Phylogenetic position of the apostome ciliates (Phylum Ciliophora, Subclass Apostomatia) tested using small subunit rRNA gene sequences*

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Abstract: The apostomes have been assigned historically to two major groups of ciliates – now called the Class Phyllopharyngea and Class Oligohymenophorea. We set about to test these competing hypotheses of relationship using sequences of the small subunit rRNA gene from isolates of five species of apostomes: *Gymnodinioides pitelkae* from Maine; *Gymnodinioides* sp. from North Carolina; *Hyalophysa chattoni* from Florida and from North Carolina; *H. lwoffi* from North Carolina; and *Vampyrophrya pelagica* from North Carolina. These apostome ciliates were unambiguously related to taxa in the Class Oligohymenophorea using Bayesian inference, maximum parsimony, and neighbor-joining algorithms to infer phylogenetic relationship. Thus, their assignment as the Subclass Apostomatia within this class is confirmed by these genetic data. The two isolates of *Hyalophysa chattoni* were harvested from the same crustacean host, *Palaemonetes pugio*, at localities separated by slightly more than 1225 km, and yet they showed only 0.06% genetic divergence, suggesting that they represent a single population.

Key words: Apostomes, crustacean, exuviotroph, *Gammarus mucronatus*, *Marinogammarus obtusatus*, Oligohymenophorea.

Introduction

Over the past 20 years, sequences of the small subunit rRNA (SSrRNA) gene have been used to confirm or reject morphologically-based hypotheses about the phylogenetic affinities of different major groups of ciliates. For example, Small & Lynn (1985) suggested that peniculines and euplotid hypotrichs were related to nassulid ciliates in the Class Nassophorea. Gene sequences refuted this hypothesis and supported the classical notions of peniculines being related to hymenostomes (Baroin-Tourancheau et al. 1998; Strüder-Kypke et al. 2000b) and euplotid hypotrichs being more closely related to stichotrich spirotrichs (Lynn & Sogin 1988). Conversely, SSrRNA gene sequences have tentatively confirmed the placement of astome ciliates in the Class Oligohymenophorea although a much more thorough sampling of taxa is needed to make a robust test (Affa'a et al. 2004).

In morphologically-based classifications, apostome ciliates have been placed with either one or the other of two major taxa, now considered classes (Bradbury 1989). Chatton & Lwoff (1935) and Corliss (1979) associated apostomes with ciliates that are now assigned to the Class Phyllopharyngea based on their possession of a mid-ventral cytostome in the trophont stage, a massive oral fibre that was considered a possible homologue to the cyrtos of phyllopharyngeans, and "glandular" organelles (e.g., canaliculi and rosette) that appear to resemble organelles in some phyllopharyngeans. By contrast, Small & Lynn (1985) placed apostomes within the Class Oligohymenophorea based on similarities between their somatic kinetids. Landers (1986) and Bradbury (1989) provided morphological confirmation of this latter hypothesis in studies of metamorphosis from the encysted tomite (phoront) to the trophont stage in the apostome *Hyalophysa chattoni* using both light and electron microscopy. They observed a paroral homologue in the oral infraciliature of the tomite that developed structures identifiable as postciliary microtubular ribbons, originating near paroral dikinetids and extending to support the cytopharynx, during metamorphosis to the macrostome feeding stage. Bradbury (1989) concluded, however, that numerous similarities between the life cycles and

* We are pleased to dedicate this paper to Professor Doctor Wilhelm "Willi" Foissner on the occasion of his 60th birthday. Willi is a leader in the revitalization of the alpha-taxonomy of ciliated protists in particular and has served as an outstanding role model for present and future generations of protist taxonomists. It has been a pleasure for several of us to collaborate with him on various research projects.

ultrastructures of hymenostomes and apostomes justified placing them in much closer taxonomic affinity to one another, with apostomes as a suborder within the Order Hymenostomatida rather than as a coequal subclass in the Class Oligohymenophorea.

We set out to test these competing hypotheses of relationship using sequences of SSrRNA genes from isolates of five species of apostomes: *Gymnodinioides pitelkae* from Maine; *Gymnodinioides* sp. from North Carolina; *Hyalophysa chattoni* from Florida and from North Carolina; *H. lwoffi* from North Carolina; and *Vampyrophrya pelagica* from North Carolina. The latter species is a histotroph that feeds on tissues of the crustacean host after it is killed by a predator, and the others are exuviotrophic, feeding on the exuvial fluid remaining on the inside surface of the host's discarded exoskeleton immediately after ecdysis. Exuviotrophy is the lifestyle of the majority of known species of apostomes.

Materials and methods

Collection of samples: *Gymnodinioides pitelkae* BRADBURY, 2005 were collected from *Marinogammarus obtusatus* at Eastport, Maine. Briefly, crustacean moults were examined to verify the presence of trophonts and removed from dishes housing hosts to clean sea water in Petri dishes. Trophonts of *Gymnodinioides pitelkae* swam from moults after feeding, settled to the bottom of the Petri dish, and attached there as encysted tomonts, which divide to form tomites that seek out new hosts once they escape from the cyst. The dish was then rinsed gently with sea water. After tomonts had divided to produce a cluster of tomites, cysts with tomites still confined within them were fixed by flooding dishes with 70 % ethanol, dislodged, and transferred by micropipette to microfuge tubes for shipping.

Trophonts of *Gymnodinioides* sp. were harvested from moults of *Gammarus mucronatus* collected from the Pamlico River near Aurora, North Carolina (NC); trophonts of *Hyalophysa chattoni* were harvested from moults of *Palaeomonetes pugio* collected from the Indian River near Fort Pierce, Florida and the Pamlico River near Aurora, NC; and trophonts of *Hyalophysa lwoffi* were harvested from moults of *Palaemonetes paludosus* collected from Yates Pond near Raleigh, NC. In all of these isolates, large, fully fed trophonts were transferred by micropipette into small plastic Petri dishes containing habitat water passed through a syringe filter (0.45 µm porosity) and from there into a second dish of filtered water, removing them from all other protists and any organic debris. Dishes of isolated trophonts were examined to verify the absence of cells other than bacteria, and any stray protists removed by pipetting.

Trophonts isolated in this way encysted on the dish as tomonts; each cyst contained a cluster of tomites after 24–48 h. Tomonts were washed 2–3 times with syringe-filtered water during this period to ensure the elimination of any other kinds of protists that may have been overlooked during the initial isolation. When tomites emerged from tomont cysts, they were pipetted into 1.5 ml microfuge tubes and centrifuged at 3000 g for 20 min to concentrate them into a pellet. Water was pipetted from the tube after centrifugation, and the sample was fixed in 95% ethanol.

Vampyrophrya pelagica was isolated from several species of marine, planktonic, calanoid copepods collected from Taylor Creek, Beaufort, NC. Trophonts of *V. pelagica* were activated by pipetting hosts into a dish of filtered sea water and squeezing them with a pair of fine forceps to kill them and release body fluids. This stimulates tomites, if they are present, to excyst and swim into the host's cadaver to feed on tissues as trophonts. Trophonts were allowed to stay within the host's body after feeding; otherwise, they did not form tomont cysts. Cadavers containing encysted tomonts were transferred to dishes of filtered sea water, and tomites were collected by pipetting when they left the host. Tomites were cleaned as described for trophont isolates above, pipetted into 1.5 ml microfuge tubes, and fixed in 95% ethanol.

Extraction and sequencing of DNA: *Gymnodinioides pitelkae* – The isolated and fixed ciliates were pelleted in 1.5-ml microfuge tubes and DNA was extracted following the modified Chelex extraction described by STRÜDER-KYPKE & LYNN (2003): 100 µl of 5% (w/v) Chelex® 100 (Sigma, Oakville, ON, Canada) and 10 µl of Proteinase K (20 mg/ml; Sigma) were added to the pelleted cells. Eight microliters of the supernatant were used in the subsequent PCR reactions. The PCR amplification was performed in a Perkin-Elmer Gene Amp 2400 thermocycler (PE Applied Biosystems, Mississauga, ON, Canada), using the universal forward primer A (5'-AACCTGGTTGATCCTGCCAGT-3', MEDLIN et al. 1988) and the universal reverse primer B (5'-TGATCCTTCTGCAGGTTCACCTAC-3', MEDLIN et al. 1988). The PCR products were excised from agarose gels and purified using the GeneClean II kit (Qbiogen, Carlsbad, CA, U.S.A.) following the manufacturer's protocol. Finally, the DNA was cloned into the PCR 2.1 TOPO vector using the TOPO TA Cloning kit (Invitrogen, Burlington, ON, Canada) and the plasmid DNA was purified with the S.N.A.P. MidiPrep kit (Invitrogen). The species was sequenced in both directions in an ABI Prism 377 automated DNA sequencer (Applied Biosystems Inc., Foster City, CA, U.S.A.) using dye terminator and Taq FS with one forward and one reverse internal SSrRNA primer (ELWOOD et al. 1985) and the amplification primers.

Other species – Pellets of fixed tomites were washed twice in phosphate-buffered saline to remove all traces of ethanol, and DNA was extracted with a DNeasy kit (Qiagen, Valencia, CA) using the protocol for animal tissues. Isolated DNA was cleaned by vacuum filtration, and resuspended in autoclaved distilled water.

Amplification of SSrRNA genes was performed in a Hybaid Express or PX2 thermal cycler (Thermo Electron Corporation, Waltham, MA) using a Titanium Taq Polymerase Kit (Clontech Laboratories, Mountain View, CA). The universal forward A and universal reverse B primers were used for most species, but the universal 82 forward primer (5'-GAAACTGCGAATGGCTC-3', ELWOOD et al. 1985) was used for some species because of poor yields with the universal forward A primer. Products of PCR reactions were cleaned by vacuum filtration and sequenced in both directions using an ABI 3730-XL DNA Analyzer (Applied Biosystems, Foster City, CA) and three forward and three reverse internal primers (GREENWOOD et al. 1991a) in addition to the end primers.

Sequence availability and phylogenetic analyses: The nucleotide sequences used in this article are available from the GenBank/EMBL databases and have the following accession numbers: *Anophryoides haemophila* U51554 (RAGAN et al. 1996), *Anoplophrya marylandensis* AY547546 (AFFA'A et al. 2004), *Bresslaua vorax* AF060453 (LYNN et al. 1999), *Bromeliophrya brasiliensis* AJ810075 (FOISSNER et al. 2003), *Carchesium polypinum* AF401522 (MIAO et al. 2004), *Cardiostomatella vermiforme* AY881632 (LI et al. 2006), *Cohnilembus verminus* Z22878 (DYAL et al., unpubl.), *Colpidium campylum* X56532 (GREENWOOD et al. 1991a), *Colpoda inflata* M97908 (GREENWOOD et al. 1991b), *Cryptocaryon irritans* (WRIGHT & COLORNI 2002), *Cyclidium glaucoma* Z22879 (EMBLEY et al. 1995), *C. plouneouri* U27816 (EMBLEY et al. 1995), *C. porcatum* Z29517 (ESTEBAN et al. 1993), *Dexiotrichides pangi* AY212805 (SONG et al. 2003), *Entodiscus borealis* AY541687 (LYNN & STRÜDER-KYPKE 2005), *Epistylis hentscheli* AF335513 (MIAO et al. 2001), *Frontonia vernalis* U97110 (HIRT et al., unpubl.), *Glaucoma scintillans* AJ511861 (FRIED et al. 2002), *Holophrya* (formerly *Prorodon*) *teres* X71140 (STECHMANN et al. 1998), *Ichthyophthirius multifiliis* U17354 (WRIGHT & LYNN 1995), *Lembadion bullinum* AF255358 (STRÜDER-KYPKE et al. 2000b), *Metanophrys similis* AY314803 (SHANG & SONG, unpubl.), *Miamiensis avidus* AY550080 (JUNG et al. 2005), *Ophryidium versatile* AF401526 (MIAO et al. 2004), *Ophryoglena catenula* U17355 (WRIGHT & LYNN 1995), *Opisthonecta henneguyi* X56531 (GREENWOOD et al. 1991a), *Paramecium bursaria* AF100314 (STRÜDER-KYPKE et al. 2000a), *P. tetraurelia* X03772 (SOGIN & ELWOOD 1986), *Paranophrys magna* AY103191 (SHANG et al. 2003), *Parauronema longum* AY212807 (SHANG & SONG, unpubl.), *Philasterides dicentrarchi* AY642280 (KIM et al., unpubl.), *Plagiopyliella pacifica* AY541685 (LYNN & STRÜDER-KYPKE 2005), *Platyophrya vorax* AF060454 (LYNN et al. 1999), *Pleuronema coronatum* AY103188 (SHANG & SONG, unpubl.), *Pseudocohnilembus marinus* Z22880 (DYAL et al., unpubl.), *Schizocaryum dogieli* AF527756 (LYNN & STRÜDER-KYPKE 2002), *Sorogena stoianovitchae* AF300285 (LASEK-NESSELQUIST & KATZ 2001), *Tetrahymena thermophila* M10932 (SPANGLER & BLACKBURN 1985), *Urocentrum turbo* AF255357 (STRÜDER-KYPKE et al. 2000b), *Uronema marinum* Z22881 (DYAL et al., unpubl.), *Vaginicola crystallina* AF401521 (MIAO et al. 2004), *Vorticella campanula* AF335518 (MIAO et al. 2001), *Zoothamnopsis sinica* AY319769 (LI & SONG, unpubl.), and *Zoothamnium arbuscula* AF401523 (MIAO et al. 2004).

The sequence fragments were imported into Sequencher ver. 4.0.5 (Gene Codes Corp.), trimmed at the ends, assembled into contigs, and checked for sequencing errors. Sequences were aligned using the Dedicated Comparative Sequence Editor (DCSE) (DE RIJK & DE WACHTER 1993) with attention paid to secondary structural features of the molecule.

Hypervariable positions were excluded from the file prepared for phylogenetic analysis, thus resulting in a data set that comprised 1764 nucleotide positions. Missing nucleotides at the beginning or end of sequences were treated as missing by MrBayes and PAUP and gaps within the alignment were regarded as a fifth character state. For the Bayesian inference analysis, MrModeltest ver. 2.2 (NYLANDER 2004; POSADA & CRANDALL 1998) was employed to find the model of DNA substitution that best fits our data. The General-Time-Reversible (GTR) model for nucleotide substitution, considering invariable sites and gamma distributed substitution rates among sites, was depicted as best model. This model (n = 6, rates = invgamma) was implemented in MrBayes ver. 3.1.2, a phylogenetic program employing Bayesian Inference (BI; HUELSENBECK & RONQUIST 2001, RONQUIST & HUELSENBECK 2003), which we used to infer a phylogenetic tree. Two parallel runs were performed and the maximum posterior probability of a phylogeny out of 1,000,000 trees, approximating it with the Markov Chain Monte Carlo (MCMC) and sampling every 50th generation, was computed, discarding the first 2000 trees as burn-in. A maximum parsimony (MP) analysis was performed with PAUP* ver. 4.0b10 (SWOFFORD 2002), using 867 parsimony-informative characters, and with the tree bisection-reconnection (TBR) branch-swapping algorithm in effect. Species were added randomly (n = 5) and the data were bootstrap resampled 1000 times. PHYLIP ver. 3.6a2 (FELSENSTEIN

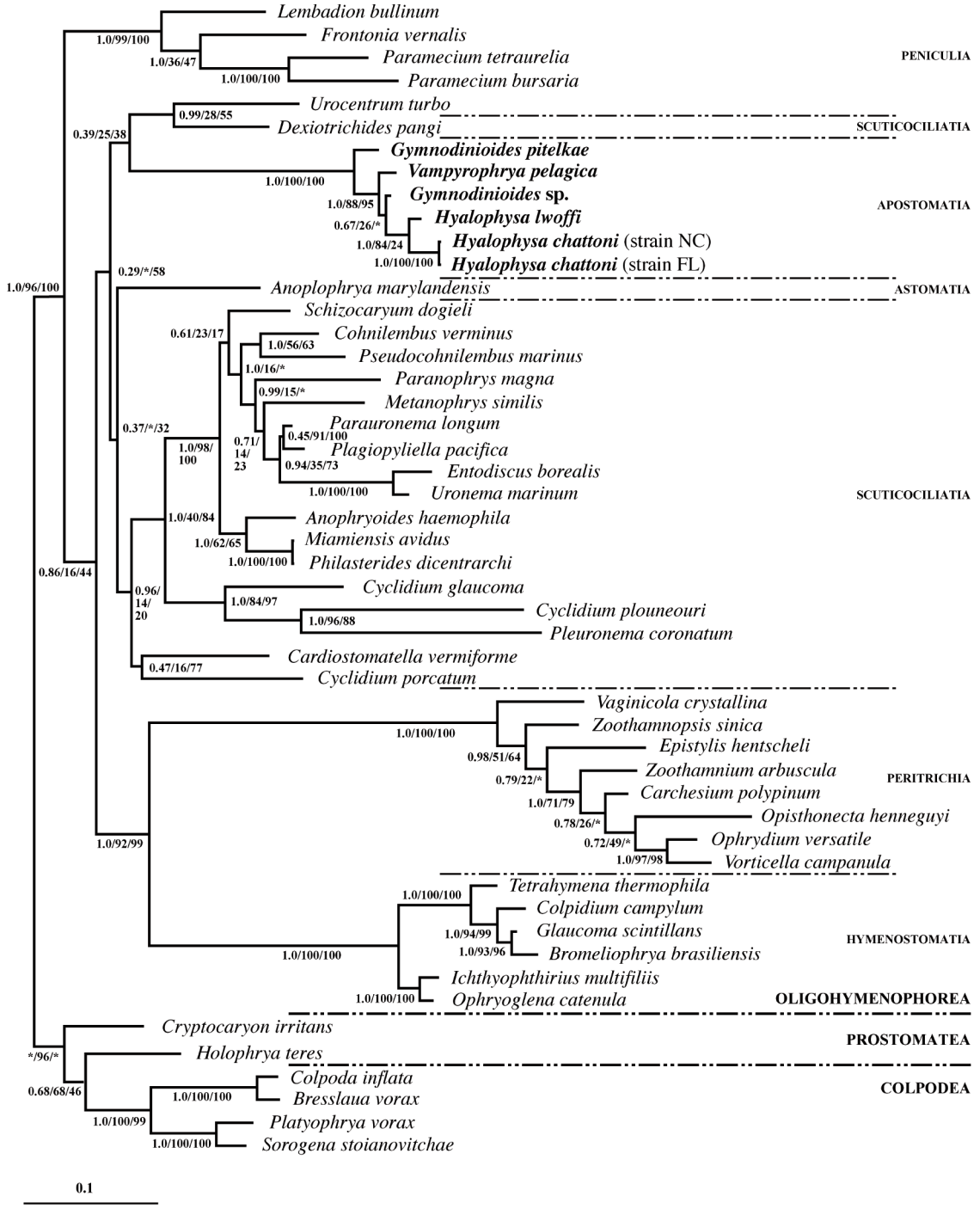

Fig. 1: Maximum likelihood tree computed with MrBayes ver. 3.1.2 (RONQUIST & HUELSENBECK 2003), based on the General Time-reversible (GTR) model with gamma-distribution and an estimate of invariable sites as determined by MrModeltest (NYLANDER 2004). The first numbers at the nodes represent the posterior probability values of the Bayesian analysis, and the second and third numbers represent bootstrap values (percent out of 1000 replicates) for maximum parsimony (SWOFFORD 2002) and neighbor joining (SAITOU & NEI 1987), respectively. An asterisk indicates bootstrap values of less than 10%. The scale bar represents 5 changes per 100 positions. New sequences appear in bold face.

2004) was employed to construct a distance matrix, using DNADIST to calculate genetic distances with the Kimura-2-parameter model (KIMURA 1980). The distance trees were constructed with NEIGHBOR, using the Neighbor Joining (NJ) algorithm (SAITOU & NEI 1987). The data were bootstrap re-sampled 1,000 times.

To test the topology of the apostome clade, the Shimodaira-Hasegawa test (SH-test; SHIMODAIRA & HASEGAWA 1999) was performed with PAUP and the log-likelihood scores were estimated using a fully optimized model.

Results

Primary structure: The length, GC (%) content, and GenBank Accession Numbers of the PCR-amplified SSrRNA of these six ciliates are as follows: for *Gymnodinioides pitelkae* – 1743, 42%, EU503534; for *Gymnodinioides* sp. – 1663, 43%, EU503535; for *Hyalophysa chattoni* (strain FL) – 1661, 43%, EU503536; for *H. chattoni* (strain NC) – 1661, 43%, EU503537; for *H. lwoffi* – 1731, 43%, EU503538; and for *Vampyrophrya pelagica* – 1662, 43%, EU503539.

Table 1: Genetic distances (%) between apostome species.

	G. pitelkae	*Gymnodinioides* sp. NC	*H. chattoni* NC	*H. chattoni* FL	*H. lwoffi*	*V. pelagica*
Gymnodinioides pitelkae	–					
Gymnodinioides sp. NC	2.22	–				
Hyalophysa chattoni NC	3.75	2.71	–			
Hyalophysa chattoni FL	3.68	2.64	0.06	–		
Hyalophysa lwoffi	3.09	1.81	1.89	1.95	–	
Vampyrophrya pelagica	2.81	1.22	2.81	2.74	2.11	–

Genetic distances among these species ranged from 0.06% (*Hyalophysa chattoni* spp.) to 3.75% (*H. chattoni* NC and *Gymnodinioides pitelkae*) (Tab. 1).

Phylogenetic analyses: All phylogenetic analyses grouped these six apostome isolates closely together in a strongly supported clade with 100% bootstrap support or a posterior probability of 1.0 (Fig. 1). The genetic distances among these apostomes were not great. However, all isolates of *Hyalophysa* grouped together, and sequences of the two isolates of *H. chattoni* were nearly identical despite being collected from sites in North Carolina and Florida, slightly more than 1,225 km apart. The *Hyalophysa* isolates were strongly separated from the *Gymnodinioides* isolates, with bootstrap support of 84% in MP and a posterior probability of 1.0 in the Bayesian analysis; neighbor joining did not always support this clade (Fig. 1). *Vampyrophrya pelagica* fell between the two *Gymnodinioides* isolates, with *G. pitelkae* being quite strongly separated from the other five isolates (Fig. 1). However, the SH-Test performed with PAUP* showed that tree topologies with various placements of the *Gymnodinioides* species (e.g., *G. pitelkae* basal, followed by *Gymnodinioides* sp. and then all other apostomes; both *Gymnodinioides* species as a sistergroup basal to all other apostomes; or *Vampyrophrya pelagica* basal, followed by both *Gymnodinioides* species) were not significantly different from the topology shown. The first branching pattern (*Gymnodinioides pitelkae* basal, followed by *Gymnodinioides* sp. and then all other apostomes) was indeed one of the 4 most parsimonious tree topologies computed by PAUP*.

There is no doubt that these apostome ciliates have strong affinities to other oligohymenophorean ciliates, here representing all the major subclasses in the class (Fig. 1). A relationship to the Subclass Scuticociliatia is not strongly supported by bootstrap values or posterior probabilities, and the density of the sampling of the Subclass Astomatia is weak; however, there is clear evidence that apostomes do not fall within the Subclass Hymenostomatia. Instead, they branch at a level and depth of divergence similar to clades representing the Subclasses Peniculia, Peritrichia, and Hymenostomatia (Fig. 1).

Discussion

Phylogenetic position of apostomes: As noted in the introduction, the apostomes have been assigned historically to two major groups of ciliates – now called the Classes Phyllopharyngea and Oligohymenophorea (Chatton & Lwoff 1935; Corliss 1979; Small & Lynn 1985). Small & Lynn (1985) placed the apostomes as the Subclass Apostomatia in the Class Oligohymenophorea primarily because the somatic kinetids of apostomes were typical for that class (Bradbury 1966; Puytorac & Grain 1975). The discovery of a presumed paroral homologue in the tomite of *Hyalophysa* led Bradbury (1989) to support the assignment by Small & Lynn (1985) to the Class Oligohymenophorea. However, Bradbury (1989) favored placing the apostomes as a suborder within the Order Hymenostomatida because the life cycle of apostomes shows so many similarities to the life cycle of histophagous hymenostomes.

Our phylogenetic analysis of six isolates of apostomes representing three genera unambiguously supports assignment to the Class Oligohymenophorea. Moreover, the distinct monophyly and deep divergence of the apostome clade argues for maintaining the subclass rank of this group rather than placing them within the hymenostomes. It is true that life cycles of apostomes and some histophagous hymenostomatids, such as *Ophryoglena* and *Ichthyophthirius* (Canella & Rocchi-Canella 1976), are similar, but life cycles of some scuticociliates, such as *Glauconema* (Small et al. 1986), and prostomes, such as *Cryptocaryon* (Colorni & Diamant 1993, Wright & Colorni 2002) and *Holophrya* (formerly *Prorodon*; Hiller & Bardele 1988), also resemble those of apostomes. A "histophagous"-type life cycle indeed may have been a trait of the common ancestor of both the Classes Prostomatea and Oligohymenophorea; however, presence of such life cycles in very different genetic lineages just as easily could represent convergent evolution of a basic ciliate life cycle adapted to similar niches.

The species of apostomes that we sequenced all belong to one family, the Foettingeriidae, in the Order Apostomatida. There are two other orders – the Astom-

atophorida and Pilisuctorida – and a total of 5 families whose assignment to the Subclass Apostomatia (LYNN 2008) remain to be confirmed using gene sequences.

Genetic diversity of apostome isolates: There is currently a debate about the biogeography of free-living protists, with two competing hypotheses seeking to explain observed patterns of distribution. On one hand, the Ubiquitous Dispersal Hypothesis (UDH) asserts that species of free-living protists should be everywhere because of their small size and almost infinite population numbers (FINLAY 2002). On the other hand, the Moderate Endemism Hypothesis (MEH) argues that as many as 30% of free-living protist species could be endemic owing to large size, very specialized niche requirements (e.g., tropical rainforest habitats, high desert soils), or both (FOISSNER 2006). The UDH and MEH both admit that symbiotic protists probably will have patterns of distribution related to those of their hosts. However, there are as yet few actual demonstrations of the phylogeography of symbiotic protists (e.g., CLAMP 1992; DA SILVA et al. 2007).

Our sampling of apostome ciliates spanned almost the entire length of the eastern seaboard of North America and included isolates from several different host species. Despite the low intensity of our sampling, two conclusions can be drawn tentatively from our results.

First, sequences of *Hyalophysa chattoni* collected from *Palaemonetes pugio* at widely separated locations are almost identical (genetic divergence 0.06%, Tab. 1). This close similarity should be confirmed using other genes, such as the cox-1 barcode region (CHANTANGSI et al. 2007), but one could infer that *Hyalophysa chattoni* shows no population subdivision across a significant geographic range when associated with one of several possible host species, *Palaemonetes pugio*.

Second, different biological species of *Tetrahymena* can be identical based on sequences of the SSrRNA gene (SOGIN et al. 1986). Thus, it is very likely that any two isolates of ciliates differing in their SSrRNA sequences by several percent represent different biological species. Isolates of *Hyalophysa* species from different crustacean hosts (*H. chattoni* from *Palaemonetes pugio* in estuarine habitats and *Hyalophysa lwoffi* from *Palaemonetes paludosus* in fresh water) show a genetic divergence of approximately 1.9% based on the SSrRNA gene sequence. These have been recognized as different morphospecies for many years (BRADBURY & CLAMP 1973) and, using *Tetrahymena* as a standard, are in all probability confirmed as different biological species by their genetic divergence. The two isolates of *Gymnodinioides* show a comparable degree of divergence (i.e., 2.2%) in the SSrRNA gene sequence, and they were isolated from different crustacean host genera – *Marinogammarus* and *Gammarus*; therefore, it is likely that they are also different biological species. A detailed morphological investigation of the *Gymnodinioides* species found on *Gammarus mucronatus* in North Carolina is underway to determine its identity as a morphospecies (J.C.C., unpubl. data). The SH-test did not refute monophyly of *Gymnodinioides*, suggesting these two isolates still may be members of the same genus. Whatever the results of these investigations, probing the genetic diversity and host-symbiont relationships of symbiotic ciliates should prove a rewarding area for further investigation.

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