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

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INTRODUCTION

The Ciliophora Doflein, 1901 comprises more than 8000 species in several highly divergent 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 systematic (e.g., Corliss, 1979; Levine et al., 1980), the Spirotrichea were subdivided into the Heterotrichida, the Odonotostomatida, 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., Euplotidae), 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 latu) into different genera. The species examined in our study now belong to the genera Euplotidae and Monoeuplotes. Members of the first genus typically
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-
sequences. 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 Intramacronucleata (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 distant 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. 1](image-url). 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.
relationships are clearly recovered within eulotids 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 *Monoeuplotes* 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 eulotids, a largely congruent branching pattern is obtained with the different reconstruction methods. Surprisingly, the oligotrich ciliate *Halteria grandinella* is separated from the other oligotrichs *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*, *Gonostoma 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 *Stylynochla lemmiae* and *S. mytilus* and 2. a heterogeneous ensemble containing *Sterkiella nova*, *Tetramena pustulata*, *Onychodromus grandis*, *O. quadricornutus*, *Steini sphagnicola*, and *Pattersoniella vitipila*. 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 *Stylynochla pustulata* into the new genus *Tetramena* (Eigner, 1997, 1999). In all trees the four clones of *Stylynochla lemmiae* and *S. mytilus* are always associated with *Laurentiella strenua* and are clearly separated from *S. pustulata* (now *Tetramena 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-
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-
phylogenetic relationships within the Spirotrichea

The genera with about 18 cirri form the oxytrichids sensu stricto (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 lato (a, b, d–g), whereas those which secondarily polymerized the cirri (c, h–j). Two subgroups (subfamilies) are recognizable in the clade, consisting of numerous fragments; arrow marks unique pouch in buccal cavity. (h) Laurentiella strenua, length 250 μm; (i) Pattersoniella vitiphila, length 240 μm; (j) Onychodromus quadricornutus, length 285 μm; arrowhead marks corona of frontal cirri; arrows denote pretransverse cirri.

**REFERENCES**


