



## 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 + Nyctotheridae, or could have diverged at the base of the Spirotrichea branch or the Intramacronucleata tree.

*Keywords:* *Caenomorpha*, *Metopus*, *Nyctotherus*, sensitivity analysis.

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### 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 Barugahare, 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  $\alpha$ -tubulin data (Israel *et al.*, 2002; Katz *et al.*, 2004) have all grouped these organisms outside the Heterotrichea 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

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*Caenomorphia* 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, including 44 armophorean sequences, were downloaded from the NCBI database (Table S1) and assembled for multiple alignment. To avoid confusion when discussing the systematics of armophoreans, only sequences that were identified at least to the level of family or order were sampled. The armophorean sequences used represented the Families Nyctotheridae Affa'a, 1987 (Order Clevelandellida Puytorac & Grain, 1976), Caenomorphidae Poche, 1913 and Metopidae Kahl, 1927 (Order Armophorida Janakowski, 1964). Many of the armophorean 18S sequences

available from the NCBI were partial so that the missing homologous regions were treated as absent.

### Sequence alignments

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	49.7616	56.5533
Overall mean <i>p</i> -distance	0.171	0.187
Mean <i>p</i> -distance within Caenomorphidae	0.045   0.109 <sup>a</sup>	0.081   0.135 <sup>a</sup>
Mean <i>p</i> -distance within Metopidae	0.068   0.066 <sup>a</sup>	0.079   0.074 <sup>a</sup>
Mean <i>p</i> -distance within Nyctotheridae	0.050	0.053
Total number of characters	2,424	2,099
Characters informative for parsimony (%)	58.3	62.5
Gaps (%)	25.3	15.6

<sup>a</sup>Value 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		
Metopidae <sup>a</sup>	0.194   0.211	
Nyctotheridae	0.198   0.224	0.099   0.106

QOA - Q-score optimized alignment; SSA - secondary structure alignment. <sup>a</sup>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

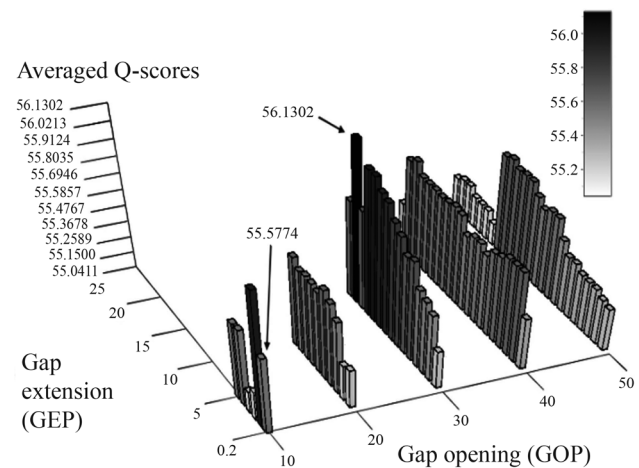
#### Sensitivity analysis

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).

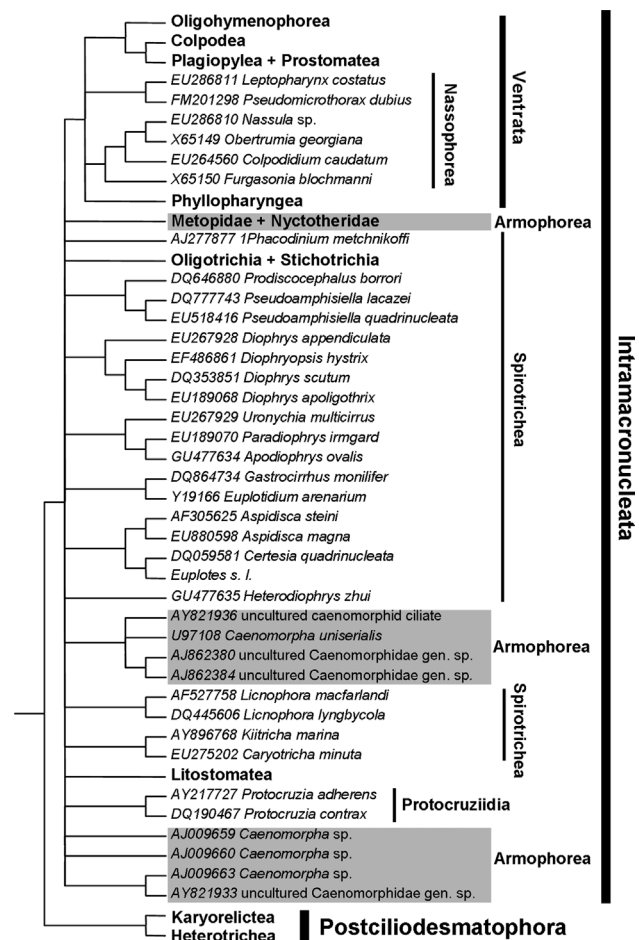
#### Analyses of the QOA and SSA data sets

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) +  $\Gamma$  (= 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-



**Figure 1** - Tridimensional bar plot showing the averaged Q-score variation across the explored parameters. The short arrow indicates the global optimum value that was further improved by manually refining the alignment (see text); the long arrow indicates the averaged Q-score obtained for ClustalW default parameters. The scale on the right defines the variation in averaged Q-scores, with darker shading indicating higher values.

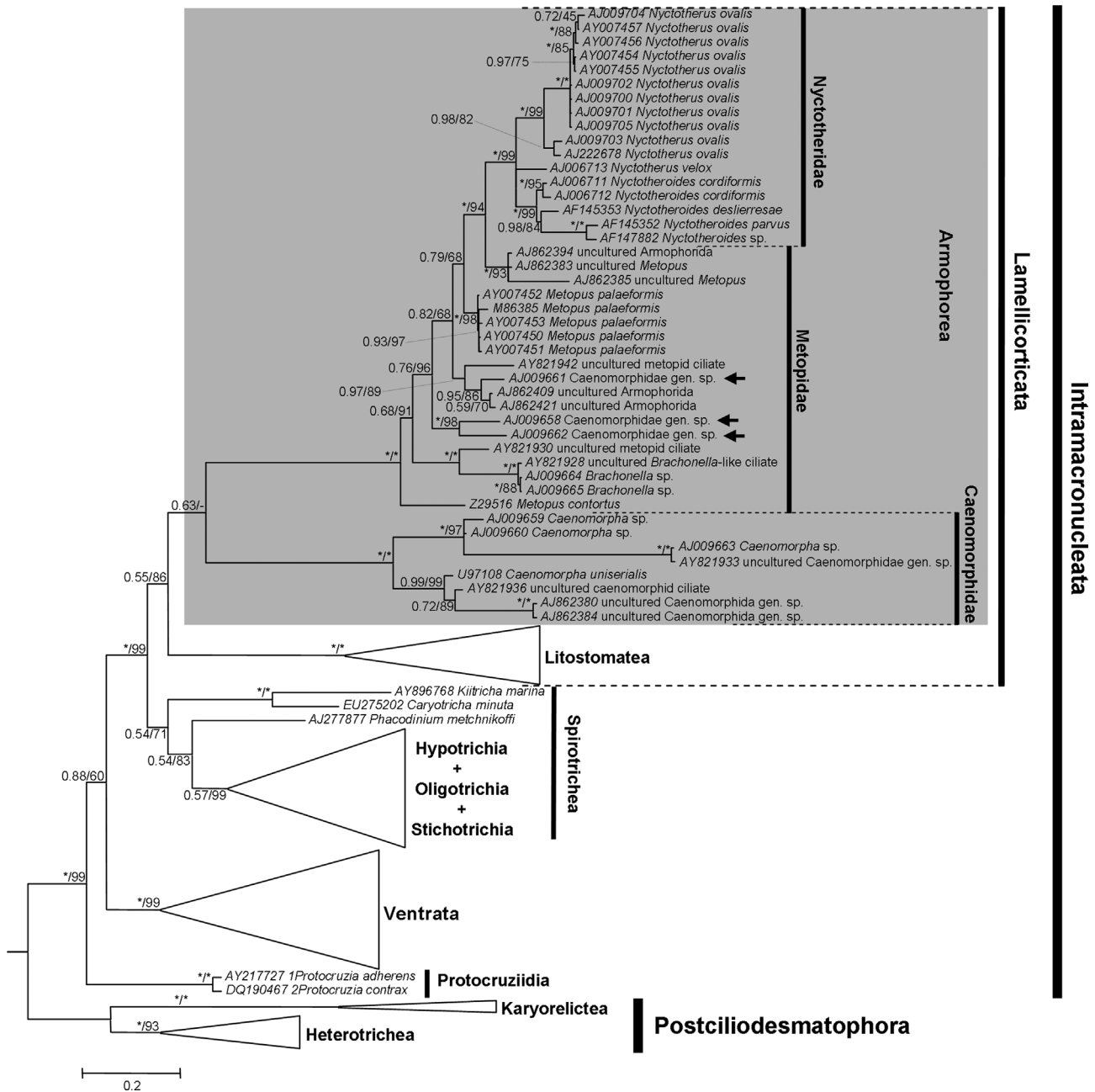


**Figure 2** - Strict consensus of 76 NJ trees, each obtained under a different combination of GOP and GEP.

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

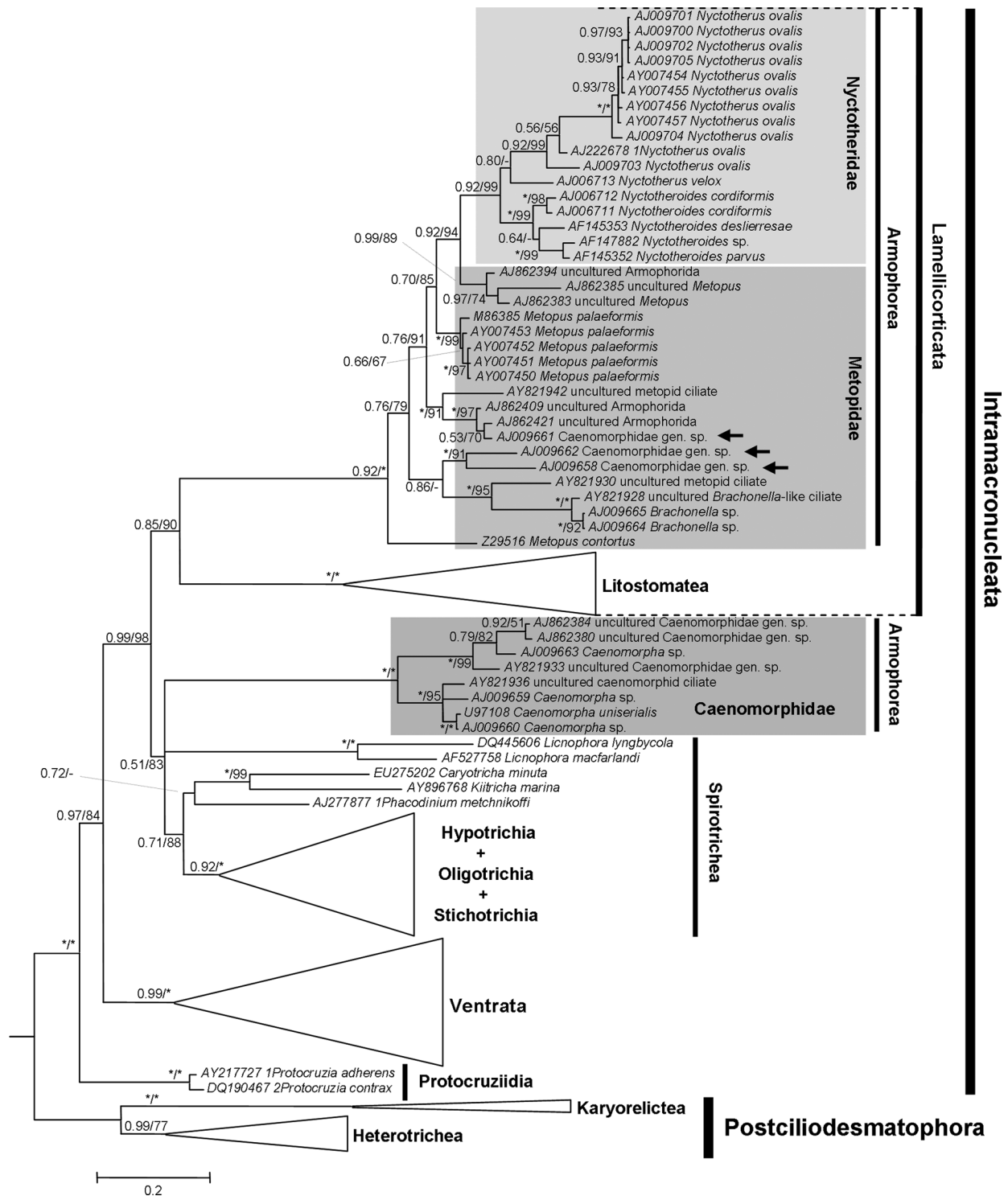
taxa displayed in the phylogenetic trees (Figures 2-6) is mostly according to Lynn (2008), although the taxonomy of the Ventrata and Lamellicorticata agrees with Vd'acny et al. (2010).

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



**Figure 3** - BI/ML tree hypothesized from the Q-score optimized alignment (-lnL = 71422.61314). Arrows indicate sequences that supposedly belong to metopids; values associated with nodes are Bayesian posterior probabilities/aLRT support; \* = full support; - = support < 50%. Scale bar = 2 substitutions per 10 nucleotide positions.

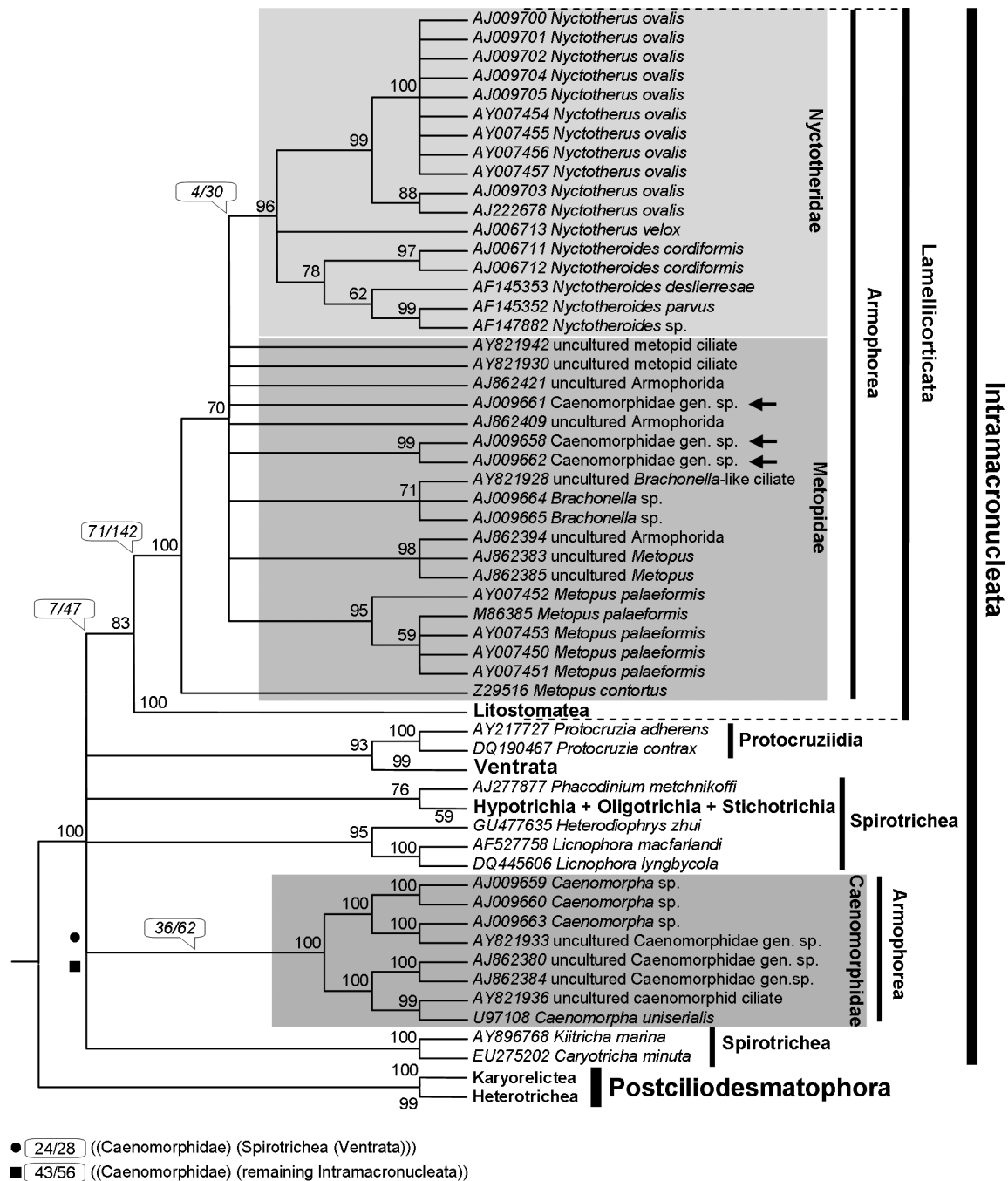




**Figure 4** - BI/ML tree hypothesized from the secondary structure alignment (-lnL = 68901.83179). Arrows indicate sequences that supposedly belong to metopids; values associated with nodes are Bayesian posterior probabilities/aLRT support; \* = full support; - = support < 50%. Scale bar = 2 substitutions per 10 nucleotide positions.

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



**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.

(Anisimova and Gascuel, 2006; Guindon *et al.*, 2010). 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-



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 (*i.e.*, 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 ob-

tained 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 adelphotaxon of some armophoreans, 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 armophoreans 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; Vd'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 caenomorphids. 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 Sawaya, 1940, a group traditionally associated with armophoreans (Jankowski, 1964a,b, 2007), clustered consistently with Class Plagiopylea 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 Foisner 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 Vd'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 <i>Licnophora</i> spp. and the remaining Spirotrichea	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 Spirotrichea (Ventrata)	No	5
SSA	MP ( $K = 1-10$ )	Diverged at the base of the Intramacronucleata	No	10

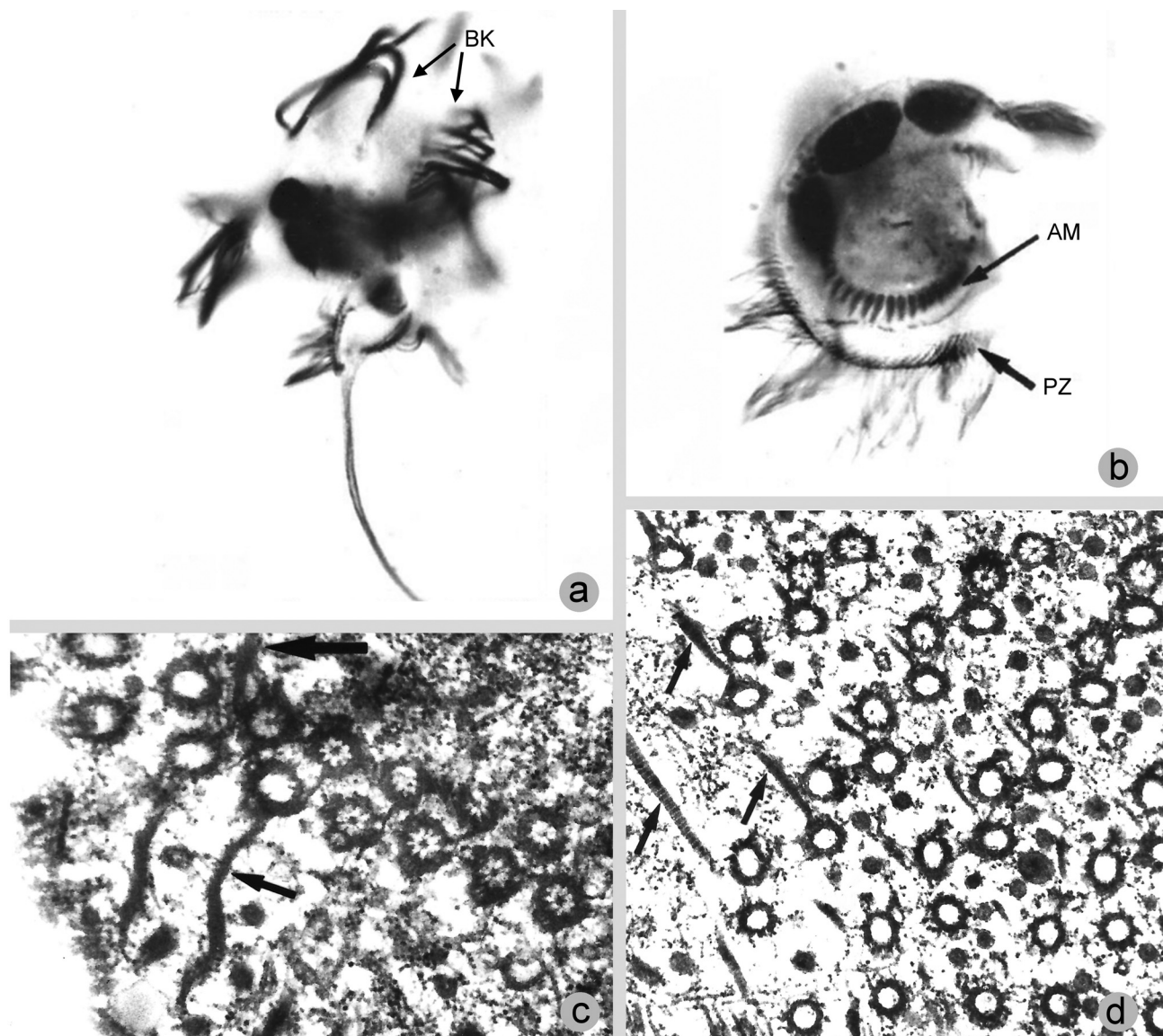
BI - Bayesian inference, ML - Maximum likelihood, MP - Maximum parsimony, QOA - Q-score optimized alignment; SSA - secondary structure alignment.

(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 ar-

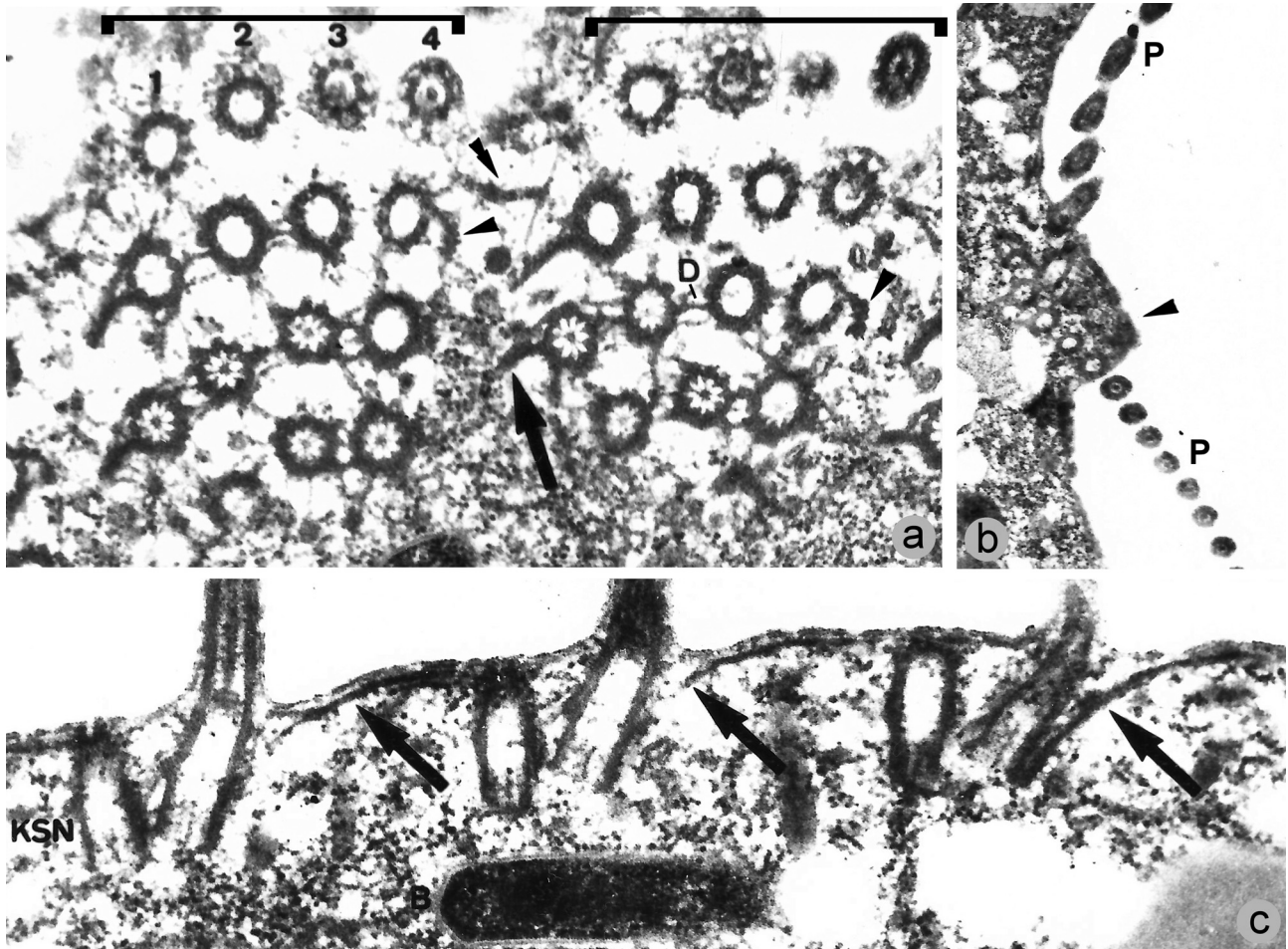
range of postciliary microtubules, the presence of such features can be assumed to be a feasibly consistent synapomorphy of the Lamellicorticata *ex* Caenomorphidae.

The classification of metopids alongside with caenomorphids 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



**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.





**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 desmosomes (D) are shown; b. Diplostichomonad paroral (P) with peristomial ridge (arrowhead); c. Longitudinal section of the kinetosomes in the perizonal region showing transverse microtubules (arrows; our interpretation), a naked (barren) kinetosome (KSN) and a prokaryote symbiont (B). Magnifications: a. 30,000x; b. 12,000x; c. 36,000x.

generates only the paroral for the opisthe, whereas the caenomorphid 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 differs 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 ciliated (Foissner and Agatha, 1999). On the other hand, in *C. medusula* all of the kinetosomes in the meridian (bell) kineties 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 evidence 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 campanulate-shaped representatives of *Brachonella* Jankowski, 1964. Furthermore, *Brachonella darwini* (Kahl, 1927) Jankowski, 1964b, exhibits a thorn-like posterior projection resembling those of the *Caenomorpha* (Jankowski, 1964b). The presence of interkinetosomal connectives joining adoral membranelles of *C. medusula* (see Figure 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 clevelandellids. However, the presence of heteromembranelles best fits the phylogenetic trees as two independent gains, viz.

one in the Caenomorphidae branch and another in the Nyctotheridae (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* by Santa-Rosa (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 *Parametopidium circumlabens* (Biggar & Wenrich, 1932) Aesch, 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  $\alpha$ -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 litostomatean 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 litostomateans, which were not included (Katz *et al.*, 2004). In any case,  $\alpha$ -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  $\alpha$ -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-GEP 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 litostomateans, 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.

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## Internet Resources

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- 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>.

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**Table S1** - The 18S-rDNA sequences used in this study and their GenBank/NCBI accession numbers.

<b>Class/species<sup>a</sup></b>	<b>Accession number</b>
Armophorea Lynn, 2004	
<i>Brachonella</i> sp.	AJ009664
<i>Brachonella</i> sp.	AJ009665
<i>Caenomorpha</i> sp.	AJ009659
<i>Caenomorpha</i> sp.	AJ009660
<i>Caenomorpha</i> sp.	AJ009663
<i>Caenomorpha uniserialis</i>	U97108
Caenomorphidae gen. sp.	AJ009658
Caenomorphidae gen. sp.	AJ009661
Caenomorphidae gen. sp.	AJ009662
<i>Metopus contortus</i>	Z29516
<i>Metopus palaeformis</i>	AY007450
<i>Metopus palaeformis</i>	AY007451
<i>Metopus palaeformis</i>	AY007452
<i>Metopus palaeformis</i>	AY007453
<i>Metopus palaeformis</i>	M86385
<i>Nyctotheroides deslierresae</i>	AF145353
<i>Nyctotheroides parvus</i>	AF145352
<i>Nyctotheroides</i> sp.	AF147882
<i>Nyctotherus cordiformis</i>	AJ006711
<i>Nyctotherus cordiformis</i>	AJ006712
<i>Nyctotherus ovalis</i>	AJ009700
<i>Nyctotherus ovalis</i>	AJ009701
<i>Nyctotherus ovalis</i>	AJ009702
<i>Nyctotherus ovalis</i>	AJ009703
<i>Nyctotherus ovalis</i>	AJ009704
<i>Nyctotherus ovalis</i>	AJ009705
<i>Nyctotherus ovalis</i>	AJ222678
<i>Nyctotherus ovalis</i>	AY007454
<i>Nyctotherus ovalis</i>	AY007455
<i>Nyctotherus ovalis</i>	AY007456
<i>Nyctotherus ovalis</i>	AY007457
<i>Nyctotherus velox</i>	AJ006713
Uncultured Armophorida	AJ862394
Uncultured Armophorida	AJ862409
Uncultured Armophorida	AJ862421
Uncultured <i>Brachonella</i> -like ciliate	AY821928
Uncultured <i>Caenomorpha</i>	AJ862380
Uncultured <i>Caenomorpha</i>	AJ862384
Uncultured <i>Caenomorpha</i> -like ciliate	AY821933
Uncultured caenomorphid	AY821936



Uncultured metopid ciliate	AY821930
Uncultured metopid ciliate	AY821942
Uncultured <i>Metopus</i>	AJ862383
Uncultured <i>Metopus</i>	AJ862385
Colpodea Small & Lynn, 1981	
<i>Bresslaua vorax</i>	AF060453
<i>Bursaria truncatella</i>	U82204
<i>Colpoda aspera</i>	EU039892
<i>Colpoda cucullus</i>	EU039893
<i>Colpoda inflata</i>	M97908
Heterotrichea Stein, 1859	
<i>Blepharisma americanum</i>	AM713182
<i>Chattonidium setense</i>	AM295495
<i>Climacostomum virens</i>	EU583990
<i>Condylostentor auriculatus</i>	DQ445605
<i>Condylostoma curva</i>	EU379939
<i>Condylostoma spatiosum</i>	DQ822483
<i>Eufolliculina uhligi</i>	U47620
<i>Fabrea salina</i>	EU583991
<i>Gruberia</i> sp.	L31517
<i>Peritromus faurei</i>	EU583993
<i>Peritromus kahli</i>	AJ537427
<i>Spirostomum ambiguum</i>	L31518
<i>Stentor coeruleus</i>	AF357145
<i>Stentor polymorphus</i>	FN659823
Karyorelictea Corliss, 1974	
<i>Geleia fossata</i>	AY187925
<i>Geleia simplex</i>	AY187927
<i>Loxodes striatus</i>	AM946031
<i>Parduczia orbis</i>	AY187924
<i>Trachelocerca ditis</i>	GQ167153
Litostomatea Small & Lynn, 1981	
<i>Amphileptus aeschtae</i>	EU242510
<i>Amylovorax dogieli</i>	AF298825
<i>Anoplodinium denticulatum</i>	AM158470
<i>Arcuospathidium muscorum</i>	DQ411859
<i>Balantidium coli</i>	AF029763
<i>Bitricha tasmaniensis</i>	AF298821
<i>Didinium nasutum</i>	U57771
<i>Dileptus</i> sp.	AF029764
<i>Enchelyodon</i> sp.	U80313
<i>Enchelys polynucleata</i>	DQ411861

<i>Epiphyllum shenzhenense</i>	GU574809
<i>Hemiophrys macrostoma</i>	AY102173
<i>Hemiophrys procera</i>	AY102175
<i>Isotricha intestinalis</i>	U57770
<i>Isotricha prostoma</i>	AM158456
<i>Lacrymaria marina</i>	DQ777746
<i>Litonotus pictus</i>	GQ351701
<i>Loxophyllum rostratum</i>	DQ411864
<i>Monodinium</i> sp.	DQ487196
<i>Ophryoscolex caudatus</i>	AM158467
<i>Phialina salinarum</i>	EU242508
<i>Polycosta turniae</i>	AF298818
<i>Polyplastron multivesiculatum</i>	U57767
<i>Spathidium stammeri</i>	DQ411862
<i>Trachelotractus entzi</i>	FJ463745

Nassophorea Small & Lynn, 1981

<i>Colpodidium caudatum</i>	EU264560
<i>Furgasonia blochmanni</i>	X65150
<i>Leptopharynx costatus</i>	EU286811
<i>Nassula</i> sp.	EU286810
<i>Obertruria georgiana</i>	X65149
<i>Orthodonella apohamatus</i>	DQ232761
<i>Pseudomicrothorax dubius</i>	FM201298
<i>Zosterodasys agamalievi</i>	FJ008926
<i>Zosterodasys transverses</i>	EU286812

Oligohymenophorea Puytorac et al., 1974

<i>Anophyroides haemophila</i>	U51554
<i>Bromeliophrya brasiliensis</i>	AJ810075
<i>Epicarchesium abrae</i>	DQ190462
<i>Frontonia didieri</i>	DQ885986
<i>Glaucoma scintillans</i>	AJ511861
<i>Glaucoides bromelicola</i>	AJ810077
<i>Ophryoglena catenula</i>	U17355
<i>Opisthonecta minima</i>	EF417834
<i>Paramecium caudatum</i>	AB252003
<i>Paranophrys marina</i>	FJ858379
<i>Paratetrahymena wassi</i>	GQ292767
<i>Pseudocohnilembus persalinus</i>	AY835669
<i>Tetrahymena corlissi</i>	U17356
<i>Urocentrum turbo</i>	EF114300
<i>Vorticella fusca</i>	DQ190468
<i>Zoothamnium pluma</i>	DQ662854
<i>Zoothamnopsis sinica</i>	DQ190469

Phyllopharyngea Puytorac et al., 1974	
<i>Chilodonella uncinata</i>	AF300284
<i>Chlamydodon excocellatus</i>	AY331790
<i>Discophrya collini</i>	L26446
<i>Dysteria procera</i>	DQ057347
<i>Ephelota</i> sp.	DQ834370
<i>Hartmannula sinica</i>	EF623827
<i>Isochona</i> sp. OOSW-3	AY242118
<i>Tokophrya quadripartita</i>	AY102174
<i>Trithigmostoma steini</i>	X71134
Plagiopylea Small & Lynn, 1985	
<i>Epalxella antiquorum</i>	EF014286
<i>Lechriopyla mystax</i>	AF527757
<i>Plagiopyla frontata</i>	Z29440
<i>Plagiopyla nasuta</i>	Z29442
<i>Trimyema compressum</i>	Z29438
<i>Trimyema minutum thermophilum</i>	AJ292526
Prostomatea Schewiakoff, 1896	
<i>Coleps hirtus</i>	U97109
<i>Coleps spetai</i>	AM292312
<i>Levicoleps biwae</i>	AB354737
<i>Prorodon teres</i>	X71140
<i>Tiarina fusa</i>	FJ858217
Spirotrichea Bütschli, 1889	
<i>Amphisiella magnigranulosa</i>	AM412774
<i>Amphisiella milnei</i>	DQ845293
<i>Apodiophrys ovalis</i>	GU477634
<i>Aspidisca magna</i>	EU880598
<i>Aspidisca steini</i>	AF305625
<i>Caryotricha minuta</i>	EU275202
<i>Certesia quadrinucleata</i>	DQ059581
<i>Diophryopsis hystrix</i>	EF486861
<i>Diophrys apoligothrix</i>	EU189068
<i>Diophrys parappendiculata</i>	EU267928
<i>Diophrys scutum</i>	DQ353851
<i>Euplotes aediculatus</i>	M14590
<i>Euplotes charon</i>	AJ305249
<i>Euplotes harpa</i>	AJ305252
<i>Euplotes minuta</i>	AJ305246
<i>Euplotes raikovi</i>	EF094974
<i>Euplotes rariseta</i>	AJ305248
<i>Euplotes trisulcatus</i>	EF690810
<i>Euplotes vannus</i>	AY361854

<i>Euplotes woodruffi</i>	AF452710
<i>Euplotidium arenarium</i>	Y19166
<i>Eutintinnus pectinis</i>	AY143570
<i>Favella ehrenbergii</i>	GU574768
<i>Gastrocirrhus monilifer</i>	DQ864734
<i>Gastrostyla steinei</i>	AF164133
<i>Heterodiophrys zhui</i>	GU477635
<i>Holosticha bradburyae</i>	EF123706
<i>Kiitricha marina</i>	AY896768
<i>Moneuplotes crassus</i>	AY361895
<i>Novistrombidium sinicum</i>	FJ422990
<i>Onychodromus grandis</i>	AJ310486
<i>Onychodromus quadricornutus</i>	X53485
<i>Oxytricha longa</i>	AF164125
<i>Parabirojimia multinucleata</i>	FJ156104
<i>Parabirojimia similis</i>	DQ503584
<i>Paradiophrys irmgard</i>	EU189070
<i>Licnophora lyngbycola</i>	DQ445606
<i>Licnophora macfarlandi</i>	AF527758
<i>Phacodinium metchnikoffi</i>	AJ277877
<i>Prodiscocephalus borrori</i>	DQ646880
<i>Protocruzia adherens</i>	AY217727
<i>Protocruzia contrax</i>	DQ190467
<i>Protogastrostyla pulchra</i>	EF194083
<i>Pseudoamphisiella lacazei</i>	DQ777743
<i>Pseudoamphisiella quadrinucleata</i>	EU518416
<i>Pseudokeronopsis flava</i>	AY881634
<i>Salpingella acuminata</i>	EU399536
<i>Spirotontonia turbinata</i>	FJ422994
<i>Strobilidium caudatum</i>	AY143573
<i>Strombidinopsis acuminata</i>	FJ790207
<i>Strombidium purpureum</i>	U97112
<i>Stylonychia mytilus</i>	EF535730
<i>Trachelostyla pediculiformis</i>	DQ057346
<i>Uroleptus gallina</i>	AF164130
<i>Uronychia multicirrus</i>	EU267929
<i>Urostyla grandis</i>	EF535731

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<sup>a</sup>Names are listed according to the NCBI/GenBank accession number.