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Symposium 4

Morphogenesis and Evolution in Ciliates

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During the last decades ciliate morphogenesis always received great attention in protistologists. The complex series of events recognizable during the formation of new oral structures prior to cell division have been subject of many publications. These investigations were mainly triggered and facilitated by the introduction of various silver-staining techniques in ciliate research and by the application of scanning and transmission electron microscopy that made it possible to elucidate details of the morphogenetic process at high resolution.

There are two main reasons to deal with ciliate ontogeny. For protistologists with special interest in developmental biology, ciliate stomatogenesis offers an opportunity to examine differentiation processes within a single cell with relative ease. Ciliates are perfect model organisms to do this since many species are easily to cultivate and multiply very rapidly. Mutants may be selected, cultivated and compared with normal cells. Details of the differentiation process can be studied by various tools including silver staining, immunofluorecence techniques and electron microscopy.

The second approach on ciliate morphogenesis is based upon ideas already published in the last century [2, 41] assuming that examination of ontogeny can help to recognize phylogenetic relationships. Although many objections can be raised against these theories, ciliate ontogeny can tell us a lot about ciliate phylogeny, if we use ontogenetic data with proper caution, as suggested by Corliss [14].

There are several examples for a successful application of ontogenetic data to phylogeny: The Suctoria are well characterized as a monophyletic group. They are sessile and show an unique mode of nutrition and reproduction. However, their affinities to other ciliate groups were discussed controversially for a long period of time. Based on investigations of Guilcher [38], who recognized that the suctorian larvae have a holotrichous ciliature derived from a field of barren kinetosomes of the adult cell, Fauré-Fremiet [23] concluded that the suctorians should be placed to the holotrich ciliates, thus solving an old problem in ciliate phylogeny.

Based on investigations of Guilcher [39] on the development of oral and somatic structures in chonotrich ciliates, this group was placed in a close relationship with cyrtophorid ciliates. Today these conclusions, based on morphogenetic characters, are widely accepted and also corroborated by results concerning cortical ultrastructure of suctorian and chonotrich larvae.

Small [72] proposed a new ciliate order, the Scuticociliatida. The key character for this new taxon is an unique assemblage of normally barren kinetosomes called 'scutica' which is recognizable at least during stomatogenesis. The scutica is a derivative of the paroral membrane of the parental cell and is involved in the formation of adoral organelles in the subsequent division cycle. Today the order Scuticociliatida based on an unique stomatogenetic pattern is widely accepted. Influenced by these ideas many investigations on stomatogenesis and ultrastructure of scuticociliates were carried out, mainly by French scientists, to further elucidate stomatogenetic details of this group [16, 35, 36, 65].

Corliss [13, 15] proposed several types of stomatogenesis that are often used for phylogenetic considerations. Within the Hymenostomes, for example, the Tetrahymenina and Ophryoglenina, characterized by a parakinetal stomatogenesis, can clearly be distinguished from the Peniculina and the Scuticociliatida that show a buccokinetal mode.

Fifteen years ago transmission electron microscopy was introduced as a tool to describe morphogenetical events at the ultrastructural level. Jerka-Dziadosz [51, 52, 53, 54] published four papers on the ultrastructure of morphogenesis in *Paraurostyla*, thus demonstrating what detailed information can be obtained using transmission electron microscopy for morphogenetical studies. Following this line many electron microscopical studies on morphogenesis in various ciliate groups, like the cyrtophorids, prostomes, nassulids and hymenostomes were carried out in the lab of Bardele [9, 19, 43, 45, 46]. These studies on ciliate ultrastructure and morphogenesis revealed many details that could be used for considerations on ciliate evolution including those presented during our symposium.

During the last years sequence comparisons derived from the 23S-like and 16S-like rRNA [6, 7, 8, 10, 34, 55, 68, 70] revealed new insights on ciliate phylogeny. Ciliate trees based upon these data can now be compared with phylogenetic considerations based upon morphological and ontogenetic characters. Some of the contributions presented in our symposium were influenced by this comparative approach.

Four lectures were presented in our symposium. The first lecture dealt with molecular phylogenies, cortical cytoskeletal organization and morphogenesis. The second lecture focused on unique morphological and ontogenetic properties of the paroral membrane and its importance for ciliate phylogeny. These presentations were followed by two lectures presenting detailed ideas on ciliate phylogeny based upon morphological and ontogenetic data. A phylogenetic system of the ciliates, mainly based on ontogenetic data, was presented and the final lecture comprised comments on phylogenetic trees reconstructed from non molecular data and on the origin of ciliates.

Extended summaries of the four lectures were prepared by the authors:

Cortical organization and molecular phylogeny

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One of the specific characters of ciliates is the many thousands of cilia anchored in a species specific pattern over the cell cortex. Associated with this morphological character are some adaptative specializations, such as increased velocity or diversification in prey prehension capabilities; this has been achieved in the course of evolution through diversified cell shapes and ciliary patterns in association with ecological adaptations. At the same time, seve-

ral problems were to be solved, such as to synchronize the ciliary beating for efficient swimming or to reproduce the cellular pattern through sexual or asexual reproduction. Because the pattern and mode of ciliary implantation is fundamentally a question of organization of the cell cortex, it is clear that the evolution of ciliate morphology is predominantly a matter of evolution of their cortex. However, the cortex of ciliates is not a homogeneous entity: a number of structural differences have long been described between the various species. The establishment of a first broad phylogeny of ciliates allowed to rationalize these differences: we were led to identify a small number of fundamentally distinct modes of cortical organization in ciliates [8, 24]. In this paper, we rediscuss this hypothesis in the light of more recent 18S and 28S ribosomal RNA data [10, 55, Baroin-Tourancheau et al., in prep].

Biological interpretation of molecular trees.

Ciliate monophyly .

As expected from all morphological and morphogenetical data, ciliates appear as a monophyletic group in ribosomal RNA trees of eukaryotes. The most specialized cytoskeletal innovation of ciliates is the system of three rootlets associated with the basal bodies [33]. Because all ciliates possess this system which leads to a polarization of the basal body, we can assume that it is ancestral and could have been a key event in ciliate emergence and diversification.

Special attention had been devoted to this system which had suggested ciliate evolutionary mechanisms, formalized by Seravin and Gerassimova [71], who defined at least two types of organization according to the relative deployment of the rootlets, and Lynn, who used it in the framework of his "structural conservatism" hypothesis [57, 58, 74]. While these approaches allowed the identification of high level taxa within ciliates (class level), it did not provide information on the actual interrelationships between these taxa. This was the major breakthrough provided by the molecular phylogenetic approach.

Species clustering.

One of the most salient information yielded by 18S and 28S rRNA trees (which are very congruent) resides in the deep branching of various ciliates genera into four major clusters. A 28S phylogenetic tree which depicts the characteristic features of these branching topologies is shown in Fig. 1. There are two striking points in this tree: first, most of the clusters correspond to one or more ciliate classes as defined by the morphological approach, i.e. there is good congruence between the two approaches at fairly high taxonomic levels. Second, as a result of new biochemical, immunocytochemical and ultrastructural studies, each from the four clusters appears to correspond to a distinct cytoskeletal organization, characterized by the hypertrophy of one cytoskeletal element subtending the cortex:

The postciliodesmy (cluster A), which is the extensive use of the postciliary microtubules. Basal bodies are patterned into rows and maintained by large overlapping bundles of postciliary microtubules coming from the posterior basal body of each pair. As a rule, during division, new basal bodies appear next to parental ones, in a strictly determined location with respect to the polarization of the basal body [11]. Because longitudinal microtubules are detected throughout the cycle, the new post-ciliary fibers are most probably inserted between parental ones, acting as a structural guide for microtubule assembly.

The ectofibrillar system (cluster B), in which basal bodies are linked to the ecto-endoplasmic boundary built up with centrin-like proteins [80]. During division, new basal bodies also appear next to parental ones and the ecto-endoplasmic boundary could act in patterning newly assembled basal bodies, as suggested by studies on regeneration [31].

The free microtubule system (cluster C) This organization corresponds to the extensive development of microtubules independent from basal bodies, patterned in form of lattices and underlying the whole cortex; microtubules originating from basal bodies or clusters of basal bodies are inserted into this subpellicular lattice. During division, almost all of the kine

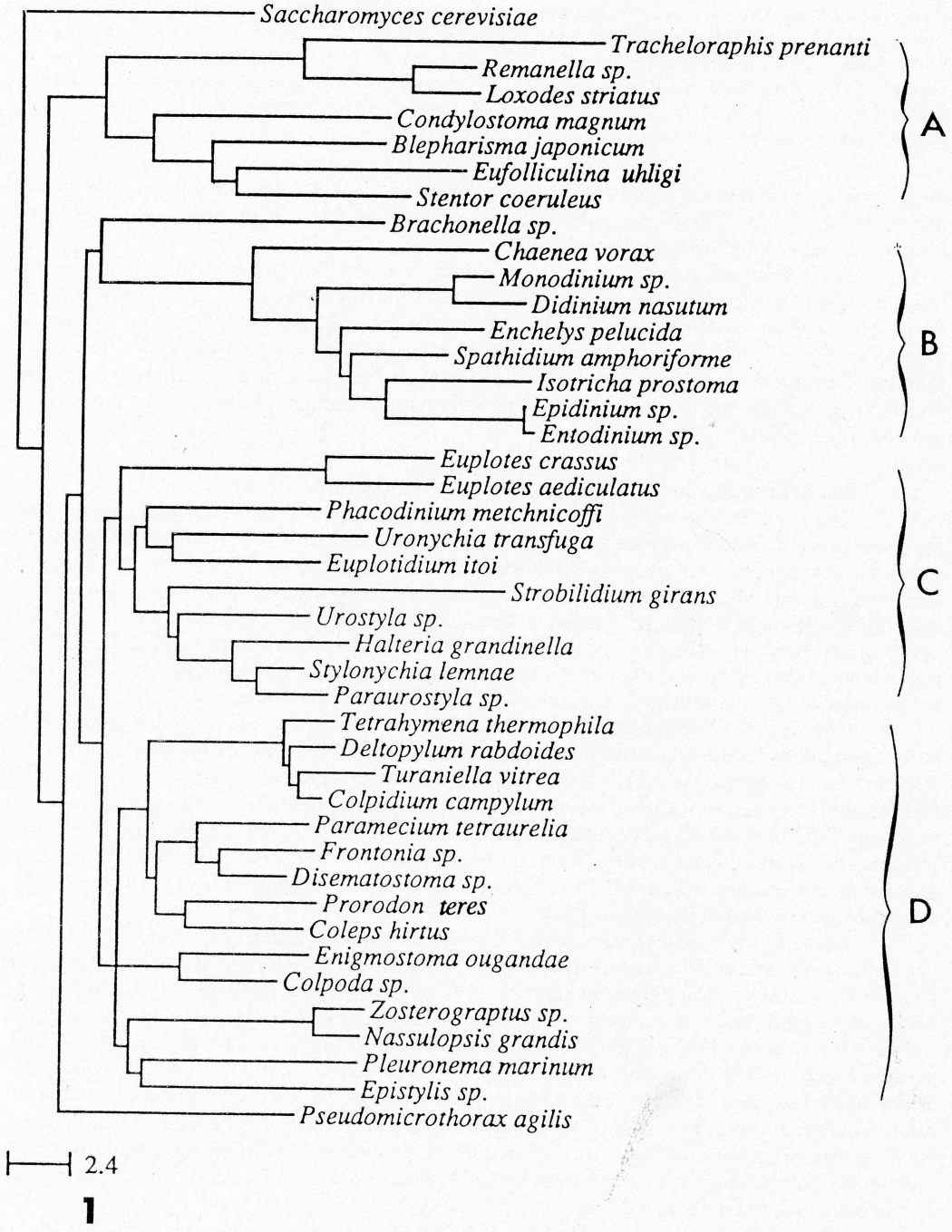

Fig. 1: Phylogenetic tree of the ciliates generated by the neighbour-joining method. The analysis is restricted to the 5' end of the 28S rRNA molecule. Saccharomyces cerevisiae is taken as an outgroup. Among the 267 unambiguously aligned sites, 171 are variable and taken into account. Construction of the tree is performed as described in [8]. The Heterotrichs and Karyorelictids are associated (cluster A) and emerge as a sister group to all other ciliates. The other

ciliates separate into three major monophyletic groups (designated as B, C and D) whose respective order of diversification is not resolved. This lack of resolution is also observed in 18S rRNA trees. B and C correspond respectively to the Litostomes and the hypotrichs. D corresponds to a monophyletic unit comprising the Oligohymenophorans, with a deep split between hymenostomes and peniculines, the Colpodeans and the Nassophoreans. The position of Pseudomicrothorax is not solid. In 18S rRNA trees, it emerges along the Nassophoreans in the cluster D.
Scale bar: percent of nucleotide substitutions.

tome is renewed and basal bodies often appear far away from parental ones in relation to the microtubular pattern [25]; microtubules of the superficial system most probably originate from these newly assembled basal bodies (unpublished data).

The epiplasmic strategy (cluster D), which extensively uses interactions between the basal body and associated rootlets, and the epiplasm for morphogenetical mechanisms. The basic scheme is an insertion of the basal bodies within the epiplasm, supplemented by microtubules or hyperdevelopped kinetodesmal fibers to ensure the longitudinal cohesion of the kineties. During kinety elongation, new basal bodies appear next to parental ones and then become inserted into the epiplasm; the spacing of kinetosomes then results from an anisotropic growth of the epiplasm [49].

Branching topology versus cytoskeletal organization.

The resulting phylogenetic pattern of the cortical organization shows that a number of representatives do not display the basic cytoskeletal organization of the cluster they belong to. Immunocytochemical and ultrastructural analyses show that two or three of the cytoskeletal elements (microtubules, ecto-endoplasmic boundary and epiplasm) may coexist in many species. Thus, although homology has been demonstrated for tubulins and centrins but not for epiplasmins, one hypothesis is that the three elements were present at the emergence of the phylum, and each of the four organizations described corresponds to the tendency developed in each cluster for a predominant, but not exclusive, use of one of these elements.

The organization found in the first divergent cluster (A) is the postciliodesmy. This early emergency can be related to the fact that this organization is basically very simple both structurally, it uses predominantly one of the rootlets, and regulatively, because a single cytoskeletal element is involved. If we refer to morphogenesis, post-ciliary fibers appear to be very important in many respects; their assembly takes place before that of other rootlets, and is followed by basal body orientation when localized in an anarchic field [11]; in addition, they are permanent systems which could have specific biochemical properties related to basal body assembly as observed in hypotrichs [26].

Some of the classical heterotrichs (*Condylostoma* and related species) exhibit this organization on both ventral and dorsal sides; they are devoid of kinetodesmal fibers and possess large oral structures coming from an anarchic field of kinetosomes. A slightly different organization is found in heterotrich species clustering in other parts of the tree; this is for example the case for *Metopus* [44] and *Brachonella* (Fig. 1), which branch off deeply in the tree, and possess kinetodesmal fibers [50] and also probably an epiplasm (unpublished data). *Phacodinium*, which branches off at the base of the hypotrich ciliates, shares a subpellicular network of microtubules covering the whole cell [17], as hypotrichs have. These data suggest that, heterotrichs sensu largo are a primitive group; accordingly, species exhibiting the postciliodesmy character in association with other cytoskeletal elements are found as early diverging representatives of several groups.

The free-microtubule organization is found in some hypotrich species (*Paraurostyla* [25] and related species). But postciliodesmy and free-microtubule organization in fact coexist in many species. This is for example the case in karyorelictids, in which the postciliodesmy is restricted to the ventral locomotory side where most of the ciliature is located, while the microtubule free system extends onto the very poorly ciliated non locomotory side (unpublished

observations). An opposite realization is found in some hypotrichs which also exhibit a juxtaposition of free-microtubule, covering the locomotory side, and postciliodesmy, covering the other one (*Uronychia* [27] and related species). Oligotrichs, characterized by a reduced somatic ciliature branch off in the hypotrichs, but are not clustered. They could represent several distinct pelagic adaptations corresponding to the two types of microtubular organizations.

Species using the ecto-endoplasmic boundary to anchor the ciliature are found in one very robust cluster. The basic scheme described in the previous section appears modified in endosymbiotic species, such as entodiniomorphids in which the functional locomotory ciliature is reduced. Non ciliated basal bodies, from which ciliary crowns originate during morphogenesis, are anchored onto the ecto-endoplasmic boundary and remain in the ectoplasmic space [29]. An epiplasmic cortical scaffold sustains the unciliated cortical membrane without any clear relation or role in kinetome anchoring.

Various cortical organizations and deeply branched lineages characterize the ciliates which possess an epiplasm, thus suggesting that this structure correlates with a large panel of cytoskeletal innovations. In some species, the epiplasm appears as a continuous system (*Tetrahymena* [81] and related species), sometimes very thick (*Pseudomicrothorax*, [64]) or very thin (*Colpoda*, [58]). In other ones, the epiplasm is segmented, into scales (Peniculids, [48]) or in alveolocysts (*Nassula* [21] and related species). The longitudinal continuity may be ensured either by subkinetal microtubules (Scuticociliates [1], Phyllopharyngea [45]), transverse microtubules (*Colpoda* [58]), or by hyperdevelopped kinetodesmal fibers relayed by microtubules in the course of division (Peniculids [48]). Most of the groups obtained up to date by molecular phylogeny correspond to one specific combination of these two characters.

In summary, since the publication of our initial correlation between species clusters in molecular phylogenies and cortical organization, which involved 20 species, we have increased the number of species to 41 and find that the correlation:

1) still stands for all species strongly inserted within each cluster;

2) is less strong for several basal species, suggesting that they still reflect today a point in time when the shift toward hypertrophy of one of another element had now been achieved.

Perspectives

As stated in a previous paper [24] different morphogenetical properties of each group may be related to the properties of their cytoskeletal components. The existence of clusters of species of different cytoskeletal organizations means that, during evolution, the commitment into one cytoskeletal strategy was irreversible. This process can be understood as the expression of the "cytotaxy", defined by Sonneborn in 1964 [77], at the evolutionary level, i.e. the emergence of a cytoskeletal organization generating structural constraints under further evolution of the system. Further studies on mechanisms of cytoskeletal morphogenesis in ciliates would probably provide new informations on these constraints.

The paroral membrane, its implication in morphogenesis, and its importance in ciliate evolution

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It is often assumed that the kinetome of the ciliate stem group exclusively consisted of somatic kineties inherited from the flagellar apparatus of a flagellate ancestor. The occurrence of a true oral ciliature therefore is assumed to represent an apomorphous character state [62, 73]. In recent years, however, results on ciliate ultrastructure and stomatogenesis [30, 42, 43, 46, 47, 60] clearly demonstrate that such gymnostome ciliates probably have evolved from

ancestors equipped with an elaborate oral ciliature composed of a paroral membrane and several adoral organelles [4]. At the same time it could be shown by molecular data that ciliates with a ventrostome oral opening equipped with a paroral and adoral ciliature branch off very early in the ciliate tree [8, 68, 70]. Against this background it is now also possible to assume that certain elements of the oral structure instead of the somatic kineties are remnants of the very first ciliature of the ciliate ancestor. As illustrated below, there is good evidence that the paroral membrane of extant ciliates with its unique morphological properties and its important functions during stomatogenesis can be interpreted as homologous to the first ciliature of the ciliate ancestor.

The basic ultrastructural pattern of the paroral membrane

Although highly modified in adult cells, a basic pattern of the paroral membrane can be recognized: The paroral membrane basically is composed of paired kinetosomes arranged perpendicular to the longitudinal axis of the paroral membrane. In contrast to somatic dikinetids there are no kinetodesmal fibres. At least the posterior one of the paired paroral kinetosomes is orientated perpendicular to the longitudinal axis of the entire organelle. This unique orientation, first recognized by Noirot-Timothée [in 32], enables the postciliary microtubules at triplet 9 to run towards the cytostome and to participate in the formation of the cytopharyngeal apparatus. According to Noirot-Timothée [in 32] these elements forming a paroral membrane may be called dyads to distinguish them from somatic kinetosomal pairs where the axis of the posterior kinetosome is in line with the longitudinal axis of the kinety (fig. 2a). The anterior kinetosomes of paroral dyads may have four different orientations in different ciliate groups and during stomatogenesis these kinetosomes may even change their orientation [20, 69].

As a consequence of this a paroral membrane should not be called an oralized somatic kinety. If a somatic kinety would build up a structure comparable with a paroral membrane on the right side of the oral opening (fig. 2b), the resulting orientation of the paired kinetosomes would be almost opposite to the orientation of the paroral dyads. The postciliary microtubules never could participate in the formation of a cytopharyngeal apparatus.

Typical paroral membranes composed of dyads are found in adult cells of many ciliates. If ontogenetic data are also taken into account, this pattern is recognizable in karyorelictids, heterotrichs, hypotrichs, colpodids, prostomes, nassulids, hymenostomes, scuticociliates and peritrichs. It is remarkable that, even if this pattern is absent in adult cells, it is present during the stage of stomatogenesis when the new cytopharyngeal apparatus is formed, thus demonstrating that this pattern is a prerequisite for the formation of one of the most important organelles of a heterotrophic organism with its vital functions in food uptake.

The paroral membrane and morphogenesis

Probably in all ciliates that have not completely lost their paroral ciliature the paroral membrane is involved in the formation of the cytopharyngeal apparatus. In many ciliates the paroral membrane also provides anlagen for the new oral apparatus for the posterior offspring cell the opisthe. This process may occur either directly or indirectly.

The direct participation of the paroral membrane in the formation of the oral apparatus for the opisthe is realized in ciliates with a buccokinetal mode of stomatogenesis like the scuticociliates, hymenostomes, peritrichs, the nassulid ciliate *Furgasonia* [18, 19, 22] and the karyorelictid ciliate *Loxodes* [5].

The indirect participation of the paroral membrane is only recognizable, if two subsequent division cycles are observed. During the first cycle the paroral membrane in both offspring cells produces kinetofragments or fields of kinetosomes. During the subsequent division cycle these kinetosomes participate in the formation of the oral anlage for the opisthe.

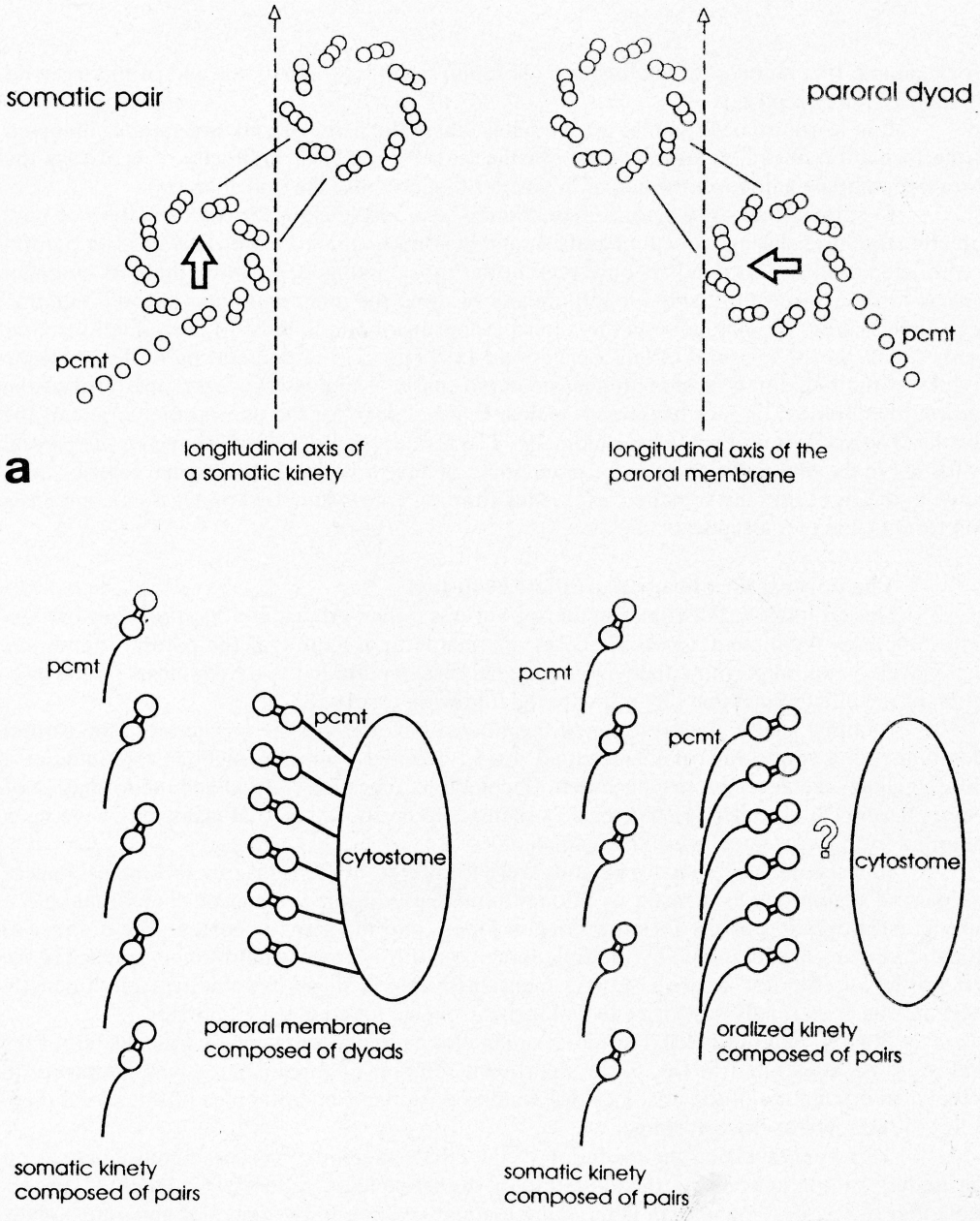

Fig. 2: a) Somatic pair and paroral dyad (according to Noirot-Timothée, in [32]). In somatic pairs the longitudinal axis of the posterior kinetosome is in line with the longitudinal axis of the kinety. In paroral dyads the longitudinal axis of the posterior kinetosome is perpendicular to the longitudinal axis of the paroral membrane.

b) Paroral membrane composed of dyads, versus oralized kinety composed of pairs. In contrast to an oralized somatic kinety, in a paroral membrane the postciliary microtubules of the posterior kinetosomes of the dyads can participate in the formation of a cytopharyngeal apparatus.

For example this mode of stomatogenesis is found in the scuticociliates and in the nassulid ciliate *Nassula citrea* [22].

It is worth to mention that in all ciliates where the parental oral apparatus is involved in the formation of oral anlagen for the opisthe, either directly or indirectly, it is always the paroral membrane and never the adoral organelles that provides the oral anlagen.

In some ciliates the paroral membrane is also responsible for the formation of new somatic structures during stomatogenesis. In the hypotrich ciliate *Paraurostyla* [53] a paroral membrane composed of dyads is only present during stomatogenesis when the new cytopharynx is formed. Later on it splits longitudinally forming the inner and outer paroral membranes. At the end of stomatogenesis the outer paroral membrane builds up the somatic frontal cirrus no. 1. In the nassulid ciliate *Furgasonia* [19] (fig. 3) the parental paroral membrane divides at the beginning of stomatogenesis in two parts. The posterior part, one third of the paroral membrane, migrates backwards and serves as anlage for the paroral membrane of the opisthe. The anterior part splits longitudinally. The former posterior kinetosomes of the paroral dyads serve as anlage for the paroral membrane of the proter and the former anterior ones move to the right into the somatic cortex, thus forming a new somatic kinety. As a compensation for this kinety n disappears.

The paroral membrane and ciliate evolution

Due to its broad distribution among various ciliate groups and its important ontogenetic functions mentioned above, it seems reasonable to assume that the paroral membrane also played a central part in ciliate evolution. This assumption led to a hypothesis on the evolution of the ciliate kinetome [20, 69] with the following essentials:

The first step in the evolution of the ciliate kinetome was the formation of the paroral membrane as a single row of kinetosomal dyads, evolved from the flagellar apparatus of a dinoflagellate-like ancestor, responsible for locomotion, ingestion of food and the formation of a cytopharyngeal tube (fig. 4). The basic structures to perform this vital tasks may have been inherited from a dinoflagellate-like organism.

In a second step somatic kineties were formed from the right row of kinetosomes of the paroral membrane as a result of a longitudinal splitting of the paroral membrane and a subsequent migration of the forming somatic kinety into the somatic cortex. The number of somatic kineties has increased by multiple repetition of this process until kinety n reached the left border of the oral area(fig. 5). As mentioned above, these hypothetical phylogenetic mechanisms are strongly supported by ontogenetic events found in extant ciliates.

Finally, in a third step, the adoral organelles evolved from somatic kineties left of the oral area. As illustrated in fig. 5, no significant rotation of kinetosomes was necessary to achieve an orientation of kinetosomes that enables postciliary microtubules of the adoral organelles to run towards the cytostome.

This hypothesis on the evolution of the ciliate kinetome has some consequences on our understanding concerning the evolution of stomatogenetic patterns in ciliates. If one accepts that the paroral membrane is the homologous structure to the flagellar apparatus of the ancestor, a primitive buccokinetal mode of stomatogenesis should be considered as ancestral which allocates the kinetosomes of the paroral membrane equally to both offspring cells like it is done with the kinetosomes in flagellates. Such a stomatogenetic pattern, probably representing the most primitive mode of buccokinetal stomatogenesis present in extant ciliates, was recently discovered by Bardele and Klindworth [5] in the karyorelictid ciliate *Loxodes*. The karyorelictid ciliates together with the heterotrichs are assumed to represent the earliest branch in the ciliate tree, in ultrastructural based systems and molecular based trees as well [8, 10, 55, 68].

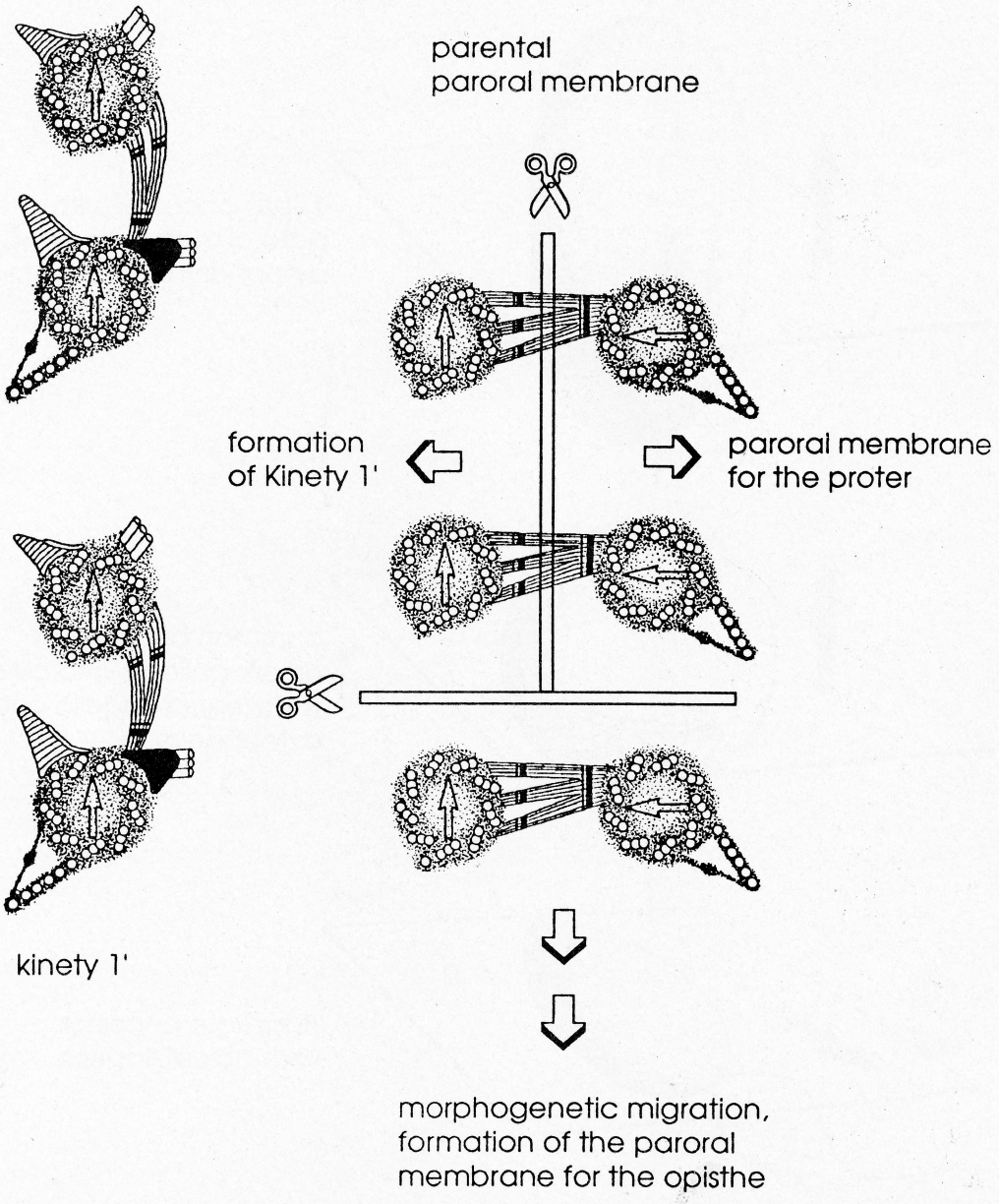

Fig. 3: Participation of the parental paroral membrane in stomatogenesis in the nassulid ciliate Furgasonia blochmanni. *Further explanations are given in the text.*

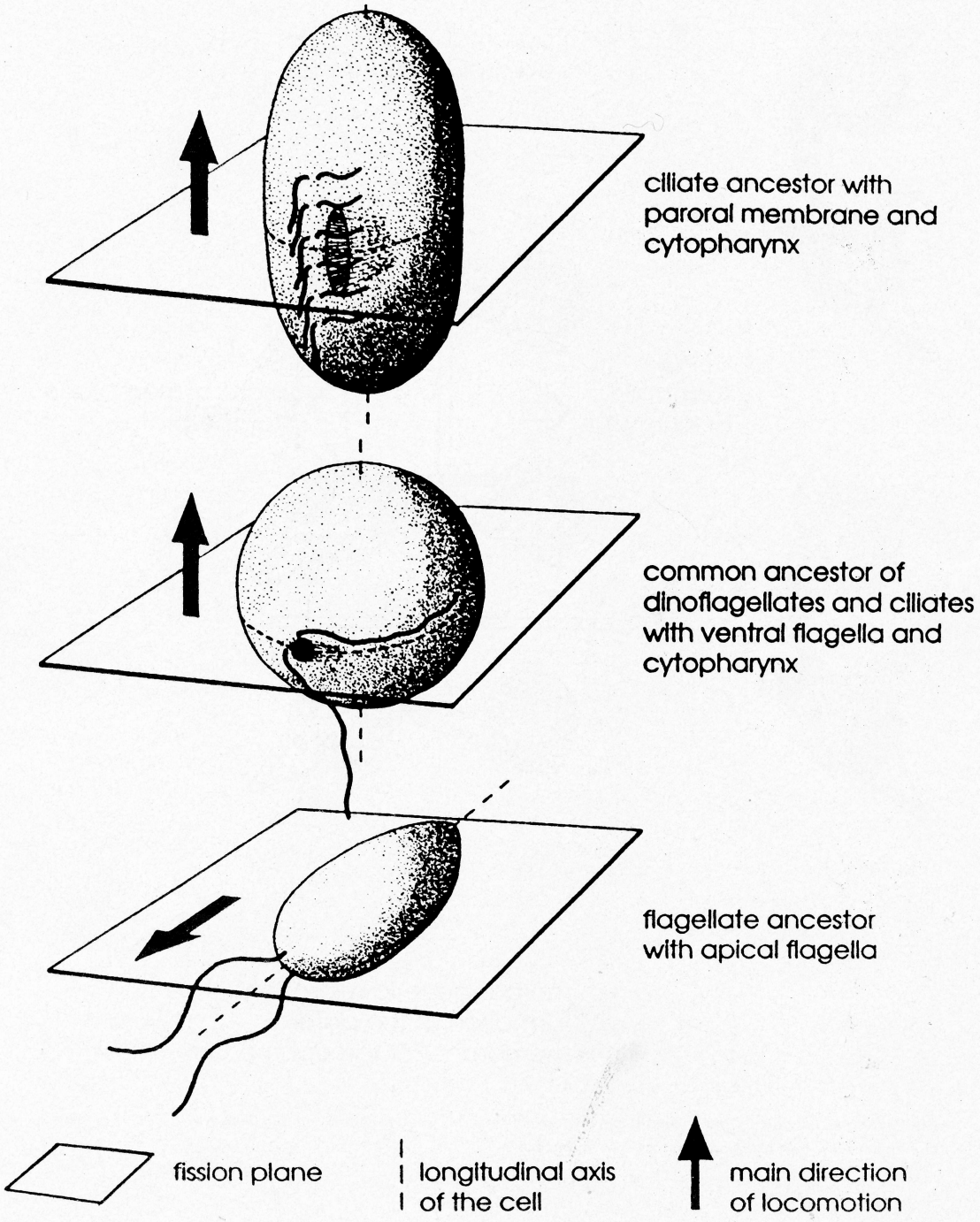

Fig. 4: First step in the evolution of the ciliate kinetome, the formation of the paroral membrane derived from the flagellar apparatus of a flagellate ancestor. (modified from [20]).

Fig. 5: Second and third step in the evolution of the ciliate kinetome, the acquisition of somatic kineties and adoral organelles. (modified from [69])

Ciliate phylogeny inferred from ontogeny

by W. Foissner, Universität Salzburg, Institut für Zoologie, Salzburg, Austria

Dr. Wilhelm Foissner, Salzburg University, reviewed the phenomenology of ciliate ontogenesis, with emphasis on stomatogenic data published between 1870 and 1993. These and other data (cortical ultrastructure etc.) were used to construct a cladogram showing possible main pathways in ciliate evolution (Fig. 6). Foissner emphasized that he does not believe that this scheme is correct in all details, but it at least shows the problems we face if we try to harmonize different data sources.

The heterotrichs and karyorelictids cannot be founded as a monophyletic group based on ontogenetic data because those available on the karyorelictids are too scanty and uncertain. The heterotrichs, though reduced by the oligotrichs, are very likely still a melting pot, as indicated by their diverse stomatogenic patterns. The typical forms, however, have parakinetal subtypes. This suggests that the monoparakinetal and the teloparakinetal subtypes, which occur in many hymenostomes, belong to another main type (buccokinetal?) or evolved convergently.

The heterotrich/karyorelictid assemblage is probably the sister group of the hypotrich/oligotrich clade. Most have a distinct adoral zone of membranelles, which was formerly used to unite heterotrichs, hypotrichs, and oligotrichs. The hypotrichs and oligotrichs are well-founded as a monophyletic group by the macronuclear reorganization band although a similar structure is found in the orthomere of the heteromeric macronucleus of some cyrtophorids and chonotrichs. Furthermore, the hypotrichs and oligotrichs are probably the only ciliates having a true apokinetal stomatogenesis. This is well-founded in oligotrichs, but still uncertain in euhypotrichs.

The Postciliodesmatophorea (at least the heterotrichids) and Spirotrichea both have elaborate oral structures, viz. a distinct zone of adoral membranelles. All other ciliates have few (usually three, e.g., most oligohymenophorans) or none (haptorids). This seems to be a main difference and is thus used for the gross distinction of the six main groups recognized. The Oligohymenophora, which possibly reside at the base of this clade, retained the ancestral buccokinetal stomatogenesis. The scheme suggests that the parakinetal subtypes found, e.g. in tetrahymenids, evolved convergently to those present in heterotrichs or, more likely, are special buccokinetal subtypes.

The Cyrtophorea, Litostomatea, and Colpodea have telokinetal or, rarely, mixokinetal (nassulids) stomatogenic subtypes. The pleurotelokinetal mode is probably ancestral because of its similarity with the pleurotelokinetal (?) subtype found in some heterotrichs. The ciliates I unite under the Cyrtophorea have a distinct ("polymerized") homonomous cyrtos, a highly characteristic organelle not found in this form in any other ciliate group, and a merotelokinetal or mixokinetal stomatogenesis. Both characters are highly modified in chonotrichs and suctorians. The cyrtophorids and chonotrichids are clearly more closely related to each other than to the suctorians because of their heteromeric macronucleus.

The nematodesmal bundles detach during the late stomatogenic stages in nassulids and cyrtophorids, whereas they remain attached to the paroral dikinetids in prostomatids. This appears to be a rather fundamental difference which not only links prostomatids and hymenostomes but can also be used to distinguish two main evolutionary lines within the Cyrtophorea.

A convincing apomorphy between litostomes and colpodids is still lacking. However, both are sharply defined, the colpodids by the LKm fibre, and the litostomes by the dorsal brush and the rhabdos type oral apparatus. The merotelokinetal stomatogenesis in the colpodids s. str. is probably related to their reproduction in cysts and very likely evolved convergently in the cyrtophorids.

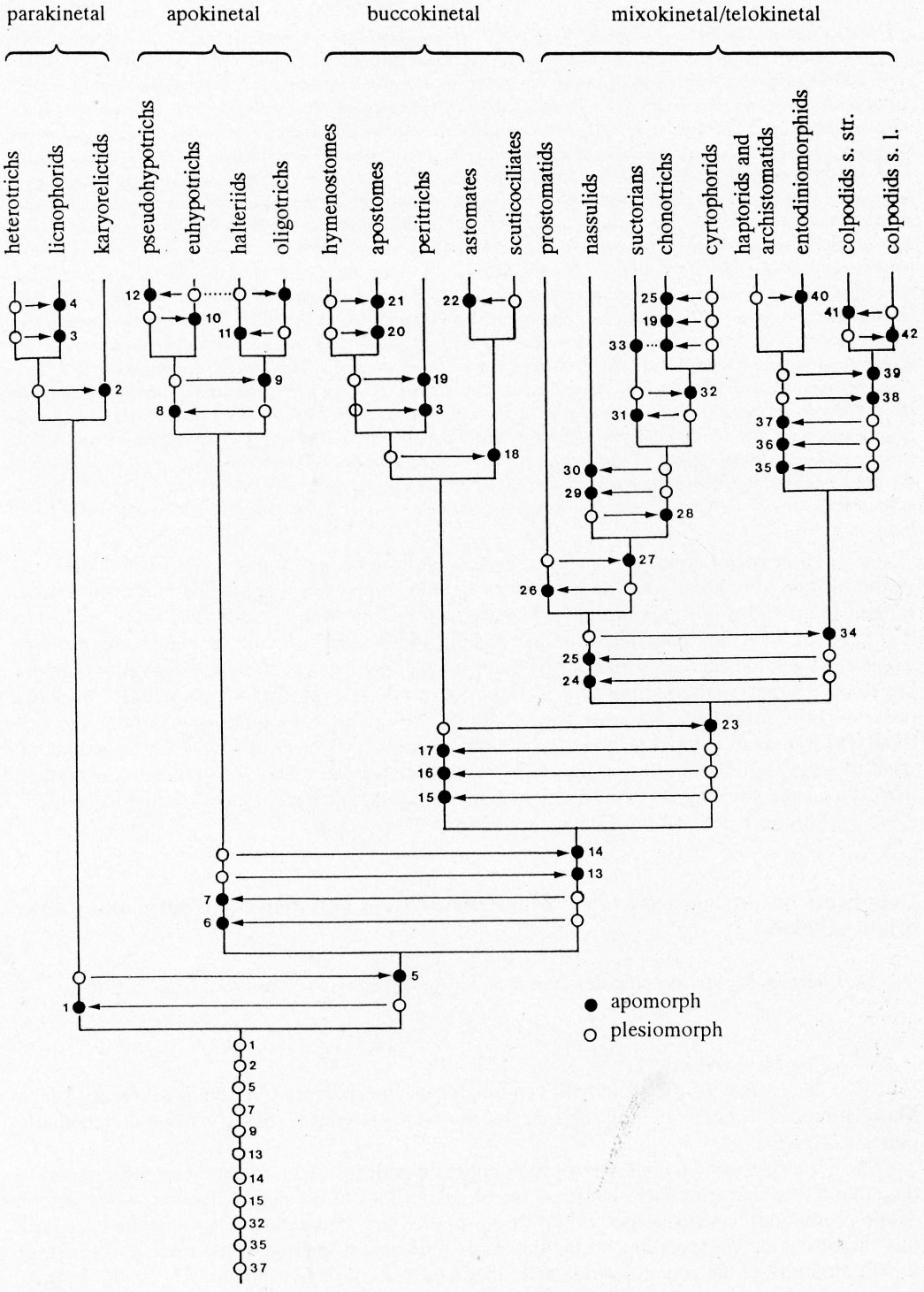

Fig. 6: A phylogenetic (cladistic) system of the ciliates mainly based on ontogenetic data. Note that I could not find reliable apomorphies for all taxa included, e.g. for licnophorids and heterotrichs; the lack is not indicated for the sake

of clarity. Character states (apomorph/plesiomorph): 1, stomatogenesis parakinetal/buccokinetal; 2, macronucleus non-dividing/dividing; 3, fission parallel/homothetogenic; 4, stomatogenesis epiapokinetal/parakinetal; 5, fibrillar system different/postciliodesmata; 6, stomatogenesis apokinetal/buccokinetal; 7, homomeric macronucleus with/without reorganization band; 8, cirri/cilia; 9, division enantiotropic/homothetogenic; 10, kinetodesmal fibre transient/permanent; 11, somatic infraciliature originates de novo/intrakinetally; 12, stomatogenesis hypoapokinetal/epiapokinetal; 13, somatic monokinetids/dikinetids; 14, adoral zone of membranelles partially or completely reduced/well developed; 15, silverline system striated/reticulate; 16, kinetodesmal fibres well developed/lacking or inconspicuous; 17, scutica or stomatogenic kinety/other; 18, stomatogenesis scuticobuccokinetal/ophryobuccokinetal; 19, loss or distinct reduction of somatic infraciliature/with somatic infraciliature; 20, with/without rosette; 21, stomatogenesis mixokinetal/ophryobuccokinetal; 22, without/with oral apparatus; 23, stomatogenesis mixokinetal or telokinetal/parakinetal, apokinetal or buccokinetal; 24, stomatogenesis merotelokinetal or mixokinetal/pleurotelokinetal, monotelokinetal or cryptotelokinetal; 25, cyrtos polymerized ("nasse")/indistinct; 26, oral apparatus polar/ventral; 27, cyrtos not connected/connected with oral kineties in adults; 28, without/with paroral and adoral ciliary fields; 29, cortex with/without alveolocysts; 30, stomatogenesis mixokinetal/merotelokinetal; 31, with/without suctorian tentacles; 32, macronucleus heteromeric/homomeric; 33, budding/normal fission; 34, transverse microtubules of somatic kinetids well/weakly developed; 35, rhabdos (transverse microtubules)/cyrtos (postciliary microtubules) type oral apparatus; 36, with/without dorsal brush; 37, oral kinetids not organized/organized to distinct fields indistinctly/distinctly separate from somatic ciliature; 38, with/without I.Km fibre; 39, somatic dikinetids/monokinetids; 40, stomatogenesis inter- or cryptotelokinetal/holotelokinetal or pleurotelokinetal; 41, stomatogenesis merotelokinetal/pleurotelokinetal; 42, I.Km fibre and transverse microtubular ribbon of anterior basal body form V-shaped figure/I.Km fibre distinctly longer than transverse microtubular ribbon of anterior basal body.

Three major conclusions are suggested by the scheme discussed (i) A subphyletic division of the Ciliophora based on a cyrtos or rhabdos type of oral apparatus is not supported. Rather, the rhabdos is an apomorphy of a single group, the Litostomatea. The same applies to the cortical fibrillar systems, i.e. the Postciliodesmatophora and Kinetodesmatophora suggested by some Russian workers. (ii) Some stomatogenic types evolved either convergently or are only superficially similar, viz. at light microscopic level. (iii) The "eociliate" possibly possessed the following character constellation: a dividing, homomerous macronucleus without a reorganization band; a cyrtos-type oral apparatus composed of a well developed adoral zone of membranelles and a paroral membrane; somatic dikinetids with postciliodesmata; a narrow-meshed silverline system; homothetogenic fission, and buccokinetal stomatogenesis.

For more detailed information see [28].

Comments on phylogenetic trees reconstructed from non-molecular data, and on the origin of ciliates

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The Phylogenetic Trees

The history of the systematics of ciliates was very lively over the past twenty years. Many attempts at reconstructing their phylogeny were elaborated, based on two conceptually opposite systems.

The first system consists of privileging one multiinformative character with regard to the others; this character allows to draw the broad outlines of the diversification, while details of the ultimate diversification could be based on the other characters. That was the case with the buccal characters (position, infraciliature complexity, stomatogenesis; review in [56]), or for the structure of the somatic cortex and kinetid (structural conservatism; [57, 59, 74, 75]), or more recently for the sequences of rRNAs. An attempt to establish a correlation between the molecular phylogeny and characteristics of the cortical cytoskeleton was made by Fleury et al.

[24], but their mathematical analysis only concerned the molecular data obtained with a single criterion, the rRNA sequences.

The second system consists of using not only one multiinformative character, but the combination of numerous multiinformative characters, all simultaneously integrated in the same analysis:

- an attempt is realized by Foissner (the present symposium) for the whole phylum, in which ontogenetic and structural characters are associated.

- we tried to reconstruct phylogenetic trees [66] from data covering 56 species and 23 morphological, nuclear, morphogenetical and ultrastructural multistate characters (for a total of 86 states). We used the parsimony MIX analysis which combines the Camin-Sokal method for characters whose polarity (plesiomorph to apomorph) could be defined. and the Wagner method for those whose polarity was not defined.

Since ciliates are considered as a monophyletic group and since no real-world outgroup could be assumed, we tried to root our trees on an hypothetic ancestor. Only one of our hypotheses was valid, where only the presence of somatic dikinetids was considered as a plesiomorphic character, while nothing was assumed for the ancestral position of the oral apparatus and for the buccal infraciliature. This hypothesis gave two trees:

- in tree A (fig. 7a), two main branches early separate from each other: one leads to 2 sister-groups (group 1 with karyorelictids, heterotrichs and spirotrichs; group 2 with colpodids); the second leads to 3 groups in which we find the Oligohymenophora and Nassophorea distributed on the 3 groups, and the old Kinetofragminophora (Litostomatea and Vestibulifera) on a single group 5.

- in tree D (fig. 7c) there is an earlier separation of the group 1 (karyorelictids, heterotrichs, spirotrichs) that is in agreement with the molecular trees; the 4 other groups differentiate later, but with the same elements as in tree A.

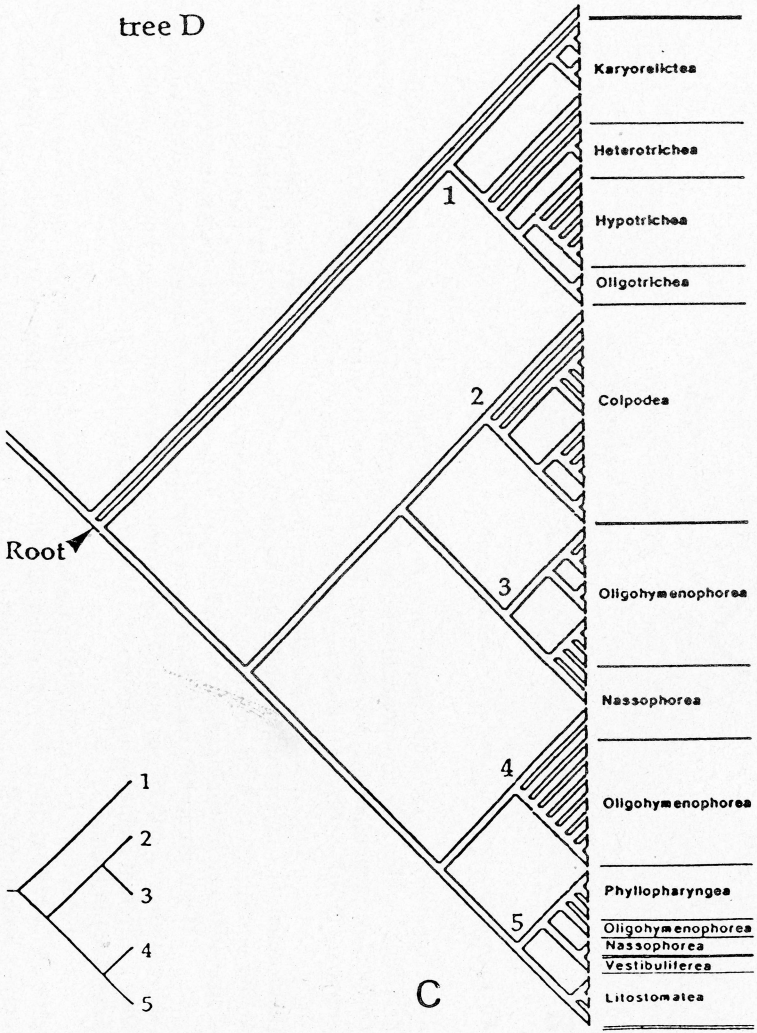
Comparing our analysis with the conclusions of other authors, we can discuss about the probable evolution of three characters:

- **the nuclei**: in molecular trees, ciliates with paradiploid macronuclei emerge early, that agrees with the idea of Orias [63] (paradiploidy seems to be a plesiomorphic character). Probably, the protociliates had 2 diploid nuclei dividing by mitosis. One of them subsequently differentiated into a macronucleus (Mn) by elimination of some genes and amplification of those that remained; this amplification first remained weak, giving the paradiploid state; this paradiploid Mn was first incapable of division; the ability to divide according to an amitotic process was then gained, concomitant with a stronger amplification of the remaining DNA, leading to the polyploid state; these 2 acquisitions perhaps were established in several steps and in different lines independently. This hypothesis can be applied to our trees. In tree A (fig. 7b), paradiploidy was maintained in the first branch, and polyploidy had to appear 3 times all along the ciliates' diversification. In tree D (fig. 7d), polyploidy had to be established only 2 times, that is more parsimonious than in tree A.

- **the position of the oral opening** (fig. 8): in our trees, ciliates with an apical mouth differentiate late. So, it is evident that the primitive ciliate was a 'ventrostome' according to Bardele [3] and that the first line which differentiated (karyorelictids + hetrotrichs + spirotrichs) gave forms with a ventral mouth and a dissymetric infraciliature, with, sometimes, a secondary state with an apical mouth and an homogenous oral infraciliature as in *Trachelorophis*. Such an apical position of the mouth was also gained secondarily in lines which appeared later, such as haptorians (a part of litostomes) and prostomes.

- **the cortical cytoskeleton**: according to Fleury et al. [24] and their 'shell theory', the protociliate had a cortical cytoskeleton composed of microtubules (Mt), epiplasm and non-actin microfilaments (NAMF) at once; the diversification during the evolution in each line would have privileged the development of only one of these elements. The first branch which appeared (karyorelictids + heterotrichs) based its strategy on the development of Mt associated

Fig. 7: Possible evolution of the macronucleus (b, d), with regard to different phylogenetic trees (a, c). (a, c: from [66])


tree D
Karyorelictea
Heterotrichea
Hypotrichea
Oligotrichea
Colpodea
Oligohymenophorea
Nassophorea
Oligohymenophorea
Phyllopharyngea
Oligohymenophorea
Nassophorea
Vestibuliferea
Litostomatea
Root
1
2
3
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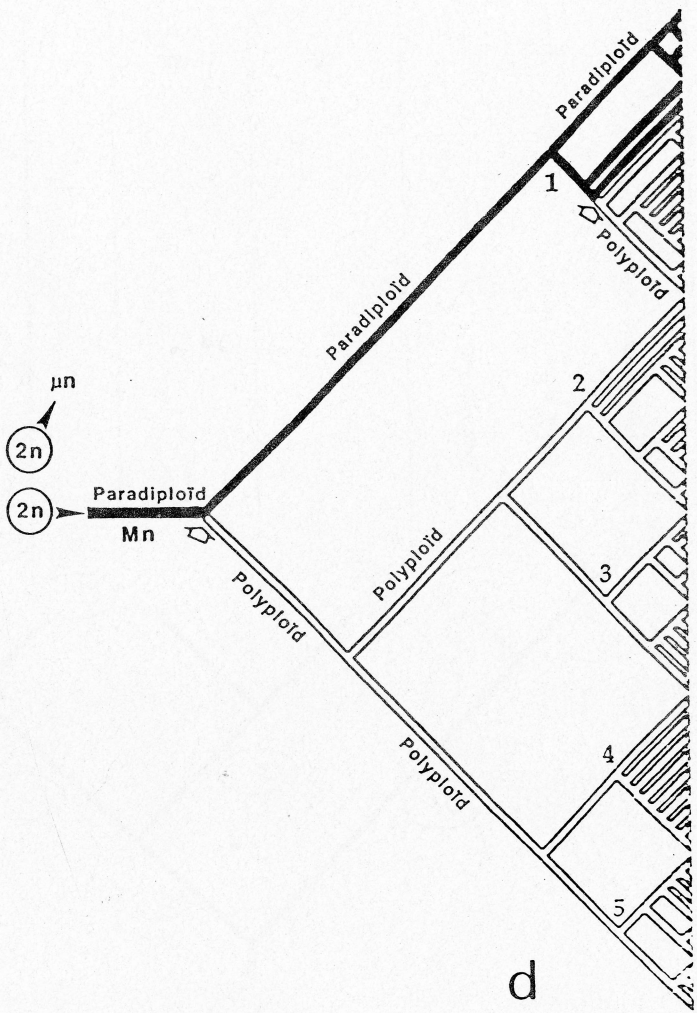
5
c


Paradiploïd
Polyploïd
Paradiploïd
μn
2n
2n
Paradiploïd
Mn
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Polyploïd
Polyploïd
1
2
3
4
5
d

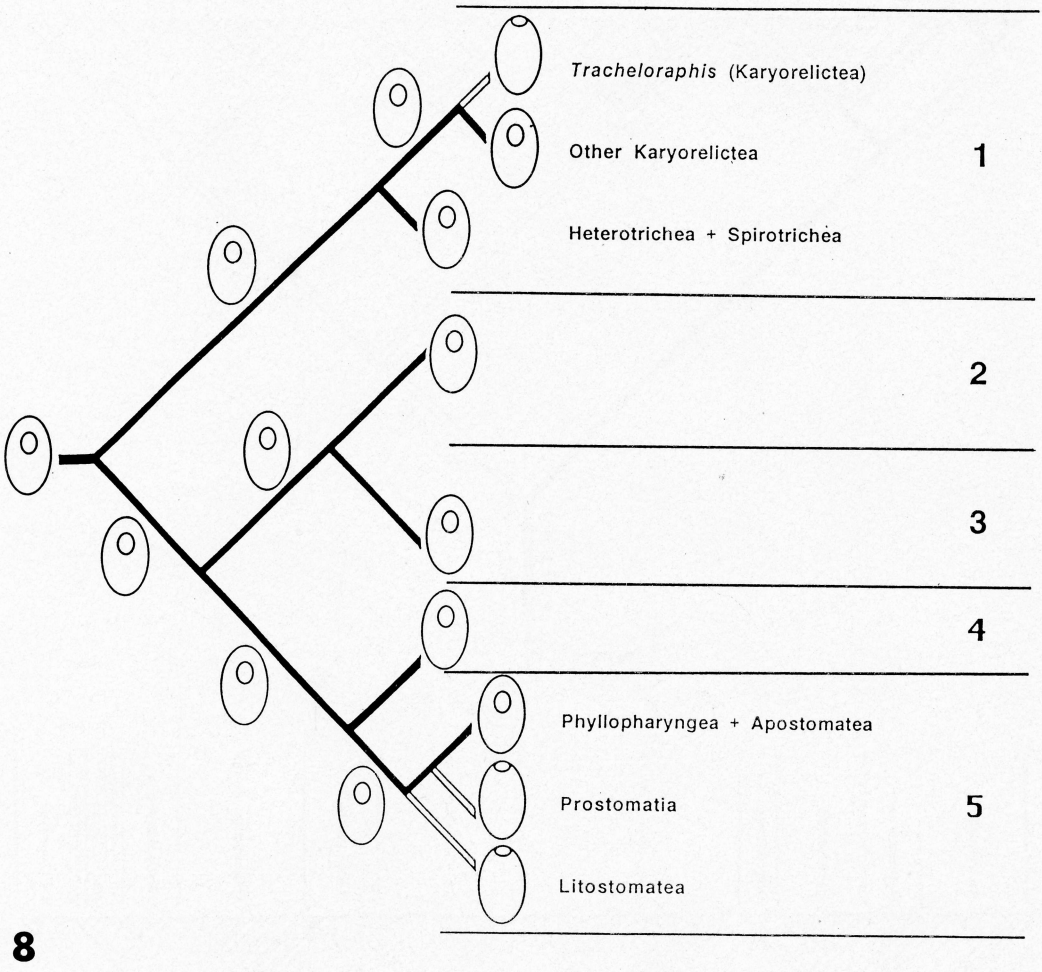

Fig. 8: Hypothesis on the evolution of the buccal location.

to the kinetosomes (Ks), the postciliary (Pc) fibres. The subsequent stages of diversification privileged either Mt independent of the Ks (hypotrichs + oligotrichs), or the epiplasm (colpodids, peniculians, tetrahymenians, scuticociliates, Nassophorea), or an ecto-endoplasmic boundary (EEB) of NAMF (litostomes, Vestibuliferea, prostomes).

According to our own phylogenetic trees (fig. 9), it appears that the group 1 (karyorelictids + heterotrichs), which developed Mt associated with the Ks (Pc fibres) is closer to the spirotrichs (which developed Mt non associated with the Ks) than to the group 2 (colpodids) which developed another category of Ks associated Mt, the transverse fibres.

If our group 4 is well-characterized by the epiplasm as major cytoskeletal structure, on the contrary a part of our group 5 (Litostomatea) shows that a unique line could privilege at once 2 different elements (epiplasm and NAMF), that means their near ancestor had conserved these 2 major elements.

Finally, if we consider the entodiniomorphids (Vestibuliferea), which were not treated by Fleury et al. [24], we notice that they all possess simultaneously a thick epiplasm, numerous longitudinal Mt and a well developed EEB made of NAMF; this means that the near

ancestor of this group 5, in spite of its recent appearance, had conserved 3 basic systems already present in the protociliate; in fact, these 3 systems are found at the present time in ciliates whose somatic ciliature is reduced.

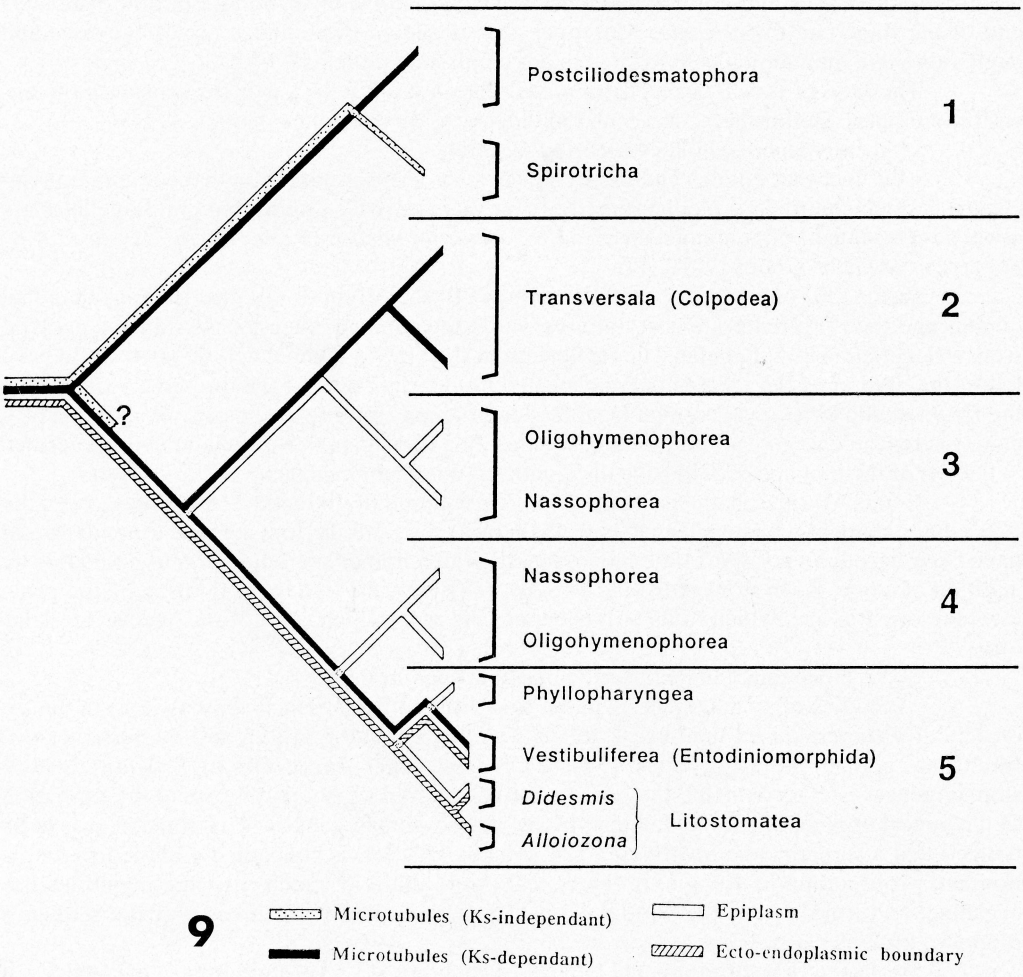

Fig. 9: Hypothesis on the evolution of the cytoskeleton.

These remarks allow us to say that it is difficult to build an evolutionary systematics from only a single character (here the cortical cytoskeleton) and that a phylogenetic reconstruction, even molecular, can lead to a wrong idea of the evolution of any character if the number and variety of species included in the analysis are insufficient.

In conclusion, as in other groups of organisms, the evolution of ciliates shows that the different characters had different rates of evolution and could have reached different states in different lines. For example, it is impossible to find an actual parallelism between the respective evolutions of the cortical cytoskeleton, nuclei and position and structure of the oral apparatus.

The problem of the origin of ciliates

As far back as 1976, Taylor [78] postulated strong phylogenetic relationships between ciliates and dinoflagellates. Lynn and Small [59] hypothesized that ciliates evolved from a 'corticoflagellate' which had cortical alveoli and a kinetid similar to those of dinoflagellates and of the flagellate *Colponema*. Moreover, it was shown that ciliates, dinoflagellates and euglenids share immunologically related major epiplasmic proteins [79].

The lack of fossils, except tintinnids, does not allow to postulate what was the ancestor of ciliates. But, from the molecular phylogenies, we can notice that:

- the emergence of ciliates occurred recently;
- the fact that ciliates and dinoflagellates are sister-groups (i. e. have a common ancestor) is often controversed: either the trees give them as sister-groups, but dinoflagellates are always represented by the unique species *Prorocentrum micans* [6, 34, 40, 76, 82]; or they do not appear as sister-groups [7, 12, 67].

Eisler [20] once again posed the problem of the origin of ciliates. He considers that ciliates and dinoflagellates are sister-groups whose the common ancestor would have evolved from a flagellate with 2 flagella. The replication of the kinetid without cell division could have led to the paroral of the ciliate ancestor located on the right side of the oral area. Afterwards the first somatic kinety was formed from the longitudinal splitting of the paroral, and this process was repeated to give all the somatic kineties. In a third step, those somatic kineties located at the left of the oral area would have differentiated into membranelles.

In this hypothesis, the paroral, which is composed of dikinetids, should have been the first ciliary element which appeared as a ciliate character, and the first somatic kinetids would have been during a very short time monokinetids which immediately turned into dikinetids by addition of a new Ks in front of the old one. So, for Eisler, the right buccal infraciliature generates the somatic one, which in its turn generates the second (left) part of the buccal infraciliature.

Some objections can be raised against this hypothesis:

1) Studies of stomatogenesis in the first differentiated branch show that: a) in the karyorelictid *Protocruzia* all the buccal organelles have a somatic origin, and the left ones differentiate before the right ones [37]; b) in the other karyorelictid *Loxodes*, whose stomatogenesis is buccokinetal, the somatic infraciliature does not participate to the formation of the buccal organelles [61]; c) in heterotrichs, whose stomatogenesis is parakinetal, it is to be noticed that a somatic area involving a few somatic kineties, is concerned with dedifferentiation and proliferation of Ks, giving the buccal primordium in which, first the membranelles organize, and a little later the paroral. This sequence is totally the reverse of the sequence proposed by Eisler (fig. 10).

So, in the first differentiated ciliates, such as some karyorelictids and heterotrichs, it is the somatic infraciliature which generates the buccal one or the buccal infraciliature became autonomous, but the oral infraciliature never gives rise to somatic ciliary structures.

2) In the majority of other ciliates (even when the paroral seems to longitudinally split, such as in *Tetrahymena*, and the scuticociliates), the buccal infraciliature is never at the origin of somatic kineties, but the reverse often occurs.

3) Only 2 cases could fit well with Eisler's hypothesis: a) in *Furgasonia* [19, 22], a buccal organelle, the paroral gives rise to a somatic kinety on the proter, i. e. on a cell whose the oral apparatus does already exist; so, this process seems like a kind of reorganization, or regulation. On the opisthe, on which a new oral apparatus is entirely built, it is a part of the old paroral which gives the new paroral, but subsequently this new born paroral does not give any somatic structure; b) in *Paraurostyla* [53], a unique primordium gives, in its right part a somatic structure (the frontal cirrus 1), and in the rest the 2 paroral organelles (IPM and OPM). Here, we can consider that it is not a buccal structure that gives a somatic structure, but a unique primordium which is born in a destabilized cortical area, and which secondarily gives 2

different kinds of organelles (one somatic, one buccal), according to the location of the kinetosomes, when this area restabilizes in a new pattern.

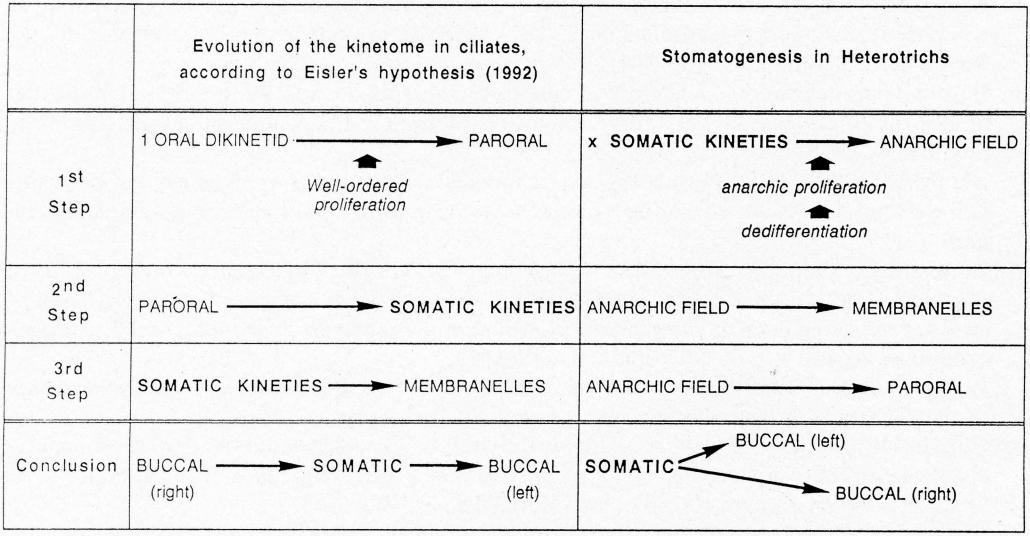

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Fig. 10: Sequences of events leading to the construction of the kinetome: on the left part for the ciliate ancestor; on the right part for heterotrichs.

It seems difficult to conceive that the ability of the paroral to give the whole ciliature would have been completely lost in the first emerging line, while conserved in the lines whose differentiation occurred later (those which include *Paraurostyla* and *Furgasonia*. Ciliates of the first emerging line seem to be the best candidates to reflect, in their own ontogeny, the ontogeny of the ciliate ancestor, i. e. what happened for the origin of the somatic kineties during the evolution from a protociliate to a true ciliate.

To conclude, I think that we have now a good idea of the order of appearance of the diverse groups of ciliates, owing to the studies on molecular and non-molecular phylogenies, but there is still a great deal of uncertainty about the origin of ciliates.

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