

# A broad molecular phylogeny of ciliates: Identification of major evolutionary trends and radiations within the phylum

(large subunit rRNA/sequence/evolution)

ANNE BAROIN-TOURANCHEAU, PILAR DELGADO\*, ROLAND PERASSO, AND ANDRÉ ADOUTTE

Laboratoire de Biologie Cellulaire 4, Centre National de la Recherche Scientifique, Unité Associée 1134, Bâtiment 444, Université Paris-Sud, 91405 Orsay Cedex, France

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**ABSTRACT** The cellular architecture of ciliates is one of the most complex known within eukaryotes. Detailed systematic schemes have thus been constructed through extensive comparative morphological and ultrastructural analysis of the ciliature and of its internal cytoskeletal derivatives (the infraciliature), as well as of the architecture of the oral apparatus. In recent years, a consensus was reached in which the phylum was divided in eight classes as defined by Lynn and Corliss [Lynn, D. H. & Corliss, J. O. (1991) in *Microscopic Anatomy of Invertebrates: Protozoa* (Wiley-Liss, New York), Vol. 1, pp. 333–467]. By comparing partial sequences of the large subunit rRNA molecule, and by using both distance-matrix and maximum-parsimony-tree construction methods (checked by bootstrapping), we examine the phylogenetic relationships of 22 species belonging to seven of these eight classes. At low taxonomic levels, the traditional grouping of the species is generally confirmed. At higher taxonomic levels, the branching pattern of these seven classes is resolved in several deeply separated major branches. Surprisingly, the first emerging one contains the heterotrichs and is strongly associated with a karyorelictid but deeply separated from hypotrichs. The litostomes, the oligohymenophorans, and the hypotrichs separate later in a bush-like topology hindering the resolution of their order of diversification. These results show a much more ancient origin of heterotrichs than was classically assumed, indicating that asymmetric, abundantly ciliated oral apparatuses do not correspond to “highly evolved” traits as previously thought. They also suggest the occurrence of a major radiative explosion in the evolutionary history of the ciliates, yielding five of the eight classes of the phylum. These classes appear to differ essentially according to the cytoskeletal architecture used to shape and sustain the cellular cortex (a process of essential adaptive and morphogenetic importance in ciliates).

The phylum Ciliophora constitutes a large group of unicellular eukaryotes containing over 7000 species which have colonized a remarkable diversity of ecological niches. The typical ciliate cell displays one of the most highly differentiated and elaborate organizations among eukaryotes both in terms of the variety of physiological functions carried out by the single cell and in terms of the diversity and complexity of the organelles making up the cell. In addition, a bewildering variety of cell shapes has been elaborated within the phylum. Members of the group, however, are united by several clear synapomorphies which have long been considered as a testimony of its monophyly. Foremost among these are the nuclear dimorphism, with germinative micronuclei and vegetative macronuclei; a sexual process of reproduction involving conjugation; and a complex pellicular and subpellicular structure comprising cilia, often organized along longitudinal anteroposterior rows (kineties), with basal bodies associated

with a typical set of cytoskeletal fibers (see refs. 1 and 2). Within the phylum, diversification is first manifested by the overall pattern of implantation of the cilia over the cell surface and in a region specialized for food ingestion, the oral apparatus. This has formed the basis of all the early systematics of the groups (3) and of the “classical” phylogenetic hypotheses, which viewed ciliate evolution as progressing from cells with simple, apical, and symmetrical oral apparatuses with homogeneously distributed cilia, to cells with complex, dissymmetrical oral apparatuses and uneven distribution of cilia. The organization of the oral apparatus led to the widely adopted three-subphylum system (4)—Kinetofragminophora, Oligohymenophora, and Polyhymenophora, cited in the order thought to reflect an increase in complexity and evolutionary trend. Karyorelictids, ciliates with a very peculiar, nearly diploid nondividing macronucleus, were later erected as a fourth subphylum, thought to correspond to the earliest emerging line among ciliates (5). The detailed comparative analysis of ultrastructural organization of basal bodies and their derivatives led to a substantially different three-subphylum system—Rhabdophora, Postciliodesmatophora, and Cyrtophora (6). Although quite different at very high taxonomic levels, the two systems presently recognize almost the same classes, which amount to eight different ones in Lynn and Corliss’ most recent treatment (1).

The updating of these phylogenies with rRNA molecular analyses has started (7–12). Small- and large-subunit (SSU and LSU) rRNA phylogenies appeared to be promising because the phylum was sufficiently old and molecularly diversified. Some major groups have hence been identified which, to a large extent, are in good agreement with the traditional systematics at “low” and “intermediate” taxonomic levels. The depth of their divergence also confirmed the large intra- and intergroup genetic distances observed through the study of enzyme polymorphism (13), structural protein comparisons (14), and DNA-DNA hybridization (15). Major questions concerning high-level (i.e., interclass) relationships remain open, however. It is such questions that are addressed in the present work. Partial sequences of the 28S LSU rRNA from 22 species belonging to seven classes (all the classes except the Phyllopharyngea) and including one karyorelictid have been obtained<sup>†</sup> and treated with distance-matrix and parsimony tree-building algorithms to yield phylogenetic schemes, the robustness of which was evaluated by bootstrap analysis. There are three major interests in such an approach. (i) It allows an extensive test of the congruence between morphologically and molecularly based phylogenetic schemes in a group of unicellular eukaryotes especially rich in morphological traits. (ii) It should provide insights on

Abbreviations: LSU, large subunit; SSU, small subunit.

\*Present address: Departamento de Microbiología, Facultad de Biología, Universidad de Sevilla, Apdo 1095, 41080 Seville, Spain.

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a remarkable experience of diversification at the unicellular level, instructing us on how the major morphological and morphogenetic strategies of ciliates evolved and, in particular, how their hyperdeveloped cytoskeleton was elaborated. (iii) It may shed light on the evolution of the peculiar genetic code observed within the phylum (reviewed in ref. 16).

## MATERIALS AND METHODS

**Sources of Ciliate Cultures.** Fresh cultures of *Blepharisma japonicum*, *Chaenea vorax*, *Loxodes striatus*, and *Uronychia* sp. were generously supplied by G. Fryd-Versavel (Laboratoire de Biologie Evolutive et Dynamique des Populations, Université Paris-Sud, Orsay, France). Fresh cultures of *Colpidium campylum*, *Didinium nasutum*, *Enchelys pelucida*, *Pseudomicrothorax* sp., *Stylonychia lemnae*, *Urostyla* sp., *Euplotes aediculatus*, *Stentor coeruleus*, *Coleps* sp., *Paraurostyla* sp., and *Pleuronema marinum* were grown in the laboratory. Identification by protargol impregnation was carried out by F. Iftode (this laboratory) and reference slides have been conserved. Frozen cells of *Isotricha prostoma* were a gift from B. Viguès (Laboratoire de Zoologie et Protistologie, Université Blaise Pascal, Clermont-Ferrand, France). Total RNA from *Halteria grandinella* and *Colpoda inflata* was a kind gift from D. H. Lynn (University of Guelph, ON, Canada). Total RNA from *Paramecium primaurelia* was kindly provided by F. Caron (Ecole Normale Supérieure, Paris).

**RNA Isolation and Sequencing.** Total RNA was extracted and sequenced by the reverse transcriptase method essentially as described (8, 17). Five oligonucleotides, complementary to the conserved 28S rRNA segments 51–75, 278–302, 382–404, 2627–2647, and 3255–3277 (numbers refer to *Mus musculus* sequence coordinates) were used systematically as primers.

**Analysis of the Data.** We have sequenced the 370 5'-terminal nucleotides of the 28S rRNA molecule. This portion of the molecule contains two highly conserved stretches of nucleotides which have been shown to provide a good phylogenetic index over broad evolutionary distances (8, 18, 19). These two domains, yielding a total of 222 unambiguously aligned nucleotides, bracket a rapidly evolving area which is readily alignable over its entire length only between very closely related species. Within the ciliates, sequence

comparison in this area can also be carried out in two subdomains in which the variations in rate of substitution and in length are moderate. An additional 100-nucleotide conserved region, 2000 nucleotides downstream from the 5' end, has also been sequenced for a subset of species. Therefore, depending on the species sample under analysis, increasing lengths of sequence can be analyzed. (Sequences have been submitted to the GenBank/EMBL data bank and an aligned set is available upon request to the authors.) The sequences taken from the EMBL data bank are those of *Tetrahymena thermophila* (X54512) and *Tetrahymena pyriformis* (X54004).

Alignment of sequences, computation of the observed and the corrected numbers of nucleotide differences obtained by using Kimura's Knuc correction (20), derivation of the resulting matrices, and formatting for the various tree-building programs were carried out with the MUST package developed in our laboratory by H. Philippe (unpublished work). Dendrograms were constructed by two distance-matrix methods [neighbor-joining according to Saitou and Nei (21) and programmed by H. Philippe; FITCH program of Felsenstein's PHYLIP 3.2 package (Department of Genetics, University of Washington, Seattle, WA)] and by the maximum-parsimony method [through the PAUP 2.1.4 program provided by D. Swofford (Illinois National History Survey, Champaign, IL)]. To test the reliability of the inferred phylogenetic trees, the bootstrap method was applied on the parsimony treatment (DNABOOT program of PHYLIP). The bootstrap sampling was repeated 1000 times.

## RESULTS

As previously reported in several rRNA sequence analyses and not shown here, the ciliates form a monophyletic unit and branch off from the eukaryotic lineage during a relatively late and intense eukaryotic diversification stage shortly preceding the emergence of multicellular organisms (19, 22–25).

**Global Phylogeny of the Ciliates.** The most complete ciliate phylogeny we have constructed is depicted in Fig. 1. The outline of the distance tree indicates four major clusters, which are consistently observed in distance and in parsimony trees whatever the outgroups chosen and ciliate sample analyzed and which correspond to the four classes heterotrichs, hypotrichs, oligohymenophorans, and litostomes. Examination of the distance matrix reveals a great depth of

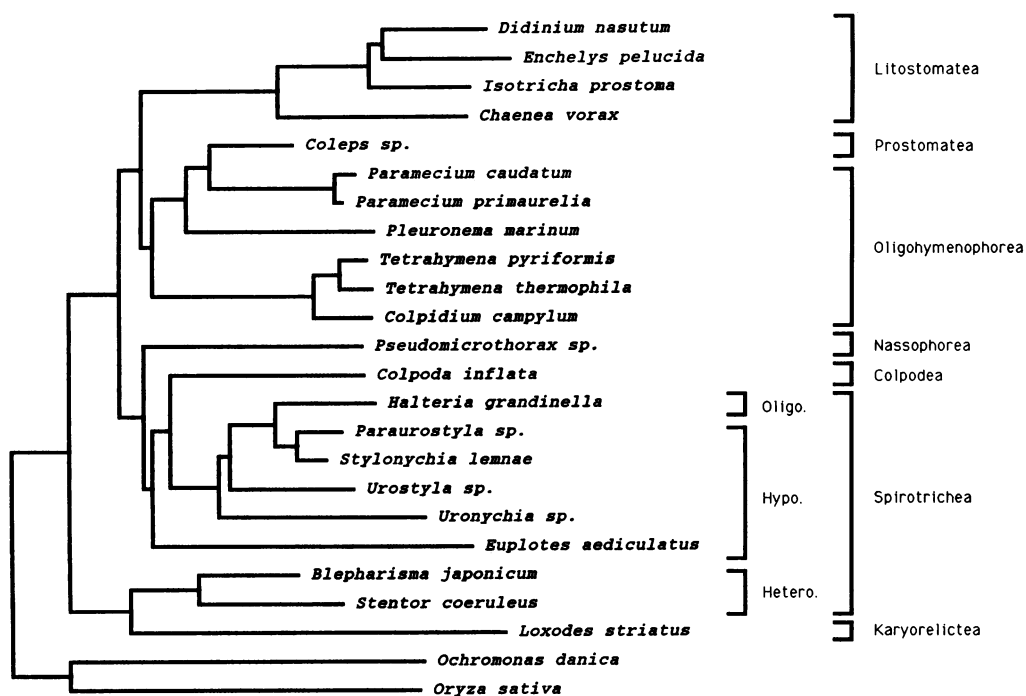


FIG. 1. Phylogenetic tree of the ciliates generated by the neighbor-joining method. The analysis is restricted to the 5' end of the molecule, for which we have the largest data base. On the right, the groups are designated according to Lynn and Corliss' systematics (1). The orders heterotrichs, hypotrichs, and oligotrichs *sensu* Corliss (3) are specified. Among the 222 alignable nucleotide sites of the two conserved domains, 122 are variable, and of these, 84 are informative. Horizontal distances are proportional to evolutionary distances. The same topology is obtained with the FITCH distance-matrix program or when distances are connected using Kimura's (20) model. Varying the sample of outgroup species also does not modify the topology.

molecular divergence between, for example, *Euplotes aediculatus* and *Paramecium caudatum* (15.43) or *Tetrahymena pyriformis* (14.51), in agreement with their separation into distinct classes (hypotrichs and oligohymenophorans, respectively) in classical systematics. By comparison, the distance between *Oryza sativa* (a plant) and *Xenopus laevis* (a vertebrate) is 15.08. Similarly, within the class Oligohymenophorea, the distance between *Paramecium caudatum* and *Tetrahymena pyriformis* is larger than the distance between *Oryza sativa* and *Citrus limon* (8 vs. 5.56).

The confrontation between the two types of data treatment also reveals the weakness of some associations. This is illustrated in Fig. 2, where a parsimony method with bootstrap analysis allows one to estimate the robustness of some of the groupings.

The salient points of the dendrograms can be summarized as follows.

The heterotrichs (*Blepharisma japonicum* and *Stentor coeruleus*) group together and emerge associated with the single representative of the karyorelictids, *Loxodes striatus*. The monophyly of heterotrichs is supported in >70% of the cases. Whatever the type of tree-building algorithm used, the ciliate sample analyzed, and the outgroups chosen, heterotrichs and karyorelictids systematically diverge prior to the hypotrich-oligohymenophoran-litostome radiation. Greenwood *et al.* (11) also noted from SSU rRNA data that *Blepharisma japonicum*, the single heterotrich they analyzed, always emerged as the earliest branch within ciliates.

The monophyly of the litostomes is supported in 96.4% of the cases. *Chaenea vorax*, a marine species, is the earliest one to emerge, prior to the cluster comprising *Isotricha prostomata*, *Didinium nasutum*, and *Enchelys pelucida*. The specific branching pattern within the cluster is unknown because in bootstrap analysis, *Didinium nasutum* is the sister group of *Enchelys pelucida* in only 43% of the replicates. As also observed within the hymenostomes (see below), the two conserved domains do not contain enough variable sites to resolve the branching orders of such closely related lines. For such close relationships, the divergent domain is more informative.

Oligohymenophorans emerge as a monophyletic unit with a deep split between the hymenostomes (*Colpidium campylum*, *Tetrahymena pyriformis*, and *Tetrahymena thermophila*) and

the peniculines (*Paramecium caudatum* and *Paramecium primaurelia*). This deep split is also observed with SSU rRNA (11). *Coleps*, which was previously thought to belong to a distinct class, that of prostomes, systematically groups with oligohymenophorans, whatever tree construction method is used. In bootstrap analysis the branching pattern is similar to that of the distance tree, but the bootstrap values for these associations are very low (21% and 22%, respectively). Omitting *Pleuronema marinum* from the analysis strengthens the monophyly of the oligohymenophorans. In this case, the bootstrap value increases to 40% (data not shown).

Within the hypotrichs *Urostyla* sp., *Stylonychia lemnae*, and *Paraurostyla* sp. cluster together in distance and parsimony trees. They correspond to the euhypotrichs *sensu* Fleury (26), a group which she has separated from the pseudohypotrichs on the basis of morphological and morphogenetic criteria. The oligotrich *Halteria grandinella* is also systematically associated with this branch, as found by Lynn and Sogin (9). Two representatives from the pseudohypotrichs (*sensu* Fleury) (*Euplotes aediculatus* and *Uronychia* sp.) emerge also associated with the hypotrich lineage. The large divergence observed between *Euplotes aediculatus* and the euhypotrichs strongly supports its separation from the group and is also observed with SSU rRNA (9). *Euplotes aediculatus* is so highly divergent that in bootstrap analysis it is no longer a sister group of the hypotrichs and emerges independently at the base of the oligohymenophoran-litostome-hypotrich trichotomy. This would render the hypotrichs paraphyletic and constitutes the major discrepancy observed between the two types of data treatment. Another striking difference between the distance-matrix and the parsimony treatment of the data concerns *Uronychia* sp., whose sister group relation to the euhypotrichs is not robust. Additional data are thus needed to confirm the position of *Euplotes aediculatus* and to increase the resolution of the relationships within the hypotrichs (see below). Deeply separated from the hypotrich branch, emerge also *Colpoda inflata* and *Pseudomicrothorax* sp., the single representatives of the Colpodea and Nassophorea, respectively, in Lynn's scheme. The values in the bootstrap analysis indicate that *Pseudomicrothorax* sp. and *Colpoda inflata* are poorly associated with all other branches of the oligohymenophoran-prostome-hypotrich radiation. Their positions in the tree alongside the hypotrich lineage in Fig. 1 are not robust and are in fact dependent upon the ciliate sample analyzed, suggesting that they indeed constitute distinct branches, in agreement with their taxonomic status. With SSU rRNA, *Colpoda inflata* emerges as a sister group of the oligohymenophorans, albeit deeply split from them (9).

In summary, the dendrograms allow the clear identification of an early emerging group (heterotrichs and karyorelictids) and of three clusters (oligohymenophorans, litostomes, and hypotrichs) whose branching order is not resolved. As uncertainties may arise from insufficient data, the relative branching order of several lineages of this multifurcation has been further investigated by using longer sequences.

**Detailed Analysis of a Limited Sample of Species.** Fig. 3 shows the topology and bootstrap values of a tree that is rooted on *Stentor coeruleus* and that was obtained by using a larger data set. The tree confirms many features observed in Figs. 1 and 2. Within the hypotrichs, we observe an improvement in the resolution of the branching pattern. The monophyly of euhypotrichs is now reasonably well supported (89.9%) and *Euplotes aediculatus* indeed emerges as their sister group but with a low bootstrap value (35.8%). At the base of this lineage, the pseudohypotrichs *Uronychia* sp. and *Euplotes aediculatus* form a paraphyletic group. The monophyly of litostomes is again strongly supported (99.2%) and that of the peniculines and hymenostomes is fairly well supported (65.7%). In contrast, the bootstrap values of the

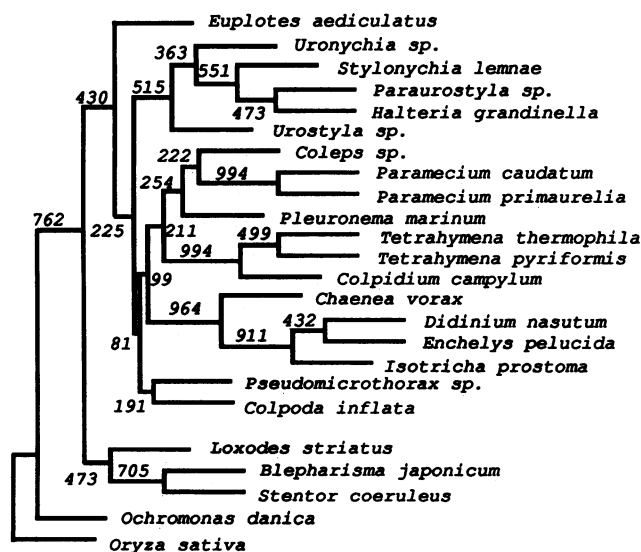


FIG. 2. Ciliate phylogeny inferred from bootstrap analysis. The molecular data analyzed are the same as in Fig. 1. The numbers at the forks refer to the number of times these forks occurred among the 1000 bootstrap replicates.

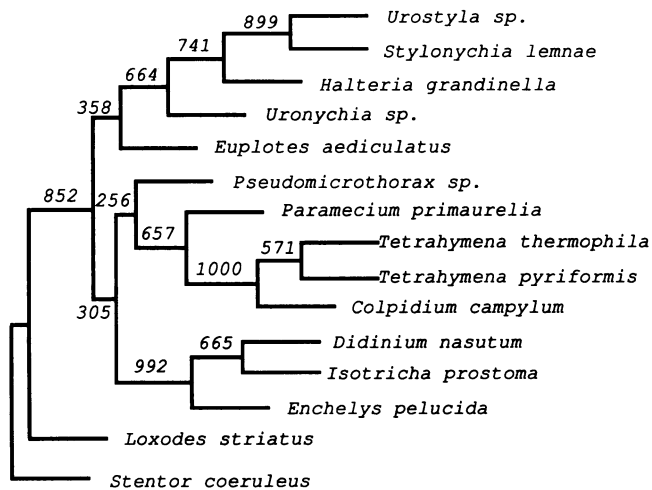


FIG. 3. Ciliate phylogeny of a limited sample of species inferred from bootstrap analysis. The analysis is restricted to 15 species. Additional conserved stretches of the molecule are analyzed (see *Materials and Methods*). Among the 369 alignable nucleotide sites, 213 are variable. *Stentor coeruleus* is taken as outgroup. The numbers at the forks refer to the number of times these forks occurred among the 1000 bootstrap replicates. Identical topologies are obtained when distance-matrix methods are used.

position of *Pseudomicrothorax sp.* as sister group of the oligohymenophorans is still very low (25.6%), leaving the branching point of this species unresolved. Similarly, the sister group relationship of the *Pseudomicrothorax*-oligohymenophoran cluster to litostomes depicted on the dendrogram is also weakly supported (30.5%), indicating that a multifurcation between these three groups would better represent the consensus observations. Finally, the distinctness of all the groups with respect to the *Stentor*-*Loxodes* pair is well confirmed (85.2%). Thus, increasing the length of the sequences analyzed, in general, raised the bootstrap values at most of the nodes but did not significantly resolve a number of previously unsolved deep multifurcations, suggesting that in these cases the limits in the resolution could reflect a true biological explosive radiation.

## DISCUSSION

The molecular data display a set of remarkable points of agreement with morphologically based systematics at low or intermediate taxonomic levels. First, many species group together and form monophyletic lines as predicted by classical schemes. For example, the whole group of Oligohymenophorea is confirmed [with the exception, with respect to Lynn's classification, that *Paramecium* is included in it, in agreement with previous schemes (3, 27)]; all the sequenced representatives of Litostomatea also group together; the euhypotrichs form a solid monophyletic group, and the same holds for the heterotrichs, etc. One of the most remarkable agreements concerns *Coleps*—a ciliate previously thought to belong to the Prostomatea because of its apparently simple, apical oral apparatus—which, following a careful analysis of its oral morphogenesis (28), was identified as being closer to Oligohymenophorea, a conclusion which is fully confirmed by the molecular data. Second, some lines that were expected to be distant from each other or that were expected to be difficult to link to other lines indeed turn out to be so. Such is the case, for example, for the deep split between pseudohypotrichs and euhypotrichs, between the single representatives of Colpodea and Nassophorea and the other lines, and the great distances observed between the various clearly monophyletic lines.

In general, all these cases correspond to those in which congruence was observed between oral apparatus characteristics, ultrastructure of the kinetid, and morphogenetic properties. This internal consistency therefore testifies to the validity of the molecular approach and, equally, confirms the quality of the morphological and morphogenetic characters that were used. One final major point of agreement with recent schemes is the early emergence of the karyorelictid *Loxodes striatus*. Because their macronuclei are nearly diploid and nondividing, in contrast to all other ciliates, karyorelictids have been considered in all schemes as derived from the most ancestral ciliate stock. Our data provide direct confirmation for this assumption, therefore providing an important element in the discussion of the origin of the typical ciliate macronucleus (see ref. 29).

A number of major surprises are also observed in the molecular phylogeny. The first one is the early emergence of heterotrichs as a sister group of a karyorelictid, and separated from hypotrichs. While the taxonomic status of heterotrichs has varied from order to subclass to class level, in all traditional and more recent classification schemes, they were united to hypotrichs to form a group known as the "spirotrichs," or to a restricted set of hypotrichs to form the "stichotrichs." Classically, this was based on the sharing of a hyperdeveloped spiral oral apparatus. Both our work and that of Greenwood *et al.* (11)—who, through complete 18S rRNA sequencing, found the heterotrich *Blepharisma americanum* to be the earliest emerging ciliate, deeply split from hypotrichs—call for a major revision of the classical view. It appears that the apparent complexity of the oral apparatus was misleading both as a derived character and as a synapomorphy between hypotrichs and heterotrichs. Instead, we suggest that asymmetry of the oral apparatus is an ancestral character in ciliates (in fact shared by some karyorelictids) and that abundance of oral cilia, typical of heterotrichs and hypotrichs, is either a symplesiomorphy or a convergent trait.

Support for this suggestion comes from the second unexpected result, that of the relatively late emergence of the Prostomatea and Litostomatea, the ciliates with "simple," apical and symmetrical oral apparatuses classically considered to correspond to an early emerging branch. For these two "simple" groups, the argument would be the reverse of that just used for heterotrichs: here apparent simplicity was misleading. In sum, the molecular data indicate that the gross characteristics of oral apparatus organization, while useful as taxonomic traits, are not reliable *phylogenetic* indicators and that their traditional polarization (from "simple" to "complex") must be turned almost upside-down. Similar conclusions have been reached by Bardele (30) on the basis of detailed analysis of oral morphogenesis in several ciliate lines.

Additional support for splitting heterotrichs from hypotrichs is provided by the fact that they form a strong monophyletic group with *Loxodes striatus*, the single karyorelictid whose rRNA we have sequenced. This is a third unexpected result in terms of the older taxonomy, but it fits quite well with Small and Lynn's (6) suggestion of grouping heterotrichs with karyorelictids within the Postciliodesmatophora on the basis of the sharing of hyperdeveloped postciliary microtubules. In this case, the greater emphasis put on a somatic kinetid ultrastructural characteristic appears to have been quite appropriate. In fact, this may have been quite significant evolutionarily in the light of the model developed by our group (31) attributing great emphasis to the procedure adopted to reinforce the cortex. In the present case, the high significance of this ultrastructural character would stem from the fact that it corresponds to a key cellular device developed early by this particular lineage.

The broad lines of morphological diversification in ciliates and the tempo of this diversification can now be suggested on the basis of the consensus tree. The molecular trees can be summarized as indicating the early emergence of one major line comprising the heterotrichs and a karyolictid, followed by an unresolved multifurcation of several major lines. That the order of emergence within these lines is difficult to establish does not seem to result from lack of data or inappropriate treatment of the data. First, increasing the length of the 28S rRNA sequences did not significantly resolve the multifurcation, while the branching orders *within* each lineage were increasingly well defined. Second, Greenwood *et al.* (12), who used complete 18S rRNA sequences (although the total number of lineages analyzed was lower than that presented here), found a deep split between the oligohymenophorans and the hypotrichs, identical to the split observed here. Third, both distance and parsimony analyses yielded similar topologies with deep splits between the four lineages and very short common stems. The corresponding bootstrap values were low, indicating lack of statistical significance of the branching orders. Finally, systematic analysis of the effect of sampling of in-group and out-group species on the topology also confirmed the instability of the branching order at the base of the multifurcation. We therefore suggest that a true evolutionary radiation occurred at this second step of ciliate evolution. Examination of the morphologies within each of the four lineages shows, in contrast, usually well-resolved branching orders and, more important, a great homogeneity of cellular organization corresponding to the "body plans" of oligohymenophorans, nassulids, colpodeans, hypotrichs, and litostomes. We have proposed elsewhere (31) a detailed model of the morphogenetic choices that may have led to this two-step diversification. Basically, the model suggests that in a first step, ciliates have adopted a strategy of cortex reinforcement and of patterning of the infraciliature through hyperdevelopment of basal body-related appendages; the postciliodesmatophorans (i.e., heterotrichs and karyorelictids), in which post-ciliary microtubules form a scaffold for the cortex, would be the present-day representatives of such a crucial period. Then a rapid diversification into distinct lines followed, each of these lines adopting a somewhat different strategy to strengthen the cortex and to anchor the infraciliature: an epiplasm in oligohymenophorans and nassophoreans, a contractile network of Ca<sup>2+</sup>-binding proteins in litostomes (the "ecto-endoplasmic boundary"), and long and continuous subcortical microtubules in hypotrichs.

A true radiation seems to have occurred in ciliate history at a point where major morphogenetic decisions were made. Following this radiation, the species, within each of the lineages, appear to have remained constrained around a similar cell body plan. This is quite reminiscent of analogous situations for several other major evolutionary transitions: the radiation of coelomates (32), that of "late-emerging" unicellular eukaryotic diversification (19, 33), that of the actinopterygian-chondrychtian-tetrapod radiation (H. L. V. Le, G. Lecointre, and R.P., unpublished work), and that of the pecoran (ruminant artiodactyl) radiation (34). We suggest, therefore, that unresolved multifurcation in molecular phylogenies may be revealing (or confirming) a fundamental characteristic of the evolutionary process. Such an explosive pattern has also been deduced from the paleontological analysis of the early Cambrian radiation of metazoa (35) as recently popularized by Gould (36).

Finally, hypotheses concerning the evolution of the genetic code, which appears to have occurred within the phylum (8, 16, 37), possibly under A-T pressure (38), can now be tested against the phylogeny proposed in this paper.

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