SCIENCE CHINA
Life Sciences

• RESEARCH PAPER •

January 2010 Vol.53 No.1: 131–138
doi: 10.1007/s11427-010-0012-9

Protocruzia, a highly ambiguous ciliate (Protozoa; Ciliophora): Very likely an ancestral form for Heterotrichea, Colpodea or Spirotrichea? With reevaluation of its evolutionary position based on multigene analyses

LI LiFang[1,2], Stoeck Thorsten[3], Shin Mann Kyoon[4], Al-Rasheid Khaled A. S.[5], Al-Khedhairy Bdulaziz A.[5] & SONG WeiBo[2*]

[1] *Marine College, Shandong University at Weihai, Weihai 264209, China;*
[2] *Laboratory of Protozoology, Ocean University of China, Qingdao 266003, China;*
[3] *Department of Ecology, University of Kaiserslautern, D-67663 Kaiserslautern, Germany;*
[4] *Department of Biology, University of Ulsan, Ulsan 680-749, Korea;*
[5] *Zoology Department, King Saud University, P. O. Box 2455, Riyadh 11451, Saudi Arabia*

Received August 6, 2009; accepted September 10, 2009

The ciliate genus *Protocruzia* belongs to one of the most ambiguous taxa considering its systematic position, possible as a member of the classes Heterotrichea, Spirotrichea or Karyorelictea, which is tentatively placed into Spirotrichea in Lynn's 2008 system. To test these hypotheses, multigene trees (Bayesian inference, evolutionary distance, maximum parsimony, and maximum likelihood) were constructed using the small subunit rRNA (SSU rRNA) gene, internal transcribed spacer 2 (ITS2) and a protein coding gene (histone H4). All analyses agree that: (1) four morphotypes of *Protocruzia* from different geographical origins group together and form a monophyletic clade, which cannot be assigned to any of the eleven described ciliate classes; (2) it is invariably positioned on an isolated branch separated from the class Spirotrichea suggesting that this clade should be clearly removed from Spirotrichea; (3) this leads us to hypothesize that this taxon may indeed represent a lineage on a class rank. Based on the fact that it is, both morphologically and in molecular features, closely related to heterotrichs, Colpodea and Oligohymenophorea, Protocruziida might be an ancestral form for the subphylum Intramacronucleata in the evolutionary line from the class Heterotrichea (subphylum Postciliodesmatophora) to higher taxa.

SSU rRNA gene, internal transcribed spacer 2 (ITS2) gene, histone H4 gene, phylogenetic position, *Protocruzia*, Protocruziidea, new class

Citation: Li L F, Thorsten S, Kyoon S M, et al. *Protocruzia*, a highly ambiguous ciliate (Protozoa; Ciliophora): Very likely an ancestral form for Heterotrichea, Colpodea or Spirotrichea? With reevaluation of its evolutionary position based on multigene analyses. Sci China Life Sci, 2010, 53: 131−138, doi: 10.1007/s11427-010-0012-9

The well-known ciliate *Protocruzia* has been repeatedly described with regard to such aspects as morphology, physiology, taxonomy, ultrastructure and molecular biology [1–8]. Yet its systematic position remains ambiguous and uncertain [4,8–11].

Previous classification efforts of *Protocruzia* demonstrate the uncertainties accompanying its systematic position: In 1979, Corliss transferred *Protocruzia* from the family Spirostomidae, placing it incertae sedis in the suborder Philasterina [10]. The kinetid of *Protocruzia* was reconsidered and found to be heterotrich- and karyorelictean-like [2,3]. Thus, both Small and Lynn [9,12] and de Puytorac [13] assigned

*Corresponding author (email: wsong@ouc.edu.cn)

© Science China Press and Springer-Verlag Berlin Heidelberg 2010

Protocruzia to the most primitive class Karyorelictea, despite striking differences in macronuclear features, Puytorac *et al.* [14] elevated *Protocruzia* to a subclass rank: Protocruziidia. Song and Wilbert [6] based its general morphology and infraciliature preferred to retain *Protocruzia* at least temporarily in the class Heterotrichea, which is consistent with the earlier arrangement by Corliss [10]. Based on initial phylogenetic analyses of SSU rRNA gene sequences, Hammerschmidt *et al.* [5] and Shin *et al.* [8] suggested that Protocruziidia be transferred to the class Spirotrichea. However, bootstrap support for this conclusion was insignificant. Current classification systems assign the subclass Protocruziidia to the class Spirotrichea [11,15,16]. Lynn [11] considered if Protocruziida might represent the as yet exclusive member of a new class. This notion was based on the tenuous relationship that this order shows to the spirotrichs. In sum, based on various considerations, there are three current phylogenetic hypotheses for *Protocruzia*: (1) It is a heterotrich or (2) it is a spirotrich or (3) it is a karyorelict or (4) it is a new class.

Previous studies have shown that multigene analyses enable resolution of the phylogenetic position of taxa of uncertain classification [17–19]. In order to determine its most likely systematic position to provide more information about its phylogenetic analysis, we took advantage of this strategy and phylogenetic analyses employing Bayesian inference, evolutionary distance, maximum parsimony, and maximum likelihood based on the multigene sequences of small subunit rRNA (SSU rRNA) gene, Internal transcribed spacer 2 (ITS2) and a protein coding gene (histone H4 gene).

1 Material and methods

1.1 Ciliate collection and identification

Protocruzia contrax Mansfeld, 1923 was found in samples collected from the open water of a scallop-farm off Qingdao (Tsingtao, 36°08′N; 120°43′E), China in 2004. After isolation, specimens were maintained in Petri dishes in the laboratory for about 4 days. Clonal cultures were then established and maintained at room temperature in boiled seawater amended with rice grains to enrich natural bacteria as food for the ciliates. Observations of living cells were carried out with bright field and differential inference contrast microscopy[20]. The protargol silver staining method [21] was used to reveal the infraciliature. Our result showed that this isolate was morphologically identical to previous descriptions [6] (Figure 1 A–D). General systematic arrangement and terminology are mainly according to Lynn [4].

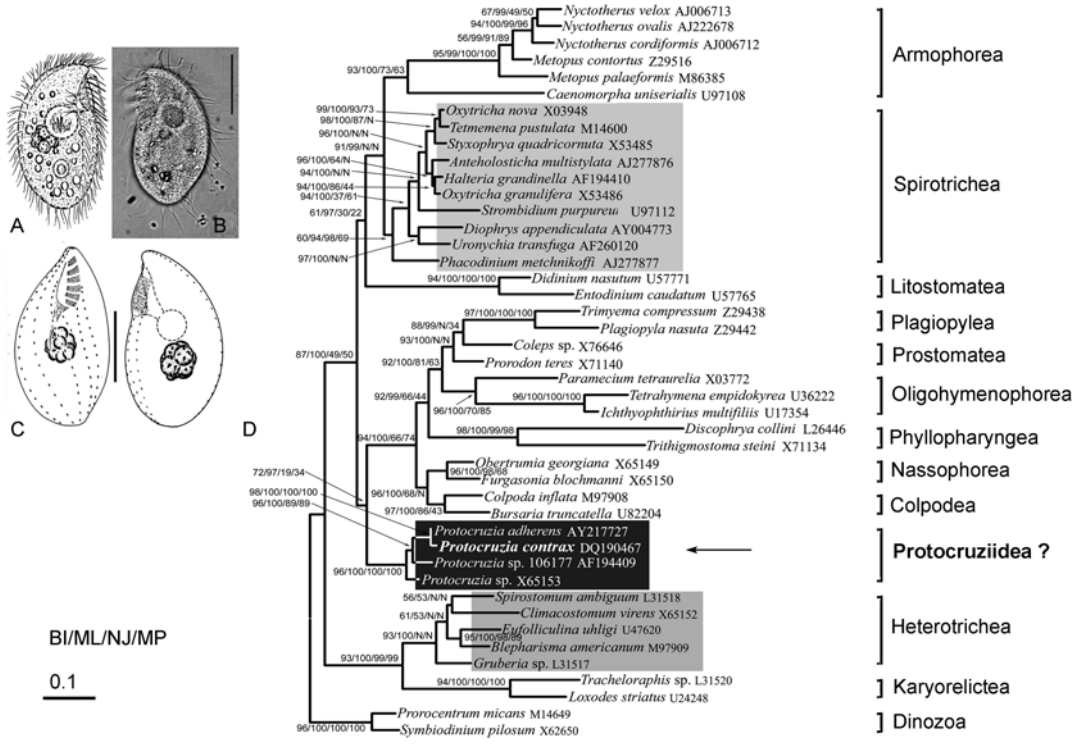

Figure 1 A Bayesian tree inferred from the nucleotide sequences of complete small subunit rRNA (SSrRNA) of ciliated protozoa. Numbers at nodes represent bootstrap values (in %) out of 1,000 replicates: the first number is the Bayesian credibility value using the MrBayes (BI) algorithm, the second number is from the maxinuim likelihood (ML) method, the third number is derived from the distance matrix based on the neighbor joining (NJ) method and the forth number is from maximum parsimony (MP) method. *Prorocentrum micans* and *Symbiodinium pilosum* (Kingdom Dinozoa) were selected as the outgroup taxon. 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. The new sequence is highlighted in boldface. Insets: Morphology and infraciliature of *Protocruzia contrax*, from life (A–B) and after protargol impregnation (C–D). Scale bar: 15 μm. Reproduced with permission from the detailed account by Song and Wilbert (1997).

1.2 PCR amplification and sequencing

Cells were starved overnight, rinsed with sterile artificial sea water, and then concentrated by low speed centrifugation. Genomic DNA was extracted following Gao *et al.* [22]. To minimize PCR amplification errors, high-fidelity TaKaRa ExTaq (TaKaRa, Otsu, Japan) was used to amplify the SSU rRNA gene using the universal oligonucleotide primers (forward 5′-AACCTGGTTGATCCTGCCAGT-3′; reverse 5′-TGATCCTTCTGCAGGTTCACCTAC-3′) designed by Medlin *et al.* [23] and resulting in a fragment of ca. 1700 bp. PCR reactions were according to Miao *et al.*[24] using 50 ng genomic template DNA. The typical amplification profile consisted of 30 cycles of 1 min at 94°C, 2 min at 60°C and 2 min at 72°C, followed by 10 min at 72°C for final extension. PCR products were inserted into the pUCm-T vector (Sangon, Toronto, Ontario, Canada). The plasmid mini-prep spin column kit (Sangon Bio. Co.) was used to harvest and purify plasmid DNA from one clone of transformed *E. coli* over-night cultures. The DNA sequencing was accomplished using an ABI Prism 3730 Automated DNA Sequencer (Applied Biosystems Inc., Dalian, China). All sequences were confirmed from both strands. Sequence fragments were assembled into continuous sequences and edited with the Sequencher 4.0 software package (GeneCodes Corp. Ann Arbor, MI).

1.3 Phylogenetic analyses inferred from the nucleotide sequences

Sequences of SSU rRNA and ITS2 genes sequences used were available from the NCBI GenBank database. Sequences were aligned with Clustal X 1.81 [25], after which the alignment was refined by eye with consideration of the conservation of secondary structures. The computer program, MrBayes v3.0b4 [26] was used for the Markov Chain Monte Carlo (MCMC) algorithm to construct a Bayesian tree under the GTR+T+R evolutionary model indicated by MrModeltest v.2 [27] and considering a gamma-shaped distribution of the rates of substitution among sites. The chain length for our analysis was 1000000 generations with trees sampled every 100 generations. The first 250000 generations were discarded as burn-in. The PHYLIP package, version 3.57c [28] was used to calculate the sequence similarity and evolutionary distances between pairs of nucleotide sequences using the Kimura (1980) two-parameter model [29]. Distance-matrix trees were then constructed using the neighbor-joining [NJ] method[30]. The distance data was bootstrap resampled 1000 times. For the maximum-parsi- mony (MP) analysis, a consensus tree was generated by heuristic tree searches using the parsimony ratchet in the PAUP (v. 4.0b10)[31] program. Bootstrap values were also generated in PAUP (v. 4.0b10). For maximum parsimony analysis, data was bootstrap resampled 1000 times. A maximum likelihood (ML) tree was constructed with PAUP using the GTR+G+I evolutionary model selected by Modeltest[32] and an input file created by performing an accelerated likelihood surface exploration [33,34] with the program PAUPRat [35]. A 50% majority-rule consensus ML tree was derived from the output of 200 trees generated by PAUP utilizing the input file from PAUPRat. For combined data sets (e.g., SSU rRNA +H4 nucleotide sequences), individual coding regions were treated as ‘unlinked’, so that separate parameter estimates (SSU rRNA: GTR+ T+R; H4: GTR) were obtained for each region for all runs.

1.4 Construction of the Bayesian tree based on the histone H4 amino acids sequences

Amino acids for the histone H4 gene from the NCBI GenBank database were aligned using Clustal X 1.81 [25], and then trimmed to the same lengths. MrBayes v3.0b4 [26] was used to construct a Bayesian tree using the Markov Chain Monte Carlo (MCMC) algorithm under the JTT [36] evolutionary model and considering a gamma-shaped distribution of the rates of substitution among sites. The chain length for our analysis was 100000 generations with trees sampled every 50 generations [37].

1.5 Secondary structure predictions

Secondary structures were predicted using the RNAViz program[38] inferred from the alignment of the SSU rRNA and ITS2 genes sequences included in the analyses[39].

2 Results

2.1 SSU rRNA sequence and comparison of *Protocruzia contrax*

The partial SSU rRNA gene sequence for *Protocruzia contrax* (GenBank Accession Number: DQ190467) is 1788 nucleotides in length and its GC content is 43.34%. The structural similarities between *P. contrax* and other ciliates have been calculated. The sequence of *Protocruzia contrax* differs in 23 nucleotides from the sequence of *P. adherens* (structural similarity 96%). 230 sites are different from that in *Protocruzia* sp. (structural similarity 93%), while 233 sites differ from *Protocruzia* sp. 106177 (structural similarity 93%). The structural similarities of *Protocruzia* to other groups are: 78.01%–83.61% to spirotrichs; 74.10%–81.16% to armophorous ciliates; 76.85%–80.56% to litostomats; 74.66%–77.59% to plagiopylous ciliates; 79.94%–81.58% to prostomats; 72.69%–77.16% to oligohymenophous ciliates; 73.62%–74.46% to phyllopharyngs; 78.72%–82.49% to colpods; 73.32%–78.49% to karyorelicts, and 78.39%–84.79% to heterotrichs.

2.2 Secondary structure predictions

The predicted secondary structures of the SSU rRNA gene of the studied ciliates are in agreement with the generalized eukaryotic SSU rRNA model [40]. All the conserved universal helices are present. Inspections of the secondary structures reveal great differences among *Protocruzia* and other ciliates in the Helix E10-1 regions. In order to illustrate this variability the main features of the Helix E10-1 regions are shown in Figure 2. The Helix E10-1 region consists of 35-60 nucleotides, indicating that the Helix E10-1 region highly variable between different classes. Thus, this region serves as a class-specific characteristic.

As shown in Figure 2, four *Protocruzia* spp. share two distinct bulges in the Helix E10-1 region, which is to some extent similar to *Stentor coeruleus*, suggesting a relatively close relationship of *Protocruzia* to heterotrichs.

2.3 Phylogenetic analyses based on SSU rRNA and ITS2 sequences

To determine the systematic position of *Protocruzia*, we constructed phylogenetic trees using multiple algorithms, which included the comparable sequences analyzed by Shin *et al*.[8] to ensure an accurate comparison with that earlier study (see Figure 1).

The results reconfirm: (1) The monophyly of the phylum Ciliophora Doflein 1901 and its two subphylum: Postciliodesmatophora and Intramacronucleata which confirmed as expected. (2) The recovery of 10 monophyltic out of 11 classes in Lynn's (2003) [4] with rejection of monophyly for the class Prostomatea. (3) All *Protocruzia* spp. clustered together and formed a monophyletic clade with high posterior probability in a Bayesian tree (96% BI) and high bootstrap support in other construction methods (100%ML, 100%NJ, 100%MP), which is the basal lineage in the Subphylum Intramacronucleata sensu Lynn, 1996 [41], but are not placed within the cluster of the class Spirotrichea sensu Lynn 2003 [4].

The phylogenetic tree inferred from the secondary structure of internal transcribed spacer 2 is shown in Figure 3. *Protocruzia adherens* forms a sister clade to the heterotrichous genus, *Spirostomum*, but repeatedly does not branch within the spirotrichous clade.

2.4 Phylogenetic analyses based on histone H4 gene sequences and the combined SSU rRNA gene and histone H4 gene sequences

Phylogenetic analysis of the histone H4 gene sequences (Figure 4A) and the combined SSU rRNA gene and histone H4 gene sequences (Figure 4B) provide further evidence for

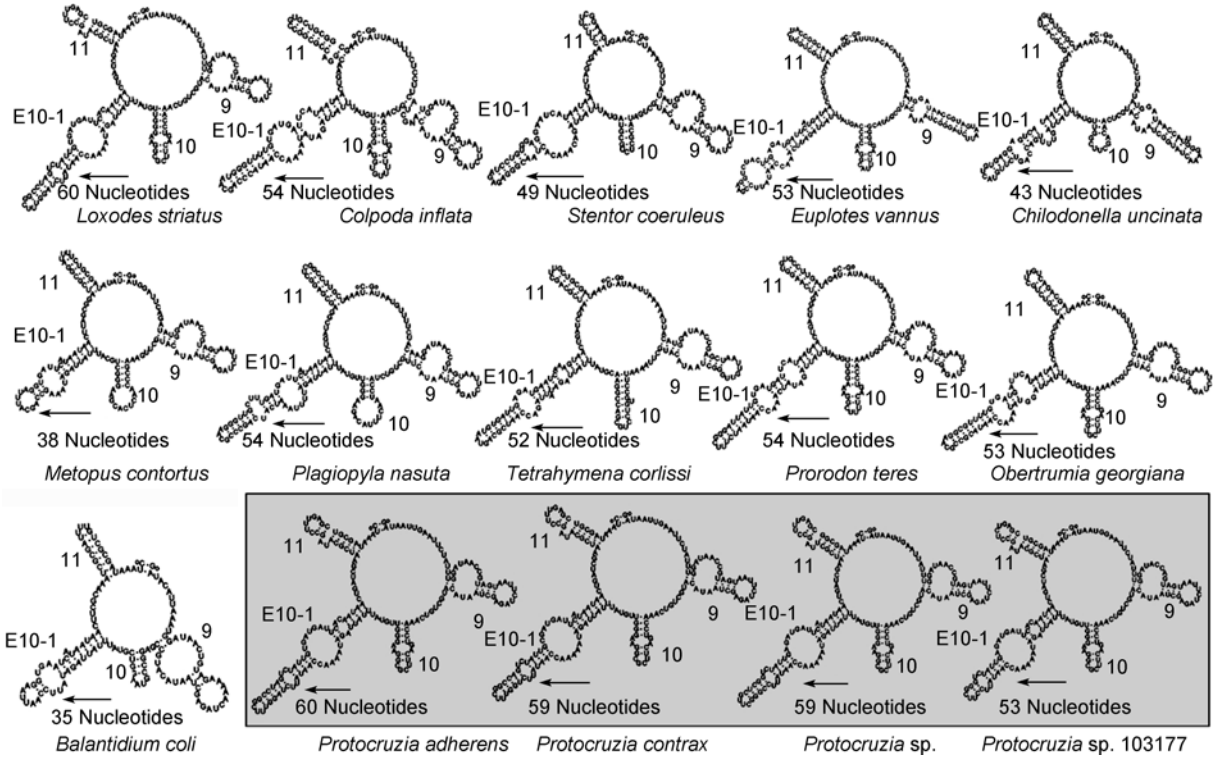

Figure 2 Secondary structures of the SSUrRNA gene molecule in the region of the Helices 9-11 for *Protocruzia* spp. and representative species of 11 classes in the Phylum Ciliophora: *Loxodes striatus* – Class Karyorelictea; *Colpoda inflata* – Class Colpodea; *Stentor coeruleus* – Class Heterotrichea; *Euplotes vannus* – Class Spirotrichea; *Chilodonella uncinata* – Class Phyllopharyngea; *Metopus contortus* – Class Armophorea; *Plagiopyla nasuta* – Class Plagiopylea; *Tetrahymena corlissi* – Class Oligohymenophorea; *Prorodon teres* – Class Prostomatea; Obertrumia georgiana – Class Nassophorea; *Balantidium coli* – Class Litostomatea. Number of nucleotides in Helix E10-1 for each species is indicated below the arrow.

a sister relationship of *Protocruzia* to the class Heterotrichea. As in previous analyses, the taxon does not cluster within the Spirotrichea.

3 Discussion

The genus *Protocruzia* was established by Faria da Cunha and Pinto in 1922. Small and Lynn [9] recognized the family Protocruziidae Jankowski in Small and Lynn, 1985 and established this as the type family for the order Protocruziida Jankowski in Small and Lynn, 1985 [11]. Puytorac *et al.* [14] elevated it to a subclass rank: Protocruziidia. The assignment of *Protocruzia* to higher taxonomic level is regarded as distinct but unclear.

The early molecular work on *Protocruzia* began in 1996 when Hammerschmidt *et al.* [5] sequenced and phylogenetically analyzed the SSU rRNA gene of one *Protocruzia* species. The authors found that the taxon forms an individual independent clade, which was branching as a sister to the class Spirotrichea. Shin *et al.* [8] sequenced the SSU rRNA gene of a second species of *Protocruzia*, and found that in two distance approaches (i.e., LS, NJ), maximum likelihood, and parsimony, the *Protocruzia* species were consistently associated with the spirotrich clade, and always in a basal position. However, bootstrap support was insignificant in their analyses, presumably as a result of an overly limited taxon sampling. Based on their results, the authors suggested that the subclass Protocruziidia might be transferred to the class Spirotrichea. Also in the taxonomic systems of Lynn and Small [15] and Adl *et al.* [16], the subclass Protocruziidia was assigned to Spirotrichea. These latter classification schemes are currently widely used and accepted. The same placement of Protocruziidia existed in Lynn [11], although the author noticed the tenuous relationship that this order showed to the spirotrichs. Based on our phylogenetic data, this hypothesis has been rejected.

The present study based on the SSU rRNA gene sequences shows that four morphotypes of *Protocruzia* from different geographical origins group together and form a monophyletic clade with strong support in all tree construction methods. All analyses agree that *Protocruzia* does not cluster with spirotrichs, but forms a basal lineage in the subphylum Intramacronucleata sensu Lynn, 1996 [41]. Based on the analyses of several genes, *Protocruzia* cannot be assigned to a described class and may represent an individual taxonomic entity on class-level.

There may be an underlying reason as to why these results are incongruent with former assignments of *Protocruzia*: First, an insufficient taxon sampling in previous phylogenetic analyses may have lead to erroneous conclusions as outlined earlier [42]. Second, utilizing a single gene as a phylogenetic marker may make phylogenetic inferences inaccurate. Specifically in ciliates, the exclusive use of the SSU rRNA gene may be insufficient, as this gene does not resolve the relations between the different ciliate classes [4]. (c) Differences in alignments, choice of outgroup the treeing algorithm, and the clustering method may contribute to in-

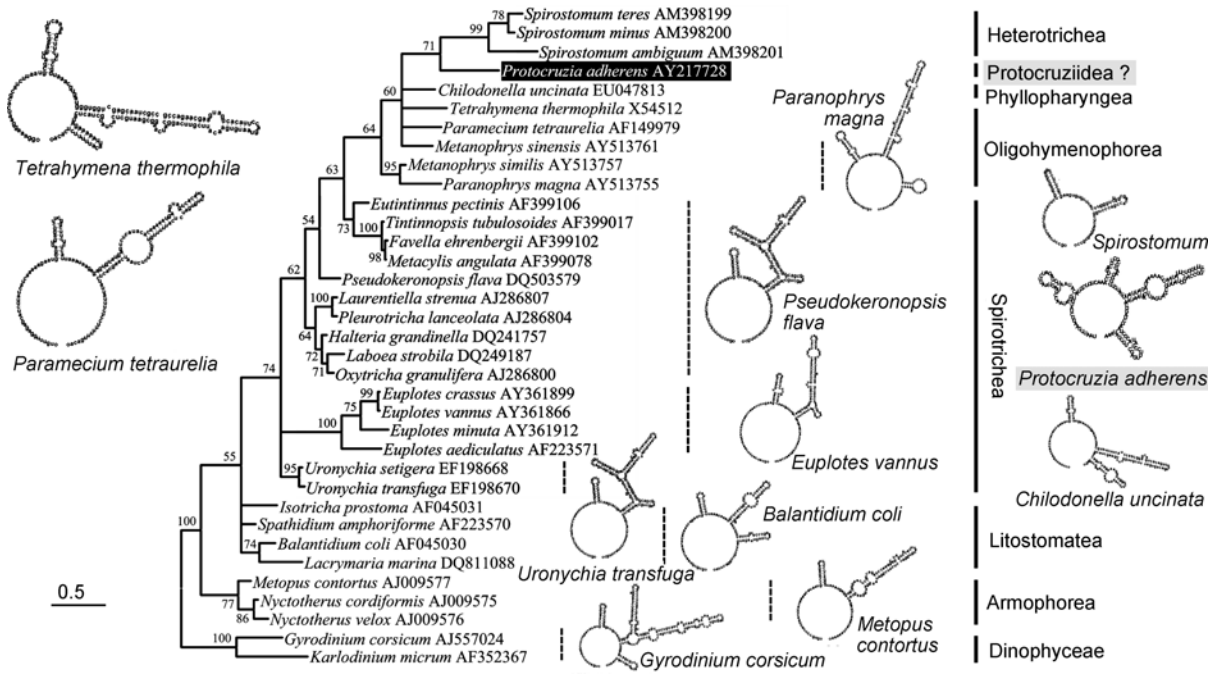

Figure 3 A Bayesian tree constructed based on alignment of internal transcribed spacer 2 sequences using the secondary structure, indicating the systematic position of *Protocruzia*. The numbers at the forks indicate the percentage of times that specific branch pattern occurred in 1,000 trees. Evolutionary distance is represented by the branch length to separate the species in the figure. The scale bar corresponds to twenty substitutions per 100 nucleotide positions.

congruent phylogenetic trees [42].

Phylogenetic analysis of the internal transcribed spacer 2, histone H4 gene sequences and the combined SSU rRNA gene and histone H4 gene sequences provide further evidence for a sister relationship of *Protocruzia* to the class Heterotrichea. As the result of SSU rRNA gene analysis, the taxon does not cluster within the Spirotrichea.

There are various minor topologies, which do not affect our interpretation of the taxonomic assignment of *Protocruzia*, which are incongruent among the SSU rRNA, internal transcribed spacer 2, and histone H4 gene trees. We mainly attribute these to different evolutionary rates and/or the different resolving power of the genes under consideration [43,44].

In sum, our current data suggests that *Protocruzia* would be inappropriately assigned to the spirotrichs, but rather presents itself as a monophyletic taxon with a close relationship to the class Heterotrichea. It may represent an ancestral form for the subphylum Intramacronucleata in the evolutionary line from the subphylum Postciliodesmatophora (i.e. class Heterotrichea).

We find further evidence for this hypothesis in the morphology and ultrastructure of *Protocruzia*. For example, *Protocruzia* exhibits a unique nuclear complex that is unlike that in any of the other described ciliate classes [5,45].

According to the general morphology and infraciliature, viz. the buccal apparatus and dikinetid somatic ciliature, Song and Wilbert [6] considered *Protocruzia* to be an intermediate form between heterotrichs and colpods and suggested that it might be reasonable to keep it temporarily under the Heterotrichea. The authors justified this argument mainly with a lack of the silverline system, which is consistent with the present molecular results. Based on Lynn [11], the replication band that appears in the macronuclear DNA S-shape is the strongest morphological synapomorphy for the class Spirotrichea. However, the replication band has

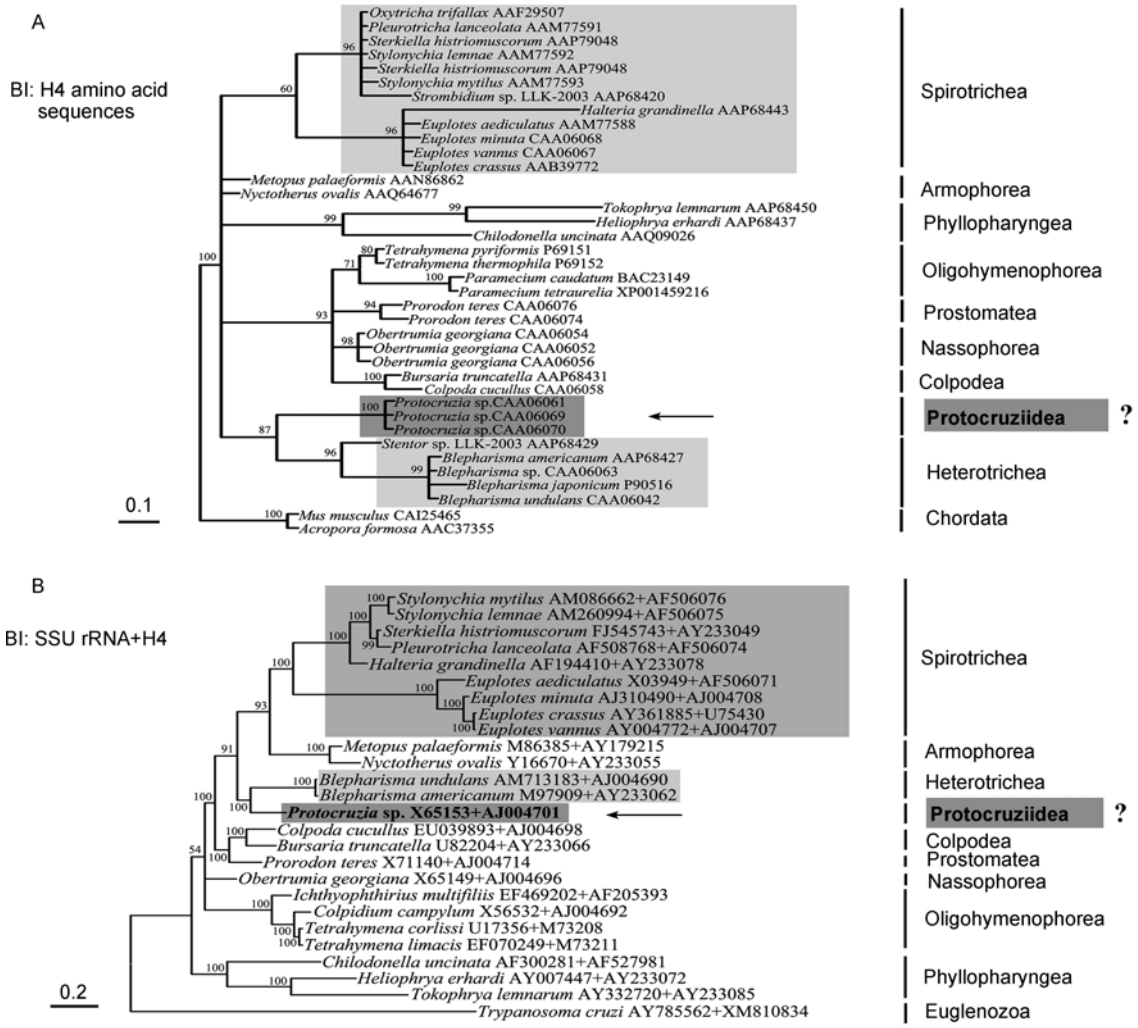

Figure 4 Bayesian trees constructed from histone H4 gene sequences (A) and the combined SSU rRNA gene and histone H4 gene sequences (B) indicating the systematic position of *Protocruzia*. The numbers at the forks indicate the percentage of times that specific branch pattern occurred in 1,000 trees. Evolutionary distance is represented by the branch length to separate the species in the figure. The scale bar corresponds to five substitutions per 100 amino acid (A) or 200 nucleotide (B) positions.

never been observed in protocruziids [45,46].

Therefore, with the compositive information inferred from the phylogenetic analysis of the SSU rRNA gene, internal transcribed spacer 2 gene sequences, histone H4 gene, supported by previously revealed ultrastructural and morphological peculiarities, the subclass Protocruziidia is unlikely to be a member of one of the eleven described (ribo)classes of the phylum Ciliophora but may deserve its own taxonomic rank at the class level. To confirm this hypothesis further data is needed, which includes extended gene sampling as well as possibly the discovery of further taxa or environmental sequences clustering in the Protocruziidia clade. A thorough redescription of the morphology of *Protocruzia* with a focus on the oral infraciliature is necessary.

This work is supported by the "Natural Science Foundation of China" (Grant No. 30870264), the Deutsche Forschungsgemeinschaft (DFG) STO414/2-4, and partially supported by the Center of Excellence in Biodiversity Research and the DNA Research Chair, King Saud University.

1 Kahl A. Urtiere oder Protozoa I: Wimptiere oder Ciliata (Infusoria), 3. Spirotricha. In: Dahl F. (ed.), Die Tierwelt Deutschlands Fischer, Jena. 1932, 25: 399–650
2 Grolière C A, Puytorac P De, Detcheva R. A propos d'observations sur la stomatogenèse et l'ultrastructure du cilié *Protocruzia tuzeti* Villeneuve-Brachon, 1940. Protistologica, 1980, 16: 453–466
3 Lynn D H. The implications of recent descriptions of kinetid structure to the systematics of the ciliated protists. Protoplasma, 1991, 164: 123–142
4 Lynn D H. Morphology or molecules: How we identify the major lineages of ciliates (Phylum Ciliophora)? Eur J Protistol, 2003, 39: 356–364
5 Hammerschmidt B, Schlegel M, Lynn D H, *et al.* Insights into the evolution of nuclear dualism in the ciliates revealed by phylogenetic analysis of rRNA sequences. J Eukaryot Microbiol, 1996, 43: 225–230
6 Song W, Wilbert N. Morphological investigations on some free living ciliates (Protozoa, Ciliophora) from China seas with description of a new hypotrichous genus, *Hemigastrostyla* nov. gen. Arch Protistenkd, 1997, 148: 413–444
7 Bernhard D D, Schlegel M. Evolution of histone H4 and H3 gene in different ciliate lineages. J Mol Evol, 1998, 46: 344–354
8 Shin M K, Hwang U W, 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. Eur J Protistol, 2000, 36: 293–302
9 Small E B, Lynn D H. Phylum Ciliophora, 1985, 393－575. In: Lee J J, Hutner S H & Bovee E C. (eds.) An Illustrated Guide to the Protozoa. Society of Protozoologists, Lawrence, Kansas.
10 Corliss J O. The Ciliated Protozoa: Characterization, Classification and Guide to the Literature. 2nd Edition. New York: Pergamon Press. 1979
11 Lynn D H. The Ciliated Protozoa: Characterization, Classification, and Guide to the Literature. 3rd edn. Dordrecht: Springer, 2008
12 Small E B, Lynn D H. A new macrosystem for the Phylum Ciliophora Doflein, 1901. BioSystems, 1981, 14: 387–401
13 De Puytorac P. Phylum Ciliophora Doflein, 1901. In: P. P. de, (ed.) Traité de Zoologie, Tome II, Infusoires Cilie´s, Fasc. 2, Systématique. Masson, Paris. 1994.
14 De Puytorac P, Grain J, Mignot J P. Précis de Protistologie. Société Nouvelle des Editions Boubée, Paris. 1987.
15 Lynn D H, Small E B. Phylum Ciliophora Doflein, 1901. In: Lee J J , Bradbury P C, Leedale G F. (Eds.) An illustrated guide to the protozoa. 2nd Ed. Lawrence: Allen Press Inc., 2002. 371–656
16 Adl S M, Simpson A G B, Farmer M A, *et al.* The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. J Eukaryot Microbiol, 2005, 52: 399–451
17 Longet D, Burki F, Flakowski J, *et al.* Multigene evidence for close evolutionary relations between Gromia and Foraminifera. Acta Protozool, 2004, 43: 303–311
18 Simpson A G B, Inagaki Y, Roger A J. Comprehensive multi-gene phylogenies of excavate protists reveal the evolutionary positions of 'primitive' eukaryotes. Mol Biol Evol, 2006, 23: 615–625
19 Grant J, Tekle Y I, Anderson O R, *et al*. Multigene evidence for the placement of a heterotrophic amoeboid lineage *Leukarachnion* sp. among photosynthetic stramenopiles. Protist, 2009, 160: 376–385
20 Hu X. Cortical structure in non-dividing and dividing *Diophrys japanica* spec. nov. (Ciliophora, Euplotida) with notes on morphological variation. Eur J Protistol, 2008, 44: 115–129
21 Wilbert N. Eine verbesserte Technik der Protargolimprägnation für Ciliaten. Mikrokosmos, 1975, 64: 171–179
22 Gao S, Chen Z, Shao C, *et al*. Reconsideration of the phylogenetic position of *Frontonia*-related Peniculia (Ciliophora, Protozoa) inferred from the small subunit ribosomal RNA gene sequences. Acta Protozool, 2008, 47: 47–54
23 Medlin L, Elwood H J, Stickel S, *et al*. The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. Gene, 1988, 71: 491–499
24 Miao M, Song W, Clamp J C, *et al*. Further consideration of the phylogeny of some "traditional" heterotrichs (Protista, Ciliophora) of uncertain affinities, based on new sequences of the small subunit rRNA gene. J Eukaryot Micribiol, 2009, 56: 244–250
25 Jeanmougin F, Thompson J D, Gouy M, *et al*. Multiple sequence alignment with Clustal X. Trends. Biochem Sci, 1998, 23: 403–405
26 Ronquist F, Huelsenbeck J P. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics, 2003, 19: 1572–1574
27 Nylander J A A. MrModeltest 2.0. Program distributed by the author. Evolutionary Biology Centre, Uppsala University, Sweden. 2004.
28 Felsenstein J. "PHYLIP: Phylogeny Inference Package," Version 3.57c. Department of Genetics, University of Washington, Seattle, WA. 1995.
29 Kimura M. A simple method of estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol, 1980, 16: 111–120
30 Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol, 1987, 4: 406–425
31 Swofford D L. PAUP*. Phylogenetic analysis using parsimony (* and other methods). Sunderland, Sinauer. 2002.
32 Posada D, Crandall K A. Modeltest: testing the model of DNA substitution. Bioinformatics, 1998, 14: 817–818
33 Nixon K C. The Parsimony Ratchet, a new method for rapid parsimony analysis. Cladistics, 1999, 15: 407–414
34 Vos R A. Accelerated likelihood surface exploration: the likelihood ratchet. Syst Biol, 2003, 52: 368–373
35 Sikes D S, Lewis P O. Beta software, version 1. PAUPRat: PAUP implementation of the parsimony ratchet. Department of Ecology and Evolutionary Biology, University of Connecticut, Storrs. 2001.
36 Jones D T, Taylor W R, Thornton J M. The rapid generation of mutation data matrices from protein sequences. Comput Appl Biosci, 1992, 8: 275–282
37 Yi Z, Song W, Warren A, *et al*. A molecular phylogenetic investigation of *Pseudoamphisiella* and *Parabirojimia* (Protozoa, Ciliophora, Spirotrichea), two genera with ambiguous systematic positions. Eur J Protistol, 2008, 44: 45–53
38 De Rijk P, De Wachter R. RNAViz, a program for the visualisation of RNA secondary structure. Nucl Acids Res, 1997, 25: 4679–4684
39 Li L, Song W, Warren A, *et al*. Reconsideration of the phylogenetic positions of five peritrich genera - *Vorticella, Pseudovorticella, Zoothamnopsis, Zoothamnium and Epicarchesium* (Ciliophora; Peritrichia; Sessilida), based on small subunit rRNA gene sequences. J Eu-

karyot Microbiol, 2008, 55: 448–456
40 Neefs J M, Van De Peer Y, De Rijk P, *et al*. Compilation of small ribosomal subunit RNA structures. Nucl. Acids Res, 1993, 21: 3025–3049
41 Lynn D H. Systematics of Ciliates. In Hausmann, K., Bradbury, P.C. (eds.) Ciliates: Cells as Organisms. Gustav Fischer Verlag, Stuttgart. 1996, pp. 51–72.
42 Foissner W, Moon-Van Der Staay S Y, Van Der Staay G W M, *et al*. Reconciling classical and molecular phylogenies in the stichotrichines (Ciliophora, Spirotrichea), including new sequences from some rare species. Eur J Protistol, 2004, 40: 265–281
43 Clements K D, Gray R D, Choat J H. Rapid evolutionary divergences in reef fishes of the family Acanthuridae (Perci-formes: Teleostei). Mol Phylo Evol, 2003, 26: 190–201
44 Moreira D, Le Guyader H, Philippe H. Unusually high evolutionary rate of the elongation factor lar genes from the ciliophora and its impact on the phylogeny of eukaryotes. Mol Biol Evol, 1999, 16: 234–245
45 Ruthmann A, Hauser M. Mitosis-like macronuclear division in a ciliate. Chromosoma, 1974, 45: 261–272
46 Ammermann D. Die Kernverhältnisse des Ciliaten *Protocruzia depressa* n. sp. Arch Protistenkd, 1968, 110: 434–438