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Integrative Phylogenetic Analysis of the Phylum Ciliophora with Descriptions of Putatively Novel Species

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Abstract

Armophorea, a highly specialized group of diverse ciliates with torsion in the anterior end, mostly inhabit anoxic or hypoxic biotopes, and very little information is known about their diversity, taxonomy, and systematics with advanced studies despite classical studies provided several descriptions and resulted in ambiguity. The main reason being is the oxygen toxicity to these organisms and difficulty in enrichment of clones and absolutely skill demanding to process them for laboratory experiments. In this work, these ciliates were cultured in laboratory for 3 years by maintaining oxic anoxic balance without employing any special anoxic chambers. As a result, the present work investigates three potentially new ciliates namely TBS19, KWBL12, MMP22–Metopus finlaii sp. nov. (close to Metopus contortus); KKV12–Metopus fencheli sp. nov. (close to Metopus fuscus); KB2–Metopus sp. nov. (close to Metopus paeliformides); morphological and molecular characterization of KTC18–Metopus setosus; TP4–Metopus es and KTC12, KTC16, KTC18–Brachonella spiralis spp. respectively; one Caenomorphid ciliate was described by morphological and molecular methods i.e. TP4–Caenomorpha sp. could be or contrary new combination or species described only through morphological methods. Moreover, all these species described here were new reports to India which were isolated from the putrefied zone of fresh water source, Lake Kolleru, India. and subjected to molecular characterization in order to update the phylogeny of the class. Moreover, Brachonella spiralis was described intact for the first time with several methods integrated together providing comprehensive taxonomy via light microscopy (In vivo analysis, silver and Feulgen staining respectively); electron microscopy (SEM&TEM); 18s rDNA sequencing and phylogeny.

Keywords: Torsion, Putrefied, Anoxic, Hypoxic, Armophorea, Metopus, Brachonella, Caenomorpha.

INTRODUCTION

Ciliates belonging to the class Armophorea are very restricted in their distribution, despite being cosmopolitan, they are confined to sediments, both aquatic and terrestrial, and the water column where oxygen tension is extremely reduced or absent (Foissner, 1987; Fenchel, 1993; Fenchel & Finlay, 1995). They are also reported as endocommensalic symbionts in the digestive tracts of a variety of invertebrates and vertebrates (Hackstein & Stumm, 1994; Affa'a et al., 1995; Bhatia & Sewell, 1936). Free living Armophoreans live in the anoxic part of the water column, often nearby sapropel–like sediment (dark–colored sediments, rich in organic matter), due to the presence of hydrogenosomes in their cytoplasm (Finlay B. J & Fenchel, 1989; Hackstein et al., 2008) and methanogen Archaea, prokaryotic organisms that are able to gain energy from methane production through different metabolic pathways (van Bruggen et al., 1983; Borrel et al., 2013). The name of the class, Armophorea, is derived from the name originally proposed by Jankowski (1964) to include only the Caenomorphidae that have the appearance of military helmets. Indeed, it derived from the Latin arma, meaning weapons. The class Armophorea includes two orders namely Armophorida and Clevelandellida. The order Armophorida comprised of two families namely Metopidae and Caenomorphidae. The order Clevelandellida composed of 5 families namely Clevelandellidae, Inferostomatidae, Neonyctotheridae, Nyctotheridae and Sicuophoridae.

There is no conspicuous synapomorphy for members of this class. They have three common features:1) They are restricted to anaerobic habitats and are typically dependent upon methanogenic symbionts.

2) Clevelandellida and Armophorida share pleurotelokinetal stomatogenesis of the adoral polykinetids, (a feature shown by members of other classes) (Foissner & Agatha, 1999).3) They show strong similarities in the sequences of their small subunit rRNA genes (Embley et al., 1995; Hackstein et al., 2001; van Hoek et al., 1998).

Indeed, this class could be called the first "riboclass" of ciliates, since its monophyly is supported by sequence analyses of the SSU rRNA genes. However, there is not yet a signature sequence that would characterize the class. Such anaerobic ciliates are interesting organisms to study, although they are not so easy to detect and manipulate: they are never very abundant at a first glance and most species are relatively rare, if compared to aerobic ciliates. They are able to produce cysts and manifest their presence only when favorable conditions arise. Furthermore, anoxic ciliates require special care to be cultured in laboratory condition, especially for their sensitivity to oxygen exposure. In this work a really straightforward method was followed for keeping and growing anaerobic ciliate populations in laboratory as a part of the original sample. This simple but useful method helped to analyze anaerobic ciliates collected from the freshwater Kolleru Lake, Andhra Pradesh, India. On–line databases lack many sequences belonging to Armophorean at present. For this reason, the present study was focused on the molecular characterization of Armophorean ciliates such as Metopus and Brachonella species. The 18S rDNA gene was sequenced to produce an updated phylogeny of the class. Moreover, some of them appeared of singular interest, being potentially new species.

2.Materials and Methods:

2.1Methods for Morphological species identification and Taxon sampling and culturing:

Sampling was carried out in India's largest freshwater lake, Kolleru, (Andhra Pradesh, India) (16°36' 48.4"N & 081°18'32.7"E) where sediment samples together with some aliquot of surface water were collected, using sterile 50 ml Falcon tubes. Monoclonal cultures were established from a single cell and subsequently maintained in the laboratory for a period of one year from the date of sampling. However, in most of the period they were observed as non-tropic forms i.e. resting cysts.

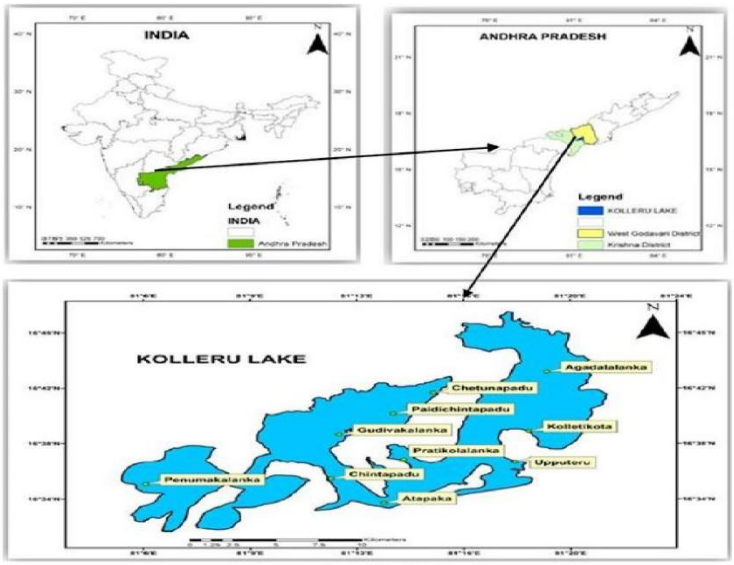

Fig 1: Study Area.

Molecular analysis and phylogeny:

Total genomic extractions were performed according to the protocol. 18S rDNA sequencing for the present isolates were carried out following the same methodology described. Output was used to generate maximum likelihood tree (PHYML software) of class Armophorea. In total 83 sequences were selected: 9 sequences from Kolleru populations, other 50 sequences belonging to Armophorea, plus 7 to Spirotrichea, 10 to Litostomatea and 8 sequences were selected as outgroup (from Postciliodesmatophora). Lengths of sequences was reduced according to shortest one obtaining a 1,039 characters matrix. Tree was built on the modified character matrix employing the GTR+I+G (4 discrete categories) model as indicated by AIC parameter, calculated by j-Model Test. We calculated bootstrap values for maximum likelihood analysis (100 pseudo replicates).

3.Results:

Class Armophorea Lynn, 2004 Order Metopida Jankowski, 1980 Family Metopidae Kahl, 1927

Genus Metopus Claparède and Lachmann, 1858.

Type locality:

Type locality KB2– Metopus sp. was collected from fresh water, Kolleru lake, Andhra Pradesh, India.

Description:

Cells die quickly as soon as placed on the microscope and highly sensitive to oxygen. Hence data was collected from fixed specimens by SEM. Body size 103 112 19–22 µm. Width at the cytostome was 12–17 µm. It appeared narrowly oblong or vase like i.e., length: width ratio near 5:6 on average, Right margin of the body is straight and left margin is bow shaped, slightly twisted anteriorly; preoral dome rostrate, flattened, distinctly projecting from body proper. Nuclear apparatus usually positioned between proximal portion of adoral zone and second third of body. Macronucleus is sausage shaped with the ends slightly conical and measured in a range of 41–72 µm after Feulgen staining, several large globular nucleoli occupied the chromatin mostly and recognizable after Feulgen staining. Micronucleus faintly stained after Feulgen and remained attached to macronucleus in a depression at the level of the half of the length. Contractile vacuole in posterior body end with round to triangle shape. Cortical granules were round and yellow in color (Figs 3J). Cytoplasm colorless. Somatic ciliature composed of monokinetids with only one basal body is ciliated (Figs 9.3J, K, 9.4G, J). caudal cilia absent. Perizonal stripe distinctively invariably composed of five rows: rows 1–3 more narrowly spaced than rows 4 and 5 and several false kineties were present.

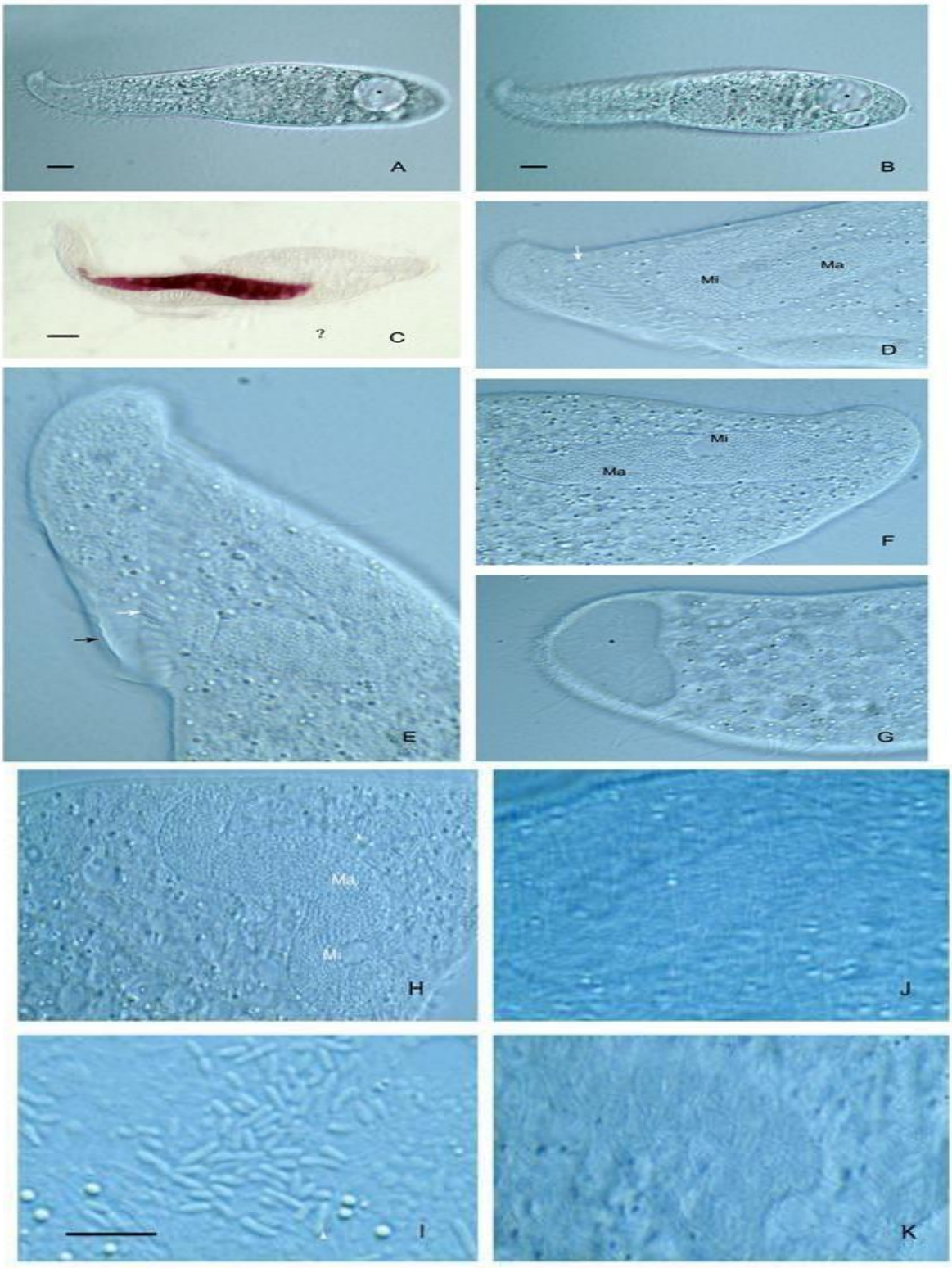

Figure 9.3.(continued). Photomicrographs of *Metopus sp.* Kolleru lake population (KB2) from life (A–B and (D–K) and Feulgen staining (C). A, B, ventral view of representative specimen; (C) general view of Feulgen stained individual with sausage shaped macro nucleus; (D) distal end of adoral zone of membranelles (pointed with white arrow); (E) details of preoral dome (yellow asterisk) and paroral membrane (black arrow); (F) details of nuclear apparatus; (G) caudal region showing contractive vacuole (black asterisk); (H) details of nuclear apparatus and rod–shaped endosymbionts (white arrow head) in cytoplasm; (I) details of endosymbionts in squashed cell. (J) details of ventral cilia (one cilia from each unit); (K) details of dorsal cilia (one cilia from each unit). Abbreviations. Ma, macronucleus; Mi, micronucleus. Bars stand for 5µm.

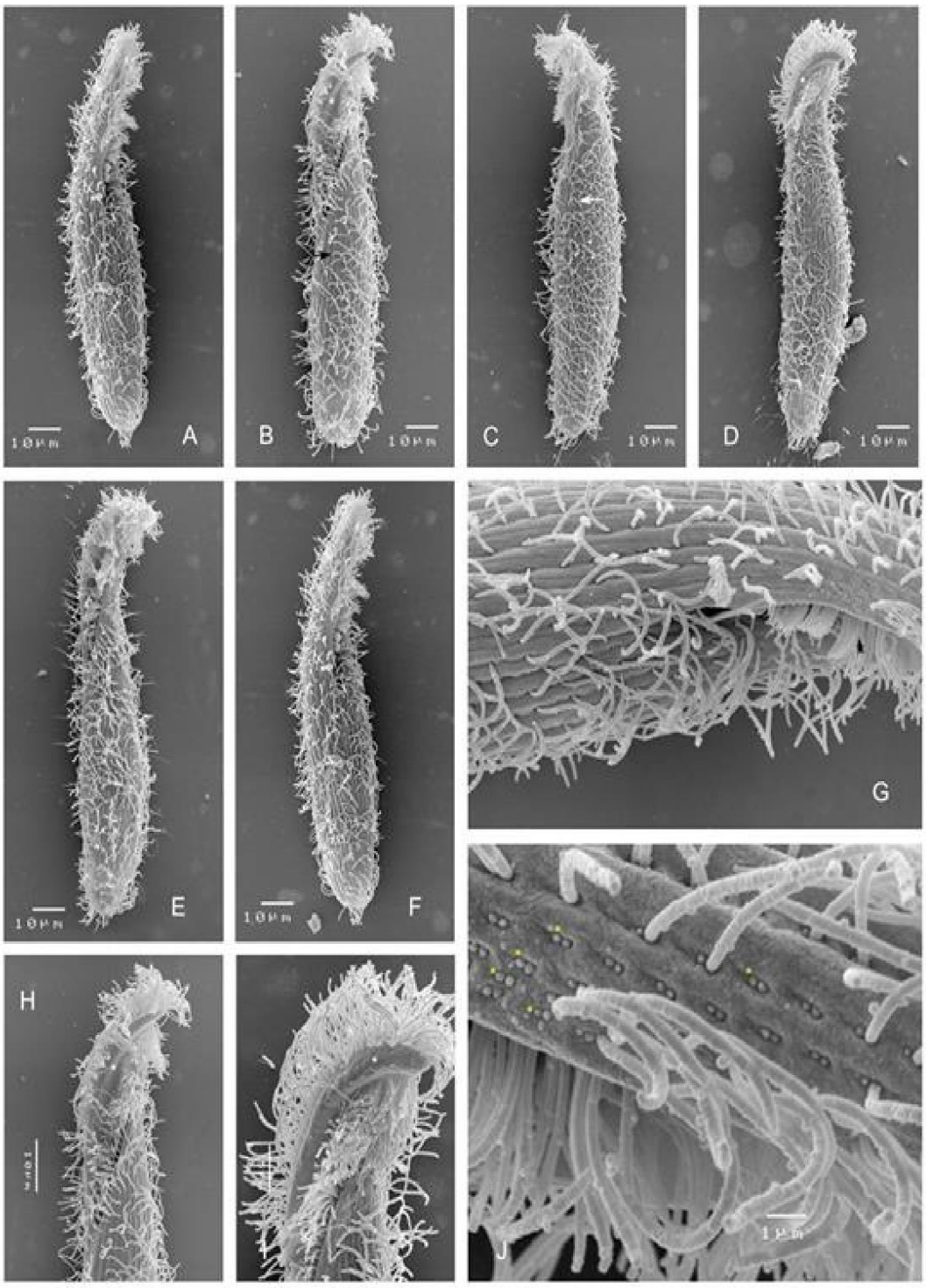

Figure 9.4. Photomicrographs of *Metopus sp*. Kolleru lake population (KB2) after SEM (A–J). A, F, lateral views of the representative; (B, D, E) ventral view of a representative specimen with ventral cilia (black arrow).; (C) dorsal view of the representative specimen with dorsal cilia (white arrow); (G) details of paroral membrane (double arrow head); H, I, details of buccal region with paroral membrane, pre–oral dome (white asterisk) and adoral zone of membranelles (black arrow head); (J) perizonal ciliary stripe kinetomes (yellow asterisks).

Class Armophorea Lynn, 2004 Order Metopida Jankowski, 1980 Family Metopidae Kahl, 1927 Genus Brachonella Spiralis, 1964.

Brachonella Spiralis (Levander, 1894) Jankowski, 1964 (Original Combination Metopus Contortus Levander, 1894)

Diagnosis:

Body size about 84 – 107 × 58 − 81 μm in vivo. Body bullet–shaped, massive conical preoral dome nearly obscures short bluntly truncate postoral part. Body is dorsoventrally biconvex. Dense olive green colored cytoplasmic granule aggregate at the anterior end of preoral dome invariably present across all specimens stained black after silver impregnation. Macronucleus globular, in anterior body half with dense chromatin shifted to 80% and the rest appeared less densed and several nucleoli. Somatic cilia usually comprised of about 53 dorso–ventral, 5 perizonal and 9 preoral domes. Elongated evenly distributed cilia encircle posterior end. Perizonal ciliary stripe invariably comprises five rows, never arranged in false kineties, same length as adoral zone proximally, slightly longer distally. Paroral stichomonad. Adoral zone makes 360◦ spiral around long axis, usually comprises about 64 polykinety rows alternated with ridges.

Type locality:

Three populations of Brachonella contortus (KTC12, KTC16, KTC18) were obtained from Kolleru lake.

Gene sequence:

18S rDNA sequences of Brachonella contortus (KTC12, KTC16, KTC18) were obtained from Kolleru lake, India and have been deposited in NCBI GenBank with the accession number.

3.1. Description of Brachonella spiralis:

Color golden yellow or golden brown, prominent olive-green colored spot at anterior poles across all specimens in the population due to large cytoplasmic granule aggregate (Fig. 9.20A–C, 9.21A–C). Swimming pace steady, rather moderate and rotates in anti–clockwise direction corresponding to the longitudinal axis. Body bullet–shaped or dome shaped, slightly curved to right on long axis, conical preoral dome, occupies 80% of body length, posterior end bluntly truncate (Figs 9.20A–C, 9.21A–C, 9.22 A–D). Size in vivo about 84–107 × 58−81 μm, 72–86 × 53–67 μm in silver–impregnated specimens and even shrined to 43–76 × 33–53 μm. Length: width ratio including preoral dome was 1:2 on an average. Preoral dome was dorsoventrally biconvex separated to posterior region by a large, ribbed preoral dome canopy, underneath this massive dome canopy structure, adoral zone polykineties were present and both these structures

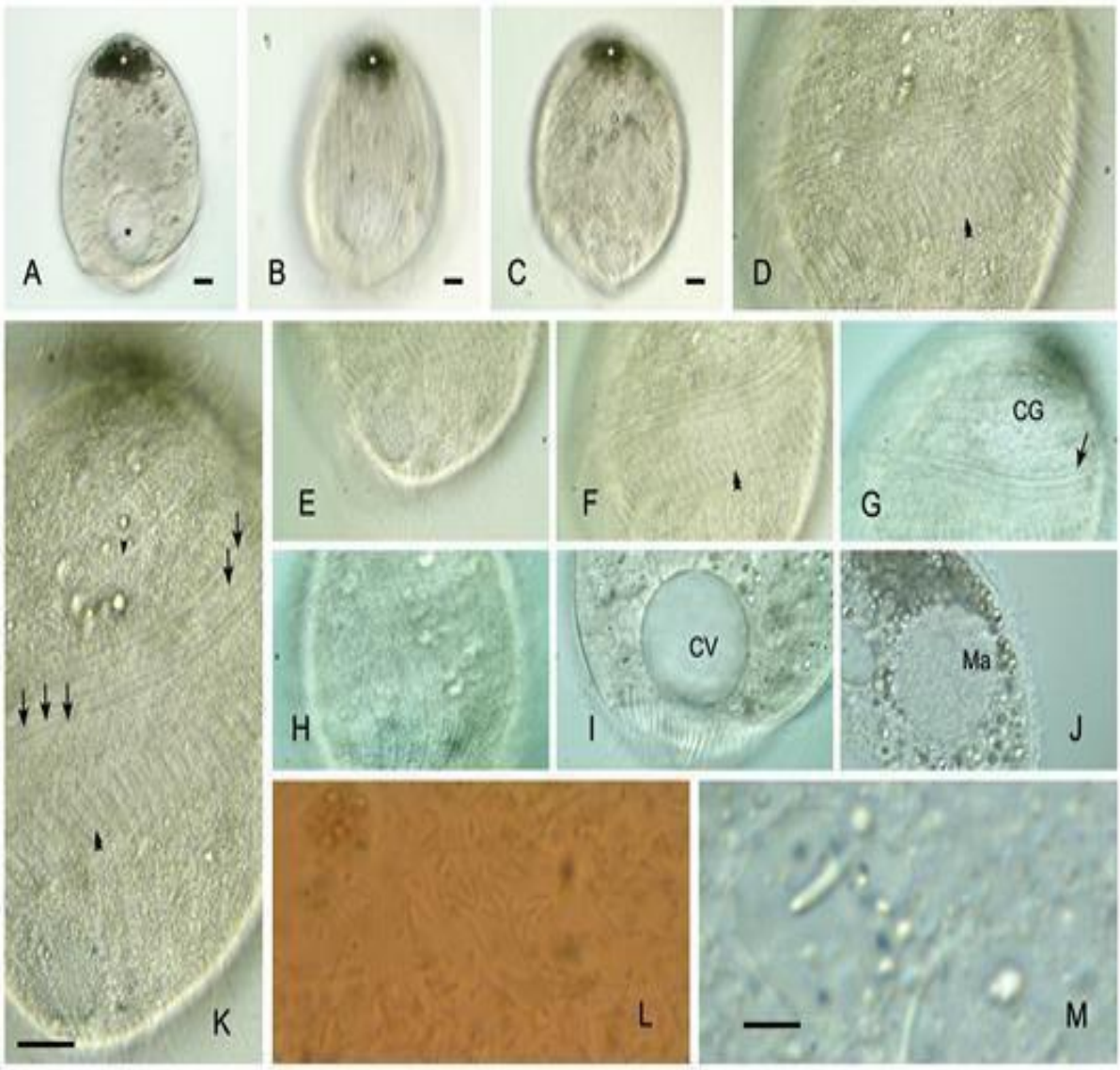

Figure 9.20. Photomicrographs of *Brachonella spiralis* population (KTC18) from life (A–L). A, dorsal view showing the anterior dense aggregate of brown–black refractive granules (white asterisk) and contractive vacuole (black asterisk); (B) dorsal view of typical specimen; (C) ventral view of a typical specimen; D, F, K, ventral view showing peripheral most preoral dome kinety (black arrowhead), perizonal ciliary stripe rows 1 to 5 (black arrows) and details of adoral zone of membranelles (double arrow head); (E) details of caudal cilia and cortical granular stripes; (G) dorsal view showing preoral dome and perizonal ciliary stripe row 1 to 5 (black arrows); (H) dorsal view showing dorsal cilia and interkinetal cortical granular stripes; (I) details of contractive vacuole and proximal adoral zone of membranelles; (J) macronucleus (Ma); (L) rod shaped cytoplasmic endosymbionts (M) squashed cell showing the details of endosymbiont. **Bars stand for A-C, 10µm; M, 5µm.**

Characteristic[a]	Method[b]	Mean	Min	Max	SD	CV	n
				107			
	in vivo	93	84	86	7	8	15
Body, length	S	78	72	76	6	7	6
	SEM	63	43		8	13	14
	in vivo	66	58	81	7	10	15
Body, maximum width at preoral dome	S	61	53	67	5	9	6
	SEM	44	33	53	9	21	14
	in vivo	1	1	2	0	8	15
Body, length:width ratio	S	1	1	2	0	13	6
	SEM	1	1	2	0	16	14
	in vivo	28	21	33	4	13	10
Posterior end width	S	22	12	33	7	32	6
	SEM	16	11	23	4	24	14
Anterior cell end to proximal end of adoral zone on ventral side, distance	S	66	62	71	3	5	6
	SEM	51	39	59	7	14	8
Anterior cell end to proximal end adoral zone distance-body length, ratio (%)	S	85	78	93	6	7	6
	SEM	89	73	105	12	13	8
Anterior cell end to distal end of adoral zone on ventral side, distance	S	29	20	38	7	24	6
	SEM	21	11	31	8	36	8
Anterior cell end to distal end adoral zone distance:body length, ratio in %	S	37	27	49	8	21	6
	SEM	36	25	60	14	37	8
Anterior cell end to posterior end of macronucleus, distance	L	47	39	56	6	13	7
Macronucleus, length	L	24	20	29	3	12	10
	F	19	16	28	4	20	11
Macronucleus,	L	24	20	30	3	11	10
	F	18	14	25	3	20	11
Macronucleus, length:width ratio	L	1	1	1	0	7	10
	F	1	1	1	0	6	11
Micronucleus, diameter	F	2	2	3	0	15	
Adoral membranelles, number[c]	S	64	61	75	5	8	6
Length of longest mid-ventral Adoral membranelle base	S	11	10	12	1	7	6
Perizonal ciliary stripe rows, number	S	5	5	5	0	0	6
Caudal ciliary dikinetids on both sides , number	S	30	27	34	3	10	6
ventral preoral dome kineties, number	S	9	9	9	0	0	6
Total Somatic kineties, number	S	43	41	48	3	6	6

[a] All measurements in µm; [b] methods applied to get the morphometric data; [c] Photos of Silver stained speicmens were taken in different depths with different focal length and realised the cumulative number of Adoral zone of polykinetids from properly oriented cells after fixation with Silver impregnation technique.
Abbreviations: standard deviation of the arithmetic mean; CV, coefficient of variation (%); Mean, arithmetic mean; M, median; Max, maximum value; Min, minimum value; n, number of cells studied; S, Silver impregnation; SD,

travelling parallelly in the ventral region in the mid–region of the body in a longitudinal way (Figs 9.20C–D, 9.21B, C, 9.22C, D). The adoral zone polykinety rows (AZP) started from the dorsal side and made one spiral on the ventral side obliquely and terminated at the anterior margin of the dome in the dorsal side (Figs 9.21A, C, 9.22B-D). After SEM analysis, the undersurface of dome composed of finely ribbed surface and continued as ridges in the buccal cavity and appeared as septa separating each adoral zone polykinety rows (Fig. 9.22C, E, G). Perizonal stripe row 1 and the buccal region was separated by preoral dome canopy (Figs 9.21B, C). Distally row 1 constructed along the edge of preoral dome without any interruption. Posterior region of the body, beyond the preoral dome was obconical in shape; circular after TEM analysis and appeared truncated at the terminal end with deep terminal concavity formed by assemblage of all the cortical ridges (Figs 9.21B, C). Macronucleus (Ma) resides underneath the preoral dome cortex in the cytoplasm (Fig. 9.20C). It appeared globular and deeply stained the chromatin granules

with several nucleoli scattered among the chromatin (Fig. 9.21D). Size was about 20–29 x 20–30 µm in live and 16–28 x 14–25 µm in stained specimens. Single, round micronucleus (Mi) measured about 2–3 µm in diameter, often resided in the proximity of macronucleus with negligible gap. Single terminal contractile vacuole (CV) spherical in shape, pulsates slowly and expels the fluids to the outside with a large excretory pore lies in the terminal concavity formed by the union of all the body rides as a whorl in the posterior end of the body (Fig. 9.21B). Cortex occupied with prominent kinetal furrows intervened with less pronounced ridges (Figs 9.21A–C,9.22A–D). Cortical granules (mucocysts) were interkinetic and highly refractile; densely aggregated; golden yellow color in vivo and always stained deeply with silver impregnation (Fig. 21A–C). Granule aggregate located in the anterior pole was large, crescentic, comprised of densely packed olive green colored spherical granules in different sizes (Figs 9.20A–C, 9.21B, C). Somatic cilia and perizonal stripe cilia beat in metachronal waves; On average 43 (usually 41–48) somatic kineties including the dorso–lateral kineties and ventro–lateral kineties up to the level of buccal apparatus in the mid–body length (Figs 9.21A–C, 9.22A–D). Perizonal stripe invariably composed of dikinetids arranged in five equally long rows, same length as adoral zone proximally, slightly longer distally; 9 preoral dome kineties all stripe rows densely ciliated, rows 1–3 closely spaced, rows 4 and 5 less so, separated by gap from rows1 to 3; Somatic kineties comprised of ordinarily spaced dikinetids often with only posterior basal body ciliated. Perizonal stripe and most dome kineties have both basal bodies ciliated; perizonal stripe dikinetids never form “false kineties”. Adoral zone comprises 64 polykinetid rows on average (range 61–75), parallel to dome brim, spirals 360◦ to end on ventral surface, proximal portion overhung by preoral dome margin but not covered by buccal lip (Figs 9.21B, C, 9.22C–G). Mid adoral zone polykinetid rows were longest (length of base about 10–12 µm), distal membranelles composed of three long files, two basal bodies at the anterior end of membranelle (Fig. 9.21B, C). Prominent paroral membrane originates at posterior end of adoral zone in a slit–like depression on undersurface of preoral dome, comprised of single file of ciliated basal bodies (Figs 9.22D–F). Conjugation was observed. Resting cysts in globular shape were observed. Cytoplasm and nucleus were inhabited with symbionts (Figs 9.20L, M, 9.21E, F).

Fluorescence in-situ hybridization:

It detected the presence of Methanogenic Archae in the cytoplasm by giving positive signals to the universal Archae probes ARC915_Cy3 (Fig. 9.19E) throughout the cell.

Transmission electron Microscopy (TEM):

TEM revealed the presence of rod–shaped Methanogenic Archae localized in a hollow in the cytoplasm and associated with bilayer organelles called hydrogenases which appeared circular in cross section distinctive electron gradience from the cytoplasm (Fig. 9.23A–C). No trichocysts were detected. However, several cortical granules or mucocysts were decorating the outer margin of the cortex (Fig. 9.23D). The nucleus was inhabited with bacteria rod shaped bacteria with a conical end and in cross section appeared star–shape may be due to the pressure exerted by the chromatin granules during the TEM fixation (Fig. 9.23E–I). A big food vacuole figured with lots of food bacteria and diatoms (Fig. 9.23L).

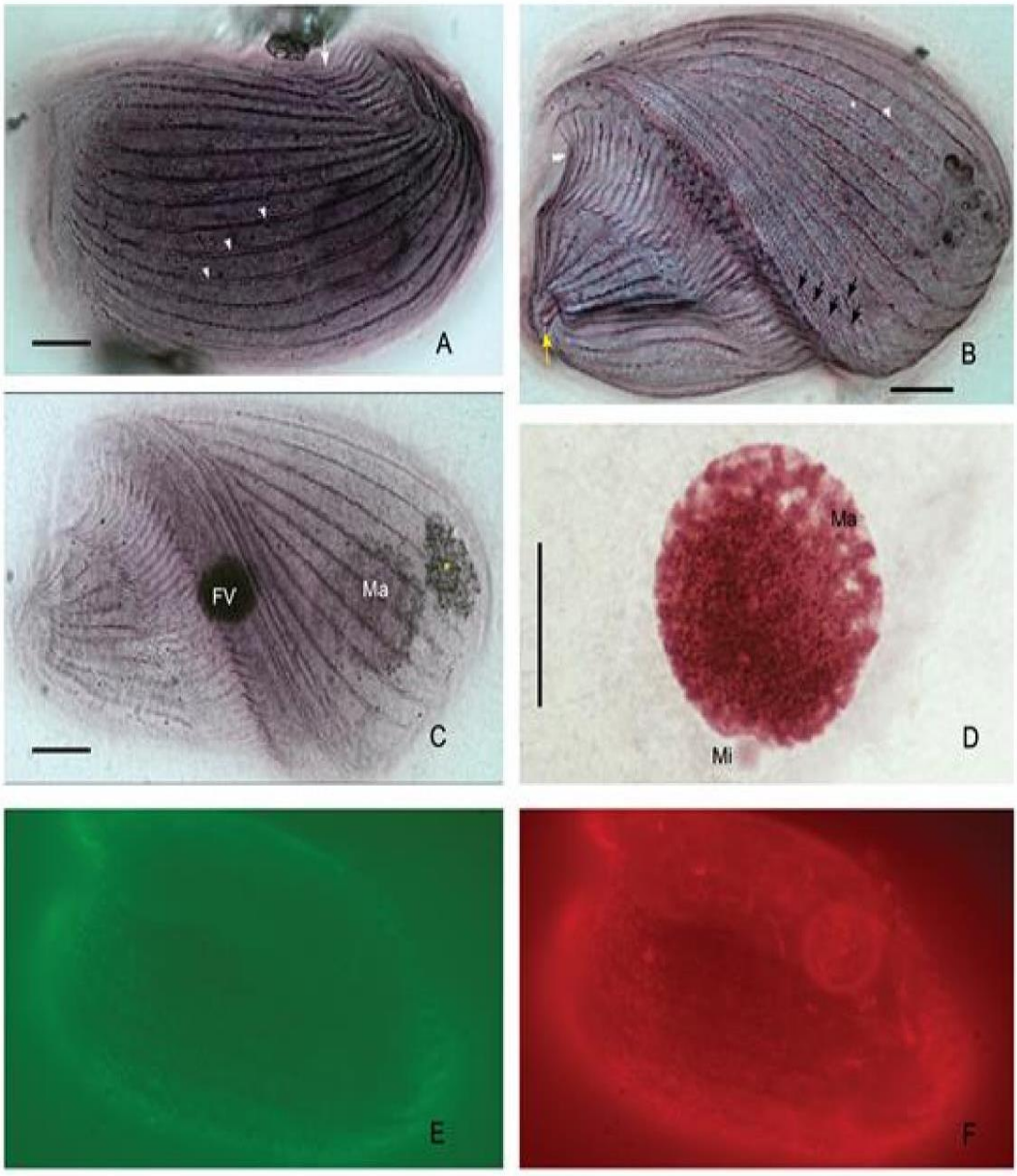

Figure 9.21. Photomicrographs of *Brachonella spiralis* population (KTC18) after silver staining (A–C) and after Feulgen staining (D) and after FISH analysis (E–F). A, dorsal view showing the preoral dome overhangs the proximal adoral zone membranelles (small arrow in white); (B) ventral view showing peripheral most preoral dome (white asterisk), preoral dome kinety (white arrow head), perizonal ciliary stripe rows 1–5 (white arrows) and details of the portion of adoral zone of membranelles (double arrow head in white) and posterior whorl of kineties (yellow arrow). (C) ventral view showing the anterior dense aggregate of brown–black refractive granules (yellow asterisk), macronucleus and a food vacuole (FV). (D). details of nuclear apparatus showing Macronucleus (Ma) and micronucleus (Mi) after Feulgen staining. (E) negative signaling to Eub338I probe; (F) emitting positive signal to Arch 915 probe. Bars stand for 10μm.

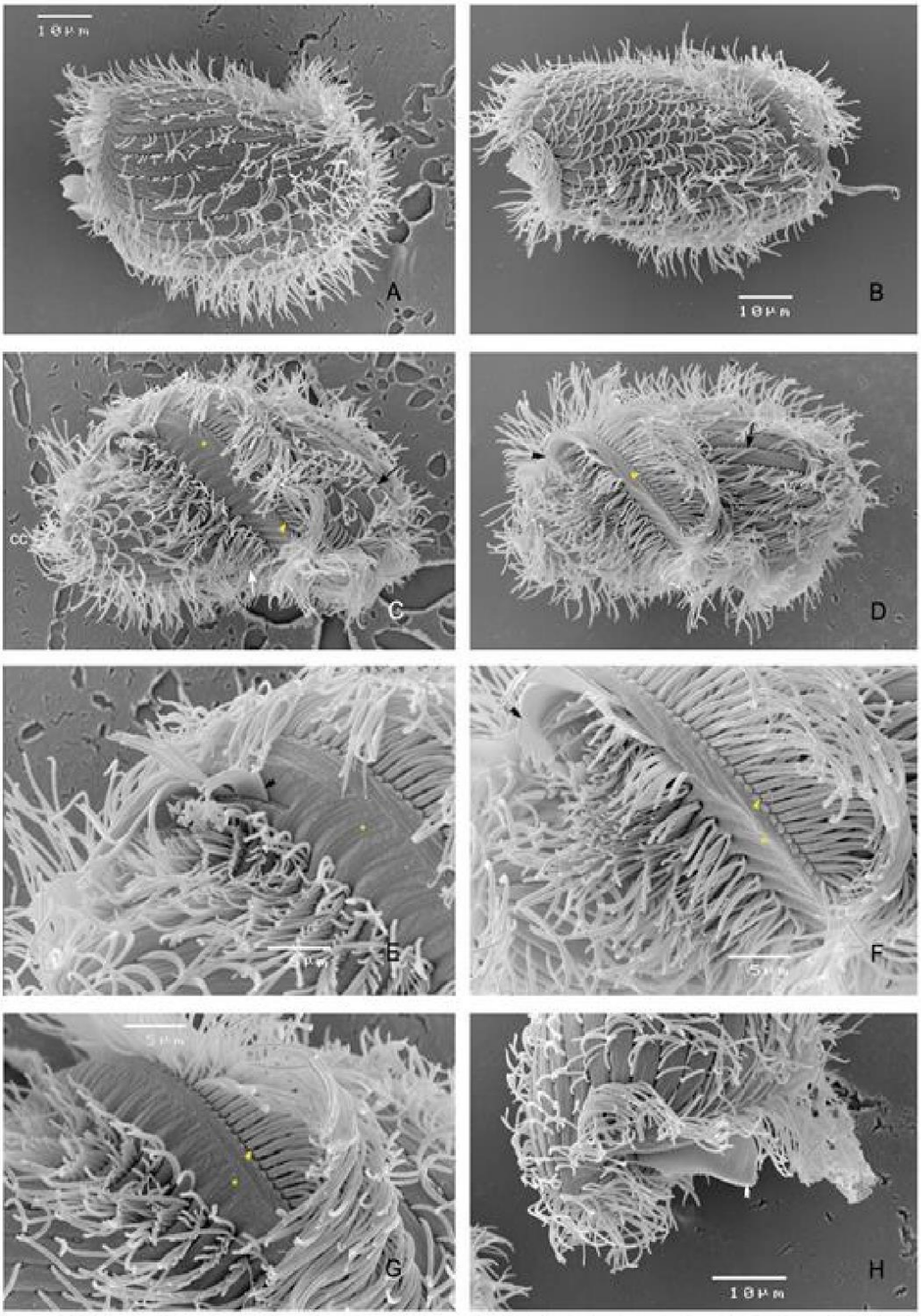

Figure 9.22. Photomicrographs of *Brachonella spiralis* population (KTC18) after SEM analysis (A– H). (A) dorsal view of typical specimen with dorsal cilia; (B) left dorsal lateral view showing details of distal region of adoral zone of membranelles; C, D, ventral view of typical representative showing details of ribbed pre–oral dome (asterisk), preoral dome kinities (black arrow), perizonal ciliary stripe rows 1 (yellow arrowhead), adoral zone of membranelles (white arrow), ventral cilia and caudal cilia (CC); E, F, detailed view of paroral membrane (double arrow head); (G) details of perizonal ciliary row showing two cilia in each unit and portion of ribbed oral dome; (H) posterior region of dorsal side showing the paroral membrane (double arrow head).

Details of 18S rDNA gene sequences and BLAST results of ciliates in analysis are shown in Table

Species	Sequence length (bp)	Blast result	ID	MM	GAP
Metopus sp. KKV12	1,676	*Metopus fuscus* (KF607083)	98.20%	29	1
Metopus sp. KWBL12	1,673	*Metopus contortus* (Z29516)	98.31%	27	–
Metopus sp. KTC18	1,675	*Metopus setosus* (KF607087)	99.34%	11	–
Metopus sp. TBS19	1,673	*Metopus contortus* (Z29516)	98.22%	27	–
Metopus sp. TP5	1,672	*Metopus es* (KX776457)	100%	–	–
Brachonella sp. KTC12	1,673	*Brachonella spiralis* (KF607090)	99.46%	9	–
Brachonella sp. KTC16	1,673	*Brachonella spiralis* (KF607090)	99.46%	9	–
Brachonella sp. KTC18	1,673	*Brachonella spiralis* (KF607090)	99.46%	9	–
Caenomorpha sp. TP4	1,647	Uncultured *Caenomorpha*–like ciliate (AY821933)	99.34%	10	–

ID= identity; MM= mismatches in basis alignment; in bold: reliable species attribution, conformed by molecular and/or morphological data.

Metopus es (KX776457), and for Metopus KTC18, which showed 99.34% identity with Metopus setosus (KF607087). 18S rDNA gene sequences of Brachonella populations, KTC12, KTC16 and KTC18, resulted identical among each other, and showed 99.46% identity with Brachonella spiralis (KF607090). Metopus KKV12 sequence shared 98.20% identity with Metopus fuscus (KF607083), while Metopus KWBL12 and Metopus TBS19 sequences resulted close to Metopus contortus one (Z29516), although with high number of mismatches, suggesting that these three ciliates could constitute a novel species. Caenomorpha TP4 sequence matched with an unknown species of Caenomorphidae (AY821933), and showed 93.30%identity with Caenomorpha uniserialis (U97108). Also, in this case it could constitute a novel species or one species described only at morphological level.

Phylogeny of Class Armophorea:

Class Armophorea appeared paraphyletic with genus Caenomorpha branching out of the Class and Being sister clade to Litostomatea. The main part of class Armophorea (with members of family Metopidae and order Clevelandellida) resulted sister to class Spirotrichea, together forming the sister clade of Litostomatea + Caenomorpha group. The inner nodes inside the Armophorea class did not support the monophyly of order Armophorida, especially regarding the family Metopidae. Order Clevelandellida resulted monophyletic, including Clevelendella spp., Nyctotherus spp. and Nyctotheroides spp. and supported by high bootstrap value. Genus Metopus is clearly paraphyletic, split in several clades. The type species of the genus, Metopus es formed a separate and well supported

clade, sister of Brachonella genus, which resulted monophyletic, including B. spiralis and B. contorta. All other Metopus species were scattered in different clades all over the Armophorea class.

For example, Metopus contortus clustered with some species from India (KWBL12, TBS19) and other uncultured organisms, being sister of Metopus es and Brachonella clades. Whereas, M. setosus and M. fuscus, clustered together with Palmarella lata and Heterometopus palaeformis. The recently described species from soil, M. hasei and M. yantainensis, clustered with Parametopidium circumlabens, Atospira galeata, and A. violacea. This clade resulted sister to the one of the order Clevelandellida, with Metopus laminarius sequence basal to the two mentioned groups. Genus Atospira resulted paraphyletic from our analysis. Metopus striatus sequence resulted basal to Clevedellida + Metopus – Atospira – Parametopidium + Metopus – Palmarella – Heterometopus clades.

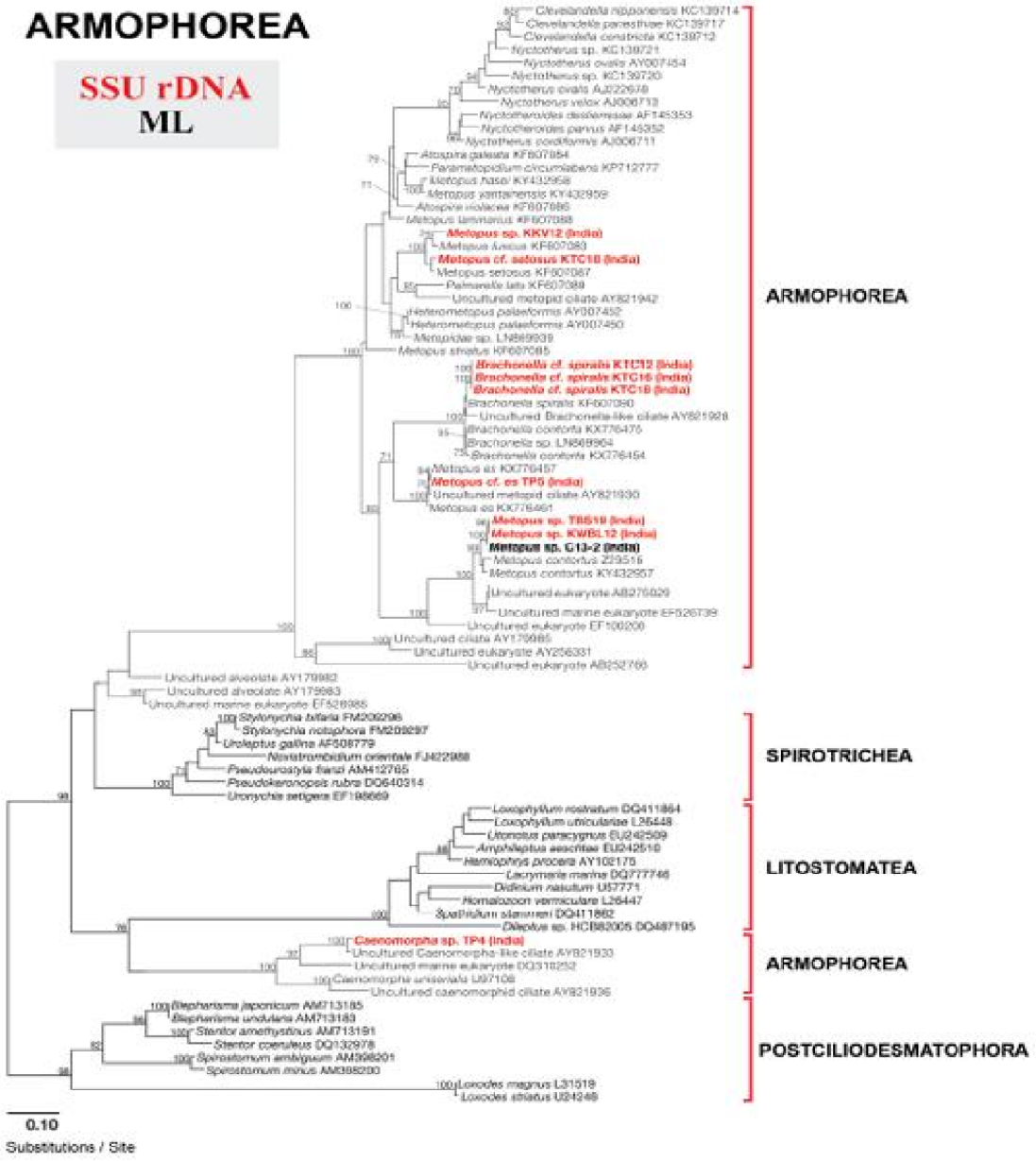

Figure 9.24. Maximum-likelihood (ML) tree of the class Armophorea inferred from the small subunit ribosomal RNA (SSU rRNA) gene sequences. Numbers at the nodes represent the bootstrap values of ML (values above 70% are shown). Sequences obtained in the present work are shown in bold text highlighted with red color.

Position of Indian species in the tree:

The phylogenetic analysis reflected the preliminary data from BLAST results. Metopus sp. KTC18 clustered with M. setosus with a minimal divergence, Metopus sp. KKV12 clustered in the same clade, being sister species of M. fuscus.

Instead, Metopus sp. TP5 resulted embedded in M. es clade, confirming the species attribution. Metopus spp. KWBL12, TBS19 and C13–2 resulted sister species to the M. contortus clade: the identity between 18S rDNA sequences of Indian Metopus and sequences of M. contortus in database (they differ by 20–28 nucleotides) suggests that our organisms could constitute another species respect with M. contortus. Further morphological analysis has been presently undergoing to fix this issue. Sequences of Brachonella spp. KTC12–16–18 resulted imbedded in the clade of B. spiralis, forming the sister clade of B. contorta. Instead Caenomorpha TP4 sequence showed a close association with an uncultured Caenomopha sp. (AY821933), suggesting that it could represent a novel species or one species already described only morphologically.

Discussion:

Considerations on morphological descriptions recorded in this study:

The present isolate has been diagnosed as Metopuses and has been compared with the previous literature. Although, it had shown slight variations, it was in agreement with the redescription from Bourland et al., (2016).

Metopus finlaii sp. nov:

Although the shape of the Metopus finlaii is comparable to Metopus contortus morphologically, Metopus finlaii can be comparable with Metopus contortus based on the shape and composition of macronucleus. However, it deviated by the presence of Y– junction forming beneath the margin of proximal buccal cavity and almost at the right ventro–lateral side where some kineties travel to anteriad and contributed to the construction of perizonal ciliary rows and this pattern of somatic ciliature forming Y junction is absent in Metopus contortus (Esteban et al., 1995; Jankowski, 1964) it deviated from several it by the position of the proximal region of peristome behind the equator (vs beyond the equator) and also deviated from it by several features reported in Table 9.4. The morphological deviations were strongly supported by molecular differences too and could be treated as novel comb. inside to Genus Metopus.

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