Molecular Phylogenetics and Evolution
Vol. 21, No. 1, October, pp. 86–92, 2001
doi:10.1006/mpev.2001.0997, available online at http://www.idealibrary.com on IDEAL®

Phylogenetic Relationships within the Class Spirotrichea (Ciliophora) Inferred from Small Subunit rRNA Gene Sequences

Detlef Bernhard,* Alexandra Stechmann,* Wilhelm Foissner,† Dieter Ammermann,‡ Manuela Hehn,§ and Martin Schlegel*

*Spezielle Zoologie, Institut für Zoologie, Universität Leipzig, Talstrasse 33, D-04103 Leipzig, Germany; †Institut für Zoologie, Universität Salzburg, Hellbrunnerstrasse 34, A-5020 Salzburg, Austria; ‡Zoologisches Institut, Universität Tübingen, Auf der Morgenstelle 28, D-72076 Tübingen, Germany; and §Scheffelweg 54, D-72793 Pfullingen, Germany

Received October 24, 2000; revised April 25, 2001

The small subunit rDNAs of five species belonging to the Euplotidae and eight species of the Oxytrichidae were sequenced to obtain a more detailed picture of the phylogenetic relationships within the Spirotrichea (Ciliophora). Various tree reconstruction algorhythms yielded nearly identical topologies. All Euplotidae were separated from the other Spirotrichea by a deep split. Further, a large genetic distance between the marine genus *Moneuplotes* and the freshwater species of *Euplotoides* was found. Differences between the methods used occurred only within the Oxytrichidae. Whereas the monophyly of the Stylonychinae was supported in all trees, the monophyly of the Oxytrichinae was not. However, the molecular data support the morphological and ontogenetic evidence that the pattern of 18 frontal–ventral–transversal cirri evolved in the stemline of the Oxytrichidae and was modified several times independently. Our results are also in agreement with taxonomic revisions: the separation of both *Sterkiella nova* from *Oxytricha* and *Tetmemena pustulata* from *Stylonychia*.

Key Words: **small subunit rDNA; phylogeny; Ciliophora; Spirotrichea; Oxytrichidae; Euplotidae.**

INTRODUCTION

The Ciliophora Doflein, 1901 comprises more than 8000 species in several highly diverse lineages. One of the most diverse classes is the Spirotrichea, which has been subjected to numerous revisions resulting in contradictory classification schemes (e.g., Corliss, 1979; Levine *et al.*, 1980; Small and Lynn, 1981, 1985; Lynn and Corliss, 1991; Lynn, 1996). In traditional systematics (e.g., Corliss, 1979; Levine *et al.*, 1980), the Spirotrichea were subdivided into the Heterotrichida, the Odontostomatida, the Oligotrichida, and the Hypotrichida. However, as was shown by molecular analyses of rRNA genes, the heterotrichs do not belong to the Spirotrichea (Greenwood *et al.*, 1991). Together with the karyorelicteans, the heterotrichs are the sister group of all other ciliates (Baroin-Tourancheau *et al.*, 1992; Hirt *et al.*, 1995; Hammerschmidt *et al.*, 1996). In recent classifications (Lynn, 1996; Lynn and Small, 1997), the Spirotrichea comprise the hypotrichs (e.g., *Euplotes*), the stichotrichs (e.g., *Oxytricha, Stylonychia*), the choreotrichs (e.g., *Strombidium*), and the oligotrichs (e.g., *Halteria*).

In this study we focus on the evolution of the stichotrichs and hypotrichs. Therefore, we analyzed the small subunit (ssu) rDNA sequences of eight species belonging to the Oxytrichidae (Stichotrichia) and five species of the Euplotidae (Hypotrichia).

Classification of and within the family Oxytrichidae is still confusing, despite the huge amount of morphologic and ontogenetic information available (for a review see Berger, 1999). Recently, two highly conflicting cladistic systems have been proposed on a similar body of evidence (Berger and Foissner, 1997; Eigner, 1997). Briefly, Berger and Foissner (1997) consider the 18 frontal–ventral–transversal cirral pattern which is present in many oxytrichids to be an important shared derived character (autapomorphy) of the Oxytrichidae. In contrast, Eigner (1997) assumes that this pattern evolved several times independently. We thus determined gene sequences from eight representatives of this group and compared the resulting tree with the trees of Berger and Foissner (1997) and Eigner (1997, 1999). Furthermore, we focused on the genera that were classified in different families by these authors. To analyze how deep the Euplotidae are separated from the rest of the Spirotrichea, we chose freshwater and marine species since Schlegel *et al.* (1988) have shown a deep split between these groups by allozyme electrophoresis. Furthermore, based on morphological data, Borror and Hill (1995) split the genus *Euplotes* (sensu lato) into different genera. The species examined in our study now belong to the genera *Euplotoides* and *Monoeuplotes*. Members of the first genus typically

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TABLE 1

Origin of Species Investigated

Species	Strain	Geographic origin	Source	EMBL Accession No.
Euplotoides eurystomus (Wrzesniowski, 1870)	40a	Marl, Germany	Prof. Heckmann, Münster	AJ310491
Euplotoides octocarinatus (Carter, 1972)		Münster, Germany	Prof. Heckmann, Münster	AJ310489
Monoeuplotes minuta (Yocum, 1930)	A23	Villefranche sur Mer, France	Prof. Heckmann, Münster	AJ310490
Moneuplotes vannus (Müller, 1786)	AL_3	Alghero, Italy	Prof. Luporini, Camerino	AJ310488
Moneuplotes crassus (Dujardin, 1841)	DT	Unknown	Zool. Institute, Tübingen	AJ310492
Laurentiella strenua (Dingfelder, 1862)		Darwin, Australia	Prof. Foissner, Salzburg	AJ310487
Steinia sphagnicola Foissner, 1989		Murrey River flood plain, Australia	Prof. Foissner, Salzburg	AJ310494
Pattersoniella vitiphila Foissner, 1987		João Pessoa, Brazil	Prof. Foissner, Salzburg	AJ310495
Paraurostyla weissei (Stein, 1859)		Austria	Prof. Foissner, Salzburg	AJ310485
Gonostomum strenuum (Engelmann, 1862)		Albury, Australia	Prof. Foissner, Salzburg	AJ310493
Onychodromus grandis Stein, 1859		Pet Shop Aquarium, Boulder, USA	Prof. Prescott, Boulder	AJ310486
Stylonychia lemnae Ammermann & Schlegel, 1983	20	Federsee, Germany	Prof. Ammermann, Tübingen	AJ310497
Stylonychia lemnae Ammermann & Schlegel, 1983	1W	Harbin, China	Prof. Ammermann, Tübingen	AJ310496
Stylonychia mytilus (Müller, 1773)	48	Entringen, Germany	Prof. Ammermann, Tübingen	AJ310498
Stylonychia mytilus (Müller, 1773)	Ek-11	Cross from strains isolated in Radolfzell and Tübingen	Prof. Ammermann, Tübingen	AJ310499

occur in freshwater, whereas all species of the latter genus are marine.

MATERIALS AND METHODS

Origin of Samples

The 13 species investigated in this study are listed in Table 1. Two strains of both *Stylonychia lemnae* and *Stylonychia mytilus* from different locations were examined. The *Stylonychia* and *Euplotoides* species were grown in Pringsheim solution and fed with *Chlorogonium elongatum*. The marine species of the genus *Moneuplotes* were cultivated in sea water with *Dunaliella salina* as food organism. All other species were grown in Eau Volvic (French table water) with some wheat grains to support bacterial growth. Starved cells were concentrated by low-speed centrifugation or hand-picked by micropipette from the culture fluid and then fixed in 70% ethanol until the number of cells was sufficient for DNA extraction.

Extraction of DNA, Isolation of ssu rRNA Genes, and DNA Sequencing

Extraction of ciliate DNA, amplification, and cloning of ssu rRNA genes was carried out as described previously (Stechmann *et al.*, 1998). Cloned ssu rDNA was sequenced in both directions with dye-labeled universal ssu rDNA primers (Elwood *et al.*, 1985) and M13 forward and reverse primers. Sequencing reactions were performed with the 7-deaza-dGTP sequencing kit (Amersham) and separated on an automated LI-COR DNA sequencer.

Phylogenetic Analyses

Additional sequence data were retrieved from EMBL and GenBank. We used only published sequences for our analyses, although in the GenBank database some other sequences of spirotrich ciliates are deposited (D. M. Prescott *et al.*, unpublished). A few of them are from the same species as ours. We found only slight differences (<1%) between those and our sequences.

Alignments were carried out with CLUSTAL W, v. 1.8 (Thompson *et al.*, 1994) with default parameters and refined by eye. The complete alignment was used for different phylogenetic analyses. Neighbor-joining trees were constructed with the program TREECON, v. 1.3b (Van de Peer and De Wachter, 1994) with the Kimura (1980) two-parameter model. Maximum-parsimony analyses were carried out with the program DNAPARS in PHYLIP, v. 3.57c (Felsenstein, 1993) and with PAUP* 4.0 (Swofford, 1998) with the heuristic search method with 10 random stepwise additions of the sequences and the TBR branch swapping option. To test the robustness of the resulting trees, bootstrap analyses with 1000 replicates were performed in all methods described above. Maximum-likelihood trees were calculated with the programs PAUP* 4.0 (Swofford, 1998) and PUZZLE 4.0.2 (Strimmer and von Haeseler, 1996). The HKY85 model (Hasegawa *et al.*, 1985) was used with both base frequencies and the transition/transversion ratio being estimated by each program. In PAUP* 4.0 the heuristic search method was invoked with 10 random stepwise additions of the se-

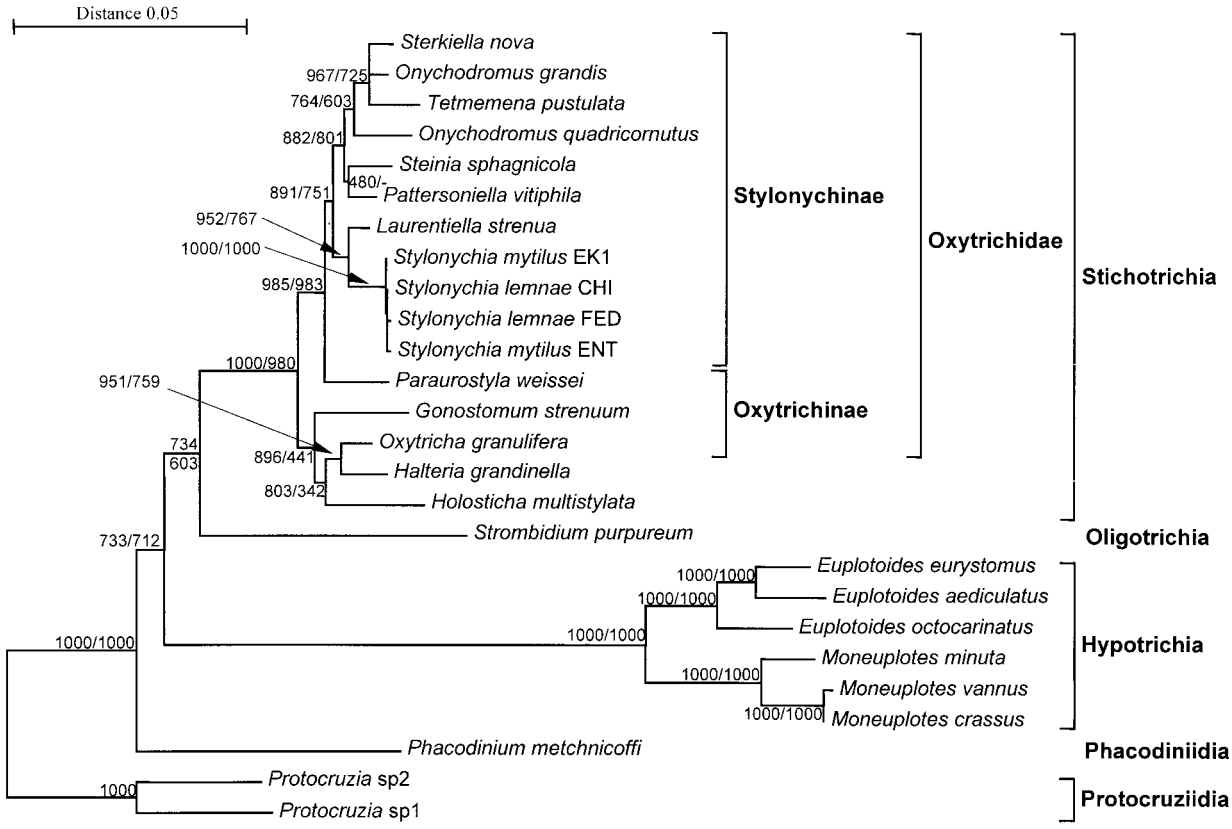

FIG. 1. Distance matrix tree of the Spirotrichea inferred from complete ssu rDNA sequences. The tree was calculated with the Kimura (1980) two-parameter model. The consensus tree of 1000 bootstrap resamplings was constructed by the neighbor-joining method (Saitou and Nei, 1987). The first numbers at the nodes represent the real values for the group. The scale bar equals 0.05 nucleotide substitutions per site. The second numbers at the nodes represent the real values for the group out of 1000 trees in maximum-parsimony analyses with PAUP* 4.0 (Swofford, 1998). The same tree topology was inferred with the maximum-likelihood method in the PAUP package.

quences. Because of the enormous computational time, bootstrap analysis could not be carried out. The program PUZZLE 4.0.2 uses the quartet puzzling method to infer maximum-likelihood trees and calculates quartet puzzle support values for each branch, which are comparable to bootstrap values (Strimmer and von Haeseler, 1996).

RESULTS

We have sequenced the ssu rDNA of 13 species belonging to the Spirotrichea (see Table 1). The amplified region of all euplotid species is longer than that of other ciliates. Including the PCR primers, the ssu rDNA sequences range from 1875 to 1898 bp in the euplotid species, whereas the sequences of all other spirotrichs are 1767- to 1775-bp long. The additional length is based on a few insertions which are scattered throughout the genes. However, the lengths and the sequences of the insertions vary between the euplotid species.

We have carried out several alignments, including ciliates from all classes, with members of dinoflagellates and apicomplexans representing the outgroup. In all analyses performed, the ciliates form a monophyletic group with two main branches: the Postciliodesmatophora (Heterotichea and Karyorelictea) and the Intramacronuleata (not shown). As in previous molecular analyses (Lynn *et al.*, 1999; Shin *et al.*, 2000; Stechmann *et al.*, 1998), the relationships among the ciliate classes within the Intramacronucleata could not be resolved unambiguously. However, the Spirotrichea always form a monophyletic group, showing *Protocruzia* to be the sister taxon to this lineage. Therefore, we reduced our data set using the published sequences of spirotrich ciliates with the *Protocruzia* species as outgroup representatives (Figs. 1 and 2). The exclusion of distantly related taxa that are not of interest in our analyses also decreases the number of ambiguous sites. The alignment obtained was nearly unambiguous and removal of slightly ambiguous sites from the alignment did not affect tree topology. The length variation of the euplotid sequences is caused by distinct insertions and did not affect the alignment. Therefore, we used the complete sequences for all analyses.

Within the Spirotrichea the new sequence data from representatives of the genera *Monoeuplotes* and *Euplotoides* confirm the deep split between the Euplotidae and the other Spirotrichea (Figs. 1 and 2). Phylogenetic

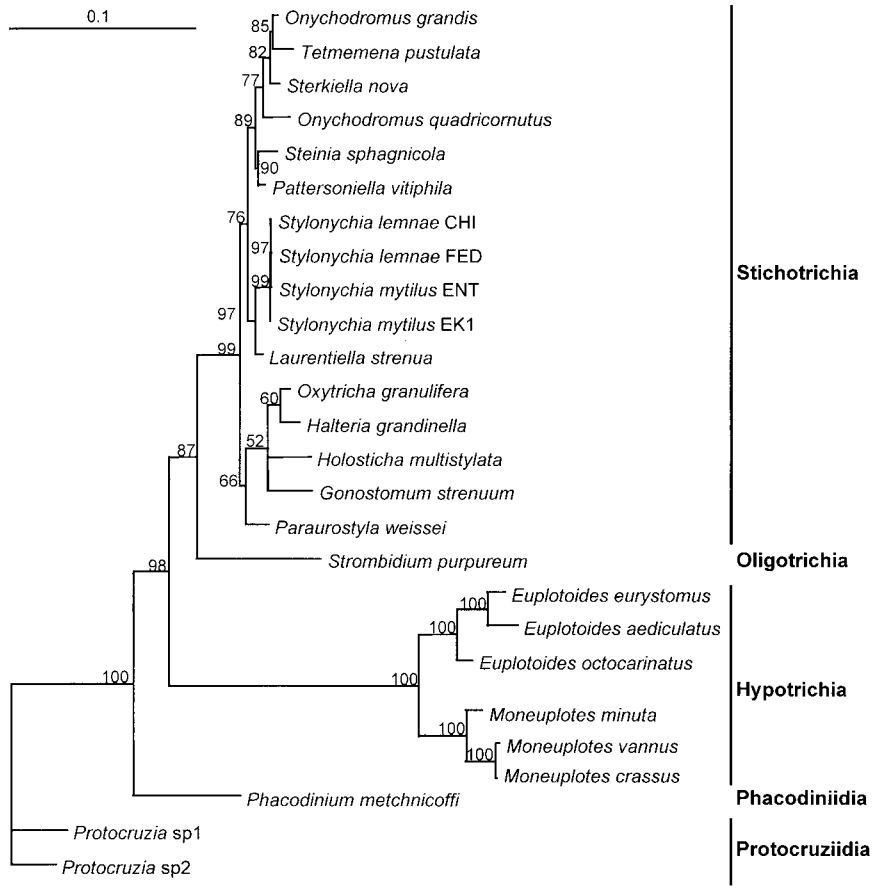

FIG. 2. A maximum-likelihood tree obtained with PUZZLE 4.0.2 (Strimmer and von Haeseler, 1996) with 1000 puzzling steps and showing maximum-likelihood branch lengths. The scale bar equals 0.1 nucleotide substitutions per site.

relationships are clearly recovered within euplotids because all trees show the same topology supported by high bootstrap values (Figs. 1 and 2). Our results show a clear separation between the marine species of *Moneuplotes* and the freshwater species of *Euplotoides.* Moreover, the genetic distances within these genera are large.

Compared to the Euplotidae, the genetic distances are smaller within the Stichotrichia. Although resolution in this group is not as good as that in the euplotids, a largely congruent branching pattern is obtained with the different reconstruction methods. Surprisingly, the oligotrich ciliate *Halteria grandinella* is separated from the other oligotrich *Strombidium purpureum* and branches together with *Holosticha multistylata* within the Oxytrichidae, as also shown by Shin *et al.* (2000).

In the neighbor-joining and maximum-parsimony trees (Fig. 1) *Oxytricha granulifera, Gonostomum strenuum, Halteria grandinella,* and *Holosticha multistylata* branch off first from this lineage followed by *Paraurostyla weissei,* whereas in the puzzle tree (Fig. 2) these species form a common group. The remaining species belong to the Stylonychinae sensu Berger and Foissner (1997). They are divided into two stable clusters: (1) *Laurentiella strenua* together with *Stylonychia lemnae*/*S. mytilus* and (2) a heterogeneous ensemble containing *Sterkiella nova, Tetmemena pustulata, Onychodromus grandis, O. quadricornutus, Steinia sphagnicola,* and *Pattersoniella vitiphila.* The separation of *Oxytricha nova* (now *Sterkiella nova;* see Foissner and Berger, 1999) from *O. granulifera* was already described by Schlegel *et al.* (1991). The molecular data also confirm the translocation of *Stylonychia pustulata* into the new genus *Tetmemena* (Eigner, 1997, 1999). In all trees the four clones of *Stylonychia lemnae* and *S. mytilus,* which differ in only one nucleotide from each other, group with *Laurentiella strenua* and are clearly separated from *S. pustulata* (now *Tetmemena pustulata*). The latter species is always associated with *S. nova, Onychodromus grandis,* and *O. quadricornutus.* The branching pattern within this group is not resolved unambiguously.

DISCUSSION

In our analyses the Spirotrichea form a monophyletic group with *Protocruzia* as the sister taxon, congruent with the trees reported by Shin *et al.* (2000). *Protocruzia* is well known for its unusual macronu-

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                          510         520         530         540         550
                           *           *           *           *           *
Sterk nova       TTGCAATGAG-TAGAATTTAAACCCCTTTA----CGAGGATCAATTGGAGGGCAAGTCTG
Eupl octo        .G.GCT...T-.T.C.AACTTTA.AT..AG----......A..................
Eupl aedic       .CA.....G.C.T..T..GC..A.A...AG----......A..................
Eupl eury        .CA.....G.C.T..T..GC..A.A....G----......A..................
Moneu minuta     .CA.....G.-CTTG....GC.AA.T..ATTTAG......A..................
Moneu vannus     .CA.....G.-CTTG....GC.AA.T..ATTTAG......A..................
Moneu crassus    .CA.....G.-CTTG....GC.AA.T..ATTTAG......A..................


                          1220        1230        1240        1250
                           *           *           *           *
Sterk nova       TGACAGATTGATAGCTCTTTCTTGATTCTATGGGT----------------GGTGGTGC
Eupl octo        ..................................ATTTT------------........
Eupl aedic       ..................................ATTTT------------........
Eupl eury        ..................................ATTTT------------........
Moneu minuta     ..................................ATTTATTTTAATTTT--........
Moneu vannus     ..................................ATTTTTTATATATTTTT........
Moneu crassus    ..................................ATTTTTTATATATTTTT........
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FIG. 3. Examples of genus-specific insertions in euplotid ciliates. Two parts of the ssu rDNA gene sequences of all euplotids aligned with those of *Sterkiella nova* are shown.

cleus which plays a key role in the understanding of ciliate macronucleus evolution (Hammerschmidt *et al.*, 1996). In general, the Spirotrichea have several macronuclei or several parts of one macronucleus. They always fuse to one nucleus prior to division. In contrast, the macronuclear vesicles of *Protocruzia* do not fuse prior to division but separate in a mitosis-like manner. The interpretation of these macronuclear vesicles is different. Whereas Ammermann (1968) believes that division is amitotic as in all other ciliates, Ruthmann and Hauser (1974) favor the interpretation that each macronuclear vesicle develops into two chromosomes which are separated mitotically. A decision between these different views is difficult, since the observed structures are very small. Whereas all spirotrich ciliates investigated to date have small gene-sized DNA (reviewed in Prescott, 1994), in *Protocruzia* no low-molecular-weight DNA was found (D. Ammermann, unpublished results). However, this finding is not in contrast with our phylogenetic trees, with *Protocruzia* branching at the base of the spirotrichs. The similarity in DNA size between other ciliate taxa, such as the heterotrichs and *Protocruzia,* can be plesiomorphic and thus would not account for the phylogenetic relationship. On the other hand, the separation of *Protocruzia* from the karyorelictids indicates that the macronuclear organization of *Protocruzia* may be more likely a derived than a plesiomorphic character state.

The enlarged data set confirms the relationship among the Hypotrichia (Euplotidae), Stichotrichia (Oxytrichidae), and Oligotrichia and the deep splits among them (Figs. 1 and 2). This may support the elevation to the order level as proposed by Lynn (1996) and Lynn and Small (1997). Our results also support the classification of *Phacodinium* within the Spirotrichea (Shin *et al.*, 2000). However, in our trees *Phacodinium* always branches off first from the spirotrich lineage, whereas in their analyses a later branching was found.

All euplotid ciliates examined show remarkably extended ssu rDNA sequences which are based on insertions scattered throughout the genes, as found in *Euplotes aediculatus* (Sogin *et al.*, 1986). Sequences and lengths of the insertions vary between the species, making an alignment of these regions difficult. However, a few of them are genus specific (Fig. 3).

The deep split found between the marine species and the freshwater species of the former genus *Euplotes* (Fig. 1) supports the separation into two different genera of *Moneuplotes* and *Euplotoides* as proposed by Borror and Hill (1995). This is in congruence with older studies based on enzyme electrophoresis and with multivariate morphometric studies (e.g., Schlegel *et al.*, 1988; Valbonesi *et al.*, 1988). The ssu rDNA analyses also confirm the branching pattern within the subgroups found by these authors. Valbonesi *et al.* (1985, 1988) demonstrated that within the marine *Euplotes crassus–minuta–vannus* group *E. vannus* and *E. crassus* are closely related, but represent true species despite their very similar morphology (Gates, 1978). The ssu rRNA genes of *E. vannus* and *E. crassus* differ only in nine positions, while 83 differences occur between *E. minuta* and *E. vannus.*

Interestingly, the euplotid species even show differences in their histone H4 amino acid sequences (Bernhard and Schlegel, 1998), although the histones H4 belong to the most conserved proteins in eukaryotes. Thus, the high genetic distances between the Euplotidae and the other spirotrichs and those within the Euplotidae are not based on the phylogenetic age of this group. Likely, they are a consequence of an unusu-

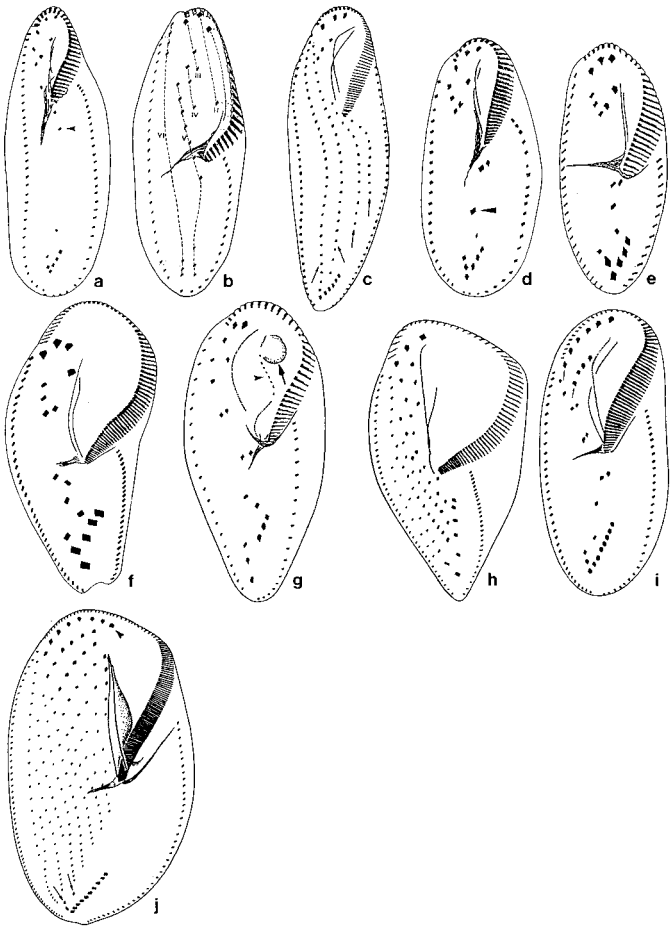

FIG. 4. Ventral cirral pattern of the Oxytrichidae investigated (from Berger (1999), where the original sources are referenced). These genera comprise a monophyletic assemblage, according to the gene sequence analyses and the conventional cladistic analysis by Berger and Foissner (1997). Main derived characters (apomorphies) are the 18 frontal–ventral–transversal cirral pattern and the fragmentation of a dorsal ciliary row during ontogenesis. The genera with about 18 cirri form the oxytrichids sensu stricto (a, b, d–g), whereas those which secondarily polymerized the cirri in several evolutionary lines comprise the oxytrichids sensu lato (c, h–j). Two subgroups (subfamilies) are recognizable in the cladistic approach (Berger and Foissner, 1997): the Oxytrichinae (a–c; with flexible body and participation of cirrus V/3 in anlagen formation) and the Stylonychinae (d–j; body inflexible, cirrus V/3 not involved in anlagen formation). (a) *Oxytricha granulifera*, length 85 μm; arrowhead marks cirrus V/3. (b) *Gonostomum strenuum*, length 100 μm; lines connect cirri originating from same anlage (II–VI). (c) *Paraurostyla weissei*, length 155 μm; arrows mark rows of frontoventral cirri. (d) *Sterkiella nova* (formerly *Oxytricha;* Foissner and Berger, 1999), length 130 μm; arrowhead denotes cirrus V/3, which is not involved in anlagen formation in this and the following species. (e) *Tetmemena pustulata* (formerly *Stylonychia;* Eigner, 1999), length 60 μm. (f) *Stylonychia mytilus*, length 215 μm. (g) *Steinia sphagnicola*, length 155 μm; arrowhead denotes endoral membrane which, uniquely, consists of numerous fragments; arrow marks unique pouch in buccal cavity. (h) *Laurentiella strenua*, length 250 μm. (i) *Pattersoniella vitiphila*, length 240 μm; arrows mark corona of frontal cirri. (j) *Onychodromus quadricornutus*, length 285 μm; arrowhead marks corona of frontal cirri; arrows denote pretransverse cirri.

ally high mutation rate detected in all molecular markers studied so far.

Compared to the Euplotidae the genetic distances and the bootstrap values within the Stichotrichia are lower, indicating that phylogenetic relationships are more difficult to resolve within this group with ssu rDNA sequences. Recently, Shin *et al.* (2000) showed that *Halteria grandinella* and *Holosticha multistylata* are associated with the stichotrichs. Our analyses with the enlarged data set of stichotrich ciliates confirm these results. Whereas the separation of *Halteria* from the Oligotrichia was proposed earlier by Petz and Foissner (1993), the position of *Holosticha* within the Oxytrichidae is surprising and contradicts all morphologic and ontogenetic data (Borror, 1972; Petz and Foissner, 1993). Based on morphological evidence, a sistergroup relationship to the oxytrichid ciliates was expected. We agree with Shin *et al.* (2000) that more sequence data are necessary to clarify the position of the Urostylida within the Stichotrichia.

There are two highly conflicting cladistic systems of the Oxytrichidae, based on a very similar body of morphostatic and ontogenetic evidence (Berger and Foissner, 1997; Eigner, 1997, 1999). Eigner's cladogram is based on the assumption that the conspicuous pattern of 18 frontal–ventral–transversal cirri evolved several times. In contrast, Berger and Foissner (1997) consider the 18 frontal–ventral–transversal cirral pattern (see Fig. 4) and the fragmentation of at least one dorsal kinety during cell division to be apomorphies of the Oxytrichidae. The sequence data favor the view of Berger and Foissner (1997), although differences occur in the position of *Paraurostyla weissei*, depending on the tree-reconstruction methods used. In all trees a subfamily Stylonychinae can be identified (Figs. 1 and 2). However, the monophyly of the subfamily Oxytrichinae is supported by the puzzle tree only (Fig. 2) and is distorted by the unusual grouping of *Halteria* and *Holosticha* (see above).

Obviously, the ssu rDNA sequences do not provide sufficient information to resolve all branching orders correctly. Faster-evolving sequences (e.g., the internal transcribed spacer) may be more informative to resolve phylogenetic relationships within the Stichotrichia.

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