J. Eukaryot. Microbiol., 51(4), 2004 pp. 441–450
© 2004 by the Society of Protozoologists

Molecular Phylogeny of Phyllopharyngean Ciliates and their Group I Introns

OONA L.O. SNOEYENBOS-WEST,[a] JEFFREY COLE,[b] ANNA CAMPBELL,[a] D. WAYNE COATS[c] and LAURA A. KATZ[a,d]

[a]Department of Biological Sciences, Smith College, Northampton, Massachusetts 01063, and
[b]Protistology Collection, American Type Culture Collection, Manassas, Virginia 20110-2209, and
[c]Smithsonian Environmental Research Center, P.O. Box 28, Edgewater, Maryland 21037, and
[d]Program in Organismic and Evolutionary Biology, University of Massachusetts-Amherst, Amherst, Massachusetts 01003, USA

ABSTRACT. We analyzed small subunit ribosomal DNA (ssu-rDNA) sequences to evaluate both the monophyly of the ciliate class Phyllopharyngea de Puytorac et al. (1974), and relationships among subclasses. Classifications based on morphology and ultrastructure divide the Phyllopharyngea into four subclasses, the Phyllopharyngia, Chonotrichia, Rhynchodia, and Suctoria. Our analyses of ssu-rDNA genealogies derived from sequence data collected from diverse members representing three of the four subclasses of Phyllopharyngea (Suctoria: *Ephelota* spp., *Prodiscophyra collini*, *Acineta* sp.; Phyllopharyngia: *Chlamydodon exocellatus*, *Chlamydodon triquetrus*, *Dysteria* sp.; and Chonotrichia: *Isochona* sp.) provide strong support for the monophyly of the Phyllopharyngea, and show that the Chonotrichia emerge from within the Phyllopharyngia. Based on this initial sampling, suctorian budding types are monophyletic, and exogenous budding appears to be basal to evaginative and endogenous budding. Further, we report the discovery of a group I intron at position 891 in the Suctoria *Acineta* sp. and *Tokophrya lemnarum*, and a second group I intron at position 1506 in *T. lemnarum*. These introns represent only the second examples of group I introns in a ciliate ribosomal gene, since the discovery of ribozymes in the LSU rRNA gene of *Tetrahymena thermophila*. Phylogenetic analyses of Group I introns suggest a complex evolutionary history involving either multiple loses or gains of introns within endogenously budding Suctoria.

Key Words. Chonotrichia, Ciliophora, group I introns, molecular genealogy, Phyllopharyngea, ssu-rDNA, Suctoria.

THE Phyllopharyngea, an understudied class in the phylum Ciliophora, is named for the radially arranged microtubular structures (phyllae) around the cytopharynx (Lynn 1996; Lynn and Corliss 1991; Lynn and Small 1997, 2002; de Puytorac 1994; de Puytorac et al. 1974). In addition to phyllae, phyllopharyngean ciliates have reduced ciliature and a synapomorphic (shared derived) kinetid structure in at least some stage in their life cycle (Lynn and Corliss 1991). Current classification schemes based on ultrastructure of the somatic kinetid and ontogenetic data consistently unite four subclasses within the class Phyllopharyngea; the Phyllopharyngia, Chonotrichia, Rhynchodia, and Suctoria (Table 1; Lynn and Small 2002; de Puytorac 1994, using terminology based on Lynn and Small (2002). One difference between these schemes is that the order Chilodonellida of de Puytorac 1994 is subsumed as a family Chilodonellidae in the order Chalmydodontida in Lynn and Small (2002; Table 1).

There is considerable diversity among the four Phyllopharyngea subclasses. The Cyrtophoria (Fauré-Fremiet in Corliss (1956)) are predominantly motile, aquatic forms that contain heteromeric macronuclei—macronuclei with DNA-rich and DNA-poor areas (Corliss 1979; Lynn and Corliss 1991). Adult stages of the Chonotrichia (Wallengren 1895) are sessile vase-shaped cells and are typically found as symbionts or ectocommensals permanently attached to their crustacean host's cuticle. Chonotrichia, like Phyllopharyngia, have a heteromeric macronucleus, suggesting these two taxa may be sister to one another (Grell and Meister 1982; Fig. 1a); however, Chonotrichia divide by budding while Phyllopharyngia divide homothetogenically (Corliss 1979; Foissner 1996; Lynn and Corliss 1991). The Rhynchodia (Chatton and Lwoff 1939), the only major lineage of Phyllopharyngea not sampled for this paper, have homomeric nuclei, lack oral ciliature, and are obligate parasites of marine invertebrates (Corliss 1979; Foissner 1996; Lynn and Corliss 1991).

The Suctoria (Claparède and Lachmann 1859), perhaps the most unusual lineage within the Phyllopharyngea, were originally not even recognized as ciliates due to the lack of cilia in the adult stage—adult forms of Suctoria have tentacles (reviewed in Corliss 1979). Suctoria have homomeric nuclei, and tentacled adult forms reproduce by budding to generate ciliated swarmers (Corliss 1979; Jankowski 1979). Unlike most ciliates, Chonotrichia and Suctoria reproduce by various types of 'live birth' or budding, where a ciliated swarmer cell emerges from the non-motile adult (Corliss 1979; Jankowski 1979). The formation of the swarmer in these groups can take place exogenously, endogenously or, in some cases of Suctoria, invaginatively. Both Chonotrichia and Suctoria are sessile and divide by budding, consistent with these lineages being sister taxa (Fig. 1b).

Like all ciliates, Phyllopharyngea exhibit a life cycle that is virtually unique among eukaryotes in that both a transcriptionally inactive germline micronucleus (MIC) and a functional macronucleus (MAC) with a processed genome develop from a zygotic nucleus following conjugation. At least two of the four groups of Phyllopharyngea extensively fragment their genomes and to some extent amplify genes in their MACs (Ménier and Hufschmid 1988; Riley and Katz 2001).

The sparse molecular data from this group limit the ability to evaluate morphological hypotheses in the context of molecular genealogies. Previously published data exist for only a few Phyllopharyngea (e.g. Bernhard et al. 1995; Leipe et al. 1994; Riley and Katz 2001). Our analyses of alpha-tubulin (Israel et al. 2002) and histone H4 (Katz et al. 2004) from a subset of the Phyllopharyngea indicate that at least these protein-coding genes fail to provide topologies consistent with morphology, ssu-rDNA phylogenies, and with each other. Hence, we focus this paper on analyzing additional ssu-rDNA sequences from diverse Phyllopharyngea as this gene has been proven to provide valuable insights into relationships among some ciliates (e.g. Hammerschmidt et al. 1996; Leipe et al. 1994; Lynn and Strüder-Kypke 2002; Shin et al. 2000; Snoeyenbos-West et al. 2002; Strüder-Kypke and Lynn 2003). Moreover, the large number of previously characterized ciliate ssu-rDNA sequences provides outgroups, as well as a few ingroup sequences. During the course of this study, we also identified and analyzed three putative Group I introns in the ssu-rDNAs of two Suctoria.

MATERIALS AND METHODS

Specimens. We chose ciliates to represent three of the four subclasses of the Phyllopharyngea, based on the classification of Lynn and Small (2002; Table 1). We characterized ssu-rDNA from the suctorians *Ephelota* spp., collected from the marine hydrozoan *Tubularia* (Marine Biology Laboratory, Woods Hole, MA; MBL, # 240), *Prodiscophyra collini* from a culture originally obtained from the Culture Collection of Algae

Corresponding Author: L. Katz—Telephone number: 413-585-3825; FAX number: 413-585-3786; Email: Lkatz@Smith.edu.

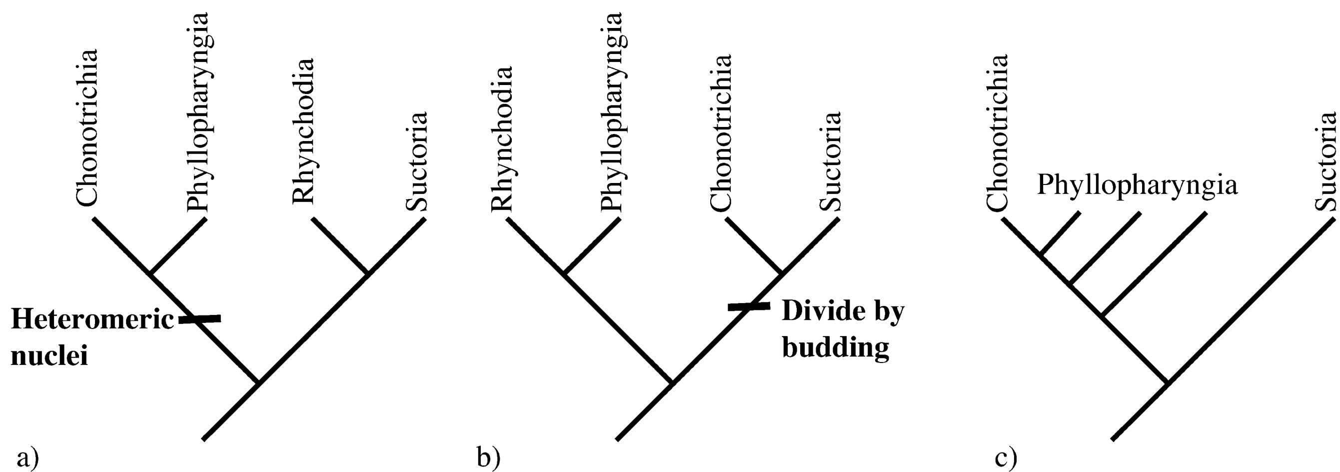

Fig. **1**. Schematic representation of competing hypotheses of evolution within the Phyllopharyngea: **a)** based on shared feature of heteromeric nuclei in Chonotrichia and Phyllopharyngia; **b)** based on shared features of division by budding and sessile life style in Suctoria and Chonotrichia; and **c)** based on the results of our ssu-rDNA genealogies (see Fig. 3).

and Protozoa, Ambleside, UK (CCAP 1618/2), and *Acineta* sp., collected from slide traps (coverslips attached to corks that were floated in water for 24 h) at the Harbor Branch Oceanographic Institute, Florida. Three species of Phyllopharyngia, *Chlamydodon exocellatus*, *Chlamydodon triquetrus*, and *Dysteria* sp. were collected from marshy sediments, also at the Harbor Branch Oceanographic Institute, Florida, and were identified using protargol staining. For protargol staining, specimens were collected using a drawn-glass micropipette, fixed in modified Bouin's solution (Coats and Heinbokel 1982), and processed by the quantitative staining procedure of Montagnes and Lynn (1993). Stained specimens were examined using Zeiss optics, with digital images obtained using a Zeiss Axiocam interfaced with a personal computer. Finally, the chonotrich *Isochona* sp. was collected from the pleopods of gammarid amphipods purchased from the MBL (MBL # 1620). A small number of these pleopods containing a number of chonotrichs were fixed, stained, and examined as above. We also chose representative ciliates from different classes for use as outgroup taxa (Table 2).

DNA extraction, amplification, cloning and sequencing. Between 30–100 cells were hand-picked, washed, and transferred to lysis buffer solution, and DNA was isolated following standard protocols (Ausubel et al. 1993). Products were generated by PCR (Saiki et al. 1988) as described in Riley and Katz (2001) using ssu-rDNA primers from Medlin et al. (1988) plus three additional internal primers used in sequencing (Snoeyenbos-West et al. 2002).

Table **1**. Classification schemes of Phyllopharyngea:

Lynn and Small 2002	de Puytorac 1994
Subclass Phyllopharyngia	Subclass Cyrtophoria
Order Chlamydodontida	Order Chlamydodontida
Order Dysteriida	Order Dysteriida
	Order Chilodonellida
Subclass Rhynchodida	Subclass Rhynchodia
Order Hypocomatida	Order Hypocomatida
Order Rhynchodida	Order Rhynchodida
Subclass Chonotrichia	Subclass Chonotrichia
Order Exogemmida	Order Exogemmida
Order Cryptogemmida	Order Cryptogemmida
	Order Chilodochonida
Subclass Suctoria	Subclass Suctoria
Order Exogenida	Order Podophryida
Order Endogenida	Order Exotropida
Order Evaginogenida	Order Entotropida

PCR products were cleaned using the Qiaquick PCR Purification System (Qiagen, Valencia, CA), and cloned using either the pAMP1 System (Gibco-BRL, Grand Island, NY) or the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). Plasmid DNA was purified using the Qiagen MiniPrep Kit (Qiagen, Valencia, CA, USA 27106). Direct sequencing of PCR products, as well as cloned plasmid DNA, was accomplished in both directions using gene-specific primers and the Big Dye terminator kit (Applied Biosytems, Foster City, CA). Sequences were run on an ABI 377 or 3100 automated sequencer.

Data analysis. Contigs were constructed and edited using SeqMan (DNAStar). The multisequence alignment algorithm Clustal W (Thompson et al. 1994) as implemented in Megalign (DNAStar), was used to align sequences obtained in this study with sequences obtained from GenBank. The alignment used a gap penalty of 15 and a gap length penalty of 6.66. Pairwise differences were calculated as uncorrected distances in PAUP* 4.0b4a (PPC) (Swofford 2002).

Alignments were imported into MacClade where further adjustments were made by eye. Ambiguous regions of the ssu-rDNA alignment were masked both conservatively and semi-conservatively to assess effects on subsequent genealogical analyses. The conservative alignment is available at: (http://www.science.smith.edu/departments/Biology/lkatz/aligns.html). Phylogenetic analyses of nucleotide data used parsimony (MP), maximum likelihood (ML) and neighbor-joining (NJ) algorithms of PAUP* version 4.0b4a (PPC) (Swofford 2002), in order to determine the stability of tree topologies under different evolutionary models. Distances for neighbor joining were estimated with the LogDet paralinear model excluding invariant sites. Maximum likelihood analyses used parameters estimated by hierarchical ratio tests in Modeltest Version 3.0 (Posada and Crandall 1998). Parsimony analyses used 10 random addition sequences in heuristic searches. Bootstraps were calculated using 100 replicates under all models.

Intron sequences were aligned by adding our ciliate intron sequences (annotated within GenBank entries AY332717 through AY332719), the original *Tetrahymena thermophila* (V01416), and the *Acrasis rosea* sequence that is also found at

Table **2**. Small subunit rDNA sequences from Genbank used in this study.

Organism	GB #	Organism	GB #	Organism	GB #
Anophryoides haemophila	U51554	*Halteria grandinella*	AY007441	*Tetrahymena thermophila*	X56165
Blepharisma americanum	M97909	*Heliophrya erhardi*	AY007447	*Tokophyra quadripartita*	AY102174
Bresslaua vorax	AF060453	*Isotricha intestinalis*	U57770	*Tracheloraphis* sp.	L31520
Caenomorpha uniserialis	U97108	*Loxodes magnus*	L31519	*Trithigmostoma steini*	X71134
Chilodonella uncinata	AF300281	*Loxodes striatus*	U24248	Uncultured ciliate clone AT12	AF530529
Climacostomum virens	X65152	*Metopus contortus*	Z29516	Uncultured ciliate clone AT723	AF530530
Coleps hirtus	U97109	*Metopus palaeformis*	AY007450	Uncultured ciliate clone AT737	AF530531
Coleps sp.	X76646	*Nyctotheroides deslierresae*	AF145353	Uncultured clone BOLA439	AF372787
Colpoda inflata	M97908	*Nyctotherus ovalis*	AY007454	Uncultured clone LEMD069	AF372828
Cryptosporidium parvum	X64341	*Obertrumia georgiana*	X65149		
Didinium nasutum	U57771	*Ophryoglena catenula*	U17355		
Diplodinium dentatum	U57764	*Ophryoscolex purkynjei*	U57768		
Discophrya collini	L26446	*Paramecium tetraurelia*	X03772		
Ephelota sp. Antarctica	AF515610	*Platyophrya vorax*	AF060454		
Ephelota sp. 1	AF326357	*Prorodon teres*	X71140		
Epidinium caudatum	U57763	*Prorodon viridis*	U97111		
Eufolliculina uhligi	U47620	*Pseudomicrothorax dubius*	X65151		
Euplotes crassus	AY007438	*Pseudoplatyophrya nana*	AF060452		
Frontonia vernalis	U97110	*Sorogena stoianovitchae*	AF300286		
Furgasonia blochmanni	X65150	*Spirostomum ambiguum*	L31518		
Glaucoma chattoni	X56533	*Sterkiella nova*	M14601		
Gruberia sp.	L31517	*Strombidium purpureum*	U97112		
Gymnodinium mikimotoi	AF009131	*Stylonychia lemnae*	AF164124		

position 891 (AF011458) to an alignment analyzed in Oliveira and Bhattacharya (2000). The alignment was adjusted by eye, and is available at: (http://www.science.smith.edu/departments/Biology/lkatz/aligns.html). Genealogies were generated using MP and NJ algorithms as described above. Bootstrap values were calculated using 100 replicates under both models.

RESULTS

Morphological description of *Chlamydodon*, *Dysteria*, and *Isochona* samples. We were able to identify the *Chlamydodon* species and the genus *Dysteria* using light microscopy and protargol-stained specimens. Specimens of *Chlamydodon exocellatus* conformed to the original description of the species provided by Ozaki and Yagiu (1941), except for the presence of a diffuse, sometimes inconspicuous eyespot at the anterior left margin of the cell. Since the presence of a stigma in ciliates, including species of *Chlamydodon*, depends on the nutritional status of the cells (Kuhlmann 1998), we conclude that the occurrence of the diffuse eyespot in our specimens does not exclude their identification as *Chlamydodon exocellatus*. Individuals in this Florida population were 150–230 μm in length, exhibited a reddish-orange to greenish coloration due to ingested food particles, and had a diffuse reddish-orange pigment spot at the anterior-left margin of the cell. The cross-striated band (Fig. 2a) formed an oblique ellipse that crossed onto the dorsal anterior surface of the cell. Protargol-stained specimens averaged 138 ± 3.5 μm by 80 ± 1.4 μm (n = 22; range 114–170 μm by 71–93 μm) , and had an ovoid macronucleus positioned near the cell equator and measuring 31 ± 0.9 μm by 19 ± 0.7 μm (n = 21; range 25–43 μm by 14–23 μm) . The macronucleus was clearly heteromeric in protargol preparations, with half of its volume containing many small, densely stained granules, while the other half usually contained two large, conspicuous nucleoli. Somatic kinety number ranged from 83–97 (mean 89 ± 1.4; n = 12), with 42–48 in the right field, 33–45 in the left field, and 4–7 in a postoral group. Several kineties of the right field course onto the dorsal cell surface paralleling the striated band. Oral ciliature (Fig. 2b) consisted of the inner and outer circumoral kineties and a preoral kinety that was typically separated into 2–4 fragments. The elliptical cytostome was encircled by 17–21 barren kinetosomes that were underlain by a cytopharyngeal basket composed of 14–19 relatively short (15–25 μm long) nematodesmata. Numerous contractile vacuole pores were distributed over the ventral surface of the cell.

Specimens of *Chlamydodon triquetrus* fit the description of the species provided by Kahl (1931). Individuals in this Florida population were 90–120 μm long in vivo and lacked color except for a conspicuous reddish-orange pigment spot at the anterior left margin of the cell. The cross-striated band coursing around the cell margin was consistently incomplete at the posterior end of the cell (Fig. 2c). Protargol-impregnated specimens were 56–74 μm long by 20–30 μm wide (n = 4) and had an ovoid heteromeric macronucleus measuring 12–15 μm by 7–8 μm. The 28–31 ventral kineties were arranged as 12–16 in the right field, four postoral, and 11–12 in the left field. Two circumoral and a preoral kinety were set anterior to the elliptical cytostome (Fig. 2d). The number of cytopharyngeal nematodesmata was not determined, but the cytostome was encircled by 15–16 barren kinetosomes, suggesting the presence of 12–14 nematodesmata.

We were unable to identify this isolate of *Dysteria* to species. Individuals in this Florida population measured 50–70 μm long in vivo, had a prominent podite, and were colorless (Fig. 2e). Protargol-impregnated specimens averaged 56 ± 4.5 μm by 28 ± 1.8 μm (n = 17; range 33–83 μm by 18–37 μm) and had an elliptical, heteromeric macronucleus measuring 18 ± 1.6 μm by 7 ± 0.5 μm (Fig. 2f). The 13–14 somatic kineties consisted of two frontoventral kineties, two right ventral kineties, a right equatorial kinety, and 7–8 left equatorial kineties (for terminology, see Gong et al. 2002). Two contractile vacuole pores were located to the left of the right ventral kineties, with one positioned in the anterior half and one in the posterior half of the cell.

The chonotrichs were identified as *Isochona* species (Cryptogemmida, Isochonidae). Members of the genus *Isochona* Jankowski (1973) are distinguished by cryptogemmous tomitogenesis and a simple funnel. Ciliates in our sample were attached to the pleopodial bristles of the amphipods. Cells had an av-

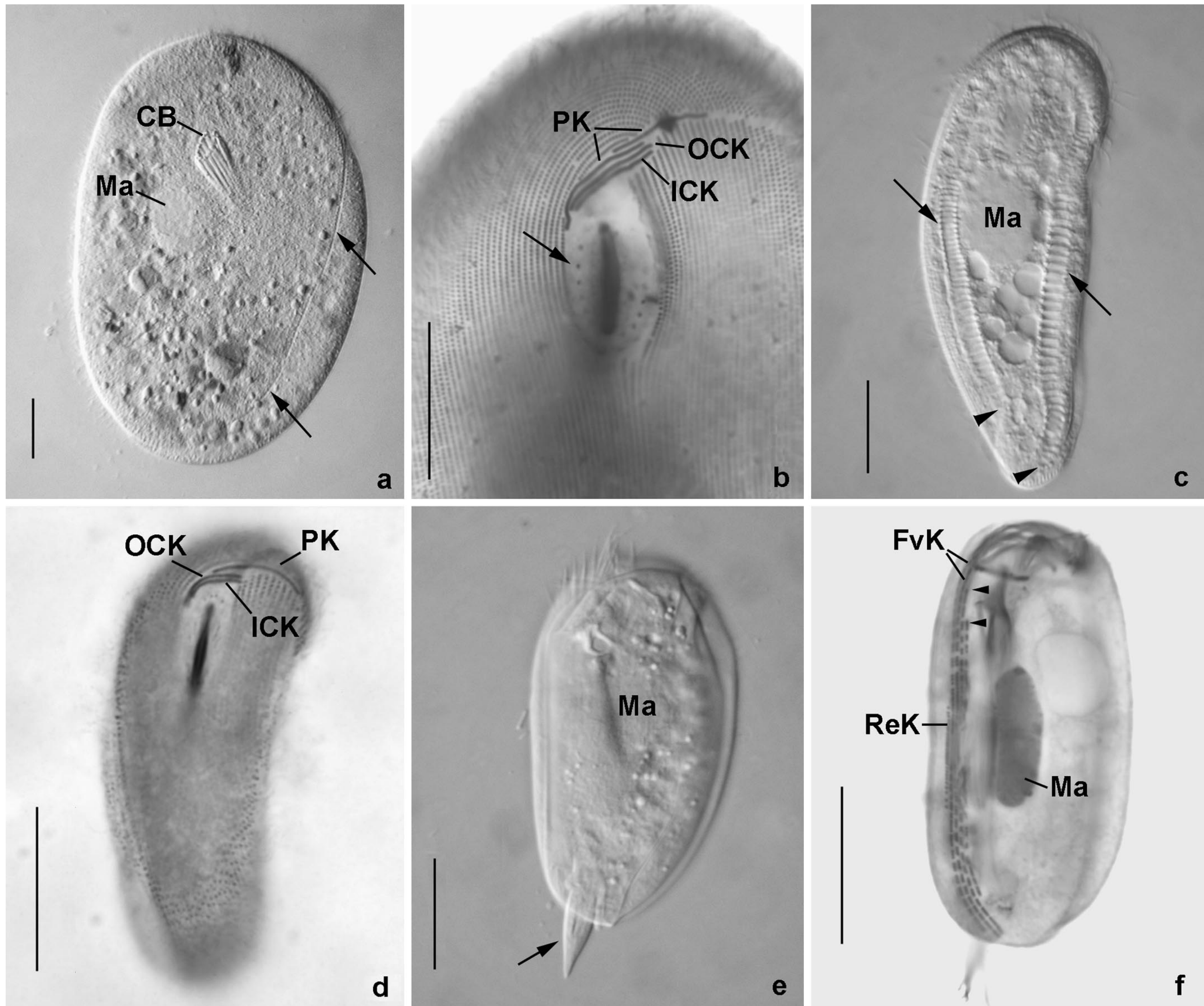

Fig. **2a–f**. Phyllopharyngean ciliates from the Indian River Lagoon, Florida. **a-b**. *Chlamydodon exocellatus*. **a**. Living specimen slightly distorted due to flattening. Arrows mark the cross-striated band. **b**. Oral region of protargol-stained specimen; arrow indicates barren kinetosomes associated with oral nematodesmata. **c-d**. *Chlamydodon triquetrus* in vivo (**c**) and after protargol impregnation (**d**); arrows indicate cross-striated band; arrow heads indicate the posterior termination of the cross-striated band. **e-f**. *Dysteria* sp. in vivo (**e**) and following protargol impregnation (**f**); arrow indicates podite; arrow heads indicate right ventral kineties. Scale bars = 20 μm; CB, cytopharyngeal basket; Fvk, frontoventral kinety; ICK, inner circumoral kinety; Lek, left equatorial kinety; Ma, macronucleus.

erage length of 45 μm and width of 16 μm (n = 10; range 42–51 μm by 14–18 μm). The funnel averaged 17 μm in width and 10 μm in depth from the margin to the cytostome (range 16–19 μm by 9–10 μm). A single large ovoid macronucleus was located near the base of the neck and averaged 10 μm by 5 μm (range 8–12 μm by 5–7 μm). Micronuclei, only visible in a few individuals, occurred in pairs and were not closely associated with the macronucleus. The interior of the funnel contained two fields of cilia: (1) the epichonal field (EF) was confined to a small rectangular area approximately 8 by 4 μm and contained 10 very short kineties 4–5 μm long, and (2) the hypochonal field (HF) contained 7–8 kineties situated perpendicularly to the long axis of the cell. Tomites averaged 30 μm in length by 11 μm in width (n = 5, range 23–35 μm by 9–13 μm). The right field (RF) was comprised of 8–9 kineties, which were aligned along the right edge of the cell body, while the left field (LF) was composed of 5–6 very short kineties (3–5 μm). A short transpodial field (TF) at the posterior extreme of RF contained 6–8 kineties or kinetofragments 4–6 μm in length.

ssu-rDNA. We amplified ~ 1.6 kb of the ssu-rDNA gene from nine samples of ciliates in the class Phyllopharyngea (Table 3). Between two and four ssu-rDNA clones per sample were sequenced and the average pairwise distances among clones for all but one sample were less than or equal to 0.4% (Table 3). The one exception is the genus *Ephelota* where we obtained two phylotypes from 11 clones. The first phylotype is represented by one sequence (AY331805), which is identical to nine clones collected from this DNA sample for a previous study by

Table **3**. Intraspecific variation and accession numbers for sequences collected in this study.

Taxon	Classification	# Clones	% Divergence	Genbank #s
Acineta sp.	Suctoria	3	0.40	AY332717
				AY332718
				AY332719
Chlamydodon exocellatus	Phyllopharyngia	4	0.1	AY331790
				AY331791
				AY331792
				AY331793
Chlamydodon triquetrus	Phyllopharyngia	3	0.2	AY331794
				AY331795
				AY331796
Dysteria sp. 2001	Phyllopharyngia	3	0.35	AY331797
				AY331798
				AY331799
Dysteria sp. 2002	Phyllopharyngia	2	0.25	AY331800
				AY331801
Ephelota sp. 1	Suctoria	2	6.3	AY331805
Ephelota sp. 2				AY331804
Isochona sp.	Chonotrichia	4	0.3	AY242116
				AY242117
				AY242118
				AY242119
Prodiscophyra collini	Suctoria	2	0.13	AY331802
				AY331803
Tokophrya lemnarum	Suctoria	2	0.04	AY332720
				AY332721

Underlined accession numbers indicate sequences used in phylogenetic analyses.

Riley and Katz (2001), and is labeled *Ephelota* sp. 1. The second phylotype, represented by one clone (AY331804), differs from the first by 6.3% (Table 2) and is labeled *Ephelota* sp. 2.

Comparisons of uncorrected average pairwise differences among specific clades can provide a rough estimate of relative divergence times. The average pairwise distance between the three *Ephelota* species used in our analysis is 6.57%. The *Chlamydodon* and *Tokophyra* congeners differ at the nucleotide level by 2.97% and 4.18% respectively. Two dysteriid populations, one field caught in 2001 and the other in 2002, are morphologically identical and differ at the nucleotide level by only 0.54%. Average pairwise distances are 16.5% within the Suctoria and 13.6% within the Cyrtophoria. The average pairwise differences between the *Chlamydodon* spp. and the *C. uncinata* + *T. steini* clade is 16.4%, while the difference between the *Chlamydodon* spp. and the *Dysteria* sp. is 12.5%.

Genealogies. Genealogical analyses of our conservatively masked alignment, using a variety of evolutionary models and phylogenetic algorithms generated similar topologies for relationships within the Phyllopharyngea (Figs. 1c, 2). For example, our analyses provide strong support for the monophyly of the Phyllopharyngea and the Suctoria (Fig. 3) with bootstrap support under all models of over 94% and 90%, respectively. The topology also indicates that the chonotrich *Isochona* sp.

Table **4**. Group I intron sequences from Genbank used in this study.

Organism	GB #	Organism	GB #
Acrasis rosea	AF011458	*Protoderma sarcinoidea*	Z47998
Ankistrodesmus stipitatus	X56100	*Protomyces inouye*	D11377
Cenococcum geophilum	Z11998	*Rhodosporidium dacryoidum*	D13459
Characium saccatum	M84319	*Scenedesmus producto-capitatus*	X91266
Chlorella ellipsoidea	X63520	*Tetrahymena thermophila*	V01416
Chlorella luteoviridis	X73997	*Tilletiopsis flava*	D82819
Chlorella mirabilis	X74000	*Trebouxia arboricola*	Z68705
Chlorella saccharophila	AB058310	*Trebouxia usneae*	Z68702
Chlorella sorokiniana	X73993	*Urospora penicilliformis*	AB049417
Choricystis minor	X89012	*Ustilago maydis*	X62396
Claviocorona pyxidata	U59066	*Zyngema circumcarinum*	X79495
Dunaliella parva	M62998		
Dunaliella salina	M84320		
Fusicladium effusum	U63629		
Hymenoscyphus ericae	U06868		
Klebsormidium flaccidum	X75520		
Mesotaenium caldariorum	X75763		
Muriella aurantica	AB005748		
Nannochloris sp.	X81965		
Panellus stipticus	U59090		
Plasmodiophora brassicae	U18981		

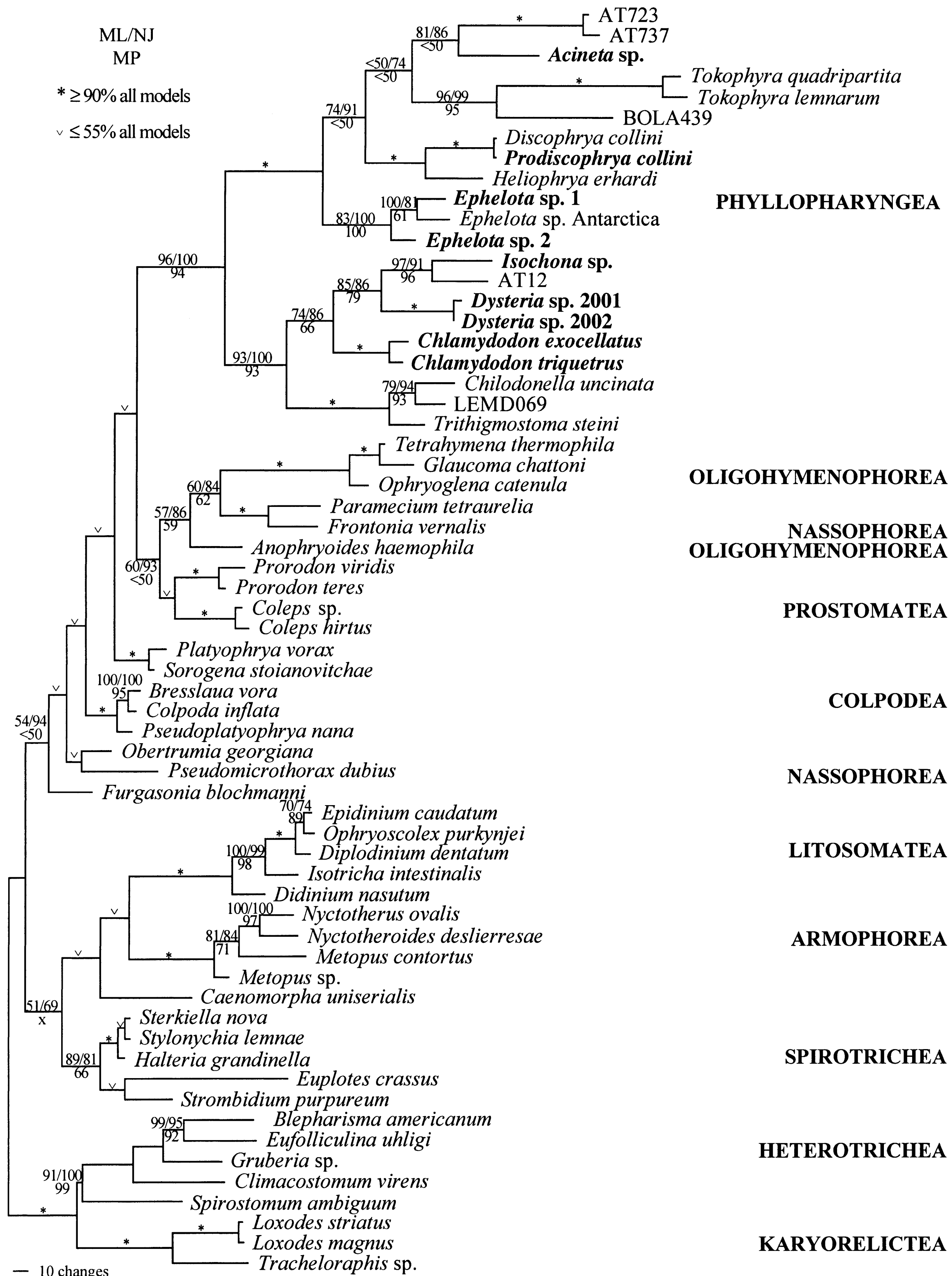

Fig. 3. Genealogy for small subunit rDNA sequences, with new sequences indicated in bold. Bootstrap values greater than 50% (100 replicates) from all analyses (ML=maximum likelihood; NJ = neighbor-joining; MP = maximum parsimony (see text for further details)) are shown at branch nodes. Accession numbers are in Tables 2 and 3.

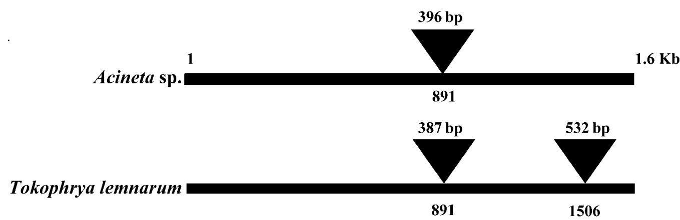

Fig. **4**. Insertion positions of Group I introns in the small subunit rRNA gene of *Acineta* sp. and *Tokophrya lemnarum*. The positions of the introns are indicated and numbers correspond to position in *Escherichia coli* (Cannone et al. 2002; Johansen and Haugen 2001).

evolved from within the Phyllopharyngia clade and is sister to the *Dysteria* sp. sequences (Fig. 3). The Suctoria are monophyletic and the exogenous budders (*Ephelota* spp.) are basal. Three sequences from environmental surveys also fall within this clade; two (AT7 23 and AT7 37) are from ssu-rDNA surveys of Mid-Atlantic Ridge hydrothermal sediment (Lopez-Garcia et al. 2003), and the other (BOLA 439) is from anoxic sediment (Dawson and Pace 2002). Although there is less support for relationships among classes, our analyses consistently place the class Phyllopharyngea sister to members of the classes Colpodea, Oligohymenophorea, Prostomatea, and Nassophorea. No substantial change was seen in topology using a semi-conservative mask (data not shown).

Introns. We discovered three relatively large (396–532 nucleotides) group I introns in the ssu-rDNA of *Acineta* sp. and *T. lemnarum* (Fig. 4). *Acineta* sp. has one intron (396 bp) at position 891 in the ssu-rDNA of *Escherichia coli* (J01695), the reference standard for ssu-rRNA intron positions. In *T. lemnarum*, two introns (387 bp and 532 bp in size) interrupt the ssu-rDNA at sites 891 and 1506 respectively (Fig. 4). The first nucleotide in the 3′- end of the exon at the 5′- splice junction is a T and the 3′- end terminates with a G residue, which is universally conserved among group I introns. Sequences in the catalytic core are also conserved and the introns do not appear to encode a mobility-conferring open reading frame (ORF), as no signature sequences of such an ORF could be found. These data suggest the Suctoria introns are affiliated with the IC1 group I introns. Further evidence for the affiliation of at least one of our introns in *T. lemnarum* with the IC1 group is its insertion site position of 1506, as all other introns with this insertion site are within the IC1 structural group. The other two suctorian introns occur at position 891 and until now, only one other group I intron has been found at this position (*Acrasis rosea* (AF011458); Comparative RNA web site (CRW) (http://www.rna.icmb.utexas.edu/)).

The introns at position 891 in *Acineta* sp. and *T. lemnarum* differ by 10.6%. Genealogical analyses indicate these newly discovered introns at position 891 form a weakly supported clade with the 891 intron in *Acrasis rosea* and the *T. thermophila* large subunit rRNA introns. The second *T. lemnarum* intron (position 1506) is highly divergent from previously characterized introns, as demonstrated by its long branch and poorly supported position in our genealogy (Fig. 5).

DISCUSSION

Relationships among phyllopharyngean ciliates. The aim of this study was to test the monophyly of, and determine relationships among taxa in three of the four subclasses of the Phyllopharyngea. The monophyly of the Phyllopharyngea is well supported in ssu-rDNA analyses (Fig. 3). This finding is in accordance with the accepted taxonomy based on the shared morphological characters of the Suctoria, Phyllopharyngia, and Chonotrichia (Table 1). Because of the similar divergences among the *Dysteria*, *C. uncinata* + *T. steini* and *Chlamydodon* spp. sequences, our ssu-rDNA genealogy indicates that the Order Chilodonellida be retained as in the taxonomic scheme of de Puytorac et al. (1994) and not subsumed as an order of Chlamydodontida as in Lynn and Small (2002)

The ssu-rDNA genealogy also places the Chonotrichia within the Phyllopharyngia, suggesting that heteromeric nuclei and the somatic ciliature are phylogenetically informative (Corliss 1979; Foissner 1996; Grell and Meister 1982; Puytorac et al. 1994). Also, several workers including Kent (1880–1882) and Dobrzánska-Kaczanowska (1963) have recognized the resemblance of the migratory ''larval'' form of chonotrichs to adult Phyllopharyngia. The paraphyly of the Phyllopharyngia with respect to the single sequence we have from the subclass Chonotrichia, suggests that the latter subclass might be derived from within the Phyllopharyngia (Lynn and Small 2002) or the subclass Cyrtophoria (de Puytorac 1994) (Fig. 1c, 3). The presence of a podite in dysterids and the tomite or swarmer cells of chonotrichs provide morphological support for the ssu-rDNA genealogy, which suggests that the chonotrichs are derived from within the Dysteriida. This also implies that morphological difference between adult chonotrichs and Phyllopharyngia may be misleading taxonomically, and that the morphology of tomites is more conserved and therefore a more reliable indicator of common ancestry.

Within the Phyllopharyngea, our analyses support a monophyletic origin of Suctoria, as this subclass is distinct from the Chonotrichia and Phyllopharygnia. In addition, our data, while admittedly only a preliminary sampling of suctorian diversity, show that budding types within the Suctoria are monophyletic, consistent with the idea that ''simple'' exogenous budding evolved first (cf. Fig. 3, 6). Endogenous budding, and the even more complex evaginative budding, appear to be derived (Fig. 6).

Relationships between Phyllopharyngea and other ciliates. Our genealogical analyses consistently place the Phyllopharyngea as sister to the classes Colpodea, Oligohymenophorea, Nassophorea, and Prostomatea, although support is weak at deep nodes (Fig. 3). There is no support for a close relationship between Phyllopharyngea and either Litostomatea, Armophorea, or Spirotrichea. According to Lynn and Small (2002), similarities in the pharyngeal basket (cyrtos) of Protostomatea, Colpodea, Nassophorea, and Phyllopharyngea, correlated with similarities in somatic kinetids, suggest that these four classes may be related. Our analyses indicate that the Oligohymenophorea, with their very different oral structures, also fall within this larger group of ciliates. In contrast, our data do not support the suggestion by de Puytorac (1994) that the Phyllopharyngea are a sister group to the Oligohymenophorea, Litostomatea and Colpodea; the Litostomatea do not show close relationship to the Phyllopharyngea under any evolutionary model or phylogenetic algorithm that we used.

Group I introns in Suctoria. Group I introns act as ribozymes, catalyzing their self-splicing from precursor transcripts (Cech 1988; Cech and Herschlag 1996), and were first characterized from the ciliate *Tetrahymena thermophila* (Grabowski et al. 1981). No group I introns had been reported in ciliates rDNAs since their Nobel prize-winning discovery over twenty years ago. On the basis of intron conserved secondary structure characteristics (Cech 1988; Michel et al. 1982), conserved core nucleotide regions (Golden et al. 1998; Michel and Westhof 1990), tertiary interactions and phylogenetic analysis, group I introns have been classified into twelve subclasses within five major groups as follows: IA1–3, IB1–4, IC1–3, ID, and IE (Michel and Westhof 1990; Sub et al. 1999). Moreover group

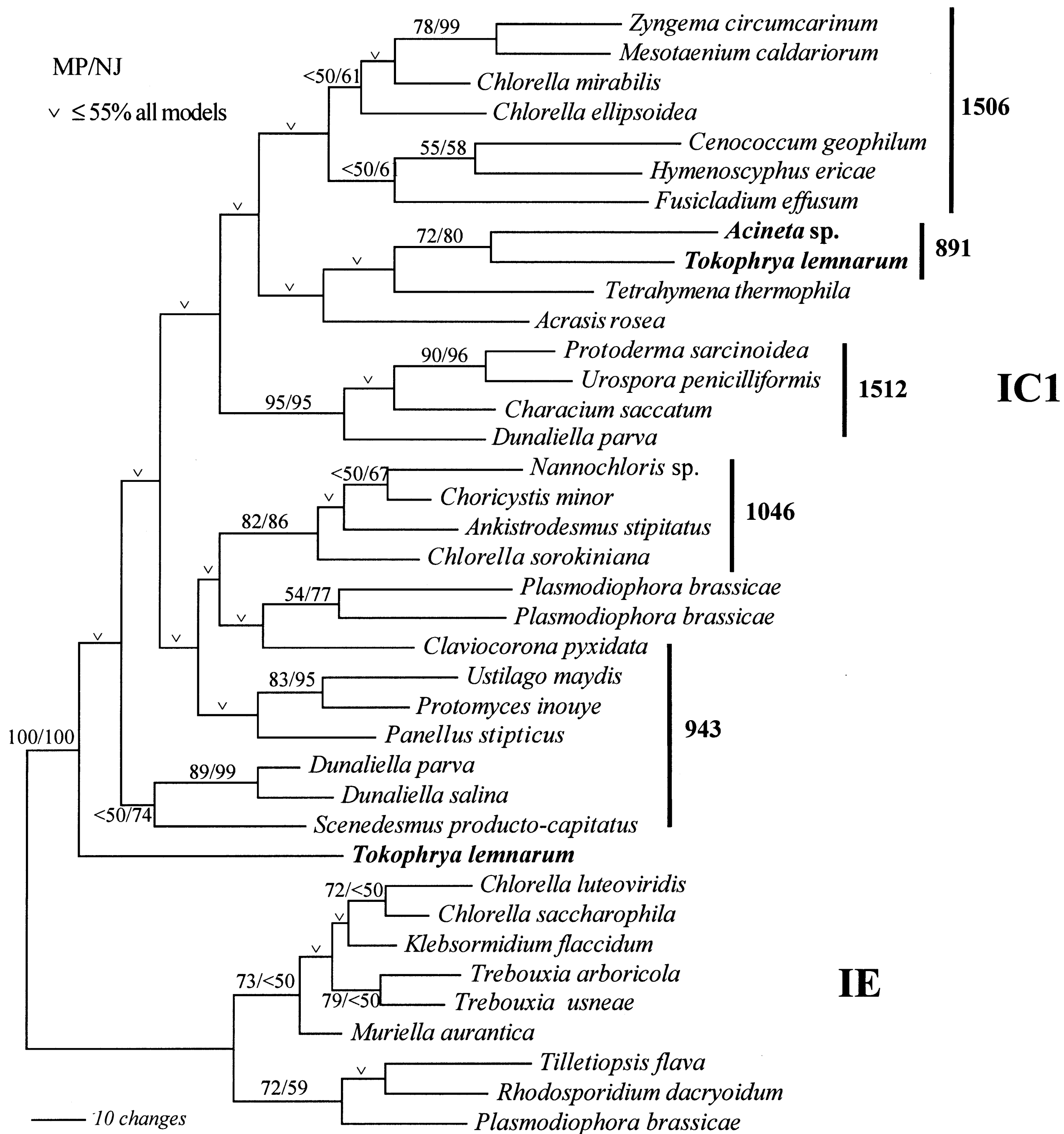

Fig. 5. Maximum Parsimony (MP) genealogy of group I introns, based on an alignment from Oliveira and Bhattacharya (2000). Bootstrap values greater than 50% from both analyses (NJ = neighbor-joining; MP = maximum parsimony (see text for further details)) are shown at branch nodes and new ciliate introns are shown in bold. Two main subclasses of group I introns (IC1 and IE) and insertion sites are also indicated. Accession numbers are in Table 4.

I introns are highly mobile and have a complex distribution in the rDNA and organellar genes of many microbial eukaryotes including green and red algae, fungi, ciliates, and some amoebae (e.g. Angata et al. 1995; Bhattacharya et al. 1996; Boucher et al. 1996; Sub et al. 1999; Wang et al. 2003). Group I introns are known from more than 1,200 taxa and integrate at eighty different sites in the ribosomal genes of protists and fungi (Cannone et al. 2002; Jackson et al. 2002). Given the apparent rapid rate of insertion and deletion of group I introns in many clades, thorough taxonomic sampling is necessary to determine the tempo and mode of intron transfer among eukaryotes.

Our characterization of introns in *Acineta* sp. and *T. lemnarum* is only the second example of group I introns in a ciliate ribosomal gene, since the discovery of a ribozyme in the large subunit rDNA gene of *T. thermophila*. This suggests that group I introns are relatively rare in ciliates. The two introns at position 891 cluster together in our genealogical analysis, albeit with only low to moderate bootstrap support (Fig. 5), suggest-

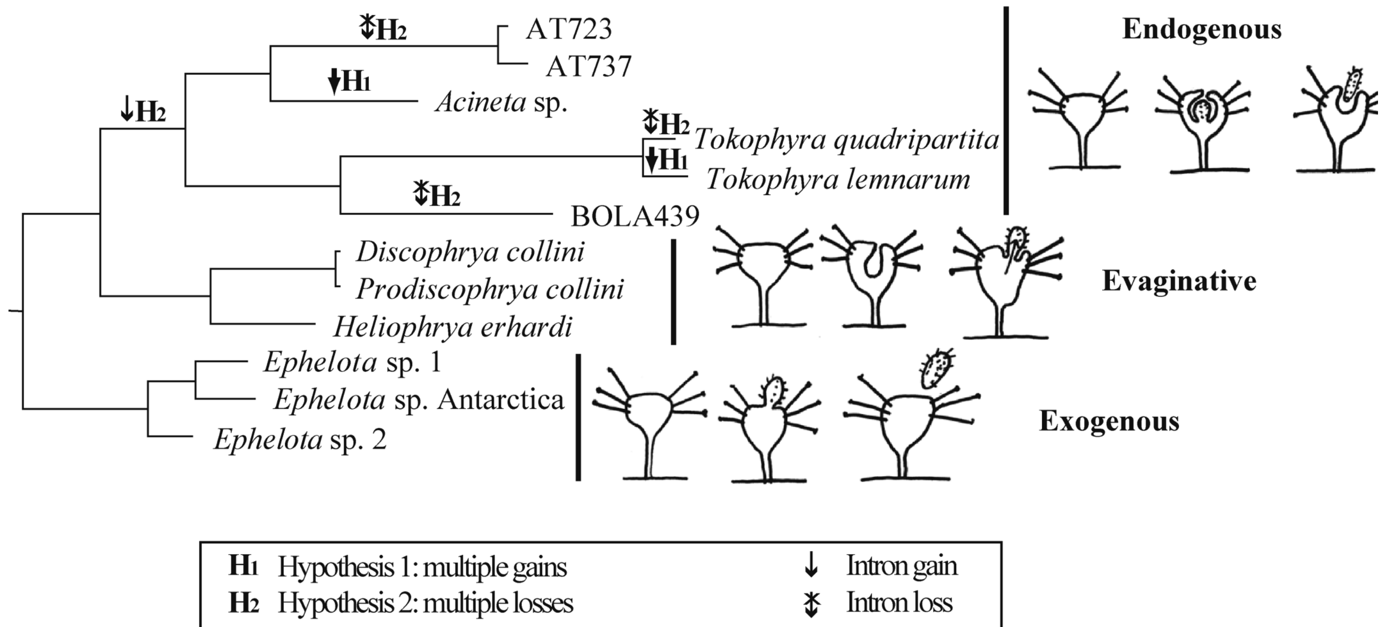

Fig. **6**. Budding types in Suctoria and two hypotheses of intron evolution mapped onto the suctorian phylogeny. The two hypotheses are: (H_1) multiple gain of the 891 intron; or (H_2) a single gain followed by multiple losses.

ing that there may have been an intron at this position in the common ancestor of these taxa. Mapping the distribution of this intron onto our suctorian genealogy reveals a more complex picture as the sister taxa to *T. lemnarum* lack an intron and the environmental samples, which are sister taxa to *Acineta* sp. also lack an intron (Fig. 6). There are two hypotheses for the evolution of the position 891 intron in Suctoria: (H_1) the 891 introns were gained independently in *Acineta* sp., and *T. lemnarum* (Fig. 6); or (H_2) the intron was present in the common ancestor of endogenous budders and was lost in *Tokophrya quadripartita* and the taxa characterized by environmental sampling (Fig. 6). Further sampling of ssu-rDNAs is required to distinguish between these hypotheses, and to elucidate the timing of the acquisition of the 1506 intron in *T. lemnarum*.

ACKNOWLEDGMENTS

This work was support by a grant from the Tomlinson Fund at Smith College to AC, and NSF grants DEB-0092908 and DEB-0079325 to LAK. We also are grateful for funding from the Smithsonian Marine Station at Fort Pierce, FL (SMS contribution # 588). The authors thank Debasish Bhattacharya for advice on analyzing introns.

LITERATURE CITED

Angata, K., Ogawa, S., Yanagisawa, K. & Tanaka, Y. 1995. A Group-I intron in the mitochondrial large-subunit ribosomal RNA encoding gene of *Dictyostelium discoideum*—same site localization in alga and *in vitro* self-splicing. *Gene,* **153**:49–55.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. 1993. Current Protocols in Molecular Biology. Wiley-Liss, New York.

Bernhard, D., Leipe, D. D., Sogin, M. L. & Schlegel, K. M. 1995. Phylogenetic relationships of the Nassulida within the phylum Ciliophora inferred from the complete small subunit rRNA gene sequences of *Furgasonia blochmanni*, *Obertrumia georgiana*, and *Pseudomicrothorax dubius*. *J. Eukaryot. Microbiol.,* **42**:126–131.

Bhattacharya, D., Friedl, T. & Damberger, S. 1996. Nuclear-encoded rDNA group I introns: origin and phylogenetic relationships of insertion site lineages in the green algae. *Mol. Biol. Evol.,* **13**:978–989.

Boucher, H., Mercure, S., Montplaisir, S. & Lemay, G. 1996. A novel group I intron in *Candida dubliniensis* is homologous to a *Candida albicans* intron. *Gene,* **180**:189–196.

Cannone, J. J., Subramanian, S., Schnare, M. N., Collett, J. R., D'Souza, L. M., Du, Y. S., Feng, B., Lin, N., Madabusi, L. V., Muller, K. M., Pande, N., Shang, Z. D., Yu, N. & Gutell, R. R. 2002. The Comparative RNA Web (CRW) Site: an online database of comparative sequence and structure information for ribosomal, intron, and other RNAs. *BMC Bioinformatics,* **3**:

Cech, T. R. 1988. Conserved sequences and structures of Group-I Introns—building an active-site for RNA catalysis—a review. *Gene,* **73**:259–271.

Cech, T. R. & Herschlag, D. 1996. Group I ribozymes: substrate recognition, catalytic strategies, and comparative mechanistic analysis. *In:* Eckstein, F. & Lilley, J. (ed.), Catalytic RNA. Nucleic Acids and Molecular Biology. Springer Verlag, Berlin/Heidelberg/New York. p. 1–17.

Chatton, E. & Lwoff, A. 1939. Sur la systématique de la tribu des thigmotriches rhynchoides. Les deux familles des Hypocomidae Bütschli et des Ancistrocomidae n. fam. Les deux genres noveaux, *Heterocoma* et *Parahypocoma*. *Compt. Rend. Acad. Sci.,* **209**:429–431.

Claparède, E. & Lachmann, J. 1859. Etudes sur les infusoires et les rhizopodes. *Mém. Inst. Nat. Genèvois,* **6**:261–482.

Coats, D. W. & Heinbokel, J. F. 1982. A study of reproduction and other life-cycle phenomena in planktonic protists using an acridine-orange fluorescence technique. *Mar. Biol.,* **67**:71–79.

Corliss, J. O. 1956. On the evolution and systematics of ciliated protozoa. *Syst. Zool.,* **5**:68–91.

Corliss, J. O. 1979. The Ciliated Protozoa: Characterization, Classification and Guide to the Literature. Pergammon Press, Oxford.

Dawson, S. C. & Pace, N. R. 2002. Novel kingdom-level eukaryotic diversity in anoxic environments. *Proc. Natl. Acad. Sci. USA,* **99**:8324–8329.

Dobrzaprñska-Kaczanowska, J. 1963. Comparaison de la morphogenèse des ciliés: *Chilodonella uncinata* (Ehrbg.), *Allosphaerium paraconvexa* sp. n. et *Heliochona scheuteni* (Stein). *Acta Protozool.,* **1**:353–394.

Foissner, W. 1996. Ontogenesis in ciliated protozoa with emphasis on stomatogenesis. *In:* Hausmann, K. & Bradbury, P. C. (ed.), Cells as Organisms. Gustav Fisher, Stuttgart. p. 95–178.

Golden, B. L., Gooding, A. R., Podell, E. R. & Cech, T. R. 1998. A

preorganized active site in the crystal structure of the *Tetrahymena* ribozyme. *Science,* **282**:259–264.

Gong, J., Song, W. B. & Warren, A. 2002. Redescriptions of two marine cyrtophorid ciliates, *Dysteria cristata* (Gourret and Roeser, 1888) Kahl, 1931 and *Dysteria monostyla* (Ehrenberg, 1838) Kahl, 1931 (Protozoa, Ciliophora, Cyrtophorida), from China. *Eur. J. Protistol.,* **38**:213–222.

Grabowski, P. J., Zaug, A. J. & Cech, T. R. 1981. The intervening sequence of the ribosomal RNA precursor is converted to a circular RNA in isolated nuclei of *Tetrahymena*. *Cell,* **23**:467–476.

Grell, K. G. & Meister, A. 1982. On the taxonomic position of Hypocomidae (Ciliata). *Z. Naturfosch.,* **37**:1050–1052.

Hammerschmidt, B., Schlegel, M., Lynn, D. H., Leipe, D. D., Sogin, M. L. & Raikov, I. B. 1996. Insights into the evolution of nuclear dualism in the ciliates revealed by phylogenetic analysis of rRNA sequences. *J. Eukaryot. Microbiol.,* **43**:225–230.

Israel, R. L., Kosakovsky Pond, S. L., Muse, S. V. & Katz, L. A. 2002. Evolution of duplicated alpha-tubulin genes in ciliates. *Evolution,* **56**: 1110–1122.

Jackson, S. A., Cannone, J. J., Lee, J. C., Gutell, R. R. & Woodson, S. A. 2002. Distribution of rRNA introns in the three-dimensional structure of the ribosome. *J. Mol. Biol.,* **323**:35–52.

Jankowski, A. W. 1973. Fauna of the USSR: Infusoria subclass Chonotrichia. *Akad. Nauk. SSSR, Leningrad.* 356 pp.

Jankowski, A. W. 1979. Phylogeny and divergence of suctorians. Translated from *Dokl. Aka. Nauk. SSSR,* **242**:493–496.

Johansen, S. & Haugen, P. 2001. A new nomenclature of group I introns in ribosomal DNA. RNA—a Publication of the RNA Society. **7**:935–936.

Kahl, A. 1931. Urtiere oder Protozoa. I. Wimpertier oder Ciliata (Infusoria), a). Holotricha. *In:* Dahl, F. (ed.), Die Tierwelt Deutschlands. Gustav Fischer, Jena. p. 181–398.

Katz, L. A., Lasek-Nesselquist, E., Bornstein, J. & Muse, S. V. 2004. Dramatic diversity of ciliate histone H4 genes revealed by comparisons of patterns of substitutions and paralog divergences among eukaryotes. *Mol. Biol. Evol.,* **21**:555–562.

Kent, W. S. 1880–1882. A Manual of the Infusoria. David Bogue, London.

Kuhlmann, H. W. 1998. Do phototactic ciliates make use of directional antennas to track the direction of light? *Eur. J. Protistol.,* **34**:244–253.

Leipe, D. D., Bernhard, D., Schlegel, M. & Sogin, M. L. 1994. Evolution of 16S-like ribosomal RNA genes in the ciliophoran taxa Litostomatea and Phyllopharyngea. *Europ. J. Protistol.,* **30**:354–361.

Lopez-Garcia, P., Philippe, H., Gail, F. & Moreira, D. 2003. Autochthonous eukaryotic diversity in hydrothermal sediment and experimental microcolonizers at the Mid-Atlantic Ridge. *Proc. Natl. Acad. Sci. USA,* **100**:697–702.

Lynn, D. H. 1996. Systematics of ciliates. *In:* Hausmann, K. & Bradbury, P. C. (ed.), Ciliates: Cells as Organisms. Gustave Fischer Verlag, Stuttgart.

Lynn, D. H. & Corliss, J. O. 1991. Ciliophora. *In:* Harrison, F. W. (ed.), Microscopic Anatomy of Invertebrates. John Wiley & Sons, Inc., New York. p. 333–467

Lynn, D. H. & Small, E. B. 1997. A revised classification of the phylum Ciliophora Doflein, 1901. *Rev. Soc. Mex. Hist. Nat.,* **47**:65–78.

Lynn, D. H. & Small, E. B. 2002. Phylum Ciliophora Doflein, 1901. *In:* Lee, J. J., Leedale, G. F. & Bradbury, P. C. (ed.), An Illustrated Guide to the Protozoa, 2nd ed. Society of Protozoologists, Lawrence, Kansas. p. 371–656

Lynn, D. H. & Strüder-Kypke, M. 2002. Phylogenetic position of Licnophora, Lechriopyla, and Schizocaryum, three unusual ciliates (Phylum Ciliophora) endosymbiotic in echinoderms (Phylum Echinodermata). *J. Eukaryot. Microbiol.,* **49**:460–468.

Medlin, L., Elwood, H. J., Stickel, S. & Sogin, M. L. 1988. The characterization of enzymatically amplified eukaryotes 16S-like ribosomal RNA coding regions. *Gene,* **71**:491–500.

Méténier, G. & Hufschmid, J. D. 1988. Evidence of extensive fragmentation of macronuclear DNA in two non-hypotrichous ciliates. *J. Protozool,.* **35**:71–73.

Michel, F., Jacquier, A. & Dujon, B. 1982. Comparison of fungal mitochondrial introns reveals extensive homologies in RNA secondary structure. *Biochimie,* **64**:867–881.

Michel, F. & Westhof, E. 1990. Modeling of the 3-dimensional architecture of Group-I catalytic introns based on comparative sequence-analysis. *J. Mol. Biol.,* **216**:585–610.

Montagnes, D. J. S. & Lynn, D. H. 1993. A quantitative protargol stain (QPS) for ciliates and other protists. *In:* Kemp, P. F., Sherr, B. F., Sherr, E. B. & Cole, J. J. (ed.), Handbook of Methods in Aquatic Microbial Ecology. Lewis Publishers, Boca Raton. p. 229–240

Oliveira, M. C. & Bhattacharya, D. 2000. Phylogeny of the Bangiophycidae (Rhodophyta) and the secondary endosymbiotic origin of algal plastids. *Am. J. Bot.,* **87**:48-2–492.

Ozaki, Y. & Yagiu, R. 1941. Studies on the marine ciliates of Japan, mainly from the Setonaikai (the Inland Sea of Japan). *J. Sci. Hiroshima Univ. series B, Div. 1,* **8**:165–184.

Posada, D. & Crandall, K. 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics,* **14**:817–818.

Puytorac, P. de 1994. Phylum Ciliophora Doflein, 1901. *In:* P. P. de, (ed.) Traité de Zoologie, Tome II, Infusoires Ciliés, Fasc. 2, Systématique. Masson, Paris. p. 1–15.

Puytorac, P. de, Grain, J. & Legendre, P. 1994. An attempt at reconstructing a phylogenetic tree of the Ciliophora using parsimony methods. *Eur. J. Protist.,* **30**:1–17.

Puytorac, P. de, Batisse, A., Bohatier, J., Corliss, J. O., Deroux, G., Didier, P., Dragesco, J., Fryd-Versavel, G., Grain, J., Groliére, C.-A., Iftode, F., Laval, M., Roque, M., Savoie, A. & Tuffrau, M. 1974. Proposition d'une classification du phylum Ciliophora Doflein, 1901. *C. R. Acad. Sci. Paris,* **278**:2799–2802.

Riley, J. L. & Katz, L. A. 2001. Widespread distribution of extensive genome fragmentation in ciliates. *Mol. Biol. Evol.,* **18**:1372–1377.

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. & Erlich, H. A. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA-polymerase. *Science,* **239**:487–491.

Shin, M. K., Hwang, U. W., Kim, W., Wright, A. D. G., Krawczyk, C. & Lynn, D. H. 2000. Phylogenetic position of the ciliates *Phacodinium* (Order Phacodiniida) and *Protocruzia* (Subclass Protocruziidia) and systematics of the spirotrich ciliates examined by small subunit ribosomal RNA gene sequences. *Eur. J. Protistol.,* **36**:293–302.

Snoeyenbos-West, O. L. O., Salcedo, T., McManus, G. B. & Katz, L. A. 2002. Insights into the diversity of choreotrich and oligotrich ciliates (Class: Spirotrichea) based on genealogical analyses of multiple loci. *Int. J. Syst. Evol. Microbiol.,* **52**:1901–1913.

Strüder-Kypke, M. C. & Lynn, D. H. 2003. Sequence analyses of the small subunit rRNA gene confirm the paraphyly of oligotrich ciliates *sensu lato* and support the monophyly of the subclasses Oligotrichia and Choreotrichia (Ciliophora, Spirotrichea). *J. Zool.,* **260**:87–97.

Sub, S. O., Jones, K. G. & Blackwell, M. 1999. A Group I intron in the nuclear small subunit rRNA gene of *Cryptendoxyla hypophloia*, an ascomycetous fungus: evidence for a new major class of Group I introns. *J. Mol. Evol.,* **48**:493–500.

Swofford, D. 2002. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Sinauer Assoc., Sunderland, MA.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. 1994. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucl. Acids Res.,* **22**:4673–4680.

Wallengren, H. 1895. Studier öfver Ciliata infusorier. II. Slägtet *Heliochona* Plate, slägtet *Chilodochona* n.g., slägtet *Hemispeira* Fabre-Domergue. *Acta Univ. Lund.,* **1**:77.

Wang, C. S., Li, Z. Z., Typas, M. A. & Butt, T. M. 2003. Nuclear large subunit rDNA group I intron distribution in a population of *Beauveria bassiana* strains: phylogenetic implications. *Mycol. Res.,* **107**:1189–1200.

Received 01-16-04, 04-21-04; accepted 03-16-04