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Molecular Phylogeny of the Heterotrichea (Ciliophora, Postciliodesmatophora) Based on Small Subunit rRNA Gene Sequences

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ABSTRACT. A comprehensive molecular analysis of the phylogenetic relationships within the Heterotrichea including all described families is still lacking. For this reason, the complete nuclear small subunit (SSU) rDNA was sequenced from further representatives of the Blepharismidae and the Stentoridae. In addition, the SSU rDNA of a new, undescribed species of the genus *Condylostomides* (Condylostomatidae) was sequenced. The detailed phylogenetic analyses revealed a consistent branching pattern: while the terminal branches are generally well resolved, the basal relationships remain unsolved. Moreover, the data allow some conclusions about the macronuclear evolution within the genera *Blepharisma*, *Stentor*, and *Spirostomum* suggesting that a single, compact macronucleus represents the ancestral state.

Key Words. Blepharismidae, Condylostomatidae, Heterotrichea, macronuclear evolution, phylogeny, Stentoridae.

REPRESENTATIVES of the Heterotrichea are often characterized by a rather large cell size and a conspicuous cell shape. Moreover, many species are brightly coloured by specific pigments that occur in the cortical granules just below the cell surface. In the past, the Heterotrichea were classified as a subgroup of the Spirotrichea (e.g. Corliss 1979). However, based on morphological characters (i.e. differences in the adoral zone of membranelles; structure of the somatic kinetids with postciliodesmata), the heterotrichs were separated from the Spirotrichea (e.g. de Puytorac, Grain, and Mignot 1987; Small and Lynn 1985). Sequence analyses of large subunit rDNA (LSU rDNA) and small subunit rDNA (SSU rDNA) confirmed the deep split between the Heterotrichea and Spirotrichea (Baroin-Tourancheau et al. 1992; Greenwood et al. 1991). Furthermore, SSU rDNA data (Hammerschmidt et al. 1996; Hirt et al. 1995) supported the close relationship between the (aerobic) Heterotrichea and the Karyorelictea. Both taxa had been integrated into the subphylum Postciliodesmatophora established by Gerassimova and Seravin (1976), which forms the sister group of all remaining ciliates (subphylum Intramacronucleata) (Lynn 1996, 2003a). These sequence analyses also revealed the separation of the anaerobic (e.g. *Metopus*) from the aerobic heterotrichs (e.g. *Spirostomum*) (Embley et al. 1992; Hirt et al. 1995). Therefore, they are now treated as an own group, the Armophorea, within the Intramacronucleata (e.g. Adl et al. 2005; Lynn 2003a).

In recent years, analyses of SSU rDNA sequences caused several changes in the constitution of the Heterotrichea. While the classification of *Peritromus kahli* and *Maristentor dinoferus* as representatives of the Heterotrichea was affirmed (Miao et al. 2005; Rosati et al. 2004), the genera *Licnophora* and *Plagiotoma* were excluded and integrated into the Spirotrichea (Affa'a et al. 2004; Lynn and Strüder-Kypke 2002). In addition, a close relationship between *M. dinoferus* and the Folliculinidae was revealed (Miao et al. 2005), supporting the establishment of a new family, the Maristentoridae (Lobban et al. 2002; Miao et al. 2005).

Currently, the class Heterotrichea comprises nine families (Lynn 2003b; Miao et al. 2005). Conclusions about the phylogenetic relationships between these families depend on the analysed data. For example, based on morphological and ontogenetical similarities, Mulisch (1987) suggested a close relationship between Stentoridae and Folliculinidae, which was not confirmed using molecular data. Similarly, the kinship of Blepharismidae and Spirostomidae, as it was discussed by Aescht and Foissner (1998), was not revealed by SSU rDNA sequence analyses, which always resulted in a common grouping of *Blepharisma americanum* together with representatives of the Stentoridae (Gong et al. 2007; Lynn and Strüder-Kypke 2002; Miao et al. 2005; Modeo et al. 2006; Rosati et al. 2004).

At the moment, the sequence database is too small to establish a comprehensive hypothesis of the phylogenetic relationships within the Heterotrichea. Thus, we provide detailed phylogenetic analyses of this group including 10 new SSU rRNA gene sequences from representatives of the families Stentoridae, Blepharismidae, and Condylostomatidae.

MATERIALS AND METHODS

Collection of organisms. *Stentor amethystinus* and *Stentor polymorphus* were isolated from environmental water samples collected from ponds of the Dosenmoor, Neumünster, Germany (kindly provided by T. U. Berendonk, Universität Leipzig). Species identification was based on morphological characters through in vivo observation, different staining methods, and in comparison with current literature (Berger and Foissner 2003; Foissner, Berger, and Kohmann 1992; Foissner and Wölfl 1994). Cultures of *Stentor coeruleus*, *Blepharisma japonicum*, and *Blepharisma undulans* were kindly provided by K. Eisler (Universität Tübingen, Germany). The culture of *Blepharisma hyalinum* was purchased at ''culturecollection of algae and protozoa'' (CCAP 1607/4). *Blepharisma elongatum* was isolated from sphagnum of a fen in the surroundings of the village of Überlingen nearby Salzburg, Austria. *Blepharisma steinii* was found in soil of the Dominican Republic. *B. americanum* and *Condylostomides* n. sp. were isolated from a soil and mud sample on the north coast of Venezuela (i.e. from very flat grassland ponds in the Maracay National Park in the surroundings of the village of Chichirivice). *Condylostomides* n. sp. is similar to *C. etoschensis*, which was described by Foissner, Agatha, and Berger (2002). The description of *Condylostomides* n. sp. will be presented in a separate paper. The four last-mentioned species were collected and identified by in vivo observation and after protargol preparations by W. Foissner.

DNA isolation, amplification, and sequencing. DNA was isolated from single cells following a Chelex 100 extraction method (Regensbogenova et al. 2004) or from fixed cells (in 80% EtOH) using a modified Kavenoff and Zimm procedure (e.g. Steinbrück and Schlegel 1983). The nuclear SSU rDNA was amplified with universal Eukarya-specific primers (e.g. Korte et al. 2004) following a polymerase chain reaction (PCR)-protocol published previously (Schmidt et al. 2006). PCR products were purified using either the Rapid PCR Purification System® of Marligen Biosciences Inc. (Biocat, Heidelberg, Germany) or the

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Table **1.** Classification of the species analysed in the present study in accordance with Lynn (2003b) and Miao et al. (2005).

Family	Species	Accession No.	Reference
Loxodidae	*Loxodes magnus*	L31519	Hirt et al. (1995)
	Loxodes striatus	U24248	Hammerschmidt et al. (1996)
Trachelocercidae	*Tracheloraphis* sp.	L31520	Hirt et al. (1995)
Blepharismidae	*Blepharisma americanum*[1]	M97909	Greenwood et al. (1991)
	***Blepharisma americanum*[2]**	AM713182	**This study**
	Blepharisma elongatum	AM713186	**This study**
	Blepharisma hyalinum	AM713184	**This study**
	Blepharisma japonicum	AM713185	**This study**
	Blepharisma steinii	AM713187	**This study**
	Blepharisma undulans	AM713183	**This study**
Chattonidiidae	*Chattonidium setense*	AM295495	Modeo et al. (2006)
Climacostomidae	*Climacostomum virens*	X65152	Hammerschmidt et al. (1996)
	Fabrea salina LesF	DQ168805	Angeli et al. (unpubl. observ.)
	Fabrea salina TorF	DQ168806	Angeli et al. (unpubl. observ.)
Condylostomatidae	*Condylostoma minutum*	DQ822482	Guo & Song (unpubl. observ.)
	Condylostoma spatiosum	DQ822483	Guo & Song (unpubl. observ.)
	*Condylostoma wangi**	DQ445605	Miao & Song (unpubl. observ.)
	Condylostoma sp.	AM295496	Modeo et al. (2006)
	***Condylostomides* n. sp.**	AM713188	**This study**
Folliculinidae	*Eufolliculina uhligi*	U47620	Hammerschmidt et al. (1996)
Maristentoridae	*Maristentor dinoferus*	AY630405	Miao et al. (2005)
Peritromidae	*Peritromus kahli*	AJ537427	Rosati et al. (2004)
Spirostomidae	*Gruberia* sp.	L31517	Hirt et al. (1995)
	Spirostomum ambiguum[1]	L31518	Hirt et al. (1995)
	Spirostomum ambiguum[2]	AM398201	Schmidt et al. (2007)
	Spirostomum minus	AM398200	Schmidt et al. (2007)
	Spirostomum teres	AM398199	Schmidt et al. (2007)
Stentoridae	*Stentor coeruleus*[1]	AF357145	Gong et al. (2007)
	***Stentor coeruleus*[2]**	AM713189	**This study**
	Stentor polymorphus[1]	AF357144	Gong et al. (2007)
	***Stentor polymorphus*[2]**	AM713190	**This study**
	Stentor roeselii	AF357913	Gong et al. (2007)
	Stentor amethystinus	AM713191	**This study**

All newly investigated species are marked in bold. Representatives of the Karyorelictea (*Loxodes* spp., *Tracheloraphis* sp.) were used as outgroup.
*Published as *Stentor auriculatus* at GenBank database. Numbering of the species refers to Fig. 1.

NucleoSpin® Extract Kit II of Macherey-Nagel (Düren, Germany) and sequenced directly. Sequencing reactions were performed for both DNA strands using the same universal Eukarya-specific primers and different internal primers (Wylezich et al. 2002) on an ABI PRISM® 3100 Genetic Analyzer (Darmstadt, Germany).

Phylogenetic analyses and sequence availability. The analysed data set (Table 1) comprises 30 SSU rDNA sequences of heterotrichous ciliates and three sequences from representatives of the Karyorelictea, which served as outgroup in all analyses. Alignments were carried out with CLUSTAL X 1.83 (Thompson et al. 1997) with default parameters. Primer sequences were removed from the alignment prior to phylogenetic analyses using BioEdit (Hall 1999).

Maximum-likelihood (ML) analysis was performed with PAUP* v4.0b10 (Swofford 2002) with 100 replications and using the evolutionary model of Tamura and Nei (1993) with $I = 0.4738$ and $\Gamma = 0.4724$, selected by Modeltest 3.6 (Posada and Crandall 1998). 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. Neighbour-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 10,000 replication steps. Maximum parsimony (MP) analysis was performed with 2,000 resamplings also using the program package MEGA 3.1 (Kumar et al. 2004).

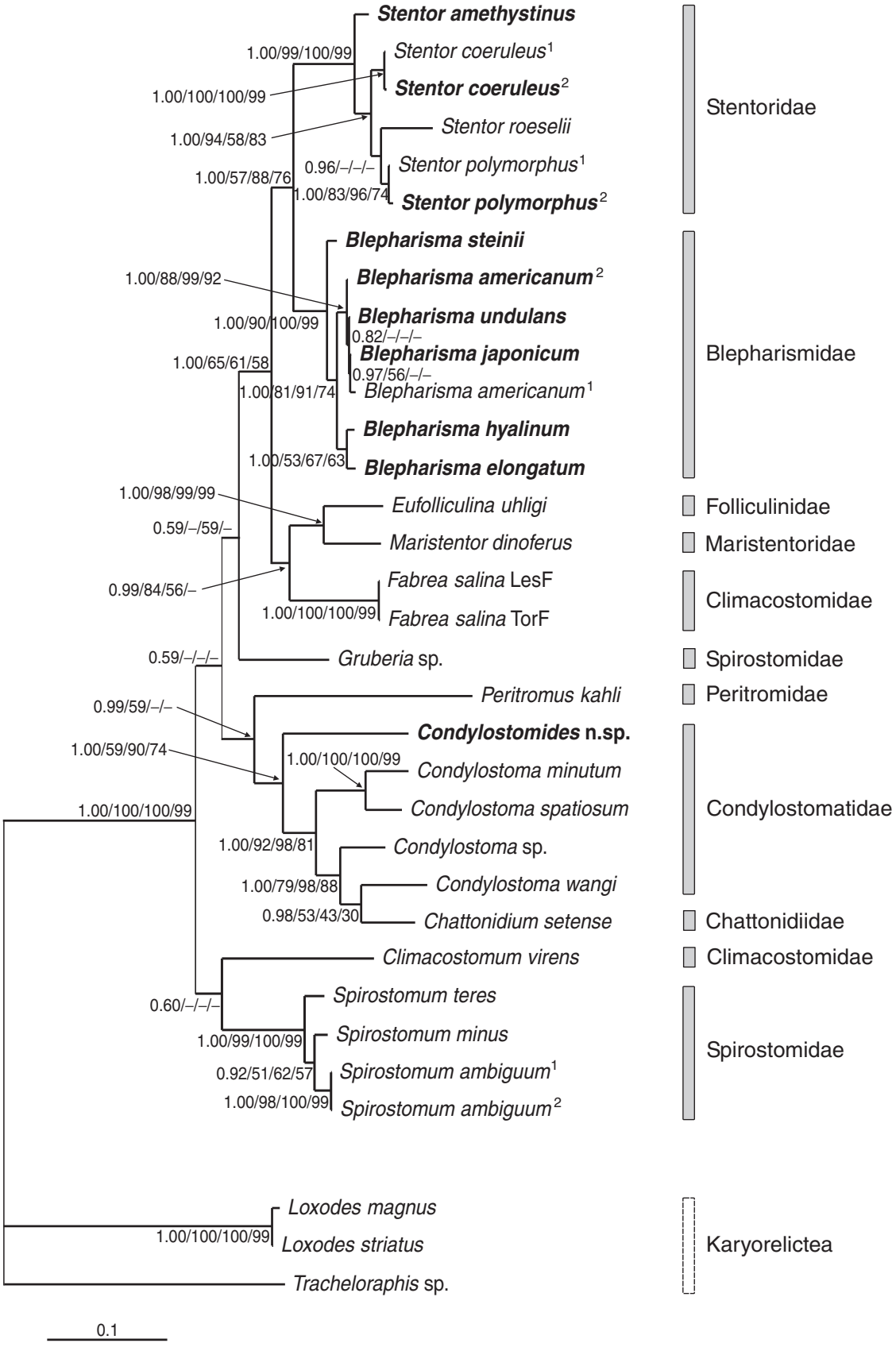
Nucleotide sequences. All new SSU rDNA sequences are deposited at GenBank database. Accession numbers are listed in Table 1.

RESULTS

The data set comprises 30 SSU rDNA sequences of 25 heterotrichous species, which are relatable to all nine families (cf. Table 1). The alignment contains 1,699 positions (578 variable; 470 parsimony informative), which could be unambiguously aligned. Therefore, no positions had to be excluded.

The Heterotrichea are always supported as a monophylum with highest bootstrap values and posterior probabilities, respectively (Fig. 1). Within the Heterotrichea, the basal branching varied between the different methods and could not be resolved unambiguously. Particularly, the position of *P. kahli* was unstable: Bayesian and ML analyses revealed a common grouping of *P. kahli*, *Chattonidium setense*, and the Condylostomatidae (Fig. 1); MP analysis showed *P. kahli* as a discrete branch within a basal polytomy; whereas *P. kahli* branched off separately as the sister taxon to all other heterotrichous ciliates in the NJ analysis (data not shown).

Invariably, *C. setense* and all members of the Condylostomatidae grouped together (Fig. 1). Within this clade, the new species assigned to the genus *Condylostomides* formed the sister taxon to all other species. Whereas *Condylostoma minutum* and *Condylostoma spatiosum* grouped together highly supported, *C. setense*


Stentor amethystinus
1.00/99/100/99
Stentor coeruleus1
Stentor coeruleus2
1.00/100/100/99
Stentor roeselii
1.00/94/58/83
0.96/–/–/–
Stentor polymorphus1
1.00/57/88/76
1.00/83/96/74
Stentor polymorphus2
Stentoridae
Blepharisma steinii
1.00/88/99/92
Blepharisma americanum2
Blepharisma undulans
1.00/90/100/99
0.82/–/–/–
Blepharisma japonicum
1.00/65/61/58
0.97/56/–/–
1.00/81/91/74
Blepharisma americanum1
Blepharisma hyalinum
1.00/53/67/63
Blepharisma elongatum
Blepharismidae
1.00/98/99/99
Eufolliculina uhligi
Folliculinidae
0.59/–/59/–
Maristentor dinoferus
Maristentoridae
Fabrea salina LesF
0.99/84/56/–
1.00/100/100/99
Fabrea salina TorF
Climacostomidae
0.59/–/–/–
Gruberia sp.
Spirostomidae
Peritromus kahli
Peritromidae
0.99/59/–/–
Condylostomides n.sp.
1.00/59/90/74
1.00/100/100/99
Condylostoma minutum
Condylostoma spatiosum
1.00/100/100/99
1.00/92/98/81
Condylostoma sp.
1.00/79/98/88
Condylostoma wangi
0.98/53/43/30
Chattonidium setense
Chattonidiidae
Climacostomum virens
Climacostomidae
Spirostomum teres
0.60/–/–/–
1.00/99/100/99
Spirostomum minus
0.92/51/62/57
Spirostomum ambiguum1
1.00/98/100/99
Spirostomum ambiguum2
Spirostomidae
Loxodes magnus
1.00/100/100/99
Loxodes striatus
Tracheloraphis sp.
Karyorelictea
0.1

(family Chattonidiidae) was found closely related to *Condylostoma wangi* and *Condylostoma* sp. (Fig. 1).

The cluster comprising the *Spirostomum* species and *Climacostomum virens* was only found by Bayesian analysis. In contrast, all other analysis methods found *C. virens* as a separate branch within the basal polytomy (data not shown). None of the analyses revealed a cluster containing all species of the Spirostomidae, because *Gruberia* sp. branched off independently from the three *Spirostomum* species (Fig. 1). Within the always highly supported *Spirostomum* cluster, *S. teres* formed the first branch, followed by *S. minus* and both isolates of *S. ambiguum*.

Analogous to the Spirostomidae, the family Climacostomidae (represented by *C. virens* and *Fabrea salina*) did not form a monophyletic group in any of our analyses. While *C. virens* branched off inconsistently, both isolates of *F. salina* always grouped together in a close relationship to *Eufolliculina uhligi*, *M. dinoferus*, the Blepharismidae, and the Stentoridae (Fig. 1). The kinship of *E. uhligi* and *M. dinoferus* was revealed by all analyses, whereas their close relationship to both isolates of *F. salina* was only the result of Bayesian, ML, and NJ analyses.

The Stentoridae and Blepharismidae were revealed as sister groups by all analyses. Within the Stentoridae, all analyses showed *S. amethystinus* as the first branch. Moreover, both isolates of *S. coeruleus* constantly grouped together, just as the two isolates of *S. polymorphus*. Only the position of *Stentor roeselii* differed between the analyses. Bayesian analysis indicated a closer relationship of *S. roeselii* and *S. polymorphus* (Fig. 1). In contrast, NJ and MP analyses showed *S. roeselii* as the second branch outside the common group formed by *S. polymorphus* and *S. coeruleus* (data not shown). ML analysis did not resolve the relationships between *S. roeselii*, *S. coeruleus*, and *S. polymorphus* unambiguously (data also not shown).

Similarly, not all relationships within the Blepharismidae were consistently resolved. *B. steinii* branched off first in all analyses, and a separation of the remaining five species into two groups was found consistently (Fig. 1). The first group comprised *B. hyalinum* and *B. elongatum*, whereas the second group contained *B. undulans*, *B. japonicum*, and both isolates of *B. americanum*. Within the second group the newly sequenced *B. americanum* did not cluster with the isolate of *B. americanum* published by Greenwood et al. (1991). Both SSU rDNA sequences differ in 13 nucleotides, most of them are uncertain positions in the sequence of Greenwood
et al. (1991).

DISCUSSION

Various molecular studies (e.g. Lynn and Strüder-Kypke 2002; Schlegel and Eisler 1996; Stechmann, Schlegel, and Lynn 1998) confirmed that the heterotrichous and karyorelictean ciliates belong to the Postciliodesmatophora as it was already indicated by morphological characters: (1) somatic dikinetids with postciliodesmata, and (2) division of the macronuclei by extramacronuclear microtubules (de Puytorac et al. 1987; Lynn 1996, 2003a; Lynn and Small 2002; Raikov 1982; Small and Lynn 1985).

At the moment, the class Heterotrichea Stein, 1859 (order Heterotrichida) is divided into nine families (Lynn 2003b; Miao et al. 2005), and our study includes SSU rDNA sequences from representatives of all these families. While the terminal branches are generally well resolved, the basal relationships differ between the analysis methods and therefore remain doubtful. Particularly, the phylogenetic positions of *C. virens* (Climacostomidae), *P. kahli* (Peritromidae), and *Gruberia* sp. (Spirostomidae) are unsolved and only found by one or two analysis methods.

The close relationship between the Condylostomatidae and the Chattonidiidae as proposed by Modeo et al. (2006) could be verified in the present study. However, in all analyses *C. setense* branched off within the Condylostomatidae close to *C. wangi*, which challenges the taxonomic status of *C. setense*. This problem should be addressed in further studies.

The species *C. wangi* Foissner and Wölfl 1994 was originally described by Kahl (1932) as *Stentor auriculatus*. After a reinvestigation, Fauré-Fremiet (1936) transferred it to the genus *Condylostoma*. Later on, Jankowski (1978, 1980) proposed the assignment of this species to the newly established genus *Condylostentor* (family Condylostentoridae), which was challenged by Foissner and Wölfl (1994). The present sequence analyses revealed a kinship of *C. wangi* (published at Genbank database as *S. auriculatus*) and all other representatives of the Condylostomatidae. Furthermore, *Condylostomides* n. sp. was found closely related to the genus *Condylostoma*, which confirms the affiliation as a member of the Condylostomatidae.

In agreement with other molecular analyses (e.g. Baroin-Tourancheau et al. 1998; Gong et al. 2007; Lynn and Strüder-Kypke 2002; Miao et al. 2005; Rosati et al. 2004), our study revealed high support for a sister group relationship between the Blepharismidae and Stentoridae. However, morphological and ontogenetic features supporting this relationship are lacking, because representatives of the genus *Blepharisma* show a higher morphological similarity to *Spirostomum* than to *Stentor* species. For instance, representatives of the genus *Stentor* are characterized by a large, frontal oral field with semicircular ciliary rows, while *Blepharisma* and *Spirostomum* have a narrow, longitudinally oriented, unciliated oral field. Furthermore, ontogenesis differs between these genera (Foissner 1996), viz., polyparakinetal in *Spirostomum* and *Blepharisma* (several postoral kineties are involved in the formation of the oral anlage), and amphiparakinetal in *Stentor* (the curved oral primordium intersects many postoral kineties at two sites and thus encloses few to many short, nonproliferating parental kinety fragments). For a detailed discussion see Aescht and Foissner (1998). Thus, the SSU rDNA-based result should be reanalysed using additional gene sequences.

Maristentor dinoferus and *Eufolliculina uhligi* form a common branch. This result is in agreement with Miao et al. (2005) and endorses the separation of *M. dinoferus* from representatives of the genus *Stentor*. Furthermore, our analyses confirm the position of *E. uhligi* (Folliculinidae) and *M. dinoferus* (Maristentoridae) as a sister group to the Stentoridae and Blepharismidae (Gong et al. 2007; Lynn and Strüder-Kypke 2002; Miao et al. 2005; Modeo et al. 2006; Rosati et al. 2004). The position of *Fabrea salina* as well as the paraphyly of the Climacostomidae (including the genera *Fabrea* and *Climacostomum*) was unexpected. This family was established by Repak (1972) based on morphological and morphogenetic characters (e.g. inconspiuous paroral membrane, bipartite adoral zone of membranelles, similar stomatogenesis, ventrally located buccal cavity, uniform and complete body

Fig. **1.** Phylogenetic tree of the Heterotrichea inferred from nuclear small subunit (SSU) rDNA sequences based on a Bayesian analysis. The karyorelictean species *Loxodes magnus*, *Loxodes striatus*, and *Tracheloraphis* sp. were chosen as outgroup taxa. The numbers at the nodes represent in order the posterior probabilities of the Bayesian analysis (first number; 1,000,000 generations), the support values of the maximum likelihood analysis after 100 replication steps (second number), the bootstrap values of the neighbour joining analysis (third number; 10,000 resamplings), and bootstrap values of the maximum parsimony analysis (fourth number; 2,000 resamplings). All newly investigated species are in bold. Numbering of the species refers to Table 1. Please note that *Condylostoma wangi* is published in GenBank as *Stentor auriculatus*.

ciliation; see also Lynn and Small 2002). *Fabrea salina* branched off in close relationship to the folliculinids (Fig. 1). However, both differ considerably in morphology and ontogenesis (Foissner 1996; Mulisch 1987; Villeneuve-Brachon 1940). Folliculinids are highly contractile, usually live in a lorica, and possess two conspicuous peristomial wings, whereas *Fabrea* is acontractile and lacks both lorica and peristomial wings. Furthermore, *Fabrea* has a complex paroral membrane not found in any other heterotrich (Da Silva Neto and Groliere 1993). The ontogenesis is rather similar to that of *Stentor* because the ciliated peristomial field is of somatic origin (Foissner 1996; Villeneuve-Brachon 1940).

The relationship between *C. virens* and *Spirostomum* spp. is only weakly supported by Bayesian analysis (Fig. 1). Indeed, the morphology of *Climacostomum* differs extremely from *Spirostomum*, so it is difficult to find a common feature at all, except of those characterizing the heterotrichs in general (Corliss 1979, Foissner et al. 1992). For instance, *Spirostomum* is strongly contractile and has a single paroral membrane right of the adoral zone of membranelles, while *Climacostomum* is acontractile and possess a minute, circular paroral membrane at the proximal end of the feeding tube, an unique organelle extending deeply into the cell. Accordingly, ontogenesis is also quite different (Aescht and Foissner 1998; Dubochet, Peck, and De Haller 1979).

None of our analyses revealed a cluster containing all representatives of the family Spirostomidae (*Gruberia* sp., *Spirostomum* spp.). The paraphyly of the Spirostomidae has been observed in other molecular analyses (Gong et al. 2007; Hammerschmidt et al. 1996; Miao et al. 2005; Modeo et al. 2006; Rosati et al. 2004), and the relationship of *Gruberia* within the heterotrichous ciliates remains unsolved.

The kinship of the species *B. americanum*, *B. undulans*, and *B. japonicum* was expected from several morphological studies. Suzuki (1954) investigated different forms of *B. undulans* characterized by a high macronuclear variation and described consequently three subspecies: *B. undulans undulans*, *B. undulans americanus*, and *B. undulans japonicus*. Later on, Bhandary (1962) continued these morphological investigations and raised each subspecies to species status. Sequence analyses of the whole SSU rDNA of *B. japonicum*, *B. undulans*, and the newly investigated *B. americanum* revealed only one or two single nucleotide differences between each other. This result was unexpected due to the morphological differentiation between these species. The separation of the two *B. americanum* isolates appears to be due primarily to a number of uncertain positions in the sequence of Greenwood et al. (1991). Considering that the three *Blepharisma* species analyzed newly by us are so genetically close, we recommend that the *B. americanum* sequence of Greenwood et al. (1991) should not be used in subsequent research since the number of unknown nucleotides now makes this sequence essentially useless.

In addition to the phylogenetic results, the sequence data can be used to follow macronuclear evolution within the genera *Blepharisma*, *Stentor*, and *Spirostomum*. These genera have different types of macronuclei (e.g. single compact, vermiform, moniliform; e.g. Aescht and Foissner 1998; Foissner and Wölfl 1994). Our analyses invariably revealed a basal position of those species having a single compact macronucleus (*S. amethystinus*, *B. steinii*, *B. hyalinum*, *B. elongatum*, and *Spirostomum teres*). Therefore, we conclude that a single, compact macronucleus might be the ancestral state, whereas other types seem to be derived. This is also supported by ontogenetic data, which show that other shapes of the macronucleus (e.g. moniliform) become ''single compact'' during the middle division stages. The evolutionary significance of this morphological character has to be proven in future analyses.

Summarizing our results, the SSU rDNA sequence analyses offer some potentialities to solve the relationships between heterotrichous ciliates. Whereas the relationships of terminal branches are generally well resolved, the basal relationships are inconsistent in the different analyses and therefore remain unsettled. For this reason, analyses of other gene sequences and taxa will be needed to resolve the basal polytomy.

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