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# An Attempt at Reconstructing a Phylogenetic Tree of the Ciliophora Using Parsimony Methods

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## SUMMARY

Phylogenetic trees were constructed for the Ciliophora using a parsimony analysis that applies the Camin-Sokal method to characters of known polarity and the Wagner method (which requires no knowledge of the ancestral state) to the other characters. The data covered 56 species and 23 morphological, nuclear and ultrastructural multistate characters.

Since no real-world outgroup can be assumed with certainty to root the ciliophoran tree, we used three hypothetical ancestor hypotheses; only one of them (hypothesis 3: somatic kinetosomes in pairs considered ancestral; no character transformation series assumed for the position of the buccal area or for the organization of the buccal infraciliature) produced interesting trees. Two trees, called A and D, have been retained because they were shorter than the others and were equally optimal for different codings of the hypothetical ancestor. In tree A, there is an early separation in two main branches. The first one contains two groups: the Karyorelictea-Heterotrichea (Postciliodesmatophora) and the Hypotrichea-Oligotrichea (Spirotricha) on the one hand, and the colpodids (Transversala) on the other. The second branch leads to 3 groups containing all other ciliates. In tree D, the Postciliodesmatophora and Spirotricha are first separated from all other ciliates; this is in agreement with molecular phylogenies.

Despite these differences, the same five major groups appear in both trees; the main difference is in the position of the colpodid group. Class Karyorelictea appears to be polyphyletic, with (a) a Loxodia-Trachelocercia line whose genera share the same type of somatic cortex and nuclear organization, and (b) a Protoheterotrichia-Protocruziidia line which is closer to the Heterotrichia. *Nyctotherus* is closer to the hypotrichs than to the heterotrichs. As in the molecular trees, the heterotrichs are closer to some of the Karyorelictea, with which they share the same main type of cortical cytoskeleton (postciliary ribbons), than to the hypotrichs and oligotrichs, where the cortical microtubules are not postciliary fibers. So, there are two competing types of reinforcement of the cell cortex by microtubules, and these were selected as early as the first (in tree D) or the second branching (in tree A); this is justification enough to consider the subphylum Tubulicorticata as totally artificial. The validity of the subphylum Filocorticata is also discussed, considering the cortical cytoskeleton of some of the Vestibuliferea (Blepharocorythida and Entodiniomorphida). The Litostomatea, Vestibuliferea and Phyllopharyngea emerge as a sister-group of the Oligohymenophorea.

In the phyllopharyngids, macronuclear DNA is gene-sized, as in the hypotrichs; this means that DNA fragmentation occurs independently in different lineages. Macronuclear characters concerning chromatin organization that depend on the size of the DNA molecules have become diversified into paraphyletic lines such as the phyllopharyngids, oligotrichs and hypotrichs for the character "DNA duplication in replication bands". *Nassula* is separated from the *Furgasonia-Pseudomicrothorax* group, which is close to the scuticociliates. *Nassula* is close to *Coleps*. The peniculids branch away markedly from the tetrahymenids and are closer to the scuticociliates and peritrichs.

The results are discussed with reference to some other new data, phylogenetic reconstructions and molecular trees.



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## Introduction

Over the past ten years, new data have become available at an increasing rate on the structure, ultrastructure, biology, ecology and genetics of ciliates. As a result, classification schemes have been proposed that attempt to unify this newly acquired knowledge [11, 12, 13, 31, 44, 45, 64, 65, 76, 77].

These different schemes emphasize, somewhat intuitively, criteria that are specific to the particular group of taxa under study. Thus, ultrastructural characteristics of either the buccal or the somatic cortex have been used as the basis for the division of the Ciliophora into subphyla or superclasses. In the first case [64], the authors considered the occurrence of complex infraciliary buccal structures to be indicative of phyletic kinship rather than of a convergence linked to feeding modes, though they recognized various possibilities for adaptation of buccal structures in a particular phylum. Hence they made a distinction between the Kinetophragminophora (with a polar cytostomal area and originally weakly differentiated oral infraciliature) and the Oligohymenophora and Polyhymenophora (with a ventral cytostomal area and oral infraciliature organized in organelles of varying structural complexity). In the second case [76, 77], the authors considered that the buccal structures are too strongly subject to variation to afford reliable phylogenetic interpretation, at least with regard to the higher taxa, and they postulated the ultrastructural conservation of the somatic cortex [52]. Lynn and Small [54] argued to the plesiomorphy of the dikinetid in the somatic cortex, whereby the most stable cortical organization from the evolutionary standpoint is the pair of kinetosomes. On the basis of variations in the ectoplasmic fibrillar systems associated with the somatic kinetid, Small and Lynn [76, 77] made a distinction between the Postciliodesmatophora, characterized by the development of postciliary fibers in long overlapping arrays, and the Rhabdophora and Cyrtophora.

In another piece of work, Bardele [2] compared these results to his phylogenetic reconstruction of the group using data based on the organization of the particles of the ciliary membrane in 68 ciliate genera. With his co-workers, using details of the morphogenetic processes as the basis of arguments, he discussed the phylogenetic relationships of different groups, adhering strongly to von Baer's principles of embryonic resemblance [3]. In a later paper with Huttenlauch [43], he demonstrated that the oral infraciliature of *Coleps* had been misinterpreted and that this ciliate (the stomatogenesis of which is ventral and not apical) is an oligohymenophorid.

In order to render the choice of the classification criteria more objective, a phenetic classification was proposed by de Puytorac et al. [66] based on 122 ultrastructural morphological characters of: the somatic cortex (70 characters), the buccal cortex (39 characters), the nuclear apparatus (5 characters), stomatogenesis (6 characters) and asexual reproduction (2 characters), in 59 species belonging to the main groups of ciliates. Although this type of numerical approach is interesting, it takes into consideration characters that may have resulted from convergence and so can lead to a non-phylogenetic classification. As the authors themselves stressed, the results obtained (3 subphyla: Kinetophragminophora, Karyorelicta and Hymenophora) are therefore open to debate. More recently, Lipscomb and Riordan [51] used a HENNIG 86 cladistic analysis to produce a phylogeny of the haptorid ciliates after examination of 43 characters for 21 genera.

Another interesting approach involves the analysis of variations in the sequences of some types of rRNA [1, 4, 5, 17, 27, 35, 36, 39, 55, 58, 71, 79, 80, 81]. However, the heterogeneity of the groups of species analyzed so far (several species from the same small taxon, placed on a par with a heterogeneous group containing a small number of species each representing a large taxon) makes the phylogenetic reconstitution of a set as large as the ciliates unfeasible at present on this basis. Moreover, nothing is known about a possible correspondence between the evolution of rRNA molecules and the selection of phenotypes in the cells in question; it is recognized that molecular homologies are no more strictly accurate than morphological homologies. So, the paper of Fleury et al. [27] is particularly interesting. These authors clearly show a correlation between the data of molecular phylogeny and the characteristics of the cortical shell on which the infraciliature is anchored: the main cortical cytoskeleton is made up either of microtubules, or of the ectoendoplasmic boundary or epiplasmic layer.

From this, we conclude that any phylogenetic reconstruction based on a large set of correctly coded characters covering a large number of species belonging to a wide variety of ciliate taxa remains of interest in the present state of our knowledge on the evolution of ciliates. So we undertook submitting the data of our previous phenetic analysis to methods of phylogenetic reconstruction.

## Material and Methods

The data used in this study cover 56 ciliophoran species (Table 1) and represent 23 morphological, nuclear, and ultrastructural traits for a total of 86 states, recoded into 70 or 72 binary variables (Table 2) depending on our evolutionary assumptions.

We did not choose dinoflagellates as an outgroup because, even if some molecular phylogenies suggest that dinoflagellates and ciliates are sister-groups (see review and trees in [71]), some recent others do not corroborate this conclusion [10, 27, 68].

So, since no real-world outgroup can be assumed with certainty to root the ciliophoran character trees, we relied on transformation series hypotheses for most of the characters, thus hypothesizing a theoretical ancestor for the ciliates; we did not manage to formulate satisfactory hypotheses in the case of a few characters and left the states of these characters unordered. For three of the characters, our uncertainty translated in the formulation of three different hypotheses about the evolution of (a) the grouping of somatic kinetosomes (character II), (b) the position of the buccal area (character XII), and (c) the arrangement of the buccal infraciliature (character XIV); reconstructing a phylogenetic tree from each of these hypotheses will be our way of studying their implications. In hypothesis 1, we considered the following to be the ancestral (= plesiomorphic) states: (a) isolated kinetosomes, (b) apical buccal area, (c) homogeneous buccal infraciliature. In

Table 1. Species of ciliophora included in the present study

| Classes (according to [60]) | Species |
|---|---|
| Karyorelictea | *Trachelonema sulcata* Kovaleva, 1972 |
| | *Tracheloraphis prenanti* Dragesco, 1960 |
| | *Loxodes magnus* Stokes, 1887 |
| | *Remanella multinucleata* Kahl, 1933 |
| | *Protocruzia tuzeti* Villeneuve-Brachon, 1940 |
| | *Geleia nigriceps* Kahl, 1933 |
| Heterotrichea | *Climacostomum virens* (Ehrenberg, 1933) |
| | *Condylostoma magnum* Spiegel, 1926 |
| | *Nyctotherus ovalis* Leidy, 1850 |
| Hypotrichea | *Euplotes eurystomus* Wrzesniowski, 1870 |
| | *Paraurostyla weissei* (Stein, 1959) |
| | *Plagiotoma lumbrici* Dujardin, 1841 |
| | *Gastrostyla steini* Engelman, 1861 |
| | *Stylonychia mytilus* Ehrenberg, 1838 |
| Oligotrichea | *Halteria grandinella* (O.F.M., 1786) |
| | *Petalotricha ampulla* (Fol, 1881) |
| Colpodea | *Bresslaua vorax* Kahl, 1931 |
| | *Tillina magna* Grüber, 1880 |
| | *Colpoda steini* Maupas, 1883 |
| | *Colpoda maupasi* Enriquez, 1908 |
| | *Colpoda simulans* Kahl, 1931 |
| | *Colpoda cavicola* Kahl, 1931 (= *C. spiralis* Novotny, Lynn and Evans, 1977) |
| | *Bursaria truncatella* O.F.M., 1786 |
| | *Cyrtolophosis mucicola* Stokes, 1888 |
| | *Platyophrya spumacola* Kahl, 1926 |
| | *Woodruffia metabolica* (Johnson et Larson, 1938) |
| | *Enigmostoma dragescoi* (Njiné, 1978) |
| | *Bryophrya bavariensis* Kahl, 1931 |
| Litostomatea | *Alloiozona trizona* Hsiung, 1930 |
| | *Spathidium* sp. |
| | *Monodinium balbiani* (Fabre-Domergue, 1888) |
| Vestibuliferea | *Paraisotricha colpoidea* Fiorentini, 1890 |
| Phyllopharyngea | *Brooklynella hostilis* Lom and Nigrelli, 1970 |
| | *Chilodochona quennerstedti* Wallengren, 1895 |
| | *Sphenophrya* sp. |
| Nassophorea | *Trimyema compressum* Lackey, 1925 |
| | *Coleps hirtus* Nitzsch, 1817 |
| | *Nassula tumida* Maskell, 1887 |
| | *Furgasonia protectissima* Pénard, 1922 |
| | *Pseudomicrothorax dubius* (Maupas, 1883) |
| Oligohymenophorea | *Frontonia atra* Ehrenberg, 1833 |
| | *Disematostoma tetraedrica* (Fauré-Fremiet, 1924) |
| | *Paramecium aurelia* Ehrenberg, 1838 |
| | *Urocentrum turbo* (O.F.M., 1786) |
| | *Myxophyllum steenstrupi* (Stein, 1861) Raabe, 1934 |
| | *Parauronema virginianum* Thompson, 1967 |
| | *Conchophthirus curtus* Engelman, 1862 |
| | *Proboveria rangiae* de Puytorac et al., 1978 |
| | *Trichodina nigra* Lom, 1960 |
| | *Tetrahymena pyriformis* Ehrenberg, 1830 |
| | *Glaucoma chattoni* Corliss, 1959 |
| | *Turaniella vitrea* (Brodsky, 1925) |
| | *Espejoia mucicola* (Pénard, 1922) |
| | *Colpidium campylum* (Stokes, 1886) |
| | *Ophryoglena mucifera* Mugard, 1948 |
| | *Collinia orchestiae* de Puytorac et Grain, 1975 |

Table 2. The characters considered (S = Camin Sokal; W = Wagner)

## SOMATIC CORTEX

### I. Distribution of kinetosomes (Ks) (1)

| | | | | | |
|---|---|---|---|---|---|
| S | over whole cell surface → | on short meridians → | on short meridians + absence of some meridians → | absence of some meridians → | absence of some meridians + erratic Ks |
| | 0000 | 1000 | 1100 | 1110 | 1111 |

### II. Grouping of kinetosomes (Ks) : 1st hypothesis

| | | | | | |
|---|---|---|---|---|---|
| S | isolated Ks → | isolated Ks + pairs → | only pairs of Ks → | pairs of Ks + cirri → | only cirri |
| | 0000 | 1000 | 1100 | 1110 | 1111 |

### 2nd and 3rd hypotheses

| | | | |
|---|---|---|---|
| S | pairs of Ks 0000 → | pairs + isolated Ks 1000 → | only isolated Ks 1100 |
| | | pairs + cirri 0001 → | only cirri 0011 |

### III. Postciliary fibers

| | | | |
|---|---|---|---|
| S | presence on all Ks → | presence on some Ks → | absence |
| | 00 | 10 | 11 |

### IV. Organization of the postciliary fibers

| | | | |
|---|---|---|---|
| W | in ribbons parallel to the cell surface | triangular arrangement | in ribbons perpendicular to the cell surface |
| | 100 | 010 | 001 |

(1) Although some authors argue that an ancestral stage would be "ciliature on one face", in the samples here considered, the reduction of the infraciliature seems most probably a secondary character.

hypothesis 2, the following were considered ancestral: (a) somatic kinetosomes in pairs, (b) ventral buccal area, (c) homogeneous buccal infraciliature. In hypothesis 3, (a) somatic kinetosomes in pairs were considered ancestral; no character transformation series were assumed for (b) the position of the buccal area or for (c) the organization of the buccal infraciliature.

The traits in Table 2 are either binary or multistate characters recoded in binary form. Multistate characters for which transformation series were hypothesized were recoded using the method of Kluge and Farris [46] and marked as "irreversible" (S type in Table 2, following the code used by the PHYLIP package), meaning that the only authorized evolutionary change is a change from 0 to 1. Other characters, for which no ancestral state nor transformation series are hypothesized, were recoded by attributing a binary variable (presence = 1, absence = 0) to each state, and marked as "reversible" (W type in Table 2), meaning that they can change either from 0 to 1, or from 1 to 0. All binary characters, "reversible" or "irreversible", were coded in a single digit (0/1).

A transformation series is recoded as follows. The hypothesized evolutionary sequence of states in the series can be represented by a "character tree" in which the states are joined by arrows indicating which state derives from which. Numbers are attributed to these arrows in any order to specify the binary variable that each arrow represents. When coding a character state, code 1 is attributed by convention to all the binary variables corresponding to arrows that are ancestral to that state, while all the others are coded 0. Thus for character XVII, "number of adoral organelles" (Table 2), if the first binary variable corresponds to the arrow joining states "absence" to "more than three", the second to the arrow joining "more than three" to "three", and the third to the arrow joining "three" to "one", then the state "more than three" will be coded "100" since the first arrow only is ancestral to it, while state "one" will be coded "111" since all three arrows are necessary to derive it from the ancestral state "absence".

Four main parsimony methods have been proposed for phylogenetic tree reconstruction from binary morphological data: the Camin-Sokal [9], Wagner [82], Dollo [18], and polymorphism [19, 20] methods; they have been compared by Felsenstein [20, 21]. The Camin-Sokal and Wagner methods are especially adapted to our date. The Camin-Sokal parsimony method [9, 78] requires information about the ancestral state to be known for each binary variable, and reversions of characters to the ancestral state (from 1 to 0) are not permitted. That method looks for the rooted tree minimizing the number of evolutionary steps (changes from 0 to 1); the root represents the ancestor with state 0 for all binary variables. This method is well adapted to characters for which one is willing to make evolutionary assumptions (type S).

Table 4. Treelengths obtained by combining trees A to D with the various ancestors described in Table 3. The shortest trees in each row are underscored

| Ancestors: | 3.1 | 3.2 | 3.3 | 3.4 |
|---|---|---|---|---|
| Tree A | 165.5 | 165.5 | 165.5 | 165.5 |
| Tree B | 165.5 | 167.0 | 167.0 | 167.0 |
| Tree C | 167.0 | 165.5 | 166.0 | 166.0 |
| Tree D | 166.0 | 166.0 | 166.0 | 166.0 |

The Wagner network parsimony method [82], on the other hand, does not require knowledge of the ancestral state of the characters, and it allows character reversions; it tries to find the network involving the smallest possible number of evolutionary changes from 0 to 1 or from 1 to 0. The method is well adapted to characters for which no evolutionary assumption was made (type W). Since our data (Table 2) contain variables pertaining to both the S and W types, an intermediate algorithm available in the PHYLIP package was used that allows type S variables to be dealt with following the Camin-Sokal method, while type W variables are processed according to the Wagner method.

Mixtures of character types can be analyzed by the PHYLIP (parsimony program MIX) and MacCLADE packages; other widely available packages, such as PAUP, HENNIG 86 or PHYSIS, do not allow treatment of mixed-type characters. Both PHYLIP and MacCLADE can also recognize missing values, coded "?" in a data file, and exclude them from the computations; out of 4032 data items, our data table contained 137 missing values (3,4 %). The PHYLIP package for inferring phylogenies, which contains a variety of parsimony and compatibility methods, was written and is distributed by Dr. J. Felsenstein, Department of Genetics, University of Washington, Seattle, Washington 98195, USA; version 2.9 was used in the present study. MacCLADE was written and is distributed by W. Maddison and D. Maddison, Museum of Comparative Zoology, Harvard University, Cambridge, Massachusetts 02138, USA; we used version 2.1. MacCLADE is not a parsimony analysis program. It allows the user to illustrate evolutionary hypotheses (trees), to compute their lengths (number of evolutionary steps) given the type and weight of each character, and to improve trees by branch swapping.

When computing the length of an evolutionary tree, we must count "1" for every evolutionary change. With "irreversible" characters, any change along the transformation series produces a number of 0-to-1 changes equal to the number of evolutionary steps. With multistate "reversible" characters on the contrary, any change of state produces one 0-to-1 change and one 1-to-0

Table 3. Coding of the four "Ancestor" character state hypotheses used to root trees A to D. These hypotheses differ in the coding of the ancestral states of characters 14 to 17 (italics). Character numbers are written vertically at the top of the table

| Character no.: | 1111111111111111111111111111111111111111111111112222 |
|---|---|
| | 1111222233444556667789990111222333*4445555555556666666666677788888999012 3* |
| Ancestor 3.1 | 0000000000100001000001000100100000*1001000000001000000000000000001000110* |
| Ancestor 3.2 | 0000000000100001000001000100100000*1000000000010000000000101000000001000110* |
| Ancestor 3.3 | 0000000000100001000001000100100000*1000000100000000000001010000000001000110* |
| Ancestor 3.4 | 0000000000100001000001000100100000*1001000000000100000000100000001000110* |

change, for a total count of two. To compensate for this effect, a weight of 2 was attributed to all "irreversible" characters (code S in Table 2), both in the PHYLIP and the MacCLADE runs, while the multistate "reversible" characters (code W) received a weight of 1. All binary characters received a weight of 2 because they had all been coded into a single state irrespective of them being "reversible" or "irreversible", so that every evolutionary step corresponds to a single 0-to-1 change or the reverse. As a consequence of this weighting, all tree lengths reported in the PHYLIP and MacCLADE outputs were twice their actual value and had to be divided by 2.

The PHYLIP package was used to search for the most parsimonious tree. However, since this is an NP-complete problem (i.e., the most parsimonious solution is certainly found only if all possible solutions have been examined), the algorithm in PHYLIP may only find a "local minimum" solution. In order to minimize this problem, six PHYLIP runs were made for each of the three data tables (three hypotheses): the first run with the original OTU placement, and five more after randomly permuting the order of the OTUs in the data table. Each run also provided an "Ancestor" character configuration; these were slightly reworked, when needed, to make sure that their character combinations made biological sense. The most parsimonious solutions obtained for each character hypothesis were selected and used as input for optimization by MacCLADE.

Using MacCLADE, we looked for even shorter trees by local and global branch swapping, in combination with the reworked "Ancestor" hypotheses described above. Treelengths were computed by MacCLADE and divided by 2 for the reason stated above; trees may be obtained with fractional lengths (ex. 331/2 = 165.5 mutations) because of the treatment of the unknown states (codes "?"). Besides trees, MacCLADE allows to represent the data sets in the form of data boxes (state "O" = white square, state "1" = black, unknown = blank).

## Results

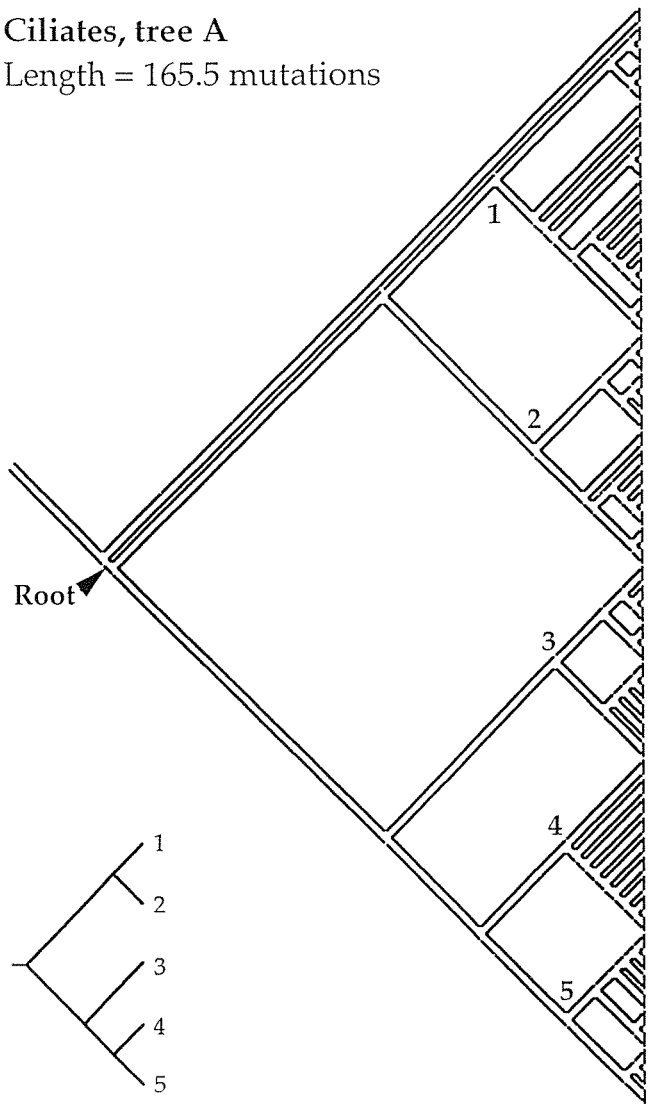
The shortest trees generated under hypothesis 1 (ancestral states = isolated kinetosomes, apical buccal area, homogeneous buccal infraciliature) were longer than those generated under hypotheses 2 and 3; they also differed so markedly from the most recent findings that they were discarded. For example, there is an early separation of the Litostomatea, Vestibuliferea from the rest. The Karyorelictea are distributed in different lines: one which gives *Monodinium,* the Trachelocercia-Loxodia, the later as a sister-group of Phyllopharyngea; the second with only *Protocruzia* grouped with the Heterotrichea; the third with *Geleia* mixed with the Colpodea. Hypothesis 2 (ancestral states = somatic kinetosomes in pairs, ventral buccal area, homogeneous buccal infraciliature) produced shorter trees than hypothesis 1 but several relationships, again, were not acceptable. Here again, the Karyorelictea are distributed in two different lines and appear later in the tree (3rd and 4th branchings). The Colpodea are not grouped and belong to two separated lines (*Platyophrya* and *Cyrotolophosis* as a sister-group of *Geleia-Protocruzia*-Heterotrichea-Hypotrichea). The early separation isolates the Litostomatea-Vestibuliferea-Phyllopharyngea. The Scuticociliata are early on separated from the Peniculia.

Hypothesis 3 (somatic kinetosomes in pairs considered ancestral; no ancestral state chosen for the position of the mouth or for the organization of the oral infraciliature) produced the four most interesting trees; trees A, B, C are shorter (165.5 changes) than tree D (166 changes), although this could change if the unknown states of the data table were resolved. Four "Ancestor" character combinations were tested with these trees. They correspond to the ancestor suggested by the PHYLIP run, modified to remove the uncertainties (codes "?") and to include only biologically meaningful character combinations. Several ancestral solutions seemed equally likely for characters XIV to XVII; they are called 3.1 to 3.4 and are reported in Table 3. It is interesting to examine the "Ancestor" solutions suggested for characters IV, VI, VIII to XII, XIV to XVI, XIX, XXI and XXII for which no ancestral state had first been imposed (type W in Table 2). Table 4 shows the treelengths obtained when testing each tree topology against each of these "Ancestor" hypotheses.

Tree A (Fig. 1) is optimal (shortest) for all four codings of the ancestor and it preserves the same length (165.5 changes) in all variants, which indicates a robust structure, since it can accomodate four different outgroups without change of length or topology. There is an early separation in two main branches. The first one, in the upper portion of the Figure, is further divided in two sub-branches: group (1), containing the Karyorelicta, Heterotrichea, Hypotrichea and Oligotrichea, forms a sister-group to the Colpodea group (2). The second main branch, in the lower half of the Figure, contains three sister-groups: (3) the Peniculia, Scuticocilia, Peritrichia, *Pseudomicrothorax* and *Furgasonia*; next (4) are the Hymenostomatia, *Urocentrum, Nassula* and *Coleps*; and finally (5) the Litostomatea, Vestibulifera, Phyllopharyngea, *Trimyema* and *Collinia* group. These five groups are formed at about the same level in the tree (species of these groups fan out from branching levels 3 and 4 above the root, not counting the Ancestor). In tree A, groups 1 and 2 have an origin clearly distinguishable from groups 3, 4 and 5.

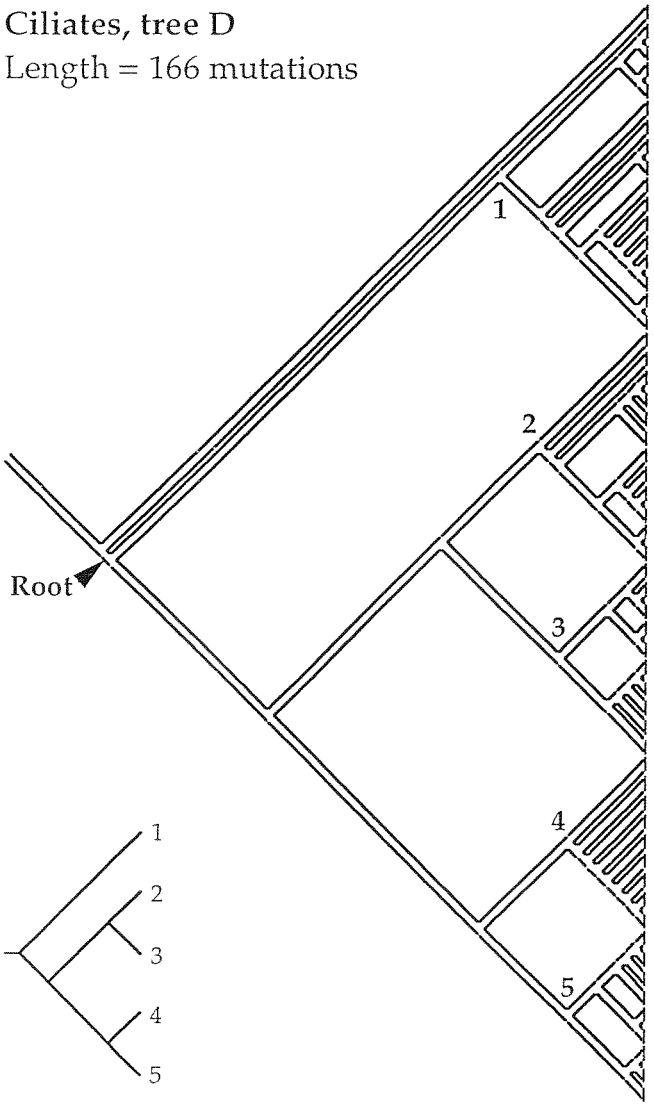
Tree B differs from A only by the early separation of group 5 that forms an independent lineage close to the Ancestor; it is not worth discussing it further. Tree C is discarded because the outgroup would be derived from one of the main groups; its length increases when Ancestor hypothesis 3.2 is forced to branch out from the root.

Tree D (Fig. 2) is also quite interesting; like tree A, it preserves the same length (166 changes) with all four Ancestor hypotheses, which indicates a robust structure. One can recognize the same five major groups as in tree A, but their higher-level topology differs, as shown in the insets of Figures 1 and 2. In this evolutionary topology, group 1 separates first from all the other Ciliophora (species of this group fan out from the second branching level above the root), while the other four groups are formed at branching level 4. Notice that tree D can be obtained from tree A simply by relocating group 2 on the stem of group 3; there is a length increase of one-half of a change in the process. There are also small differences between trees A and D in the arrangement of the species of




| | Order | Subclass | Class | Superclass | Subphylum |
|---|---|---|---|---|---|
| Ancestor 3.3 | | | | | |
| Remanella | Loxodida | Loxodia | Karyorelictea | Postciliodesmatophora | TUBULICORTICATA |
| Loxodes | | | | | |
| Trachelonema | Trachelocercida | Trachelocercia | | | |
| Tracheloraphis | | | | | |
| Geleia | Protoheterotrichida | Protoheterotrichia | | | |
| Protocruzia | Protocruziida | Protocruziidia | | | |
| Climacostomum | Heterotrichida | Heterotrichia | Heterotrichea | | |
| Condylostoma | | | | | |
| Nyctotherus | Clevelandellida | Clevelandellidia | | | |
| Euplotes | Euplotida | Euplotia | Hypotrichea | Spirotricha | |
| Paraurostyla | Urostylida | Oxytrichia | | | |
| Stylonychia | Oxytrichida | | | | |
| Plagiotoma | | | | | |
| Gastrostyla | | | | | |
| Halteria | Oligotrichida | Oligotrichia | Oligotrichea | | |
| Petalotricha | Tintinnida | Strobilia | | | |
| Cyrtolophosis | Cyrtolophosida | Colpodia | Colpodea | Transversala | |
| Platyophrya | | | | | |
| Woodruffia | | | | | |
| Enigmostoma | | | | | |
| Bursaria | Bursariomorphida | | | | |
| Bryophrya | Bryophryida | | | | |
| Bresslaua | Colpodida | | | | |
| Tillina magna | | | | | |
| Colp. simulans | | | | | |
| Colp. spiralis | | | | | |
| Colp. steini | | | | | |
| Colp. maupasi | | | | | |
| Paramecium | Peniculida | Peniculia | Oligohymenophorea | Membranellophora | EPIPLASMATA |
| Frontonia | | | | | |
| Disematostoma | | | | | |
| Myxophyllum | Philasterida | Scuticociliatia | | | |
| Conchophthirus | Pleuronematida | | | | |
| Proboveria | | | | | |
| Parauronema | Philasterida | | | | |
| Trichodina | Mobilida | Peritrichia | | | |
| Furgasonia | Parahymenostomatida | Nassulia | Nassophorea | | |
| Pseudomicroth. | Microthoracida | | | | |
| Nassula | Nassulida | | | | |
| Coleps | Prorodontida | Prostomatia | | | |
| Ophryoglena | Ophryoglenida | Hymenostomatia | Oligohymenophorea | | |
| Urocentrum | Urocentrida | Peniculia | | | |
| Turaniella | Tetrahymenida | Hymenostomatia | | | |
| Espejoia | | | | | |
| Colpidium | | | | | |
| Tetrahymena | | | | | |
| Glaucoma | | | | | |
| Chilodochona | Chilodochonida | Chonotrichia | Phyllopharyngea | Ciliostomatophora | |
| Brooklynella | Dysteriida | Cyrtophoria | | | |
| Sphenophrya | Rhynchodida | Rhynchodia | | | |
| Collinia | Apostomatida | Apostomatia | Oligohymenophorea | Membranellophora | |
| Trimyema | Prorodontida | Prostomatia | Nassophorea | | |
| Paraisotricha | Trichostomatida | | Vestibuliferea | | |
| Alloiozona | Haptorida | | Litostomatea | | FILICORTICATA |
| Monodinium | Spathidiida | | | | |
| Spathidium | | | | | |

Fig. 1. Phylogenetic reconstruction of 165.5 mutations in length (tree A) obtained from hypothesis 3 character coding. All four "Ancestors" in Tables 3 and 4 produce the same tree.




| Ancestor 3.1 | Order | Subclass | Class | Superclass | Subphylum |
|---|---|---|---|---|---|
| Remanella | Loxodida | Loxodia | Karyorelictea | Postciliodesmatophora | TUBULICORTICATA |
| Loxodes | | | | | |
| Trachelonema | Trachelocercida | Trachelocercia | | | |
| Tracheloraphis | | | | | |
| Geleia | Protoheterotrichida | Protoheterotrichia | | | |
| Protocruzia | Protocruziida | Protocruziidia | | | |
| Climacostomum | Heterotrichida | Heterotrichia | Heterotrichea | | |
| Condylostoma | | | | | |
| Nyctotherus | Clevelandellida | Clevelandellidia | | | |
| Euplotes | Euplotida | Euplotia | Hypotrichea | Spirotricha | |
| Paraurostyla | Urostylida | Oxytrichia | | | |
| Stylonychia | Oxytrichida | | | | |
| Plagiotoma | | | | | |
| Gastrostyla | | | | | |
| Halteria | Oligotrichida | Oligotrichia | Oligotrichea | | |
| Petalotricha | Tintinnida | Strobilia | | | |
| Cyrtolophosis | Cyrtolophosida | Colpodia | Colpodea | Transversala | |
| Bryophrya | Bryophryida | | | | |
| Platyophrya | Cyrtolophosida | | | | |
| Woodruffia | | | | | |
| Enigmostoma | | | | | |
| Bursaria | Bursariomorphida | | | | |
| Bresslaua | Colpodida | | | | |
| Tillina magna | | | | | |
| Colp. simulans | | | | | |
| Colp. spiralis | | | | | |
| Colp. steini | | | | | |
| Colp. maupasi | | | | | |
| Paramecium | Peniculida | Peniculia | Oligohymenophorea | Membranellophora | EPIPLASMATA |
| Frontonia | | | | | |
| Disematostoma | | | | | |
| Myxophyllum | Philasterida | Scuticociliatia | | | |
| Conchophthirus | Pleuronematida | | | | |
| Proboveria | | | | | |
| Parauronema | Philasterida | | | | |
| Trichodina | Mobilida | Peritrichia | | | |
| Furgasonia | Parahymenostomatida | Nassulia | Nassophorea | | |
| Pseudomicroth. | Microthoracida | | | | |
| Nassula | Nassulida | | | | |
| Coleps | Prorodontida | Prostomatia | | | |
| Ophryoglena | Ophryoglenida | Hymenostomatia | Oligohymenophorea | | |
| Urocentrum | Urocentrida | Peniculia | | | |
| Turaniella | Tetrahymenida | Hymenostomatia | | | |
| Espejoia | | | | | |
| Colpidium | | | | | |
| Tetrahymena | | | | | |
| Glaucoma | | | | | |
| Chilodochona | Chilodochonida | Chonotrichia | Phyllopharyngea | Ciliostomatophora | |
| Brooklynella | Dysteriida | Cyrtophoria | | | |
| Sphenophrya | Rhynchodida | Rhynchodia | | | |
| Collinia | Apostomatida | Apostomatia | Oligohymenophorea | Membranellophora | |
| Trimyema | Prorodontida | Prostomatia | Nassophorea | | |
| Paraisotricha | Trichostomatida | | Vestibuliferea | | |
| Alloiozona | Haptorida | | Litostomatea | | FILICORTICATA |
| Monodinium | Spathidiida | | | | |
| Spathidium | | | | | |

Fig. 2. Phylogenetic reconstruction of 166 mutations in length (tree D) obtained from hypothesis 3 character coding. All four "Ancestors" in Tables 3 and 4 produce the same tree.

group 2, but these are of little consequence on the lengths of the trees: tree D retains the same length of 166 changes, whether the order of the species in group 2 is as shown in Figures 1 or 2; the total length of tree A, however, increases from 165.5 to 166 changes if the fine topology of group 2 of tree D is forced onto it (results not illustrated in the Figures).

Notwithstanding these differences, the following relationships among genera are noticeable and similar in both trees:

a) *Nassula* is well-separated from *Pseudomicrothorax* and *Furgasonia*.

b) *Nassula* is close to *Coleps*, and these two genera are close to the Tetrahymenida.

c) *Nyctotherus* is closer to the Hypotrichea than to the Heterotrichea.

d) *Urocentrum* is close to the Hymenostomatia and far from the Peniculia.

e) *Trimyema* is close to *Paraisotricha*, but new data that have only recently appeared [71] about the stomatogenetic process in *Trimyema* have not been included in our coding. So, the position of this ciliate is provisional and should be reconsidered in some further study.

The position of some groups is also noticeable:

a) The Tetrahymenida are remote from the Peniculida and Scuticocilia.

b) The *Pseudomicrothorax-Furgasonia* group, the Peritrichia and some Scuticocilia (*Proboveria, Parauronema*) are in the same group.

c) The Karyorelictea are close to the Heterotrichea, Hypotrichea and Oligotrichea.

Finally, notice the composition of some of the groups:

a) The Colpodea always form a homogeneous group [2] containing two sub-branches.

b) The Nassophorea are divided in two subgroups pertaining to distinct groups (3 and 4).

c) The Karyorelictea (in group 1) are clearly composed of two subgroups: the Loxodia and Trachelocercia on the one hand, and *Geleia* and *Protocruzia* on the other.

d) Among the Heterotrichea (in group 1), the origin of *Nyctotherus* is clearly distinct from that of the *Climacostomum-Condylostoma* subgroup.

e) The Oligohymenophorea artificially contain subgroups that are found in groups 3 and 4.

## Discussion

The phylogenetic reconstructions reported here depend entirely, of course, on the choice of the characters and on the phylogenetic assumptions that we made. Cladistic analysis is a way to synthesize these various assumptions and to look for their consequences in terms of relationships among taxa; this will help to determine how good the assumptions were, and how adequate the characters.

With respect to the ciliates, if we stick to morphological considerations (structural and ultrastructural), cortical and nuclear characters are available. Among the latter, the degree of differentiation of the macronucleus with respect to the micronucleus is relevant since it has implications on the organisation of chromatin, its mechanism of distribution of the DNA during macronuclear division, as well as the degree of ploidy. It can be assumed, for example, that the existence of two identical nuclei is a plesiomorphic (ancestral) state, while the gradual distribution of the genome between a micronucleus that holds all the genes of the species and a macronucleus only containing a limited fraction of the genes is the derived state; it is likely to be the end result of successive steps, and it may have arisen in separate lineages to different degrees and at different periods.

Considering the cortex characters, what is noteworthy among ciliates is the presence of a juxtaposition of territories or cortical units each centred on a kinetosome or a pair of kinetosomes, with tangential fibrillar derivatives (kinetodesmal, transverse, postciliary fibres). As Fleury et al. [27] have shown, the anchoring of the kinetosomes and the dynamic properties of the cortex are dependent upon the superficial cytoskeleton. The cortical diversification of unicellular ciliates can only occur through the realisation of different adaptive constructions from a single starting material: the cortical unit. The question arises: Is the cortex basically composed of identical plesiomorphic units? In all cases, differentiation of certain units occurs fast at the level of the cytostome, resulting in a functional buccal apparatus.

In the tree D, the early emergence of a branch which derives straight from the hypothetic common ancestor (protociliate) and which contains the karyorelicts, heterotrichs, hypotrichs and oligotrichs is in agreement with molecular phylogeny [27] and with the idea that the paradiploidy of the macronucleus is a plesiomorphic character. A probable hypothesis would be that protociliates had two diploid nuclei dividing by mitosis, and that the subsequent differentiation of one of those nuclei into a macronucleus occurred by elimination of certain genes, amplification of those that remained, loss of the capacity to organise the microtubules in a mitotic spindle, and acquisition of an amitotic process of unequal distribution of genetic material between the daughter nuclei. Nevertheless, the question could be raised of whether the character "DNA-poor macronucleus" is plesiomorphic with respect to the usual polyploid macronucleus. It can be noted: a) that the differentiation of the macronucleus includes a phase of elimination of DNA [47] as well as a phase of synthesis of DNA, as in the macronuclear differentiation of other ciliates; b) that macronuclei are completely incapable of distributing their chromatin among two daughter nuclei by any type of division (except in *Protocruzia*) that could explain the separation of *Protocruzia* from the subgroup Loxodia-Trachelocercia; and c) that they have an abnormally high content of ribonucleoproteins [69], which is suggestive of a process of functional deregulation. So, the question arises as to whether those nuclei are those that have kept the primitive character of differentiation of the macronucleus to the largest degree (elimination of DNA and amplification of the remaining genes, unequal distribution by an intricate amitotic process), or whether they are a later product of this process of differentiation

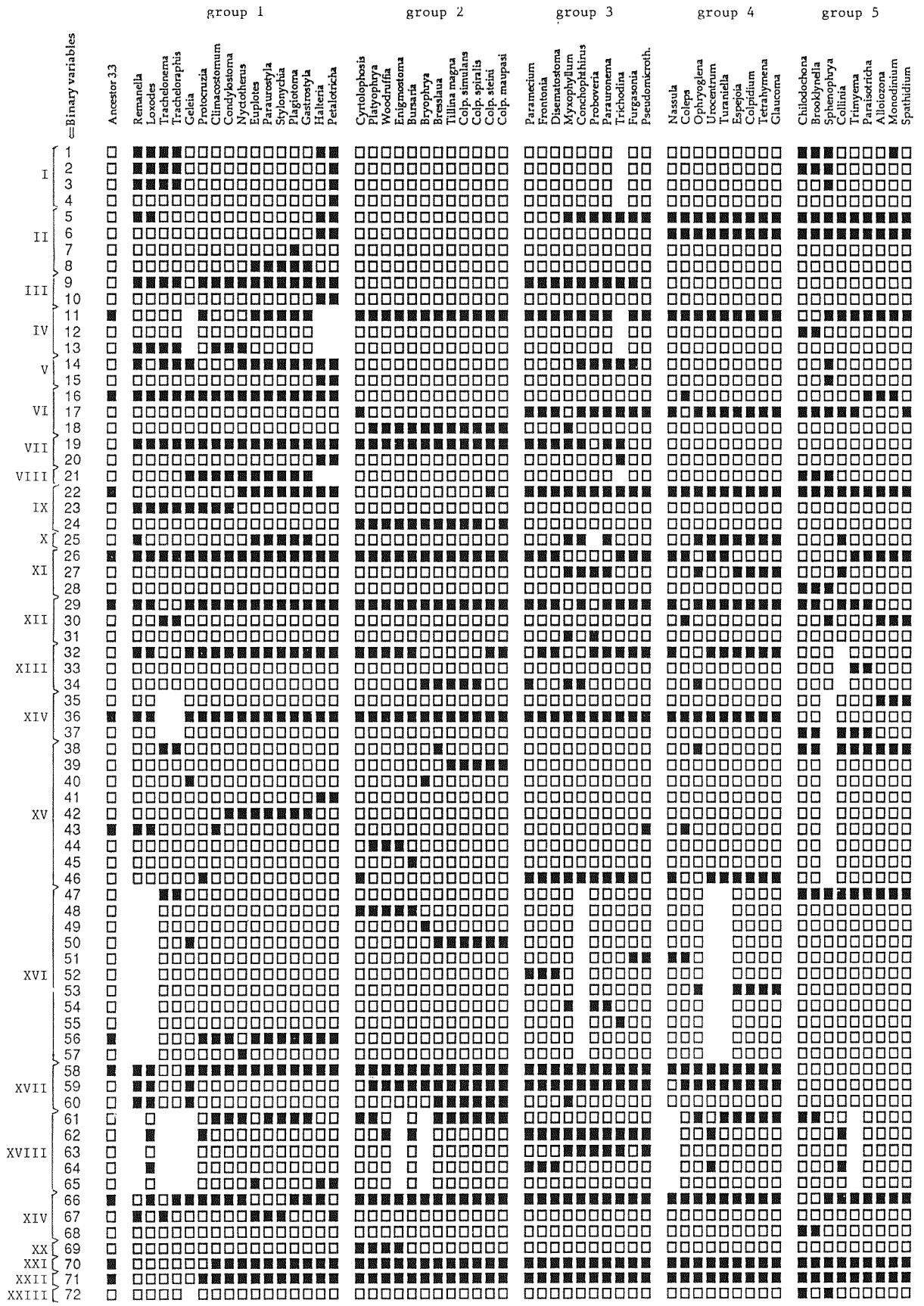



Fig. 3. Character coding for the 56 ciliates in the order that they appear in tree A (Fig. 1). The 23 morphological, nuclear and ultrastructural traits (I to XXIII) are recoded into 72 binary variables. Blanks are missing values. The "Ancestor" represented at the extreme left is 3.3.

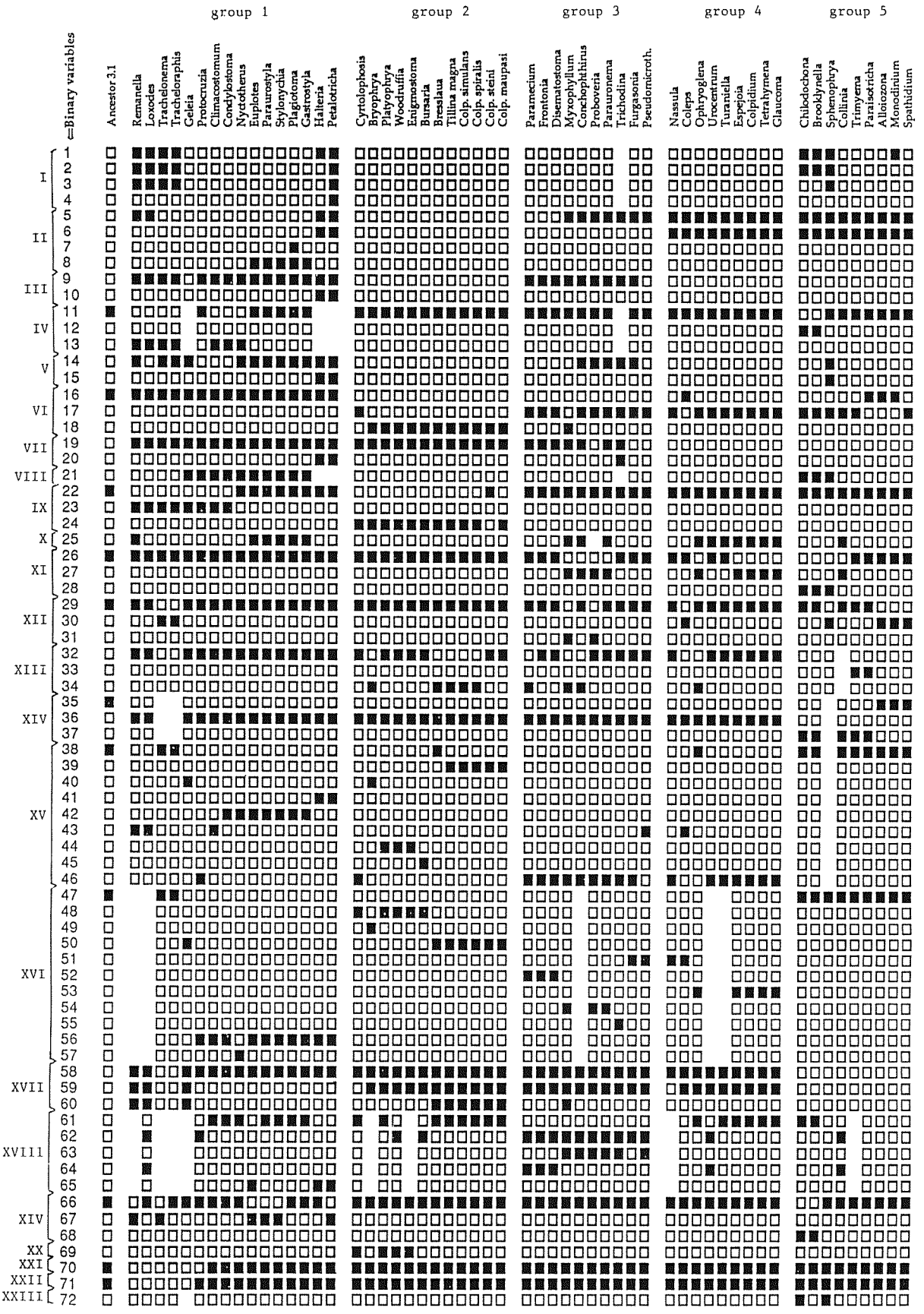



Fig. 4. Character coding for the 56 ciliates in the order that they appear in tree D (Fig. 2). The 23 morphological, nuclear and ultrastructural traits (I to XXIII) are recoded into 72 binary variables. Blanks are missing values. The "Ancestor" represented at the extreme left is 3.1.

(the elimination of DNA and subsequent amplification only reestablishing a quasi-diploid state, and the deregulation leading to a complete inability to share genetic material between the two daughter nuclei).

The answer given by our reconstructed tree D and by the molecular phylogeny is clear: ciliates with DNA-poor diploid macronuclei are primitive. It follows: a) that the fragmentation of the DNA of micronuclear chromosomes during the differentiation of the macronucleus is a general situation among ciliates, although in *Loxodes* nuclear DNA molecules are longer than in the other ciliates [56]; probably in all ciliates, macronuclear DNA molecules are subchromosomic; b) that non dividing macronuclei are primitive; this result confirms Orias' hypothesis [61] that ancestral ciliate macronuclei (as is some karyorelicts) were incapable of division while higher ciliate macronuclei gained the ability to divide, perhaps in several steps, and independently in different lines. An original proposition for the generation of the karyorelict non dividing macronuclei and life cycle was recently raised which involves heterophasic ancestors [62]. The emergence of the karyorelicts first does not disagree with Bardele's hypothesis [3] that the primitive ciliate is a ventrostome.

However, in our tree A, the emergence of the branch which leads to group 1 is posterior to a first branching. In that case, this group has a sister-group, the colpodids (group 2), while in tree D, it has no sister-group, and the branching that gives rise to the colpodids is later on.

Since the position of *Colpoda* is also highly variable in the molecular trees [27, 35] we cannot ascertain that either tree A or D conforms best to the molecular phylogenic trees. But, if we disregard the Colpodea, we find a great similarity between our two trees A and D, with an actual early separation of group 1 from the branch that leads to all the other ciliates.

It appears that the subgroup *Tracheloraphis – Trachelonema – Loxodes – Remanella* may be distinct from *Geleia-Protocruzia* which are close to the free-living heterotrichs. This conclusion had already been drawn from our earlier phenetic analysis [64]. It confirms the idea of Nouzarede [60] that the subgroup composed of Protoheterotrichia (*Geleia*) and Protocruziidia (*Protocruzia*) is closer to the heterotrichs than to the other karyorelicts. It suggests the the parapolyploid state of the macronucleus has been preserved in different lineages. Thus we can infer the existence of a subgroup of ciliates (Karyorelictea) from which we know only a few survivors that have subsisted by virtues of their psammic habitat and have evolved into diverging lines. This accounts for the marked differences between *Tracheloraphis* which has an apical mouth (but this may be a secondary state) and buccal infraciliature with a circumoral dikinety row [83] and *Loxodes* with its antero-ventral mouth and complex buccal asymmetric infraciliature with possibly autonomous stomatogenesis [59]. Within the same cortical and nuclear type, different stages of evolution of the buccal structures and morphogenetic processes have been reached, whence the separation of two classes more recently changed into two subclasses by de Puytorac et al. [63]: the Trachelocercia Jankowski, 1980 and the Loxodia Jankowski, 1980.

Consequently, the class Karyorelictea is polyphyletic and contains four subclasses: Loxodia and Trachelocercia, which are on the same branch, Protoheterotrichia and Protocruziidia, which are closer to the Heterotrichia.

The relationship of the Karyorelictea, Heterotrichea, Hypotrichea and Oligotrichea would indicate that they belong to a single group. However, our trees show that the Hypotrichea (and *Nyctotherus*) and Oligotrichea originate from the same branch, which differs from the Karyorelictea and Heterotrichea branches. This result fits well with the molecular tree of Fleury et al. [27] in which the Heterotrichea (*Stentor coeruleus* and *Blepharisma japonicum*) are closer to the Karyorelictea than to the Oligotrichea and Hypotrichea. Moreover the cortical cytoskeleton in the Karyorelictea and Heterotrichea is mainly composed of long postciliary ribbons of microtubules associated with the kinetosomes, while in the hypotrichs the cortical microtubules are not made of postciliary fibers and are not related to the kinetosomes [27]. It is also noticeable that observations on the nuclear apparatus confirm these relationships. According to Miyake et al. [57], micronuclei, in *Blepharisma* as well as in the Karyorelictea, can start differentiating into macronuclei at times other that the stages of postkaryogamic mitosis. On the other hand, the Oligotrichea and Hypotrichea have gene-sized DNA molecules and reorganization bands for the macronuclear division. So, it seems reasonable to group together the Karyorelictea and Heterotrichea in the same super-class Postciliodesmatophora Gerassimova and Seravin, 1977, according to their common cortical cytoskeleton [27, 31, 72, 73], while the Hypotrichea and Oligotrichea would be united in super-class Spirotricha Bütschli, 1889.

*Nyctotherus* constitutes a line closer to the hypotrichs than to the heterotrichs (*Climacostomum-Blepharisma*). This is in agreement with the distinction of a subclass Clevelandellidia [64], with heteromembranelles and a diplostichomonad as paroral organelle, separated from the subclass Heterotrichia with paramembranelles and variable paroral organelle structure (stichodyad, or one or two infraciliary rows). The heterotrich subgroup is probably polyphyletic.

The oligotrichs (*Halteria, Petalotricha*) are found in our trees close to hypotrichs. That agrees with the molecular tree [27] where *Halteria* is close to *Sylonychia* and *Paraurostyla* (Oxytrichida). In the opinion of Peuto-Moreau and Deroux (personal communication) the tintinnids would be close to *Euplotes*, and *Halteria* close to Oxytrichida. This assumption is not evident in our trees. In this interpretation, the oligotrichs would be a polyphyletic subgroup with a planktonic life. But if this is the case, the perilemma would have appeared twice in divergent lineages, as well as particular extrusomes (capsules of the tintinnids, trichites of the strombidids) which are absent in the hypotrichs. The ultrastructure of the oligotrichs must be subjected to further studies [37, 59].

Our classification includes *Plagiotoma* among the hypotrichs, as proposed by Fleury et al. [24]. For Small and Lynn [76, 77], however, subclass Stichotricha of the hypotrichs should be included in the Postciliodesmatopho-

ra, while the other subclass (Hypotrichea, order Euplotida) should be placed in class Nassophorea of subphylum Cyrtophora. This considerably diverging view receives no support in our classification and is not in agreement either with the molecular phylogeny. The ultrastructural and morphogenetic heterogeneity of the hypotrichs, in which stomatogenesis is accompanied by cortical rearrangements restricted to the ventral face or extending over the two faces, as demonstrated by Fleury et al. [25, 26], does not split up the group in our classification, contrary to Small and Lynn's proposition, despite the large difference of *Euplotes* from other hypotrichs.

The Colpodea group forms a sister-group of the Peniculia + Scuticocilia + Peritrichia + *Furgasonia* + *Pseudomicrothorax* in tree D, and of the Karyorelictea, Heterotrichea, Hypotrichea and Oligotrichea in tree A. This double possibility is found again in molecular trees where *Colpoda* may be a sister-group now of the peniculians and tetrahymenians [55] or of the hypotrichs (except *Euplotes*) [27]. According to Hofmann-Münz [42], considering the arrangement of kinetosomes, the paroral field of *Colpoda* resembles a paroral membrane accompanied by an anarchic field; so phylogenetic relationships would bring it closer to *Paramecium* than to *Tetrahymena*. The evolution of the Colpodea is dominated by the occurrence of division and resistance cysts, which allow an extreme diversification in edaphic media [28]. This diversification has occurred by way of widely differing buccal organelles, though by morphogenetic processes that remain essentially of the kinetophragmophorous type and with conservation of a cortex type (dikinetids with transverse fibres in arrays directed posteriorly). Possession of a micronucleus included in the same envelope as the macronucleus may have been acquired only once, since it is found in a monophyletic subgroup in tree A (*Enigmostoma – Woodruffia – Cyrtolophosis – Platyophrya*), as proposed also in the tree diagram of Foissner [28], or twice in tree D (in *Enigmostoma – Woodruffia – Platyophrya* on the one hand, in *Cyrtolophosis* on the other). Another hypothesis is that the common envelope of the two nuclei is a character which was acquired by the ancestor of the Colpodea and that there was a secondary loss in some genera but not in *Enigmostoma – Woodruffia – Cyrtolophosis* and *Platyophrya*.

As the Colpodea are characterized by a somatic cortical cytoskeleton mainly composed of longitudinal tractus of transverse fibers (microtubules associated with the kinetosomes), it is justified to consider that they belong to a new super-class called Transversala, which is independent of the Postciliodesmatophorea and Spirotrichea superclasses. If we want to group all the ciliates which have a cortical cytoskeleton composed of microtubules in a subphylum Tubulicorticata, as it was done by de Puytorac et al. [63], it appears that this grouping is justified by tree A where the three super-classes originate from the same branch, while it is polyphyletic in tree D, which would be paradoxical for a subphylum. In that sense, we have to consider that the character of the cortical cytoskeleton is not a good criterion for the phylogeny in tree D, while it is a good criterion in tree A, where, however, the early emergence of the Postciliodesmatophorea is not realized, which is in contradiction with the molecular trees. If we want to respect these molecular trees, it becomes evident that different solutions of reinforcement of the cell cortex by microtubules were selected as early as the first branching, and later during the diversification of the ciliates, and that it is impossible to consider that the Transversala could be grouped with the other super-classes Postciliodesmatophorea and Spirotrichea in the same subphylum.

In both trees A and D, the place of emergence of the Litostomatea and Vestibuliferea disagrees with the classical scheme of an ancestral ciliate with apical mouth and regular meridian kineties with monokinetids. However, it agrees with the molecular tree of Fleury et al. [27] in which the group *Chaenea, Isotricha, Enchelys* and *Didinium* emerges at the same time as ciliates usually considered as highly evolved (oligohymenophorids, hypotrichs, oligotrichs and nassophorids).

In both trees, the endocommensal ciliate *Alloiozona* is close to the free-living haptorid ciliate *Monodinium*. In *Alloiozona* [29, 32], the pericytostomial infraciliature consists only of somatic oralized monokinetids; this is justification enough for a taxon called archistomatids by de Puytorac and Grain [64], which is also characterized by the absence of toxicysts. In *Monodinium* on the contrary, the pericytostomal infraciliature consists of the anterior part of all the somatic kineties which differentiate into two or three oral dikinetids, and toxicysts are present. In some other haptorids such as *Enchelydium* [29], the oral monokinetids appear to be a secondarily acquired state [51].

*Spathidium* [7, 84] and *Monodinium* come out, in our trees, close to *Alloiozona*. So the Spathidiida and the Haptorida (*Alloiozona)* are to be joined in a subgroup called Litostomatea Small and Lynn, 1981. This subgroup and the Vestibuliferea (*Paraisotricha*) are separated on distinct branches which justifies the separation in two classes. In the class Litostomatea, the cytostomal aperture is superficial, while it is in a vestibulum in the class Vestibuliferea. In both classes, the somatic kinetid is a monokinetid with two ribbons of transverse fibres, the cytopharynx is strengthened by a rhabdos made of transverse microtubular ribbons and nemadesms, both originating from oral or oralized mono- or dikinetids, and the stomatogenesis is telokinetal.

In our trees, we have not considered any species from the Entodiniomorphida and Blepharocorythida which are true Vestibuliferea. De Puytorac et al. [63] had grouped the Litostomatea and Vestibuliferea in the same subphylum Filicortica, which is characterized by a cortical cytoskeleton consisting of an ecto-endoplasmic-boundary (EEB) of non-actin microfilaments. However a certain heterogeneity has to be noticed.

In most cases in the Litostomatea, the EEB is the main somatic cortical cytoskeletal element, in which basal bodies are anchored (except in *Isotricha);* however, the EEB is very thin in *Didesmis* [32] or remains undetected in *Chaenea, Fuscheria* [30] and *Acropisthium*. Moreover, in *Didesmis,* longitudinal microtubules whose origin remains unknown are present under the cell membrane.

In the Vestibuliferea, the Trichostomatida also have an EEB as the main component of their cytoskeleton, while in the Blepharocorythida and Entodiniomorphida an important epiplasmic layer is present, accompanied with underlying longitudinal microtubules and then by an EEB. So, why are these two latter orders placed by de Puytorac et al. [63] in the subphylum Filicorticata rather than in the subphylum Epiplasmata? These two orders, which emerge later in the diversification of the ciliates, would have retained all the basic components of the cortical cytoskeleton of the "protociliate" (microtubules, epiplasm, EEB) as defined by Fleury et al. [27], without any particular development of one of them, but with an equal development of each of them. This only characteristic would have authorized to create a new subphylum for these two orders. According to Fleury et al. [27], the diversification of ciliates led to several different major lines in which "once the cells had adopted a given shell strategy, they remained constrained within the choice made ...". If we agree with this hypothesis, we can ascertain that the choice was to keep all the basic elements in those ciliates that have a very poor somatic infraciliature without any well-developed kinetosomal associated fibers, and to use the maximum of cytoskeletal elements to maintain their cell shape, that gave a very rigid cortex.

For Small and Lynn [77], a subclass Trichostomatia in the Litostomatea would group together all the parasitic trichostomes whose diversification has mostly taken place in the fermentation organs of herbivorous vertebrates. The Plagiopylidae and Sonderiidae would be included in a subclass Plagiopylia, in class Oligohymenophorea. The interpretation of the cortical ultrastructure of *Lechriopyla* and *Plagyopyla* (course of the transverse microtubules) by Berger and Lynn [6], suggesting that the Plagiopylidae are not related to the classical trichostomids, has been discussed by de Puytorac et al. [67] who nevertheless recognise the particular ultrastructural characteristics of these ciliates.

In both trees, the Phyllopharyngids *Brooklynella* – *Chilodochona* (with postciliary fibres arranged in triangles and macronucleus heteromeric) – *Sphenophrya* form a branch with well-defined cortical (dissymmetry of the cortical fields, isolated kinetosomes, absence of transverse fibres, dextral orientation of the kinetodesmal fibres, transverse tract, subkinetosomal microtubules) and buccal characters (lamellar pharynx), and well defined stomatogenetic processes (Kinetophragmophora type: [40, 41]). In the phyllopharyngids, evolution is dominated by the development of thigmotactism and adaptation to the benthic habitat, culminating in fixation as an epizoon concomitant with the disappearance of somatic ciliature and subsequently of the buccal area (suctorids). In suctorids, the macronucleus is homomeric whereas that of the cyrtophorids and chonotrichs is heteromeric. In these ciliates macronuclear DNA is gene-sized, as in the hypotrichs [48]. This means that the DNA of the macronuclear genome has been fragmented in very different lines. The phyllopharyngids are closer to the Vestibuliferea and Litostomatea than to the oligohymenophorids (tetrahymenids).

On one side, the cortical point of initiation of the stomatogenetic processes clearly defines a ventral face in the phyllopharyngids; stomatogenesis is telokinetal and the transformation of the extremities of the somatic kineties, consisting of monokinetids, into segments of dikinetids making up the inverted kineties [40, 41], is a relatively simple process of haptoridian type, complicated here by the accompanying movement to rotation. On the other side, cytopharyngeal lamellae originate from the postciliary microtubules of the non-ciliated oral kinetosomes [40], as in the oligohymenophorids. The phyllopharyngids have an epiplasmic layer like the oligohymenophorids.

In all the previous classifications, the Apostomatia were considered as members of the oligohymenophorids. Recently, de Puytorac et al. [63] persisted in this opinion, according to Bradbury [8]. However, our trees clearly show that the Apostomatia *Collinia* is placed close to the Phyllopharyngea, with which it represents one branch separated from the branch leading to the Oligohymenophorea. This position agrees with the idea of Chatton and Lwoff, but not with the opinion of Bradbury [8] who suggested that the apostomes should more properly be considered a suborder of the hymenostomatids. In our coding, x, y, z have been considered as buccal kineties; in Bradbury's interpretation, the falciform and ogival fields are considered to be the adoral ciliature, an anterior row of barren kinetosomes being the paroral infraciliature. Even assuming this to be true, the affinities of the apostomes remain subject to discussion.

The separation of *Nassula* (Nassulida) from *Furgasonia* (Parahymenostomatida) and *Pseudomicrothorax* (Microthoracida) in our trees is surprising. Indeed the cyrtos of *Nassula, Furgasonia* and *Pseudomicrothorax* have the same structure and functioning. In all of them, the alveolocysts are regular components of the somatic cortex; the paroral organelles are autonomous while the adoral organelles depend ontogenetically on the somatic kineties [15, 16]. So the idea of a subclass Nassulia grouping the Nassulida, Microthoracida and Parahymenostomatida [34] might be accepted, although this subclass appears at least diphyletic. The position of *Pseudomicrothorax* in these trees entirely disagrees with the molecular trees of Fleury et al. [27] and Baroin-Tourancheau et al. [4] in which this ciliate diverges earlier than hypotrichs.

In both trees *Coleps* is close to *Nassula*. In *Coleps* [74], stomatogenetic processes involve a ventral cortical destabilisation resulting in a crown of pairs of pericytostomal kinetosomes (= peribuccal kinety) on the opisthe, which is homologous to a paroral kinety. The brush is homologous to the adoral organelles and analogous to the brush of the haptorids. In *Coleps* as in *Nassula,* the cytopharyngeal apparatus is lined by postciliary microtubules, but this is a general characteristic of a larger group of ciliates (Cyrtophora Small, 1976). So, the artificial class Nassophorea would group together the Nassulia and Prostomatia. The cortical ultrastructure of *Coleps* (absence of two tangential transverse ribbons) allowed Lynn [52] to separate the prostomatid genera *Coleps, Urotricha* and *Placus* from the litostomate genera in with he distinguishes two assem-

blages, one corresponding to the haptorids (*Monodinium, Spathidium, Lepiotrachelophyllum*), the other to the trichostomids (*Balantidium, Isotricha, Eudiplodinium, Epidinium*). This separation is confirmed in our analysis. In our reconstructed trees, *Coleps* is closer to the tetrahymenids while in the molecular tree of Fleury et al. [27] and Baroin-Tourancheau et al. [4], it is closer to the peniculids. This would support the inclusion of the Nassulida and Prostomatia into the Oligohymenophorea.

In both trees, the scuticociliates (*Myxophyllum – Conchophthirius, Proboveria, Parauronema*) are closer to the peniculids than to the tetrahymenids. This is partially in agreement with the molecular trees [4, 27] in which *Pleuronema* is closer to *Paramecium* than to *Tetrahymena* and *Colpidium*. The proposition of Small and Lynn [76] to separate the peniculids (in class Nassophorea) from the tetrahymenids and scuticociliates is not supported by our findings. However, here again we have a subclass (Scuticociliatia) which is composed of two groups coming from two distinct branches.

In both trees, the peritrichs (*Trichodina*) are closer to the scuticociliates and peniculids than to the tetrahymenids; in this they differ from the phylogenetic construction of Lynn and Sogin [55] in which *Opisthonecta* is more closely related to the *Colpidium – Glaucoma – Tetrahymena* group than to *Paramecium*. Our results are in agreement with the presence in *Paramecium* of an anarchic field of kinetosomes remaining at the origin of all the buccal organelles, and with the stomatogenetic processes of the peritrichs (with a germinal row homologous to a scutica of the scuticociliate type).

*Turaniella and Espejoia* are included in the tetrahymenid group, which is generally accepted; curiously, *Urocentrum* is also found in the same group, and that is contrary to the classical scheme in which *Urocentrum* is seen as a peniculid, considering the fact that its autonomous stomatogenesis may be found in different lineages. However, its position in our trees would be justified by its cortex of *Tetrahymena*-type.

The overall scheme confirms that in the course of the evolution of the ciliates, as it is the case also in other groups, various cell characters evolved at different rates and have now reached different states in different lineages. Thus, the macronuclear characters, concerning the organisation of the chromatin probably being a function of the size of the DNA molecules [56], have diversified in different paraphyletic lines, so that the karyorelicts and protoheterotrichs now have a reduced amount of DNA with long molecules, while in the phyllopharyngids, as in the oligotrichs and hypotrichs, there is gene-sized DNA and duplication of DNA in replication bands. The micronucleus and macronucleus included in the same envelope is a character found in the prostomids (*Enchelys*) as well as in some colpodids.

For the same cortical features, widely differing adaptations of the buccal structure can occur, as illustrated by the diversity of buccal types in colpodids. The apical position of the mouth has been secondarily obtained, so that it is found in lineages as divergent as the karyorelicts, haptorids and prostomids. A system of feeding by filtration with numerous organelles bordering the left side of the peristome and perpendicularly oriented to it in the oligotrichids, heterotrichids and hypotrichids, retaining relatively large particles (> 2 μm), is most likely primitive; the evolution of the buccal organelles in the filter-feeding ciliates appears to be an optimisation of the filtration by selectivity of smaller and chemically identified particles [22, 23]. Raptorian feeding, which is common among the karyorelicts, appears again in other lineages such as the haptorids. Histiophagy has also developed independently in several different lines (*Coleps, Ophryoglena* and the scuticociliates).

In the buccal area again, similar adaptive features are found in distinct lineages (e.g., the sucker of the rhynchodines and the sucking tentacles of the suctorians). The same evolutionary stage in the development of autonomy of the buccal system may also have been reached in different lines.

Our phylogenetic reconstructions based on certain ultrastructural characters are, in general, in good agreement with the molecular phylogeny derived by others. So, the type of phylogenetic analysis presented in this paper should be pursued; it will allow to statistically compare protozoan phylogenies derived from independent classes of characters (ultrastructural and molecular data, in the present case), using for instance the new technique of Lapointe and Legendre [49].

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*Key words:* Ciliates – Parsimony methods – Phylogeny

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CORRIGENDUM

Erroneously, Table 2 of the article on ciliates parsimony phylogenetic tree by P. de Puytorac, J. Grain, and P. Legendre [Europ. J. Prostistol. *30*, 1–17 (1994)] was printed incompletely. The following 4 pages should be added. The pages are also enclosed separately to insert them at the corresponding place in issue 30/1 of the EJP.

Klaus Hausmann (Managing Editor)

# An Attempt at Reconstructing a Phylogenetic Tree of the Ciliophora Using Parsimony Methods

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## V. Transverse fibers

| | | | |
|---|---|---|---|
| S | presence on all Ks → 00 | presence on some Ks → 10 | absence 11 |

## VI. Parasomal sacs (PS)

| | | | |
|---|---|---|---|
| W | absence 100 | 1PS per Ks or group of Ks 010 | more than 1PS per Ks or group of Ks 001 |

## VII. Kinetodesmal fibers

| | | | |
|---|---|---|---|
| S | presence on all Ks → 00 | presence on some Ks → 10 | absence 11 |

## VIII. Organization of kinetodesmal fiber

| | | |
|---|---|---|
| W | directed forwards 0 | directed transversally 1 |

## IV. Well developed longitudinal tracts of tangential microtubules

| | | | |
|---|---|---|---|
| W | absence 100 | presence due to postciliary fibers 010 | presence due to transverse fibers 001 |

## X. Kinetosome-independent superficial longitudinal microtubules in the interphase cell

| | | |
|---|---|---|
| W | absence 0 | presence 1 |

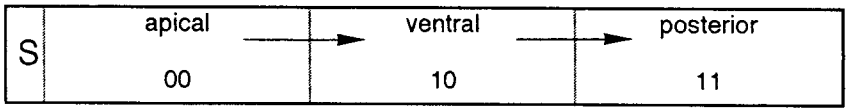
## XI. Deep longitudinal microtubules

| | | | |
|---|---|---|---|
| W | absence 100 | parakinetal 010 | subkinetal 001 |

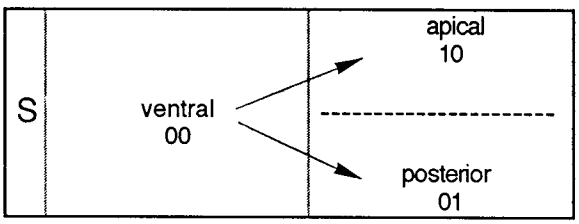
## BUCCAL CORTEX

### XII. Position of the buccal area

#### 1st hypothesis



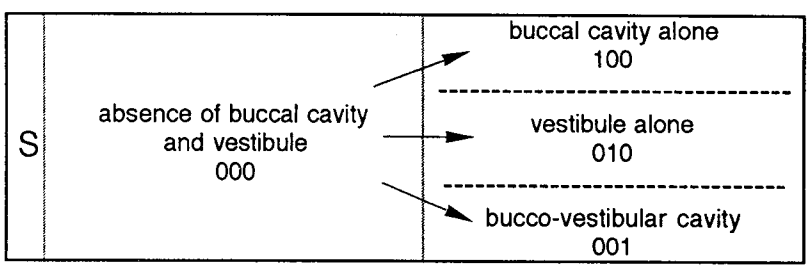

#### 2nd hypothesis




#### 3rd hypothesis

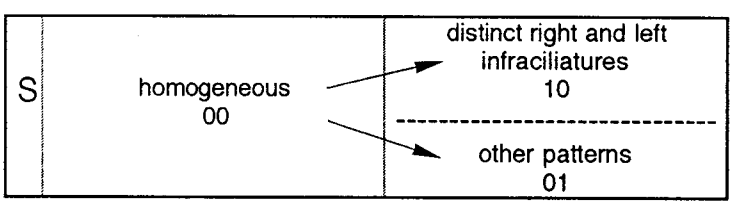
| W | ventral | apical | posterior |
|---|---|---|---|
| | 100 | 010 | 001 |

### XIII Constitution of the buccal area




### XIV. Arrangement of the buccal infraciliature

#### 1st and 2nd hypotheses




#### 3rd hypothesis

| W | homogeneous | distinct right and left infraciliatures | other patterns |
|---|---|---|---|
| | 100 | 010 | 001 |

## XV. Pattern of right buccal field

| | | | | |
|---|---|---|---|---|
| W | absence | 100000000 | one diplokinety with pairs of Ks, normally oriented | 000001000 |
| | anarchic field | 010000000 | one inverted diplokinety | 000000100 |
| | ordered field | 001000000 | more than one stichodyad | 000000010 |
| | one monostichomonad | 000100000 | one stichodyad | 000000001 |
| | one polystichomonad | 000010000 | | |

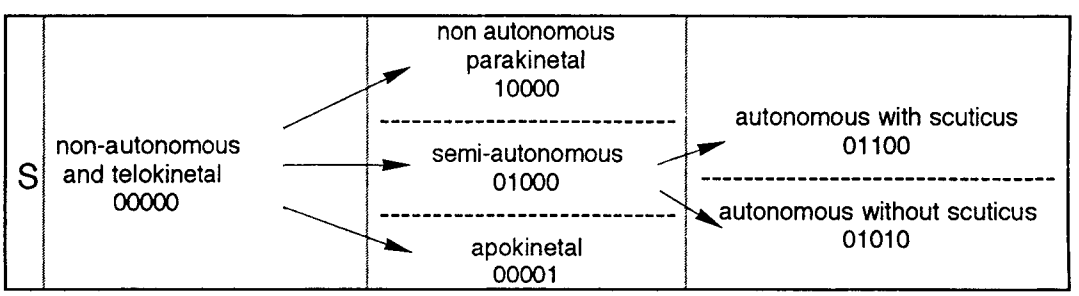
## XVI. Pattern of left buccal field

| | | | | |
|---|---|---|---|---|
| W | absence | 10000000000 | peniculi | 00000100000 |
| | several cirro-membranelles | 01000000000 | membranelles | 00000010000 |
| | several cirro-membranelles and a field | 00100000000 | membranoids | 00000001000 |
| | one field | 00010000000 | polykineties | 00000000100 |
| | pavés | 00001000000 | paramembranelles | 00000000010 |
| | | | heteromembranelles | 00000000001 |

## XVII. Number of adoral organelles

| | | | | |
|---|---|---|---|---|
| S | absence → | more than three → | three → | one |
| | 000 | 100 | 110 | 111 |

## XVIII. Mode of stomatogenesis




# NUCLEI

## XIX. Constitution of the macronucleus

| | | | |
|---|---|---|---|
| W | homomeric without replication band | homomeric with replication band | heteromeric |
| | 100 | 010 | 001 |

## XX. Relative positions of micronucleus and macronucleus

| | | |
|---|---|---|
| S | in separate nuclear envelopes → | in the same envelope |
| | 0 | 1 |

### XXI. Degree of polyploidy of the macronucleus

| | | |
|---|---|---|
| W | Weakly polyploid<br>0 | polyploid<br>1 |

### XXII. Division of the macronucleus

| | | |
|---|---|---|
| W | does not divide<br>0 | divides<br>1 |

## ASEXUAL REPRODUCTION

### XXIII. Mode of cell division

| | | |
|---|---|---|
| S | binary division<br>0 | budding<br>1 |