Univerzita Karlova v Praze

Přírodovědecká fakulta

Katedra zoologie

Morfologická a molekulární diverzita volně žijících zástupců čeledi Metopidae a objev nové linie anaerobních nálevníků

Morphological and molecular diversity of the free-living representatives of the family Metopidae and the discovery of a new lineage of anaerobic ciliates

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Diplomová práce

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Praha, 2015

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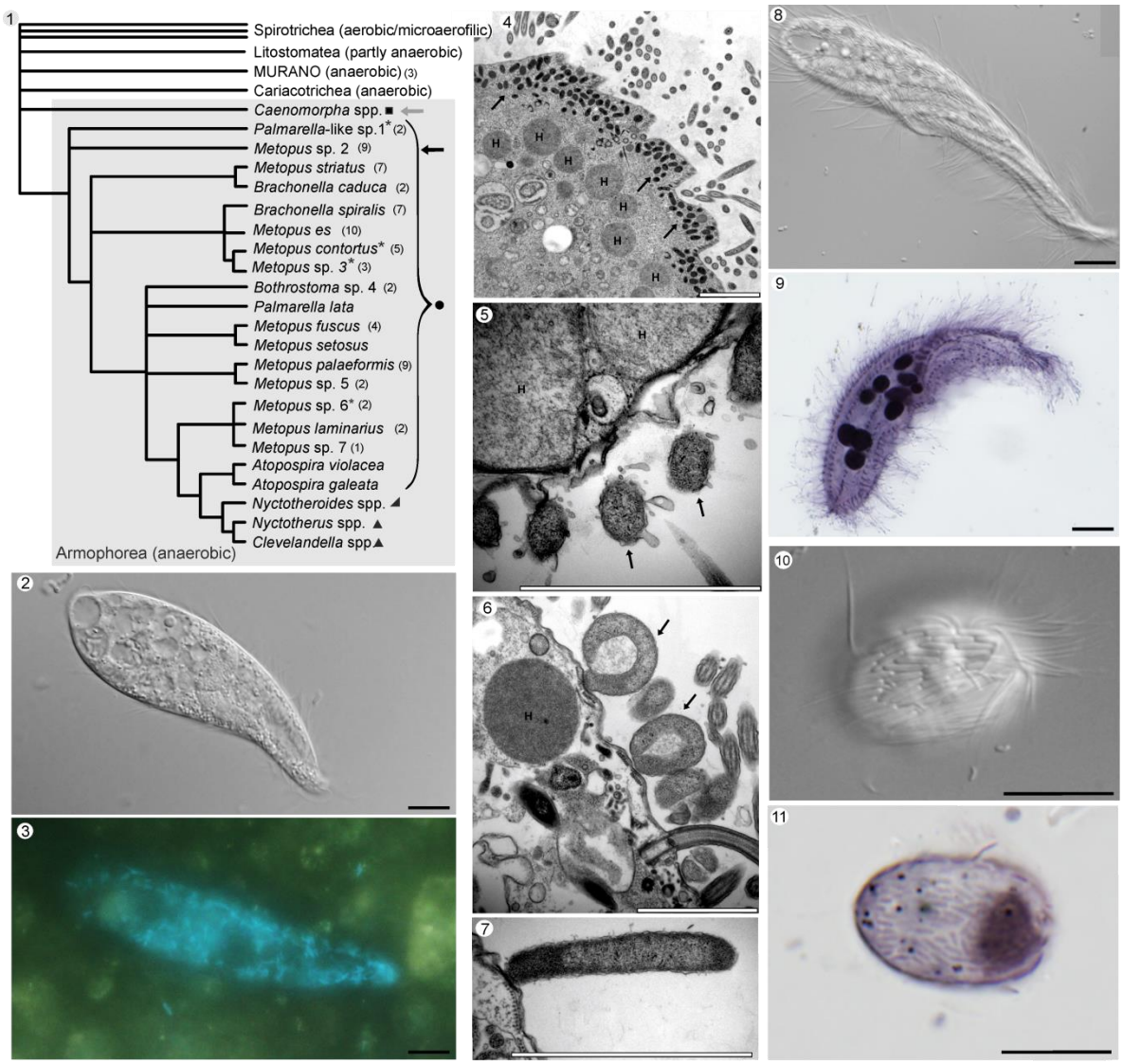

1. Phylogenetic tree; 2. Metopus palaeformis, strain CSS(Ndjoko); 3. M.palaeformis, strain CSS, methanogenic endosymbionts; 4. marine M.contortus methanogenic endosymbionts, TEM; 5. Cyclidium sp., strain Larnaka, ectosymbionts, TEM; 6. MURANO, strain Ridka, ectosymbionts; 7. MURANO, strain Ridka, ectosymbionts; 8. MURANO; 9. MURANO, protargol stained cell; 10. Cyclidium sp., strain Coorong, ectosymbionts; 11. Cyclidium sp., strain Larnaka2An, ectosymbionts, protargol stained cell. Symbol legend: *- marine, ▲ - endobiotic Clevelandellida, ● - free-living Metopidae, ■ - Caenomorphidae, black scale bar - 10 µm, white scale bar - 2 µm

Na tomto místě bych chtěla poděkovat svému školiteli Doc. RNDr. Ivanu Čepičkovi, Ph. D. za pomoc a užitečné rady při psaní této práce a odborné vedení během jejího řešení. Dále bych ráda poděkovala Mgr. Tomášovi Pánkovi a Mgr. Petru Táborskému za pomoc v začátcích mé laboratorní práce a za konzultace k této práci. Poděkování patří všem členům našeho výzkumného týmu za odborné rady v průběhu práce a rovněž všem, jež nám z expedic přivážejí početné vzorky sedimentů, z kterých vznikají naše kultury. V neposlední řadě chci samozřejmě velmi poděkovat své rodině za podporu po celou dobu studia.

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ABSTRAKT

Anoxické sedimenty slouží jako životní prostředí mnohým nálevníkům. Přestože je známo, že anaerobióza vznikla nezávisle v několika liniích z 11 hlavních linií Ciliophora, a anaerobové byli nalezeni v nejméně osmi liniích nálevníků, diverzita anaerobních nálevníků třídy Armophorea je podstatně přehlížena. Podobně, jen velmi málo je známo o jejich hydrogenosomech. Abychom prohloubili vědomosti o diverzitě volně žijících zástupců třídy Armophorea, kultivovali jsme více než 100 armophoridních kmenů ze sladkovodních, brakických, i mořských anoxických sedimentů z celého světa. Stanovili jsme jejich SSU rDNA sekvence, provedli barvení protargolovou technikou, a studovali jejich morfologii ve světelné mikroskopii. Kromě toho jsme použili transmisní elektronovou mikroskopii pro posouzení ultrastruktury některých kmenů. Identifikovali jsme několik nových linií, které pravděpodobně představují nové druhy. Významnou součástí práce je objevení nové hluboké linie mořských anaerobních nálevníků - Muránů. Podle analýzy SSU rDNA se větví mezi třídami skupiny SAL (Spirotrichea, Armophorea a Litostomatea), ale tvoří samostatnou hlubokou linii, případně novou třídu. Došli jsme k závěru, že anoxické sedimenty ukrývají velkou diverzitu dosud nepopsaných anaerobních nálevníků.

Klíčová slova: Anerobióza, Armophorea, nálevníci, hydrogenosomy, diverzita, SSU rDNA

ABSTRACT

Anoxic sediments host a wide variety of ciliates. Although it is known that anaerobiosis has independently arisen in several lineages of the main 11 lineages of Ciliophora and anaerobes have been found in at least eight lineages of ciliates, the diversity of anaerobic ciliates of the class Armophorea is severely understudied. Similarly, not much is known about their hydrogenosomes. To deepen our knowledge about the diversity of Armophorea, we have cultivated more than 100 armophorid strains from fresh water, brackish, and marine anoxic sediments worldwide. We determined their SSU rDNA sequences, performed protargol staining techniques, and studied light-microscopic morphology. In addition, we used transmission electron microscopy to assess the ultrastructure of some of the strains. Several novel clades of metopids, the free-living anaerobic ciliates of the class Armophorea, were identified. Importantly, a new deep lineage of marine anaerobic ciliates, muranes, was discovered. According to the SSU rDNA analysis, it is related to SAL group (Spirotrichea, Armophorea, and Litostomatea) with Cariacotrichea, but forms a separate lineage, possibly a novel class. We conclude that anoxic sediments harbour a high diversity of undescribed anaerobic ciliates.

Key words: Anaerobiosis, Armophorea, ciliates, hydrogenosomes, diversity, SSU rDNA

1. INTRODUCTION, AIMS AND OBJECTIVES OF THESIS

1.1. INTRODUCTION

Ciliates are one of the most studied groups of unicellular eukaryotic organisms, and yet there are lineages that have been heavily overlooked despite their ecological importance, and even more surprisingly - despite their nigh ubiquitous distribution. Ciliates inhabit the most various environments, and through their evolution they have multiply forayed even into biotopes with extreme living conditions, and successfully occupied them.

Numerous extreme environments, such as hydrothermal vent sites with outrageous temperatures, areas polluted by acid mine drainage, acidic geysers and sulfuric pools, or environments with anaerobic conditions as the digestive system of many metazoans or diverse anoxic sediments (Hu, 2014), are inhabited by ciliates more frequently than one could expect.

As the old paradigm, saying that life is not possible without light or oxygen, fades away, there are still more and more organisms being discovered - surviving, or even keening on such conditions (Orsi et al., 2012; Fenchel, 2012), a major part of them being microorganisms. Remarkably many ciliates live in marine or freshwater anoxic sediments. The ecological importance of these ciliates is indisputable, yet understanding of the diversity and their role in anoxic sediments is still very limited (Dopheide et al., 2009). It is known that out of the current 11 main ciliate lineages, anaerobiosis has independently arisen at least in eight of them – in Phyllopharyngea, Prostomatea, Plagiopylea, Oligohymenophorea, Litostomatea, Spirotrichea, Cariacotrichea, and Armophorea (Esteban et al. 1964; Fenchel et al., 1977; Esteban et al., 1993; Batisse, 1994; Baumgartner et al., 2002; Edgcomb et al., 2014). Although the relationships of some inner lineages within the two subphyla of Ciliophora (Postciliodesmatophora and Intramacronucleata) have not been fully resolved and there are still many lineages with uncertain position, a recent multigene analysis revealed that Intramacronucleata consist of groups CONThreeP (Colpodea, Oligohymenophorea, Nassophorea, Phyllopharyngea, Prostomatea, and Plagiopylea), SAL (Spirotrichea, Armophorea, Litostomatea) and Protocruziida (Gentekaki et al., 2014).

After a diversity survey of anaerobic ciliates performed by Msc. Ludmila Nováková in 2011, together with only several papers concerning the same topic published in the last years, a need for deeper study of particular groups has ensued/arisen/appeared in our minds. Thus, this work focuses on Armophorea, an important but severely overlooked group of solely anaerobic, both endobiotic and free-living ciliates, where the free-living ones are the major issue.

The class Armophorea has a recent origin, although many species inside the two orders, Clevelandellida and Armophorida are long known ciliates. Armophorea can also be called a "riboclass", as the major characteristics joining two orders together are phylogenetic analyses based on 18S rDNA sequences. Other than that, there are only few known common features. An important one is undoubtedly that both of the orders live under anoxic conditions, in spite of the substantial difference in their natural habitats. The order Clevelandellida comprises exclusively endobiotic representatives, ranging from various invertebrates, mainly from arthropod phylum to few particular vertebrates. In contrary, all representatives of the order Armophorida are free-living ciliates, inhabiting marine and freshwater sediments all over the world. Despite their cosmopolitism, there is nearly nothing known about them.

In this work, we would like to map the diversity of the free-living representatives of the class Armophorea, the order Armophorida, perform 18S rDNA phylogenetic analysis of cultivated strains, study their morphological features as well as assess ultrastructure of several species.

Interestingly, during our survey, we have discovered new deep lineage of anaerobic ciliates. It does not belong to any of the currently known classes of ciliates, therefore may form a new class.

1.2. AIMS AND OBJECTIVES OF THESIS

- Mapping the diversity of anaerobic ciliates with main emphasis on the class Armophorea.
- Establishing cultures of acquired isolates for a long term cultivation maintenance.
- Molecular assessment of SSU rDNA sequences of all isolated strains and their phylogenetic analysis.
- Morphological study of selected isolated strains with emphasis on taxonomical relevant features.
- Ultrastructural study of selected isolated strains using transmission electron microscopy, with emphasis on mitochondrion like organelles, methanogenic endosymbionts, and cortical structures.
- Confirming presence of methanogenic symbionts in selected cultivated strains using fluorescence microscopy for further analysis.

2. LITERATURE OVERVIEW

2.1. CURRENT TAXONOMY AND PHYLOGENETICS OF ARMOPHOREA

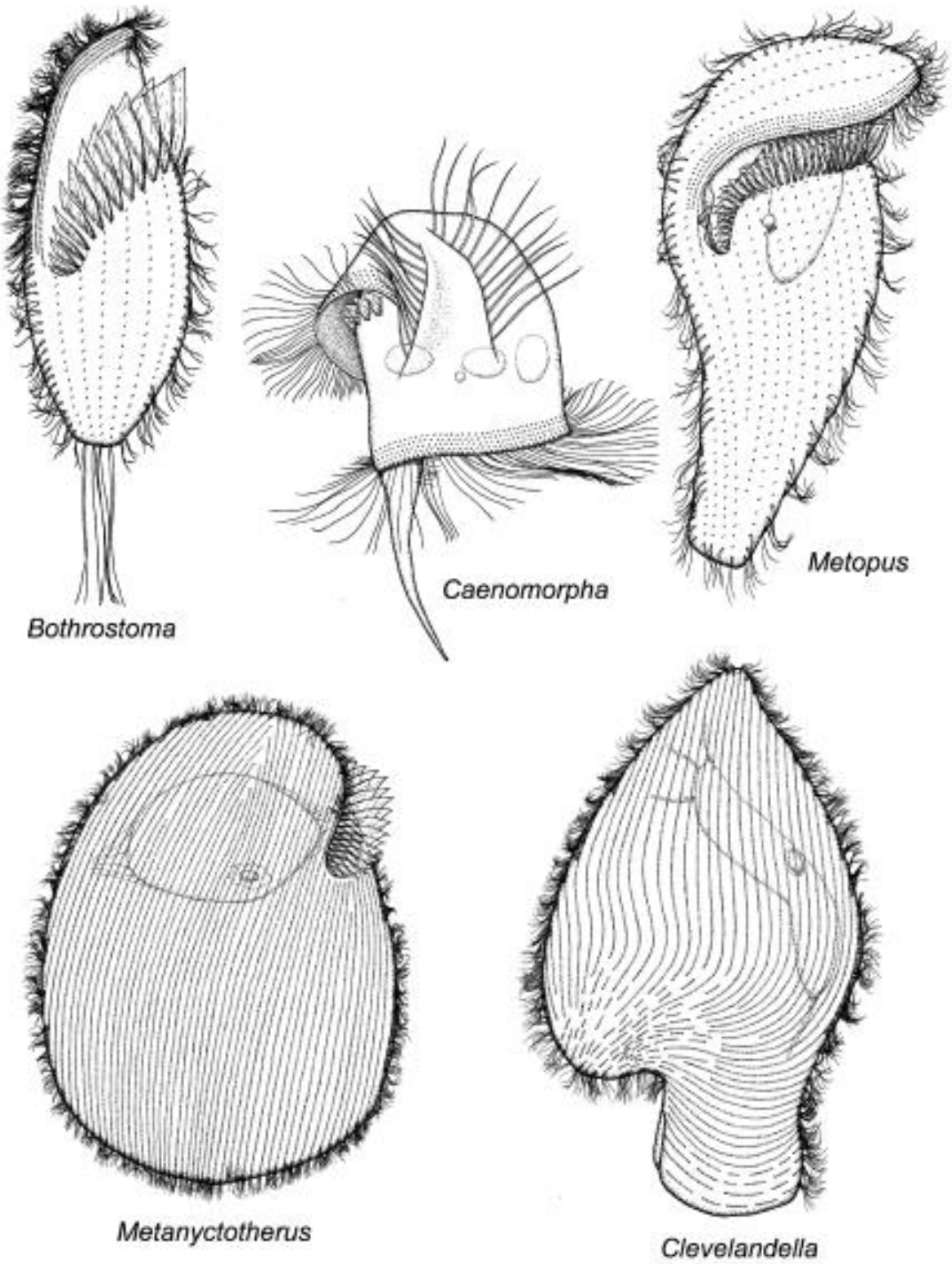

Fig. 1. Representatives of the class Armophorea. Lynn (2008)

The class Armophorea currently comprises two orders (Fig. 1), Armophorida Jankowski, 1964 and Clevelandellida de Puytorac & Grain, 1976 (Lynn, 2008). The free-living Armophorida consist of two families, Metopidae Kahl, 1927 and Caenomorphidae Poche, 1913 (Lynn, 2008). The order Clevelandellida includes solely endobiotic representatives and is divided into five families: Clevelandellidae Kidder, 1938, Nyctotheridae Amaro, 1972, Sicuophoridae Amaro, 1972, Inferostomatidae Ky, 1971, and Neonyctotheridae Affa'a, 1987 (Lynn, 2008, see 2.8.).

Metopidae comprise genera *Atopospira* Jankowski, 1964 (type species *Metopus galeatus* Kahl, 1927), *Bothrostoma* Stokes, 1887 (type species *Bothrostoma undulans* Stokes, 1887), *Brachonella* Smith, 1878 (type species *Brachonella contorta* (Levander, 1894) Jankowski, 1964, *Metopus* Müller, 1786 (type species *Metopus es* Müller, 1786), *Palmarella* (Gaievskaia, 1925) Jankowski, 1975 (type species *Palmarella salina* (Gaievskaia, 1925) Jankowski, 1975), and *Tropidoatractus* Levander, 1894 (type species *Tropidoatractus acuminatus* Levander, 1894). The type species of *Metopus* and *Brachonella* will need further investigation due to synonymization of the type species with a congeneric species possibly not eligible for type fixation (Aescht, 2001).

Caenomorphidae contain genera *Caenomorpha* Perty, 1852 (type species *Caenomorpha medusula* Perty, 1852), *Cirranter* Jankowski, 1964 (type species *Trochella mobilis* Penard, 1922), *Ludio* Penard, 1922 (type species *Ludio parvulus* Penard, 1922), and *Sulfonecta* Jankowski, 1978 (type specis *Caenomorpha uniserialis* Levine, 1894).

The molecular data for species of Armophorea are still mostly missing. Ciliate phylogenetic analyses including Armophorea that have been performed throughout the history, were based on 18S rDNA and usually included only *Metopus palaeformis*, *Nyctotherus ovalis*, and *Caenomorpha uniserialis*. The recent phylogenetic analysis of da Silva Paiva (2013) showed several ambiguities in the taxonomy of this "ribo-class". The internal relationships of this class remain poorly understood due to the lack of molecular data and also because only few representatives have been studied in detail using proper morphological methods (Bourland et al., 2014a). However, it has been revealed that the genera *Metopus* and *Brachonella* are likely non-monophyletic (Bourland et al., 2014a). Caenomorphidae have been shown to be possibly unrelated to the rest of Armophorea according to phylogenetic analyses based on SSU rDNA (da Silva Paiva et al., 2013).

2.2. SPECIES LIST OF ORDER ARMOPHORIDA

Species list includes all species (bold italic font) that we were able to find throughout the literature with the original name and year of description and we were able to track their synonyms in literature. However, we cannot exclude the possibility that we have missed some. We state the type species and the found synonyms (normal italic font).

CLASS ARMOPHOREA Lynn, 2004

Order Armophorida Jankowski, 1964

Metopina

Family Caenomorphidae Poche, 1913

Gyrocoridae , Gyrocorycidae , Gyrocorythidae, Ludiidae , Ludioidae

Genus *Caenomorpha* Perty, 1852

Caenomorpha medusula Perty, 1852 - type species
Caenomorpha aculeata Wetzel, 1928
Caenomorpha capucina Kahl, 1927
Caenomorpha lauterborni Kahl, 1927
Caenomorpha parva Wetzel, 1928
Caenomorpha levanderi Kahl, 1927
Caenomorpha medusula* var. *dentata Wetzel, 1928
Caenomorpha medusula* var. *duplex Wetzel, 1928
Caenomorpha medusula* var. *lata Kahl, 1927
Caenomorpha medusula* var. *trinucleata Kahl, 1927
Caenomorpha sapropelica Kahl, 1927
Caenomorpha steini Bütschli, 1879
Caenomorpha uniserialis Levander, 1894

Genus *Cirranter* Jankowski, 1964

Cirranter mobilis (Penard, 1922) Jankowski, 1964

Metopus mobilis Penard, 1922

Trochella mobilis Kahl, 1935 – type species

Genus *Ludio* Penard, 1922

Ludio parvulus Penard, 1922 – type species

Genus *Sulfonecta* Jankowski, 1978

Sulfonecta uniserialis (Levine, 1894) Jankowski, 1978 - type species (as *Caenomorpha***)**

Sulfonecta simplex (Levine, 1894) Jankowski, 1978

Family Metopidae Kahl, 1927

Genus *Atopospira* Jankowski, 1964

Atopospira violacea (Kahl, 1926) Bourland, 2014

Metopus violaceus Kahl, 1926

Atopospira galeata (Kahl, 1927) Bourland, 2014

Metopus galeatus Kahl, 1927 – type species

Caenomorphina heinrici Blochmann, 1894 – *nomen oblitum* (Corliss, 1979)

Brachonella galeata (Kahl, 1927) Jankowski, 1964

Genus *Bothrostoma* Stokes, 1887

Bothrostoma undulans Stokes, 1887 - type species by monotypy

Metopus undulans Stokes, 1887

Blepharisma bothrostoma Mermod, 1914

Metopus bothrostoma Kahl, 1927

Metopus bothrostoma var. *longior* Kahl, 1927

Metopus undulans var. *ovalis* (Kahl, 1932)

Pleuronema undulans (Bütschli, 1889)

Bothrostoma extensa (Kahl, 1926)

Metopus extentus Kahl, 1926

Metopus extentus var. *steini* Kahl, 1927

Bothrostoma mirabilis (Kahl, 1927) Jankowski, 1964

Metopus mirabilis Kahl, 1927

Genus *Brachonella* Smith, 1897

Brachonella spiralis (Smith, 1897) Jankowski, 1964 - invalid type species (Aescht, 2001)

Metopus spiralis Smith, 1897

Metopus contortus Levander, 1894

Brachonella contorta (Levander, 1894) Jankowski, 1964 – type species (Aescht, 2001)

Metopus contorta Levander, 1894 – type species by original designation

Metopus circumlabens Biggar & Wenrich, 1932 (*Parametopidium*)

Brachonella caduca (Kahl, 1927) Jankowski, 1964

Metopus caducus Kahl, 1927

Brachonella caenomorphoides Foissner, 1980

Brachonella campanula (Kahl, 1932) Jankowski, 1964

Metopus campanula Kahl, 1932

Metopus intercedens var. *minor* Kahl, 1927

Brachonella cydonia (Kahl, 1927) Jankowski, 1964

Metopus cydonia Kahl, 1927

Brachonella darwini (Kahl, 1927) Jankowski, 1964

Metopus darwini Kahl, 1927

Brachonella elongata Jankowski, 1964

Brachonella fastigata (Kahl, 1927) Jankowski, 1964

Metopus fastigatus Kahl, 1927

Brachonella intercedens (Kahl, 1927) Jankowski, 1964

Metopus intercedens Kahl, 1927

Brachonella mitriformis Alekperov, 1984

Brachonella lemani (Dragesco, 1960) Esteban, Fenchel & Finlay, 1995

Metopus lemani Dragesco, 1960

Brachonella pyriformis (Levander, 1894)

Metopus pyriformis Levander, 1894

Genus *Metopus* Claparede & Lachmann, 1858

Metopus es (O. F. Müller, 1786) Kahl, 1932 – type species by synonymy (as *Trichoda*)

Metopus sigmoides Claparede & Lachmann, 1858 – type species by monotypy

Metopus caucasicus Alekperov, 1984

Metopus daphnides Jankowski, 1964

Trichoda es. O. F. Müller, 1776

Metopus es var. *pinguis* Kahl, 1927

Metopus acidiferus Kahl, 1935

Metopus entorhipidioides Jankowski, 1964

Metopus mathiasi Viulleneuve-Brachon, 1940

Metopus alpestris Foissner, 1980

Metopus barbatus Kahl, 1927

Metopus bothrostomiformis Foissner, 1980

Metopus contortus (Quennerstedt, 1867) Kahl, 1932

Metopides contorta Quennerstedt, 1867

Metopus bivillus Tucolesco, 1962

Metopus sapropelicus Tucolesco, 1962

Metopus contortus var. *major* Kahl, 1932

Metopus contortus var. *pellitus* Kahl, 1932

Metopus contractus Penard, 1922

Metopus ridiculus Kahl, 1927

Metopus fuscus Kahl, 1927

Metopus fuscoides Alekperov, 1984

Metopus jankowskii Dragesco, 1968

Metopus halophila (Kahl, 1925) Corliss, 1960

Metopus halophilus Kahl, 1925

Metopus hasei Sondheim, 1921

Metopus fuscus Vuxanovici, 1962

Metopus latusculisetus Tucolesco, 1962

Metopus inversus (Jankowski, 1964) Foissner & Agatha, 1999

Brachonella inversa Jankowski, 1964

Metopus laminarius Kahl, 1927

Metopus trichocystiferus Jankowski, 1964

Metopus laminarius f. *minor* Kahl, 1932

Metopus major Kahl, 1932

Metopus micrans Jankowski, 1964

Metopus minor Kahl, 1932

(*Metopus mobilis* (Penard, 1922)

Cirranter mobilis Jankowski, 1964

Trochella mobilis Kahl, 1935)

Metopus mucicola Kahl, 1927

Bothrostoma mucicola (Kahl, 1927)

Metopus nasutus Da Cunha, 1915

Copemetopus nasutus (Da Cunha, 1915)

Metopus vexilliger Penard, 1922

Metopus nivaaensis Esteban, Fenchel & Finlay, 1995

Metopus ovalis Kahl, 1927

Metopus ventrosus Vuxanovici, 1962

Metopus palaeformis Kahl, 1927

Metopus hyalinus (Kahl, 1927) Kahl, 1935

Metopus rostratus Kahl, 1927

Metopus tenuis Kahl, 1927

Tesnospira alba Jankowski, 1964

Metopus palaformis Kahl, 1927

Metopus propagatus Kahl, 1927

Metopus ovatus Dragesco & Dragesco-Kerneis, 1986

Metopus strelkowi Jankowski, 1964

Metopus rectus Kahl, 1927

Metopus rotundus Scott Lucas, 1934

Metopus setosus Kahl, 1927

Metopus setifer Kahl, 1935

Metopus setosus var. *minor* Kahl, 1927

Metopus spinosus Kahl, 1927

Metopus attenuatus Penard, 1931

Metopus caudatus Penard, 1922

Metopus caudatus Da Cunha, 1915

Metopus vestitus Kahl, 1935

Metopus convexus Kahl, 1927

Metopus curvatus Kahl, 1927

Metopus striatus McMurrich, 1884

Metopus bacillatus var. *caudatus* Kahl, 1927

Metopus bacillatus Levander, 1894

Metopus denarius Kahl, 1927

Metopus bacillatus var. *denarius* Kahl, 1927

Metopus dentatus Kahl, 1927

Metopus fastigatus Kahl, 1927

Metopus gibbus Kahl, 1927

Metopus pulcher Kahl, 1927

Metopus pulcher var. tortus Kahl, 1927

Metopus pullus Kahl, 1927

Metopus recurvatus Vuxanovici, 1962

Metopus recurvatus var. *pusillus* Vuxanovici, 1962

Metopus acuminatus Stokes, 1886

Metopus acutus Kahl, 1927

Metopus minimus Kahl, 1927

Metopus violaceus Kahl, 1927

Urostomides Jankowski, 1964

Metopus tortus (Kahl, 1927) Kovalchuk, 1980

Metopus turbo Dragesco & Dragesco-Kerneis, 1986

Metopus verrucosus (Da Cunha, 1915) Kahl, 1935

Spirorhynchus verrucosus Da Cunha, 1915

Genus *Palmarella* Jankowski, 1964

Palmarella salina (Gaievskaia, 1925) Jankowski, 1975 – type species (as *Palmarium*)

Palmarium salinum (Gaievskaia, 1925)

Palmarella lata (Kahl, 1927) Jankowski, 1975

Metopus latus Kahl, 1927

Metopus angustus Kahl, 1927

Palmarium latum (Kahl, 1927) Jankowski, 1975

Palmarella angusta (Kahl, 1927) Jankowski, 1975

Palmarella mucicola (Kahl, 1927) Jankowski, 1975

Metopus mucicola Kahl, 1927

Genus *Tropidoatractus* Levander, 1894

Tropidoatractus acuminatus Levander, 1894 – type species

Metopus cuspidatus Penard, 1922

2.3. MORPHOLOGICAL DESCRIPTION OF ARMOPHORIDA

Although there are two orders within the anaerobic class Armophorea, there is no obvious synapomorphy between them. The free-living representatives are usually small to medium-sized ciliates, whose size ranges from 30 μm up to 300 μm. According to the literature, all armophoreans are always free-swimming (i.e., they do not attach to the substrate), which applies also for the period of cell division. Stomatogenesis is assumed to be pleurotelokinetal in all armophoreans (as in some other classes), although it has been described only in *Metopus* and *Caenomorpha* (Foissner & Agatha, 1999; Lynn, 2008). Alveoli are visible only in caenomorphids (Fenchel et al., 1977), and they are highly reduced or even absent in metopids (Fenchel & Finlay, 1991a) and clevelandellids (de Puytorac & Grain, 1969). Typically more than ten, sometimes many more, adoral polykinetids are present throughout the class (Lynn, 2008).

Family Metopidae is characterized by anterior part of body being twisted towards left, while the posterior one bears caudal cilia differing in length (sometimes tufted) and sometimes possesses a pointy „tail“; by large but inconspicuous adoral (buccal) membranelles; uniform somatic ciliature, which can be dense or sparse; compact macronucleus located usually in the centre of the cell and anaerobic lifestyle. The genera of Metopidae (see above) differ from each other mainly by the position of the cytostome, the constitution, shape, and size of peristomal area, paroral membrane, mono- or bipartite adoral zone, as well as the size and shape of the body and macronucleus. Particular species within these genera differ from each other by the size and shape of body, size of paroral membrane, length of adoral zone of membranelles, and the size and shape of preoral dome but, contrarily, other species share these features despite assignment in different genera. Therefore, the particular genera names need further analysis at least within *Metopus* and *Brachonella* (Aescht, 2001; Bourland et al., 2014b).

The genus *Metopus* is characterized by a twisted anterior part and a frontal lobe that overhangs an obliquely upward positioned adoral zone of membranelles, resulting often into an S-shape (Kahl, 1927; Jankowski, 1964). The body is relatively flexible (Esteban et al. 1995, Bourland et al., 2014a). The anterior twisted part usually contains five to ten kineties, from which some form a perizonal ciliary stripe by getting allied closely to each other at the end of the overhanging lobe, and some may form sutures (the secant system) (Jankowski, 1964).

Species of *Metopus* have been separated into five groups by Kahl, (1935) and later on resummarized by Esteban et al. (1995) based on the morphology of adoral zone of membranelles, cell shape and other features into different five groups (Fig. 2). Group I contains thin and elongate *Metopus palaeformis*-like species, whose equatorial part of the cell is not wider than the posterior part. Group II contains bell-shaped *Metopus striatus*-like species, whose anterior and equatorial cell parts are wider than the posterior part with a roundish projection. Group III is formed by species resembling distinct *Metopus contortus* and *M. es*, whose equator must be wider than the anterior and posterior parts. Group IV comprises species like *Metopus ovalis* with oval shaped cells. Group V comprises species like *Metopus vestitus*, whose posterior part of the cell is narrower than the equator and the anterior part, ending with a conspicuous spine-like posterior extension.

The genus *Bothrostoma* has been defined as "freshwater, free-swimming, ovate, soft and flexible ciliate with peristome-field obliquely directed in longitudinal depression, situated on the left-hand side of the body, extending beyond the body-centre, and continued inward as a short, ciliated, pharyngeal passage; the left-hand border of the peristome bearing a series of large cilia, the posterior portion of the right-hand margin supporting an undulating membrane; a cluster of long setose cilia projecting from the posterior extremity; contractile vesicle and nucleus conspicuous; anal aperture postero-terminal" (Stokes, 1887).

The genus *Brachonella* are medium-sized, bulky metopids that usually contain cortical granules with large preoral dome overhanging the adoral zone of membranelles, which is spiralling along the entire cell, and cytostome displaced posteriorly (Kahl, 1927; Bourland et al., 2014b).

The genus *Palmarella* was described as rare, approximately 30 μm long ciliate with the anterior end curved to the left, possessing long cilia on the anterior top; wide peristomal area with membranelles; sparse somatic ciliature with a single long caudal cilium on the rounded posterior end (Post et al., 1983).

The single species in genus *Tropidoatractus* was described as dorso-ventrally flattened elongate ciliate, with distinctly twisted body, which is covered with translucent longitudinal ribs following the shape of the body, allowing easy distinction from other metopids. Its peristomal

area is restricted to the top of the rounded anterior region and the perizonal cilia are located on a distinct hoop-like structure. The posterior part is ended with a spike (Curds et al., 1983).

Recently, the genus *Atopospira* has been morphologically defined by Bourland et al., (2014b) by diplostichomonad paroral membrane and bipartite adoral zone of membranelles with a short buccal part composed of ordinary membranelles and a longer distal part composed of much smaller membranelles sometimes bearing a single cilium extending by the entire length of perizonal ciliary stripe.

Caenomorphids have a left-torsed, roundish bolete- or cone-shaped cells with relatively rigid pellicle and a perizonal stripe formed by five ciliary rows coiled around the cell above the adoral zone of membranelles. The cell is endowed with multiple processes and spines, differing between individual species. Contrarily to metopids, the somatic ciliature is reduced, patchy, and formed by cirrus-like tufts or kineties, creating an "armoured" impression (Jankowski, 1964; Small & Lynn, 1985; Decamp & Warren, 1997).

All armophoreans are characterized by somatic dikinetids that have been relatively well studied in clevelandellids (*Nyctotherus, Sicuophora, Paracichlidotherus*) (Paulin, 1967; de Puytorac & Grain, 1969; Grim, 1998), whereas there is not much evidence for the ultrastructure of the dikinetids in metopids and no available data for caenomorphids (Schrenk & Bardelle, 1991; Lynn, 2008) (Fig. 3).

However, it is known that the armophorid kinetids do not give rise to postciliodesmata as in the unrelated, but morphologically somehow similar, heterotrichs, and there is a prominent left-directed, striated or non-striated cathetodesmal-like fibril arising adjacent to the anterior kinetosome, while the postciliary microtubular ribbons run along each other in a cortical ridge (Paulin, 1967; de Puytorac & Grain, 1969, 1976; Foissner & Agatha, 1999). The armophorid somatic dikinetid consists of one anterior kinetosome bearing a cilium, with a marginal anterior transverse ribbon at triplets 3, 4, and 5 (which can be accompanied by a group of microtubules) and one posterior kinetosome bearing a cilium, with a diverging postciliary ribbon and a laterally-directed kinetodesmal fibril at triplets 5, 6, 7 that are sometimes non-striated, with several transverse microtubules connecting the two kinetosomes (Schrenk & Bardele, 1991; Esteban et al., 1995; Lynn, 2008).

Complex filamentous structures and microtubules underlie the oral structures of armophoreans and enable motion of vesicles containing food particles towards the food vacuole (Lynn, 2008; Eichenlaub-Ritter & Ruthmann, 1983). Various mucocysts in the cortex and a contractile vacuole of type I in the posterior part of the cell are present in all armophoreans, as well as a cytoproct, which is usually well visible (Paulin, 1967; de Puytorac & Grain, 1969; Esteban et al., 1995, Bourland et al., 2014a). The colour of the cells varies among species and

populations from clear through yellowish to light brown. Some armophorids contain distinctive dark particles inside of the anterior part of the cell (Bourland et al., 2014a).

There is typically a single nucleus in the cells of armophorids, according to the literature usually of a simple, oval or roundish shape, although caenomorphids may possess over four macronuclei. The factors initiating conjugation in armophorids are unknown. However, it has been described that conjugants typically fuse within the whole anterior region or even within the entire two cells, while the gametic nucleus (nuclei) and cytoplasm of the first cell pass into the second cell (Noland, 1927). The micronuclei are supposed to go through three maturation divisions (two meiotic divisions and a mitosis of one of the four haploid products) (Raikov, 1972; Martín-González et al., 1987). Micronuclear mitosis is a typical ciliate endomitosis (Raikov, 1982). Development of a macronuclear anlage in armophoreans may take up to two weeks (Noland, 1927).

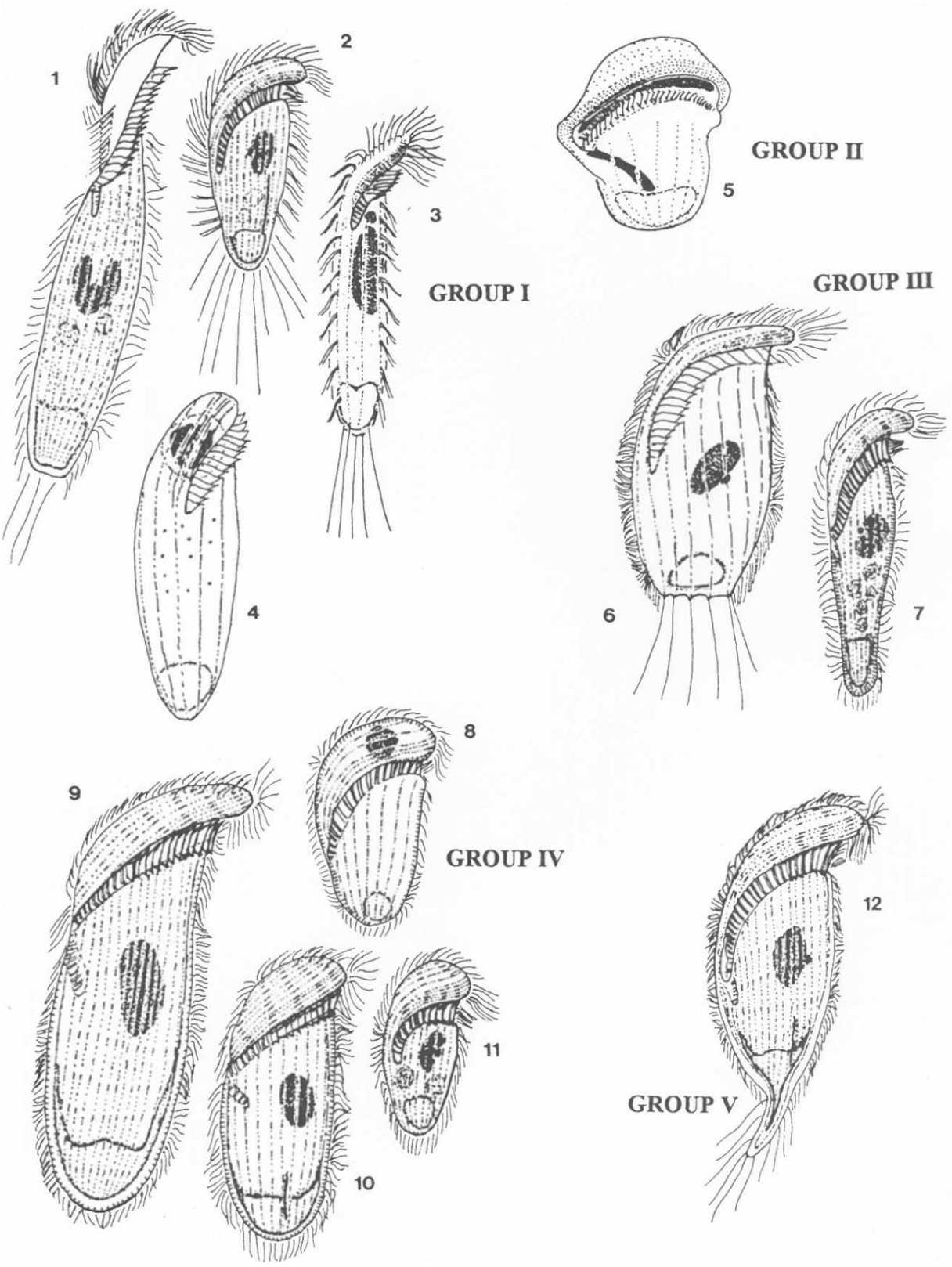

Fig. 2. Groups of *Metopus* morphospecies divided by Esteban et al. (1995).

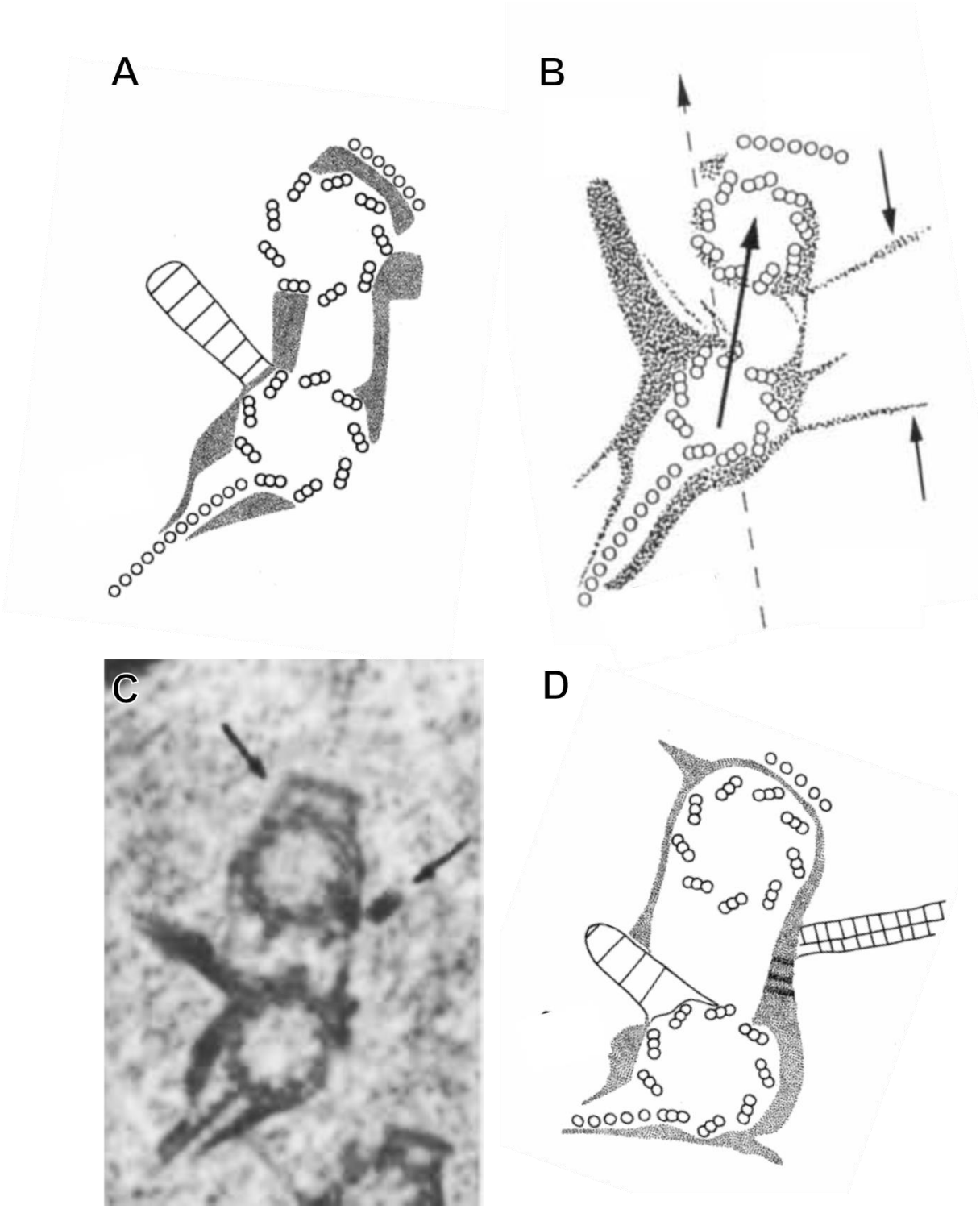

Fig. 3. Somatic dikinetids in Armophorea. Schema (A, B, D) or TEM micrograph (C). A - Metopus sp., B - *Metopus es*, C - *Metopus contortus*, D - *Nyctotherus sp*., Schrenk & Bardele (1991) and Foissner & Agatha (1999) (B), Esteban et al. (1995) (C), and Lynn (2008) (A, D).

2.4. HISTORY OF CLASSIFICATION

The first idea of affinity between free-living armophorids and endobiotic clevelandellids had originated in the mind of the French scientist Simone Villeneuve-Brachon (1940), although the establishment of the class Armophorea had a long journey ahead at that moment. Representatives of the former group (armophorids) have a tortuous history of classification. Although several attempts to organize (and reorganize) armophorid species into morphologically related groups with type species have passed (Kahl, 1932; Esteban & Finlay, 1995), the performed morphospecies groupings have not made the structure of Armophorida clear, but rather even more chaotic.

The first described armophorid species is *Trichoda es* (Müller, 1776) (syn. *Metopus es*). Afterwards, *Metopus sigmoides* was described by Claparède & Lachmann (1858), who placed it within the then family Bursarina (together with, for example, *Frontotia, Chaetospira, Spirostomum, Plagiotoma, Bursaria*), while Kahl (1927, 1932) synonymized it with *Metopus es* and assigned it into the then suborder Heterotricha within the then order Spirotricha. Closer to the end of the 19th century, several more species of *Metopus* have been described (*M. inflatus* Fromentel, 1876; *M. bacillatus* Levander, 1894; *M. pyriformis* Levander, 1894; *M. acuminatus* Stokes, 1886; *M. striatus* McMurrich, 1884; *M. acuminatus* Stokes, 1886; *M. tesselatus*, Diesing, 1886). Subsequently, Kahl (1927, 1932), da Cunha (1915), Penard (1922, 1931), and Powers (1935) described over another 50 species of *Metopus*, many of which have been synonymized throughout the history, concurrently with other new species being described (Jankowski,et al., 1964).

Other generic names, such as *Bothrostoma* Stokes, 1887, *Spirorhynchus* da Cunha, 1915, *Tesnospira* Jankowski, 1964, and *Tropidoatractus* Levander, 1894 have been proposed, however, all of them have sooner or later fallen into the whirl of synonymization or resynonymization with the generic name *Metopus*, specifically *Bothrostoma extensa* (syn. *Metopus extensus*) (Kahl, 1926), *Bothrostoma mucicola* (syn. *Metopus mucicola*, syn. *Palmarella mucicola*) (Kahl, 1927) or *Metopus bothrostoma* var. *longior* (Kahl, 1927), synonymized with *Metopus undulans* (syn. *Bothrostoma undulans)* (Stokes, 1887; Kahl, 1932), and *Metopus mirabilis* (Kahl, 1927) with *Bothrostoma mirabilis* (Jankowski, 1964). Species name *Tesnospira alba* (Jankowski, 1964) is synonymous with *Metopus palaeformis* (Kahl, 1927). A single species has been described in the genus *Tropidoatractus*, i.e. *Tropidoatractus acuminatus* (Levander, 1894), although it supposedly is synonymous to *Metopus cuspidatus* (Penard, 1922). Genus *Palmarium* (Gajewskaja, 1925) is a synonym of *Palmarella* (Jankowski, 1975). Altogether four species have been assigned into this genus over multiple synonymizations – *Palmarella salina* (syn. *Palmarium salinum* Gajewskaja,

1925), *Palmarella lata* (syn. *Palmarium latum*, syn. *Metopus latus* Kahl, 1927), and *Palmarella mucicola* (Kahl, 1927).

Brachonella contorta (Levander, 1894) Jankowski, 1964 and *Brachonella pyriforme* (Levander, 1894) Jankowski, 1964 were firstly described as *Metopus contortus*, respectively *Metopus pyriformis*, and later transferred to the genus *Brachonella*, whilst the ambiguity within the order has accreted. The generic names *Metopus* and *Brachonella* overlap in many cases, resulting into just three *Brachonella* species that do not possess a synonymous name in the *Metopus* genus (*Brachonella elongata* Jankowski, 1964; *Brachonella mitriformis* Alekperov, 1984; *Brachonella caenomorphoides* Foissner, 1980) out of the fourteen historically described species. Esteban et al. (1995) have revised the genus *Metopus* to reduce 76 nominal species to 22 morphospecies, while also discovering a new species (*Metopus nivaaensis*). It is still not clear whether that could be a final arrangement, although currently there are strong voices against, and the monophyly of the genera *Metopus* and *Brachonella* might be overall rejected (Bourland et al., 2014a,b).

Caenomorphidae were classified as closely related to Metopidae already by Kahl (1932) who has assigned the three genera known at that time (*Caenomorpha*, *Ludio*, and *Cirranter* included as *Trochella mobilis* within the genus *Metopus*) within Metopidae. The genera were included in the family due to their anaerobic lifestyle, feeding on rhodobacteria, and the typical body torsion and the adoral zone of membranelles coiling along the body. Kahl (1932) classified the genera due to: holotrichous somatic ciliatures, soft and pliant ectoplasm, contractile terminal vacuole, typical peristome in *Metopus*, one or two dorsal rows of cirri in *Caenomorpha*, two separated rows of cirri in *Ludio*, and the reduced body ciliature in *Trochella* (i.e. *Cirranter*). Only Jankowski (1964) has recognized differences between genera of the current Metopidae and Caenomorphidae and created a separate family for genera *Caenomorpha, Ludio, Cirranter,* and *Sulfonecta* to separate them from Metopidae, and created the order Armophorida. However, in phylogenetic analysis, some caenomorphid species were shown to be likely paraphyletic (van Hoek et al., 2000b). Additionally, the whole family may not even be a member of the order Armophorida and class Armophorea, as its phylogenetic position within ciliates is uncertain (da Silva Paiva et al., 2013).

The endobiotic lineage of Armophorea, clevelandellids, has seemingly followed an easier path. The Family Clevelandellidae was established by Kidder (1937, 1938) due to distinctive characteristics of these endocommensals of the digestive system of wood-feeding roaches of the genus *Panesthia*, while he already observed similarities with the genus *Nyctotherus* (Leidy, 1849, 1853), belonging into the Family Nyctotheridae. Jankowski (1968) speculated an origin of the clevelandellids from nyctotherids and at the same time had seen a correlation with the free-living

metopids, whom he attributed ancestral origin. Albaret (1975) with Njiné (1976) united clevelandellids and nyctotherids into a superfamily, which was by de Puytorac and Grain (1976) defined as the Suborder Clevelandellina. Corliss (1979) defined the five families (Nyctotheridae, Sicuophoridae, Clevelandellidae, Inferostomatidae, Nathellidae) of the Order Clevelandellida.

However, there also have some been some transfers on the genus level, as for example the transfer of *Nyctotherus cordiformis* (Ehrenberg, 1838) to the genus *Nyctotheroides* due to its morphological features (Albaret, 1975). The genus *Nyctotheroides* was established by Grassé (1928), the genus *Nyctotherus* by Kidder (1937), and the genus name *Clevelandia* (Kidder, 1937) was changed to *Clevelandella* by Kidder (1938), while Yamasaki (1939) has established another new genus *Emmaninius*, which, however, has later been synonymized again with *Clevelandella* (Corliss, 1961).

The original opinion that clevelandellids are solely endosymbionts of invertebrates has been disproved when new genera with species inhabiting the intestines of anurans (Wichterman, 1934), lizards (Amrein, 1952), and fish have been described (Affa'a, 1980, 1983, 1988, 1989; Earl, 1991; Grim, 1992). On the other hand, not until the phylogenetic analysis of nyctotherids the species inhabiting anurans were taxonomically separated from those in arthropods (Hackstein et al., 2002; Affa'a et al., 2004), although it had corresponded with conclusions in previous morphological studies (Albaret, 1975).

For a very long period, armophorids and clevelandellids (suborders Armophorina and Clevelandellina) have been among other groups interpreted as Heterotrichida that were ranked as a subgroup of Spirotrichea (Corliss, 1979). Only the arrival of molecular phylogenetics has enabled to shed a light on the relationships between these ciliate groups, together with emphasis on the cortical ultrastructure. One of the early phylogenetic analyses has revealed that "traditional" heterotrichs are in fact distant from other ciliate groups as Spirotrichea, and therefore the class Heterotrichea has been established (de Puytorac et al., 1993) and forthwith greatly accepted (Lynn & Small, 1988). Former "traditional" heterotrichs were split into "true heterotrichs" of the class Heterotrichea and others that have remained part of Spirotrichea.

Van Hoek et al. (1998, 2000b), Hackstein et al. (2002), and Affa'a et al. (2004) have also positively conveyed the relation between the two groups, and Villeneuve-Brachon's (1940), Jankowski's (1968), and Albaret's (1975) inkling that armophorids are a sister taxon to the clevelandellids was confirmed in their phylogenetic analyses. Armophorids were finally separated and placed together with clevelandellids into a new "ribo-class" Armophorea (Lynn, 2004), which was supported by numerous phylogenetic analyses based on SSU rDNA (Small & Lynn, 1985; ; de Puytorac, 1994; Lynn & Small, 1988, 2002; van Hoek et al., 1998, 1999; Riley and Katz, 2001; Affa'a et al. 2004).

The class Armophorea was defined by Lynn (2004), who named it according to Jankowski to honour his long-term work on these ciliates, maybe as well as recognizing the possibility of relation even without the molecular approach. The class name is derived from a Latin word *arma*, which means "weapon", and an adopted Latin word *phoros* (originally a Greek word *foros*), meaning "bearing", which hints the armour-like shape of caenomorphid cells (Jankowski, 1964). Armophorea is considered a ''riboclass'', as the representatives do not share morphological justification as a group despite strong phylogenetic support of metopids and clevelandellids. Even on an ultrastructural level, Schrenk and Bardele (1991) have discovered significant differences between the somatic and oral structures of armophorids (*Metopus*) and clevelandellids (*Nyctotherus*).

As mentioned above, it has been suggested several times throughout the historical classification revisions that clevelandellids may have derived from metopids (Villeneuve-Brachon, 1940; Jankowski, 1968; Albaret, 1975), during transmission from the outer environment into the gut of various invertebrates or vertebrates. The main traces lie in the transformation of the cortical patterning (Jankowski, 1968). Consistent with the hypothesis that Clevelandellida are descendants of Armophorida, based on morphology, they appeared as an internal group of Metopidae in phylogenetical analyses based on SSU rDNA (Lynn et al., 2013). Contrarily, Caenomorphidae have branched outside of the class Armophorea in phylogenetic analyses based on SSU rDNA (da Silva Paiva et al., 2013). Therefore, the entire class needs a proper revision.

2.5. ECOLOGY OF THE FREE-LIVING ARMOPHORIDS

Ciliates in general have a huge impact on various ecosystems. Free-living ciliates play a significant role in aquatic ecosystems, functioning as top predators in microbial food webs and main grazers of bacteria and protists, contributing to decomposition of organic detritus and providing nutrition for other organisms, therefore having a considerable impact on carbon sources (Kemp, 1988). They are also frequently used as bioindicators of water quality (Jiang et al., 2011).

Representatives of the order Armophorida always need microaerobic or anoxic conditions, but otherwise they inhabit diverse environments, from hypolimnion of lakes, stagnant water reservoirs, through terrestrial habitats to freshwater and marine sediments. Armophorids generally serve as good indicators of anoxic environments (Lynn, 2008).

There have been numerous ecological studies mentioning armophorid ciliates (Taylor, 1979; Kovalchuk, 1999; Mazei et al., 2005; Jiang et al., 2005), however most of them have not performed a detailed taxonomical analysis and therefore mostly revolve around just few species, particularly *Metopus palaeformis*, *M. contortus*, *M. striatus*, *M. es*, *Brachonella spiralis*, rarely *M.*

setosus, or do not state a species at all. The ecological data for other armophorid species are scarce.

Armophorids are most likely cosmopolites and have been described on almost all continents but Antarctica (Foissner, 1998). Various species have been frequently found in anoxic hypolimnion of thermally-stratified lakes, stagnant water reservoirs, ponds, or bays (Kahl, 1927, 1932, Esteban et al., 1995). Relatively rich armophorid populations have been encountered in rice fields (Schwarz & Frenzel, 2003, 2005), as well as other various terrestrial habitats such as soils. Metopids have been recorded also from sandy sediments (Finlay et al., 1993b), wastewater or even chloride lakes (Madoni, 1990). Caenomorphids are mostly found in freshwater sediments, although several marine species exist (Kahl, 1935; Decamp & Warren, 1997). In one site, there are usually several armophorid species inhabiting the same environment, the highest recorded number from one sample being ten armophorid species from soil in evergreen rainforests (Foissner, 1997; Schwarz & Frenzel, 2003; Schwarz & Frenzel, 2005).

They are bacterivorous, feeding mainly heterotrophic and phototrophic purple bacteria (Massana et al., 1994). *Caenomorpha* feeds especially on *Thiopedia*, a purple sulphur bacterium, whose populations may supposedly be controlled by these ciliates in some habitats (Guhl & Finlay, 1993; Lynn, 2008). The only recorded clearance rate of *Metopus* sp. was 32 nL ciliate^{-1} h^{-1} (Massana et al., 1994). Even though armophorids can reach abundances over 5,000 cells per liter in the water column, most usually their abundances are much lower (Fenchel et al., 1990; Fenchel & Finlay, 1991a; Guhl & Finlay, 1993; Guhl et al., 1996). The biovolume ratio of anaerobic ciliates and their bacterial prey ranges from 5% to 10% (Guhl & Finlay, 1993).

Chemosensory in armophorids has been studied just briefly, resulting into two conclusions. Firstly, the chase after bacterial prey may be driven by chemosensory. Secondly, there is also a chemosensory response for oxygen concentration changes. Not just that there is a change to a higher swimming speed if oxygen concentrations increase, but also the cilia movement is reversal (Fenchel & Finlay, 1990a). As typical for anaerobes, the cell growth of armophorids is significantly slower than in aerobic ciliates (Fenchel & Finlay, 1990b).

Environmental factors such as predation, water and food availability, salinity, and temperature can influence the cell sizes as well as a growth rate and cell volume (Fenchel, 1987; Decamp & Warren, 1997). If the conditions get suboptimal and the ciliates starve, they divide into significantly smaller cells within one or two generations (Fenchel, 1987). The other way around, ciliates grow to larger maximum sizes in environments with rich bacteria populations, e.g. wastewaters (Decamp & Warren, 1997). Such conclusion corresponds with a wide range of cell sizes in environments with various different microhabitats present at one site (Decamp & Warren, 1997).

Esteban et al. (1995) noted that most armophorid species are rather rare, at least in compare to aerobic ciliates, while other authors consider some metopid species as common ciliates (Kahl, 1928). Armophorids probably have a vertical distribution and patched abundance throughout the year. In the cold temporal area, their abundance is low and they are homogeneously distributed in early spring, but their numbers drop after the establishment of stratification (Massana et al., 1994). In sites with seasonal anoxia, the abundance of metopids in sediments highly increases during the periods of anoxia (Finlay, 1982).

Foissner (1997) polemized if soil ciliates populations might be patchily distributed as he had observed higher species richness more likely close to cultivated soils and arid deserts, or the reason for the ciliate species paucity could be the usual low nutrient status of soils. Nevertheless, he concluded another idea, which is the methodology of sampling in laboratory-remote places. He compared a soil sample that had been dried and rewetted as usual, and a fresh soil sample from a Venezuelan rainforest. The latter comprised of significantly richer ciliate species numbers.

Undoubtedly, an important stage of the life cycle of metopids is encystment. It is possible that in some cases metopids spend most of their life as a cyst (Lynn, 2008). When dry mud samples from temporarily dried out water body were examined, cysts of metopids have been found (Foissner & Agatha, 1999). Encystment enables metopids to endure suboptimal conditions and be able to commonly inhabit soils (Foissner, 1987, 1995b). The cycle of encysting and excysting depends on water and food availability (Finlay & Fenchel, 1991). The cysts were described as flask-shaped (Esteban et al., 1995).

2.6. HYDROGENOSOMAL METABOLISM

Mitochondria of some organisms inhabiting anoxic environments have been transformed into organelles called hydrogenosomes. The transformation has independently happened multiple times throughout various eukaryotic groups, mainly including protists (Tachezy et al., 2007). Hydrogenosomes have been so far described from many unrelated protistan lineages, e.g. in heteroloboseans of the genera *Psalteriomonas* and *Sawyeria* (Broers et al., 1993; de Graaf et al., 2009; Barbera et al., 2010; Pánek et al., 2012), trichomonads (Lindmark and Müller, 1973; Dyall et al., 2000a; Hrdý et al., 2004; Pütz et al., 2006; Smutná, et al., 2009; Schneider et al., 2011), chytridiomycetes of the genera *Neocallimastix* and *Piromyces* (Yarlett et al., 1986; Gelius-Dietrich et al., 2007; Akhmanova et al., 1998; James et al. 2006), and various rumen-dwelling (Yarlett et al. 1984, Paul et al., 1990) and free-living ciliates (Embley et al., 1995). Recently, they have been discovered also in the cells of parasitic diplomonad *Spironucleus salmonicida* (Jerlström-Hultqvist et al., 2013).

The most reduced mitochondrion related organelles, mitosomes, were firstly found in the parasitic archamoeba *Entamoeba histolytica* (Mai et al. 1999; Tovar et al., 1999), subsequently it has been discovered that mitosomes are also present in other protists, e.g. microsporidia *Encephalitozoon cuniculi*, *Trachipleistophora hominis* (Katinka et al. 2001, Williams et al., 2002; Vávra, 2005), apicomplexan *Cryptosporidium parvum* (Riordan et al.1999, 2003, Šlapeta a Keithly 2004), diplomonad *Giardia intestinalis* (Tovar et al. 2003).

Mitochondrion- and hydrogenosome-related organelles were found in several protists as stramenopilean *Blastocystis* or *Proteromonas lacerate* (Pérez-Brocal et al., 2010), and armophorean endobiotic ciliate *Nyctotherus ovalis* (Akhmanova et al., 1998). The level of mitochondrion transformation varies between different groups of anaerobic eukaryotes. All hydrogenosomes have a double membrane, while the inner membrane is folded into cristae in some cases but not in others (Bui et al., 1996). Hydrogenosomes usually do not possess a genome, with a couple of exceptions (Akhmanova et al., 1998; Stechman et al., 2008; Pérez-Brocal et al., 2010).

Hydrogenosomal metabolism takes place under anaerobic conditions without the typical mitochondrial oxidative phosphorylation. Instead, ATP is synthesised by substrate phosphorylation, where the electron acceptor is a proton instead of oxygen, and the final product is hydrogen instead of water. Although hydrogenosomes still generate ATP, generally alternative enzymes such as pyruvate:ferredoxin oxidoreductase (PFO) are used (Dyall et al., 2000b), instead of the regular pyruvate dehydrogenase (PDH) from aerobic mitochondria. Pyruvate or malate as entry substrates are then oxidatively decarboxylated to acetyl-CoA. The formation of acetate and ATP can be mediated by acetate:succinate CoA transferase and succinate thiokinase. After the decarboxylation of pyruvate, hydrogenases remove the reduction equivalents and the reduced protons participate in formation of molecular hydrogen (Müller, 1993, 1998).

Mitochondrial heat-shock proteins, components of mitochondrial membrane transport complexes, and various mitochondrial proteins for FeS centre assembly, as frataxine or cystein-desulphurase (IscS), are mostly present. Protein transport also shares many similarities with the mechanisms in classical mitochondrion (Doležal et al., 2007; Mentel et al., 2008; Lithgow & Schneider, 2010). Sometimes, the metabolic pathways as pyruvate dehydrogenase complex, tricarboxylic acid cycle, electron transport chain, and F1FO-ATPase are absolutely or partially missing (Müller et al., 2012).

As mentioned earlier, ciliates have been able to seize anoxic environments multiple times in their evolution and hydrogenosomes have independently evolved in at least 6 out of the 11 main ciliate lineages (Fenchel and Finlay, 1995). It is generally accepted that anaerobic ciliates have evolved secondarily from aerobic ancestors (Embley et al., 1995, 1997, 2003; Fenchel &

Finlay, 1995; Hackstein et al., 2001, 2002). The hydrogenosomes have been so far studied in only several ciliate groups. Hydrogenosomes of some rumen ciliates (e.g. *Dasytricha, Isotricha, Epidinium, Eudiplodinium*) can use malate as a substrate in anaerobic conditions but are able to utilize oxygen if present, and also contain the complete pathway from malate through pyruvate to H_2 (Yarlett et al., 1984; Paul et al., 1990). Obligately anaerobic plagiopylids (*Plagiopyla, Sonderia, Trimyema*) contain functioning hydrogenases (Zwart et al., 1988). *Trimyema* possesses a hydrogenosome that produces formate as a major end product, with minor amounts of acetate and lactate (and no hydrogen or ethanol) under micro-aerobic conditions (Goosen et al., 1988). Contrarily, in strictly anaerobic conditions, it produces ethanol as the main end product, and smaller amounts of formate, acetate, lactate, and hydrogen are formed (Goosen et al., 1990). Odonstomatids (*Myelostoma, Saprodinium*) also possess hydrogenosomes (Bardele & Schrenk, 1991). Hydrogenosomes have also been discovered in scuticociliate species *Cyclidium porcatum*, some pleurostomatids, and litostomatean *Lacrymaria sapropelica* (Esteban & Finlay, 1994; Esteban et al., 1993; Fenchel & Finlay, 1995).

The free-living representatives of Armophorea possess hydrogenosomes that have not been metabolically compared to that of *Nyctotherus ovalis*, but are at least morphologically different from them (Biagini et al., 1997a). The hydrogenosomes of free-living armophorids morphologically vary even between individual species. Those that have been studied were all shown to be accompanied by symbiotic methanogens. Oval-shaped hydrogenosomes of the marine species *Metopus contortus* show a presence of subsurface cristae (Embley & Finlay, 1994; Esteban et al., 1995), while smaller roundish hydrogenosomes of the freshwater species *M. palaeformis* seem to lack them in micrographs by Esteban et al. (1995), where it, however, has not been explicitly described. It has been revealed that hydrogenosomes of *M. palaeformis* are hydrogenase-positive and release hydrogen (Finlay, 1990; Embley et al., 1992). Hydrogenosomal transmembrane electrochemical potential has been proved in *M. contortus* thanks to the presence of the cationic lipophilic cyanine dye $DiOC_7(3)$ (Biagini et al., 1997b). Further studies about metopids hydrogenosomes are needed to fully understand the anaerobiosis within Armophorea.

Finlay (1981) has encountered organelles resembling hydrogenosomes also in *Caenomorpha*. Nevertheless, further study has not been performed yet, partially due to the scarce abundance of this ciliate. Despite the uncertain phylogenetic relation of *Caenomorpha* (da Silva Paiva et al., 2013), some think that the hydrogenosomes of this ciliate are of common origin with those in other armophoreans (van Hoek et al., 2000b)

The hydrogenosomes of the endobiotic clevelandellid species *Nyctotherus ovalis* have a unique character (Gijzen et al., 1991; Akhmanova et al., 1998; van Hoek et al., 1998; 1999; 2000a). These organelles also possess a genome, which is quite unusual feature for

hydrogenosomes. So far, it is the only case within ciliates where a hydrogenosomal genome has been discovered, although no further surveys have been performed. Only two other cases of a putative hydrogenosomes with a genome within protists have been found in the stramenopiles *Blastocystis hominis* (Stechmann et al., 2008) and *Proteromonas lacertae* (Pérez-Brocal et al., 2010). The metabolism of *N. ovalis* hydrogenosome shows similarities to hydrogenosomal metabolism of those two phylogenetically very distant protists, with present respiratory chain complexes, Fe-Fe dehydrogenase, and an inner membrane capable of forming cristae (Nasirudeen & Tan, 2004; Perez-Brocal & Clark, 2008; Stechmann et al., 2008; Wawrzyniak et al., 2008) (Fig. 4).

As shown by transmission electron microscopy, the hydrogenosomes of *Nyctotherus ovalis* also contain cristae, putative 70S ribosomes and appear in various numbers in each cell, always surrounded by archaeal endosymbiotic methanogens that utilize hydrogen produced by the hydrogenosomes (Akhmanova et al., 1998; Hackstein et al., 2001). Voncken et al. (2002) have shown that cardiolipin, which is a characteristic compound of membranes in aerobic mitochondria, is present. Insight into its genome has revealed a lot more (Akhmanova et al. 1998; van Hoek et al. 2000a; Boxma et al. 2005; de Graaf et al., 2011).

Analysis of the almost complete genome allowed a preliminary reconstruction of the hydrogenosomal metabolism (de Graaf et al., 2011). A number of genes encoding mitochondrial proteins are coded also in the macronuclear genome (Boxma et al., 2005). The electron transport chain is probably reduced into just two steps through mitochondrial complex I and II, as almost all of the genes coding the complex I, which pumps protons out of the organelles, and complex II, that allows fumarate (electron acceptor) reduction, were identified, but genes coding complexes III, IV and V, have not been discovered (de Graaf et al., 2011). Many genes for proteins participating in the metabolism of amino acids, protective mechanisms against oxidative stress, mitochondrial protein synthesis, mitochondrial protein import and processing, transport of metabolites through mitochondrial membrane, and two genes for hypothetical proteins linked to mitochondrial disease in humans (MPV17 and ACN9), as well as certain genes encoding Krebs cycle enzymes such as malate dehydrogenase, succinate dehydrogenase, succinyl-CoA synthetase or α-ketoglutarate dehydrogenase have been also found (Boxma et al., 2005; Hackstein et al., 2006).

No evidence for the presence of the usual hydrogenosomal enzyme pyruvate:ferredoxin oxidoreductase (PFO) was found. Surprisingly, genes for nuclear-encoded mitochondrial pyruvate dehydrogenase (PDH) were discovered. That led to conclusion that PDH plays a crucial role in the hydrogenosomal metabolism of *N. ovalis*. Supportively, various experiments have revealed the presence of an oxidative carboxylation step and three out of the four genes encoding the E1 alpha,

E1 beta, and the E2 subunit of the mitochondrial-type PDH. These all possibly encode a mitochondrial targeting signal at the N-terminus of the particular gene, and also cluster with the orthologues of their mitochondriate relatives (Boxma et al., 2005). The main final metabolic product is succinate, which is typical for anaerobic mitochondria, alternatively small amounts of acetate (Tielens et al. 2002).

Nyctotherus ovalis possesses a complex [FeFe]-hydrogenase (Akhmanova et al., 1998). It represents a unique type among all known hydrogenases in eukaryotes. Other studied eukaryotic [FeFe]-hydrogenases and hydrogenase-like proteins as "NARFs" differ, for example from those in breviates (Stairs et al., 2014) or those from rumen ciliates, anaerobic chytrids, green algae or trichomonads, which have been shown to be monophyletic (Florin et al., 2001; Horner et al., 2002; Nixon et al., 2003; Boxma, 2004), although the statistical support was poor (Voncken et al., 2002; Embley et al., 2003). This polyprotein is encoded by a macronuclear gene-sized chromosome with a mosaic character. [FeFe]-hydrogenase is fused from two modules, which are similar with a 24kDa and 51kDa subunits of mitochondrial complex I (Boxma et al., 2007). The paralogous mitochondrial 24 kDa and the 51 kDa modules are closely related to homologous modules with a function in bacterial [NiFe]-hydrogenases. The [FeFe]-hydrogenase plays an important role in connecting the fermentative glucose metabolism with the basic components of the mitochondrial electron transfer, as it is covalently linking to a NAD-possessing protein with FMN binding sites, and a ferredoxin-like module allowing transfer of electrons to the catalytic site of the hydrogenase (Akhmanova et al., 1998; Vignais et al., 2001; Voncken et al., 2002). In other words, it allows direct reoxidation of reduced NAD(P) in complex I (thanks to the presence of mitochondrial paralogues of the accessory 24kDa and 51kDa modules, while other hydrogenases need ferredoxine), which enables creating an energy storage that can be later used for expulsion of protons allowing the formation of molecular hydrogen.

An iron-only [Fe]-hydrogenase module, which are typical for methanogenic Arachae and had been suggested to not be widely distributed in eukaryotes, is also present in *Nyctotherus ovalis* (Horner et al., 2000). It supposedly originated from δ-proteobacteria, but the ferredoxin-like subunits are putatively of a β-proteobacterial origin (Akhmanova et al., 1998; Voncken et al., 2002). The 5′ part of the gene is homologous to the corresponding domain of the hydrogenase from *Clostridium* and *Desulfovibrio*, but the middle and 3′ part of the gene most resemble the nuclear genes nuoE and nuoDF, which encode components of the NADH dehydrogenases in the mitochondrial respiratory chain complex (de Graaf et al., 2011). Therefore, the [Fe]-hydrogenase was most likely acquired by a concerted lateral gene transfer from different sources (Boxma et al., 1995, 2007).

The hydrogenosomes of rumen ciliates and plagiopylids likely independently evolved from aerobic mitochondria (Embley et al., 1995). Although it is generally accepted that endobiotic clevelandellids have evolved from the free-living armophorids (van Hoek et al., 2000a; Bourland et al., 2014a), there is no study about the evolution of mitochondria during the switch to the endobiotic lifestyle of these ciliates.

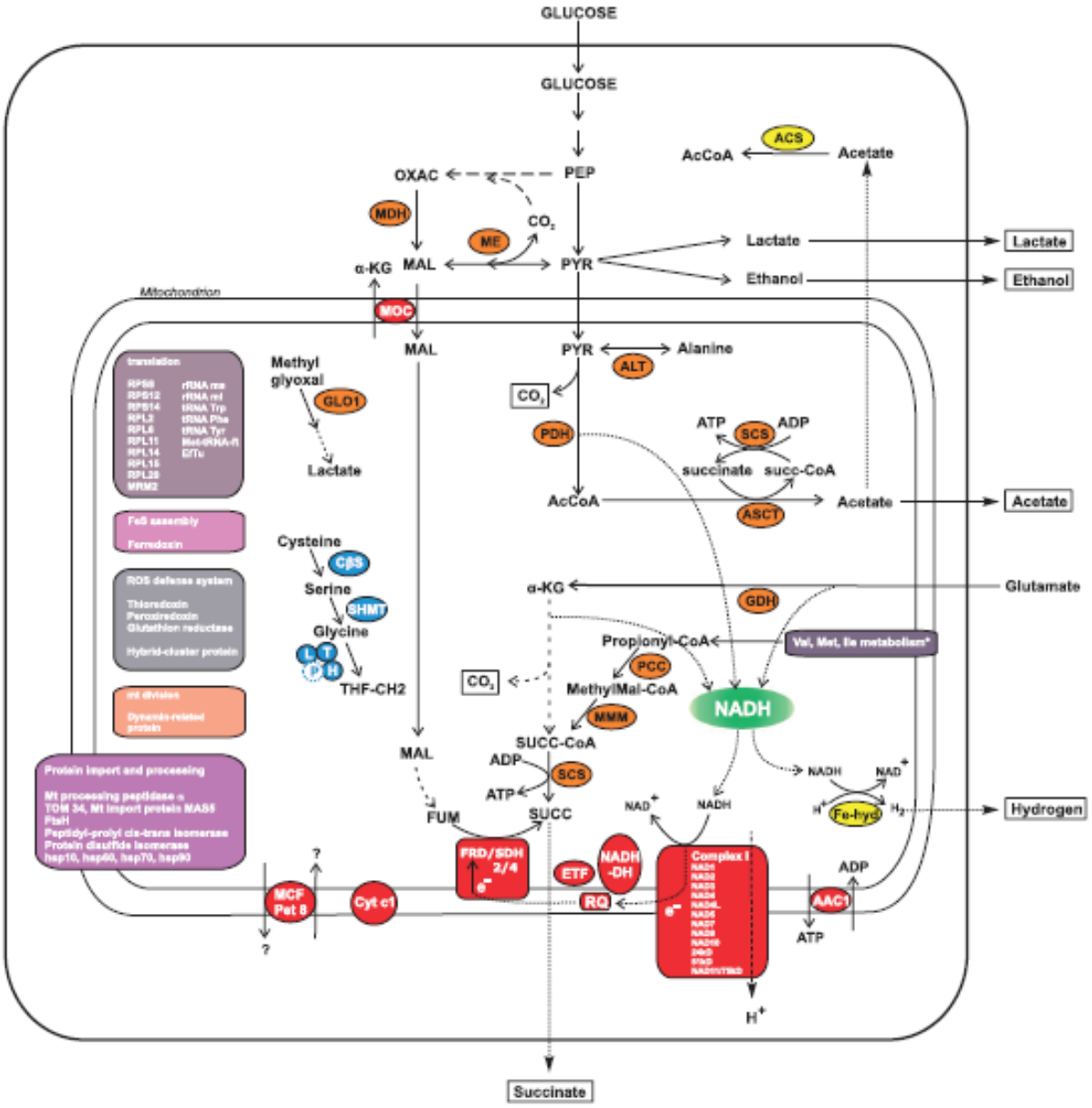

Fig. 4. Tentative reconstruction of *Nyctotherus ovalis* hydrogenosomal metabolism map. De Graaf et al. (2011)

2.7. METHANOGENESIS

Many protists, including ciliates, embrace endosymbiotic prokaryotes within their cells, some of which were identified as methanogenic Archaea associated with protist hydrogenosomes (Fenchel & Finlay, 2010; Hackstein et al., 2002; 2010; Ushida et al., 2010). Archaea are prokaryotic organisms that were first found inhabiting isolated and extreme environments, but later it has been revealed that this prokaryotic domain is almost ubiquitous (van Hoek et al., 2000b). Archaean methanogens are generally capable to survive in various extreme conditions with wide temperature ranges from 5 °C to 110 °C and anoxic sediments in fresh-water and marine ecosystems (Ferry, 1993).

Methanogens use the hydrogen produced by hydrogenosomes to generate methane (Hungate et al., 1970). Methane formation from formate occurs by the decomposition of formic acid into CO_2 and H_2 followed by a reduction of CO_2 by H_2 (Thauer et al., 1993). Methane gets partially oxidized to CO_2 within the aerobic environment, but possibly huge amounts of it escape in the upper atmosphere. The methane that is released into the atmosphere represents one of the main greenhouse gases (Ferry, 1993; Shine et al., 2005; Howarth et al., 2011). The amounts of atmospheric methane are rising every year mainly due to the influence of humans, also counting their need to keep rising the size of ruminant populations worldwide (Ferry, 1993; Blazewicz et al., 2012). Concurrently, the amount of attention to methane levels is growing exponentially in recent years, as methane plays a crucial role in enhancing the greenhouse effect (Blazewicz et al., 2012). Despite the fact that more attention is still paid to carbon dioxide, new study shows that methane is significantly more potent as a heat-trap (Yvon-Durocher et al., 2014).

From the eight ciliate lineages containing anaerobic representatives (out of the 11 main ones), from which hydrogenosomes have been studied in six of them, only Oligohymenophorea, Litostomatea, Plagiopylea, and Armophorea have been recorded to host methanogenic endosymbionts (Fenchel & Finlay, 1995; van Hoek et al., 2000b; Hackstein et al., 2002). They have been studied mainly in plagiopylids and rumen ciliates.

Methanogenesis in anaerobic ciliates represents a significant share in a green-house gas accumulation (Holmes et al. 2014). The methane production by anaerobic ciliates varies significantly depending on the host environment, from minute contributions of freshwater anaerobic ciliates (e.g. *Caenomorpha* sp., *Metopus palaeformis*, *Plagiopyla* sp., *Cyclidium* sp.) to over 90 % of the total methane production in oceans in marine anaerobic ciliates (e.g. *Metopus contortus*) (Fenchel & Finlay, 2010). The endosymbionts of rumen ciliates are responsible for up to 40 % of the total methane amount produced in the rumen, however, they also seem to vary case to case in production from 9 % to 40 % of total methane amount (Finlay et al., 1993a; Newbold et

al., 1995). Each year, 86 million tonnes of enteric methane is estimated to be generated by methanogens of domestic ruminants, mainly cattle, while the methane production from manure has been estimated to over "just" 18 million tonnes, where a little over a half is still due to the manure production by pigs and small ruminants (McMichael et al., 2007; Hook et al., 2010). The amounts of methane produced each year are rising (Ferry, 1993; McMichael et al., 2007). Therefore, rumen methanogens and the increases in cattle farming may hold a major part in the important environmental processes. Majority of the methane produced in the hindgut of the cockroach species *Periplaneta americana* has been attributed to methanogens hosted by endobiotic armophorean *Nyctotherus ovalis* (Gijzen & Barugahare, 1992).

Host specificity of methanogens seems to correlate with the host environment, as the endosymbionts of the free-living ciliates are related to but clearly distinct from the free-living methanogens and vice versa, which would suggest acquisition of the endosymbionts in the early ciliate evolution and therefore, an exclusive vertical transmission, which is also supported by the recorded methanogens within ciliate cysts and random distribution during a cell division (van Hoek et al., 2000b). However, host switches have probably occurred several times as there is no significant correspondence between the ciliate and archaean phylogeny (van Hoek et al., 2000b; Hackstein, 2010). Conclusions on this matter are furthermore blurred due to certain confusion in determinations of the archaean taxa and their position within ciliates due to the methods used (van Hoek et al., 2000), as well as a new study opposing the previous results (Narayanan et al. 2009). The methanogens of endobiotic ciliates, both clevelandellids and rumen ciliates, are related to various endobiotic species within two clades of Methanobacteriaceae, while those hosted by the free-living armophorids seem to have an unrelated origin within Methanocorpusculaceae, Methanosaetaceae, and Methanobacteriaceae, and, possibly, Methanomicrobiaceae (van Hoek et al., 2000b; Narayanan et al., 2009).

Plagiopylid *Trimyema compressum* hosts *Methanobrevibacter arboriphilicus* (Shinzato et al., 2007) and *Trimyema* sp. hosts species related to *Methanocorpusculum parvum* (Finlay et al., 1993a). The prokaryote cells are there usually allied by hydrogenosomes and enclosed by a cell wall, forming levelled stocks, similarly as in endobiotic *Plagiopyla minuta* and *Lechriopyla mystax*, while in freshwater or brackish, free-living *Plagiopyla nasuta*, the endosymbionts referred to as *Methanobacterium formicum* are different, long shaped individual rods nigh the ciliate cell surface (Goosen et al., 1988), again differing from a case in marine *Plagiopyla frontata*, whose endosymbionts were related to the free-living species *Methanolobus tindarius* (Stetter et al., 1983; Embley et al., 1995). Also an anaerobic scuticociliate *Cyclidium porcatum* contains methanogenic endosymbionts within its cells close to the hydrogenosomes (Clarke et al., 1993). However, those have been assigned to a taxonomical rank besides identifying their archaean origin, which was

confirmed by autofluorescence. Among rumen ciliates, large numbers of methanogens that have been found mainly in entodiniomorphids (Stumm et al., 1982) are closely related to *Methanobrevibacter smithii*, a common human hindgut commensal (Irbis & Ushida, 2004), whereas trichostomatids had either lower amounts of other methanogenic Archaea, or only bacterial symbionts present (Lloyd et al., 1996).

The archaean methanogen in clevelandellid *Nyctotherus ovalis* has been identified as belonging into the genus *Methanobrevibacter* (Gijzen et al., 1991). There has been only a little attention paid to the hydrogenosomes of the free-living representatives of the class Armophorea, although it has been shown that hydrogenosomes of several armophorid species are associated with endosymbiotic methanogens as well (Finlay & Fenchel, 1989; Biagini et al., 1997a). Already in one of the early studies of armophorean ciliates of the genera *Metopus* and *Caenomorpha*, there have been endosymbiotic as well as ectosymbiotic prokaryotes found (Fenchel et al., 1977).

It is not well known what amount of methane might be produced by methanogenic symbionts of free-living armophorean ciliates worldwide. All symbionts in one ciliate cell can produce up to 7.4 pmol of methane per hour at maximum ciliate growth rates (Fenchel & Finlay, 1992). Apparently, the rate of methane production per ciliate is strongly influenced by the amount of methanogenic cells within. The numbers of methanogens per cell in armophorids strongly vary species to species. According to van Bruggen (1986), the marine species *Metopus contortus* can possess 4,500 methanogens per cell; however, Fenchel et al. (1977) have counted an over six times higher amount (28,000). Freshwater species *Metopus striatus* contains around 2,000 methanogens per cell (van Bruggen et al., 1983). In *Metopus es,* the number of methanogens is significantly lower and varies around 637 ± 12 per cell (Schwarz & Frenzel, 2005), and *Metopus palaeformis* hosts just 360 endosymbionts per cell (Fenchel & Finlay, 1992).

All those differ from symbionts inhabiting the rumen ciliates and are closely related to the free-living methanogenic Archaea (Van Bruggen et al., 1986; Embley et al., 1992a, b, 1994; Narayanan et al., 2009). The host specificity of methanogens in free-living anaerobic ciliates is still matter of debate as the data on this topic are scarce, yet there are metabolic differences known between various methanogenic endosymbionts of anaerobic ciliates, which may suggest an independent origin of such endosymbioses (Hackstein et al., 2008).

Although the first encounter of endosymbiotic Archaea in free-living armophorids has been described already in the 70s as mentioned earlier (Fenchel et al., 1977), it took more than half a decade until the first methanogens were isolated from them (Van Bruggen et al., 1984, 1986). Fluorescence microscopy can enable visualizing the methanogenic endosymbionts within the ciliate cells due to their possession of autofluorescent coenzymes deazaflavin F 420 or pterin F342 (van Bruggen et al., 1983), and thus confirm their presence (Doddema et al., 1978; Vogels et

al., 1980, Tokura et al., 1999), but to identify what species of methanogen is present in the cell, a whole cell *in situ* hybridization, specific fluorescent oligonucleotide probes with specific primers designed for Archaea are needed (Finlay & Fenchel, 1991; Embley et al., 1992a, 1994; Embley & Finlay, 1994; Narayanan et al., 2009).

From the high current number of the recognized metopid species, only four of them have been properly examined for the presence of endosymbionts. *Metopus striatus* presumably hosts a so-far undescribed archaean species (Embley et al., 1995), although ten years earlier *Methanobacterium formicicum* has been described in this ciliate species (Van Bruggen et al., 1984). However, the symbiosis has not been proven in this case and it is not clear whether the Archaean cells were not ingested as food (Embley et al., 1995). Another archaean species related to *Methanobacterium formicicum* is hosted by *Metopus palaeformis* (Embley et al., 1992a, 1994). A more recent analysis showed that a cultured strain of *Metopus es* host *Methanosaeta* sp. (Narayanan et al., 2009). Marine metopids host different Archaea from the order Methanomicrobiales, as described in case of a cultured strain of *Metopus contortus* possibly containing cells of *Methanoplanus endosymbiosus* (Van Bruggen et al., 1986) or *Methanocorpusculum parvum*-related species that has been encountered there just four years later in an another study (Finlay & Fenchel 1991; Embley et al., 1992a), and which is also closely related to *Trimyema* sp. endosymbiont (Embley & Finlay, 1994). Recently, a study on the genus *Atopospira* has shown that its two species, *Atopospira galeata* and *A. violacea*, may not harbour endosymbionts at all (Bourland et al., 2014b).

The relationship within the endosymbiont and its armophorid host is not fully revealed yet, but according to Finlay (1991), the methanogens might support the growth rate of its host, as the growth rates of armophorids experimentally void of endosymbionts have been reported as slower; the reason could be a supply of organic excretions produced by methanogens. On the other hand, ciliates are not fully dependent on the presence of its methanogenic endosymbionts. It has been discovered that ciliates can also support the methane production of free-living methanogens by producing organic acids as propionate and acetate (Biagini et al., 1998b).

The phenotype of methanogenic Archaea is often restricted, but, on the other hand, one species can vary in its shapes and sizes throughout the life cycle (Embley et al., 1992b; Finlay & Fenchel, 1991). Methanogens are of various shapes from elongate rods, up to 7 μm in length, to coccoid forms, about 0.5 μm in diameter. Studied endosymbionts of the freshwater species *Metopus palaeformis* were always rod-shaped and unattached to the hydrogenosomes despite their nigh presence. As cells of some methanogens might show the same morphology between species (Garcia et al., 1990), it was decided that the polymorphic methanogens represent stages of a single species in case of the marine metopid species *Metopus contortus* (Finlay & Fenchel, 1991), where

the methanogen cells are tightly attached to hydrogenosomes and enclosed just in a conspicuous ciliate vacuolar membrane, as their own cell wall was putatively lost (Finlay & Fenchel, 1991b; Embley & Finlay, 1994).

The endosymbionts of the free-living ciliates are related to but clearly distinct from the free-living methanogens and vice versa, which would suggest acquisition of the endosymbionts in the early ciliate evolution and, therefore, an exclusive vertical transmission, which is also supported by the recorded methanogens within ciliate (nyctotherid) cysts and random distribution during a cell division (van Hoek et al., 2000b). However, host switches have probably occurred several times as there is no significant correspondence between the ciliate and archaean phylogeny (van Hoek et al., 2000b; Hackstein, 2010). Conclusions on this matter are furthermore blurred due to certain confusion in determinations of the archaean taxa and their position within ciliates due to the methods used (van Hoek et al., 2000b; Narayanan et al., 2009).

Species that are known to host methanogenic endosymbionts had the endosymbionts in all examined cells of the uncultivated population. In non-armophoreans, long-term cultivation caused a loss of the endosymbionts in a case of *Trimyema* sp. (Shinzato & Kamagata, 2010). On the other hand, it was shown possible to reinfect a symbiont-free strain of *Trimyema compressum* with *Methanobacterium formicicum* (Wagener et al., 1990), although the newly established endosymbiosis has never regained the same methanogenic effectivity, and according to a phylogenetic analysis of the methanogens, it probably does not happen on a regular occurrence (Hackstein, 2010). It is expected that these symbioses have also occurred already during an early evolution of anaerobiosis in Armophorea, and the last common ancestor of free-living, and endobiotic representatives have already contained some methanogenic endosymbionts (van Hoek et al., 2000).

Van Bruggen et al. (1986) claimed that methanogenic archaean taxa are of an opportunistic character and that these endosymbioses happen continually, while the ciliates acquisite endosymbionts from various free-living methanogens, although phylogenetic analyses of SSU rDNA sequences have later revealed that the methanogenic endosymbionts in ciliates often differ from the free-living ones (Embley & Finlay, 1994).

For example, *Methanobacterium formicicum* and related species seem to be able to colonize various hosts throughout all eukaryotes. However, the case of *Methanobacterium formicicum* described in *M. striatus* may have been a mistake, but other closely related species inhabit various ciliates (Embley & Finlay, 1994). The other way around, methanogens from the genus *Metopus* and the unrelated prostomatean of the genus *Plagiopyla* are closer related to free-living methanogenic archaean species than to each other in some cases (Fenchel & Finlay, 2010). Nevertheless, *Metopus contortus* and the unrelated *Trimyema* sp. both hosted a relative of

Methanocorpusculum parvum) (Finlay et al., 1993a). It seems that methanogenic archaean taxa are generalists, capable of assessing symbiotic relationship with various hosts. Thus, endosymbiotic archaean species diversity presumably depends mainly on the habitat type and the time of sampling (Embley & Finlay, 1993a, 1994).

Nonetheless, methanogenic archaea are not exclusive symbionts of anaerobic ciliates. Various anaerobic ciliates (e.g. *M. contortus*, cariacotricheans, several anaerobic scuticociliates) also host ectobiotic bacteria, mostly recorded in marine or sulphate-rich environments (Fenchel et al., 1995, Orsi et al., 2012, Edgcomb & Pachiadaki, 2014). The ciliates harbouring methanogens usually possess smaller volume of ectosymbionts or are totally absent of them (Fenchel & Finlay, 2010). Within armophoreans, the ectosymbionts of *Metopus contortus* and *Caenomorpha levanderi* were described as unidentified sulphate reducers (Fenchel & Ramsing, 1992). Also *Caenomorpha capucina*, and *Metopus vestitus* were recorded to possess ectosymbionts, although these were never identified (Fenchel et al., 1977). Fenchel and Finlay (2010) have suggested it could be the case for other ectosymbionts of marine anaerobic ciliates too. Ectosymbionts of armophoreans have never been referred to as methanogens.

2.8. ENDOBIOTIC REPRESENTATIVES OF ARMOPHOREA

The class Armophorea also contains endobiotic representatives that have been all grouped into a separate order. The Order Clevelandellida contains five families: Clevelandellidae Kidder, 1938, Nyctotheridae Amaro, 1972, Sicuophoridae Amaro, 1972, Inferostomatidae Ky, 1971, and Neonyctotheridae Affa'a, 1987 (Lynn, 2008). So far, more than 100 species have been described within this order, most of them belonging into the family Nyctotheridae. The nyctotherid species that have been molecularly analysed do not show a strong support as a monophyletic clade in phylogenetic analysis; however, more data are needed for such conclusion (Lynn et al., 2013). More data allowing final conclusions are needed, as our knowledge of clevelandellid genetic diversity is still highly limited. Nevertheless, it has been very recently revealed that the family Clevelandellidae is unambiguously monophyletic, while Nyctotheridae is not (Lynn et al., 2013).

Clevelandellids are ciliates of medium size, ranging around 100 μm (de Puytorac & Grain, 1976). Unlike armophorids, the clevelandellids are densely ciliated, possessing closely allied somatic kineties. Their oral cavity forms an infundibulum with heteromembranelles present, which are formed by three rows of kinetosomes hexagonally packed with a fourth shorter row in the opposite direction. Their adoral polykinetids are multiplied several times contrary to the free-living armophoreans. The diplostichomonad paroral zone contains two divided sets of

kinetosomes. The macronucleus is usually elongated or irregularly shaped, sometimes attached by karyophore to the cortex (Lynn, 2008).

Clevelandellids are intestine endocommensals that inhabit a wide variety of invertebrate and vertebrate hosts. The invertebrate hosts include cockroaches (Hackstein & Stumm, 1994), annelids (Télesphore et al., 2014), millipedes (Albaret, 1970; Hackstein & Stumm, 1994), molluscs (Laval & Tuffrau, 1973), or sea urchins (Biggar & Wenrich, 1932). The vertebrate hosts are amphibians (Wichterman 1937; Albaret, 1975; Affa'a et al., 1995), fish (Grim, 1998; Grim et al., 2002; Jankowski, 1974), and reptiles (Bhatia & Gulati 1927; Lucas 1927; McKean 1972). Clevelandellids of amphibians and millipedes form a monophyletic clade, separated from clevelandellid endocommensals of cockroaches (Lynn et al., 2013). Besides the family Nyctotheridae, not much is known about the representatives of each family.

The family Sicuophoridae Amaro, 1972 contains 7 genera - *Geimania* Albaret, 1975, *Metasicuophora* Albaret, 1973, *Parasicuophora* Albaret, 1968, *Prosicuophora* de Puytorac & Oktem, 1967, *Spiroperistomatus* Amaro & Sena, 1967, *Sicuophora* de Puytorac & Grain, 1969, syn. *Wichtermania*, and relatively newly *Albaretia* Affa'a in Aescht, 2001. These cilitates inhabit amphibians and reptiles (Lynn, 2008). The family Inferostomatidae Ky, 1971 (syn. Nathellidae) comprises species of three genera, *Ichthyonyctus* Jankowski, 1974, *Inferostoma* Ky, 1971, and *Nathella* Singh, 1953 (*nomen nudum* – Aescht, 2001; Lynn, 2008) inhabiting only freshwater fish. *Neonyctotherus* Affa'a, 1983 has been described as a single genus of the family Neonyctotheridae Affa'a, 1983, an endocommensal of amphibians.

The family Nyctotheridae, represented mainly by species of the genus *Nyctotherus* Leidy, 1849, can be found in insects, mainly in the hindgut of cockroaches *Periplaneta americana* and *Blaberus* sp. (*Nyctotherus ovalis* Leidy, 1849, *N. periplanetae* Lalpotu, 1980, *N. spirostreptae* Lalpotu, 1976), myriapods and millipeds (*N. velox* Leidy, 1849, *N. cordiformis* Stein, 1862, *N. spirostreptae*), and oligochaetes, frogs (*N. cordiformis*, *N. oektemae* Senler & Yildiz, 2000), lizards (*N. beltrani* Hegner, 1940, *N. woodi* Amrein, 1952) and snakes (*N. uscae* Galaviz-Silva & Jimenéz-Guzmán, 1986) (Hegner, 1940; Amrein, 1952; Galavíz-Silva & Jiménez-Guzmán, 1986; van Hoek et al., 2000b; Şenler & Yildiz, 2000; Bhandari et al., 2012; Bhamare, 2014). *Nyctotherus* morphologically differs from *Nyctotheroides* Grassé, 1928 only by the presence of a left-caudal suture (Affa'a et al., 2004). Species in the genus *Nyctotheroides* (*N. parvus* Walker, 1909, *N. cordiformis* Ehrenberg, 1838, and *N. deslierresae* Affa'a 1991, *N. cacopusi* Uttangi, 1951, *N. spirotomatus* Amaro & Sena, 1968, *N. tejerai* Pinto, 1926) inhabit frogs, adults as well as toads (Hackstein et al., 2002; Affa'a et al., 2004).

Van Hoek et al. (2000b) have revealed that species from intestinal tracts of frogs and millipedes, such as *Nyctotherus ovalis* and its closely related *Nyctotherus cordiformis*, form a

monophyletic clade within Nyctotheridae. On the other hand, position of *Nyctotherus velox* within the genus *Nyctotherus* was expressed uncertain (Lynn et al., 2013), although that would actually mean that the entire taxonomy of all other species of *Nyctotherus* is uncertain, as *Nyctotherus velox* is the type species for the genus. Interestingly, even ciliates from the same species of a cockroach, isolated from a different site, can significantly differ in their DNA sequences (van Hoek et al., 2000b). *Clevelandella* Kidder, 1938 occurs in wood roaches and termites (van Hoek et al., 2000b). Genera inhabiting fish have been also described (Grim, 1992). Genera *Spirocytopharynxa* Li et al., 2002 and *Macrocytopharynxa* Li et al., 2002 have been described from anurans in China (Li et al., 2002), however, Lynn (2008) does not mention them.

Clevelandellids do not affect their amphibian hosts. However, those inhabiting cockroaches may increase the host growth rate and body weight (Gijzen & Barugahare, 1992). Abundances of clevelandellids are also dependent on the host, for example the hindgut of the *Periplaneta americana* can host from ca 3200 to almost 4000 cells of *Nyctotherus ovalis*, while frogs were found to host just hundreds of *Nyctotheroides* (Gijzen & Barugahare, 1992). It seems that the key step in the transmission between hosts of the clevelandellids is encystment; although the possibility of motile ciliate infections has been mentioned as well, as the feces of adult frogs show an abundance of ciliates (Sadek, 1979; Lynn, 2008). Conjugation processes may be initiated among other unknown factors by host-produced gonadotropins (Wichterman, 1934).

According to studies by Affa'a et al., (1988, 199) the infaunation can happen in three ways. Species as *Nyctotheroides brachystomus*, *Neonyctotherus reticulatus*, and *Parasicuophora aberrans* can be found only in the juvenile or tadpole stages; species *Nyctotheroides heterostomus* and *Prosicuophora basoglui* infaunate just in the adults. Species *Nyctotheroides teochii* can infect both tadpoles and adults. The controlling factors in acquiring and losing the ciliates from the tadpole to adults are unknown, although gonadotropins may induce encystment in *Nyctotheroides* and *Prosicuophora* (Affa'a, 1988).

Very recently, four novel species of *Nyctotherus* have been described, although no sequence data has been analysed and they were considered as members of the class Heterotrichea. All four of the proposed species (*N. orthostomatus*, *N. ndoumeleleensis*, *N. ngassami*, and *N. atunibaensis*) were isolated from the mid- and hindgut of an annelid earthworm of the genus *Eupolytoreutus* from the northwest region of Cameroon (Télesphore et al., 2014), and characterized as novel species according to their ciliary pattern. The authors have also noted that there were other unspecified nyctotherids present. Moreover, a species of *Nyctotheroides bambuiensis* has been described from an anuran of the species *Bufo regularis*, also from the northwest of Cameroon, together with a description of a novel species of of Sicuophoridae, *Prosicuophora cyclostomatus*, isolated from the same frog (Télesphore et al., 2013).

One would say that, when compared to other ciliates, the representatives of the class Armophorea are neglected by researchers. However, some species of the genus *Nyctotherus* have obviously gained a merited morphological attention even multiple times, as for example *Nyctotherus spirostreptae* Lalpotu, 1976, which has been morphologically re-described even twice after its original description (Bhamare, 2014). Apart from that there are absolutely no sequence data for this species. At the end, one can rather say that it is the molecular data of armophoreans that need more attention.

3. MATERIALS AND METHODS

3.1. ISOLATION AND CULTIVATION OF ANAEROBIC CILIATES

3.1.1. Methods of sampling and cultivation

Samples containing free-living representatives of the class Armophorea and other anaerobic ciliates were collected from anoxic and microoxic, freshwater, brackish and marine sediments. Freshwater strains were isolated in Sonneborn's *Paramecium* medium (ATCC medium 802); marine strains were isolated in seawater 802 medium (ATCC medium 1525), brackish strains were isolated in the mixture of both media in different ratios (1:1 or 4:1). Approximately 2 ml of the samples were initially inoculated into the medium and followingly 1 ml of the culture was inoculated into a new tube with fresh media every second week. Cultivation of the isolates was carried out at room temperature in polyxenic agnotobiotic conditions (in the presence of unidentified bacteria and other protists).

Most cultures were not monoeukaryotic and contained various other protists besides the ciliates. Monoeukaryotic cultures were established by limiting dilution and by filtering.

3.1.2. Composition and preparation of used culture media

Sonneborn's *Paramecium* medium (ATCC medium 802)

Table 1. Sonneborn's Paramecium *medium (ATCC # 802)*

Cereal Grass Media (Scholar Chemistry)	1.25 g
Na_2HPO_4	0.25 g
distilled water	add volume to 1000 ml

2.5 g of Cereal Grass Media (Scholar Chemistry, USA) was added to 1000 ml of distilled water. The suspension was brought to boiling, allowed to boil for five minutes, and cooled down. Subsequently, the suspension was filtered through filter paper and distilled water was added to reach 1000 ml. As a last step, 0.5 g of Na_2HPO_4 was added to 1000 ml of this suspension. After preparation, the medium was sterilized by autoclaving (20 minutes at 121 ° C) and stored at 4 °C.

Seawater ATCC medium 1525

Table 2. Twice concentrated artificial sea water (ASW)

NaCl (used in Solution A)	24.71 g
KCl (used in Solution A)	0.68 g
$CaCl_2 \cdot 2H_2O$ (used in Solution A)	1.36 g
$MgCl_2 \cdot 6H_2O$ (Solution B)	4.66 g
$MgSO_4 \cdot 7H_2O$ (Solution B)	6.3 g
$NaHCO_3$ (Solution C)	0.18 g
distilled water	add volume to 1000 ml

Firstly, the medium ASW (artificial sea water) was mixed from three separate solutions (A, B, C) which were separately autoclaved (20 minutes at 121 ° C) to prevent the precipitation of the salts. Solutions were prepared as described in Table 2. Then, twice concentrated ATCC medium # 802 (see Table 1.) was prepared. Subsequently, twice concentrated ASW (see Table 2.) was prepared and filtered through a sterilizing filter into the cold twice concentrated ATCC # 802 medium. After preparation, the medium was sterilized by autoclaving (20 minutes at 121 ° C) and stored at 4 °C.

Preparation of artificial anaerobic environment

An artificial anaerobic environment was obtained using AnaeroGen (Oxoid) sachets, which were inserted into AnaeroJar 2.5 l (Oxoid Limited, Great Britain) together with selected cultures and closed.

3.2. LIGHT MICROSCOPY

3.2.1. Native preparations, observation of living and fixed cells

Living cells of the cultured strains were observed under BX51 light microscope (Olympus Corporation, Tokyo, Japan) using differential interference contrast. The microscope is equipped with camera Olympus DP70 (Olympus Corporation, Tokyo, Japan). Living cells were documented either during their natural movement, alternatively some cells were put in methylcellulose solution to minimize their movement. Cells of some freshwater or brackish strains were documented also fixed in 10% aqueous formalin.

3.2.2. Protargol staining

The morphology of isolated organisms was also examined in protargol-stained preparations. Two different protocols were used.

Staining protargol protocol according to Bodian, modified by Nie (1950) and Ptáčková (2010)

The staining must be performed under hydrated conditions, as the cells must not be allowed to dry during the whole process to avoid deformation.

1. Preparation of Bouin - Hollande's fluid:

25 g of copper acetate was dissolved in distilled water. 40 g of picric acid was then added to the mixture. Subsequently, 100 ml of 40% formaldehyde was added to the solution and finally 1.5 ml of glacial acetic acid was carefully added to the final solution.

2. Fixation:

1.5 ml of a sample from a well-grown culture was pipetted from the bottom and centrifuged in a centrifuge for 8 minutes at 500 g. The supernatant was discarded and the pellet was in resuspended with the rest of the supernatant. Meanwhile, an egg white (albumen) was prepared in sterile conditions and mixed with cultivation media (1:2 or 1:3 ratio depending on the sample cells size). Approximately 1 µl drop of the culture sample and 0.75 µl of the egg albumen were pipetted to a 15 x 15 mm clean cover slip next to each other, quickly stirred together and gently spread on the whole size of the slide, and immediately placed onto a surface of the prepared fixative facing the smeared side down. After a while, the slides were turned, with smeared side

facing the bottom for ca 5 – 14 hours to fix. For easier handling, the slides were mounted on ca 1cm polyethylene rods on which were formed notches of about 3 mm, which were gently inserted onto slides. Preparations were passed through a 50% ethanol to 70% ethanol to wash fixative. 70% ethanol was changed several times.

3. Staining:
Fixed samples preserved in 70% ethanol were passed through a 50% ethanol into distilled water. Slides were collected in a jar with 0.5% potassium permanganate solution for 5 minutes and then rinsed 5 times for 30 seconds in distilled water. Preparations were then left for 5 minutes in a 5% solution of oxalic acid and followingly 5 times rinsed for 30 seconds in distilled water.

4. Protargol preparation:
First, refined curled copper wires were placed at the bottom of a small beaker with 25 ml of distilled water. Then, 0.25 g of protargol powder (Bayer, IG Farbenindustrie Actinengesellschaft) was dispersed on the surface of the distilled water and was left to be dissolved spontaneously. Slides were gently folded in a vertical position into a beaker with the solution of 1% protargol. Thin copper wires were inserted among the stacked slides and sealed beaker was placed for 48 hours in 37 °C.

5. Preparations were rinsed twice for 5 seconds in distilled water.

6. Slides were placed in a freshly prepared reducing solution (solution 1% hydroquinone and 5% Na_2SO_3) for 10 minutes.

7. Slides were rinsed 5 times for 30 seconds in distilled water and subsequently immersed for 5 minutes in 0.5 - 1% solution of $AuCl_3$ and rinsed for 5 seconds in distilled water. Then, they were immersed for 5 minutes in 2% oxalic acid solution.

8. Slides were rinsed 5 times for 30 seconds in distilled water and subsequently immersed for 10 minutes in a 5% solution of $Na_2S_2O_3$ and consequently washed for 20 minutes under running tap water. After that, slides were transferred through series of alcohol (50 %, 70 %, 80 %, 96 %, 100% ethanol) into xylene for two times.

9. Finally, the slides were mounted in DPX Mountant for histology (Sigma).

Staining protargol protocol following Foissner (2014), modified by William Bourland (2014a, personal communication)

Reagents:

c) formalin solution - 5 ml formalin (HCHO; commercial concentration, about 37%)

d) albumin-glycerol - 15 ml egg albumin, 15 ml concentrated (98 – 100%) glycerol ($C_3H_8O_3$)

e) 0.2% potassium permanganate solution

f) 2.5 % oxalic acid solution

g) 0.4 – 0.8% protargol solution

h) ordinary developer (sodium sulfite must be dissolved before hydroquinone is added) - 95 ml distilled water, 5 g sodium sulfite (Na_2SO_3), 1 g hydroquinone ($C_6H_6O_2$)

1. Organisms were fixed in formalin (~4%) for 30 min. The ratio of fixative to sample fluid was 1:1. Approximately 1 ml of ciliate culture was centrifuged at 500 g for 8 minutes, the supernatant was removed and subsequently, the fixative was poured in using a pipette and the whole suspension vortexed immediately to bring the organisms in contact with the fixative as fast as possible.

2. Specimens were concentrated by centrifugation and washed 3 times in tap water.

3. A small drop each of albumin-glycerol and concentrated organisms were placed in the centre of a clean slide. Drops were mixed with amounted needle and spread over the middle-third of slide. Glycerol prevents cell distortion with drying. Fixed, washed ciliates were collected in 1 ml of water in a micro tube and 2 – 3 drops of very dilute egg albumin were added and swirled until mixed well. Using a micropipette with a lumen large enough to avoid damaging the ciliates, suck them up under the stereomicroscope and place them in a drop in the centre of the slides. Cells were allowed to dry for 2 hours or overnight. Thanks to glycerol, cells will not be damaged by drying.

4. Slides were placed in a jar filled with 95% ethanol for 30 min. A jar with protargol solution was placed into a heating bath (60 °C).

5. Slides were rehydrated through 70% alcohol and two tap water steps for 5 min each.

6. Slides were placed in 0.2% potassium permanganate solution for 120 s, rinsed in tap water and placed in 2.5% oxalic acid for 180 s, then washed in tap water two times for 3 min and once in distilled water for 3 min.

7. Slides were placed in the warm (60 °C) protargol solution and impregnated for 10 – 15 min or for 48 hours. One slide at a time was removed from the jar with the warm (60 °C) protargol solution and dipped into distilled water for 1 – 2 s and then transferred into an acetone developer. As soon as the albumin turned yellowish, the slide was removed, dipped into two tap water steps for about 2 s each, and the impregnation was controlled with the compound microscope. If the cells appeared impregnated, the slide was submerged into the fixative (sodium thiosulfate) for 5 min.

8. Slides were fixed in sodium thiosulfate for at 5 min and washed in tap water three times for about 3 min each.

9. Slides were transferred to 70% – 100% – 100% ethanol for 3–5 min each and subsequently cleared by two 10 min transfers through xylene and mounted in synthetic neutral medium.

3.2.4. Fluorescence microscopy

Endosymbiotic methanogenic archaea, present in ciliate cells, can be identified by autofluorescence based on the presence of enzymes within their cells (Doddema et al., 1978), particularly fluorescing coenzymes F350 and F420. For autofluorescence observation, Olympus BX51 with WU filter (excitation 330 – 385 nm, bs 400 nm, emission 420 nm LP) was used, alternatively CFP/SEMROCK filter.

3.3. TRANSMISSION ELECTRON MICROSCOPY

3.3.1. Preparation of samples

The samples for electron microscopy were fixed with glutaraldehyde in cacodylate buffer and osmium tetroxide or by cryosubstitution. Cells of chosen strains were pelleted by centrifugation at 500 g for 8 minutes, resuspended in a solution containing 2.5% glutaraldehyde (Polysciences) and 5 mM CaCl2 in 0.1 M cacodylate buffer (pH 7.2). After washing in 0.1 M cacodylate buffer (three times per 15 minutes), the cells were post-fixed with 2% OsO4 in 0.1 M cacodylate buffer

for 3 hours. After washing with an excess volume of 0.1 M cacodylate buffer (three times per 15 minutes) the fixed cells were dehydrated in acetone series and embedded in EPON (Poly/Bed 812, Polysciences) or SPURR resin (Sigma Aaldrich).

For cryosubstitution, the cell suspension with addition of 20% BSA was frozen using the high-pressure freezer (Leica EM Pact II) and then transferred to the freeze substitution unit (Leica EM AFS). The ice in the specimen was replaced by anhydrous acetone containing 2% osmium tetroxide. The samples were embedded in EMbed-812 (EMS) and polymerized at 62 ˚C for 48 h.

The ultrathin sections were stained with uranyl acetate (2%) and lead citrate in both methods. The concentration of chemicals was optimized for individual isolates. The samples were further processed by standard methodology at the EM service laboratory at the Faculty of Science, Charles University in Prague, which includes enhancing the contrast with uranyl acetate, dehydration, embedding in EPON-Araldite, being cut on an ultramicrotome, mounted on the mesh and coloured with heavy metal compounds.

3.3.2. Observation, photo documentation

Observations of the ultrastructure were performed in the Laboratory of Electron Microscopy (Faculty of Science, Charles University in Prague). All samples were examined using a TEM JEOL 1011 transmission electron microscope.

3.4. MOLECULAR APPROACHES

DNA was isolated from the cultures, subsequently amplified by specific PCR primers, amplified DNA fragments were then purified, and selected strains were cloned into plasmids, from which several plasmids from a single PCR were sequenced.

3.4.1. DNA isolation

1.5 ml of sample was centrifuged for 8 minutes at 500 g. 1400 µl of the supernatant was removed, and the remaining 100 µl was resuspended and used for DNA isolation. Genomic DNA was isolated from the cultures using the DNA isolation kit (Genomic DNA Minikit, Geneaid; DNeasy Blood & Tissue Kit, Qiagen; or ZR Genomic DNA Kit II TM, Zymo Research) according to the manufacturer's instructions. The DNA was stored at -20 ° C.

3.4.2. DNA amplification, electrophoresis and PCR products purification

DNA amplification

Table 3. PCR primers used for SSU rDNA gene amplification

Medlin A	5’ AYCTGGTTGAYYTGCCAG 3’
Medlin B	5’ TGATCCATCTGCAGGTTCACCT 3’
ArmF1	5’ GCGAYATRTCATTCAAGT 3’
ArmR4	5’ GWGGTTWTCCACACAGTC 3’

Table 4. Composition of the reaction mixtures using polymerase LA, cycle temperatures in thermocycler for LA polymerase.

(10x conc., + MgCl2)	5 µl
dNTP (10 mM each)	1.5 µl
DMSO	1 µl
Medlin A	12.5 pmol
Medlin B	12.5 pmol
LA polymerase	0.5 µl
DNA	5 ng
PCR H_2O	added to 50 µl

Table 5. Setting the temperature cycle when using polymerase LA the number of cycles the temperature of the cycle time

1x 94 °C for 1 min initial denaturation
94 °C 20 sec denaturation
31x 55 °C 30 sec annealing
68 °C 2 min 30 sec polymerization
1x 68 °C for 10 min final polymerization

Almost complete sequence of 18S rDNA was amplified by specific PCR primers that we have designed for the class Armophorea, and we have confirmed that they cover the whole known range of armophorid ciliates. The specific primers ArmF1 and ArmR4 (Tab. 3) had an annealing temperature of 60 °C. Alternatively, the 18S rDNA of non-armophorid ciliate strains was amplified using eukaryotic primers MedlinA and MedlinB (Tab. 3; Medlin et al., 1988) with an

annealing temperature of 50 ◦C. DNA amplification was performed using LA polymerase (5 U / ml; top-Bio).

Electrophoresis of amplified DNA

Electrophoresis was performed on a 1% agarose horizontal gel. Ethidium bromide (final concentration 0.5 mg / ml) was added to visualize DNA fragments. Followingly, the electrophoresis gel was photographed under a transilluminator with a digital camera and using Alpha DigiDoc RT (JH BIO Innovations Pvt. Ltd.) and the photograph was transferred to a computer and processed.

Purification of PCR products

Amplified DNA fragments were purified using the PCR products purification kit (Gel/PCR DNA fragments extraction kit, Geneaid; ZymocleanTM GEL DNA Recovery Kit, Zymo Research; or QIAquick PCR Purification Kit, Qiagen). DNA was eluted in 25 µl of EB buffer and stored at -20 °C. The final DNA concentration in the sample was measured on a NanoDrop (ND-1000, NanoDrop Technologies, Inc.). Subsequently, the purified DNA was either directly sequenced or selected strains were cloned.

3.4.3. PCR products cloning

Composition and preparation of used media in PCR products cloning

LB Broth medium

Table 6. Composition of LB Broth medium

LB Broth (Sigma)	5 g
distilled water	add to volume 500 ml

LB Broth powder was dissolved in distilled water and then autoclaved (20 minutes at 121 ° C). Medium was later used in cloning procedures as a selective media for bacteria.

Solid LB Broth medium

Table 7. Composition of LB Broth agar Petri dishes

LB Broth (Sigma)	10 g

Bacteriological agar (Oxoid Limited, UK)	6 g
distilled water	add volume to 500 ml

LB broth powder was dissolved in distilled water and bacteriological agar was added to the solution. The medium was autoclaved (20 minutes at 121 ° C) and dispensed into Petri dishes. These were then used for cloning.

Cloning

Tab. 8. Composition of ligation mixture

2x ligation buffer	5 µl
pGEM T-easy	1 µl
ligase	1 µl
DNA	ca 50 ng

In cases where there were several related ciliate strains present in the culture, it was necessary to clone selected strains to obtain the desired sequence. Cloning has been done using cloning kit (pGEM-T Easy Vector System I, Promega) and JM109 competent cells of *Escherichia coli* (High Efficiency Competent Cells, Promega). The sample was briefly lurched after mixing and left overnight in a refrigerator at 4 °C. Several plasmids from a single PCR were sequenced.

Preparation of bacterial colonies

Table 9. Composition of mixture for bacterial colony on Petri dishes

IPTG	(23.83 mg / ml) 100 µl
ampicillin	(100 mg / ml) 25 µl
X - gal	(50 mg / ml) 20 µl

10 µl of the ligation mixture was cautiously added to the melted competent cells and left for 20 minutes on ice.

The cells, kept in a 1.5 ml tube, were shocked at 42 ° C for 45 seconds. After that, the tube was placed back on ice for 2 minutes.

After 2 minutes, 800 µl of LB medium was cautiously added and the cells were left on a shaker (37 °C, 220 rpm) for 1.5 hours to incubate.

Meanwhile, a mixture of IPTG, ampicillin and X-gal was prepared and it was applied onto Petri dishes with solid LB Broth medium and agar. After removed from a shaker, 100 µl of the cells was evenly spread onto a Petri dish.

The remaining amount of cells was centrifuged at 10,000 g for 5 minutes, the supernatant was removed so the remnant volume would equal 100 – 200 µl, which were evenly spread onto another Petri dish. The plates were placed overnight in a thermostat at 37 °C.

Colony PCR

Table 10. Setting the degradation temperature cycle

1x	96 ° C	5 min
1x	50 ° C	1 min 50 sec
1x	96 ° C	1 min 50 sec
1x	45 ° C	1 min
1x	96 ° C	1 min
1x	40 ° C	1 min

Table 11. Primers used

Primer SP6	5’ GATTTAGGTGACACTATAG 3’
Primer T7	5’ TAATACGACTCACTATAGGG 3’

Table 12. Composition of reaction mixture (using Combi PPP Master Mix, Top-Bio)

Combi PPP Master Mix	10 µl
primer SP6	10 pmoles
T7 primer	10 pmoles
bacterial lysate	9 µl

Table 13. Setting the temperature cycle when using PPP Combi Master Mix (Top-Bio)

number of cycles	temperature	cycle time
1x	94 ° C	4 min initial denaturation
	94 ° C	denaturation for 30 sec
31x	50 ° C	30 sec annealing
	72 ° C	2 min 30 sec polymerization
1x	72 ° C	10min final polymerization

Using a sterile pipette tip, ca 10 white colonies from each isolate were picked. Colonies were resuspended in 9 µl of sterile water which had previously been pipetted into micro tubes. The

samples were put in a thermocycler that was set on degradation cycle. DNA dissolved in water was used for PCR reaction. During PCR amplification, T7 and SP6 primers (complementary to the vector) were used.

The presence of amplified fragment was verified on a horizontal electrophoresis. If a positive result appeared, the sample was purified using a kit. The DNA concentration was measured on the Nanodrop and sent to sequencing. If the concentrations of DNA were too low, bacterial colonies were grown and plasmids were isolated the next day.

Isolation of plasmids

Selected colonies were transferred to a tube 4 ml of LB medium using an automatic pipette tip, with 4 µl ampicillin added and left growing overnight in a shaker (37 °C / 220 rpm). Isolation of plasmids was performed using kit Wizard Plus SV Minipreps DNA Purification System (Promega). The amount of DNA was measured on a Nanodrop and was sent to sequencing. Remaining plasmid DNA was stored at -20 °C.

3.4.4. DNA sequencing

Table 14. Sequencing mix composition

DNA 100 ng / 1 kbp
3.2 pmol of primer
H2O added to 8 µl

Table 15. Primers used

Medlin A	5’ AYCTGGTTGAYYTGCCAG 3’
Medlin B	5’ TGATCCATCTGCAGGTTCACCT 3’
SP6	5’ GATTTAGGTGACACTATAG 3’
T7	5’ TAATACGACTCACTATAGGG 3’
577F	5’ GCCAGCMGCCGCGGT 3’
577R	5’ ACCGCGGCKGCTGGC 3’

Sanger DNA sequencing took place in the Laboratory of DNA sequencing at Charles University, which is equipped with an ABI PRISM 3100 sequencer (Applied Biosystems). Samples for the sequencing reaction were prepared in microtubes by mixing template DNA, primer, and water.

3.5. SEQUENCE ANALYSIS

3.5.1. Sequence editing

Firstly, after we have determined the SSU rDNA sequences of each armophorid strain, we have used BLAST (Basic Local Alignment Search Tool) set on "blastn" using GenBank as a source database, to have a rough idea about the identity of the/each particular sequence. DNA fragments sequences were assembled into a complete contiguous sequence (Seqman, Lasergene), from which the primer sequences were removed. After all the sequences were obtained, we have created several datasets using also the available related (and unrelated sequences to serve as outgroup) SSU rDNA sequences from GenBank, which were subsequently aligned. The sequences were aligned using the MAFFT method (Katoh et al., 2002) with the help of the MAFFT 6 server http://align.bmr.kyushuu.ac.jp/mafft/online/server/ with G-INS-i algorithm at default settings. Alignments were manually edited using BioEdit 7.0.9.0 (Hall, 1999). The final dataset of unambiguously aligned characters consisted of 1481 positions. Afterwards, we have added "n" letter in the beginning/ending of each sequence that lacked a proper beginning/ending to avoid blank spaces, manually trimmed the alignment (BioEdit) and removed introns and highly variable regions of the sequences (especially for the pan-ciliate analysis).

We have prepared two datasets in total:

1. Armophorean analysis dataset, including all SSU rDNA sequences of our strains, GenBank sequences of known armophorean species as well as environmental armophorean sequences, together with sequences of representatives of other main ciliate lineages as outgroup. This dataset had 151 sequences in total.

2. Pan-ciliate analysis dataset, including all SSU rDNA sequences of our MURANO strains, representatives of all main ciliate lineages, with a more detailed sampling in the lineages Litostomatea, Spirotrichea, Armophorea, and Cariacotrichea, together with related environmental SSU rDNA sequences from GenBank, and particularly including environmental sequences AY179982 and AB505525. This dataset had 169 sequences in total.

From the two datasets, we have performed three phylogenetic analyses. The first one used the armophorean dataset; the second one used the pan-ciliate dataset. The third analysis was performed subsequently due to two environmental ciliate sequences AY179982 and AB505525 which alternate positions of other ciliates, which we have excluded from the aligned and cut dataset in the third analysis.

3.5.2. Phylogenetic analysis of SSU rDNA

SSU rDNA phylogenetic trees were constructed by maximum likelihood (ML) and Bayesian methods. They were performed in programs RAxML 7.0.3 and MrBayes 3.2.2 (Huelsenbeck & Ronquist, 2001; Stamatakis, 2006). Node support was assessed by ML analysis of 1000.

Bayesian analysis was performed using the GTR + I + Γ + covarion model with four discrete categories. Four MCMCs were run for 3 million generations in the analysis of Armophorea and 1 million generations for both pan-ciliate analyses, with sampling frequency each 500 generations in all (until average standard deviation of split frequencies was lower than 0.01). First 25% of trees were removed as burn-in.

4. RESULTS

4.1. MAPPING THE DIVERSITY OF ARMOPHOREA

4.1.1. Sampling and cultivation

We have encountered armophorid representatives in over a hundred samples of hypoxic/anoxic sediments from hundreds of collected samples and established cultures from over 90 of them (see Table 16.). The samples were collected mostly by our colleagues from the Faculty of Science, Charles University in Prague, during their expeditions and come from ecologically and geographically very diverse sites. The sampling was performed in a worldwide range; we obtained samples from all continents besides Antarctica. The samples were usually collected from freshwater or marine anoxic sediments with various depths, frequently in shallow standing waters, but sometimes also streams, rivers or the ocean shore (Fig. 5). If we ignore the fact that anoxic environment itself is a type of extreme environment, we can say that some samples come from environments with very extreme conditions, e.g. SAMOSIR comes from a volcanic hot spring with volcanically heated water continuously erupting from the ground with temperatures estimated over 50 °C, SIBOLANGIT was collected in a river sediment with hot volcanic spring in the direct vicinity of an active volcano, with temperatures estimated over 70 °C in the sampling site (however, the water temperature may change seasonally and dependently on the volcano activity). Several samples (e.g. API and STRPJEZ) come from water bodies in high altitudes, often over 4000 mamsl. Strain COLORADA comes from a thermal lagoon in 4293 mamsl with conspicuously red-coloured water (probably due to the presence of algae, as there were also flamingos inhabiting the lagoon), where other water bodies were all frozen (personal communication with the sample collector). Some samples were collected in urban polluted areas (e.g. KU10, personal communication with the sample collector). Interestingly, samples resulting into cultures with strains of *Metopus* sp. 2 come from the southern hemisphere (share a Gondwanian distribution), besides one sample from an aquarium with South-American tortoise species.

Table 16. List of strains included in the study.

Legend: B – brackish, F – freshwater, M – marine, h. – habitat, O. L. – original location, grey font – exterminated strain

STRAIN	h.	LOCALITY	COORDINATES	O. L.
2ELI	F	n.a.	n.a.	F sediment
3ELI	F	n.a.	n.a.	F sediment
ADANIA	F	Adana, Turkey	39°59'10"N 35°20'00"E	river sediment
ALOOA1	F	Alooa, Nias, Indonesia	22°35'07"N 63°39'51"E	rainforest marsh soil
ALOOA3	F	Alooa, Nias, Indonesia	22°35'07"N 63°39'51"E	rainforest marsh soil
API	F	Apivanak yayla, Turkey	40°50'02"N 41°06'20"E	mountain strain, 2930 m.a.s.l.
ARAGON	F	Pozuel del Campo, Aragón, Spain	40°46'29"N001°31'16"W	F sediment
BEAVERC	F	Beaver lake, Vancouver, Canada	49°18'N 123°08'W	F sediment
BEAVER5	F	Beaver lake, Vancouver, Canada	49°18'N 123°08'W	F sediment
BHARA	F	Keoloadeo NP, India	27°02'29"N 77°49'43"E	F sediment
BOTANKA4AN	F	Botanical Garden, Prague, Czech Republic	50°04'20"N 14°24'24"E	pond
BOTANKA2	F	Botanical Garden, Prague, Czech Republic	50°04'20"N 14°24'24"E	pond
BUSSPRAND	M	Busselton, Australia	33°38'S 115°11'E	sediment at the sea opening
BUSSELTONP	M	Busselton, Australia	33°38'52"S 115°11'33"E	sediment at the sea opening
CANC	F	Canada	n.a.	n.a.
CASTILLO	F	Cerro Castillo, Ands, Chile	45°59'S 72°4'W	F sediment
CIDLINA	F	Vysoké Veselí, Czech Republic	50˚20'11"N 15˚26'09"E	stream sediment
CHACOL2	F	Chaco, Argentina	24°23'S 53°51'W	F sediment
CHITWANM.	F	Chitwan, Nepal	27°30'24"N 84°39'53"E	forest rhino pond
CHITWANV.	F	Chitwan, Nepal	27°30'24"N 84°39'53"E	forest rhino pond
CHITWANB	F	Chitwan, Nepal	27°30'24"N 84°39'53"E	forest rhino pond
COLORADA	F	Laguna Colorada, Bolivia	22°14'13"S 67°45'26"W	red coloured thermal lagoon, 4293 m.a.s.l.
COLOMBIA	F	Colombia	n.a.	F sediment
CSS	F	Jetřichovice, Czech Republic	50°51'N 14°22'E	pond
DRINGARI2	F	Dringari, Cameroon	n.a.	F sediment
EVROS3	F	Evros river, Greece	40°48'N 26°00'E	F sediment
EVROS4B	F	Evros river, Greece	40°48'N 26°00'E	F sediment
FRIOJESKYNE	**F**	Ribeiro Frio, Madeira	32°44'N 16°53'E	F sediment
GDUKABASM	F	Gdukabas, Bhangan, India	n.a.	F sediment
GDUKABASB	F	Gdukabas, Bhangan, India	n.a.	F sediment
GUAT	F	Guatemala	14°41'43"N 91°16'W	vulcanic lake sediment
HRAD2	F	Hradiště, Czech Republic	50°27'N 13°20'E	F sediment
IND5	F	Lake Telaga Warna, India	27°05'N 76°17'E	lake sediment

IZR	F	Galilei, Israel	32° 36'N 35° 33'E	small lake
IZRBR	F	Galilei, Israel	32° 36'N 35° 33'E	small lake
KAMERUN2B	F	Cameroon	n.a.	F sediment
KAN2	F	Canada	n.a.	F sediment
KALALAO	F	Kalalau, Hawai	n.a.	F sediment
KAO	F	n.a.	n.a.	n.a.
KU10	F	Prague, Czech Republic	50˚3′2″N 14˚18′57'E	F sediment
KUBAMA 1,2,3	M	Cuba	23°11'42"N81°09' 33"W	mangrooves
KORRISSION	B	Korission, Corfu, Greece	39°27'10"N 19°52'35"E	lake by the sea
TADDOUSAC	F	Taddousac, Quebec, Canada	48°09'11"N69°41'52"W	lake
LAGUNDRI	M	Lagundri, Nias, Indonesia	0°34'45"N 97°44'03"E	dark muddy beach sediment in lagoon
LISTOPADBS	F	French Guyana	3°51'12"N 53°25'54"W	F sediment
LISTOPADBC	F	French Guyana	3°51'12"N 53°25'54"W	F sediment
LISTOPADM	F	French Guyana	3°51'12"N 53°25'54"W	F sediment
LUKIFOREST	F	Luki Forest Reserve, DR Congo	n.a.	forest strain
LUOBIAO	F	Luo Biao, Sichuan, China	28°05'43"N104°58'41"E	strain in polluted area
MAREK4C	F	Kozlovice,Czech Republic	49°36'09"N 8°14'47"E	unused cesspit
MAREK4D	F	Kozlovice, Czech Republic	49°36'09"N 8°14'47"E	unused cesspit
MEJONA	F	Mejono, Java, Indonesia	7°43'S 112°08'E	F sediment
MOLUKY	F	Moluky, Indonesia	2°57'53"S 129°15'18"E	rainforest dark mud
MONTEBREH	S	Montericco, Guatemala	13°53'N 90°29'W	mangroves
MORETESB	F	Morretes, Brazil	25°26'S 48°47'W	F sediment
NELSON2	F	Nelson, New Zealand	43°09'50"S 174°20'50"E	F sediment
NDJOKO	F	Ndjoko, Ondu forest, CGO	n.a.	F sediment
OKREHEK	F	Botanical Garden, Prague, Czech Republic	50°04'20"N 14°24'24"E	pond
OLSRYB	F	Olšanský Rybník, Prague, Czech Republic	50°0'N, 14°29'E	pond
OROBINEC	F	Botanical Garden, Prague, Czech Republic	50°04'20"N 14°24'24"E	pond
PANT3	F	NP Pantanal, Brazil	16°47'S, 56°51'W	F sediment
PETRYB	F	Petrovice, Czech Republic	48°55'56"N 14°41'07"E	F sediment
QDUSW	M	n.a.	n.a.	n.a.
QDU	F	n.a.	n.a.	n.a.
RADUN	F	Raduň, Czech Republic	49°53'N 17°56'E	lake
RAJCA	F	Rajča Vas, Croatia	45°25'36"N 14°05'44"E	lake
REU1	F	Réunion, France	n.a.	pond
REU4B	B	Réunion, France	n.a.	seashore sediment
RIOTM	F	Rio de Janeiro, Brazil	22°58'06"S 43°13'31"W	pond
RIOTB	F	Rio de Janeiro, Brazil	22°58'06"S 43°13'31"W	pond
RIOZ	F	Rio de Janeiro, Brazil	22°58'06"S 43°13'31"W	F sediment
QUEENSLAND	B	Cooktown, Australia	15°27'S 145°58'E	river opening to sea
QUMBASCHI	F	Qumbaschi, Ázerbaidjan	38°55'57"N 48°48'40"E	river sediment
SALKALUZ	M/H	Sal, Cape Verde	16°35'23"N 22°55'29"W	pond near seashore
SALKAM	F	n.a.	n.a.	F sediment
SAMOSIR	F	Toba, Sumatra, Indonesia	2°31'47"S 98°44'4"E	vulcanic thermal

				pond (geyser-like)
SCHOONER5S	F	Pacific Rim NP, Vancouver Island, Canada	49°04'N 125°46'W	F sediment
SIBOLANGIT	F	Sibolangit,Sumatra, Indonesia	3°17'50"N 98°34'11"E	vulcanic lake sediment
SIPEK1APA	M	Spiridonisos, Corfu, Greece	39°48'56"N 19°51'35"E	M sediment
SIPEK1C	M	Spiridonisos, Corfu, Greece	39°48'56"N 19°51'35"E	M sediment
SKADARSKE	F	Skadar, Albania	41°24'45"N 21°33'49"E	F sediment
STORCH6	F	Cameroon	n.a.	F sediment
STORCH7	F	Cameroon	n.a.	F sediment
STOVIK	F	Botanical Garden, Prague, Czech Republic	50°04'20"N 14°24'24"E	pond
STRM2	F	Strmilov, Czech Republic	49°09'60"N 15°11'34"E	lake
STRPJEZ	F	Štrbské pleso, Tatry, Slovakia	49°07'19"N 20°03'20"E	lake
SULMOK	F	Danau delta, Romania	45°8'42"N 29°40'45"E	marsh soil
SULMOK2	F	Danau delta, Romania	45°8'42"N 29°40'45"E	marsh soil
SUSBARBBO	F	Bako NP, Malaysia	1°43'00"N 110°28'00"E	F sediment
SUSBARBBS	F	Bako NP, Malaysia	1°43'00"N 110°28'00"E	F sediment
SWAN4B	F	Perth, Australia	31°56'S 115°53'E	F sediment
TIKAL1,2,3,4,BO	F	Tikal, Guatemala	17°13'27"N 89°36'46"W	lake
TROODOS	F	Troodos, Cyprus	34°54'9"N 32°52'14"E	F sediment
VIT9	F	Kamenice, Czech Republic	49°54'N, 14°35'E	F sediment
VLADA	F	Germany	n.a.	F sediment
VLADECH	F	Czech Republic	n.a.	F sediment
VLH1	F	n.a.	n.a.	aquarium with southamerican turtle
WACT07	F	n.a.	n.a.	n.a.

Fig. 5. Sampling sites. A – strain GUAT; B – strain SAMOSIR, C – strain ALOOA1, D – strain – MONTEBREH, E – strain TIKAL1; F – strain ALOOA3.

In most cultures there were other protists present. They represented other anaerobic lineages, mostly Heterolobosea, Archamoebae, Preaxostyla, Diplomonadida, Euglenoidea, and Kinetoplastea. We have been able to reduce or eliminate those by enlarging the intervals between inoculations for a certain time period (usually several months) or by reinoculating small volume of the culture (i.e. 0.3 ml) into a tube with fresh media.

We have managed to establish monociliate cultures in many cases, as well as monoeukaryotic ones several times (i.e. MORETESB, NDJOKO, COLORADA, BUSSPRAND, and MURANO). On the other hand, several different species of armophorid ciliates have been co-cultured in one culture for a long period of time. Culture CHITWANMALY contains *Metopus* sp. 2 and *Brachonella spiralis*, CHITWANVELKY contains *Metopus* sp. 2, *Metopus* sp. 4, and CHITWANB contains *Metopus* sp. 2, *Metopus* sp. 4, and *Brachonella spiralis*, culture GDUKABAS contains *Metopus es* and *Brachonella spiralis*, OKREHEK contains *Metopus* sp.4 and *Brachonella spirals*, RIOTB contains *Metopus* sp. 2 and *Brachonella spiralis*. Cultures IZR (strains IZR, IZRBR), SUSBARB (strains SUSBARB, SUSBARBO), and TIKAL (strains TIKAL1, TIKAL4) each contain one (diverse) species of *Bothrostoma* and one (diverse) species of *Brachonella*. SIPEK1C contains *Metopus contortus* and *Palmarella salina*. Culture LISTOPAD (strains LISTOPADM, LISTOPADBS, and LISTOPADBC) contains *Metopus laminarius*, *Brachonella* sp. 1, and *Brachonella spiralis*, although the culture is not stable yet and the final arrangement may differ, as *Brachonella* sp. 1 has a rather scarce abundance.

The freshwater isolates are cultivated in ATCC 802 medium (Sonneborn's *Pamamecium* medium), whereas the marine isolates are cultivated in ATCC 1525 medium with artificial seawater. Brackish strains are cultivated in 1:1 ratio of ATCC 802 and ATCC 1525 media combination. Several strains started growing well only after adjustment of the media ratio.

We have performed an experiment with freshwater strains COLOMBIA, KAO and QDU after we had found out that they are phylogenetically related to the marine species *Metopus contortus* (see below). Surprisingly, the strains survive well transfer into brackish medium. We have started at 1:9 ATCC 1525 and ATCC 802 combination and increased the volume of ATCC 1525 each few weeks during the inoculation (1:4, 3:7, 2:3). Currently, we cultivate these strains in 1:1 ATCC 1525 and ATCC 802 media combination and we will continue with a transfer into a full ATCC 1525 medium.

Another strain (MAREK4), assigned to species *Metopus palaeformis*, was cultivated on Dobell and Laidlaw's biphasic medium (Dobbel, 1935). This strain showed a different morphology than the other strains of this species ("swollen" cells), but when we inoculated it in the usual freshwater medium (ATCC 802), the ciliates changed their shape to typical *M. palaeformis*, after ca two months cultivation (Fig. 31, I, J).

4.1.2. Phylogenetic analysis of Armophorea

Phylogenetic tree of Armophorea is depicted in Fig. 6. Armophorea (excluding Caenomorphidae) split into eleven more or less robust lineages, A1 – A11, whose interrelationships were mostly unsupported. The lineage A1 comprised *Palmarella salina* and three species of *Metopus* (*M.* sp. 1, *M. mucicola*, and *M.* sp. 2; for species description see 4.1.4.). In addition, four environmental sequences affiliated to *P. salina* and the environmental sequence AB252766 forming an independent branch belong to this lineage as well. Although the monophyly of A1 remains unsupported, we consider it a clade due to morphological similarities of *P. salina* and *M. mucicola*. The three *Metopus* species with the lineage A1 form a moderately supported clade (BS 75, BPP 0.97). *M.* sp. 2 and *M. mucicola* are strongly supported sister species (maximum support). The environmental sequence AB252766 belongs to an uncultivated ciliate from saline meromictic lake, while our *Metopus* strains come from freshwater sediments.

Lineages A2 – A11 formed a highly supported clade (BS 97, BPP 1). Although the relationships within this clade were generally unresolved, its overall topology was consistent with results of Bourland et al. (2014a). The lineage A2 was relatively robustly monophyletic (BS 81, BPP 1) and appeared to be sister to the remaining lineages, though this position was not supported. It consisted of *Brachonella* sp.1, *Metopus striatus*, and the environmental sequence AJ009658, which is in GenBank labelled as to belong to an uncultured caenomorphid.

Lineages A3 – A5 formed a clade, though without any support; lineages A4 and A5 appeared closely related with some support (BS 60, BPP 1). The lineage A3 consisted of strains of *Brachonella spiralis*. The lineage A4 included *Metopus es* and *M.* sp. 3. The lineage A5 included *M. nivaaensis, M. contortus*, *M. halophila*, *M.* sp. 3, and numerous environmental sequences.

Lineages A6 – A11 formed a clade that was unsupported in ML analysis and relatively highly supported in BI analysis (BPP 0.99). The lineage A6 represented by *Palmarella lata*, appeared sister to the rest of lineages, though this position was supported only in BI analysis (BPP 1). Lineages A7 and A8 formed an unsupported clade. The lineage A7 contained *Metopus nasutus*, *M. bothrostomiformis*, *Bothrostoma undulans*, and an environmental sequence; the lineage A8 comprised *M. fuscus*, *M.* sp. 4, and *M. setosus*. The lineage A9 was represented by *Metopus palaeformis*. The lineages A10 and A11 appeared closely related, though the relationship received some support only in BI analysis (BPP 0.98). The lineage A10 contained *Metopus* spp. 5 and 6.

The lineage A11 was the most species-rich among armophorean lineages comprising *Metopus* spp. 7 – 11, *M. laminarius*, genus *Atopospira*, and the entire Clevelandellida. Clevelandellida appeared closely related to *Metopus laminarius*, though the relationships were not

supported in ML analysis (BS 50, BPP 1). Consistently, there was also the clade formed by of *M. laminarius, Atopospira* spp., and Clevelandellida in the analysis by Bourland et al. (2014a), although, differently, the clade received high support (BS 90, BPP 1) there and Clevelandellida were closer related to *Atopospira* spp. than to *M. laminarius* with relatively high support (BS 79, BPP 1). Although Clevelandellida show a strong support as a monophyletic group, their internal phylogeny remained unresolved. The genus *Nyctotheroides* appeared paraphyletic with respect to *Nyctotherus*, which, in turn, was paraphyletic with respect to the genus *Clevelandella.*

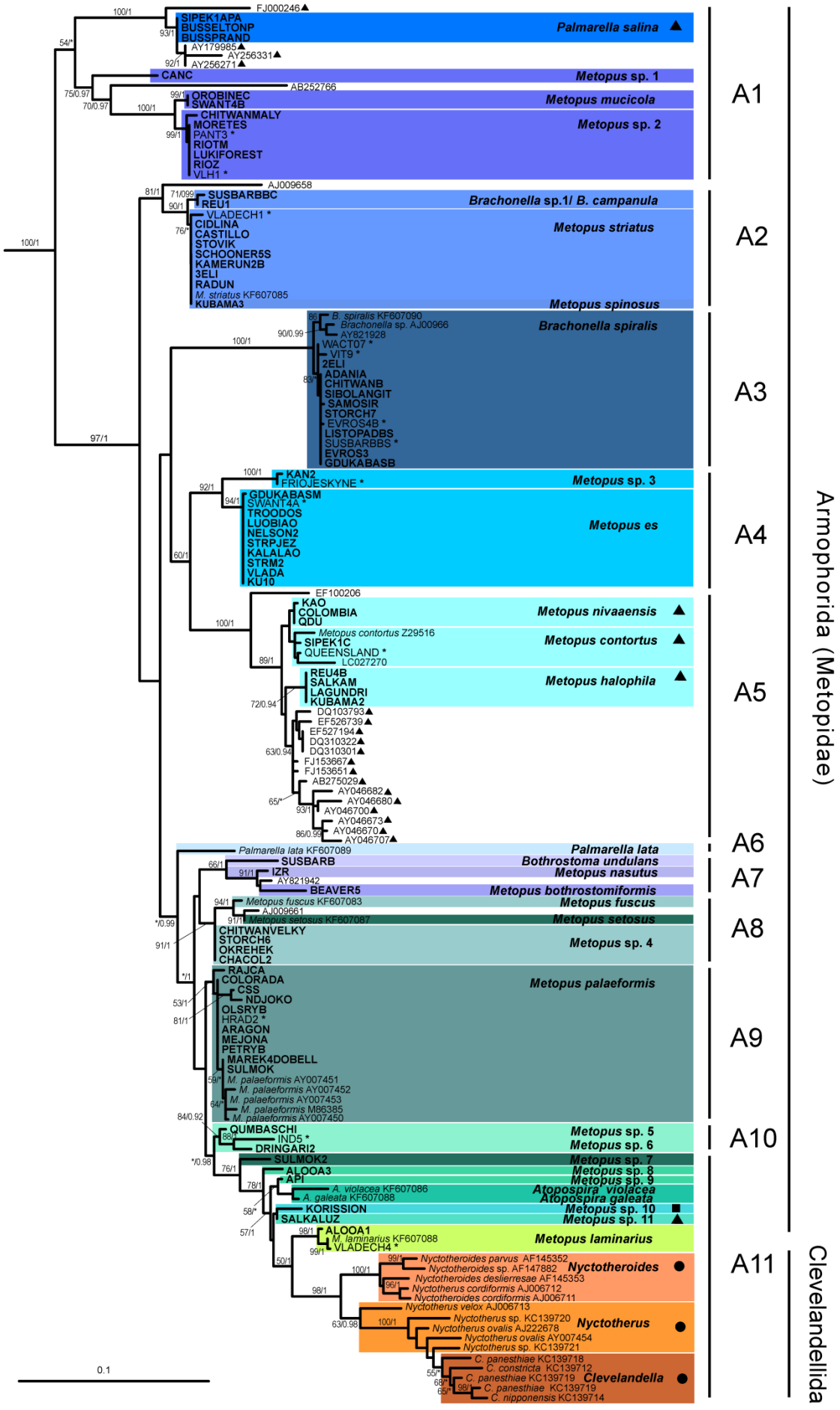
Palmarella salina
Metopus sp. 1
Metopus mucicola
Metopus sp. 2
Brachonella sp.1/ B. campanula
Metopus striatus
Metopus spinosus
Brachonella spiralis
Metopus sp. 3
Metopus es
Metopus nivaaensis
Metopus contortus
Metopus halophila
Palmarella lata
Bothrostoma undulans
Metopus nasutus
Metopus bothrostomiformis
Metopus fuscus
Metopus setosus
Metopus sp. 4
Metopus palaeformis
Metopus sp. 5
Metopus sp. 6
Metopus sp. 7
Metopus sp. 8
Metopus sp. 9
Atopospira violacea
Atopospira galeata
Metopus sp. 10
Metopus sp. 11
Metopus laminarius
Nyctotheroides
Nyctotherus
Clevelandella
A1
A2
A3
A4
A5
A6
A7
A8
A9
A10
A11
Armophorida (Metopidae)
Clevelandellida
0.1

Fig. 6. Phylogenetic tree of the class Armophorea based on SSU rDNA sequences. The tree was constructed by the maximum likelihood method and was rooted by representatives of the other ciliate classes and Caenomorphidae (the outgroups were removed). The numbers at the nodes represent BS ≥ 50/BPP ≥ 0.9. Lower are not shown or are denoted by an asterisk. Environmental sequences are represented by GenBank accession numbers. New sequences are in bold. Legend: ▲ - marine, ■ - brackish, ● - endobiotic, * - sequence determined by Nováková (2011).

4.1.3. Morphology of armophorids

We have studied morphology of most of the cultivated strains. We used light microscopy techniques (DIC, BF) and observed living cells and fixed cells as well as protargol-stained cells. Observation of living cells allows us to see the natural state of living cells (e.g. movement, contractile vacuoles movement, type of feeding, food vacuoles path, cell division, and conjugation) and compare them with the fixed state. Besides that, protargol staining technique is necessary to enable detailed visualisation of particular morphological characteristics such as the size and shape of macronucleus and micronucleus (which are sometimes visible also in the light microscope, although not always), sometimes also epibiotic symbionts, or the number of adoral zone membranelles, perizonal stripe kineties, and overall the cortical structures, and is generally used for species determination. We usually chose one or two representative strains for each species to employ protargol staining.

We determined species partially using the traditional morphometrics and, mainly, the inter-specific determinative morphological features, which are the size and shape of cells, macronucleus and micronucleus, length and number of membranelles in the adoral zone, presence or absence (sometimes also number) of caudal cilia, which were all observed using the oil immersion objective and DIC in living cells and BF in protargol-stained cells. We relied on determination techniques for armophorids used by Foissner & Agatha (1999), Vďačný (2007), and Bourland (2014a, b) and species descriptions by Kahl (1927, 1929, 1930, 1931, 1932, 1935), Jankowski (1964, 2007), Foissner (1980), Esteban et al. (1995), and Bourland (2014a, b).

Our strains ranged in size from 25 μm to 245 μm in living state and 20 μm to 80 μm when stained with protargol, which corresponds with the previous studies on armophorids (see 2.1.). The smaller size of protargol-stained cells is caused by shrinkage during fixation. The shapes and other features differ between species, sometimes also between conspecific strains and in some cases there are differences even between cells of a single strain, which is caused by high intraspecific polymorphism in armophorids (see 4.4., 5.1.). Additionally, we have recorded cystic stages; dividing cells or conjugation in some strains (see 4.5.).

In the present study, we use the terminology used by Jankowski (1964), Foissner & Agatha (1999), Lynn (2008), and mainly Bourland et al. (2014a) (Fig. 7), who explicitly defined

the ventral side of the cell in armophorids as “the surface on which the terminus of the adoral zone (and the cytostome) is at the right margin” and the dorsal, left and right side accordingly to this.

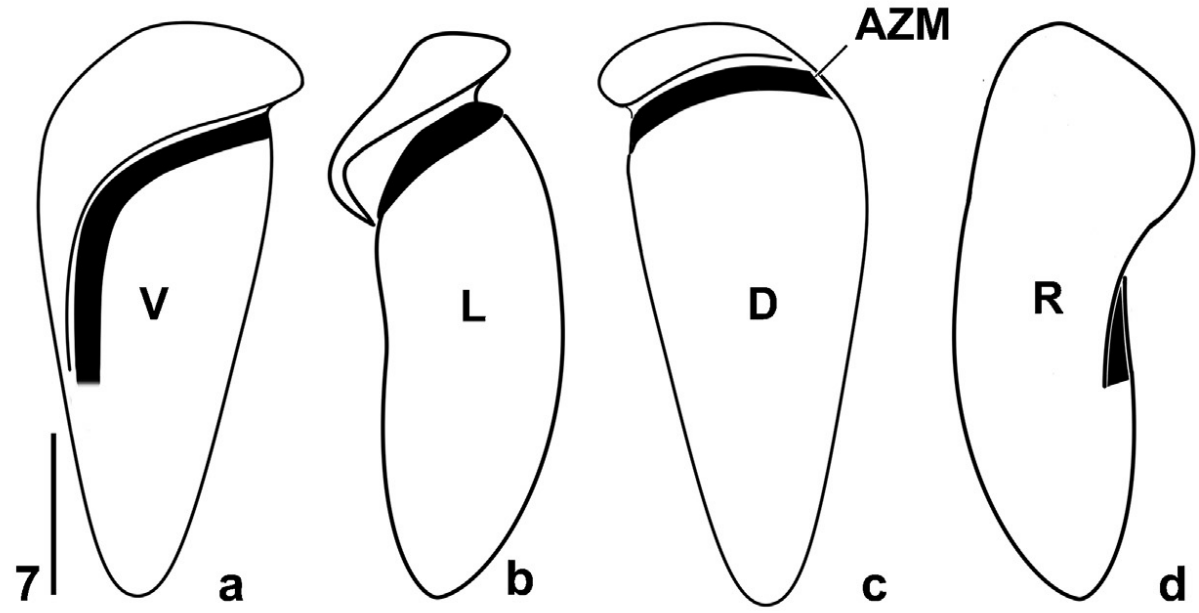

Fig. 7. Ventral (a), left (b), dorsal (c), and right (d) sides of *Metopus fuscus*, a terminology generally accepted in this study; Bourland et al. (2014a).

4.1.4. Species identification and brief description

Bothrostoma mirabilis (Kahl, 1927) Jankowski, 1964 (Fig. 8, G, H, I)

Strain: **TIKAL1**

The TIKAL1 strain was identified as *Bothrostoma mirabilis* (Kahl, 1927) Jankowski, 1964 according to the description by Kahl (1932), although the cells are smaller than in the original description and also smaller compared to other species of *Bothrostoma*.

The long–ovate cells of strain TIKAL1 are ca 40 μm long and ca 20 μm wide in living specimens. The oval macronucleus is located in the anterior part of the cell, with a small round micronucleus allied. The adoral zone of membranelles (AZM) reaches a cell equator, allied by a large paroral membrane. The posterior contractile membrane and caudal cilia are present.

Bothrostoma undulans Stokes, 1887 (Fig. 9)

Strain: **SUSBARBBO**

The freshwater ciliates of the strain SUSBARBBO, very distinctive from other metopids, were identified as *Bothrostoma undulans* Stokes, 1887, according to the original description, as well as Kahl’s (1932). The main characteristics lie in the presence of a conspicuous paroral membrane

with unique triangular shape, starting at the proximal end of AZM and ending at the posterior third of AZM, and an almost straightforward AZM lying in an unusually (for metopids) wide and open peristomal area. As described by Stokes (1887), the peristomal area starts on the left-side of the cell, obliquely inwards beyond the cell centre, and directs towards the apical end of the cell.

The long–ovate cells of strain SUSBARBBO are 80 – 100 μm long and 25 – 30 μm wide in living specimens and 40 – 50 μm long and ca 15 μm wide in protargol-stained specimens. The macronucleus is roundish oval ca 22 x 15 μm (6 x 12 μm in protargol-stained cells) big, located in the anterior (or sometimes central) part of the cell, with a small round micronucleus (ca 3 μm) lying by the centre of the left side. The AZM is ca 35 μm long (23 μm in protargol-stained cells) and contains 19 membranelles. A single posterior contractile vacuole is present, as well as long caudal cilia.

***Brachonella* sp.1** (Fig. 10)

Strains: SUSBARBBC, REU1, LISTOPADBC

The freshwater ciliates of strains SUSBARBBC, REU1, and LISTOPADBC are easily distinguished from other metopids thanks to their peculiar shape reminiscent of a mushroom. However, due to a group of several nearly identical species described by Kahl (1927, 1932), we were not able to unequivocally determine the strains into species. They seem to be morphologically closest to *Brachonella campanula* (syn. *Brachonella intercedens* var. *minor*) (Kahl, 1927) Jankowski, 1964. This species has matching size and shape, but the shape of macronucleus and the position of the perizonal stripe were depicted differently by Kahl (1927). The strains also lacks the group of anterior cilia (tuft) described in (Kahl, 1927). Cells of our strains have a large anterior part comprising of a preoral dome, which is doubled in compare with the posterior part, and in dorsolateral view is horizontally overlapping the posterior part. From the ventral view, we can see the buccal part of AZM in the posterior part of the cell, directing anteriorly and then horizontally spiralling under the preoral dome. The cells measure 35 x 30 μm to 52 x 40 μm in vivo (ca 25 x 17 μm in protargol-stained cells) at the longest and widest point, respectively. The round macronucleus is located in the cell centre and measures ca 15 μm in diameter. The micronucleus is round and, interestingly, is allied either on the right-lateral anterior or the right-lateral posterior of macronucleus.

The AZM is ca 20 μm long, starts proximately at the first fifth of the posterior in the smaller cells and in the proximal half of the posterior in the larger cells, and usually contains 18 or 19 membranelles. The paroral membrane is present and contains five wider membranelles at the buccal part of AZM. The kinetosomes in membranelles are vertically multiplied, creating an impression of wider AZM and paroral membrane in all our strains. The perizonal stripe is located

closer to anterior than in all described species and contains only four perizonal kineties, three of them closely allied, while the fourth is isolated and placed further to the anterior. There are three somatic kineties in the preoral dome. The number of somatic kineties is reduced compared to other metopids. The posterior of the cell is oval to round, with a contractile vacuole within, and group of caudal cilia present. Interestingly, there is an anterior kinety (bell kinety) located on the top of the anterior part, comprising ca 8 or 9 kinetosomes that create membranelles.

These ciliates are significantly faster swimmers compared to other metopids. The last two unique features among metopids, together with the position of perizonal stripe and the wider AZM, indicate a resemblance with *Cirranter mobilis* (Penard, 1922) Jankowski, 1964 (syn. *Metopus mobilis* Penard, 1922, Kahl, 1927; syn. *Trochella mobilis* Kahl, 1932), which was, however, placed within caenomorphids by Jankowski (1964) and its micronucleus is placed beneath macronucleus, contrary to the situation *Brachonella campanula*. However, in our strains the macronucleus can be placed in both positions

Brachonella darwini (Kahl, 1927) Jankowski, 1964 (Fig. 11, A - D)

Strain: **TIKAL2**

We have identified *Brachonella darwini* (Kahl, 1927) Jankowski, 1964 according to the original description, even though the cell size is more than halved compared to Kahl's (1927) description. We have encountered this species only once, namely in the culture TIKAL2. Unfortunately, the culture has not become stable yet, and we have not been able to determine SSU rDNA sequence or assess better morphological characterization of this ciliate. However, this species is very distinctive and easily recognizable. The cells are dorsoventrally flattened and measure ca 40 x 12 μm. There is a conspicuous, pointy, ca 12 μm long end at the posterior of the cell. It seems rigid. The AZM is left-spiralling around the cell as in *B. spiralis*, which highly resembles this ciliate if we imagine it with a pointy ending. There is a group of dark brown granules at the anterior of the cell. The macronucleus is round and fills the anterior part of the cell, having ca 10 μm in diameter. The posterior contractile vacuole is present.

Brachonella spiralis (Smith, 1897) Jankowski, 1964 (Fig. 12)

Strains: **2ELI, ADANIA, EVROS3, EVROS4B, CHITWANB, GDUKABASB, SAMOSIR, SIBOLANGIT, STORCH7, SUSBARBB, VIT9, WACT07, OKREHEKB, TIKAL4, GUAT, LISTOPADBS**

The strains were identified as *Brachonella spiralis* (Smith, 1897) Jankowski, 1964 according to the description in Kahl (1932) and Jankowski (1964). We have not isolated DNA from strains OKREHEK, TIKAL4, GUAT, LISTOPADBS, as these cultures are very new and not stable yet,

but we were able to assign them into the same species according to the typical morphological features. *Brachonella spiralis* was one of the most frequent ciliate in our samples and is easily cultured. This species has several synonyms and also resembles *Metopus tortus* Kahl, 1927.

The cells measure ca 60 x 35 μm to 90 x 50 μm and are slightly dorsoventrally flattened (ca 20 – 40 μm wide) in some strains (e.g. CHITWANB, SAMOSIR, TIKAL4) while spatial and roundish in other strains. The body shape and size is highly variable within a single population. The macronucleus is round and measures ca 15 μm in diameter. The long AZM spirals around the body, as the species name suggests, and measures ca 75 x 7 μm. The anterior part contains dark granules in some strains (e.g. SAMOSIR). The paroral dome is conspicuously wide and ranges from 20 to 30 μm in the narrowest (anterior) part. The perizonal stripe contains five perizonal kineties. There are ca 30 somatic kineties placed in well visible grooves (even if observed in light microscopy). There is a short posterior part that is narrowed to ca 15 μm in some strains (e.g. SAMOSIR, SIBOLANGIT). There is a group of short caudal cilia at the posterior part. The posterior contractile vacuole is present.

Metopus bothrostomiformis Foissner, 1980 (Fig. 8, J - M)

Strain: **BEAVER5**

Strain BEAVER5 was easily identified as *Metopus bothrostomiformis* Foissner, 1980, according to its original description. The cells are dorsoventrally flattened and not left-towards twisted as is so typical for *Metopus*. The preoral dome is relatively large. The oblique AZM reaches the cell equator, while the peristomal area is still very wide, as in the genus *Bothrostoma*. Another feature resembling *Bothrostoma* is the presence of a conspicuous paroral membrane, which is shortened to the first third of the peristome in this species. The posterior contractile vacuole and caudal cilia are present. The only differences from Foissner's description are the shape of the macronucleus, which is kidney-shaped with a relatively large micronucleus seated in the macronuclear isthmus, compared to the small oval macronucleus with an apically located micronucleus described in Foissner (1980), and the nearly double size of the ciliates in our culture (ranging around 100 x 30 μm).

Metopus contortus Quennerstedt, 1867 (Fig. 13, A - D)

Strains: **SIPEK1C, QUEENSLAND**

Ciliates from strains SIPEK1C and QUEENSLAND were determined as the marine species *Metopus contortus* Quennerstedt, 1867 according to the redescription of this species by Esteban et al. (1995). Contrarily to the marine strain SIPEK1C, the strain QUEENSLAND inhabited

brackish environment. The morphological description was assessed only in the strain SIPEK1C (see 5.1.)

The cells of strain SIPEK1C are ca 120 μm long and 40 μm wide. The anterior part of the cell is significantly wider than the posterior part, widening from the cell equator, ending with a large preoral dome extending the cell body. The oblong macronucleus is large, extending to almost one half of the cell size (ca 50 μm), and occupies the anterior part of the cell while it reaches the proximal end of the AZM. We have not confirmed the position and shape of the micronucleus. The peristome with five kineties is relatively narrow compared to the size of the ciliate. The AZM reaches two thirds of the cell and contains ca 40 membranelles. There are ca 40 somatic kineties. The posterior contractile vacuole and long caudal cilia are present.

Metopus es O. F. Müller, 1776 (Fig. 14)

Strains: **GDUKABASM, KALALAO, KU10, LUOBIAO, NELSON2, STRM2, STRPJEZ, SWAN4A, TROODOS, VLADA**

The freshwater strains listed above have characteristic, S–shaped cells and belong to the species *Metopus es* O. F. Müller, 1776, which is the type species for the genus. Ciliates of this species are characteristic for their frequent abundance, which is confirmed by our results, as this species was the most frequently encountered one during the study. This species has a variable morphology with two main morphotypes, from which one is represented by thinner cells with narrowed posterior part, while cells of the second morphotypes are more oval and their posterior part is rounder.

The cells generally range from 80 x 30 μm (strain KALALAO) to 170 x 45 μm, with variations also from strain to strain. Conspicuously, ciliates in the strain KALALAO are always smaller and form rounder shapes. The oval macronucleus measures ca 30 x 15 μm with frequent variations. The AZM is long and contains ca 24 membranelles. The oval macronucleus is positioned in the anterior part of the cell and contains conspicuous granular structures. The micronucleus is allied on the top of it. The perizonal stripe consists of five kineties. We have frequently recorded well-visible food vacuoles adjacent to the posterior contractile vacuole.

Metopus halophila (Kahl, 1925) Corliss, 1960 (Fig. 15)

Strains: **KUBAMA2, LAGUNDRI, REU4B, SALKAM**

Marine strains KUBAMA2, LAGUNDRI, REU4B, and SALKAM, were identified as *Metopus halophila* (Kahl, 1925) Corliss, 1960, according to the description of Kahl (1925) and Esteban et al. (1995). This species is considered relatively rare, as we have encountered it only four times. Also the cultures never evince a high abundance.

These fast-swimming ciliates are dorsoventrally flattened and measure from 50 x 20 μm to 90 x 30 μm. The oval macronucleus is located in the centre of the anterior part of the cell. They possess conspicuous cilia growing from the anterior part of the cell, i.e. the preoral dome. Long caudal cilia and posterior contractile vacuole are also present.

Metopus laminarius Kahl, 1927 (Fig. 16)

Strains: **ALOOA1, LISTOPADM, VLADECH4**

We have been able to culture three strains from the freshwater species *Metopus laminarius* Kahl, 1927. The strains were assigned to this species according to its original description by Kahl (1927). These ciliates are never very abundant in the culture. Cells of the strain ALOOA1 are 100 – 120 μm long and ca 15 μm wide. They may resemble *M. palaeformis* at first sight; however, their preoral dome overhangs the AZM, which easily distinguishes those two species. The oblong macronucleus, located in the cell centre, is ca 35 – 40 μm long and has a small round micronucleus closely allied by its side. The AZM measures ca 40 μm and contains ca 20 – 24 membranelles. The preoral dome is twisted towards left of the cell. The posterior contractile vacuole is present.

Metopus mucicola Kahl, 1927 (Fig. 17)

Strains: **OROBINEC, SWAN4B**

Strains OROBINEC and SWAN4B were identified as *Metopus mucicola* Kahl, 1927 according to the descriptions of this species by Kahl (1927, 1932). Both strains morphologically closely resemble the strains of *Metopus* sp. 2 (see below); however, the cells are generally shorter and wider, and they never form the bigger long forms. The ovate cells range from 50 x 20 μm to 70 x 25 μm. The oblong macronucleus is 20 x 8 μm large and is located in the anterior part of the cell, usually leaned towards to equator, with a round micronucleus allied at the top of it. The shortened perizonal area is wider than in other species of *Metopus*, and the AZM is also shortened, measuring 15 - 25 μm, both features are similar to *Palmarella salina*. A short paroral membrane is present. The posterior contractile vacuole is present.

Metopus nasutus da Cunha, 1915 (Fig. 8, A - D)

Strains: **IZR, TIKALBO**

These ciliates were identified as *Metopus nasutus* da Cunha, 1915 (syn. *Metopus vexiliger* Penard, 1922), according to its original descriptions (da Cunha, 1915), as well as Kahl's descriptions (1927, 1932). The cells measure 80 x 20 μm to 100 x 25 μm in the strain IZR and 90 x 20 μm to 110 x 30 μm in the strain TIKALBO. The roundish granular macronucleus is ca 13 x 16 μm large,

and the adjacent macronucleus is small and not well visible. The most conspicuous morphological feature is a thin pointy protrusion on the apical end of the cell, which seems flexible. Its length is variable, from 10 μm in the strain IZR up to 25 μm in the strain TIKALBO. The AZM extends the cell equator, is ca 65 μm long, and contains 15 – 18 membranelles. A high paroral membrane is present, ca 20 μm wide, starting at the AZM initiation and extending in length of ca 30 μm towards the second third of the AZM. The posterior contractile vacuole and 5 long caudal cilia (up ca 30 μm) are present.

Metopus nivaaensis Esteban, Fenchel & Finlay, 1995 (Fig. 13, E - L)

Strains: **COLOMBIA, KAO, QDU**

Strains COLOMBIA, KAO, and QDU were identified as the marine species *Metopus nivaaensis* Esteban, Fenchel & Finlay, 1995, according to the original description by Esteban et al. (1995) with unclarities. Those strains are also closely related to another marine species - *Metopus contortus*, yet they were all collected in freshwater sediments. Startlingly, they all survive brackish medium. The cells are slightly dorsoventrally flattened and measure from 65 x 35 μm (strain QDU) to 75 x 40 μm (strain KAO) or 80 x 30 μm (some elongate cells in the strain COLOMBIA). The macronucleus is oblong, measures ca 30 x 10 μm, and has a granular structure. These ciliates have densely allied somatic kineties. The paroral dome is narrow, ca 8 μm wide. The perizonal stripe contains five perizonal kineties. The AZM is ca 45 μm long. A short (ca 13 μm), reduced paroral membrane is present. There are several long (ca 30 μm) caudal cilia, as well as a small (ca 5 μm in diameter) posterior contractile vacuole present.

Metopus palaeformis Kahl, 1927 (Fig. 18)

Strains: **ARAGON, COLORADA, CSS, HRAD2, NDJOKO, OLSRYB, MAREK4, MEJONA, PETRYB, RAJCA, SULMOK**

The freshwater strains listed above were assigned to *Metopus palaeformis* Kahl, 1927, according to the description in Esteban et al. (1995). These ciliates are relatively common and usually get very abundant in the culture. The cells highly vary in size (50 x 5 μm to 150 x 25 μm), shape (long and thin, sometimes twisted, pear-shaped, simple elongate), resulting into several morphotypes. The most common morphotype is an elongate thin cell. The macronucleus in very long and sometimes may be tangled or even segmented by strangulations, measuring ca 25 μm in the tangled form and up to 75 μm if uncoiled. The small round micronucleus is allied on the side of it. The AZM is ca 40 μm long, going through the central ventral side of the anterior part of the cell, not as twisted as, for example, in *Metopus es*. The preoral dome is reduced and not

overhanging the AZM. Also the perizonal area is restricted, straightforward and thin. The posterior contractile vacuole is present.

Metopus striatus McMurrich, 1884 (Fig. 19)

Strains: **3ELI, CASTILLO, CIDLINA, KAMERUN2B, RADUN, SCHOONER5S, STOVIK, VLADECH4**

Eight strains obviously belong to the species *Metopus striatus* McMurrich, 1884, according to the description in Kahl (1932) and Esteban et al. (1995). This species is easily distinguished by its typical boxing glove-like shape, characterized by a conspicuously large, overhanging preoral dome and a pointy end at the posterior side. The cells are dorsoventrally flattened and measure 60 x 40 μm to 120 x 60 μm at the widest spot. The round macronucleus with ca 15 to 30 μm in diameter is located in the centre of the cell, with an underlying small round micronucleus. The posterior end is filled with a contractile vacuole and there are many long caudal cilia present, reaching ca 40 μm length. The perizonal zone contains 5 kineties as typical for other metopids, even despite the size of the preoral dome. The length of AZM ranges between 40 and 60 μm and it contains ca 45 membranelles. There are significant variations in the sizes and shapes both between and within the strains. Similarly to Esteban et al. (1995) and Bourland et al. (2014a), we have noticed conspicuous extrusomes within the cortex of all strains. Nevertheless, we were not able to specify their function. The morphology of the strain VLADECH4 could not be determined, as the culture of this strain was exterminated in the initiation of this study. However, in a micrograph in Nováková (2011), the cells differed from the other strains of this species and had roundish shape with a rounded shorter posterior ending, distinctly resembling *Brachonella* sp. 1 (possible *Brachonella campanula*).

Metopus spinosus Kahl, 1927 (Fig. 11, E - H)

Strains: **KUBAMA3 (MOLUKY1, MONTEBREH, TIKAL3)**

We have encountered this distinctive species only few times, interestingly only in samples collected during early spring (February to April) in tropical environments. We have been trying to establish cultures from strains KUBAMA3, TIKAL3, MOLUKY1, and MONTEBREH, although all of them are still unstable, and the abundance of this species is very low. This species is characterized by the posterior pointy ending of the cell and a large preoral dome. The cells are elongate and relatively large compare to other metopids, measuring from 150 x 40 μm to 200 x 50 μm. The pointy end is ca 40 μm long and seems rigid. The AZM is ca 60 μm long. The cells contain a tuft of cilia on the anterior ventral part. The posterior contractile vacuole is located at the anterior base of the pointy end.

***Metopus* sp. 1** (Fig. 20)

Strain: **CANC**

We have cultured only a single strain belonging to this species, and we have never encountered it otherwise. This freshwater strain could not be assigned to any known species (at least known to us), and yet it is a very distinctive ciliate. The cells widely vary in sizes, ranging from 50 x 5 μm to 85 x 20 μm. The elongate macronucleus is ca 22 x 8 μm large. Frequently, the macronucleus is strangulated in the anterior first third and creates the shapes of number one, while the small oval micronucleus is nesting in the constriction site. The AZM is relatively straight and short, measuring ca one fifth of the cell. The cells are curved and have a slight S or D shape (question-mark-like shape), and, although the body is flexible, it keeps the shape while swimming or feeding. The posterior contractile vacuole is present.

***Metopus* sp. 2** (Fig. 21)

Strains: **MORETESB, RIOZ, RIOTM, VLH1, PANT3, LUKIFOREST, CHITWANMALY**

Ciliates from the listed strains were identified as *Metopus* sp. 2, although they were initially assigned to *M. hasei* Sondheim, 1929, according to the conservative approach. The reasons for the original assignment are morphological features of this species, including the body shape in protargol stained cells, the length and width, the position, size and shape of macronucleus and micronucleus, the number and shape of AZM, and the morphotype variations, as all described in Foissner & Agatha (1999) and Vďačný (2007). However, there are several notable differences from the descriptions, i.e. the shape of the body in vivo, where the preoral dome in our strains is not projecting above ventral and lateral body surface, also due to the lack of long caudal cilia in our strains and the presence of another morphotype in our cultures (**Fig** x). Therefore, our strains may also represent a novel species and were identified as *Metopus* sp. 2 for now.

The cells measure ca 60 x 15 μm (30 x 8 μm when stained with protargol). The peristomal area is wider than in other metopids. The macronucleus is long and slim, and its size is ca 20 x 5 μm. It is located in the anterior part of the cell, and the micronucleus is placed on top of it. The AZM is long 12 – 22 μm and consists of 16 – 19 membranelles. The perizonal area contains 4 or 5 perizonal stripes. The posterior contractile vacuole is present.

***Metopus* sp. 3** (Fig. 22)

Strains: **KAN2, FRIOJESKYNE**

We were not able to undoubtedly identify the strain KAN2 into a known species. The ciliates in the strain KAN2 resemble *Metopus es*, although they differ from it as well. This species has a distinctive round particle in the anterior part of each cell, well visible in the light microscope.

These ciliates are large, measuring ca 250 x 45 μm. The macronucleus is located in the centre of the cell, oval and measures ca 60 x 35 μm. The AZM is ca 70 μm long. The posterior contractile vacuole is present.

***Metopus* sp. 4** (Fig. 23)

Strains: **CHACOL2, CHITWANVELKY, OKREHEK, STORCH6**

We culture four strains of a metopid that resembles *Metopus fuscus* Kahl, 1927. However, due to differences we identify these strains as *Metopus* sp. 4. The cells of our strains measure from ca 130 x 30 μm (STORCH6) to 155 x 52 μm (CHITWANVELKY) in vivo (50 x 20 μm to 65 x 25 μm in protargol-stained cells). The macronucleus is typically oval, located at the base of the anterior part of the cell, and measures from ca 30 x 10 μm (STORCH6) to 40 x 20 μm (CHITWANVELKY) in vivo (20 x 15 in protargol-stained cells). The AZM is usually 70 μm (20 – 40 μm in protargol-stained cells) long and contains ca 35 membranelles, although we have observed cells with shortened AZM in the strain STORCH6, measuring ca 45 – 55 μm. The posterior contractile vacuole is present.

Palmarella salina (Gaievskaja, 1925) Jankowski, 1964 (Fig. 24)

Strains: **BUSSPRAND, BUSSELTON, SIPEK1APA**

Marine strains BUSSPRAND, BUSSELTON, and SIPEK1APA were identified as the species *Palmarella salina* (Gaievskaja, 1925) Jankowski, 1964 with uncertainties. There are two morphotypes, both highly distinct from other metopids. The cells of morphotype 1 are ovate, ca 30 x 22 μm large. The cells of morphotype 2 are elongate, ca 50 μm x 13 μm large. Other than these characteristics, the shape and size, both morphotypes mostly share identical features. Also, we have encountered cells which seem to represent a transition between these two morphotypes. The peristome area is wide and straight. The AZM is ca 18 μm long and contains only nine membranelles. The peristome area is enlarged in the morphotype 2, while the AZM stays the same. There are numerous cilia on the perizonal area. The macronucleus is oval and ca 13 x 6 μm large, with a round macronucleus adjacent on the top of it. There is a very long single caudal cilium on the posterior end of the cell, reaching 40 μm, surrounded by shorter caudal cilia. A small (3 μm) posterior contractile vacuole is present.

***Metopus* sp. 5** (Fig. 25, A - G)

Strain: **QUMBASCHI**

We were not able to assign this strain to species. The cells measure ca 80 x 20 μm. The macronucleus is positioned in the central part of the cell and seems oval, but is actually squished

from a long thin shape into the shape of letter U, which measures ca 15 x 9 μm. The preoral dome is reduced. The AZM measures ca 20 μm. The posterior contractile vacuole is present. These ciliates are morphologically similar to the *Metopus* sp. 8 (strain ALOOA3), beside the conspicuous differences in their macronuclei.

***Metopus* sp. 6** (Fig. 25, H, I)

Strains: **DRINGARI2, IND5**

We were not able to assign these strains to species. However, they resemble *Metopus palaeformis* and *M. laminarius*. Some cells resembled *M. major* Kahl, 1932 from micrographs in Esteban et al. (1995). The cells are elongate and measure from ca 90 x 18 μm (rarely 80 x 15 μm) to ca 130 x 20 μm. The macronucleus is elongate and measures ca 38 x 11 μm. The AZM is typically ca 35 μm long, but rarely can reach 50 μm. The preoral dome is twisted towards left as in *M. laminarius.* The posterior contractile vacuole is present.

***Metopus* sp. 7** (Fig. 26)

Strain: **SULMOK2**

The single strain of this species closely resembled *Metopus setosus* Kahl, 1927. However, several uncertainties remain, thus it was determined as *Metopus* sp. 7. The cells measure 40 x 20 μm to 60 x 30 μm in vivo (23 x 11 μm in protargol stained cells). Roundish macronucleus is large 20 x 13 μm (11 x 5 μm in protargol stained cells), located in the centre of the cell, with a large round micronucleus (4 μm) allied to the central part of it. There are several similar structures inside of the macronucleus. AZM is long, ca 25 um (13 um in protargol stained cells) and contains ca 13 membranelles. The present caudal cilia are very long and numerous (20 μm). The posterior contractile vacuole is present.

***Metopus* sp. 8** (Fig. 27, A - D)

Strain: **ALOOA3**

We were not able to identify the strain ALOOA3 and place it among described species. It resembles *Metopus laminarius* and is similar also to the ciliates in *Metopus* sp. 5 (strain QUMBASCHI). The elongate cells measure ca 80 x 15 μm. The macronucleus is long and thin, sometimes crooked, measuring ca 25 x 6 μm. The preoral dome is reduced. The AZM measures ca 20 μm. The single posterior contractile vacuole is present.

***Metopus* sp. 9** (Fig. 27, E - H)

Strain: **API**

We were not able to determine the species of the strain API. The elongate cells measure ca 75 x 15 μm. The macronucleus is oblong and measures ca 16 x 9 μm. The AZM is ca 25 μm long. These ciliates are morphologically similar to *Metopus* sp. 8 (strain ALOOA3) and *Metopus* sp. 5 (strain QUMBASCHI), as well as *M. laminarius.* The posterior contractile vacuole is present.

***Metopus* sp. 10** (Fig. 28)

Strain: **KORRISSION**

The brackish strain KORRISSION could not be assigned to any known species, although it is very easy to distinguish it from other species. Nevertheless, it may resemble *Metopus laminarius* or *M. palaeformis* on the first sight, due to the shorter peristomal area, which is not as twisted as in other species. The cells range in the size from 80 x 5 μm to 130 x 15 μm in vivo (72 x 9 μm to 110 x 15 μm in protargol-stained cells). The body is twisted, often even multiple times, creating the impression of two strangled spots in the anterior and posterior part of the cell. However, we have recorded also cells lacking this conspicuous body torsion and strangulation, and also the fixation while using protargol staining techniques causes the cells to untwist. The macronucleus is very long and can be tangled in the cell, in the tangled form it measures ca 20 – 30 μm, but the real length of the twisted macronucleus is over 40 um. The AZM is relatively short compare to the body size, ca 13 – 29 μm long and contains 15 – 17 membranelles. The paroral membrane is well visible (4 x 10 μm). The perizonal area contains only three kineties. There are two to four long caudal cilia present, reaching up to 14 μm. The posterior contractile vacuole is small.

***Metopus* sp. 11** (Fig. 29)

Strain: **SALKALUZ**

We have obtained only a single strain from this species, and we have never encountered it otherwise. It is cultivated in medium for other marine ciliates; however, we are not sure about the marine origin of this ciliate (see Discussion). The cells are 65 x 20 μm to 95 x 50 μm (35 x 10 μm to 65 x 16 μm in protargol-stained cells). The elongate macronucleus is tangled into a U or J shape, and its size is ca 13 x 2 μm to 22 x 4.5 μm. The micronucleus is placed on the top of the anterior end of the macronucleus. The macronucleus itself is often constricted so it creates an impression of two macronuclei. The AZM measures ca 70 μm (18 – 30 μm in protargol-stained cells) and contains 15 – 19 membranelles. The paroral membrane is 20 – 30 μm long. The perizonal area contains four kineties. The posterior contractile vacuole is present. These ciliates possess eight to ten very long caudal cilia, reaching up to 42 μm.

***Caenomorpha* spp.** (Fig. 30)

We have cultured two strains (**BEAVERC, BHARA**) of *Caenomorpha* spp. for over a year. We have not been able to determine the species morphologically, nor assess molecular data. *Caenomorpha* of strain **BEAVERC** resembled the most *Caenomorpha uniserialis* described also in Kahl (1932). The strain **BHARA** did not resemble any known *Caenomorpha* species. Both the species were rapidly moving, medium sized ciliates with a spiky posterior end.

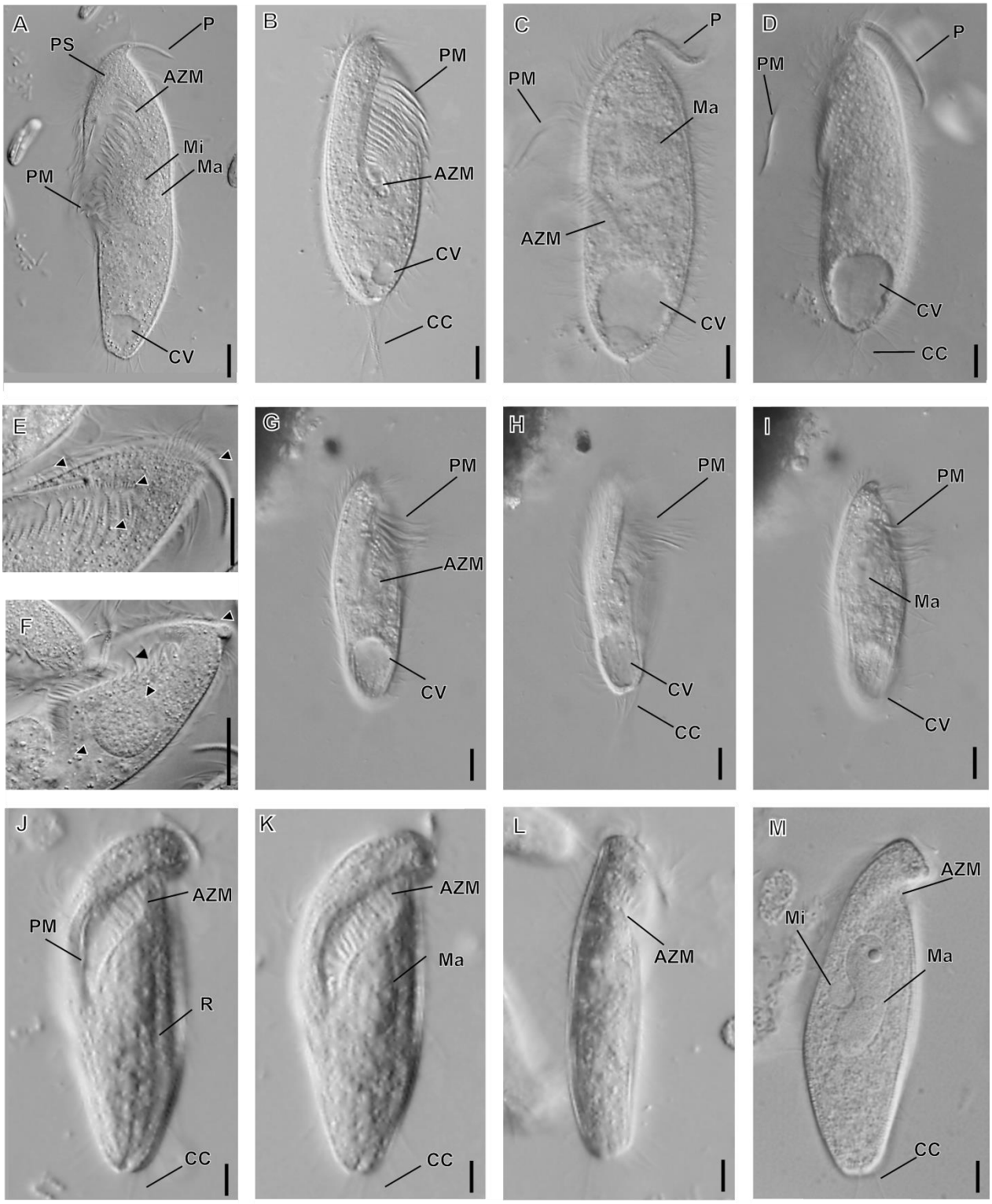

Fig. 8. Genera *Metopus*, *Bothrostoma;* A, B - *Metopus nasutus* (syn. *Metopus vexiliger*), strain IZR; C, D - *Metopus nasutus* (syn. *Metopus vexiliger*), strain TIKAL1; E, F - detail on oral structures, strain IZR, G, H, I - *Bothrostoma* sp. (*B. mirabilis*), strain TIKAL2; J - M - *Metopus bothrostomiformis*, strain BEAVER5; legend: AZM - adoral zone of membranelles; CC - caudal cilia; CV - contractile vacuole; Ma - macronucleus; Mi - micronucleus; P - protrusion, PM - paroral membrane, PS - perizonal stripes; scale bars - 10 μm.

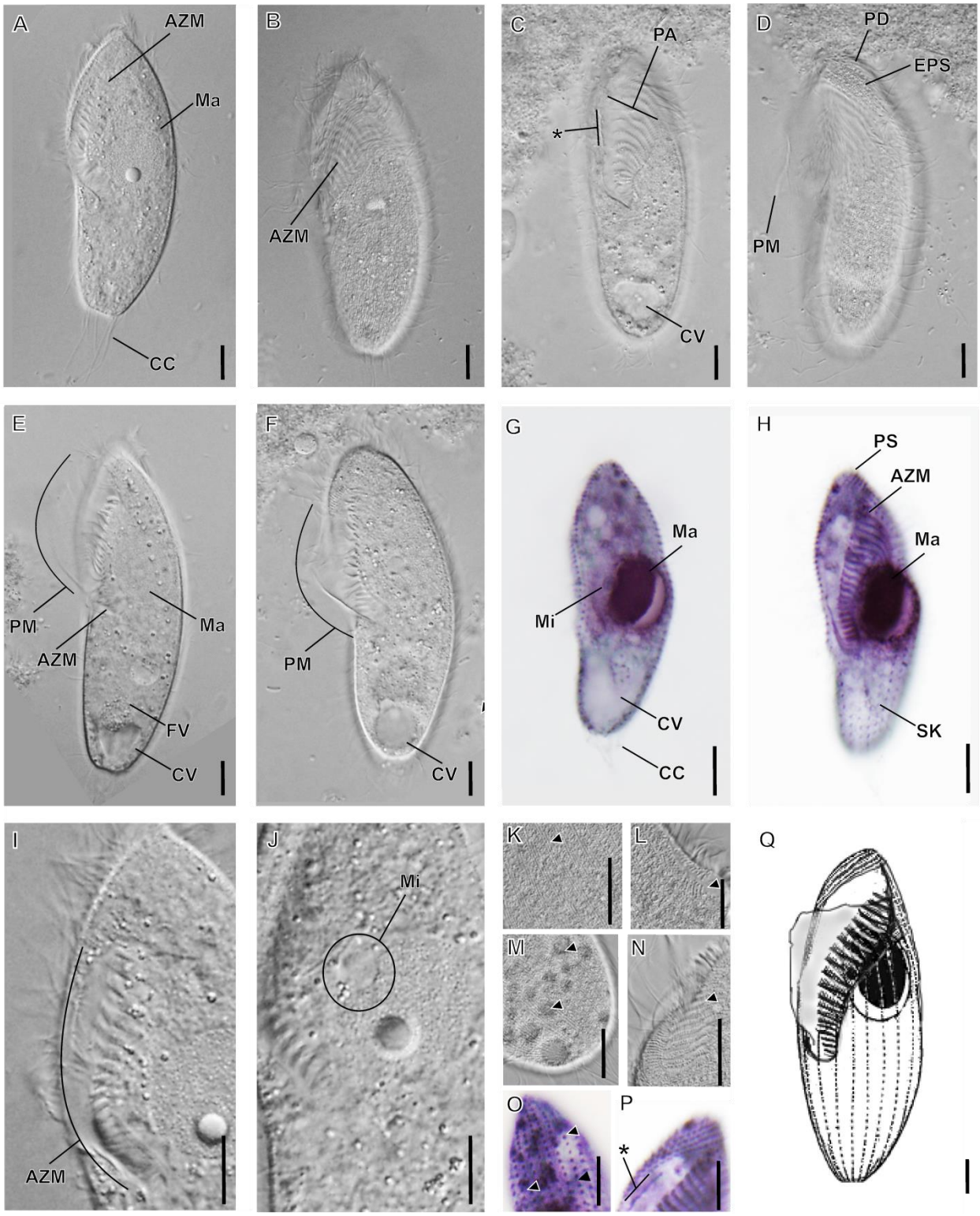

Fig. 9. Genus *Bothrostoma*. A, B - *Bothrostoma undulans*, strain SUSBARBBO, living cell; G - protargol stained cell; H - M - details (H - squished cell, cortical structures; I - squished cell, oral structure; J - protargol stained cell, anterior cell part, vacuoles, black arrowhead somatic kineties; K - squished cell, particles; L - protargol stained cell, paroral membrane; M - squished cell, adoral zone of membranelles; legend: AZM - adoral zone of membranelles; CC - caudal cilia; CV - contractile vacuole; EPS - end of perizonal stripe, FV - food vacuole, Ma - macronucleus; Mi - micronucleus; PA - wide perizonal area, PD - preoral dome, PM - paroral membrane, PS - perizonal stripe, SK - somatic kinety, *- microtubular structure; scale bars 10 μm.

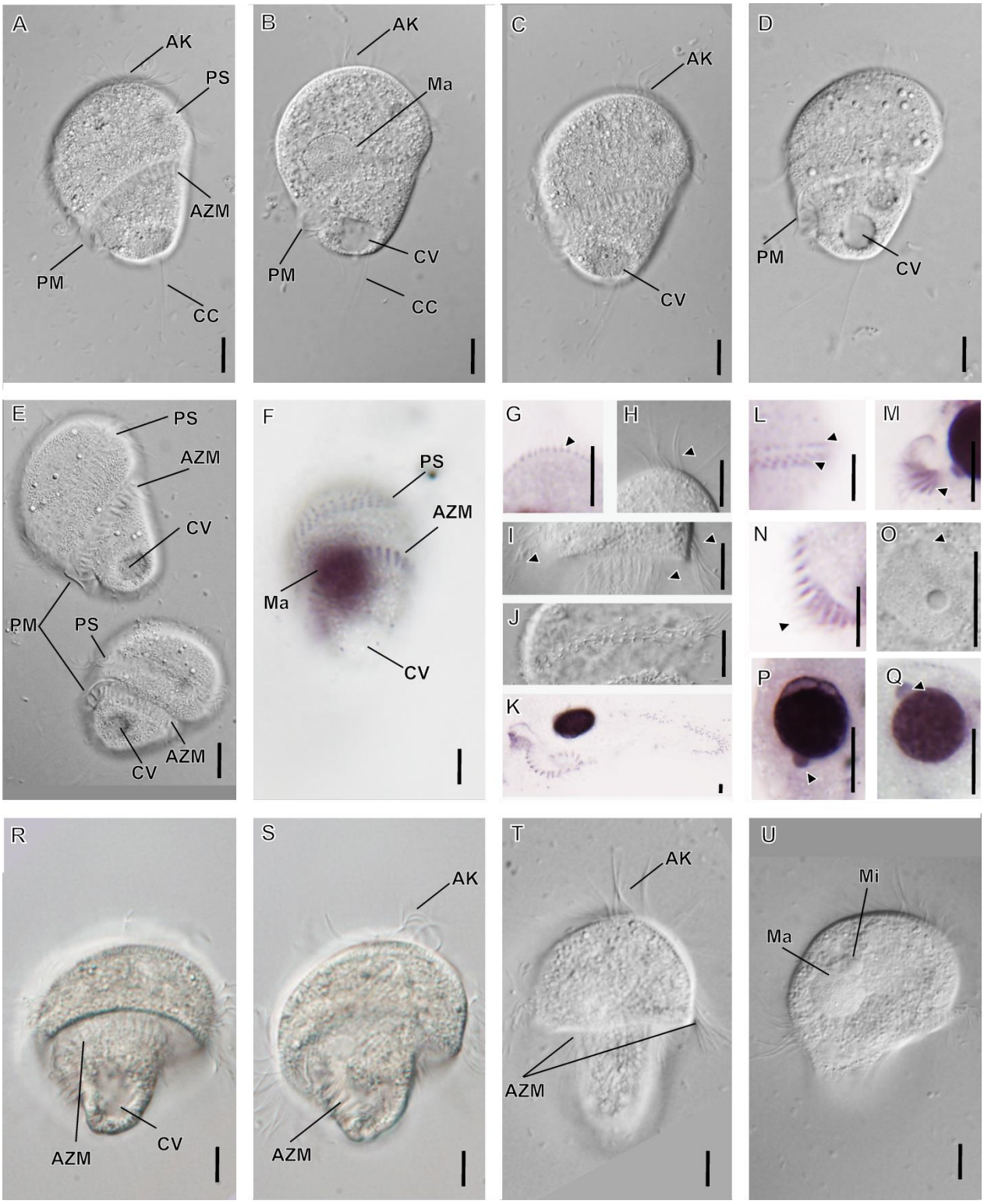

Fig. 10. Genus *Brachonella*, *Brachonella* sp.1, A - Q strain SUSBARB; A - E living cell; F - protargol stained cell; G - detail on AK (arrowhead), protargol stained cell; H - detail on AK (arrowhead), living cell; I - detail on AZM (arrowheads), living cell, J - detail on AZM (arrowheads), dying cell, K - losen cortical structures, protargol stained cell, M, N - detail on PM (arrowheads), protargol stained cell; O - detail on macronucleus and micronucleus (arrowheads), living cell; P, Q - detail on macronucleus and micronucleus (arrowheads), protargol stained cell, R, S - strain LISTOPADBC; T, U - strain REU1; legend AK - anterior kinety, AZM - Adoral zone of membranelles; CC - Caudal Cilia; CV - contractile vacuole; Ma - Macronucleus; Mi - micronucleus; PM - paroral membrane, PS - Perizonal stripe; scale bars 10 μm.

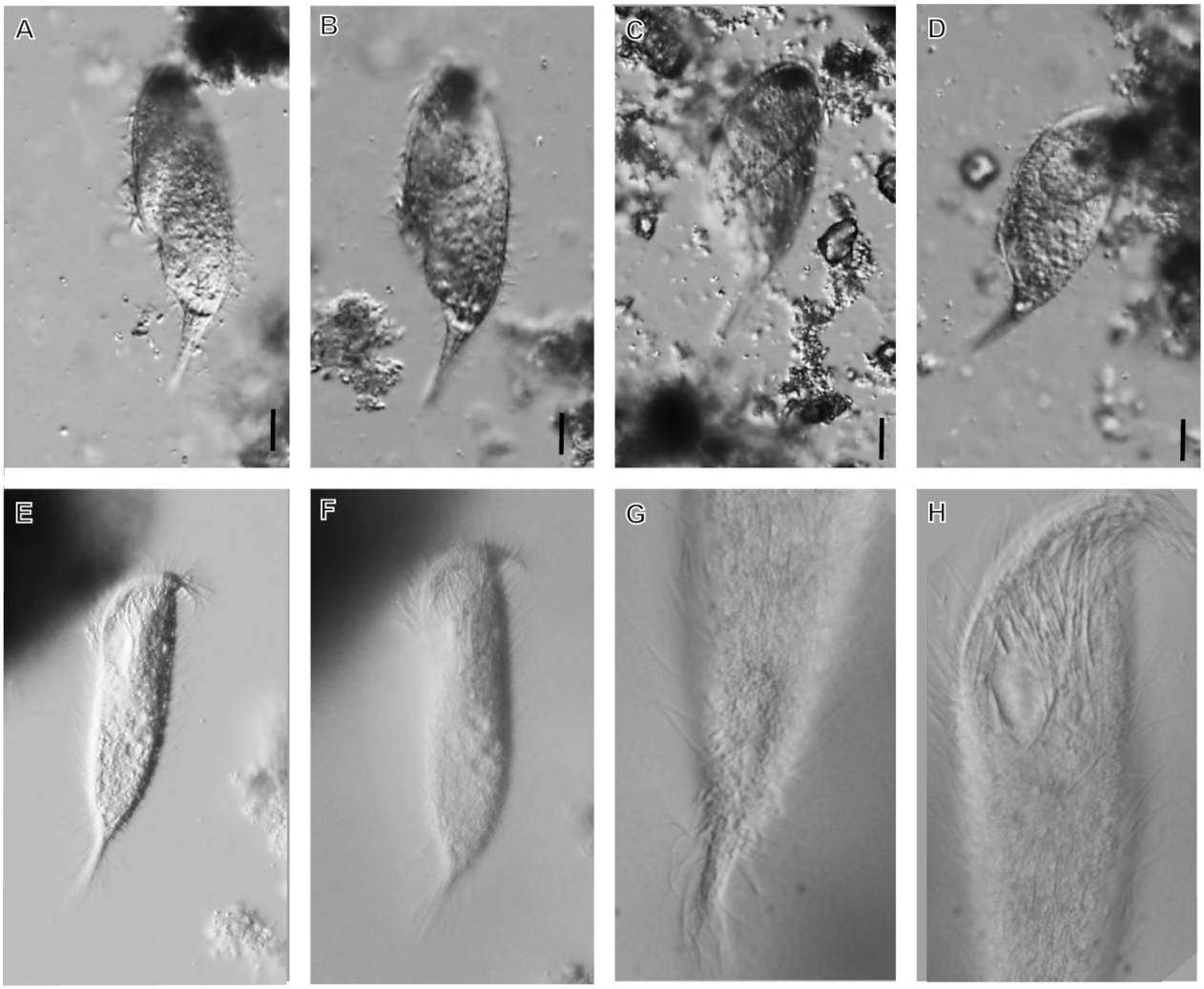

Fig. 11. Genus *Metopus, Brachonella*. A, B C, D - *Brachonella darwini,* strain TIKAL**3**; E, F, G, H - *Metopus spinosus*, strain TIKAL2 (scale unknown), all living cells; scale bar 10 μm.

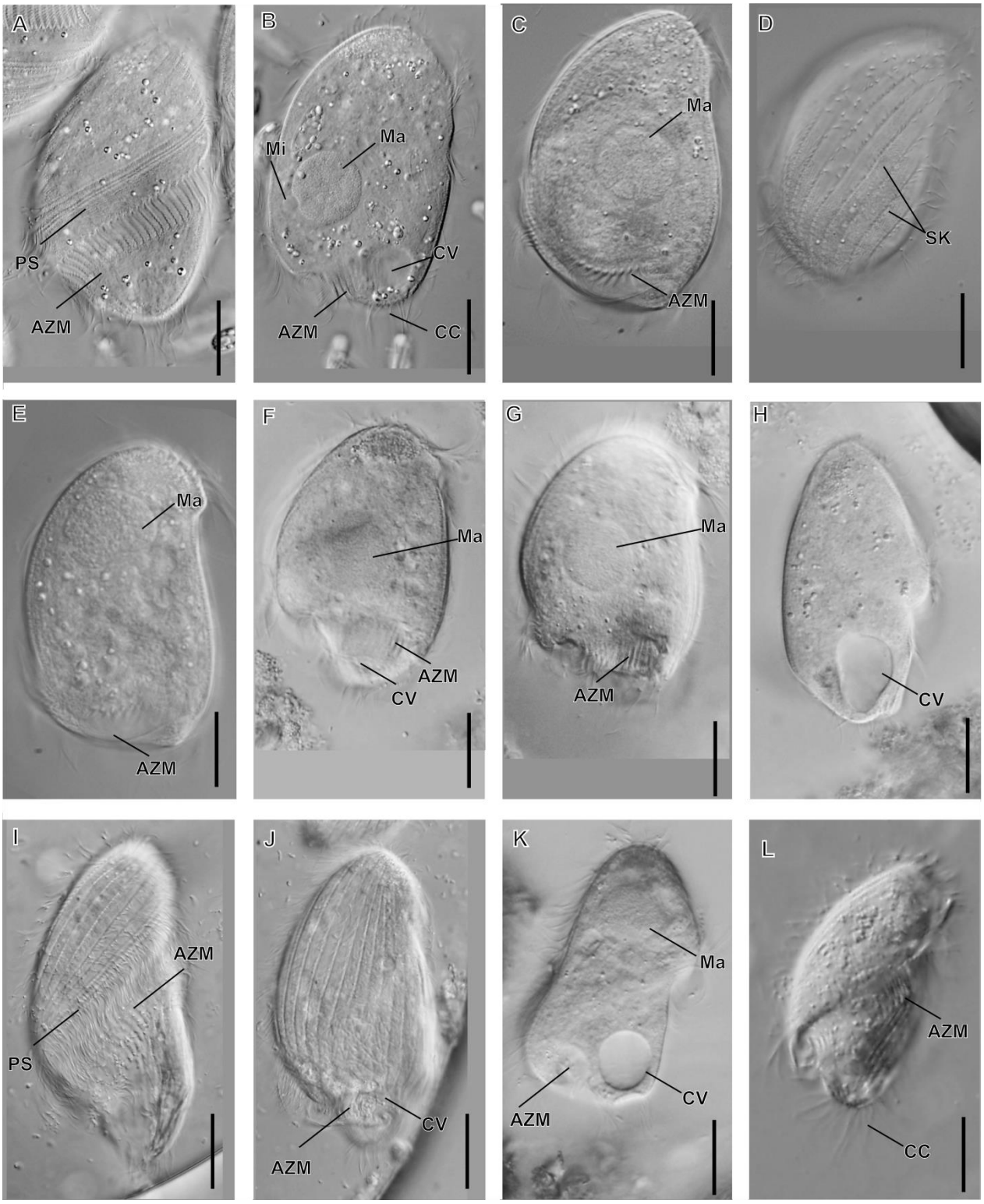

Fig. 12. Genus *Brachonella*, *Brachonella spiralis*, living cells, A, B - strain IZRB; C, D - strain RIOTB; E - strain GDUKABASB; F, G, H, strain SAMOSIR; I, J - strain SIBOLANGIT, K- strain LISTOPADBS, L - strain TIKAL4; legend: AZM - adoral zone of membranelles; CC - caudal cilia; CV - contractile vacuole; Ma - macronucleus; Mi - micronucleus; PS - perizonal stripe, SK - somatic kineties; scale bar 10 μm.

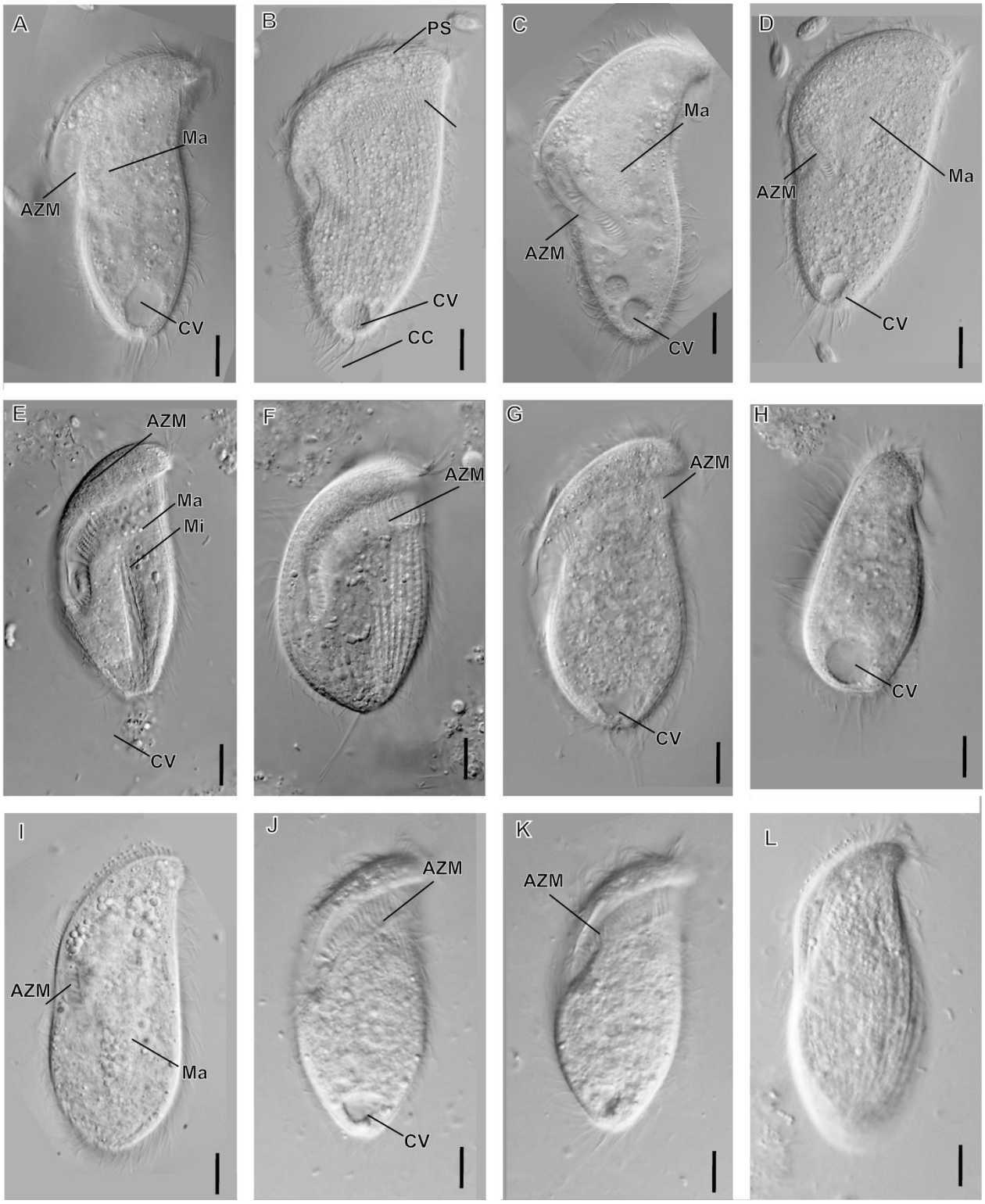

Fig. 13. Genus *Metopus*, A, B, C, D- *Metopus contortus*, strain SIPEK1C; E, F, G, H, I, J, K, L - *Metopus nivaaensis*, strain KAO; QDU, COLOMBIA; legend: AZM - adoral zone of membranelles; CC - caudal cilia; CV - contractile vacuole; Ma - macronucleus; Mi - micronucleus; scale bar 10 μm.

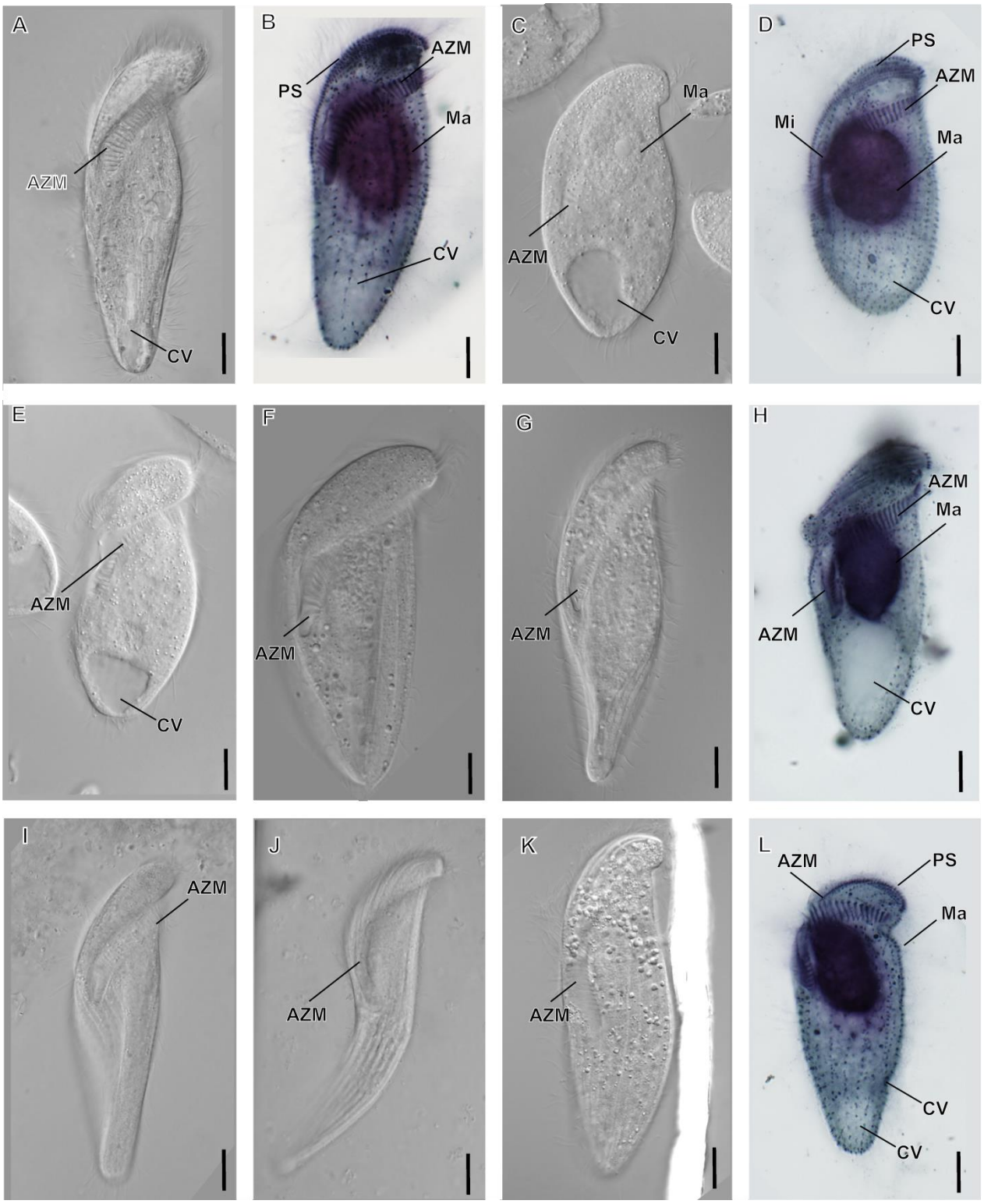

Fig. 14. Genus *Metopus*, *Metopus es*, A - strain TROODOS, living cell; B - strain TROODOS, protargol stained cell, C - strain KALALAO, living cell, D - strain KALALAO, protargol stained cell; E - strain LUOBIAO, living cell, F - strain GDUKABAS living cell; G - strain STRM2, living cell; H - strain STRM2, protargol stained cell; I - strain NELSON2, living cell; J, K - strain STRPJEZ, living cell; L - strain STRPJEZ, protargol stained cell; legend: AZM - adoral zone of membranelles; CC - caudal cilia; CV - contractile vacuole; Ma - macronucleus; Mi - micronucleus; scale bar 10 μm.

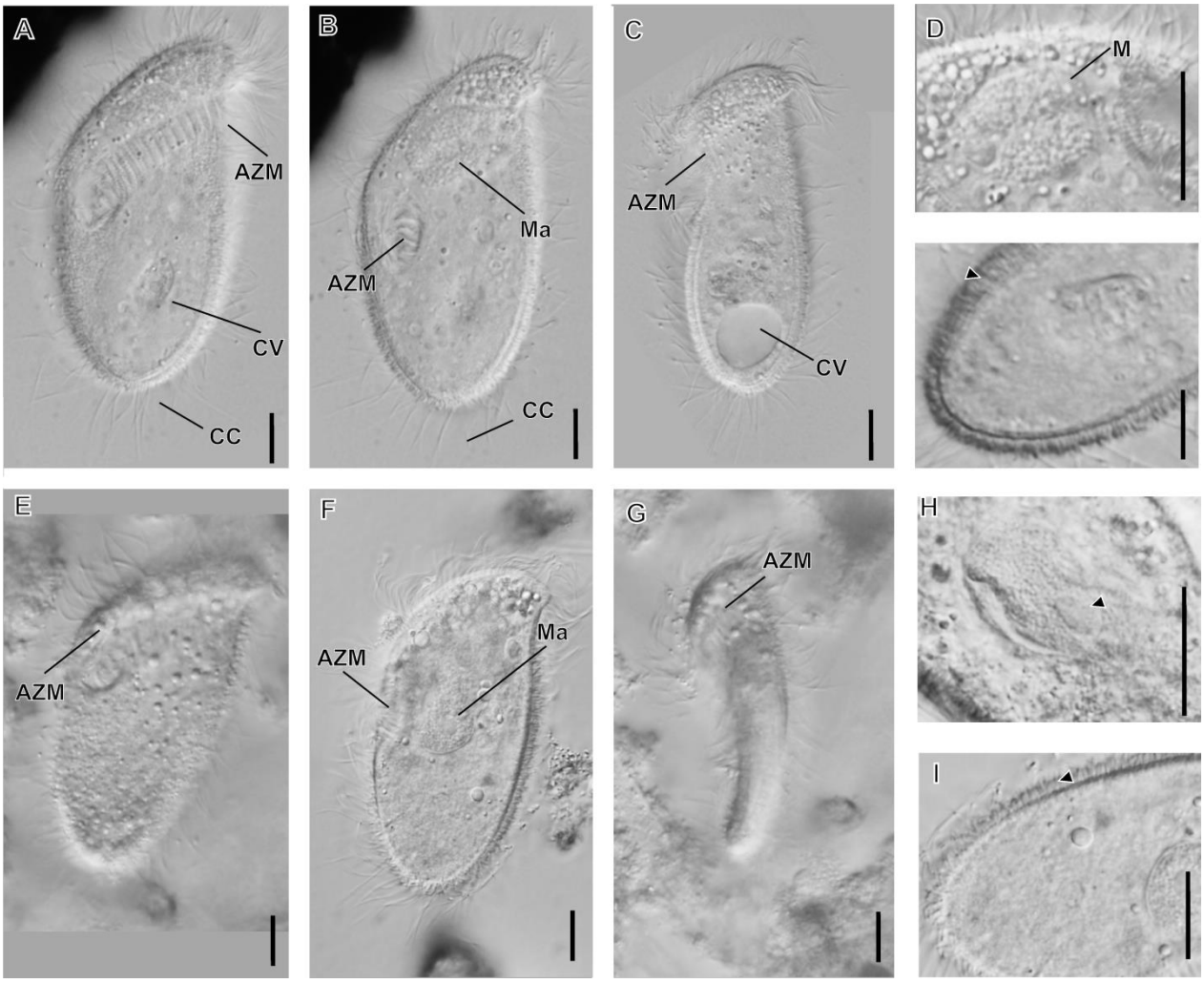

Fig. 15. Genus *Metopus*, *Metopus halophila*, A, B, C - strain LAGUNDRI; D - strain LAGUNDRI, detail on ectosymbionts; E, F strain REU4B, living cell; G - strain REU4B - lateral view; legend: AZM - adoral zone of membranelles; CC - caudal cilia; CV - contractile vacuole; Ma - macronucleus; Mi - micronucleus; scale bars 10 μm.

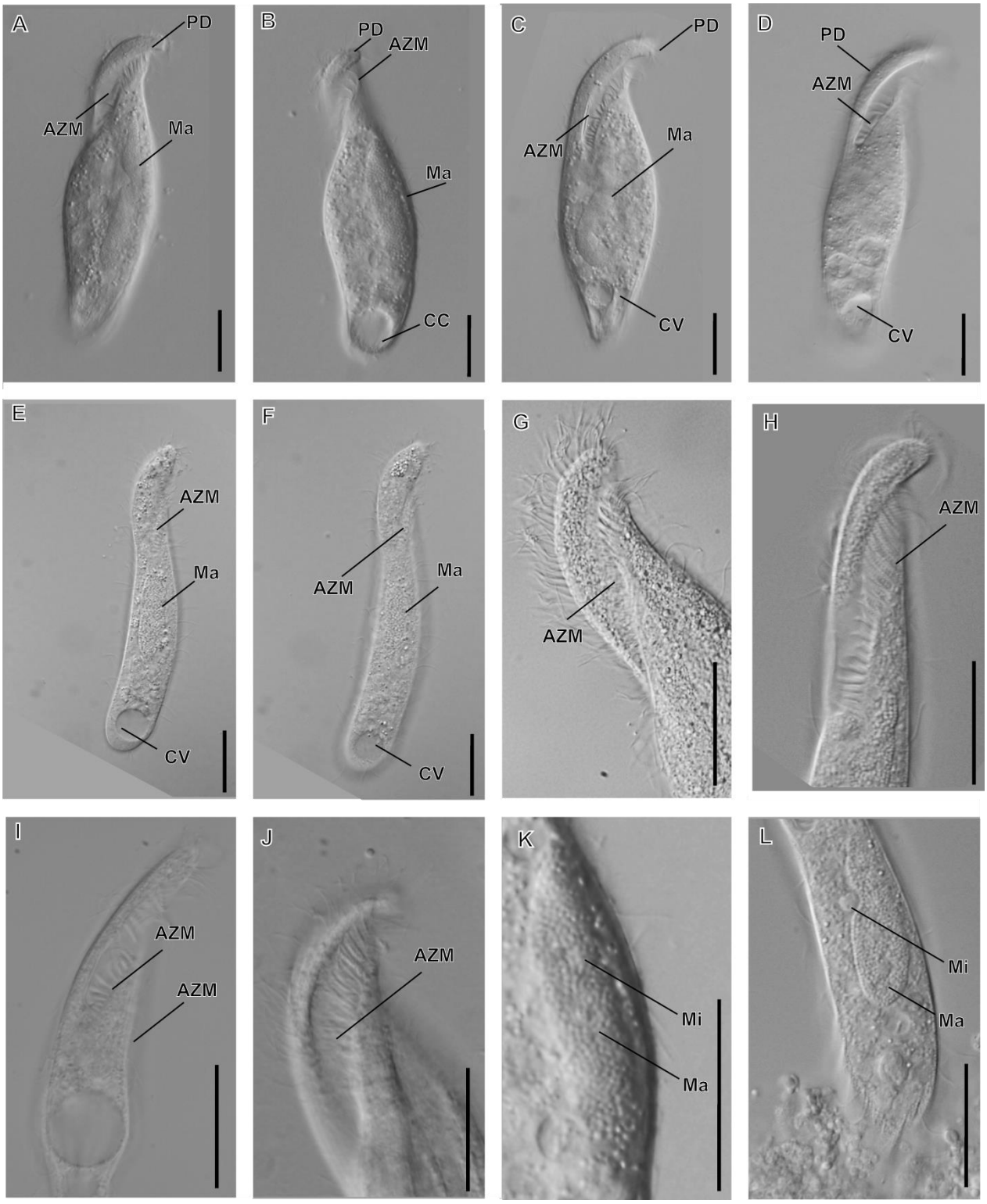

Fig. 16. Genus *Metopus*; *Metopus laminarius*, A, B, C, D - strain LISTOPADM, living cells; E, F - strain ALOOA1, living cell; G - J, detail of AZM, living cell; G - strain ALOOA1, H, I - strain VLADECH4, J - strain LISTOPADM, K, L - detail on macronuclei, living cell; K - strain LISTOPADM, L - strain VLADECH4; legend: AZM - Adoral zone of membranelles; CC - Caudal Cilia; CV - contractile vacuole; Ma - Macronucleus; Mi - micronucleus; PD - paroral dome; scale bars - 10 μm.

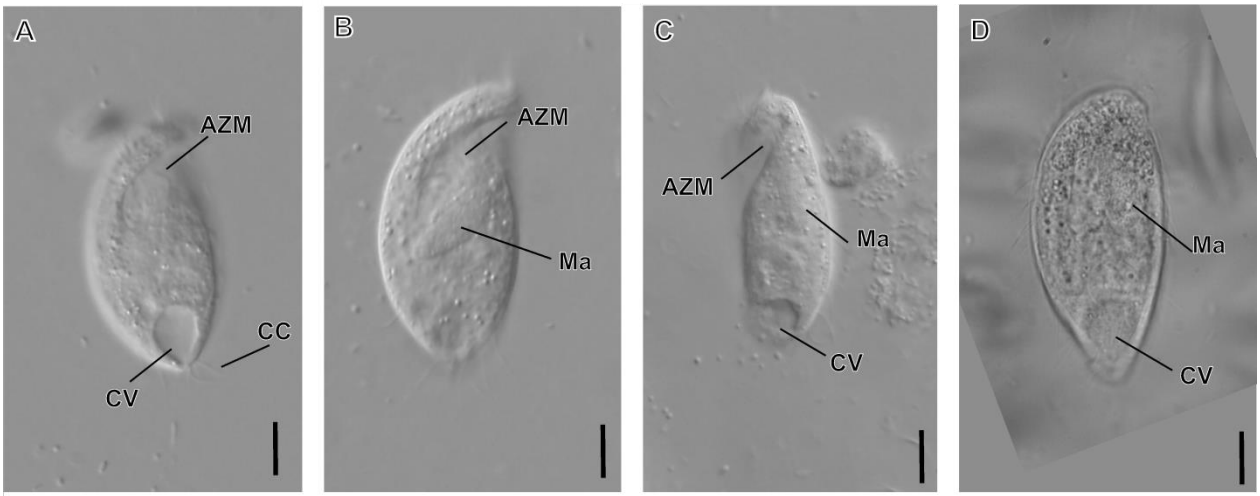

Fig. 17. Genus *Metopus*; *Metopus mucicola*, A, B, C - strain OROBINEC, living cells; D - strain SWAN4B, living cell; legend: AZM - Adoral zone of membranelles; CC - Caudal Cilia; CV - contractile vacuole; Ma - Macronucleus; Mi - micronucleus; PD - paroral dome; scale bar - 10 μm.

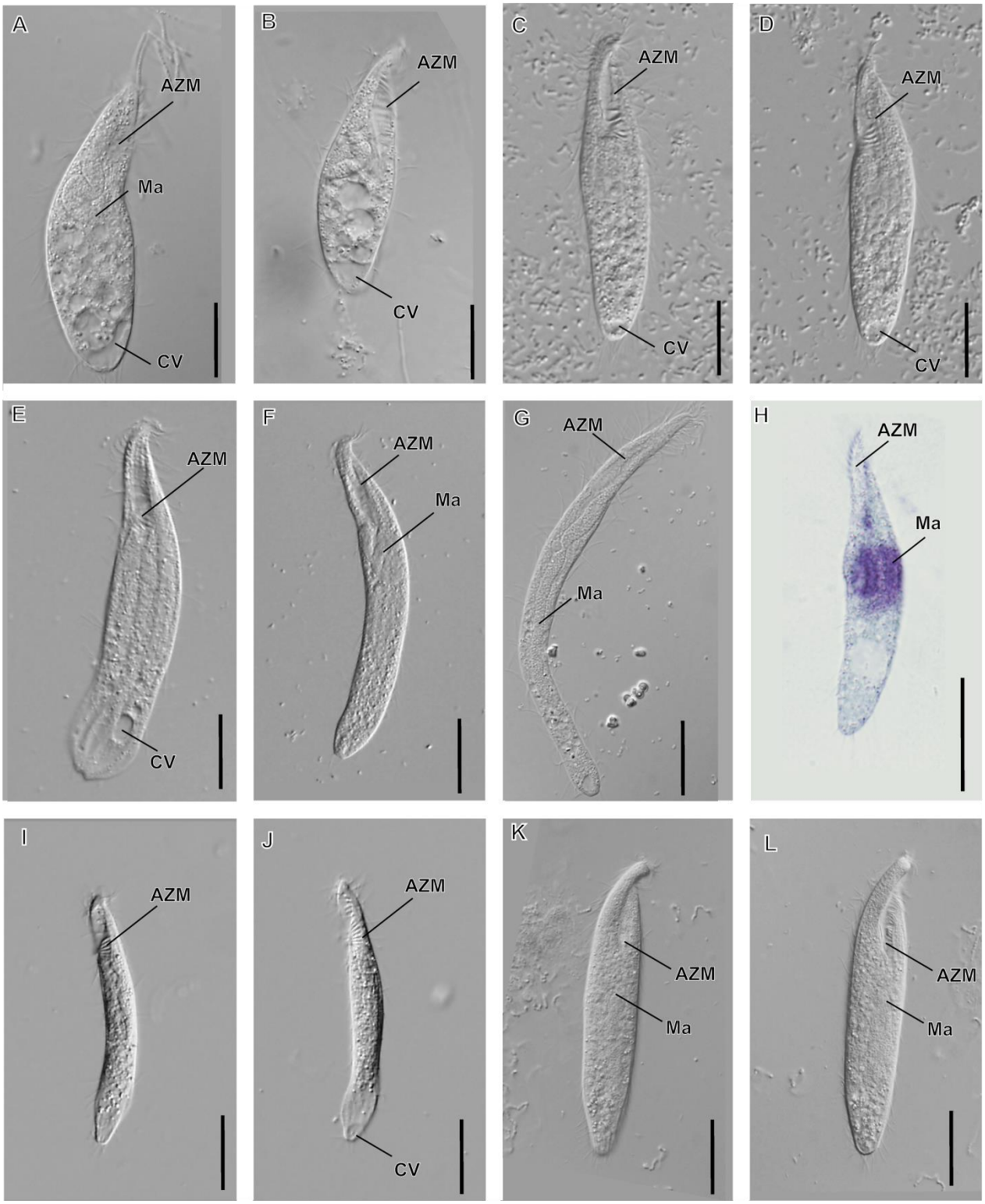

Fig. 18. Genus *Metopus*, *Metopus palaeformis*, A, B - strain MEJONA, living cells; C, D - strain OLSRYB, living cells; E, F, G - strain COLORADA, living cells; H - strain CSS, protargol stained cell; I, J - strain RAJCA; K, L - strain - SULMOK; legend AZM - Adoral zone of membranelles; CC - Caudal Cilia; CV - contractile vacuole; Ma - Macronucleus; Mi - micronucleus; scale bars - 10 μm.

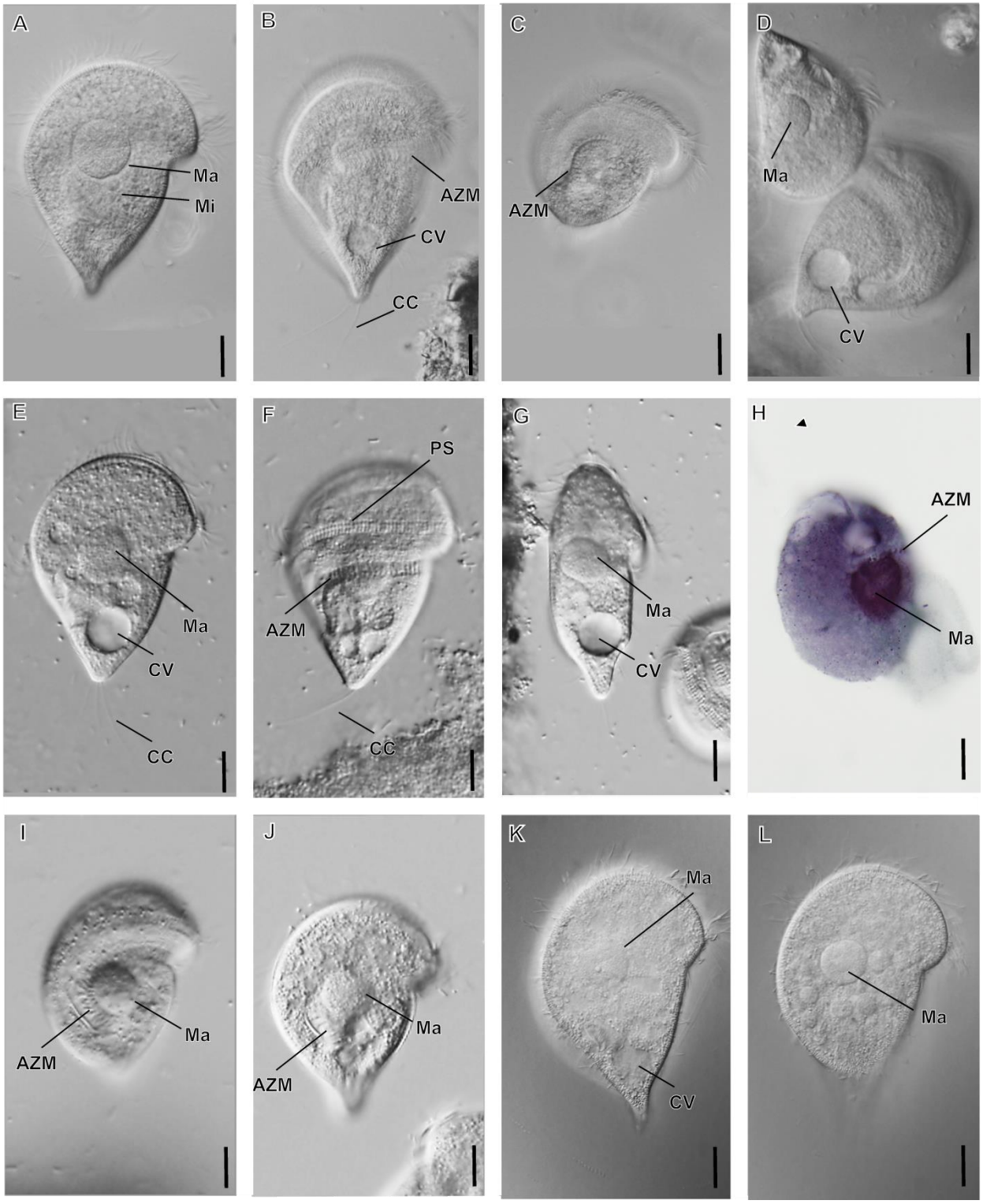

Fig. 19. Genus *Metopus*, *Metopus striatus*; A, B - strain STOVIK; C, D - strain KAMERUN2B; E, F, G - strain CIDLINA, living cell; H - strain STOVIK, protargol stained cell; I - strain CASTILLO, J - strain SCHOONER5S; K - strain RADUN, L - strain 3ELI; legend: AZM - adoral zone of membranelles; CC - caudal cilia; CV - contractile vacuole; Ma - macronucleus; Mi - micronucleus; PS - perizonal stripe; scale bars 10 μm.

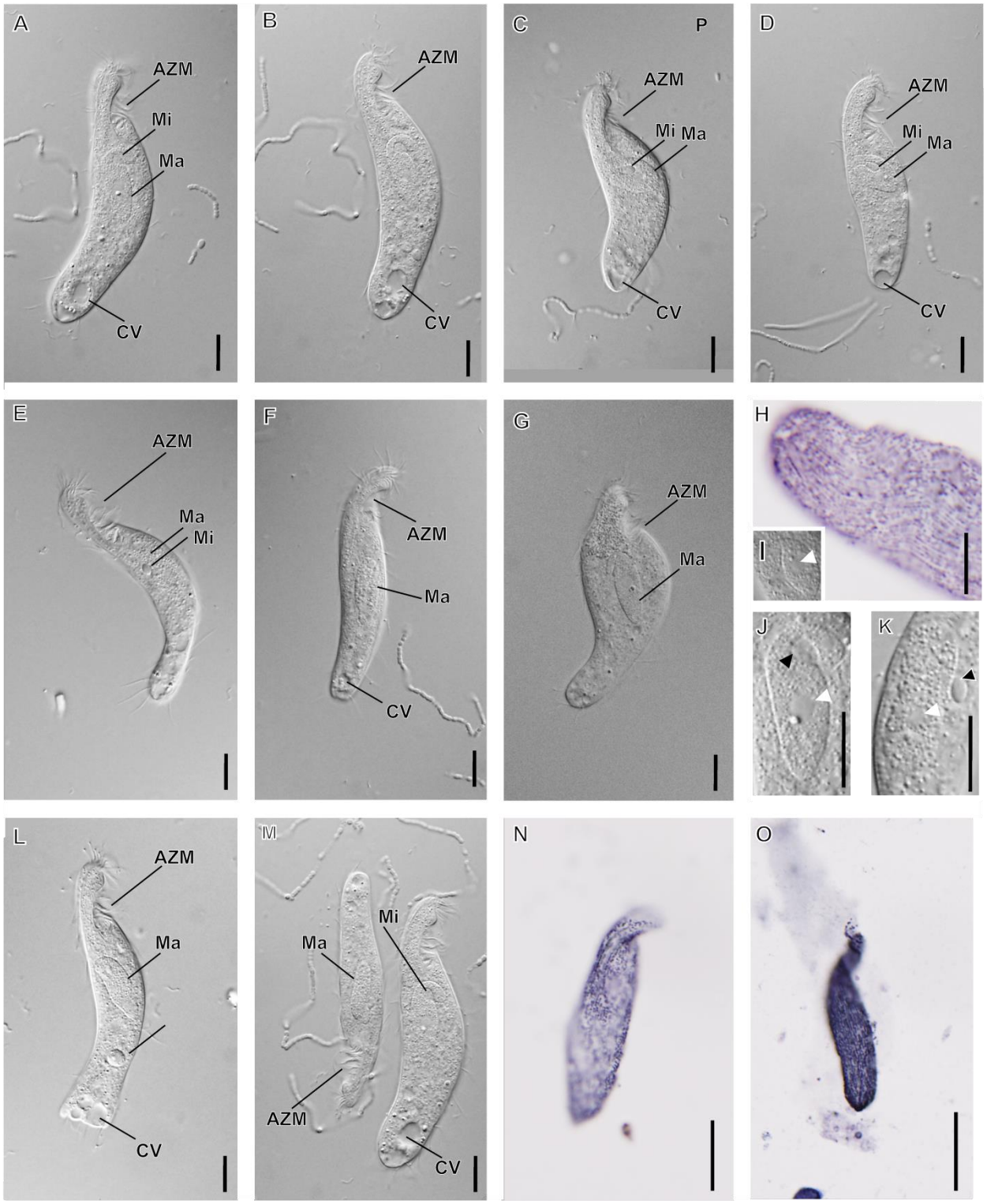

Fig. 20. Genus *Metopus*, *Metopus* sp, 1, strain CANC; A, B, C, D, E, F, G, I, J, K, L, M - living cells; H, N, O - protargol stained cells; H detail on cortical structures; - detail on macronucleus, putative nucleolus - white arrowhead, black arrowhead – micronucleus, legend AZM - Adoral zone of membranelles; CC - Caudal Cilia; CV - contractile vacuole; Ma - Macronucleus; Mi - micronucleus; scale bars 10 μm.

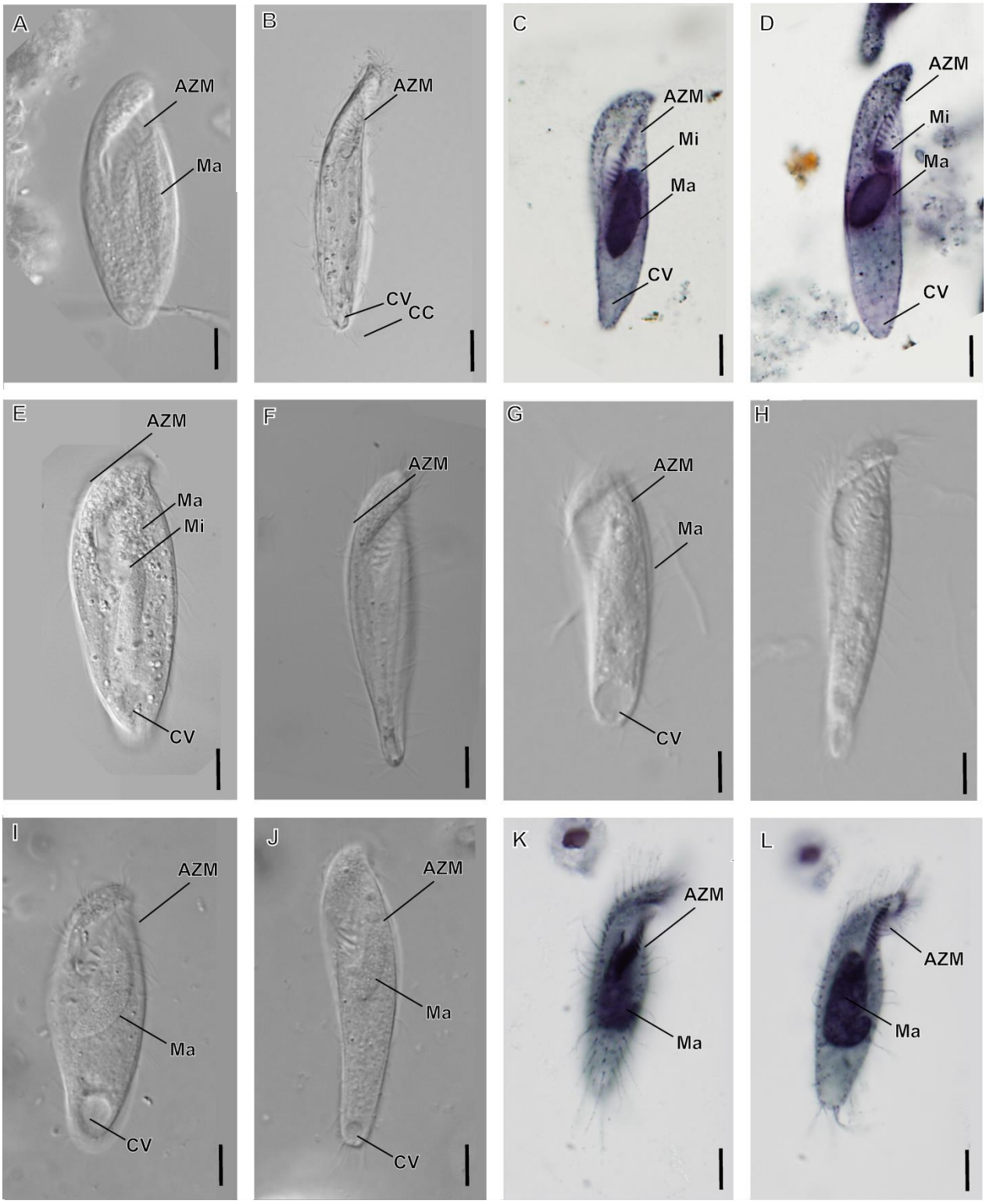

Fig. 21. Genus *Metopus*, *Metopus* sp. 2, A, B - strain RIOZ, living cells; C, D - strain RIOZ, protargol stained cells; E, F - strain RIOTM, living cells; G, H - strain LUKIFOREST, living cells; I, J - strain CHITWANMANLY, living cells; K, L - strain CHITWANMALY, protargol stained cells; legend: AZM - Adoral zone of membranelles; CC - Caudal Cilia; CV - contractile vacuole; Ma - Macronucleus; Mi - micronucleus; scale bar 10 μm.

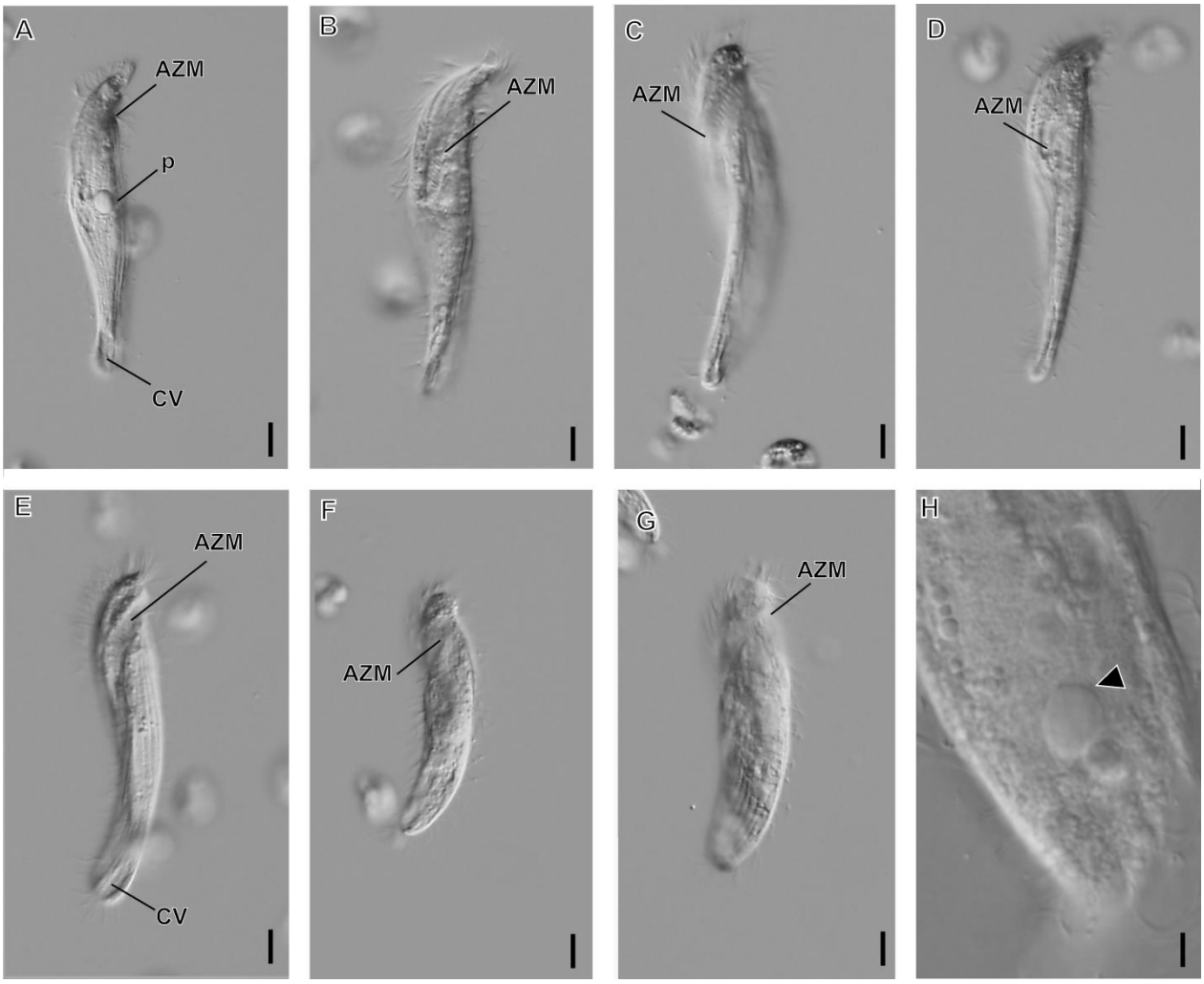

Fig. 22. Genus, A - H - *Metopus* sp. 3, strain KAN2; living cells; legend: AZM - Adoral zone of membranelles; CV - contractile vacuole; p - particle; scale bar 10 μm.

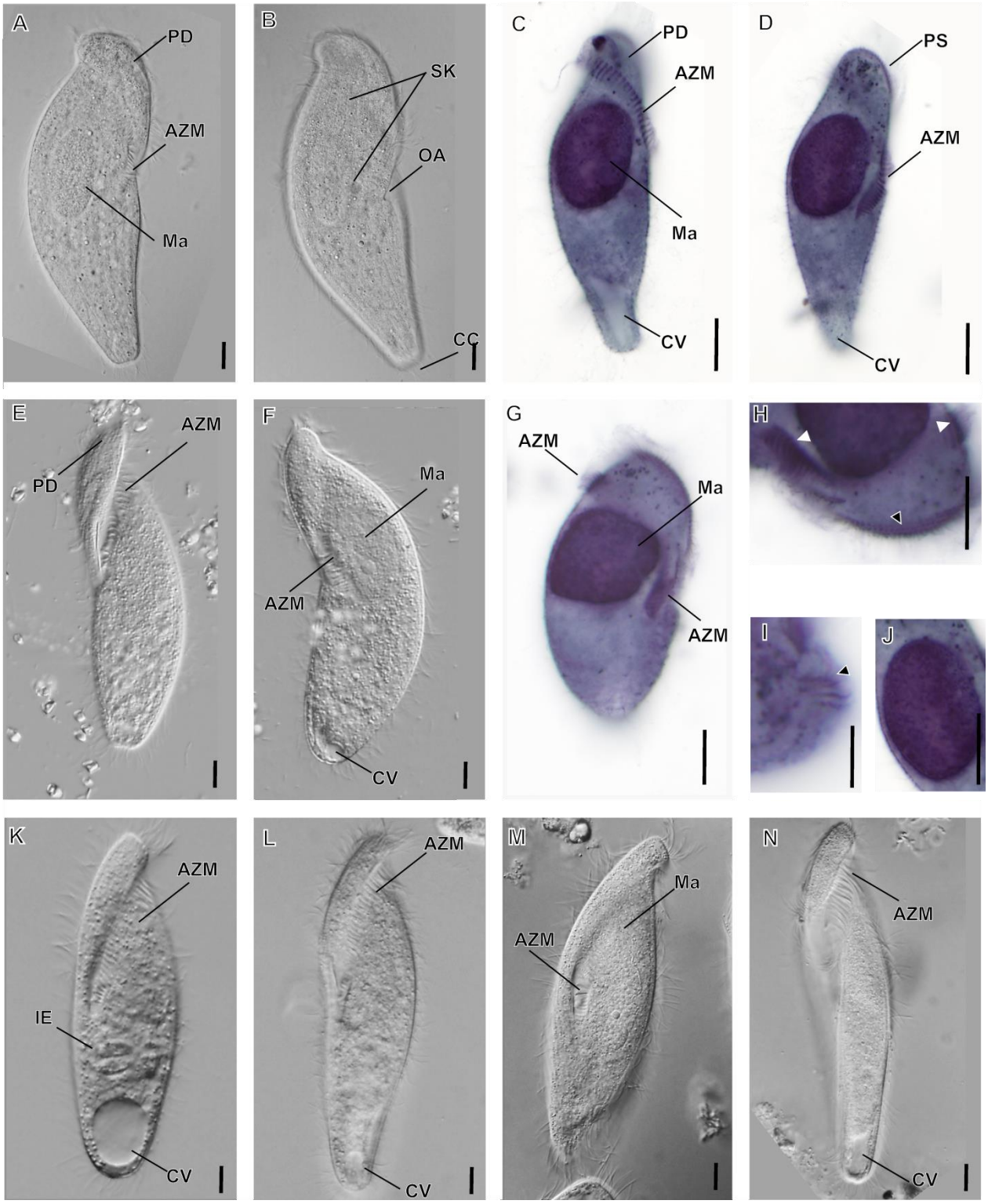

Fig. 23. Genus *Metopus*; *Metopus* sp. 4, A, B - strain CHITWANVELKY, living cells, C, D - strain CHITWANVELKY, protargol stained cells; E, F - strain CHACOL2, living cells; G - strain CHACOL2 - protargol stained cell, H - J - strain protargol stained cells in details, black arrowhead - perizonal stripe, white arrowhead - AZM; J – detail on macronucleus in protargol stained cell; K, L - strain OKREHEK, living cell, M, N - strain STORCH6; legend: AZM - adoral zone of membranelles; CC - caudal cilia; CV - contractile vacuole; IE - ingested eukaryote, Ma - macronucleus; OA – oral opening; PD - preoral dome, PS - perizonal stripe; SK – somatic kineties; scale bars 10 μm.

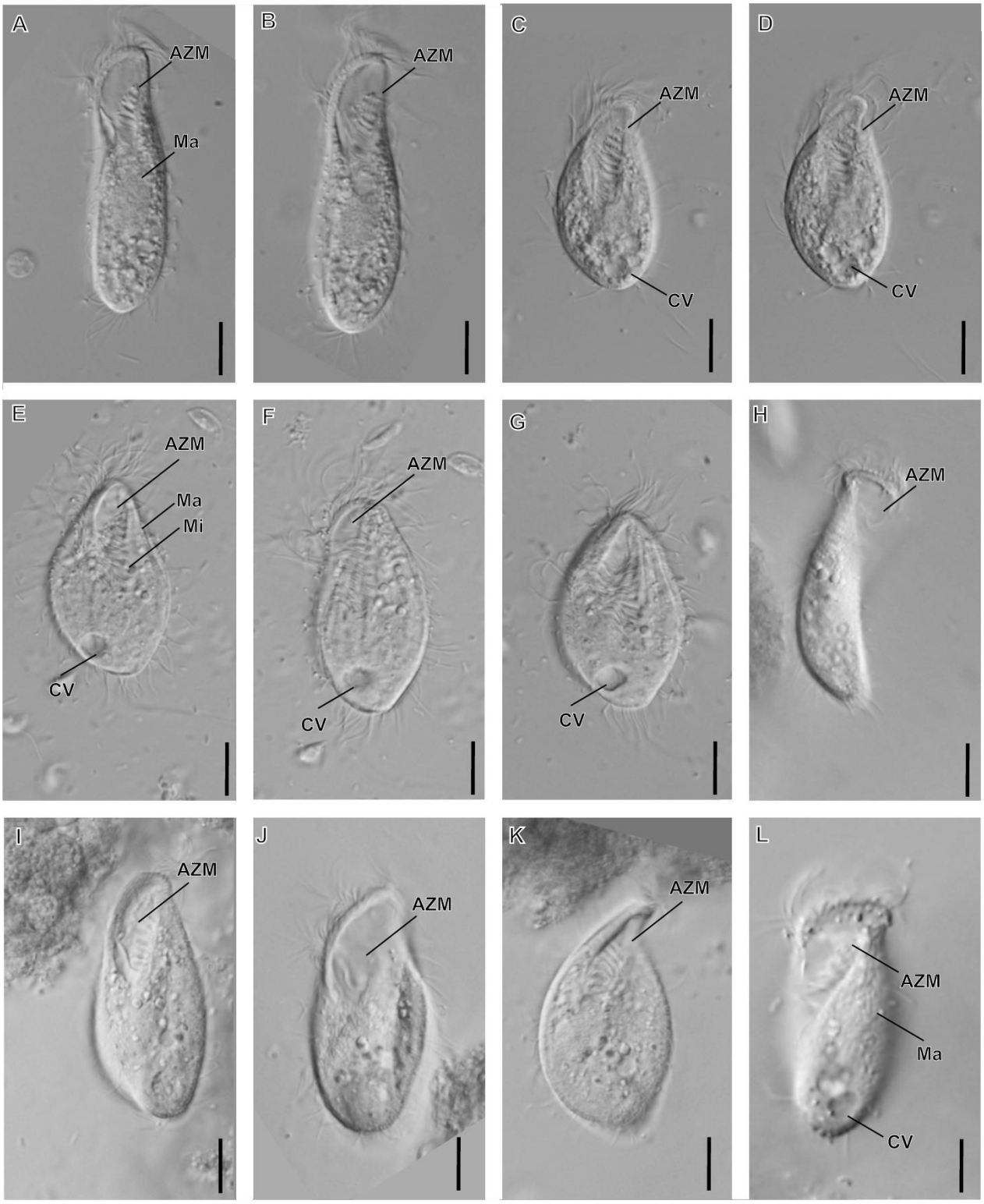

Fig. 24. Genus *Palmarella*, *Palmarella salina*, A, B, C, D - strain BUSSELTON, morphotype 1 (A, B) and 2 (C, D); E, F, G - strain BUSSPRAND; H, I, J, K, L - strain SIPEK1APA, living cell; legend AZM - Adoral zone of membranelles; CC - Caudal Cilia; CV - contractile vacuole; Ma - Macronucleus; Mi - micronucleus; scale bar 10 μm.

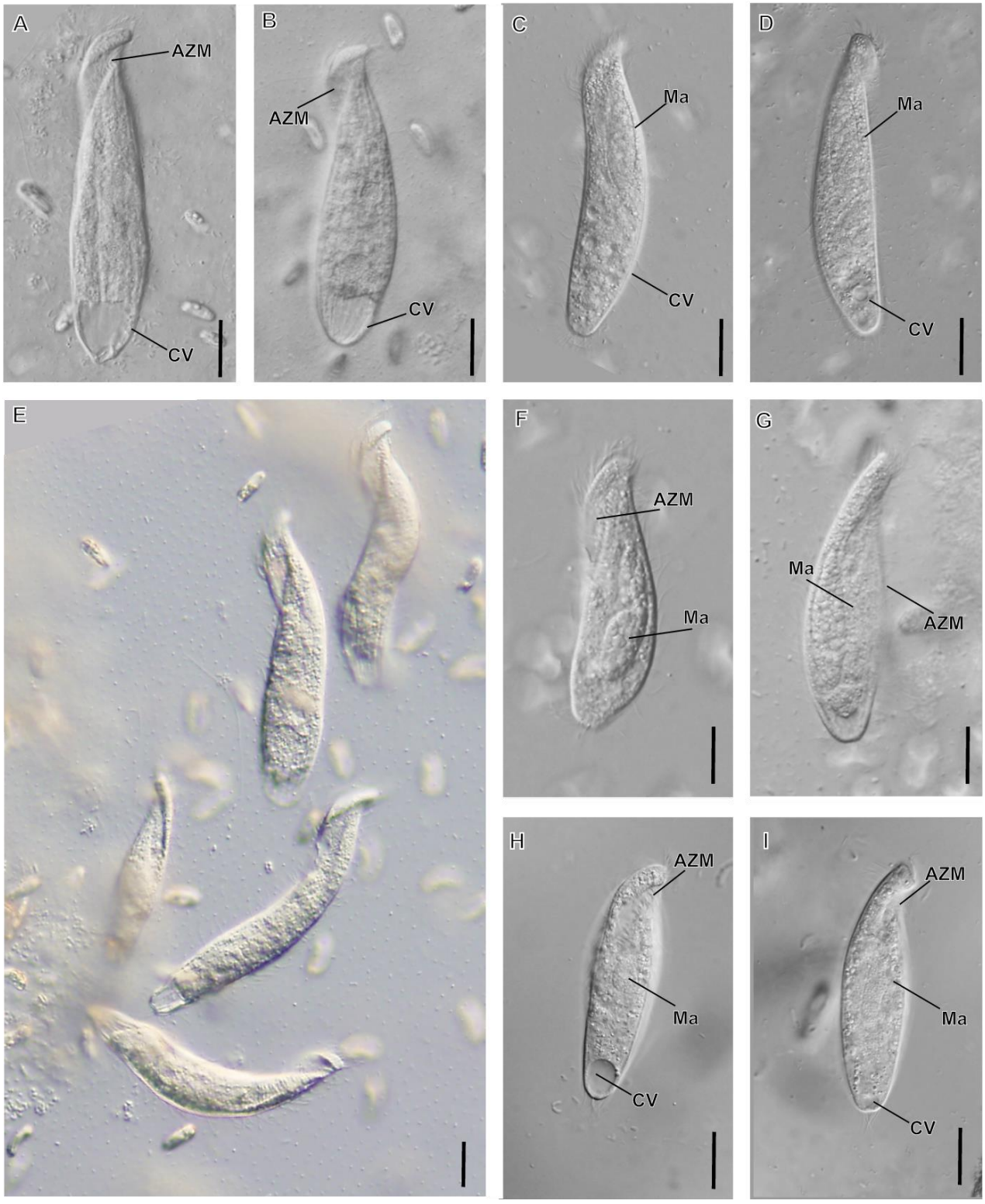

Fig. 25. Genus *Metopus*, A – G - *Metopus* sp. 5, strain QUMBASCHI; living cells; H , I - *Metopus* sp. 6, strain DRINGARI2; legend: AZM - Adoral zone of membranelles; CC - Caudal Cilia; CV - contractile vacuole; Ma - Macronucleus; Mi - micronucleus; scale bars 10 μm.

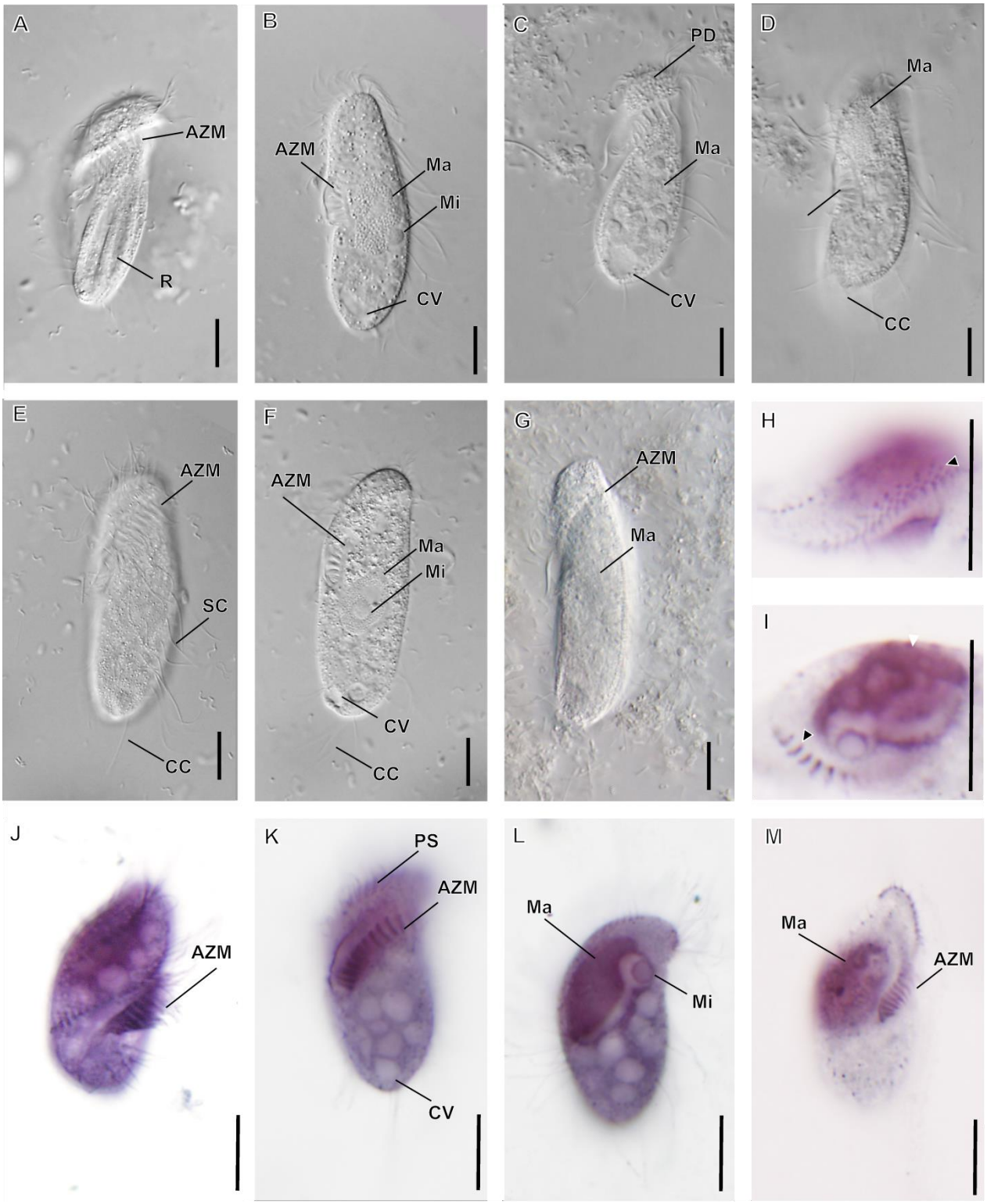

Fig. 26. Genus *Metopus*, *Metopus* sp. 7, strain SULMOK2; A, B C, D, E, F, G living cell; H - M protargol stained cells; H - black arrowhead – perizonal stripe; I – black arrowhead – AZM, white arrowhead – macronucleus; legend – AZM - Adoral zone of membranelles; CC - Caudal Cilia; CV - contractile vacuole; Ma - Macronucleus; Mi – micronucleus, PD – preoral dome; PS – perizonal stripe; R – ridges; scale bars 10 μm.

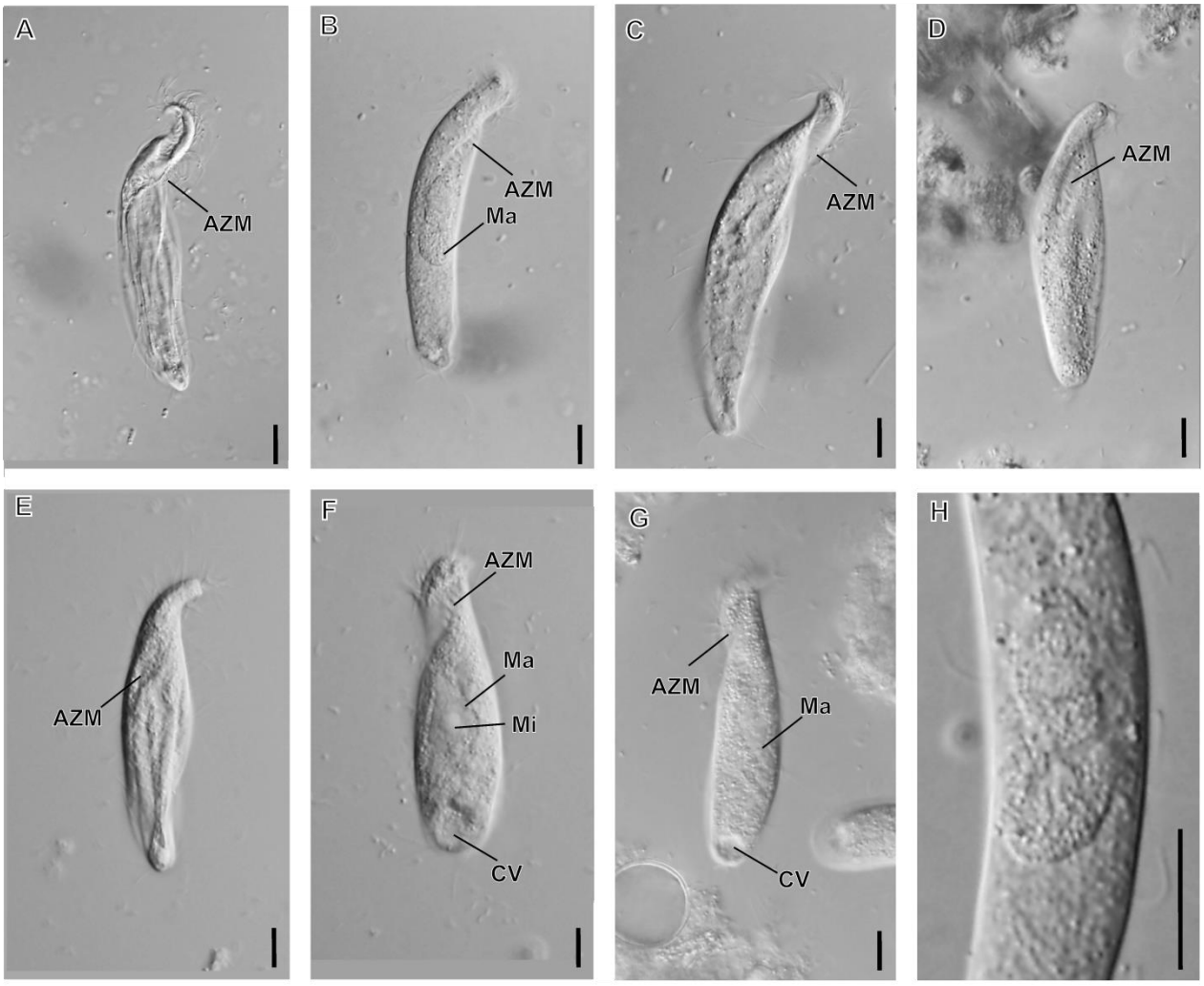

Fig. 27. Genus *Metopus*, A, B, C, D - *Metopus* sp. 8; strain ALOOA3; E, F, G - *Metopus* sp. 9, strain API; H – detail on macronucleus, strain ALOOA3; all living cells; legend AZM - adoral zone of membranelles; CC - caudal cilia; CV - contractile vacuole; Ma - macronucleus; Mi - micronucleus; scale bars 10 μm.

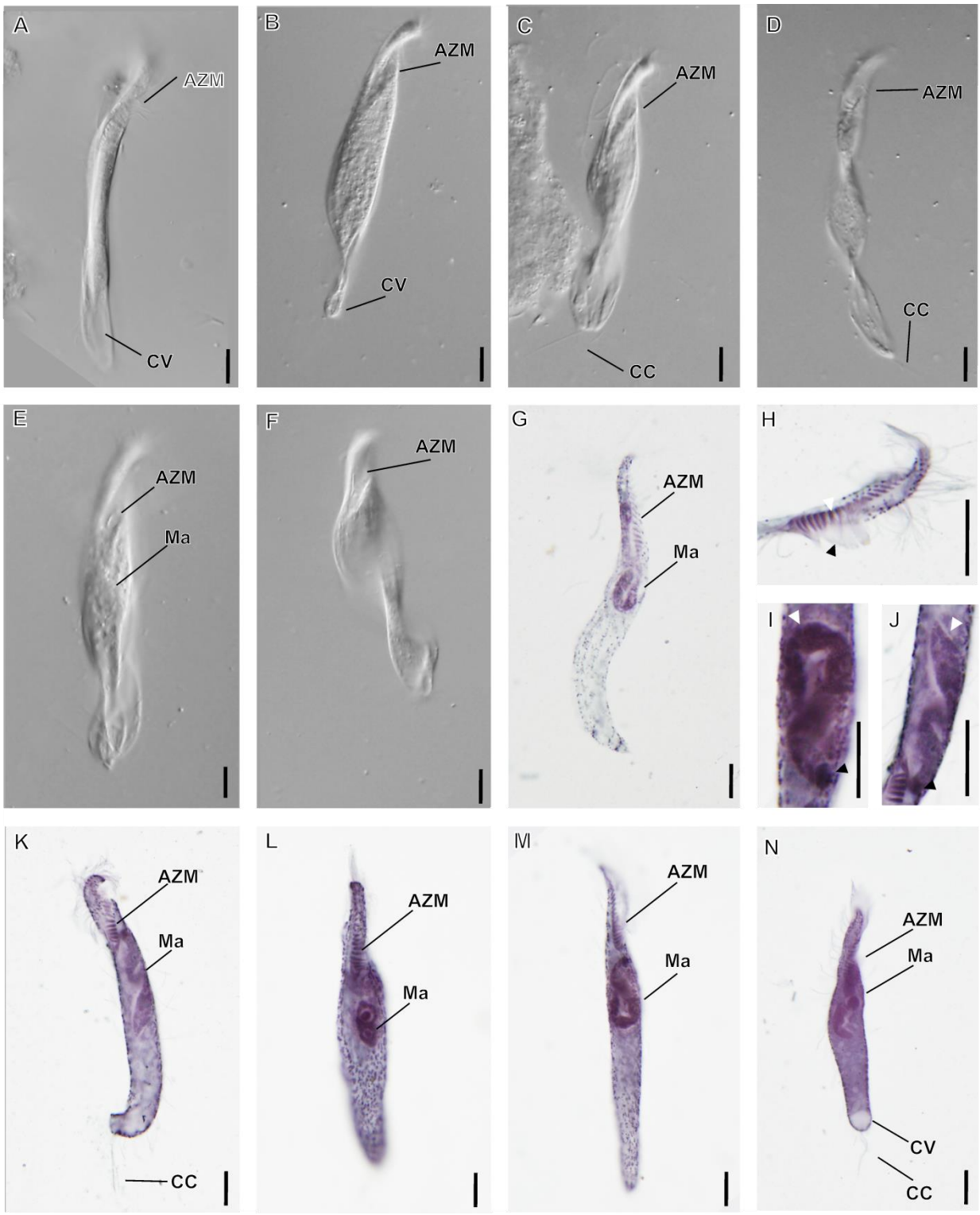

Fig. 28. Genus *Metopus*, *Metopus* sp. 10, strain KORRISSION, A, B, C, D, E, F - living cell; G - N protargol stained cells; H oral part, white arrowhead – AZM, black arrowhead – paroral membrane, I, J – detail on macronucleus (white arrowhead), black arrowhead – micronucleus; legend: AZM - Adoral zone of membranelles; CC - Caudal Cilia; CV - contractile vacuole; Ma - Macronucleus; Mi - micronucleus; scale bars 10 μm.

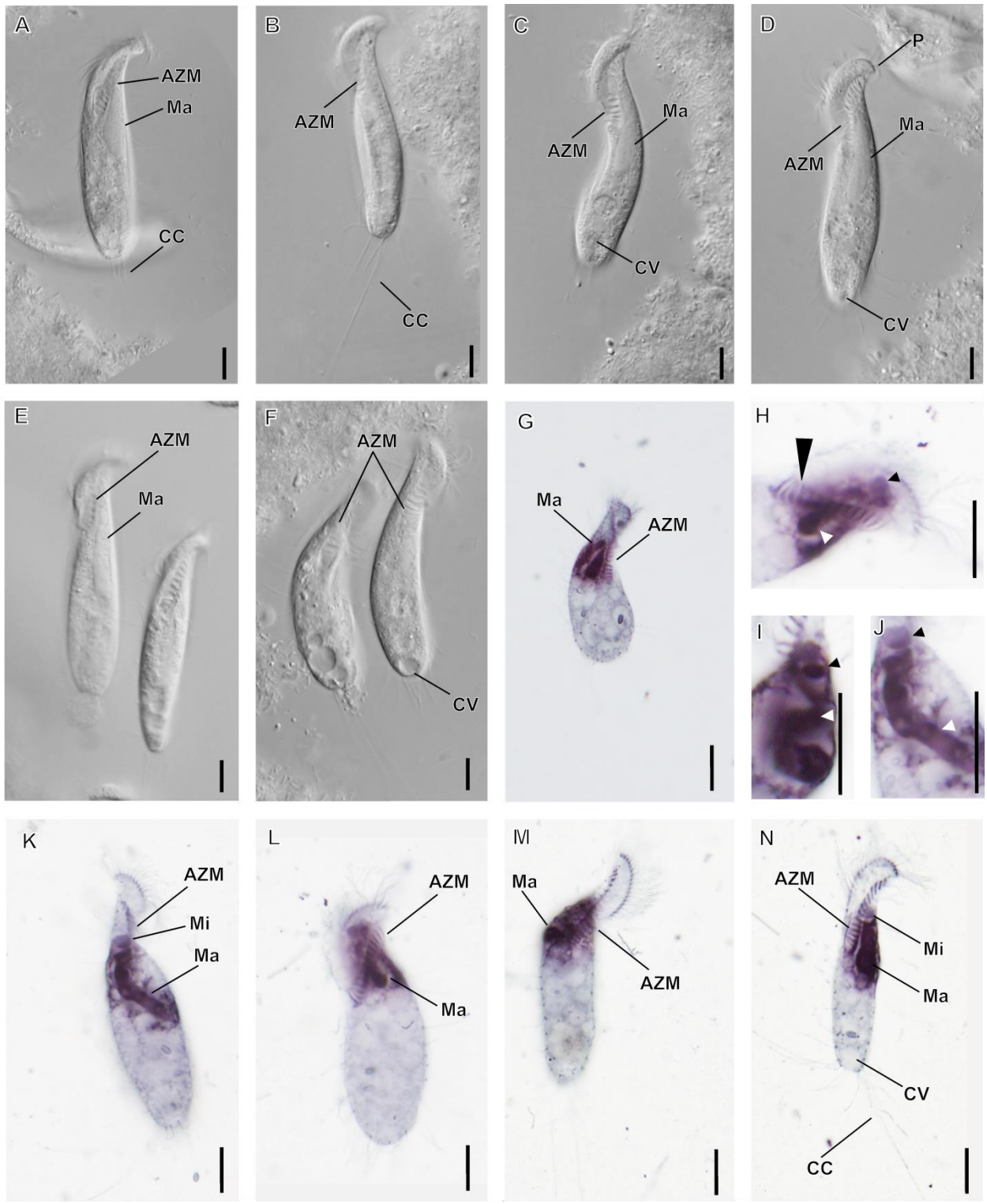

Fig. 29. Genus *Metopus*, *Metopus* sp. 11., strain SALKALUZ; A, B C, D, E, F living cell; G - O protargol stained cell; H, I, J - detail on macronucleus (white arrowhead), black arrowhead – micronucleus, long arrowhead – AZM; legend: AZM - Adoral zone of membranelles; CC - Caudal Cilia; CV - contractile vacuole; Ma - Macronucleus; Mi - micronucleus; P - protrusion; scale bars 10 µm.

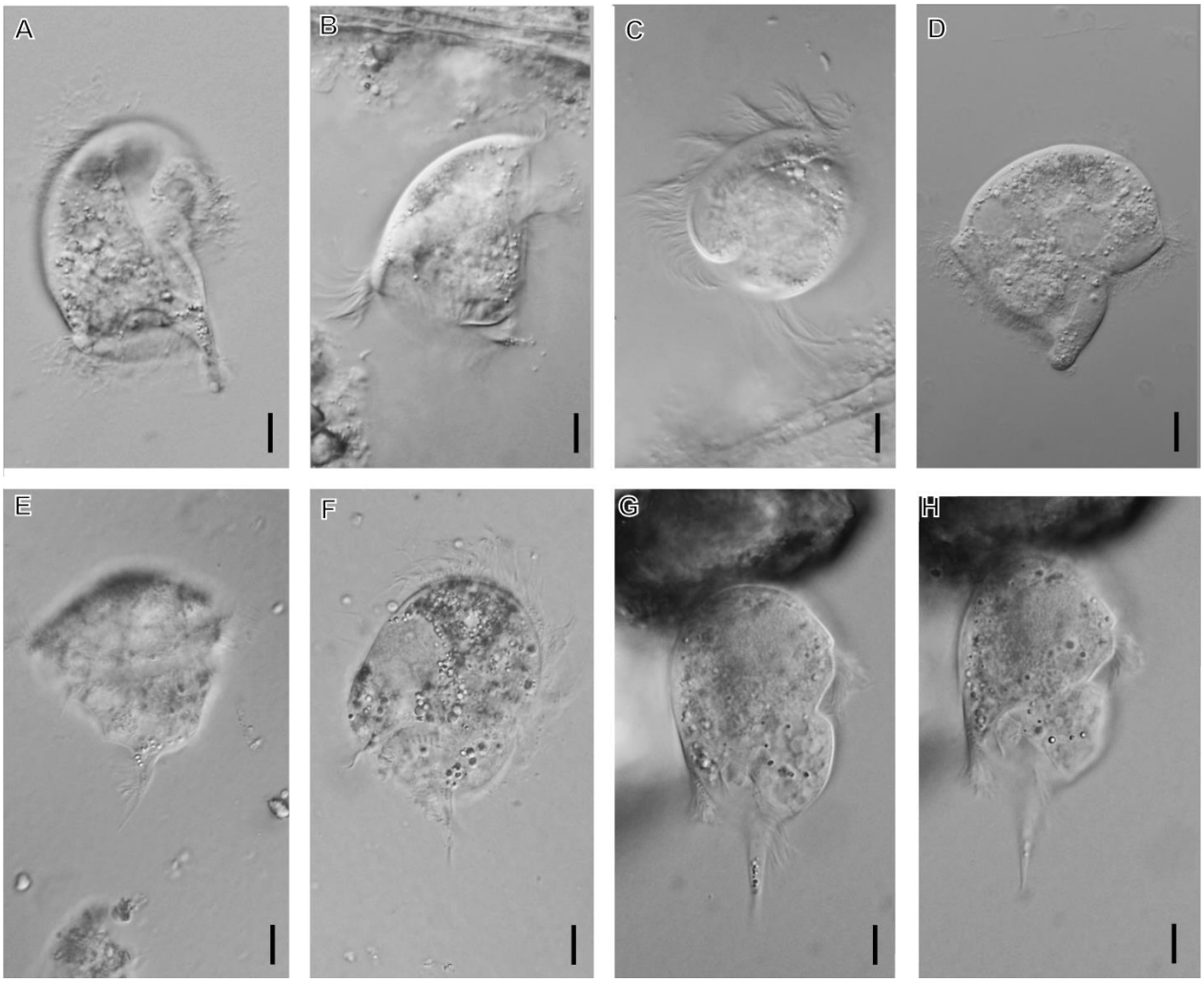

Fig 30. *Caenomorpha* spp. – A – D – strain BEAVERC, E – H strain BHARA, scale bar 10 µm.

4.1.5. Behaviour, cysts, and cell division in armophorids

We have observed some characteristics in armophorids, which have either never been recorded or have been described very sporadically (e.g. in a single species) in them before. One of them is the ability to attach to a solid surface. We have documented several metopids (and observed even more of them) to use somatic dorsal cilia to attach to the surface (e.g. organic material) and filter bacteria. If the ciliate is attached, the oral ciliature movement causes spiral motions of medium with bacteria towards the ciliate's buccal cavity. We can only guess if this saves energy or increases the efficiency of feeding. We have documented this behaviour in *Brachonella spiralis* (strain IZRBR), *Metopus* sp. 3 (strain KAN2), *M. halophila* (strains QDU, KAO), and *Metopus* sp. 4 (strain STORCH6) (Fig. 31, E – H). When disturbed, the ciliate rapidly discontinues the attachment and swims away.

Another interesting finding is that, despite the known bacteriovory in armophorids, they are also able to be eukaryovores, i.e. feed on other protists. We have documented *Metopus nasutus* (strain IZR) and *Brachonella spiralis* (strain IZRBR) to ingest euglenids (Fig. 31, A – C).

We have performed experiments with altering the cultivation conditions on several metopid strains to see possible triggers of encystment. The sub-optimization of the conditions had variable effects on different strains. If the time of reinoculation exceeded three weeks, some metopids have partially encysted (i.e. certain amount of the population remained free-swimming and the other cells have encysted), or fully encysted, if the time of sub-optimization was prolonged to over a month or more. The cysts of metopids have either a shape of a flask (Fig. 32, A – G), which are nearly identical across unrelated strains; or smaller, round cysts with a mucous layer over it (Fig. 32, H). The cysts are yellowish, greenish or brownish.

Another result of suboptimization was an increase of conjugating cells in several strains. Metopids in our cultures conjugate while fused at the anterior oral area. Several times, we have observed peculiar shapes of cells in the cultures that were doubled in width but had nearly the same length as normal cells. We cannot confirm that as we have not performed any detailed study of such cells, but we came down to an impression that these cells may be fully fused conjugants. We have not been able to document these observations.

We have also observed binary fission in several strains and documented it in two species, *Metopus palaeformis* (strain ARAGON) and *Metopus* sp. 8 (ALOOA3) (Fig. 33). The binary fission in metopids was pictured before, for example in *M. palaeformis* by Esteban et al. (1995). The process in our strains seems to be identical.

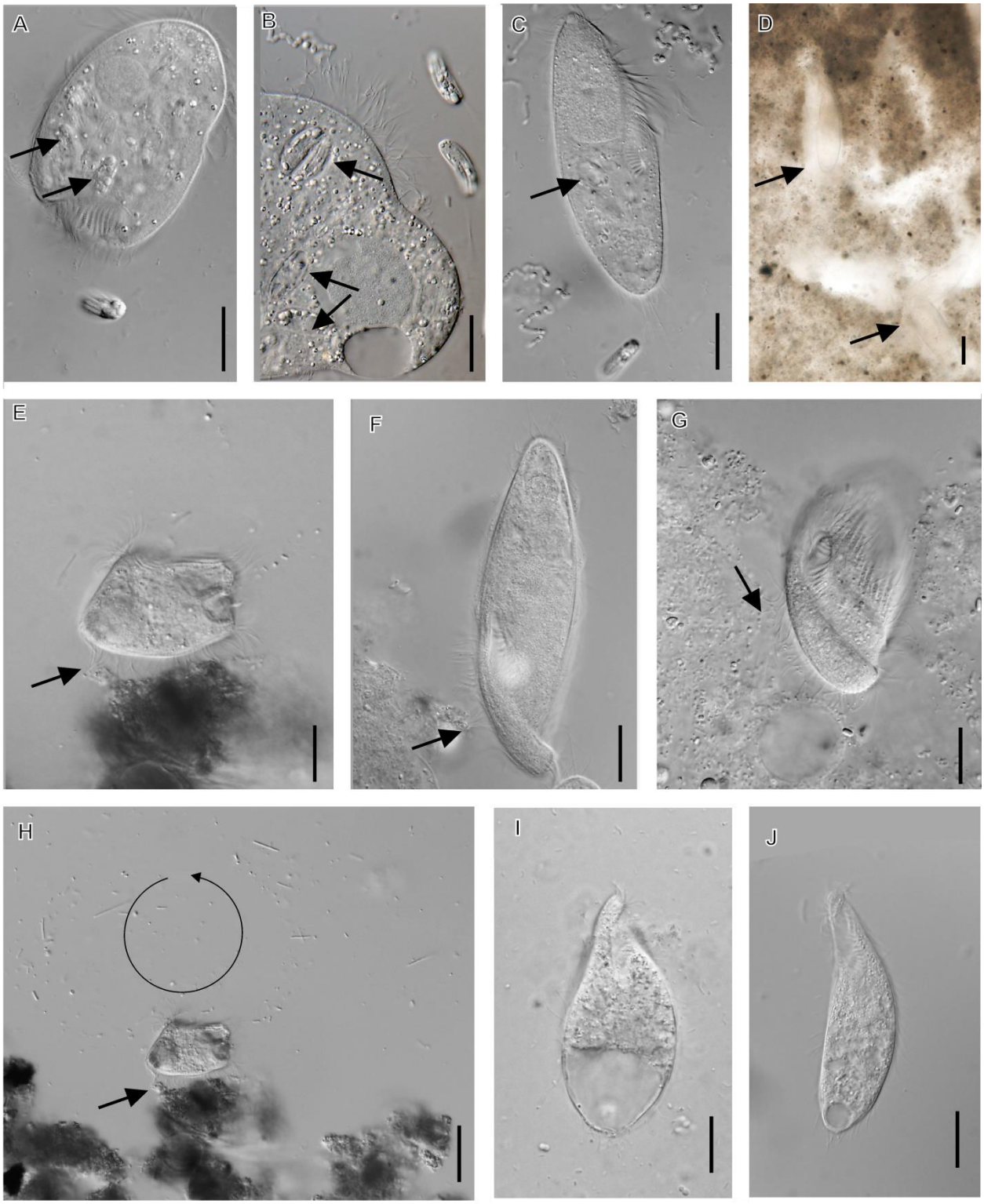

Fig. 31. A, B, C – eukaryovory (A, B – *Brachonella spiralis*, B – squished cell; strain IZRB; C – *Metopus nasutus*, strain IZR), D – tunnels in sediment, *Metopus nasutus* (strain IZRB); E – H – attachment to surface, E - *Brachonella spiralis*, strain TIKAL4; F – *Metopus* sp. 3 (strain STORCH6); G – *Metopus nivaaensis* (strain KAO); H - *Brachonella spiralis*, strain TIKAL4, filtering food; I, J – change of morphotype due to change of media, strain MAREK4; scale bars 10 μm.

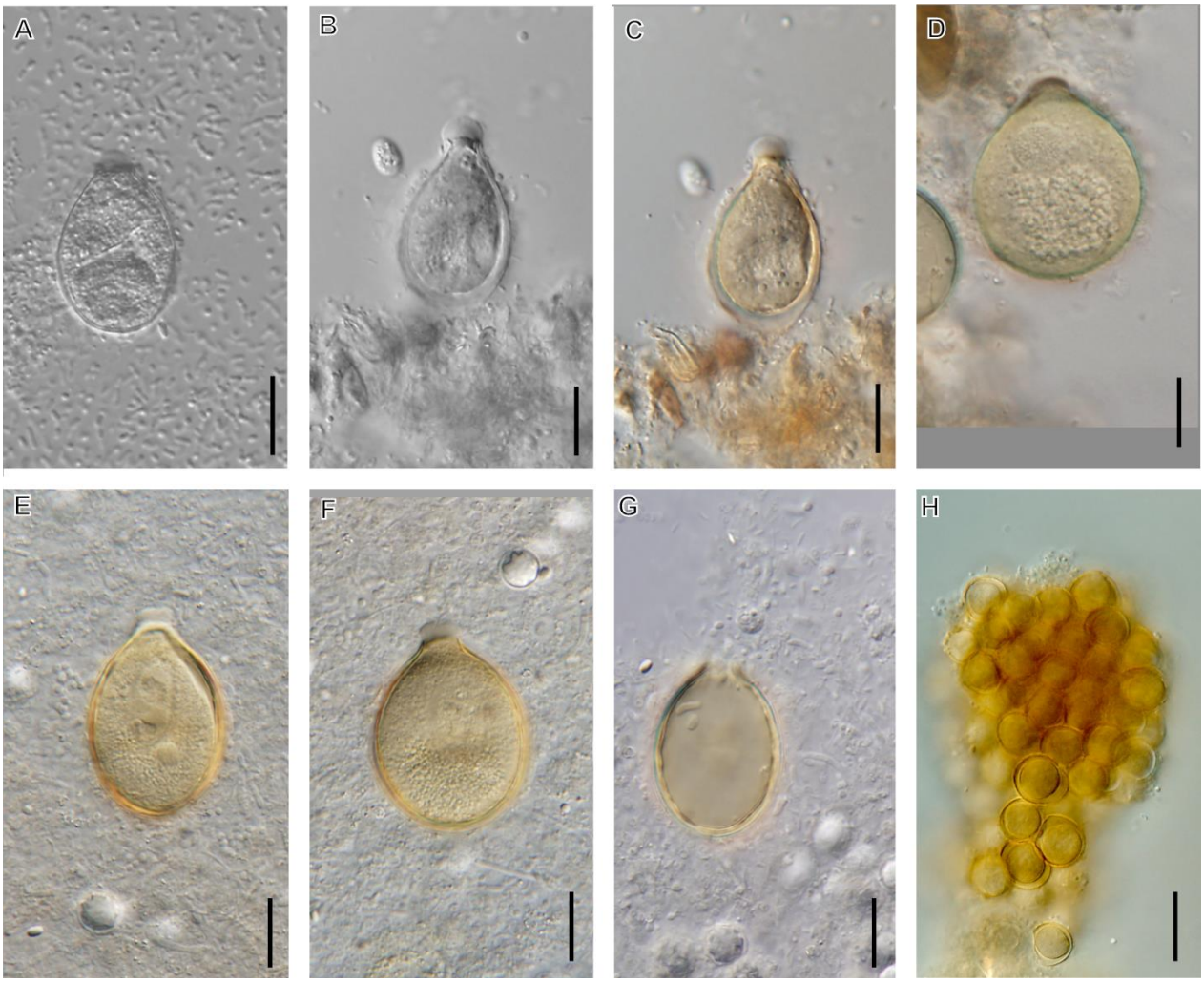

Fig. 32. Cysts, A – *Metopus palaeformis*, strain OLSRYB; B, C – *Metopus* sp. 7 strain ALOOA3; D – *Metopus* sp. 8, strain API; E, F, G – *Metopus* sp. 6, strain SULMOK2, H– empty cyst, *Metopus* sp. 6, strain SULMOK2; scale bar 10 μm.

Fig. 33. Dividing cell, *Metopus* sp. 7, strain ALOOA3; A – K – dividing cell; L, M – two daughter cells after division, scale bar 10 μm.

4.1.6. Ultrastructure of metopids

We studied the ultrastructure of four species – *Metopus* sp. 1 (strain CANC), *Metopus* sp. 2 (strain RIOZ), *M. es* (strain TROODOS), and *M. contortus* (strain SIPEK1C). During this study, we focused on hydrogenosomes and basic characterization of the cortical structure of the studied organisms, especially somatic dikinetids and oral polykinetids. Additionally, we observed other features, such as structure of macronucleus and micronucleus, extrusomes, vacuoles, and lipid particles. Importantly, we have also detected endosymbiotic and ectosymbiotic prokaryotes.

4.1.6.1. Nuclei

We have observed nuclei of all four studied species. There is always an oval macronucleus and a smaller micronucleus allied in the direct vicinity of it (Fig. 34). We were able to see dense chromatin bodies in macronuclei distributed in the karyoplasm and a nuclear envelope enclosing the organelle. We have observed structures that we have identified as putative nucleoli. Similar structure was identified as nucleolus in other ciliate studies (Karajan et al., 2003; Raikov, 2013). The macronuclei had a larger ratio to the body in *Metopus es* and *M. contortus* compared to *M.* sp. 2.

4.1.6.2. Hydrogenosomes

The ultrastructure of hydrogenosomes of our strains differs between species (Fig. 35). The hydrogenosomes of species *Metopus* sp.1 (strain CANC) and related *M.* sp. 2 (strain RIOZ) are both rounded with a diameter of ca 500 nm (with maximal recorded diameter ca 600 nm). There are well visible subsurface cristae present in hydrogenosomes of both strains.

The same situation applies also for the organelles of *Metopus es* (strain TROODOS). However, the hydrogenosomes of this strain are of a different shape, ranging from long ovals to various irregular forms, while the size also varies from 300 to 1500 nm long and 200 to 500 nm wide.

Different situation was observed in the hydrogenosomes of the only studied marine species, *Metopus contortus* (strain SIPEK1C). The organelles are round or slightly oval and significantly greater, ranging from 1000 to 1600 nm long and 1200 to 800 nm wide, which may correspond with the greater average size of the entire cell in comparison to the other three strains. The subsurface cristae are present, but rather inconspicuous and thin in the strain SIPEK1C. Both

attributes correspond to results of the study of Biagini et al. (1997b), where the observed hydrogenosomes had, however, a bigger size.

Hydrogenosomes of all strains are enveloped by a double membrane and contain small dark particles (Fig. 35, C, D). These are very conspicuous and always present in *Metopus* sp.1 and *M.* sp. 2. Usually, several particles are visible on each organelle section. They are also conspicuous, when present, in *Metopus es*, but there are often no visible particles on many sections. In *M. contortus*, they are numerous but discreet.

4.1.6.3. Cortex and other structures

Cortex of all examined strains lacks visible alveoli, a feature so characteristic for ciliates (see 5.1.). Nonetheless, the cell surface is ruffled with various seemingly irregular invaginations, dents, and apertures in all of the species, particularly in *Metopus* sp. 2 (Fig. 36, A, C, D, E). An interesting oddity appears in the cortex of *Metopus* sp. 1 (and less in *Metopus* sp. 2), where we can see small round alveoli, either empty or containing an undefined matter that seem to be irregularly distributed along the inner cell surface (not shown).

Metopids have somatic dikinetids and oral polykinetids, which were confirmed also in our study (Fig. 37). Somatic dikinetids in the studied species consisted of two (anterior and posterior) kinetosomes both bearing cilia. There is a transverse microtubular ribbon allied above the anterior kinetosome at triplets 3, 4, and 5. A long postciliary ribbon is diverging from the posterior kinetosomes, allied conically by dark fibrils at each side. From an opposite direction, there is laterally-directed kinetodesmal fibril, which seemed to be striated in *Metopus* sp. 2, but unstriated in *M. es* and *M. contortus* (that can be an artefact caused by improper fixation).

We have observed differences from previous studies and from the currently accepted schematic structure of the dikinetid in *Metopus* (Lynn, 2008) in the connection of the two dikinetids. Contrarily to the literature describing one row of transverse microtubules (or two parallel ones while one is thin), there are two rows, starting at the base of the anterior kinetosomes directing divergently towards the posterior kinetosomes, to the triplets 4 and 6, respectively (Fig. 37, B). Alternatively, we also found two rows diverging to triplets 3 and 5 (Fig. 37, F).

Various extrusomes are present in the cortex of metopids (Fig 38). In several sections in *Metopus* sp. 2 and *M. es*, we have recorded the extrusion of unspecified mucocysts (e.g. Fig. 38, A). Interestingly, the putative mucocysts in *M. es* seem to be enveloped in a vesicle of a cytoplasmatic membrane (Fig. 38, A). We have also observed other various particles within the cells, such as roundish or oval lipid droplets (Fig. 38, C, D), which are especially frequent and well visible in the cells of *M. es*, where they range in size from 1000 to 1500 nm long and 700 to

1500 nm wide. We have also observed various endosymbiotic prokaryotes within the studied metopid cells, of which at least some were confirmed by autofluorescence to be methanogenic (see 4.3.).

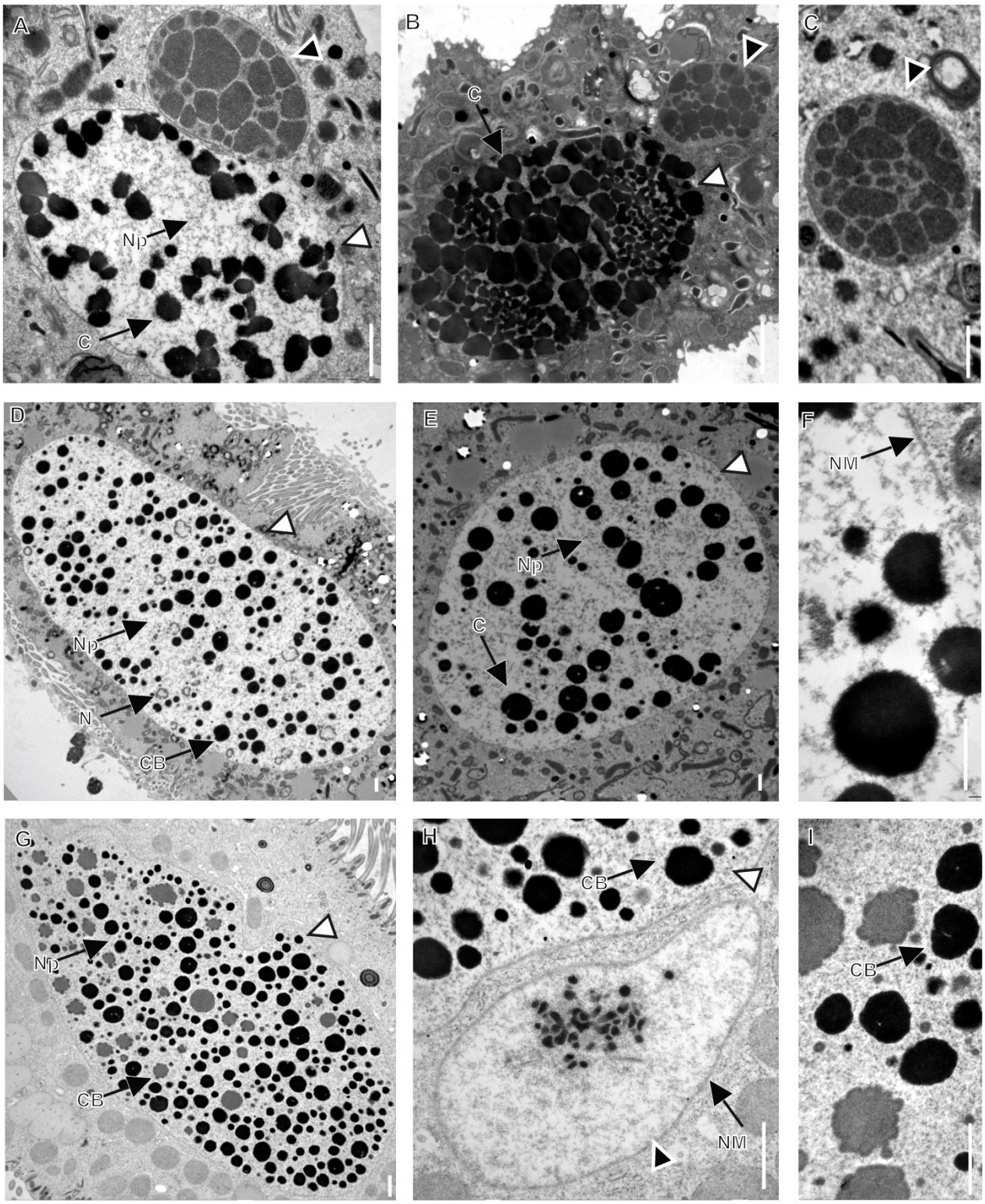

Fig. 34. Nuclei ultrastructure in genus *Metopus*. A, B, C - *Metopus* sp. 2, strain RIOZ; D, E, F - *Metopus es*, strain TROODOS; G, H, I - *Metopus contortus*, strain SIPEK1C. Legend: White arrowhead - macronucleus, black arrowhead - micronucleus, CB - dense chromatin bodies, N - putative nucleoli, NM - nuclear membrane, Np – nucleoplasma; scale bars 0.5 μm.

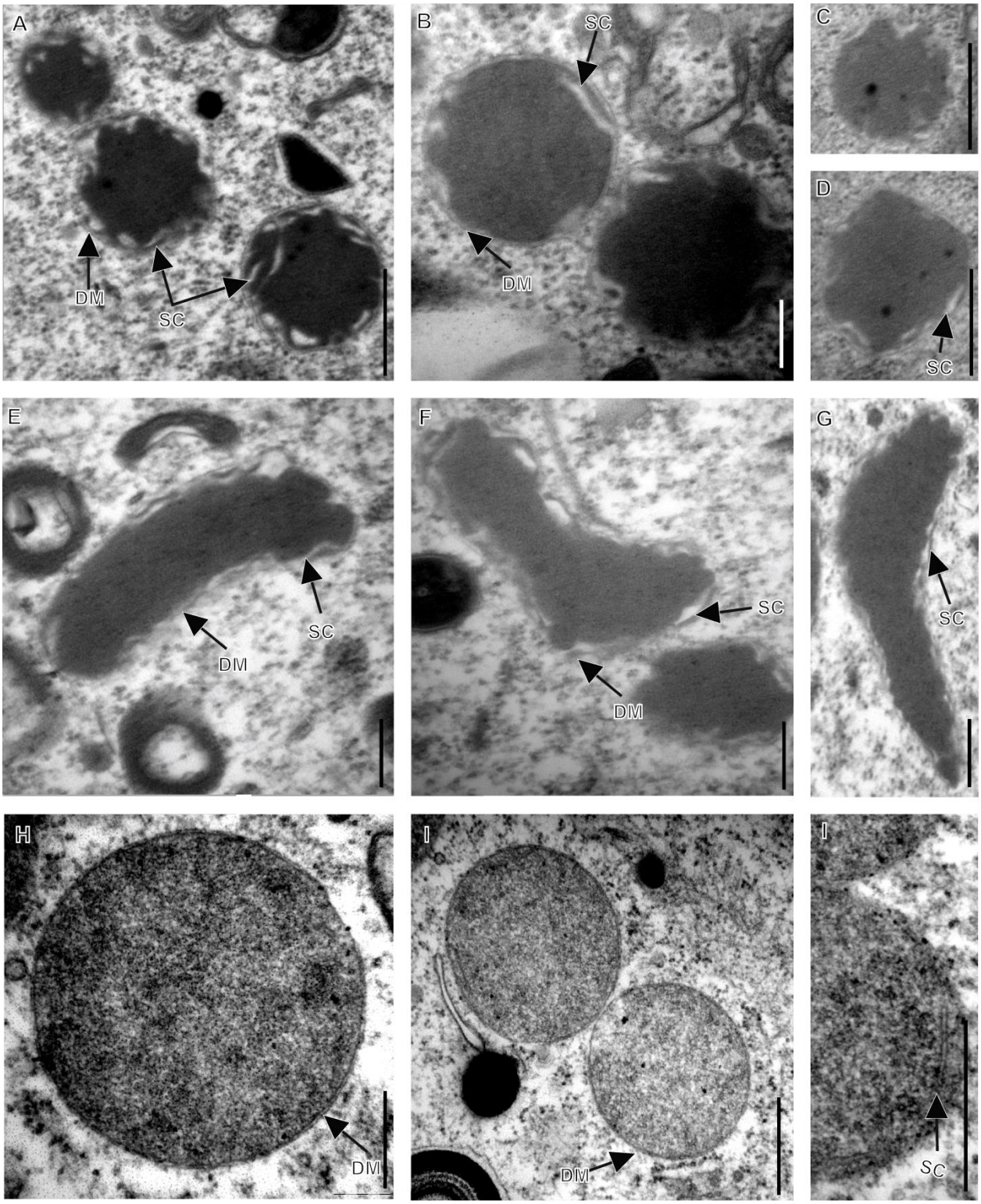

Fig. 35. Ultrastructure of putative hydrogenosomes in genus *Metopus*. A, B, C - *Metopus* sp. 2, strain RIOZ; D, E, F - *Metopus es*, strain TROODOS; G, H, I - *Metopus contortus*, strain SIPEK1C. Legend: DM - double membrane, SC - surface cristae; scale bars 0.5 μm.

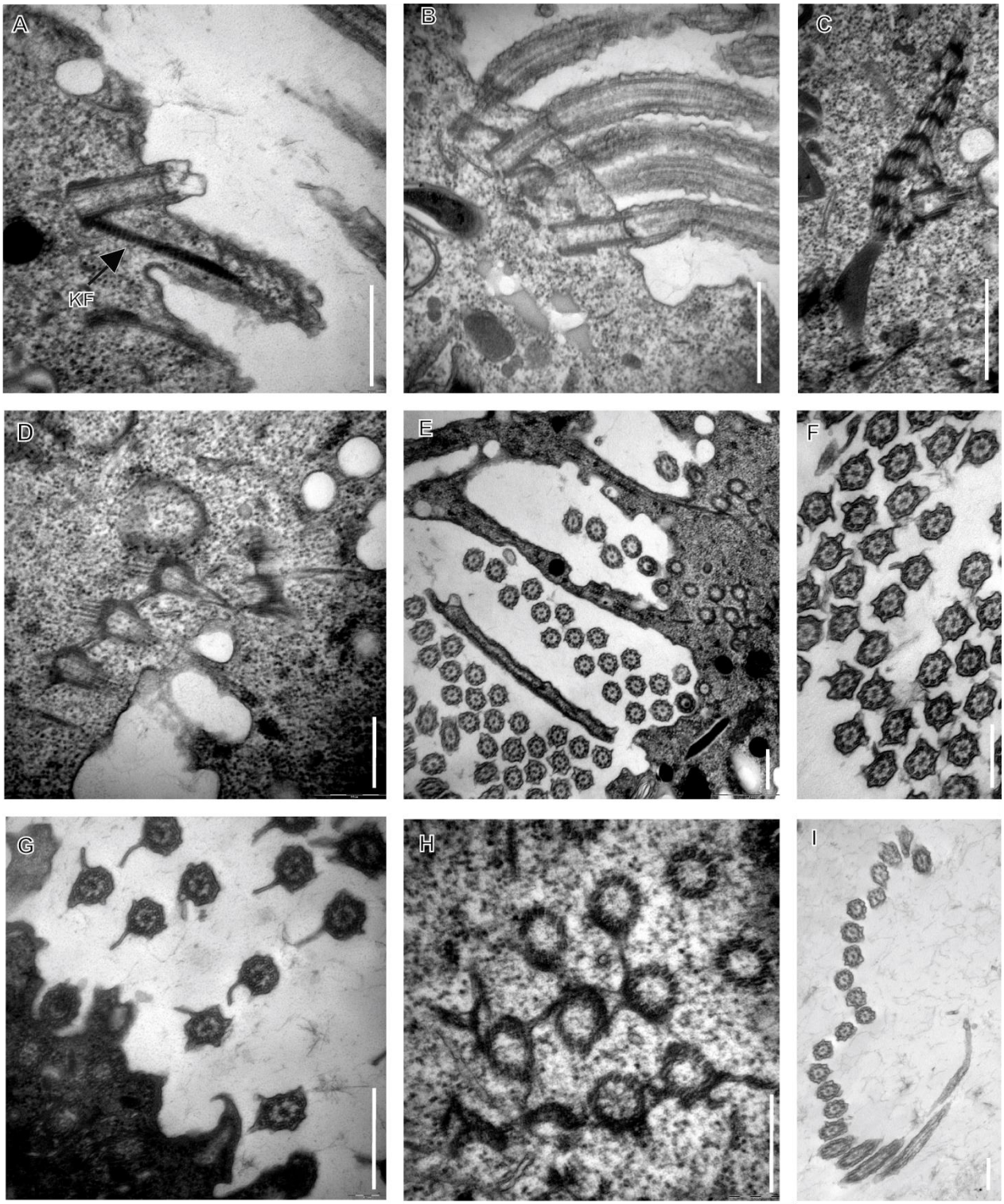

Fig. 36. Ultrastructure of somatic structures in *Metopus* sp. 2. (strain RIOZ), A, B, C, D, E, F Legend: DC - dikinetid connection, KF - kinetodesmal fibre, PMT - postciliary microtubule ribbon, TE - transverse extending, TM - transverse microtubular ribbon; scale bars 0.5 μm.

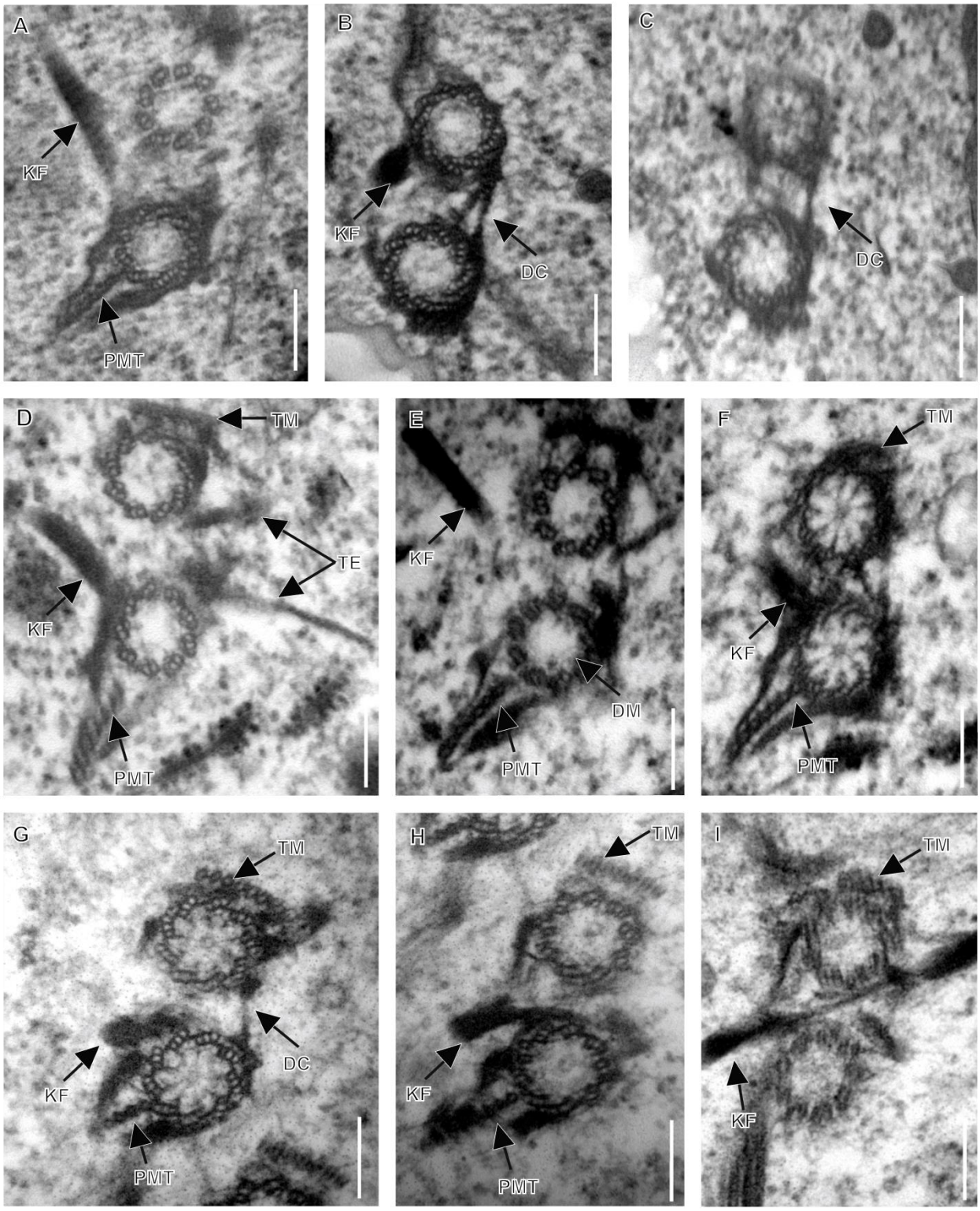

Fig. 37. Ultrastructure of somatic dikinetids in *Metopus es* and *M. contortus*. A, B, C D, E, F - *Metopus es*, strain TROODOS; G, H, I - *Metopus contortus*, strain SIPEK1C. Legend: DC - dikinetid connection, KF - kinetodesmal fibre, PMT - postciliary microtubule ribbon, TE - transverse extending, TM - transverse microtubular ribbon; scale bars 0.5 μm.

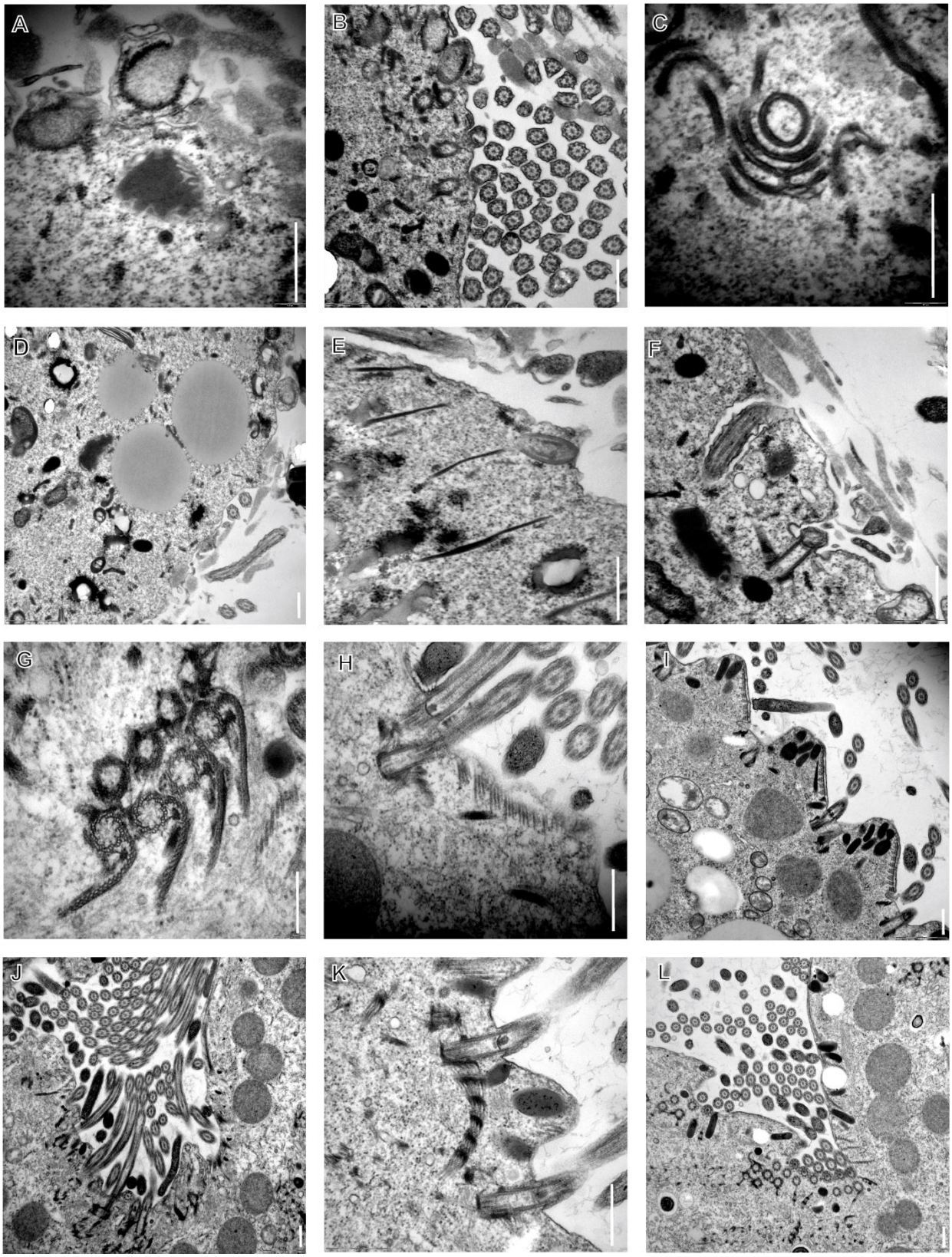

Fig. 38. Ultrastructure of somatic structures in *Metopus* es and *M. contortus*. A, B, C D, E, F - *Metopus es*, strain TROODOS; G, H, I - *Metopus contortus*, strain SIPEK1C; scale bars 0.5 µm.

4.2. DISCOVERY OF A NOVEL MARINE ANAEROBIC CILIATE LINEAGE – THE MURANES

During mapping of the diversity of Armophorea, we have encountered other anaerobic ciliates that resembled metopids or stichotrichs at a first sight. In this study, we call this lineage murane (singular) or muranes (plural), which is derived from the name of the first established culture with this ciliate, following the name of the sample collection location (Murano Island, Venetian lagoon, Italy).

4.2.1. Sampling and cultivation of muranes

We observed these ciliates in five samples and were able to establish four strains in culture (MURANO, RIDKA, SIPEK, and SUMMARTIN). All samples were collected by our colleagues from the Faculty of Science, Charles University in Prague. The samples were obtained from marine water (MURANON) or anoxic sediments, in shallow shore waters in vicinity of a lagoon or brackish lake (RIDKA, SIPEK), but also marine sediment from open sea (e.g. SUMMARTIN strain comes from marine sediment collected 30 meters under the sea level). These ciliates are probably cosmopolites, as we obtained them from two continents (Europe, North America). We use 4:1 ratio of marine medium ATCC 5225 to freshwater medium ATTC 802 to routinely culture the strains. We tried to culture muranes in pure marine medium, which is possible, but the cells grow the best in the 4:1 media ratio.

We have confirmed that muranes are anaerobes by placing them into an anaerobic chamber, where they survive and successfully grow. Contrarily, we tried cultivating them under aerobic conditions on Petri dishes. All cells were dead the next day, when we inspected the dish. We have been able to establish one monoeukaryotic culture of muranes (strain MURANON). The other three cultures contained also novel lineages of anaerobic scuticociliates and other anaerobic protists besides the muranes.

Table 17. List of strains included in this study

Legend: H – habitat, M – marine, O. L. – original location.

STRAIN	H	PLACE OF ORIGIN	COORDINATES	O. L.
MURANON	M	Murano, Venetia, Italy	45°27'09"N 12°21'15" E	marine lagoon
RIDKA	M	Florida, USA	24°39'36"N 81°16'24"W	shore sediment
SIPEK1A	M	Agios Spiridonisos, Corfu	39°48'56"N 19°51'35"E	marine lagoon
SUMMARTIN	M	Summartin, Brač, Croatia	47°17'N 16°52'E	35 m deep sea

4.2.2. Phylogenetic analysis of muranes

We have carried out a pan-ciliate phylogenetic analysis based on SSU rDNA sequences in order to assess the phylogenetic position of the muranes (Fig. 39). We included representatives of all main ciliate lineages, four newly determined sequences of the muranes, and also environmental sequences representing clades closely related to Cariacotrichea, Caenomorphidae, and muranes, which were obtained by blast against nr GenBank database. Some of the sequences had to be removed from the dataset, because they were shown to be chimeric or of poor quality (i.e. AB256205, AB725338, GU819259, GU819740, GU819618, GU819413, GU824618, GU823902, AB725338, and GU823902, AY882478, GU819545, GU819555, AY179984, AY179982).
AY179982 AB505525

Ciliophora robustly split into Postciliodesmatophora and Intramacronucleata. The two postciliodesmatophoran classes, Karyorelictea and Heterotrichea, were recovered monophyletic. Intramacronucleata split into three lineages with unresolved relationships. There was robust subclass Protocruziidia (Lynn, 2003, 2008) with the only genus *Protocruzia*. Intramacronucleata further divided into robust CONThreeP (Adl et al., 2012; Lynn, 2008) and SAL (Gentekaki et al., 2014).

The lineage of CONThreeP received high support in our analysis (bootstrap 98; posterior probability (PP) 1). Within, Oligohymenophorea and Phyllopharyngea are monophyletic and well supported. Oligohymenophorea was robustly closely related to Prostomatea, which appeared monophyletic in ML analysis, though without support, and paraphyletic in BI where it had strong support. The classes Nassophorea and Colpodea did not appear monophyletic, which corresponds to the results of previous studies (Zhang et al., 2014). Part of Nassophorea was branching with Phyllopharyngea with moderate support (bootstrap 83; posterior probability (PP) 1). *Discotricha* appeared as *incertae sedis* out of core microthoracids. However, the core microthoracids cluster with Phyllopharyngea. Both results are consistent with previous studies (Fan et al., 2014; Zhang et al., 2014).

Both the ML and BI analyses have weakly supported the monophyly of the SAL lineage, which is consistent with previous studies (Riley and Katz, 2001; Vďačný et al., 2010; Gentekaki et al., 2014). Spirotrichea + Armophorea + Cariacotrichea + Muranes form an unsupported clade. The "core Spirotrichea" (i.e. Spirotrichea excl. Licnophorida, Protospirotrichea, Phacodiniida) formed a robust clade. The order Phacodiniida resulted related to Spirotrichea with low support, similarly to Protospirotrichea. *Licnophora* forms a separate lineage with no obvious relationship to any particular SAL group, consistently with the previous studies (Lyn& Strüder-Kypk, 2002).

Armophorea without Caenomorphidae are robustly monophyletic. Cariacotrichea formed a weakly supported clade with the closely related environmental sequences.

Caenomorphidae appeared as a robust monophyletic clade out of Armophorea, which corresponds to the phylogenetic analysis by da Silva Paiva et al. (2013). It forms a sister clade to the well supported Litostomatea. Nevertheless, this relationship has no support.

Muranes appeared as a robust clade with two environmental sequences, AY179982 and AB505525. They appeared closely related to Armophorea, though with no support, and the clade of Armophorea + muranes was closely related to Cariacotrichea, though with no support as well

Considering results of our preliminary analyses, which did not contain the two environmental sequences closely related to muranes (AY179982, AB505525) and where Muranes were always closely related to Cariacotrichea and Spirotrichea, we performed another phylogenetic analysis using the pan-ciliate dataset, from which the two environmental sequences were removed. The phylogenetic tree is depicted in Fig. 40. Consistently with the previous analysis, Ciliophora split into the two subphyla (Postciliodesmatophora and Intramacronucleata), and Intramacronucleata was further divided into Protocruziida, CONThreeP, and SAL. Within CONThreeP, classes Colpodea with Nassophorea formed an unsupported clade without *Discotricha.* Prostomatea and Oligohymenophorea formed a robust clade, but Prostomatea was paraphyletic both in ML and BI analyses. The relationships between particular lineages within the SAL clade considerably differed from the previous analysis. Muranes, Cariacotrichea, and Spirotrichea (including *Licnophora*) formed an unsupported clade. Muranes were closely related to Cariacotrichea with low support (bootstrap 64; posterior probability (PP) 0.98). This whole group was sister to robust monophyletic lineage of Metopidae and Clevelandellida (Armophorea excl. Caenomorphidae).

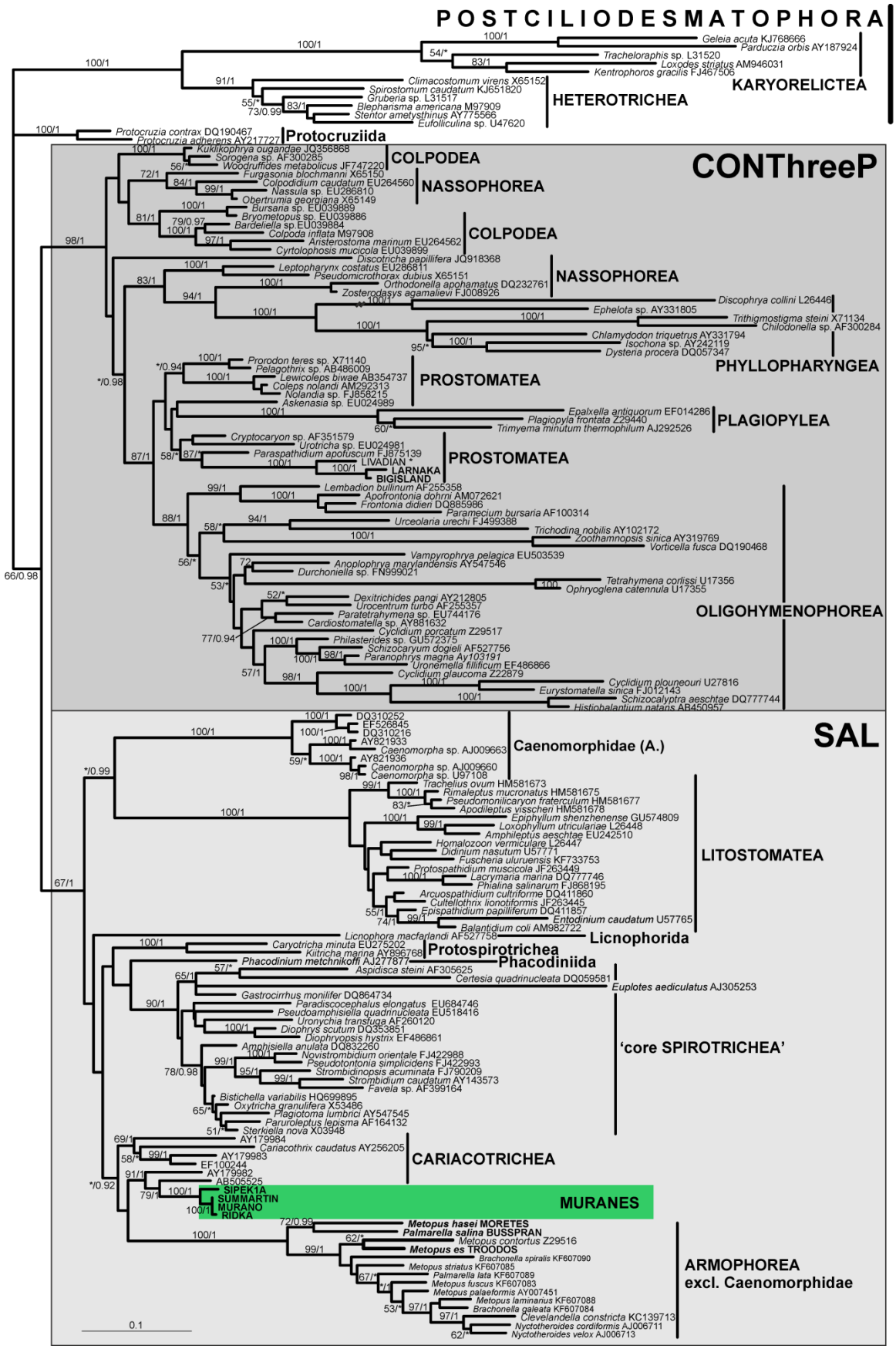
POSTCILIODESMATOPHORA
Geleia acuta KJ768666
Parduczia orbis AY187924
Trachelotaphis sp. L31520
Loxodes striatus AM946031
Kentrophoros gracilis FJ467506
KARYORELICTEA
Climacostomum virens X65152
Spirostomum caudatum KJ651820
Gruberia sp. L31517
Blepharisma americana M97909
Stentor amethystinus AY775566
Eufolliculina sp. U47620
HETEROTRICHEA
Protocruzia contrax DQ190467
Protocruzia adherens AY217727
Protocruziida
CONThreeP
Kuklikophrya ougandae JQ356868
Sorogena sp. AF300285
Woodruffides metabolicus JF747220
COLPODEA
Furgasonia blochmanni X65150
Colpodidium caudatum EU264560
Nassula sp. EU286810
NASSOPHOREA
Obertrumia georgiana X65149
Bursaria sp. EU039889
Bryometopus sp. EU039886
Bardeliella sp EU039884
Colpoda inflata M97908
COLPODEA
Aristerostoma marinum EU264562
Cyrtolophosis mucicola EU039899
Discotricha papillifera JQ918368
Leptopharynx costatus EU286811
Pseudomicrothorax dubius X65151
NASSOPHOREA
Orthodonella apohamatus DQ232761
Zosterodasys agamalievi FJ008926
Discophrya collini L26446
Ephelota sp. AY331805
Trithigmostigma steini X71134
Chilodonella sp. AF300284
Chlamydodon triquetrus AY331794
Isochona sp. AY242119
Dysteria procera DQ057347
PHYLLOPHARYNGEA
Prorodon teres sp. X71140
Pelagothrix sp. AB486009
Lewicoleps biwae AB354737
PROSTOMATEA
Coleps nolandi AM292313
Nolandia sp. FJ858215
Askenasia sp. EU024989
Epalxella antiquorum EF014286
Plagiopyla frontata Z29440
Trimyema minutum thermophilum AJ292526
PLAGIOPYLEA
Cryptocaryon sp. AF351579
Urotricha sp. EU024981
Paraspathidium apofuscum FJ875139
PROSTOMATEA
LIVADIAN *
LARNAKA
BIGISLAND
Lembadion bullinum AF255358
Apofrontonia dohrni AM072621
Frontonia didieri DQ885986
Paramecium bursaria AF100314
Urceolaria urechi FJ499388
Trichodina nobilis AY102172
Zoothamnopsis sinica AY319769
Vorticella fusca DQ190468
Vampyrophrya pelagica EU503539
Anoplophrya marylandensis AY547546
Durchoniella sp. FN999021
Tetrahymena corlissi U17356
Ophryoglena catennula U17355
OLIGOHYMENOPHOREA
Dexitrichides pangi AY212805
Urocentrum turbo AF255357
Paratetrahymena sp. EU744176
Cardiostomatella sp. AY881632
Cyclidium porcatum Z29517
Philasterides sp. GU572375
Schizocaryum dogieli AF527756
Paranophrys magna Ay103191
Uronemella filificum EF486866
Cyclidium glaucoma Z22879
Cyclidium plouneouri U27816
Eurystomatella sinica FJ012143
Schizocalyptra aeschtae DQ777744
Histiobalantium natans AB450957
SAL
DQ310252
EF526845
DQ310216
AY821934
Caenomorpha sp. AJ009663
Caenomorphidae (A.)
AY821936
Caenomorpha sp. AJ009660
Caenomorpha sp. U97108
Trachelius ovum HM581673
Rimaleptus mucronatus HM581675
Pseudomonilicaryon fraterculum HM581677
Apodileptus visscheri HM581678
Epiphyllum shenzhenense GU574809
Loxophyllum utriculariae L26448
Amphileptus aeschtae EU242510
Homalozoon vermiculare L26447
Didinium nasutum U57771
Fuscheria uluruensis KF733753
Protospathidium muscicola JF263449
Lacrymaria marina DQ777746
Phialina salinarum FJ868195
Arcuospathidium cultriforme DQ411850
Cultellothrix lionotiformis JF263445
Epispathidium papilliferum DQ411857
Entodinium caudatum U57765
Balantidium coli AM982722
LITOSTOMATEA
Lichnophora macfarlandi AF527758
Licnophorida
Caryotricha minuta EU275202
Kiitricha marina AY896768
Protospirotrichea
Phacodinium metchnikoffi AJ277877
Phacodiniida
Aspidisca steini AF305625
Certesia quadrinucleata DQ059581
Euplotes aediculatus AJ305253
Gastrocirrhus monilifer DQ864734
Paradiscocephalus elongatus EU684746
Pseudocamphisiella quadrinucleata EU518416
Uronychia transfuga AF260120
Diophrys scutum DQ353851
Diophryopsis hystrix EF486861
Amphisiella anulata DQ832260
Novistrombidium orientale FJ422988
Pseudotontonia simplicidens FJ422993
Strombidinopsis acuminata FJ790209
Strombidium caudatum AY143573
Favela sp. AF399164
'core SPIROTRICHEA'
Bistichella variabilis HQ699895
Oxytricha granulifera X53486
Plagiotoma lumbrici AY547545
Paruroleptus lepisma AF164132
Sterkiella nova X03948
AY179984
Cariacothrix caudatus AY256205
CARIACOTRICHEA
AY179983
EF100244
AY179982
AB505525
SIPEK1A
SUMMARTIN
MURANO
RIDKA
MURANES
Metopus hasei MORETES
Palmarella salina BUSSPRAN
Metopus contortus Z29516
Metopus es TROODOS
Brachonella spiralis KF607090
Metopus striatus KF607085
Palmarella lata KF607089
Metopus fuscus KF607083
Metopus paleiformis AY007451
Metopus laminarius KF607088
Brachonella galeata KF607084
Clevelandella constricta KC139713
Nyctotheroides cordiformis AJ006711
Nyctotheroides velox AJ006713
ARMOPHOREA
excl. Caenomorphidae
0.1
INTRAMACRONUCLEATA

Fig. 39. Phylogenetic tree of Ciliophora based on SSU rDNA sequences. The tree was constructed by the maximum likelihood method and is unrooted. The numbers at the nodes represent BS ≥ 50/BPP ≥ 0.9. Lower are not shown or are denoted by an asterisk. Environmental sequences are represented by GenBank accession numbers. New sequences are in bold.

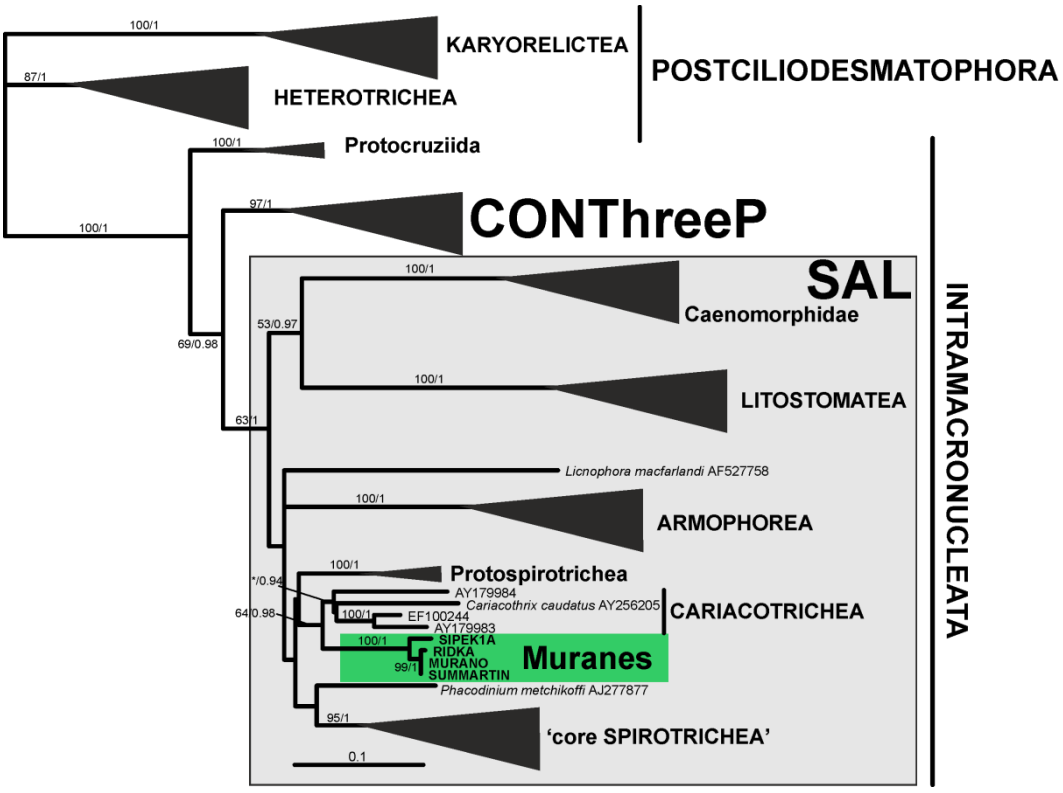

Fig. 40. Phylogenetic tree of Ciliophora based on SSU rDNA sequences, excluding environmental sequences AY179982, AB505525. The tree was constructed by the maximum likelihood method and is unrooted. The numbers at the nodes represent BS ≥ 50/BPP ≥ 0.9. Lower are not shown or are denoted by an asterisk. Environmental sequences are represented by GenBank accession numbers. New sequences are in bold.

4.2.3. Morphology of muranes

All strains of muranes are morphologically rather similar (Figs 41, 42). The cells are flexible but not contractile, cylindrical, and elongate, with a single posterior contractile vacuole, caudal cilia, and a shallow buccal cavity surrounded by the posterior beginning of an adoral zone of membranelles (AZM) and an extended paroral membrane that lead on the right of the buccal cavity, with the AZM twisting from the ventral towards the dorsal anterior end of the cell. The anterior of the cell ends with a narrowed "proboscis", which is dorsally inclined to the left. The end of the "proboscis" contains a stripe of four perizonal kineties as in metopids. Typical cells of muranes are of a relatively uniform size measuring from 90 x 12 μm to 120 x 20 μm in vivo. Protargol-stained cells are shrunk, standardly ranging from 52 x 8 μm to 81 x 16 μm. Besides, we frequently observed also "small forms", which measured only approximately 11 x 6 μm and may be a manifestation of a complex life cycle (see 4.2.4.). The following part of is chapter focuses on the morphology of typical, mature cells. In the present study, we use the same cell description terminology for muranes as for metopids (Fig. 7.). We have not shown morphometrics, as it has been done only partially.

Muranes possess eleven small, round macronuclei with diameter of 4 – 6 μm in vivo (2.5 – 4 μm in protargol-stained cells) that are randomly distributed in the central part of the cell (Fig. 42). We have not been able to find micronuclei in living cells, but we have recorded a single small, round micronucleus in protargol-stained cells with ca 1.3 μm in diameter. We have also identified the micronucleus using transmission electron microscopy (see 4.2.5.).

The oral opening is located just below the centre of the cell (in smaller cells it is moved towards the anterior end of the first posterior third), at the base of the "proboscis", similarly as in litostomatean rhynchostomatids (Vďačný et al., 2011). However, muranes probably do not possess the litostomatean oral bulge. Rather, the oral opening is surrounded by four membranelles from each side and a row of kinetids bearing cilia (Figs 41, 42), and is positioned on the right and below from the posterior start of AZM, which is accompanied by an extended paroral membrane and leads towards the anterior end of the cell, while spiralling left around the proboscis, towards the right side of the distal end (Figs 41, 42). The AZM contains ca 16 – 20 membranelles. There is a closely allied somatic kinety that disconnects in the first third and spirals right around the "proboscis". The paroral membrane contains 16 or 17 membranelles. On the opposite (right) side of the buccal cavity, there is another zone of ca 10 membranelles. We have discovered that muranes contract the opening of the buccal cavity while filtering food, supposedly at the moment of the food vacuole detachment (not shown). The buccal cavity is conspicuous and has a pocket–like shape (3.5 – 6.5 μm wide) when opened and a droplet–like shape when closed (not shown).

The somatic ciliature is similar as in spirotrich stichotrichs, although no murane strain possesses any prominent transverse cirri that are so typical for spirotrichs. The cell is grooved with a regular pattern, and from light microscopy micrographs it seems that the somatic kineties are located in these grooves (Figs 41, 42). Muranes bear six or seven somatic kineties that are possibly formed by di- or trikinetids, in which we could not find any regular pattern. Oddly, we have found also single kinetosomes in TEM, and the light microscopy shows isolated single somatic cilia that are longer than the rest of the ciliature (Figs 41, 42). Similar situation was described in *Phacodinium metchnikoffi*, which has uncertain phylogenetic position but is currently placed within the class Spirotrichea (Shin et al., 2000).

The posterior end of the cell contains a single contractile vacuole, which is oval or round and measures ca 13 μm in diameter or ca 13 x 4.5 μm when elongate. There is a tuft of numerous short caudal cilia, ca 6.5 – 11 μm long, and a very long single cilium (bristle) growing from the centre of the tuft, ca 30 – 45 μm long. Interestingly, there are ectosymbiotic prokaryotes present on the cell surface (see 4.3.).

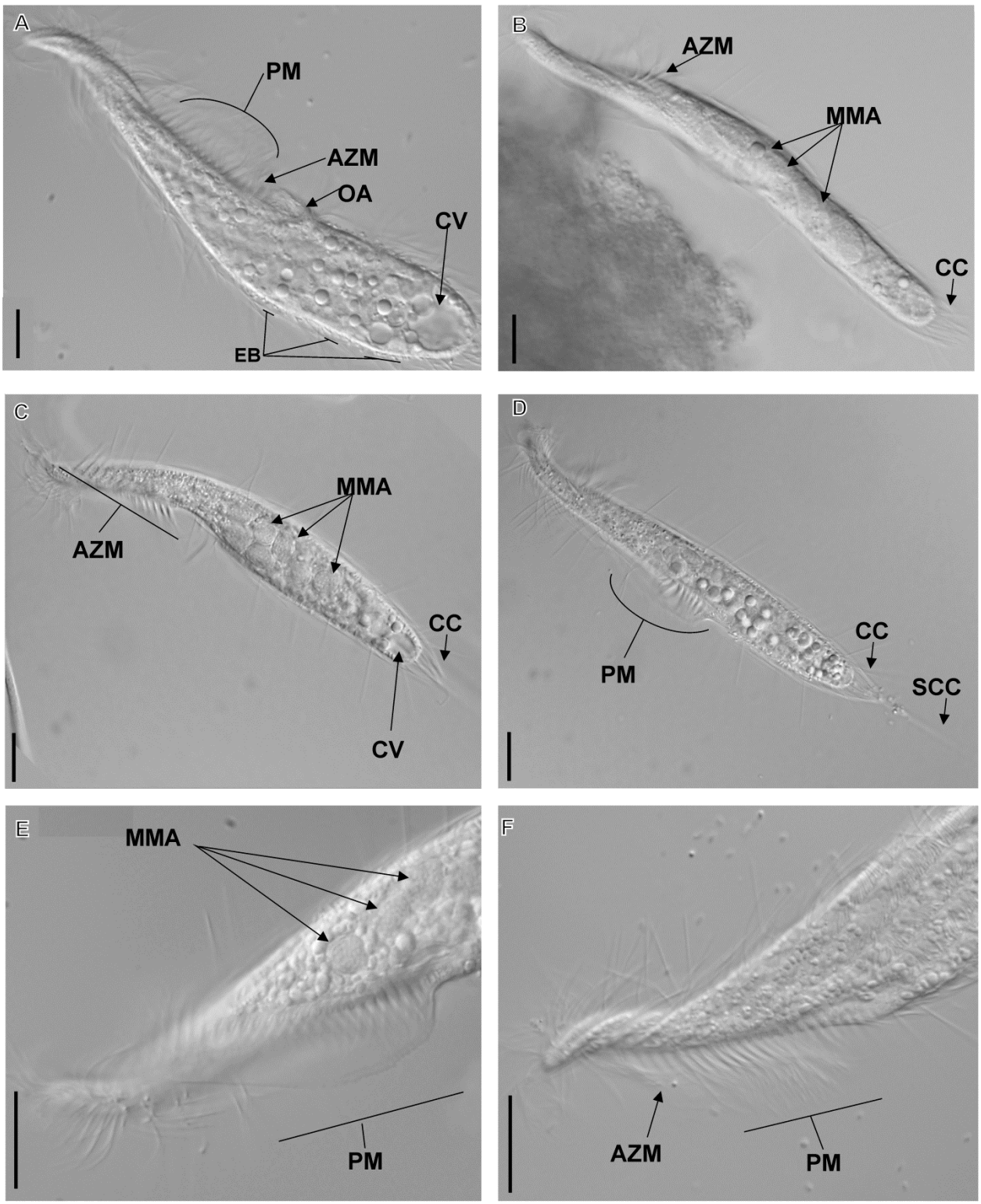

Fig. 41. Muranes; isolate MURANON, B - isolate SIPEK1A, C - isolate RIDKA, D - isolate SUMMARTIN, DIC 100x, living cell; E, F - Isolate MURANON, details of anterior part, living cells, DIC 100x; AZM- adoral zone of membranelles, CC - caudal cilia, EB - tufts of epibacteria, MMA - multiple macronuclei, PM - paroral membrane, SC - somatic cilia, SCC - long single caudal cilium; scale bars 10 μm.

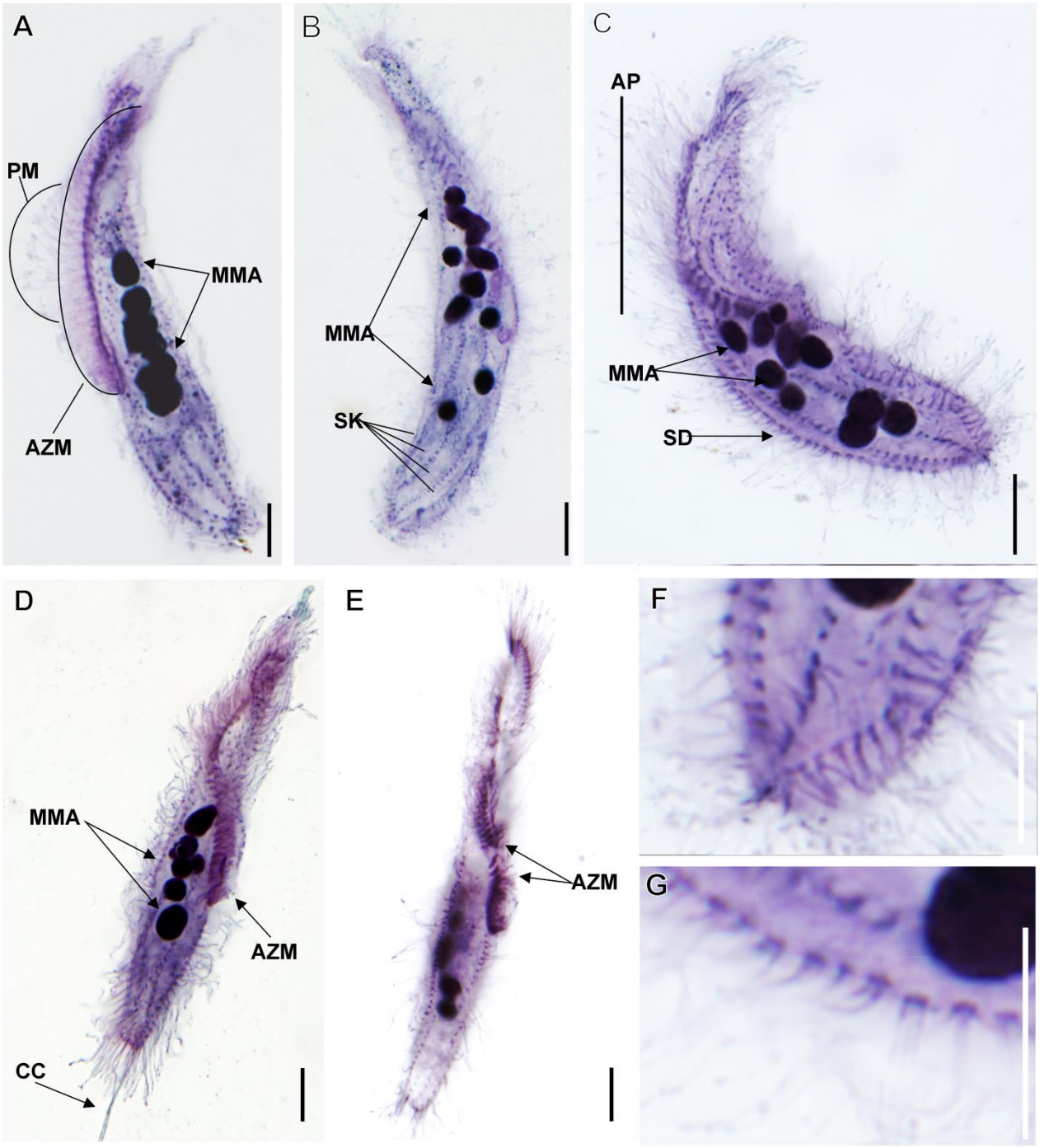

Fig. 42. Muranes; protargol stained cells, A - C isolate MURANON, D - E isolate RIDKA, F, G – somatic kinetids. AP - twisted anterior part, AZM - adoral zone of membranelles, CC - caudal cilia, MMA - multiple macronuclei, PM- paroral membrane, SD - somatic dikinetids, SK - somatic kineties. Black scale bars 10 μm, white scale bars 5 μm.

4.2.4. Morphological polymorphism and life cycle

Although muranes usually show morphological uniformity, they may possess a complex life cycle. It is represented by various very small or bigger forms that highly differ from the typical shape of murane cells. However, they usually possess the same basic morphological features, as AZM, tuft of caudal cilia or multiple macronuclei. They constantly appear in monoeukaryotic as well as non-monoeukaryotic cultures. These cells range from only 11 x 6 μm (Fig. 43, B – D, F – H), over medium forms large ca 40 x 10 μm (Fig. 43, A, E), to larger cells that measure up to 70 x 16 μm (Fig. 43). The smallest forms possess many cilia and do not seem to contain macronuclei (Fig. 43, B – D, F – H). The medium and larger forms contain the typical macronuclei, usually in smaller number (4 – 8) than the typical cells do (Fig. 43, A, E). Some of these forms have a well-developed AZM that can reach the posterior part of the cell. Contrarily, in other forms the AZM is reduced and there is the typical tuft of caudal cilia, with a single long cilium. We have also observed peculiar round forms (Fig. 43, L – P), which had a trace of AZM on one side and the tuft of cilia on the other side, containing various numbers of macronuclei. We have never observed cysts in our cultures of muranes. We have recorded dividing cells several times (Fig. 43, I). In this process, the anterior end of the opisthe (the posterior daughter cell), i.e. the narrowed proboscis-like part, was inserted to the posterior part of the proter (the anterior cell).

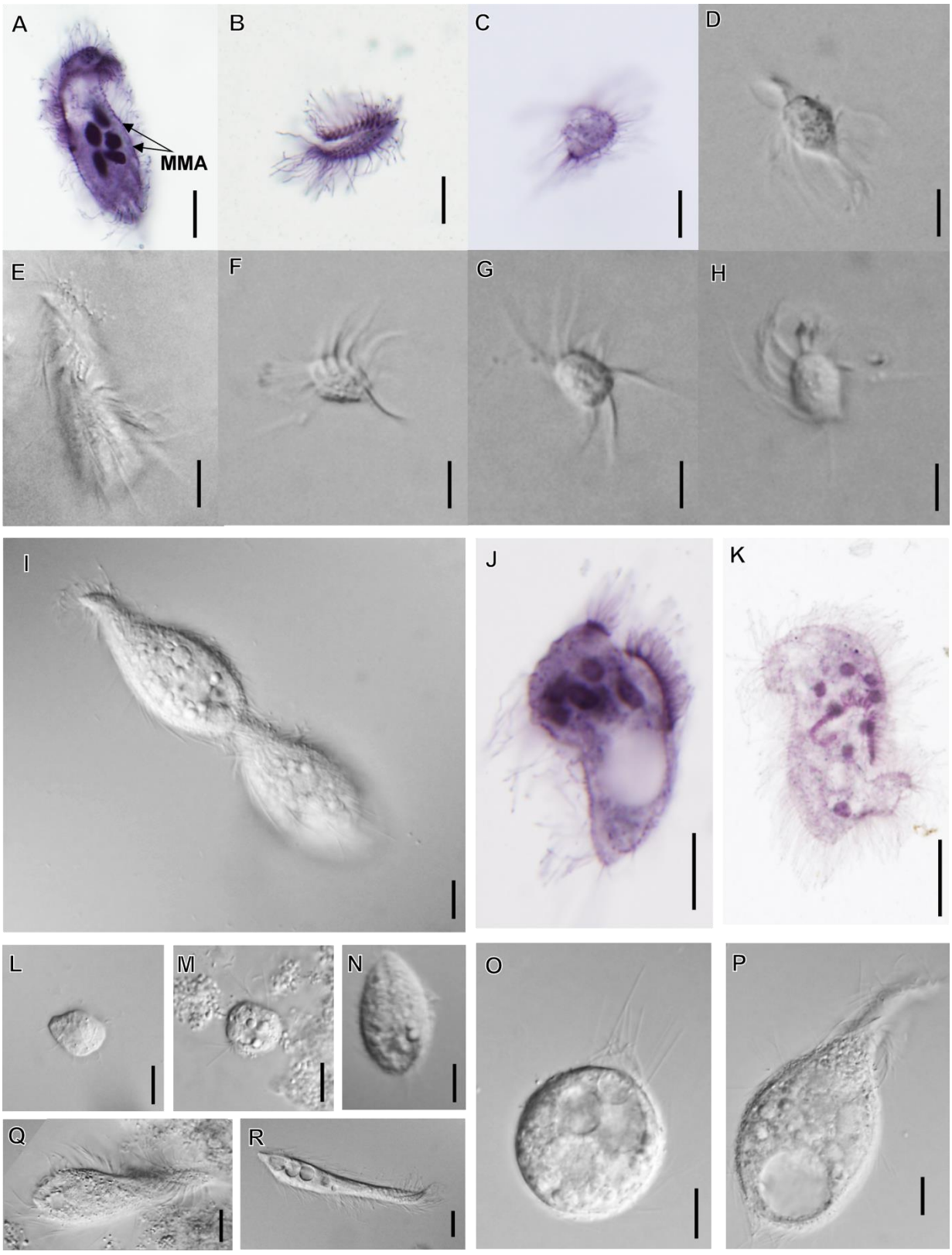

Fig 43. Various forms of muranes. A, B - strain MURANON, protargol stained cells, C, D - living small cell forms, E – larger living cell form, F, G - small living forms, H - strain RIDKA, protargol stained cell, possibly just empty cell with membrane and ciliature, I - strain MURANON, dividing cells, J, K - strain RIDKA - strange cell, maybe damaged cell, L - R - various small forms of MURANON ciliates, strains RIDKA, MURANON, SUMMARTIN; scale bars 10 μm.

4.2.5. Ultrastructure of muranes

We have studied the ultrastructure of two strains of muranes (MURANON, RIDKA). The ultrastructure of the two strains is consistent. We have observed the cells thoroughly, even though we have focused especially on searching for mitochondria and observing the nuclei and cortical structures. Unfortunately, the fixation was suboptimal, which is, however, common situation in ultrastructural studies of marine protists, as the fixation in those is frequently worse than in freshwater ones (personal communication with I. Čepička).

We have confirmed that there are multiple macronuclei present (Fig. 44). We have also observed a putative micronucleus, which we have never seen in living or protargol stained cells (Fig. 44, C, G). We have found mitochondrion-related organelles. They have a well visible double membrane and resemble hydrogenosomes of metopids but do not seem to possess cristae (Fig. 45). These organelles are small and round or slightly oval and occur in the cell in high numbers.

In TEM, we have recorded only somatic dikinetids and possibly monokinetids (Fig. 46), despite the fact that protargol technique shows somatic trikinetids (Fig. 42, F, G). The position and connectors between the two kinetosomes are different from other classes, although they most resemble the ones described in our metopid species (Figs 36, 47). We have recorded two kinetosomes both bearing cilia, with the anterior kinetosomes being allied by a row of microtubules in some sections. Due to the suboptimal fixation, we were not able to recognize if the microtubules are underlined by a fibrillary structure as in metopids or not (as in heterotrichs, *Protocruzia*, and *Euplotes*, Lynn, 2008). The posterior kinetosome showed a long diverging microtubular row, as in metopids and heterotrichs, which was allied by fibrillary structures from both sides as in metopids. The connection between the two kinetosomes was represented by two diverging rows towards the posterior kinetosomes, similarly as in metopids. There was also a laterally directed kinetodesmal fibril, which seemed unstriated, but had a different shape than in metopids (i.e. shorter and "conical"). However, we could see also a fibril diverging dorsally from the posterior kinetosomes, and another laterally directed fibril allied by the entire length of the dikinetid (which could be an artefact). The oral ciliature is formed by oral polykinetids. Some dikinetids were unevenly positioned, with each kinetosomes directing to a different side.

There are multiple mucocysts present within the cell cortex (Fig. 47, H). Structures similar to trichocysts described in ciliates (Hausmann & Radek, 2014) were observed as well. We have observed also a conspicuous rippled glycocalyx and flattened alveoli. Similarly as in metopids, we have also discovered endosymbionts and ectosymbionts (see 4.3.).

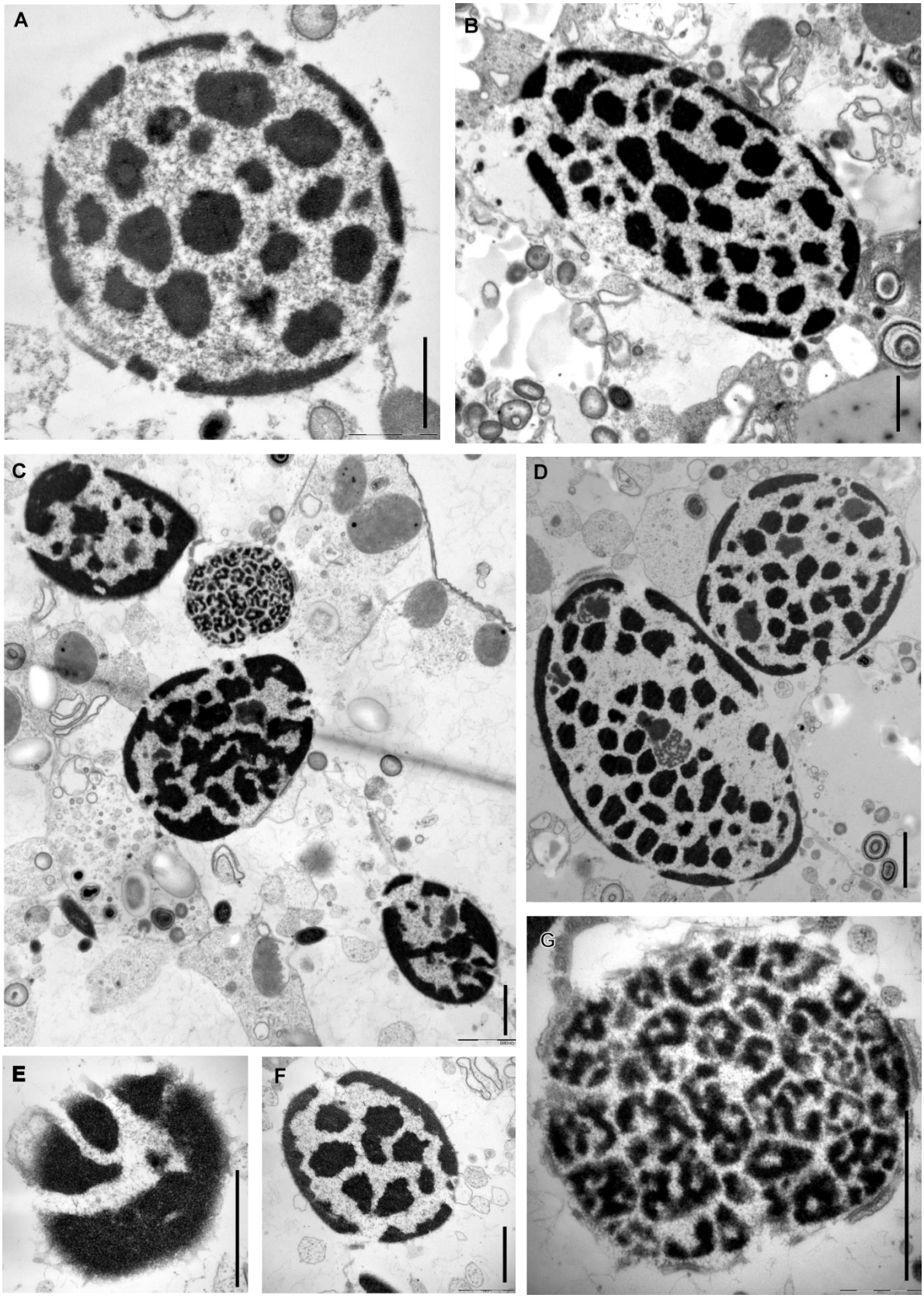

Fig. 44. TEM of murane nuclei; A - strain MURANON, macronucleus, B - F - strain RIDKA, macronucleus; G – micronucleus, scale bars 0.5 μm.

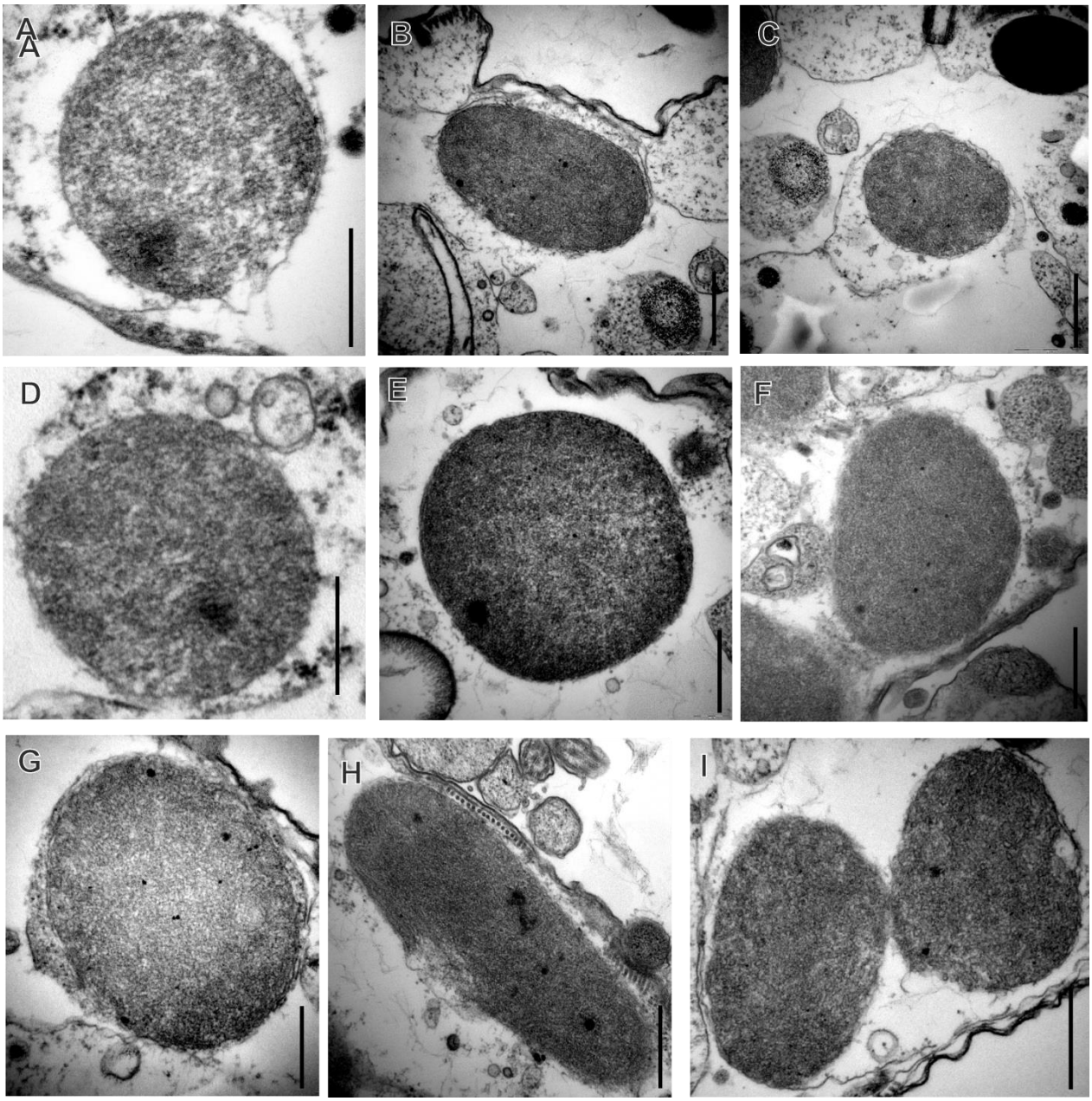

Fig. 45. TEM of murane MROs; A, D - strain MURANON; B, C, E, F, G, H, I - strain RIDKA, scale bars 0.5 μm.

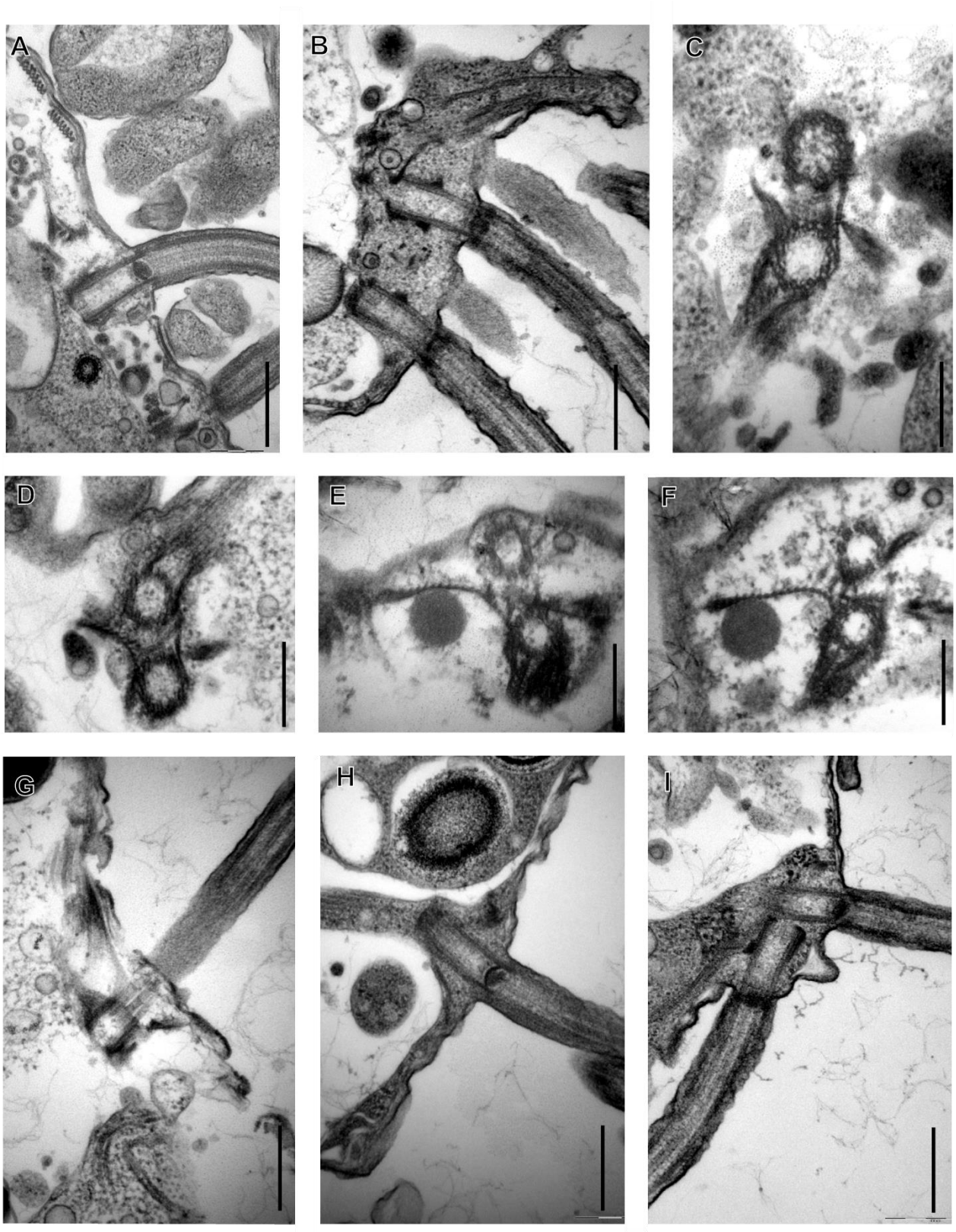

Fig. 47. TEM of murane somatic kinetids, A, B - strain RIDKA, somatic dikinetids, longitudinal section; C - strain RIDKA, somatic dikinetid, transversal section; D, E, F - strain MURANON, somatic dikinetid, transversal section; G, H - strain RIDKA, somatic monokinetid; I – strain RIDKA, somatic dikinetids in diverging position; scale bars 0.5 μm.

4.3. SYMBIONTS OF ANAEROBIC CILIATES

We have observed prokaryotic symbionts in our strains of armophorids (Fig. 48) as well as muranes (Fig. 49). We have studied the symbionts in four armophorid strains (CANC, RIOZ, TROODOS, SIPEK) and two murane strains (MURANON, RIDKA) using transmission electron microscopy (TEM). The presence of endosymbionts in cells of Armophorea and other anaerobic ciliates has been studied and confirmed multiple times (Embley et al., 1992a; Embley et al., 1992b; Narayanan et al., 2009; Van Bruggen et al., 1986; Finlay & Fenchel, 1989; Biagini et al., 1997a; Finlay et al., 1993a; Lloyd et al., 1996; van Hoek et al., 2000a; Hackstein et al., 2010).We have observed both endosymbionts and ectosymbionts. The endosymbionts are present in the most of our studied armophorid strains and in all murane strains. The ectosymbionts were observed in marine representatives of Armophorea (i.e. *Metopus contortus*) and in muranes. Interestingly, we have discovered putative ectosymbionts also in one freshwater strain of *Metopus es* (strain TROODOS).

Those endosymbionts were present in all cells, while varying in shapes and types from species to species. There were even several different prokaryote morphotypes present in the cells of *Metopus* sp. 2 (Fig. 48, I – K) and *M. es* (Fig. 48, E, F). Those endosymbionts are always in the near presence of hydrogenosomes, which supports the idea of their methanogenic character, although they are not directly allied together as in other anaerobic ciliates (including *Metopus contortus*) described in Embley & Finlay (1993). Additionally, there are undefined prokaryotes within the ciliature of *M. es*, although it is unclear whether those are ectosymbionts or free-living organisms, as there is no attachment visible (Fig. 48, G). On the other hand, there are obvious rod-shaped prokaryotic ectosymbionts attached to the cell surface of the marine *M. contortus* (Fig. 48, H).

In the freshwater armophorid strains of *Metopus* sp. 2 (RIOZ) and *M. es* (TROODOS), there were two similar morphotypes of prokaryotes present in both species. The first morphotype is represented by moon-sickle shaped cells which are frequently paired (Fig. 48, E, J, K). The second morphotype is represented by larger elongate rods (Fig. 48, F, I, K). Both of them, especially the former morphotype, are always in close association with hydrogenosomes.

We have observed endosymbionts of different shapes in murane ciliates (Fig. 49). These were rod-shaped or ovate, occurring near the mitochondrion-related organelles (Fig. 49). We have also discovered ectosymbionts (Fig. 49), which were represented by elongate rods allied in groups (strain MURANON), or by individual cells attached to the surface of the cell (strain RIDKA).

Due to the close association of the prokaryotes with hydrogenosomes in the cells of armophorids and the known presence of methanogenic archaea in some anaerobic ciliates (Van Bruggen et al., 1986), we have decided to use autofluorescence microscopy to confirm methanogens, which is possible thanks to the presence of autofluorescent cofactors F420 and F350 within their cells (Doddema et al., 1978). We were able to use the filter enabling emission of F350, which enables a view with less contrast, but still good/functional results (Doddema et al., 1978). We confirmed that at least some endosymbionts of metopids (*Metopus es, Metopus contortus, Metopus* sp. 10, *Metopus* sp. 11) (Fig. 48, A - D), as well as murane ciliates (strains MURANON, RIDKA, SUMMARTIN) are methanogens.

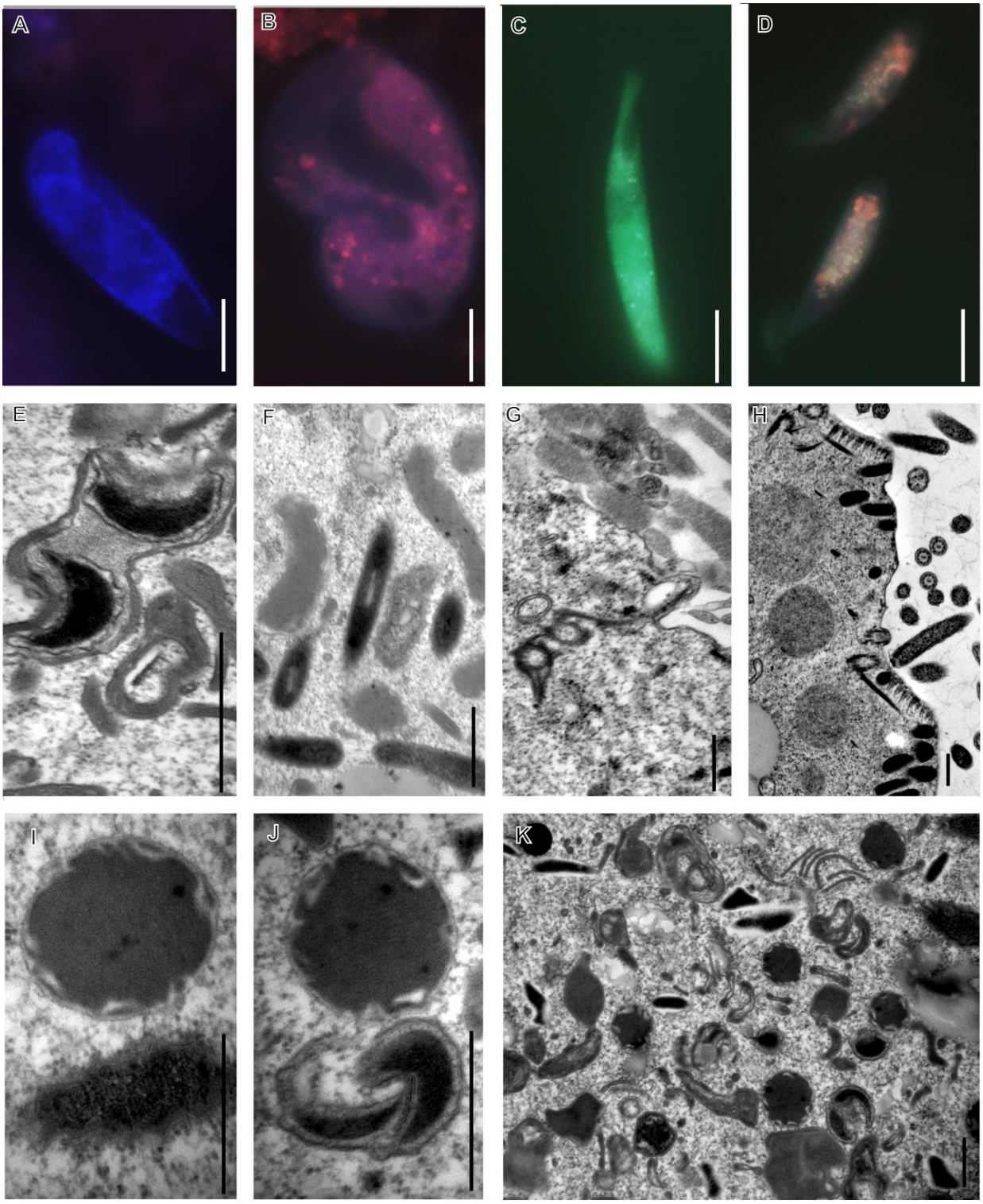

Fig. 48. Symbionts of armophorids, A – D - autofluorescing methanogens; A – *Metopus es*, strain TROODOS; B – *Metopus contortus*, strain SIPEK1C; C – *Metopus* sp. 11, strain KORRISSION; D – *Metopus* sp. 10, strain SALKALUZ; E – K – TEM; E, F – endosymbionts in *Metopus es*, strain TROODOS; G – putative ectosymbionts in *Metopus es*, strain TROODOS; H – ectosymbionts in *Metopus contortus*, strain SIPEK1C, I – K - endosymbionts in *Metopus* sp.2, strain RIOZ; white scale bars – 50 μm, black scale bars 500 nm.

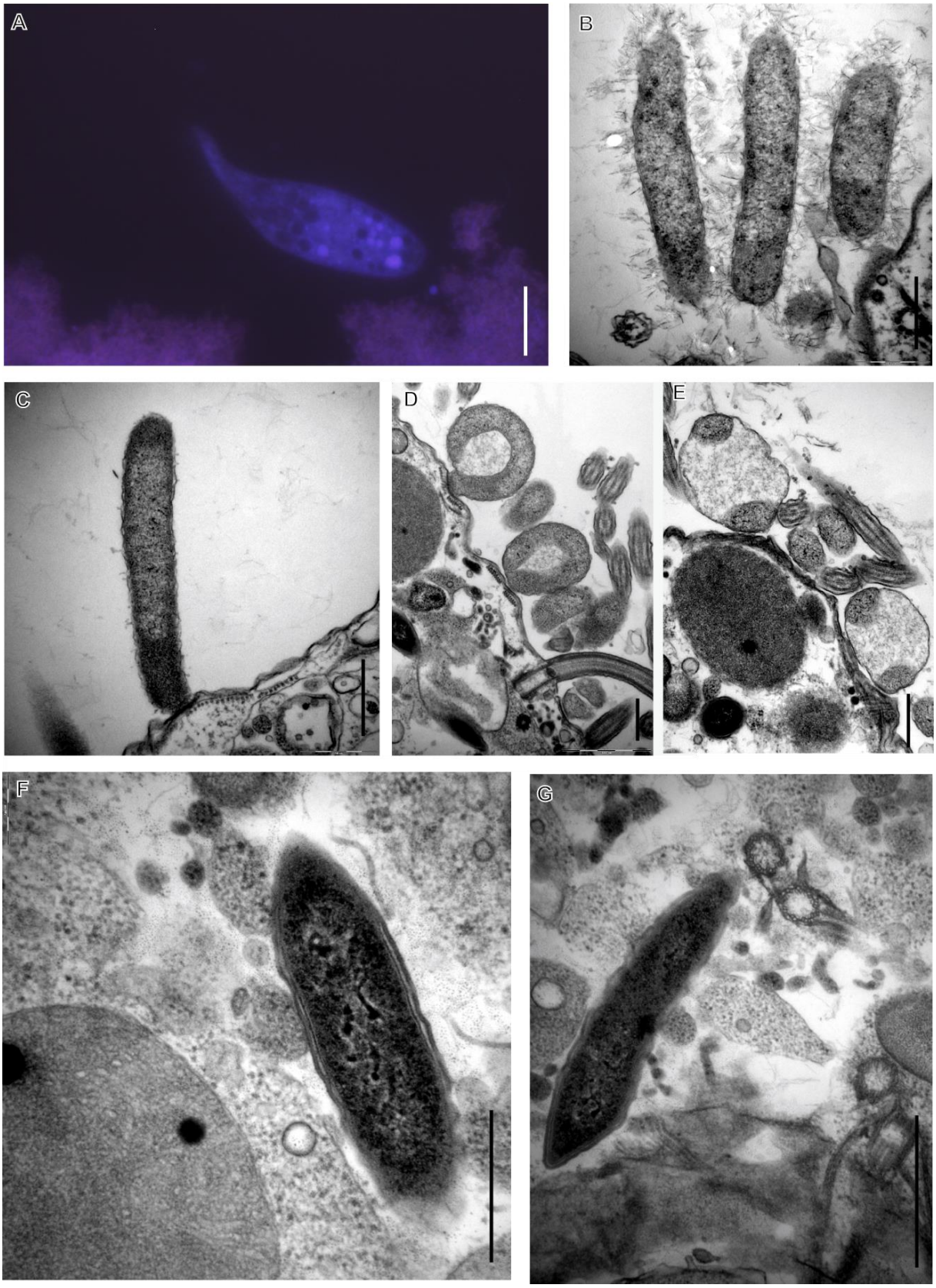

Fig. 49. Symbionts of muranes, A - autofluorescing methanogens, strain MURANON; B – ectosymbionts, strain MURANON; C, D, E – ectosymbionts, strain RIDKA; F – endosymbionts, strain MURANON; G – endosymbionts, strain RIDKA; white scale bar – 50 μm, black scale bars 500 nm.

5. DISCUSSION

5.1. CULTIVATION OF ANAEROBIC CILIATES

The cultivation of anaerobic ciliates in our laboratory has been started by Msc. Ludmila Nováková, who had focused her master's thesis on initial collection of cultures of anaerobic ciliates and studying their molecular diversity, during which she has (among other ciliates) collected 16 armophorid isolates. She has confirmed suitability of the cultivation method (Nováková, 2011). We have significantly expanded this collection by obtaining many more samples and establishing new cultures. Currently, our collection of metopids comprises 93 cultured strains, which is, to our knowledge, the only current and historically largest collection of metopid cultures in the world, and we continue in obtaining samples. Our collection contains also strains that are maintained in culture for years, as well cultures containing more co-cultured species, and, interestingly, the ciliates keep their endosymbionts and ectosymbionts in our cultures for years. These circumstances allow us to perform further studies (e.g. host specificity of the symbionts) in future.

Thanks to our advantage of establishing and maintaining cultures of nearly all studied strains, we were allowed to observe the cells in the natural sample, sometimes shortly after the moment of isolation, during the culture establishment process and, of course, periodically during the entire time of the cultivation. Therefore, we were able to document if the cultivation process has any impact on the biology, morphology and behaviour of the ciliates. The impact of cultivation on anaerobic ciliates, and protists in general, is a topic for forever going discussion (Esteban et al., 1995; Narayanan et al., 2007). There have been various methods of cultivation of anaerobic ciliates carried out and published. Usually, there are brief notes about changes of shape or size, or loss of endosymbionts (Esteban et al., 1995). We could only speculate what were the triggers for such changes, as there are certainly numerous factors influencing these features in all protists. However, in our cultures, the changes seem rather minor, and we have not observed the changes described by others (even in the same species). The variation in morphotype seen during of shortly after isolation mostly persists in the culture for years. We are not able to confirm this for the cultures that have been established long before the initiation of this study, although these are mostly identical to cultures established later on or even recently.

Even though we mostly cultivate freshwater armophorids, we commonly establish also brackish and marine cultures. In few cases, (i.e. strains COLOMBIA, KAO, QDU, SALKAM) we had a ciliate isolated from a freshwater locality that has, however, been shown to be closely related to a marine strain or assigned to a species known only from marine habitats. Therefore, we

have tried a transfer into a medium for marine ciliates, and we have succeeded. The strains COLOMBIA, KAO, QDU are currently cultivated in brackish medium, and we will continue with the transfer into marine medium. Right after the first inoculation (and every following inoculation into medium with higher salinity) the ciliates compress/shrink, some die, but the majority of the cells survives and reproduces. When observed after a week or two, the ciliates showed the normal morphology. Thus, at least some metopid species obviously have a high tolerance for environmental changes. Out of curiosity, we have also tried similar process with other freshwater species (e.g. strain TROODOS of *Metopus es*) or, vice versa, analogically with marine species (e.g. strain LAGUNDRI of *Metopus halophila*). All of them died already in the first passage.

Interestingly, *Metopus palaeformis* (strain MAREK4), which has been cultivated in Dobell and Laidlaw's biphasic medium, had a different morphology than conspecific strains cultivated in ATCC medium 802. It resembled *Tesnospira alba* (Jankowski, 1964), which was later synonymized with *M. palaeformis*. The ciliates of the strain MAREK4 have changed their "swollen" morphology to an ordinary *Metopus palaeformis* after ca two months of cultivation in ATCC medium 802.

5.2. MORPHOLOGY OF ARMOPHORIDS AND USED METHODS

The reason for not carrying out a morphological study of certain strains (BOTANKA2, EVROS4B, IND5, PANT3, VLH1, WACT07) was the extermination of the cultivated strains before the beginning of this study, although there was partial information from the thesis of Msc. Nováková (Nováková, 2011).

For protargol technique, we initially used a protocol that was not developed for ciliates but for intestinal flagellates (Nie, 1950). We have gained some results using this technique in a quality good enough to be able to determine targeted characteristics and therefore obtain the data we needed. Results in some ciliates are sufficient with this protocol. However, the standard quality for species description is usually higher than our average quality and therefore, we have tried protocol by Foissner (2014), modified by Bourland (personal communication), which is specifically designed for ciliates. Using this protocol, we have not gained much better results yet, but it is supposedly a matter of small adjusting, and we will try again.

There were also differences between the results given using 10% aqueous formalin or Bouin and Hollande's fixative. Both work well with freshwater ciliates, but the former deforms marine ciliates, and sometimes also brackish ones. Therefore, the latter fixative is more suitable for marine ciliates.

5.3 SPECIES IDENTIFICATION

It was impossible to determine many strains on the species level with certainty. The first reason may be the insufficient amount of data obtained from these strains, for example due to the recent establishment of the culture, or, oppositely, the extermination of the culture in the beginning of this study. However, the second, and more prevailed, reason is the utter chaos in the literature about the taxonomy of free-living armophorids, the boom of descriptions in the first half of the 20th century persisting all the way to the end of the century, which includes numerous wrong synonymizations and numerous parallel descriptions of a single species (e.g. 16 synonyms of *Metopus striatus*), and the fact that some species descriptions were rather brief and based only one simplified drawing; or, contrarily, multiple descriptions of one species, with more less significant differences between the descriptions. In addition, many species have never been recorded again after the original description. The third reason, which applies especially for the newer descriptions, is the astounding lack of molecular data for armophorids (and all armophoreans in general). In the beginning of our study, there were identified species of just two metopid species whose SSU rDNA sequences were available in GenBank, namely *Metopus contortus* and *M. palaeformis* (Embley et al., 1992a; Hirt et al., 1995), besides several environmental or unidentified armophorid sequences (e.g. Riley & Katz, 2001). During our study, sequence data from seven additional species together with detailed morphological descriptions of three and reliable micrographs of all were obtained and published by Bourland (2014a, b, c).

The issues described above highly hinder the process of determination for everyone who wants to avoid misidentifications and have led to initial confusion in our study about several species, namely *Metopus contortus*, *M. mucicola*, *M. rostratus*, *M. undulans*, and *Palmarella salina*. For example, the species *Metopus mucicola* has been transferred to the genus *Bothrostoma* by Esteban et al. (1995), and we have not found any motive for such change. Contrarily, we would argue for transferring it to the genus *Palmarella*, as this species resembles other species of the genus *Palmarella* by, for example, the wide peristomal area, short zone of adoral membranelles, and smaller and shortened cells, rather than genus *Bothrostoma*.

The identification of strain TIKAL1 as ***Bothrostoma mirabilis*** was based on a small number of cells as the culture was obtained very recently and is not stable yet, this ciliate is not very abundant there, and we were not able to amplify DNA of this strain. Thus, more data will be needed to be certain about the species identity of this strain.

We have examined more thoroughly the morphology of three strains that we identified as ***Brachonella* sp. 1** or ***Brachonella campanula*** with several doubts. We were not able to determine whether our strains of this species belong to *B. campanula* or another similar species, due to

several differences (see 4.1.2.). This species is closely related to *Metopus striatus* in our phylogenetic trees, which is consistent with their similar body shape, the large preoral dome, and the shape of AZM.

Jankowski (1964) transferred *Metopus darwini* to the genus *Brachonella*, renaming it ***Brachonella darwini***. It is still a question, whether the reason for such a transfer was the size of the preoral dome, typically large in *Brachonella* spp. and usually thinner in *Metopus* spp. Otherwise, this species is morphologically similar to some *Metopus* species, namely *M. spinosus* and *M. propagatus*, which also have a pointy, elongated posterior end, or distantly also to *M. striatus*, which has a smaller posterior elongation. However, we have not been able to obtain sequence data from the only strain of this species in our collection due to the instability of the newly obtained culture.

Even though we were able to isolate only a single strain of ***Metopus bothrostomiformis***, we suppose that this species is a cosmopolite, because our strain comes from North America (small lake in the direct vicinity of the ocean), while Foissner's (1980) *M. bothrostomiformis* comes from Europe (high mountains). We also suggest its future transfer to the genus *Bothrostoma* due to the morphological similarity and, mainly, close relationship of this species with *Bothrostoma* spp.

Despite the missing morphological description of the strain QUEENSLAND, which was exterminated prior this study due to incorrect choice of freshwater medium, we have assigned it to *Metopus contortus* due to the nearly identical SSU rDNA sequence to the one of properly examined *Metopus contortus* (strain SIPEK1C). We have also not been able to carry out morphological description of our strain SALKAM (***Metopus halophila***) due to the premature extermination of an instable culture. Nevertheless, we can confirm that this strain was almost identical with the strain LAGUNDRI, at least when the general morphology of living cells is concerned. The culture possibly did not survive due to the incorrect choice of freshwater medium, for which we decided according to instructions from a colleague who established the culture with this strain but did not have information about the place of collection of the original sample, which was collected by an another colleague. Thus, this strain was likely marine according to the phylogenetic analysis. We have not been able to obtain sequence data from the strain LISTOPADM due to the instability of this freshly established culture and scarce numbers of the cells within, but we still assign this strain to the species ***Metopus laminarius*** on the basis of its typical morphological features.

Initially, we cultured several strains of ***Metopus* sp. 2** (MORETESB, RIOZ, RIOTM, PANT3) that were all isolated from samples collected in South America by different collectors during several years. Therefore, we assumed that this species may have a geographically limited

distribution. Later on, we obtained strains of this species from samples collected in Africa (DR Congo, strain LUKIFOREST), Asia (Nepal, strain CHITWANMALY). Another strain (VLH1**)** comes from a sediment sample from an aquarium with putative South American species of turtle (unknown). However, we do not know the origin of this particular turtle and we cannot conclude that it may have or have not carry (e.g. encysted) the ciliate from the original South American environment and thus fit in the hypothesis about a limited geographic distribution, as it would be just a pure speculation. Our findings suggest that at least some metopids may not be cosmopolites. However, further sampling is needed to confirm such hypothesis to avoid premature and unjustified conclusions.

Strains of ***Metopus* sp. 2** were initially identified as ***Metopus hasei*** Sondheim, 1929, according to several morphological features (see 4.1.) that we have not seen elsewhere. However, due to different anterior part, i.e. the narrowed anterior part with overlapping preoral dome in *M. hasei*, which is not present in our strains, we have abandoned this intention and identified this species as *Metopus* sp. 2. Kahl (1927) also described ***Metopus setifer*** Kahl, 1927, which is depicted similarly to our strains but was; however, later synonymized with (unrelated to our strains) *Metopus setosus* by Esteban et al. (1995). Nevertheless, its anterior part was described as wider, and overall the species could be related or identical to our strains of *Metopus* sp. 2. Additionally, the stains also resemble another species – ***Metopus rostratus*** Kahl, 1927. There are no references suggesting these two species could be related or even synonymous. Contrarily, *M. rostratus* has been synonymized with *M. palaeformis* (Esteban et al., 1995), but we disagree and are convinced that the species Kahl (1927) described was different from *M. palaeformis* due to the differently shaped AZM. Unfortunately, there are no reliable descriptions of this species, besides a simple drawing in Kahl (1927).

We have assigned two strains to the species ***Metopus mucicola*** based on a simple but distinctive drawing of Kahl (1927). This species has been transferred to the genus *Bothrostoma* by Esteban et al. (1995), but we argue against, as we could not find any morphological features suggesting the relation to the genus *Bothrostoma* and as our strains are not closely related to this genus. Rather, if there is any genus that *M. mucicola* should be transferred into; we would suggest *Palmarella* due to the common morphological features (wide peristomal area, short zone of adoral membranelles, small cell size and its shortened ovate shape) and a close phylogenetic relation to our marine strains of *P. salina.* This species is also closely related to the morphologically similar species *Metopus* sp. 2.

Esteban et al. (1995) transferred ***Metopus nasutus*** to the genus *Copemetopus* (da Cunha, 1915). However, *Copemetopus* is a member of heterotrichous climacostomids (Al-Rasheid, 2001). Al-Rasheid (2001) rejected the affiliation of this ciliate to climacostomids and, therefore, the

transfer itself. Already Kahl (1927) expressed doubts about assigning this ciliate into genus *Metopus*, as it was reminiscent of *Bothrostoma* to him, and we concur, having studied the morphology of strains IZR, and TIKALBO. Moreover, our strains assigned to this species are closely related to several *Bothrostoma* species.

After we discovered that our strains COLOMBIA, KAO, and QDU are phylogenetically related to the marine species *Metopus contortus*, with which they also share morphological features, we decided to try to transfer them into a brackish and subsequently marine medium. They all survive brackish medium without difficulties. Due to their morphological features, we have assigned the strains as ***Metopus nivaaensis***. We will continue with the transfer to marine medium. The sample collectors have not measured the salinity of the original environment. Thus, it is yet to answer, whether this species is able to inhabit either both freshwater and marine environment; or whether it is actually a freshwater species but evinces a high tolerance to the environment. Anyway, the ciliates have survived the freshwater medium in a time range from several months to almost a year. Contrarily, from our experiments it is obvious that if any other freshwater, respectively marine species present in our collection is inoculated into a medium with higher, respectively lower salinity, they die almost immediately.

We have assigned the strain SULMOK2 to species ***Metopus* sp. 7** and concluded that it is morphologically very similar to ***Metopus setosus***. However, in our phylogenetic analysis, the sequence of our strains is not closely related to the GenBank sequence assigned to *Metopus setosus* by Bourland et al. (2014c), although the micrograph in the same publication resembles our strain SULMOK2. Thus, these species either have evolved convergently to a similar morphotype, or there was a mistake during determining the DNA sequences.

Contrary to Esteban's et al. (1995) claim that ***Metopus striatus*** loses its posterior pointy end when cultured, cells of all eight strains in our collection assigned to this species, maintain their pointy posterior end even during years of culturing. However, there are various morphotypes present in the culture (already at the time of isolation and, therefore, most probably not caused by the cultivation process), including smaller rounder forms or longer thinner forms, which may have caused the multiple independent descriptions of this species.

Recently, we have obtained several new cultures containing ***Metopus spinosus***. However, due to the instability of the fresh cultures, we were able to obtain sequence data only from a single strain, KUBAMA3. Quite surprisingly, we obtained SSU rDNA sequence almost identical to *M. striatus*, which was never observed in the culture. We did not assign this sequence to the KUBAMA3 strain of *M. spinosus* as we could not exclude contamination. Yet, we also consider that such relation is possible and, therefore, we are going to repeat the sequencing when this culture gets stable.

The only description that resembles metopids in the strain CANC (***Metopus* sp. 1**) is a simple drawing in Kahl (1927) (page 131, **Fig**. 3c), which was however, identified as *Metopus palaeformis* by him. Therefore, we think that either the drawing is too simple to allow determination of a species from it, or the drawing actually does not represent *M. palaeformis* but the species the strain CANC belongs to. Interestingly, the species was placed in the same group with *M. mucicola*, which is in our phylogenetic study related to the strain CANC, and also shares common morphological features, e.g. the wider peristomal area.

The strain SALKALUZ (***Metopus* sp. 11**) was collected from a small temporary pond, only few meters away from the seashore on an arid island locality, and it grows only in the marine medium, which makes us conclude this species is a marine ciliate. Nevertheless, the second hypothesis is that this strain is a hypersaline soil ciliate inhabiting arid localities, as hypersaline and arid metopids have already been recorded (Foissner & Agatha, 1999).

Strains KAN2 and FRIOJESKYNE (***Metopus* sp. 3**) were not assigned to a particular species, although we may suggest they represent a variation of the species *Metopus es* using a conservative approach, as these two strains are closely related to our strains of this species. On the other hand, the two lineages are separated from each other by a relatively long branch suggesting that they represent separate species. We were not able to study the morphology of the strain FRIOJESKYNE, as the culture was prematurely exterminated, before the beginning of this study but we have assigned it to a species *Metopus* sp. 3 with the strain KAN2 due to its close phylogenetic relation.

We have had several difficulties identifying the marine strains BUSSPRAND, BUSSELTON, and SIPEK1APA. Originally, we speculated whether the two morphotypes present in the first established culture, BUSSPRAND, represented two separate species, although the PCR product cloning would generate only sequences of a single species. However, the same variations occur in the strain BUSSELTON. Later on, we have obtained another sample from another location, and established a culture of SIPEK1APA strain. There were again the same two morphotypes present in the culture, which we also managed to establish as monoeukaryotic. We have also performed PCR product cloning of this culture and just as before, we have obtained only sequences of one species. Thus, we have concluded that these are two morphotypes of a single species, ***Palmarella salina***, despite there is only very scarce morphological data available for this species (and for the entire genus in general). Surprisingly, our strains of *Palmarella salina* are not phylogenetically related to the freshwater species *P. lata*, whose 18S rDNA sequence was determined by Bourland et al. (2014c). Considering the latter and the very closely related freshwater metopid species (*Metopus* sp. 1 and 2, *M. mucicola*), the genus *Palmarella* is polyphyletic and *Metopus* is paraphyletic.

The strains QUMBASCHI (***Metopus* sp. 5**), DRINGARI2 and IND5 (***Metopus* sp. 6**), ALOOA3 (***Metopus* sp. 8**), and API **(*Metopus* sp. 9**) are morphologically similar to *Metopus laminarius* and *M. palaeformis,* although they probably represent distinctive species. Surprisingly, all the typically thin elongate ciliates (with an elongate macronucleus, as well as other common features) from species *Metopus laminarius, M. palaeformis*, *M. setosus,* and the strains ALOOA3 (*Metopus* sp. 7), API (*Metopus* sp. 9), DRINGARI2 and IND5 (*Metopus* sp. 6), QUMBASCHI (*Metopus* sp. 5), as well as the marine strains KORRISSION (***Metopus* sp. 10)** and SALKALUZ (*Metopus* sp. 11) form a branch where are also *Atopospira* spp., although they have no close morphological resemblance with the two species in this genus. This branch also includes the entire order Clevelandellida, which has resulted as a terminal lineage of Metopidae in our phylogenetic analysis.

Our goal in this study was not to revise the taxonomy of Armophorea. Nevertheless, we have observed that the class needs a taxonomic revision due to the prevalent chaos. We have observed species which we could not assign to any known ciliates, sometimes also because we have not obtained sufficient data to do so. However, several species, which we have studied relatively deeply, were not possible to be determined as a known species. We conclude that these may be novel species, although we will need more data to confirm that. Obviously, the diversity of Armophorea is underestimated and even though there were over 70 described species throughout the history, we have still observed undescribed morphotypes. Additionally, there are environmental clades which are unrelated to any species with available molecular data. Those may also represent at least some novel species, as the clade branching with *Metopus contortus* includes solely marine sequences and there were no other marine species described beside those that we have observed and molecularly define.

The ancestors of Armophorea were likely freshwater ciliates and thus we conclude that they have occupied the marine environment independently at least three times (*Palmarella salina*, *Metopus contortus* and its relatives, *Metopus* sp. 10 and 11), or possibly four and more times, for examples if *Metopus spinosus* is really closely related to freshwater *Metopus striatus*. Clevelandellids likely originated from the freshwater armophorids.

5.4. ULTRASTRUCTURE OF ARMOPHORIDA

The first finding while observing the ultrastructure of metopids was the lack of visible alveoli in the cells of *Metopus es* (strain TROODOS) and *M. contortus* (strain SIPEK1C), which was conspicuous, as it is a feature characteristic for ciliates and even one of the main features of all alveolates. Firstly, we assumed it was an artefact caused by suboptimal fixation. However,

Fenchel & Finlay (1991) and Lynn (2008) mention the compression or even total absence of alveoli in metopids, although they noted those are conspicuous in caenomorphids. It is yet to answer what is the reason for such modification and whether it is correlative of their adaptations for anaerobic lifestyle. Nevertheless, we have discovered interesting vesicle-like structures in the cortex of *Metopus* sp. 1 (strain CANC) and *Metopus* sp. 2 (strain RIOZ). It is a matter of discussion whether those were formed by an improper fixation or they represent actual alveolar layer, which would be contrary to the previous findings, or simply have a different purpose that needs to be resolved.

The hydrogenosomes of our studied strains also differ from previously described hydrogenosomes in *Metopus palaeformis* (Fenchel & Finlay, 1991), which supports our hypothesis that the morphology of hydrogenosomes differ inter-specifically in metopids.

Our study contributes to the knowledge about the cortical structures of metopids, especially because we have studied several species and were able to compare one to each other. The present study has already revealed several differences from the current recognized dikinetid connection in metopids, from the ones described in Schrenk & Bardele (1991), Foissner & Agatha (1999) and explained in Lynn (2008). The structure of somatic dikinetids of metopids, their position and connection have not been studied in detail before and there have been calls about the necessity of such study (Lynn, 2008). The connection between the two dikinetids may be formed by two rows of transverse microtubules and not one as described previously. However, on some sections that were not showing the entire connecting structure, it seemed like just one microtubular row, which may explain the conclusions from the previous studies. Therefore, it is always necessary to conclude these structures from larger number of samples, sections, and mainly to compare them between various strains and species.

5.5. CAENOMORPHIDAE - NOT ARMOPHOREANS?

We have encountered *Caenomorpha* ciliates in our samples, although we were able to establish cultures from only two samples with strains BEAVERC and BHARA. Both cultures do no longer exist, although the strain BEAVERC had been cultivated almost for one year and, therefore, we can confirm that it is possible to cultivate at least some *Caenomorpha* species using our methods. However, we consider these ciliates (caenomorphids) relatively rare, as we have encountered them only a few times compared to many metopids. We have not been able to amplify DNA of any caenomorphids and we have obtained only limited morphological data.

As we have already mentioned (2.1.), caenomorphids are formally still part of the class Armophorea, as a family within the order Armophorida. Nevertheless, in our phylogenetic

analysis, Caenomorphidae form a clade that is unrelated to the rest of Armophorea. Instead, they appeared to be closely related to the class Litostomatea, though without any support. This result is not inconsistent with the study by da Silva Paiva et al. (2013), which showed a relation of caenomorphids with Litostomatea.

There were two GenBank environmental sequences that were identified only as "Caenomorphidae gen. sp." They appeared in two different positions within metopids. The first was AJ009658, closely related to *Metopus striatus* and, interestingly, *Brachonella* sp. 1. We see morphological resemblance of caenomorphids and the latter species (e.g. the presence of bell kinety). Thus, we conclude that this sequence was misidentified as a caenomorphid and actually represents unidentified metopids. The second sequence, AJ009661, appeared within the clade of *Metopus fuscus* and *Metopus setosus*. Both of these sequences were published by van Hoek et al. (1999), who speculates "the paraphyly of *Caenomorpha* species" due to these two sequences. We do not have information with what techniques were these two sequences assigned to belong to *Caenomorpha* species, but it seems rather unlikely, even if ignoring the fact that van Hoek et al. were actually speaking about possible polyphyly of *Caenomorpha* species, not paraphyly, concluding from their phylogenetic trees (van Hoek et al., 2000). Similarly to the previous case, we are convinced that this environmental sequence was misidentified, and we highly doubt that any caenomorphid would branch between these two species.

Considering that caenomorphids are phylogenetically not part of the class Armophorea, we must challenge the name of the class Armophorea, which was named after the armour of caenomorphids. If caenomorphids are not sister to Metopidae and the order Armophorida is polyphyletic, the class Armophorea should be represented only by a single family Caenomorphidae, and the rest of the current representatives (Metopidae + Clevelandellida) need a new uniting class name.

5.6. MURANES

We have cultured four strains of a novel anaerobic lineage of ciliates, the muranes. Morphologically, they are somehow similar to spirotrich genera *Stichotricha* Perty, 1849 and *Trachelostyla* Cohn, 1866 or the heterotrich *Parablepharisma collare* Kahl, 1932, or might be misidentified as the metopid *Metopus verrucosus* da Cunha, 1915. However, the muranes do not possess comprehensive characteristics of any representative of Armophorea, Spirotrichea or Heterotrichea, nor any other of the current ciliate classes, when studied in detail. Possible confusion may result from the size, shape of the body as well as the zone of adoral membranelles (AZM) and their common occurrence in marine sediments. We conclude that no species of the

murane lineage has been described yet, despite their relatively high abundance and frequent encounter, simply because they have always been confused with the ciliates named above, even by excellent researchers.

In the case of *Metopus verrucosus*, we speculate whether the species described exactly a century ago (da Cunha, 1915) with a simple drawing and one paragraph (fitting to metopids characteristics), could actually be a description of a muranes species. Incorrect taxonomic assignments happen very frequently in the ciliate taxonomy and we can only guess if this is also the case, or if there is a real metopid *Metopus verrucosus*. The latter hypothesis is supported by a TEM micrograph of Bernhard et al. (1997), which shows very different structures in *Metopus verrucosus* than seen in the TEM study of our murane strains. That, however, can also be caused by a different (or improper, in our case) fixation.

In cases of *Stichotricha* sp. and *Trachelostyla pediculiformis*, we tried to investigate all possible details and differences to make sure muranes can be distinguished from them, as there are many common features. Besides the similar AZM, the body size and shape, muranes also possess multiple macronuclei, have a narrowed proboscis-like anterior part, and also the ciliature pattern is similar, including the dorsal bristles, respectively long somatic isolated single cilia that are well visible in light microscopy.

However, muranes also differ from these spirotrichs in many features. Firstly, they seem not to possess the conspicuous anterior and posterior cirri, or any other somatic cirri. We have observed somatic mono – di - and trikinetids, which has been described in other classes, for example in unrelated plagiopyleans, where the prevalent somatic dikinetids are sometimes supplemented by inserted mono- and trikinetids (Xu et al., 2013). However, we will need to perform a further analysis to confirm the somatic kinetid structure and to confirm an absolute lack of cirri. Thus, for the clarification and confirmation of the somatic ciliature structure of muranes, deeper research is needed.

Secondly, we have not any confirmation about the anaerobiosis of these species. Finlay et al. (1993b) mention an occurrence of unidentified anaerobic stichotrich; however, they did not provide any micrograph or morphological description of it. Similarly, Sacca et al. (2008) have recorded *Trachelostyla* sp. in anoxic waters, and Mazei & Burkovsky (2003) have listed *Trachelostyla pediculiformis* (Cohn, 1866) Borror, 1972 as a ciliate entering anoxic zones of sediment, but they have not provided any evidence about the identity of this species.

Importantly, the buccal cavity of muranes is placed in the centre of the cell and the AZM starts considerably closer to the posterior end of the cell. Also the behaviour is different, as the spirotrichs are supposed to swim fast forward, but muranes swim relatively slowly, they change speed, and they are able to swim backwards or spirally.

As last but not least difference, there is a published SSU rDNA sequence of *Trachelostyla pediculiformis* in GenBank (which does not apply for *Parablepharisma collare* or *Metopus verrucosus*), and we have confirmed that muranes are not phylogenetically closely related to any stichotrichs, including *Trachelostyla pediculiformis*.

One of the most interesting features in muranes is their morphological polymorphism and putatively complex life cycle, which have confused us in the beginning. We have established monoeukaryotic culture of a single strain, but after some time, we found out small ciliated cells, with a unique movement, as well as bigger ciliates of various shapes and sizes, which we were not able to identify. Later on, we have found out that all these cells belong to muranes. Firstly, after such discovery, we have concluded that these represent cell pieces after imperfect dividing process. However, those are perfectly viable cells and they appear constantly in the culture, the more frequent after ca a week of the reinoculation. Also, we have confirmed that these cells are not a result of the cultivation technique, as we have seen them also in a sample of another murane strain (SUMMARTIN), before the stable culture establishment.

The smallest forms may represent a type of ciliate reproduction - budding. That means departing of small ciliated forms (larvae) from the parent cell. They initially possess only the parts of the either anterior or posterior part of the cell. However, such situation occurs for example in suctorids, which do not show any relation to muranes. These small parts can also represent tomits, which is similar process that appears in other ciliates.

We have assessed the ultrastructural study of two murane strains to find out if they possess ultrastructural characteristics of representatives of the known classes. The mitochondrion-related organelles that we have observed were not similar to any described mitochondrion-related organelles or hydrogenosomes in ciliates. After observing the cells of the strain MURANON, we had considered that it may have been caused by improper fixation. However, the fixation of the cells of the strain RIDKA is better and the organelles look similar. Despite the fact that we have seen somatic trikinetids in protargol stained cells of muranes, we could observe only somatic dikinetids in TEM. There were also monokinetids in some sections, which we, however, consider to possibly be parts of the dikinetids, as in muranes, the two kinetosomes in the dikinetids may evince divergent direction from each other and thus, the second kinetosome may not have to be seen on some sections and it can form an impression of a monokinetid. This conclusion, however, would have to be confirmed by studying larger sample of muranes.

The position of muranes within ciliates has not been resolved. The presence or absence of the two environmental sequences AY179982 and AB505525 have altered positions of many lineages within ciliates, including muranes. If these sequences were included in the dataset, muranes appeared related to Armophorea and Spirotrichea, while if these sequences were

removed, muranes formed a lineage with Cariacotrichea, distant from other classes. Similarly, the positions of Cariacotrichea have been appearing in different topologies from various authors, with some concluding that Cariacotrichea is part of Spirotrichea (Lynn et al., 2008; Hausmann & Radek, 2014). SSU rDNA phylogenetic analyses cannot resolve phylogenetic relationships within main ciliate lineages, therefore, multigene analyses are necessary (Gentekaki et al., 2014). However, in consistency with both the phylogenetic trees and importantly also by the distinct morphological features in muranes, possessing characteristics for distinct classes, we conclude that muranes are a deep novel lineage of anaerobic ciliates, possibly not less than a novel subclass, if not a new class. We also need to confirm the ultrastructure of the somatic kinetids, which is an important determining feature in ciliates and could hint us the assignment of muranes within the classes of Ciliophora. Currently, it seems that the dikinetids are the most similar to armophoreans; however, there are substantial differences. Interestingly, the dikinetids seem different between the two strains MURANON and RIDKA. We need to study the ultrastructure on more samples and additionally on more strains to confirm the kinetid structure. From the current results, we are not able to confirm if the anaerobiosis in muranes is another independent one within ciliates or if there was a common origin either with cariacotricheans, spirotrichs or armophoreans.

5.7. SYMBIONTS OF ANAEROBIC CILIATES

Consistent with the published literature, we have confirmed methanogenic endosymbionts in our armophorid strains, which keep these endosymbionts even throughout years of cultivation, which has not been recorded yet. We could not confirm a presence of multiple prokaryote endosymbiotic species hosted by one ciliate species as we were not able to determine whether the two diverse endosymbiotic prokaryote morphotypes, present both in *Metopus es* and *Metopus* sp. 2, represent various prokaryotes or contrarily, a single species. The latter would be supported by the study of Embley et al. (1992b), who have suggested that endosymbiotic prokaryotes in metopids can actually be polymorphs of a single methanogen species. Therefore, further analysis is needed, such as obtaining DNA of all endosymbionts present in a ciliate cell.

The ectosymbionts have been observed only in marine species, which is a generally accepted conviction. However, there are numerous rods within the dense ciliature of the strain TROODOS, which are suspected to possibly also represent ectosymbionts of undefined character. There were, however, no connections spotted, which may argue for an ordinary presence of free-living prokaryotes within the ciliate ciliature.

6. CONCLUSIONS

We have globally mapped the diversity of free-living Armophorea, mainly Metopidae. We were able to do so also thanks to successful cultivation of more than a hundred armophorid strains from the extensive collection of anaerobic protist cultures established and maintained in our laboratory. Our samples come from all continents. We have briefly described the morphology of the studied strains and characterized them molecularly, as well as a basic study of their ultrastructure, which revealed the presence of hydrogenosomes with surface cristae and ecto – and endosymbionts, at least some of which are methanogens, confirmed by autofluorescent microscopy. We have carried out phylogenetic analyses based on SSU rDNA which showed that the diversity of Armophorea was severely undervalued even despite the high number of historically described species. We have discovered and briefly described several new clades of Metopidae, some of which may represent novel species. Additionally, our phylogenetic analyses demonstrated that Caenomorphidae are not part of the class Armophorea, respectively, Metopidae and Clevelandellida may not be part of the class Armophorea, which was named after the armour like pellicle of caenomorphids.

There are certainly numerous insufficiencies in the individual species determination and description. However, the aim of this study was not a detailed description of studied strains and putative novel species but mapping the general armophorid diversity and deepening our knowledge about the free-living Armophorea.

Importantly, we have also discovered a novel deep lineage of anaerobic ciliates - muranes, which may represent a novel class or a subclass. We have cultured four murane strains, obtained from marine anoxic sediments from different parts of the world, which suggests that members of this novel lineage are widespread but overlooked. In our phylogenetic analyses, this lineage is placed within the SAL group but not assigned to any known class, which is consistent with overall morphological disparateness from other classes. We have shown that muranes are anaerobic and possess MROs without cristae. We have also proved that muranes host endosymbiotic and ectosymbiotic prokaryotes in basic ultrastructural study.

Majority of the cultivated strains studied here is still maintained in our laboratory and thus available for further research, in which we plan to study the current topics deeper as well as focus on other related topics to extend the current state of knowledge.

Further research aims resulting from the present study:

- Proper description of the possible novel species of Metopidae in cooperation with Dr. Bourland (University of Boise, Idaho, USA)
- Proper description of the possibly novel class of ciliates – muranes
- Mapping the diversity of Caenomorphidae
- Searching for other strains of muranes
- Revision of cortical hypothesis in Armophorea
- Generating and analysis of EST data from transcriptomes of the novel lineages
- Research of methanogens and other endo- and ectosymbionts associated with anaerobic ciliates, together with studying their host specifity other features in cooperation with Dr. Edgcomb (WHOI, Massachussets, USA)

7. LIST OF ABBREVIATIONS

ASW – artificial sea water

ATP – adenosine triphosphate

AZM – adoral zone of membranelles

BF – bright field

BI analysis – bayesian inference analysis

BLAST – Basic Local Alignment Search Tool

BPP – bayesian posterior probability

BS – bootstrap support

DIC – differential interference contrast

IPTG – isopropyl β-D-1-thiogalactopyranoside

LB medium – lysogeny broth medium

ML analysis – maximum likelihood analysis

n.n. – nomen nudum

NADH – reduced coenzyme nicotinamide adenine dinucleotide

PCR – polymerase chain reaction

PDH – pyruvate dehydrogenase

PFO – pyruvate:ferredoxin oxidoreductase

SSU rDNA – small subunit ribosomal DNA

TEM - transmission electron microscopy

8. GLOSSARY OF TERMS (partially from Lynn, 2008)

Adoral zone of membranelles – orderly arrangement of three or more oral polykinetids serially arranged typically along the left side of the oral region

Cathetodesmal-like fibril – periodically striated, subpellicular fiber, transversely oriented, arising from or near the anterior right region of the posterior somatic kinetosome of a somatic dikinetid, literally, "cutting" to the left toward the next kinety

Diplostichomonad – type of double paroral whose infraciliature is composed of two parallel rows or files of kinetosomes – the inner or endoral membrane and the outer or paroral membrane; the kinetosomes are never in dyads nor do they form a zigzag pattern, and all are ciliferous; a type of diplokinety

EPON - type of epoxy resin used in sample preparation for electron microscopes

Glycocalyx – glycoprotein-polysaccharide covering that surrounds the cell membranes of some bacteria, epithelia and other cells

Holotrichous – having somatic cilia evenly distributed over the body surface

Infundibulum – lower or inner or posterior part or section of the buccal cavity; an often long, funnel-shaped tube or canal; may contain some of the oral ciliature and its infraciliature

Karyophore – strands or sheets of specialized and generally conspicuous fibers emanating from subpellicular locations and surrounding and suspending the macronucleus

Kinetid – elementary repeating organellar complex of the typical ciliate cortex, consisting of a kinetosome (or two more kinetosomes) and its fibrillary associates, which include cilium, unit membranes, alveoli, kinetodesma, and various ribbons, bands, or bundles of microtubules, including some nematodesmata, and sometimes also microfibrils, myonemes, parasomal sacs, and extrusomes

Kinetodesmal fibril – typically periodically striated, subpellicular fiber arising close to the base of a somatic kinetosome, near triplets numbers 5–8, and extending right or anteriad and toward or parallel to the organism's pellicular surface and on the right side of the kinety involved

MAFFT method – multiple sequence alignment program for amino acid or nucleotide sequences

Paroral – preferred term, used in a broad sense, for the ciliary organelle lying along the right side or border of the oral region; its cilia may be undulatory or membrane-like, behaving as a single unit because of their fully or partially coalescent nature

Perizonal ciliature – somatic ciliature , usually to the right of the oral region , the rows of which appear to run transversely, the often closely packed cilia are said to function in intensification of the food-carrying water currents that are being directed toward the oral region

Pleurotelokinetal – telokinetal stomatogenesis in which the oral anlage is derived by subequatorial proliferation of kinetosomes within several right lateral somatic kineties

Microtubular ribbon – set of microtubules aligned laterally to form a flat "ribbon-like" structure; the most striking microtubular ribbons include the **transverse** and **postciliary** microtubules , and the microtubular arrays in the suctorial tentacle

Postciliodesma –the conspicuous fiber, running posteriorly on the right side of the associated kinety and composed of stacked ribbons of overlapping postciliary microtubules, and involved in extension of the body following contraction by the myonemes

Preoral dome – anterior part often overhanging adoral zone of membranelles

Somatic kinety – kinety confined to the somatic region

SPURR - type of epoxy resin used in sample preparation for electron microscopes

Suture lines – simply folds or creases in the pellicle; preferably associated with the important concept of the secant system, the converging of kineties from different areas of the surface of the ciliate onto suture lines forming a pattern consistent within a given taxonomic group

Transverse Microtubules – microtubular ribbon arising at the left anterior side of the kinetosome close to triplet numbers 3, 4, and sometimes 5; the ribbon, which may be composed of 4–6 cross-linked microtubules, may originate tangentially or radially to the kinetosomal perimeter, first extends upward toward the pellicle and then continue to the left

9. LITERATURE

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