J. Eukaryot. Microbiol., 54(2), 2007 pp. 201–209

DOI: 10.1111/j.1550-7408.2007.00250.x

Phylogeny of the Stichotrichia (Ciliophora; Spirotrichea) Reconstructed with Nuclear Small Subunit rRNA Gene Sequences: Discrepancies and Accordances with Morphological Data

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ABSTRACT. The Stichotrichia, known as an especially various and taxonomically difficult group, were intensely studied with morphological, morphogenetic, and molecular methods in the last years. Nevertheless, a consistent classification is lacking and several important questions about the phylogenetic relationships within this group remain unsolved. In order to gain deeper insights into these relationships, the nuclear small subunit rRNA genes of seven species of the Stichotrichia, representatives of the families Oxytrichidae, Amphisiellidae, and Pseudourostylidae, were phylogenetically analysed. Although our analyses resulted in a poor resolution of the phylogenetic relationships, some conclusions can be drawn. Firstly, following the current classification systems the Oxytrichidae as well as their subfamilies seem to be paraphyletic and the basic 18 FVT cirral pattern has been modified several times independently. Secondly, sequence analyses of several *Oxytricha* species resulted in a high molecular diversity, which does not support monophyly of this genus. Thirdly, several families of the order Urostylida (Urostylidae, Pseudokeronopsidae, and Pseudourostylidae) also do not form monophyletic groups.

Key Words. Evolution, *Oxytricha*, Oxytrichidae, phylogeny, small subunit rDNA, Stichotrichia, Urostylida.

THE Spirotrichea represents one of the most diverse classes of the Ciliophora Doflein (1901) and has been the subject of many revisions (e.g. Corliss 1979; Levine et al. 1980; Lynn and Corliss 1991; Lynn 1996, 2003a; Lynn and Small 2002; Small and Lynn 1981, 1985). Currently, the Spirotrichea are divided into seven subclasses (sensu Adl et al. 2005; Lynn 2003a). However, their phylogenetic relationships are poorly understood. Moreover, Lynn (2003a) stated that the Spirotrichea are weakly supported, although in sequence analyses of the small subunit rDNA (SSU rDNA) (e.g. Bernhard et al. 2001; Shin et al. 2000) the class Spirotrichea appeared monophyletic.

Different studies focussed on kinships within several subgroups of the Spirotrichea (e.g. Croft et al. 2003; Hewitt et al. 2003; Snoeyenbos-West et al. 2002; Strüder-Kypke and Lynn 2003). For example, Snoeyenbos-West et al. (2002) used multi-gene sequence analyses to resolve the genealogy of choreotrich and oligotrich ciliates. Bernhard et al. (2001) and Hewitt et al. (2003) investigated rRNA gene evolution and phylogeny of the Stichotrichia and Hypotrichia.

The Stichotrichia sensu Small and Lynn (1985) appears to be an especially diverse and taxonomically difficult group (Adl et al. 2005; Chen and Song 2002; Foissner et al. 2004; Lynn 2003a) of which 13 families were described (Lynn and Small 2002; Lynn 2003b). Previous molecular studies focussed on the Oxytrichidae and Urostylidae (Bernhard et al. 2001; Croft et al. 2003; Foissner et al. 2004; Hewitt et al. 2003). Particularly for the Oxytrichidae, SSU rRNA gene sequences of at least 20 species are available in the GenBank database. Berger (1999) included 169 valid species in this family, which are characterized by the typical fronto-ventral-transverse (FVT) cirral pattern that usually comprises 18 cirri (Fig. 1A). Furthermore, the Oxytrichidae have been divided into two subfamilies, the ''flexible'' Oxytrichinae and the ''rigid'' Stylonychinae (Berger 1999; Berger and Foissner 1997). This subdivision was confirmed by sequence analyses of the SSU rRNA gene, with strong support for the Stylonychinae and weaker support for the Oxytrichinae (Bernhard et al. 2001; Hewitt et al. 2003). The genus *Oxytricha*, eponymous for this systematic group, represents the largest taxon within the oxytrichids sensu Berger (1999). At the moment, about 50 valid species are known for this genus (Berger 1999), but unambiguous species identification is difficult. Therefore, sequence data seem to be necessary to confirm morphological species affiliation, as has been demonstrated for other groups within the Ciliophora (e.g. Petroni et al. 2003; Schmidt et al. 2006).

Berger (1999) suggested that the genus *Oxytricha* is not monophyletic. Molecular data provided support for this assumption (Bernhard et al. 2001), and several former *Oxytricha* species were transferred into other genera (e.g. *Oxytricha nova* to *Sterkiella nova*; *Oxytricha trifallax* to *Sterkiella histriomuscorum*; Foissner and Berger 1999).

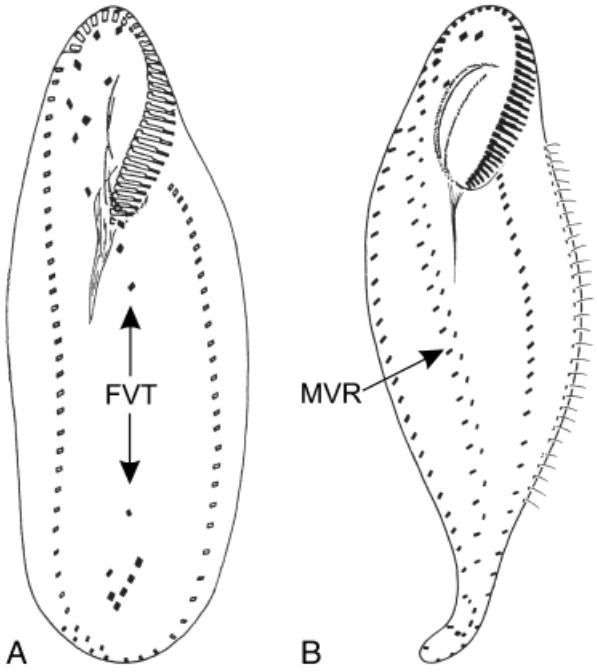

Fig. **1.** Schematic drawing of the oxytrichid *Oxytricha granulifera* (**A**) and the urostylid *Uroleptus lepisma* (**B**) Like typical oxytrichids, *O. granulifera* has the 18 fronto-ventral-transverse (FVT) cirral pattern, while the urostylids possess the so-called midventral row (MVR) composed of zigzagging cirri. Drawings are based on Foissner and Adam (1983) and Berger and Foissner (1989).

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Although molecular data, such as SSU rRNA gene sequences, have contributed significantly to the reconstruction of phylogenetic relationships within the Stichotrichia, important questions still remain. For examples, the weak support for the monophyly of the subfamily Oxytrichinae and the phylogenetic assessment of the species summarized under the genus *Oxytricha* are unresolved. Therefore, we sequenced the SSU rRNA genes of *Oxytricha elegans*, *Oxytricha lanceolata*, *Oxytricha longigranulosa*, and five isolates from the type species *Oxytricha granulifera* (Foissner 1989). Moreover, we analysed the SSU rRNA gene of *Amphisiella magnigranulosa*, *Onychodromopsis flexilis*, and *Pseudourostyla franzi*, to gain further insights into the phylogenetic relationships within the Stichotrichia.

MATERIALS AND METHODS

Sources of species investigated. The origin of strains and source of DNA were as follows: (1) *O. longigranulosa*—originally isolated and identified by W. Foissner; genomic DNA provided by M. Schlegel; (2) *O. elegans*—isolated from soil from the margin of a Mangrove forest in the Dominican Republic and identified by W. Foissner; (3) *O. lanceolata*—isolated from soil of Namibia (cf. Foissner, Agatha, and Berger 2002) and identified by W. Foissner; (4) *O. granulifera* (2)—isolated from the Neuhaus pond in the surroundings of Salzburg City, Austria and identified by W. Foissner; (5) *O. granulifera* (3)—isolated from a pond in the village of Michelbeuern, Salzburg, about 50 km away from the Neuhaus pond mentioned above and identified by W. Foissner; (6) *O. granulifera* (4)—isolated from slightly saline soil from the north coast of Venezuela, Morrocoy National Park, surroundings of the village of Chichiriviche and identified by W. Foissner; (7/8) *O. granulifera* (SK2; SK3)—subclonal cultures of *O. granulifera* from soil in Baumgarten, Austria (type locality; Foissner and Adam 1983) and originally identified by W. Foissner; genomic DNA provided by M. Schlegel; (9) *A. magnigranulosa*—isolated from dune soil in the surroundings of Nijmegen, the Netherlands, and identified by W. Foissner; (10) *O. flexilis*—isolated from Neuhaus pond in the surroundings of Salzburg City, Austria, and identified by W. Foissner; and (11) *P. franzi*—isolated from a pond at foot of Pöstlingberg, Linz, Upper Austria, and identified by W. Foissner. Family assignment of these species following Lynn (2003b) is shown in Table 1.

DNA isolation, amplification of the Small Subunit rRNA gene and sequencing. DNA from fixed cells (in 80% EtOH) was isolated using a modified Kavenoff and Zimm procedure (e.g. Steinbrück and Schlegel 1983).

The nuclear SSU rDNA was amplified with universal Eukarya-specific primers (Elwood, Olsen, and Sogin 1985) following a PCR protocol published previously (e.g. Schmidt et al. 2006). The PCR products were purified using the Rapid PCR Purification System® of Marligen Biosciences Inc. (Biocat, Heidelberg, Germany) and sequenced directly. Sequencing reactions were performed for both DNA strands using the primers of Elwood et al. (1985) on an ABI PRISM® 3100 Genetic Analyzer.

Phylogenetic analyses and sequence availability. Additional sequence data used for the analyses were taken from GenBank and are listed in Table 1.

Species identification of stichotrichous ciliates is not easy and several of the SSU rDNA sequences deposited at GenBank are potentially from misidentified taxa (see Foissner et al. 2004). Therefore, we did not include all available SSU rDNA sequences of stichotrichous species in our analyses and reduced the data set to those sequences from unambiguously identified species according to Foissner et al. (2004).

Nevertheless, the dataset analysed comprises 55 SSU rRNA gene sequences of stichotrichous ciliates and five SSU rRNA gene sequences from representatives of the subclasses Choreotrichia and Oligotrichia (see Table 1) as outgroups. Alignments were carried out with CLUSTAL × 1.83 (Thompson et al. 1997) with default parameters. Primer sequences were removed from the alignment before phylogenetic analyses using BioEdit (Hall 1999).

Maximum-likelihood (ML) and maximum-parsimony (MP) analyses were performed with PAUP* v4.0b10 (Swofford 1998) with 100 replications. The evolutionary model of Tamura and Nei (1993) with $I = 0.4767$ and $\gamma = 0.4027$, selected by Modeltest 3.6 (Posada and Crandall 1998), was used for ML analysis. Bayesian analysis was conducted with MrBayes v3.1 (Huelsenbeck and Ronquist 2001), using the same model of substitution and parameters, 1,000,000 generations, and an initial burn in of 2,500. Neighbor-joining (NJ) analysis was performed with the program package MEGA 3.1 (Kumar, Tamura, and Nei 2004) also using the TrN model of substitution (Tamura and Nei 1993) with 1,000 replication steps.

Nucleotide sequences. All new SSU rDNA sequences are deposited at GenBank database. Accession numbers are listed in Table 1.

RESULTS

The phylogenetic analyses of the present study included 11 new SSU rRNA gene sequences of seven stichotrichous species: *A. magnigranulosa*, *O. flexilis* (Salzburg), *P. franzi*, *O. elegans*, *O. granulifera*, *O. lanceolata*, and *O. longigranulosa*. Moreover, the species *O. granulifera* is represented by sequences from five new isolates.

Altogether, the analysed dataset comprises 60 SSU rDNA sequences, in which the genus *Oxytricha* is represented by a total of 13 sequences belonging to seven species. Conspicuously, these species are spread over nearly all stichotrichous subgroups in the phylogenetic analyses (Fig. 2, 3).

Monophyly of the Stichotrichia? Within this study, the subclass Stichotrichia is represented by eight out of 13 families (after Lynn and Small 2002; Lynn 2003b). Only Bayesian and ML analyses recovered the Stichotrichia as a monophyletic group (Fig. 2, 3). Maximum-parsimony analysis resulted in a basal polytomie including all representatives of the Oligotrichia and the Stichotrichia, whereas in NJ analysis the stichotrich *Diaxonella trimarginata* branched off first, followed by a cluster of both *Strombidium* species (Oligotrichia) and a polytomy, including all remaining stichotrichous species. Overall, it is difficult to relate the subgroups occurring in the dendrograms clearly to single families (Fig. 2, 3). Furthermore, some of these subgroups were only supported from Bayesian analysis (Fig. 2), whereas the other analysis methods yielded less resolution (Fig. 3). Nevertheless, our analyses provide some relevant information about the phylogenetic relationships within the Stichotrichia.

No molecular support for the monophyly of the family Oxytrichidae. The Oxytrichidae did not occur as a monophyletic group in any of the phylogenetic analyses performed (Fig. 2, 3). Also, the subfamily Oxytrichinae was never found as a monophylum, whereas the subfamily Stylonychinae seemed to be monophyletic, except that *Gastrostyla steinii*, assigned to the Amphisiellidae, and *Pattersoniella vitiphila* (unclassified; see also Table 1) branched off within this group. All analyses revealed a group, containing all representatives of the Stylonychinae, some species belonging to the Oxytrichinae (e.g. *Cyrtohymena citrina*), *G. steinii* (Amphisiellidae), *Plagiotoma lumbrici* (Plagiotomidae), and *Paraurostyla weissei* (Kahliellidae) (Fig. 2, 3).

Within the Stylonychinae, the cluster containing *Stylonychia mytilus*, *Stylonychia lemnae*, and *Laurentiella strenua* branched off first. The remaining species of the Stylonychinae formed a

Table **1.** Classification of the species included in the present study following Lynn (2003b).

Subclass	Family	Species	Accession number
Choreotrichia	Codonellidae	*Tintinnopsis fimbriata*	AY143560
		Tintinnopsis dadayi	AY143562
Oligotrichia	Strombidiidae	*Strombidium purpureum*	U97112
		Strombidium inclinatum	AJ488911
	Halteriidae[a]	*Halteria grandinella*	AF194410
Stichotrichia	Plagiotomidae	*Plagiotoma lumbrici*	AY547545
	Amphisiellidae	***Amphisiella magnigranulosa***	**AM412774**
		Gastrostyla steinii[b]	AF508758
		Orthoamphisiella breviseris	AY498654
	Kahliellidae	*Engelmanniella mobilis*	AF508757
		Paraurostyla weissei[b]	AJ310485
	Oxytrichidae	*Cyrtohymena citrina*	AF508755
		Cyrtohymena citrina	AY498653
		Laurentiella strenua	AJ310487
		Onychodromopsis flexilis (Antarctic population)	AY498652
		***Onychodromopsis flexilis* (Salzburg population)**	**AM412764**
		Onychodromus grandis	AJ310486
		Oxytricha elegans	**AM412767**
		Oxytricha ferruginea (= *Cyrtohymena ferruginea*)	AF370027
		Oxytricha granulifera S	X53486
		Oxytricha granulifera H	AF508762
		***Oxytricha granulifera* 2**	**AM412770**
		***Oxytricha granulifera* 3**	**AM412771**
		***Oxytricha granulifera* 4**	**AM412772**
		***Oxytricha granulifera* SK2**	**AM412768**
		***Oxytricha granulifera* SK3**	**AM412769**
		Oxytricha lanceolata	**AM412773**
		Oxytricha longa	AF508763
		Oxytricha longigranulosa	**AM412766**
		Oxytricha saltans	AF370028
		Pleurotricha lanceolata	AF508768
		Steinia sphagnicola	AJ310494
		Sterkiella histriomuscorum	AF508770
		Sterkiella nova	AF508771
		Stylonychia lemnae CHI	AJ310496
		Stylonychia mytilus EK11	AJ310499
		Styxophrya quadricornutus (= *Onychodromus quadricornutus*)	X53485
		Tetmemena pustulata	X03947
		Tetmemena pustulata	AF508775
	Trachelostylidae	*Gonostomum namibiense*[b]	AY498655
		Gonostomum strenuum[b]	AJ310493
		Trachelostyla pediculiformis	DQ057346
	Pseudokeronopsidae	*Pseudokeronopsis carnea*[d]	AY881633
		Pseudokeronopsis flava	AY881634
		Pseudokeronopsis qingdaoensis	DQ359728
		Pseudokeronopsis rubra	DQ640314
	Pseudourostylidae	*Pseudourostyla cristata*	DQ019318
		Pseudourostyla franzi	**AM412765**
	Urostylidae	*Holosticha diademata*	DQ059583
		Holosticha heterofoissneri	DQ059582
		Holosticha manca	DQ503578
		Holosticha multistilata	AJ277876
		Holosticha warreni	DQ059582
		Uroleptus gallina[b]	AF508779
		Uroleptus lepisma[b,d]	AF508765
		Uroleptus piscis[b,d]	AF508780
		Urostyla grandis	AF508781
Unclassified species		*Diaxonella trimarginata*[c]	DQ190950
		Hemiurosoma terricola[b]	AY498651
		Pattersoniella vitiphila[b]	AJ310495

The newly investigated species or isolates are marked in bold.
[a]Classification controversial, for details see Foissner et al. (2004).
[b]Representatives of the Oxytrichidae following Berger and Foissner (1997, 1999) and Foissner et al. (2004).
[c]Representative of the Urostylida following Berger (2006).
[d]The spelling of these names differs from those published at GenBank.

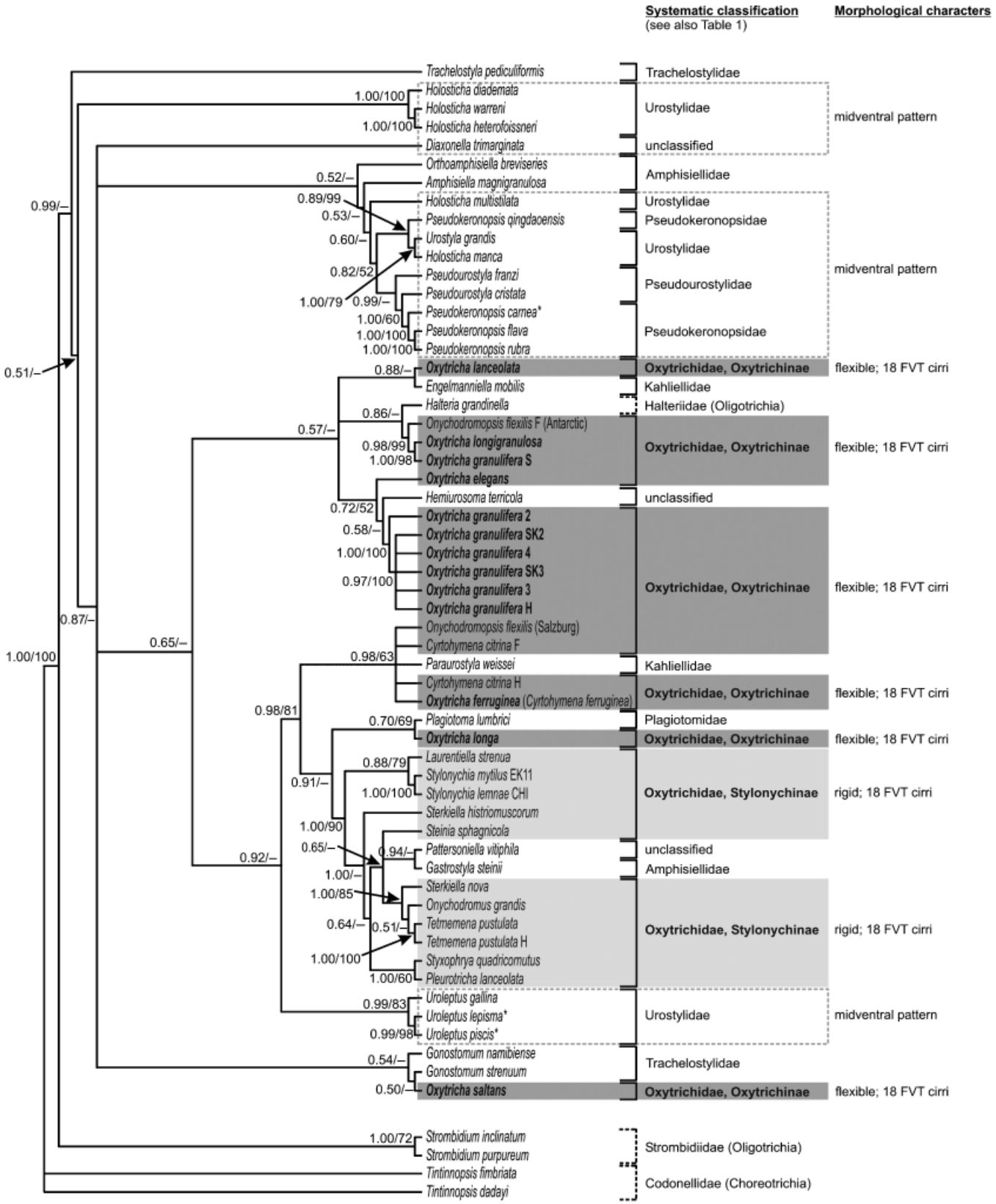

Fig. **2.** Phylogenetic analyses of the Stichotrichia inferred from the nuclear small subunit (SSU) rRNA gene sequences based on Bayesian analysis. Representatives of the subclasses Choreotrichia and Oligotrichia served as outgroups. The numbers at the nodes represent the posterior probabilities of Bayesian analysis (first number) and the bootstrap values of Neighbor-joining analysis (second number). All species of the genus *Oxytricha* are in bold. Systematic classification follows Lynn (2003b). The abbreviations S, H, and F identify the species corresponding to their reference: S, Schlegel et al. (1991); H, Hewitt et al. (2003); F, Foissner et al. (2004). Please note that the spelling of the species names (marked with an asterisk) differs from those published at GenBank.

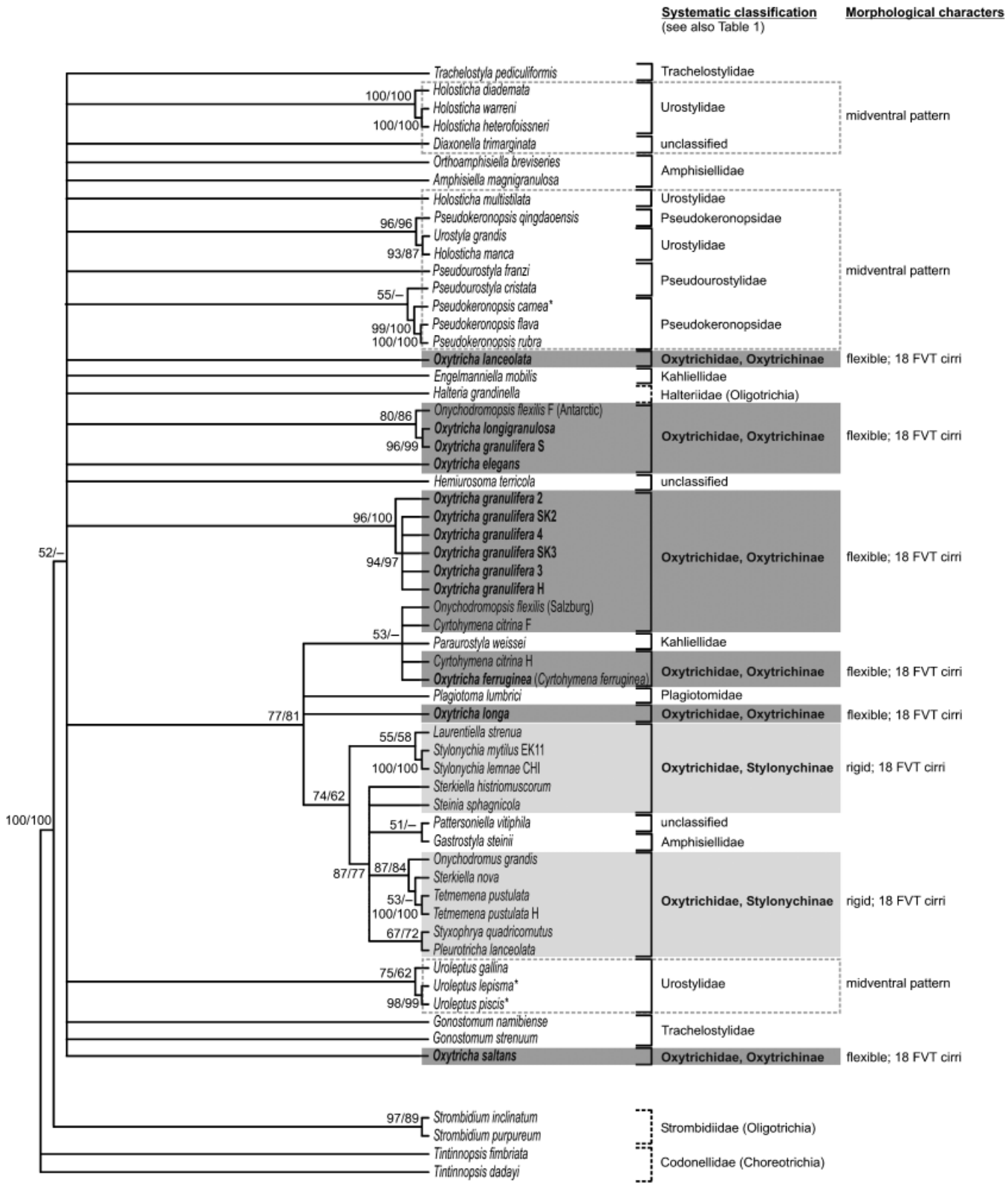

Fig. **3.** Phylogenetic analyses of the Stichotrichia inferred from the nuclear small subunit (SSU) rRNA gene sequences based on Maximum-likelihood (ML) analysis. Representatives of the subclasses Choreotrichia and Oligotrichia served as outgroups. The numbers at the nodes represent the bootstrap values of ML analysis (first number) and of Maximum-parsimony analysis (second number). All species of the genus *Oxytricha* are in bold. Systematic classification follows Lynn (2003b). The abbreviations S, H, and F identify the species corresponding to their reference: S, Schlegel et al. (1991); H, Hewitt et al. (2003); F, Foissner et al. (2004). Please note that the spelling of the species names (marked with an asterisk) differs from those published at GenBank.

common group in Bayesian, ML, and MP analyses, with *Sterkiella histriomuscorum* as the first branch in Bayesian analysis. Maximum-likelihood and MP analyses resulted in a polytomy for this group. The close relationships between *Styxophrya quadricornutus* and *Pleurotricha lanceolata* on the one hand, and *Tetmemena pustulata*, *Sterkiella nova*, and *Onychodromus grandis* on the other hand was indicated by all phylogenetic analyses (Fig. 2, 3).

As already mentioned, some species of the subfamily Oxytrichinae (e. g. *Oxytricha longa* and *C. citrina)*, but also *P. lumbrici*, and *P. weissei* occurred in close relationship to the Stylonychinae, irrespective of the analysis method applied. Among oxytrichines, two subgroups were revealed by Bayesian and NJ analyses (Fig. 2). The first subgroup consisted of *Oxytricha longa* and the endosymbiotic *P. lumbrici* (family Plagiotomidae; sensu Lynn 2003b), whereas the second group was formed by *P. weissei*, *O. flexilis* (Salzburg), *C. citrina*, and *Oxytricha ferruginea* (*Cyrtohymena ferruginea* sensu Foissner 1989). The latter group was also recovered by ML analysis (Fig. 3), while MP analysis resulted in a polytomy.

All other representatives of the subfamily Oxytrichinae were found either as separate branches within the basal polytomy (Fig. 3) or formed the sister group to the afore-described Oxytrichinae/Stylonychinae cluster with the exception of *Oxytricha saltans* (Fig. 2).

Phylogeny of *Oxytricha*. Species assigned to the genus *Oxytricha* appeared at different positions within the dendrograms and never formed a monophyletic group. As already mentioned, the species *O. ferruginea* (*C. ferruginea* sensu Foissner 1989) and *O. longa* were found in close relationship to representatives of the Stylonychinae.

With one exception, all isolates of *O. granulifera* formed a common and well-supported cluster. Within this cluster, *O. granulifera* 2 branched off first. This conspicuous position was caused by a difference of 15 nucleotides in comparison with all other isolates of this cluster, which differed in zero to six nucleotides from each other (data not shown). In contrast and also strongly supported by all phylogenetic analyses, the sequence of *O. granulifera*, published by Schlegel, Elwood, and Sogin (1991), grouped separately with *O. longigranulosa* and *O. flexilis* (Antarctic population; Foissner et al. 2004). The close relationship of *Halteria grandinella* to these species was revealed by Bayesian analysis only (Fig. 2).

Some other groupings were also only found by one or two analysis methods: the group containing *O. elegans*, *Hemiurosoma terricola*, and the *O. granulifera* isolates occurred in Bayesian and NJ analyses (Fig. 2), while the newly investigated species *O. lanceolata* and *Engelmanniella mobilis* (family Kahliellidae; sensu Lynn 2003b) only clustered in the Bayesian analysis.

Finally, the phylogenetic position of *O. saltans* (Chen and Song 2002) could not be resolved unambiguously.

Phylogeny of the families Trachelostylidae (*Trachelostyla, Gonostomum*) and Amphisiellidae (*Amphisiella*, *Orthoamphisiella*). The representatives of the Trachelostylidae (sensu Lynn 2003b), species of the genera *Trachelostyla* and *Gonostomum*, did not form a common cluster in any of our analyses. Instead, these species occurred as separate branches in the basal polytomy (Fig. 3). Only Bayesian analysis showed a relationship between *Gonostomum strenuum* and *Gonostomum namibiense*, but clearly separated from *Trachelostyla pediculiformis*, which formed the first branch within the Stichotrichia (Fig. 2).

Within this study, the family Amphisiellidae (sensu Lynn 2003b) was represented by the species *G. steinii*, *A. magnigranulosa*, and *Orthoamphisiella breviseries*. *Gastrostyla steinii* grouped constantly within the Stylonychinae (family Oxytrichidae), while the positions of *A. magnigranulosa* and *O. breviseries* could not be resolved (Fig. 2, 3).

Phylogeny of the families Pseudokeronopsidae (*Pseudokeronopsis*), Pseudourostylidae (*Pseudourostyla*), and Urostylidae (*Urostyla*, *Holosticha*, *Uroleptus*). Sequence analyses of the present study included 15 SSU rDNA sequences of representatives of the order Urostylida, following the classification of Lynn (2003b). A common cluster containing all 15 species did not occur in any of the analyses. Fragmentation into four groups occurred only in the Bayesian analysis (Fig. 2), while all other analyses resulted in more than four subgroups branching off independently in the polytomy (Fig. 3).

Nonetheless, some subgroups comprised species of the same family. All analyses revealed a highly supported group for *Pseudokeronopsis carnea*, *Pseudokeronopsis flava*, and *Pseudokeronopsis rubra* (family Pseudokeronopsidae; sensu Lynn 2003b), while *Pseudokeronopsis qingdaoensis* always clustered together with *Urostyla grandis*, and *Holosticha manca* (Fig. 2, 3). A connection between both *Pseudourostyla* species (family Pseudourostylidae; Lynn 2003b) was only hinted by Bayesian analysis.

The representatives of the family Urostylidae (genera *Urostyla*, *Holosticha*, and *Uroleptus*; sensu Lynn 2003b) were split up into four clades in our analyses: *Holosticha diademata*, *Holosticha warreni*, and *Holosticha heterofoissneri* grouped consistently together with highest support (Fig. 2, 3), while *H. manca*, always clustered together with *U. grandis*. With the exception of Bayesian analysis, all other analysis methods resulted in a conspicuous separation of *Holosticha multistilata* from all other species of the same genus (Fig. 2, 3). The species *Uroleptus gallina*, *Uroleptus piscis*, and *Uroleptus lepisma* always formed a distinct cluster, whereas the close relationship to the Oxytrichidae was only indicated by Bayesian analysis (Fig. 2).

DISCUSSION

The phylogenetic relationships within the spirotrichous ciliates have been investigated in many studies. However, the results of the sequence analyses (e.g. analyses of the SSU rDNA) differ markedly from the classification systems based on morphology and ontogeny (Bernhard et al. 2001; Foissner et al. 2004; Hewitt et al. 2003; for review see Lynn 2003a).

Oxytrichidae and the genus *Oxytricha*. Our data clearly show that the family Oxytrichidae is a paraphyletic assemblage and endorse the assumption of Berger and Foissner (1997) and Berger (1999) that the 18 FVT cirral pattern (Fig. 1A), which is characteristic for representatives of this family, has been modified several times independently. Bernhard et al. (2001) provided some molecular evidence for the separation of the Oxytrichidae into the ''flexible'' Oxytrichinae and the ''rigid'' Stylonychinae, as was also proposed by morphological and morphogenetic characters (for review see Berger 1999). The monophyly of the Stylonychinae as discussed by Bernhard et al. (2001) was not confirmed, because *G. steinii*, a representative of the Amphisiellidae (sensu Lynn 2003b), grouped consistently within the Stylonychinae. The same situation occurred in the SSU rRNA gene analyses of Hewitt et al. (2003) and Foissner et al. (2004), and in the phylogenetic analyses of actin genes (Croft et al. 2003). Therefore, Foissner et al. (2004) established *Gastrostyla* as a ''stichotrichine oxytrichid''. The common grouping of *G. steinii* and *P. vitiphila* (a representative of the Stylonychinae sensu Berger 1999), revealed by Bayesian and ML analyses, corresponds to morphological data (e.g. an increased number of FVT cirri). This relationship was already discussed in detail by Foissner et al. (2004). In consideration of the assignment of *G. steinii* to the Oxytrichidae (cf. Foissner et al. 2004), the monophyly of the ''rigid'' Stylonychinae might be confirmed by the present analyses.

In accordance with previously published results (e.g. Foissner et al. 2004; Hewitt et al. 2003), representatives of the Oxytrichinae

are never found as a monophyletic group in our analyses. Some species, assigned to this subfamily, constitute a common cluster with representatives of the Stylonychinae (e.g. *Stylonychia*, *Tetmemena*, *Sterkiella*). However, not all relationships are also supported by morphological and morphogenetic data (e.g. *C. citrina* and *P. weissei*; cf. Foissner et al. 2004).

The wide molecular separation of the *O. flexilis* populations (Salzburg and Antartic) is an unexpected result, because specimens of both populations are morphologically very similar, indicating a cryptic speciation, likely due to the wide geographic distance. For this reason, detailed descriptions of both populations and taxonomic consequences will be provided in a separate paper.

Berger (1999) suggested that the representatives of *Oxytricha* probably do not form a monophylum. However, corresponding to morphological and morphogenetic characters, which were described for the genus *Oxytricha*, the dispersion of the species investigated in the present study among different clades was not expected at all. Our results clearly show that the large genus *Oxytricha* does not form a monophyletic group, although not all branches are well supported by the different analyses performed. The misleading inclusion of some species to this genus was already shown in previous studies (e.g. Bernhard et al. 2001; Foissner and Berger 1999).

Oxytricha longa groups close to *P. lumbrici*, albeit the support for this relationship is low (0.70 in Bayesian analysis and 69% in NJ analysis). A close relationship of *P. lumbrici* to the Oxytrichidae, especially with representatives of the Oxytrichinae, was already shown by Affa'a et al. (2004). However, the molecular data contrast with the morphological characters (sensu Lynn and Small 2002): representatives of the genus *Plagiotoma* (family Plagiotomidae) have a ventral cirral pattern of longitudinal rows (Lynn and Small 2002), whereas the Oxytrichidae are characterized by the 18 FVT cirral pattern.

Oxytricha ferruginea (*C. ferruginea* sensu Foissner 1989) is also clearly separated from all other species of the same genus based on SSU rDNA sequences. The close relation to *C. citrina* supports the species affiliation of *O. ferruginea* to the genus *Cyrtohymena* as proposed by Foissner (1989). Indications for the assignment of this species to the genus *Rubrioxytricha* (cf. Berger 1999) could not be found with the sequence data available. Regardless of this problem, the genus *Cyrtohymena* does not form a monophyletic group in our analyses.

The species *O. longigranulosa* and the isolate *O. granulifera* (S) published by Schlegel et al. (1991) group consistently together, whereas all other isolates of *O. granulifera* form a common group, branching off separately. Sequence analyses revealed differences of up to 15 nucleotides between these isolates. Recent studies showed that a single nucleotide difference within the whole SSU rDNA allows the differentiation of species (e.g. species of the *S. mytilus* complex; Schmidt et al. 2006). Referring to *O. granulifera*, these nucleotide differences may also indicate the existence of a species complex. However, the separation of *S. mytilus* and *S. lemnae* was confirmed by morphological data and additional molecular markers (e.g. Ammermann and Schlegel 1983; Haentzsch et al. 2006; Wirnsberger, Foissner, and Adam 1986). Therefore, detailed investigations of different isolates of *O. granulifera* are necessary to clarify this question.

One possible explanation for the separation of *O. granulifera* (S) from all other isolates of the same species might be that *O. longigranulosa* and *O. granulifera* (S) were mixed up by Schlegel et al. (1991). In spite of this, it is indeed unexpected that *O. longigranulosa* and *O. granulifera* appear to be so distantly related, because they show a high morphological similarity and are only distinguishable by the shape and the arrangement of the cortical granules (Berger 1999).

Urostylida. Phylogenetic analyses of SSU rRNA and actin gene sequences resulted in the fragmentation of the order Urostylida (sensu Lynn 2003b) into different clades (Croft et al. 2003; Foissner et al. 2004; Hewitt et al. 2003; present study). The hypothesis of ''convergent evolution of urostylid and uroleptid'' ciliates (CEUU hypothesis), as proposed by Foissner et al. (2004), is in so far supported by the present analyses that ''uroleptid'' and ''urostylid'' ciliates branch off independently in the trees, albeit our data do not confirm monophyly for the ''urostylids''. Our data also reveal the close relationship of the *Uroleptus* spp. to the Oxytrichidae despite their morphological similarity to the Urostylidae (i.e. midventral row composed of zigzagging cirri; Fig. 1B; for details see Foissner et al. 2004).

Since the study of Foissner et al. (2004) was published, at least ten new SSU rDNA sequences from representatives of the Urostylida became available, including now the genera *Pseudokeronopsis* and *Pseudourostyla*. The *Pseudokeronopsis* species, like the species of the genus *Pseudourostyla*, group on separate branches in our analyses. Therefore, both genera and their respective families (Pseudokeronopsidae and Pseudourostylidae sensu Lynn 2003b) are not monophyletic based on SSU rDNA phylogenies.

As already discussed for the Oxytrichidae, the morphological classification of some species is also inconsistent for the Urostylidae in the current literature. Berger (2003) revised the genus *Holosticha*, which he described as ''a melting pot for all urostylids with three distinct frontal cirri, a midventral complex composed of cirral pairs only, transverse cirri, and one left and one right marginal row.'' After revision (Berger 2003), the genus *Holosticha* comprises only seven species. All other species were transferred into other, partly newly established genera (e.g. *Anteholosticha*; Berger 2003). In the present study five species assigned to the ''melting pot'' *Holosticha* were analysed. Whereas *H. diademata* and *H. heterofoissneri* still belong to this genus, *H. manca*, *H. multistilata*, and *H. warreni* are now assigned to the new genus *Anteholosticha* (cf. Berger 2003). Our phylogenetic analyses constantly reveal a highly supported cluster consisting of *H. diademata*, *H. heterofoissneri*, and *H. warreni* (*Anteholosticha warreni* sensu Berger 2003), in which *H. diademata* branches off first. The remaining three representatives of the new genus *Anteholosticha* never group together in our analyses and also in the analysis of Shao et al. (2006). These results indicate that neither *Holosticha* nor *Anteholosticha* (both sensu Berger 2003) form monophyletic groups.

CONCLUSIONS

The present study demonstrates for different systematic subgroups of the Stichotrichia that an increased taxon sampling alone does not improve the resolution of phylogenetic relationships sufficiently. The poor resolution of the relationships in all phylogenetic methods used in the present study suggests either that the systematic hypotheses based on morphological evidence are incorrect or that there is still insufficient phylogenetic information in the molecular data (i.e. the SSU rDNA sequences). That these molecular markers contain at least in part phylogenetic information is supported by the constant recovering of the subfamily Stylonychinae. A promising approach to further analyse phylogenetic relationships of higher taxa is the combination of different gene sequences (Baldauf et al. 2000; Bapteste et al. 2002).

Although the relationships within the Stichotrichia are poorly resolved, some general conclusions can be drawn: (1) the genera *Holosticha* and *Anteholosticha* (sensu Berger 2003) as well as the family Urostylidae are not monophyletic; (2) the families Pseudokeronopsidae and Pseudourostylidae, and their eponymous genera are not monophyletic; (3) the family Oxytrichidae is

paraphyletic and the basic 18 FVT cirral pattern, which is characteristic for representatives of this family, has been modified several times independently; (4) in consideration of the assignment of *G. steinii* to the Oxytrichidae as proposed by Foissner et al. (2004), the rigid Stylonychinae might form a monophyletic group; (5) the monophyly of the subfamily Oxytrichinae is not confirmed; and (6) isolates assigned to the genus *Oxytricha* show a considerable morphological similarity, and this contrasts with the molecular diversity of their SSU rDNA, which do not support monophyly of the genus *Oxytricha*. This genus needs further revision based on combined morphological and molecular investigations.

ACKNOWLEDGMENTS

We would like to thank two anonymous reviewers for constructive comments on the manuscript. This work was supported by the German Research Foundation (DFG) project Schl 229/12-1 and the Austrian Research Foundation (FWF) project P-15017. S. Schmidt was supported by a grant of the German National Academic Foundation.

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Received: 07/10/06, 10/26/06, 01/09/07; accepted: 01/10/07