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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

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Summary

The small subunit rRNA (SSrRNA) genes were sequenced for *Protocruzia* sp2, *Phacodinium metchnicoffi*, *Holosticha multistylata*, and *Halteria grandinella*. All four genera are placed within the Class Spirotrichea with strong bootstrap support in both distance matrix and parsimony tree construction methods and by maximum likelihood analysis using quartet puzzling. *Protocruzia* sp2 groups with *Protocruzia* sp1 with 100% bootstrap support, and the 5.6% genetic difference between them strongly argues that they are different species although they are morphologically quite similar. The *Protocruzia* species branch first in the spirotrich clade at a deep level, supporting their recognition as a Subclass Protocruziidia. *Phacodinium* branches after *Euplotes* at a deep level, confirming the conclusion that others have reached that this genus is related to the hypotrich and stichotrich spirotrichs. *Phacodinium* is assigned to its own family and order, and we conclude that the deep branching within the spirotrichs argues for its own subclass, the Subclass Phacodiniidia. Consistent with the partial SSrRNA sequences and with the sequence of a polymerase gene, *Halteria* groups within the stichotrich clade, supporting the argument that the oligotrichs are not monophyletic as currently conceived. Finally, *Holosticha*, which has been assigned to the Order Urostylida, groups outside the stichotrichs with parsimony analysis, which is consistent with this ordinal assignment. However, it is associated with *Halteria* and *Oxytricha granulifera* in the other analyses. Additional stichotrich sequences obviously are required before we can confidently begin revision of the Subclass Stichotrichia.

Key words: PCR; Ciliophora; Stichotrich; Hypotrich; Puzzling; *Halteria*.

Introduction

The hypotrich (e.g., euplotid) and stichotrich (e.g., oxytrichid) ciliates are among the most easily recognizable ciliates. They are characterized by a dorsoventral flattening of the body with the presence of numerous compound somatic ciliary structures or cirri on the ventral surface and a wreath-like set of oral polykinetids along the left side of the oral region [6]. Of the 8,000 species of described ciliates, nearly 500 species belong to these two groups, which also typically show a replication band that passes through the macronucleus during the S-phase of DNA synthesis [41]. This replication band is also exhibited during macronuclear S-phase in choreotrich and oligotrich ciliates, although they do not possess typical cirri and dorsoventrally-flattened bodies. This suggests that these two groups should be included in the same clade as the hypotrich and stichotrich ciliates [33]. Lynn and Small [34] have thus included the hypotrichs (e.g., *Euplotes*), sti-

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chotrichs (e.g., *Oxytricha*, *Stylonychia*), choreotrichs (e.g., *Tintinnopsis*, *Strobilidium*), and oligotrichs (e.g., *Halteria*, *Strombidium*) within the Class Spirotrichea.

Ultrastructural studies of some unusual taxa have suggested that they might also be associated with this spirotrich clade. *Protocruzia* has an unusual nuclear complex [1, 43], reminiscent of the complex chromosomes seen during macronuclear development in hypotrichs and stichotrichs. It has a relatively sparse somatic ciliature [24, 55], and the ultrastructure of the somatic kinetids suggested relationships to the heterotrichs [24]. Hammerschmidt et al. [25] sequenced the small subunit rRNA (SSrRNA) gene of a *Protocruzia* species and found that it associated with the spirotrich clade in distance matrix analyses only. Bernhard and Schlegel [3] also could not resolve its position using histone genes. Since the density of species in a clade can stabilize that clade's position in the topology [51], we undertook to sequence another species of *Protocruzia* to assess whether the association with the spirotrichs was confirmed.

Another potential spirotrich, *Phacodinium metchnicoffi*, was redescribed in the early 1970s by Roque [42] and Dragesco [11], who described linear groupings of kinetosomes in the somatic kineties. Dragesco [11] concluded that *Phacodinium* was a highly unusual heterotrich. Didier and Dragesco [9, 10] described the ultrastructure of *Phacodinium* and confirmed the highly unusual nature of the linear somatic polykinetids compared to the somatic dikinetids of heterotrichs, but still maintained the genus in its own family within the heterotrichs, pending careful redescription of its oral structures. Fernández-Galiano and Calvo [18] undertook the redescription of the somatic and oral structures using the silver carbonate technique. They made two important observations that led them to conclude that *Phacodinium* ought to be associated with the hypotrichs and stichotrichs: 1) they observed that some of the somatic polykinetids were actually composed of two rows of kinetosomes, reminiscent of the simpler cirri in stichotrichs; and 2) they reported a highly unusual, polykinetid-like paroral that was similar to the paroral of *Euplotes* [18]. In an ultrastructural investigation, da Silva Neto [49] described the somatic polykinetids with two rows, confirmed the complex structure of the paroral, and concluded that *Phacodinium* was intermediate between the heterotrichs and the hypotrichs. To our knowledge, *Phacodinium* does not exhibit a replication band during interphase, although its macronuclear shape is very similar to that of *Euplotes*. We have undertaken to sequence the SSrRNA gene of *Phacodinium* to determine whether it is related to the spirotrich clade in the subphylum Intramacronculeata.

We have provided complete SSrRNA sequences of two additional genera in this study, *Halteria* and *Holosticha*. Lynn and Sogin [36] first presented evidence using reverse transcriptase and an incomplete gene sequence that *Halteria* was closely related to the stichotrichs. This relationship has been confirmed using polymerase sequences [28]. We have completed the SSrRNA gene sequence of *Halteria* to determine if its position remains stable in the SSrRNA gene tree.

During the past two decades, there is increasing evidence that unravelling evolution within the hypotrich and stichotrich lineages will be difficult. Each investigator who interprets the morphostatic and morphogenetic features of these ciliates arrives at a slightly different classification scheme, often conflicting in major aspects [5, 6, 12, 20, 22, 29, 34, 35, 50, 60, 63]. This may be taken as evidence that a new set of characters, potentially molecular ones, might aid in resolving these differences. To begin to increase the species sampling of stichotrichs, we have sequenced the SSrRNA gene from *Holosticha multistylata*. This genus has been classified with urostyline ciliates and so should be expected to be basal to the other stichotrichs that have been sequenced and that are all grouped as sporadotrichs [35].

Materials and Methods

Ciliate collection and culture. *Holosticha multistylata* and *Phacodinium metchnicoffi* were collected from mosses in Seoul, Korea. Clonal cultures were established and maintained in neutral Pringsheim solution or autoclaved commercial mineral water with the appropriate prey. *Halteria grandinella* (strain H6-1) was obtained from Dr. David Prescott (University of Colorado, Boulder) and cultured on 0.05–0.1% (w/v) cereal grass leaves infusion previously bacterized with *Enterobacter aerogenes*. *Protocruzia* sp2 was obtained from Dr. T. Nerad (ATCC) and grown on a 0.05–0.1% (w/v) cereal grass leaves infusion diluted with double strength Instant Ocean® to a final salinity of about 3% (w/v) and also previously bacterized with *Enterobacter aerogenes*. All cultures were maintained at room temperature.

Identification of species. Living specimens were observed without coverslip pressure using cotton fibres and Protoslo® (Carolina Biological Supply Co.) to slow down their movement. The infraciliature was also examined by staining using protargol silver proteinate [38, 64] and silver carbonate methods [21]. Specimens were compared to drawings in Dragesco [11], Small and Lynn [50], and Shin and Kim [48].

Extraction of genomic DNA. 500 cells or more of each of *Phacodinium* and *Holosticha* were washed 3 times with autoclaved Pringsheim solution and frozen in a final volume of 500 µl. Samples were subsequently melted and centrifuged. The pellet was suspended in 500 µl DNA extraction buffer (10 mM Tris-HCl, pH 8.0, 0.1 M EDTA, pH 8.0, 20 mg/ml pancreatic RNase, 0.5% SDS), and disintegrated using a sonicator for a few minutes, with intervals of 30 sec for cooling the tube on ice. Proteinase K (final concentration 0.1 mg/ml) and 1% SDS were added. The lysed cells were incubated for 3 h at 50 °C. After incubation, DNA was extracted with an equal volume of phenol:chloroform-isoamyl alcohol, repeated once or twice, and precipitated with ethanol. Cells of *Hal-*

teria were collected and lysed as described previously [36]. *Protocruzia* were harvested by centrifugation and DNA extracted following procedures outlined in Wright and Lynn [65]. After incubation, DNA was extracted with an equal volume of phenol:chloroform-isoamyl alcohol, repeated once or twice, and precipitated with ethanol.

PCR amplification. For *Phacodinium* and *Holosticha*, PCR conditions were as follows: 30 cycles with denaturation at 94 °C for 1 min (initial denaturation for 5 min), primer annealing at 52 °C for 2 min, and primer extension at 72 °C for 3 min (final extension for 10 min) using the forward primer 5'-CCTGGTTGATCCTGCCAG-3' and the reverse primer 5'-TAATGATCCTTCCGCAGGTTA-3' [39]. Amplification of *Halteria* and *Protocruzia* SSrRNA genes followed procedures in Stechmann et al. [57]. Typically, amplified products were extracted with Gene Clean II (Bio 101 Inc.) and diluted appropriately prior to cloning or sequencing.

Cloning. The blunt end PCR products for *Phacodinium* and *Holosticha* were prepared with T4 nucleotide polymerase and T4 kinase and then inserted into a pUC19 plasmid (Promega, Madison, WI). Transformations were accomplished using *Escherichia coli* JM82 competent cells (Promega). The plasmid mini-prep spin column kit (Qiagen Inc., Chatsworth, CA) was used to harvest and purify the plasmid DNA for sequencing. Cloning of *Halteria* was performed by blunt-end ligation of the PCR product into a specially prepared plasmid using the Sure Clone ligation kit (Pharmacia, Baie d'Urfé, Québec) and *Escherichia coli* XL-1 Blue (Stratagene, La Jolla, ca) competent cells. The Flexi-prep 100 kit (Qiagen Inc., Chatsworth, ca) was used to harvest and purify plasmid DNA from confirmed clones for sequencing.

Sequencing of SSrRNA gene. DNA sequencing for *Phacodinium* and *Holosticha* was accomplished using the Sanger dideoxynucleotide chain termination protocol [46]. DNA sequencing for *Protocruzia* and *Halteria* was accomplished using the ABI Prism 377 Automated DNA Sequencer (Applied Biosystems Inc.) using dye terminator and Taq FS with three forward and three reverse internal universal 18S primers [13] and the PCR primers [57]. All sequences were confirmed by reads from both strands.

Sequence availability. The nucleotide sequences in this paper are available from the GenBank/EMBL databases under the following accession numbers: *Blepharisma americanum* M97909 [23], *Bursaria truncatella* U82204 [57], *Caenomorpha uniserialis* U97108 (Hirt et al., unpubl.), *Climacostomum virens* X65152 [25], *Coleps hirtus* X76646 [57], *Colpoda inflata* M97908 [23], *Didinium nasutum* U57771 [66], *Discophrya collini* L26446 [32], *Entodinium caudatum* U57765 [67], *Eufolliculina uhligi* U47620 [25], *Euplotes aediculatus* X03949, M14590 [53], *Furgasonia blochmanni* X65150 [4], *Gruberia* sp. L31517 [27], *Ichthyophthirius multifiliis* U17354 [65], *Loxodes striatus* U24248 [25], *Metopus contortus* Z29516 (Embley et al., unpubl. data), *Metopus palaeformis* M86385 [14], *Nyctotherus cordiformis* AJ006712 [61], *Nyctotherus ovalis* AJ222678 [61], *Nyctotherus velox* AJ006713 [61], *Obertrumia georgiana* (= *Obertrumia aurea*) X65149 [4], *Onychodromus quadricornutus* X53485 [47], *Oxytricha granulifera*, X53486 [47], *Paramecium tetraurelia* X03772 [52], *Plagiopyla nasuta* Z29442, Z29543 [15], *Prorocentrum micans* M14649 [26], *Prorodon teres* X71140 [57], *Protocruzia* sp1. X65153 [25], *Spirostomum ambiguum* L31518 [27], *Sterkiella nova* (=*Oxytricha nova*) X03948, M14601 [13], *Strombidium purpureum* U97112 (Hirt et al., unpubl.), *Stylonychia pustulata* M14600, X03947 [13], *Symbiodinium pilosum* X62650, S44661 [44], *Tetrahymena empidokyrea* U38622 [30], *Trachelocerca* sp. L31520 [27], *Trimyema compressa* Z29438, Z29556 [15], and *Trithigmostoma steini* X71134 [32].

Phylogenetic analyses. Two dinoflagellates (*Prorocentrum*, *Symbiodinium*) were used as the outgroups for the ciliates [37, 51, 56, 62]. Alignment of sequences used the Dedicated Comparative Sequence Editor (DCSE) program [8] and was refined by considering secondary structure features of the SSrRNA molecule.

Calculation of sequence similarity and evolutionary distances used the Kimura [31] two-parameter model in the PHYLIP (Version 3.51C) package [17]. The Fitch and Margoliash [19] least-squares (LS) method and the neighbor-joining (NJ) method [45] were used in the construction of distance trees. PAUP [59] was used for the maximum parsimony (MP) analysis. Of the 1874 sites, 854 characters were phylogenetically informative and used to find the most parsimonious tree using the heuristic search option. Parsimony and distance data were bootstrap resampled [16] 1,000 times. Maximum likelihood (ML) tree reconstruction used PUZZLE Version 4.0.2 [58] with 10,000 puzzlings quartets.

Results

The complete SSrRNA sequences were determined for *P. metchnicoffi* (1772 nucleotides; GenBank Accession No. AJ277877), *Protocruzia* sp2 (1791 nucleotides; GenBank Accession No. AF194409), *H. multistylata* (1778 nucleotides; GenBank Accession No. AJ277876), and *H. grandinella* (1775 nucleotides; GenBank Accession No. AF194410).

The SSrRNA genes of the two *Protocruzia* species are 1791 nucleotides in length, but there were 100 differences between them, corresponding to 5.6% difference.

Distance Matrix Analysis

The two major ciliate clades, designated as the subphyla Postciliodesmatophora and Intramacronucleata are resolved in this analysis (Fig. 1). The heterotrichs, with which both *Protocruzia* and *Phacodinium* had been classified, are the sister taxon to the karyorelicts, both of which are members of the subphylum Postciliodesmatophora. The armophorids and clevelandellids, long considered to be heterotrichs, are not grouped with the other postciliodesmatophorans, but form the sister clade to the spirotrichs (Fig. 1). Moreover, the four new sequences cluster within the spirotrich clade: *Protocruzia* sp2 supported by 100% bootstrap as the sister taxon to *Protocruzia* sp1; *P. metchnicoffi* basal to the stichotrich and oligotrich clade with more than 93% bootstrap support; and both *H. multistylata* and *H. grandinella* are members of a strongly supported (i.e., > 97% bootstrap) stichotrich clade that includes *O. granulifera* (Fig. 1).

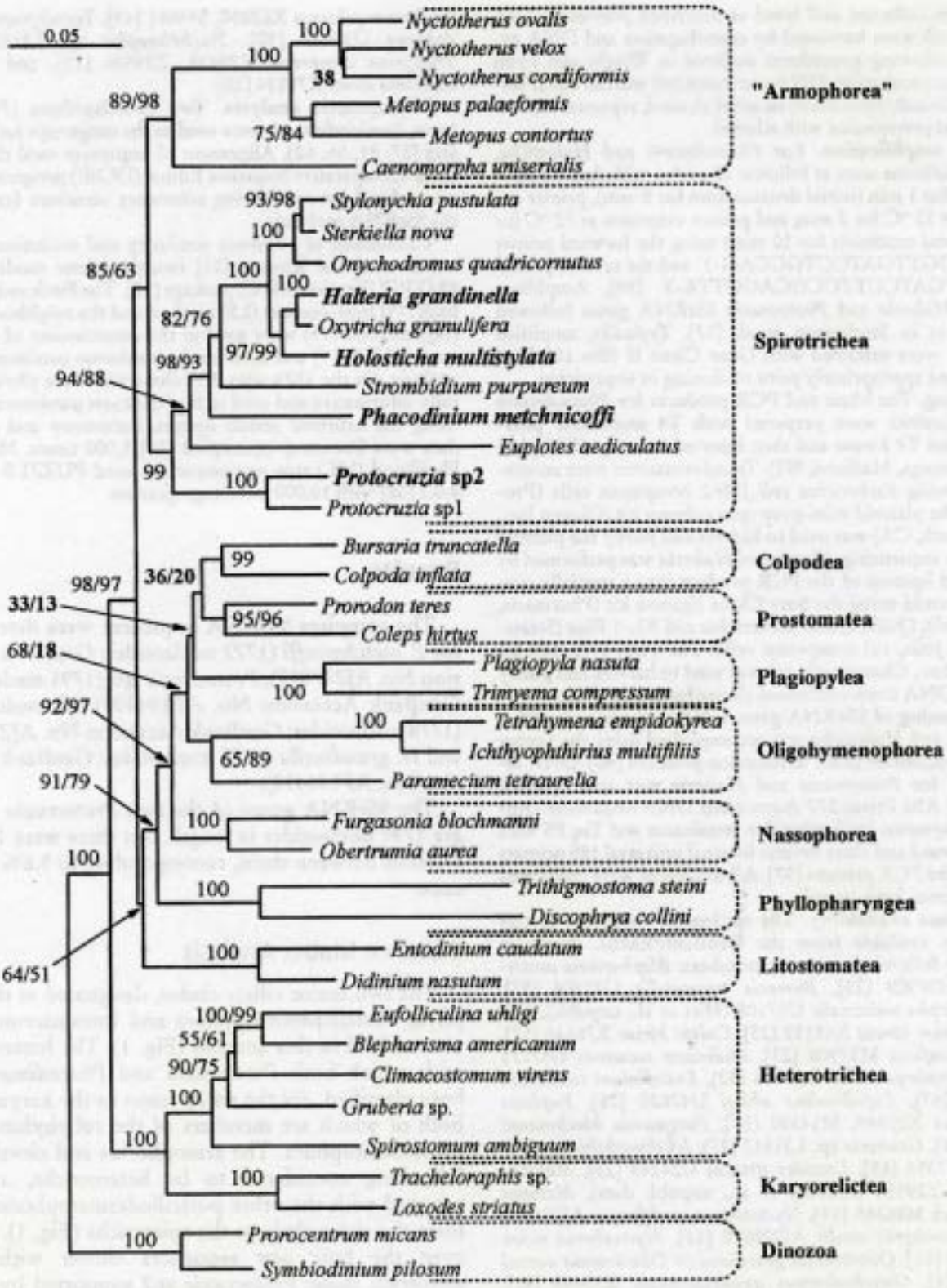

Fig. 1. Distance matrix tree of the ciliates based on small subunit rRNA sequences. Evolutionary distances were calculated using the Kimura [30] two-parameter correction model and constructed using the Fitch and Margoliash [18] least-squares (LS) method. The bootstrap percentages for LS are followed by bootstraps for neighbor-joining (NJ) [44], based on consensus trees for 1,000 bootstrap resamplings. Evolutionary distance is represented by the horizontal component separating species in the figure. The scale bar corresponds to 5 changes per 100 positions. The sequences for new species are represented in *boldface* as are the names for suprageneric groupings.

Maximum Parsimony Analysis

The major aspects of the topology of the MP tree are similar to those of the distance matrix trees (compare Fig. 1, 2): the two major subphyletic clades; the separation of the armophorids and clevelandellids from the heterotrichs, but here as the basal intramacronucleate clade; and the placement of the four new sequences within the spirotrich radiation. The only difference in the topology is the emergence of *H. multistylata* as the basal stichotrich.

Maximum Likelihood Analysis

The likelihood mapping analysis of the alignment used default parameters estimated from the data set and 10,000 quartets. The distribution of 97.9% of the quartets in regions 1, 2 and 3 of the simplex triangle indicated that fully resolved quartets were found and supported use of this data set in tree construction. The topology of the ML tree (Fig. 3) is very similar to the distance (Fig. 1) and parsimony (Fig. 2) trees. With respect to the Spirotrichea, the ML topology is identical to the distance tree topologies (Fig. 1).

Discussion

Phylogenetic Position of *Protocruzia*

The first molecular phylogenies to include a *Protocruzia* species could not establish its position consistently using different tree construction methods, either with SSrRNA sequences [25] or with histone nucleotide or amino acid sequences [3]. The sequence of a second *Protocruzia* species is reported here: in two distance approaches (i.e., LS, NJ), maximum likelihood, and parsimony, the *Protocruzia* species are consistently associated with the spirotrich clade, and always in a basal position. *Protocruzia* clustered basally with the intramacronucleate clade based on histone H4 amino acid sequences but with the karyorelicteans based on histone nucleotide sequences [3]. Sequencing of other genes may help to resolve the position of *Protocruzia*.

Others have used molecular characters to establish new species of ciliates among sibling species complexes: for example, for the *Paramecium aurelia* complex [2] and for the *Tetrahymena pyriformis* species complex [30]. Within the *T. pyriformis* complex, biological species can have identical SSrRNA gene sequences [54], and differences of less than 1% have been used to justify the designation of new species [30]. Thus, the difference of 5.6% between the two isolates of *Protocruzia*, Species 1 from Germany and Species 2 from the east coast of the United States, suggests that they are sufficiently genetically distinct, relative to *Tetrahymena* species, to be considered new species. We were unable to obtain high enough quality silver stains to describe morphologically these isolates, and so at this time we will not name them. However, it is appropriate to point out that Song and Wilbert [55] may not be correct in synonymizing all previously described *Protocruzia* species with *Protocruzia contrax* based on morphology alone. It will be important to determine the genetic diversity among isolates of this small marine benthic ciliate before drawing any further conclusions about the taxonomy within the genus.

Phylogenetic Position of *Phacodinium*

This is the first molecular phylogenetic analysis for *Phacodinium*, and it provides a strong and unambiguous result: *Phacodinium* is a member of the spirotrich clade. Thus, in agreement with Fernández-Galiano and Calvo [18], we conclude that this genus warrants its own family, its own order, and it would appear from our analysis, its own subclass. Lynn and Small [34] considered the Order Phacodiniida Small & Lynn, 1985 as *sedis mutabilis* within the Subphylum Intramacronucleata. Our molecular analysis leads us to conclude that the phacodiniids should be recognized with subclass status within the Class Spirotrichea. Since the conception of this subclass does not change from that of the Order Phacodiniida Small & Lynn , 1985, we suggest elevation of this name to subclass rank as Subclass Phacodiniidia Small & Lynn, 1985.

Phylogenetic Relationships within the Class Spirotrichea

With recognition of a Subclass Phacodiniidia, there are now six subclasses within the Class Spirotrichea: the Subclass Protocruziidia, the Subclass Phacodiniidia, the Subclass Hypotrichia, the Subclass Choreotrichia, the Subclass Stichotrichia, and the Subclass Oligotrichia. The first two classes are monotypic, represented by only one order, one family, and one genus. With the complete sequence of the oligotrich *Halteria* and the new sequence of the oligotrich *Strombidium*, we can make some assessment of the monophyly of the oligotrichs.

Dale and Lynn [7] argued in support of the separation of oligotrichs and choreotrichs and suggested that halteriids and strombidiids remain a clade on the basis of their enantiotropic cell division. This was contrary to the view put forward by Petz and Foissner [40] who separated the halteriids from the oligotrichs and choreotrichs using features of division morphogenesis. Although the species sampling is still limited to one genus to represent each family, the molecular evidence so far supports the separation of halteriids from strom-

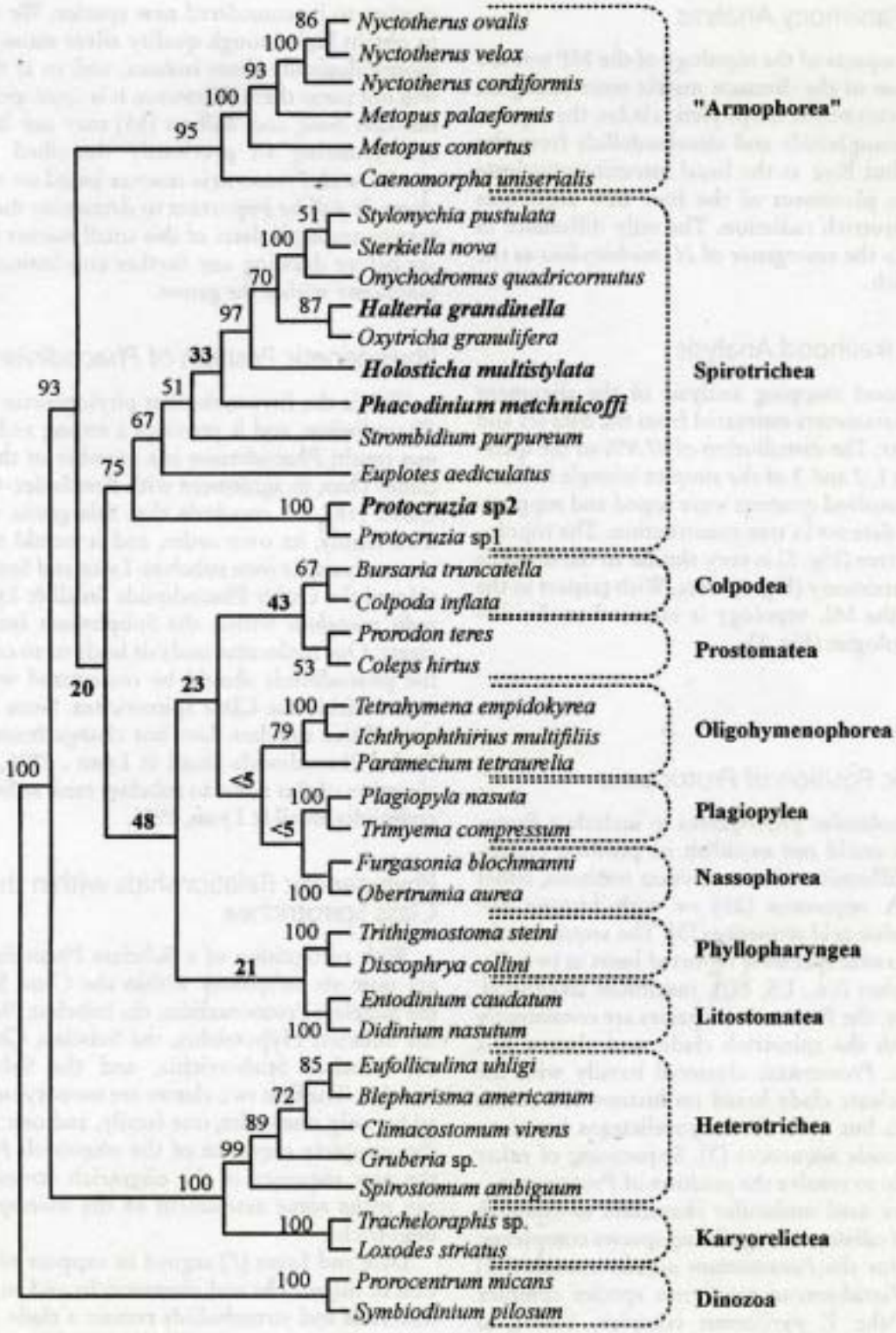

Fig. 2. A maximum parsimony tree of the ciliates constructed from complete small subunit rRNA gene sequences. The numbers at the bifurcations represent the percentage of times the group occurred out of 1,000 trees. No significance is placed on the lengths of the branches connecting the species. The sequences for new species are represented in *boldface* as are the names for suprageneric groupings.

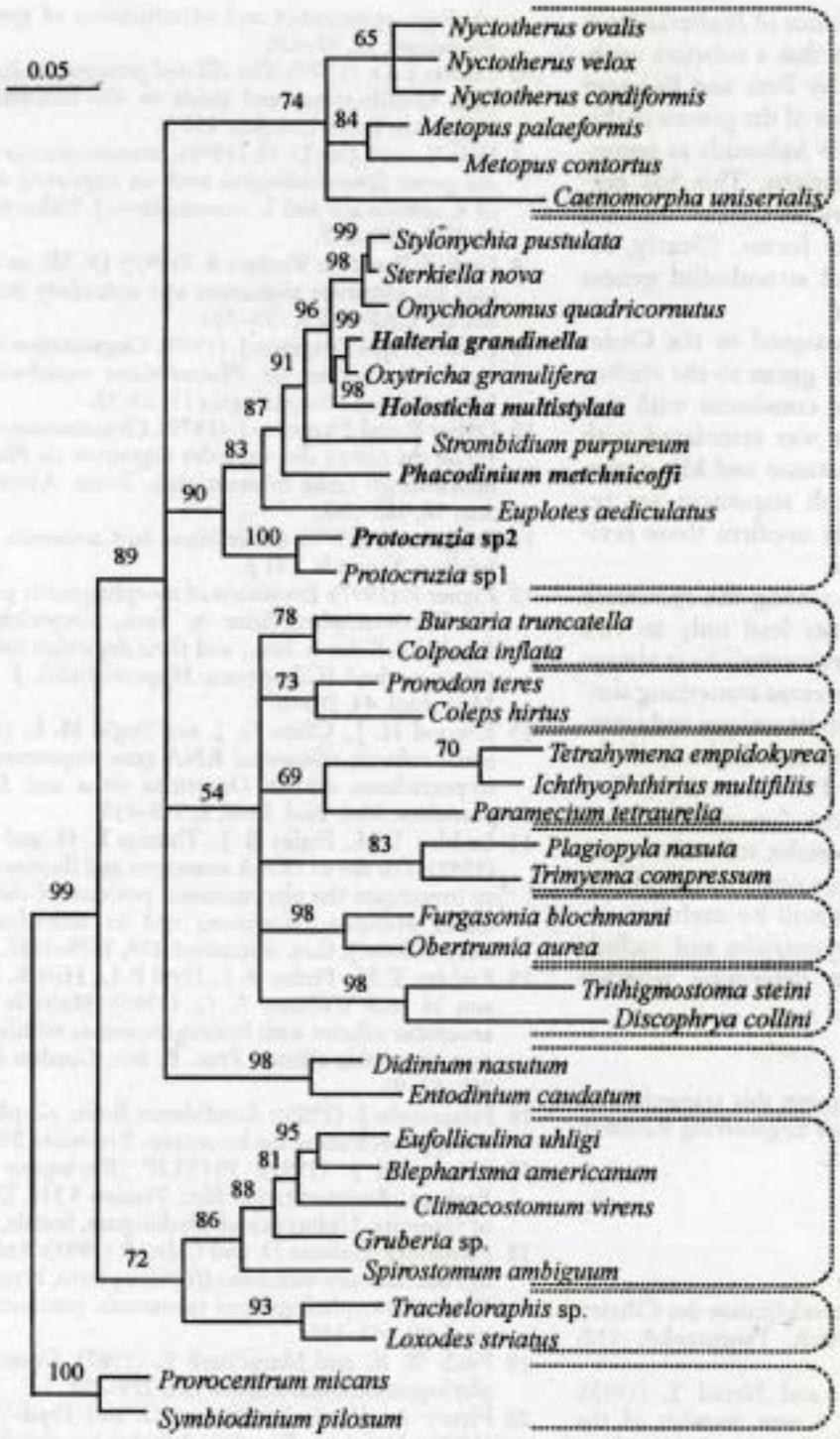

Fig. 3. A maximum likelihood tree reconstruction based on 10,000 puzzling quartets. The numbers at the nodes represent estimates of support for each internal branch, analogous to bootstrap values [58]. Evolutionary distance is represented by the horizontal component separating species in the figure. The scale bar corresponds to 5 changes per 100 positions. The sequences for new species are represented in *boldface* as are the names for suprageneric groupings.

bidiids. However, the genetic distance of *Halteria* from its stichotrich sister taxa suggests that a subclass rank, the Subclass Halteria, proposed by Petz and Foissner [40] may be elevating the difference of the genera in this family too highly: one might view halteriids as secondarily-evolved planktonic stichotrichs. This has certainly occurred among the hypotrichs: *Cytharoides* and *Gastrocirrhus* are planktonic-like forms. Clearly, sequences from other halteriid and strombidiid genera are required to resolve this matter.

Holosticha, which has been assigned to the Order Urostylida [35, 50], was the sister genus to the stichotrichs in the parsimony analysis, consistent with this ordinal assignment. However, it was associated with *Halteria* and *Oxytricha* in the distance and ML analyses. Clearly, additional stichotrich sequences are required before we can confidently confirm these revisions of the Subclass Stichotrichia.

With respect to relationships among the spirotrich subclasses, our molecular analyses lead only to two conclusions. First, the subclass Protocruziidia is always basal, suggesting that it might represent something similar to the ancestral spirotrich with its unique and interesting macronuclear "chromosomes." Indeed, if the amino acid sequences of histone H4 are used [3], *Protocruzia* may represent the most "ancestral" intramacronucleate. Second, the hypotrichs, still only represented by *E. aediculatus*, are always next, and highly divergent from the stichotrichs. It will be useful to increase the species sampling of hypotrichs and include some choreotrich sequences to determine whether these relationships remain stable.

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