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Further Consideration of the Phylogeny of Some ''Traditional'' Heterotrichs (Protista, Ciliophora) of Uncertain Affinities, Based on New Sequences of the Small Subunit rRNA Gene

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ABSTRACT. The systematic relationships and taxonomic positions of the traditional heterotrich genera *Condylostentor*, *Climacostomum*, *Fabrea*, *Folliculina, Peritromus*, and *Condylostoma*, as well as the licnophorid genus *Licnophora*, were re-examined using new data from sequences of the gene coding for small subunit ribosomal RNA. Trees constructed using distance-matrix, Bayesian inference, and maximum-parsimony methods all showed the following relationships: (1) the ''traditional'' heterotrichs consist of several paraphyletic groups, including the current classes Heterotrichea, Armophorea and part of the Spirotrichea; (2) the class Heterotrichea was confirmed as a monophyletic assemblage based on our analyses of 31 taxa, and the genus *Peritromus* was demonstrated to be a peripheral group; (3) the genus *Licnophora* occupied an isolated branch on one side of the deepest divergence in the subphylum Intramacronucleata and was closely affiliated with spirotrichs, armophoreans, and clevelandellids; (4) *Condylostentor*, a recently defined genus with several truly unique morphological features, is more closely related to *Condylostoma* than to *Stentor*; (5) *Folliculina*, *Eufolliculina*, and *Maristentor* always clustered together with high bootstrap support; and (6) *Climacostomum* occupied a paraphyletic position distant from *Fabrea*, showing a close relationship with Condylostomatidae and Chattonidiidae despite of modest support.

Key Words. *Climacostomum, Condylostentor, Folliculina, Licnophora, Peritromus, phylogenetic relationships.*

TRADITIONAL heterotrichs are a huge, diverse assemblage of species with a complex systematic history that remain one of the most confusing groups of ciliates. There have been a series of major efforts to define taxa within this group. It was widely accepted for many years that ''traditional'' heterotrichs were a subgroup of typical spirotrichs comprising six suborders: Heterotrichina, Clevelandellina, Armophorina, Coliophorina, Licnophorina, and Plagiotomina (Corliss 1979). Based on ultrastructure of the cortex, Small and Lynn (1981) proposed a close relationship between the class Karyorelictea, presumably the most basal ciliate group based on reproductive characteristics, and ''traditional'' heterotrichs (as part of Spirotrichea) and placed them within the subphylum Postciliodesmatophora. The ''traditional'' heterotrichs were still ranked as a subclass of spirotrichs, however. De Puytorac et al. (1993) were the first to rank ''traditional'' heterotrichs as a class separate from spirotrichs, and their distinctiveness was supported by the first phylogenetic analysis using molecular markers (Hirt et al. 1995). Subsequently, this concept of the Heterotrichea gained wide acceptance (Lynn and Small 1997). From then on, phylogenetic analyses among ''traditional'' heterotrichs have been carried out to obtain which taxa are ''true'' heterotrichs (the class Heterotrichea). The ''traditional'' heterotrichs *Protocruzia*, *Phacodinium*, *Licnophora*, and *Plagiotoma* have been included within Spirotrichea (now within the subphylum Intramacronucleata), based on a combination of ultrastructural and molecular data (Affa'a et al. 2004; Lynn and Strüder-Kypke 2002; Shin et al. 2000). Armophorids and clevelandellids are transferred out of the Heterotrichea to the new ribo-class Armophorea (Lynn and Small 2002). ''Traditional'' heterotrichs sensu Corliss (1979) now comprise several divergent clades spread among three major taxa (the classes Heterotrichea sensu stricto, Armophorea, and Spirotrichea).

Despite the stabilization of higher-order taxa remaining within Heterotrichea (''true'' heterotrichs), well-supported evidence for kinship among its lower-level taxa (i.e. families and genera) remains inconsistent. Based on small subunit ribosomal RNA (SSU rRNA) gene sequences, Rosati et al. (2004) found that *Peritromus* branched off separately as a sister taxon to all other ''true'' heterotrichous ciliates; but Schmidt et al. (2007) observed this genus to group with *Chattonidium* or *Condylostoma*. Lobban, Schefer, and Simpson (2002) hypothesized a close relationship between the new genus *Maristentor* and stentorids based on morphological similarities, but instead, a relationship with folliculinids was indicated by analyses of SSU rRNA gene sequences (Miao et al. 2005; Schmidt et al. 2007). To sum up, neither morphological characters nor molecular information have allowed consistent placement of many taxa at the familial level. Furthermore, the molecular data from investigations consist of only SSU rRNA gene sequences of a relatively low number of taxa: one in Greenwood et al. (1991), two in Baroin-Tourancheau et al. (1992), five in Hirt et al. (1995), eight in Hammerschmidt et al. (1996), nine in Rosati et al. (2004), 12 in Modeo et al. (2006), 11 in Gong et al. (2007), and 30 in Schmidt et al. (2007).

In the present paper, we have performed an expanded analysis of phylogenetic relationships among the ''traditional'' heterotrichs based on addition of new SSU rRNA gene sequences of six species in five genera for which sequences have already been reported and two species in two genera never before sequenced. Other than these, five additional sequences from the GenBank were included in our analysis for a total of 44 species in 22 genera. Finally, we used features of the secondary structure of sequences to extend analyses of relationships that were still ambiguous based on the analysis of primary sequence characters alone.

MATERIALS AND METHODS

Taxon sampling and DNA manipulations. All isolates were obtained from the coastal area of Qingdao (36°08′N, 120°43′E), China from 2005 to 2007 (Fig. 1–18). Species isolation, morphological investigation, and identifications were carried out according to Shao et al. (2007).

Cells were picked from pure cultures with a micropipette and starved in sterilized seawater for up to 1 day to prevent DNA contamination by food vacuole contents. DNA of collected cells was isolated by phenol extraction and precipitated by ethanol. The entire coding region of the SSU rRNA gene was amplified by PCR

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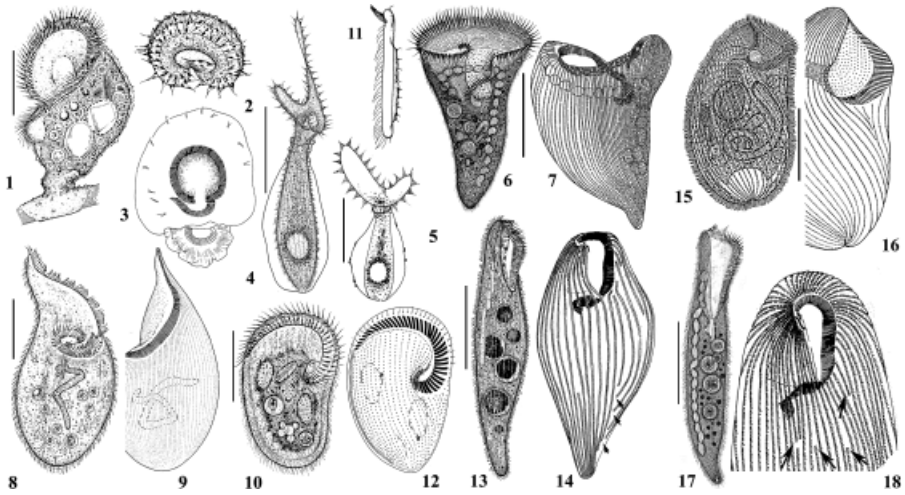

Fig. **1–18.** Morphology of ''traditional'' heterotrich ciliates mentioned in the present study. **1–3.** *Licnophora lyngbycola* (after Song et al. 2003). **4, 5.** *Folliculina simplex* (after Song et al. 2003). **6, 7.** *Condylostentor auriculatus* (after Chen et al. 2007). **8, 9.** *Fabrea salina* (after Song and Packroff 1997), **10–12.** *Peritromus faurei* (after Song and Wilbert 1997). **13, 14.** *Condylostoma minutum* (after Chen et al. 2007). Arrows indicate sutures. **15, 16.** *Climacostomum virens* (after Foissner, Berger, and Kohmann 1992). **17, 18.** *Condylostoma spatiosum*. Note that arrows in Fig. 18 mark the ''extra'' kineties (after Shao et al. 2006). Scale bar = 50 μm in Fig. 1; 30 μm in Fig. 10; 100 μm in Fig. 4, 6, 8, 13, 15, 17.

using two universal primers (Medlin A and Medlin B) that are complementary to the 5′- and 3′-termini of eukaryotic SSU rRNA genes (Medlin et al. 1988). Cycling parameters were the same as in Miao et al. (2007). Polymerase chain reaction products were verified by agarose gel electrophoresis, inserted in pUCm-T plasmids (Sangon, Toronto, ON, Canada), and two individual colonies were picked and sequenced in both directions on an ABI 3730 automated sequencer with a set of universal end and internal primers for eukaryotic SSU rRNA genes and universal M13 sequencing primers (Yi et al. 2008).

Alignment and phylogenetic analysis. Nucleotide sequences other than ones resulting from the present study were obtained from the GenBank/EMBL databases. Sequences were aligned using CLUSTAL X, ver. 1.83 (Thompson, Higgins, and Gibson 1994). The alignment was refined by considering features of the secondary structures of molecules, and the final matrix included 1,879 unambiguously aligned nucleotide positions. Bayesian inference (BI) was performed with MrBayes version 3.1.1 (Huelsenbeck and Ronquist 2001) using the general time-reversible model (Rodríguez et al. 1990) with discrete gamma-distributed rate variation among sites with four categories and allowing for invariant sites as the model chosen by MrModeltest v2 (Nylander 2004). Two parallel runs of 1,000,000 generation, were performed, sampling every 100th generation and discarding the first 1,000 trees as burn-in. A maximum parsimony (MP) analysis was performed with PAUP* ver 4.0b10 (Swofford 2002) using 924 parsimony-informative characters with the TBR branch-swapping algorithm in effect. For distance-matrix methods, evolutionary distances were calculated with the Kimura two-parameter model in the PHYLIP package V3.6.6 (Felsenstein 2006), and trees were constructed using the neighbor-joining (NJ) (Saitou and Nei 1987) and Fitch and Margoliash least-squares (LS) methods (Fitch and Margoliash 1967). Bootstrapping with 1,000 replicates was performed for MP, NJ, and LS analyses.

Mesquite 2.01 (Maddison and Maddison 2007) was used to generate constraint trees representing the following hypotheses: *Condylostentor auriculatus* clusters in a single clade either with species of *Stentor* or species of *Condylostoma*. The resulting trees were compared with unconstrained BI and MP results using the Shimodaira–Hasegawa (S–H) test (Shimodaira and Hasegawa 1999) implemented in PAUP.

Prediction of secondary structure. Secondary structures of the variable and insertion regions of SSU rRNA genes were computed by submission of primary sequences to the RNA folding website supporting MFOLD version 3.2 (http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1-2.3.cgi; Zuker, Mathews, and Turner 1999), using the default parameters for folding except $T = 25\ ^{\circ}C$. Graphic output was generated with the RNAViz program (De Rijk and De Wachter 1997) for aesthetic purposes.

RESULTS

Small subunit ribosomal RNA gene sequences and secondary structures. The SSU rRNA sequences and their length, GC content, and GenBank accession numbers of the eight species that we sequenced are as follows *Licnophora lyngbycola*—1,700 nt, 49.59%, DQ445606; *C. auriculatus*—1,651 nt, 46.52%, DQ445605; *Fabrea salina*-QD—1,672 nt, 47.19%, EU583991; *Folliculina simplex*—1,680 nt, 47.14%, EU583992; *Climacostomum virens*-QD—1,668 nt, 48.26%, EU583990; *Peritromus faurei*—1,681 nt, 44.62%, EU583993; *Condylostoma minutum*—1,619 nt, 46.39%, DQ822482; and *Condylostoma spatiosum*—1,614 nt, 46.47%, DQ822483.

A slight difference in primary structure and GC content (range 41.27–49.59%) among ''traditional'' heterotrichs was evident when sequences were inspected in the course of adjusting the alignment. Complete sequences, with the exception of those of *Condylostoma* spp., ranged from 1,651 to 1,684 nucleotides long with a mean length of 1,675 nucleotides, GC content ranged from 44.38% to 48.26% with an average of 46.49%—typical for SSU rRNA genes of ''true'' heterotrichs (class Heterotrichea). The SSU rRNA sequences of *Licnophora*, *Phacodinium*, *Plagiotoma*, and *Protocruzia* were slightly longer than those of ''true'' heterotrichs, and those of *Licnophora* spp. had a higher GC content (*Licnophora macfarlandi*—48.06%, *L. lyngbycola*—49.59%) than that observed for ''true'' heterotrichs.

So far, the secondary structure of SSU rRNA genes in ''traditional'' heterotrichs has not been investigated. To address this, we focused on 22 species (compared with *Tetrahymena canadensis*) belonging to different genera in which the predicted secondary structure agreed with the generalized eukaryotic SSU rRNA gene model (Neefs et al. 1993). All 50 universal helices were identified,

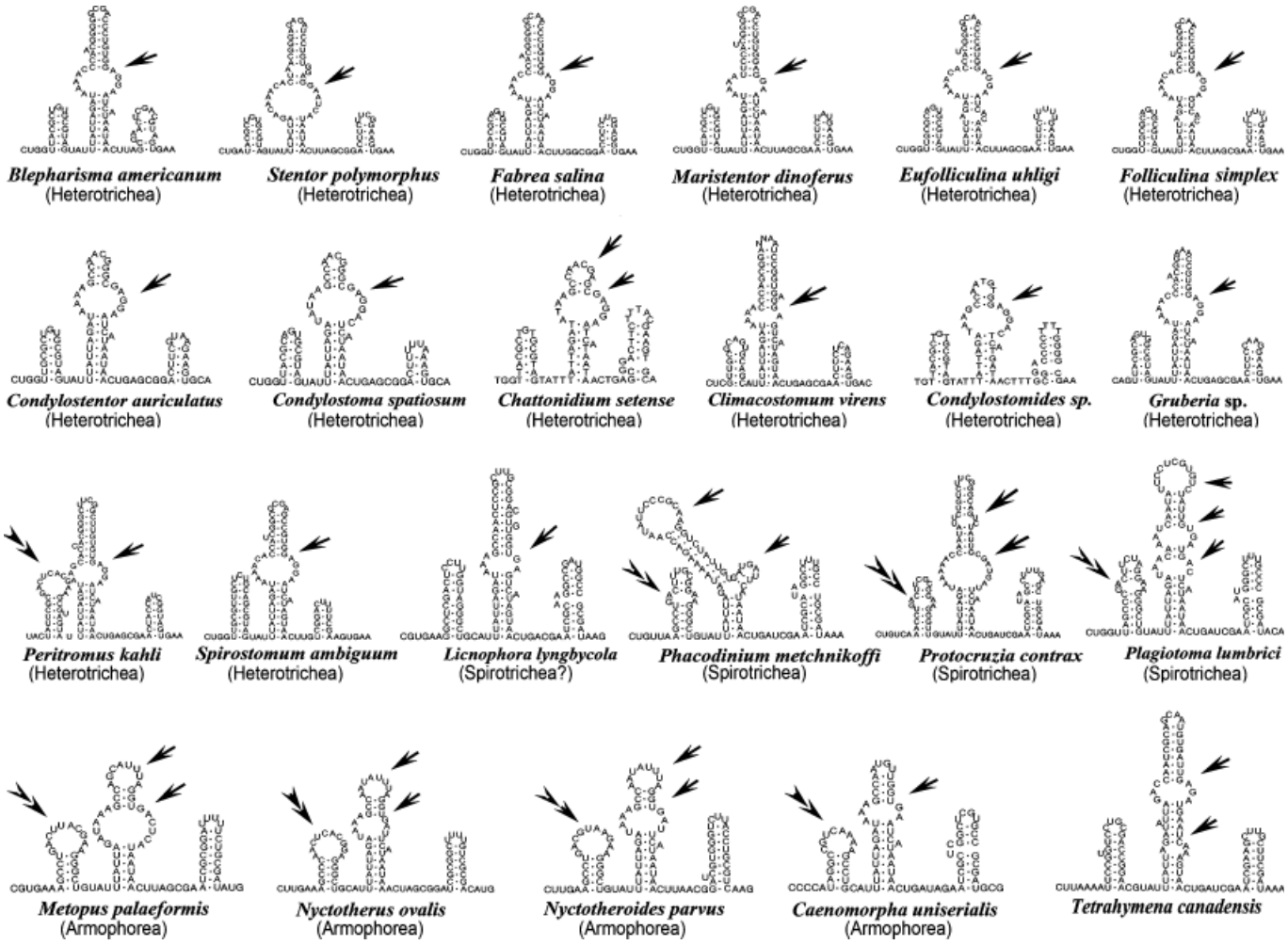

Fig. **19.** Secondary structure of the small subunit rRNA molecule in the region of the Helices 10, E10-1, and 11 (from left to right) in some ''traditional'' heterotrichs (*s.l.*). Arrows mark differences in the shape, numbers, and/or position of main loops in Helix E10-1 among taxa, and double-arrowheads indicate the loops in helix 10 that are present only in some species.

and all the common eukaryotic-specific helices were present in the sequences.

Inspection of the alignment showed some differences among ''traditional'' heterotrichs in the variable region 2, including Helix 10, Helix E10-1, and Helix 11(Fig. 19). The nucleotides comprising Helix E10-1 in all ''true'' heterotrichs appeared to form a linear helix with one bulge, although *Condylostentor*, *Condylostoma*, and armophoreans shared the derived character of a shorter Helix E10-1 (Fig. 19). *Protocruzia* and *Plagiotoma* had one more bulge in Helices 10 and E10-1, and the branched Helix E10-1 of *Phacodinium* was markedly different from all the others (Fig. 19). There was little variation between taxa with respect to the base composition of Helices 10 and 11. The nucleotides comprising Helix 11 in ''true'' heterotrichs formed the most conserved sequence in the V2 region and could potentially form a helix with a terminal loop. By contrast, *Protocruzia*, *Phacodinium*, *Plagiotoma*, and *Licnophora* had 2–9 nt more nucleotides in the middle of Helix 11 than did ''true'' heterotrichs (Fig. 19). The armophorids (*Metopus* and *Caenomorpha*) and clevelandellids (*Nyctotherus* and *Nyctotheroides*) had a larger terminal bulge in Helix 10 than ''true'' heterotrichs (Fig. 19).

Small subunit rRNA phylogeny of the heterotrichs. Trees constructed using different methods all depicted the ''traditional'' heterotrichs, including the licnophorids and *Protocruzia* as a polyphyletic assemblage (Fig. 20, 21). *Phacodinium* always occupied a basal branch in the Class Spirotrichea, and *Protocruzia* was related to spirotrichs although with only moderate support. The BI tree placed licnophorids as a sister group to litostome ciliates, armophorids, and clevelandellids, but in the MP analysis, licnophorids clustered with both armophorids and clevelandellids (data not shown).

Our study confirmed the class Heterotrichea as monophyletic, with high bootstrap values and posterior probabilities (0.99BI, 79%MP, 100%LS, 100%NJ). Six genera dealt with in the present work (*Condylostentor*, *Climacostomum*, *Fabrea*, *Folliculina*, *Peritromus*, and *Condylostoma*) were confirmed as belonging to a clearly outlined, monophyletic assemblage and thus should be regarded as ''true'' heterotrichs. Highly supported associations between different genera were as follows: *Blepharisma-Stentor*; *Folliculina-Eufolliculina-Maristentor*; and *Condylostoma-Condylostentor-Chattonidium*. Also, the basal positions of *Peritromus* and *Spirostomum* were generally supported.

DISCUSSION

''Traditional'' heterotrichs are not a monophyletic assemblage. Previous morphological studies identified all ciliates with an adoral zone of membranelles and non-specialized somatic

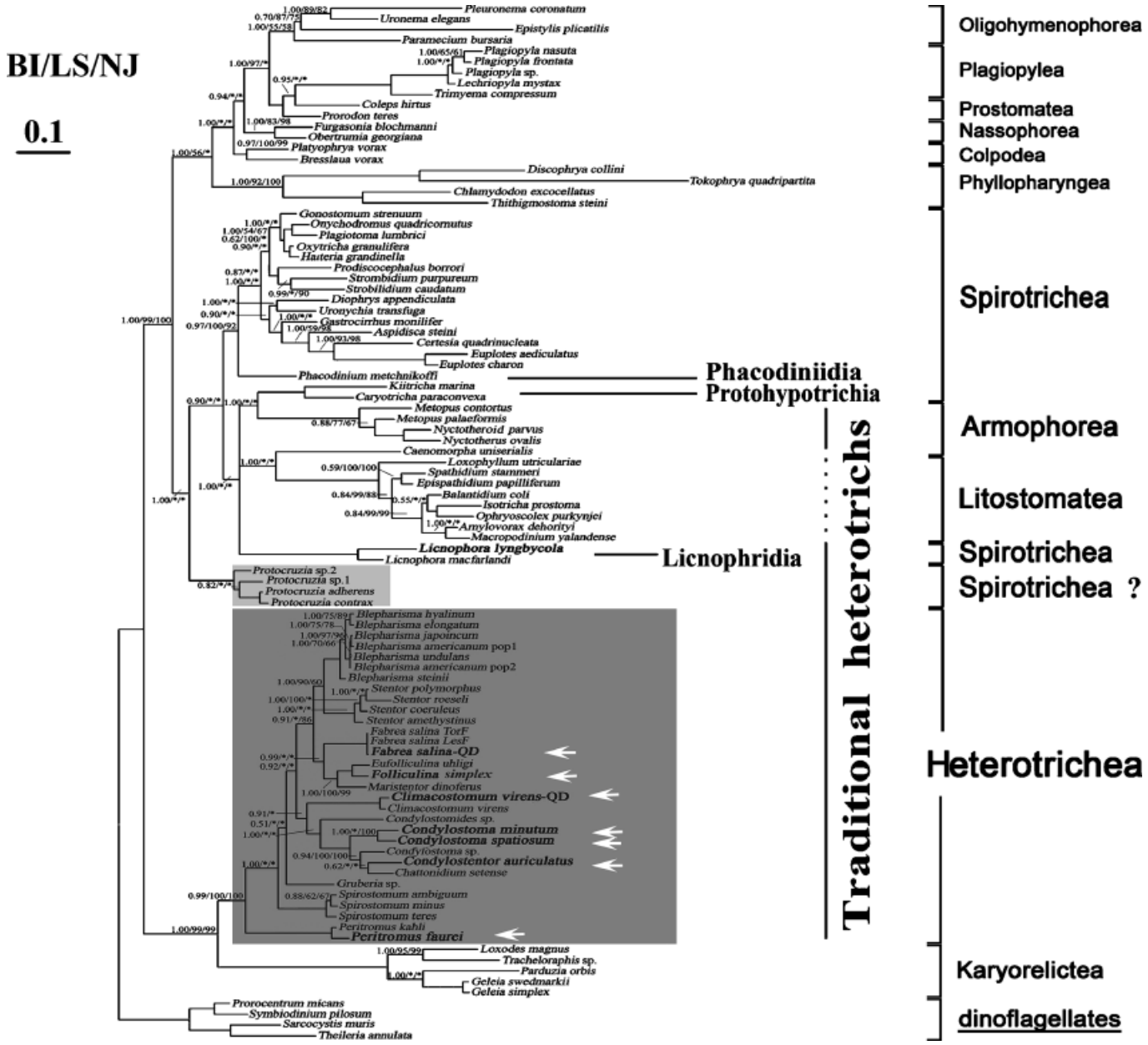

Fig. **20.** Bayesian tree inferred from complete sequences of small subunit (SSU) rRNA. The first of the three numbers at the nodes represents the Bayesian posterior probability values; the second and third numbers represent bootstrap values for the LS and NJ analyses, respectively, derived from 1,000 replicates. Asterisks at nodes indicate bootstrap values $< 50\%$ and disagreement between the reference Bayesian tree and another method of inference at a given node. The average number of nucleotide substitutions between two nodes is represented by length of the branch between them; the scale bar equals 0.1 nucleotide substitutions per site. New sequences added by the present study are in bold type. Nodes without numbers have values of 1.00/100/100. LS, least squares; NJ, neighbor-joining.

ciliature as heterotrichs (Corliss 1979; Jankowski 1980; Small and Lynn 1981), but this assemblage has been divided recently into two main lineages based on new information from biological as well as molecular characters (Lynn and Strüder-Kypke 2002; Schmidt et al. 2007). According to this new understanding, many ''traditional'' heterotrichs have been removed from the class Heterotrichea. For example, the well-known genera *Metopus* and *Nyctotherus* were assigned to the class Armophorea (Lynn 2004), *Licnophora* was placed into Spirotrichea as the subclass Licnophoridia (Lynn and Strüder-Kypke 2002), and *Plagiotoma* was revealed to be a member of Spirotrichea (Affa'a et al. 2004).

When the secondary structure of the V2 region was constructed for ''traditional'' heterotrichs, three lineages were recognized with the following distinguishing features: Helix E10-1 with one bulge vs. Helix E10-1 with two to three bulges or branched (''true'' heterotrichs vs. *Protocruzia–Phacodinium–Plagiotoma*); large bulge at the end of Helix 10 vs. small bulge at the end of Helix 10 (armophoreans vs. ''true'' heterotrichs except *Peritromus*); short Helix 11 with two to nine deletions vs. long Helix 11 (''true'' heterotrichs vs. *Protocruzia–Phacodinium–Plagiotoma–Licnophora*).

We confirmed the research of others that ''traditional'' heterotrichs are a polyphyletic assemblage of four major clades:

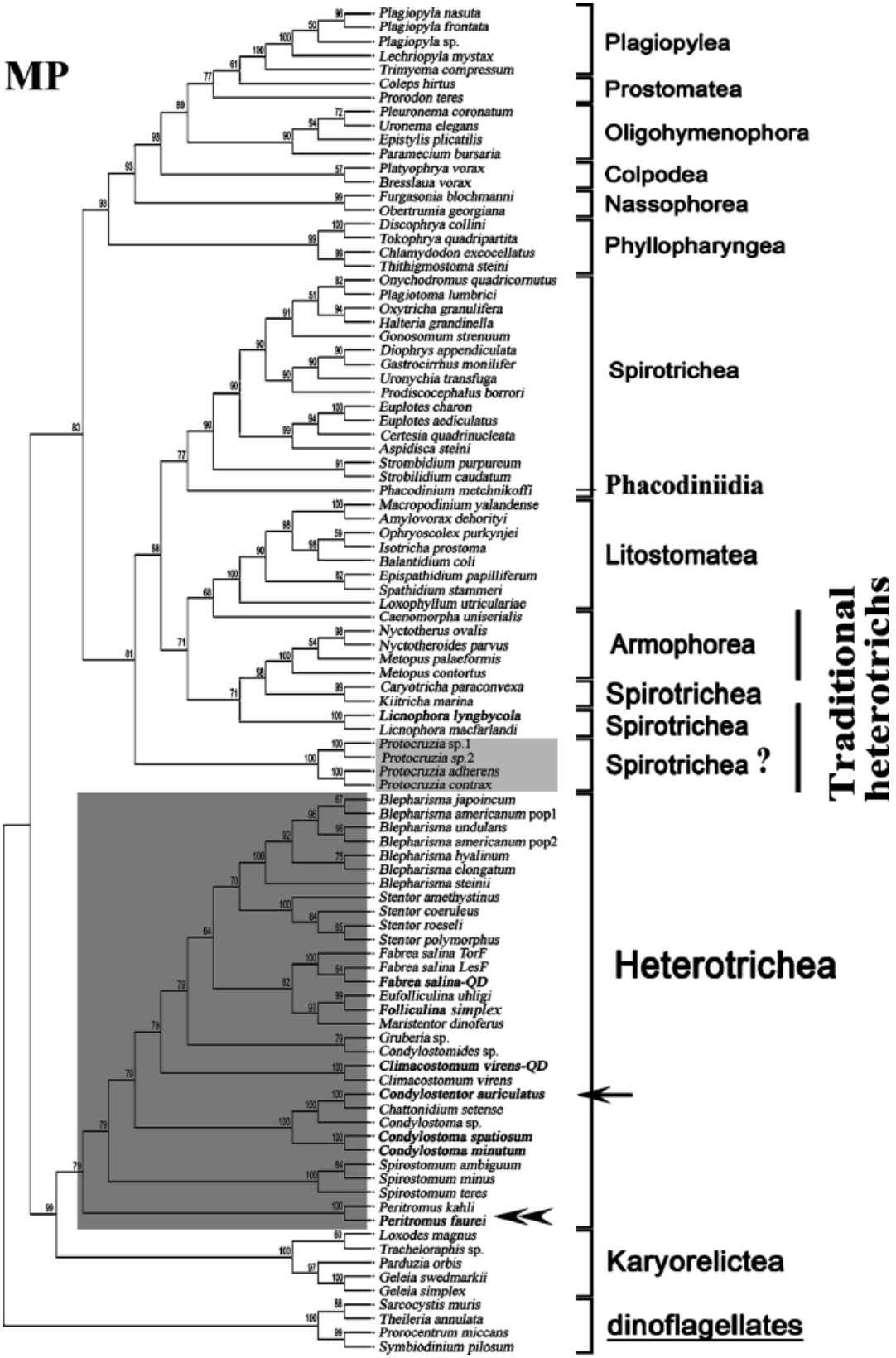

Fig. **21.** A maximum-parsimony tree inferred from complete sequences of the small subunit (SSU) rRNA. Numbers at nodes represent bootstrap values derived from 1,000 replicates. Nodes that are not supported by more than 50% bootstrap proportions are labeled with asterisks. New sequences added by the present study are in bold type.

armophoreans, licnophorids, protocruziids, and the ''true'' heterotrichs (the class Heterotrichea) (Lynn 2008). In our analyses, protocruziids are a basal clade of spirotrichs affiliated with two other clades—the subclass Licnophoridia and the class Armophorea—supported by a posterior probability exceeding 90% in Bayesian analyses and high bootstrap values in MP and NJ analyses.

Currently, *Licnophora* is placed in the subclass Licnophoridia, which in turn has been placed in Spirotrichea, primarily on the basis of two lines of evidence—the presence of macronuclear replication bands and the SSU rRNA gene sequence (Lynn and Strüder-Kypke 2002). The density of species in a clade can stabilize that clade's position in the topology; therefore, we sequenced another species of *Licnophora* to assess whether the association with the spirotrichs was confirmed. An interspecific divergence of 152 nt was revealed between these two species of *Licnophora*. This genus, as a basal clade, had an affiliation with the classes Spirotrichea (i.e. Protohypotrichia) and Armophorea, with a posterior probability exceeding 90% in our Bayesian analysis and moderate bootstrap value in parsimonous analysis. However, the branching pattern of the genus *Licnophora* obtained from LS/NJ analysis did not show a common topology with BI tree. For a more reliable phylogenetic analysis the number of sequenced species must be enlarged. Thus, our work supports a conclusion that *Licnophora*, indeed, should be taken out of the Heterotrichea, but it would be a highly divergent and peripheral branch if placed into the Spirotrichea. Therefore, additional analysis is necessary to resolve its position.

Phylogenetic position of *Peritromus*. Lynn (2008) assigned the rarely investigated genus *Peritromus* to its own family based only on morphological data. Rosati et al. (2004) argued that the dorso-ventral differentiation of *Peritromus* is a convergent characteristic and hypothesized that it is a true member of the class Heterotrichea, which diverged early from ''true'' heterotrichs. This was supported by the morphological and molecular analysis of Modeo et al. (2006), and very recently, Schmidt et al. (2007) noticed an association of *Peritromus* with *Chattonidium* and *Condylostoma* in some molecular trees.

We obtained a sequence from another species of *Peritromus*, which showed 48 nt difference from *Peritromus kahli*. Nevertheless, adding just one more species of *Peritromus* to analyses resulted in trees with consistent topology in which *Peritromus* branches off as a well-supported clade basal to all other ''true'' heterotrichs. However, *Peritromus* showed the features of ''true'' heterotrichs in the secondary structure of its SSU rRNA gene: one bulge in Helix E10-1 and relatively short Helix 11, although it did show the larger loop in Helix 10, similar to that of armophoreans.

Phylogenetic position of *Condylostentor*. *Condylostentor auriculatus* is a poorly known species, which was originally regarded as a species of *Stentor* Kahl, 1932. After several reinvestigations (Fauré-Fremiet 1936; Foissner and Wölfl 1994; Jankowski 1980; Schmidt et al. 2007), Chen et al. (2007) carried out silver staining as well as living observations, redefined the genus *Condylostentor*, and revealed that it can be clearly separated from *Stentor* by the presence of a dominant buccal cavity and from *Condylostoma* by its distinctive oral ciliature.

In our Bayesian trees, *Condylostentor* and *Chattonidium* grouped together into a small clade with moderate support (0.62BI) that then clustered with species of *Condylostoma*, *Condylostomides*, and *Climacostomum* in a larger, well-supported clade (0.91BI) that was invariably separated from another large clade that contained *Stentor* and its closer relatives (e.g. folliculinids, *Fabrea*, and *Blepharisma*). Furthermore, BI and MP trees with *Condylostentor* grouped with *Chattonidium* scored significantly better in the Shimodaria-Hasegawa test than one in which *Condylostentor* was grouped with species of *Stentor* or *Condylostoma*, respectively (P value <0.05). This finding is also consistent with morphological features as the clade containing *Condylostoma* and *Chattonidium* presents the similar organization of ciliary structures. All of this evidence leads to a firm conclusion that *Condylostentor* is closely related to *Chattonidium* and together possibly derived from *Condylostoma*, despite the similarity of its oral ciliature to that of *Stentor*.

Phylogenetic position of *Folliculina*. Folliculinids are a uniquely differentiated, clearly defined group (Corliss 1979) that consists of mostly loricate species possessing a conspicuously expanded peristome with prominent ''wings.'' In our analyses, *Folliculina producta* formed a close, strongly supported relationship to *Eufolliculina* (1.00BI, 99%MP, 100%LS, 100%NJ), which then clustered with the *Stentor*-like genus *Maristentor*. These topologies are thus in good agreement with and support the results of some recent works (Miao et al. 2005; Schmidt et al. 2007). Furthermore, both our Bayesian and MP trees show *Maristentor* as basal to folliculinids. This argues against *Maristentor* being a secondarily aloricate form derived from folliculinids, one of the possible hypotheses presented by Miao et al. (2005). More evidence is needed to clarify the relationship of *Maristentor* to other heterotrichs; however, our results indicate that it may be an offshoot of the line leading to folliculinids.

Phylogenetic position of *Climacostomum*. Though the genera *Climacostomum* and *Fabrea* were placed together in the family Climacostomidae by Repak (1972), *Climacostomum* differs conspicuously from *Fabrea* in many morphological characters (e.g. the somatic ciliature, oral cavity, cytopharynx). Neither our analyses nor those of Schmidt et al. (2007) clustered *Climacostomum* spp. with *Fabrea* spp. Schmidt et al. (2007) found the cluster comprising *Climacostomum* and *Spirostomum* in the Bayesian tree, while all other analyses placed *Climacostomum* as an isolated branch. When we included more species, we demonstrated a close relationship of *Climacostomum* to Condylostomatidae as well as Chattonidiidae with modest support demonstrated only by Bayesian analyses. There is morphological support for this as *Climacostomum* has prominent, *Condylostoma*-like frontal cirri.

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