A molecular phylogeny of the Thaliacea

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Thaliaceans are gelatinous holoplanktonic tunicates comprised of around 72 described species in three orders: the Pyrosomatida (pyrosomes), Salpida (salps) and Doliolida (doliolids). Despite their interesting biology and important role in ocean ecosystems, relatively little is known about their evolutionary relationships with other tunicate lineages and with each other. Here, using 40 newly obtained 18S rDNA sequences, we present a molecular phylogeny of the Thaliacea. Our results show the pyrosomes branching off first and support their division into the Pyrostremmatinae and Pyrosomatinae. Within the Salpida, we found the subfamily Salpinae is paraphyletic with the morphologically divergent Cyclosalpinae. We also resolved the uncertainty regarding *Weelia* (*Salpa*) *cylindrica*, finding it to be relatively distantly related to *Salpa aspera*, *Salpa maxima*, *Salpa fusiformis* and *Salpa thompsoni*.

INTRODUCTION

Thaliaceans are gelatinous holoplanktonic tunicates that include three orders: the Pyrosomatida (pyrosomes), Salpida (salps) and Doliolida (doliolids). There are around 72 described species, many with worldwide distributions (Table I). Thaliaceans are known for their complex life cycles (Alldredge and Madin, 1982). Salps alternate between solitary (also called oozoid) and aggregate (also called blastozooid) stages, and an even higher degree of zooid specialization is observed in doliolids (Table I; Alldredge and Madin, 1982). Thaliaceans also play an important role in ocean food chains and in the transfer of organic material to the deep sea (Wiebe et al., 1979; Madin, 1982; Lebrato and Jones, 2009; Phillips et al., 2009). Furthermore, thaliacean abundance may be increasing due to climate change. For example, recent years have seen an increase in *Salpa thompsoni* and a corresponding decrease in krill in parts of the Southern Ocean that may be related to the decline in sea ice cover, and has the potential to disrupt Southern Ocean food chains (Loeb et al., 1997; Atkinson et al., 2004; Moline et al., 2004).

From an evolutionary perspective, there is significant interest in tunicate phylogeny in order to understand the origin of the chordates (Garstang, 1928; Wada and Satoh, 1994; Swalla et al., 2000; Winchell et al., 2002; Zeng and Swalla, 2005; Stach, 2008; Swalla and Smith, 2008; Tsagkogeorga et al., 2009). Furthermore, the Tunicata is an excellent group in which to examine the evolution of coloniality and life history (Wada, 1998; Zeng et al., 2006). The Tunicata includes thaliaceans, ascidians and appendicularians and contains both solitary and colonial forms. The holoplanktonic Thaliacea all exhibit coloniality but differ significantly from benthic ascidians and from each other in their life cycles and degree of colony integration (Table I). Notably, the Thaliacea are the only tunicate lineage to exhibit zooid polymorphism, which is best developed in the doliolids (Berrill, 1950; Harvell, 1994).

Despite their interesting biology and relevance to ocean ecosystems, very little is known about the

Table I: Thaliacean taxa and characteristic features

	Number of taxa	Life cycle	Feeding and propulsion
Salpida	~48 species in 2 subfamilies and 12 genera (Godeaux, 1998; Madin and Madin, unpublished)	Alternation between sexual aggregate and asexual solitary generations	Water pumped by muscle bands through mucous filter; exiting water provides propulsion
Pyrosomatida	8 species in 3 genera (Godeaux, 1998)	Bioluminescent, hollow cylindrical colonies with internal fertilization and embryonic development	Water pumped by ciliary action past mucous filter; exiting water provides propulsion
Dolioida	23 species in 4 families (Godeaux, 1998; Godeaux and Harbison, 2003; Robison et al., 2005a, b)	Complex life cycles and zooid specialization; asexual oozoids produce asexual phorozooids, which produce sexual gonozooids	Water pumped by ciliary action past mucous filter; muscle bands provide propulsion

Characteristic life cycles, feeding and propulsion mechanisms summarized from Berrill (Berrill, 1950) and Alldredge and Madin (Alldredge and Madin, 1982).

evolutionary relationships within the Thaliacea and between the Thaliacea and other tunicates. Previous genetic studies based on 18S rDNA have placed the Thaliacea within the Ascidiacea; however, the exact relationships between thaliacean and ascidian lineages and within the Thaliacea are not known (Swalla et al., 2000; Zeng and Swalla, 2005; Yokobori et al., 2005; Tsagkogeorga et al., 2009). Relationships within the Thaliacea are even less well studied, and have been inferred from morphological characters for pyrosomes (Van Soest, 1981), doliolids (Godeaux, 2003) and salps (Metcalf, 1918, Madin, 1974, Godeaux 1998). Tsagkogeorga et al. (Tsagkogeorga et al., 2009) examined relationships between salps, pyrosomes and doliolids using 18S rDNA, but only included 10 thaliacean sequences in their analysis. A major problem for conducting genetic studies is the lack of appropriately preserved specimens available for molecular analysis. Thaliaceans are relatively difficult to collect intact, and there are few experts capable of accurate species identification.

The goal of our work is to provide an evolutionary context for thaliaceans based on an 18S rDNA phylogeny. Here, we present a molecular phylogeny of the Thaliacea that includes 40 new salp, pyrosome and doliolid 18S rDNA sequences, and compare our results with the current morphologically based classification.

METHOD

Sample collection

Thaliacean specimens were collected from locations around the world, including the Northeast Pacific (near Panama and California, USA), Indian (near Northwest Australia) North and South Atlantic, Southern Ocean (Western Antarctic Peninsula region), the Celebes Sea (near the Philippines) and the Red Sea. Because thaliaceans are extremely delicate and are easily damaged in plankton nets, the majority of specimens were collected through blue-water diving or by remotely operated vehicles (Table II). Immediately after collection, specimens were identified and preserved in ethanol or RNA*later* (Applied Biosystems), or frozen at −80°C.

DNA extraction, PCR and sequencing

For most specimens, DNA was extracted using DNEasy extraction kits (Qiagen) following the manufacturer's protocol. For a few species, a CTAB/phenol/chloroform protocol (Coffroth et al., 1992) was used. The 18S rDNA gene was amplified using standard PCR and sequencing primers and protocols described in Halanych et al. (Halanych et al., 1998) and Stach and Turbeville (Stach and Turbeville, 2002). In some cases, $0.4–0.5\ \mu g/\mu L$ BSA was added to the PCR mixture. PCR products were purified using Qiagen PCR purification kit according to the manufacturer's protocol. Purified PCR products were sequenced bidirectionally at the Center of Marine Molecular Analysis (COMMA) at the University of Connecticut. Sequences were assembled with Sequencher 4.8 (Gene Codes Corp., 2007).

Outgroup selection, alignment and phylogenetic analysis

Several tunicate and other chordate 18S rDNA sequences from GenBank were selected as outgroups (Table II). Of the available aplousobranch sequences on GenBank, only *Pycnoclavella aff. detorta* and *Clavelina meridionalis* were included, as the other available aplousobranch sequences are extremely divergent and can confound analysis (Tsagkogeorga et al., 2009). Sequences were aligned using MUSCLE (Edgar, 2004). Ambiguously aligned regions were removed for

Table II: Thaliacean and outgroup species, sampling locality (new sequences)/author (published sequences), and GenBank accession numbers

Species	Family	Sampling locality/Author	GenBank accession no.
Salpida			
Soestia zonaria	Salpidae	NW Atlantic	HQ015389
Pegea confoederata	Salpidae	Celebes Sea	HQ015386
Pegea confoederata	Salpidae	NW Atlantic	HQ015387
Pegea bicaudata	Salpidae	NW Atlantic	HQ015388
Thetys vagina	Salpidae	NW Atlantic	HQ015390
Ihlea racovitzai	Salpidae	Southern Ocean (West Antarctic Peninsula)	HQ015378
Ihlea racovitzai	Salpidae	Tsagkogeorga *et al.* (2009)	FM244865
Gen. nov. sp. nov. A	Salpidae	NE Pacific (Monterey, CA, USA)	HQ015377
Ritteriella retracta	Salpidae	Celebes Sea	HQ015411
Ritteriella retracta	Salpidae	NE Pacific (Panama)	HQ015410
Weelia cylindrica	Salpidae	Celebes Sea	HQ015399
Weelia cylindrica	Salpidae	Indian (NW Australia)	HQ015402
Weelia cylindrica	Salpidae	NW Atlantic	HQ015400
Weelia cylindrica	Salpidae	Red Sea	HQ015401
Weelia cylindrica	Salpidae	Tsagkogeorga *et al.* (2009)	FM244866
Brooksia rostrata	Salpidae	Celebes Sea	HQ015403
Brooksia rostrata	Salpidae	NE Pacific (Panama)	HQ015404
Salpa aspera	Salpidae	NE Pacific (Panama)	HQ015405
Salpa maxima	Salpidae	NW Atlantic	HQ015407
Salpa maxima	Salpidae	Celebes Sea	HQ015408
Salpa thompsoni	Salpidae	Southern Ocean (West Antarctic peninsula)	HQ015406
Salpa thompsoni	Salpidae	Tsagkogeorga *et al.* (2009)	FM244867
Salpa fusiformis	Salpidae	NE Pacific (Panama)	HQ015409
Thalia orientalis	Salpidae	Southern Ocean	HQ015412
Thalia democratica	Salpidae	Indian (NW Australia)	HQ015414
Thalia democratica	Salpidae	Celebes Sea	HQ015415
Thalia democratica	Salpidae	North Atlantic	HQ015413
Thalia democratica	Salpidae	Wada and Satoh (1994)	D14366
Cyclosalpa floridana	Salpidae	Celebes Sea	HQ015393
Cyclosalpa sewelli	Salpidae	Celebes Sea	HQ015398
Cyclosalpa sewelli	Salpidae	NE Pacific (Panama)	HQ015395
Cyclosalpa polae	Salpidae	Atlantic	HQ015394
Cyclosalpa polae	Salpidae	NE Pacific (Panama)	HQ015396
Cyclosalpa quadriluminis	Salpidae	Indian (NW Australia)	HQ015397
Cyclosalpa quadriluminis	Salpidae	Tsagkogeorga *et al.* (2009)	FM244864
Cyclosalpa affinis	Salpidae	Celebes Sea	HQ015392
Cyclosalpa affinis	Salpidae	Atlantic	HQ015391
Doliolida			
Doliolum denticulatum	Doliolidae	Indian (NW Australia)	HQ015376
Doliolum denticulatum	Doliolidae	Tsagkogeorga *et al.* (2009)	FM244861
Doliolum nationalis	Doliolidae	Wada (1998)	AB013012
Pyrosomatida			
Pyrosomella verticillata	Pyrosomidae	Celebes Sea	HQ015382
Pyrosomella verticillata	Pyrosomidae	Indian (NW Australia)	HQ015280
Pyrosomella verticillata	Pyrosomidae	Tsagkogeorga *et al.* (2009)	FM244863
Pyrosomella verticillata	Pyrosomidae	Celebes Sea	HQ015383
Pyrosoma godeauxi	Pyrosomidae	Indian (NW Australia)	HQ015384
Pyrosoma godeauxi	Pyrosomidae	Tsagkogeorga *et al.* (2009)	FM244862
Pyrosoma atlanticum	Pyrosomidae	Celebes Sea	HQ015385
Pyrosoma atlanticum	Pyrosomidae	NW Atlantic	HQ015381
Pyrosoma atlanticum	Pyrosomidae	Wada (1998)	AB013011
Pyrostremma spinosum	Pyrosomidae	Celebes Sea	HQ015379
Phlebobranchia			
Chelysoma siboja	Corellidae	Swalla *et al.* (2000)	AF165821
Perophora sagamiensis	Perophidae	Okuyama *et al.* (unpublished)	AB104873
Ciona intestinalis	Cionidae	Wada (1998)	AB013017
Ascidia ceratodes	Ascidiidae	Hadfield *et al.* (1995)	L12378
Megalodicopia hians	Octacnemidae	Kurabayashi *et al.* (unpublished)	AB075543
Aplousobranchia			
Pycnoclavella aff. detorta		Tsagkogeorga *et al.* (2009)	FM244841
Clavelina meridionalis		Tsagkogeorga *et al.* (2009)	FM244840
Stolidobranchia			

Continued

Table II: Continued

Species	Family	Sampling locality/Author	GenBank accession no.
Molgula manhattensis	Molgulidae	Hadfield *et al.* (1995)	L12426
Halocynthia igaboja			
Appendicularia			
Oikopleura dioica	Oikopleuridae	Wada (1998)	AB013014
Cephalochordata			
Branchiostoma floridae	Branchiostomidae	Stock and Whitt (1992)	M97571
Craniata			
Myxine glutinosa	Myxinidae	Stock and Whitt (1992)	M97574
Echinorhinus cookei	Echinorhinidae	Bernardi and Powers (1992)	M91181

Twenty-four thaliacean species from 15 genera are published for the first time here.

phylogenetic analysis using Gblocks (Castresana, 2000). Default Gblocks parameters were used except the allowed gap positions was set at with half.

A maximum likelihood approach was used to estimate phylogeny. Model parameters were obtained using ModelTest 3.7 (Posada and Crandall, 1998) using the Akaike information criterion (Posada and Buckley, 2004). The best-fit model selected was the GTR + I + G with the parameters: Base = 0.2495, 0.2320, 0.2878, Nst = 6, Rmat = (0.7652, 1.7774, 1.0295, 0.6019, 3.9722), Rates = gamma, Shape = 0.4861 and Pinvar = 0.3784. A heuristic search was conducted with PAUP* (Swofford, 2003) using starting trees obtained by stepwise addition with 10 random addition sequence replicates and tree bisection-reconnection (TBR) branch swapping. Support for the topology was obtained by bootstrapping and Bayesian approaches. A maximum likelihood bootstrap analysis was conducted using RAxML 7.0.3 (Stamatakis *et al.*, 2007) under the GTRGAMMA option and a complete random starting tree for the 10 000 bootstrap replicates (Pattengale *et al.*, 2009). For ML optimization, every fifth bootstrap tree was used as a starting point to search for maximum likelihood trees.

Bayesian posterior probabilities were obtained with MRBAYES 3.12 (Huelsenbeck and Ronquist, 2001). Bayesian analyses were run for 1 000 000 generations with four chains and a sampling frequency of 100. Burn-in was set at 250. The substitution model was selected with MrModeltest (Nylander, 2004) using the Akaike information criterion and was GTR + I + G.

RESULTS

The 1565 base pair multiple sequence alignment contained 63 sequences, including 40 new thaliaceans. The new thaliaceans included 32 salps belonging to 11 genera, 7 pyrosomes belonging to 3 genera and one doliolid. For some thaliacean species, sequences were obtained from multiple geographic locations. Sequence divergence among conspecific individuals was very small even for specimens collected from widely separated regions (Table III).

The Thaliacea formed a sister group to a clade containing the Aplousobranchia and Phlebobranchia (bootstrap and Bayesian posterior probability values 100 and 99, respectively. The aplousobranchs clustered together (bootstrap and Bayesian posterior probability values of 100) and formed a sister group to a clade containing *Megalodicopia hians* and *Chelysoma siboja* (bootstrap and Bayesian posterior probability values of 100), altogether forming a moderately supported (bootstrap and Bayesian posterior probability values of 62 and 86, respectively) clade.

Monophyly of the Thaliacea was strongly supported by bootstrap and Bayesian posterior probability values

Table III: Intraspecific divergences (given as uncorrected p-distance) for species collected from multiple locations

Species	Collection localities	Uncorrected *p*-distances
Pyrosoma atlanticum	North Atlantic, Celebes Sea	0.00119
Pyrosomella verticillata	Australia, Celebes Sea	0–0.00116
Pegea confoederata	North Atlantic, Celebes Sea	0.00934
Thalia democratica	North Atlantic, Australia, Celebes Sea	0–0.00177
Weelia cylindrica	North Atlantic, Australia, Celebes Sea, Red Sea	0–0.00482
Salpa maxima	North Atlantic, Celebes Sea	0.00059
Ritteriella retracta	Panama (Pacific side), Celebes Sea	0.00293
Brooksia rostrata	Panama (Pacific side), Celebes Sea	0
Cyclosalpa affinis	North Atlantic, Celebes Sea	0.00117
Cyclosalpa polae	North Atlantic, Panama (Pacific side)	0
Cyclosalpa sewelli	Panama (Pacific side), Celebes Sea	0

(98 and 99, respectively), as was monophyly of each of the Salpida (bootstrap and posterior probability values of 100), Doliolida (bootstrap and posterior probability values of 100) and Pyrosomatida (bootstrap and posterior probability values of 98 and 100, respectively) lineages. The pyrosomes branch off first, and the doliolids and salps form a weakly supported clade (bootstrap and posterior probability values of 59 and 69, respectively).

Within the Pyrosomatida, *Pyrostremma spinosum* branched off from a well-supported pyrosome clade containing the *Pyrosoma* and *Pyrosomella* lineages (bootstrap and posterior probability values of 100). *Pyrosoma* and *Pyrosmella* sequences exhibited minimal divergence from each other and branching order could not be completely resolved. The three doliolids, belonging to two species in the genus *Doliolum*, also exhibited minimal divergence from each other but were separated by a relatively long branch from the other thaliaceans.

Within the Salpida, *Soestia*, *Thetys* and *Pegea* branch off first, although this clade was not supported by bootstrapping or Bayesian analysis. *Soestia* and *Thetys* formed a strongly supported clade (bootstrap and posterior probability values of 99 and 100, respectively), and *Pegea* monophyly was strongly supported (bootstrap and posterior probability values of 100). Within the remaining Salpida, *Ihlea racovitzae* and *Gen. nov. sp. nov.A* clustered strongly together (bootstrap and posterior probability values of 100). At the generic level, monophyly of *Weelia*, *Thalia*, *Salpa*, *Brooksia*, *Riteriella* and *Cyclosalpa* was supported, and together these genera form a clade (bootstrap and posterior probability values of 89 and 100, respectively). *Weelia cylindrica*, which some authors place in the genus *Salpa* (Madin, 1974), formed a distinct, strongly supported monopohyletic clade (bootstrap and posterior probability values of 100) that falls outside the *Salpa* clade. The morphologically divergent *Cyclosalpa*, which is traditionally placed in the subfamily Cyclosalpinae, was nested with the subfamily Salpinae in a clade including *Salpa*, *Ritteriella* and *Brooksia* (bootstrap and posterior probability values <50 and 95, respectively).

DISCUSSION

Tunicate framework

Our increased sampling and analysis of thaliacean 18S rDNA sequences fills an important gap in the study of tunicate evolution. Thaliaceans have been poorly represented in molecular studies likely because appropriately preserved and identified samples are difficult to obtain. Most recently, Tsagkogeorga et al. (Tsagkogeorga et al., 2009) included 10 representatives in a phylogeny of the Tunicata. Here, we present 40 new thaliacean 18S rDNA sequences, primarily from salps and pyrosomes. Future efforts should focus on acquiring additional doliolid samples.

The nested position of thaliaceans within the larger tunicate phylogeny recovered here and elsewhere (Swalla and Smith, 2008; Tsagkogeorga et al., 2009) indicates that their holoplanktonic lifestyle evolved at least once from benthic ascidian ancestors. However, questions linger on the relationships between the major tunicate lineages, including the positions of the pyrosomes, salps and doliolids. Morphological and molecular investigations have led to conflicting hypotheses regarding the monophyly of the Thaliacea and their relationship to ascidians. In particular, a separate origin for the doliolids has been proposed by several authors. Based on sperm ultrastructure, Holland (Holland, 1988, 1989, 1990) proposed that salps and pyrosomes may be derived from colonial ascidians such as the aplousobranchs, while doliolids may have arisen independently from primitive solitary ascidians. Compère and Godeaux (Compère and Godeaux, 1997) reported simpler endostyles in doliolids than in salps, pyrosomes and ascidians. Godeaux (Godeaux, 1996) proposed reclassifying the Thaliacea into two orders: the "true" Thaliacea containing salps and pyrosomes, and the Doliolida, containing two suborders of doliolids. Godeaux (Godeaux, 1998) additionally justified the separate evolution of doliolids from the pyrosomes and salps, possibly from a solitary aplousobranch ancestor, on the basis of morphology and oozoid development.

In contrast to an independent doliolid lineage, Hirose et al. (Hirose et al., 2001) noted that tunic net cells are found only in pyrosomes and aplousobranch ascidians but not in salps, doliolids or phlebobranch ascidians. Based on a parsimony analysis of a morphological character matrix, Stach and Turbeville (Stach and Turbeville, 2002) grouped together salps and doliolids separate from the pyrosomes, and suggested that the unique endostyle ultrastructure reported by Compère and Godeaux (Compère and Godeaux, 1997) is an autapomorphy and did not warrant an independent doliolid order.

Molecular studies based on 18S rDNA have suggested that thaliaceans are monophyletic and may have a close relationship to the phlebobranch and aplousobranch ascidians (Swalla et al., 2000; Stach and Turbeville, 2002; Zeng and Swalla, 2005; Yokobori et al., 2006). Phlebobranchs are positioned as a sister group to the Thaliacea (Stach and Turbeville, 2002; Zeng and Swalla, 2005; Yokobori et al., 2005; Swalla

and Smith, 2008). Aplousobranch ascidians may also be closely related to thaliaceans, but their exact relationship has not been well supported probably because of their exceptionally long branch lengths and the possibility that at least some published aplousobranch sequences (Stach and Turbeville, 2002) may represent microbial contaminants (Yokobori et al., 2006). Moreover, a major drawback to these 18S studies is that at most only one salp, one pyrosome and one doliolid 18S sequence have been included in the data sets. Most recently, Tsagkogeorga et al. (Tsagkogeorga et al., 2009) using a larger data set that included 10 thaliaceans was unable to resolve a trichotomy between the thaliaceans, aplousobranchs and phlebobranchs.

Finally, additional support for a nested position of the Thaliacea within the traditional Ascidiacea and a close relationship with the phlebobranch ascidians comes from the highly divergent Doliolum (Doliolida) mitochondrial genome. Unusual tRNAs and codon usage were shared between ascidians and doliolids (Yokobori et al., 2005). Furthermore, phylogenetic analysis of 12 mitochondrial protein coding genes supported a close relationship between Doliolum and the phlebobranch ascidians (Yokobori et al., 2005).

Using phlebobranch and basal aplousobranch ascidians as outgroups, our phylogenetic results support a monophyletic Thaliacea with pyrosomes branching off first (Fig. 1). An association between the salps and doliolids is weakly supported. However, the long branches observed in most aplousobranchs (Stach and Turbeville, 2002; Yokobori et al., 2006; Tsagkogeorga et al., 2009) suggest that molecular markers other than 18S will be necessary to fully resolve tunicate phylogeny (Tsagkogeorga et al., 2009). Additional sampling of the Doliolida is also critical for clarifying the relationship between the salp, pyrosomes and doliolid lineages within the Thaliacea. Our results, however, provide several new insights on relationships within the Salpida and Pyrosomatida.

Pyrosome relationships

Our 18S rDNA results strongly support the taxonomic division of Pyrostremma from other pyrosomes, and are consistent with Van Soest's hypothesis that it is similar to the ancestral pyrosomes. The order Pyrosomatida consists of one family, Pyrosomatidae, split into two subfamilies (Van Soest, 1981). The Pyrostremmatinae contains a single genus, Pyrostremma, and the Pyrosomatinae contains the genera Pyrosomella and Pyrosoma. The Pyrostremmatinae and Pyrosomatinae differ fundamentally in numerous reproductive and anatomical characters. Van Soest's (Van Soest, 1981) cladistic analysis

suggested that the Pyrostremmatinae branched off first. Strong evidence comes from Pyrostremma's developmental similarities to salps and doliolids. In the pyrosome life cycle, the initial zooid (the cyathozooid) gives rise to subsequent zooids and is homologous to the oozooid in the salp life cycle (Van Soest, 1981). In Pyrostremma, the cyathozooid contains most organs present in typical zooids and produces 30–80 primary zooids, while in Pyrosomella and Pyrosoma, the cyathozooid is greatly reduced, lacking many organs and producing only four primary zooids (Van Soest, 1981). However, in Pyrostremma, the newly formed zooids are fixed in position. In Pyrosomella and Pyrosoma, as in salps and doliolids, the zooids are moved within the budded colony (Van Soest, 1981).

In contrast to the divergence between Pyrostremma and the Pyrosomatinae, there was almost no divergence within the Pyrosomatinae. Our sampling included both Pyrosomella and Pyrosoma representatives. Additional research, including the development of new genetic markers, is necessary to elucidate the relationships between these species.

Salp relationships

In existing morphology-based classifications, the Order Salpida contains one family, the Salpidae, which comprises two subfamilies, the Cyclosalpinae and Salpinae (Yount, 1954; Godeaux, 1998). The Cyclosalpinae is distinguished from the Salpinae in the solitary generation by the presence in most of so-called luminous organs (which are probably not bioluminescent but have an undetermined function; Godeaux et al., 1998) and the form of the body muscles and intestine, and in the aggregate generation by "luminous organs" and a single ventral attachment organ that connects the zooids to others in various species-specific patterns (Yount, 1954; Van Soest, 1974; Godeaux, 1998). The Cyclosalpinae contains the genera Cyclosalpa and Helicosalpa, while the Salpinae contains about 10 genera (Godeaux, 1998).

There are about 48 described salp species. Many have broad or cosmopolitan distributions (Van Soest, 1998), while some are known from only a few specimens, or only one of the two generations. Whether subtle morphological differences in otherwise identical organisms with overlapping distributions represent environmental variation or species distinctions has been debated. Some of the variation in salps, such as in the number of muscle fibers, appears to be related to latitude (Van Soest, 1972, 1975a) and sympatric speciation seems unlikely (Van Soest, 1975a). However, when examined at the molecular level, many plankton species presumed to have cosmopolitan distributions exhibit significant genetic
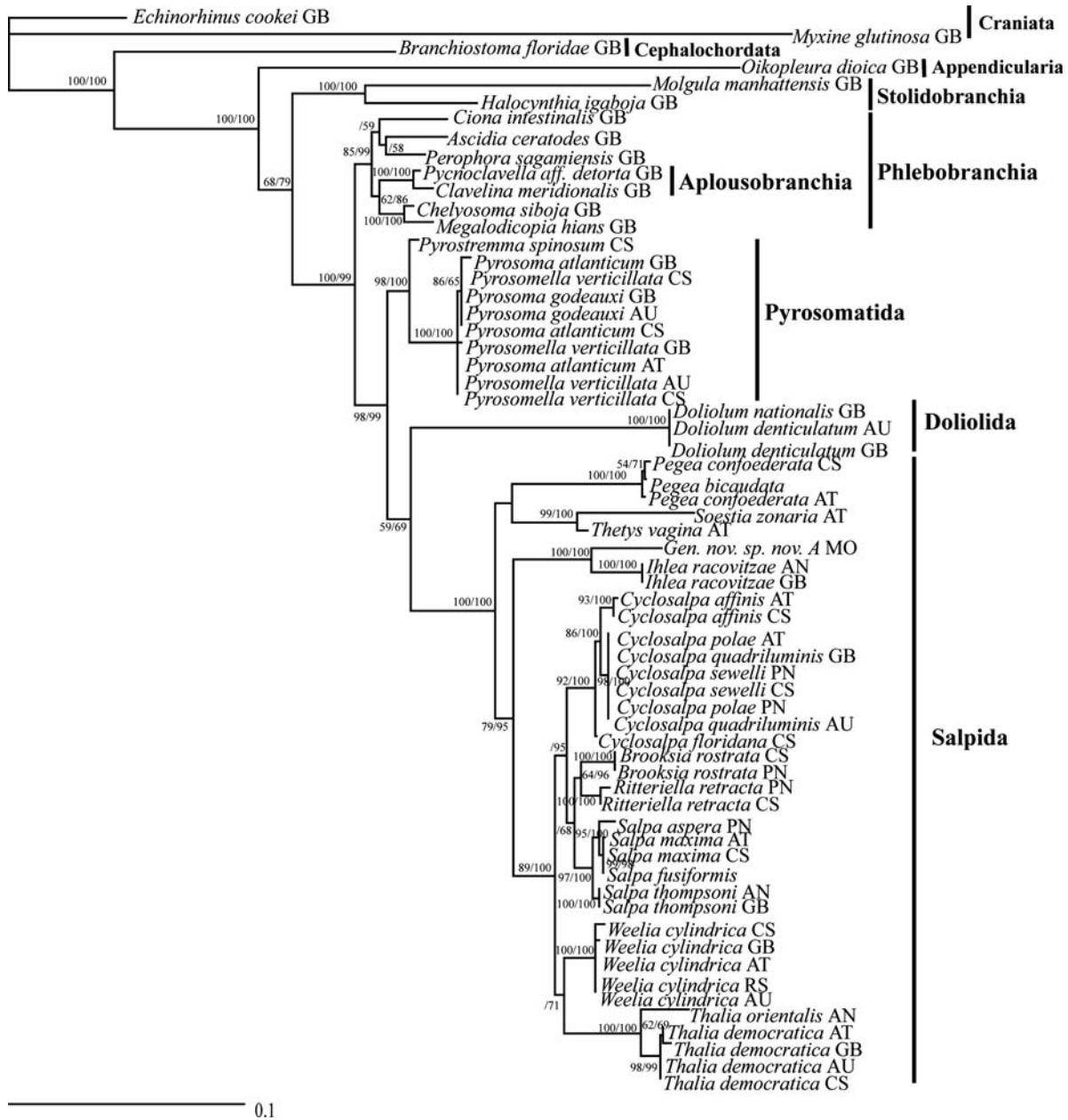
Fig. 1. Maximum likelihood topology. Nodal support is indicated by maximum likelihood bootstrap values (first number) and Bayesian posterior probabilities (second number). Values <50 not shown. Sample source code follows species name: AT, North Atlantic Ocean; CS, Celebes Sea; AN, West Antarctic Peninsula (Southern Ocean); AU, NW Australia (Indian Ocean); MO, Monterey, CA, USA (Pacific Ocean); PN, Panama (Pacific Ocean); RS, Red Sea; GB, GenBank.

structure and may possibly represent cryptic species complexes (Bucklin *et al.*, 2007 for krill; Goetze, 2003 for copepods; Dawson and Jacobs, 2001 and Holland *et al.*, 2004 for Scyphozoa; Govindarajan *et al.*, 2005 for Hydrozoa and Darling and Wade, 2008 for Foraminifera). This study included a number of putatively cosmopolitan species with specimens collected from widely separated locations across their geographic ranges. Our results did

not reveal any significant intraspecific variation of 18S rDNA, suggesting that morphological variation is environmentally induced and that cryptic speciation is unlikely. However, analysis of other genetic markers that are more variable at the species level will be critical for examining population genetic structure.

Metcalf (Metcalf, 1918) hypothesized relationships among salp genera (Fig. 2) based on musculature, gut
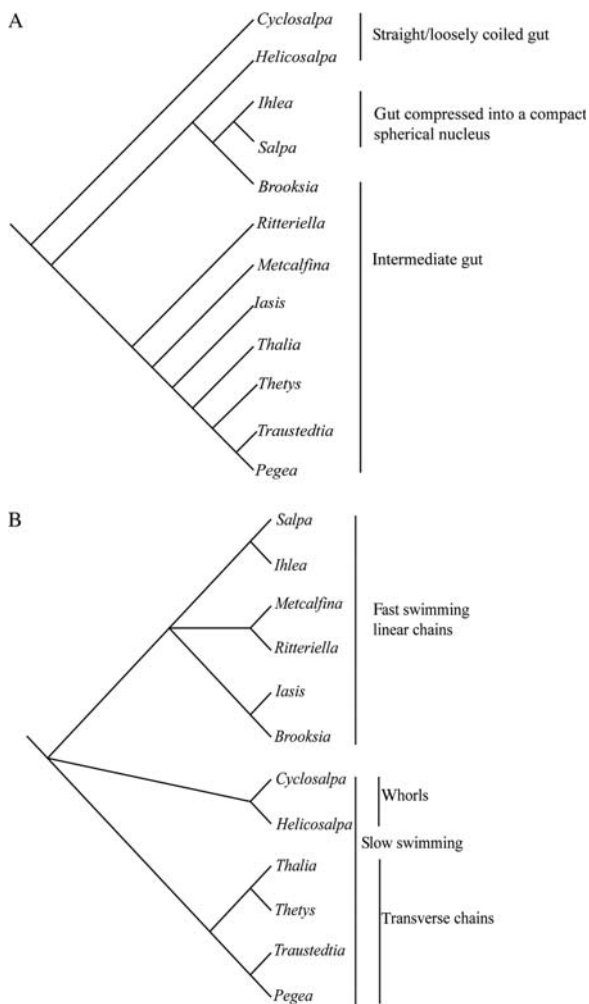
Fig. 2. Salp phylogenies constructed from hypothesized relationships based on (A) gut structure (Metcalf, 1918) and (B) swimming speed and chain structure (Madin, 1974). Nomenclature updated from Metcalf (Metcalf, 1918). Note in Metcalf (Metcalf, 1918) and Madin (Madin, 1974), ancestor-descendant relationships are depicted for genera. Our interpretation depicts genera as descending from common ancestors.

structure and symmetry, and eye characteristics, the characters which most authors have used to distinguish species. He defined three groups based on the degree to which the gut was compressed and compacted. In the Dolichodaea, which he considered closest to the ancestral form, the gut was straight or loosely coiled (*Cyclosalpa* and *Helicosalpa*), in the Sphaerodaea it was compressed into a compact spherical nucleus (*Salpa* and *Ihlea*), while the Circodaea was a catchall group of remaining genera with intermediate gut structures.

Another approach was taken by Madin (Madin, 1974) based partly on symmetry of the aggregate chains and its implications for swimming behavior. In all salp species, the young aggregate salps are formed

by transverse strobilation of the strands of tissue that make up the stolon, and soon form a biserial chain of zooids oriented at right angles to the stolon (or chain) axis. In *Pegea*, the aggregates remain in this orientation, but in most species the zooids shift their orientation in the chain just before it is released from the solitary, forming into whorls or clusters in species of *Cyclosalpa* or shifting to lie more nearly parallel to the chain axis (linear chains), as in *Salpa*. Swimming capability (speed and maneuverability) is weakest in those with whorls or which retain the original transverse orientation, and greatest in species with linear chains. A classification based on these differences considers *Pegea* and *Cyclosalpa* closer to the ancestral salp form and the fast swimming linear species (*Salpa*, *Iasis*) as more derived and specialized for greater locomotory ability, affecting both their feeding efficiency and capacity for diel migration (Madin, 1974, 1990).

Our results clearly demonstrate the usefulness of the 18S gene for illuminating relationships among salp species, but suggest some conclusions that conflict with the morphology-based schemes of Metcalf (Metcalf, 1918) or Madin (Madin, 1974). Two key findings are the placement of the Cyclosalpinae and relationships within the genus *Cyclosalpa*, and relationships within the Salpinae, especially in the genus *Salpa*.

The Cyclosalpinae

Our phylogeny places *Cyclosalpa* nested within the Salpinae in contrast to most previous authors (Metcalf, 1918; Madin, 1974; Van Soest, 1975a) who considered cyclosalps, and particularly *C. pinnata* and similar forms, to reflect the ancestral salp condition based on the symmetry of the solitary body and muscles the simple structure of the gut, and the form of the aggregate whorls. In our phylogeny, the Cyclosalpinae is nested within the Salpinae, as a sister group to *Salpa* [exclusive of *Weelia (Salpa) cylindrica*, see below], whereas *Ritteriella*, *Pegea* and *Thalia*, which are considered to be relatively specialized by Metcalf (Metcalf, 1918) and Van Soest (Van Soest, 1975a), branch off earlier. On the other hand, our phylogeny is consistent with the expectations of Garstang and Platt (Garstang and Platt, 1928) who, on the basis of the marginal bands of the endosytle, concluded that *Cyclosalpa* and *Salpa* were derived and *Pegea*, *Thalia* and *Thetys* were similar to the ancestral condition.

Within the Cyclosalpinae, the genus *Cyclosalpa* is further distinguished by the arrangement of the aggregate individuals in whorls, rather than in chains (Van Soest, 1974). Van Soest (Van Soest, 1974) describes four groupings of *Cyclosalpa* based on morphological similarity: one group containing *C. pinnata*, *C. sewelli*, *C. polae*

and *C. quadriluminis*, a group containing *C. affinis*, a group containing *C. floridana* and a group containing *C. bakeri*, *C. foxtoni*, *C. strongylenteron* and *C. ihlea*. The *C. pinnata* group (represented here by *C. sewelli*, *C. polae* and *C. quadriluminis*) occupies the most nested position in the *Cyclosalpa* clade. Our results for branching order within the *Cyclosalpa* clade agrees with the morphological groupings described by Van Soest (Van Soest, 1974) and his predictions from a morphological cladistic analysis (Van Soest, 1998).

The Salpinae

Our results show that the Salpinae, as currently used, is paraphyletic due to the nested position of *Cyclosalpa*. Of the traditional Salpiniid genera, Van Soest (Van Soest, 1998) suggested that *Salpa*, *Weelia* and *Ritteriella* are closely related, based on their "fusiform" aggregates. He placed *Weelia* as a sister group of *Salpa*, and *Ritteriella* as a sister group of both. Our results show that *Salpa* and *Ritteriella* are sister groups, but *Weelia* (*Salpa*) *cylindrica* is more distantly related.

The taxonomic position of *Weelia (Salpa) cylindrica* has been controversial. It was first described in the genus *Salpa* (Cuvier, 1804), but Yount (Yount, 1954) placed it in the monotypical genus *Weelia*. Solitary generation *Weelia* have either eight or nine body muscles, compared with nine in *Salpa*, and aggregate generation have five, versus six in *Salpa* (Van Soest, 1975b). There are also differences from *Salpa* spp. in the ciliation of the branchial bar. In *Weelia*, the ciliated bands touch one another on the dorsal side of the bar, and diverge toward the ventral side, whereas in *Salpa* they are more nearly separate and parallel. However, this generic distinction has not been universally accepted (Madin, 1974) and the name *Salpa cylindrica* continues to appear in the literature. Our results, supported by specimens obtained from different geographic regions, demonstrate that "*Salpa*" *cylindrica* is indeed a distinct lineage and that placement in the separate genus *Weelia* is warranted.

Our analysis includes one newly discovered but as yet undescribed salp, *Gen nov. sp. nov. A*. This species, which is limited to the meso- and bathypelagic zones, differs in many morphological respects from other species. In Fig. 1, it is most closely allied with *Ihlea racovitzai*, and does share one morphological trait, twin embryos, with species of *Ihlea*.

The phylogeny presented here on the basis of 18S rDNA sequences supports some of the prior ideas based on morphology and contradicts others. The suggested nesting of Cyclosalpinae within the Salpinae is particularly intriguing since these two groups are quite different with respect to both morphology and behavior, and have previously been considered phylogenetically distant. Other close relationships such as between *Thetys* and *Soestia* are also perplexing given the many morphological differences, including very different musculature, between those genera. Our results also supported the early divergence and distinctiveness of *Pyrostremma* from other pyrosomes. A full understanding of the relationships and evolution of the Thaliacea, including doliolids and pyrosomes, will require the use of additional genetic, developmental and morphological characters.

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