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Phylogenetic Positions of *Aspidisca steini* and *Euplotes vannus* within the Order Euplotida (Hypotrichia: Ciliophora) Inferred from Complete Small Subunit Ribosomal RNA Gene Sequences

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Summary. The small subunit rRNA (SSrRNA) genes were sequenced for the hypotrichous ciliates, *Aspidisca steini* and *Euplotes vannus*. These two genera form a monophyletic clade and branch first in the euplotid clade at a long level with strong bootstrap support in both distance matrix and maximum parsimony tree construction methods. The phylogenetic trees further suggest the postulated relationships among families within the order Euplotida that (1) the order Euplotida, represented by *Uronychia*, *Diophrys*, *Euplotidium*, *Euplotes* and *Aspidisca*, forms a paraphyletic group; (2) the families Euplotidae and Aspidiscidae, likely as a monophyletic clade, share a common ancestor; (3) two other "related" genera, *Uronychia* and *Diophrys*, which were usually placed in the family Uronychiidae, branch later and share closer relationship each other than they are to other euplotids. On the contrary, *Euplotidium arenarium*, placed in the family Gastrocirrhidae, might be more closely related to *Uronychia-Diophrys* than to the *Aspidisca-Euplotes* group.

Key words: *Aspidisca steini, Euplotes vannus*, monophyletic, paraphyletic, phylogenetic positions, SSrRNA.

INTRODUCTION

Members of the hypotrichous genera, *Euplotes*, *Aspidisca*, *Diophrys* and *Uronychia*, are among the best known and most readily recognized ciliates with cirri on the functional ventral surface-"hypotrichs" within the order Euplotida Small and Lynn, 1985. They are united by many morphological, morphogenetic, ultrastructural and life history characters, e.g. patterns of ciliature, structure of oral apparatus, number and arrangement of frontal, ventral as well as caudal cirri (Fleury and Fryd-Versavel 1981, Foissner 1982, Fleury *et al.* 1986, Song and Packroff 1993, Berger 2001).

Morphological attributes, features of the life cycle and physiological properties are used to deduce relationships among the families within the order Euplotida (Borror 1972, Curds and Wu 1983, Borror and Hill 1995, Song 1995). However, the euplotid phylogeny still remains confusing considering their evolutionary process and systematic positions of many well-known groups. This is due to the high diversity of the morphology, the difficulty in recognizing which similarities are due to convergent evolution, and the loss of intermediate forms during the long period of time euplotids have existed.

Sequence information from homologous macromolecules shared by all members of a group can be used to

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measure the extent of genetic relationships between organisms (Zuckerlandl and Pauling 1965). In the last few years, molecular characters and ribosomal RNA in particular have been used to reevaluate ciliate phylogeny (Elwood *et al.* 1985, Sogin and Elwood 1986, Lynn and Sogin 1988, Greenwood *et al.* 1991, Schlegel *et al.* 1991, Shin *et al.* 2000). These studies revealed many different results from traditional morphological and ontogenetic characters. Within the ciliates, molecular data indicate that the heterotrich ciliates represent a very basic branch of the tree, and prostome and haptorid ciliates branch off later (Schlegel and Eisler 1996, Lynn and Small 1997).

Including descriptions of the ultrastructure of both morphostatic and morphogenetic states and analyses of gene sequences, particularly the small (SSrRNA) and large subunit ribosomal RNA (LSrRNA) genes, Lynn and Small (1997) presented a revised classification of the phylum Ciliophora Doflein, 1901 which includes 10 classes, 17 subclasses and 57 orders. Together with other three subclasses (Protocruziidia, Choreotrichia and Oligotrichia), the Hypotrichia Stein, 1859 (including the order Kiitrichia and Euplotida) and Stichotrichia Small and Lynn, 1985 (including the order Plagiotomida, Stichotrichida, Urostylida and Sporadotrichida) have been placed in the class Spirotrichea Bütschli, 1889. However, this revision for hypotrichs and stichotrichs (*sensu* Lynn and Small 1997) was mainly based on few published analyses of SSrRNA gene sequences (Elwood *et al.* 1985, Schlegel *et al.* 1991, Sogin *et al.* 1986). With the supplement of more molecular data, particularly the SSrRNA and LSrRNA genes of hypotrichous and stichotrichous ciliates, the more detailed description of their phylogenetic relationships might be proposed.

As part of a comprehensive analysis of ciliate phylogeny, we have studied recently the SSrRNA gene sequences from two "critical" marine hypotrichous ciliates, *Aspidisca steini* and *Euplotes vannus*, in order to provide more information of their phylogenetic positions. Together with sequences of other hypotrichs and stichotrichs, our molecular evolution studies further explore the phylogenetic relationships within this "highly evolved" group. Meantime, the relationships among families within the order Euplotida based on molecular data are preparatory postulated in our work.

MATERIALS AND METHODS

Ciliate collection and culture. *Aspidisca steini* (Buddenbrock, 1920) Kahl, 1932 and *Euplotes vannus* (Müller, 1786) Diesing, 1850 were collected from the coast of Qingdao, China (salinity about 32-34 ‰). Clonal cultures were established and maintained in autoclaved marine water at room temperature with rice grains to enrich natural bacteria as food for the ciliates.

Identification of species. Live specimens were observed with phase contrast and differential interference microscopes at various magnifications. Protargol silver impregnation technique (Wilbert 1975) was applied to reveal the infraciliature. The silverline system was impregnated with Chatton-Lwoff method introduced by Corliss (1953). Specimens were compared to previous papers (Curds 1975, Wu and Curds 1979, Song and Packroff 1996/97, Song and Wilbert 1997). Authorship of species is according to Berger (2001). Systematic and terminology at the ordinal level and above are mainly based on Lynn and Small (1997).

Extraction of genomic DNA. Cells were rinsed three times with sterile artificial marine water after being starved overnight and then pelleted by centrifugation. 0.5 ml lysis buffer (10mM Tris-HCl, pH 8.3; 50mM KCl; 2.5mM $MgCl_2$; 0.6% Tween 20; 0.6% Nonidet P40; 60μg/ml Proteinase K) was added to extract DNA at 56 °C for 2 h. After incubation, DNA was extracted with an equal volume of phenol:chloroform-isoamyl alcohol (25:24:1) and precipitated with 70% alcohol. DNA was stored at -20 °C (Kusch and Heckmann 1996, Chen *et al.* 2000, Chen and Song 2001).

PCR amplification. Amplifications by PCR were carried out in a total volume of 100 μl containing 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 0.1% Triton X-100; 3 mM $MgCl_2$; 0.2 mM dNTP; 0.5 mM of each oligonucleotide primer (16s-like F: 5'-AACCTGGTTGATCCT GCCAGT-3'; 16s-like R: 5'-TGATCCTTCTGCAGGTTCACC TAC-3'); 50 ng of genomic DNA and 5U Taq Pfu DNA polymerase (Sangon Bio. Co., Canada). The reaction mixtures were denatured at 94 °C for 5 min before the polymerase added, followed by the first 5 cycles consisting of denaturation for 1 min at 94 °C, primer annealing for 2 min at 56 °C, and extension for 2 min at 72 °C. In the subsequent 35 cycles, the annealing temperatures were rise to 62 °C. The circulation was followed by a final extension step for 5 min at 72 °C (Elwood *et al.* 1985, Medlin *et al.* 1988, Chen and Song 2001).

Cloning and Sequencing of SSrRNA gene. The amplified products were extracted with UNIQ-5 DNA Cleaning Kit (Sangon Bio. Co., Canada) and inserted into a pUCm-T vector. The plasmid mini-prep spin column kit (Sangon Bio. Co., Canada) was used to harvest and purify plasmid DNA. DNA sequencing for *Aspidisca steini* and *Euplotes vannus* was accomplished using the ABI Prism 377 Automated DNA Sequencer (Applied Biosystems Inc.) with three forward and three modified reverse 16S sequencing primers (Elwood *et al.* 1985, Medlin *et al.* 1988) as well as the RV-M and M13-20 primers. All sequences were confirmed from both strands.

Sequence availability. The nucleotide sequences in this paper are available from the GenBank/EMBL databases under the following accession numbers: *Diophrys appendiculata* AY004773 (Chen and Song 2001), *Euplotidium arenarium* Y19166 (Petroni *et al.* 2000), *Euplotes aediculatus* X03949 (Sogin *et al.* 1986), *Holosticha multistylata* AJ277876 (Shin *et al.* 2000), *Onychodromus quadricornutus* X53485 (Schlegel *et al.* 1991), *Oxytricha granulifera* X53486 (Schlegel *et al.* 1991), *Sterkiella nova* (= *Oxytricha nova*) X03948 (Elwood *et al.* 1985), *Stylonychia pustulata* X03947 (Elwood *et al.* 1985), *Uronychia transfuga* AF260120 (Chen and Song 2001). *Protocruzia* sp1 X65153 (Hammerschmidt *et al.* 1996), *Protocruzia* sp2 AF194409 (Shin *et al.* 2000) and *Blepharisma americanum* M97909 (Greenwood *et al.* 1991) were used as the outgroup species.

Phylogenetic analyses. The sequences were aligned with other SSrRNA gene sequences using a computer assisted procedure, Clustal W, ver. 1.80 (Thompson *et al.* 1994), and refined by considering the conservation of both primary and secondary structures (Elwood *et al.* 1985). PHYLIP package, ver. 3.57c (Felsenstein 1995) was used to calculate the sequence similarity and evolutionary distances between pairs of nucleotide sequences using the Kimura (1980) two-parameter model. Distance-matrix trees were then constructed using the Fitch and Margoliash (1967) least-squares [LS] method and the neighbor-joining [NJ] method (Saitou and Nei 1987). For the maximum-parsimony [MP] analysis, sequence data were reduced from 1790 sites to 648 phylogenetically informative sites. The DNAPARS program in PHYLIP was used to find the most parsimonious tree (Kluge and Farris 1969). Both parsimony and distance data were bootstrap resampled 1,000 times (Felsenstein 1985).

RESULTS

Sequences and Comparisons

The complete SSrRNA gene sequences were determined for *Aspidisca steini* (1746 nucleotides, GenBank/EMBL accession number AF305625) and *Euplotes vannus* (1890 nucleotides, GenBank/EMBL accession number AY004772) (Fig. 1). The GC content (44.96% *A. steini*; 43.81% *E. vannus*) is in the similar range as in other ciliates (Elwood *et al.* 1985, Sogin *et al.* 1986, Schlegel *et al.* 1991, Chen and Song 2001).

Structural similarity and evolutionary distance values were calculated pairwise as described (Jukes and Cantor 1969, Elwood *et al.* 1985) between the sequences aligned in Fig. 1 and those of other hypotrichs as well as *Blepharisma americanum* (Table 1). The sequence of *E. vannus* differed in 132 nucleotides from the sequence of *E. aediculatus* (structural similarity 90.93%). 225 sites are different between *A. steini* and *E. vannus* (structural similarity 82.95%), and 229 sites differ between *A. steini* and *E. aediculatus* (structural similarity 82.86%).

Distance Matrix Analysis

Both least-squares [LS] and neighbor-joining [NJ] analyses provide strong bootstrap support for the monophyly of the class Spirotrichea *sensu* Lynn and Small 1997 (100% [LS], 100% [NJ], Fig. 2), as well as the stichotrichs (e.g. *Sterkiella nova*, *Stylonychia pustulata*, *Onychodromus quadricornutus*, *Holosticha multistylata* and *Oxytricha granulifera*) (100% [LS], 98% [NJ], Fig. 2). The subclass Protocruziidia, represented by *Protocruzia*, forms a sister clade to other spirotrichs (100% [LS], 100% [NJ], Fig. 2). However, the sister group relationship between hypotrichs (e.g. *Uronychia transfuga*, *Diophrys appendiculata*, *Euplotidium arenarium*, *Aspidisca steini*, *Euplotes aediculatus* and *E. vannus*) and stichotrichs is not bootstrap supported.

As shown in Fig. 2, *Euplotes* and *Aspidisca* branch first from the hypotrichous clade at a very long level and form a monophyletic clade as a sister group to all other hypotrichous / stichotrichous taxa with strong bootstrap support (100% [LS], 100% [NJ]). *Euplotidium*, *Diophrys* and *Uronychia* represent other branching lineage though some bootstrap values are not very high. The stichotrichs might diverge later from hypotrichous line. Hence, the subclass Hypotrichia, as well as the order Euplotida, is supported as a paraphyletic clade. However, the separations between some genera within the euplotids are very deep and difficult to be resolved, e.g. the large distance between *E. aediculatus* and *U. transfuga* or *Aspidisca steini* and *Diophrys appendiculata* (Table 1).

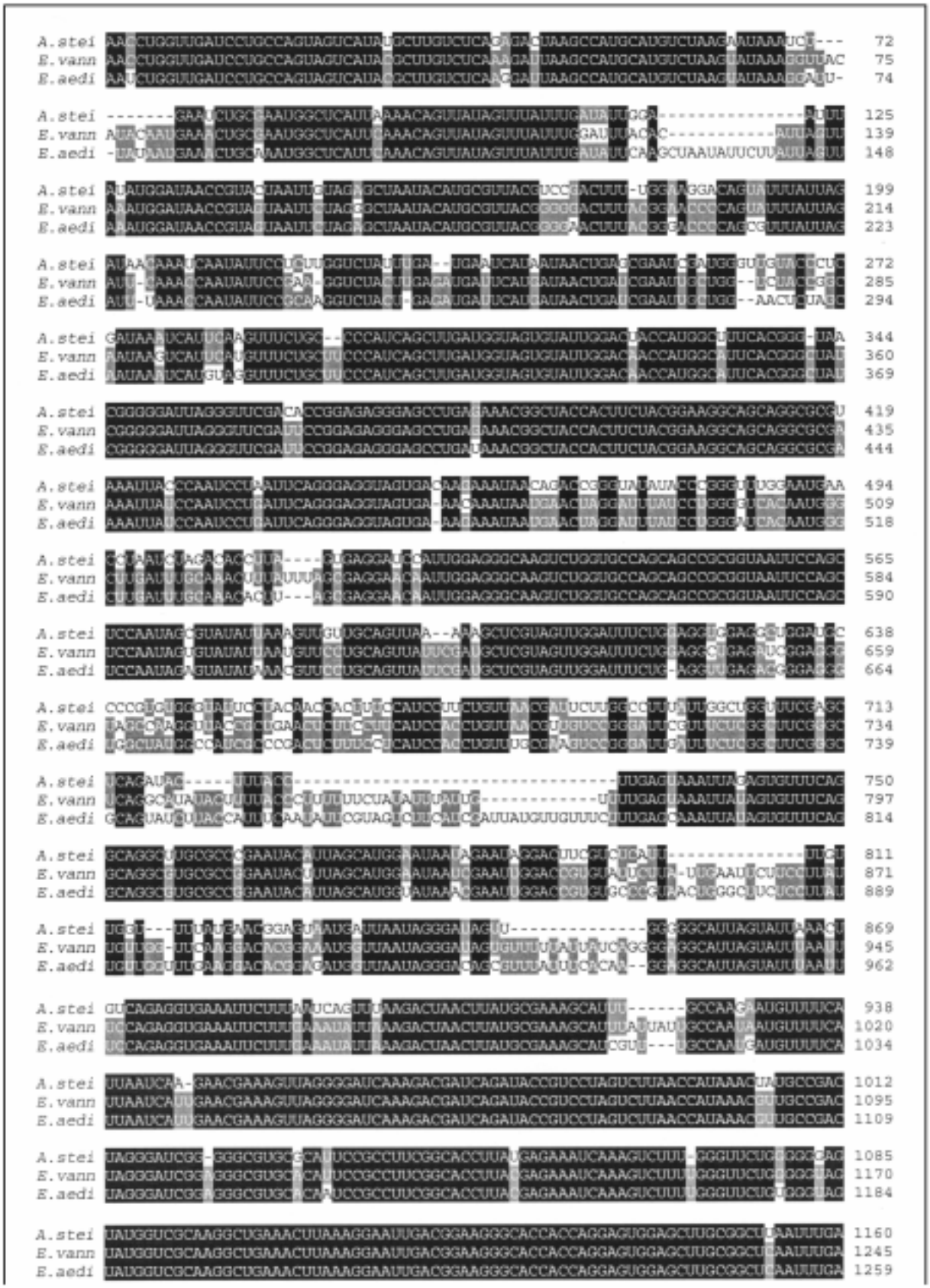
Maximum Parsimony Analysis

The major aspects of the topology of the maximum parsimony trees (Fig. 3) are similar to those of the distance matrix trees (Fig. 2).

DISCUSSION

Phylogenetic Positions of *Euplotes* and *Aspidisca*

Since the density of species in a clade can stabilize that clade's position in the topology (Smith 1994), we hence sequenced another *Euplotes* species, *E. vannus*, to assess its systematic position within the subclass Hypotrichia. Together with the new SSrRNA gene sequence for *Aspidisca steini*, the molecular data provide a strong and unambiguous result: the order Euplotida (*Uronychia*, *Diophrys*, *Euplotidium*, *Euplotes* and *Aspidisca et al.*) should be placed as the earliest diverging taxon after the hypotrichs separated from the main line (Figs 2, 3). Further, the long branching of *Euplotes* and *Aspidisca* in our trees might be the consequence of unusually high genetic substitution rates or "fast evolutionary clock speeds" in Euplotida (Sogin *et al.* 1986). To our knowledge, both of them are likely more evolved than other sister groups within the subclass Hypotrichia.


A.stei
E.vann
E.aedi

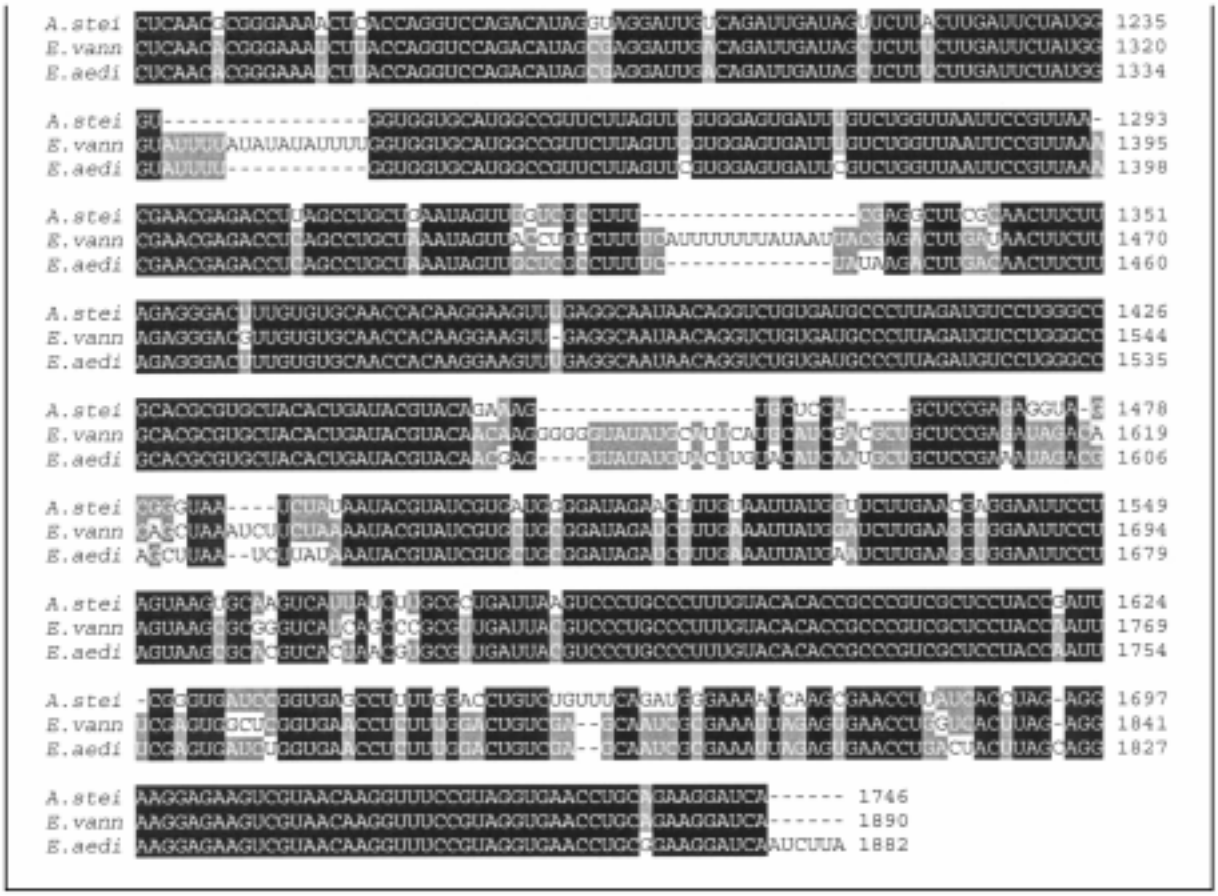


Fig. 1. Small subunit rRNA gene sequences of the euplotid ciliates *Aspidisca steini* (*A. stei*) and *Euplotes vannus* (*E. vann*) aligned with the sequences from *Euplotes aediculatus* (*E. aedi*) (Sogin *et al.* 1986). Numbers at the end of lines indicate the number of nucleotides. The differences in sequence length were compensated for by introducing alignment gaps (-) in the sequences. Matched sites are marked with black color, while unmatched are gray

Postulated evolutionary order for the families within the order Euplotida

Borror and Hill (1995) divided the order Euplotida into five families: Gastrocirrhidae, Certesiidae, Uronychiidae, Aspidiscidae and Euplotidae on the basis of morphology, stomatogenesis, ultrastructure, cyst structure, and behavior. Including some morphological and ontogenetic characters involved in early differentiation of members of the order Euplotida (e.g. short and stubby dorsal cilia, relatively stable number of five developmental streaks in the fronto-ventral cirri field, lower ratio of cell length and width), they postulated phylogenetic relationships among these families. Our current work supports their hypothesis basically: (1) Euplotidae and Aspidiscidae diverge first from the euplotid line and share a common ancestor as a monophyletic clade (Figs 2, 3); and (2) *Uronychia* and *Diophrys*, placed in the family Uronychiidae by Borror and Hill 1995, branch later and share closer relationship each other (with structural similarity 0.9119) than they are to other euplotids (Table 1).

However, our molecular data indicate that *Euplotidium arenarium*, a member of the family Gastrocirrhidae by Borror and Hill (1995), is more closely related to *Uronychia-Diophrys* than to *Aspidisca-Euplotes* group (Figs 2, 3). Considering the unique pattern of ciliature, specialized macronuclei, absence of the caudal cirri and the single left marginal cirrus, *Euplotidium* shows evidently closer relation to *Euplotes* than to other euplotids (Song 1995). Since there is only

Table 1. 16s-like SSrRNA structural similarity (upper half) and evolutionary distance (lower half) data determined by the Elwood *et al.* (1985) and Jukes and Cantor (1969) formulas for conversion of structural similarity for available hypotrichous ciliates. Sources of data for the aligned SSrRNA gene sequences are listed in Materials and Methods

	S. nov	*S. pus*	*O. qua*	*O. gra*	*H. mul*	*U. tra*	*D. app*	*E. are*	*A. ste*	*E. aed*	*E. van*
S. nov	-	0.9853	0.9774	0.9642	0.9563	0.9275	0.9121	0.9191	0.8905	0.8278	0.8304
S. pus	0.0148	-	0.9752	0.9608	0.9473	0.9196	0.9109	0.9157	0.8888	0.8262	0.8282
O. qua	0.0241	0.0270	-	0.9603	0.9462	0.9241	0.9155	0.9169	0.8894	0.8240	0.8304
O. gra	0.0353	0.0418	0.0406	-	0.9679	0.9257	0.9120	0.9213	0.8916	0.8246	0.8310
H. mul	0.0436	0.0527	0.0533	0.0316	-	0.9172	0.8973	0.9173	0.8706	0.8148	0.8162
U. tra	0.0777	0.0796	0.0827	0.0780	0.0851	-	0.9119	0.9098	0.8898	0.8167	0.8302
D. app	0.0924	0.0956	0.0872	0.0941	0.1065	0.0910	-	0.9085	0.8900	0.8254	0.8258
E. are	0.0776	0.0789	0.0808	0.0762	0.0801	0.0880	0.0958	-	0.8959	0.8258	0.8372
A. ste	0.1221	0.1255	0.1262	0.1232	0.1344	0.1240	0.1208	0.1088	-	0.8286	0.8295
E. aed	0.1779	0.1837	0.1816	0.1833	0.1830	0.1903	0.1833	0.1653	0.1667	-	0.9093
E. van	0.1746	0.1811	0.1732	0.1728	0.1757	0.1742	0.1738	0.1569	0.1552	0.0781	-

Abbreviation: *S. nov - Sterkiella nova*; *S. pus - Stylonychia pustulata*; *O. qua - Onychodromus quadricornutus*; *O. gra - Oxytricha granulifera*; *H. mul - Holosticha multistylata*; *U. tra - Uronychia transfuga*; *D. app - Diophrys appendiculata*; *E. are - Euplotidium arenarium*; *A. ste - Aspidisca steini*; *E. aed - Euplotes aediculatus*; *E. van - Euplotes vannus*

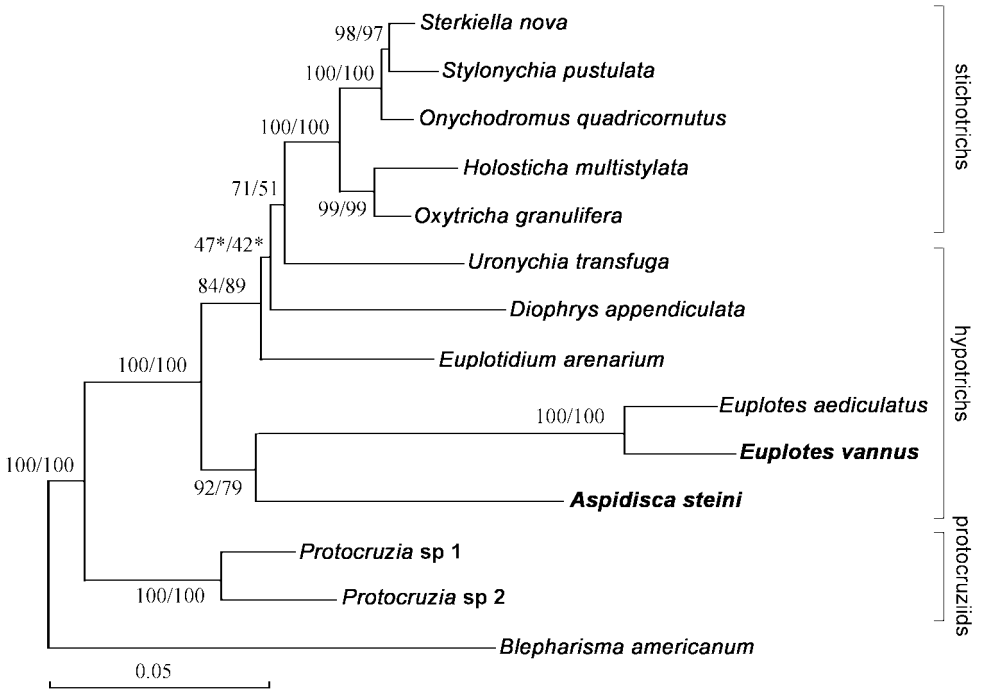


Fig. 2. A distance tree of the hypotrichous ciliates inferred from complete 16s-like small subunit ribosomal RNA gene sequences showing the systematic position of *Aspidisca steini* and *Euplotes vannus* and phylogenetic relationships among the available hypotrichs. Evolutionary distances were calculated by the Kimura (1980) two-parameter correction model and constructed by the Fitch and Margoliash (1967) least-squares [LS] method. The numbers at the nodes represented the bootstrap percentages of 1,000 for the LS method followed by the bootstrap values for the Saitou and Nei (1987) neighbor-joining [NJ] method. Asterisks indicate bootstrap values less than 50%. Evolutionary distance is represented by the branch length to separate the species in the figure. The scale bar corresponds to 5 substitutions per 100 nucleotide positions. The new sequences are represented in boldface

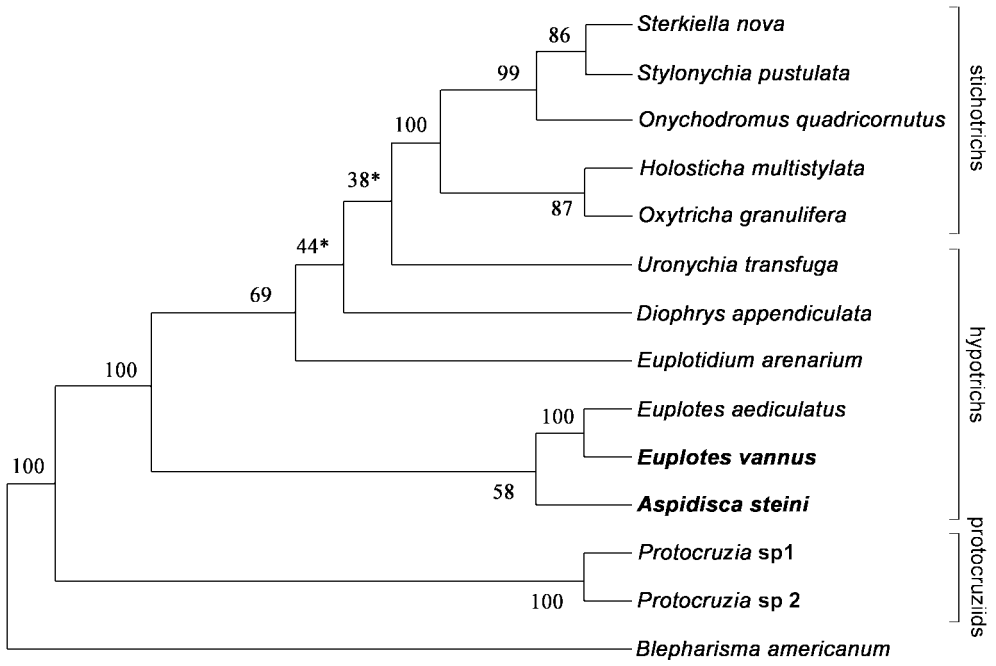


Fig. 3. A maximum-parsimony tree of the hypotrichous ciliates constructed from complete 16s-like small subunit ribosomal RNA sequences indicating the systematic position of *Aspidisca steini* and *Euplotes vannus* and phylogenetic relationships among the available hypotrichs. The numbers at the forks exhibit the percentage of times the group occurred out of the 1,000 trees. No significance is placed on branch lengths connecting the species. The new sequences are represented in boldface

one published analysis of SSrRNA sequence of *Euplotidium* within the family Gastrocirrhidae, it may be premature to define its exact phylogenetic position among Euplotida before more molecular data for euplotids, such as *Gastrocirrhus*, *Cytharoides*, *Certesia*, are available.

Postulated relationships between the subclass Hypotrichia and Stichotrichia

Historically, protozoologist classified *Euplotes* and its presumed relatives with other "highly developed" ciliates in hypotrichs. Based mostly upon ultrastructural characteristics of the dikinetid, Small and Lynn (1985) aligned the order Euplotida in the "primitive" subclass Nassophorea. However, Sogin *et al.* (1986) considered *Euplotes* more closely allied to oxytrichids than to *Paramecium* and *Tetrahymena* based on the SSrRNA gene sequences. Martin (1982) even suggested that *Euplotes*-like ciliates arose from *Oxytricha*-like hypotrichs by increase in size of the buccal cavity and reduction in number of cirri. Later, Lynn and Sogin (1988) described that hypotrichs, stichotrichs, and choreotrichs might be of the same evolutionary branch. According to the molecular data, Lynn (1996) placed oligotrichs, hypotrichs and stichotrichs in the class Spirotrichea that contains five subclasses: Protocruziidia, Hypotrichia, Choreotrichia, Stichotrichia and Oligotrichia (Lynn and Small 1997). Both distance-matrix and maximum-parsimony analyses in our work (Figs 2, 3) support: (1) the monophyly of the class Spirotrichea; (2) the monophyly of the subclass Protocruziidia and Stichotrichia; and (3) the paraphyly of the subclass Hypotrichia, as well as the order Euplotida. Since (1) there is no sister group relationship between hypotrichs and stichotrichs from our trees, (2) *Diophrys* and *Uronychia*, placed in the family Uronychiidae, likely do not branch with the hypotrichs, but with the stichotrichs, and (3) stichotrichs might diverged later from hypotrichous line within the class Spirotrichea, our results suggest that the subclass Stichotrichia and Hypotrichia might be incorporated together.

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