Zoological Systematics, 40(1): 31–40 (January 2015), DOI: 10.11865/zs.20150102

ORIGINAL ARTICLE

Molecular data suggests the ciliate *Mesodinium* (Protista: Ciliophora) might represent an undescribed taxon at class level

Xiao Chen[1, 2], Hong-Gang Ma[1], Khaled A.S. Al-Rasheid[3], Miao Miao[2 *]

[1] *Institute of Evolution & Marine Biodiversity, Ocean University of China, Qingdao 266100, China*
[2] *College of Life Sciences, University of Chinese Academy of Sciences, Beijing 100049, China*
[3] *Zoology Department, College of Science, King Saud University, Riyadh 11451, Saudi Arabia*
[*] *Corresponding author, E-mail: doublemiao@126.com*

Abstract The well-known ciliate, *Mesodinium* Stein, 1863, is of great importance to marine microbial food webs and is related to the "red tides". However, it is possibly one of the most confusing ciliate taxa in terms of its systematic position: either the morphological or the molecular data excluded it from all the other known assemblages or groups. In the current work, the sequences of small subunit ribosomal RNA (SSU rRNA) genes for all isolates available are analysed and an examination of the secondary structure patterns of related groups is carried out. The results indicate that (1) *Mesodinium* invariably represents a completely separated and isolated clade positioned between two subphyla of ciliates with very deep branching, which indicates that they should be a primitive or ancestral group for the subphylum Intramacronucleata; (2) the secondary structure of the SSU rRNA of *Mesodinium* species is unusual in that, while the secondary structure of V4 in *Mesodinium* sp. has the deletions common to all litostome ciliates, it has more extensive deletions in helix E23_8 and a longer helix E23_1; (3) combining the phylogenetic and morphological information, we suggest establishing Mesodiniea **cl. nov.** , including the order Mesodiniida Grain, 1994, belonging to the subphylum Intramacronucleata.

Key words Ciliophora, *Mesodinium*, phylogenetic analysis, secondary structure, SSU rRNA.

1 Introduction

The *Mesodinium*-complex plays a unique role in marine microbial food webs as a fast-swimming primary producer (Lynn, 2008). They are partly or entirely autotrophic and can reach high densities under "normal" conditions in coastal waters, sufficient to produce "red tides" (Johnson *et al.*, 2004). Furthermore, most species of the genus *Mesodinium* contains cryptophyceae endosymbiont and the organelles of fourteen microtubules, both of which make them an organism of unusual evolutionary significance (Gustafson *et al.*, 2000; Lindholm *et al.*, 1988; Moestrup *et al.*, 2012). The morphological characters of these species remain confusing, however, due to incomplete or incorrect descriptions of the infraciliature and silverline system (Strüder-Kypke *et al.*, 2006). In the latest classification system of Ciliophora, *Mesodinium*-complex occupies an ambiguous phylogenetic position and is usually regarded as one cluster of Litostomatea (Adl *et al.*, 2012; Lynn,

urn:lsid:zoobank.org:pub:792F41B5-5288-4B8E-B495-C4A59233CD0B
Received 12 July 2014, accepted 8 January 2015
© *Zoological Systematics*, 40(1): 31–40

2008). Recently, partial sequences of the small subunit ribosomal RNA (SSU rRNA) gene for isolated cells of *Mesodinium* have also been obtained, and the unreliable nature of the derived position is likely to be due to long branch attraction near the base of the trees (Strüder-Kypke *et al.*, 2006).

In recent years, the small subunit rRNA (SSU rRNA) genes have been widely used in investigations of molecular phylogeny and evolution among ciliates (Dunthorn *et al.*, 2011; Gao *et al.*, 2012a; Gao *et al.*, 2012b; Gong *et al.*, 2009; Quintela-Alonso *et al.*, 2011; Zhang *et al.*, 2012; Zhang *et al.*, 2014; Zoller *et al.*, 2012). Since the general structure of rRNA is universally conserved across all taxa that have been examined, the secondary structure of rRNA, even when not universally identical across taxa, is more highly conserved than primary nucleotides (Wuyts *et al.*, 2004). Structural changes of SSU rRNA variable regions have been used for more and more studies and have appeared to be phylogenetically informative at lower taxa (above genus) as well as higher levels (above order) (Hwang and Kim, 1999; Vogler and Pearson, 1996).

Here, we are interested in (1) conducting a detailed examination of the secondary structure in the unusual SSU rRNA of *Mesodinium* species; (2) the phylogenetic implications of the debate as to whether *Mesodinium* is a primitive early branching genus during the evolution of the ciliates.

2 Materials and methods

2.1 Biological material and DNA extractions

Mesodinium sp. (Fig. 1) was collected from the coast of Qingdao, China. Organisms were isolated under a dissecting microscope using glass micropipettes. Whole genomic DNA was extracted from cells using REDExtract-N-Amp Tissue PCR Kit (Sigma, St. Louis, USA) using the method described by the manufacturer's protocol, with some modifications (Huang *et al.*, 2012).

2.2 Amplification and sequencing of the SSU rRNA genes

SSU rRNA coding regions were amplified in PCR using two primers, EukA and EukB, which are complementary to the 5' and 3' termini of eukaryotic 16S-like rRNA genes (Medlin *et al.*, 1988). The polymerase chain reaction (PCR) followed the protocol of Miao *et al.* (2011). The full-length product of the amplification was purified by agarose gel electrophoresis, cloned into the pUCm-T vector (Sangon. Toronto, ON, Canada) and sequenced on both strands by the Takara sequencing facility in Shanghai, China.

2.3 Alignment, secondary structure prediction and analysis of structural elements

The cloned sequence obtained from the *Mesodinium* sp. was aligned with one previously reported (GenBank accession No. FJ687221) and a variety of other *Mesodinium* sequences that have been published (Johnson *et al.*, 2004; Strüder-Kypke *et al.*, 2006). The sequences were aligned using the Clustal X multiple alignment program (Thompson *et al.*, 1997). From the resultant alignment, large indel regions were carefully searched by eye. RNA structures were decomposed into sub-structural components and their features characterized and coded using an alphanumerical format, based on the model proposed by Van de Peer and de Wachter (1997). Preliminary modelling of blocks with a high degree of positional variation by energy minimisation was carried out using the MFOLD (http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form) (Zuker, 2003). The secondary structure model of the SSU rRNA V4 region in *Mesodinium* sp. and one species from other each class/subclass (11 classes/subclasses in total) were predicted and constructed. These structures were compared with the European Ribosomal RNA database models (http://bioinformatics.psb.ugent.be/webtools/rRNA/secmodel/index.html) (Wuyts *et al.*, 2004); and manually adjusted to ensure retention of conserved core elements, taking into account predicted tertiary interaction (Alkemar & Nygard, 2004). Folding results were displayed using RnaViz2 (de Rijk *et al.*, 2003).

ML (Maximum likelihood) methods were used to construct a phylogenetic tree, using Karyorelictea as the outgroup. Modeltest (Posada & Crandall, 1998) was used to choose the model, and it found the GTR+I+G model (I=0.23, G=0.56) to be the best mathematical model.

Constraint trees were constructed using PAUP* 4.0 (Swofford, 2003). In each condition, one constraint tree was compared with one best ML tree and one hundred bootstrap trees, which were randomly picked from 1 000 ML bootstrap trees. The total of 102 trees was used to run an AUtest and the final AUtest values were calculated in Consel (Shimodaira & Hasegawa, 2001).

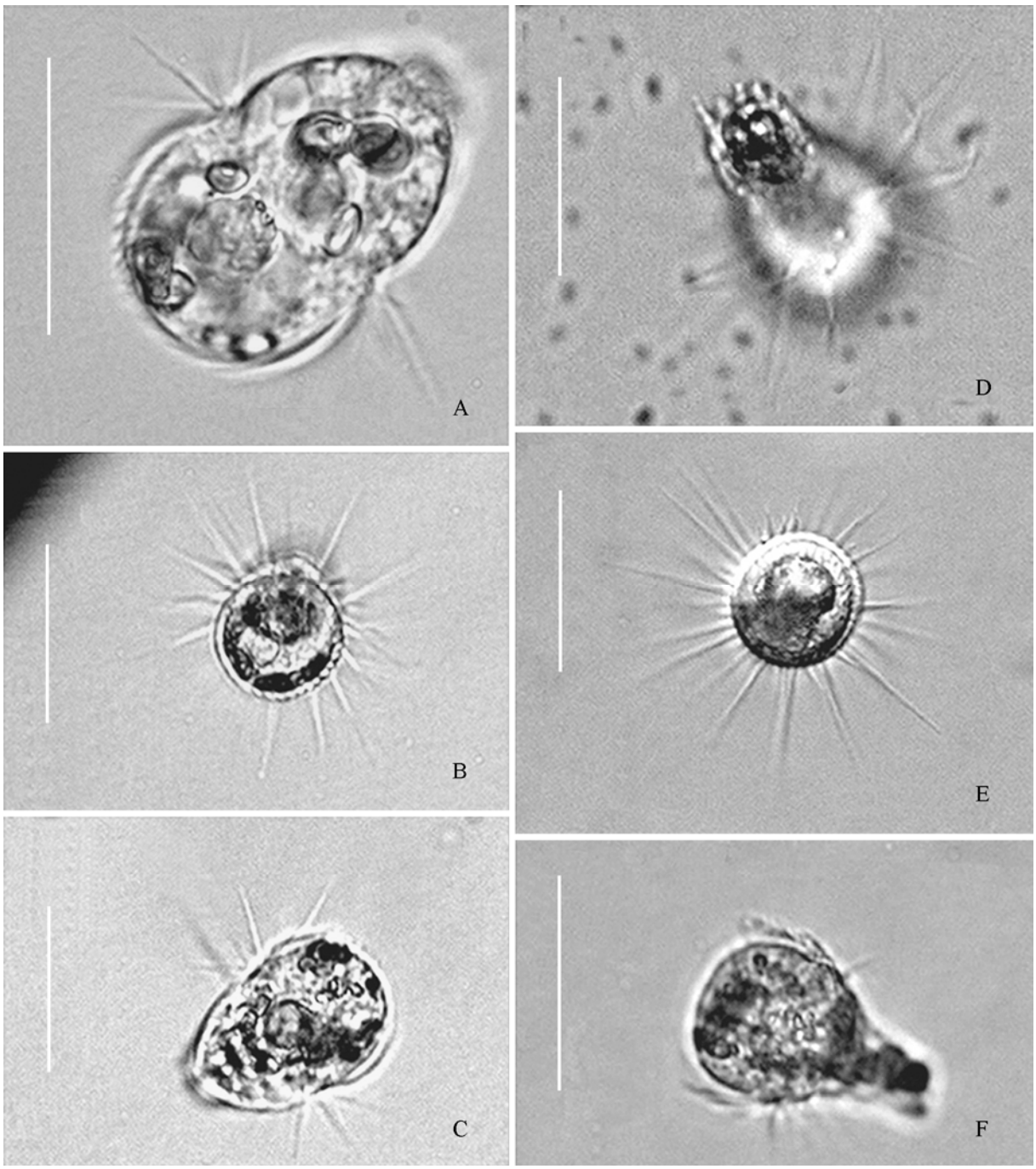

Fig. 1. Photomicrographs of *Mesodinium* sp. *in vivo*. A. Representative individual, front view. B–E. Body shape, top/bottom view. F. Lateral view. Scale bars = 20 μm.

3 Results

3.1 Primary sequence comparisons among SSU rRNAs of *Mesodinium* species

The full SSU rRNA sequence of *Mesodinium* sp., 1 595 bp, whose G+C content was 48.21%, was deposited in GenBank under Accession No. FJ687221. The SSU rRNA sequences of the six populations (*Mesodinium pulex*, *Myrionecta rubra*, *Mesodinium* sp.) were aligned and compared (GenBank accession No. see supplemental Table S1, similarity matrix data not shown). The similarity among SSU rRNA sequences of *Mesodinium* species varied from 88.4% to 99.6%.

3.2 Structural pattern in the hypervariable region

The 50 universal helices that can be distinguished are given a single number corresponding to the order of occurrence of their 5'-proximal strand (Wuyts *et al.*, 2000).

Referring to secondary structure of the SSU rRNA sequence of *Tetrahymena canadensis* which is one of the eukaryotic SSU rRNA secondary structure models in the European Ribosomal RNA database, the secondary structure of the SSU rRNA of *Mesodinium* sp. which we predicted and constructed is correspond to that of widely accepted eukaryotic SSU rRNA (Neefs *et al*., 1993; Wuyts *et al.*, 2000) with hypervariable regions peculiar to eukaryotic SSU rRNA genes (Fig. 2). But the SSU rRNA of *Mesodinium* sp. has obvious deletions in regions E23_1, E23_2 and E23_9 (Fig. 2).

Within the genus *Mesodinium*, few variations occurred in hypervariable region E23. Length and structural differences occur mainly in the variable region 4 (V4) which usually forms a complex structure in most eukaryotes whereas the corresponding area in prokaryotes is considerably shorter and forms a single hairpin (Wuyts *et al.*, 2000). The model of the secondary structure of V4 of the small subunit rRNA molecule of *Mesodinium* sp. has a similar pattern to the other 11 representative species picked from the other 11 classes/subclasses (as set out in Fig. 2). However, as Fig. 2 shows, although the secondary structure of V4 in *Mesodinium* sp. has the deletions common to all litostome ciliates (litostomes have characteristic deletions in helices E23_1 and E23_8, and lack helix E23_5 (Strüder-Kypke *et al.*, 2006; Wuyts *et al.*, 2000)), *Mesodinium* has more extensive deletions in helix E23_8 compared to *Enchelyodon* (the other Litostome studied here) and the total length of Helix E23_1 in *Mesodinium* is markedly greater than in *Enchelyodon* (36 bp versus 25 bp--compare Fig. 2).

3.3 Phylogenetic analyses

In order to compare with the system of Lynn 2008, we tried to pick one or two species from each order so as to construct a phylogenetic tree with greater resolution. 64 SSU rRNA sequences of almost all orders were downloaded from GenBank/EMBL databases to construct this tree. The results of distance analysis and MP (Maximum Parsimony) analysis indicated that the clade of *Mesodinium/Myrionecta* always clustered independently while at the same time showing an unusually long branch (MP tree not shown). Building on this initial analysis we therefore removed ambiguously aligned regions of the data set (mainly the V4 area) and proceeded with ML (Maximum Likelihood) methods (Fig. 3).

According to the ML tree, which used SSU rRNA gene sequence information, subphylum Intramacronucleata and subphylum Postciliodesmatophora, which belong to phylum Ciliophora, clustered into two independent clades. In subphylum Intramacronucleata, class Litostomatea, Colpodea, Oligohymenophorea, Plagiopylea and Protomatea were monophyletic groups. Similar results were found by recent phylogenomic analysis (Gao & Katz, 2014). The genus *Protocruzia* was the earliest branching lineage of Spirotrichea, and also of subphylum Intramacronucleata (Fig. 3).

The clade of genus *Mesodinium/Myrionecta* was located between subphylum Intramacronucleata and subphylum Postciliodesmatophora, with a low bootstrap value. *Mesodinium* sp. clustered with three populations of *Mesodinium pulex*, then clustered together with two populations of *Myrionecta rubra*, with bootstrap values higher than 98% on both occasions (Fig. 3). The results of the AUtest, however, cannot reject the hypothesis that *Mesodinium/Myrionecta* is monophyletic with various other classes in the SSU rRNA phylogenetic trees (Table 1).

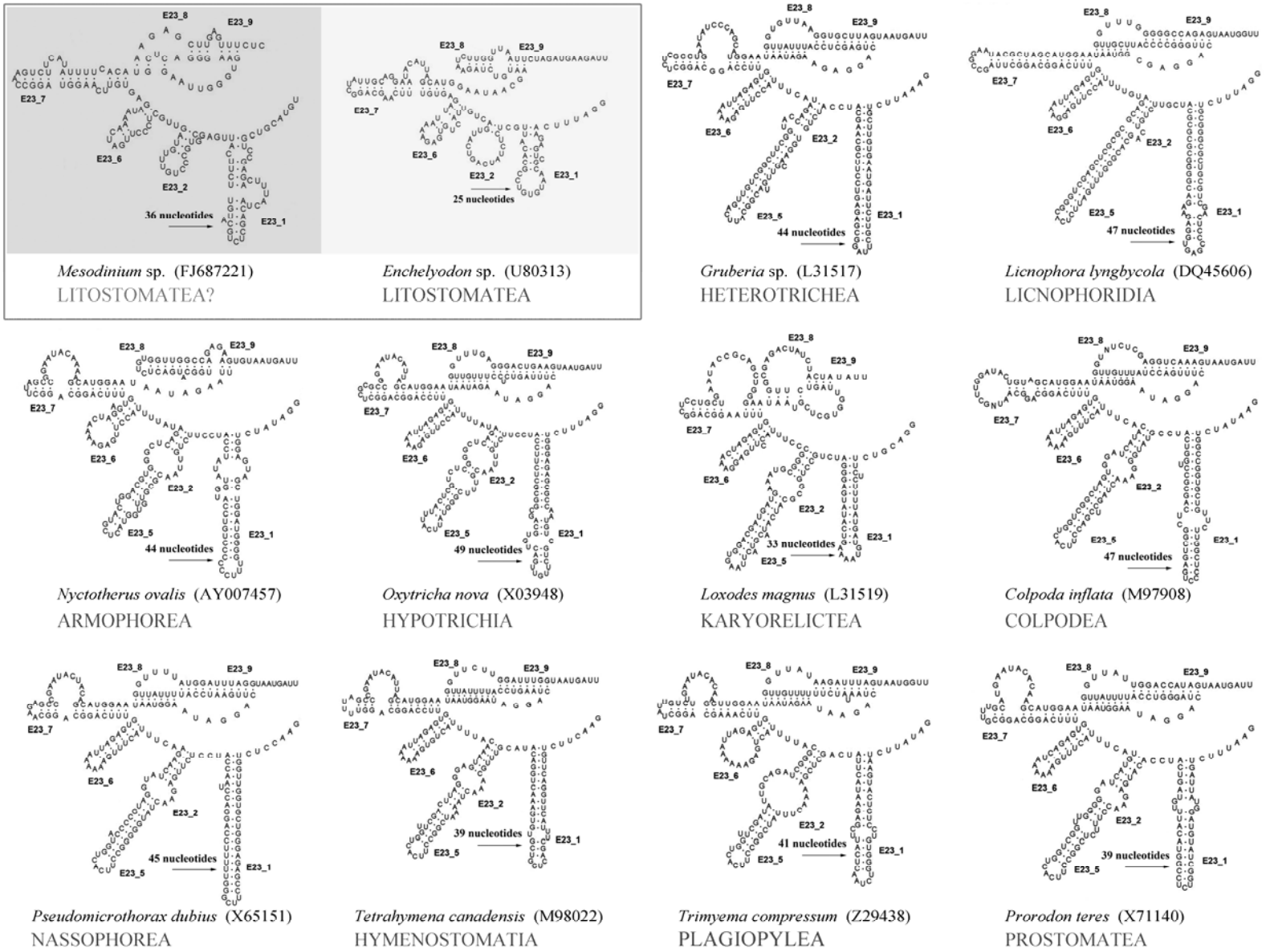

Fig. 2. Models of the secondary structure of variable region 4 (V4) of the small subunit rRNA molecule, comparing helices 23_1, 23_2, 23_5, 23_6, 23_7, 23_8, and 23_9 for the ciliate species**.** GenBank/EMBL accession numbers are enclosed in brackets. The number of nucleotides in Helix E23_1 for each species is given above the long arrow. The classes or subclasses which these species represent are marked in blue below each illustration.

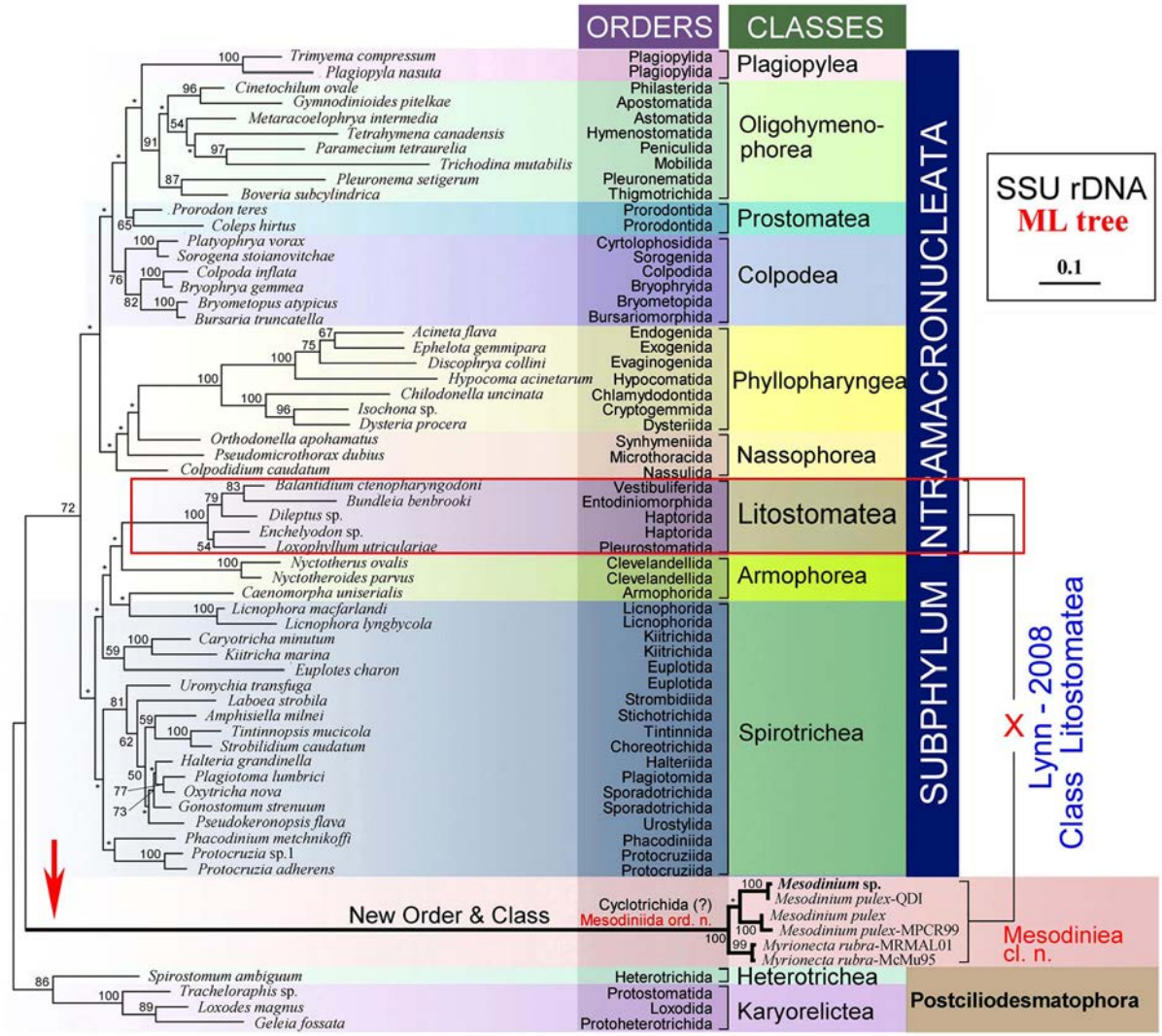

Fig. 3. The comprehensive phylogenetic tree inferred from SSU rRNA gene sequences using Maximum Likelihood analysis with the model selected by AIC in MRMODELTEST for Bayesian analysis. Numbers at the nodes represent the bootstrap percentages from 1 000 replicates for ML analysis. Asterisks indicate bootstrap values less than 50% at a given node. Evolutionary distance is represented by the branch length separating the species in the figure. The scale bar corresponds to ten substitutions per 100 nucleotide positions.

4 Discussion

4.1 Phylogenetic analyses

In this study, we have made an attempt to use four methods of phylogenetic analysis (Maximum Likelihood, Bayesian Inferences, Neighbour-Joining and Maximum Parsimony) to construct the phylogenetic trees. Regardless of which method was used, *Mesodinium*/*Myrionecta* species always clustered in a group independently, and were both separated from other ciliates and formed an unusually long branch. This phenomenon was also identified and described in the analyses of Johnson *et al.* (2004) and Strüder-Kypke *et al.* (2006). In regard to the analysis in the cases of long-branch attraction (LBA), ML outperforms MP over a wide range of conditions (Philippe *et al.*, 2005), and ML outperforms NJ under comparable conditions with a general superiority (Huelsenbeck, 1995). Long-branch attraction has been used to explain the phenomena of seemingly unrelated but

fast-evolving taxa being drawn to one another in a tree (Philippe & Laurent, 1998). In the context of the presence of long-branch attraction, therefore, this fact has a great influence on the procedure for phylogenetic analyses when we use MP and NJ methods. As a result, these phylogenetic trees are unreliable, and thus we adopted the ML method in the end. In the AUtest results of SSU rRNA phylogenetic relationships, all the values of hypotheses that *Mesodinium/Myrionecta* and other each Ciliophora class/subclass were monophyletic were greater than 0.74 (Table 1). So the hypotheses *Mesodinium/Myrionecta* together with any one other class/subclass were monophyletic could not be rejected. In another word, *Mesodinium/Myrionecta* seems to have an identical phylogenetic level with all of these classes/subclasses. Moreover, considering *Mesodinium/Myrionecta* species always form a steady, long and isolated branch, these imply that *Mesodinium/Myrionecta* is in fact an unusual and unknown taxon at the class level.

Table 1. AUtest results under constraints of *Mesodinium/Myrionecta* with one another classes in SSU rRNA phylogenetic trees.

Class	AUtest value
Plagiopylea	0.947
Oligohymenophorea	0.888
Prostomatea	0.895
Colpodea	0.940
Phyllopharyngea	0.908
Nassophorea	0.859
Litostomatea	0.918
Armophorea	0.914
Spirotrichea	0.745
Heterotrichea	0.915
Karyorelictea	0.949

4.2 SSU rRNA gene sequence/primary structure

According to Strüder-Kypke's research, the typical length of litostome SSU rRNA is approximately 1 640 nucleotides (Strüder-Kypke *et al.*, 2006). This is a consequence of several deletions across the SSU rRNA gene (Leipe *et al.*, 1994; Wright *et al.*, 1997; Wright and Lynn, 1997a, b). This study has found that the *Mesodinium/Myrionecta* sequence showed further deletions and had an average length of 1 569 nucleotides.

A comparison of the secondary structure of the variable region 4 (V4) for *Mesodinium* sp. and other species representing different classes or subclasses has showed that *Mesodinium* has a similar pattern with most other ciliates (Fig. 2), apart from more extensive deletions in Helix E23_8 and a longer helix E23_1 compared with common litostome ciliates (Fig. 2). This agrees with the Strüder-Kypke's findings (Strüder-Kypke *et al.*, 2006). Lynn and Small (2002) recognized these two genera within a single class, Litostomatea, but the secondary structure patterns revealed here obviously challenge this arrangement. Taken together, these findings would suggest that there are good reasons in favour of the establishment of a new class, Mesodiniea **cl. nov**.

Our sequence of *Mesodinium* sp. collected from Qingdao, China, was 18 nucleotides longer (1 595 bp vs. 1 577 bp) than the sequence of *M. pulex* collected from Puget Sound by Strüder-Kypke *et al.* (2006), probably due to the use of different PCR primers. Over the common length, however, the sequences were 94.3% identical. Our *Mesodinium* sp. and the published *Myrionecta rubra* by Johnson *et al.* (2004) SSU rRNA gene sequence shared 92.2% similarity, while the published *M. pulex* by Strüder-Kypke *et al.* (2006) shared 92.9% similarity with *M. rubra*.

4.3 A new class

According to Lynn's classification, the genus *Mesodinium* (family Mesodiniidae) belongs to the class Litostomatea, subclass Haptorida, order Cyclotrichida, and is adjacent to order Haptorida (Lynn, 2008). This attempt to assign *Mesodinium*

reflects the practice, common for a long time, in which a large number of low grade taxa, including *Mesodinium*, were placed temporarily within the subclass Haptorida, which was like a "melting pot" due to the lack of phylogenetic or ontogenic information. All the information now available, however, indicates that this arrangement has no reliable evidence to support it. Similarly, Lynn's attempt to put another four genera within Mesodiniidae is also groundless since. Actually, due to its conspicuous overall morphology *Mesodinium* forms a well-defined group light microscopically (Garcia-Cuetos *et al.*, 2012).

Based on the unusual secondary structures and phylogenetic analyses of SSU rRNA genes in *Mesodinium*, we have found that *Mesodinium*/*Myrionecta* branches early in the history of ciliate evolution. Our evidence also shows that *Mesodinium* represents a completely separate clade which is located between subphyla Intramacronucleata and Postciliodesmatophora, and this represents a substantial great difference with Lynn's 2008 system. All the evidences above confirm that there are good reasons support the establishment of a new class. This new class, Mesodiniea **cl. nov.** , only includes Mesodiniida Grain, 1994 at the moment, and belongs to subphylum Intramacronucleata.

Following Jankowski (1980), Lynn arranged the family Mesodiniidae in the newly established monotypic order Cyclotrichida. However, the well-known genus *Cyclotrichium* Jankowski (1980) was then assigned by Lynn into the order Haptorida Corliss, 1974. Thus, we suggest to moving the family Mesodiniidae from order Cyclotrichida to the current order Mesodiniida Grain, 1994.

In summary, combining the phylogenetic and morphological information, we suggest establishing a new class, Mesodiniea **cl. nov.** , including order Mesodiniida Grain, 1994, belonging to the subphylum Intramacronucleata.

Mesodiniea cl. nov.

Diagnosis. Somatic ciliature dimorphic and mostly bipartite and dimorphic: in vivo cirrus-or membrane-like, arranged in girdles/ belt around the body and reduced to the equatorial area. Conical oral area, typically with specialized capitate tentacles, no buccal ciliature. Symbionts often present. Pelagic in both marine and fresh water.

Type order. Mesodiniida Grain, 1994.

Systematic arrangement. The new class is a new member of the subphylum Intramacronucleata.

Funding This research was supported by the National Natural Science Foundation of China (31272285, 41276139), King Saud University Deanship of Scientific Research (Research Group Project No. RGP-083) and Special Foundation B of President of the Chinese Academy of Sciences (Y25102EN00).

References

Adl, S. M., Simpson, A. G., Lane, C. E., Lukes, J., Bass, D., Bowser, S. S., Brown, M. W., Burki, F., Dunthorn, M., Hampl, V. *et al.* 2012. The revised classification of eukaryotes. *Journal of Eukaryotic Microbiology*, 59: 429–493.

Alkemar, G. and Nygard, O. 2004. Secondary structure of two regions in expansion segments ES3 and ES6 with the potential of forming a tertiary interaction in eukaryotic 40S ribosomal subunits. *RNA*, 10: 403–411.

de Rijk, P., Wuyts, J. and de Wachter, R. 2003. RnaViz 2: an improved representation of RNA secondary structure. *Bioinformatics*, 19: 299–300.

Dunthorn, M., Foissner, W. and Katz, L. A. 2011. Expanding character sampling for ciliate phylogenetic inference using mitochondrial SSU-rDNA as a molecular marker. *Protist*, 162: 85–99.

Gao, F and Katz, L. A. 2014. Phylogenomic analyses support the bifurcation of ciliates into two major clades that differ in properties of nuclear division. *Molecular Phylogenetics and Evolution*, 70: 240–243.

Gao, F, Struder-Kypke, M., Yi, Z, Miao, M, Al-Farraj, S. A. and Song, W 2012a. Phylogenetic analysis and taxonomic distinction of six genera of pathogenic scuticociliates (Protozoa, Ciliophora) inferred from small-subunit rRNA gene sequences. *International Journal of Systematic and Evolutionary Microbiology*, 62: 246–256.

Gao, S, Huang, J, Li, J and Song, W 2012b. Molecular phylogeny of the cyrtophorid ciliates (Protozoa, Ciliophora, Phyllopharyngea). *PLoS One*, 7: e33198.

Garcia-Cuetos, L., Moestrup, O. and Hansen, P. J. 2012. Studies on the genus *Mesodinium* II. Ultrastructural and molecular investigations of five marine species help clarifying the taxonomy. *Journal of Eukaryotic Microbiology*, 59: 374–400.

Gong, J, Stoeck, T., Yi, Z, Miao, M, Zhang, Q, Roberts, D. M., Warren, A. and Song, W 2009. Small subunit rRNA phylogenies show that the class Nassophorea is not monophyletic (Phylum Ciliophora). *Journal of Eukaryotic Microbiology*, 56: 339–347.

Gustafson, D. E., Jr., Stoecker, D. K., Johnson, M. D., Van Heukelem, W.F. and Sneider, K. 2000. Cryptophyte algae are robbed of their organelles by the marine ciliate *Mesodinium rubrum*. *Nature*, 405: 1049–1052.

Huang, J, Dunthorn, M. and Song, W 2012. Expanding character sampling for the molecular phylogeny of euplotid ciliates (Protozoa, Ciliophora) using three markers, with a focus on the family Uronychiidae. *Molecular Phylogenetics and Evolution*, 63: 598–605.

Huelsenbeck, J. P. 1995. The robustness of two phylogenetic methods: four-taxon simulations reveal a slight superiority of maximum likelihood over neighbor joining. *Molecular Biology and Evolution*, 12: 843–849.

Hwang, U. W. and Kim, W. 1999. General properties and phylogenetic utilities of nuclear ribosomal DNA and mitochondrial DNA commonly used in molecular systematics. *The Korean Journal of Parasitology*, 37: 215–228.

Jankowski, A. W. 1980. Conspectus of a new system of the phylum Ciliophora. *Proceedings of the National Academy of Sciences (USSR)*, 94: 103–121.

Johnson, M. D., Tengs, T, Oldach, D. W., Delwiche, C. F. and Stoecker, D. K. 2004. Highly divergent SSU rRNA genes found in the marine ciliates *Myrionecta rubra* and *Mesodinium pulex*. *Protist*, 155: 347–359.

Leipe, D. D., Bernhard, D., Schlegel, M. and Sogin, M. L. 1994. Evolution of 16S-like ribosomal RNA genes in the ciliophoran taxa Litostomatea and Phyllopharyngea. *European Journal of Protistology*, 30: 354–361.

Lindholm, T., Lindroos, P. and Mork, A. C. 1988. Ultrastructure of the photosynthetic ciliate *Mesodinium rubrum*. *Biosystems*, 21: 141–149.

Lynn, D. H. 2008. *Ciliated Protozoa:* Characterization, Classification, and Guide to the Literature. Springer Verlag, Dordrecht. 605 pp.

Lynn, D. H. and Small, E. B. 2002. Phylum Ciliophora, Doflein, 1901. Society of Protozoologists, Lawrence, Kansas. pp. 371–676.

Medlin, L., Elwood, H. J., Stickel, S. and Sogin, M. L. 1988. The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. *Gene*, 71: 491–499.

Miao, M, Shao, C, Chen, X and Song, W 2011. Evolution of discocephalid ciliates: molecular, morphological and ontogenetic data support a sister group of discocephalids and pseudoamphisiellids (Protozoa, Ciliophora) with establishment of a new suborder Pseudoamphisiellina subord. n. *Science China (Life Science)*, 54: 634–641.

Moestrup, Ø., Garcia-Cuetos, L., Hansen, P. J. and Fenchel, T. 2012. Studies on the genus *Mesodinium* I: ultrastructure and description of *Mesodinium chamaeleon* n. sp., a benthic marine species with green or red chloroplasts. *Journal of Eukaryotic Microbiology*, 59: 20–39.

Neefs, J. M., Van de Peer, Y., de Rijk, P., Chapelle, S. and de Wachter, R. 1993. Compilation of small ribosomal subunit RNA structures. *Nucleic Acids Research*, 21: 3025–3049.

Philippe, H. and Laurent, J. 1998. How good are deep phylogenetic trees? *Current Opinion In Genetics & Development*, 8: 616–623.

Philippe, H., Zhou, Y, Brinkmann, H., Rodrigue, N. and Delsuc, F. 2005. Heterotachy and long-branch attraction in phylogenetics. *BMC Evolutionary Biology*, 5: 50.

Posada, D. and Crandall, K. A. 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics*, 14: 817–818.

Quintela-Alonso, P., Nitsche, F. and Arndt, H. 2011. Molecular characterization and revised systematics of *Microdiaphanosoma arcuatum* (Ciliophora, Colpodea). *Journal of Eukaryotic Microbiology*, 58: 114–119.

Shimodaira, H. and Hasegawa, M., 2001. CONSEL: for assessing the confidence of phylogenetic tree selection. *Bioinformatics*, 17: 1246–1247.

Strüder-Kypke, M. C., Wright, A. D., Foissner, W., Chatzinotas, A. and Lynn, D. H. 2006. Molecular phylogeny of litostome ciliates (Ciliophora, Litostomatea) with emphasis on free-living haptorian genera. *Protist*, 157: 261–278.

Swofford, D. L. 2003. PAUP*: phylogenetic analysis using parsimony, version 4.0 b10.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. and Higgins, D. G. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, 25: 4876–4882.

Van de Peer, Y. and de Wachter, R. 1997. Evolutionary relationships among the eukaryotic crown taxa taking into account site-to-site rate variation in 18S rRNA. *Journal of Molecular Evolution*, 45: 619–630.

Vogler, A. P. and Pearson, D. L. 1996. A molecular phylogeny of the tiger beetles (Cicindelidae): congruence of mitochondrial and nuclear rDNA data sets. *Molecular Phylogenetics and Evolution*, 6: 321–338.

Wright, A. D. G., Dehority, B. A. and Lynn, D. H. 1997. Phylogeny of the rumen ciliates *Entodinium*, *Epidinium* and *Polyplastron* (Litostomatea: Entodiniomorphida) inferred from small subunit ribosomal RNA sequences. *Journal of Eukaryotic Microbiology*, 44: 61–67.

Wright, A. D. G. and Lynn, D. H. 1997a. Monophyly of the Trichostome ciliates (Phylum Ciliophora: Class Litostomatea) tested using new 18S rRNA sequences from the Vestibuliferids, *Isotricha intestinalis* and *Dasytricha ruminantium*, and the Haptorian, *Didinium nasutum*. *European Journal of Protistology*, 33: 305–315.

Wright, A. D. G. and Lynn, D. H. 1997b. Phylogenetic analysis of the rumen ciliate family Ophryoscolecidae based on 18S ribosomal RNA sequences, with new sequences from *Diplodinium*, *Eudiplodinium*, and *Ophryoscolex*. *Canadian Journal of Zoology*, 75: 963–970.

Wuyts, J., de Rijk, P., Van de Peer, Y., Pison, G., Rousseeuw, P. and de Wachter, R. 2000. Comparative analysis of more than 3000 sequences reveals the existence of two pseudoknots in area V4 of eukaryotic small subunit ribosomal RNA. *Nucleic Acids Research*, 28: 4698–4708.

Wuyts, J., Perriere, G. and Van de Peer, Y. 2004. The European ribosomal RNA database. *Nucleic Acids Research*, 32: D101–103.

Zhang, Q, Simpson, A. and Song, W 2012. Insights into the phylogeny of systematically controversial haptorian ciliates (Ciliophora, Litostomatea) based on multigene analyses. *Proceedings of the Royal Society Biological sciences*, 279: 2625–2635.

Zhang, Q, Yi, Z, Fan, X, Warren, A., Gong, J and Song, W 2014. Further insights into the phylogeny of two ciliate classes Nassophorea and Prostomatea (Protista, Ciliophora). *Molecular Phylogenetics and Evolution*, 70: 162–170.

Zoller, S. D., Hammersmith, R. L., Swart, E. C., Higgins, B. P., Doak, T. G., Herrick, G. and Landweber, L. F. 2012. Characterization and taxonomic validity of the ciliate *Oxytricha trifallax* (Class Spirotrichea) based on multiple gene sequences: limitations in identifying genera solely by morphology. *Protist*, 163: 643–657.

Zuker, M. 2003. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Research*, 31: 3406–3415.