

Phylogenetics of Trachylina (Cnidaria: Hydrozoa) with new insights on the evolution of some problematical taxa

ALLEN G. COLLINS¹, BASTIAN BENTLAGE², ALBERTO LINDNER³, DHUGAL LINDSAY⁴,
STEVEN H.D. HADDOCK⁵, GERHARD JARMS⁶, JON L. NORENBURG⁷, THOMAS JANKOWSKI⁸
AND PAULYN CARTWRIGHT²

¹NMFS, National Systematics Laboratory, National Museum of Natural History, MRC-153, Smithsonian Institution, PO Box 37012, Washington, DC 20013-7012, USA, ²Department of Ecology and Evolutionary Biology, University of Kansas, 1200 Sunnyside Avenue, Lawrence, KS 66045, USA, ³Centro de Biologia Marinha—USP—Rodovia Manoel Hipólito do Rego, Km 131, 5—São Sebastião, SP, Brazil, ⁴Japan Agency for Marine-Earth Science and Technology (JAMSTEC), Yokosuka, Japan, ⁵Monterey Bay Aquarium Research Institute, 7700 Sandholdt Road, Moss Landing, CA 95039, USA, ⁶Biozentrum Grindel und Zoologisches Museum, Universität Hamburg, Martin-Luther-King Platz 3, 20146 Hamburg, Germany, ⁷Smithsonian Institution, PO Box 37012, Invertebrate Zoology, NMNH, W-216, MRC163, Washington, DC 20013-7012, USA, ⁸Federal Institute of Aquatic Science and Technology, Dübendorf 8600, Switzerland

Some of the most interesting and enigmatic cnidarians are classified within the hydrozoan subclass Trachylina. Despite being relatively depauperate in species richness, the clade contains four taxa typically accorded ordinal status: Actinulida, Limnomedusae, Narcomedusae and Trachymedusae. We bring molecular data (mitochondrial 16S and nuclear small and large subunit ribosomal genes) to bear on the question of phylogenetic relationships within Trachylina. Surprisingly, we find that a diminutive polyp form, Microhydrula limopsicola (classified within Limnomedusae) is actually a previously unknown life stage of a species of Stauromedusae. Our data confirm that the interstitial form Halammohydra sp. (Actinulida) is derived from holopelagic direct developing ancestors, likely within the trachymedusan family Rhopalonematidae. Trachymedusae is shown to be diphyletic, suggesting that the polyp stage has been lost independently at least two times within trachylina evolution. Narcomedusae is supported as a monophyletic group likely also arising from trachymedusan ancestors. Finally, some data, albeit limited, suggest that some trachylina species names refer to cryptic species that have yet to be sorted taxonomically.

Keywords: phylogenetics, Trachylina, problematic taxa

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INTRODUCTION

Molecular data continue to enhance our understanding of the evolutionary history of life at all hierarchical levels. Nevertheless, many questions about cnidarian phylogenetic relationships remain unanswered (Daly *et al.*, 2007). To address these questions, and thereby provide a robust phylogenetic framework for addressing biological questions involving cnidarians, is the primary aim of the Cnidarian Tree of Life project (<http://cnidarian.info>). To achieve this goal, this large, collaborative effort is compiling molecular data from both nuclear and mitochondrial markers from as many cnidarian species as possible. This paper summarizes progress in understanding the phylogeny of Trachylina and provides greater insight into the evolution of several problematical cnidarians.

Within Hydrozoa (Cnidaria), an ancient phylogenetic divergence appears to have given rise to two primary clades with extant representatives, Trachylina and Hydroidolina

(Collins, 2002; Collins *et al.*, 2006a). In terms of species richness, the vast majority of hydrozoan diversity is contained within Hydroidolina (=Leptolina *sensu* Schuchert, 2007). Trachylina encompasses roughly 150 valid species, whereas Hydroidolina contains more than 3000 (Schuchert, 1998). Nevertheless, because of lack of study and generally simple and plastic morphology, the true species richness of Trachylina remains obscure, and significant levels of cryptic species are possible. Interestingly, the numbers of higher taxa classified within Trachylina and Hydroidolina are comparable. Limnomedusae, Narcomedusae, Trachymedusae and Actinulida (though it has not been sampled for molecular data until this study) are the main taxa, usually ranked as orders, within Trachylina (Figures 1–3). Hydroidolina (see Cartwright *et al.*, this volume) contains three relatively diverse clades, Anthoathecata (athecate hydroids and anthomedusae), Leptothecata (thecate hydroids and leptomedusae) and Siphonophora.

The diversity of life history and morphological features across Trachylina makes the group particularly interesting from an evolutionary perspective. For instance, the trachylina species that perhaps first come to mind are the oceanic,

Corresponding author:

A.G. Collins

Email: CollinsA@SI.edu

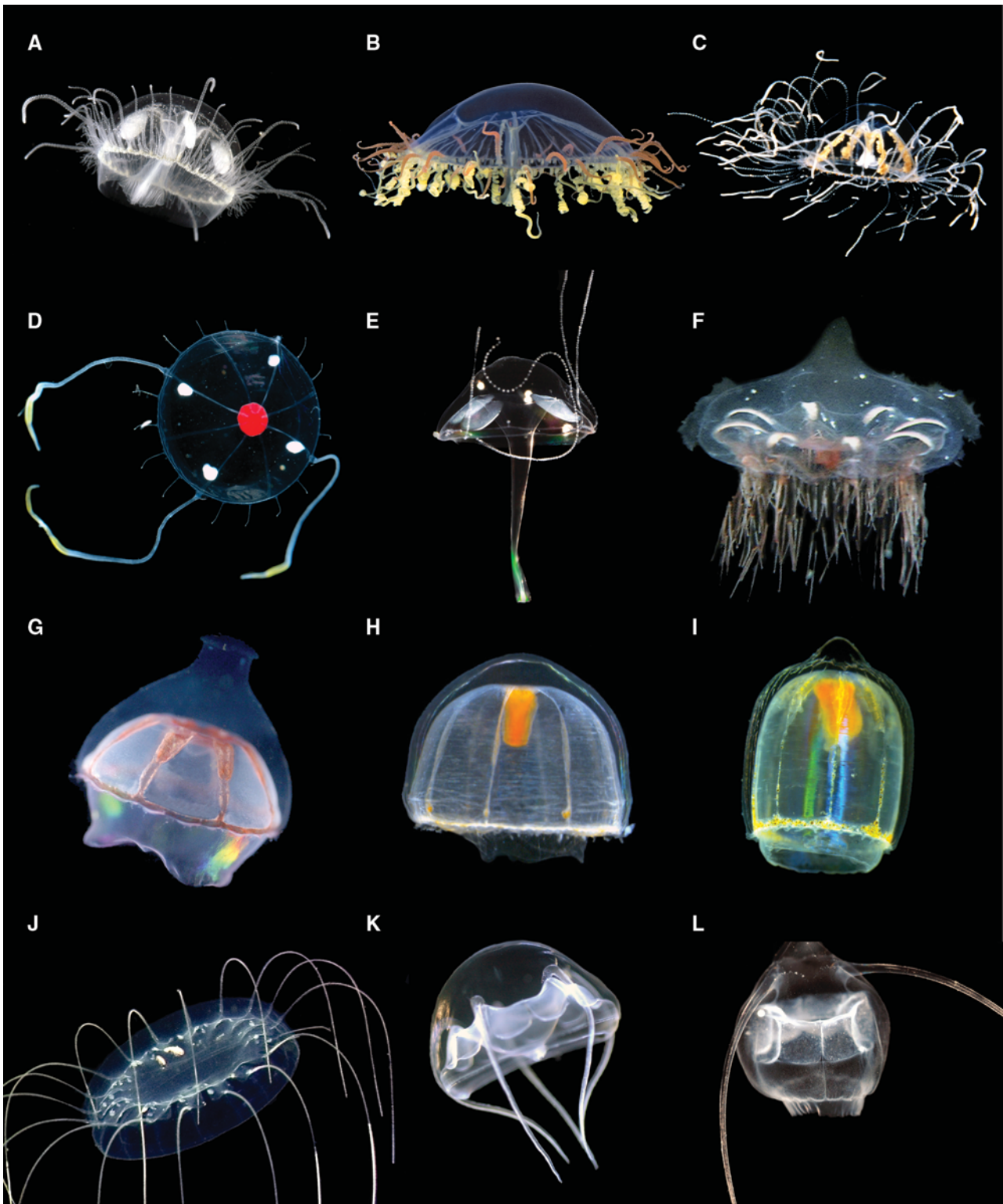


Fig. 1. Images of representative trachylines species. Note that the specimens depicted are not the ones DNA samples were obtained from. (A–C) Limnomedusae; (D–I) Trachymedusae; (J–L) Narcomedusae. (A) *Craspedacusta* sp.; (B) *Olindias sambaquiensis*; (C) *Gonionemus vertens*; (D) *Tetrorchis erythrogaster*; (E) *Liriope tetraphylla*; (F) *Halicreas minimum*; (G) *Botrynema brucei*; (H) *Pantachogon* ‘white-red’; (I) *Pantachogon* ‘orange’; (J) *Solmissus incisa*; (K) *Aegina rosea*; (L) *Solmundella bitentaculata*. A, B and E from A.E. Migotto (Centro de Biologia Marinha, Universidade de São Paulo); C from P. Schuchert (Muséum d’Histoire Naturelle de la Ville de Genève); D and L from S.H.D. Haddock (images captured in California); F–K from D. Lindsay (images captured in Japan).

holopelagic members of Trachymedusae and Narcomedusae, which usually develop directly from planula to adult. Even within these groups there are exceptions, e.g. the benthopelagic trachymedusans of the family Ptychogastridae, narcomedusans

with secondarily derived polypoid, parasitic life stages (Bouillon, 1987; Collins, 2002), and the highly unusual worm-shaped narcomedusan *Tetraplatia* (Hand, 1955; Collins *et al.*, 2006b) that bears four sets of swimming flaps. The only

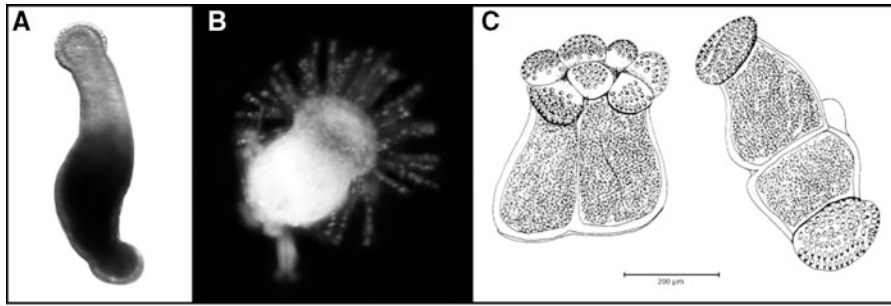


Fig. 2. (A) Polyp of *Craspedacusta sowerbii*; (B) polyp of *Astrohydra japonica*; (C) *Microhydrula limopsicula* modified from Jarms & Tiemann (1996); left: cauliflower shaped polyp; right: polyps closely attached to their bivalve host.

species of Trachylina that possess a true polyp stage are those classified within Limnomedusae. Limnopolyps are generally tiny (less than 1 mm in length) and simple, laterally bud medusae like those of the hydroidolitan groups, and usually produce frustrules, creeping asexual larvae that disperse and metamorphose into additional polyps. Because the medusa stages of limnomedusans are very similar in morphology to those of Trachymedusae, the presence of a polyp stage is critical to differentiating the two taxa. Limnomedusae is also of note because several species live in fresh water, a relatively unusual habit for cnidarians (Jankowski *et al.*, 2008).

Perhaps the most unusual trachylina species are those of Actinulida. These minute tentacled, ciliated and solitary forms spend their lives creeping between sand grains. Actinulidans, which develop directly from planulae to adults, were hypothesized to be living remnants of interstitial ancestral hydrozoans (Swedmark & Tessier, 1966). In contrast, however, their possession of ecto-endodermal statocysts, and sometimes brood chambers have led most researchers in recent decades to conclude that they are more likely derived medusae that have been highly modified for their interstitial existence (e.g. Werner, 1965; Salvini-Plawen, 1987; Bouillon & Boero, 2000; Marques & Collins, 2004). However, their simple and derived morphology has prevented any definitive conclusions about their precise phylogenetic position within Trachylina, hindering our understanding of how these interesting species evolved.

A key step in understanding the evolution of trachylina diversity is the development of a robust hypothesis of phylogenetic relationships within and between the various trachylina taxa. By sampling numerous new species (including

a member of Actinulida), for sequences of mitochondrial and nuclear markers, this study continues the effort to unravel the phylogeny of Trachylina, thereby providing insight into the evolutionary patterns underlying the diversity of trachylina life cycles and life history strategies.

MATERIALS AND METHODS

Sampling

Specimens included in this study have come from a diversity of sources (Table 1). Tissue samples were preserved in pure ethanol or frozen in liquid nitrogen and stored at -80°C . DNA was extracted using either commercially available extraction kits (DNAzol, Molecular Research Center, Inc.; Invisorb Spin Tissue kit, Invitex) or phenol/chloroform. For the latter, a small amount of tissue was incubated at 55°C for several hours or overnight in 500 μl lysis buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA), 25 μl SDS (20%) and 12.5 μl of Proteinase-K (20 mg/ml). An equal volume of equilibrated phenol/chloroform (1 : 1) was then added to the homogenate. The reaction tube was subsequently inverted several times to allow mixing of the solution followed by 5 min of centrifugation at maximum speed. The upper, aqueous layer was then transferred into a new reaction tube. Optionally, 200 μl of TE (10 mM Tris-HCl [pH 7.4], 1 mM EDTA [pH 8]) were added to the leftover layer of phenol/chloroform also followed by mixing and centrifugation in order to increase the overall yield of the extraction ('back-extraction' of the residual aqueous phase). An additional washing step with chloroform was then performed by adding solely chloroform to the recovered aqueous phases followed by mixing and centrifugation for five minutes. The aqueous phases of initial and 'back-extraction' were pooled and the dissolved DNA was precipitated with ethanol (e.g. Mühlhardt, 2003 for a protocol), and the resulting pellet was eluted in 15–30 μl of TE.

Genes coding for the large subunit of the mitochondrial ribosome (16S) and the nuclear genes coding for both the small (18S or SSU) and large (28S or LSU) ribosomal subunits were amplified and sequenced as described below. All PCR reactions contained 50 vol% *Taq* PCR Master Mix (Qiagen, Valencia, California) and 0.5 μM of each respective primer. A region of mitochondrial 16S (approximately 640 bp) was amplified and sequenced with the primers F2 and R2 (Cunningham & Buss, 1993), using a modified PCR profile ($94^{\circ}\text{C}/5$ min; ($94^{\circ}\text{C}/50$ s, $45^{\circ}\text{C}/50$ s, $72^{\circ}\text{C}/60$ s) \times 5; ($94^{\circ}\text{C}/50$ s, $50^{\circ}\text{C}/50$ s, $72^{\circ}\text{C}/60$ s) \times 30; $72^{\circ}\text{C}/5$ min). Near complete



Fig. 3. Still image taken from a video of *Halammohydra* sp. by J. Norenburg.

Table 1. Samples with GenBank accession and voucher numbers when available; *, derived for this study.

Taxonomic hierarchy and species	16S	18S	28S	Voucher
Stauromedusae				
Cleistocarpida	<i>Craterolophus convolvulus</i>	AY845344	AY920781	USNM 1073330
Eleutherocarpida	<i>Haliclystus octoradiatus</i>	AY845346	AH014894	
Scyphozoa				
Coronatae	<i>Atolla vanhoeffeni</i>	AF100942	AY026368	
Sematostomeae	<i>Chrysaora melanaster</i>	AF358099	AY920780	
Cubozoa				
Carydeida	<i>Carybdea rastonii</i>	AF358108	AY920787	
Chirodrpida	<i>Chironex fleckeri</i>	AF358104	AY920785	
Hydrozoa				
Hydroidolina				
Candelabridae	<i>Candelabrum cocksii</i>	AY512520	AY920758	AY920796
Proboscidactylidae	<i>Fabienna sphaerica</i>	AM183133.1	AY920767	AY920797
Moerisiidae	<i>Moerisia</i> sp.	AY512534	AF358083	AY920801
Porpitidae	<i>Porpita</i> sp.	AY512529	AF358086	AY920803
Polyorchidae	<i>Scrippisia pacifica</i>	AY512551	AF358091	AY920804
Laodiceidae	<i>Melicertissa</i> sp.	AY512515	AF358075	AY920798
Prayidae	<i>Nectadamas diomedea</i>	AY512512	AF358068	AY026377
Trachylina				
Actinulida				
Halammohydridae	<i>Halammohydra</i> sp.	EU293991*	EU301622*	EU301623*
Limnomedusae				
Olindiasidae	<i>Aglauroopsis aeora</i>	EU293973*	AY920754	AY920793
Olindiasidae	<i>Astrohydra japonica</i>	EU293975*		AY920794
Olindiasidae	<i>Craspedacusta sowerbii</i>	EU293971*	AF358057	USNM 1105483
Olindiasidae	<i>Craspedacusta sinensis</i>	AY512507	EU247815*	
Olindiasidae	<i>Craspedacusta ziguensis</i>	EU293974		USNM 1102057
Olindiasidae	<i>Gonionemus vertens</i>	EU293976*		
Olindiasidae	<i>Limnocnida tanganyicae</i>	EU293972*	AY920755	AY920795
Olindiasidae	<i>Maeotias marginata</i>	AY512508	AF358056	EU247810*
Olindiasidae	<i>Olindias phosphorica</i>	EU293978*	AY920753	EU247808*
Olindiasidae	<i>Olindias sambaquiensis</i>	EU293977*	EU247814*	EU247809*
Microhydrulidae	<i>Microhydrula limopisicola</i>	EU294003*	EU247811*	G. Jarms culture
Monobrachiidae	<i>Monobrachium parasiticum</i>	EU292970*	AY920752	USNM 1074993
Narcomedusae				
Aeginidae	<i>Aegina citrea</i>	EU293997*	AF358058	AY920789
Aeginidae	<i>Aegina rosea</i>		EU247813*	
Aeginidae	<i>Solmundella bitentaculata</i>	EU293998*		USNM 1107456
Aeginidae	<i>Solmundella bitentaculata</i>		EU247812*	EU247795*
Cuninidae	<i>Cunina frugifera</i>		AF358059	
Cuninidae	<i>Sigiweddellia</i> sp.	EU293996*		EU247796*
Cuninidae	<i>Solmissus incisa</i>	EU294002*		USNM 1111080
Cuninidae	<i>Solmissus marshalli</i>	EU294001*	AF358060	AY920790
Tetraplatiidae	<i>Tetraplatia volitans</i>	EU293999*	DQ002501	DQ002502
Tetraplatiidae	<i>Tetraplatia</i> sp.	EU294000*		KUMIP 314322
Trachymedusae				
Geryoniidae	<i>Geryonia proboscidalis</i>	EU293979*	EU247816*	EU247807*
Geryoniidae	<i>Liriope tetrphylla</i>	AY512510	AF358061	
Geryoniidae	<i>Liriope tetrphylla</i>	EU293980*	AY920756	USNM 1107457
Halicreatidae	<i>Botrynema brucei</i>	EU293982*	EU247822*	EU247798*
Halicreatidae	<i>Haliscera conica</i>	EU293981*	EU247825*	EU247797*
Halicreatidae	<i>Halicreas minimum</i>	EU293983*	EU247826*	
Rhopalonematidae	<i>Aglantha digitale</i>	EU293985*	EU247821*	AY920791
Rhopalonematidae	<i>Aglaura hemistoma</i>		EU247818*	EU247803*
Rhopalonematidae	<i>Aglaura hemistoma</i>	EU293984*	EU247820*	EU247802*
Rhopalonematidae	<i>Amphogona apicata</i>	EU293994*		EU247801*
Rhopalonematidae	<i>Crossota rufobrunnea</i>	EU293986*	EU247824*	EU247800*
Rhopalonematidae	<i>Crossota rufobrunnea</i>	EU293987*	EU247823*	EU247799*
Rhopalonematidae	<i>Pantachogon haeckeli</i>		AF358062	AY920792
Rhopalonematidae	<i>Pantachogon haeckeli</i>	EU293988*		USNM 1111078
Rhopalonematidae	<i>Pantachogon 'white-red'</i>	EU293989*	EU247817*	EU247805*
Rhopalonematidae	<i>Pantachogon 'orange'</i>	EU293990*		EU247806*
Rhopalonematidae	<i>Rhopalonema velatum</i>	EU293992*	EU247819*	EU247804*
Rhopalonematidae	<i>Rhopalonema</i> cf. <i>velatum</i>	EU293993*		USNM 1107461
Rhopalonematidae	<i>Tetrorchis erythrogaster</i>	EU293995*		USNM 1111077

USNM, National Museum for Natural History, Smithsonian Institution; KUMIP, University of Kansas Natural History Museum, Division of Invertebrate Paleontology; MHNG INVE, Muséum d'Histoire Naturelle de la Ville de Genève.

SSU (approximately 1800 bp) was amplified and sequenced according to Medlin *et al.* (1988). The original PCR profile was modified to 94°C/4 min; (94°C/20 s, 57°C/20 s, 72°C/1 min 45 s) × 35; 72°C/7 min. Nearly complete LSU (approximately 3200 bp) was amplified in two parts using the primers F63mod (Medina *et al.*, 2001) and R2084 (5'-AGA GCC AAT CCT TTT CC-3') in one PCR reaction and the primers F1383 (5'-GGA CGG TGG CCA TGG AAG T-3') and R3238 (5'-SWA CAG ATG GTA GCT TCG-3') in a second reaction. The temperature profile of each PCR was the following: 94°C/5 min; (94°C/30 s, 45°C/60 s, 72°C/3 min) × 30; 72°C/10 min (modified from Medina *et al.*, 2001). The LSU sequences were obtained with the previously published primers F63sq, F635, R635, R1411sq, R1630, F2076sq, F2766 (all from Medina *et al.*, 2001), R2800 (Voigt *et al.*, 2004) and the newly developed primers F1383, F1689 (5'-CTA AGM SRY AGG GAA AYT C-3'), R2084 and R3238. Purification of PCR products and sequencing were carried out at the laboratories of Cogenics (Houston, Texas). Sequences and collection data for the source specimens have been deposited to GenBank (see Table 1).

Datasets

In order to investigate different phylogenetic questions, assess levels of correspondence among the different phylogenetic markers, and derive the most robust phylogenetic hypothesis of Trachylina possible, we created five datasets. First, partial mitochondrial 16S, nuclear SSU, and nuclear LSU data for all taxa (Table 1) were aligned using MUSCLE (multiple

sequence alignment with high accuracy and high throughput) (Edgar, 2004). No adjustments were made by eye. 'Conserved' positions for each of the alignments were identified using Gblocks vo.91 (Castresana, 2000) with default parameters, except that half the taxa were allowed to be gaps for any given position. Positions deemed non-conserved by Gblocks were excluded from phylogenetic analysis.

The first dataset analysed was constructed in order to conduct initial investigations of the phylogenetic placement of the actinulidan *Halammohydra* sp., which had been hypothesized by some authors to represent an anciently diverging lineage of Hydrozoa (Swedmark & Tessier, 1966). Therefore, for this dataset, we combined both SSU and LSU data (for the regions over which we were able to derive data from *Halammohydra*, 1298 and 1032 nucleotides, respectively) from a diverse set of trachylines, seven representatives of Hydroidolina, and six non-hydrozoan representatives of Medusozoa, used as outgroups (see Figure 4). Non-hydrozoans were not included in any of the other datasets, which were constructed to conduct more detailed analyses of Trachylina phylogeny and to investigate the degree of correspondence between the different markers. The second through fourth datasets consisted of all available trachylina sequences for mitochondrial 16S, SSU, and LSU markers (Table 1), along with seven species representing the breadth of hydroidolinan (non-trachylina hydrozoan) diversity for outgroups (Figures 5–7).

The fifth and final dataset was used to derive the most robust phylogenetic hypothesis possible for Trachylina. This dataset combined data from all three markers for which at least SSU

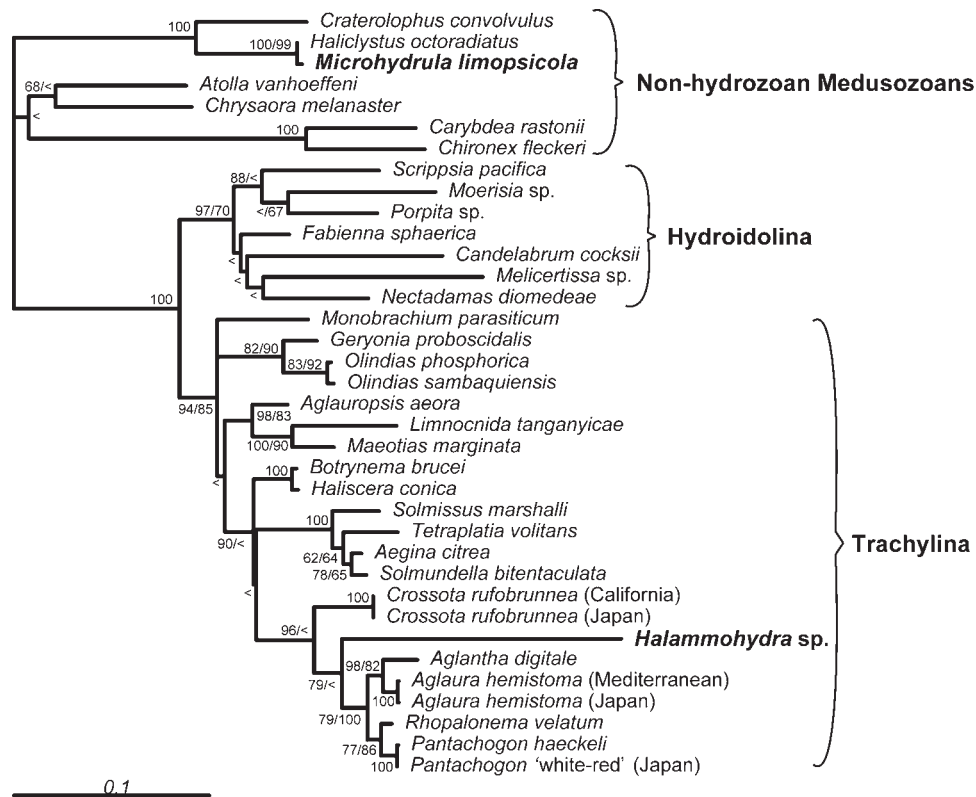


Fig. 4. Phylogenetic hypothesis with non-hydrozoan medusozoan outgroups to investigate the position of *Halammohydra* sp. within Hydrozoa. Note also the position of *Microhydrula limopsisicola*. Phylogram of ML topology based on combined SSU and LSU data (dataset 1) with bootstrap indices under both ML and MP at each node. '<' indicates a bootstrap index of less than 61; a single 100 or < was placed at nodes if indices under both criteria were 100 or less than 61, respectively. For ML analyses, the assumed model of nucleotide evolution was GTR + I + G. Scale bar is equivalent to 0.1 substitutions per site.

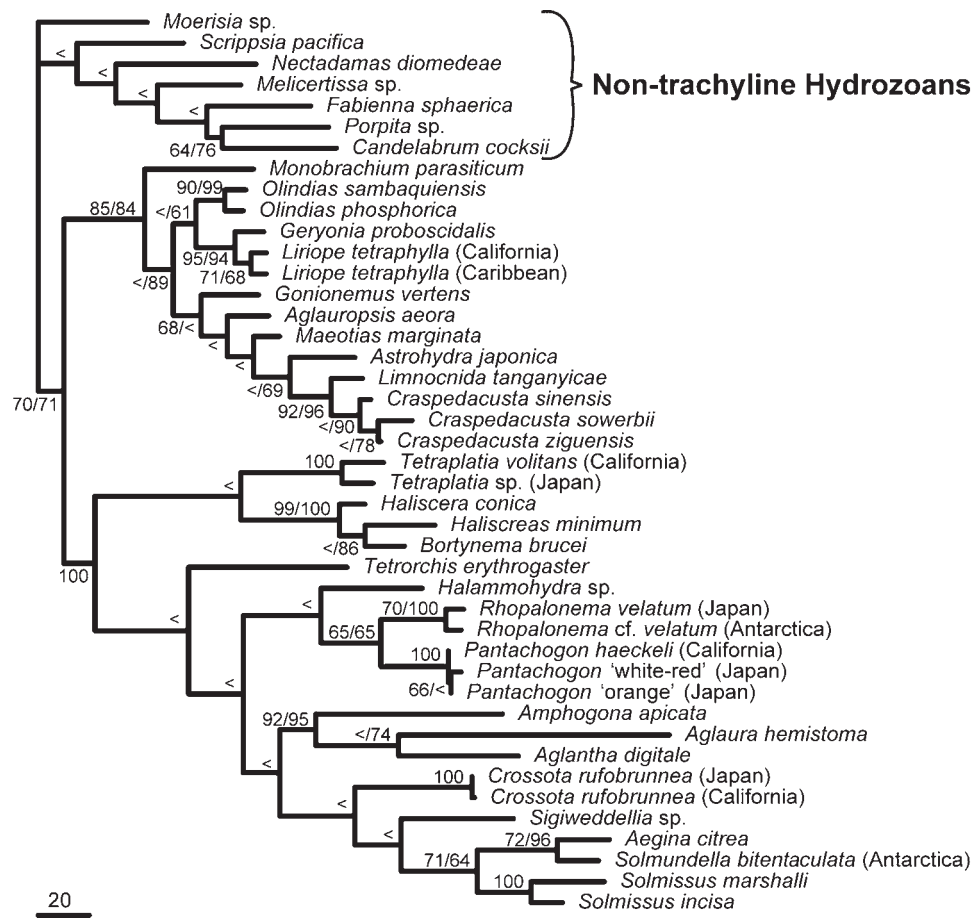


Fig. 5. Phylogenetic hypothesis based on mitochondrial 16S data (dataset 2). Phylogram of one of the 20 most parsimonious trees, with bootstrap indices under both ML and MP at each node. ‘<’ indicates a bootstrap index of less than 61; a single 100 or < was placed at nodes if indices under both criteria were 100 or less than 61, respectively. For ML analyses, the assumed model of nucleotide evolution was K81uf + I + G. Scale bar is equivalent to 20 substitutions.

or LSU had successfully been derived (Table 1; Figure 8), as well as the seven outgroup hydroidolins for which all three markers were available. For this dataset, two chimerical sequences were constructed. Mitochondrial 16S from *Solmundella bitentaculata* from Antarctica was combined with nuclear SSU and LSU of *S. bitentaculata* from the Mediterranean Sea. Mitochondrial 16S from *Pantachogon haeckeli* from the central coast of California was combined with un-voucher Genbank SSU and LSU sequences derived from *P. haeckeli* from Californian coastal waters.

Phylogenetic analyses

Phylogenetic hypotheses were constructed under both maximum likelihood (ML) and maximum parsimony (MP) optimality criteria. Modeltest (Posada & Crandall, 1998) was used, employing the Akaike information criterion, to choose the most appropriate model for each of the datasets. Assuming these models, PAUP* (Swofford, 2002) was used to search (with 100 random addition replicates) for the topology with the greatest likelihood and GARLI v0.951 (Zwickl, 2006) was used to calculate ML bootstrap indices from searches for 500 replicate datasets. PAUP* was further employed to find most parsimonious topologies, with 100 random replicate searches and to calculate bootstrap indices (500 replicates, with 10 searches per replicate).

RESULTS

In general, ML and MP topologies are congruent, only differing where bootstrap values are low under one or both criteria. Topologies, either ML or MP, for each of the five datasets are presented as Figures 4–8. In addition, both ML and MP bootstrap indices are reported on these figures at each of the relevant nodes. Two main results are drawn from Figure 4 showing the ML topology for the combined SSU and LSU analysis including non-hydrozoan taxa as outgroups. First, the representative of Actinulida, *Halammohydra* sp., does not appear to be an early diverging lineage of Hydrozoa, but is instead revealed as a member of Trachylina. Second, *Microhydrula limopsisicola* (classified in the family Microhydrulidae within Limnomedusae) is shown to not be a member of Hydrozoa at all, but instead to have a very close relationship to the stauromedusan *Haliclystus octoradiatus*.

Figure 5 presents one of 20 MP trees based on our 16S data. Generally, nodes in the topology do not receive high support. There is a well-supported split between two clades, one containing species of Limnomedusae plus species of the trachymedusan family Geryoniidae and the other comprising all the remaining trachymedusans, narcomedusans and the actinulidan *Halammohydra* sp. Other phylogenetic associations that receive method-independent (i.e. both MP and ML) support include: the limnomedusan genera *Limnocrnida* and

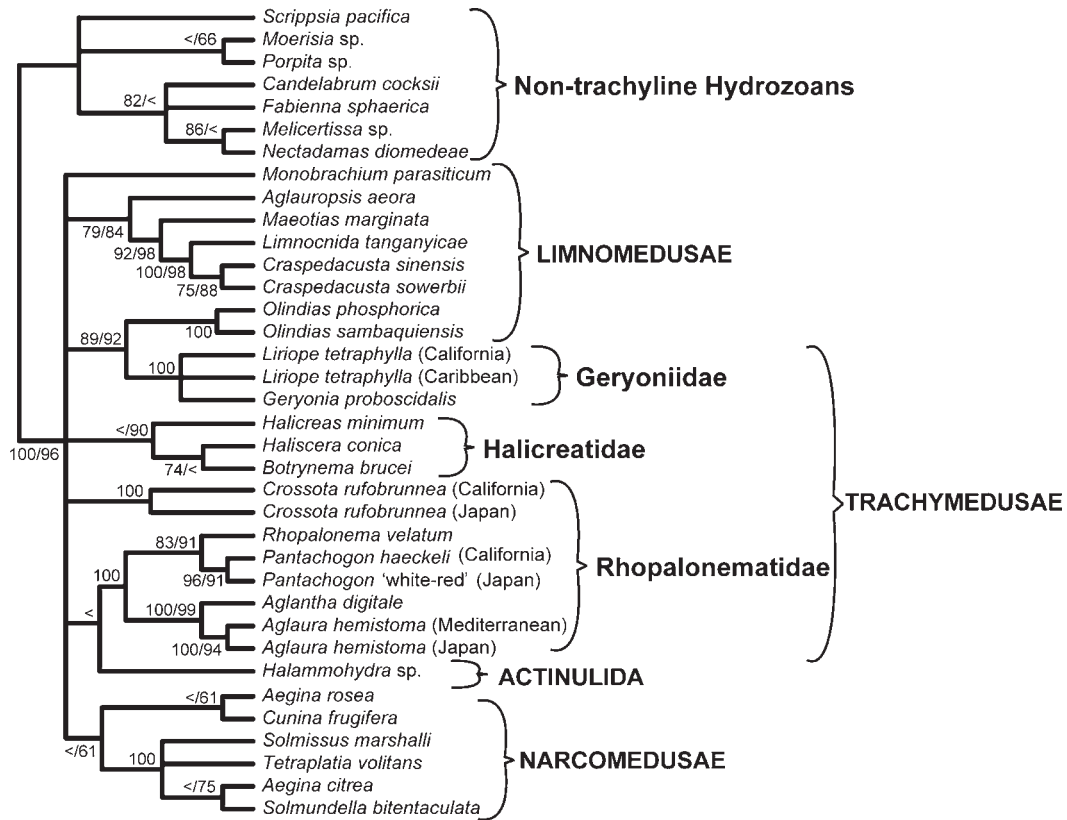


Fig. 6. Phylogenetic hypothesis based on SSU data (dataset 3). Strict consensus of the 21 most parsimonious trees, with bootstrap indices under both ML and MP at each node. ‘<’ indicates a bootstrap index of less than 61; a single 100 or < was placed at nodes if indices under both criteria were 100 or less than 61, respectively. For ML analyses, the assumed model of nucleotide evolution was GTR + I + G.

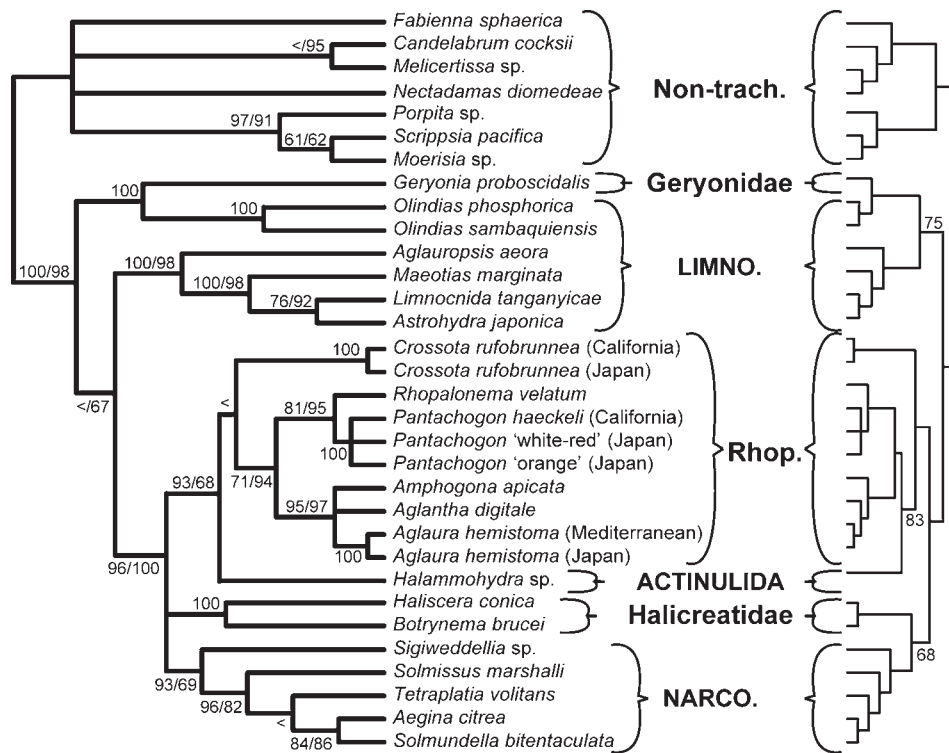


Fig. 7. Phylogenetic hypotheses based on LSU data (dataset 4). Left: strict consensus of the 12 most parsimonious trees with bootstrap indices under both ML and MP at each node. ‘<’ indicates a bootstrap index of less than 61; a single 100 or < was placed at nodes if indices under both criteria were 100 or less than 61, respectively. For ML analyses, the assumed model of nucleotide evolution was GTR + I + G. Right: ML topology with ML bootstrap indices for three nodes receiving support under ML but not under MP; terminal taxa in the same order as shown on the left.

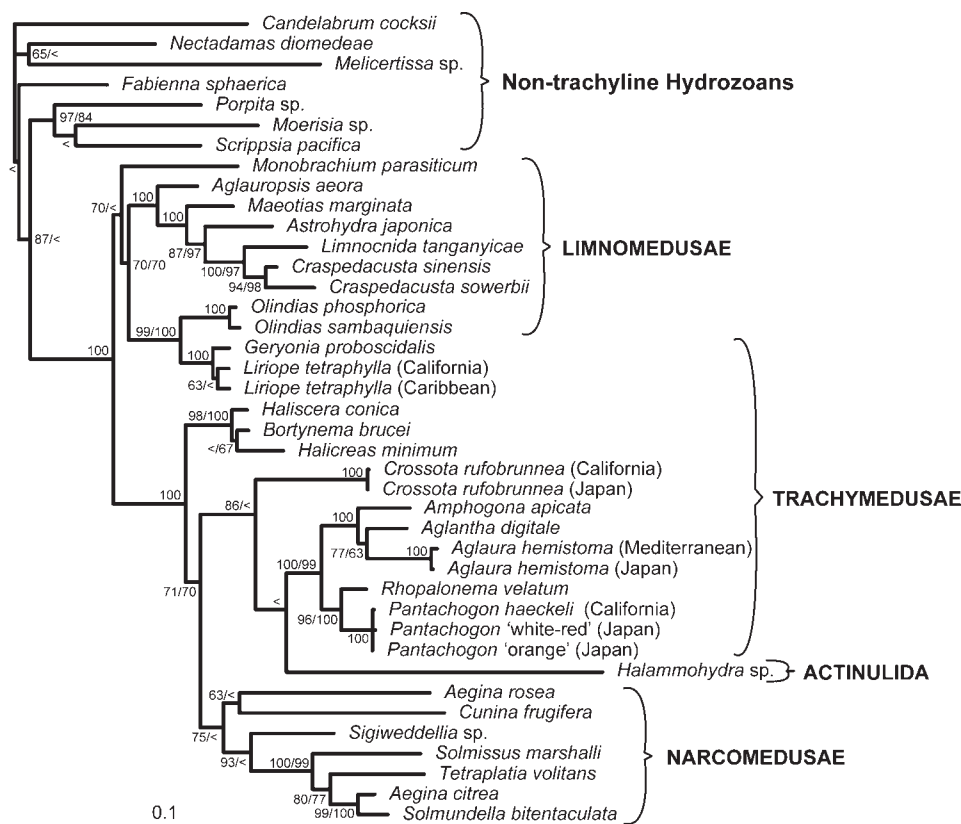


Fig. 8. Phylogenetic hypothesis based on combined 16S, SSU, and LSU data (dataset 5). Phylogram of ML topology, with bootstrap indices under both ML and MP at each node. '<' indicates a bootstrap index of less than 61; a single 100 or < was placed at nodes if indices under both criteria were 100 or less than 61, respectively. For ML analyses, the assumed model of nucleotide evolution was GTR + I + G. Scale bar is equivalent to 0.1 substitutions per site.

Craspedacusta; the trachymedusan genera *Geryonia* and *Liriope* (Geryoniidae); the trachymedusan genera *Haliscera*, *Halicreas* and *Botrynuma* (Halicreatidae); the trachymedusan genera *Rhopalonema* and *Pantachogon*; the trachymedusan genera *Amphogona*, *Aglaura* and *Aglantha*; and, the narcomedusan genera *Aegina*, *Solmundella* and *Solmissus*.

Figure 6 shows the strict consensus of 21 MP trees found in searches using the SSU dataset. With three exceptions, all of the relationships strongly supported by the mitochondrial 16S analyses (Figure 5) receive method independent support from the SSU data. The first exception is that there is no support for the splitting of Trachylina into two main clades. The second exception is that the ML bootstrap support for the node linking *Haliscera*, *Halicreas* and *Botrynuma* (Halicreatidae) is only 54. The third exception is that the clade linking *Aegina citrea*, *Solmundella* and *Solmissus* also includes *Tetraplatia volitans*, which had an uncertain position based on the 16S data. Additional clades receiving support, as measured by both MP and ML bootstraps are: the limnomedusan taxa *Aglauroopsis* (marine) and *Maeotias* (brackish) with the freshwater *Craspedacusta* and *Limnocyda*; the limnomedusan genus *Olindias* with Geryoniidae; and, the two trachymedusan clades (*Rhopalonema* + *Pantachogon* and *Amphogona* + *Aglaura* + *Aglantha*).

The strict consensus of 12 MP trees based on the LSU data supports many of the relationships also indicated by the 16S and SSU analyses (Figure 7). One of the two main trachyline clades (non-geryoniid trachymedusans, narcomedusans and the actinulidan *Halammohydra* sp.) suggested

by 16S is also supported by LSU data. Two additional clades that receive high bootstrap support from LSU data under both MP and ML criteria are: all sampled representatives of Rhopalonematidae plus *Halammohydra* sp.; and, all sampled representatives of Narcomedusae.

Finally, Figure 8 shows the ML topology using the combined dataset. Given the great amount of correspondence between the different single-marker analyses, it is not surprising that this combined analysis contains nearly all of the associations enumerated above. In total comparing across all the single-marker analyses, there is only one instance of relationships receiving strong contradictory support. The SSU, LSU and combined data support an alliance of *Aegina citrea*, *Solmundella*, *Solmissus* and *Tetraplatia volitans*, whereas the 16S analyses suggested that the first three taxa form a well-supported clade exclusive of *Tetraplatia*.

DISCUSSION

Scope of Limnomedusae

Limnomedusae has a somewhat complicated taxonomic history that reflects uncertainty about the phylogenetic affinities of the groups that constitute it, as well as confusion arising from the historical use of separate taxonomic systems by experts on hydromedusae and hydrozoans. The taxon was created in the late 1930s (Kramp, 1938; Browne & Kramp, 1939) for species

of the families Moerisiidae, Olindiasidae (=Olindiidae) and Proboscidiactylidae. The underlying concept of Limnomedusae was that it should receive species with a biphasic life cycle, whose medusae did not fit the Anthoathecata (=Anthomedusae or Athecata) because they had either ecto-endodermal statocysts or gonads along their radial canals, and whose polyps did not fit the Leptothecata (=Leptomedusae or Thecata) because they lacked a theca. Subsequently, Monobrachiidae, which also meets the criteria above, has been added to Limnomedusae (Naumov, 1960), as have the small and simple species of Armorhadridae and Microhydrulidae, for lack of better alternatives (Bouillon, 1985). More detailed analysis that included information on the cnidome indicated that Moerisiidae is more likely to share a recent common ancestry with members of the anthoathecate group Capitata (Rees, 1958; Petersen, 1990). Likewise, the absence of statocysts and the presence of desmonemes strongly favour the hypothesis that Proboscidiactylidae is closely related to anthoathecate species classified as Filifera (Edwards, 1973; Schuchert, 1996). Recently, molecular data have confirmed that Moerisiidae and Proboscidiactylidae do not belong in Limnomedusae (Collins *et al.*, 2006a), thereby limiting its scope to Armorhadridae, Microhydrulidae, Monobrachiidae and Olindiasidae. To a certain extent, our results (Figures 4–8) challenge this view of Limnomedusae, as further discussed below.

***Microhydrula limopsicola* is a species of Stauromedusae**

Microhydrula limopsicola (originally classified within Limnomedusae) is an Antarctic species that possesses minute polyps found living on bivalve shells (Jarms & Tiemann, 1996; Figure 2C). The polyps are less than 0.5 mm in length and 0.35 mm in diameter and solitary, though they do form aggregates. The morphology of the polyps is quite simple as they lack tentacles and possess just a single type of nematocyst, microbasic euryteles. These polyps were observed to rest on a thin periderm and produce creeping, asexual frustules (Jarms & Tiemann, 1996). The polyps of Limnomedusae (where known) are tiny, often without tentacles (e.g. Figure 2A), capable of producing frustules, and typically armoured with microbasic euryteles. This suite of features therefore suggests that *M. limopsicola* should be part of Limnomedusae. Thus, it is quite surprising that genetic data taken from samples of *M. limopsicola* strongly indicate that it is actually part of the non-hydrozoan Stauromedusae (Figure 4).

Concern that this result was a product of contamination prompted a re-sampling of a culture of *Microhydrula limopsicola* maintained in the Jarms laboratory. This yielded the same data, indicating that *M. limopsicola* is a member of Stauromedusae. In fact, in an ongoing study of Stauromedusae, mt16S data (Collins *et al.*, unpublished data) identify *M. limopsicola* as a member of the genus *Haliclystus*, with a very close relationship to the species of *Haliclystus* from southern Chile reported on by Zagal (2004).

The most straightforward way to reconcile the interpretation of *M. limopsicola* as described by Jarms & Tiemann (1996) is that it likely represents a microscopic life cycle stage not previously known in Stauromedusae. This would

explain why no gonads were observed by Jarms & Tiemann (1996). Life cycles of stauromedusans (and indeed most cnidarians) are not well known or documented. In particular, the juvenile stages of very few stauromedusan species are known. The only case for which the actual habitat of juvenile stauropolyps is known is for two species of *Stylocoronella*, whose polyps live interstitially (Salvini-Plawen, 1987; Kikinger & Salvini-Plawen, 1995). Euryteles are widely distributed across Medusozoa, including Stauromedusae, so this character is not difficult to reconcile. However, this would appear to be the first time that a periderm and frustules have been recognized in any species of Stauromedusae. Both features are known from species within Discomedusae (Scyphozoa) and Limnomedusae (Hydrozoa) suggesting that they have evolved independently at least two times.

It is unclear if this result could be extended to other species of Microhydrulidae, the limnomedusan family to which *M. limopsicola* was assigned. The family contains just three species in two genera, and no adults or sexual reproduction have been reported from any of them. *Microhydrula pontica* is very similar in morphology to *M. limopsicola* and it may be that this species too, known from the northern Atlantic, represents another species of Stauromedusae. *Rhaptapagis cantacuzeni*, the third representative of the family, is differentiated from the other species by the possession of an unusual form of microbasic euryteles known as semiophores (Bouillon & Deroux, 1967). DNA sampling of these taxa should help to resolve phylogenetic affinities of these species.

Freshwater limnomedusans

We present some new mt16S data for freshwater limnomedusans. These data further reinforce (albeit with low support) the hypothesis that freshwater limnomedusans had a single evolutionary origin (Figure 5, but note the robust support in Figure 8). Among our 16S samples is a representative of *Craspedacusta ziguensis*. As reviewed by Jankowski (2001), the validity of this species has been in doubt. However, our mt16S data, which include representatives of two other readily accepted species of *Craspedacusta*, *C. sowerbii* and *C. sinensis*, indicate that *C. ziguensis* has comparable divergences from these two species (*P* distances of 7.4% and 6.1%, respectively) as they do from each other (8.8%). The SSU and 16S data show *Craspedacusta* to have a close relationship to the freshwater genus *Limnocrnida*. The polyps of the two genera are nearly indistinguishable: they are diminutive (1 mm or less in length), form small colonies up to seven individuals, lack tentacles, possess papillae with eurytele nematocysts at their oral ends, and produce creeping frustules that develop into additional polyps (Bouillon, 1957). The other freshwater species sampled here, *Astrohydra japonica*, also produces frustules, but the polyps are smaller (up to 0.3 mm in length), solitary, and have 10 to 20 nematocyst-rich tentacles composed of single cells that may be homologous with the papillae of *Craspedacusta* and *Limnocrnida* polyps (Hashimoto, 1981). The polyps of *Astrohydra japonica* and those of another freshwater genus, *Calpasoma* (not yet sampled for molecular data), are indistinguishable, and if it were not for the presence of a poorly known medusa stage in the former (Hashimoto, 1985), the two would have to be considered conspecific.

Geryoniidae (Trachymedusae) is derived from within Limnomedusae

The medusae of Trachymedusae and Limnomedusae are quite similar, with gonads typically on the radial canals and statocysts of ecto-endodermal origin. The two differences between the groups are the presence or absence of a polyp stage and hollow or solid marginal tentacles in Limnomedusae and Trachymedusae, respectively (Schuchert, 2007). As a practical matter, the two groups can often be separated based on the number of radial canals, with most species of Limnomedusae possessing four (rarely six) and most species of Trachymedusae having eight (but some with four or six). We have found strong evidence that Geryoniidae is the sister group to species of the limnomedusan genus *Olindias* (Figures 4–8), whereas our analyses weakly favour the hypothesis that Limnomedusae plus Geryoniidae forms a clade with *Monobrachium* as its earliest diverging lineage (Figures 4 & 8).

Interestingly, Geryoniidae is one of the two families of Trachymedusae with species not characterized by having eight radial canals, but rather four (*Liriope*) or six (*Geryonia*). Moreover, species of Geryoniidae have both solid and hollow marginal tentacles, as well as centripetal canals. Therefore, in most respects Geryoniidae can readily be incorporated within Limnomedusae. It has long been known that members of Geryoniidae lack a polyp stage (e.g. Brooks, 1886) and this appears to be the major difference between the group and members of Limnomedusae. Given our phylogenetic results, it would appear that a polyp stage was likely lost independently in Geryoniidae and the lineage leading to the other trachymedusans.

Scope of Trachymedusae

As presently constituted, Trachymedusae comprises five families, Geryoniidae, Halicreatidae, Petasidae, Ptychogastridae and Rhopalonematidae. Our analyses contain members of three of these families; we have yet to sample Petasidae and Ptychogastridae. Besides Geryoniidae, the other group of trachymedusans without eight radial canals is Petasidae. Petasids have four radial canals, raising the possibility that they could potentially fall with Geryoniidae within Limnomedusae. However, they lack other features (hollow tentacles, centripetal canals) that could give credence to such a hypothesis. Ptychogastrids are somewhat more typical of Trachymedusae, possessing eight radial canals. But these species are benthic-pelagic and have features (such as tentacles with adhesive discs) that appear to be adapted for such an existence. Several members of Limnomedusae (*Olindias*, *Gonionemus* and *Vallentinia*, etc.) also spend considerable time resting on substrates, prompting one to question if ptychogastrids too might be closely related to or derived from within Limnomedusae.

Our analyses find strong support for the hypothesis that Halicreatidae and Rhopalonematidae form a clade with Narcomedusae and the interstitial *Halammohydra* sp. (Figures 7 & 8). The best sampled family in our analyses is Rhopalonematidae. A close alliance appears to exist between *Aglantha*, *Aglaura* and *Amphogona*, all of which have a gastric peduncle and pendant gonads. Our data also suggest a relatively close relationship between *Pantachogon* and *Rhopalonema*, and that these two genera form a clade with

Aglantha plus *Aglaura* plus *Amphogona*. Among our samples of Rhopalonematidae, *Crossota* appears to be the earliest diverging lineage. Interestingly, in many of our analyses, the unusual interstitial *Halammohydra* (Actinulida) is shown as having had an origin within Rhopalonematidae. In all of our analyses, representatives of Halicreatidae form a well supported clade. Our combined data analyses suggest that this family is the sister group of a clade containing Narcomedusae, *Halammohydra* and the rhopalonematids (Figure 8).

Halammohydra may be derived from within Rhopalonematidae (Trachymedusae)

Species of *Halammohydra* were among the first cnidarians discovered to inhabit the interstices of sand grains (Remane, 1927). Subsequent studies have led to the discovery of additional species of interstitial hydrozoans (e.g. Swedmark, 1964; Clausen, 1971; Norenburg & Morse, 1983). *Otohydra*, discovered off Roscoff, was grouped with *Halammohydra* in the order Actinulida by Swedmark & Tessier (1958). In their view (Swedmark & Tessier, 1966), these small and simple animals were representatives of the earliest diverging hydrozoan lineages, which themselves were inhabitants of the interstitial environment. In contrast, the presence of features including solid tentacles, ecto-endodermally derived statocysts, and lack of asexual reproduction, have been regarded as evidence that actinulids may be related to free-swimming Narco- and Trachymedusae (e.g. Remane, 1927; Werner, 1965; Salvini-Plawen, 1987; Bouillon & Boero, 2000; Marques & Collins, 2004). In particular, it has been suggested that the small and simple bodies of adult actinulids resemble early developmental stages of trachylines (e.g. Swedmark, 1964; Bouillon & Boero, 2000). Gould (1977), for example, holds that actinulids and other interstitial organisms may have attained small body sizes by accelerating sexual maturation (paedomorphosis through progenesis), which he regarded as an important adaptive response for inhabiting minute interstitial spaces.

Paedomorphic species have been notoriously difficult for phylogenetic analyses based on morphology (Wiens *et al.*, 2005) and molecular data can be particularly important for such cases. Our analyses consistently place *Halammohydra* within Trachylina (Figures 4–8) and confirm earlier suggestions that actinulids are related to Narco- and Trachymedusae. Specifically, ML analyses of LSU (Figure 7, right) and combined data (Figure 8) indicate that *Halammohydra* falls within the family Rhopalonematidae (Trachymedusae). Although adult rhopalonematids are typical planktonic trachyline medusae, some early developmental stages are similar to the adult *Halammohydra*. For example, developing medusae of the rhopalonematid *Aglaura hemistoma* resemble adult actinulids in having an elongated and flagellated actinuloid body shape bearing long tentacles (Metschnikoff, 1886). Although trachyline medusae are usually planktonic, the evolution of the interstitial *Halammohydra* from a trachyline ancestor may have followed a shift from a holopelagic to a pelago-benthic or holobenthic life cycle. As mentioned above, several trachyline species live pelago-benthic lifestyles. It is conceivable that the evolution of a similar epibenthic habitat in the ancestor of actinulids may have preceded the invasion of the interstitial environment.

It remains to be seen how our understanding of actinulidan evolution will be refined when samples of *Otohydra* become

available for DNA-based analyses. Species of the latter genus share with *Halammohydra* the flagellated/ciliated actinuloid body with ontogenetically similar (ecto-endodermal) statocysts. However, the two groups have some significant differences, with *Otohydra* lacking a nerve ring and an adhesive organ, being hermaphroditic, and possessing two types of tentacles (Swedmark & Teissier, 1958; Swedmark, 1964; Clausen, 1971). Elucidating the phylogenetic placement of *Otohydra* remains an important step towards a better understanding of the patterns and processes that resulted in the invasion of the interstitial environment within Trachylina.

Scope of Narcomedusae

The enigmatic intracellular parasitic cnidarian *Polypodium hydriforme* (see Raikova, 1994) has sometimes been referred to Narcomedusae (e.g. Hyman, 1940), but unpublished analyses suggest that it is not part of this group (Evans *et al.*, in preparation). Therefore, just four families make up Narcomedusae (Schuchert, 2007). Three families, Aeginidae, Cuninidae and Tetraplatiidae, are sampled here, whereas Solmarisidae is not. The name Tetraplatiidae first appeared in print in Daly *et al.* (2007) as a *nomen nudum*, but the taxonomic authority was erroneously stated to be Schuchert, 2007 in reference to Schuchert's (2007) *Hydrozoan Directory* website. We take the opportunity to formally establish the new family Tetraplatiidae, with its type genus being *Tetraplatia* Busch, 1851. Its members are readily distinguished by the possession of four distinctive swimming lappets arising from a groove that divides their worm-like bodies into aboral and oral halves.

Our analyses suggest that our sampled representatives of Narcomedusae are monophyletic, though support for this hypothesis is only moderate (Figures 6–8). We find no strong evidence for the monophyly of Aeginidae or Cuninidae. However, support for most relevant nodes is weak and so it does not appear that our analyses contradict monophyly of these families, with one exception. In most of our analyses, the worm-shaped *Tetraplatia* is shown to have a close relationship with *Aegina citrea* and *Solmundella bitentaculata*. The sister to these three species, among our samples, appears to be *Solmissus marshalli*. Thus, *Tetraplatia* and by extension the family Tetraplatiidae appears to fall within Aeginidae. As with all the subgroups dealt with in the present paper, additional sampling should further enhance understanding of the phylogeny and evolution of Narcomedusae.

Concluding remarks

Important questions remain about how to best classify trachylina taxa in light of the phylogenetic analyses presented here. Because our taxon sampling is relatively sparse, formal recommendations seem inappropriate at this time. However, our limited sampling suggests that Trachymedusae is (at least) diphyletic, suggesting that this taxon will need to either have its meaning modified or be discarded. Because Limnomedusae is paraphyletic with respect to the trachymedusan family Geryoniidae, Limnomedusae will also need to have its meaning altered to take these, and forthcoming, findings into account.

Finally, future sampling of molecular data will no doubt be used to clarify species boundaries within trachylina cnidarians. Our data are extremely limited in this regard, but

reporting of a few observations are warranted. We note several substantial genetic divergences within what are currently thought to be single species. First, both mitochondrial 16S and nuclear SSU data taken from *Liriopse tetraphylla* collected in California and the Caribbean (Panama) show significant divergences (*P* distances >11% for 16S and >0.3% for SSU) likely indicating at least some cryptic speciation in this cosmopolitan species. Similarly, *Tetraplatia volitans* and *Tetraplatia* sp. from California and Japan, respectively, have mitochondrial 16S sequences that differ by nearly 6%, exceeding typical intraspecific differences measured for other hydrozoans (Schuchert, 2005; Govindarajan *et al.*, 2005; Miglietta *et al.*, 2007). Visual inspection of *Tetraplatia* sp. specimens from Japan suggests that these do not fit the description of *T. chuni*, the only other named species in the genus, but rather that of *T. volitans*, suggesting that this latter name may be being used to refer to at least two distinct species. In contrast, very little to no genetic divergence was revealed in samples of *Crossota rufobrunnea* and *Pantachogon haeckeli* (including some unusual colour forms; Figure 1H, I) from California and Japan, and *Aglaura hemistoma* samples from Japan and the Mediterranean Sea. The geographical distributions of trachylina hydrozoans, as well as the factors controlling them, remain open and interesting questions for further research.

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Correspondence should be addressed to:

Allen G. Collins
 NMFS, National Systematics Laboratory
 National Museum of Natural History
 MRC-153, Smithsonian Institution
 PO Box 37012, Washington
 DC 20013-7012, USA
 email: CollinsA@SI.edu