Phylogenetic study of Class Armophorea (Alveolata, Ciliophora) based on 18S-rDNA data

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Abstract

The 18S rDNA phylogeny of Class Armophorea, a group of anaerobic ciliates, is proposed based on an analysis of 44 sequences (out of 195) retrieved from the NCBI/GenBank database. Emphasis was placed on the use of two nucleotide alignment criteria that involved variation in the gap-opening and gap-extension parameters and the use of rRNA secondary structure to orientate multiple-alignment. A sensitivity analysis of 76 data sets was run to assess the effect of variations in indel parameters on tree topologies. Bayesian inference, maximum likelihood and maximum parsimony phylogenetic analyses were used to explore how different analytic frameworks influenced the resulting hypotheses. A sensitivity analysis revealed that the relationships among higher taxa of the Intramacronucleata were dependent upon how indels were determined during multiple-alignment of nucleotides. The phylogenetic analyses rejected the monophyly of the Armophorea most of the time and consistently indicated that the Metopidae and Nyctotheridae were related to the Litostomatea. There was no consensus on the placement of the Caenomorphidae, which could be a sister group of the Metopidae + Nyctorheridae, or could have diverged at the base of the Spirotrichea branch or the Intramacronucleata tree.

Keywords: Caenomorpha, Metopus, Nyctotherus, sensitivity analysis.

Received: April 15, 2013; Accepted: July 18, 2013.

Introduction

The Class Armophorea Lynn, 2004 is represented by ciliates that live in anoxic environments, have mitochondria that transformed into hydrogenosomes during the course of evolution, and harbor symbiotic methanogenic prokaryotes (Jankowski, 1964a,b; Fenchel and Finlay, 1991; Gijzen and Barugaheare, 1992; van Hoek et al., 2000a,b; Lynn, 2008). Many armophoreans occur as free-living forms, such as Brachonella Jankowski, 1964, Caenomorpha Perty, 1852, and Metopus Claparède & Lachmann, 1858, while others, such as Nyctotherus Leidy, 1849 and Nyctotheroides Grassé, 1928, inhabit the digestive system of animals, particularly insects and amphibians (Lynn, 2008). In the past, the armophoreans were classified as heterotrichs based on their morphology (Corliss, 1979). However, phylogenetic analyses of the 18S-rDNA (Embley et al., 1995; Hirt et al., 1995; van Hoek et al., 1998; Shin et al., 2000; Affa’a et al., 2004; Gong et al., 2009; Miao et al., 2009a,b; Vd’acny et al., 2010) and of histone H4 and α-tubulin data (Israel et al., 2002; Katz et al., 2004) have all grouped these organisms outside the Heterotrichia Stein, 1859, and within the Intramacronucleata Lynn, 1996. Hence, Armophorea is now considered as a molecular class, sometimes referred to as a “riboclass”, for which morphological synapomorphies are unknown (Lynn, 2008).

The phylogenetic affinities of the armophoreans to other intramacronucleates remain unclear, with recent classifications suggesting an uncertain placement near the Spirotrichea Bütschli, 1889 or the Litostomatea Small & Lynn, 1981 (Riley and Katz, 2001; Cavalier-Smith, 2004; Lynn, 2008; Katz and Kovner, 2010); this uncertainty reflects divergent competing phylogenetic hypotheses (Shin et al., 2000; Gong et al., 2009; Miao et al., 2009a,b; Li et al., 2010; Vd’acny et al., 2010; Zhang et al., 2010; Lynn and Wright, 2013). In addition, the monophyly of armophoreans is sometimes rejected when 18S data of
Caenomorpha are considered (Miao et al., 2009a,b; Lynn and Wright, 2013). Statistical support for the branching pattern of armophoreans and the intervening taxa is also variable.

As indicated by various authors (Rannala et al., 1998; Zwickl and Hillis, 2002; Bergsten, 2005; Heath et al., 2008), the quality of taxon and character sampling is an important factor that interferes with phylogenetic hypotheses. This was shown by Vd’acny et al. (2010), who found that the stability of the Armophorea is to some extent dependent on the number of sequences from representatives of other taxa included in the alignment. However, the sequences of armophorean representatives have never been broadly sampled to adequately evaluate their phylogenetic stability. The sensitivity of data to nucleotide alignment parameters and character weighting (Wheeler, 1995; Morrison and Ellis, 1997; Hall, 2005; Kjer et al., 2007; Goloboff et al., 2008; Dessimoz and Gil, 2010) is related to differences in phylogenetic hypotheses. This has already been demonstrated for ciliates by Kivimaki et al. (2009), although their study did not include data on armophoreans. Inconsistencies among phylogenies may reflect the properties of the analytical frameworks used, i.e., different sets of premises, concepts and processes underlying the phylogenetic analyses (Hillis, 1987; Huelsenbeck and Kirkpatrick, 1996; Bruno and Halpern, 1999; Swofford et al., 2001).

In this study, we examined the phylogenetic relationships of armophoreans based on 18S-rDNA sequences available in the NCBI/GenBank database and used a broad sample that included various sequences from unidentified armophoreans. We also explored the usefulness of two nucleotide alignment criteria and three phylogenetic frameworks, in addition to undertaking a sensitivity analysis. The systematics of the Armophorea is discussed based on these results and data from the literature.

Material and Methods

Sequence acquisition

Since the monophyly of the Armophorea is questionable and the phylogenetic affinities are variable (Shin et al., 2000; Miao et al., 2009a,b; Vd’acny et al., 2010), we broadly sampled ciliophoran 18S sequences to include representatives of all recognized ciliate classes (Lynn, 2008). One hundred and ninety-five ciliate 18S rDNA sequences, representing the phylogenetic affinities are variable (Shin et al., 2009a,b; Lynn et al., 2009a,b; Vd’acny et al., 2010), we

There is generally little agreement on how to treat ‘ambiguously alignable’ regions. Some authors recommend elimination of the nucleotide positions of ambiguous alignments as a means of improving phylogenetic hypotheses (Olsen and Woese, 1993; Swofford et al., 1996; Talavera and Castresana, 2007), while others consider that such positions contain information that is potentially useful for phylogenetic reconstructions (Lutzoni et al., 2000; Aagesen, 2004; Redelings and Suchard, 2009). In this study, we opted to preserve this information and to explore different alignments (Wheeler, 1995; Doyle and Davis, 1998). To assess how different alignment criteria might influence phylogenetic hypotheses obtained from the ciliate 18S data, the sequences were multiple-aligned using the ClustalW algorithm and the 18S rRNA secondary structure. The resulting alignment files were inspected in BioEdit v7.0.5 (Hall, 1999) to code leading and trailing gaps as missing data. The overall and mean p-distances displayed in Tables 1 and 2 were calculated with the program MEGA

Table 1 - Informational content of the secondary structure alignment (SSA) and Q-score optimized alignment (QOA).

| Content                        | SSA | QOA |
|--------------------------------|-----|-----|
| Averaged Q-score               | 49.7| 56.5|
| Overall mean p-distance        | 0.171| 0.187|
| Mean p-distance within Caenomorphidae | 0.045| 0.109a |
| Mean p-distance within Nyctotheridae | 0.068| 0.066a |
| Total number of characters     | 2424| 2099 |
| Characters informative for parsimony (%) | 58.3| 62.5 |
| Gaps (%)                       | 25.3| 15.6 |

*aValue obtained when sequences AJ009658, AJ009661 and AJ009662 were subtracted from the Metopidae and added to the Caenomorphidae; see section on “Bayesian inference, Maximum-likelihood and Maximum parsimony results”.

Table 2 - Mean p-distances between armophorean families.

| Families/alignment criteria | 1     | 2     |
|-----------------------------|-------|-------|
| SSA | QOA | SSA | QOA |
| Caenomorphidae              | 0.194| 0.211|
| Metopidae *                 | 0.198| 0.224|
| Nyctotheridae               | 0.099| 0.106|

QOA - Q-score optimized alignment; SSA - secondary structure alignment. *Calculated by considering sequences AJ009658, AJ009661 and AJ009662 as metopids (see section on “Bayesian inference, Maximum-likelihood and Maximum parsimony results”).
5 (Tamura et al., 2011), using pairwise deletion as a treatment for gaps and missing data.

ClustalW alignment

The sequences were aligned with the program ClustalW 1.81 through the CIPRES Science Gateway (Miller et al., 2010). Gap-opening (GOP) and gap-extension (GEP) penalties for multiple-alignment were optimized based on the averaged Q-scores, calculated with the program TuneClustalX (Hall, 2004) and used as a benchmark for alignment accuracy (Hall, 2005). The range explored for the parameters included GOP values of 10, 20, 30 and 40, each combined with integer GEP values that varied evenly from 1 to GOP/2. For comparison, we also included the ClustalW default values, i.e., GOP = 10 and GEP = 0.2, to yield 76 combinations (Figure 1). The alignment of the highest averaged Q-score was inspected and refined by eye in BioEdit as a means of improving the averaged Q-score. This alignment is referred to as the Q-score optimized alignment (QOA).

Secondary structure alignment

As an alternative approach, the sequences were aligned based on the eukaryotic 18S rRNA secondary structure using the SINA web aligner (Pruesse et al., 2007) with its default settings. The alignment was inspected in BioEdit to remove gap-only columns followed by further refinement by eye that took into account the structural similarity among the sequences. This alignment is referred to as the secondary structure alignment (SSA).

Phylogenetic analyses

Sensitivity analysis

To evaluate whether differences in the GOP-GEP choices in the ClustalW alignment affected the hypotheses for ciliate 18S phylogeny, the BioNJ algorithm (Gascuel, 1997) implemented in the program PAUP* 4b10 (Swofford, 2003) was used to build neighbor-joining (NJ) trees from p-distance matrices of each alignment. For this purpose, the alignments were not refined manually in order to prevent altering the decisions made by ClustalW for each GOP-GEP combination. The resulting trees were gathered with PAUP* and a strict consensus tree was built to show the insensitive branches, with emphasis on ciliate higher taxa (Figure 2).

Analyses of the QOA and SSA data sets

Both data sets were independently analyzed using Bayesian inference (BI), maximum likelihood (ML) and maximum parsimony (MP) frameworks. In all resulting trees, the root was placed a posteriori at the Intramacronucleata-Postciliodesmatophora split (Lynn, 2008). The BI and ML routines employed a GTR + I (= 0.19) + F (= 0.6) model, selected based on the Akaike information criterion (AIC) (Akaike, 1974; Bos and Posada, 2005) in MODELTEST 3.7 (Posada and Crandall, 1998). To test whether the optimal trees were significantly differ-
ent from suboptimal ones, all of the trees were statistically compared based on the data sets and optimality criteria by which they had been generated, with emphasis on the monophyly/non-monophyly of the Armophorea; this comparison was done using the approximately unbiased (AU) test for maximum likelihood and the Templeton test for maximum parsimony (Templeton, 1983; Shimodaira, 2002). The tests were done using the package CONSEL v0.1i (Shimodaira and Hasegawa, 2001; Shimodaira, 2004) and PAUP* 4b10, respectively. The taxonomy of higher taxa displayed in the phylogenetic trees (Figures 2-6) is mostly according to Lynn (2008), although the taxonomy of the Ventrata and Lamellicorticata agrees with Vd’acny et al. (2010).

Bayesian inference was determined with MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) and was based on two independent Markov chain Monte Carlo (MCMC) simulations that were run with four chains of 1,000,000 generations. The trees were sampled every 200 generations (temperature of heat chains = 0.2), and the first 100,000

![Phylogenetic tree](image-url)
generations were discarded as burn-in. A 50% majority rule consensus of the trees remaining after burn-in was used to calculate the Bayesian posterior probabilities of the recovered kinships; these probabilities were used as node support measures for BI (Schneider, 2007).

For ML, the data sets were analyzed with the program PhyML 3.0 (Guindon and Gascuel, 2003), starting with a BioNJ tree for which the likelihood was improved via SPR branch-swapping to generate the ML tree. Node stability was evaluated via the SH-like aLRT branch support.
The MP analyses were done with the program TNT 1.1 (Goloboff et al., 2003, 2008), using a strategy that combined routines of parsimony-ratchet (Nixon, 1999) with tree-drifting, tree-fusing and sectorial searches (Goloboff, 1999) in order to find optimal cladograms. Gaps were coded as a “fifth base” to accommodate their phylogenetic information (Giribet and Wheeler, 1999; Schneider, 2007), and only parsimony-informative characters were analyzed. We agree with Goloboff (1993) and Platnick et al. (1991, 1996) concerning the incompleteness of equal-weighted cladistics and thus applied the implied-weighting approach of Goloboff (1993) to the data. For this, Goloboff’s parameter $K$ was explored at integer intervals varying evenly from 1 to 10. The resulting trees were summarized via strict consensus. The common synapomorphies of the nodes of interest were assessed by optimizing the characters of each data set onto the trees using TNT. Node support was mea-

Figure 5 - Strict consensus of 10 MP cladograms obtained from the Q-score optimized alignment (CI = 0.194-0.196; RI = 0.728-0.732). Arrows indicate sequences that supposedly belong to metopids; values associated with nodes are symmetric resampling percentages (values < 50% are omitted); values in balloons represent the number of synapomorphies common to all trees and the total possible synapomorphies of a given node, respectively.
Results and Discussion

Sequences and alignments

The global optimum for the averaged Q-scores distribution was found at GOP = 30, GEP = 14 (Figure 1), and the corresponding alignment was selected as optimal among the GOP-GEP combinations that were explored. However, regions with higher averaged Q-scores may exist for wider GOP-GEP intervals. Manual refinement of the optimal alignment improved the averaged Q-score by ~0.75% (56.5533). The ClustalW default parameters produced a suboptimal result (55.5774) within the GOP-GEP space explored (Figure 1).
The alignment based on the corresponding 18S rRNA secondary structure generated a data set for which the averaged Q-score was 49.7616. Although this value appeared to be considerably suboptimal, it should not be interpreted as strictly in SSA since the Q-score is a measure of alignment quality that reflects the minimization of changes among nucleotides, whereas alignments based on secondary structure tend to minimize the changes among RNA structures (Thompson et al., 1994; Hall, 2005; Kjer et al., 2007).

The G + C content of the ciliate 18S data was 44.5 mol%. QOA yielded 2,099 characters, of which 1,312 (62.5%) were parsimony-informative, 408 were constant and 379 variable, but parsimony-uninformative. On the other hand, SSA yielded 2,424 characters, of which 1,412 (58.3%) were parsimony-informative, 455 were constant, and 557 variable, but parsimony-uninformative. Thus, although QOA provided fewer characters it contained slightly more information for parsimony analysis than SSA. The quantitative difference in the number of characters between the two data sets is explained by SSA having more gap entries than QOA (25.6% vs. 15.3%).

The overall mean p-distance of the 18S data was 0.187 for QOA and 0.171 for SSA; the within- and among-group distances also varied according to the alignment criteria. For the armophorean families examined here, the lowest within-group mean distances were found within the Caenomorphidae in SSA whereas this same family had the largest mean distance in QOA (Table 1). Inter-group distance comparisons of the 18S sequences indicated that the Metopidae and Nyctotheridae were genetically closer to each other than to the Caenomorphidae (Table 2).

Sensitivity to GOP-GEp variation

An analysis of sensitivity to GOP-GEp variation (Figure 2) indicated that most higher taxa relationships in the Intramacronucleata (especially those of spirotrich clusters) depended upon how indels were estimated during multiple alignments and the influence of such estimates on the calculation of distance matrices in NJ methods. This situation is aggravated by differences in the placement of ‘difficult’ positions for each combination of parameters. These findings not only corroborate previous observations based on cladistic analyses of implied-aligned matrices of ciliate 18S rRNA (Kivimaki et al., 2009), but also emphasize the need for thorough indel parameter exploration during automated nucleotide alignments (Morrison and Ellis, 1997; Carroll et al., 2006; Smythe et al., 2006; Kjer et al., 2007).

Bayesian inference, Maximum likelihood and Maximum parsimony results

Although a considerable number of sequences from all major ciliate groups was analyzed in this work, the following discussion focuses on the kinships of the Armophorea. BI and ML yielded two topologies (one for each alignment), with the log likelihood of the ML tree obtained with SSA being slightly higher than that for QOA (Figures 3 and 4). In MP analyses, the strict consensus of cladograms resulting from SSA provided more resolution than that from QOA. This finding suggested that the former was slightly more robust to variation in Goloboff’s K parameter than the latter as it dealt with the relationships among higher taxa and affinities within Metopidae and Nyctotheridae (Figures 5 and 6). The matrices resulting from both QOA and SSA showed considerable character incongruence, as indicated by the relatively low ensemble consistency index of their resulting cladograms. On the other hand, the ensemble retention index was relatively high, indicating that most nucleotide primary homologies contributed to synapomorphy. The cladograms from QOA were rather more consistent and showed slightly more homology than those from SSA. Clades representing the main divergences of armophorean lineages hypothesized from the QOA matrix were united by more synapomorphies than those from SSA, except for the Caenomorphidae (Figures 5 and 6).

In all analyses (BI, ML and MP), the Litostomatea was adelphtaxon of some armathoreans, with high support (> 80%) most of the times. However, a completely monophyletic but relatively weakly supported Armophorea was only hypothesized by the BI and ML trees from QOA (Figure 3), which were significantly different (AU test; p < 0.05) from those in which the armophoreans were not monophyletic. For all other trees, the Armophorea were polyphyletic, and Litostomatea was sister to Metopidae + Nyctotheridae. In these trees, the Caenomorphidae branched outside the Lamellicorticata and diverged at the base of Intramacronucleata or near Licnophora spp. and the remaining spirotrich branch (Table 3). These topologies also differed significantly from those in which the armophoreans were monophyletic (AU test, Templeton test; p < 0.05).

The Caenomorphidae were distributed in a fully supported symmetric group, dichotomized in branches containing four terminals, the position of which varied slightly depending on the alignment criteria and analytic framework. Three Caenomorphid terminals that were classified to family level in NCBI/GenBank, namely AJ009658, AJ009661 and AJ009662, unambiguously branched within the Metopidae (Figures 3-6). Moving these sequences into the main Caenomorphidae branch consistently augmented the mean p-distance of this group but had little effect on that of the Metopidae (Table 1). We therefore suppose that such sequences might belong to actual metopids. They were originally mentioned in a paper by van Hoek et al. (1999) as Caenomorpha-“like” species, so their identity is unknown.

The Metopidae comprised a pectinate line of branches, paraphyletic in relation to the monophyletic Nyctotheridae, and showed the least stable phylogenetic pattern among armathoreans in MP analyses; the latter was seen as polytomies in the consensus trees (Figures 5 and 6),
whereas the BI-ML trees of both alignments yielded little inconsistency (Figures 3 and 4). Remarkably, *Metopus contortus* (Quennerstedt, 1867) Kahl, 1932, always diverged at the base of the Metopidae + Nyctotheridae, and *Metopus palaeformis* Kahl, 1927, was consistently monophyletic, even though the genus *Metopus* was not. This situation not only reflects the position of these species in relation to nyctotherids, but also the finding that the branch containing *Brachonella* arose from within *Metopus*. The Nyctotheridae were hypothesized to be monophyletic in all analyses, with the affinities of *Nyctotherus ovalis* Leidy, 1950, sequences varying according to the alignment criteria and phylogenetic framework. The monophyly of *Nyctotherus* depended on the position of its type species *N. velox*, which was unstable, although *Nyctotheroides* was always monophyletic.

**Systematics of the Armophorea**

Our results rejected the classification of Armophorea within the heterotrichs (Corliss, 1979) and corroborated previous studies based on 18S data (Embley et al., 1995; Hirt et al., 1995; van Hoek et al., 1998; Gong et al., 2009; Miao et al., 2009a,b; V’d’acny et al., 2010) and other molecular markers (Israel et al., 2002; Katz et al., 2004; Lynn, 2008) that indicated their status as a separate class.

Regarding their internal kinships, the monophyly of Armophorea was rejected in all but two analyses (Figures 4-6; Table 3) in which it was weakly supported by the data (Figure 3). Miao et al. (2009b) also found armophoreans to be not monophyletic, but in a different scenario than that hypothesized here. Thus, these authors found *Caenomorpha uniserialis* to branch off the base of the Litostomatea, while the Metopidae + Nyctotheridae were a sister group of the protohypotrichs. This contrasts with the study by Shin et al. (2000), in which *C. uniserialis* branched off the base of a *Metopus + Nyctotherus* group with moderate to high support (74-98) in distance-based, ML and MP trees.

In his recent classification of the Ciliophora, Lynn (2008) considered the Armophorea to contain two orders (Armophorida and Clevelandellida). The former included the Families Metopidae and Caenomorphidae, while the latter included the Family Nyctotheridae plus five other families that unfortunately were not represented in our study (see Lynn, 2008). The affinity of the Metopidae to the Nyctotheridae contradicts the Order Armophorida proposed by Lynn (2008), who considered metopids to be closely related to caenomorphs. On the other hand, our results seem to fit the system of Jankowski (2007) better, with the caenomorphids, metopids and nyctotherids placed in three separate orders, viz. Armophorida, Metopida Jankowski, 1980, and Clevelandellida, respectively. The sequence of *Epalxella antiquorum* (Penard, 1922) Corliss, 1960, representative of the Order Odontostomatida Saway, 1940, a group traditionally associated with armophoreans (Jankowski, 1964a,b, 2007), clustered consistently with Class Plagiopyleae Small & Lynn, 1985 (Ventrata) in all analyses (not shown). These findings corroborate a previous study by Stoeck et al. (2007) who found *E. antiquorum* to be related to trimyemids and plagiopylids. Lynn (2008) thus tentatively transferred the odontostomatids from the Armophorea to the Plagiopylea, but considered that phylogenetic analyses of further representatives and of other markers were necessary in order to decide on their affinity. The placement of *Brachonella* within the pectinate assemblage of *Metopus* terminals casts some doubt on the validity of the former, as it involves their morphological separation (see Esteban et al. (1995) and Foissner and Agatha (1999)).

Although the non-monophyly of armophoreans has been reported in the literature (e.g. Miao et al., 2009a,b), it has never been discussed in detail. The unambiguous proximity of the Metopidae and Nyctotheridae is frequent (e.g., Riley and Katz, 2001; Affa’a et al., 2004; Gong et al., 2009), and their position as an adelphotaxon of Litostomatea agrees with the recent study by V’d’acny et al. (2010), who proposed the name Lamellicorticata for the taxon formed by Armophorea and Litostomatea. Accordingly, one putative morphological synapomorphy of this group is the plate-like organization of the postciliary microtubules that form a layer right and between the ciliary rows.

**Table 3 - Phylogenetic position of the Caenomorphidae branch according to different alignment criteria and analytic frameworks.**

| Alignment criterion | Analytic framework | Position of the Caenomorphidae branch | Monophyletic Armophorea? | Number of trees |
|---------------------|--------------------|---------------------------------------|--------------------------|----------------|
| QOA                 | BI/ML              | Adelphotaxon of Metopidae + Nyctotheridae | Yes                      | 2              |
| SSA                 | BI/ML              | In a trichotomy among *Licnophora* spp. and the remaining Spirotrichae | No                       | 2              |
| QOA                 | MP (K = 1-5)       | Diverged at the base of the Intramacronucleata | No                       | 5              |
| QOA                 | MP (K = 6-10)      | Diverged at the base of a clade formed by Spirotrichae (Ventrata) | No                       | 5              |
| SSA                 | MP (K = 1-10)      | Diverged at the base of the Intramacronucleata | No                       | 10             |

BI - Bayesian inference, ML - Maximum likelihood, MP - Maximum parsimony, QOA - Q-score optimized alignment; SSA - secondary structure alignment.
(Foissner and Agatha, 1999; Lynn, 2008; Vd’acny et al., 2010).

In a detailed fine structure study of _Caenomorpha medusula_ Perty, 1852 (Figures 7 and 8), Santa-Rosa (1975) found that postciliary microtubules were not developed in the somatic kinetids, thus precluding the organization mentioned above (Figures 7C,D). Consequently, if the Armophorea are to be considered monophyletic, then a loss of the plate-like arrangement of postciliary microtubules is assumed to have occurred after the Caenomorphidae lineage diverged at the base of the armophorean cluster (Figure 3). On the other hand, assuming that caenomorphids are distantly related to armophoreans and that no further traditional armophoreans are found to lack the plate-like arrangement of postciliary microtubules, the presence of such features can be assumed to be a feasibly consistent synapomorphy of the Lamellicorticata ex Caenomorphidae.

The classification of metopids alongside with caenomorphs is generally based in the assumption of homology of the perizonal ciliary stripe by Small and Lynn (1985) and Puytorac (1994), as discussed by Foissner and Agatha (1999). Foissner and Agatha (1999) described and compared the morphogenetic process in _Metopus hasei_ Sondheim, 1929 and _M. inversus_ (Jankowski, 1964) Foissner and Agatha, 1999 to that described for _C. medusula_ by Martin-Gonzalez et al. (1987) and concluded that they have different morphogenetic origin and function (Foissner and Agatha, 1999). Accordingly, the metopid perizonal stripe

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**Figure 7** - Micrographs of _Caenomorpha medusula_ from Santa-Rosa (1975). a-b. Protargol impregnated specimens; c-d. Transmission electron microscopy sections. a. Specimen in lateral view showing meridian (bell) kineties (BK); b. Specimen in aboral view showing adoral membranelles (AM) and perizonal stripe kineties (PZ). c. Kinetid organization of a bell kinety. Arrows indicate cathetodesmal fibers departing in opposite directions; d. Kinetid organization in the perizonal stripe. Arrows indicate cathetodesmal fibers. Magnifications: a. 650x; b. 570x; c-d. 30,000x.
generates only the paroral for the opisthe, whereas the
calenomorphid stripe generates the paroral plus the adoral
membranelles and the opisthe’s juvenile perizonal stripe
(Martin-Gonzalez et al., 1987; Foissner and Agatha, 1999).
Additionally, the kinetome organization of metopids dif-

Figure 8 - Transmission electron micrographs of Caenomorpha medusula, from Santa-Rosa (1975). a. Tangential section of two adoral membranelles, delimited by brackets at the anterior region and with ciliary rows of one membranelle numbered 1-4. Postciliary microtubules (arrow), transverse microtubules (arrowheads), interkinetosomal connective (double arrowhead; our interpretation), and desmoses (D) are shown; b. Diplostichomonad paroral (P) with peristomial ridge (arrowhead); c. Longitudinal section of the kinetosomes in the perizonal region showing transverse microtubules (ar-
rows; our interpretation), a naked (barren) kinetosome (KSN) and a prokaryote symbiont (B). Magnifications: a. 30,000x; b. 12,000x; c. 36,000x.

ters from that of caenomorphids (Santa-Rosa, 1975; Sola
et al., 1990; Silva-Neto, 1993; Decamp and Warren, 1997;
Foissner and Agatha, 1999). In M. hasei and M. inversus
the somatic dikinetids have a barren anterior kinetosome,
while in perizonal dikinetids both kinetosomes were cili-
ated (Foissner and Agatha, 1999). On the other hand, in C.
medusula all of the kinetosomes in the meridian (bell)
kinetemes are ciliated whereas the posterior kinetosome of
perizonal dikinetids is barren (Santa-Rosa, 1975) (Figures
7D and 8C). The three lowermost perizonal dikinetids in
M. hasei and M. inversus are not positioned equidistantly,
compared to the equidistant placement in C. medusula.

While the foregoing features can be used to support
hypotheses that the Caenomorphidae are not closely related
to the Metopidae + Nyctotheridae, there is morphologic ev-
dence to support the monophyly of Armophorea, although
sometimes ambiguously. The most obvious morphological
characteristic is the body torsion present in metopids and
caenomorphids (Jankowski, 1964b; Corliss, 1979). Among
the metopids, this torsion is conspicuous in the campa-
nulate-shaped representatives of Brachonella Jankowski,
1964. Furthermore, Brachonella darwini (Kahl, 1927)
Jankowski, 1964b, exhibits a thorn-like posterior projec-
tion resembling those of the Caenomorpha (Jankowski,
1964b). The presence of interkinetosomal connectives
jointing adoral membranelles of C. medusula (see Figu-
re 8A) characterizes them as heteromembranelles
(Puytorac and Grain, 1976; Lynn, 2008) that also occur in
clevelandellids (Lynn, 2008).

Such evidence might be considered support for a

close relationship between caenomorphids and cleveland-
elids. However, the presence of heteromembranelles best
fits the phylogenetic trees as two independent gains, viz.
one in the Caenomorphidae branch and another in the Nyctotherididae (representing the clevelandellids). This also applies even to trees in which Armophorea is monophyletic, given the paraphyly of Metopidae (Figure 3). A diplostichomonad paroral, in which a ridge separates two rows of kinetosomes (Figure 8B) was found in C. medusula (1975), thus matching this structure’s configuration in clevelandellids (Paulin, 1967; Puytorac and Grain, 1976; Takahashi and Imai, 1989; Grim, 1998), but also in the metopid Parametopodium circumlabens (Biggar & Wenrich, 1932) Aescht, 2001 (Silva-Neto, 1993). However, this possibly differs from the seemingly linearly arranged oral dikinetids in Metopus (Esteban et al., 1995; Foissner and Agatha, 1999; Lynn, 2008).

The BI-ML trees inferred from the secondary structure alignment and the MP cladograms (Figures 5 and 6; Table 3) also show the possibility of caenomorphids having either diverged at the base of the Intramacronucleata tree, as suggested by Lynn and Wright (2013) or to be related to Spirotrichea. The phylogeny of ciliates based on other markers exhibit different branching patterns (Lynn, 2008) in which metopids and nyctotherids are closely related to spirotrichs. Based on α-tubulin amino acids, Israel et al. (2002) hypothesized a neighbor-joining cluster formed by M. palaeformis + N. ovalis with the spirotrich Euplotes spp. distantly placed from the litostomean cluster. Moreover, based on histone H4 data, a neighbor-joining tree was hypothesized by Katz et al. (2004) in which M. palaeformis and N. ovalis branched off a trichotomy formed by Spirotrichea and the remaining ciliate clusters (the rooting method was not specified), except for litostomeans, which were not included (Katz et al., 2004). In any case, α-tubulin and H4 phylogenies must be interpreted cautiously because of paralogy (Israel et al., 2002; Katz et al., 2004). These results corroborate the close affinity of metopids to nyctotherids shown by our analyses. However, further data, especially on caenomorphids, is still required to improve our understanding of armophorean kinships based on α-tubulin and H4 phylogenies.

Concluding Remarks

The present study has shown that different nucleotide alignment criteria and the use of different phylogenetic frameworks provided different hypotheses to explain the evolutionary affinities of armophoreans based on the 18S marker. This and the sensitivity of some basal branching patterns of the Intramacronucleata to GOP-GEU variation highlight the importance of explicitness in nucleotide alignment criteria. Whereas the 18S phylogeny of the Armophorea results in an apparently stable placement of metopids and nyctotherids near the litostomeans, the same cannot be said for caenomorphids. Moreover, assumptions regarding the evolution of morphological features based on 18S phylogeny are quite general and ambiguous and must not be over-interpreted since various aspects of the life cycle (which includes morphogenesis) and fine structure of most representatives of Armophorea (Foissner and Agatha, 1999; Lynn, 2008) remain unknown. Improvements in taxon sampling for phylogenetic analyses, the use of additional molecular markers, and advances in our knowledge of the life cycle and fine structure of armophoreans should shed some light on the natural history of these organisms.

Acknowledgments

The authors thank Prof. Dr. Milden Rodrigues de Santa-Rosa for kindly allowing the use of his micrographs of Caenomorpha medusula, and the anonymous reviewers for their comments and suggestions. This study was financed by a post-doctoral fellowship to TSP by CNPq (PDJ) and CAPES (PRODOC) via the project PROTAX (no. 52/2010).

References

Aagesen L (2004) The information content of an ambiguously alignable region, a case study of the trnl intron from the Rhamnaceae. Org Divers Evol 4:35-49.

Affa’a FM, Hickey DA, Struder-Kypke M and Lynn DH (2004) Phylogenetic position of species in the genera Anaplephyra, Plagiota, and Nyctotheroides (Phylum Ciliophora), endosymbiotic ciliates of anemids and anurans. J Euk Microbiol 51:301-306.

Akaike HA (1974) A new look at the statistical model identification. IEEE Trans Automat Contr 19:716-723.

Anisimova M and Gascuel O (2006) Approximate likelihood-ratio test for branches: A fast, accurate, and powerful alternative. Syst Biol 55:539-552.

Bergsten J (2005) A review of long-branch attraction. Cladistics 21:163-193.

Bos DH and Posada D (2005) Using models of nucleotide evolution to build phylogenetic trees. Dev Comp Immunol 29:211-227.

Bruno WJ and Halpern AL (1999) Topological bias and inconsistency of maximum likelihood using wrong models. Mol Biol Evol 16:564-566.

Carroll H, Ridge P, Clement M and Snell Q (2006) Effects of gap open and gap extension penalties. In: Clement M and Snell Q (eds) Proceedings of the Third Biotechnology and Bioinformatics Symposium. Brigham Young University, Utah, pp 19-23.

Cavalier-Smith T (2004) Chromalveolate diversity and cell megagvolution: Interplay of membranes, genomes and cytoskeleton. In: Hirt RP and Horner DS (eds) Organelles, Genomes and Eukaryote Phylogeny. CRC Press, Boca Raton, pp 75-108.

Corliss JO (1979) The Ciliated Protozoa. Characterization, Classification and Guide to the Literature. Pergamon Press, Oxford, 445 pp.

Decamp O and Warren A (1997) Observations on the morphology of Caenomorpha uniserialis Levander, 1894 (Ciliophora, Heterotrichida) isolated from a wastewater treatment plant. Acta Protozool 36:105-110.
Dessimoz C and Gil M (2010) Phylogenetic assessment of alignments reveals neglected tree signal in gaps. Genome Biol 11:R37.

Doyle JJ and Davis JJ (1998) Homology in molecular phylogenetics: A parsimony perspective. In: Solis DE, Solitis PS and Doyle JJ (eds) Molecular Systematics of Plants. II. DNA Sequencing. Kluwer Academic Publishers, Boston, pp 101-131.

Embley TM, Finlay BJ, Dyal PL, Hirt RP, Wilkinson M and Williams AG (1995) Multiple origins of anaerobic ciliates with hydrogenosomes within the radiation of aerobic ciliates. Proc R Soc B: Biol Sci 262:87-93.

Esteban G, Fenchel T and Finlay B (1995) Diversity of free-living morphospecies in the ciliate genus *Metopus*. Arch Protistenkunde 146:137-164.

Fenchel T and Finlay BJ (1991) Synchronous division of an endosymbiotic methanogenic bacterium in the anaerobic ciliate *Plagioïdyla frontata* Kahl. J Protozool 38:22-28.

Foissner W and Agatha S (1999) Morphology and morphogenesis of *Metopus hasei* Sondheim, 1929 and *M. inversus* (Jankowski, 1964) nov. comb. (Ciliophora, Metopida). J Euk Microbiol 46:174-193.

Gascuel O (1997) BIONJ: An improved version of the NJ algorithm based on a simple model of sequence data. Mol Biol Evol 14:685-695.

Gijzen HJ and Barugahare M (1992) Contribution of anaerobic protozoa and methanogens to hindgut metabolic activities of the American cockroach, *Periplaneta americana*. Appl Environ Microbiol 58:2565-2570.

Giribet G and Wheeler WC (1999) On gaps. Mol Phylogenet Evol 2:268-272.

Goloboff PA (1993) Estimating character weights during tree search. Cladistics 9:83-91.

Goloboff PA (1999) Analyzing large data sets in reasonable times: Solutions for composite optima. Cladistics 15:415-428.

Goloboff PA, Carpenter JM, Arias JS and Esquivel DRM (2008) Weighting against homoplasy improves phylogenetic analysis of morphological data sets. Cladistics 24:1-6.

Gong J, Stoeck T, Yi Z, Miao M, Zhang Q, Roberts DM, Warren A and Song W (2009) Small subunit rRNA phylogenies show that the Class Nassophorea is not monophyletic (Phylum Ciliophora). J Euk Microbiol 56:339-347.

Grim JN (1998) A comparison of three populations of the ciliate genus, *Paracichlidotherus* Grim, 1992. New fish hosts, and biogeography; Revised genus description. J Euk Microbiol 45:40-44.

Guindon S and Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol 52:696-704.

Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W and Gascuel O (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: Assessing the performance of PhyML 3.0. Syst Biol 59:307-321.

Hall BG (2005) Comparison of the accuracies of several phylogenetic methods using protein and DNA sequences. Mol Biol Evol 22:792-802.

Hall TA (1999) BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Series 41:95-98.

Heath TA, Hedtke SM and Hillis DM (2008) Taxon sampling and the accuracy of phylogenetic analyses. J Syst Evol 46:239-257.

Hillis DM (1987) Molecular vs. morphological approaches to systematics. Annu Rev Ecol Syst 18:23-42.

Hirt RP, Dyal PL, Wilkinson M, Finlay BJ, Roberts DM and Embley TM (1995) Phylogenetic relationships among karyorelictids and heterotrichs inferred from small subunit rRNA sequences: Resolution at the base of the ciliate tree. Mol Phylogenet Evol 4:77-87.

Huelsenbeck JP and Kiprick M (1996) Do phylogenetic methods produce trees with biased shapes? Evolution 50:1418-1424.

Israel RL, Pond SLK, Muse SV and Katz LA (2002) Evolution of duplicated alpha-tubulin genes in ciliates. Evolution 56:1110-1122.

Jankowski AW (1964a) Morphology and evolution of Ciliophora. I. The new system of sapropelebiotic Heterotrichida. Zoologichesky Zhurnal 43:503-517.

Jankowski AW (1964b) Morphology and evolution of Ciliophora. III. Diagnoses and phylogenesis of 53 sapropelebionts, mainly of the Order Heterotrichida. Arch Protistenkunde 107:185-294.

Jankowski AW (2007) Phylum Ciliophora Doflein, 1901. In: Krylow MV and Frolov A0 (eds) Protista: Handbook on Zoology, Part 2. Nauka, St. Petersburg, pp 415-976.

Katz LA and Kovner AM (2010) Alternative processing of scrambled genes generates protein diversity in the ciliate *Chilodonella uncinata*. J Exp Zool, Part B, Mol Develop Evol 314:480-488.

Katz LA, Bornstein JG, Lasek-Nesselquist E and Muse SV (2004) Dramatic diversity of ciliate histone H4 genes revealed by comparisons of patterns of substitutions and paralog divergences among eukaryotes. Mol Biol Evol 21:555-562.

Kivimaki KL, Bowditch BM, Riordan GP and Lipscomb DL (2009) Phylogeny and systematic position of *Zosterodasys* (Ciliophora, Synhymeniida): A combined analysis of ciliate relationships using morphological and molecular data. J Euk Microbiol 56:323-338.

Kjer KM, Gillespie JJ and Ober KA (2007) Opinions on multiple sequence alignment, and an empirical comparison of repeatability and accuracy of POY and structural alignment. Syst Biol 56:133-146.

Li LF, Stoeck T, Shin MK, Al-Rasheid KAS, Al-Khedhairy BA and Song W (2010) *Protozooa*, a highly ambiguous ciliate (Protozoa; Ciliophora): Very likely an ancestral form for Heterotrichia, Colpodia or Spirotrichea? With reevaluation of its evolutionary position based on multigene analyses. Science China (Life Sci) 53:131-138.

Lutzoni F, Wagner P, Reeb V and Zoller S (2000) Integrating ambiguously aligned regions of DNA sequences in phylogenetic analyses without violation positional homology. Syst Biol 49:628-651.

Lynn DH (2008) The Ciliated Protozoa: Characterization, Classification, and Guide to the Literature. 3rd edition. Springer, Dordrecht, 606 pp.

Lynn DH and Wright AD (2013) Biodiversity and molecular phylogeny of Australian *Clevelandella* species (Class Armophorea, Order Clevelandellida, Family Clevelandellidae), intestinal endosymbiotic ciliates in the wood-feeding roach
Panesthia cribrata Saussure, 1864. J Euk Microbiol 60:335-341.

Martin-Gonzalez A, Serrano S and Fernandez-Galiano D (1987) Cortical morphogenesis and conjugation process in Caenomorpha medusula (Ciliophora, Heterotrichida). Eur J Protistol 23:111-121.

Miao M, Shao C, Jiang J, Li L, Stoeck T and Song W (2009a) Caryotricha minuta (Xu et al., 2008) nov. comb., a unique marine ciliate (Protista, Ciliophora, Spirotrichea), with phylogenetic analysis of the ambiguous genus Caryotricha inferred from the small-subunit rRNA gene sequence. Int J Syst Evol Microbiol 59:430-438.

Miao M, Song W, Clamp JC, Al-Rasheid KAS, Al-Khedhairy AA and Al-Arifi S (2009b) 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 Euk Microbiol 56:244-250.

Miller MA, Pfeiffer W and Schwartz T (2010) Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In: Proceedings of the Gateway Computing Environments Workshop. IEEE, New Orleans, pp 1-8.

Morrison DA and Ellis JT (1997) Effects of nucleotide sequence alignment on phylogeny estimation: A case study of 18S rDNAs of Apicomplexa. Mol Biol Evol 14:428-441.

Nixon KC (1999) The Parsimony Ratchet, a new method for rapid Parsimony analysis. Cladistics 15:407-414.

Olsen GJ and Woese CR (1993) Ribosomal RNA: A key to phylogeny. FASEB J 7:113-123.

Paulin JJ (1967) The fine structure of Nyctotherus cordiformis (Ehrenberg). J Protozool 14:183-196.

Platnick NI, Coddington JA, Forster RR and Griswold CE (1991) Spinneret morphology and the phylogeny of haplogyne spiders (Araneae, Araneomorphae). Am Mus Novitat 3016:1-73.

Platnick NI, Humphries CJ, Nelson GJ and Williams DM (1996) Is Farris optimization perfect? Three-taxon statements and multiple branching. Cladistics 12:243-252.

Posada D and Crandall KA (1998) Modeltest: Testing the model of DNA substitution. Bioinformatics 14:817-818.

Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J and Glockner FO (2007) SILVA: A comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res 35:7188-7196.

Puytorac P de (1994) Phylum Ciliophora Doflein, 1901. Traité Zoologie 2:1-15.

Puytorac P de and Grain J (1976) Ultrastructure du còrtex buccal et evolution chez les ciliés. Protistologica 12:49-67.

Rammala B, Huelsenbeck JP, Yang Z and Nielsen R (1998) Taxon sampling and the accuracy of large phylogenies. Syst Biol 47:702-710.

Redelings BD and Suchard MA (2009) Robust inferences from ambiguous alignments. In: Rosenberg M (ed) Sequence Alignment: Methods, Concepts, and Strategies. University of California Press, Berkeley, pp 209-270.

Riley JL and Katz LA (2001) Widespread distribution of extensive genome fragmentation in ciliates. Mol Biol Evol 18:1372-1377.

Ronquist F and Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572-1574.

Santa-Rosa MR de (1975) Contribution a l’Ultrastructure Compare de Quelques Espèces de Ciliés Appartenant a Divers Orders. PhD Thesis. Université de Clermont-Ferrand.

Schneider H (2007) Méthodes de Analyse Filogénétique - Um Guia Prático. 3rd edition. SBG and Holos, Ribeirão Preto, 200 pp.

Shimodaira H (2002) An approximately unbiased test of phylogenetic tree selection. Syst Biol 51:492-508.

Shimodaira H (2004) Approximately unbiased tests of regions using multistep-multiscale bootstrap resampling. Ann Stat 32:2616-2641.

Shimodaira H and Hasegawa M (2001) CONSEL: For assessing the confidence of phylogenetic tree selection. Bioinformatics 17:1246-1247.

Shin MK, Hwang UW, Kim W, Wright ADG, Krawczyk C and Lynn DH (2000) Phylogenetic position of the ciliates Phacodinium (Order Phacodinida) and Protocruzia (Subclass Protocruzoida) and systematic of the spirotrich ciliates examined by small subunit ribosomal RNA gene sequences. Eur J Protistol 36:293-302.

Silva-Neto ID da (1993) Estrutura e ultraestrutura de cinco espécies de ciliados heterotróquicos e um estudo comparativo das estruturas infraciliares corticais e bucais da classe Heterotrichae Stein, 1859. PhD thesis. Universidade de Sao Paulo.

Small EB and Lynn DH (1985) Phylum Ciliophora Doflein, 1901. In: Lee J, Hutner SH and Bovee EC (eds) An Illustrated Guide to the Protozoa. Allen Press, Kansas, pp 393-575.

Smythe AB, Sanderson MJ and Nadler SA (2006) Nematode small subunit phylogeny correlates with alignment parameters. Syst Biol 55:972-992.

Sola A, Guinea A, Longís JE and Fernandez-Galiano D (1990) Nouvelles données sur l’infra-ciliature somatique et buccale de Caenomorpha uniserialis Levander, 1894 (Ciliophora, Heterotrichida). Arch Protistenkunde 138:233-238.

Stoeck T, Brummer F and Foissner W (2007) Evidence for local ciliate endemism in an alpine anoxic lake. Micro Ecol 54:478-486.

Swofford DL (2003) PAUP* Phylogenetic Analysis Using Parsimony (* and Other Methods) Version 4. Illinois Natural History Survey, Champaign, 179 pp.

Swofford DL, Olsen GJ, Waddell PJ and Hillis DM (1996) Phylogenetic inference. In: Hillis DM, Moritz C and Mable BK (eds) Molecular Systematics. Sinauer Associates, Sunderland, pp 407-514.

Swofford DL, Waddell PJ, Huelsenbeck JP, Foster PG, Lewis PO and Rogers JS (2001) Bias in phylogenetic estimation and its relevance to the choice between parsimony and likelihood methods. Syst Biol 50:525-539.

Takahashi EI and Imai S (1989) Light and scanning electron microscopy of Nyctotherus kyphodes (Ciliophora, Phacotrichidae) from the Galapagos giant tortoise, Testudo sp. Bull Nippon Vet Zootechnical College 38:9-15.

Talavera G and Castresana J (2007) Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. Syst Biol 56:564-577.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M and Kumar S (2011) MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol Biol Evol 28:2731-2739.
Templeton AR (1983) Phylogenetic inference from restriction endonuclease cleavage site maps with particular reference to the humans and apes. Evolution 37:221-244.

Thompson JD, Higgins DG and Gibson TJ (1994) CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673-4680.

van Hoek AHAM, van Alen TA, Sprakel VSI, Hackstein JHP and Vogels GD (1998) Evolution of anaerobic ciliates from the gastrointestinal tract: Phylogenetic analysis of the ribosomal repeat from *Nyctotherus ovalis* and its relatives. Mol Biol Evol 15:1195-1206.

van Hoek AHAM, Sprakel VSI, van Alen TA, Theuvenet APR, Vogels GD and Hackstein JHP (1999) Voltage-dependent reversal of anodic galvanotaxis in *Nyctotherus ovalis*. J Euk Microbiol 46:427-433.

van Hoek AHAM, Akhmanova AS, Huynen MA and Hackstein JHP (2000a) A mitochondrial ancestry of the hydrogenosomes of *Nyctotherus ovalis*. Mol Biol Evol 17:202-206.

van Hoek AHAM, van Alen TA, Sprakel VSI, Leunissen JAM, Brigge T, Vogels GD and Hackstein JHP (2000b) Multiple acquisition of methanogenic archaeal symbionts by anaerobic ciliates. Mol Biol Evol 17:251-258.

Vd’acny P, Orsi W and Foissner W (2010) Molecular and morphological evidence for a sister group relationship of the Classes Armophorea and Litostomatea (Ciliophora, Inframacronucleata, Lamellicorticata infraphyl. nov.), with an account on basal haptorid litostomateans. Eur J Protistol 46:298-309.

Wheeler WC (1995) Sequence alignment, parameter sensitivity, and the phylogenetic analysis of molecular data. Syst Biol 44:321-331.

Zhang Q, Yi Z, Song W, Al-Rasheid KAS and Warren A (2010) The systematic position of *Paraspashiidiium* Noland, 1937 (Ciliophora, Litostomatea?) inferred from primary SSU rRNA gene sequences and predicted secondary rRNA structure. Eur J Protistol 46:280-288.

Zwickl DJ and Hillis DM (2002) Increased taxon sampling greatly reduces phylogenetic error. Syst Biol 51:588-598.

Internet Resources

Goloboff P, Farris J and Nixon K (2003) T.N.T.: Tree Analysis Using New Technology, http://www.zmuc.dk/public/phylogeny/TNT (November 13, 2012).

Hall BG (2004) Tune ClustalX. Computer software and manual, http://homepage.mac.com/barryghall/TuneClustalX.html (November 15, 2012).

Supplementary Material

The following supplementary material is available for this article:

Table S1 - The 18S-rDNA sequences used in this study.

This material is available as part of the online version of this article from http://www.scielo.br/gmb.

Associate Editor: Guillermo Orti

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