

Short communication

Reconsideration of the phylogenetic positions of three stichotrichous genera *Holosticha*, *Anteholosticha* and *Pseudokeronopsis* (Spirotrichea: Ciliophora) inferred from complete SSU rRNA gene sequences

Lifang Li^{a,b,c}, Weibo Song^{b,*}, Mann Kyoon Shin^c, Alan Warren^d, Khaled A.S. Al-Rasheid^e, Abdulaziz A. Al-Khedhairi^e, Saud Al-Arifi^e

^a Marine College, Shandong University at Weihai, Weihai 264209, China

^b Laboratory of Protozoology, KLM, Ocean University of China, Qingdao 266003, China

^c Department of Biology, University of Ulsan, Ulsan 680-749, Korea

^d Department of Zoology, The Natural History Museum, Cromwell Road, London SW7 5BD, UK

^e Zoology Department, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia

Received 13 May 2008; received in revised form 18 June 2008; accepted 20 June 2008

Abstract

The small subunit rRNA (SSU rRNA) gene for six marine stichotrichs, *Pseudokeronopsis carnea*, *P. flava*, *Holosticha heterofoissneri*, *H. diademata*, *H. bradburyae* and *Anteholosticha manca*, was sequenced and characterized. Using this molecular information, the phylogenetic positions of three related genera, *Pseudokeronopsis*, *Holosticha* and *Anteholosticha* were determined. The results indicate that the family Urostylidae is clearly separated from the family Pseudokeronopsidae. The present phylogenetic analyses unambiguously placed *Pseudokeronopsis* close to *Holosticha*, and support the conclusion that these genera should be regarded as the members of the order Urostylidae within the stichotrich clade. Furthermore, the results of this study also support the monophyly of the genus *Pseudokeronopsis*, the redefinition of *Holosticha* s. str. and the placement of *Anteholosticha* in a clade separate from *Holosticha*.

© 2009 National Natural Science Foundation of China and Chinese Academy of Sciences. Published by Elsevier Limited and Science in China Press. All rights reserved.

Keywords: Urostylidae; Clade; Evolution; Taxonomy

1. Introduction

Stichotrichous ciliates are commonly found in marine, limnetic and terrestrial biotopes. Studies on these have concentrated mainly on their morphology, ecology, taxonomy and systematic classification. A large part of the remaining spirotrichs comprises the hypotrichs (s. l.), which were split, mainly according to DNA sequence data [1,2], into hypotrichs s. str. (the former euplotids) and stichotrichs (all other former hypotrichs). The taxonomy and phylog-

eny of the subclass Stichotrichia are, however, among the most confused within the phylum Ciliophora, mainly due to the paucity of morphological and morphogenetic data [3]. The genera *Pseudokeronopsis* and *Holosticha* (s. l.) are highly and particularly differentiated at the intrageneric level, which indicates that they might be pivotal organisms in terms of their phylogenetic positions [4–7].

Sequence information from homologous macromolecules shared by all members of a group can be used to measure the extent of genetic relationships among them [8]. The evolutionary relationships of and within the class Spirotrichea based on the molecular data have been discussed by several authors [9–11]. These reports mainly focused on order level and higher. Interesting information related to

* Corresponding author. Tel./fax: +86 532 8203 2283.
E-mail address: wsong@ouc.edu.cn (W. Song).

lower level taxa within the group (e.g. the composition and the branching orders of families and genera) is, however, now being generated as the molecular data increase.

We have sequenced the SSU rRNA gene for six marine stichotrichous ciliates, *Pseudokeronopsis carnea*, *P. flava*, *Holosticha heterofoissneri*, *H. bradburyae*, *H. diademata* and *Anteholosticha manca*. The main aims of this study are to determine the systematic positions of these taxa and to enhance our understanding of the phylogenetic relationships within the subclass Stichotrichia.

2. Materials and methods

2.1. Ciliate collection and identification

All specimens used are from the culture store of the Laboratory of Protozoology (OUC), which were isolated from the coastal waters near Qingdao, China. Clonal cultures were established and maintained in autoclaved marine water with the appropriate prey at room temperature. Identification was performed according to Song and Wilbert [12].

2.2. DNA extraction, PCR and cloning

Cells were rinsed three times with sterile artificial marine water after being starved overnight and then pelleted by centrifugation. Genomic DNA was extracted as described previously [13]. A 1:1 mixture of the regular DNA polymerase (Promega, USA) and the *Pfu* Taq DNA polymerase (High Fidelity, Sangon, Canada) was utilized for the PCR [3]. Oligonucleotide primer sequences used were 16s-like F: 5'-AACCTGGTTGATCCT GCC AGT-3' and 16s-like R: 5'-T GATCCTTCTGCAGGTTACC TAC-3'.

The amplified products were purified (UNIQ-5 DNA Cleaning Kit, Sangon, Canada), inserted into a pUCm-T vector (Sangon, Canada) and sequenced on an ABI Prism 377 automated DNA sequencer. Subsequent sequencing was performed using primer walking.

2.3. Sequence availability and phylogenetic analysis

The nucleotide sequences referred to in this work are available from the GenBank/EMBL databases. A karyorelictid ciliate, *Loxodes magnus* L31519, was selected as the outgroup species. All methods used for phylogenetic analyses were performed as previously described [13].

3. Results

3.1. Sequences and comparisons

The SSU rRNA gene sequences of six stichotrichs were submitted to the GenBank database. Their length, GC content (%) and the accession numbers are as follows: *Pseudokeronopsis carnea*, 1770 bp, 44.35%, AY881633; *P. flava*, 1770 bp, 44.69%, AY881634; *Holosticha heterofoissneri*, 1776 bp, 45.49%, DQ059582; *H. bradburyae*, 1783 bp,

44.97%, EF123706; *H. diademata*, 1784 bp, 45.74%, DQ059583, and *Anteholosticha manca*, 1772 bp, 43.9%, DQ503578. Alignment of these sequences clearly showed similarities in the primary structure and GC content of the SSU rRNA gene in stichotrichs to other Spirotrichous ciliates. The GC contents are in the same range as in other ciliates [3,13,14].

The sequence of *P. carnea* differed in 35 nucleotides from the sequence of *P. flava* (structural similarity 98%). Seventy-one sites are different between *H. heterofoissneri* and *H. diademata* (structural similarity 96%); 104 sites are different between *H. heterofoissneri* and *H. bradburyae* (structural similarity 94%); 52 sites are different between *H. bradburyae* and *H. diademata* (structural similarity 97%); 110 sites are different between *A. manca* and *A. multistylata* (structural similarity 93%). The structural similarities were 89–91% between *Pseudokeronopsis* and *Holosticha* specimens; 88–92% between *Anteholosticha* and *Holosticha* specimens; and 90–92% between *Pseudokeronopsis* and *Anteholosticha* specimens.

3.2. Bayesian inference and distance-matrix analyses

Trees inferred from the SSU rRNA gene sequences were constructed using multiple algorithms. Both Bayesian inference and distance-matrix trees indicate that the specimens belonging to the same genus cluster together with strong bootstrap support (Fig. 1). Interestingly, *Holosticha* (s. str.) clusters with *Amphisiella*, which is consistent with the morphological data [15], and is separated from *Anteholosticha*, which clusters in a clade with *Diaxonella* and *Urostyla*. *Pseudokeronopsis* groups with *Pseudourostyla*, which branches within the stichotrich clade just after *Holosticha-Amphisiella*.

Phylogenetic analyses provide a strong bootstrap support for the monophyly of the classes Spirotrichea, Oligohymenophorea and Heterotrichea *sensu* Lynn and Small 2002 (Fig. 1). In the Bayesian tree, the subclass Hypotrichia branches first from the spirotrichean clade at a very deep level (93% Bayesian credibility). The subclass Stichotrichia is divided into two orders, Stichotrichida and Urostylida, and forms a sister group to the clade Hypotrichia (99% Bayesian credibility) (Fig. 1). However, both Stichotrichida and Urostylida seem to be paraphyletic. In the stichotrich clade, *Pseudoamphisiella* diverges first from the main group followed in turn by *Pseudokeronopsis-Pseudourostyla*, *Anteholosticha-Diaxonella-Urostyla*, *Holosticha-Amphisiella*, *Hemigastrostyla*, with the remaining stichotrich ciliates clustering together and to form a monophyletic group. Interestingly, the clade comprising *Uroleptus*, *Paruroleptus* and *Paraurostyla* is more closely related to oxytrichids (s. l.) than to other typical urostylids.

3.3. Maximum parsimony analyses

As shown in Fig. 2, the major aspects of the topology of the maximum parsimony tree (MP) are generally similar to

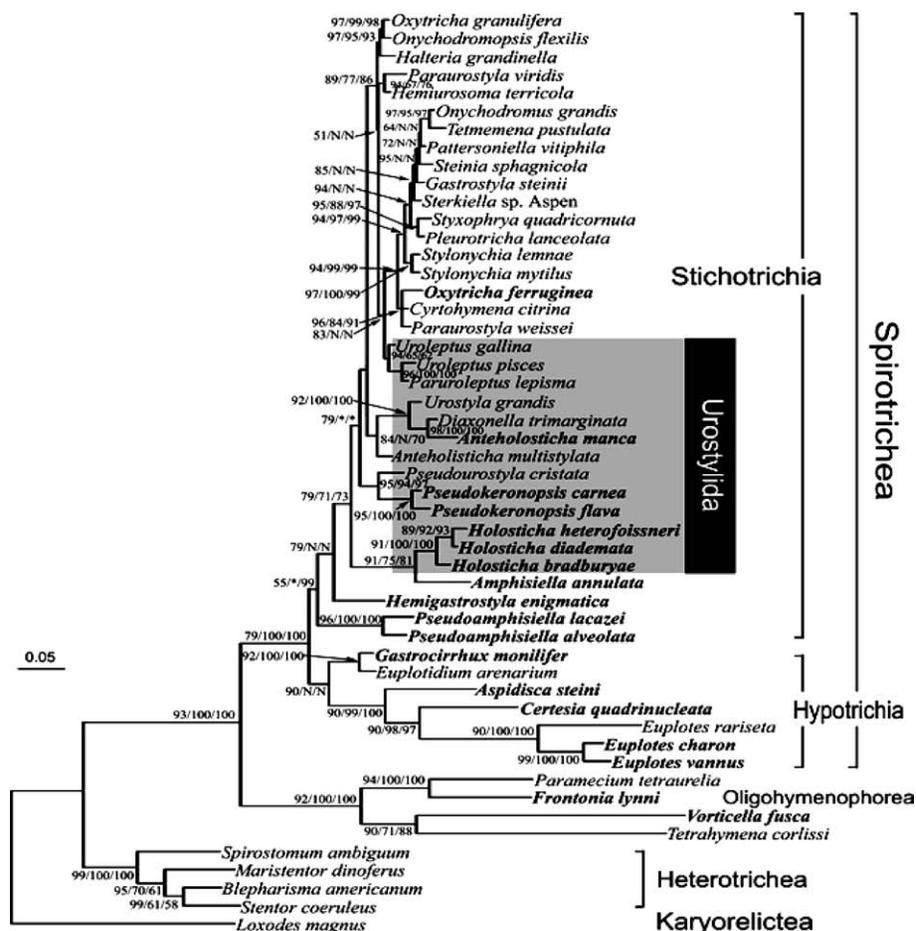


Fig. 1. Bayesian tree inferred from the nucleotide sequences of complete SSU rRNA of ciliated protozoa. Numbers on branches indicate support indices from 1000 bootstrap estimations using each of the algorithms in the following order: Bayesian credibility value using the MrBayes; distance-matrix based least-squares (LS); neighbor joining (NJ) bootstrap percentage using the Phylip package. Asterisks indicate bootstrap values less than 50%. “N” reflects disagreement between a method and the reference Bayesian tree at a given node. Evolutionary distance is represented by the branch length to separate the species in the figure. The scale bar corresponds to five substitutions per 100 nucleotide positions. Numbers at nodes represent bootstrap values (in %) out of 1000 replicates; first No. = Bayesian credibility, second No. = LS, third No. = NJ. The taxa in bold typeface were sequenced at the Laboratory of Protozoology, OUC.

those of the Bayesian tree (Fig. 1). For example, *Pseudokeronopsis* (represented by *P. carnea* and *P. flava*) and *Holosticha* (s. str., represented by *H. heterofoissneri*, *H. bradburyae* and *H. diademata*) cluster together in the subclass Stichotrichia, while *Holosticha* clusters with *Amphisiella* and is separated from *Anteholosticha*.

4. Discussion

As demonstrated in this work and elsewhere [1,2], each of the classes Spirotrichea, Oligohymenophorea, and Heterotrichia is confirmed as monophyletic groups based on SSU rRNA gene sequences. Furthermore, our analyses also support the monophyly of the subclass Stichotrichia *sensu* Lynn and Small. Stichotrichia comprises four orders: Plagiotomida, Sporadotrichida, Urostylida and Stichotrichida. Only representatives of the latter two were included in this study, both of which were found to be polyphyletic, as demonstrated by some recent investigations and the systematic relationship among these highly diverse taxa needs

further re-estimation and reevaluation (Shao et al. unpublished, personal communication).

The genus *Pseudokeronopsis*, which was erected by Borror and Wicklow [16], is characterized by its ciliature that is of the *Holosticha*-pattern apart from the frontal cirri, which form the so-called “bicolorona”, i.e. comprising two curved and parallel rows of frontal cirri that are continuous posteriorly with the midventral complex (=midventral rows) [7,16]. Our results indicate that the family Urostylidae (including the subfamily Holostichinae) is clearly separated from the family Pseudokeronopsidae, as suggested by Borror and Wicklow [16]. Furthermore, our analysis unambiguously places *Pseudokeronopsis* close to *Holosticha*, which supports the conclusion that these genera should be regarded as the members of the order Urostylida within the stichotrich clade; and confirms the monophyly of the genus *Pseudokeronopsis*.

Holosticha Wrzesniowski, 1877 is a large genus within the subclass Stichotrichia. It has long been assumed that *Holosticha* (s. l.) is monophyletic because its members share

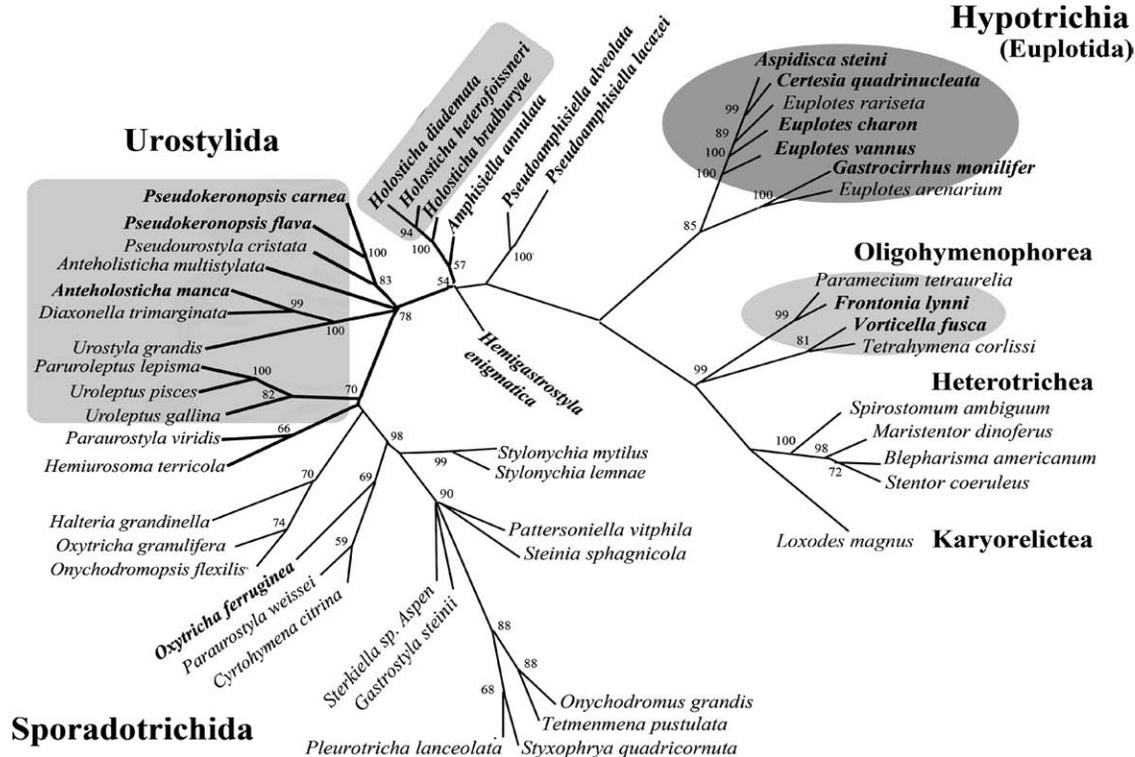


Fig. 2. Maximum Parsimony (MP) tree of the ciliated protozoa. The numbers at the forks show the percentage of times which occurred out of the 1000 trees. The taxa in bold typeface were sequenced at the Laboratory of Protozoology, OUC.

very similar patterns of infraciliature. Furthermore, it is generally considered that most hypotrichs (s. l.) share a similar pattern of morphogenesis at the generic level [4].

Species within the genus *Holosticha*, however, exhibit a range of morphogenetic processes, which led to the suggestion that these taxa may have evolved from different lineages and that *Holosticha* is therefore not monophyletic [5]. In his review of the genus, Berger [17] recognized only seven species of *Holosticha*, these being unified by a highly characteristic combination of synapomorphies (i.e. anterior end of the left marginal row curved rightwards, adoral zone bipartite, buccal cirrus distinctly anterior of the paroral) and plesiomorphies. The seven species are: *H. bradburyae* Gong et al., 2000; *H. diademata* (Rees, 1884) Kahl, 1932; *H. foissneri* Petz et al., 1995; *H. gibba* (Müller, 1786) Wrzesniowski, 1877; *H. heterofoissneri* Hu and Song, 2001; *H. pullaster* (Müller, 1773) Foissner et al., 1991; *H. spindleri* Petz et al., 1995. All other species formerly included in *Holosticha* were assigned to the genera *Anteholosticha* (29 species), *Caudiholosticha* (10 species), and *Biholosticha* (2 species) [8]. This work supports this arrangement in the sense that (1) *H. bradburyae*, *H. diademata* and *H. heterofoissneri* clustered together; (2) *Holosticha* was confirmed as being monophyletic; and (3) *Anteholosticha* clustered separately from the *Holosticha* clade. More molecular data, including SSU rRNA gene sequences for related genera (e.g. *Caudiholosticha*, *Biholosticha*), are needed to ascertain the precise relationships within the Holostichidae.

Moreover, details of the deep branching pattern within the order Stichotrichida still remain unresolved. In order

to determine these relationships, more molecular data are required, including complete sequences of the large subunit ribosomal RNA and sequences of protein genes such as tubulins and DNA-dependent RNA polymerase.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant Nos. 30670280 and 40676076), and partially supported by the Center of Excellence in Biodiversity Research and the DNA Research Chair, King Saud University. We gratefully acknowledge the help of Dr. David McL. Roberts (Natural History Museum, London) whose comments substantially improved the manuscript.

References

- [1] Lynn DH, Small EB. A revised classification of the phylum Ciliophora Doflein 1901. *Rev Soc Mex Hist Nat* 1997;47:65–78.
- [2] Lynn DH, Small EB. Phylum Ciliophora Doflein, 1901. In: An illustrated guide to the protozoa. Lawrence, Kansas: Society of protozoologists; 2002. p. 371–656.
- [3] Chen Z, Song W. Phylogenetic positions of *Aspidisca steini* and *Euplotes vannus* within the order Euplotida (Hypotrichia: Ciliophora) inferred from complete small subunit ribosomal RNA gene sequences. *Acta Protozool* 2002;41:1–9.
- [4] Hu X, Song W. On morphology of the marine hypotrichous ciliate, *Holosticha diademata* (Ciliophora, Hypotrichida), with comparison of its related species. *J Ocean Univ Qingdao* 1999;29:469–73, (in Chinese).
- [5] Hu X, Song W. Morphology and morphogenesis of *Holosticha heterofoissneri* nov. spec. from the Yellow Sea, China (Ciliophora, Hypotrichida). *Hydrobiologia* 2001;448:171–9.

- [6] Hu X, Song W, Suzuki T. Morphogenesis of *Holosticha bradburyae* (Protozoa, Ciliophora) during asexual reproduction cycle. *Europ J Protistol* 2003;39:173–81.
- [7] Song W, Warren A, Roberts D, et al. Comparison and redefinition of four marine, coloured *Pseudokeronopsis* spp. (Ciliophora, Hypotrichida), with emphasis on their living morphology. *Acta Protozool* 2006;45:271–87.
- [8] Zuckerlandl E, Pauling L. Molecules as documents of evolutionary history. *J Theor Biol* 1965;8:357–66.
- [9] Schlegel M, Elwood HJ, Sogin ML. Molecular evolution in hypotrichous ciliates: sequence of the small subunit RNA genes from *Onychodromus quadricornutus* and *Oxytricha granulifera* (Oxytrichidae, Hypotrichida, Ciliophora). *J Mol Evol* 1991;32:64–9.
- [10] Shin MK, Hwang UW, Kim W, et al. Phylogenetic position of the ciliates *Phacodinium* (order Phacodiniida) and *Protocruzia* (subclass Protocruziidia) and systematics of the spirotrich ciliates examined by small subunit ribosomal RNA gene sequences. *Europ J Protistol* 2000;36:293–302.
- [11] Bernhard D, Stechmann A, Foissner W, et al. Phylogenetic relationships within the class Spirotrichea (Ciliophora) inferred from small subunit rRNA gene sequences. *Molec Phylogen Evol* 2001;21:86–92.
- [12] Song W, Wilbert N. Morphological studies on some free living ciliates (Ciliophora: Heterotrichida, Hypotrichida) from marine biotopes in Qingdao, China, with descriptions of three new species: *Holosticha warreni* nov. spec. *Tachysoma ovata* nov. spec. and *T. dragescoi* nov. spec. *Europ J Protistol* 1997;33:48–62.
- [13] Li L, Song W. Phylogenetic position of the marine ciliate, *Certesias quadrimucleata* (Ciliophora; Hypotrichia; Hypotrichida) inferred from the complete small subunit ribosomal RNA gene sequence. *Europ J Protistol* 2006;42:55–61.
- [14] Shang H, Song W, Warren A. Phylogenetic positions of two ciliates, *Paranophrys magna* and *Mesanophrys carcini* (Protozoa, Ciliophora, Oligohymenophorea), within the subclass Scuticociliatia inferred from complete small subunit rDNA gene sequence. *Acta Protozool* 2003;42:171–81.
- [15] Corliss JO. The ciliated Protozoa: characterization, classification and guide to the literature. 2nd ed. Oxford: Pergamon Press; 1979.
- [16] Borror AC, Wicklow BJ. The suborder Urostyliina Jankowski (Ciliophora, Hypotrichida): morphology, systematics and identification of species. *Acta Protozool* 1983;22:97–126.
- [17] Berger H. Redefinition of *Holosticha Wrzesniowski, 1877* (Ciliophora, Hypotricha). *Europ J Protistol* 2003;39:373–9.