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Comparative phylogenetic study of Stichotrichia (Alveolata: Ciliophora: Spirotrichea) based on 18S-rDNA sequences

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ABSTRACT. Since molecular phylogenies of stichotrich ciliates started to be published, some remarkable contradictions to morphology-based classifications have been reported, such as the *C*onvergent *E*volution of *U*rostylids and *U*roleptids (CEUU) hyphothesis, the *Halteria* paradox, the polyphyly of *Oxytricha* and of Stichotrichia. We hypothesized the internal phylogeny of 18S-rDNA from 53 morphological species of stichotrichs and their relationships with Hypotrichia and Oligotrichia using parsimony and neighbor-joining methods, including new data from *Pseudouroleptus caudatus* and *Strongylidium pseudocrassum*. Competing phylogenetic scenarios were compared using statistical tests, and the results suggest the reconsideration of both CEUU and the position of *Halteria* among flexible-body oxytrichids. The polyphyly of *Oxytricha* was not rejected and the monophyly of Stichotrichia was accepted based on parsimony analysis if *Pseudoamphisiella* is considered an external (discocephalid related) taxon.

Key words: CEUU; *Halteria*; *Oxytricha*; *Strongylidium*; *Pseudouroleptus*

INTRODUCTION

During the last years, attempts to hypothesize the evolutionary relationships among the Stichotrichia (= Hypotricha in Berger, 2006) and closely related groups based on molecular data (e.g., Bernhard et al., 2001; Hewitt et al., 2003; Strüder-Kypke and Lynn, 2003; Foissner et al., 2004; Foissner and Stoeck, 2006) have given results that generally contradict other hypotheses and classification systems based on morphological data (e.g., de Puytorac et al., 1994; Berger and Foissner, 1997; Eigner, 1997, 1999, 2001; Lynn and Small, 2002; Lynn, 2003; Agatha, 2004; Foissner et al., 2007).

Molecular phylogenies have raised challenging inconsistencies with stichotrich systematics based on morphology. The most remarkable are: i) the placement of *Halteria grandinella* among the flexible-body oxytrichids (Bernhard et al., 2001; Strüder-Kypke and Lynn, 2003), referred to as the "*Halteria* paradox" (see Foissner et al., 2007); ii) the separation of *Uroleptus* from the urostylids (Croft et al., 2003; Hewitt et al., 2003; Foissner et al., 2004; Foissner and Stoeck, 2006, 2008), which led to the "*C*onvergent *E*volution of *U*rostylids and *U*roleptids" (CEUU) hypothesis, and the polyphyly of the non-uroleptid urostylids, suggested in subsequent studies (Schmidt et al., 2007; Yi et al., 2008a,b); iii) the polyphyly of *Oxytricha* (Schmidt et al., 2007), and iv) the splitting of the Stichotrichia, which were polyphyletic in Yi et al. (2008b).

Furthermore, the position of Stichotrichia as a sister group of the Oligotrichia, often based on molecular phylogenies, is not entirely consensual with morphological analyses (Petz and Foissner, 1992; Eisler and Fleury, 1995; Agatha, 2004). Some of these suggest Hypotrichia (= Euplota in Berger, 2006) as the adelphotaxon of Stichotrichia (but see Foissner et al., 2007 as an exception) while others, less commonly, suggest the sister relationship of Hypotrichia and Oligotrichia (Snoeyenbos-West et al., 2004).

This study aims to contribute to the systematics of the Stichotrichia by hypothesizing its internal phylogeny and their relationship with Hypotrichia and Oligotrichia based on analyses of 18S-rDNA data and statistical comparisons of competing phylogenies. For the first time, sequences of *Strongylidium pseudocrassum* and *Pseudouroleptus caudatus*, species of contrasting taxonomy based on morphology (Borror, 1972; Tuffrau and Fleury, 1994; Eigner, 1997, 1999; Berger, 1999; Lynn and Small, 2002; Lynn, 2003; Paiva and Silva-Neto, 2007), were included and their systematic position was determined based on molecular data.

MATERIAL AND METHODS

Species identification and terminology

Strongylidium pseudocrassum was obtained from samples of water containing bottom sediments from Cabiúnas Lagoon (Parque Nacional da Restinga de Jurubatiba, Macaé, RJ, Brazil, geographic coordinates: S 22° 17' 46.7" W 41° 41' 32.3"). *Pseudouroleptus caudatus* was found in cultures of hydrated mud from the margins of the Alma River (Tocantins, Brazil, exact geographic coordinates unknown), kindly provided by a collaborator. These organisms were identified through standard microscopy techniques widely used to investigate ciliate morphology, including *in vivo* observations (Dragesco and Dragesco-Kernéis, 1986; Foissner, 1991), protargol-impregnation (Dieckmann, 1995) and scanning electron microscopy (Silva-Neto, 1994). The terminology adopted follows mainly the works of Eigner and Foissner (1994) and Berger (1999, 2006). In the text

we refer to taxons of conflicting delimitation in the literature using vernacular names.

DNA extraction, amplification and sequencing

Approximately 30 cells of each species were picked from their respective cultures and transferred to staining jars, where sediment particles, small protists and other visible contaminants were carefully removed with micropipettes. The cells were washed several times with mineral water and left starving for about 2 h. The excess water was removed until the cells became concentrated in a drop-sized pool in the middle of the stain jar. The cells were then fixed with two or three droplets of alcoholic Bouin mixture, prepared according to Dieckmann (1995), for about 5 min, and then washed with distilled water until the medium became clear transparent. After this step, the fixed cells were collected with a micropipette and transferred to microtubes for DNA isolation.

The cells were treated with lysis buffer, homogeneity buffer and proteinase K (10 mg/mL) at 55°C for 2 h, followed by the phenol-chloroform protocol described in Sambrook et al. (1989). The DNA was eluted overnight in TE solution and then stored at -20°C.

For amplification of 18S-rDNA gene, primers were designed based on conserved regions found in sequences of Stichotrichia based on the literature (Table 1). Two independent polymerase chain reactions (PCRs) were run to amplify the gene, resulting in overlapped fragments, using the primers: Cil18SF 5' GTCATATGCTTGTCTCAAAGACTAAGCC 3' and Cil18SR 5' CGACTTCTCCTTCCTCTAAGTGATATGG 3' for the first segment, and Cil910 5' TTAGAGTGTTCCAGGCAGGC 3' and Cil2279 5' ACTTCGCTGCGTTCTTCATCG 3' for the second one. The conditions of each reaction were: 5 min at 94°C for initial denaturation, followed by 40 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 30 s. The final extension was at 72°C for 5 min. Amplified fragments were purified with the phenolic method (Sambrook et al., 1989) and sequenced by the dideoxyterminal method (Sanger et al., 1977) using the *ABI 377* (Applied Biosystems) automatic sequencer equipment using these primers and three internal ones for the second gene segment: Cil1239 5' CCGACTAGGGATCGGAGG 3', Cil1561 5' GAGACCTTAGCCTACTAA 3' and Cil1838 5' TTGGAATTATAGATCTTGAA 3'.

Sequence alignment and optimization

Initially, a data base containing 63 sequences of Stichotrichia (including those of *S. pseudocrassum* and *P. caudatus*) were aligned with 23 outgroup sequences (Heterotrichia, Hypotrichia, *Licnophora* and *Phacodinium*) through the "progressive approach of multiple sequence alignment" of Feng and Doolittle (1987) implemented in the Clustal X 1.81 computer software (Thompson et al., 1997). Penalties for gap-opening and -extension were adjusted to improve the average alignment quality score (Q-score), calculated with TuneClustalX (Hall, 2004). The resulting alignment was further refined and trimmed manually to mount a character matrix, using the BioEdit v7.0.5 software (Hall, 1999). The quality of the resulting matrix was then assessed by checking its degree of saturation with the DAMBE software (Xia and Xie, 2001). To minimize reconstruction artifacts such as long-branch attraction, which can be associated with signal noise caused by mutational saturation (Felsenstein, 1978; Philippe, 2000; Lartillot et al., 2007), one outgroup sequence was removed at a time, commencing from the most distant (Philippe and Laurent, 1998; Brinkmann et al., 2005), and the whole sequence alignment procedure was redone until the matrix was clean of saturation, reducing the outgroup assemblage to nine sequences (Table 1).

Table 1. Sequences used in our study and their GenBank accession numbers.

Species	Accession numbers
Amphisiella magnigranulosa	AM412774
Anteholosticha manca	DQ503578
Anteholosticha multistylata	AJ277876
Apokeronopsis crassa	DQ359728
Aspidisca steini	AF305625
Blepharisma americanum	M97909
Certesia quadrinucleata	DQ059581
Codonellopsis americana	AY143571
Cyrtohymena citrina (1)	AF508755
Cyrtohymena citrina (2)	AY498653
Diaxonella pseudorubra polystylata	AF508760
Diaxonella trimarginata	DQ190950
Diophrys appendiculata	AY004773
Engelmanniella mobilis	AF508757
Eufolliculina uhligi	U47620
Euplotes aediculatus	M14590
Euplotes crassus	AJ310492
Euplotes eurystomus	AJ310491
Euplotes minuta	AJ310490
Favella panamensis	AY143572
Gastrostyla steinii	AF508758
Gonostomum namibiense	AY498655
Gonostomum strenuum	AJ310493
Halteria grandinella	AF194410
Hemiurosoma terricola	AY498651
Holosticha diademata	DQ059583
Holosticha heterofoissneri	DQ059582
Laurentiella strenua	AJ310487
Licnophora macfarlandi	AF527758
Maristentor dinoferus	AY630405
Novistrombidium testaceum	AJ488910
Onychodromopsis flexilis (1)	AY498652
Onychodromopsis flexilis (2)	AM412764
Onychodromus grandis	AJ310486
Orthoamphisiella breviseries	AY498654
Oxytricha elegans	AM412767
Oxytricha ferruginea	AF370027
Oxytricha granulifera (1)	AM412769
Oxytricha granulifera (2)	X53486
Oxytricha granulifera (3)	AM412772
Oxytricha granulifera (4)	AM412771
Oxytricha granulifera (5)	AF508762
Oxytricha lanceolata	AM412773
Oxytricha longa	AF508763
Oxytricha longigranulosa	AM412766
Oxytricha saltans	AF370028
Parabirojimia similis	DQ503584
Paraurostyla viridis	AF508766
Paraurostyla weissei (1)	AJ310485
Paraurostyla weissei (2)	AY294648
Pattersoniella vitiphila	AJ310495
Peritromus kahli	AJ537427
Phacodinium metchnikoffi	AJ277877
Plagiotoma lumbrici	AY547545
Pleurotricha lanceolata	AF508768

Continued on next page

Table 1. Continued.

Species	Accession numbers
Prodiscocephalus borrori	DQ646880
Pseudoamphisiella alveolata	DQ503583
Pseudoamphisiella lacazei	DQ777743
Pseudokeronopsis carnea	AY881633
Pseudokeronopsis flava	AY881634
Pseudokeronopsis rubra	DQ640314
Pseudouroleptus caudatus	DQ910904
Pseudourostyla cristata	DQ019318
Pseudourostyla franzi	AM412765
Rigidothrix goiseri	DQ490236
Steinia sphagnicola	AJ310494
Stentor polymorphus	AF357144
Sterkiella histriomuscorum	AF508770
Sterkiella nova	AF508771
Strombidinopsis jeokjo	AJ628250
Strombidium inclinatum	AJ488911
Strombidium purpureum	U97112
Strongylidium pseudocrassum	DQ910903
Stylonychia lemnae	AJ310496
Stylonychia mytilus	AJ310499
Styxophrya quadricornuta	X53485
Tetmemena pustulata (1)	X03947
Tetmemena pustulata (2)	AF508775
Tintinnopsis fimbriata	AY143560
Trachelostyla pediculiformis	DQ057346
Uroleptus gallina	AF164130
Uroleptus lepisma	AF508765
Uroleptus piscis	AF164131
Uronychia transfuga	AF260120
Urostyla grandis (1)	AF164129
Urostyla grandis (2)	AF508781

Species marked in grey were excluded from the final nucleotide character matrix due to mutational saturation. Species initially assumed as outgroups are marked in bold.

Phylogenetic analyses

Our initial assumption of ingroup monophyly circumscribed the Stichotrichia *sensu* Berger (2006). The outgroups Hypotrichia and Oligotrichia correspond to euplotids (including the discocephalid *Prodiscocephalus*) and oligotrichs + choreotrichs, respectively.

The character matrix was analyzed with maximum parsimony (MP) and neighbor-joining (NJ) methods using the PAUP* 4b10 software (Swofford, 2003). Alignment gaps were treated as a "fifth base" to preserve their information in MP analyses; uncertain bases were coded as missing data ("?" in PAUP*). According to Giribet and Wheeler (1999), NJ methods are unable to handle indel information in their calculations, so gaps were treated as "missing data" in these analyses. The sequences of Hypotrichia and Oligotrichia were analyzed simultaneously with the Stichotrichia. Two different root placements were attempted *a posteriori*: rooting according to the outgroup position (Nixon and Carpenter, 1993) and midpoint rooting (Farris, 1972).

Optimization of character transformations followed the accelerated transformation (ACCTRAN) criterion. Inspection of character transformations over trees was based on PAUP* output log files containing apomorphy and character state change lists. The Mesquite 2.01 software (Maddison and Maddison, 2007) was used to trace the changes of the morphological character "presence of midventral complex" over trees, using parsimony criterion. Trees were visualized and edited for publishing on the MEGA 4 software (Tamura et al., 2007). The descriptive statistics and the Mann-Whitney test in Table 4 were calculated with the GraphPad Prism 4 software (Motulsky, 1999). In the text, we refer to the dendrograms generated by MP as "cladograms", and by NJ as "NJ trees".

Maximum parsimony

Initially, fundamental most parsimonious cladograms (FMPC) were obtained from an equal-weighted character matrix, through the parsimony ratchet strategy (Nixon, 1999) implemented in PAUP*, using a command block wrote by the accessory PaupRat program (Sikes and Lewis, 2001), which was adjusted to "tree bisection and reconnection" (TBR) branch-swapping algorithm and 200 ratchet iterations. Independent runs were conducted until the total number of FMPC stabilized. Successive weighting (Farris, 1969) was applied by re-weighting the characters according to their maximum rescaled consistency index in the FMPCs, and a heuristic search (iteration) conducted using TBR with 300 replicates of random sequence addition. This procedure was repeated until the character weights stabilized for two consecutive iterations (Kitching et al., 1998). Character weights were scaled from 0 to 1. Spuriously resolved branches were collapsed to polytomies whenever present.

Neighbor-joining

Prior to the analysis, a nucleotide substitution model (see Li, 1997; Schneider, 2007) was chosen through the "minimum theoretical information criterion" (Akaike, 1974; Bos and Posada, 2005) using the MODELTEST 3.7 software (Posada and Crandall, 1998). The NJ analysis was then performed using the BioNJ algorithm, which according to Gascuel (1997), produces trees with topologies more accurate than those from conventional NJ (Saitou and Nei, 1987). Likelihood was used as a criterion to calculate a pairwise distance matrix based on which phylogenetic tree was built, considering the substitution model parameters selected. Branch length estimates and tree-score calculation by minimum-evolution function were conducted with the default settings of PAUP* (Schneider, 2007).

Nodes reliability assess

For both MP and NJ optimal trees, the reliability of the internal nodes was assessed through 1000 bootstrap pseudo-replicates generated by PAUP*. Under the parsimony criterion, only parsimony-informative characters (Carpenter, 1996) were analyzed by heuristic tree search with TBR and simple sequence addition. Characters were sampled with equal probability, but their final weights were considered. The BioNJ algorithm was employed under the distance criterion. Support values inferior to 50% were not assigned to the optimal trees (Figures 1, 2). Tree metrics for bootstrap analyses were based on 50% majority rule consensus trees (Table 2).

Table 2. Tree metrics for maximum parsimony (MP) and neighbor-joining (NJ) analyses.

Trees	CI	HI	RC	RI	TL(E)	TL(S)	ME score	-lnL
FMPC	0.427	0.578	0.252	0.596	2458	-	-	-
SW iteration 1	0.685	0.315	0.486	0.710	2460	677.50548	-	-
MPC	0.686	0.314	0.489	0.712	2460	675.89890	-	-
MP bootstrap consensus tree	0.678	0.322	0.475	0.700	2520	684.50824	-	-
NJ tree	-	-	-	-	-	-	1.68867	13896.06912
NJ bootstrap consensus tree	-	-	-	-	-	-	1.70518	13977.92946

CI = consistency index; FMPC = fundamental most parsimonious cladogram(s); HI = homoplasy index; ME = minimum evolution; MPC = most parsimonious cladogram(s); RC = rescaled consistency index; RI = retention index; TL(E) = tree length under equal-weighted matrix; TL(S) = tree length under successive-weighted matrix; SW = successive weighting.

Test of hypotheses

Unrooted trees with topological constraints were built using parsimony (heuristic search with TBR and 300 random sequence addition replicates) and distance (BioNJ, with the same parameters used to obtain the optimal tree) criteria using PAUP*. Each constraint corresponded to a different scenario enforced to obtain trees that contradict the CEUU hypothesis (I-IV), force the monophyly of the urostylids (V-VIII), the branching of *Halteria grandinella* among the Oligotrichia (IX), the monophyly of *Oxytricha* (X), and the monophyly of Stichotrichia (XI-XIII). The topological constraints are given in Table 3.

Table 3. P values of statistical tests for tree comparisons considering different topological constraint scenarios.

Scenarios	Constrained topologies	SH	WSH	Templeton	WS
I	*Rigidothrix goiseri* + *Uroleptus* spp	0.82913	0.621	0.0459	0.1338
II	*Rigidothrix goiseri* + *Uroleptus* spp + Core urostylids	0.78676	0.522	0.1492	0.1000
III	*Uroleptus* spp + Core urostylids	0.41369	0.108	0.0422	0.1078
IV	*Pattersoniella vitiphila* + *Rigidothrix goiseri* + *Uroleptus* spp	0.00015	<0.0001	<0.0001	<0.0001
V	Core urostylids + *Holosticha* spp *Parabirojimia similis* + *Pseudoamphisiella* spp	0.01301	<0.0001	<0.0001	<0.0001
VI	Core urostylids + *Holosticha* spp *Parabirojimia similis* + *Pseudoamphisiella* spp + *Uroleptus* spp	0.00606	<0.0001	<0.0001	<0.0001
VII	Core urostylids + *Holosticha* spp *Parabirojimia similis* + *Pseudoamphisiella* spp + *Rigidothrix goiseri* + *Uroleptus* spp	0.00052	<0.0001	<0.0001	<0.0001
VIII	Core urostylids + *Holosticha* spp *Parabirojimia similis* + *Pattersoniella vitiphila* + *Pseudoamphisiella* spp + *Rigidothrix goiseri* + *Uroleptus* spp	<0.0001	<0.0001	<0.0001	<0.0001
IX	*Oxytricha* spp	<0.0001	<0.0001	<0.0001	<0.0001
X	*Halteria grandinella* + Oligotrichia	0.38575	0.119	0.0255	0.0059
XI	Stichotrichia *sensu* Berger (2006)	0.16105	0.003	<0.0001	<0.0001
XII	Core Stichotrichia + *Holosticha* spp + *Oxytricha saltans*	0.98290	0.939	-	-
XIII	Core Stichotrichia + *Pseudoamphisiella* spp	0.57696	0.056	0.0003	0.0018

Values for rejection of null hypotheses are marked in gray. SH = Shimodaira-Hasegawa; WS = winning sites; WSH = weighted Shimodaira-Hasegawa. $\alpha = 0.01$.

To examine the support of the data for such competing hypotheses, we tested whether shortest trees (unconstrained) differed significantly from the constrained ones, comparing them in likelihood and parsimony frameworks, based on the criteria with which they were built, and considering a conservative significance level (α) of 0.01. The NJ trees were compared through

the "Shimodaira-Hasegawa" (SH) test implementation in PAUP*, with the SH distribution generated with 10,000 bootstrap pseudo-replicates by the "resampled estimated log-likelihood" method (Kishino et al., 1990), and the "weighted Shimodaira-Hasegawa" (WSH) test (Shimodaira, 1993, 1998; Shimodaira and Hasegawa, 1999; Buckley et al., 2001) using the CONSEL software package (Shimodaira and Hasegawa, 2001) with 1,000,000 multiscale bootstrap pseudo-replicates (Shimodaira, 2005). Cladograms were compared using two sitewise tests: the "Wilcoxon signed-ranks" (Templeton, 1983) and the "winning sites" test (WS) (see Prager and Wilson, 1988), both ran in PAUP*, following Schneider (2007), and considering characters' final weights.

RESULTS AND DISCUSSION

Sequences and alignment

The 18S-rDNA sequences of *P. caudatus* and *S. pseudocrassum* had 1717 nucleotides and were deposited in GenBank (accession codes in Table 1). The final character matrix contained 72 sequences of 63 different morphological species, which after they were trimmed, had 1679 characters, of which 1071 were constant, 204 were variable but parsimony-uninformative, and 404 parsimony-informative. Gap opening and extension penalties were set to 30.0 and 2.00, respectively, resulting in an optimum Q-score of 90.9506.

Maximum parsimony

After four parsimony ratchet runs, the number of FMPC stabilized in 12 cladograms (not shown) each measuring 2458 steps. Character weights stabilized at the second successive iteration, producing a single most parsimonious cladogram (MPC), which was not among the 12 FMPCs and was two steps longer than those, when measured based on an equal-weighted matrix (Figure 1, Table 2). The resulting matrix had 1338 characters with weights equal to 1, and 341 characters that received other weights. As a favorable side-effect (but not an obligatory one), the process reduced the number of MPCs to one fully resolved topology (Kitching et al., 1998). The initially equal-weighted matrix produced cladograms with large amounts of homoplasy, expressed by the ensemble homoplasy index in FMPCs (igual to 0.578). Application of *a posteriori* character weighting resulted in a more consistent tree (Farris, 1969, 1989a; Kitching et al., 1998), which had a homoplasy index igual to 0.314. In this context, we agree with Platnick et al. (1991, 1996), Goloboff (1993, 1997) and Goloboff et al. (2008), who defend that parsimony analyses require weighting to achieve self-consistent results. For a contrary view, see Turner and Zandee (1995), Källersjö et al. (1999), and Kluge (1997a,b; 2005).

Neighbor-joining

The 18S-rDNA data were best fit to the "General Time Reversible" (GTR) nucleotide substitution model (Rodriguez et al., 1990), with 44.99% invariable sites (I) and gamma distribution (γ) of rate equivalent to 0.1542 (GTR + I + G; negative log likelihood [-lnL] = 16678.7188). This model assumed a symmetric substitution rate (R) matrix with six possible transformations (Hillis et al.,

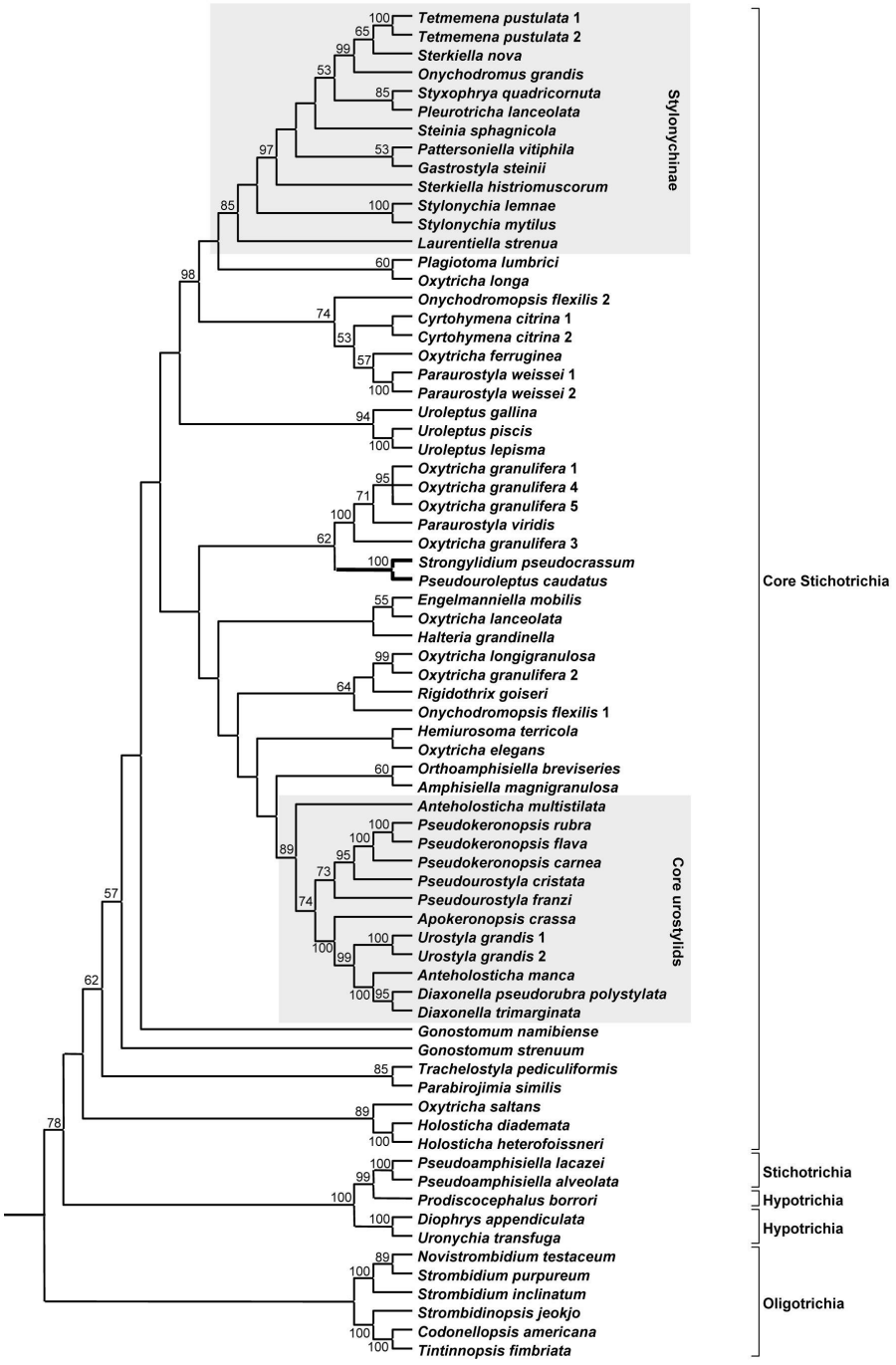

Figure 1. Single most parsimonious cladogram built using parsimony ratchet search strategy and successive weighting. Branches with new sequences are marked in bold. Values associated with nodes correspond to bootstrap support in %.

1996; Li, 1997) (a, b, c, d, e, f), which were: R(a) [A-C] = 1.5507; R(b) [A-G] = 3.2390; R(c) [A-T] = 1.5154; R(d) [C-G] = 1.1415; R(e) [C-T] = 5.5128; R(f) [G-T] = 1.0000. Nucleotide frequencies of 25.43, 19.22, 26.21, and 29.14% were found for bases A, C, G, T, respectively. The resulting tree (Figure 2, Table 2) had a minimum evolution score of 1.68867 and -lnL = 13896.06912.

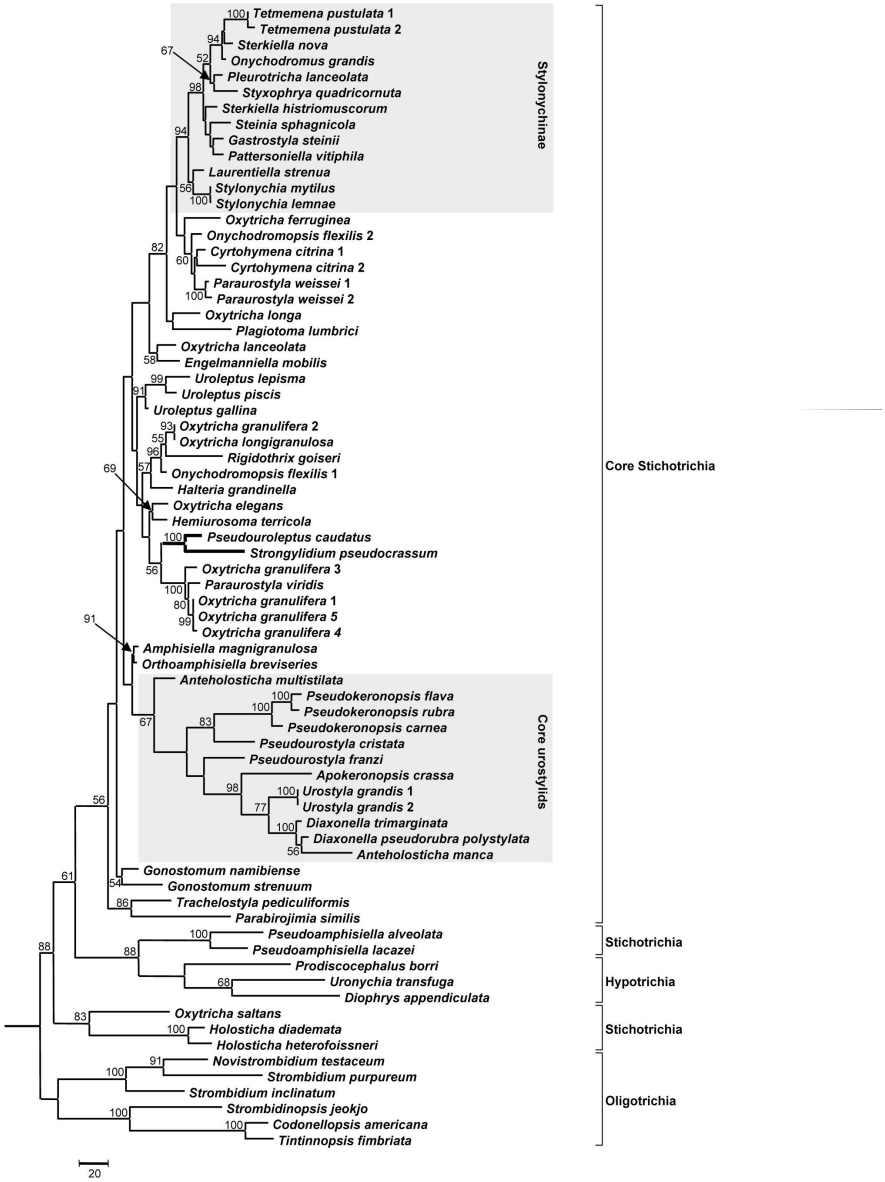

Figure 2. Neighbor-joining tree built using the BioNJ algorithm and the GTR + I + G model of nucleotide substitution. Branches with new sequences are marked in bold. Values associated with nodes correspond to bootstrap support in %. Scale bar: substitutions per site.

Root placement

In both unrooted trees, monophyly of the Stichotrichia was not supported. In the MPC, *Pseudoamphisiella* spp formed a clade with *Prodiscocephalus borrori*, standing between the core Stichotrichia + Oligotrichia and the Hypotrichia. Yi et al. (2008b) found similar results regarding the placement of *Pseudoamphisiella* spp and *Prodiscocephalus*, which grouped with 64% bootstrap support (99% in our MPC). In the NJ tree, however, *P. borrori* branched from the base of Hypotrichia independently from *Pseudoamphisiella* spp, with <50% bootstrap support. Additionally, the group formed by *Holosticha* spp + *Oxytricha saltans* remained outside of the core Stichotrichia, but with bootstrap <50%.

The relative position of the outgroup taxons thus did not corroborate the initial premise of a monophyletic ingroup, therefore, rooting the trees according to outgroup comparison criterion produced an arbitrary choice of which group would be the most distant from the stichotrichs.

Considering the euplotid hypotrichs as an outgroup leads to the suggestion of the union of Stichotrichia and Oligotrichia, perhaps due to the presence of the perilemma (Foissner et al., 2007). On the other hand, rooting with the oligotrichs as an outgroup would suggest the common ancestrality of Hypotrichia and Stichotrichia, morphologically supported by the presence of cirri (Petz and Foissner, 1992; Agatha, 2004). Either way, we agree with Berger (2006) that more data are needed to determine the relationships among these three groups. Moreover, if the root is placed following the relationships recovered in molecular studies, then it should be placed at the point of divergence between *Diophrys appendiculata* + *Uronychia transfuga* and the remaining taxons. However, one must be aware of the possibility that artifacts caused by mutational saturation could have misled the understanding of the relationship of the Stichotrichia with more distant groups (including hypotrichs, oligotrichs, and basal spirotrichs) in those studies. Therefore, we used another root placement criterion, viz. midpoint rooting (Farris, 1972; Felsenstein, 1978; Qiu et al., 2001; Sanderson and Shaffer, 2002), which as shown by Hess and de Moraes Russo (2007), can be very effective in such cases where the "real" outgroup is not clearly defined. This method does not require external assumptions based on previous analyses, avoiding arbitrary outgroup choice by placing the root at the midpoint between the two most divergent operational taxonomic units (Swofford et al., 1996; Nei and Kumar, 2000).

Accordingly, the root was placed at the branching point from the Oligotrichia to the remaining taxons, in agreement with morphological studies (Petz and Foissner, 1992; Eisler and Fleury, 1995; Agatha, 2004).

Tree topologies and nodal support

Both the MPC and NJ tree were almost fully resolved (with the exception of *Oxytricha granulifera* trichotomy), but there was considerable disagreement at family and order levels. Strict consensus of both topologies (Figure 3) did not depict a monophyletic Stichotrichia due to the aforementioned position of *Pseudoamphisiella* spp and because the *Holosticha* spp + *Oxytricha saltans* group was included in the core Stichotrichia only in the MPC. The position of *Holosticha* outside a major lineage of stichotrichs was already described by Schmidt et al. (2007); however, they did not include euplotid hypotrichs in their analyses, so the branching

pattern was slightly different. Additionally, *Oxytricha saltans* grouped with *Gonostomum* spp in their study, but with <50% bootstrap support.

Figure 3. Strict consensus topology of the optimal cladogram and neighbor-joining tree. Branches with new sequences are marked in bold.

The core Stichotrichia was composed of the *Trachelostyla pediculiformis* + *Parabirojimia similis* group, also hypothesized by Gong et al. (2007); *Gonostomum* spp (which formed a monophylum in the NJ tree, with bootstrap support of only 54%) plus a larger group that consisted of a dichotomous subset of the stichotrich species. Inasmuch as the phylogenetic hypotheses generated according to parsimony or distance criteria were not entirely consensual, two major well-resolved groups were displayed in opposite sides of this dichotomy: the Stylonychinae Berger and Foissner, 1997, and the core urostylids (Figures 1, 2).

The rigid-body subfamily Stylonychinae was proposed by Berger and Foissner (1997) based on cladistic analyses of morphological traits from typical oxytrichids. This group is often recovered in molecular phylogenies (e.g., Bernhard et al., 2001; Hewitt et al., 2003; Foissner et al., 2004; Schmidt et al., 2007; Shao et al., 2007; Yi et al., 2008a,b) and so they were in both our trees, however, with discrepant placement of *Steinia sphagnicola* and *Sterkiella histriomuscorum*. Nevertheless, stylonychine external affinities did not follow the same pattern in both trees.

Closely related to the Stylonychinae, but with different placements, were the *Oxytricha longa* + *Plagiotoma lumbrici* group and the cyrtohymenids. The former is a notable odd pair, since morphologically *Plagiotoma* is assumed to be distantly related to a typical oxytrichid due to its "holotrichous" ciliature pattern. Affa'a et al. (2004) speculated that this organism could be a highly apomorphic stichotrich adapted to endosymbiotic life in the intestine of annelid worms. Among the cyrtohymenids, *Cyrtohymena citrina* was found to be paraphyletic by Foissner et al. (2004) and Foissner and Stoeck (2008). In our trees, both sequences of this species formed a monophylum, but with <50% bootstrap support, compatible with the study by Foissner and Stoeck (2006). The close relationship of *Cyrtohymena*, the *Onychodromopsis flexilis* population from Salzburg, *Oxytricha ferruginea* [*Cyrtohymena ferruginea sensu* Foissner (1989)] and *Paraurostyla weissei* was also similar to what was reported in a previous study (Schmidt et al., 2007).

Engelmanniella mobilis and *Oxytricha lanceolata* consistently formed a monophylum in our trees, agreeing with the Bayesian tree in Schmidt et al. (2007), but with bootstrap values close to 50% and branching at highly discrepant points. In the MPC, they formed a clade with *Halteria grandinella*, which in both trees branched off from different, but nearby points in relation to the monophyletic group formed by the Antartic population of *Onychodromopsis flexilis*, *Oxytricha granulifera* 2 and *Oxytricha longigranulosa* (both equal after aligned and trimmed), and *Rigidothrix goiseri*. These sequences formed monophyla in both analyses, which is compatible with the findings from other studies (Foissner and Stoeck, 2006, 2008; Schmidt et al., 2007).

The new sequences of *S. pseudocrassum* and *P. caudatus* formed a monophyletic group in both trees with high bootstrap support. Their 18S sequences shared 96.2% similarity, confirming that these two organisms are closely related (Paiva and Silva-Neto, 2007). In our analyses, the *Strongylidium* + *Pseudouroleptus* group was closely related to the remaining *Oxytricha granulifera* populations and *Paraurostyla viridis*. According to Berger (2006), the latter may be a misidentified species, because of its ambiguous phylogenetic placement (Kelminson et al., 2002; Croft et al., 2003; Hewitt et al., 2003) and given that no morphological data were provided as evidence of correct identification. We decided to include it in our study to determine how it would be placed when analyzed in a broader taxon sample context than it originally was.

We found that *Paraurostyla viridis* grouped among *Oxytricha granulifera* sequences, forming a monophylum with *Oxytricha granulifera* 1, 4 and 5 (the sequences *O. granulifera* 1 and 5 were found to be equal after being aligned and trimmed), supported by bootstrap values of 71 and 80% in MPC and NJ tree, respectively. This result is compatible with the findings of Hewitt et al. (2003) and may indicate a misidentification, this being a strain of *Paraurostyla viridis* or possibly a sample from another population of *Oxytricha granulifera*. Moreover, the group formed by *Oxytricha elegans* and *Hemiurosoma terricola*, although recovered in both trees, has affinities that are not equally established.

The kinships of *Uroleptus* were not consensually hypothesized, even though the genus was monophyletic in both trees. In the MPC it was the sister taxon of the group formed by the cyrtohymenids, *Oxytricha longa* + *Plagiotoma lumbrici* and the Stylonychinae, a pattern that is commonly reported (Foissner and Stoeck, 2006, 2008; Schmidt et al., 2007; Gong et al., 2007). In the NJ tree, however, *Uroleptus* branched off the base of the group that contained *Rigidothrix goiseri*, a species with a similar cirral pattern, but that has a rigid body and lacks cortical granules (Foissner and Stoeck, 2006). This, however, puts *Uroleptus* in a phylogenetic position that is more compatible with that found in Foissner et al. (2004).

Finally, a well-resolved taxon supported by the highest bootstrap values within the core Stichotrichia and recovered in both analyses was formed by *Amphisiella magnigranulosa* + *Orthoamphisiella breviseries*, confirming the polyphyly of the amphisiellids (Gong et al., 2007; Schmidt et al., 2007), plus the core urostylids. The latter displayed *Pseudokeronopsis* as a monophylum (Shao et al., 2007; Schmidt et al., 2007; Yi et al., 2008a,b) and the polyphyly of *Anteholosticha* Berger, 2003, which was already supposed by Berger (2003, 2006) because the genus was assembled with a combination of morphological plesiomorphies.

Additionally, the position of *Pseudourostyla franzi* was not consensually defined, inasmuch as it grouped with *Pseudokeronopsis* spp, with 73% bootstrap support in the MPC. The two sequences of *Urostyla grandis* were found to be equal after aligned and trimmed, and grouped consistently with *Diaxonella* spp + *Anteholosticha manca* in both trees.

Among the 404 parsimony-informative characters in our matrix, 345 had a retention index (RI) of >0; therefore, they were retained as synapomorphies (Farris, 1989b). Of these, 63 had RI = 1.000, 38 had RI ≥0.750 and ≤0.957, 107 had RI ≥0.556 and ≤0.743, 98 had RI ≥0.364 and ≤0.545, and 39 had RI ≥0.143 and ≤0.348. Thus, more than half of the synapomorphies that united the groups in Figure 1 were potentially homoplastic. According to Kitching et al. (1998), groups that are corroborated by small percentages of informative characters tend to exhibit low bootstrap support because they are less frequently recovered during resamplings, especially if they are united by homoplastic characters.

We observed 17 nodes with bootstrap support <50% in the MPC and 20 in the NJ tree. In the MPC the nodes with support <50 and ≥50% were corroborated on average by 4 and 18 characters, respectively. When the distribution of characters was plotted on the NJ tree, the former value changed to 7 characters. We also found that the differences in the arithmetic means of the number of characters associated with nodes with <50 and ≥50% support did not differ significantly between the MPC and the NJ tree (Table 4). Albeit using different premises, both analyses exhibited similar trends in nodal support, since in both trees, the nodes with support of ≥50% tended to be away from the tree spine and close to the terminals. Similar trends were noticed in other 18S phylogenies of unicellular eukaryotic organisms (e.g., Deane et al., 2002; Cavalier-Smith and Chao, 2003) and of land plants (Soltis et al., 1999).

Table 4. Statistics of the number of character transformations associated with nodes with bootstrap (bs) <50% ≥50% in maximum parsimony (MP) and neighbor-joining (NJ) analyses.

Groups*	Mean	M	SD	Min	Max	N	Groups	MP bs <50	MP bs ≥50	NJ bs <50
MP bs <50	4.29	4	3.24	1	13	17	MP bs <50			
MP bs ≥50	18.35	15	17.93	1	87	51	MP bs ≥50	U = 131.5		
								P < 0.0001		
NJ bs <50	7.15	5	7.68	2	32	20	NJ bs <50	U = 125.0	U = 244.0	
								P = 0.1742	P = 0.0007	
NJ bs ≥50	17.77	14	17.64	1	83	48	NJ bs ≥50	U = 135.0	U = 1177.0	U = 245.0
								P < 0.0001	P = 0.7447	P = 0.0016

Mean = arithmetic mean; M = median; SD = standard deviation; Max = maximum number of character transformations; Min = minimum number of character transformations; N = number of nodes; U = Mann-Whitney test statistics. Values for rejection of null hypothesis are marked in gray. $\alpha = 0.01$. *The node corresponding to the monophyly of the outgroup was not considered.

The CEUU hypothesis and the polyphyly of urostylids

After molecular phylogenetic studies suggested that the traditional urostylids (Borror, 1979; Lynn and Small, 2002; Lynn, 2003) were not a monophyletic assemblage because of the position of *Uroleptus* species (Croft et al., 2003; Hewitt et al., 2003), Foissner et al. (2004) conceived the CEUU hypothesis. It was proposed that *Uroleptus* is more closely related to the typical oxytrichids than to urostylids, and that its midventral complex had secondarily evolved (Foissner et al., 2004; Berger, 2006). The original CEUU was then modified after the discovery of *Rigidothrix goiseri* by Foissner and Stoeck (2006), a peculiar stichotrich with an uroleptid ciliature coexisting with typical stylonychine features (viz. a conspicuously rigid body, dorsal dikinetids encaged in fusiform structures, wide triangular peristome, and absence of cortical granules); but according to molecular phylogeny they belong to a lineage of typical flexible-body oxytrichids.

More recently, the discovery by Foissner and Stoeck (2008) of yet another stichotrich with phylogenetic affinities to the cyrtohymenids, *Neokeronopsis* (*Afrokeronopsis*) *aurea* (not included in our study), but possessing morphologic traits similar to *Pattersoniella vitiphila* (a stylonychine that has a midventral complex), expanded the complexity of the morphological foundation necessary to explain the CEUU, which was already considered weak when first proposed (Foissner et al., 2004). Accordingly, Foissner and Stoeck (2008) proposed that the midventral complex evolved independently in at least four lineages, viz. urostilids, uroleptids, rigidothrichids, and neokeronopsids (which includes *Pattersoniella*). Moreover, as aforementioned, other phylogenetic analyses decomposed the non-uroleptid urostylids into a polyphyletic group (Schmidt et al., 2007; Yi et al., 2008b).

If the presence/absence of midventral complex is traced on our optimal trees (not shown), then its independent rise in *Uroleptus*, *Rigidothrix*, *Pattersoniella*, and at least the core urostylid lineages is an acceptable hypothesis to explain its distribution in terminal taxons. However, the statistical tests that we used to compare the optimal trees with trees that were built with topological constraints failed to reject the null hypothesis in scenarios I-III (Table 3), meaning that the 18S data alone do not yield sufficient resolution to generate a phylogenetic pattern robust enough to unambiguously support the construction of hypotheses

on the evolution of *Rigidothrix* and *Uroleptus*, as well as their relation to other urostylids. Nevertheless, not only the report kinships of *Uroleptus* but also those of other "rogue" stichotrich groups vary in different studies, according to which gene data and reconstruction method are used, resulting sometimes in little resolved consensus trees (e.g., Snoeyenbos-West et al., 2002; Hewitt et al., 2003; Dalby and Prescott, 2004; Schmidt et al., 2007).

The CEUU hypothesis also finds support in a multi-gene tree from Hewitt et al. (2003, p. 264), which is roughly similar to the tree in Berger (2006, p. 33), from where uroleptids branched immediately after the urostylids. A tree in Foissner and Stoeck (2008, p. 31) shows a similar, but improved scenario with the addition of the rigidothrichids and neokeronopsids.

If the presence/absence of the midventral complex is traced over this tree, then the trend that minimizes the number of steps required to explain the distribution of this character on terminals is the presence of the midventral complex as a plesiomorphy (Figure 4), instead of multiple convergent evolution. Therefore, its absence in the remaining taxons (of the Dorsomarginalia Berger, 2006) was supposedly due to decreases in the number of ventral cirral primordia and/or increases in the number of cirri generated by each primordium.

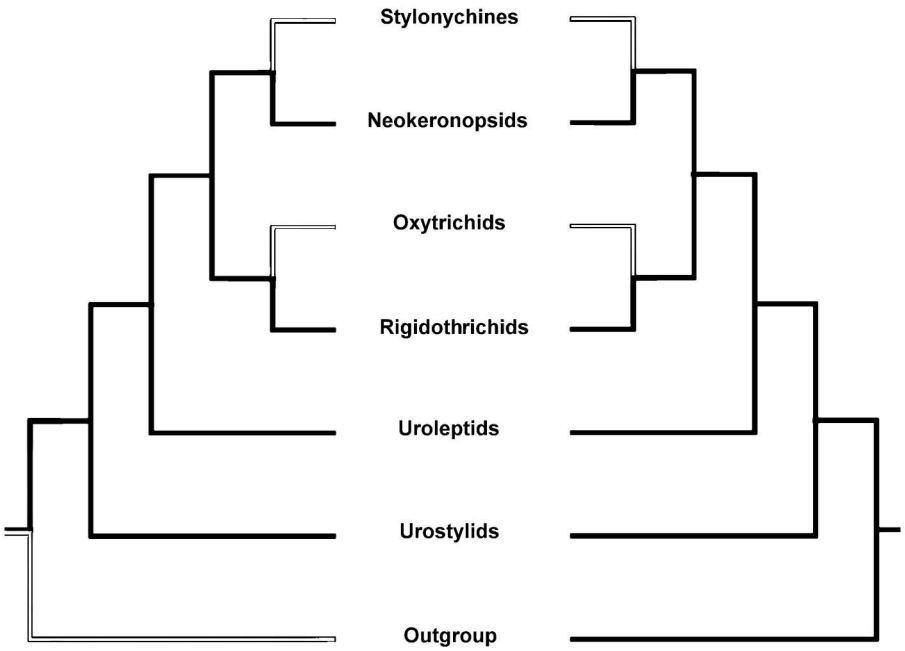

Figure 4. Trees modified from Foissner and Stoeck (2008, p. 31) showing changes of the character "presence of midventral complex" (absent = white lines; present = black lines), polarized with different outgroups: without midventral complex in the left tree (3 steps), and with midventral complex (2 steps) in the right tree. The presence of the complex in neokeronopsids may, however, be homoplastic. See "Results and Discussion".

The midventral complex occurs in some hypotrich taxons; hence, this structure could be a trait reminiscent of an ancestor with ventral architecture similar to that of a hypotrich with a midventral complex, like a gastrocirrhid or even a discocephalid in which the cirri from

the midventral pairs were not partially reabsorbed during late morphogenesis (see Wicklow, 1982), thus maintaining their pairwise configuration. This apparently raises the possibility that contrary to what is suggested by Foissner and Stoeck (2008), the same trend of maintaining the plesiomorphic midventral complex might have occurred in the neokeronopsids (Figure 4). However, *Pattersoniella* is unlikely to be sufficiently closely related to urostylids or *Rigidothrix* and *Uroleptus* (Figures 1, 2), and the statistical tests rejected the null hypothesis in scenarios IV and VIII (Table 3). So, considering the position of *Pattersoniella* in our optimal trees (Figures 1, 2), the possibility of the midventral complex in the neokeronopsids actually being a homoplasy (Foissner and Stoeck, 2008) instead of plesiomorphic is accepted in the current state of knowledge.

Finally, the tests involving trees where the global monophyly of the urostylids was enforced allowed us to reject the null hypothesis, with the exception of the SH test in scenario V; however, with a P value very close to the critical level (Table 3). These results corroborate the hypotheses that: i) *Pseudoamphisiella* is more closely related to discocephalids than to the core urostylids (Yi et al., 2008b); ii) *Parabirojimia* and *Trachelostyla* may be sister groups (Yi et al., 2008a); iii) *Holosticha* branches at the base of core Stichotrichia (Schmidt et al., 2007; Yi, et al., 2008a), and iv) discocephalids may be an intermediate taxon between urostylids and euplotids (Wicklow, 1982).

The polyphyly of *Oxytricha*

The large genus *Oxytricha* currently contains more than 50 valid species. According to Berger (1999), identification of *Oxytricha* species is a difficult task, due to their overall morphological similarity. Several species originally assigned to *Oxytricha* were relocated to other genera after detailed studies of morphology were performed (for reviews see Berger, 1999, 2001). Currently, about three fourths of the valid congeners lack investigation with modern microscopy techniques, so it is possible that the present circumscription of *Oxytricha* is artificial (Berger, 1999). Our tests rejected the null hypothesis for scenario IX, thus the monophyly of *Oxytricha* based on 18S data alone is unlikely (Table 3). The phylogenetic patterns obtained in both trees are compatible with that reported by Schmidt et al. (2007), except for *Oxytricha saltans*, which grouped with *Gonostomum* in their study.

Remarkably, *Oxytricha saltans* was the most distant congener (Table 5). This species has a peculiar oral apparatus bearing a flexible spur-like protrusion (Song and Wilbert, 1997) and its adoral zone of membranelles is slightly bipartite, with the crown membranelles pointy and longer than usual in this genus. Additionally, based on the morphology reported in Song and Wilbert (1997), it is possible to interpret the shape of the anterior region of the cell as that of a shortened trachelostylid frontal scutum. Furthermore, Berger (1999) emphasized that the cirral pattern in this species is not like that in typical oxytrichids. Even though *Oxytricha saltans* has at least one dorsomarginal kinety (Song et al., 1991; Song and Wilbert, 1997; Berger, 1999), a trait absent in urostylids (Berger, 2006), its position as sister group of *Holosticha* was strongly supported in our trees and the branching pattern at the base of the core Stichotrichia corroborated a report by Shin (2005). Thus, the results indicate that the reactivation of the old basionym *Actinotricha saltans* is suitable, but checking morphogenetic data is strongly recommended prior to adopting this convention.

Table 5. Pairwise distance matrix of *Oxytricha* 18S-rDNA sequences from our data set (in %).

		1	2	3	4	5	6	7	8	9	10
1	*Oxytricha granulifera* 1										
2	*Oxytricha granulifera* 2	2.6									
3	*Oxytricha granulifera* 3	0.8	2.5								
4	*Oxytricha granulifera* 4	0.2	2.8	1.0							
5	*Oxytricha granulifera* 5	0.0	2.6	0.8	0.2						
6	*Oxytricha longigranulosa*	3.8	0.0	2.5	2.8	2.6					
7	*Oxytricha elegans*	2.8	1.6	3.2	3.0	2.8	1.6				
8	*Oxytricha lanceolata*	3.3	1.9	3.3	3.5	3.3	1.9	2.3			
9	*Oxytricha ferruginea*	3.3	4.0	3.6	3.6	3.3	4.0	3.7	3.5		
10	*Oxytricha longa*	3.8	3.0	4.0	4.1	3.8	3.0	3.3	2.8	3.3	
11	*Oxytricha saltans*	9.6	10.2	10.1	9.9	9.6	10.2	9.4	10.4	9.8	10.3

Moreover, the results also corroborated the hypothesis of Eigner (1997) that the 18 fronto-ventral-transverse cirri pattern of oxytrichids evolved more than once. Thus, future analyses of additional sequences from other *Oxytricha* species and supposedly related organisms (e.g., *Notohymena*, *Tachysoma*, *Urosoma*, *Urosomoida*) are expected to improve the knowledge concerning the evolution of this group.

The *Halteria* paradox

One of the most controversial issues in molecular phylogeny of ciliates was the position of *Halteria grandinella*, traditionally classified as an oligotrich by morphologists (e.g., Kahl, 1932; Corliss, 1979; de Puytorac et al., 1994; Lynn and Small, 2002; Lynn, 2003), but placed alongside flexible body oxytrichids (viz. *Oxytricha granulifera*) in ribosomal molecular trees (Lynn and Sogin, 1988; Hoffman and Prescott, 1997; Bernhard et al., 2001; Snoeyenbos-West et al., 2002; Hewitt et al., 2003; Strüder-Kypke and Lynn, 2003; Foissner et al., 2004; Schmidt et al., 2007). The same case applies to the related organism *Meseres corlissi* (Foissner et al., 2004). Nevertheless, an α-tubulin based tree in Snoeyenbos-West et al. (2002) has placed *Halteria* among choreotrichid oligotrichs.

The exact phylogenetic position of *Halteria grandinella* within stichorichs varied slightly in our trees according to inference criteria and had weak bootstrap support in both analyses (Figures 1, 2). With the exception of the WS, all tests failed to reject the null hypotheses for scenario X (Table 3); hence the resolution of 18S alone was considered insufficient to unambiguously define the position of this organism. Indeed, according to Agatha (2004), the placement of *Halteria* as a sister group of *Oxytricha granulifera* would require that several probably complex morphological features (e.g., enantiotropic divisional morphogenesis) evolved in convergence with the Oligotrichia. In our constrained trees (not shown), *Halteria grandinella* always branched from the base of Oligotrichia, agreeing with phylogenetic inferences based on morphology (de Puytorac et al., 1984, 1994; Petz and Foissner, 1992; Agatha, 2004; Agatha and Strüder-Kypke, 2007; Foissner et al., 2007).

Monophyletic Stichotrichia?

The null hypothesis for scenario XI was rejected by all tests except for SH; there-

fore, the results do not support the monophyly of Stichotrichia *sensu* Berger (2006). Since the two optimal trees were not consensual concerning the phylogenetic pattern at the base of the ingroup, we compared them with two other scenarios (Table 3), independently reinforcing the inclusion of *Holosticha* spp + *Oxytricha saltans* and *Pseudoamphisiella* spp within the core Stichotrichia.

Both scenarios XII and XIII do not differ significantly from the optimal NJ tree. However, both the Tempelton and WS tests rejected the null hypothesis in scenario XIII (= scenario XII due to the position of *Holosticha* spp and *Oxytricha saltans* in the MPC). Thus, in the parsimony framework, the inclusion of *Pseudoamphisiella* spp in the core Stichotrichia made the constrained tree significantly longer than the optimal one (Table 3).

Although the two constraints are different, the P values from the SH and WSH tests for scenario XII indicate that this constrained topology is less different from the optimal NJ tree than the one from scenario XIII. Hence, the monophyly of Stichotrichia is acceptable in the parsimony analysis context when *Pseudoamphisiella* is considered as an external taxon. Actually, the possibility of *Pseudoamphisiella* being more close to discocephalids than holostichid urostylids is sustained by the morphological similarity between *Pseudoamphisiella* and *Psammocephalus faurei* in traits such as the cephalized frontal scutum and the overall developmental processes of the ciliature pattern (Wicklow, 1982; Song et al., 1997).

FINAL CONSIDERATIONS

Inconsistency among phylogenetic inferences may occur due to various causes, including differences in properties of the reconstruction methods employed (Hillis, 1987). The quality of taxon and character sampling are relevant factors that interfere in phylogenetic inferences (Bergsten, 2005). According to Berger in Foissner and Stoeck (2008), there are approximately 600 valid stichotrich species, corresponding to about 9.7 times the total of stichotrich 18S-rDNA sequences that we used in our study, albeit more than 50% of the estimated number of extant ciliates species are still unknown to science (Foissner et al., 2002). Thus, according to Berger (2006), less than 5% of the stichotrich species that probably exist have been examined in molecular phylogenies to date! Therefore, an effort to augment taxon sampling is necessary to increase the accuracy of phylogenetic estimates (Hillis, 1996, 1998; Graybeal, 1998; Ranalla et al., 1998; Bergsten, 2005). Concerning stichotrichs, this should focus on taxons that have been little sampled, such as amphisiellids, flexible-body oxytrichids, kahliellids, and spirofilids. As demonstrated by Hillis (1998), an increase in the number of characters used in phylogenetic inferences can also increase their accuracy, reaching a point where all branches in a tree are correctly estimated with 5000 nucleotides, which is about 3 times the number of characters that we used in our study.

We conclude that within the context of the data analyzed and the methodology adopted, the phylogeny of 18S-rDNA alone did not yield enough resolution to reliably estimate many of the branching patterns within stichotrichs; thus, proposal of hypotheses on evolution based on these topologies alone should be extremely cautious. In fact, some internal groups were consistently supported by the data, such as the Stylonychinae and the core urostylids, thus indicating that the 18S holds potential for resolving stichotrich systematics at low taxonomic levels. Soltis et al. (1999) reached a similar conclusion studying land plants, and attributed the low resolution at higher taxonomic levels to conflicting signals at the 18S variable sites and

insufficient signals at the conserved ones. Noteworthy, the use of a single gene in phylogeny estimates may also be problematic because if its evolution differs from that of species, the trees can yield well-supported, but inaccurate topologies concerning species evolution (Doyle, 1992). Therefore, as pointed out by Bergsten (2005), the addition of different kinds of data, preferably from genes with different proprieties or evolution rates is necessary to overcome single-gene biases. The study by Snoeyenbos-West et al. (2002) demonstrated that internal transcribed spacer (ITS), and especially α-tubulin matrices, possess higher nucleotide divergence than 18S among choreotrichs, oligotrichs and stichotrichis. Hence, new studies with these markers and that include significantly larger and more homogeneous taxon sampling are expected to better address the molecular systematics of Stichotrichia.

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