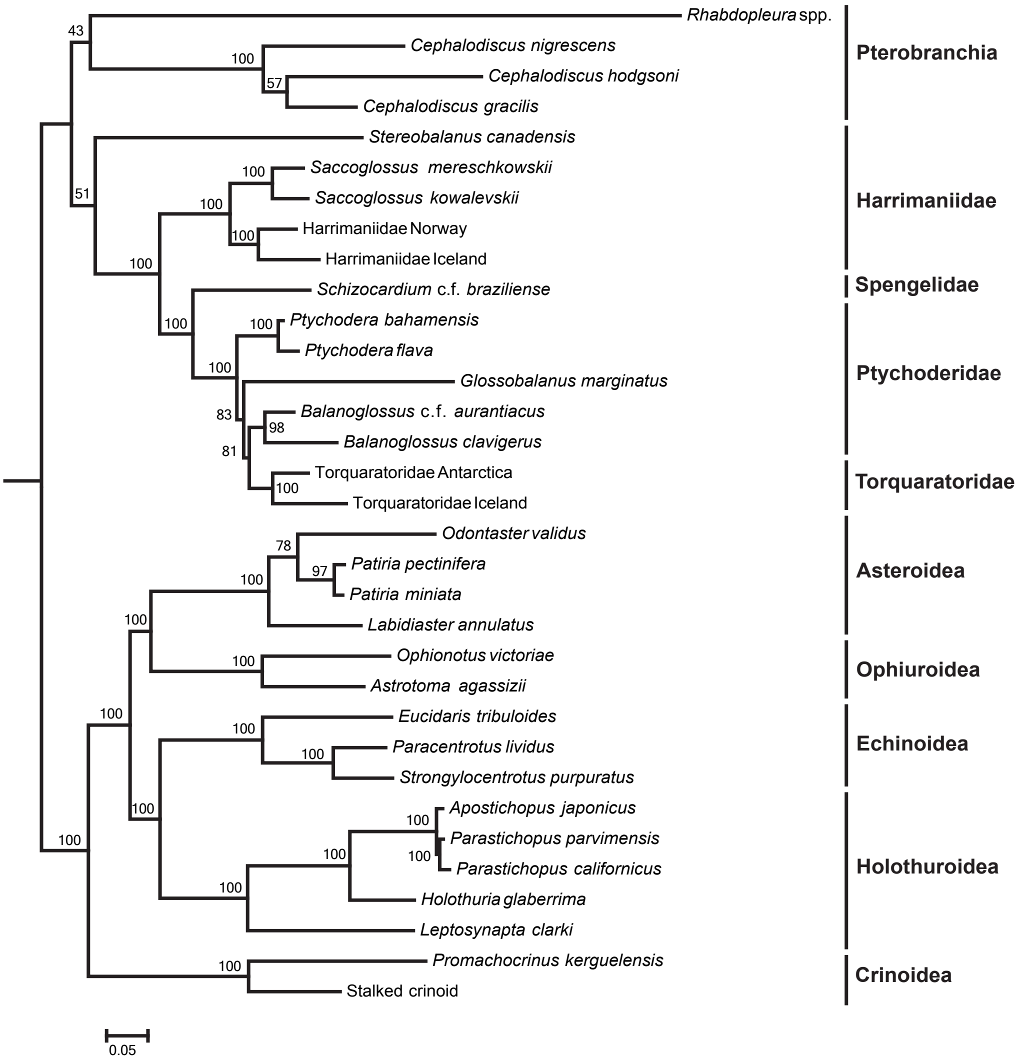

Figure 6. Ambulacrarian phylogeny based on 185 genes, all taxa represented.

Maximum likelihood tree shown, with bootstrap values indicated at nodes.

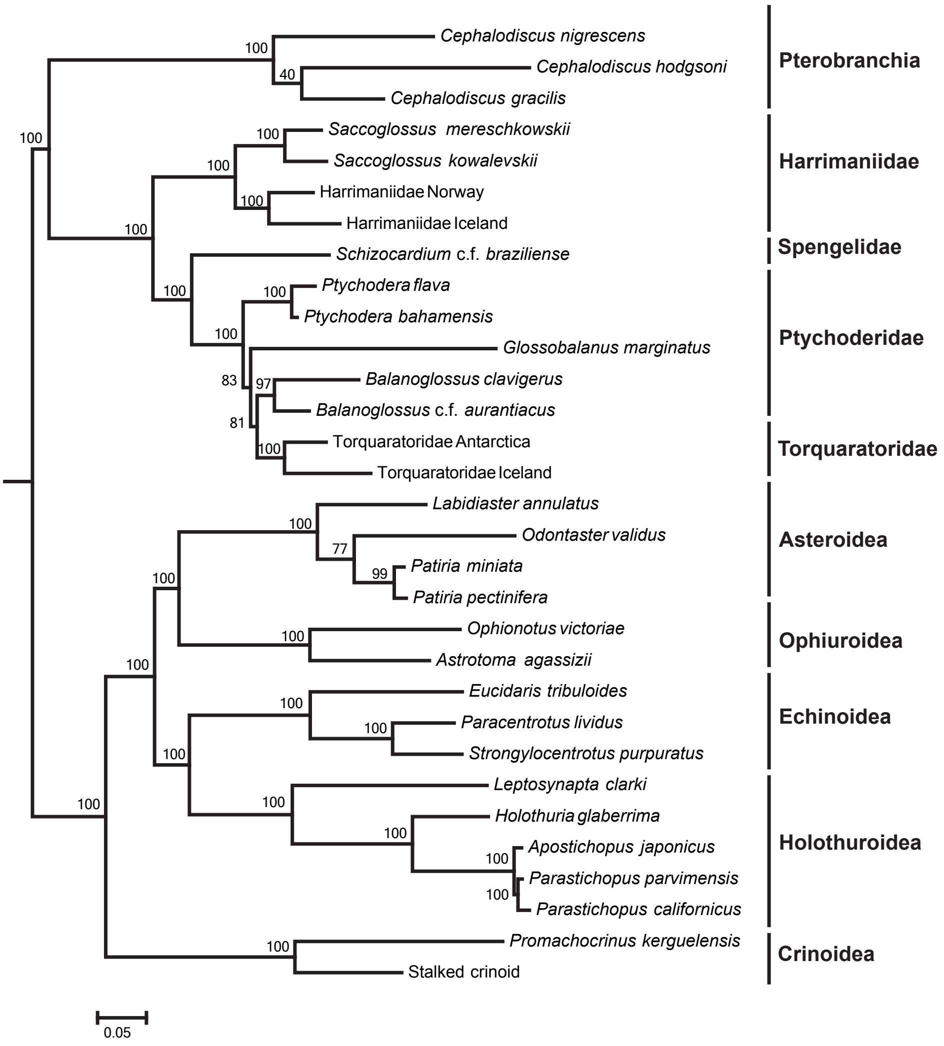

Figure 7. Ambulacrarian phylogeny based on 185 genes, *Stereobalanus canadensis* and *Rhabdopleura* spp. excluded. Maximum likelihood tree shown with bootstrap values indicated at nodes.

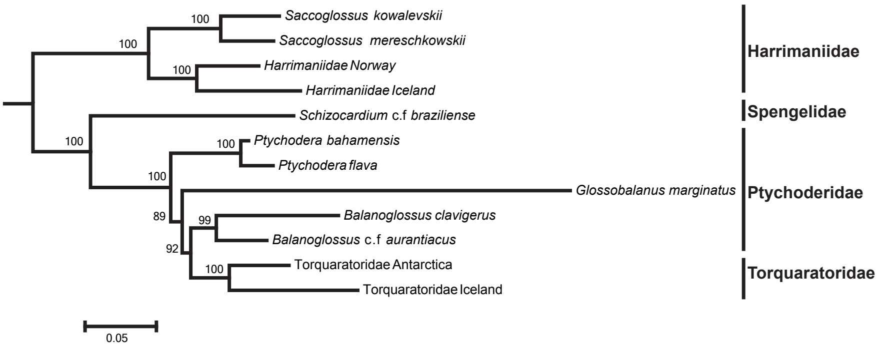

Figure 8. Phylogeny of Enteropneusta based on 185 genes. Maximum likelihood tree shown with bootstrap values indicated at nodes.

Chapter 5. Conclusions and future directions

5.1 General summary

Since the late nineteenth century, hemichordates have been central to hypotheses of chordate and deuterostome evolution, yet their own evolutionary history has received little attention. The preceding chapters have shed considerable light onto questions of hemichordate evolution and relationships. Chapter 2 provides evidence that Saxipendiidae is not a valid family, Torquaratoridae is allied to Ptychoderidae, and supports enteropneust paraphyly. In Chapter 3, we uncover a globally distributed clade of undescribed cold-water enteropneusts, and provide evidence for previously unrecognized enteropneust diversity in the deep sea and Antarctica. Phylogenomic evidence in Chapter 4 suggests that Pterobranchia and Enteropneusta are reciprocally monophyletic taxa, and Torquaratoridae is likely nested within Ptychoderidae. Lastly, within Echinodermata, the sister group to hemichordates, we find support for Asterozoa (Ophiuroidea + Asteroidea).

5.2 Enteropneust diversity

Since publication of dissertation Chapter 2 (Cannon et al., 2009), two series of papers focused on hemichordate systematics have been published. Torquaratoridae has increased from a single species in 2005 to 7 species in 5 genera today (Holland *et al.*, 2005, 2009, 2012a,b; Smith *et al.*, 2005; Osborn *et al.*, 2011, 2013; Priede *et al.*, 2012). An unfinished monograph, initiated around the turn of the last century by W.E. Ritter (1856-1944), including work by T.H. Bullock (1915-2005) and K.P. Rao (dates unknown), has finally seen print, with a series of papers describing 16 new species and 3 new genera (Deland *et al.*, 2010; Cameron *et al.*, 2010, 2013; Cameron & Perez, 2012).

Despite this renewed interest, hemichordate diversity remains underestimated, and many areas of hemichordate taxonomy remain unclear.

Based largely on phylogenetic results from Cannon et al. (2009), Deland et al. (2010) formally dissolved Saxipendiidae, and placed *Saxipendium coronatum* within Harrimaniidae. A second species of *Saxipendium* was recently described, *Saxipendium implicatum* Holland et al. (2012). The holotype of this species was collected at 3034 meters, and the authors suggest that torquaratoridae is the sole enteropneust family to have diversified in the deep sea, while other taxa may have migrated there in isolated instances (Holland et al. 2012). Results from Chapter 3 suggest that this may not the case. Subclades 3 and 4 of our novel harrimaniid clade were all collected at greater than 1500 meters, in the Atlantic and Pacific. Minute acorn worms in this group or others are likely to be more broadly distributed in the deep-sea. Biogeographic hypotheses based on current knowledge of large-bodied enteropneusts are missing what may be a major component of hemichordate biodiversity, and are likely subject to taxonomic artifacts.

In addition to the cold-water clade of harrimaniids, we collected three undescribed members of Torquaratoridae. In Chapter 2, we discuss the possibility that Torquaratoridae is nested within Ptychoderidae, a result that has been refuted by molecular phylogenies including more torquaratorid taxa based on 18S and 16S (Osborn et al., 2011; Worsaae et al., 2012; Cannon et al., accepted). However, phylogenomics results strongly support placement of torquaratorid taxa within ptychoderidae (Chapter 4). Torquaratoridae and Ptychoderidae both have hepatic caecae and genital wings, but torquaratorids lack gill bar synapticules, an apparent apomorphy of Ptychoderidae. They also have a reduced or absent proboscis skeleton and the stomochord is either absent or disconnected from the buccal cavity of the collar. If torquaratorids are within Ptychoderidae, these represent secondary losses. Additionally, although little is known about the reproductive biology of torquaratorids, some information has become available. *Allapasus aurantiacus* has been shown to possess externalized ovaries (Holland et al. 2012), and newly described *Coleodesmium karaensis* (Osborn et al., 2013) broods its embryos. Specimens collected in Antarctica were also shown to brood embryos (See Chapter 4, Figure 2). Unlike *C. karaensis*, these broods appeared to contain multiple developmental stages. These Antarctic specimens await description, and characterization of development seen in their brooded embryos is needed.

An important next step for this work will be morphological species descriptions of the undescribed taxa from Chapter 3. Morphological descriptions coupled with molecular sequence data are especially important for taxa such as enteropneusts, where specimens are easily damaged during collection, and are often sparsely distributed, with many collections consisting of a single incomplete specimen. Although the state of hemichordate systematics and taxonomy has improved with recent revisions, there are still major challenges. Most acorn worms are impossible to identify to species level without histological sectioning, and external morphology is often only sufficient to place specimens into families. There are very few living taxonomic experts, and for most researchers, DNA sequencing will provide a more tractable and reliable means of identification.

However, with few exceptions, notably within Torquaratoridae, and *Meioglossus psammophilus* (Holland *et al.*, 2009; Osborn *et al.*, 2011, 2013; Priede *et al.*, 2012; Worsaae *et al.*, 2012), recent revisions of enteropneust taxonomy have not included molecular data (Deland *et al.*, 2010; Cameron *et al.*, 2010, 2013; Cameron & Perez, 2012). Many of species described from Bullock's historical work have not been seen since their original collections, some of which date as far back as 1899. Local population extinction, habitat loss, or unknown seasonal changes may be affecting our ability to relocate these species. Thus, synthesis between molecular and morphological studies on enteropneust taxonomy will be a sustained effort, largely dependent on fortuitous collections, such as the recent rediscovery of *Glandiceps abyssicola* (Holland et al., 2013).

5.3 Pterobranch placement

As the amount of available sequence data for pterobranch taxa has increased, both in terms of number of characters and number of taxa, support for pterobranch and enteropneust reciprocal monophyly has also increased. Our results in Chapter 2 support pterobranch placement within Enteropneusta based on 18S data alone for pterobranchs. However, SH tests (but not Bayes factors) suggest that enteropneust monophyly is not significantly worse than the most likely tree. In Chapter 3, we were able to add mitochondrial 16S sequences for three species of *Rhabdopleura*, and recover monophyletic Pterobranchia and Enteropneusta. Again, however, the alternative hypothesis (enteropneust paraphyly in this case) was not significantly worse based on hypothesis testing. We take a phylogenomics approach in Chapter 4, and with 185 genes, we find consistently strong support for enteropneust monophyly. SH tests reject the alternative hypothesis of acorn worm paraphyly.

Although enteropneust and pterobranch reciprocal monophyly is supported in our phylogenomic analyses, congruence from additional sources of data will be important to validate this result. Genomic characters such as non-coding ultraconserved genomic elements (UCEs) (Faircloth et al., 2012), rare genomic changes, and gene/genome duplications could prove useful for this question. Preliminary genomic sequencing has been performed for pterobranch *Cephalodiscus hodgoni* using Illumina HiSeq, as well as enteropneusts *Schizocardium* c.f. *braziliense*, *Glossobalanus marginatus*, *Stereobalanus canadensis*, and a member of the novel harrimaniid clade from Norway using Illumina MiSeq. We plan to annotate mitochondrial genomes from these taxa, and employ gene order and mtDNA sequences as additional sources of phylogenetic data.

If further studies support enteropneust and pterobranch monophyly, pterobranchs cannot be considered a derived acorn worm offshoot, and must come to the forefront of research efforts to understand hemichordate ancestral states. Development and genetic regulation of body plan evolution has been extensively studied in enteropneusts (e.g., Luttrell et al., 2012; Miyamoto and Wada, 2013; Nomaksteinsky et al., 2009; Röttinger and Martindale 2011) and echinoderms (see McClay, 2011 for review). Within enteropneusts in particular, developmental expression of transcription factors involved in nervous system and body plan patterning have shown remarkable similarities to chordates (Lowe et al., 2003, 2006; Miyamoto and Wada, 2013; Nomaksteinsky et al., 2009). Sato et al. (2009) conducted the first gene expression study on *Rhabdopleura compacta*, and found that *hedgehog* in *Rhabdopleura* is expressed in a dramatically different pattern than in *Saccoglossus kowalevskii.* No similar studies have been conducted in *Cephalodiscus*. I have collected specimens of *Cephalodiscus densus* and *Cephalodiscus nigrescens* (including numerous larvae) from Antarctica, as well as *Cephalodiscus gracilis* and

Rhabdopleura normani from Bermuda in order to pursue this line of work in the future.

5.4 Hemichordates within Deuterostomia

Chapter 4 isolated Ambulacraria for phylogenomic analyses in order to generate robust phylogenetic hypotheses for Hemichordata and Echinodermata. In addition to results within Hemichordata discussed above, I find strong support for Asterozoa, suggesting that features uniting Cryptosyringida [such as enclosed radial components of the adult water vascular system, pluteus larvae, construction of the jaw apparatus (Smith, 1984), and neurulation of radial nerve (Heinzeller and Welsh, 2001)] are convergence or secondary losses in Asteroidea. A saccate gut and adambulacral ossicles are Asterozoan synapomorphies (Janies, 2001). These results have clarified relationships within Ambulacraria, but there are major remaining questions within deuterostome phylogeny.

One primary uncertainty is the position of Xenoturbellida and Acoelomorpha. To address this question, genomic sequencing of both groups is currently in progress. I will be directly involved in this work as a postdoctoral researcher with Dr. Ulf Jondelius at the Swedish Museum of Natural History. My postdoctoral work will involve assembly and annotation of Acoelomorph genomes, as well as phylogenomic analyses. We will take a comparative approach to search for genomic signatures suggesting protostome or deuterostome affinities for acoelomorphs, hopefully clarifying the position of this enigmatic group.

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