PHYLOGENETICS OF APLANULATA (CNIDARIA: HYDROZOA) AND THE EVOLUTION AND DEVELOPMENT OF *ECTOPLEURA LARYNX*

BY

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Submitted to the graduate degree program in Ecology and Evolutionary Biology and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

The model organism *Hydra* belongs to the hydrozoan clade Aplanulata. Despite being a popular model system for diverse fields of biological research, the morphology and development of *Hydra* are atypical of most hydrozoans. For example, most hydrozoans develop gonophores (structures housing gametes) on the body of the polyp, or release free-swimming medusae that spawn in the water column. In contrast, *Hydra* produce no gonophores or medusae and instead form gametes directly in the epithelia of the body column. Additionally, *Hydra* embryos are difficult to isolate for developmental studies (embryos encyst and are thus difficult to study), so there is currently no model species in Aplanulata for examining gene expression in developing polyps. In this dissertation, I examine the phylogenetic relationships of Aplanulata and the clade Capitata *sensu stricto*, originally thought to group with Aplanulata, and examine the evolution and development of the Aplanulata species *Ectopleura larynx*. This close relative of *Hydra* is ideally suited for evolutionary developmental studies because it develops directly in brooding structures, and produces attached gonophores. Because *Ectopleura larynx* broods on the body of the polyp, its juveniles and gonophores are easily procured for gene expression and developmental studies. My examination of *Ectopleura larynx* development reveals a unique type of colony formation that has never before been described in Hydrozoa in that *Ectopleura larynx* colonies form through sexual reproduction followed by epithelial fusion of offspring polyps to adult colonies. I characterize the expression of the *paired*-like homeobox gene *manacle* to determine polyp-colony boundaries, and suggest that stalks beneath the neck of *Ectopleura larynx* polyps do not have polyp identity and instead are specialized structures that interconnect polyps (stolons). Lastly, I characterize the canonical Wnt pathway in *Ectopleura larynx*, and examine its role in axial patterning of polyp and gonophore structures. My results are consistent with the Wnt pathway playing a role in patterning oral structures of the polyp and gonophore, and suggest that changes in expression patterns of Wnt pathway genes could explain the sexually-dimorphic morphologies of male and female gonophores of *Ectopleura larynx*, and the truncation of medusa development in this species.

INTRODUCTION

The integration of phylogenetic and developmental information in order to better understand the evolution of morphological complexity is a pivotal part of understanding organismal evolution. This dissertation integrates phylogenetic, character mapping, developmental and gene expression data to understand the evolution of morphology in the clade Aplanulata. I accomplish this by resolving phylogenetic relationships, exploring the evolution of developmental characters in a phylogenetic context, and then investigating some of the genes putatively involved in the evolution of morphology in a key Aplanulata taxon, *Ectopleura larynx*.

A phylogenetic framework for Aplanulata

Interpreting gene expression data in an evolutionary context requires a robust phylogeny of both the genes studied as well as the taxa of interest. The first two chapters of this dissertation provide well-sampled phylogenies of two hydrozoan clades in order to confirm taxonomic membership and lay the foundation for future evo-devo studies. While capitate hydrozoans (aka, Capitata *sensu* Petersen 1990) were originally described as containing most non-filiferan species, recent studies demonstrate that this clade is not monophyletic, and that instead, two well supported but non-sister clades, Capitata *sensu stricto* and Aplanulata exist (Collins, Schuchert, Marques *et al*. 2006, Collins, Winkelmann, Hadrys *et al*. 2005, Cartwright, Evans, Dunn *et al*. 2008). The freshwater model organism *Hydra* falls within the latter (Collins, Winkelmann, Hadrys *et al*. 2005). **Chapter 1** of this dissertation provides a well-sampled phylogeny of the hydrozoan sub-order Capitata, previously thought to belong with Aplanulata, and an update of the systematics of the most speciose family in this clade, Corynidae. This chapter has been published in the journal

Zoologica Scripta (Nawrocki, Cartwright and Schuchert 2010). **Chapter 2** of this dissertation investigates the phylogenetic relationships of Aplanulata. In this study, I provide the most well-sampled phylogeny of Aplanulata to date, solidify the placement of *Hydra* in Aplanulata, and discusses the morphology of the component families of Aplanulata. Lastly, I provide taxonomic recommendations for two Aplanulata species. This chapter is currently in preparation for submission for publication.

Colony development of *Ectopleura larynx*

Chapter 3 of this dissertation presents a character mapping and developmental study of colony formation in the Aplanulata species *Ectopleura larynx*. In this chapter, I examine the evolution of coloniality in Aplanulata, and demonstrate that coloniality has re-evolved in the Aplanulata genus *Ectopleura*. I then examine the ontogeny of colony formation, and find that re-evolved colonies in *Ectopleura* are unique in that they form through offspring settlement on and fusion to adult colonies. I show that this behavior is correlated with a number of life history characters. Lastly, I use the expression of a developmental regulatory gene, *manacle*, to examine polyp-colony boundaries in *Ectopleura larynx*. This chapter is in press at *Current Biology* and will shortly be published.

Developmental gene expression of *Ectopleura larynx*

One aspect that Chapter 3 revealed is that direct development and the reduction of gonophores both play a key role in the evolution of coloniality in Aplanulata. Understanding the genetic mechanisms patterning polyps and gonophore structures is vital to understanding their evolution. Wnt proteins are cysteine-rich secreted proteins that are part of a signaling pathway that is

implicated in larval formation and/or metamorphosis in the hydrozoans *Hydractinia echinata* (Duffy, Plickert, Kuenzel et al. 2010, Muller, Frank, Teo et al. 2007, Plickert, Jacoby, Frank et al. 2006), *Clytia hemaespherica* (Momose, Derelle and Houliston 2008) and *Hydra vulgaris* (Broun, Gee, Reinhardt *et al*. 2005, Guder, Pinho, Nacak *et al*. 2006, Hobmayer, Rentzsch, Kuhn *et al*. 2000, Lengfeld, Watanabe, Simakov *et al*. 2009, Philipp, Aufschnaiter, Özbek *et al*. 2009). Thus, the canonical Wnt signaling pathway serves as a good candidate pathway for examining the genetic mechanisms behind polyp and gonophore development in the Aplanulata species *Ectopleura larynx*.

Chapter 4 of this dissertation characterizes key elements of the canonical Wnt signaling pathway in *Ectopleura larynx*. Using next-generation sequencing technologies, I isolated *Wnt3*, *Wnt5*, the Wnt receptor *Frizzled1*, a secreted frizzled related protein (*SFRP*), *β-catenin*, Tcf and *GSK3β*. I then used whole-mount *in situ* hybridization and Quantitative Real-Time-PCR (qRT-PCR) to examine the expression of genes in the canonical Wnt signaling pathway during polyp and gonophore development. Expression patterns suggest that members of the Wnt pathway specify and maintain oral structures in *Ectopleura larynx* polyps, and are also involved in patterning in the different morphologies displayed in male and female gonophores. This work also reveals that a Wnt inhibitor may play a role shifting the expression of canonical *Wnt3* in gonophore development, and suggests that downregulation of the canonical Wnt pathway may be responsible for medusa loss in *Ectopleura larynx*. Lastly, I find evidence for separate roles for *Wnt5* and *Wnt3* in specifying and maintaining (respectively) developing axes in *Ectopleura larynx* polyps and gonophores. This chapter is currently in preparation for submission for publication.

Appendix

As the first three of chapters of this dissertation are formatted for publication, the process necessitated the exclusion of some data and methods from the main text. This information has been instead moved to the Appendix at the end of the document.

CHAPTER 1: Phylogenetic relationships of Capitata *sensu stricto*

ABSTRACT

Generic- and family-level classifications in Hydrozoa have historically been problematic due to limited morphological characters for phylogenetic analyses and thus taxonomy, as well as disagreement over the relative importance of polyp versus medusa characters. Within the recently redefined suborder Capitata (Cnidaria: Hydrozoa: Hydroidolina), which includes 15 families and almost 200 valid species, family-level relationships based on morphology alone have proven elusive, and there exist numerous conflicting proposals for the relationships of component species. Relationships within the speciose capitate family Corynidae also remain uncertain, for similar reasons. Here, we combine mitochondrial 16S and nuclear 18S, and 28S sequences from capitate hydrozoans representing 12 of the 15 valid capitate families, to examine family-level relationships within Capitata. We further sample densely within Corynidae to investigate the validity of several generic-level classification schemes that rely heavily on the presence/absence of a medusa, a character that has been recently questioned for its generic-level utility in classification. We recover largely congruent tree topologies from all three markers, with 28S and the combined dataset providing the most resolution. Our study confirms the monophyly of the redefined Capitata, and provides resolution for family-level relationships of most sampled families within the suborder. These analyses reveal Corynidae as paraphyletic and suggest that the limits of the family have been underestimated, thus contradicting available generic-level classification schemes for Corynidae. Classification schemes for Corynidae have been largely based on reproductive characters such as the presence/absence of a medusa, yet our results suggest that these are not valid generic-level characters for the clade. We suggest a new taxonomic structure for the lineage that includes all members of the newly redefined Corynidae, based on molecular and morphological synapomorphies for recovered clades within the group.

INTRODUCTION

The classically defined suborder Capitata is a large and diverse clade of hydrozoans that includes 26 families and 375 valid species. Capitata has been the subject of a number of classic morphological studies (e.g. Rees 1957; Petersen 1990) as well as a more recent molecular phylogenetic analysis (Collins *et al*. 2005). Capitata lacks obvious morphological synapomorphies, and molecular phylogenetic analyses question the monophyly of the clade (Collins 2002; Collins *et al*. 2005, 2006). These studies instead suggest that capitate hydrozoans comprise two well supported clades, Aplanulata (Collins *et al*. 2006) and non-aplanulate capitates, or Capitata *sensu stricto* (Cartwright *et al*. 2008). This second clade, herein defined as the new meaning of 'Capitata' and given equal weight to Aplanulata, is the subject of this analysis.

Members of the newly redefined clade Capitata are morphologically diverse. This group comprises roughly 15 families, and includes members with floating pelagic colonies, species with free-swimming medusae or fixed gonophores, as well as tropical and sub-tropical species possessing skeletonized, upright colonies. Capitata includes the family Corynidae, which is the most speciose family within the clade. To date, marker resolution, limited sampling, and the lack of obvious morphological synapomorphies have precluded the resolution of family-level relationships within Capitata. Additionally, recent molecular phylogenetic studies have provided evidence that this clade may not conform to traditional views of capitate taxonomy, and that some capitate families may be polyphyletic (Collins *et al*. 2005, 2006). These molecular studies find little evidence for classic family-level groupings, such as many of those proposed by

Petersen (1990) and suggest the need for a comprehensive study to further investigate capitate relationships.

Generic limits within some capitate families have been similarly difficult to disentangle. Corynidae is a capitate family whose members inhabit numerous shallow-water marine environments. Previous morphological analyses did not find convincing synapomorphies for the family, and suggest it may be polyphyletic (Schuchert 2001). A recent molecular phylogenetic study lends support to this hypothesis (Collins *et al*. 2005), and furthermore suggests that the three major corynid genera, *Sarsia* Lesson, 1843, *Dipurena* McCrady, 1859, and *Coryne* Gaertner, 1774 are polyphyletic. Grouping hydrozoans into appropriate genera is challenging in clades whose members lack a sufficient number of taxonomically informative morphological characters (Brinckmann-Voss 1970; Schuchert 2001, 2005; Collins *et al*. 2005). This is exacerbated in clades where the frequent reduction or disappearance of the medusa in some taxa further limits available characters for taxonomy, and has led to conflicting classification schemes (Russell 1953; Rees 1957; Naumov 1969; Brinckmann-Voss 1970; Petersen 1979, 1990; Bouillon 1985; Schuchert 2001; Bouillon *et al*. 2006).

Early generic-level classification schemes for corynid hydrozoans relied primarily on the presence/absence of a medusa, as well as on the presence/absence of two types of tentacles in the polyp stage (Stechow 1923; Russell 1953). However, rearing experiments of Picard (1960) and Brinckmann-Voss (1970) showed that tentacle types of the polyp are not reliable characters for delimiting corynid genera. Picard (1960) and Naumov (1969) merged all or most corynid species into the single genus *Coryne*, while Brinckmann-Voss (1970) followed a two genera system.

Coryne was reserved for species with sessile gonophores while *Sarsia* was reserved for species with free medusae. Later authors (e.g. Millard 1975; Bouillon 1985) added the genus *Dipurena,* separating species from *Sarsia* based on a single character: the number of gonads (Schuchert 2001). Bouillon (1985) further expanded the classification by adding *Bibrachium* Stechow, 1919, *Dicodonium* Haeckel, 1879, and *Sarsiella* Hartlaub, 1907 (Figure 1).

Classification schemes based on fixed versus free medusae have been rejected by many hydrozoan taxonomists (e.g. Levinsen 1893; Kramp 1935; Petersen 1979). Recent molecular phylogenetic analyses have additionally demonstrated that hydrozoan reproductive characters are labile amongst closely related species (Cunningham & Buss 1993; Govindarajan *et al*. 2006; Leclère *et al*. 2007, 2009, Nawrocki *et al*., in review), suggesting that classification schemes reliant on the criterion of medusa reduction are not necessarily congruent with the evolutionary history of some hydrozoan lineages. Brinckmann-Voss (1970) recognized that establishing a generic-level classification scheme based on fixed versus free medusae alone was not ideal for Corynidae, but pointed out that the lack of available characters for generic classification limited the establishment of a classification scheme based on other criteria.

Petersen recognized the problem of relying on fixed versus free medusae as a generic separator, and in his 1990 classification attempted to redefine corynid genera with other characters. His generic subdivision of Corynidae places species with both free medusae and reduced medusae within the genera *Coryne* and *Sarsia* (Petersen, 1990). His study relies on both polyp and medusa characters, including the shape of the manubrium and placement of gametes in the

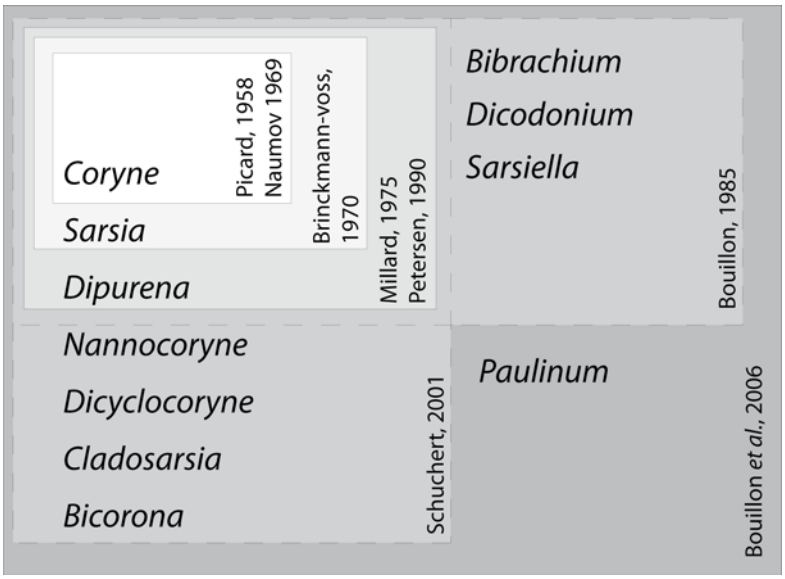

Figure 1. Major classification schemes for Corynidae proposed to date.

medusa, and the arrangement and structure of tentacles and reproductive elements on the polyp (Petersen 1990).

In contrast with Bouillon's 1985 classification, Petersen maintained a three-genus system (*Coryne, Sarsia,* and *Dipurena*). The three major corynid genera he redefined, *Dipurena*, *Sarsia*, and *Coryne*, contain the bulk of the species of the Corynidae (Figure 1). In redefining these genera, Petersen synonymized *Cladosarsia* Bouillon, 1978 with *Dipurena* and the genus *Bicorona* Millard, 1966 with *Dicyclocoryne* Annandale, 1915, but followed Bouillon (1985) in keeping *Dicyclocoryne* in a separate family (Dicyclocorynidae). Petersen's analysis represents an important step in developing a unified and phylogenetically congruent classification scheme for corynid hydroids, although his choice of characters has been questioned (Schuchert 1996, 2001). Despite this, Petersen's ideas for classifying Corynidae have gained acceptance and largely remain in use today, with some modification (e.g. Bouillon *et al*., 2006).

Schuchert (2001) examined Corynidae in detail and modified Petersen's classification scheme by returning members of Dicyclocorynidae to Corynidae, and by examining and resolving some species of uncertain affinity (Figure 1). However, he refrained from establishing a new classification scheme for Corynidae, due to the inability of available morphological characters to resolve the family, and concluded that the collection of molecular data was necessary to resolve the clade (Schuchert 2001).

In their recent synthesis of the classification of Hydrozoa, Bouillon *et al*. (2006) largely adopted classification scheme for Corynidae presented by Schuchert (2001), which includes the genera

Coryne, *Dipurena*, *Sarsia*, *Cladosarsia*, *Bicorona*, *Dicyclocoryne*, and *Nannocoryne*. Within Corynidae, *Coryne*, *Dipurena*, and *Sarsia* comprise the vast majority of all Corynidae (currently about 44 valid species of a total about 51), with the remaining three comprising a small number of species. However, Bouillon *et al*. additionally included genera in their classification that Schuchert describes as doubtful or indeterminate but that Bouillon (1985) included within Corynidae (*Bibrachium* Stechow, 1919; *Dicodonium* Haeckel, 1879; *Paulinum* Brinckmann-Voss & Arai, 1998; and *Sarsiella* Hartlaub, 1907. Figure 1 summarizes the major taxonomic classification schemes proposed for Corynidae to date.

Despite the rich taxonomic history that Corynidae embodies, a consensus on corynid classification has not been established based on morphological characters alone. In this study, a molecular phylogenetic approach, which included a comprehensive sampling of capitate taxa, was performed in order to provide further insight into family-level capitate relationships. In addition, given the conflicting hypotheses regarding the status of Corynidae (Collins *et al*., 2006) and its component genera (Schuchert 2001), a detailed sampling of Corynidae species was included in this study to further test for monophyly, and investigate whether the currently used genera *Coryne, Dipurena*, and *Sarsia* as delimited by Petersen's 1990 scheme are valid clades. We provide an alternative suggestion for generic classification for Corynidae based on our results.

MATERIALS AND METHODS

Taxonomic sampling and marker selection

Sampling for this study aimed for both representation of the diversity of capitate families, as well as dense sampling of the species-rich family Corynidae. With one exception, corynid species were identified as listed in Schuchert (2001), a classification based on the morphological phylogenetic analysis of Petersen (1990). *Coryne tricycla* Schuchert, 1996 is here used in its new combination, despite that it was transferred to the genus *Bicorona* by Schuchert (2001). Recent molecular analyses (Collins *et al*. 2005; Schuchert 2005) demonstrate that this species is clearly related to *Coryne* in the sense of Petersen (1990). Duplicate species were included if they were from different localities in order to determine if there were genetic differences between populations. A list of taxa sampled for this study can be found in Table 1. Three separate markers were chosen for this study: the small subunit nuclear ribosomal gene, 18S (SSU), the large subunit nuclear ribosomal gene, 28S (LSU), and the large subunit mitochondrial ribosomal gene, 16S (Table 2).

Table 1. Sampled Taxa. New sequences are in bold. New classification suggested by this study indicated in parenthesis.

Family	Genus	Species	Locality	16s	18s	28s	VOUCHER
Asyncorynidae	*Asyncoryne*	*ryniensis*	Japan	**EU876552**	**EU876578**	**GQ424289**	KUNHM 2639
Cladocorynidae	*Cladocoryne*	*floccosa*	Spain, Mallorca	AY512535			MHNG INVE29808
Cladocorynidae	*Cladocoryne*	*floccosa*	Brazil		EU272608	EU272551	A.Lindner: AL1407
Cladonematidae	*Cladonema*	*californicum*			AF358085		
Cladonematidae	*Cladonema* (now *Staurocladia)*	*radiatum*	Italy; Island of Elba	AM088482	EU448096	**GQ424290**	
Cladonematidae	*Cladonema*	sp.		AM088484			MHNG INVE37640
Cladonematidae	*Eleutheria*	*claparedii*	France, Roscoff	AM088486	**GQ424320**	**GQ424292**	MHNG INVE49494

Cladonematidae	*Eleutheria*	*dichotoma*	Mediterrannean	AM088485	**GQ424321**	**GQ424291**	MHNG INVE34228
Cladonematidae	*Eleutheria*	*dichotoma*	Australia; Sydney Harbor	AM159500			MHNG INVE37416
Cladonematidae	*Staurocladia*	*bilateralis*	Japan	AY512537			
Cladonematidae	*Staurocladia* (now *Cladonema)*	*oahuensis*	Japan	AY512536			
Cladonematidae	*Staurocladia*	*vallentini*	New Zealand	**GQ395332**	**GQ424322**	**GQ424293**	
Cladonematidae	*Staurocladia*	*wellingtoni*	New Zealand	AY787882	**GQ424323**	**EU879948**	MHNG INVE25379
Corynidae	*Coryne*	*tricycla*	New Zealand	AJ608640			H-641, NIWA Wellington
Corynidae	*Coryne* (now *Stauridiosarsia)*	*cliffordi*	Canada, British Columbia	**GQ395313**	**GQ424324**	**GQ424294**	MHNG INVE36025
Corynidae	*Coryne*	*epizoica*	Italy; Island of Elba	**GQ395314**		**GQ424295**	MHNG INVE37171
Corynidae	*Coryne*	*eximia*	South Africa	AJ878713	**GQ424325**	**GQ424296**	MHNG INVE34009
Corynidae	*Coryne*	*eximia*	France, Atlantic	AY512541			
Corynidae	*Coryne*	*fucicola*	France, Roscoff	AM084259	**GQ424326**		MHNG INVE36328
Corynidae	*Coryne*	*japonica*	New Zealand	AY512540			MHNG INVE27293
Corynidae	*Coryne*	*muscoides*	France, Roscoff	AJ878689	**GQ424327**	**GQ424297**	
Corynidae	*Coryne*	*muscoides*	France, Atlantic	**GQ395315**	**GQ424328**	**GQ424298**	
Corynidae	*Coryne*	*muscoides*	France, Roscoff	AY512553	AY920761		
Corynidae	*Coryne* (now *Stauridiosarsia)*	*nipponica*	Japan, Seto Marine Station	**GQ395316**	EU448096	EU305530	KUNHM 2627
Corynidae	*Coryne* (now *Stauridiosarsia)*	*nipponica*	Japan, Okinawa	**GQ395333**	**GQ424329**	**GQ424299**	
Corynidae	*Coryne*	*pintneri*	France, Villefranche	AJ878717	**GQ424330**	**GQ424300**	MHNG INVE31976
Corynidae	*Coryne*	*pintneri*	France, Marseille	AJ878718		**GQ424301**	
Corynidae	*Coryne* (now *Stauridiosarsia)*	*producta*	Norway, Raunefjord	**GQ395316**	**GQ424331**	**GQ424302**	MHNG INVE48751
Corynidae	*Coryne* (now *Codonium)*	*prolifera*	France, Roscoff	**GQ395318**		**GQ424303**	MHNG INVE49490
Corynidae	*Coryne*	*pusilla*	France, Roscoff	AY787874			MHNG INVE29386
Corynidae	*Coryne*	*pusilla*	Scotland, Firth of Lorn	AY512552		**GQ424304**	MHNG INVE35756
Corynidae	*Coryne*	*pusilla*	Korea		Z86107		
Corynidae	*Coryne*	*uchidai*	Japan	**GQ395319**		**GQ424305**	KUNHM 2809
Corynidae	*Coryne*	*uchidai*	Japan, Oshoro	**GQ395320**	**GQ424332**	**GQ424306**	MHNG INVE49102
Corynidae	*Dipurena* (now *Stauridiosarsia)*	*gemmifera*	France, Roscoff	**EU876547**	**EU876573**	**EU879945**	
Corynidae	*Dipurena*	*halterata*	France, Villefranche	AM084261	**EU883544**	**EU883550**	MHNG INVE31741
Corynidae	*Dipurena* (now *Stauridiosarsia)*	*ophiogaster*	Japan, Shirahama harbor	EU305473	EU272615	EU272560	KUNHM 2803
Corynidae	*Dipurena* (now *Stauridiosarsia)*	*ophiogaster*	France, Banyuls	AJ878721		**GQ424307**	MHNG INVE32963

Corynidae	*Dipurena* (now *Stauridiosarsia)*	*ophiogaster*	Japan		**GQ424333**		
Corynidae	*Dipurena* (now *Stauridiosarsia)*	*reesi*	Aquarium Zoo Basel	**GQ395321**	**GQ424334**	**GQ424308**	
Corynidae	*Dipurena* (now *Stauridiosarsia)*	*reesi*	Brazil	AY512546			
Corynidae	*Dipurena*	*simulans*	France, Roscoff	AY512547			
Corynidae	*Dipurena* (now *Stauridiosarsia)*	sp.	Japan	**GQ395331**	**GQ424335**	**GQ424309**	
Corynidae	*Sarsia*	*apicula*	Canada, British Columbia	**GQ395330**	**GQ424336**		MHNG INVE29806
Corynidae	*Sarsia*	*lovenii*	Iceland, Sandgerdi	AY787876			MHNG INVE29592
Corynidae	*Sarsia*	*lovenii*	Norway, Raunefjord	**GQ395329**	**GQ424337**	**GQ424310**	MHNG INVE48736
Corynidae	*Sarsia*	*lovenii*	Iceland, Sandgerdi	AJ608796			
Corynidae	*Sarsia* (now *Stauridiosarsia)*	*marii*	France, Mediterannean	AY512544			
Corynidae	*Sarsia*	*princeps*	Canada, British Columbia	**EU876549**	**EU876575**	**EU879947**	
Corynidae	*Sarsia*	*striata*	Scotland, Firth of Lorn	**GQ395328**	**GQ424338**	**GQ424311**	MHNG INVE35765
Corynidae	*Sarsia*	*tubulosa*	Scotland, Firth of Lorn	**EU876548**	**EU876574**	**EU879946**	MHNG INVE35763
Corynidae	*Sarsia*	*tubulosa*	Norway, Raunefjord	**GQ395327**	**GQ424339**	**GQ424312**	
Corynidae	*Sarsia*	*tubulosa*		AY512545			
Hydrocorynidae	*Hydrocoryne*	*miurensis*	Japan	**GQ395326**		**GQ424313**	KUNHM 2814
Hydrocorynidae	*Hydrocoryne*	*iemanja*	Brazil	**GQ389713**			
Milleporidae	*Millepora*	sp.		**EU876551**	AF358088	**EU879950**	
Moerisiidae	*Moerisia*	sp.	USA - California	AY512534	AF358083	AY920801	
Moerisiidae	*Moerisia*	*inkermanica*	Brazil		**GQ424340**		
Moerisiidae	*Odessia*	*maeotica*	France, Portiragnes	**GQ395324**	**GQ424341**	**GQ424314**	MHNG INVE53642
Pennariidae	*Pennaria*	*disticha*		AY512533	**EU883545**	**GQ424315**	
Pennariidae	*Pennaria*	*disticha*	Spain, Mallorca	AM088481	**GQ424342**	**GQ424316**	MHNG INVE29809
Pennariidae	*Pennaria*	*disticha*			AY920762		
Pennariidae	*Pennaria*	sp.			**GQ424343**		
Polyorchidae (now Corynidae)	*Polyorchis*	*haplus*	USA - California	AY512549 *	**GQ424344**	**GQ424317**	
Polyorchidae (now Corynidae)	*Polyorchis*	*penicillatus*	USA - Friday Harbor	AY512550	AF358090		
Polyorchidae (now Corynidae)	*Scrippsia*	*pacifica*	USA - California	AY512551	AF358091	AY920804	
Porpitidae	*Porpita*	*porpita*		AY935322	**GQ424319**	**EU883551**	
Porpitidae	*Porpita*	sp.	Guam	AY512529	AF358086	AY920803	
Porpitidae	*Velella*	sp.			AF358087	EU272597	
Porpitidae	*Velella*	*velella*	France, Villefranche	EU305487	**EU876576**	**EU879949**	
Solanderiidae	*Solanderia*	*ericopsis*	New Zealand	AY512530	EU272636	EU272593	MHNG INVE29593

Solanderiidae	*Solanderia*	*secunda*	Japan, Seto Marine Station	EU305484	EU305502	EU305533	KUNHM 2611
Solanderiidae	*Solanderia*	*secunda*	South Korea		AJ133506		
Sphaerocorynidae	*Sphaerocoryne*	*agassizi*	Florida	**GQ395323**		**GQ424318**	
Sphaerocorynidae	*Sphaerocoryne*	*bedoti*	Panama	**GQ395322**			KUNHM 2787
Zancleidae	*Zanclea*	*costata*	France, Mediterannean	AY512531	**EU876579**	**EU879951**	MHNG INVE26507
Zancleidae	*Zanclea*	*prolifera*	Japan, Seto Marine Station	EU305488	EU272639	EU272598	KUNHM 2793
Zancleidae	*Zanclea*	*sessilis*	Spain, Mallorca	AY512532			
OUTGROUPS							
Tubulariidae	*Ralpharia*	*gorgoniae*	Panama, Bocas del Toro, Crawl Cay	EU305482	EU272633	EU272590	KUNHM 2778
Eudendriidae	*Eudendrium*	*californicum*	USA, California, Monterey Bay, Scott Creek	EU305476	EU305492	EU305513	KUNHM 2816
Ptilocodiidae	*Hydricthella*	*epigorgia*	Japan, Hazema, Takane	EU305478	EU272622	EU272569	KUNHM 2665
Hydractiniidae	*Hydractinia*	sp.	USA, California	EU305477	EU305495	EU305518	KUNHM 2876
Oceaniidae	*Turritopsis*	sp.	Japan, Seto Marine Station	EU305486	EU305504	EU305538	KUNHM 2817
Melicertidae	*Melicertum*	*octocostatum*		EU305479	AY920757	EU272575	USNM 1073342

* Different specimen from same locality amplified

Table 2. Primer profiles

Target Gene	Primer Pair	Sequences	Reference
28s - A Fragment	F63Mod	5'-ACCCGCTGAAYTTAAGCATATHANTMAG-3'	Medina *et al*. (2001)
	R2077	5'-GAGCCAATCCTTWTCCCGARGTT-3'	
	F97	5'-CCYYAGTAACGGCGAGT-3'	Evans *et al*. (2008) and
	R2084	5'-AGAGCCAATCCTTTTCC-3'	Cartwright *et al*. (2008)
28s – B Fragment	F1383	5'-GGACGGTGGCCATGGAAGT-3'	Evans *et al*. (2008) and
	R3238	5'-SWACAGATGGTAGCTTCG-3'	Cartwright *et al*. (2008)
18s	18sA	5'-AACCTGGTTGATCCTGCCAGT-3'	Medlin *et al*. (1988)
	18sB	5'-TGATCCTTCCGCAGGTTCACCT-3'	
16s	F2	5'-TCGACTGTTTACCAAAAACATAGC-3'	Cunningham & Buss (1993)
	R2	5'-ACGGAATGAACTCAAATCATGTAAG-3'	

DNA extraction, PCR amplification and sequencing

DNA was extracted from polyp or medusa tissue using a CTAB protocol established by Coffroth *et al*. (1992), a standard phenol-chloroform procedure (available on request), or a Qiagen DNeasy Tissue kit following manufacturer's instructions (QIAGEN Inc., Mississauga, ON). A 600-bp fragment of 16S was amplified following Cunningham & Buss (1993), an 1,800-bp fragment of 18S was amplified following Medlin *et al*. (1988), and a 3,200 bp fragment of 28S was amplified following Evans *et al*. (2008) (Table 2). Purification and direct sequencing of PCR products were conducted by Cogenics, Inc. (Houston, TX). Sequencher v4.5 was used for contig assembly and editing (GeneCodes 2005). Mesquite was utilized for concatenation and matrix editing (Maddison & Maddison 2007).

Sequence alignment and model selection

Preliminary DNA alignments were generated with MUSCLE (**MU**ltiple **S**equence **C**omparison by **L**og-**E**xpectation) (Edgar 2004a, b). The alignments were then adjusted by hand in Seaview version 2.4 (Galtier *et al*. 1996) according to secondary structure models based on either *Hydra*

for 16S (Dunn *et al*. 2005), or all of Cnidaria for 18S and 28S (M. S. Barbeitos, *pers comm*). The final alignments were end-trimmed to remove characters missing in the majority of taxa. Model testing of the alignments was conducted in PAUP v 4.0 (Swofford 2003) using Model Test (Posada and Crandall, 1998) and the suggested model following the AIC (Akaike Information Criterion) value (Akaike 1987) was chosen for each analysis. Final alignments are available on Treebase (ID SN4691-25510).

Phylogenetic analyses

Parsimony analyses were conducted for each gene and on the complete, concatenated dataset in the program TNT (Goloboff *et al*. 2000). In all cases, a New Technology search was conducted, with sectorial searching and tree fusing selected. For each dataset, a strict consensus was calculated and 1000 bootstrap replicates were generated to assess support.

Maximum likelihood analyses were conducted on the three gene datasets separately, and also on the partitioned and unpartitioned concatenated dataset in the parallel version of RaxML (Stamatakis *et al*. 2005). For the partitioned dataset, data was partitioned by gene. A GTRMIX model was applied to the dataset, and 500 (16S) or 1000 (18S, 28S and combined datasets) bootstrap replicates were generated. Replicates were summarized in PAUP v4.0 (Swofford 2003). Only taxa with at least two of the three markers available were included in the combined analysis, with the exception of *Moerisia inkermanica*.

Bayesian analyses were conducted on all three gene datasets separately, and also on the partitioned and unpartitioned concatenated dataset in the parallel version of MrBayes. Two runs

of 10 million generations with one heated chain and three cold chains were cued in MrBayes. Analyses for 16S, 18S, and 28S were allowed to run for 10 million generations. Convergence was assessed and analyses stopped for the partitioned dataset at approximately 6.6 million iterations, and for the unpartitioned dataset at approximately 4.9 million iterations. The burnin in each case was set to one million. Chain convergence and the presence of a sizeable effective sample size (ESS) was assessed in Tracer (Rambaut & Drummond 2007), and topological convergence was evaluated using AWTY (**A**re **W**e **T**here **Y**et) (Nylander *et al*. 2007). A Bayes factors test was conducted between the harmonic mean of the -lnL scores from the posterior distributions of the partitioned and unpartitioned analyses to determine preference for the partitioned versus unpartitioned dataset.

RESULTS

Taxon sampling, alignment, and analyses

A summary of taxon sampling, matrix length, and parsimony tree number and length can be found in Table 3. Our complete dataset included a total of 60 specimens representing 13 capitate families, 20 capitate genera, and 46 different capitate species. For this study we contributed 99 new capitate sequences, including sequences from species representing three previously unsampled families - Hydrocorynidae, Asyncorynidae, and Sphaerocorynidae - four previously unsampled genera - *Odessia*, *Hydrocoryne*, *Asyncoryne*, and *Sphaerocoryne* - as well as over 15 previously unsampled species (Table 1). To date, this is the largest sampling of capitates collected for molecular analysis.

ModelTest selected the GTR + Γ + I model for 16S, 18S, and 28S separately and for the concatenated dataset based on AIC criterion. This model was applied for all ML (GTRMIX) and Bayesian analyses. A Bayes Factors (BF) test confirmed preference for the partitioned dataset over the unpartitioned dataset for the Bayesian analyses.

Table 3. Data Summary

Marker	Positions	Number of taxa	Tree length (parsimony)	Number of most parsimonious trees
16S	594	75	2493	8
18S	1824	57	926	10
28S	3301	55	3353	5
Concatenated	5880	60	6462	1

Phylogenetics of Capitata

16S, 18S, 28S and the combined, concatenated dataset (Figures 2-5) recover Capitata as a well-supported clade using all three optimality criteria. Separate analyses are largely congruent, both between markers as well as between optimality criterion. Some relationships differ between the

16S and the combined topology (the placement of *Cladonema radiatum* and members of Sphaerocorynidae); however, the recovered 16S placement of these taxa is not well supported. In general, 16S recovered well-supported relationships toward the tips of the tree (with deeper relationships only supported by BPP), 18S recovered a limited number of lower-level nodes, and 28S and the combined, concatenated dataset recovered topologies with most nodes supported throughout the tree. Given that the combined topology represents the most complete sampling of our data and displays the largest number of well-supported nodes, we consider this our most robust hypothesis (Figure 5).

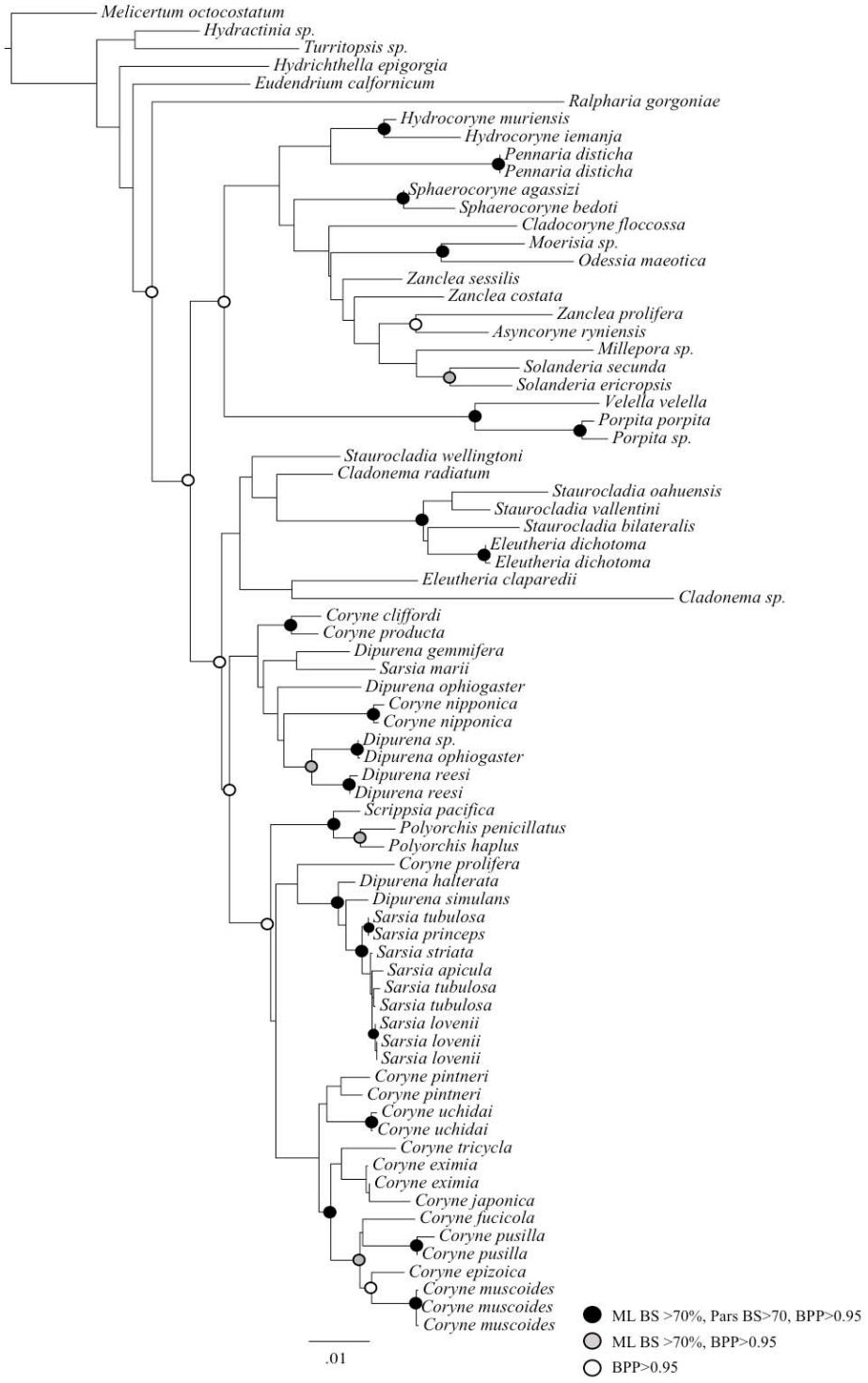

Figure 2. Hypothesis of relationships of Capitata based on mitochondrial 16S and run in RaxML under a GTR + Γ + I model.

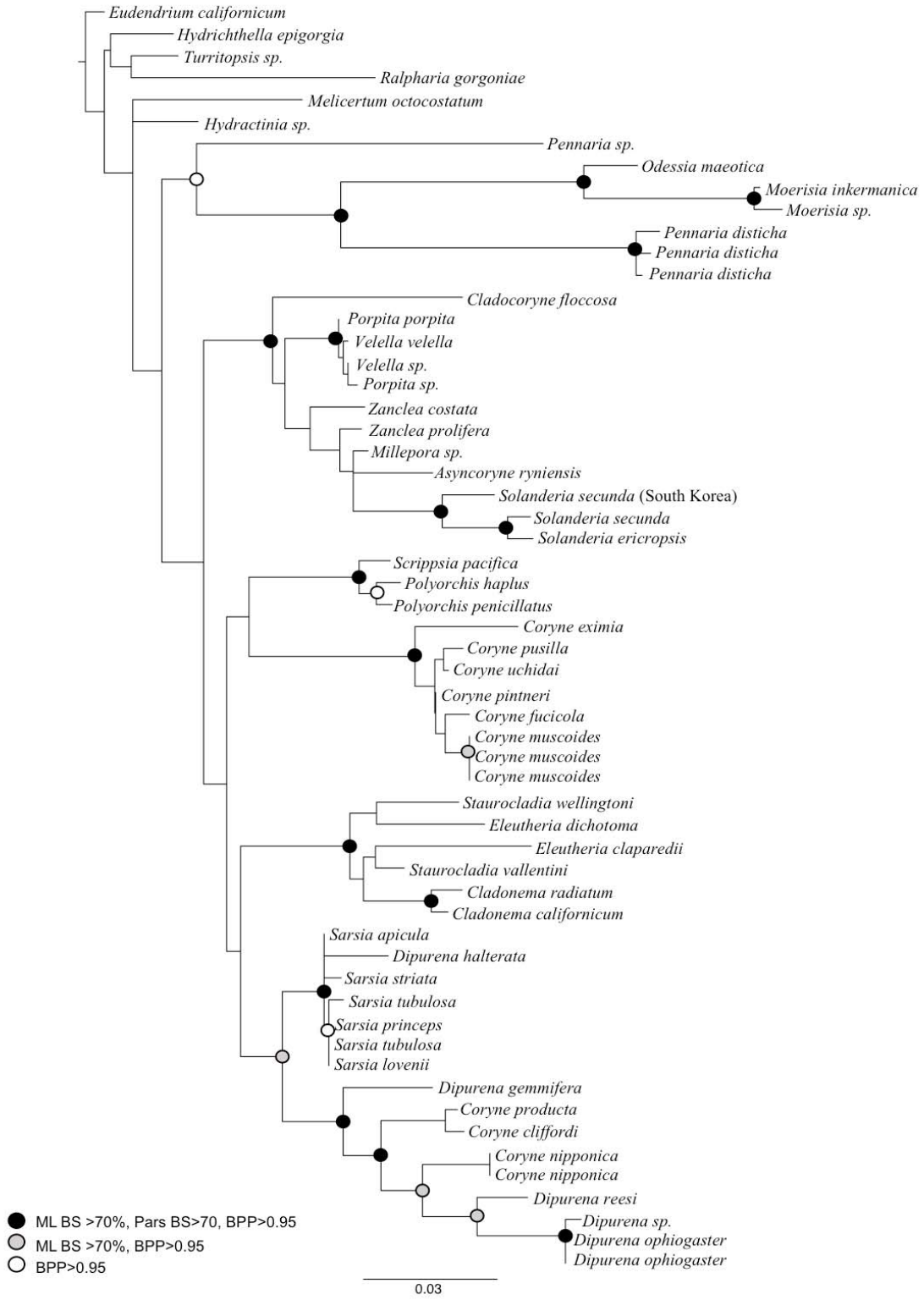

Figure 3. Hypothesis of relationships of Capitata based on 18S (SSU) and run in RaxML under a GTR + Γ + I model.

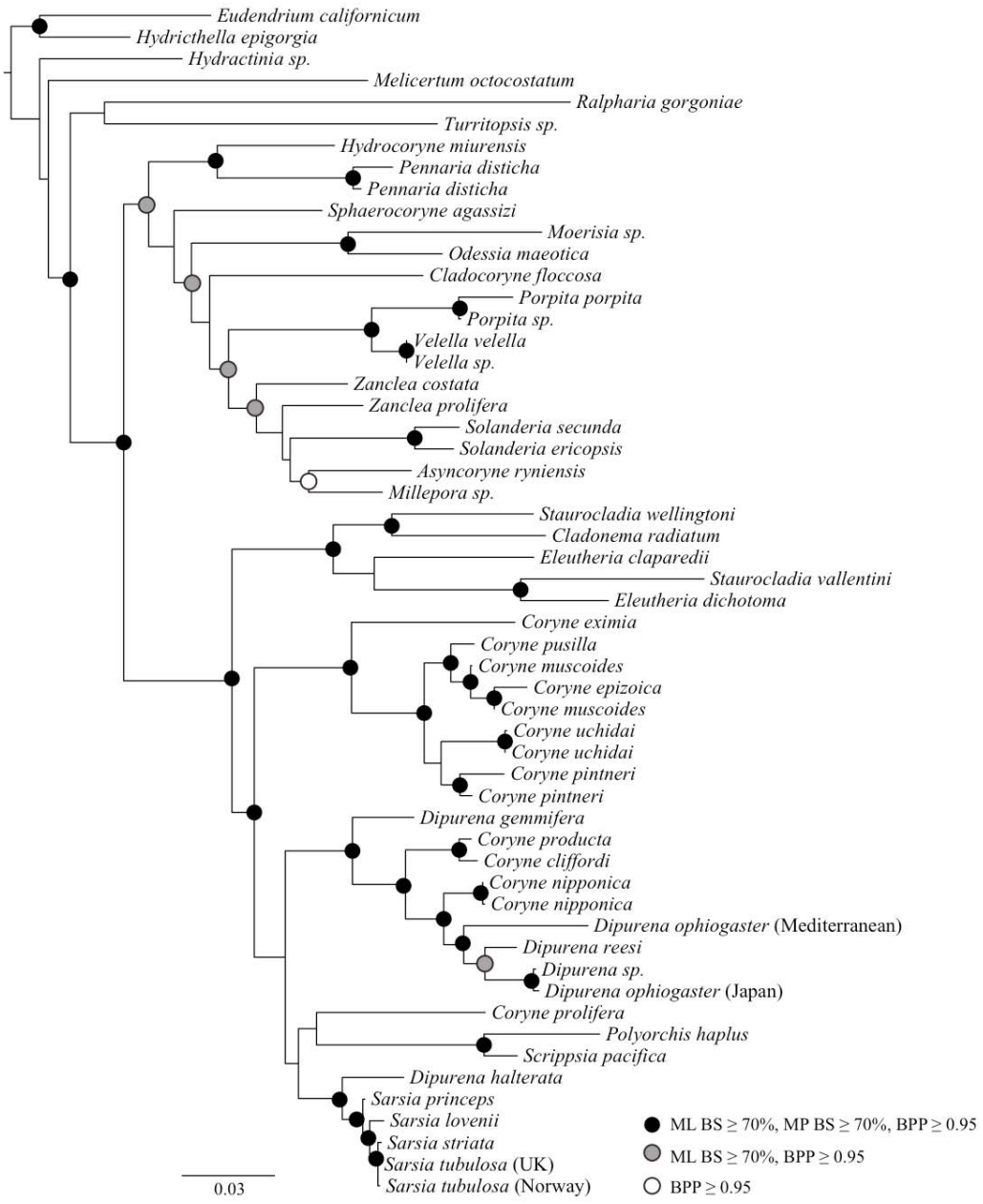

Figure 4. Hypothesis of relationships of Capitata based on 28S (LSU) and run in RaxML under a GTR + Γ + I model.

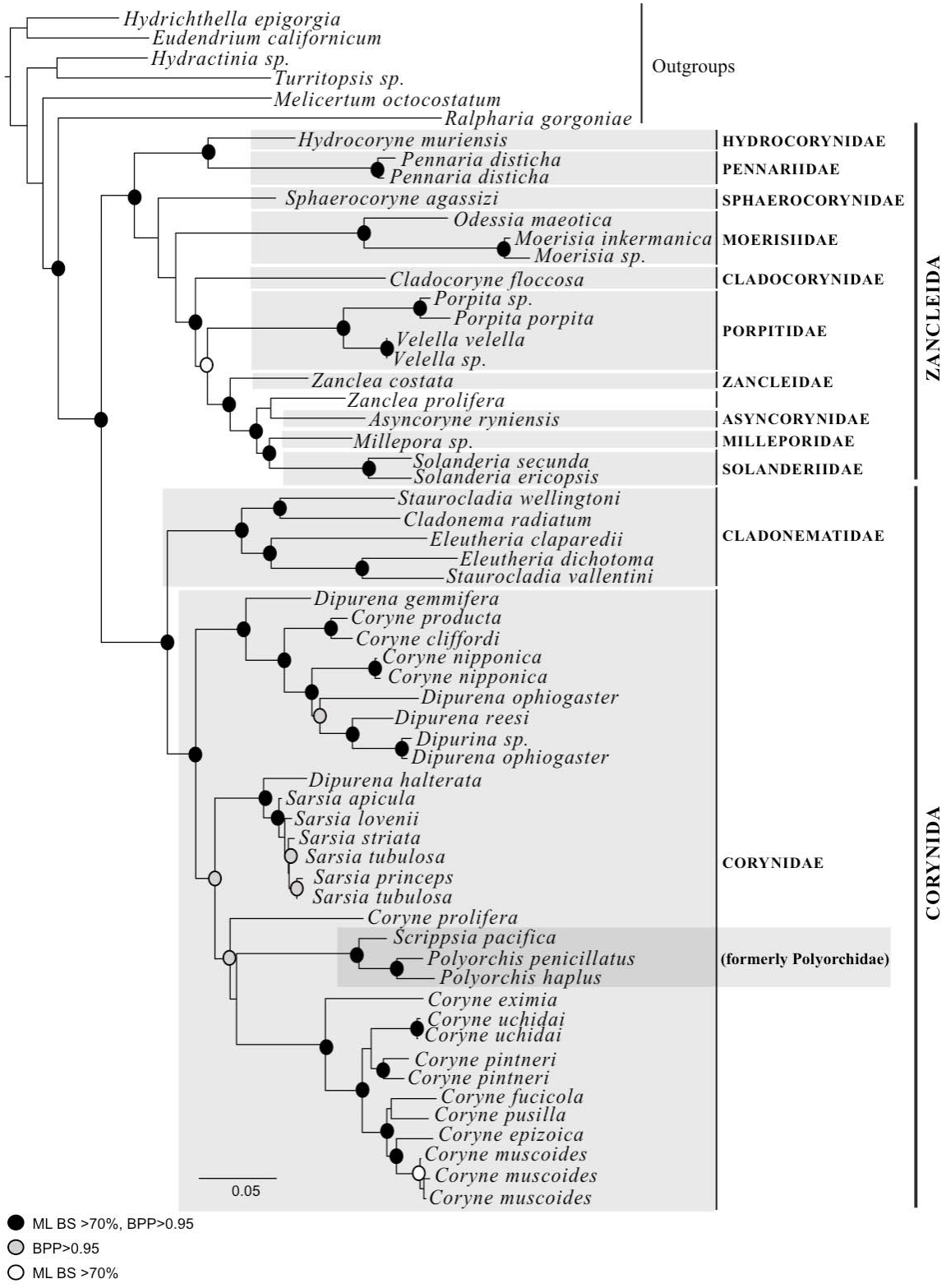

Figure 5. Combined topology of Capitata based on concatenated and partitioned 16S, 18S and 28S analysis. GTR + Γ + I model applied separately to each partition.

Our combined phylogeny of Capitata (Figure 5) questions classic relationships reliant on morphology (Petersen 1990) and is congruent with previous molecular phylogenetic analysis, while adding resolution at a number of nodes as well as including several previously unsampled families. Although the monophyly of Sphaerocorynidae, Asyncorynidae and Milliporidae could not be tested due to the inclusion of just one species, all other families, with the exception of Zancleidae and Corynidae were recovered as monophyletic with strong support. In addition, the phylogenetic relationships between the families were well resolved, except Moersiidae and Sphaerocorynidae, but they clearly belong to one of the two subclades of Capitata.

Within Capitata, there is a deep and well-supported split separating the clade Corynidae/Polyorchidae/Cladonematidae and the remaining sampled families within Capitata. Within the latter lineage, the families Porpitidae, Zancleidae, Asyncorynidae, Milleporidae, Cladocorynidae and Solanderiidae form a clade. There is also strong evidence that Pennaridae and Hydrocorynidae are sister families, as are Milleporiidae and Solanderidae. Within Corynidae/Polyorchidae/Cladonematidae clade, we recover Cladonematidae as sister to all else, and Polyorchidae nested within Corynidae. Two of the sampled genera within the Cladonematidae are polyphyletic (*Staurocladia* and *Eleutheria*), although sampling within this family is not dense enough to determine structural patterns.

Phylogeny of Corynidae

Corynidae is rendered paraphyletic by its inclusion of polyorchiid taxa (Figure 5). The exact placement of Polyorchidae within Corynidae is not well supported although it has a weakly

supported position within a clade of *Coryne* species. Strong support is recovered for most nodes within Corynidae (Figure 5), most of which challenge classic assumptions of relationships based on morphology (Figure 1). Of the three sampled genera, *Coryne* and *Dipurena* are polyphyletic and *Sarsia* forms a clade with *Dipurena halterata* as its sister taxon. Our results reject a Corynidae classification reliant on only two genera (*Sarsia* and *Coryne*, *sensu* Brinckmann-Voss 1970), a classification scheme reliant on three genera *Sarsia*, *Coryne*, and *Dipurena*, (*sensu* Millard 1975 & Bouillon 1985), as well as more recent classifications reliant on other generic limits (*sensu* Petersen 1990 and Bouillon *et al*. 2006) (Figure 1).

DISCUSSION

Phylogenetics of Capitata

The capitates are recovered as a well-supported monophlyetic group (Figure 5) and this clade, which excludes aplanulate taxa, should henceforward be used in the new meaning of Capitata. The combined and 16S topologies place *Ralpharia gorgoniae* (a member of Aplanulata) as the closest sampled outgroup to the capitates; however, the limited sampling of outgroups in this analysis does not allow for testing of the placement of Capitata within Hydroidolina. A recent higher-level analysis of Hydroidolina further indicates that the sister group relationship to Capitata is not resolved (Cartwright *et al*. 2008). We suggest that a synapomorphy for Capitata is the complex, cup-shaped ocelli found in many species (for the structure see Weber, 1980; Thomas & Edwards, 1991), although the structure appears to have been lost in the clade Zancleoida (see below). We recovered family-level relationships that are inconsistent with Petersen's 1990 hypothesis derived from a phylogenetic analysis of morphological data; however, our results are consistent with recent molecular phylogenetic analyses (Collins 2002; Collins *et al*. 2005, 2006). The approach of Petersen (1990) did not allow for the non-monophyly of Capitata (to the exclusion of aplanulate taxa) nor did it explicitly test for the phylogenetic status of its component genera.

Our analyses reveal two well supported clades of Capitata (Figure 5). The first of these is delimited by the families Porpitidae, Zancleidae, Asyncornidae, Milleporidae, Cladocorynidae and Solanderiidae. A similar association has been proposed by earlier authors and given different names: Pteronematoidea by Picard (1957), Zancleoidea by Bouillon *et al*. (1987), or Zancleida by Petersen (1990). We call this clade Zancleida (Figure 5). The scope of each of these groups differs slightly depending on the author but all basically unite a group of capitate hydroids that

possess macrobasic eurytele stinging capsules, with the exception of the Solanderiidae. Its placement within this clade indicates that members of Solanderiidae likely lost these capsules secondarily. Petersen (1990) associated the Solanderiidae with Corynidae, and Collins *et al*. (2005) associated Solanderiidae with Zancleidae and Moerisidae, although this grouping was not well supported and Milleporidae was not sampled. Our results unambiguously group the Solanderiidae with Zancleidae and Milleporidae. The sister relationship of Solanderiidae with the coral-like Milleporidae means that the large, erect colonies found in both groups is a likely a synapomorphy. Our combined analysis (Figure 5) recovers the genus *Zanclea* as paraphyletic. However, *Z. prolifera* has only been provisionally classified in the genus *Zanclea,* as its polyp stage is unknown (Uchida & Sugiura, 1976). We suggest that it could instead belong to the genus *Asyncoryne* due to its sister group relationship (Figure 5), or that one group gave rise to the other. *Asyncoryne* and *Zanclea* have identical medusae (Migotto *et al*. 1996; Bouillon *et al*. 2006) and differ only in the tentacles of their polyp stages. We find evidence for a sister relationship between Hydrocorynidae and Pennaridae, which has not been previously suggested. The second clade of capitates we recover is delimited by the families Cladonematidae, Corynidae, and Polyorchidae. We name this clade Corynida (Figure 5). Cladonematidae is the sister group to the Corynidae-Polyorchidae clade, an expected affiliation as the polyps of some Cladonematidae species are indistinguishable from Corynidae polyps (Schuchert 2001, 2006). Also, Petersen (1990) placed Cladonematidae as sister to Corynidae. A previous molecular study recovered Cladonematidae as polyphyletic and more closely related to the Zancleida than Corynidae, although this relationship, based solely on 16S data, was not well supported (Collins *et al*. 2005).

A previous study of Cladonematidae based on morphological characters has shown that the current generic-level subdivision within this family is problematic (Schuchert 2006), and a previous molecular study utilizing 16S rDNA proposed that the genus *Staurocladia* is polyphyletic (Collins *et al*. 2005). As predicted by both Schuchert (2006) and Collins *et al*. (2005), our molecular analyses renders the genus *Staurocladia* polyphyletic, grouping *Cladonema radiatum* with *Staurocladia wellingtoni,* and *Eleutheria* species with *Staurocladia vallentini* (Figures 2, 4, 5). The genus *Staurocladia* is separated from *Eleutheria* based on the number of nematocyst clusters on its tentacles: one for *Eleutheria*, more than one for *Staurocladia* (Browne & Kramp 1939; Brinckmann-Voss 1970; Schuchert 2006). Our trees suggest that this is not a valid character and that the genus may need to be redefined following further analyses with more taxa. Interestingly, Cladonematidae has two distinct subclades, one containing *Eleutheria* species and *S. vallentini* (all of which have a reduced mesoglea), and the other *C. radiatum* as well as *S. wellingtoni* (a species with a more developed umbrella). We suggest that in the interest of nomenclatural stability it is advisable to emend the diagnoses of the genera *Cladonema* and *Eleutheria* so that they correspond to the two evolutionary lineages found here. The diagnosis of *Cladonema* given by Schuchert (2006) corresponds *de facto* to this new view, while the diagnosis of *Eleutheria* can be simplified to include *Staurocladia* species that have tentacles with one or more nematocyst clusters. *Staurocladia*, the type species for which is *S. vallentini*, is thus synonymized with *Eleutheria*.

Phylogenetics of Corynidae

The second major goal of this study was to obtain a detailed phylogeny of Corynidae in order to evaluate current generic-level classification schemes; therefore, this family contributed the

majority of taxa in our analysis. In all analyses, we recover a paraphyletic Corynidae that includes the family Polyorchidae nested within it, here represented by the genera *Scrippsia* and *Polyorchis*. This result is not unexpected, as a recent phylogenetic analysis placed the Polyorchidae within Corynidae, though this relationship was not well-supported (Collins *et al*. 2005). The Polyorchidae (*sensu* Mills 2000; Bouillon *et al*. 2006) are only known from their medusa phase, which limits comparison with Corynidae. There is no apparent synapomorphy for the grouping of corynid and polyorchid species, and the medusae of both families are so distinct that it is difficult to provide a useful diagnosis for a family that comprises both of them. Currently, then, an emended diagnosis for Corynidae must be based on a phylogenetic definition alone: e.g. the least inclusive clade comprising *Coryne pusilla*, *Polyorchis penicillatus*, *Sarsia tubulosa*, and *Coryne producta*. (See Appendix).

Two genera that were previously included in the Polyorchidae, *Tiaricodon* and *Urashimea*, were recently placed back in the revised family Halimedusidae by Mills (2000). Despite this, the medusae of Halimedusidae and Polyorchidae are at least superficially rather similar (comp. Bouillon *et al*. 2006). The polyps of Halimedusidae are solitary, club-shaped, and with 4 to 5 long, capitate tentacles in one whorl (Mills 2000 for *Halimedusa*; Xu & Chen 1998 for *Tiaricodon*; Uchida & Nagao 1961 for *Urashimea*). Except for the non-colonial mode of life, they are thus identical to some polyp types of Corynidae and Cladonematidae. Another detail that links *Tiaricodon* with Corynidae is the juvenile *Tiaricodon* medusae are indistinguishable from juvenile corynid medusae (Schuchert 1996). Given these similarities, members of Halimedusidae likely belong to the Corynida, if not to Corynidae.

The recovered generic-level subdivisions of Corynidae are mostly incongruent with classification schemes that have been suggested to date. Although the genus *Sarsia* sensu Petersen (1990) was retrieved as monophyletic, the genera *Coryne* and *Dipurena sensu* Petersen were recovered as polyphyletic. Our topology furthermore rejects a generic-level subdivision based solely on the presence/absence of a medusa (Figure 6). The placement of *Sarsia marii* outside of the clade including all other *Sarsia* species is not surprising, as the species is incompletely described (the adult medusa is unknown) and it has been previously suggested that it may instead be more closely aligned to some *Dipurena* species (Schuchert 2001).

Based on the tree topology as well as supporting morphological characters, a generic-level structure emerges for Corynidae (Figure 6; See Appendix). *Sarsia sensu* Petersen is recovered as monophyletic and can retain its generic label and diagnosis, despite the placement of the incompletely described species *S. marii* in the 16S topology (see Schuchert 2001). *Scrippsia* and *Polyorchis* can be designated as genera belonging to Corynidae, although further sampling of this clade is needed to confirm the monophyly of the two genera as well as the exact placement of the two genera within the family.

The clade containing the bulk of the *Coryne* species is well supported and must retain the name *Coryne* [type species is *Coryne pusilla*]. This genus is well supported and includes members with reduced reproductive structures as well as species with free medusae (*Coryne eximia* and *Coryne japonica*). Most members of this clade share a distinct morphological synapomorphy: their gonophores arise in the upper axils of the tentacles, whereas in nearly all other Corynidae they are independent of the tentacles (comp. Schuchert, 2001). There are two exceptions to this

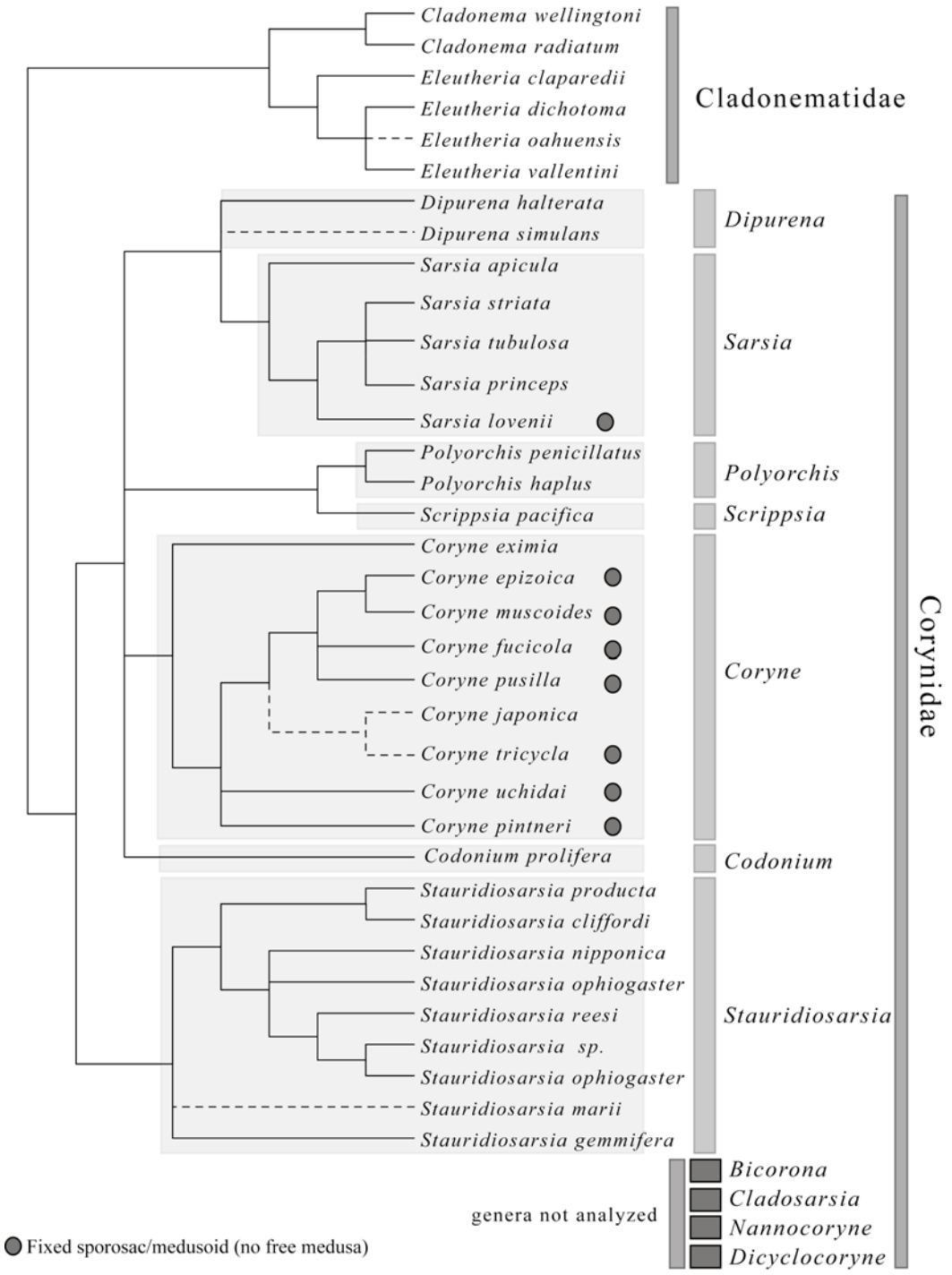

Figure 6. Phylogenetic relationships of genera within Corynide, and new classification (see Appendix). Dashed line indicates that the specimen was not included in the combined analysis.

as *Coryne tricycla* has the plesiomorphic state (Figure 2, and Schuchert 2005), and *C. japonica* is polymorphic.

The remaining *Coryne* species sampled in this analysis produce medusae and cluster either with some *Dipurena* species, or form their own lineage, like *C. prolifera*. The medusa of *Coryne prolifera* is unique in that it buds medusae on its tentacle bulbs. This apomorphy together with its isolated position in the tree justify a transfer to a separate genus. *Codonium* Haeckel, 1879 could be made available for it by designating *Codonium codonoforum* Haeckel, 1879 as the type species of the genus. *Codonium codonoforum* Haeckel, 1879 is a subjective synonym of *Sarsia prolifera* Forbes, 1848 (Mayer 1910; Schuchert 2001) (See Appendix).

Like *Coryne*, *Dipurena sensu* Petersen is also recovered as polyphyletic in our analysis. Some *Dipurena* species cluster with *C. producta* (here grouped in *Stauridiosarsia*), while *Dipurena halterata* emerges as sister to *Sarsia* (Figure 5). 16S analyses suggest that *Dipurena simulans* is also part of this clade (Figure 2). The available 16S sequence of *D. simulans* is rather short and the position of the species is not well resolved. Unfortunately, it was not possible to include the type species of the genus *Dipurena*, *D. strangulata,* in our molecular analysis. However, *D. strangulata*, *D. simulans* and *D. halterata* all possess very similar medusae with capitate tentacles and their polyps are associated with sponges (see Schuchert, 2001). These morphological and ecological similarities suggest that *D. strangulata* belongs to the same clade. This group thus retains the generic name *Dipurena*.

The association of *Coryne producta* and other similar species with a subset *Dipurena* species is unexpected and no morphological diagnosis is currently possible for this clade, which notably

contains medusae with long *Dipurena*-type and short *Coryne*-type manubria (cf. Petersen, 1990). The designated name for this genus is *Stauridiosarsia* Mayer, 1910 [type species *S. producta* by monotypy] (See Appendix).

In summary, we suggest that Polyorchidae be subsumed within Corynidae and the family Polyorchidae be disbanded, with the sub-family name Polyorchinae to refer to the grouping of the genera Scrippsia and Polyorchis.. The species within Corynidae will be distributed into the following genera: *Coryne*, *Codonium*, *Dipurena*, *Sarsia*, *Stauridiosarsia*, *Scrippsia*, and *Polyorchis* (Fig 6; see See Appendix for diagnoses). Future sampling of genera not included in this analysis (*Bicorona*, *Dicyclocoryne*, *Cladosarsia*, and *Nannocoryne*) may expand or alter these diagnoses. The current analyses includes a species that was once included in the genus *Bicorona* (*B. tricycla*, now *Coryne tryicycla*), but for a taxonomically correct evaluation of any genus, the type species of this genus must be examined. The type species, *Bicorona elegans* Millard 1966, has sporosacs in the upper axils of its tentacles and is thus potentially also a member of the genus *Coryne sensu stricto*.

This study did not include all species currently attributed to either *Coryne, Sarsia,* or *Dipurena* (see Schuchert 2001 or Bouillon *et al*. 2006 for a full listing). These species are best left in their current genus within Corynidae until their relationship has been tested by more thorough taxonomic sampling. Moreover, there exist a number of incompletely described Corynidae which cannot be attributed reliably to any of these clades (see Schuchert, 2001). We suggest including them provisionally in the genus *Coryne* pending further sampling.

CONCLUSION

We have provided the largest sampling of capitates to date and have made progress in resolving family-level relationships within this hydrozoan lineage. We designate this group of hydrozoans Capitata, and subdivide them into two clades, the Zancleida (*sensu* Petersen 1990) and Corynida. Furthermore, we have provided strong support for relationships within the well-studied family Corynidae, and suggest a new classification based on these phylogenetic relationships. Preliminarily, we propose a generic-level classification for Corynidae supported by both molecular and, in most cases, morphological characters. Although we provide molecular phylogenetic evidence for both family-level relationships within the new Capitata as well as lower-level relationships within Corynidae, future sampling is needed to uncover relationships between genera established here in order to further refine and stabilize classification of Corynidae.

CHAPTER 2: Phylogenetic placement of *Hydra* and the relationships of Aplanulata

ABSTRACT

The model organism *Hydra* belongs to the hydrozoan clade Aplanulata. Despite being a popular model system for development, little is known about the phylogenetic placement of this taxon or the relationships of its closest relatives. Previous studies have been conflicting regarding sister group relationships and have been unable to resolve deep nodes within the clade. In addition, there are several putative Aplanulata taxa that have never been sampled for molecular data or analyzed using multiple markers. Here, we combine the fast-evolving cytochrome oxidase 1 (CO1) mitochondrial marker with mitochondrial 16S, nuclear small ribosomal subunit (18S, SSU) and large ribosomal subunit (28S, LSU) sequences to examine relationships within the clade Aplanulata. We further discuss the relative contribution of four different molecular markers to resolving phylogenetic relationships within Aplanulata. Lastly, we report morphological synapomorphies for some of the major Aplanulata genera and families, and suggest new taxonomic classifications for two species of Aplanulata, *Fukaurahydra anthoformis* and *Corymorpha intermedia*, based on a preponderance of molecular and morphological data that justify the designation of these species to different genera.

INTRODUCTION

The model organism *Hydra* belongs to the hydrozoan clade Aplanulata (Collins, Schuchert, Marques *et al*. 2006, Collins, Winkelmann, Hadrys *et al*. 2005). Despite numerous studies on this species spanning as disparate fields as immunology (Bosch, Augustin, Anton-Erxleben *et al*. 2009), stem cell biology (Ambrosone, Marchesano, Tino *et al*. 1012, Hartl, Mitterstiller, Valovka *et al*. 2010) and evolutionary biology (Hemmrich, Anokhin, Zacharias *et al*. 2007, Martinez, Iniguez, Percell *et al*. 2010), little is known about the precise phylogenetic placement of *Hydra* or the relationships between major lineages of Aplanulata. Recent studies have begun to shed light on these relationships, particularly within the families Hydridae (Martinez, Iniguez, Percell *et al*. 2010) Tubulariidae (Marques and Migotto 2001) and Corymorphidae (Nawrocki and Cartwright *in press*, Cartwright and Nawrocki 2010). However there is little support for relationships between major lineages of Aplanulata, and many putative Aplanulata taxa have not been sampled or studied in a phylogenetic context with multiple markers.

Aplanulata comprises 8 families (Collins, 2006) and approximately 170 valid species (Daly, Brugler, Cartwright *et al*. 2007). These species demonstrate great morphologically diversity (Figure 1), inhabit several disparate ecological habitats, and display a wide variety of life cycles. Unlike members of all other major hydrozoan lineages, most Aplanulata species display a solitary, as opposed to a colonial, polyp stage (but see Nawrocki and Cartwright, *in press*). Species vary greatly in their morphology and habitats. The solitary polyps range in size from a few millimeters in length (i.e. *Hydra*) to over a meter in length (i.e. *Branchiocerianthus*). This clade includes species that inhabit

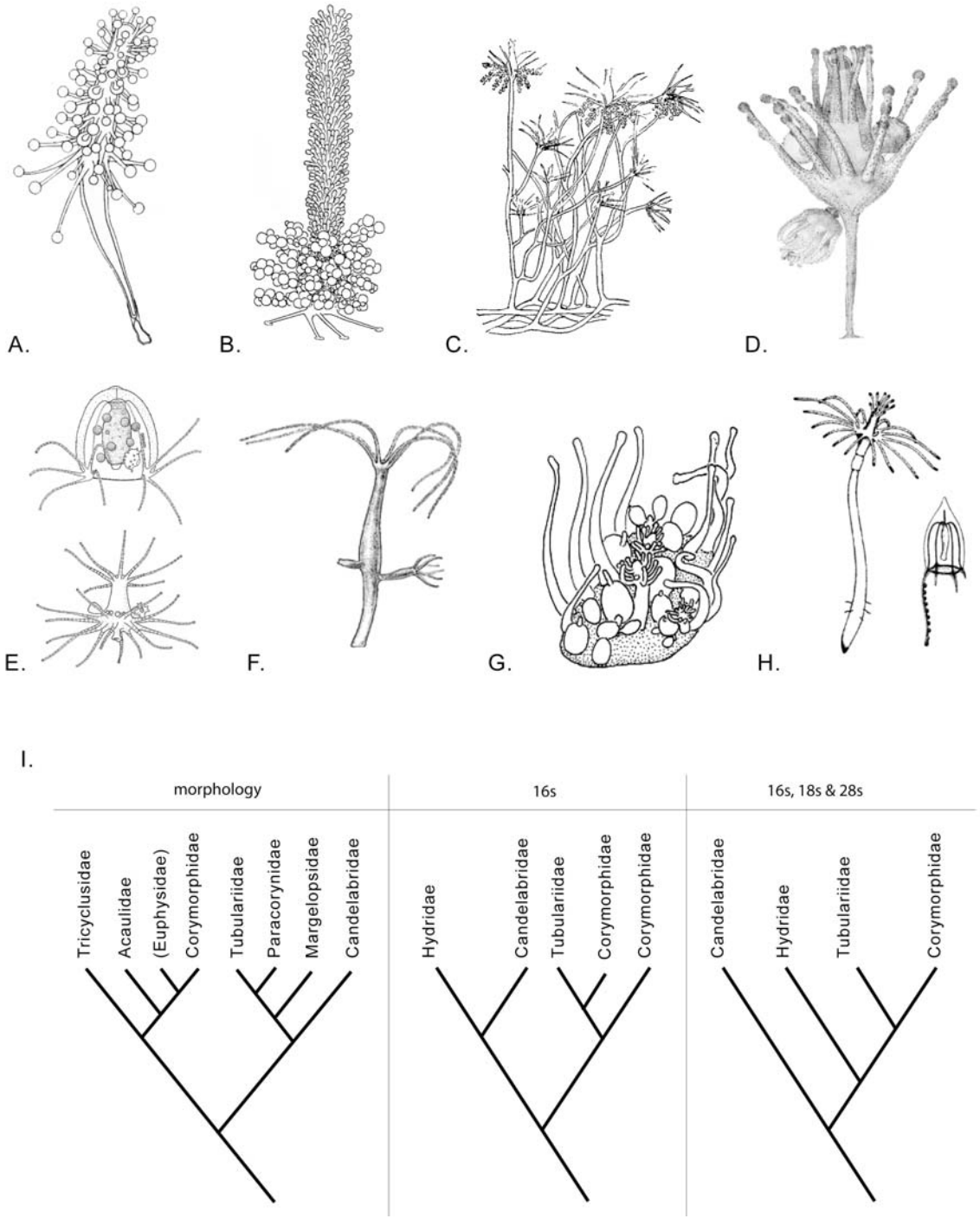

Figure 1**.** Eight Major families belonging to Aplanulata (Collins 2006) (A-H) and current hypotheses of Aplanulata relationships (I). A. *Acaulis ilonae*; Acaulidae. B. *Candelabrum phrygium*; Candelabridae. C. *Ectopleura crocea*; Tubulariidae. D. *Tricyclusa singularis*; Tricyclusidae. E. *Margelopsis haeckeli*; Margelopsidae. F. *Hydra* sp.; Hydridae. G. *Paracoryne huevi;* Paracorynidae. H. *Corymorpha bigelowi*; Corymorphidae. I. Recent morphological and molecular hypotheses of relationships of members of Aplanulata. Left: Hypothesis based on morphological data (Petersen 1990). Center: Hypothesis of

Aplanulata relationships based on mitochondrial 16S (Collins et al. 2005). Right: Hypothesis of Aplanulata relationships based on mitochondrial 16S, and nuclear 28S and 28S (Cartwright & Nawrocki 2010; Nawrocki & Cartwright *in press*). *Acaulis ilonae* modified from Brinckmann-Voss 1966; *Candelabrum phrygium* modified from Schuchert 2006; *Ectopleura crocea* modified from Hargitt 1901; *Tricyclusa singularis* modified from Schulze 1876. *Margelopsis haeckeli* modified from Schuchert 2006; *Paracoryne huevi* and *Hydra* images from Schuchert 2012, *Corymorpha bigelowi* modified from Sassman & Rees 1978.

cold and deep waters, as well as intertidal species, and tropical species that may live symbiotically with sponges or corals. And while most species in Aplanulata are marine, this clade also includes *Hydra*, one of the few hydrozoan species that inhabits fresh water environments. (Figure 1). Aplanulata species also vary greatly in their possession of a pelagic medusa (jellyfish) stage, with some species producing fully-independent, free-swimming medusae, while others exhibit various stages of medusae truncation. Reduced medusae, called gonophores, remain attached to the body of the polyp and often possess remnants of medusa morphology, such as tentacles. Despite their diversity, the group is united by a striking developmental synapomorphy: individuals bypass a planula larval stage typical of hydrozoans and instead develop directly into juvenile polyps inside a gonophore or within a cyst.

The relationships within Aplanulata families have not been thoroughly investigated within a molecular phylogenetic context, with the exception of Hydridae (Martinez, Iniguez, Percell *et al*. 2010, Nawrocki and Cartwright *in press*). Hydridae (approximately 30 valid species) (Daly, Brugler, Cartwright *et al*. 2007) includes the single genus *Hydra*, and all members of this genus are solitary and inhabit freshwater environs. Hydridae is

split into three large, well-supported clades, and the relationships between and within these major lineages are well resolved (Martinez, Iniguez, Percell *et al*. 2010).

Candelabridae is comprised of 20 valid species, most of which are solitary (but see (Brinkmann-voss and Lindner 2008)) (Daly, Brugler, Cartwright *et al*. 2007). Species in this family all have a large number of randomly scattered capitate (knobbed) tentacles along the body column, and reproductive structures are localized below the tentacles. No more that two species have been previously sampled for phylogenetic analyses.

Corymorphidae (approximately 45 valid species) (Daly, Brugler, Cartwright *et al*. 2007) is exclusively comprised of solitary species, and members possess a body column lacking both tentacles and a hard skeleton. Two whorls of filiform (elongated with tapering ends) or moniliform (elongated but with batteries of nematocysts along them) tentacles are found towards the oral end of the polyp and reproductive structures form between these two sets of tentacles. Occasionally, oral tentacles are capitate (nobbed ends). Studies examining the relationships of this clade in a molecular phylogenetic context using 16S rDNA have failed to recover this family as monophyletic (Collins, Winkelmann, Hadrys *et al*. 2005, Schuchert 2010). More recently, an analysis using a combination of 18S, 28S and 16S data, which included eleven corymorphid taxa did not recover a monophyletic Corymorphidae, and instead recovered *Corymorpha groenlandica* and *Hataia parva* separate from the rest of the Corymorphidae (Nawrocki and Cartwright *in press*). This same study sampled the massive (1-2m), deep-water species, *Branchiocerianthus imperator*, and recovered its placement at the base of the *Euphysa* clade, but this

placement was poorly supported. Its affiliation with *Euphysa* was surprising, given that members of this genus are some of the smallest polyps represented in Corymorphidae, being only 1-4 cm length (Norenburg and Morse 1983). This study also sampled *Corymorpha intermedia*, a taxon that recently was recovered as grouping with members of the genus *Euphysa* in molecular phylogenetic analyses (Cartwright and Nawrocki 2010, Nawrocki and Cartwright *in press*). *Fukaurahydra anthoformis*, an Aplanulata taxon with a unique squat polyp possessing a widened, flat platform bearing gonophores, is classified as a corymorphid but has never before been sampled for phylogenetic analyses.

Tubulariidae (approximately 60 valid species) (Daly, Brugler, Cartwright *et al*. 2007) is comprised of both solitary and colonial species (but see Nawrocki and Cartwright, *accepted*), with polyps sharing the general morphology of those of Corymorphidae, except that polyps are much smaller and have stalks covered with a hard exoskeleton (perisarc). Phylogenetic studies using 16S rDNA were unable to recover this family as monophyletic (Collins, Winkelmann, Hadrys *et al*. 2005, Schuchert 2010). A more recent study with larger sampling of this family and two additional markers (18S and 28S) recovered a monophyletic Tubulariidae and suggested three well-supported lineages in the clade - an *Ectopleura* clade, a clade comprised of *Hybocodon* and *Tubularia*, and a clade comprised of *Ralpharia* and *Zyzzyzus* (Nawrocki and Cartwright *in press*). Within the *Ectopleura* clade, there are three distinct lineages, which lack clear morphological synapomorphies. The results of this study disagree with a former phylogenetic analysis

based on morphology alone, which suggested two major groupings of *Ectopleura* species based on the presence/absence of a medusa (Marques and Migotto 2001).

Acaulidae is comprised of three genera and approximately 5 valid species (Daly, Brugler, Cartwright *et al*. 2007). All members of this family are solitary, and contain one set of tentacles surrounding the mouth, with scattered tentacles along the body column, and with or without an additional whorl of fleshy filiform tentacles at the base of the polyp (Petersen 1990, Bouillon *et al*. 2006). Gonophores form between the tentacles on the body of the polyp (Schuchert 2006, Bouillon *et al*. 2006). This family may (Cairns *et al*. 2003) or may not (Yamada and Kubota 1991, Bouillon *et al*. 2006) include the solitary species *Hataia parva* (Hirai and Yamada 1965).

Margelopsidae (three genera and approximately 5 valid species) (Daly, Brugler, Cartwright *et al*. 2007) is a family comprised exclusively of pelagic members. Species belonging to this family resemble polyp hydranths, except that they lack a hydrocaulus underneath the hydranth. Instead, individuals float freely in the ocean. Members of this family are known to encyst (Kubota 1993), but Petersen grouped this family with Paracorynidae and Tubulariidae based on the length of oral and aboral tentacles, as well as the shape of the hydranth (Petersen 1990).

Two putative Aplanulata families (Collins 2006) are not sampled in this study. Tricyclusidae is a monotypic family containing the single species *Tricyclusa singularis* Schulze 1876. This species is a solitary polyp with two whorls of tentacles with slightly

capitate ends (Schulze 1876, Schuchert 2006). Gonophores form between the two sets of tentacles, and below the aboral tentacles (Figure 1). Petersen (1990) affiliated this family with Acaulidae and Corymorphidae, based on the gelatinous perisarc and encystment that members of these families all possess. Paracorynidae is another monotypic family containing the species *Paracoryne huvei* Picard 1957 (Figure 1). *Paracoryne huvei* is described as a polymorphic colony, containing gastrozooids, gonozooids and dactylozooids (Picard 1957). However, it has recently been suggested that the colony is actually a flattened tubulariid hydranth (head) (Bouillon 1974, Bouillon 1975). Evidence for this lies in the morphology of the basal plate of the colony, which contains endodermal cavities and a large layer of parenchymatic tissue, similar to what is found in tubulariid heads (Bouillon 1974, Bouillon 1975). If this is the case, then dactlyozooids are in actuality the tentacles of the hydranth, gonozooids are the blastostyles carrying gonophores, and gastrozooids are duplicated hypostomes (Petersen 1990). Neither of these families has ever been sampled for molecular phylogenetic analyses.

Relationships between the component families of Aplanulata are also not well understood. Petersen (1990) provided a phylogenetic hypothesis based on morphological data for six of the major families (Figure 1), with the exclusion of Hydridae, which he hypothesized to belong to a different hydrozoan lineage. Petersen split Aplanulata into two major clades based on developmental mode - one major lineage for families whose members encyst (Tricyclusidae, Acaulidae and Corymorphidae) and one for members who develop directly into an actinulae, which are juvenile polyps (Tubulariidae, Paracorynidae, Margelopsidae and Candelabridae) (Petersen 1990). However, subsequent

phylogenetic analyses reveal that this division is likely not reflective of the clade's evolutionary history, and that Tubulariidae is more closely related to Corymorphidae than to Candelabridae (Figure 1) (Collins, Schuchert, Marques *et al*. 2006, Collins, Winkelmann, Hadrys *et al*. 2005, Cartwright and Nawrocki 2010, Nawrocki and Cartwright *in press*). This aligns Corymorphidae and Tubulariidae into a clade more or less consistent with Bouillon's Tubularioidea (Bouillon 1985) by the presence of two whorls of tentacles (Collins, Schuchert, Marques *et al*. 2006, Collins, Winkelmann, Hadrys *et al*. 2005, Nawrocki and Cartwright *in press*, Cartwright and Nawrocki 2010), Bouillon also included in Tubularioidea some families that we did not sample here (Margelopsidae and Paracorynidae), and one that is known to group outside of Aplanulata (Nawrocki *et al*. 2010). Here we use Tubularioidea to reflect the grouping of taxa with two distinct sets of tentacles (here Corymorphidae + Tubulariidae). Furthermore, a phylogenetic analysis with mitochondrial 16S data places Candelabridae and Hydridae as sister taxa (Collins, Winkelmann, Hadrys *et al*. 2005), which would unite them by their extensile bodies. In contrast, other studies incorporating nuclear18S and/or 28S rDNA have suggested that Candelabridae might instead be a separate early diverging lineage of Aplanulata, with Hydridae being sister to Corymorphidae + Tubulariidae (Collins, Schuchert, Marques *et al*. 2006, Cartwright and Nawrocki 2010, Nawrocki and Cartwright *in press*). This latter hypothesis would unite Hydridae, Corymorphidae and Tubulariidae by the presence of oral tentacles organized in a whorl (Figure 1).

Here, we provide the most complete sampling of 39 Aplanulata taxa, comprising members of six out of the eight described families, with four markers – and new, previously unsampled taxa including the monotypic *Fukaurahydra anthoformis*, in an effort to provide support for the major lineages of Aplanulata and better understand the phylogenetic placement of *Hydra*. We compare our modern understanding of the evolution of characters in the clade to historical concepts of the evolution of the group, and demonstrate that reproductive characters that have been previously overlooked are likely important to understanding the evolution of this lineage. Based on these reproductive and morphological characters, we hypothesize a placement for four unsampled or under-sampled Aplanulata families. Lastly, we offer new taxonomic classifications for two species of Aplanualta, *Corymorpha intermedia* and *Fukauarahydra anthoformis*, based on their recovered phylogenetic placement in our analyses, as well as a re-examination of their morphology.

METHODS

DNA isolation and sequencing

Fresh, ethanol-preserved, or RNALater-preserved tissue was extracted using the Qiagen DNeasy tissue kit following manufacturer's instructions (Qiagen, Inc., Mississauga, ON, Canada), or using a standard phenol-chloroform protocol (available on request). A 640 base pair fragment of the mitochondrial 16S, 651 base pair fragment of the mitochondrial cytochrome oxidase 1 (CO1), 1800 base pair fragment of the nuclear 18S (small ribosomal subunit, SSU), and 3201 base pair fragment of the nuclear 28S (large ribosomal subunit, LSU) markers were amplified as previously described (Cartwright, Evans, Dunn *et al*. 2008, Cunningham and Buss 1993, Dawson 2005, Evans, Lindner, Raikova *et al*. 2008, Folmer, Black, Hoeh *et al*. 1994). PCR product was purified and sequenced directly by the University of Washington High Throughput Sequencing Unit (Seattle, WA, USA), or were retrieved from Genbank. Contig assembly and sequence editing were conducted in Sequencher v4.9 (GeneCodes 2005). Concatenation and matrix editing was conducted in Mesquite v2.74 (Maddison and Maddison 2007). All new sequences generated for this study were deposited in Genbank (**Table 1**).

Sequence alignment and phylogenetic analyses

The DNA alignment for cytochrome oxidae 1 (CO1) was generated in the program Translator X (Abascal, Zardoya and Telford 2010), which uses the translated protein code to guide the generation of a nucleotide alignment. Program settings were default, except that we used a 'coelenterate-specific' mitochondrial genetic code, MUSCLE for alignment , and inferred the most likely reading frame based on the aligned data. The final

alignment was end-trimmed to remove characters missing from more than half of the sampled taxa.

DNA alignments for 16S, 18S and 28S were generated with MUSCLE (Edgar 2004a, b), and were subsequently adjusted by hand based on developed secondary structure models for Hydridae (16S) following (Nawrocki and Cartwright *in press*), or models for Cnidaria (18S and 28S) (M. S. Barbeitos, personal communication). Alignments were run through GBlocks v0.91b (Castresana 2000) to remove ambiguously aligned regions using the following settings: minimum block length = 5; gaps = with half. The final alignments were end-trimmed to remove characters missing in more than half of the aligned taxa. Analyses of partial datasets employing the doublet model, that incorporates information of secondary structure, did not show significant improvement in topology (not shown); thus, we applied a general time reversible model (GTRGamma) to all alignments used in this analysis. An additional proportion of invariant sites was not used, as the lowest rate category of the gamma distribution that accounts for rate heterogeneity in the GTRGamma model includes sites that are close to invariant (Ren, Tanaka and Yang 2005).

Analyses were run in the parallel version of RaxML v7.2.8 (Stamatakis 2006) for all markers. For CO1, a GTRGamma model was applied to three different data partitions determined by codon position. For the concatenated analysis, the data was split into data partitions by marker and by codon position (CO1 only), accounting for a total of 6 partitions in the combined analysis. 1000 bootstrap replicates were generated for each

analysis, including the concatenated analysis. Trees were visualized in Mesquite v2.75 (Maddison and Maddison 2007) and FigTree (Drummond and Rambaut 2007).

RESULTS

3.3 Taxon sampling, alignment and analyses

Sixty-five sequences were included in the 16S analysis, and a total of 508bp, or 79% of the amplified 16S, were retained after removal of ambiguously aligned region in GBlocks (Castresana 2000). For 18S, 51 sequences were included in the analysis and a total of 1398bp, or 86% of the amplified fragment were retained after GBlocks. For 28S, 51 sequences were included in the analysis and a total of 3072bp, or 86% of the amplified fragment, were retained after GBlocks. Forty-three CO1 sequences were included in this analysis and the entire amplified CO1 (645bp with ends trimmed) was analyzed. For the combined analysis, all taxa with at least three sequenced markers were included in the final combined analysis, for a total of 51 taxa and 5623 alignment positions. This study contributed 34 new DNA sequences (including a new marker, CO1), and sampled 56 species, including 39 Aplanulata species from 14 genera representing 5 out of the 8 families in the clade. Species identifications, Genbank IDs, voucher numbers, and specimen localities are reported in Table 1.

Relative contribution of markers to topology

A comparison of node support between markers (Figure 2) demonstrates that 28S accounts for the most well-supported clades (66% of the nodes with bs >70), with 16S supporting 45%, 18s supporting 34%, and CO1 supporting only 5% of nodes. In general, the mitochondrial markers (16S and CO1) recover some relationships at the tips of the trees and within Hydridae, but little support for deeper relationships, while 18S recovers some lower-level relationships and no deep nodes, and 28S provides the most resolution

Table 1: Specimens and associated Genbank accession numbers. Names of specimens included in combined analysis are bolded. Genbank accession numbers for new sequences generated for this study will be bolded.

Higher Level	Family	Species	28s	18s	16s	CO1	Voucher or Published Reference Sequence
Aplanulata	Candelabridae	*Candelabrum austrogeorgiae*	-	-	FN424120	-	Cantero et al. (2010)
Aplanulata	Candelabridae	***Candelabrum cocksii***	EU879928	EU876556	AY512520	GU812438	MHNGINVE29591
Aplanulata	Candelabridae	***Candelabrum* sp.**	EU879929	EU876557	EU876530	awaiting GBID	-
Aplanulata	Corymorphidae	***Branchiocerianthus imperator***	JN594035	JN594046	-	awaiting GBID	-
Aplanulata	Corymorphidae	***Corymorpha bigelowi***	EU272563	EU876564	EU448099	awaiting GBID	KUNHM2829
Aplanulata	Corymorphidae	***Corymorpha glacialis***	JN594036	JN594047	FN687549	awaiting GBID	MHNGINVE67050
Aplanulata	Corymorphidae	***Corymorpha groenlandica***	JN594037	JN594048	FN687551	-	MHNGINVE67051
Aplanulata	Corymorphidae	*Corymorpha groenlandica*	-	-	FN687550	-	MHNGINVE63302
Aplanulata	Corymorphidae	***Corymorpha intermedia***	EU879930	AY920759	FN687910	GU812436	Collins et al. (2006), Schuchert (2010)
Aplanulata	Corymorphidae	***Corymorpha nutans***	EU879931	EU876558	FN687546	awaiting GBID	MHNGINVE48745
Aplanulata	Corymorphidae	*Corymorpha nutans*	-	-	FN687549	-	MHNGINVE67050
Aplanulata	Corymorphidae	*Corymorpha nutans*	-	-	FN687548	-	Schuchert (2010)
Aplanulata	Corymorphidae	*Corymorpha nutans*	-	-	FN687547	-	Schuchert (2010)
Aplanulata	Corymorphidae	***Corymorpha pendula***	EU879936	EU876565	EU876538	awaiting GBID	KUNHMDIZ2962
Aplanulata	Corymorphidae	***Corymorpha sarsii***	JN594038	JN594049	-	awaiting GBID	-
Aplanulata	Corymorphidae	*Corymorpha sp.*	-	-	FN424121	-	Cantero et al. (2010)
Aplanulata	Corymorphidae	***Hataia parva***	JN594034	JN594045	JN594033	awaiting GBID	UF5407
Aplanulata	Corymorphidae	***Euphysa aurata***	EU879934	EU876562	EU876536	awaiting GBID	MHNGINVE48753
Aplanulata	Corymorphidae	*Euphysa aurata*	-	-	FN687552	-	Schuchert (2010)
Aplanulata	Corymorphidae	*Euphysa flammea*				FJ602537	
Aplanulata	Corymorphidae	*Euphysa japonica*	awaiting GBID	EU301605	awaiting GBID	awaiting GBID	Lindsay, D.J. et al. (2008)
Aplanulata	Corymorphidae	***Euphysa tentaculata***	EU879935	EU876563	EU876537	awaiting GBID	Cartwright & Nawrocki (2010)
Aplanulata	Corymorphidae	***Fukaurahydra anthiformis***	awaiting GBID	awaiting GBID	awaiting GBID		
Aplanulata	Corymorphidae	***Paragotea bathybia***				FJ602533	
Aplanulata	Hydridae	***Hydra canadensis***	JN594039	JN594050	GU722797	GU722883	Martinez et al. (2010)
Aplanulata	Hydridae	***Hydra circumcincta***	EU879939	EU876568	GU722764	GU722857	Cartwright & Nawrocki (2010) , Martinez et al. (2010)
Aplanulata	Hydridae	***Hydra hymanae***	JN594040	JN594051	GU722760	GU722849	Martinez et al. (2010)
Aplanulata	Hydridae	***Hydra oligactis***	JN594041	JN594052	GU722781	GU722871	Martinez et al. (2010)
Aplanulata	Hydridae	***Hydra utahensis***	JN594042	JN594053	GU722774	GU722861	Martinez et al. (2010)
Aplanulata	Hydridae	***Hydra viridissima***	EU879940	EU876569	GU722756	GU722845	Martinez et al. (2010)

Aplanulata	Hydridae	***Hydra vulgaris***	JN594043	JN594054	GU722817	GU722914	Martinez et al. (2010)
Aplanulata	Tubulariidae	***Ectopleura crocea***	EU879932	EU876559	EU876533	awaiting GBID	MHNGINVE34010
Aplanulata	Tubulariidae	***Ectopleura crocea***	EU883554	EU883548	EU883543	-	Cartwright & Nawrocki (2010)
Aplanulata	Tubulariidae	*Ectopleura dumorteri*	-	-	FN687542	-	Schuchert (2010)
Aplanulata	Tubulariidae	***Ectopleura dumortieri***	EU272561	EU876560	EU305474	-	Pers. Voucher: Alberto Lindner, AL525
Aplanulata	Tubulariidae	***Ectopleura dumortieri***	EU879933	EU876561	EU876534	awaiting GBID	Cartwright & Nawrocki (2010)
Aplanulata	Tubulariidae	*Ectopleura dumortieri*	-	-	FN687543	-	Schuchert (2010)
Aplanulata	Tubulariidae	***Ectopleura larynx***	EU879943	EU876572	EU876545	-	KUNHMDIZ2963
Aplanulata	Tubulariidae	***Ectopleura larynx***	EU883549	AY920760	AY787877	awaiting GBID	MHNGINVE29389
Aplanulata	Tubulariidae	*Ectopleura larynx*	-	-	FN687535	-	MHNGINVE54563
Aplanulata	Tubulariidae	*Ectopleura larynx*	-	-	FN687536	-	MHNGINVE62576
Aplanulata	Tubulariidae	***Ectopleura marina***	EU883553	EU883547	EU883542	awaiting GBID	Cartwright & Nawrocki (2010)
Aplanulata	Tubulariidae	***Ectopleura wrighti***	JN594044	JN594055	FN687541	awaiting GBID	MHNGINVE27331
Aplanulata	Tubulariidae	***Hybocodon chilensis***	EU879937	EU876566	EU876539	awaiting GBID	MHNGINVE36023
Aplanulata	Tubulariidae	***Hybocodon prolifer***	EU879938	EU876567	EU876540	awaiting GBID	Cartwright & Nawrocki (2010)
Aplanulata	Tubulariidae	***Ralpharia gorgoniae***	EU272590	EU272633	EU305482	GU812437	KUNHM2778
Aplanulata	Tubulariidae	*Ralpharia sp.*	-	JN594056	-	-	
Aplanulata	Tubulariidae	***Tubularia indivisa***	EU879942	EU876571	EU876544	awaiting GBID	Cartwright & Nawrocki (2010)
Aplanulata	Tubulariidae	*Tubularia indivisa*	-	-	FN687532	-	Schuchert (2010)
Aplanulata	Tubulariidae	*Tubularia indivisa*	-	-	FN687530	-	MHNGINVE60972
Aplanulata	Tubulariidae	*Tubularia sp.*	-	-	FN424153	-	Cantero et al. (2010)
Aplanulata	Tubulariidae	***Zyzzyzus warreni***	EU272599	EU272640	EU305489	awaiting GBID	KUNHM2777
Capitata	Corynidae	***Stauridiosarsia ophiogaster***	EU272560	EU272615	EU305473	awaiting GBID	KUNHM2803
Capitata	Solanderiidae	***Solandaria secunda***	EU305533	EU305502	EU305484	awaiting GBID	KUNHM2611
Filifera I	Proboscidactylidae	***Proboscidactyla flavicirrata***	EU305527	EU305500	EU305480	awaiting GBID	USNM1074994
Filifera I	Ptilocodiidae	***Hydrichthella epigorgia***	EU272569	EU272622	EU305478	awaiting GBID	KUNHM2665
Filifera II	Eudendriidae	*Eudendrium capillare*				awaiting GBID	
Filifera II	Eudendriidae	***Eudendrium californicum***	EU305513	EU305492	EU305475	-	KUNHM2850
Filifera II	Eudendriidae	***Eudendrium glomeratum***	FJ550440	FJ550583	AM991301	-	MHNGINVE49717
Filifera III	Hydractiniidae	***Clavactinia gallensis***	EU272553	EU272610	EU448101	-	MHNGINVE33470
Filifera III	Stylasteridae	***Lepidopora microstylus***	EU272572	EU272644	EU645329	awaiting GBID	USNM1027724
Filifera IV	Bougainvillisae	***Garveia grisea***	EU272588	EU272632	AM183131	-	MHNGINVE34436
Filifera IV	Pandeidae	***Hydrichthys boycei***	EU272570	EU305496	EU448102	awaiting GBID	MHNGINVE37417
Leptothecata	Campanulariidae	***Obelia bidentata***	FJ550446	AY789754*	AY789815*	-	MHNGINVE37294
Leptothecata	Sertulariidae	***Sertularella mediterranea***	FJ550403	FJ550546	FJ550479	awaiting GBID	MHNGINVE32948
Limnomedusae	Olindiasidae	***Olindias phosphorica***	EU247808	AY920753	AY512509	awaiting GBID	MHNGINVE29811
Siphonophorae	Clausophyidae	***Clausophyes ovata***	EU305508	AY937336	AY935294	awaiting	YPM35349

Siphonophorae	Forskaliidae	***Forskalia edwardsi***	EU305516	AY937354	AY935312	GBID awaiting GBID	YPM35036
Trachymedusae	Rhopalonematidae	***Aglaura hemistoma***	EU247803	EU247818	EU293984	-	MHNGINVE31745

throughout the entire tree. The combined analysis recovers 88% of nodes with a bs > 70. Additionally, with the combined analysis, we recover strong support for the monophyly of Aplanulata (bs = 100), strong support for a sister relationship between Hydridae and Tubularioidea , and strong support for most of the deep nodes in the phylogeny. Thus, we consider the combined analysis our most robust hypothesis of relationships of Aplanulata taxa.

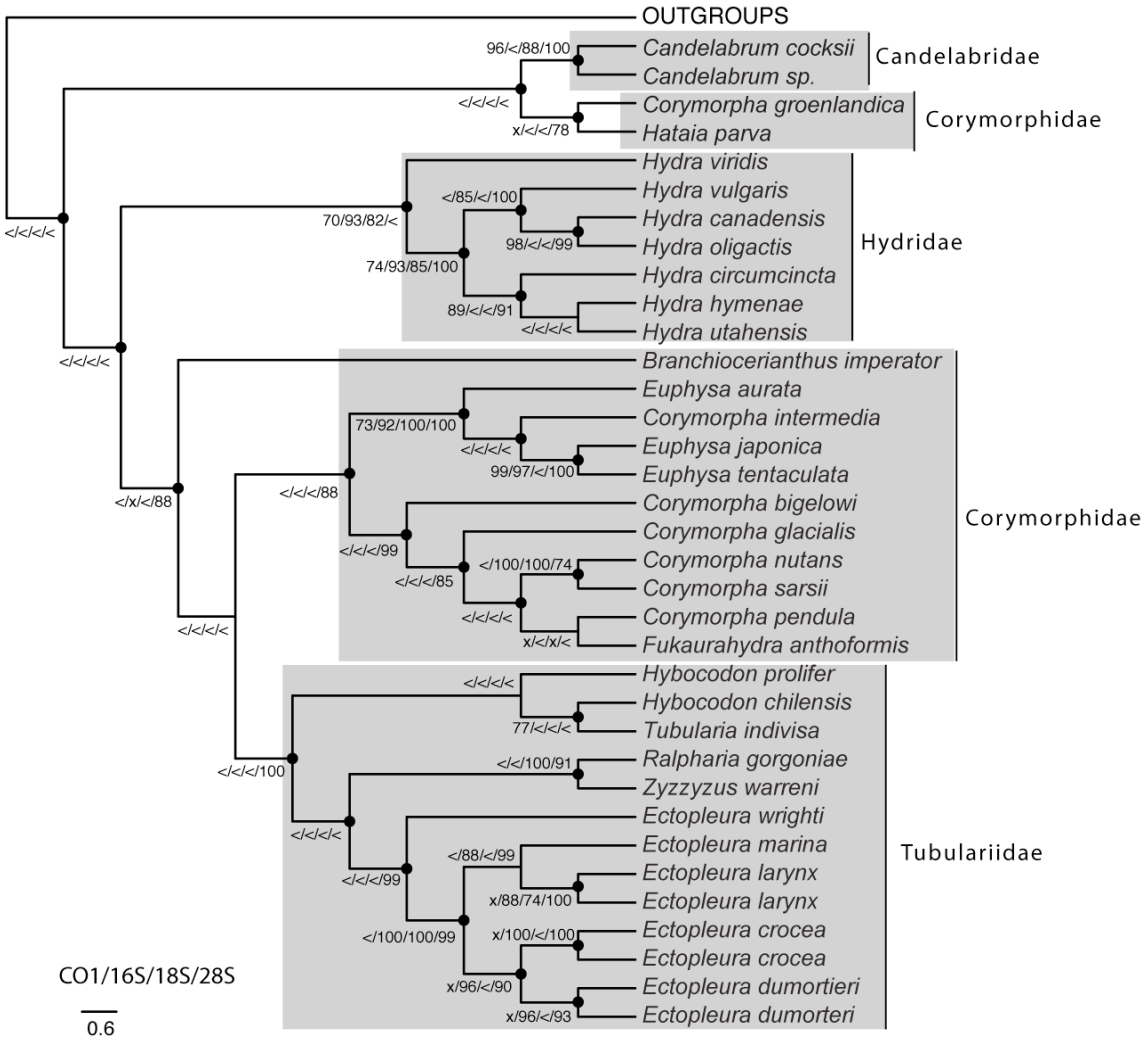

Figure 2**.** Cladogram of relationships based on combined CO1, 16S, 18S and 28S, with nodes demonstrating boostrap support for topology based on single-gene analyses. Node values are displayed if they are ≥ 70. Darkened circles on nodes indicate bootstrap values ≥ 70 on combined, partitioned analysis in RaxML. X indicates that one of the sampled taxa was not in the analysis, thus the node did not exist.

Phylogenetic relationships of Aplanulata

CO1, 16S, 18S, 28S and combined analyses recover largely congruent topologies (see Supplementary Information for single-gene analyses). Aplanulata is monophyletic in all analyses, although only the combined analysis shows strong support for the node (Figures 2-3; bs = 100). All analyses recover a monophyletic Hydridae, and our topology within *Hydra* is completely congruent with a recently published paper with much denser sampling of the family (Martinez, Iniguez, Percell *et al*. 2010).

Within Tubulariidae, the combined analysis recovers a monophyletic *Ectopleura* (also supported by 28S), a *Tubularia* + *Hybocodon* clade (but recovered with low support in all analyses), and a *Ralpharia* + *Zyzzyzus* clade (also supported by 18S and 28S).

Corymorphidae is polyphyletic in all of the analyses that we conducted. *Corymorpha groenlandica,* an unidentified *Corymorpha* species from Cantero et al. 2010 (Cantero, Sentandreu and Latorre 2010) (in 16S analyses only), and *Hataia parva* (supported by 28S and combined analyses) form a clade sister to Candelabridae. Most of the sampled *Corymorpha* species, including the type species, *Corymorpha nutans*, and all of the sampled *Euphysa* species, including the type species *Euphysa aurata* (supported by 28S and combined analyses) fall into a well-supported clade that is sister to Tubulariidae. Within this clade there is a split between members of the genus *Euphysa* + *Corymorpha intermedia* and a second group comprised of *Corymorpha* species (*C. bigelowi, C. glacialis, C. nutans, C. sarsii, C. pendula*) and *Fukaurahydra anthoformis* (Figures 2-3). We recover the corymorphid *Branchiocerianthus imperator* at the base of Tubularioidia

(Tubulariidae + most other Corymorphidae) with good support in the 28S and combined analyses (Figures 2-3).

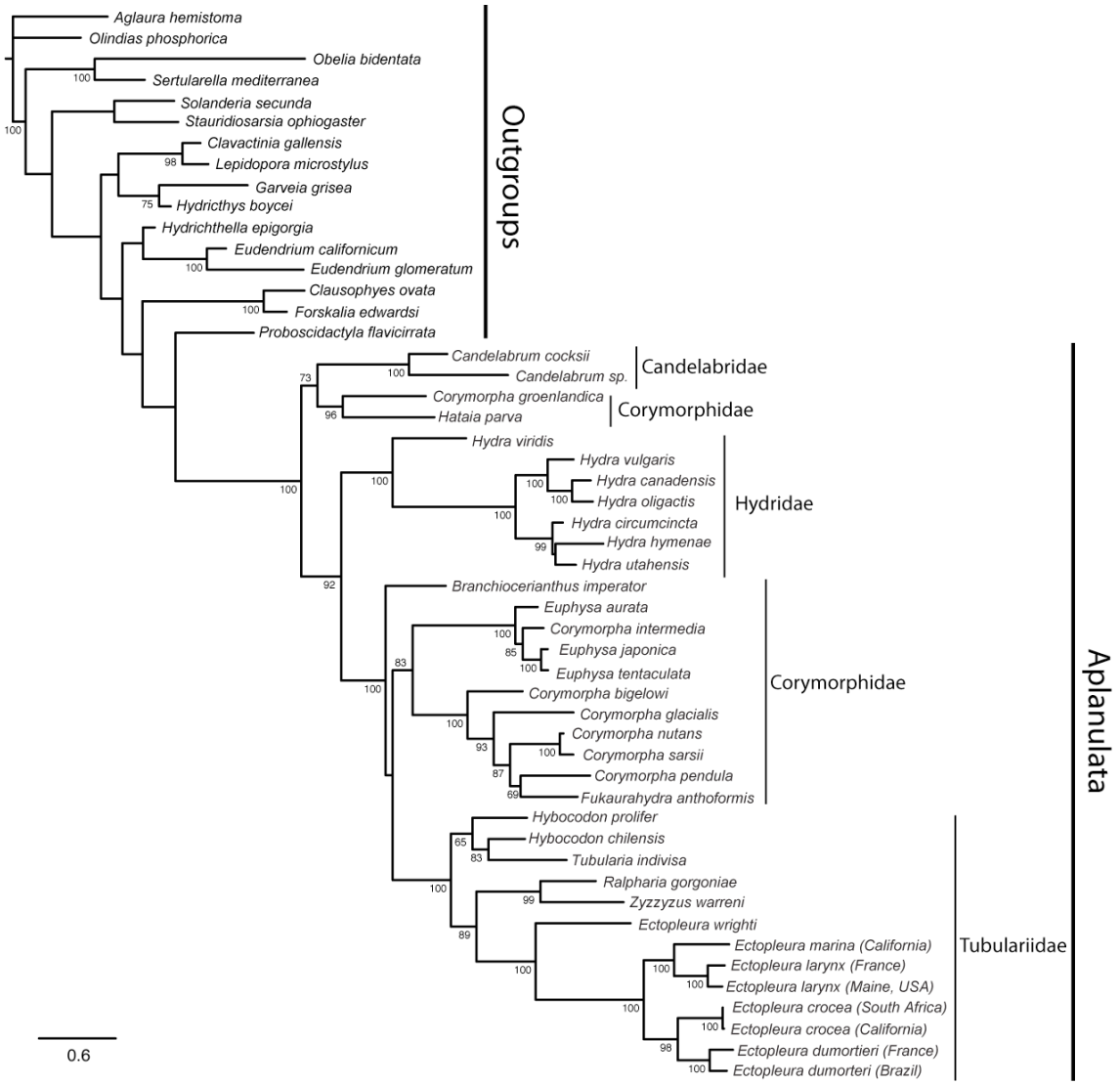

Figure 3. Phylogenetic hypothesis of relationships of Aplanulata based on combined CO1, 16S, 18S and 28S analyzed under a partitioned GTR + Γ model in RaxML. Bootstrap values reported if ≥ 70, Node values indicate bootstrap support from 1000 replicates**.** See Appendix for single gene analyses.

DISCUSSION

Relative contribution of CO1, 16S, 18S and 28S to topology

Our results suggest that faster evolving mitochondrial markers 16S and CO1 provide support at the tips of the tree, whereas ribosomal markers such as 28S provide stronger overall support at deep nodes. These are congruent with previous phylogenetic analyses utilizing CO1 or 16S (Collins, Winkelmann, Hadrys *et al*. 2005, Schuchert 2010, Nawrocki, Cartwright and Schuchert 2010, Ortman, Bucklin, Pagès *et al*. 2010), and others utilizing nuclear ribosomal markers (Collins, Schuchert, Marques *et al*. 2006, Cartwright and Nawrocki 2010, Cartwright, Evans, Dunn *et al*. 2008, Nawrocki, Cartwright and Schuchert 2010). As the combined analysis recovers the most well-supported nodes (94% of the nodes), we consider the recovered topology from the combined analysis as the strongest hypothesis of relationships of component Aplanulata taxa.

Major Aplanulata lineages

Our combined topology supports an early-diverging lineage comprised of Candelabridae and the corymorphids *Corymorpha groenlandica* + *Hataia parva*. We recover the clade Tubularioidea *sensu lato,* which includes Corymorphidae (with the exclusion of *Corymorpha groenlandica* and *Hataia parva*) and a monophyletic Tubulariidae. The corymorphid, *Branchiocerianthus imperator* is recovered as the earliest diverging branch of Tubularioidea. Tubularioidea is recovered as sister to Hydridae, with strong support in the combined analysis.

Relationships within Hydridae

The monophyly of Hydridae is well supported by ours and previous studies (Hemmrich, Anokhin, Zacharias *et al*. 2007, Martinez, Iniguez, Percell *et al*. 2010, Nawrocki and Cartwright *in press*, Cartwright and Nawrocki 2010), and members of Hydridae are united by the strong synapomorphies of their freshwater habitat and by the development of ovaries and testes directly in the epithelia of the polyp. Our combined analysis supports splitting Hydridae into three major lineages (the Viridis group, the Braueri group, and a third clade comprised of *H. oligactis*, *H. canadensis*, and *H. vulgaris*), which is congruent with a recent study with much denser sampling of Hydridae (Martinez, Iniguez, Percell *et al*. 2010). Two of the major lineages of Hydridae that we recover have strong morphological synapomorphies. The *Hydra viridis* clade (represented in our analysis by only a single specimen) is united by both its distinctive green color (due to the presence of intracellular algae) as well as the presence of an embryotheca with a cobbled surface, and the Braueri group has a flattened embryotheca and holotrichous isorhiza nematocysts (Martinez, Iniguez, Percell *et al*. 2010). The *oligactis-canadensis-vulgaris* clade is united by the presence of very long tentacles (longer than the length of the body of the animal) (Schuchert 2010, Hyman 1931).

Relationships within Tubulariidae

Tubulariidae is united by the combination of a presence of a thick skeletal covering (perisarc) over the polyp, direct development through a brooded actinula phase, and unbranched blastostyles (structures housing developing gonophores). Members of this family also have a hydrocaulus (region below the polyp head) that is clearly divided into

two distinct regions - the neck, which serves as a boundary for the polyp and stalk (Nawrocki and Cartwright *in press*), and a stalk region. 28S and combined analyses recover a monophyletic Tubulariidae, congruent with previous analyses (Figures **2-3**) (Nawrocki and Cartwright *in press*, Cartwright and Nawrocki 2010). As in a previous study (Nawrocki and Cartwright *in press*), we find support for two major groups within the family, with a third grouping only receiving low support. The earliest diverging group (recovered in 16S CO1 and combined analyses, but poorly supported) is comprised of the genera *Tubularia* and *Hybocodon*, which both contain solitary species with long, unbranched stalks covered in a hard, rigid perisarc. Members of this clade are often found in aggregates with polyps often settling on one another, and have sometimes been mistaken for colonies (Nawrocki and Cartwright *in press*). These medusae have four tentacle bulbs along the margin of their bell, but a tentacle only develops from a single one of these bulbs, giving the gonophore the appearance of bilateral symmetry. This is also the only group of tubulariids that produce bilaterally symmetric gonophores (either attached or detached).

The second clade of Tubulariidae is *Ralpharia gorgoniae* + *Zyzzyzus warreni*. Both taxa live symbiotically with other invertebrate hosts, which *Zyzzyzus warreni,* imbedded in a sponge, and *Ralpharia gorgoniae* imbedded in the body of a gorgonian coral.

The last well-supported clade of Tubulariidae (28S and combined analyses) is comprised of all sampled members of *Ectopleura*. This group is united by possession of a single whorl of oral tentacles (Petersen 1990). *Ectopleura wrighti* is the earliest diverging

sampled member of the group, followed by two sister clades comprised of *E. marina-E. larynx* and *E. crocea-E. dumortieri*. We find no support for grouping medusa-bearing species of this clade into one lineage, and gonophore-bearing species into another, as suggested previously (Marques and Migotto 2001).

Relationships within Corymorphidae

Our analyses find Corymorphidae as polyphyletic and recover three separate corymorphid lineages, two of which fall unexpectedly outside of Corymorphidae *sensu stricto*. In all of our analyses that included it, *Corymorpha groenlandica* grouped at the base of Aplanulata with *Hataia parva* and/or Candelabridae (Figures **2-3**). In the 16S analysis, we were able to include another unidentified *Corymorpha* species from Genbank, which also grouped with the *Corymorpha groenlandica* samples, but was slightly divergent in sequence (see Supplemental file). This specimen was sampled only for 16S and was collected in Antarctica off of the Antarctic Peninsula, and could be one of any number of unsampled *Corymorpha* species, or alternatively, a more divergent sample of *Corymorpha groenlandica*, since this species has a broad range (Schuchert 2010, Svoboda and Stepanjants 2001). *Hataia parva* was originally classified as a Clavidae based on the scattering of tentacles along the body (Hirai and Yamada 1965). Later the development of *Hataia parva* was characterized, and authors noted its ability to encyst (Yamada and Kubota 1991), clearly affiliating it with one of a number of Aplanulata families that have this capability. Later authors placed this species within Acaulidae (Cairns *et al*. 2003) or Corymorphidae (Bouillon *et al*. 2006), although there do not appear to be any strong synapomorphies that group it with one of these families, to

the exclusion of the other. The placement of *Corymorpha groenlandica* + *Hataia parva* *in* our analyses with Candelabridae is not completely unexpected, given the gross similarity that *Hataia parva* polyps share with members of Candelabridae (scattered capitate tentacles along the body column). However, its placement should be viewed as preliminary. While it is possible that the *Corymorpha groenlandica* + *Corymorpha* sp. + *Hataia parva* lineage is a valid grouping separate from other corymorphids given the strong support in the 16S analysis, the confident retrieval of its higher level placement will require much denser sampling of Corymorphidae.

Our results do not support the recent designation to a resurrected genus, *Monocaulus*, by Svoboda & Stepanjants (Svoboda and Stepanjants 2001) to includes *C. groenlandica*, *C. glacialis* and *C. sarsii*. Svoboda & Stepanjants (2001) suggested that these corymorphid taxa which lack branched gonophores should be classified as the separate genus Monocaulus. This however is not universally recognized due to disagreement over the importance of branched blastostyles as a valid taxonomic character (Schuchert 2010, Boullion, Gravili, Pages *et al*. 2006), and our study, though preliminary with regard to the placement of *C. groenlandica*, does provides phylogenetic evidence that the grouping of these species is not valid.

Phylogenetic placement of *Branchiocerianthus imperator*

Branchiocerianthus imperator is a morphologically-distinct and large (2m tall) deep-sea hydrozoan classified within Corymorphidae (Schuchert 2010). A previous analysis was unable to find strong support for the placement of this taxon, and instead placed it with

weak support within Corymorphidae as sister to the genus *Euphysa* (Nawrocki and Cartwright *in press*). Our analysis, which added CO1 for this species, recovers *B. imperator* as sister to Tubularioidea (Figure 3). We recover this relationship in the 28S analysis (bs = 88) and combined analysis (bs = 100), but not in the 18S or CO1 analyses (Figures 2-3). The placement of *B. imperator* outside of Corymorphidae is surprising, given the morphological synapomorphies this species shares with Corymorphidae (such as rooting filaments and a reduced perisarc). However, this species also possesses a number of unique morphological apomorphies, such as its large size (over 1m tall) and the striking bilateral symmetry of the polyp not found in any other hydrozoan group. In the interest of nomenclatural stability, we recommend keeping the current classification of *B. imperator*, pending further sampling of the genus, including its type species, *Branchiocerianthus urceolus* Mark, 1898.

Phylogenetic placement of *Fukaurahydra anthoformis*

Fukaurahydra anthoformis is a morphologically distinct corymorphid that is monotypic. This species' polyp stage has a short, squat body with a whorl of rooting filaments, in contrast to most corymorphid polyps, which have long bodies and a section at the base of the polyp with densely scattered rooting filaments. These morphological characteristics led to the erection of a new genus for the species (Yamada, Konno and Kubota 1977). Our analyses recover *F. anthoformis* as nested within the clade that includes most *Corymorpha* species (with the exception of *C. groenlandica*), and sister to the species *C. pendula* (combined analysis, bs = 69). All analyses that included *F. anthoformis* recover it as sister to *Corymorpha pendula*, but only the combined analysis provided some

support for this relationship. Regardless, *F. anthoformis* is unequivocally nested within a clade of *Corymorpha* species, suggesting that a separate generic designation is unnecessary and that this species is a member of the genus *Corymorpha* (see section *4.12 Taxonomic Recommendations*).

Phylogenetic placement of *Corymorpha intermedia*

Within Corymorphidae *sensu stricto*, there is strong support for two major clades. One of these clades includes all sampled *Euphysa* species, as well as the species *Corymorpha intermedia*. The the polyp stage of this species of *C. intermedia* is currently unknown and the medusa possesses characteristics of of both *Corymorpha* and *Euphysa* (see section 4.12 below) and was classified preliminarily in *Corymorpha* (Schuchert 1996). Our analyses recover *Corymorpha intermedia* with *Euphysa*, and thus we recommend redesignating this species as *Euphysa intermedia* (see section *4.12 Taxonomic Recommendations*). Members of *Euphysa* are morphologically distinct from other corymorphid species, in that they are markedly smaller polyps, the stalk (hydrocaulus) lacks endoderm canals characteristic of other corymorphids, they possess of a single whorl of oral tentacles on the polyp, and the medusa lacks an apical canal or a peduncle (Petersen 1990, Boullion, Gravili, Pages *et al*. 2006).

Phylogenetic placement of *Paragotea bathybia*

We sampled *Paragotea bathybia* Kramp, 1942 (Kramp 1942) for our CO1 analysis using a sequence available on genbank. This species grouped, albeit with low support, with *Euphysa*. This is an interesting result because athough *Paragotea bathybia* has been

traditionally classified within Corymorphidae (Boullion, Gravili, Pages *et al*. 2006, Pages and Bouillon 1997) *Euphysa* and *Paragotea bathybia* have medusae with a single tentacle, and their medusae also lack an apical canal. The affiliation of *Paragotea bathybia* with *Euphysa* should be viewed as preliminary and awaits sampling of additional markers.

Other Aplanulata taxa

Phylogenetic placement of Margelopsis hartlaubi

Margelopsis hartlaubi Browne 1903 (Browne 1903) is a holopelagic species with a narrow distribution and is thus difficult to sample. This species closely resembles tubulariid polyps (two whorls of tentacles between which gonophores develop), except that it lacks a long stalk under the neck likely due to is pelagic existance (Figure **1**) (Boullion, Gravili, Pages *et al*. 2006, Mayer 1910, Schuchert 2006). We were only able to sample *M. hartlaubi* using a CO1 sequence available on Genbank (Ortman, Bucklin, Pagès *et al*. 2010). Our CO1 analysis does not suggest affiliation with Tubulariidae or even Tubularioidia, as one would expect given the morphology of this species and instead we recover it as sister to the rest of Aplanulata. However, we did not get strong support for this placement nor in the nodes separating Margelopsis from Tubularioidea and thus this result should be viewed as preliminary. Thus, placement of this species and the scope of the family Margelopsidae awaits future sampling with more DNA markers and specimens, including the type species, *Margelopsis haeckelii* Hartlaub, 1897.

Phylogenetic placement of Hataia parva

Hataia parva Hirai and Yamada 1965 was originally classified within Claviidae, a clade of filiferan hydrozoans far removed from Aplanulata, based on its possession of scattered filiform tentacles (Hirai and Yamada 1965, Bouillon 1985). However, its solitary habitat and direct development through encystment clearly affiliate it with Aplanulata, likely allied to one of the families whose members undergo encystment (Margelopsidae, Acaulidae, or Corymorphidae). Recently, authors have suggested that it is affiliated with Corymorphidae (Boullion, Gravili, Pages *et al*. 2006), although there are no strong morphological synapomorphies to group it with this family. Our examination of specimens of this species acquired from Friday Harbor Laboratories as well as photographs provided by Shin Kubota (pers. comm.) reveal that the most distal ends of scattered tentacles of *Hataia parva* are slightly rounded, lending them a capitate appearance. This characteristic, in combination with its possession of a pedal disc, reduced gonophores, encystment and reduction of perisarc, align this species morphologically with the family Acaulidae, whose members possess this combination of characteristics (Schuchert 2006). Some authors have recently classified *Hataia parva* in Acaulidae, although this classification is not universally accepted (Cairns *et al*. 2003). Although we did not sample any other Acaulidae species, our analysis instead supports *Hataia parva* as grouping with the corymorphid *Corymorpha groenlandica* along with another family of Aplanulata with scattered tentacles along the body column – Candelabridae. At least one author has suggested that Acaulidae and Candelabridae are sister families (Bouillon 1985), and morphological characteristics strongly align these two families. Based on both molecular and morphological evidence, we suspect that Acaulidae taxa would fall within our recovered *Corymorpha groenlandica* + *Hataia*

parva clade. Clarification of the classification of *Corymorpha groenlandica* and *Hataia parva* and the phylogenetic affinity of these taxa to Acaulidae and Candelabridae awaits further sampling.

Phylogenetic placement of unsampled Aplanulata families

We were unable to sample other Aplanulata families for this analysis, including Paracorynidae and Tricyclusicae. Tricyclusidae has not been documented in the Mediterranean since it was first described in 1876, and has only rarely been reported in other localities (Schuchert 2006). Furthermore, we were unable to sample additional members of Acaulidae and Margelopsidae. All of these families include species that are rare and therefore difficult to sample for molecular analysis.

The strong affiliation between Corymorphidae and Tubulariidae into the superfamily Tubularioidea suggests that tentacle patterning may be an important evolutionary character for lineages in Aplanulata. Based on this character, we would hypothesize that members of Margelopsidae and Tricicyclusidae are affiliated with this superfamily (both possess tentacles organized in groups, or whorls), while Acaulidae is associated with Candelabridae (both possess scattered, capitate tentacles). Reproductive characters also appear to be evolutionarily important in this lineage and may lend insight into relationships. We also did not sample Paracorynidae, but a number of features including reproduction through encystment and lack of a brooded actinula affiliate it with the Corymorphidae or Hydridae. Additional sampling and future phylogenetic studies that integrate morphological and molecular data will assist in determining a robust hypothesis

for the phylogenetic placement of these divergent taxa, and will also likely reveal novel and interesting synapomorphies for evolutionary lineages within Aplanulata.

Taxonomic recommendations

Based on our results as well as a number of previous studies, we formally recommend the following changes to the taxonomy of Aplanulata and its component species, as compared to modern classifications reflected in Schuchert 2010 (Schuchert 2010, 2006) and Bouillon et al. 2006 (Boullion, Gravili, Pages *et al*. 2006).

(a) *Fukaurahydra anthoformis* falls within the genus *Corymorpha*, and is herein redesignated as *Corymorpha anthoformis*. We propose the following new diagnoses for the genus *Corymorpha* and for the species *Fukaurahydra anthoformis*.

Corymorpha M. Sars, 1835
TYPE SPECIES: *Corymorpha nutans* M. Sars, 1835 by monotypy.

DIAGNOSIS: Solitary hydroids with more or less vasiform hydranth and long caulus, **or with short, squat polyp with broad head**. Hydranth with one or several closely set whorls of 16 or more moniliform or filiform tentacles and one **or more** aboral whorls of 16 or more long, non-contractile filiform tentacles. Gastrodermal diaphragm parenchymatic. Hydrocaulus stout, covered by a thin perisarc, filled with parenchymatic gastrodermis, with long peripheral canals; aboral end of caulus with papillae turning more aborally into rooting filaments, **rooting filaments scattered or gathered in a whorl**, rooting filaments composed of epidermis and solid gastrodermis, sometimes tips with non-ciliated statocysts. With or without asexual reproduction through constriction of tissue from aboral end of hydrocaulus.

Gonophores develop on blastostyles arranged in a whorl over aboral tentacles. Gonophores remain either as fixed sporosacs, **medusoids**, or are released as free medusae.

Medusa bell apex dome-shaped or pointed. Four marginal bulbs present, lacking long exumbrellar spurs. With a single tentacle or three short tentacles and one long tentacle that differs not merely in size, but also in structure. Manubrium thin-walled, sausage-shaped with flared mouth rim, reaching to umbrella margin. Cnidome comprises stenoteles, desmonemes, and haplonemes.

REMARKS: This diagnosis for the most part corresponds to Schuchert 2010 (Schuchert 2010) and Petersen 1990 (Petersen 1990), but with modifications (**indicated in bold**) to polyp body shape and arrangement of rooting filaments to include *Fukaurahydra* (*Corymorpha*) *anthoformis*. Medusoids are also added to diagnosis, as a number of species of *Corymorpha* produce these structures.

Corymorpha anthoformis (Yamada, Konno & Kubota 1977)
Fukaurahydra anthoformis Yamada, Konno & Kubota 1977: 151-154, fig. 1.

MATERIAL EXAMINED: Japan, exposed coast near Senkaku Bay, Sado Island (Japan Sea); Collected by Dr. Yayoi M. Hirano on May 12, 2011. 6 mature polyps.

DIAGNOSIS: *Corymorpha* polyp with short, squat hydrocaulus, completely filled with parenchymatic endoderm. Base of polyp flat, with a ring of rooting filaments. Hydranth broad, plate-like. Live specimens with brightly-colored green, brown and red gonophores (see Figure 4.

DESCRIPTION: See Yamada et al. 1977 and Yamada and Kubota 1991.

(b) The species *Corymorpha intermedia* groups with strong support within the genus

Euphysa, and is herein redesignated as *Euphysa intermedia*.

Euphysa Forbes, 1848
TYPE SPECIES: *Euphysa aurata* Forbes, 1848 by monotypy

DIAGNOSIS: Corymorphid hydroid with hydrocaulus enveloped in gelatinous perisarc, covered by mud and detritus; hydrocaulus hollow, without peripheral longitudinal canals. Hydranth cylindrical to ovoid, with rounded hypostome, with 3-10 oral capitate tentacles and up to 20 aboral moniliform tentacles, no gastric diaphragm. Near base of hydranth papillae, each with an ecto-endodermal, statocyst-like structure. Gonophores singly or in clusters just above aboral tentacles, usually released as free medusae, rarely remaining as fixed sporosacs.

Asexual reproduction through budding of polarity-reversed polyps from the hydranth above aboral tentacles and through asexual bodies constricted off from basal end of hydrocaulus.

Medusa with an evenly rounded umbrella, **or rarely, a pointed umbrella with thickened apical mesoglea.** Umbrella without apical canal; with one to four tentacles, if more than one then usually unequally developed, but all of the same structure, usually moniliform; manubrium stout, cylindrical with small round mouth, shorter than bell cavity. **Phylogenetically, the least-inclusive clade containing *Euphysa intermedia, E. aurata, E. tentaculata, E. japonica*, and *E. intermedia*.**

REMARKS: The diagnosis of *Euphysa* follows Schuchert 2010, except for modifications made in bold to accommodate *Corymorpha* (*Euphysa*) *intermedia*.

Euphysa intermedia (Schuchert 1996)

Corymorpha intermedia Schuchert 1996: 104, fig. 62.

DIAGNOSIS: *Euphysa* medusa with apical process and a stout, cylindrical manubrium that narrows into a small, round mouth. Apical mesoglea thick. No apical canal or peduncle. Medusa with single moniliform tentacle and three non-tentacular bulbs.

DESCRIPTION: See Schuchert 1996.

NOTES: The medusa of *Euphysa intermedia* strongly resembles that of *Euphysa aurata*, with the exception of its possession of a thick, apical mesoglea and apical process.

Figure 4. Live specimen of *Corymorpha* (formerly *Fukaurahydra*) *anthoformis*. Photo taken by Y.M. Hirano.

CHAPTER 3: Colony formation in *Ectopleura* (Hydrozoa: Aplanualta) occurs through the fusion of sexually-generated individuals

ABSTRACT/INTRODUCTION

Coloniality, as displayed by most hydrozoans, is thought to confer a size advantage in substrate-limited, benthic marine environments, and affects nearly every aspect of a species' ecology and evolution (Coates and Jackson 1985, Jackson 1977). Hydrozoan colonies normally develop through asexual budding of polyps that remain interconnected by continuous epithelia and gastrovascular cavity. The clade Aplanulata is unique in that it comprises mostly solitary species, including the model organism *Hydra*, with only a few colonial species (Schuchert 2006, Schuchert 2010). We reconstruct a multi-gene phylogeny to trace the evolution of coloniality in Aplanulata, which reveals that the ancestor of Aplanulata was solitary, and that coloniality was regained in the genus *Ectopleura*. Our examination of *Ectopleura larynx* development reveals a unique type of colony formation that has never before been described in Hydrozoa in that *Ectopleura larynx* colonies form through sexual reproduction followed by epithelial fusion of offspring polyps to adult colonies. We characterize the expression of the *paired*-like homeobox gene *manacle*, which is known to be involved in foot development in *Hydra* (Bridge, Stover and Steele 2000), to determine polyp-colony boundaries. Our results suggest that stalks beneath the neck do not have polyp identity and instead are specialized structures that interconnect polyps. The ability to fuse epithelia, brooding behavior, and the presence of a skeleton, were all key factors behind the evolution of this novel pathway to coloniality in *Ectopleura*.

RESULTS AND DISCUSSION

Coloniality is a prominent feature of most hydrozoan life cycles, and the emergence of colonialty represents a key event in their evolutionary history (Cartwright and Nawrocki 2010). Hydrozoan colonies consist of asexually generated polyps that remain interconnected through continuous epithelia and a gastrovascular cavity. A typical hydrozoan life cycle consists of a free-swimming or crawling planula larva that metamorphoses into a benthic primary polyp. The primary polyp generates tube-like epithelial structures (stolons) from its base. Stolons grow and eventually bud new polyps and stolons. A chitinous exoskeleton called a perisarc is often secreted around the stolons. Upon maturity, polyps either bud medusae that disperse in the water column and release gametes, or produce gonophores, which are reproductive structures that remain attached to the polyp through sexual maturity.

The clade Aplanulata is unique in that most species are solitary, comprising a single polyp. However, a few species within the genus *Ectopleura*, display a colonial organization. *Ectopleura* colonies, depending on the species, are either small (only a few interconnected polyps) or large (hundreds of interconnected polyps on long, branched stalks) (Petersen 1979, Petersen 1990) (Figure 1A). Other instances of coloniality are reported within Aplanulata, but these do not achieve the size or level of integration of *Ectopleura* colonies, as these other 'colonies' are either loose aggregates that do not share gastrovascular tissues (Schuchert 2006, Petersen 1990, Stepanjants, Svoboda and Anokhin 2002, Galea 2006, Hughes 1983), or polyp buds that remain attached due to the presence of a soft substratum (Brinkmann-voss and Lindner 2008).

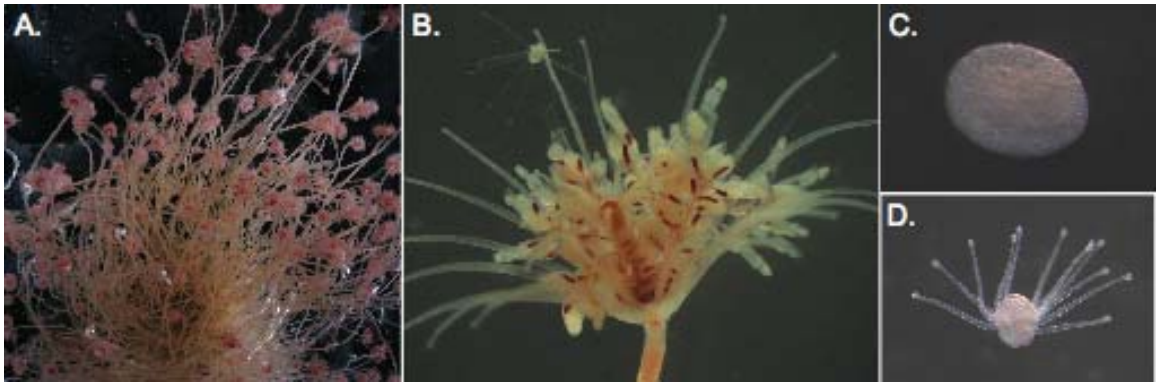

Figure 1: The Aplanulata species *Ectopleura larynx* forms large, dense colonies consisting of hundreds of interconnected polyps. (A) A colony of *Ectopleura larynx* illustrating a network of stalks connecting individual polyps. (B) A close-up of a gravid female *E. larynx* polyp with actinulae (juveniles) emerging from gonophores. (C) Early-stage *E. larynx* embryo dissected from gonophore. (D) Actinula with developed aboral tentacles and mouth.

Coloniality re-evolved in the genus *Ectopleura*

Phylogenetic analyses comprising three molecular markers (16S, 18S and 28s), in conjunction with ancestral character state reconstructions, indicate that the ancestor of Aplanulata was solitary and coloniality re-evolved in the genus *Ectopleura* (Figure 2, S1). Our recovered topology is largely congruent with previous studies that had smaller taxonomic sampling of Aplanulata (Collins, Winkelman, Hadrys and Schierwater 2005, Cartwright, *et al*. 2008, Cartwright and Nawrocki 2010).

Re-evolved *Ectopleura* colonies are chimeras

The Aplanulata species *Ectopleura larynx* Ellis & Solander 1786 produces large branched colonies (Figure 1) (Ellis and Solander 1786). Its life cycle lacks a medusa and individuals instead produce gametes within gonophores that remain attached to the polyp body (Figure 1). Colonies are generally comprised of a single sex, and male colonies release sperm and fertilize a neighboring female colony. *E. larynx*, like many other species of

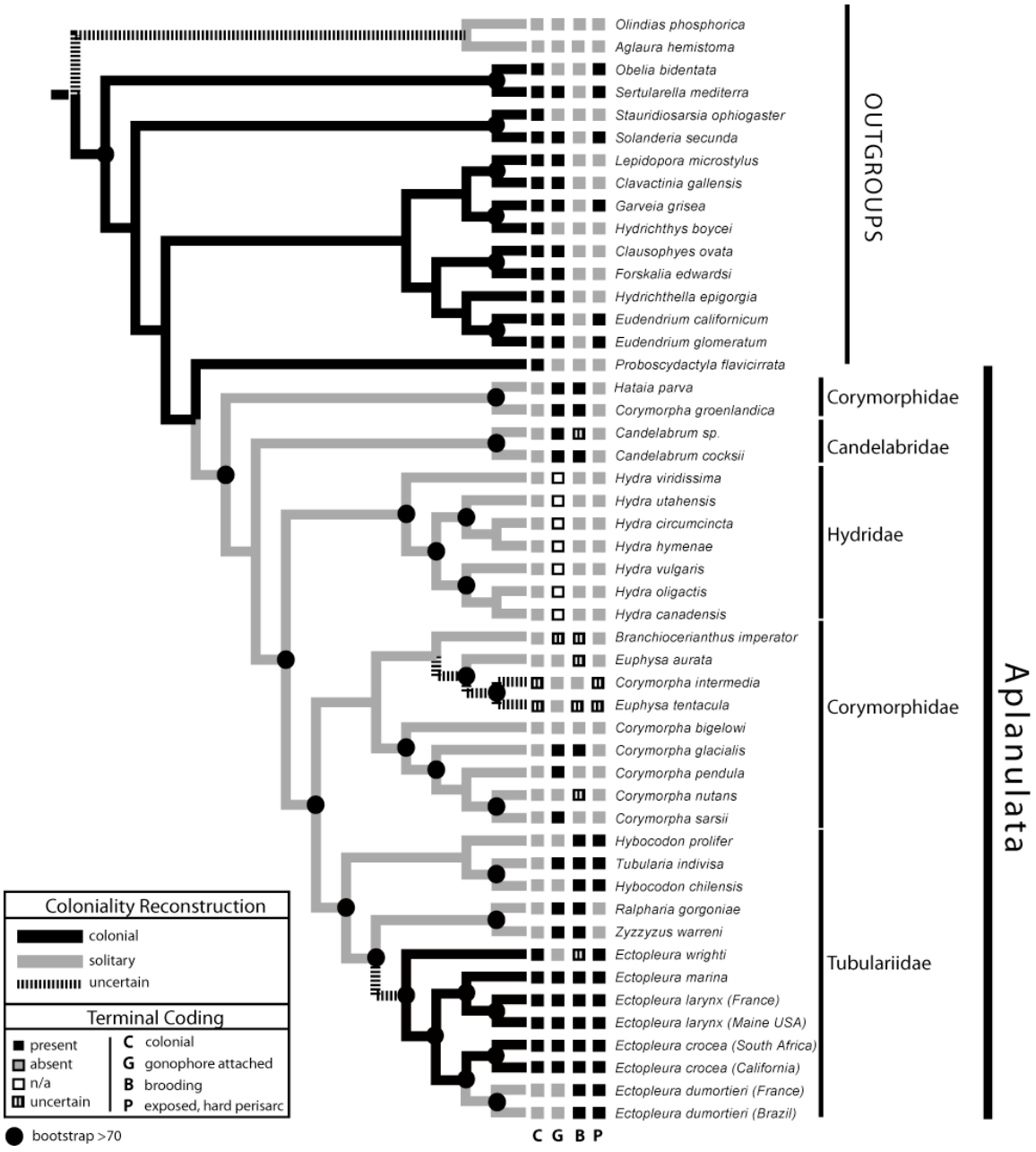

Figure 2. Phylogenetic reconstruction of Aplanulata relationships based on 16S, 18S and 28S and run under a GTR + gamma + doublet (model 16) model (RaxML) . Black circles at nodes indicate bootstrap values greater than 70%, calculated from 1000 replicates in RaxML. Ancestral character state reconstruction demonstrates that coloniality re-evolved in this clade. See also figure S1-S5.

Aplanulata, lacks a planula larva and instead broods its developing young within gonophores. Embryos develop directly into actinulae (juvenile polyps) within gonophores (Figure 1B,C). Each female releases hundreds of actinulae per reproductive cycle. Actinulae settle on a suitable substrate, elongate, and secrete perisarc over their stalks (Berrill 1952). There are reports that some *Ectopleura* spp. colonies can be hermaphroditic (Berrill 1952). It has also been observed and that actinulae can sometimes settle on parent colonies (Schuchert 2010, Petersen 1990). However, little is known about the ontogeny of *Ectopleura* colonies or the fate of polyps that settle on adults.

Colonies are traditionally defined as being composed of genetically identical zooids that develop asexually (Beklemishev 1969, Jackson, Buss and Cook 1985). Our observations suggest that *E. larynx* colonies form in a completely novel manner that does not involve asexual budding. Released actinulae exhibit two different settlement behaviors. In the lab, we observed that following settlement on a hard substrate, actinulae form a few branched stolons at their base (no more than four). New polyp heads can form at the tips of these stolons. We never observed these small, four-polyp colonies to subsequently bud new stolons or polyps. In the lab and in the field, we also observed that actinulae settle on the parent colony (Figure 3A). Following settlement, the perisarc of the parent dissolves at the point of attachment, and the juvenile epithelia and perisarc fuse with those of the parent (Figure 3B,C). The end product of these fusion events is a chimeric colony that possesses features identical to integrated, asexually-formed hydrozoan colonies: an array of polyps interconnected by a continuous epithelia, perisarc, and gastrovascular cavity.

Chimerism leaves constituents vulnerable to cell-lineage competition, where the cell lineage of one individual of the colony overcomes another that is genetically distinct (Buss 1982, Buss 1987, Buss 1990, Hughes 2002). In chimeric colonies of the ascidian *Botryllus schlosseri* the germline of one

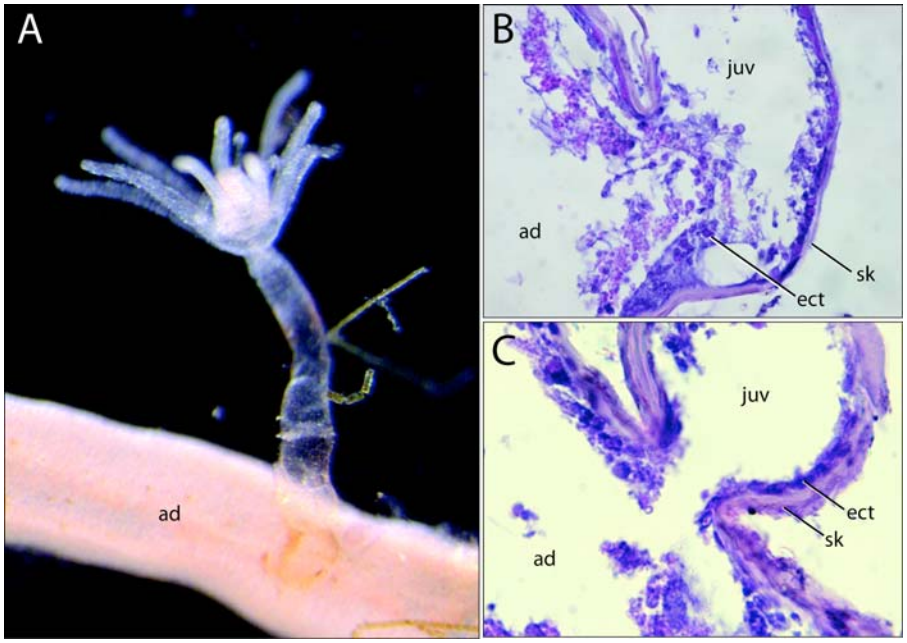

Figure 3: Juvenile polyps settle on the parent, and juvenile and parental tissues fuse. (A) Juvenile polyp settled on a parent stalk. (B-C) Histological sections demonstrating that following settlement, juvenile polyps fuse with parental polyps. (B) Newly-fused juvenile polyp showing continuous perisarc (skeleton) and epithelial tissue spanning adult-juvenile boundary, prior to complete fusion. (C) Completely fused juvenile and parent demonstrating continuous perisarc and epithelial tissue. jv = juvenile/actinula; ad = adult polyp; sk = skeleton (perisarc); ect = ectoderm.

individual can overcome the other through stem cell parasitism (De Tomaso 2006, Pancer, Gershon and Rinkevich 1995, Sabbadin and Zaniolo 1979, Stoner, Rinkevich and Weissman 1999, Laird, De Tomaso and Weissman 2005). This too may be operating in *Ectopleura larynx*. Parent-offspring fusion would be expected to result in a mixture of male and female polyps in a colony. However most colonies we observed are predominately one sex. Hydrozoans don't sequester their germ-line and instead possess a multipotent or totipotent stem cell lineage (Bosch and David 1986, Bode 1996, Müller, Teo and Frank 2004), making them susceptible to stem cell parasitism (Buss 1982, Buss 1987, Buss 1990, Hughes 2002). We suspect that the stem cell line of the parent colony parasitizes the stem cell line of the settled polyp (Buss 1987). However, reports that ageing female polyps often develop male gametes in their most distal gonophores (Schuchert 2010, Fenchel 1905, Hawes 1955, Perez 1925, Berrill 1952) suggests that competitiveness for the germline weakens in aging colonies, which could make them susceptible to stem cell parasitism from offspring.

Chimerism has been documented in many animal phyla, as well as in the hydrozoan *Hydractinia* (Buss 1987, Hughes 2002, Rosengarten and Nicotra 2011, Cadavid, Powell, Nicotra, Moreno and Buss 2004, Grossberg 1988, Ivker 1972, Rosa*, et al*. 2010, Powell*, et al*. 2007, Nicotra*, et al*. 2009, Feldgarden and Yund 1992, Hoffman, Kafatos, Janeway and Ezekowitz 1999). In *Hydractinia*, asexually-formed colonies fuse somatic tissue or display a rejection response depending their degree of relatedness (Buss 1987, Buss 1990, Feldgarden and Yund 1992, Ivker 1972, Lakkis, Dellaporta and Buss 2008, Lange, Plickert and Muller 1989), and two genes control this fusion/rejection response (Cadavid,

Powell, Nicotra, Moreno and Buss 2004, Rosa, *et al*. 2010, Powell, *et al*. 2007, Nicotra, *et al*. 2009). Although it is unknown if allorecognition operates in *E. larynx,* is it possible that it plays a role in determining whether polyps can fuse.

Ectopleura stalks function as stolons

An important characteristic of all hydrozoan colonies is the stolonal system, a series of tube-like structures that connect polyps. In *Ectopleura* colonies, polyps are connected by structures that are traditionally referred to as stalks. Following actinula settlement, the stalk develops at the most distal end of the polyp, but it is unclear if this structure is simply an extension of the polyp body column, or is instead a specialized structure functioning to interconnect polyps.

To determine the distal boundary of polyps in *E. larynx* colonies, we characterized expression patterns of *manacle,* a paired-like homeobox gene that is a marker for foot development in *Hydra* (Figure 4A) (Bridge, Stover and Steele 2000). Relative expression levels of *manacle* assayed by quantitative real-time PCR (Figure 4B) in adult polyps of *E. larynx* demonstrate that *manacle* is upregulated in neck tissue (immediately below the distal tentacles). *In situ* hybridization shows that *manacle* is expressed in a band of ectodermal tissue underneath the second whorl of tentacles, above the stalk (Figure 4C). These data are corroborated by qRT-PCR evidence that *manacle* is upregulated in developmental stages during which this structure develops (Figure 4B). Although expression of a single gene cannot confirm regions of homology, it does suggest the presence of a patterning mechanism in *Ectopleura larynx* that delineates the base of the

polyp from stalk tissue. Thus, despite their independent evolution, preliminary evidence suggests that *Ectopleura* colonies exhibit one of the hallmarks of colony structure found in other hydrozoan species - a stolonal system separate from the polyp that interconnects polyps through a shared gastrovascular cavity.

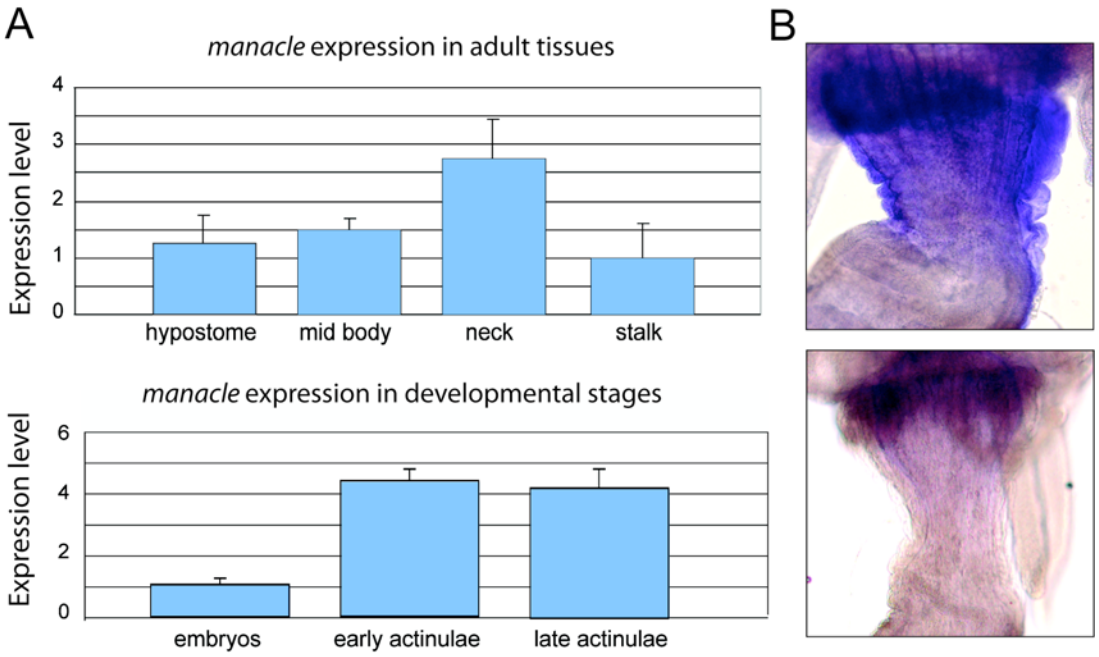

Figure 4. Relative spatial and temporal expression of *manacle* in *Ectopleura larynx*, normalized to the reference gene, ß-actin, using the comparative Ct (cycle threshold) method. (A) qRT-PCR of *manacle* in *E. larynx* adult tissue pools and in developing whole juveniles. Bars indicate standard error. (B) Spatial expression of *manacle* in adult *E. larynx* polyps. (top) Antisense probe to *manacle*. (bottom) Negative control.

Re-evolved coloniality is correlated with life history characters

We coded and mapped life history characters on the phylogeny in order to determine if particular features are correlated with coloniality (Figure 2, S1). Although many hydrozoans display a pelagic sexual medusa stage, reduction of medusae to attached gonophores is a common feature in hydrozoan evolution (Cartwright and Nawrocki 2010). Within Aplanulata, medusae have been reduced to attached gonophores multiple times (Figure 2). Another life cycle feature in Aplanulata is brooding. Many species of Aplanulata brood their embryos and release them as actinulae. The perisarc, a chitinous skeleton over the polyp stalk, is found in a number of Aplanulata species. The perisarc covers stalks and provides support for their upright form. This is in contrast to many solitary species, which either lack perisarc or instead form a thin, membranous covering over the polyp body (Schuchert 2010, Petersen 1990).

Our character mapping suggests that colony re-evolution in *Ectopleura* is associated with a combination of all three characters (Figure 2); thus, the possession of these characters is a requirement for coloniality in *Ectopleura*. Pair-wise character correlation analyses provide evidence for this. Coloniality was significantly correlated with brooding, the presence of an exposed, hard perisarc, and the simultaneous presence of all three characters (see Supplementary Material for description of statistical analyses).

The combination of attached gonophores and brooding provides an opportunity for chimeric colonies to develop, as they ensure that the actinula is in proximity to the parent upon release. Additional evidence is found in the closely related solitary species *E.*

dumorteri, which bears a medusa (Figure 2) Its actinulae are unable to settle on the parent likely due to their dispersal away from the parent.

Attached gonophores and a brooding habit, however, are not enough to allow for fusion of juvenile polyps to the parent. There also needs to be a suitable substrate for the juvenile. This suitable substrate is the exposed, hard perisarc of the parent. We sampled a number of other species in our analysis that have attached gonophores and brood, but lack an exposed hard perisarc. In these other species, the perisarc may be thin and membranous (Corymorphidae), buried in an invertebrate host (*Ralpharia gorgoniae* and *Zyzzyzus warreni*), or very small (Candelabridae) (Petersen 1990).

Although the combination of attached gonophores, brooding, and a hard, exposed perisarc are necessary for coloniality to evolve (with the exception of *E. wrighti,* see below), it does not appear sufficient, as *Tubularia indivisa* has all of these characters, but is not colonial. *Tubularia indivisa* polyps are often found in clusters, and can settle upon one another, but there is no evidence that they fuse. Further investigation into these characters may prove illuminating.

From the recovered phylogenetic pattern, the simplest interpretation is that re-evolution of coloniality evolved in a step-wise fashion. Many of the early diverging lineages of Aplanulata can bud, but the buds detach, as exemplified in *Hydra*. Species of the Corymorphidae/Tubulariidae clade do not bud, and thus we infer that the ability to bud asexually was lost following the divergence of the *Hydra* lineage (Figure 2, S1). A small

colony of a few polyps re-evolved at the base of *Ectopleura*, as exhibited by *Ectopleura wrighti* [4,8], the sister taxon to the rest of *Ectopleura* (Figure 2, S1). All colonial *Ectopleura* species start from a single primary polyp that forms up to four other polyps through the growth of stolons from its aboral end, with polyps developing at the distal ends of these stolons. The number of polyps that develop in this manner appears to be restricted by the amount of stolonal tissue that can form at the base of the primary polyp. Unlike other colonial hydrozoans, these small colonies never bud additional stolons or polyps. The extensively branched chimeric colonies consisting of hundreds of polyps evolved subsequently, in association with brooding, attached gonophores, and the presence of an exposed hard perisarc. Because *Ectopleura wrighti* has a medusa and not the attached gonophores, it doesn't develop the large chimeric colony.

This novel mode of colony formation through sexual reproduction has never before been reported in Hydrozoa. The evolution of coloniality has, however, been associated with life history traits in non-hydrozoans, such as symbiosis in corals (Barbeitos, Romano and Lasker 2010) and viviparity in ascidians (Pérez-Portela, Bishop, Davis and Turon 2008). Future work on *Ectopleura larynx* should help uncover the mechanisms involved in colony re-evolution and shed additional light on the complex evolutionary history of this ancient and diverse animal lineage.

Experimental Procedures

See Appendix for more detailed experimental procedures.

DNA sequencing, phylogenetic reconstruction and character analysis

DNA amplification and sequencing was conducted as previously described (Collins, *et al*. 2008, Collins, *et al*. 2006, Collins, Winkelman, Hadrys and Schierwater 2005, Cunningham and Buss 1993, Cartwright, *et al*. 2008), and phylogenetic analyses were conducted in the parallel version of RaxML 7.2.8 (Stamatakis 2006). Global maximum likelihood character state reconstructions and correlation analyses were performed in Mesquite (Maddison and Maddison 2010) using an MK1 model following (Cartwright and Nawrocki 2010). Correlation analyses were performed in Mesquite using Pagel's Correlation Method (Maddison and Maddison 2010, Pagel 1994), following (Cartwright and Nawrocki 2010).

***Histology and manacle gene* expression**

E. larynx was obtained from Marine Biological Laboratory (Woods Hole, MA). Fixation, sectioning and staining were carried out following standard protocols (Cielocha and Jensen 2011).

A 280bp fragment of the paired-like gene *manacle* was identified from a library of *E. larynx* cDNA with 454 pyrosequencing, and submitted to Genbank (JN594057). This fragment was amplified, cloned, transformed into E. coli, and purified following standard protocols. *In situ* hybridization and qRT-PCR was carried out following standard protocols (Gajewski, Leitz, Schlöherr and Plickert 1996).

CHAPTER 4: Expression of Wnt pathway genes in polyp and medusa-like structures of *Ectopleura larynx* (Hydrozoa: Aplanulata)

ABSTRACT

The canonical Wnt signaling pathway is highly conserved in its role in axial patterning throughout Metazoa. In hydrozoans (Phylum Cnidaria), Wnt signaling is implicated in oral-aboral patterning of the planula, polyp and medusa life history stages. Unlike most hydrozoans, members within the hydrozoan clade Aplanulata, which includes the model organism *Hydra*, lack a planula larva and the polyp instead develops from a brooded embryo. The Aplanulata species *Ectopleura larynx* broods its embryos within gonophores that represent a truncated medusa stage of the hydrozoan life cycle. *Ectopleura larynx* gonophores retain evolutionary remnants of medusa structures but remain attached to the polyps from which they bud. These gonophores differ between males and females in their degree of medusa truncation, making them an ideal system for examining different stages of truncated medusa development. Using next-generation sequencing technologies, we isolated genes belonging to the canonical Wnt signaling pathway and examined their expression in *Ectopleura larynx*. Our data are consistent with the canonical Wnt signaling pathway being involved in axial patterning of polyp and the truncated medusa during *Ectopleura larynx* development. We report a shift in the order of *Wnt5* and *Wnt3* expression, consistent with a role for *Wnt5* in initiating new axes of the polyp prior to the expression of *Wnt3*. Our data are consistent with expression patterns in *Hydra*, and together suggest that early expression of *Wnt5* prior to deployment of *Wnt3* is unique to Aplanulata, and possibly related to the loss of a larva. Gene expression patterns in *Ectopleura larynx* truncated medusae are congruent with *Wnt5* initiating new axes of the gonophore, and suggest that changes in the spatial expression of Wnt pathway genes are correlated with the development of different oral structures in male and female gonophores. Lastly, the absence of expression of components of the Wnt pathway, as well as presence of a Wnt pathway antagonist *SFRP*, in the developing

anterior end of the gonophore, suggest that downregulation of the pathway may be responsible for medusa reduction in *Ectopleura larynx*, and perhaps in the multiple instances of medusa reduction in hydrozoan evolution.

INTRODUCTION

The canonical Wnt signaling pathway is conserved in evolution, playing a critical role in axial patterning throughout Metazoa, including members of the phylum Cnidaria (Croce and McClay 2008). The role of this pathway in initiating and maintaining the oral end of the polyp has been characterized in several cnidarian species, including the anthozoan *Nematostella vectensis* (Kusserow, Pang, Sturm *et al*. 2005) and the hydrozoans, *Hydra magnipapillata* (Hobmayer, Rentzsch, Kuhn *et al*. 2000, Broun, Gee, Reinhardt *et al*. 2005, Guder, Pinho, Nacak *et al*. 2006), *Clytia hemaespherica* (Momose, Derelle and Houliston 2008), and *Hydractinia echinata* (Muller, Frank, Teo *et al*. 2007, Plickert, Jacoby, Frank *et al*. 2006, Duffy, Plickert, Kuenzel *et al*. 2010). Several recent studies additionally suggest a role for Wnt signaling in *Clytia hemaespherica* in patterning the oral end of the medusa, the free-swimming jellyfish stage of hydrozoans (Momose and Houliston 2007). Proteins involved in the Wnt signaling pathway, some of which have been characterized in cnidarians, include Wnt (e.g. *Wnt3*) ligands, transmembrane receptors of these ligands (*Frizzled* and *LCF*), a number of antagonists (including secreted frizzled related proteins, or *SFRP*s) and the downstream elements *glycogen synthase kinase 3-beta* (GSK3β), *β-catenin*, *Tcf* and others. Additional ligands, including *Wnt5*, are also implicated in this pathway (He *et al*. 1997) (**Figure 1**).

The Canonical Wnt Pathway and Polyp Development

The Wnt pathway has been implicated in patterning the oral end of the hydrozoan polyps *Hydractinia echinata* (Plickert *et al*. 2006, Duffy *et al*. 2010) and *Clytia hemaespherica* (Momose, Derelle & Houliston 2008) as they develop from an embryo into a planula larva, and then through metamorphosis into a primary polyp. In most species, polyps form following the

metamorphosis of a pelagic larva called a planula. The planula eventually settles with its anterior end touching the surface. The planula then undergoes metamorphosis, resulting in a primary polyp with an oral end where the posterior end of the planula once was.

In *Clytia hemaespherica Wnt3* is a maternally-coded secreted protein that directs oral specification in developing embryos, and remains active from early embryonic stages through planula formation, where it is expressed in the region of the embryo and planula that correspond to the future oral end of the polyp (Momose, Derelle & Houliston 2008).

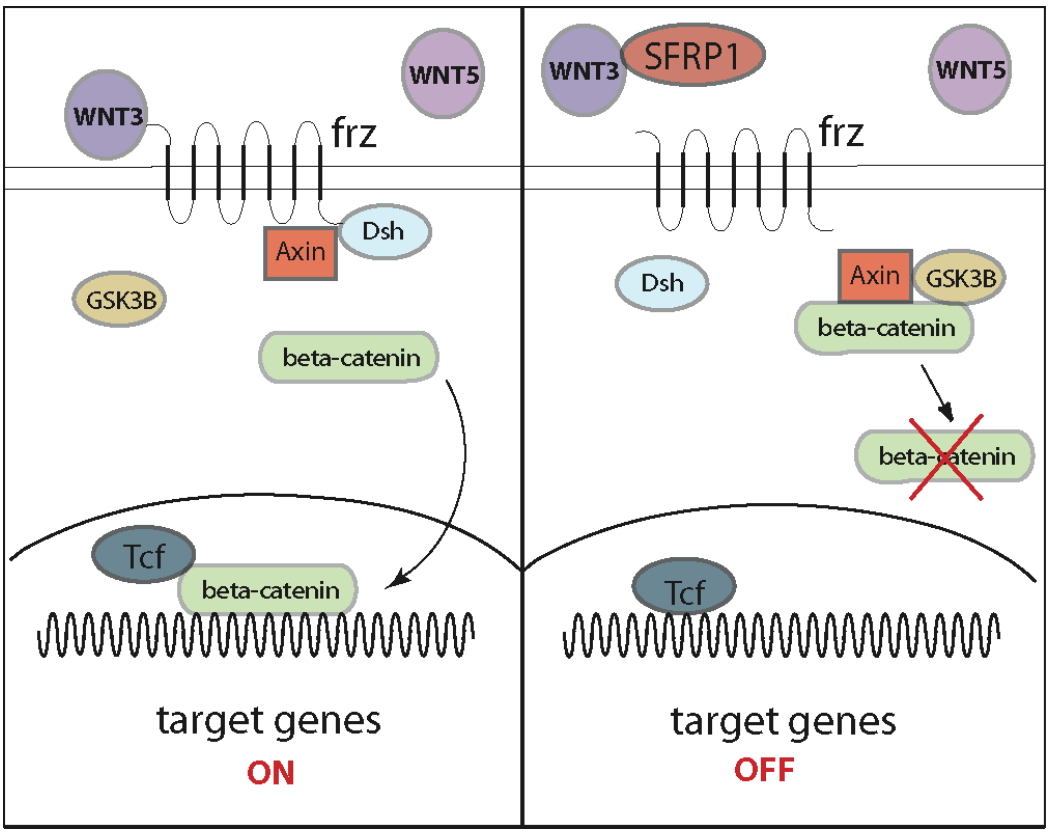

Figure 1. Schematic of the canonical Wnt signaling pathway and some of its known components. **Left**: The canonical Wnt, *Wnt3*, binds to one of its receptors, *frizzled*, allowing the movement of *β-catenin* into the nucleus, where it binds to *Tcf* and regulatory sequences, and activates downstream gene pathways. **Right**: Binding of *Wnt3* by *SFRP* prevents the activation of *Frizzled*, leading to the degradation of *β-catenin* and the inhibition of its downstream target genes. *Wnt5* may also be involved in canonical wnt signaling, but its exact role in this pathway is unknown.

Expression is similar in *Hydractinia echinata*, where *Wnt3* is a maternally-provided transcript expressed in regions of the polyp and planula that correspond to the future oral end of the polyp (Plickert *et al*. 2006). In adult stages, *Wnt3* is expressed in the structure that supports the polyp's mouth, called the hypostome (Plickert *et al*. 2006) and promotes the formation of oral structures in regenerating adult polyps (Duffy et *al*. 2010). In summary, these studies implicate *Wnt3* in polarizing the embryonic axis, maintaining the oral pole of the future polyp during metamorphosis, and initiating and maintaining the patterning of the oral region of polyps (Plickert et *al*. 2006, Momose, Derelle & Houliston 2008, Duffy *et al*. 2010).

Unlike most hydrozoans, species in the clade Aplanulata, which includes the model organism *Hydra*, do not develop through a planula larva stage. Instead, members of this clade develop directly into a primary polyp, either while inside a gonophore (e.g. *Ectopleura larynx*) (Schuchert 2010, Nawrocki and Cartwright *in press*), or in a cyst (e.g. *Hydra*). Following encystment or brooding in a gonophore, a fully-formed juvenile polyp hatches or emerges respectively (Hyman 1940, Berrill 1952). One characteristic of this development is that the primary body axis appears to be specified late in ontogeny, subsequent to the formation of polyp tentacles (**Figure 2**) (Berrill 1952). The role of the Wnt pathway axis specification has been characterized in one Aplanulata species – the model organism *Hydra vulgaris*. In *Hydra*, *Wnt3* is critical for the formation and maintenance of the oral axis during development (Hobmayer, Rentzsch, Kuhn *et al*. 2000, Lengfield *et al*. 2009), and a number of additional Wnt ligands are expressed in overlapping patterns in the head organizer (Philipp *et al*. 2009, Lengfield *et al*. 2009). However, unlike in planula-bearing species, *Wnt3* is not expressed in early embryonic stages (Frobius et *al*. 2003), and its expression appears only subsequent to the initiation of

asexual buds that eventually become new polyps (Lengfield *et al*. 2009). These data together suggest that *Wnt3* does not play a role in the initial establishment of the oral/aboral axis of the *Hydra* polyp, but rather in the maintenance of the head organizer during tissue proliferation of the bud or polyp (Lengfield *et al*. 2009). This raises the question of what genes might be implicated in the initiation of polyp axis formation in Aplanulata hydrozoans.

One candidate is a paralog of *Wnt3*, *Wnt5*. In the planula-bearing species *Clytia hemaespherica* and *Hydractinia echinata* this gene is deployed subsequent to *Wnt3* and immediately prior the initiation of metamorphosis (Momose, Derelle and Houliston 2008, Stumpf et *al*. 2010). In contrast, in the Aplanulata species *Hydra*, *Wnt5* expression appears prior to *Wnt3* during asexual budding, suggesting that it may be involved in axis initiation (Philipp *et al*. 2009, Lengfield *et al*. 2009). Additionally, in *Hydra*, the expression of *Wnt5* overlaps with *Tcf* and *β-catenin*, downstream elements of the canonical Wnt signaling pathway, suggesting *Wnt5* may be involved in canonical Wnt signaling (Philipp *et al*. 2009). This is supported by studies in *Xenopus*, which also suggest a role for Wnt5 in canonical Wnt signaling (He *et al*. 1997).

The Canonical Wnt Pathway and Medusa Development

Hydrozoans also display complex life cycles in which the polyp asexually buds jellyfish (medusae) on its body column. Depending on the species, this structure detaches and lives independently in the water column as a medusa and becomes sexually mature, or its development is arrested at an earlier stage and remains attached to the polyp throughout sexual maturity. Reduced structures that fail to develop completely into medusae are called gonophores. This reduction or loss of the pelagic medusa stage has occurred multiple times in hydrozoan evolution

(Cartwright & Nawrocki, 2010). Depending on the species, the truncated medusa, or gonophore, retains varying degrees of medusa characteristics. Gonophores, for example, may have tentacles, remnants of a mouth (called a spadix) and/or musculature. In some species, these structures are completely absent and the gonophore lacks any morphology reminiscent of a medusa. Studies examining the expression of Wnt pathway genes in hydrozoans suggest that genes in this pathway known to pattern polyp development also pattern the oral end of medusae and gonophores (Muller, Frank, Teo *et al*. 2007, Momose and Houliston 2007). For example, the Wnt receptors *Frizzled1* and *frizzled3* are expressed in oral structures of the medusa of the hydrozoan *Clytia hemaespherica*, with *Frizzled1* most strongly expressed in the tentacle bulbs of the developing medusa, and *frizzled3* expressed in the ring canal of the medusa, which is located at the bell margin, and in a band on the mouth (Momose and Houliston 2007). The expression patterns of *Wnt3* and *Wnt5* are not characterized in this developmental stage, and it is unknown if these two *Wnt* ligands initiate or maintain axes, as they are thought to do in polyp stages. In *Hydractinia echinata*, a species whose gonophores lack all medusae morphology, *Wnt3* is expressed in a small subset of ectodermal cells at the most distal end of the gonophore (Muller, Frank, Teo et *al*. 2007), while other genes in the Wnt signaling pathway, including *Frizzled*, are absent (Duffy et *al*. 2010).

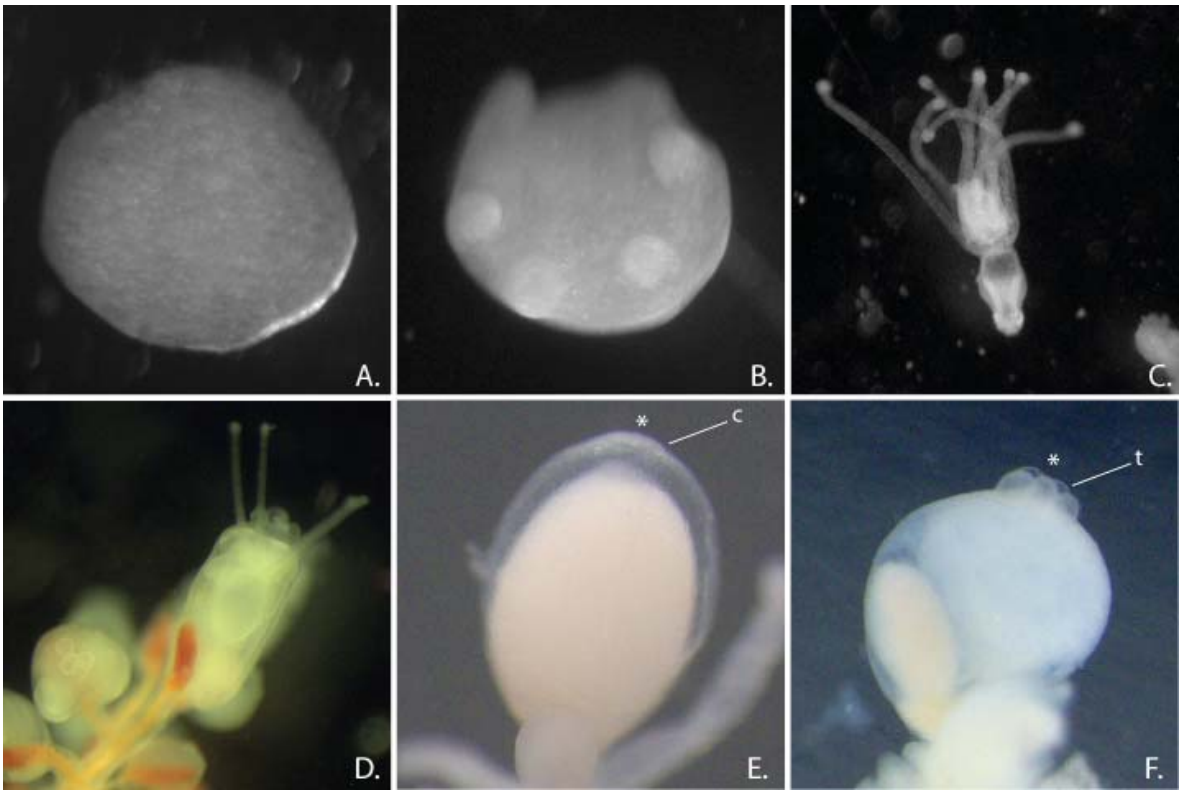

Figure 2. Life cycle stages of the Aplanulata species *Ectopleura larynx*. (A-C) Polyps develop from an embryo into a primary polyp. A. Embryo. B. Early-stage actinula (juvenile polyp). C. Actinula. D. Actinula emerging from female *Ectopleura larynx* gonophore. (E-F) Sexually-dimorphic gonophores of *Ectopleura larynx* displaying differing degrees of medusa reduction. E. Male gonophore displaying distal cap structure on the anterior/oral end of the structure. F. Female gonophore displaying tentacle bulbs on the anterior/oral end of the structure. c = distal cap; t = tentacle bulbs; * anterior/oral end of gonophore.

The Aplanulata species *Ectopleura larynx*, a close relative of the model organism *Hydra*, is a well-suited system for examining the role of the Wnt pathway in patterning hydrozoan lifecycle stages (**Figure 2**). Like many other members of Aplanulata, *Ectopleura larynx* polyps develop directly from an embryo and lack and planula larva stage (**Figure 2A-C**). As their development occurs within gonophores on the polyp body (**Figure 2D)**, developing polyps are more accessible for *in situ* hybridization studies than species whose polyps develop inside cysts (e.g. *Hydra*). *Ectopleura larynx* produces a large number of gonophores per polyp, which are easily accessible for experimental study. *Ectopleura larynx* also displays sexual dimorphism in its truncated medusae (gonophores), with male gonophores developing fewer morphological hallmarks of medusae than female gonophores. Male gonophores develop a cap on the distal end of the gonophore (**Figure 2E**) whereas female gonophores are larger and develop tentacle remnants (**Figure 2F**). This sexual dimorphism as displayed by *Ectopleura larynx* provides an opportunity to examine the development of gonophores displaying different stages of medusa truncation within the same species.

Here, we report the isolation and characterization of major elements of the canonical Wnt signaling pathway from *Ectopleura larynx*, including *Wnt3*, a paralog of *Wnt3*, *Wnt5*, a putative Wnt receptor, *Frizzled1,* and a putative Wnt pathway antagonist, *SFRP* (secreted frizzled related protein). We examine the expression of these genes in all developmental stages of *Ectopleura larynx* using *in situ* hybridization and/or qRT-PCR in order to address the question of whether the Wnt pathway patterns oral structures of the polyp and truncated medusa (gonophore) in this species that lacks a planula larva. Our results are consistent with the Wnt signaling pathway patterning oral structures of the *Ectopleura larynx* polyp and gonophore. Our data suggest that

Wnt signaling plays a role in patterning diverse oral structures in all life cycle stages of hydrozoans, and that temporal shifts in Wnt gene deployment may be implicated in evolutionary transitions between larval and direct developers. Lastly, our data shed light on the genes possibly involved in the truncation of medusa development, and offer one possible explanation for the frequency with which medusae have been lost over the evolutionary history of Hydrozoa.

MATERIALS & METHODS

Animal culturing and tissue preservation

Male and female *Ectopleura larynx* colonies were obtained from Marine Biological Laboratory (Woods Hole, MA) or near Mount Desert Island Biological Laboratory (Salisbury Cove, ME), and were observed alive in the laboratory. Colonies were then maintained at 12ºC in 32ppt artificial sea water in the dark for four weeks in order to collect and preserve embryos, liberated actinulae and adult tissues for *in situ* hybridization. During lab maintenance, animals were fed 4x weekly with *Artemia nauplii*. For fixation, animals were relaxed with $MgCl_2$, and tissues were fixed in 4% paraformaldehyde overnight and stored in 100% MeOH at -20ºC indefinitely prior to *in situ* hybridization. Preserved tissues were used for subsequent studies described in this paper.

Isolation of Wnt pathway genes from *Ectopleura larynx*

In order to identify Wnt genes and other Wnt pathway components from *Ectopleura larynx* developmental stages, we constructed a number of cDNA libraries for Illumina sequencing. Illumina libraries were constructed for three different polyp developmental stages (embryos, early actinulae, late actinulae) using Illumina's TruSeq paired-end sample preparation protocol (Illumina, San Diego, CA), and sequenced 100bp paired-end reads on three lanes of an Illumina HiSeq 2000 (University of Kansas Genome Sequencing Facility, Kansas City, KS). Resulting sequences were trimmed using the python stript **q-trim.py** (M. Shcheglovitova, pers. comm). Data was then assembled using the software package Trinity (Grabherr, Haas, Yassour *et al*. 2011) on Amazon's EC2 on a 13-processor computer with 4 cores and 34.2 GB of RAM. A BLAST database was constructed from each resulting assembly. These assemblies were then

searched locally using tBLASTx to identify major elements of the Wnt signaling pathway, including *Wnt3*, *Wnt5*, *Frizzled*, *TCF*, *β-catenin*, *GSK3β* and *SFRP*,.

Sequence retrieval, alignment, model selection and phylogenetic analyses

Following identification using BLAST searches, the putative Wnt pathway genes *Wnt3, frizzled,* and *SFRP* were used to search again against *Ectopleura larynx* transcriptome databases in order to isolate all potential orthologs and paralogs of these genes of interest. tBLASTx searches were conducted, and the top 30 hits were pulled from the assembled transcriptomes. Then, the original Wnt pathway gene sequences were used as input for tBLASTx searches against Genbank. The top 50 hits were downloaded. For Wnt genes, which are well-characterized and diverse in cnidarians, tBLASTx searches were limited to cnidarian genes only. For all other genes, tBLASTx searches were expanded to all of Metazoa. Duplicate sequences were removed, and protein alignments were generated for each gene in MUSCLE (Edgar 2004a, b). Alignments were then hand edited in SeaView v2.4 (Galtier, Gouy and Cautier 1996) to remove ambiguously aligned regions, or were run through the program Gblocks v0.91b (Castresana 2000). Final alignments were visualized in Mesquite v2.75 (Maddison and Maddison 2007) and Seaview v2.4 (Galtier, Gouy and Cautier 1996). Final alignments were run through ProtTest v.3 (Darriba, Taboada, Doallo *et al*. 2011) to determine the best fit model of protein evolution for each gene given the alignment. We used the BIC (Schwarz 1978) to select the model of protein evolution for subsequent analyses.

Phylogenetic analyses were conducted in the parallel version of RaxML v7.2.5 (Stamatakis 2006) in order to make the best estimate of gene orthology given the protein alignment. For each

analysis, we assessed node support by generating and summarizing 1000 bootstrap replicates (Stamatakis 2006). Trees were visualized in FigTree v1.3.1 (Drummond and Rambaut 2007) and Mesquite v2.75 (Maddison and Maddison 2007).

Quantitative Real-Time PCR (qRT-PCR)

RNA was isolated from *Ectopleura larynx* using the following protocol: Developmental stages were dissected from reproductive structures and placed immediately on dry ice. Samples were then frozen at -80, macerated using a mortar and pestle, and incubated overnight at 4°C in TriReagent (Life Technologies, Grand Island, NY). Remaining tissue was spun down and re-macerated until no visible tissue remained. BCP was added following manufacturer's protocol, and samples were shaken vigorously for 15-30 seconds to ensure even mixing. Tubes were incubated at room temperature for 15 minutes, and then spun down for 15 minutes. The top layer was removed and combined with isopropanol and incubated at room temperature for 20 minutes, and then overnight at -20°C. Samples were spun down and a visible pellet was washed twice with 75% EtOH. RNA was then treated with TurboDNAse (Life Technologies, Grand Island, NY) following manufacturer's protocol, and a sodium acetate back-precipitation (protocol available upon request) removed residual phenol. RNA integrity was confirmed by visualizing 18s and 28s peaks using the Agilent 2100 Bioanalyzer and quantified using the RNA Quant-It assay for the Qbit Fluorometer (Life Technologies, Grand Island, NY). For each library construction, no less than 500ng of total RNA was used going into the cDNA library preparation. Final RNA was then treated with TurboDNAse (Life Technologies, Grand Island, NY) to ensure removal of genomic DNA.

RNA was reverse-transcribed with Superscript III Reverse Transcriptase (Life Technologies, Grand Island, NY). To ensure equal relative transcript abundances, reverse transcription was carried out on equal quantities of each tissue-derived RNA pool. qRT-PCR was conducted using a DNA Engine Opticon 2 real-time PCR machine (MJ Research, Ramsey, MN, USA) in the presence of SYBR Green I (Life Technologies, Grand Island, NY) and DyNAzyme II Hot Start DNA Polymerase (Thermo Scientific, Wilmington, DE), or by using a Step One Plus Real-Time PCR Thermal Cycler (Applied Biosystems, Carlsbad, CA). For all genes, expression in each tissue was evaluated in quadruplicate, and two independent experimental replicates were conducted. Three control genes *(β-Actin, β-Tubulin* and *18S*) were evaluated for invariability across all tissue pools (**Table 1**), and the single gene *β-Actin* was selected as a control due to its invariability across tested tissue pools. Data analysis on qRT PCR data was conducted by calculating the relative expression ratios of each gene, including control gene, in each RNA pool. For each reaction, the amount of target gene cDNA expression was inferred relative to the level of the control gene using the comparative Ct method. In short, the mean expression levels of the genes of interest and control genes were calculated from multiple replicates, and the highest relative expression level per tissue pool per gene was set to one. Normalization factors were then calculated from control gene expression in each tissue pool, and used to rescale expression values of genes of interest.

Table 1. Primer Pairs for Quantitative Real-Time PCR (qRT-PCR) and *in situ* Hybridization

Gene	Forward Primer	Reverse Primer
qRT-PCR primers		
β-Actin	ATTGCTGACCGTATGCAGAA	CCAAAATAGATCCTCCGATCC
Frizzled1	GCCCTACAAGACCACGATGT	AGATAGGCGATGCAAAGGAA
SFRP	ACCGACTACCGTCAAACCTG	GAACGCTCTCCTTCTTGCAC
Tcf	GTGACGAAGACTCGTCCAAAG	CGATCTGCGAAACTGCTACA
β-catenin	GTTGGTGCCCTGCATATTCT	ATGGCATCAGCACCTTCTTT
GSK3B	TAATCATGGGGGTTTCAGGA	TCCGTGCTGTCAACAATCTT
In situ probe primers		
β-Actin	AAGCTCTTCCCTCGAGAAATC	CCAAAATAGATCCTCCGATCC
Frizzled1	GGTGCGTTTCCATAACATCTG	GGGTCCTTTCAATCCCTTTC
SFRP	ATCGATTGTTCCCCTCACCT	TTGTTTTCAGCTCACTCACACA
Wnt3	ATAAATGCAGCCAGCCAATC	CGCTGCGCCTAATCTTGTAG
Wnt5	GAATTGCACTTTTCCCGAAC	GCCGAATGAACCTTCGTCTA

*All primers are given in the 5' to 3' orientation

Gene amplification, cloning, and probe generation

Primers were designed for genes of interest, based on full-length sequences isolated from either 454 or Illumina assembled transcriptomes (Table 1). PCR products were ligated directly into Invitrogen TOPO-4-PCR vectors (Life Technologies, Grand Island, NY) and then transformed into chemically-competent *E. coli*. Amplified clones were purified using a Qiagen Miniprep Kit (Qiagen, Valencia, CA). Sequence identity was confirmed using directional sequencing with standard T7 and T3 primers (ACGT, inc., Wheeling, IL). Sense and antisense RNA probes were generated for *Wnt3*, *Wnt5*, *Frizzled1*, and *SFRP* using Ambion's T7/T3 MAXIscript kits according to manufacturer's protocol, except that probe synthesis was carried out overnight (Life Technologies, Grand Island, NY). Probes were precipitated at -20C overnight using NaAc and 100% EtOH. Probe concentration was determined with a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE), and a standard dot-blot assay on a nitrocellulose membrane was conducted to assess the efficiency of DIG incorporation.

***in situ* hybridization**

We carried out whole-mount *in situ* hybridization on embryos, actinulae, adult polyps and reproductive structures following (Nawrocki and Cartwright *in press*), except that all tissues were heat treated at 80ºC for 10 minutes following fixation to disrupt endogenous alkaline phosphatase activity, and anti-DIG-AP was added at a concentration of 1:5,000.

RESULTS

***Ectopleura larynx* Wnt gene family**

tBLASTx searches of *Ectopleura larynx* transcriptome assemblies revealed multiple Wnt genes sharing affinity to six of the seven major Wnt families characterized in Hydrozoa: WNT5/11, WNT3, WNT9/10, WNT8/11, WNT7 and WNTX1. Phylogenetic analysis of a 447 amino-acid long alignment of 72 cnidarian Wnt sequences under a WAG + Γ model in RaxML (Stamatakis 2006) confirmed the orthology of these genes (**Figure 3**). In general, we recovered strong support for the placement of each of the Wnt genes with respect to other Wnt orthologs.

Frizzled and *SFRP* gene orthology

Given that the *SFRP* gene represents a truncated version of the *frizzled* gene, we included SFRP and frizzled in the same phylogenetic analysis. We conducted a phylogenetic analysis on metazoan *frizzled* genes and include our sequence for putative *SFRP*, to better determine its orthology. tBLASTx searches of *Ectopleura larynx* transcriptome assemblies revealed multiple *frizzled* and *frizzled*-like genes sharing affinity to three of the four major *frizzled* families characterized in Hydrozoa: *Frizzled1*, *frizzled5/8*, and *frizzled4/9/10*. Phylogenetic analysis of a 443 amino-acid long alignment of 35 metazoan *frizzled* sequences under a WAG + Γ model in RaxML (Stamatakis 2006) and including our *SFRP* sequence, confirmed the orthology of these genes (**Figure 4**), although we do not recover a strong placement for *Frizzled1* or SFRP.

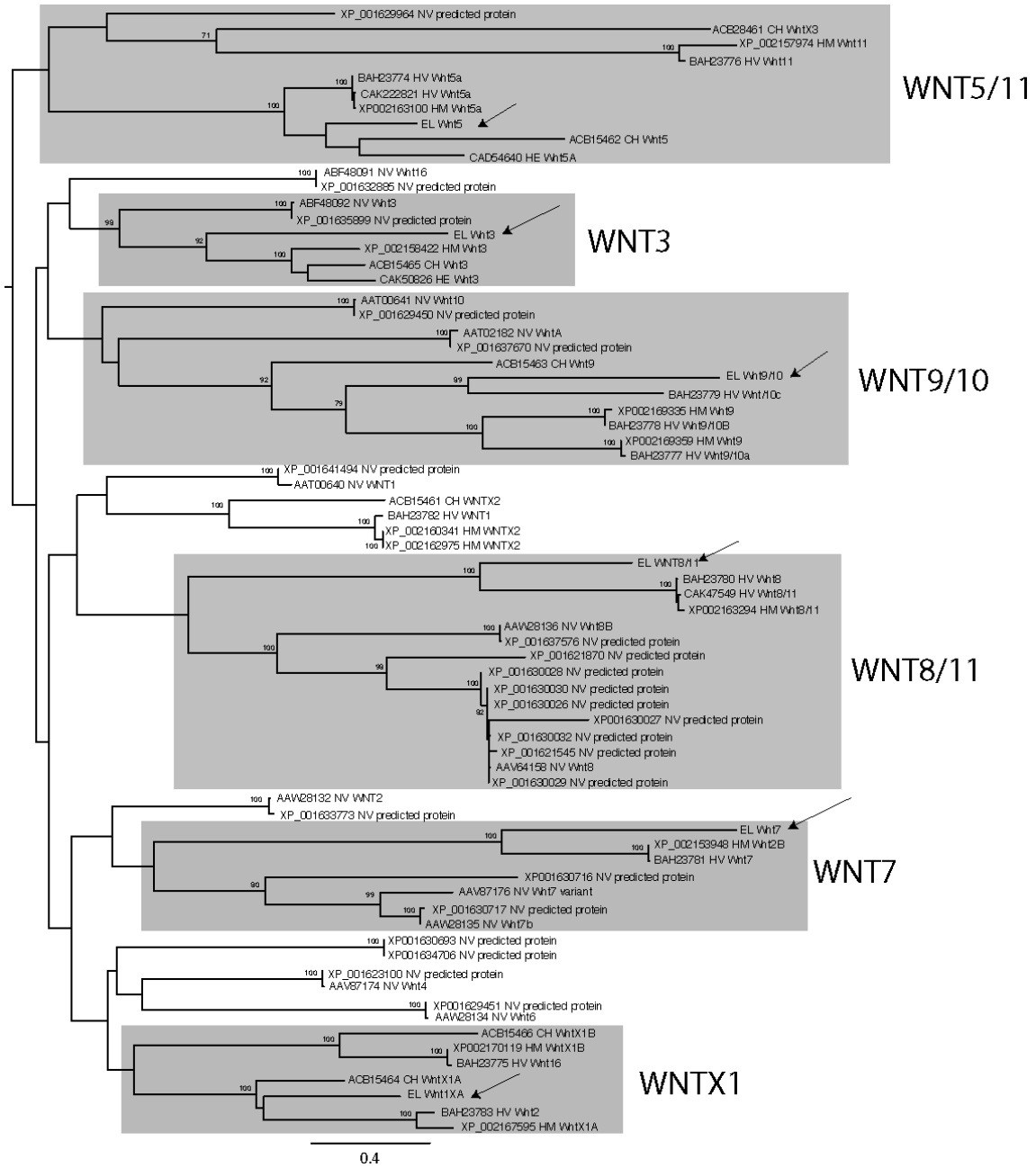

Figure 3. Phylogenetic hypothesis (midpoint-rooted) of relationships of *Ectopleura larynx* Wnt genes with those of other cnidarians. Analysis run in RaxML under a WAG + Γ model. Bootstrap (bs) support values generated with 1000 bs replicates in RaxML. BS values are reported if ≥ 70. Terminals of the tree are labeled as follows: "GenbankID species orthology". EL = *Ectopleura larynx*; NV = *Nematostella vectensis*; HM = *Hydra magnipapillata*; HV = *Hydra vulgaris*; CH = *Clytia hemaespherica*.

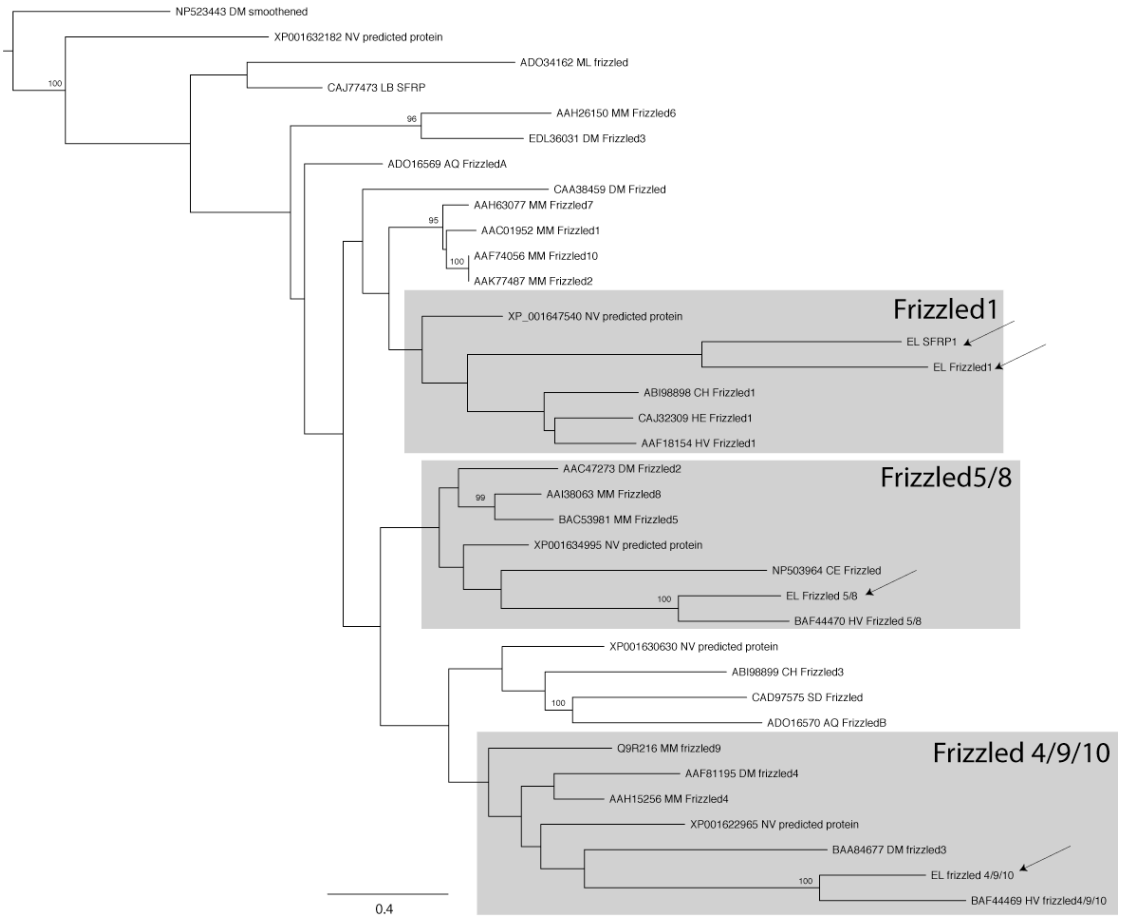

Figure 4. Phylogenetic hypothesis for relationships of *Ectopleura larynx frizzled* genes with *frizzled* genes of other metazoans. Analysis run in RaxML under a WAG + Γ model. Bootstrap (bs) support values generated with 1000 bs replicates in RaxML. BS values reported if ≥ 70. Terminals of the tree are labeled as follows: "GenbankID species orthology". EL = *Ectopleura larynx*; NV = *Nematostella vectensis*; HV = *Hydra vulgaris*; CH = *Clytia hemaespherica*. DM = *Drosophila melanogaster*; CE = *Caenorhabditis elegans*; MM = *Mus musculus*; AQ = *Amphimedon queenslandica*; ML = *Mnemiopsis leidyi*; HE = *Hydractinia echinata*; SD = *Suberites domuncula*

Wnt pathway genes are expressed in developing actinulae

During development, *Ectopleura larynx* embryos begin as a flattened disc shape. The first set of tentacles, the aboral tentacles, form along the margin of the disc and grow outward (**Figure 2**) (Berrill 1952). After tentacles are well formed, their elongation appears to stop and the body axis then begins to develop and elongate. Subsequently, a mouth and oral tentacles form (**Figure 2**) (Berrill 1952). To examine if Wnt pathway genes play a role in tissue evagination and new axis formation in *Ectopleura larynx*, we investigated the temporal and spatial expression of *Wnt3*, *Frizzled1, SFRP,* and *Wnt5* in developing *Ectopleura larynx* actinulae using qRT-PCR and *in situ* hybridization. We do not detect the expression of *Frizzled1*, *SFRP*, or *Wnt3* in embryos with *in situ* hybridization, although qRT-PCR data indicate that low levels of *SFRP* are present in this developmental stage (**Figure 5**). *Wnt5* is present in embryos in the endoderm of evaginating tentacles, and continues to be expressed in the most distal endoderm of the tentacles as they elongate (**Figure 6A-B**). Subsequent to tentacle formation, the zone of expression of *Wnt5* shifts from the endoderm of the tentacles to the hypostome, where it is expressed in a small zone at the most oral tip of the polyp during hypostome evagination and polyp elongation (**Figure 6C**). qRT-PCR data demonstrate that the downstream Wnt pathway genes *β-catenin* and *GSK3β* are abundant in embryos and increase during development (**Figure 5**). In contrast, *Tcf* transcripts are low in early developmental stages, but up-regulated in late actinula development (**Figure 5**).

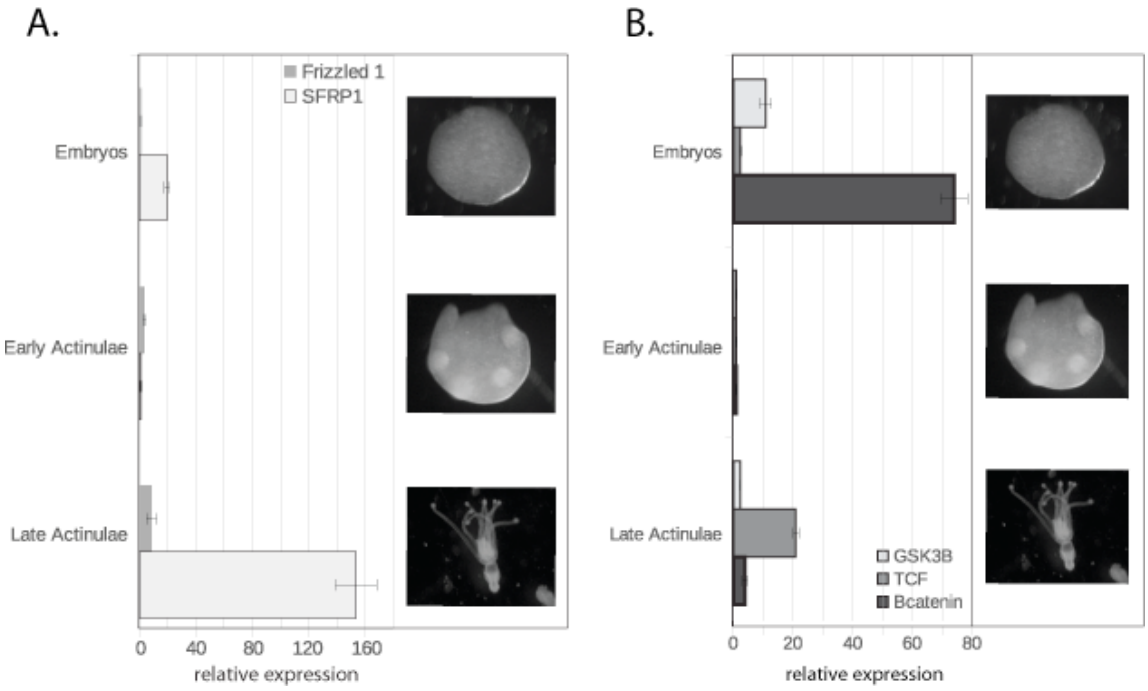

Figure 5. Expression of Wnt pathway genes in developing *Ectopleura larynx* polyps. (A) Expression of *Frizzled1* and *SFRP*. *Frizzled1* and *SFRP* are both upregulated during polyp development. (B) Expression of the downstream genes *GSK3β*, *Tcf* and *β-catenin* during polyp development. *GSK3β* and *β-catenin* are down-regulated during polyp development, while *Tcf* is upregulated during polyp development. All calculations are normalized to *β-actin* using the comparative Ct method.

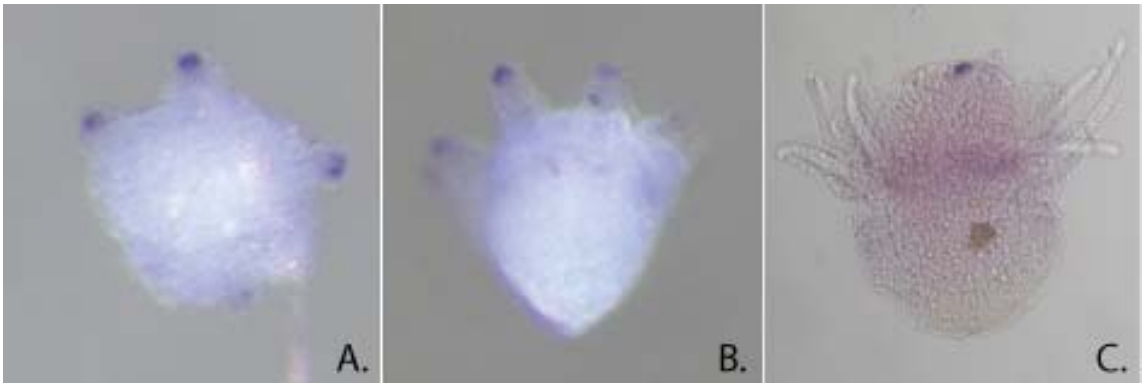

Figure 6. *Wnt5* expression during early polyp development shifts from the evaginating tentacle tips (A, B) to the oral end of the polyp (hypostome) (C). (A) View from top of late-stage embryo expressing *Wnt5* in the distal endoderm of evaginating tentacles. (B) Side view of mid-stage actinula displaying *Wnt5* expression in endoderm of the most distal tips of tentacles during tentacle elongation. (C) *Wnt5* expression in the oral endoderm of the polyp during hypostome evagination and polyp elongation. *Wnt3* expression is absent in early stages of polyp development but turns on in later stages (see **Figure 7**).

Canonical Wnt pathway genes are differentially expressed along the body axis of actinulae (juvenile polyps) and adult polyps

We examined the spatial expression of Wnt pathway genes in late-stage actinulae (juvenile polyps) and in adult polyps. In contrast to earlier stages of development where only *Wnt5* was expressed (**Figure 6**), all of the major Wnt pathway genes that we examined – *Wnt3*, *Frizzled1* and *SFRP* as well as *Wnt5* – are expressed in the late-stage actinula (**Figure 7**).

By the late stage of actinula development, *Wnt5* has shifted from the tentacles to the endoderm of the hypostome (region of the polyp containing the mouth), where it is expressed in a small subset of cells at the oral tip (**Figure 7A**). This expression zone remains as the polyp grows and elongates. *Wnt3*, which is absent in early developmental stages, is also expressed in late actinulae in the oral region of the polyp, and its expression overlaps with that of *Wnt5*, except that its expression domain is much broader, and appears to be both endodermal and ectodermal (**Figure 7B**). In late actinulae, *Wnt3* is also expressed in a small number of cells at the distal ends of the tentacles (**Figure 7C**). *Frizzled1* is expressed in a ring of endodermal cells at the base of the aboral tentacles of actinulae (**Figure 7D**) and does not overlap in expression with Wnt 3 or Wnt5. *SFRP* is expressed in the endoderm of the foot, and also in a ring of endodermal cells underneath the aboral tentacles, similar to *Frizzled1* expression (**Figure 7E**). qRT-PCR data show that *Frizzled1* may be slightly upregulated in the mid-body of adult polyps (but not significant; **Figure 8A**), and that *SFRP* is upregulated in the hypostome and mid-body of adult polyps (**Figure 8A**). We also find evidence for differential expression of the downstream Wnt pathway components *β-catenin, Tcf* and *GSK3β* in the adult polyp. *GSK3β* expression is highest in the hypostome of adult polyps, while *β-catenin* is upregulated in the oral end of the polyp

(hypostome and mid-body) (**Figure 8B**). *Tcf* expression is ubiquitous, with the lowest expression in the neck (**Figure 8B**).

Figure 7. Wnt pathway genes are differentially expressed along the body of *Ectopleura larynx* actinulae. (A) *Wnt5* is expressed in a small subset of endodermal cells in the hypostome. Side view of whole actinula. (B) *Wnt3* is expressed in the ectoderm and endoderm of the hypostome of late-stage actinulae. Side view of whole actinula. (C) *Wnt3* is expressed in clusters of ectodermal cells in the tips of the aboral tentacles of actinula. (D) *Frizzled1* is expressed at the base of the aboral tentacles of the actinula. Side view of whole polyp. (E) *SFRP* is expressed in a ring of cells below the base of the aboral tentacles and in the endoderm of the aboral end of the polyp. Side view of whole actinula. (F) Sense control (for *Frizzled1*).

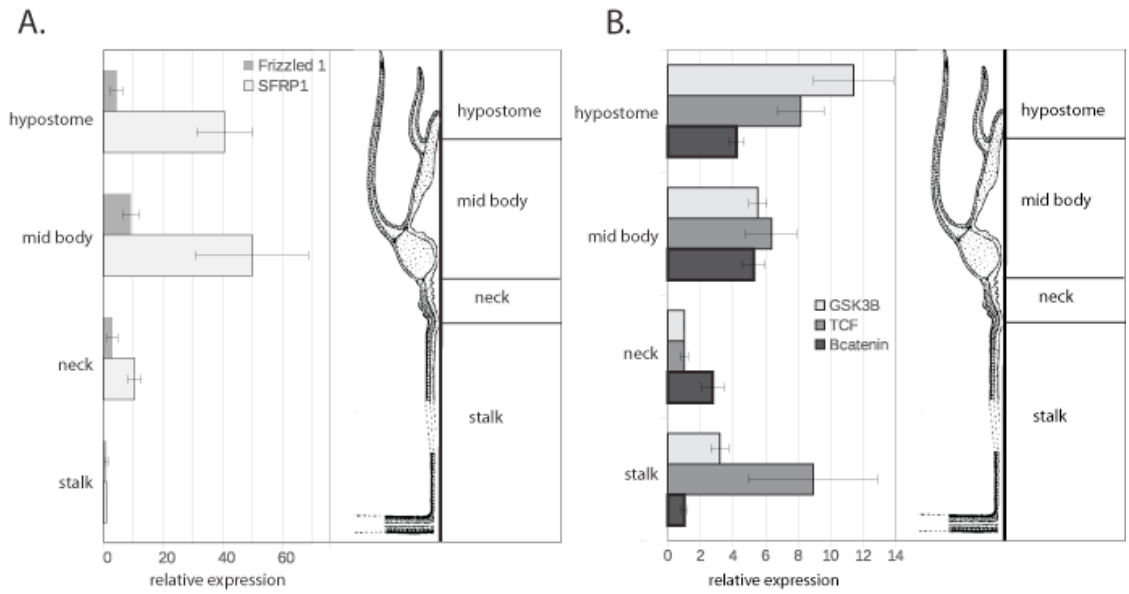

Figure 8. Wnt pathway genes are differentially expressed along the polyp body axis of adult *Ectopleura larynx* polyps. (A) Expression of *Frizzled1* and *SFRP*. *Frizzled1* is upregulated in the mid body of adult polyps (but not significant). *SFRP* is upregulated in the hypostome and mid body of adult polyps. (B) Expression *GSK3β*, *Tcf* and *β-catenin* in adult polyp tissues. *GSK3β* and *β-catenin* are upregulated in the anterior portion of the polyp (hypostome and mid body), while *Tcf* is ubiquitous in the adult polyp, with lowest expression in the neck. All calculations are normalized to *β* -actin using the comparative Ct method.

***Wnt* pathway genes are expressed in oral structures of male and female gonophores**

Sexually-mature male gonophores lack tentacles but have an enlarged cap on their most oral end (**Figure 2E**). Spatial expression data suggests that major components of the Wnt signaling pathway, as well as *Wnt5*, are expressed in the oral end the developed male gonophore (**Figure 9A-D, L**). *Wnt3* is absent from early-stage male gonophores but is expressed in a small subset of ectodermal cells at the most distal end of the gonophore and in the center of the cap as the gonophore matures (**Figure 9A**). *Wnt5* is expressed early in gonophore development (prior to *Wnt3*) in a small subset of endodermal cells (**Figure 9L**). Expression later shifts to the ectoderm, and then expands to an additional domain represented by a ring of ectodermal cells along the edge of the cap (**Figure 9D**). *Frizzled1* expression in male gonophores overlaps with that of *Wnt3* and *Wnt5*, and is confined to a larger circle of ectodermal cells at the most oral end of the gonophores (**Figure 9B**). This expression zone is always localized to the central region of the distal cap. Thus, in the center of the most oral end of male gonophores, *Wnt3*, *Wnt5* and *Frizzled1* expression domains overlap. In contrast, *SFRP* is expressed in a ring of cells denoting the proximal edge of the zone of expression of *Frizzled1*, in proximity to *Wnt5* expression (**Figure 9C**). *SFRP* has an additional expression domain in the tip of the spadix of the male gonophore (**Figure 9C**).

Mature female gonophores display between 3 and 5 tentacles in a cluster at the most oral end of the gonophore (**Figure 2F**). We found that Wnt pathway genes are also expressed in these oral structures of the female gonophore (**Figure E-H, L**). Similar to in male gonophores, *Wnt3* appears to be absent in early-stage female gonophores (not shown). In contrast, *Wnt5* expression begins early (prior to *Wnt3*) in a small subset of endodermal cells at the most oral end of the

gonophore, similar to male gonophore development (**Figure 9L**). Subsequently, *Frizzled1* and *Wnt5* become localized to endodermal circles denoting the regions that become the base of the tentacles before they develop (not shown). Subsequently, *Wnt3*, *Wnt5* and *Frizzled1* are co-expressed in the endoderm of tentacles of female gonophores as these structures develop. Eventually, expression shifts entirely to the endoderm of fully-developed tentacles (**Figure 9E-F, H**). *Wnt5* has an additional expression domain in a circle of cells at the oral end of the gonophore between the tentacles (**Figure 9F**), and *Frizzled1* has an additional expression domain in the ectoderm at the tip of the spadix (**Figure 9F**). In contrast, *SFRP* is expressed in a small cap at the most distal tip of the gonophore, centered between the tentacles (**Figure 9G**). This cap of *SFRP* expression appears early in female gonophore development as a broad expression domain, but over time is sharpened to a small subset of cells (**Figure 9G**). **Figure 10** provides a summary of the expression of these four genes in mature male and female gonophores.

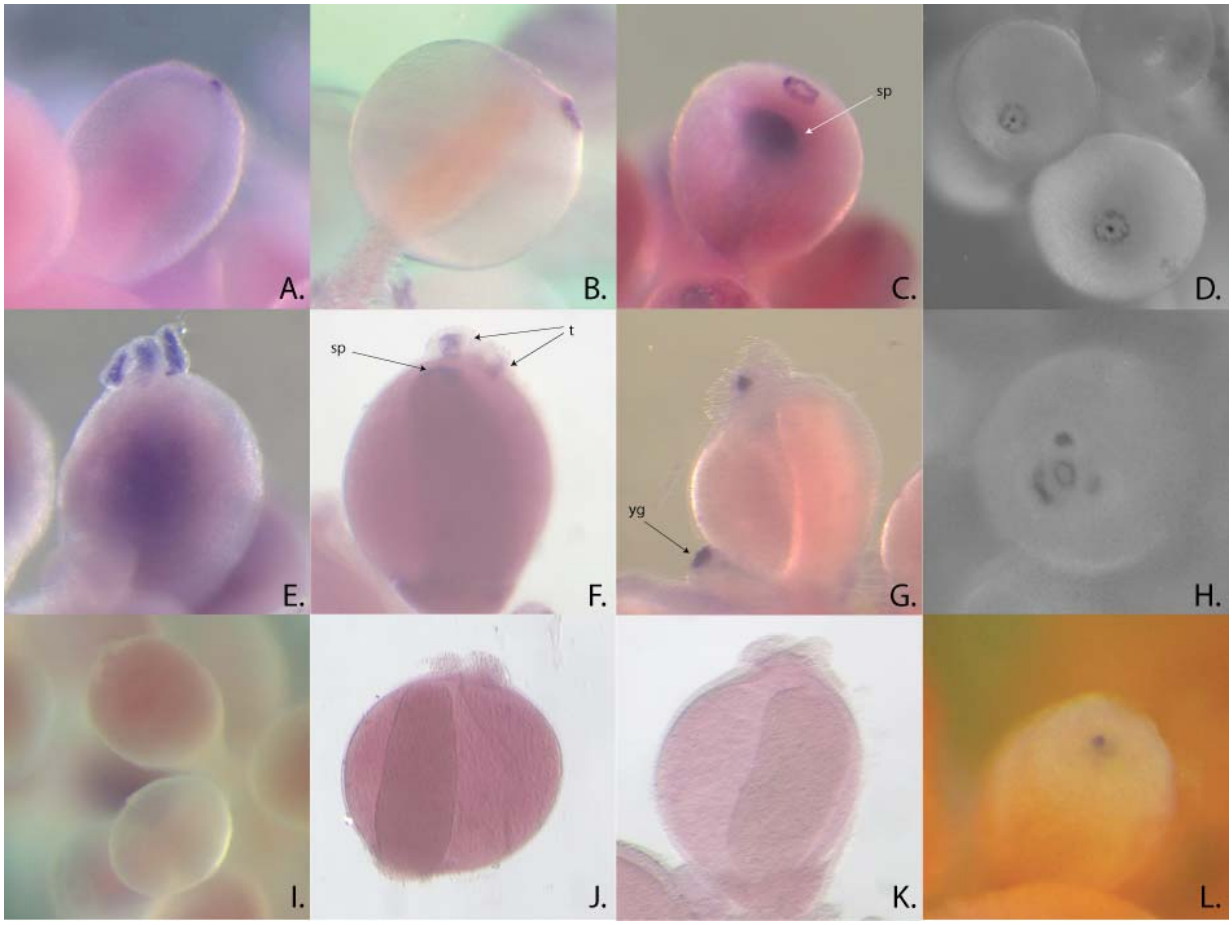

Figure 9. *Wnt3*, *Frizzled1*, *SFRP* and *Wnt5* are expressed in oral structures of *Ectopleura larynx* gonophores. A-D: Expression in male gonophores. (A) *Wnt3* is expressed in a small region in the oral tip of developing male gonophores. (B) *Frizzled1* is expressed in a subset of ectodermal cells at the most oral end of the male gonophore (oral end is offset in this figure). (C) *SFRP* is expressed in a ring at the distal end of the male gonophore. (D) *Wnt5* is expressed in a subset of ectodermal cells at the most oral end of the male gonophore, and in a ring of cells at the oral end of the male gonophore (oral view). E-H: Expression in female gonophores. (E) *Wnt3* is expressed in the endoderm of developing tentacles. *Frizzled1* is expressed in the endoderm of developing tentacles, and in the ectoderm of the spadix. (G) *SFRP1* is expressed in a subset of ectodermal cells at the most oral end of the female gonophore. (H) *Wnt5* is expressed in the endoderm of developing tentacles, and in a ring of cells at the most oral end of the gonophore (oral view). (I) *Wnt3* negative control. (J) *Frizzled1* sense control. (K) *SFRP* sense control. (L) Early-stage male gonophore showing *Wnt5* expression in the oral end of the developing gonophore. Expression of *Wnt5* in early stage male and female gonophores is identical. sp = spadix; t = tentacle buds; yg = young gonophore.

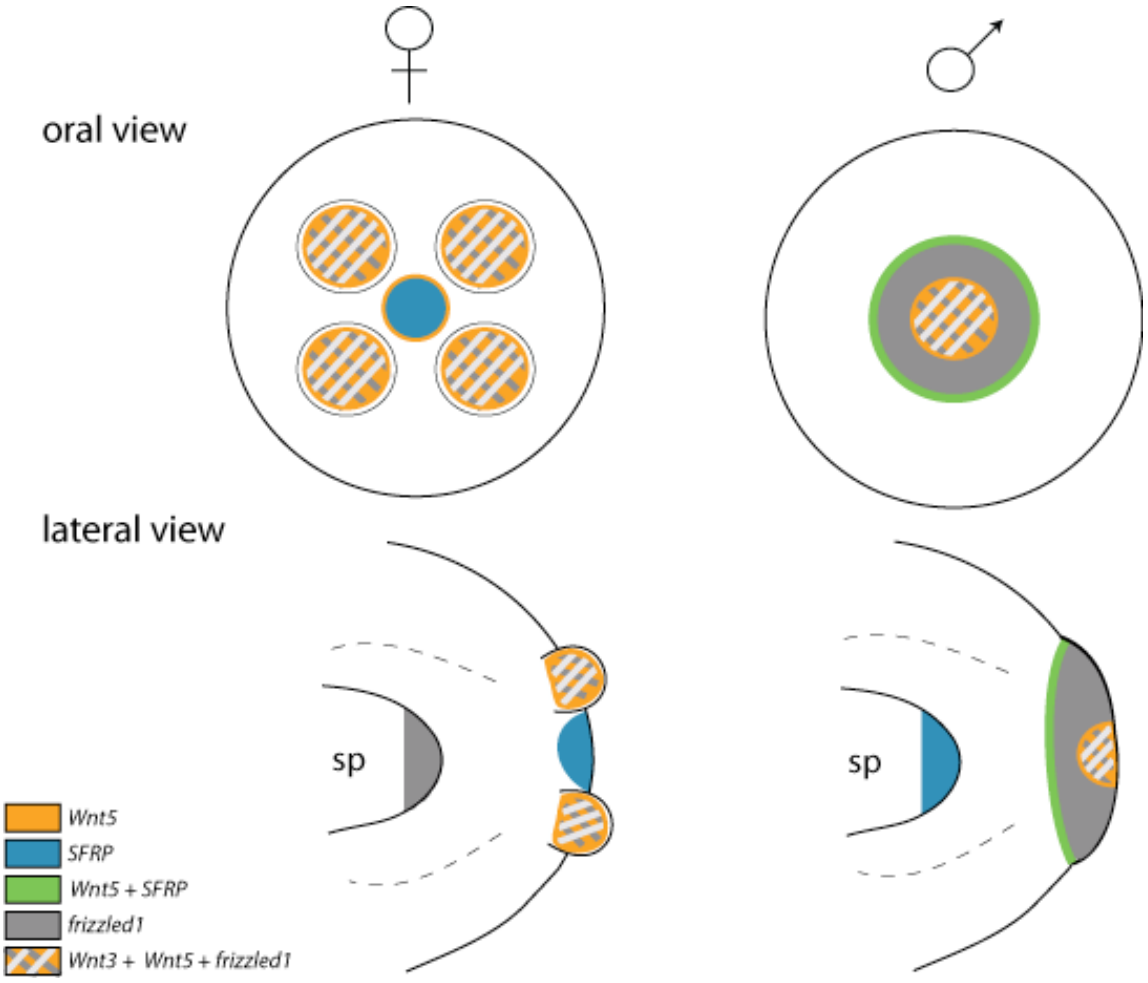

Figure 10. Schematic summarizing the expression of *Wnt3, Wnt5*, *Frizzled1* and *SFRP* in male and female gonophores of *Ectopleura larynx*. Tissues co-expressing *Wnt3*, *Wnt5* and *Frizzled* correspond to regions of the gonophore undergoing active tissue expansion. Green indicates overlapping expression of *Wnt5* and *SFRP*. Hatched indicates overlapping expression of *Wnt5*, *Wnt3* and *Frizzled1*. sp = spadix.

DISCUSSION

Wnt5 expression correlates with axis initiation while *Wnt3* expression correlates with axis maintenance during development of *Ectopleura larynx* polyps

In all cnidarians in which is has been characterized, the canonical Wnt gene, *Wnt3*, is expressed in the most oral end of the polyp at some point during polyp ontogeny (Broun, Gee, Reinhardt *et al*. 2005, Guder, Pinho, Nacak *et al*. 2006, Hobmayer, Rentzsch, Kuhn *et al*. 2000, Muller, Frank, Teo *et al*. 2007, Plickert, Jacoby, Frank *et al*. 2006, Duffy, Plickert, Kuenzel *et al*. 2010, Momose, Derelle and Houliston 2008). In *Hydractinia echinata*, *Clytia hemaespherica*, and *Nematostella vectensis*, species that develop through a larva, *Wnt3* expression patterns implicate it in polarizing the embryo early in development, maintaining the future oral end of the polyp during metamorphosis (except in *Nematostella vectensis*, which does not undergo metamorphosis), and maintaining the patterning of the mouth of the polyp (Duffy et *al*. 2010, Plickert et *al*. 2006, Momose et *al*. 2007, Lee *et al*. 2006). However, in the direct-developing species *Hydra*, *Wnt3* is not turned on until later in embryonic (Frobius et *al*. 2003) or bud (Philipp, Aufschnaiter, Özbek *et al*. 2009) development. Our expression data is consistent with data from *Hydra*, suggesting that *Wnt3* may not be involved in initiating axes during development in *Ectopleura larynx*. Instead, in *Ectopleura larynx*, *Wnt3* is expressed after tentacle formation and after the hypostome has already formed (**Figures 6-7**), congruent with a role in maintaining already-established axes.

In contrast, our data demonstrating early *Wnt5* expression during *Ectopleura larynx* embryonic development are consistent with a role for *Wnt5* in initiating the polyp axis. Both our data (here) and data from *Hydra* show that *Wnt5* is expressed early in polyp development from embryos

(*Ectopleura*) or buds (*Hydra*) (Philipp, Aufschnaiter, Özbek *et al*. 2009). Our data show that during embryonic development of polyps, *Wnt5* is first expressed in the distal axes of developing tentacles of the developing embryo, and subsequently is expressed in the oral axis of the developing polyp. In *Hydra* buds, the primary body axis forms prior to tentacle formation. In the case of *Hydra* buds, *Wnt5* is expressed first in the most distal portion of outpocketing of tissue that becomes a bud, and secondly shifts expression to developing tentacles (Philipp, Aufschnaiter, Özbek *et al*. 2009). *Ectopleura larynx* polyps form tentacles along their periphery prior to the formation of the oral/aboral axis, whereas in *Hydra*, asexually developing polyps form the polyp oral axis prior to tentacle formation. In both cases however, *Wnt5* is expressed at the time of initiation of the development of the tentacles and the polyp oral axis.

Because *Wnt3* maintains polarity of the animal during both planula formation and metamorphosis in *Hydractinia* and *Clytia* development, the observed shift in the relative order of deployment of *Wnt3* and *Wnt5* between these species and *Hydra* and *Ectopleura larynx*, which lack a planula larva stage, raises the question of whether these changes in the order of expression of Wnt genes are associated with lack of a planula larva or rather with a lack of metamorphosis. In a distant relative of hydrozoans, the direct-developing anthozoan *Nematostella vectensis*, which forms a planula but does not undergo metamorphosis, the canonical Wnt pathway is well characterized. The canonical *Wnt3* gene is turned on early in development in the gastrula stage (localized to the future oral end of the polyp), while *Wnt5* expression turns on later (Lee *et al*. 2006), consistent with the order of expression in *Hydractinia* And *Clytia*. These data together suggest that the switch in the timing and order of deployment of *Wnt5* and *Wnt3* is unique to Aplanulata taxa and likely associated with the loss of a planula larva.

***Frizzled1* and *SFRP* expression during embryonic development are consistent with late deployment of *Wnt3* and canonical Wnt signaling**

In *Hydra*, the *Wnt3* receptor *Frizzled1* is expressed throughout early embryonic development (Frobius *et al*. 2003). In *Ectopleura larynx*, we found evidence for low-level upregulation of *Frizzled1* in late polyp development, but overall it appears to be present in relatively low levels throughout embryonic development. *SFRP* expression is similarly low in early developmental stages of *Ectopleura larynx,* but is strongly upregulated in late development, coinciding with the deployment of *Wnt3* in the hypostome of late-stage actinulae. These data are in contrast with expression in *Clytia hemaespherica*, where *Frizzled1* is a maternally-localized transcript present in high levels in the earliest stages of development, and localized to the future oral end of the polyp during planula development and metamorphosis (Momose and Houliston 2007). This difference in expression is likely attributable to differences in development, where *Wnt3* signaling is not present until very late stages of *Hydra* and *Ectopleura larynx* embryonic development, whereas in *Clytia hemaespherica, Wnt3* signaling is vital early in development for establishing the oral/aboral axis of the future polyp.

We find high levels of *β-catenin* in early embryonic development, consistent with reports that *β-catenin* is a maternal transcript provided by nurse cells (Alexandrova, Schade, Bottger *et al*. 2005) or directly by the oocyte (Duffy, Plickert, Kuenzel *et al*. 2010) (**Figure 5**). However, late deployment of *Wnt3*, coupled with a lack of *Tcf* in early stages of embryonic development (**Figure 5**) suggest that canonical Wnt signaling is inactive in early stages of *Ectopleura larynx* development. This is in contrast to *Hydractinia* where there is evidence that *Tcf* is provided as a

maternal transcript (Plickert, Jacoby and Frank *et al*. 2006). We do find relatively early expression of *Wnt5*, which has been suggested to act in the canonical Wnt pathway (He *et al* 1997). However, lack of expression of other Wnt pathway elements in early developmental stages of *Ectopleura larynx* make it unlikely that *Wnt5* is acting in the canonical Wnt pathway in polyp development. While *β-catenin* is present early in the embryo, this gene is known to be additionally involved in other roles in cnidarians that do not involve the canonical Wnt pathway (Wikramanayake 2003, Momose & Schmid 2006).

Additional Wnt ligands and receptors may interact in late developmental stages of the polyp

In later developmental stages of the *Ectopleura larynx* polyp, *Frizzled1* and *SFRP* are expressed in the endoderm at the base of the aboral polyp tentacles, thus their expression does not overlap with that of *Wnt3* or *Wnt5* expression patterns in the hypostome. These data suggest that during late polyp development, *Frizzled1* and *SFRP* may not interact with either of these two Wnt genes. These data are congruent with expression in *Hydra* demonstrating that *Frizzled1* is expressed in the endoderm of the body of the adult polyp (Minobe, Fei, Yan *et al*. 1999), and that it thus does not have overlapping expression with *Wnt3* or *Wnt5* (Philipp *et al*. 2009, Lengfield *et al*. 2009).

Our transcriptome analyses revealed four additional Wnt ligands and two additional Frizzled genes not characterized here, and it is possible that one or more of these other ligands and receptors are interacting with genes characterized here in the polyp stage. *SFRP* expression is not characterized in other hydrozoans, but we suspect that this gene also interacts with one or more

Wnt ligands and inhibits Wnt pathway signaling at the base of *Ectopleura larynx* tentacles. However, it should be noted that it is also possible that Wnt proteins, which can travel, are acting in different tissues than their transcripts are present in (Christian 2000). Future studies characterizing the expression of both mRNA and protein patterns will shed further light on the this pathway in polyp development.

Downstream Wnt pathway elements in *Ectopleura larynx* also warrant future attention. In *Hydra*, *β-catenin* is upregulated in the oral end of *Hydra* polyps and is localized to nuclei (Broun, Gee, Reinhardt *et al*. 2005, Hobmayer, Rentzsch, Kuhn *et al*. 2000). qRT-PCR data demonstrates expression of *β-catenin* in *Ectopleura larynx* adult polyps in the anterior end of the polyp (hypostome and mid body), although we do not determine exact cellular localization through *in situ* hybridization (**Figure 8B**). Additionally, in *Hydra*, *Tcf* is expressed in the anterior portion of the polyp, with the strongest expression in the hypostome (Hobmayer *et al*. 2000). We report *Tcf* expression highest in the anterior portion of the polyp (the hypostome and mid body) of adult *Ectopleura larynx* polyps, while it is lowest in the aboral end of the polyp. We also find high expression in the stalk of *Ectopleura larynx* polyps, which was unexpected, but may be related to the regenerative capacity of *Ectopleura* stolons (Tardent 1963). We saw highest expression of GSK3*β* in the oral end of adult *Ectopleura larynx* polyps (**Figure 7**), in contrast to data from *Hydra*, demonstrating uniform distribution throughout the endoderm of the polyp (Hobmayer *et al*. 2000). Because the exact cellular localization of downstream elements in the Wnt signaling pathway is more important than their spatial localization in the polyp (Broun *et al*. 2005), future studies in *Ectopleura larynx* are needed to determine the cellular localization of these genes and their proteins.

***Wnt5* expression is correlated with early axis formation of *Ectopleura larynx* gonophores**

Our data demonstrate that Wnt pathway gene expression is consistent with patterning the oral end of truncated medusae structures during their early development. *Wnt3* is absent from early-stage male and female gonophores but is turned on in late stage gonophores. In contrast, *Wnt5* is expressed early in the most distal tip of both male and female gonophores, prior to *Wnt3* (**Figure 9**). Later, this expression of *Wnt5* shifts in female and male gonophores to the endoderm of evaginating tentacles (female gonophores) or to an additional domain falling along the edge of the developing cap (male gonophores) as it grows outward (**Figure 10**). These data are consistent with *Wnt5* playing a role in initiating new gonophore axes during development, similar to what is found in the polyp stage of *Ectopleura larynx*.

SFRP, which possibly prevents *Wnt3* from binding to *Frizzled1*, is expressed in the earliest stage of gonophore development at the tip of developing gonophores. Here, it may be inhibiting *Wnt3* signaling until the body axis has been established, at which point its expression becomes localized to a small zone at the oral end of female gonophores, or a ring at the end of male gonophores (**Figure 9C, G**). The proximity of *SFRP* expression to *Wnt5* in early stage gonophores, suggests that they are interacting in some way. However, *Wnt5* expression appears to be endodermal in early gonophore development while *SFRP* expression is ectodermal. While Wnt pathway inhibitors are not characterized in other hydrozoans, these data indicate that they may play a role in the formation of the primary body axis of the polyp and gonophore/medusa by delimiting regions of Wnt signaling in these developmental stages.

Wnt3, Wnt5, Frizzled1 and *SFRP* expression is associated with the development of oral structures of male and female gonophores

Ectopleura larynx is sexually dimorphic, wherein female gonophores possess tentacle buds and male gonophores instead have a cap on the most oral end of the gonophore. This cap represents tentacle tissue that has evaginated but not completely formed separate tentacles (Berrill 1952). Our study reveals that the co-expression of the ligands *Wnt3* and *Wnt5* with the receptor *Frizzled1* is directly correlated with formation of these oral structures on the most distal end of male and female gonophores. In female gonophores, co-expression of *Wnt3, Wnt5* and *SFRP* is found in the endoderm of growing tentacles (**Figure 10**), while in males these three genes are co-expressed in the endoderm of the distal cap on the end of the gonophore (**Figure 10**). The co-expression of the Wnt ligands *Wnt3* and *Wnt5* with the Wnt receptor *Frizzled1* in oral structures that are actively undergoing growth suggests that the interaction of these genes is possibly important for tissue proliferation in these structures. Co-expression of *Wnt3* and *Frizzled1* have not been previously reported in medusae or gonophores of hydrozoans. However, there is a report of co-expression of these genes in the hypostome (most oral end) of the polyp of *Hydractinia echinata* following metamorphosis (Müller *et al*. 2007), consistent with these genes interacting and activating the canonical Wnt signaling pathway in this region of the polyp.

The expression of the putative Wnt pathway antagonist *SFRP* is counter to that of the expression pattern of *Wnt3* and *Frizzled* in both female and male gonophores (**Figure 10**), and suggests a role for this gene in delimiting regions of Wnt signaling in the oral end of the gonophore. If *SFRP* competes with Frizzled1 to bind *Wnt3* and prevent *Frizzled1* activation, our data would be consistent with a role for *SFRP* in delimiting the morphogenetic gradient of *Wnt3* signaling.

While *SFRPs* have not been characterized in cnidarians, our data are consistent with data in other metazoan taxa suggesting that *SFRP* delimits morphogenetic gradients of *Wnt* signaling (Jones and Jomary 2002, Kawano & Kypta 2003).

Our data are consistent with reports of expression of Wnt pathway genes in the oral structures of *Clytia hemaespherica* medusae (Momose et *al*. 2007) and in the oral end of completely truncated *Hydractinia echinata* gonophores (Duffy et *al*. 2010). The expression patterns in *Ectopleura larynx*, a hydrozoan species displaying different degrees of medusa reduction, suggests that even though free-living medusae have been lost, the canonical Wnt pathway is being expressed in structures that are remnants of medusae, which were retained in evolution from medusae-bearing ancestors

A possible role for the canonical Wnt pathway in medusa truncation

The spadix is the region of the gonophore that corresponds to the stomach and mouth in fully-formed medusa. In gonophores, this structure fails to develop completely. The absence of *Wnt3* and *Frizzled1* co-expression in the spadix of male and female *Ectopleura larynx* gonophores suggests that the Wnt pathway is being downregulated in this structure. *Frizzled1* is expressed without a Wnt ligand counterpart in the oral end of the spadix in female gonophores. Furthermore, in male gonophores, *SFRP* is expressed in the tip of the spadix suggesting that this antagonist is active in spadix tissues. The lack of *Wnt3* and *Frizzled1* co-expression in the spadix could explain why the spadix fails to develop completely into a mouth in gonophores. These data together suggest that the Wnt pathway is involved in the truncation medusa development in *Ectopleura larynx*.

Our data are consistent with previous studies examining the expression of Wnt pathway elements in medusae (*Clytia hemaespherica*) or gonophores (*Hydractinia echinata*). Data in *Clytia hemaespherica* show that *Frizzled1* is expressed on the manubrium of the medusa (*Wnt3* expression has not been characterized in this species) (Momose and Houliston 2007). In *Hydractinia echinata*, whose gonophores that lack all elements of medusa morphology, *Wnt3* is expressed in the most oral tip of gonophores in a small subset of ectodermal cells, but other Wnt pathway elements such as *frizzled* are not co-expressed in these structures (Plickert, Jacoby, Frank *et al*. 2006). The lack of *frizzled* expression in *Hydractinina echinata* gonophores and apparent downregulation of the Wnt signaling pathway could account for lack of development of any oral structures such as tentacles or a distal cap, as we find in *Ectopleura larynx*.

CONCLUSION

Evolutionary modifications of life cycles stages is a prominent feature in the history of Hydrozoa. Information about the developmental processes dictating these modifications can provide insight in to the complex patterns of life cycle evolution in Hydrozoa. Our data presented here implicate canonical Wnt signaling in the development of the embryo, polyp, and truncated medusae of *Ectopleura larynx*. We provide evidence that temporal changes in the deployment of Wnt ligands during development may be implicated in a shift from indirect (planula) to direct (no planula) development in Aplanulata hydrozoans. We also suggest that changes in the spatial expression of Wnt pathway components are directly correlated with the differing levels of oral structure development. The absence of co-expression of *Wnt3* and *Frizzled1* in the developing stomach (spadix) of truncated medusae suggests that downregulation of Wnt pathway elements could be involved in truncation of medusa development. The differences in the expression of Wnt pathway elements in the two types of gonophores that display different degrees of medusae reduction, in conjunction with the downregulation of some of these genes, suggests that the Wnt pathway may play a role in the multiple instances of medusa reduction that have occurred in the evolution of Hydrozoa (Cartwright & Nawrocki, 2010). The prevalence of Wnt pathway involvement in the development of all life history stages in hydrozoans makes future studies of this pathway important for illuminating the evolution and development of this diverse animal lineage.

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APPENDIX

I. APPENDIX TO CHAPTER 1: Proposal for revised diagnosis of the family Corynidae and some of its genera.

Family Corynidae Johnston. 1836

SYNONYMS: Sarsiadae Forbes, 1848; Syncorynidae Allman, 1872; Codonidae Haecke, 1879; Polyorchidae A. Agassiz, 1862.

DIAGNOSIS: The least inclusive clade comprising the species *Coryne pusilla* Gaertner, 1774; *Stauridiosarsia producta* (Wright, 1858); and *Sarsia tubulosa* (M. Sars, 1835).

REMARKS: The clade includes at least the genera *Coryne, Codonium*, *Dipurena*, *Sarsia*, *Stauridiosarsia*, *Scrippsia*, and *Polyorchis* as has been shown by the present investigation.

Schuchert (2001) additionally also included some or all of the following genera in this family: *Bicorona* Millard, 1966; *Dicyclocoryne* Annandale, 1915; *Cladosarsia* Bouillon, 1978a; and *Nannocoryne* Bouillon & Grohmann, 1994. We were unable to procure DNA samples of their respective type species and there is no clear morphological synapomorphy available that would tie them unambiguously to Corynidae. Therefore, the status of these genera remains unclear and must await further molecular phylogenetic studies. For the diagnoses of these genera see Schuchert (2001) or Bouillon *et al*. (2006).

No member of the genus *Spirocodon* Haeckel, 1880 (currently in family Polyorchidae) could be included in the present study and it is unknown whether the genus also falls within the newly redefined Corynidae clade.

Species of the family Halimedusidae Arai & Brinckmann-Voss, 1980 show some similarities to the Polyorchidae and might therefore map within the redefined Corynidae. If so, then also the genera *Halimedusa* Bigelow, 1916; *Tiaricodon* Browne, 1902; and *Urashimea* Kishinouye, 1910 are potentially genera of the Corynidae. For the diagnoses of these genera see Bouillon *et al*. (2006).

Coryne Gaertner, 1774

TYPE SPECIES: *Coryne pusilla*Gaertner, 1774

DIAGNOSIS: The least inclusive clade comprising the species *Coryne pusilla* Gaertner, 1774; *C. pintneri* Schneider, 1898; and *C. eximia* Allman, 1859.

REMARKS: Most, but not all, species of this clade have axillar gonophores. *Coryne* should also be used as the default genus for those species for which not enough molecular or morphological data is available to link them confidently to one of the redefined genera.

Codonium Haeckel, 1879

TYPE SPECIES: *Codonium codonoforum* Haeckel, 1879, is herewith selected as type species of the genus. *Codonium codonoforum* is a subjective synonym of *Sarsia prolifera* Forbes, 1884 (Mayer 1910; Schuchert 2001).

DIAGNOSIS: Monotypic, comprises *Codonium prolifera* (Forbes, 1884). REMARKS: The apomorphic trait of medusa-budding from the tentacle bulbs justifies the creation of a monotypic genus.

Dipurena McCrady, 1859
TYPE SPECIES: *Dipurena strangulata* McCrady, 1859
DIAGNOSIS: The least inclusive clade comprising the species *Dipurena strangulata* McCrady, 1859; *D. simulans* Bouillon, 1965; and *D. halterata* (Forbes, 1846).

Sarsia Lesson, 1843
TYPE SPECIES: *Oceania tubulosa* M. Sars, 1835.
DIAGNOSIS: The least inclusive clade comprising the species *Sarsia tubulosa* (M. Sars, 1835); *S. lovenii* (M. Sars, 1846); and *S. apicula* (Murbach & Shearer, 1902).

Stauridiosarsia Mayer, 1910
TYPE SPECIES: *Stauridia producta* Wright, 1858
DIAGNOSIS: The least inclusive clade comprising the species *Stauridiosarsia producta* (Wright, 1858); *St. ophiogaster* Haeckel, 1879; and *St. gemmifera* (Forbes, 1848).

Scrippsia Torrey, 1909
TYPE SPECIES: *Scrippsia pacifica* Torrey, 1909.
DIAGNOSIS: Monotypic, see diagnosis in Bouillon *et al*. (2006).

Polyorchis A. Agassiz, 1862
TYPE SPECIES: *Melicertum penicillatum* Eschscholtz, 1829

DIAGNOSIS: See diagnosis in Bouillon *et al*. (2006).

II. APPENDIX TO CHAPTER 2: Supplementary single-gene phylogenetic analyses

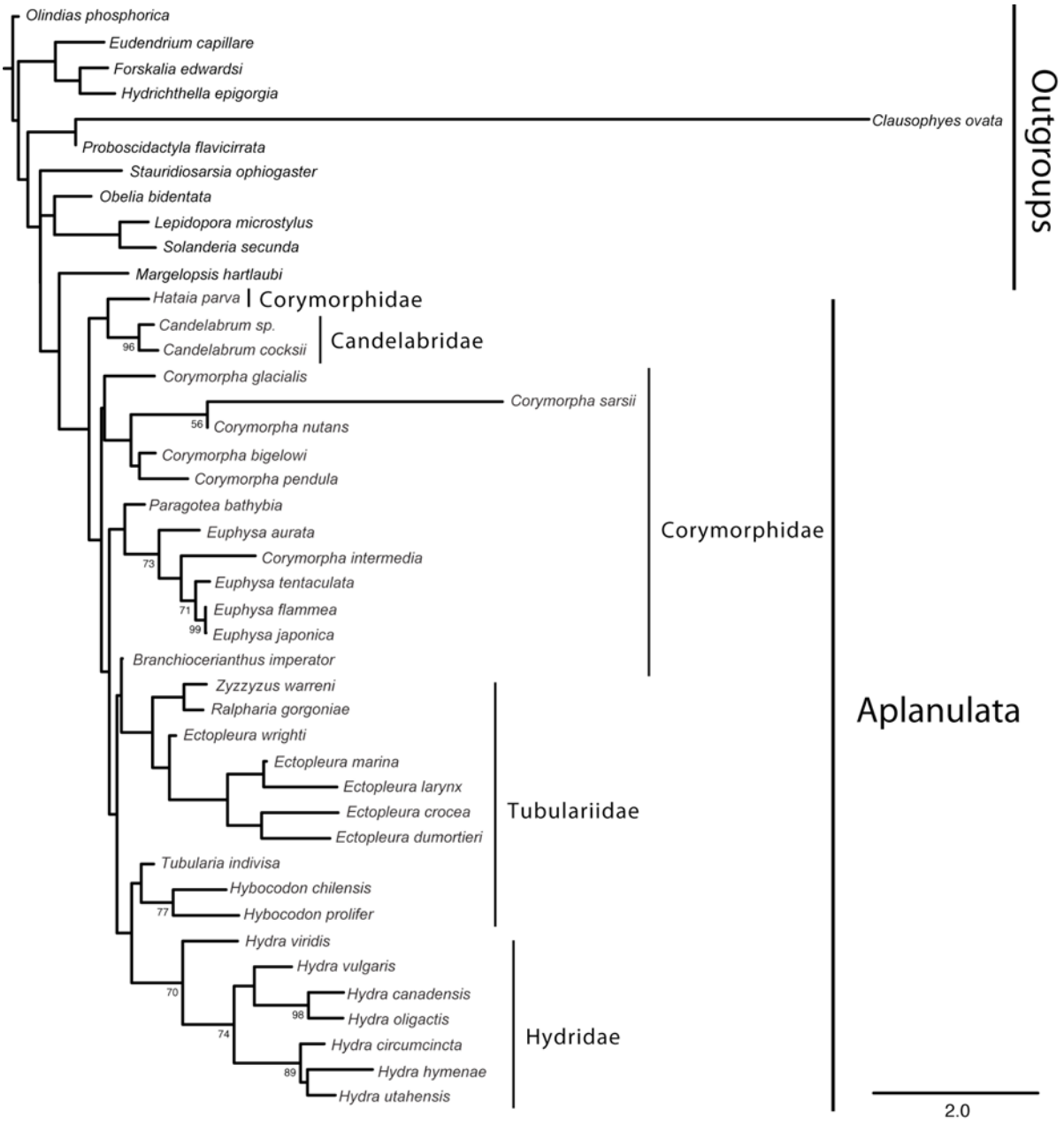

Figure S1. Phylogenetic relationships of Aplanulata based on nuclear cytochrome oxidase 1 (CO1) and analyzed under a GTR + Γ model in RaxML and partitioned by codon position. Node values indicate bootstrap support from 1000 replicates. Where not indicated, support < 70.

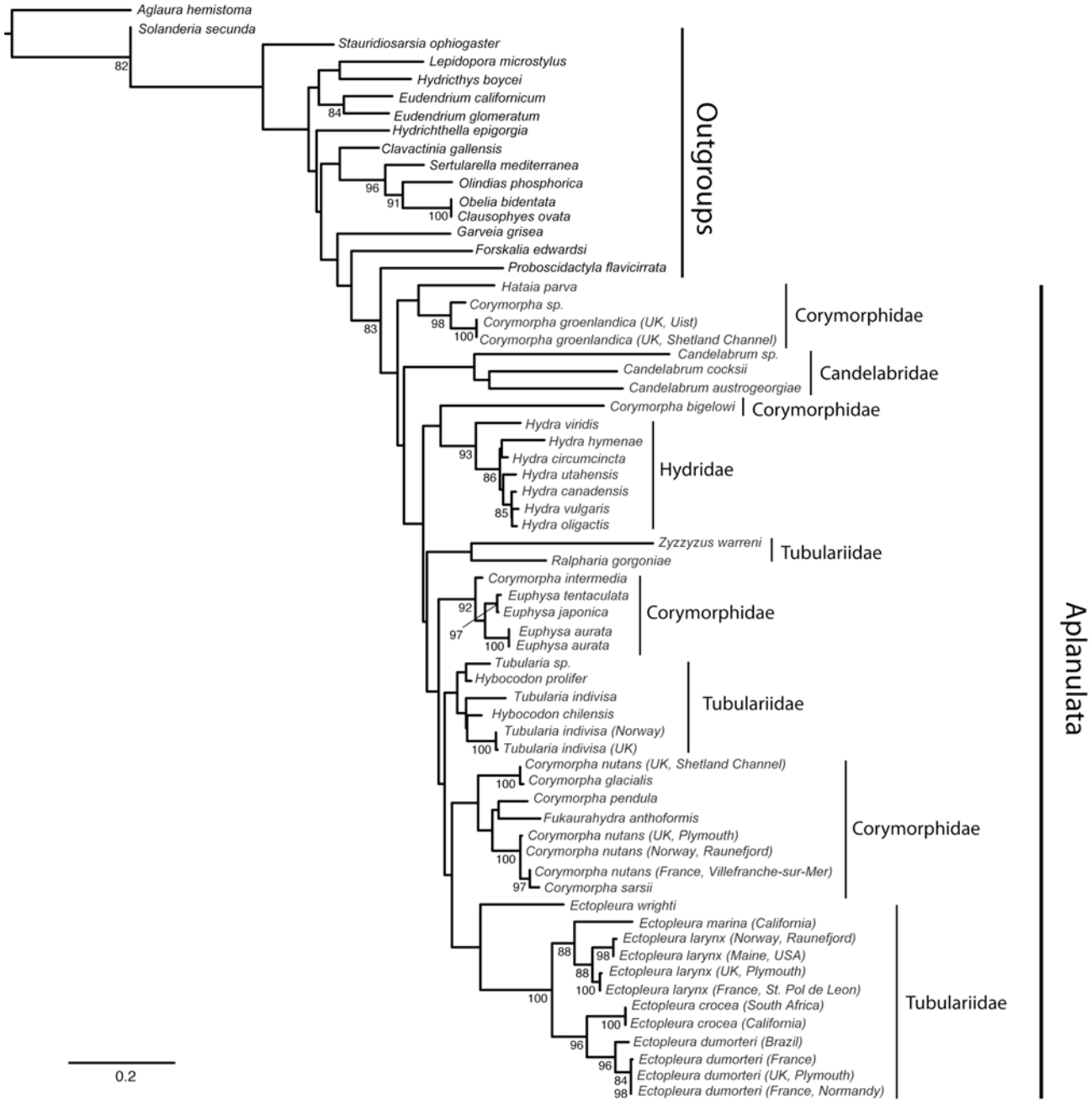

Figure S2. Phylogenetic relationships of Aplanulata based on mitochondrial 16S and analyzed under a GTR + Γ model in RaxML. Node values indicate bootstrap support from 1000 replicates. Where not indicated, support < 70.

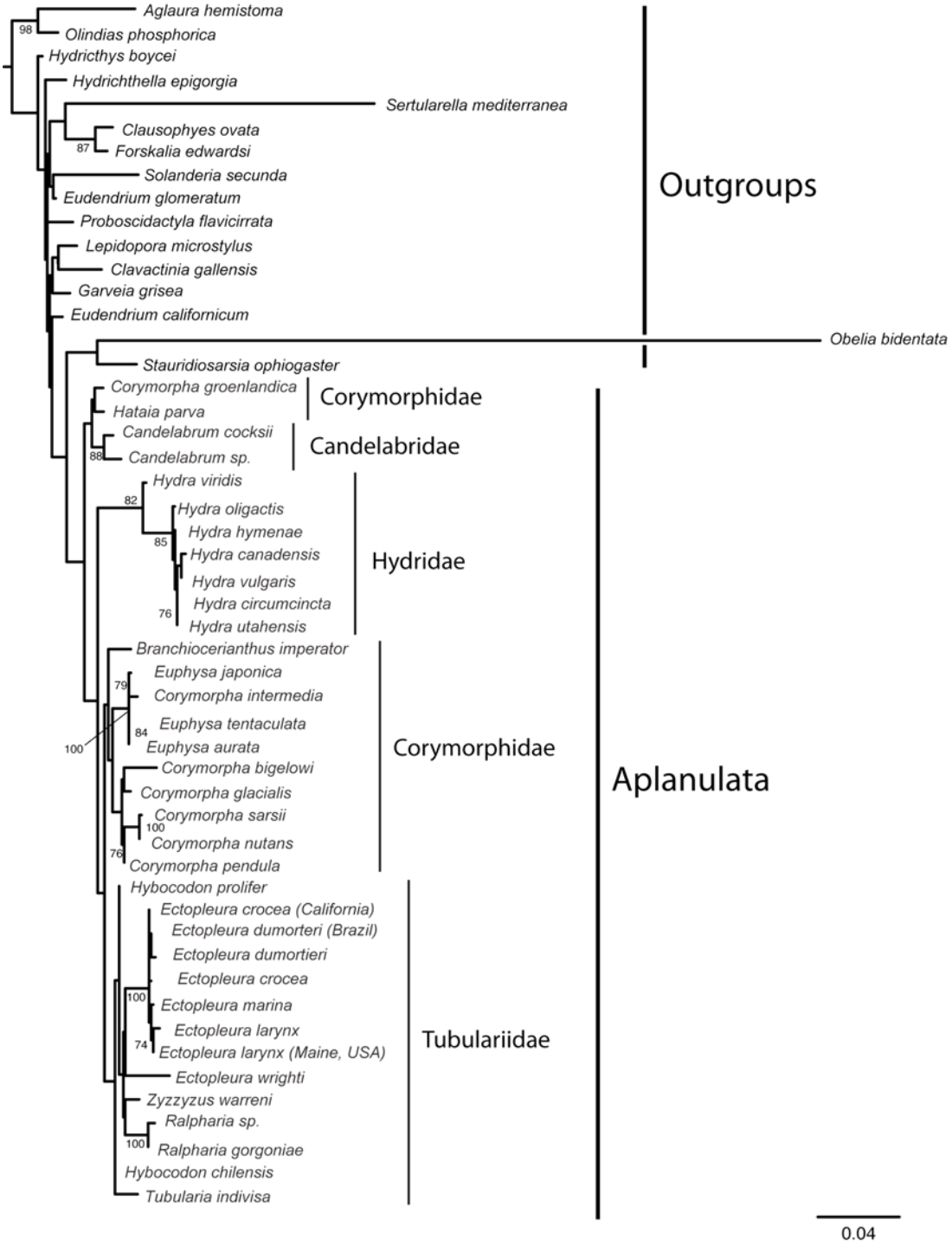

Figure S3. Phylogenetic relationships of Aplanulata based on the small ribosomal subunit (18S) and analyzed under a GTR + Γ model in RaxML. Node values indicate bootstrap support from 1000 replicates**.** Where not indicated, support < 70.

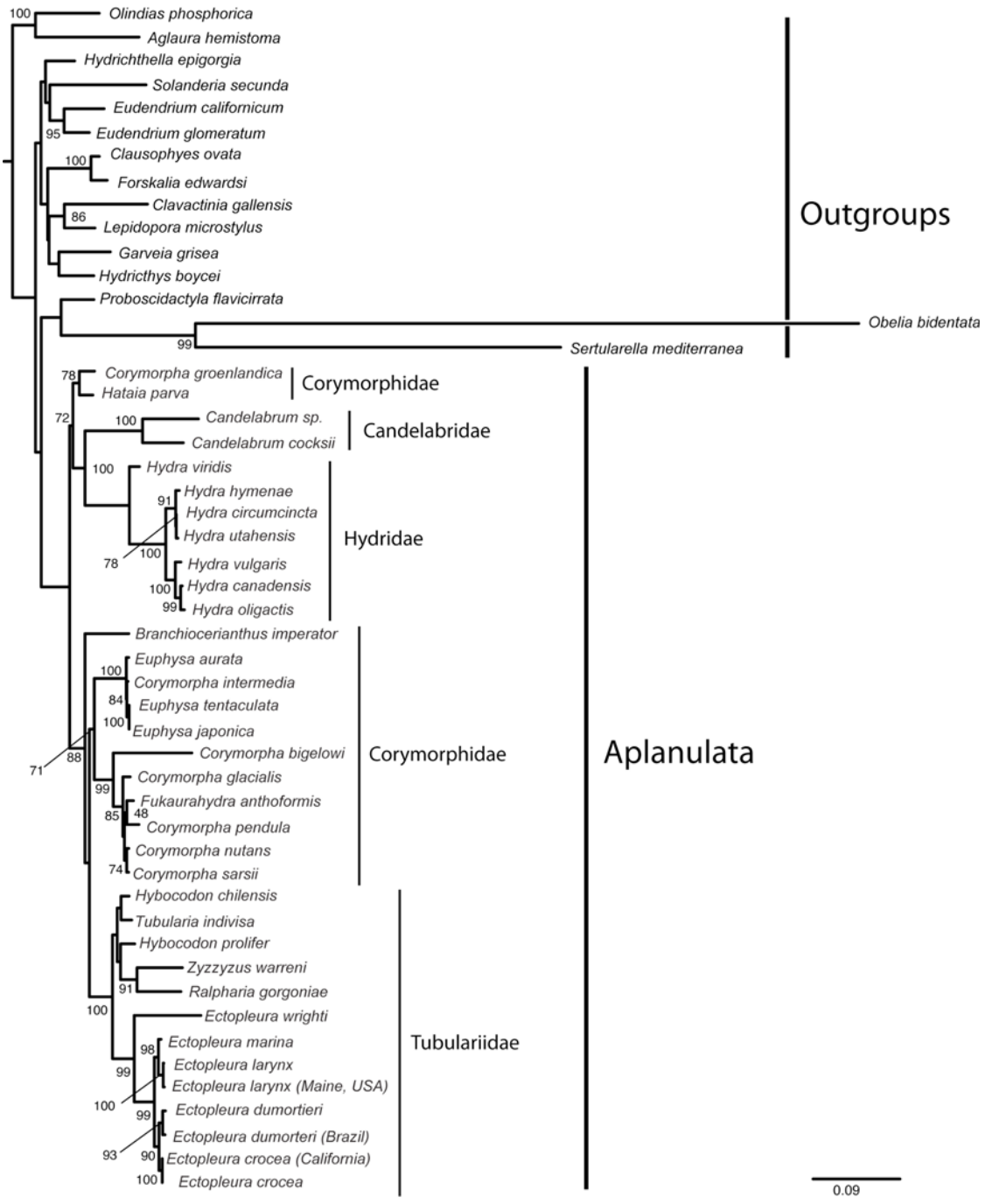

Figure S4. Phylogenetic hypothesis of relationships of Aplanulata based on the 28S large ribosomal subunit and analyzed under a GTR + Γ model in RaxML. Node values indicate bootstrap support from 1000 replicates**.** Where not indicated, support < 70.

III. APPENDIX TO CHAPTER 3: Supplementary experimental procedures and results.

1. Supplementary Experimental Procedures

Phylogenetic analyses

Phylogenetic analyses incorporating RNA secondary structure were conducted on all markers separately and on a concatenated matrix in the parallel version of RaxML 7.2.8 (Stamatakis 2006). For 18s and 28s, secondary structure models generated from large datasets of cnidarians were used (M.S. Barbeitos, pers. comm.). Phase masks from these models were generated using the perl script DCSE2jRNA.pl (M.S. Barbeitos, pers. comm.). Additionally, for 18S and 28S, a large cnidarian alignment was modified according to cnidarian RNA secondary structure using the script ReNAtonb.pl (M.S. Barbeitos, pers. comm.). A subset of taxa was taken for this analysis from larger alignments. For 16S, due to its rapid rate of evolution, no generalized cnidarian model has been developed. Instead, an RNA secondary structure model was generated from an existing *Hydra oligactis* model (Kayal and Lavrov 2008). This model was hand edited after being compared to an alignment for a number of *Hydra* species. Next, a phase mask was generated from this model using DCSE2jRNA.pl. This phase mask was used in the program RNASalsa (Stocsits, Letsch, Hertel *et al*. 2009), along with a file of 16S data of taxa for this study, to make a generalized RNA secondary structure model and corresponding alignment for 16S analyses.

For each analysis, the phase mask representing the RNA secondary structure was used in addition to the final alignment. Analyses under GTR + Γ + RNA16, RNA16A, or RNA16B model was run for each marker and for the concatenated dataset (three analyses per matrix), following model designation from the program PHASE. An AIC test was used to determine the

best model (16, 16A or 16B) per gene and for the concatenated analysis (Akaike 1987). 1000 bootstrap replicates were generated for each run. Trees were visualized in Mesquite and FigTree (Drummond and Rambaut 2007, Maddison and Maddison 2007).

Character correlation tests

Character correlation analyses on a reduced phylogeny containing only Aplanulata species were performed in Mesquite using Pagel's Correlation Method (Maddison and Maddison 2007, Pagel 1994), which tests for the independent evolution of two binary characters. 1,000 Monte Carlo simulations were conducted in order to calculate a *p* value (see Pagel 1994 for a complete description of the method). A cutoff of 0.05 was used to determine statistical significance. *p* values < 0.05 indicate that a dependent model of evolution over an independent model of evolution is strongly favored.

We recoded multistate characters as binary for each pairwise comparison between coloniality and brooding, attached gonophores, and exposed, hard perisarc. Because character state uncertaintly is not allowed for this test, we coded uncertain character states by conducting an equal weights parsimony ancestral character state reconstruction in Mesquite.

In Situ Hybridization Methods

Sense and antisense RNA probes were generated by amplifying the cloned *manacle* insert using Ambion's T7/T3 MAXIscript kits and according to manufacturer's instructions. Polyps were fixed overnight at 4°C in 4% paraformaldehyde (PFA). Following fixation, polyps were taken through 3, 10-minute washes in PBST. Polyps were incubated at 90°C for 10 minutes in PBST,

and post-fixed for 20 minutes at RT in 4% PFA in PBST. A 20mg/mL proteinase-k wash was conducted for 20 minutes to permeate tissues.

Tissues were blocked for 10 minutes in Roche blocking reagent at room temperature with rocking. Pre-hybridization was conducted by first transitioning polyps from blocking buffer into hybridization buffer (10 minute wash at room temperature), and then incubating in pre-warmed hybridization buffer for 2 hours at 50°C. Probe was heated at 80°C for 10 minutes, and added to tissues at 0.04 ng/uL. Tissues were hybridized overnight at 50°C.

Post-hybridization washes were conducted as following: One wash at 55°C for 10 minutes in hybridization buffer. One wash at 50°C for 15 minutes in 50%-SSC, 2x-SSC, 0.1% Tween. One wash at 50°C for 15 minutes in 2x-SSC, 0.1% Tween. Two washes at 50°C for 15 minutes in 0.2x-SSC, 0.1% Tween. One 5 minute wash at RT in PBST. Tissues were blocked in 1% BSA in fresh PBST for one hour at RT, followed by a one hour incubation in pre-absorbed anti-DIG-AP. Incubation was conducted overnight at 4°C a concentration of 1:10,000. Final washing was conducted as follows: four, 20 minute washes with PBST at room temperature, followed by three, 5-minute washes in AP-buffer. Colorimetric reaction was attained by NBT/BCIP staining for 15 minutes - 72 hours. Polyps were mounted in 90% glycerol.

Quantitative Real-Time PCR (qRT-PCR)

RNA was isolated using TriReagent (Invitrogen) following standard protocols, and then DNAse digested. RNA was quantified and reverse-transcribed using Superscript III RT (Invitrogen), using equal quantities for each tissue-derived pool. qRT-PCR was conducted using a DNA

Engine Opticon 2 real-time PCR machine (MJ Research, Ramsey, MN, USA), SYBR Green I (Invitrogen) and DyNAzyme II Hot Start DNA Polymerase (Finnzymes). Multiple control genes were evaluated, and Beta Actin was selected as a control for normalization based on its invariability across tissue pools. For all genes, expression in each tissue was evaluated in quadruplicate, and two independent experimental replicates were conducted. Data analysis on qRT PCR data was conducted by calculating the relative expression ratios of each gene, including control gene, in each tissue. For each reaction, target gene expression was inferred relative to control gene levels.

Morphological Character Coding

Characters were coded from relevant literature as well as lab observation. Four characters suspected to be related to colony development were coded as binary: hard, exposed perisarc (present/absent); coloniality (present/absent); brooding (present/absent); attached gonophore (present/absent). **See figure S1.**

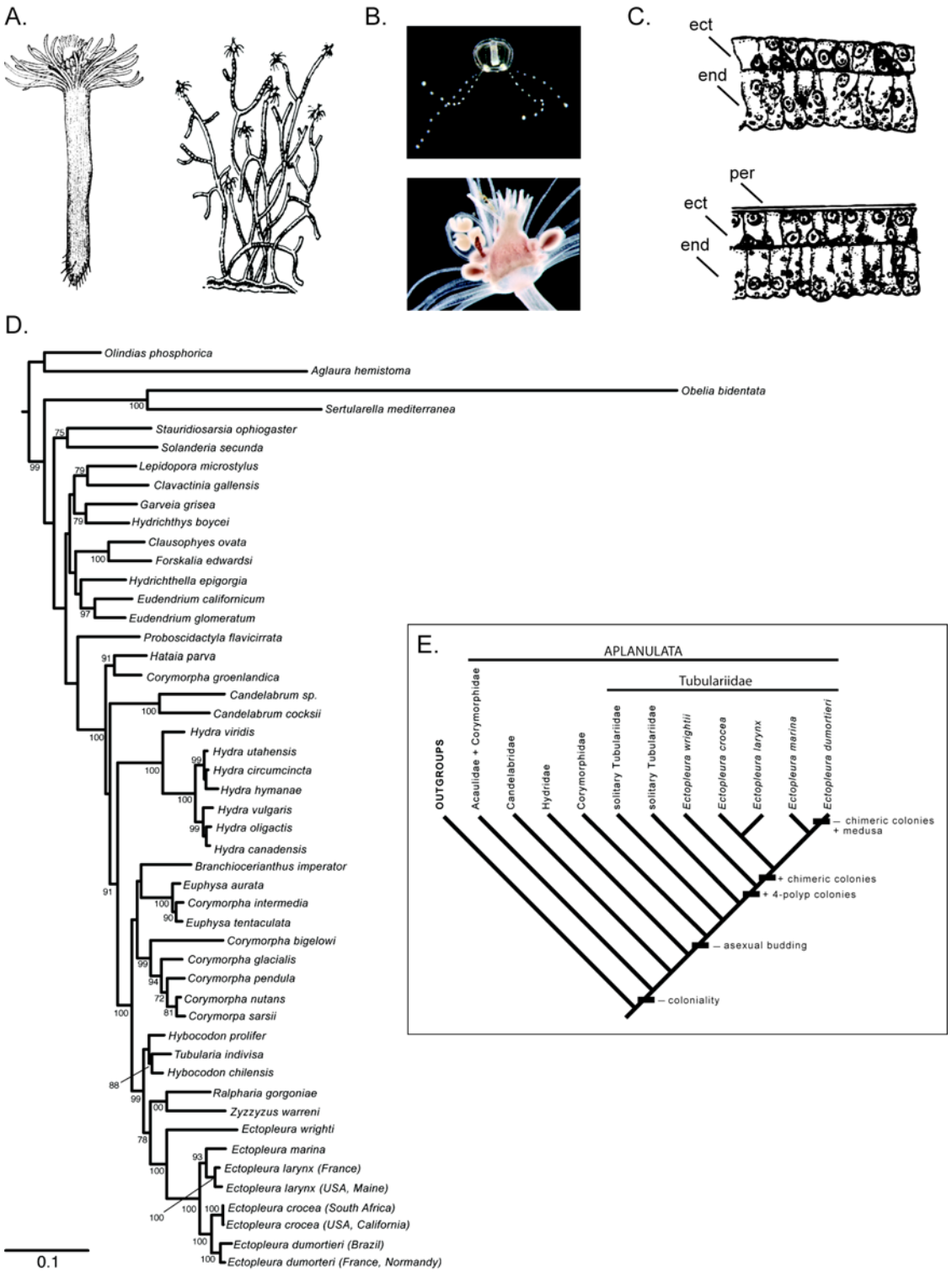

Figure S1. Morphological characters, phylogenetic relationships, and colony evolution of Aplanulata (A) Aplanulata polyp types. Left to right: solitary *Corymorpha pendula*; colonial *Ectopleura larynx*. (B) Reproductive structure types of Aplanulata. Top: free-swimming medusa of *Ectopleura dumortieri*. Bottom: Reduced reproductive structures attached to *Zyzzyzus warreni*

polyp. (C) Perisarc. Top: epithelial structure of the body column of *Hydra vulgaris*, demonstrating naked ectoderm; Bottom: epithelial structure of *Ectopleura larynx* stalk, illustrating perisarc. (D) Phylogram showing phylogenetic hypothesis based on ML analysis of 16S + 18S + 28S sequences in RaxML under a GTR + Γ + i + RNA16 model. All bootstrap support values indicated at nodes and calculated from 1000 replicates. **(E)** Evidence suggests that the re-evolution of coloniality occurred in a step-wise fashion. Following the loss of coloniality, some solitary species could still form detached buds. Subsequently, asexual budding was completely lost. 4-polyp colonies that form by aboral tissue growth (not budding) evolved at the base of the genus *Ectopleura*. The ability to form large colonies through parent-offspring fusion subsequently allowed the diversification of large colonial species in the genus *Ectopleura*. *Hydra vulgaris* and *Ectopleura larynx* illustrations modified from [9]. *Corymorpha pendula* modified from [10,11]. *Ectopleura larynx* colony illustration modified from [12]. *E. dumortieri* and *Z. warreni* photographs with permission of A.E. Migotto and C. Campos. ect = ectoderm; end = endoderm; per = perisarc. This figure is associated with Figure 2 in the main text.

Character 1 - *Polyp organization*. 0 = solitary, 1 = colonial, ? = unknown. We defined coloniality as connected polyps that share epithelial tissue and a gastrovascular cavity. Species within Aplanulata are solitary (Fig. S1A), form small colonies (comprised of up to four polyps) or form dense, bush-like colonies (many hundreds of polyps) (Fig. S1A). We coded this character as binary, with species who form dense, permanent colonies of many to a hundred polyps coded as colonial, as well as sparse colonies of only a few polyps (e.g. *Ectopleura wrighti*) coded as colonial. *Hybocodon chilensis* is described in the literature as forming small colonies, but its polyps are connected by their skeletons only and do not form true integrated colonies (Galea 2006); thus, this species was coded as solitary. Solitary species of Aplanulata that form buds that always detach (e.g. *Hydra* spp. and others) or never form buds at all (e.g. *Corymorpha* and others) were both coded as the same character state. Trachymedusae and Limnomedusae outgroups were coded as solitary, as the species sampled either form small polyps (*Olindias*), or produce an 'actinula' (*Aglaura*), which we interpret as a primary polyp. For binary coding for character correlation analyses, species were coded as reported above, except

that unknown character states were inferred from a least squared change parsimony analysis in Mesquite (Maddison and Maddison 2007).

Character 2 - *Absence/presence of a free-living, pelagic reproductive structure*. 0 = pelagic gonophore; 1 = attached gonophore, ? = unknown, n/a = no gonophore. The sexual, pelagic medusa stage is found in some members of Tubulariidae and Corymorphidae. The rest of the sampled species produce their gametes in attached gonophores, or, in the case of Hydridae that lack gonophores altogether, produce their gametes within the epidermis (Fig. S1B). Species were scored as possessing a free-living, pelagic medusa if their reproductive structure detaches and feeds in the water column. Reduced reproductive structures called medusoids were only coded as medusae if they detach from the polyp and are known to feed. All other reproductive structures were coded as 'fixed gonophore.' *Hydra* do not make a gonophore (gametes produced in the epithelia of the polyp body column), so members of this genus were coded 'n/a.' For binary coding for character correlation analyses, species were coded as above, except that *Hydra* spp. were coded as 'fixed gonophore.' Unknown character states were inferred from a least squared change parsimony analysis in Mesquite (1).

Character 3 - *Absence/presence of brooding behavior*. 0 = brooding absent, 1 = brooding present, ? = unknown. All major clades of Aplanulata, with the exception of Hydridae, have members that brood (Fig. 2). Brooding structures develop on the body of the polyp and either remain attached throughout the development of the juvenile, or are released as swimming medusae (free-living jellyfishes). In Aplanulata, young develop directly in these structures and bypass a larval stage. In contrast, indirect-developing hydrozoans spawn eggs or sperm directly from the attached or pelagic gonophore. For this character, species that brood developing young in gonophores or medusae were coded as 'brooding present,' and all others were coded as

'brooding absent.' For binary coding for character correlation analyses, species were coded as reported above, except that unknown character states were inferred from a least squared change parsimony analysis in Mesquite (Maddison and Maddison 2007).

Character 4 - *Presence of hard, exposed perisarc covering the ectoderm of the polyp or stolonal system*. 0 = hard, exposed perisarc absent, 1 = hard, exposed perisarc present, ? = unknown. Exposed, hard perisarc is present in all members of Tubulariidae, except *Zyzzyus warreni* and *Ralpharia gorgoniae* (Fig. 2). A typical hydrozoan perisarc is composed of a layer of acellular material (usually chitin) that is smooth and rigid and covers the ectoderm of the polyp body or stolonal system (Fig. S1C). The perisarc may cover a small portion of the polyp or may cover the entire length of the stolonal system (e.g. in *Ectopleura* polyps). Such a skeleton provides support for an upright growth form. Members of *Hydra* and the species *Hataia parva* completely lack a persarc. Species of the genera *Corymorpha, Euphysa, Branchiocerianthus* and *Zyzzyzus* have a thin, gelatinous or membranous covering over the polyp. Because these coverings are thin and do not function as support for an upright structure, we do not consider them a true perisarc; we coded these species as 'hard, exposed perisarc absent.' Species that possess a hard perisarc that is *not* exposed, due to ecological factors such as substrate use (*Ralpharia gorgoniae*, *Zyzzyzus warreni*) or small size (Candelabridae), were coded as 'hard, exposed perisarc absent.' All other Aplanulata taxa were coded as 'hard, exposed perisarc present.' For binary coding for character correlation analyses, coding was as reported above, except that unknown character states were inferred from a least squared change parsimony analysis in Mesquite (Maddison and Maddison 2007).

II. Supplemental Results

Supplementary Table 1 (S1). Specimens, Genbank IDs and Character Coding. Genbank IDs for new sequences generated for this study are bolded. Coding is indicated for specimens included in combined analysis only.

Higher Level	Family	Species	28s	18s	16s	Char 1	Char 2	Char 3	Char 4	Voucher or Published Reference Sequence
Aplanulata	Candelabridae	*Candelabrum austrogeorgiae*	-	-	FN424120	-	-	-	-	Cantero et al. (2010)
Aplanulata	Candelabridae	*Candelabrum cocksii*	EU879928	EU876556	AY512520	0	1	1	1	MHNGINVE29591
Aplanulata	Candelabridae	*Candelabrum* sp.	EU879929	EU876557	EU876530	0	1	?	1	-
Aplanulata	Corymorphidae	*Branchiocerianthus imperator*	**JN594035**	**JN594046**	-	0	?	?	0	-
Aplanulata	Corymorphidae	*Corymorpha bigelowi*	EU272563	EU876564	EU448099	0	0	0	0	KUNHM2829
Aplanulata	Corymorphidae	*Corymorpha glacialis*	**JN594036**	**JN594047**	FN687549	0	1	1	0	MHNGINVE67050
Aplanulata	Corymorphidae	*Corymorpha groenlandica*	**JN594037**	**JN594048**	FN687551	0	1	1	0	MHNGINVE67051
Aplanulata	Corymorphidae	*Corymorpha groenlandica*	-	-	FN687550	-	-	-	-	MHNGINVE63302
Aplanulata	Corymorphidae	*Corymorpha intermedia*	EU879930	AY920759	FN687910	?	0	0	?	Collins et al. (2006), Schuchert (2010)
Aplanulata	Corymorphidae	*Corymorpha nutans*	EU879931	EU876558	FN687546	0	0	0	0	MHNGINVE48745
Aplanulata	Corymorphidae	*Corymorpha nutans*	-	-	FN687549	-	-	-	-	MHNGINVE67050
Aplanulata	Corymorphidae	*Corymorpha nutans*	-	-	FN687548	-	-	-	-	Schuchert (2010)
Aplanulata	Corymorphidae	*Corymorpha nutans*	-	-	FN687547	-	-	-	-	Schuchert (2010)
Aplanulata	Corymorphidae	*Corymorpha pendula*	EU879936	EU876565	EU876538	0	1	0	0	KUNHMDIZ2962
Aplanulata	Corymorphidae	*Corymorpha sarsii*	**JN594038**	**JN594049**	-	0	1	0	0	-
Aplanulata	Corymorphidae	*Corymorpha sp.*	-	-	FN424121	-	-	-	-	Cantero et al. (2010)
Aplanulata	Corymorphidae	*Euphysa aurata*	EU879934	EU876562	EU876536	0	0	0	0	MHNGINVE48753
Aplanulata	Corymorphidae	*Euphysa aurata*	-	-	FN687552	-	-	-	-	Schuchert (2010)
Aplanulata	Corymorphidae	*Euphysa japonica*	-	EU301605	-	-	-	-	-	Lindsay, D.J. et al. (2008)
Aplanulata	Corymorphidae	*Euphysa tentaculata*	EU879935	EU876563	EU876537	?	0	0	?	Cartwright & Nawrocki (2010)
Aplanulata	Corymorphidae	*Hataia parva*	**JN594034**	**JN594045**	**JN594033**	0	1	1	0	UF5407
Aplanulata	Hydridae	*Hydra canadensis*	**JN594039**	**JN594050**	GU722797	0	n/a	0	0	Martinez et al. (2010)
Aplanulata	Hydridae	*Hydra circumcincta*	EU879939	EU876568	GU722764	0	n/a	0	0	Cartwright & Nawrocki (2010) , Martinez et al. (2010)
Aplanulata	Hydridae	*Hydra hymanae*	**JN594040**	**JN594051**	GU722760	0	n/a	0	0	Martinez et al. (2010)
Aplanulata	Hydridae	*Hydra oligactis*	**JN594041**	**JN594052**	GU722781	0	n/a	0	0	Martinez et al. (2010)
Aplanulata	Hydridae	*Hydra oxycnida*	-	-	GU722789	-	-	-	-	Martinez et al. (2010)
Aplanulata	Hydridae	*Hydra utahensis*	**JN594042**	**JN594053**	GU722774	0	n/a	0	0	Martinez et al. (2010)
Aplanulata	Hydridae	*Hydra viridissima*	EU879940	EU876569	GU722756	0	n/a	0	0	Martinez et al. (2010)
Aplanulata	Hydridae	*Hydra vulgaris*	**JN594043**	**JN594054**	GU722817	0	n/a	0	0	Martinez et al. (2010)
Aplanulata	Tubulariidae	*Ectopleura crocea*	EU879932	EU876559	EU876533	1	1	1	1	MHNGINVE34010
Aplanulata	Tubulariidae	*Ectopleura crocea*	EU883554	EU883548	EU883543	1	1	1	1	Cartwright & Nawrocki (2010)
Aplanulata	Tubulariidae	*Ectopleura dumorteri*	-	-	FN687542	-	-	-	-	Schuchert (2010)
Aplanulata	Tubulariidae	*Ectopleura dumortieri*	EU272561	EU876560	EU305474	0	0	0	1	Pers. Voucher: Alberto Lindner, AL525
Aplanulata	Tubulariidae	*Ectopleura dumortieri*	EU879933	EU876561	EU876534	0	0	0	1	Cartwright & Nawrocki (2010)
Aplanulata	Tubulariidae	*Ectopleura dumortieri*	-	-	FN687543	-	-	-	-	Schuchert (2010)
Aplanulata	Tubulariidae	*Ectopleura larynx*	EU879943	EU876572	EU876545	1	1	1	1	KUNHMDIZ2963
Aplanulata	Tubulariidae	*Ectopleura larynx*	EU883549	AY920760	AY787877	1	1	1	1	MHNGINVE29389
Aplanulata	Tubulariidae	*Ectopleura larynx*	-	-	FN687535	-	-	-	-	MHNGINVE54563

Aplanulata	Tubulariidae	*Ectopleura larynx*	-	-	FN687536	-	-	-	-	MHNGINVE62576
Aplanulata	Tubulariidae	*Ectopleura marina*	EU883553	EU883547	EU883542	1	1	1	1	Cartwright & Nawrocki (2010)
Aplanulata	Tubulariidae	*Ectopleura wrighti*	**JN594044**	**JN594055**	FN687541	1	0	0	1	MHNGINVE27331
Aplanulata	Tubulariidae	*Hybocodon chilensis*	EU879937	EU876566	EU876539	0	0	0	1	MHNGINVE36023
Aplanulata	Tubulariidae	*Hybocodon prolifer*	EU879938	EU876567	EU876540	0	0	0	1	Cartwright & Nawrocki (2010)
Aplanulata	Tubulariidae	*Ralpharia gorgoniae*	EU272590	EU272633	EU305482	0	1	1	1	KUNHM2778
Aplanulata	Tubulariidae	*Ralpharia sp.*	-	**JN594056**	-	-	-	-	-	
Aplanulata	Tubulariidae	*Tubularia indivisa*	EU879942	EU876571	EU876544	0	1	1	1	Cartwright & Nawrocki (2010)
Aplanulata	Tubulariidae	*Tubularia indivisa*	-	-	FN687532	-	-	-	-	Schuchert (2010)
Aplanulata	Tubulariidae	*Tubularia indivisa*	-	-	FN687530	-	-	-	-	MHNGINVE60972
Aplanulata	Tubulariidae	*Tubularia sp.*	-	-	FN424153	-	-	-	-	Cantero et al. (2010)
Aplanulata	Tubulariidae	*Zyzzyzus warreni*	EU272599	EU272640	EU305489	0	1	1	0	KUNHM2777
Capitata	Corynidae	*Stauridiosarsia ophiogaster*	EU272560	EU272615	EU305473	1	0	0	0	KUNHM2803
Capitata	Solanderiidae	*Solandaria secunda*	EU305533	EU305502	EU305484	1	1	0	1	KUNHM2611
Filifera I	Proboscidactylidae	*Proboscidactyla flavicirrata*	EU305527	EU305500	EU305480	1	0	0	0	USNM1074994
Filifera I	Ptilocodiidae	*Hydrichthella epigorgia*	EU272569	EU272622	EU305478	1	1	0	0	KUNHM2665
Filifera II	Eudendriidae	*Eudendrium californicum*	EU305513	EU305492	EU305475	1	1	0	1	KUNHM2850
Filifera II	Eudendriidae	*Eudendrium glomeratum*	FJ550440	FJ550583	AM991301	1	1	0	1	MHNGINVE49717
Filifera III	Hydractiniidae	*Clavactinia gallensis*	EU272553	EU272610	EU448101	1	1	0	0	MHNGINVE33470
Filifera III	Stylasteridae	*Lepidopora microstylus*	EU272572	EU272644	EU645329	1	1	0	0	USNM1027724
Filifera IV	Bougainvillisae	*Garveia grisea*	EU272588	EU272632	AM183131	1	1	0	1	MHNGINVE34436
Filifera IV	Pandeidae	*Hydrichthys boycei*	EU272570	EU305496	EU448102	1	0	0	0	MHNGINVE37417
Leptothecata	Campanulariidae	*Obelia bidentata*	FJ550446	AY789754	AY789815	1	0	0	1	MHNGINVE37294
Leptothecata	Sertulariidae	*Sertularella mediterranea*	FJ550403	FJ550546	FJ550479	1	1	0	1	MHNGINVE32948
Limnomedusae	Olindiasidae	*Olindias phosphorica*	EU247808	AY920753	AY512509	0	0	0	0	MHNGINVE29811
Siphonophorae	Clausophyidae	*Clausophyes ovata*	EU305508	AY937336	AY935294	1	1	0	0	YPM35349
Siphonophorae	Forskaliidae	*Forskalia edwardsi*	EU305516	AY937354	AY935312	1	1	0	0	YPM35036
Trachymedusae	Rhopalonematidae	*Aglaura hemistoma*	EU247803	EU247818	EU293984	0	0	0	0	MHNGINVE31745

Supplementary Table 2 (S2). RNA model selection data for phylogenetic analyses.

Marker	RNA Model	# Free Parameters	-ln(L) Score	AIC	AIC_{Δ}
16s	16	134	8715.32	8849.32	25.14
	16A*	19	8805.18	8824.18	n/a
	16B	15	8913.16	8928.16	103.98
18s - LSU	16*	134	4590.53	4724.53	n/a
	16A	19	4745.56	4764.56	40.03
	16B	15	5075.34	5090.34	325.78
28s - SSU	16*	134	13889.71	14023.71	n/a
	16A	19	14339.69	14358.69	334.98
	16B	15	15271.17	15286.17	1262.46
16s + 18s +	16*	134	28032.51	28166.51	n/a
28s	16A	19	32705.57	32724.57	4558.46
	16B	15	34093.72	34108.72	5942.21

AIC_{Δ} = The difference between the lowest AIC value and that of the **tested model.**
*** indicates best-fitting model based on AIC score.**

Character Correlation Analyses

Coloniality was correlated with brooding ($p = 0.02$; 1,000 simulations) and with the presence of an exposed, hard perisarc ($p = 0.0$; 1,000 simulations). Coloniality was also correlated with the simultaneous presence of all three characters ($p = 0.0$; 1,000 simulations).

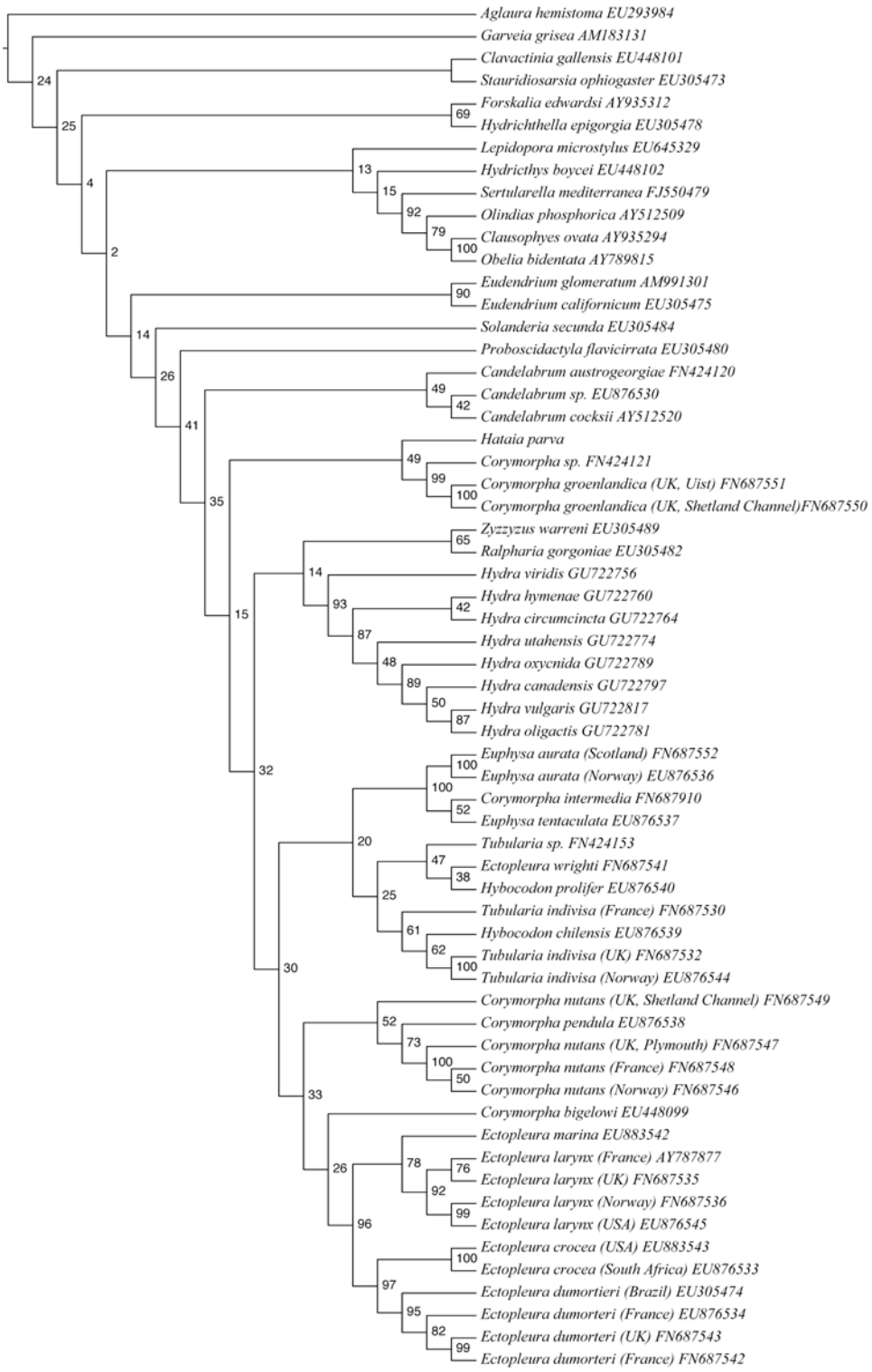

Figure S2. Cladogram showing phylogenetic hypothesis based on ML analysis of mitochondrial 16s rRNA sequences in RaxML under model GTR + Γ + i + RNA16A. All bootstrap support values indicated at nodes and calculated from 1000 replicates.

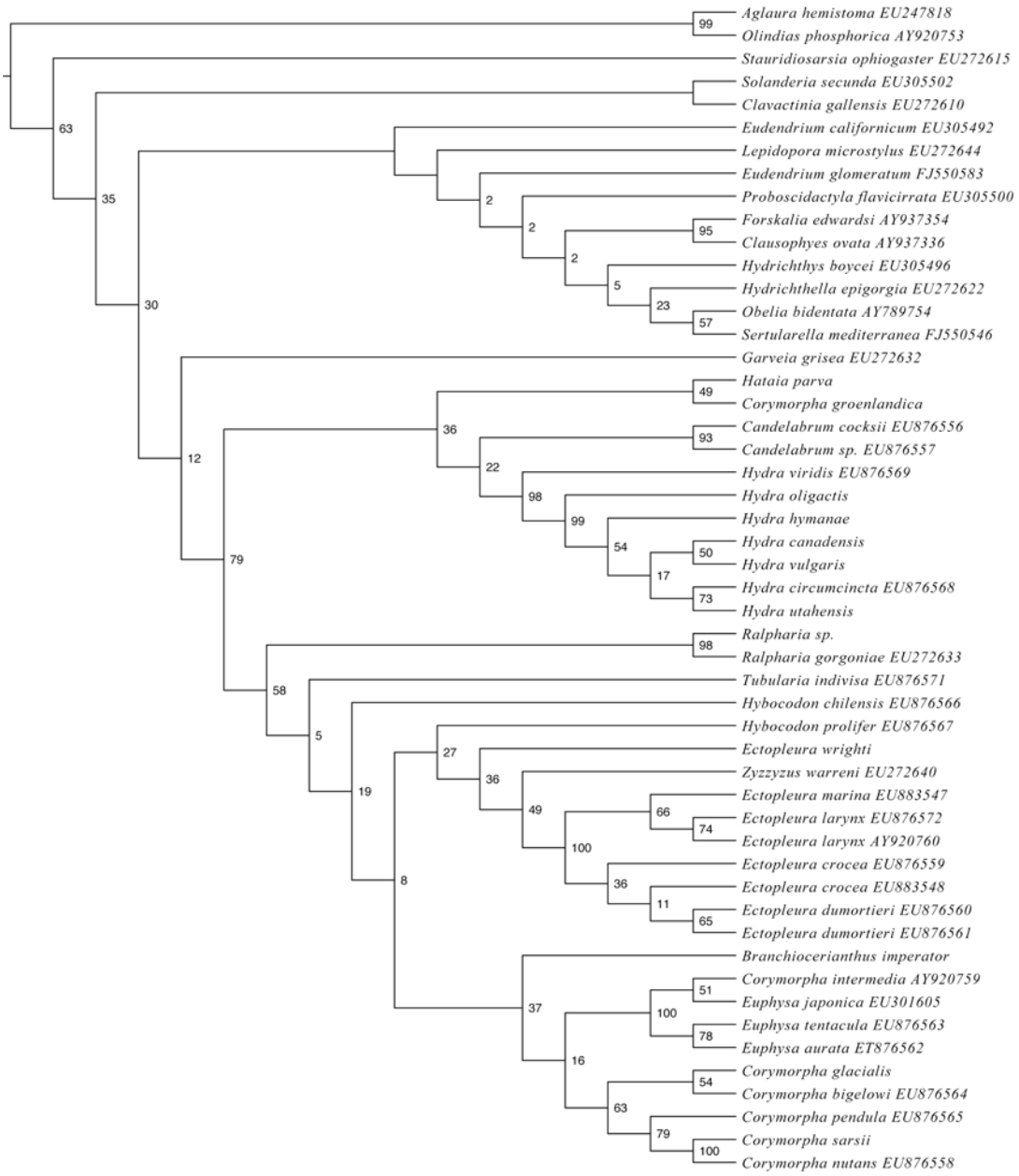

Figure S3. Cladogram showing phylogenetic hypothesis based on ML analysis of mitochondrial 18s rRNA sequences in RaxML under model GTR + Γ + i + RNA16. All bootstrap support values indicated at nodes and calculated from 1000 replicates.

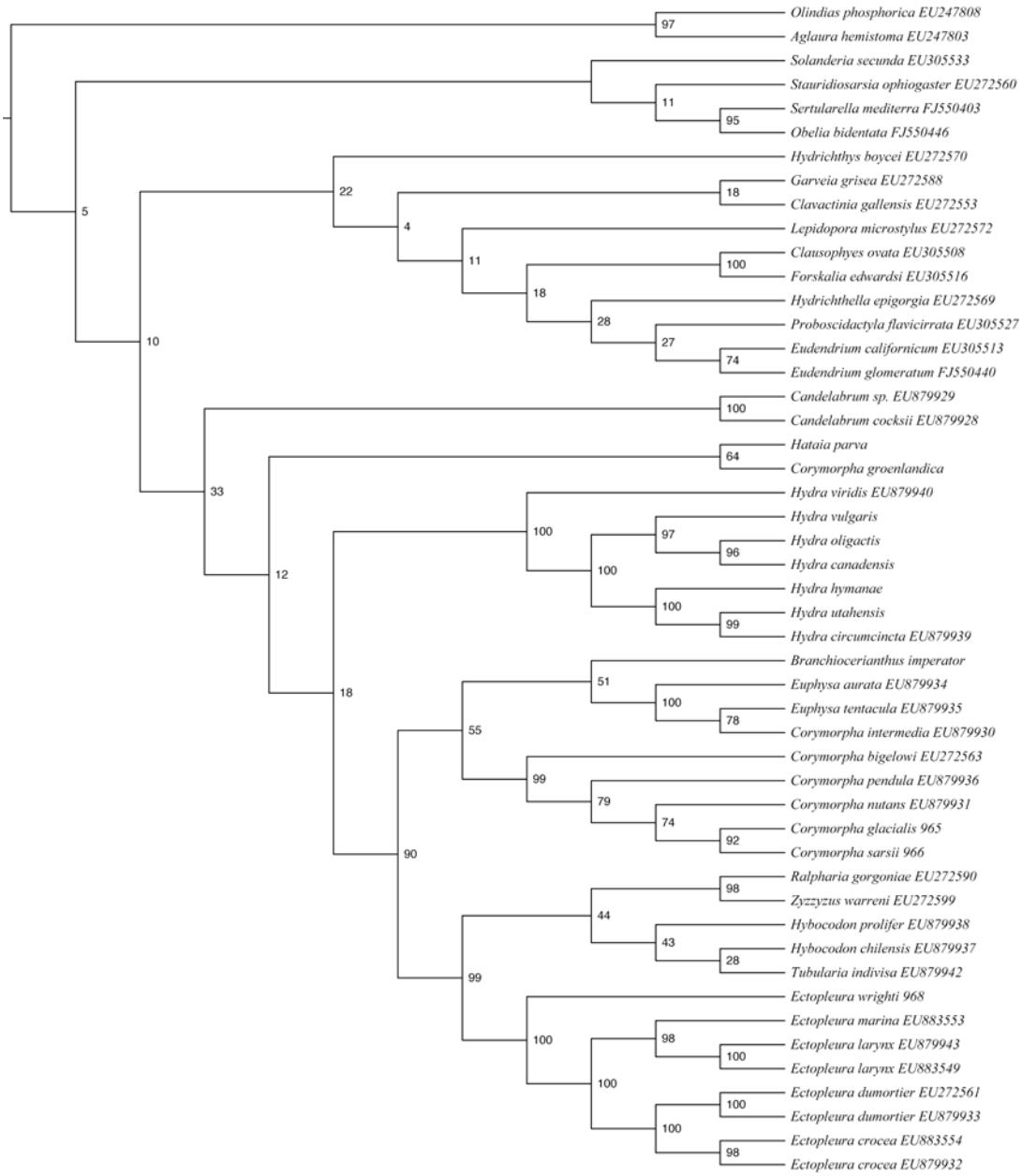

Figure S4. Cladogram showing phylogenetic hypothesis based on ML analysis of 28s rRNA (LSU) sequences in RaxML under model GTR + Γ + i + RNA16. All bootstrap support values indicated at nodes and calculated from 1000 replicates.