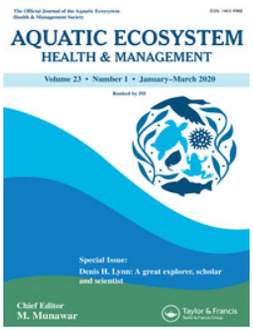

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Morphological and molecular phylogeny of the polycytopharyngeal ciliate *Pycnothrix monocystoides* Schubotz, 1908

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We reinvestigated Pycnothrix monocystoides *Schubotz, 1908 from the intestine of the African Rock Hyrax (*Procavia capensis*) collected from Saudi Arabia, using protargol impregnation and electron microscopy.* Pycnothrix *is a unique, up to 5 mm long intestinal ciliate with about two million cilia and many cytostomes (i.e. actual cytostome plus cytopharynx; polycytopharyngy) in the oral furrow (~vestibulum) that extends on both sides of the cell. Both walls of the furrow are covered by adoral membranelle-like ciliary polymerizations. To the right of the furrow and the cytostomes there are small ciliary fields, very likely homologous to the "dorsal brush" found in free-living litostomateans. The molecular investigations show* Pycnothrix *as sister to* Balantidium *and endemic Australian intestinal ciliates, e.g. the genus* Bandia*. We propose that the common ancestor had both an oral furrow and a special ciliary field both similar to that found in* Balantidium*. The unique morphology (polycytopharyngy) and the rather distinct molecular separation suggest maintenance of the family status given by Poche (1913).*

Keywords: morphology, Pycnotrichidae, Verstibuliferida

Introduction

The ciliate class Litostomatea is divided into the subclasses Haptoria, Rhynchostomatia, and Trichostomatia (Adl et al., 2019); the former two consist solely of free-living species while the latter includes only endosymbiotic and parasitic genera (Lynn, 2008). The main morphological synapomorphy of the class Litostomatea is the ultrastructure of the somatic kinetid (Lynn, 2008). Molecular markers depict the class Litostomatea and the subclasses Rhynchostomatia and Trichostomatia as monophyletic, but not the subclass Haptoria, some haptorian taxa are sister to all trichostomes (Strüder-Kypke et al., 2006; Vd'ačný et al., 2011, 2014). Many of the free-living genera are predatory, consuming flagellates, ciliates, and even small metazoans, such as

[†]deceased

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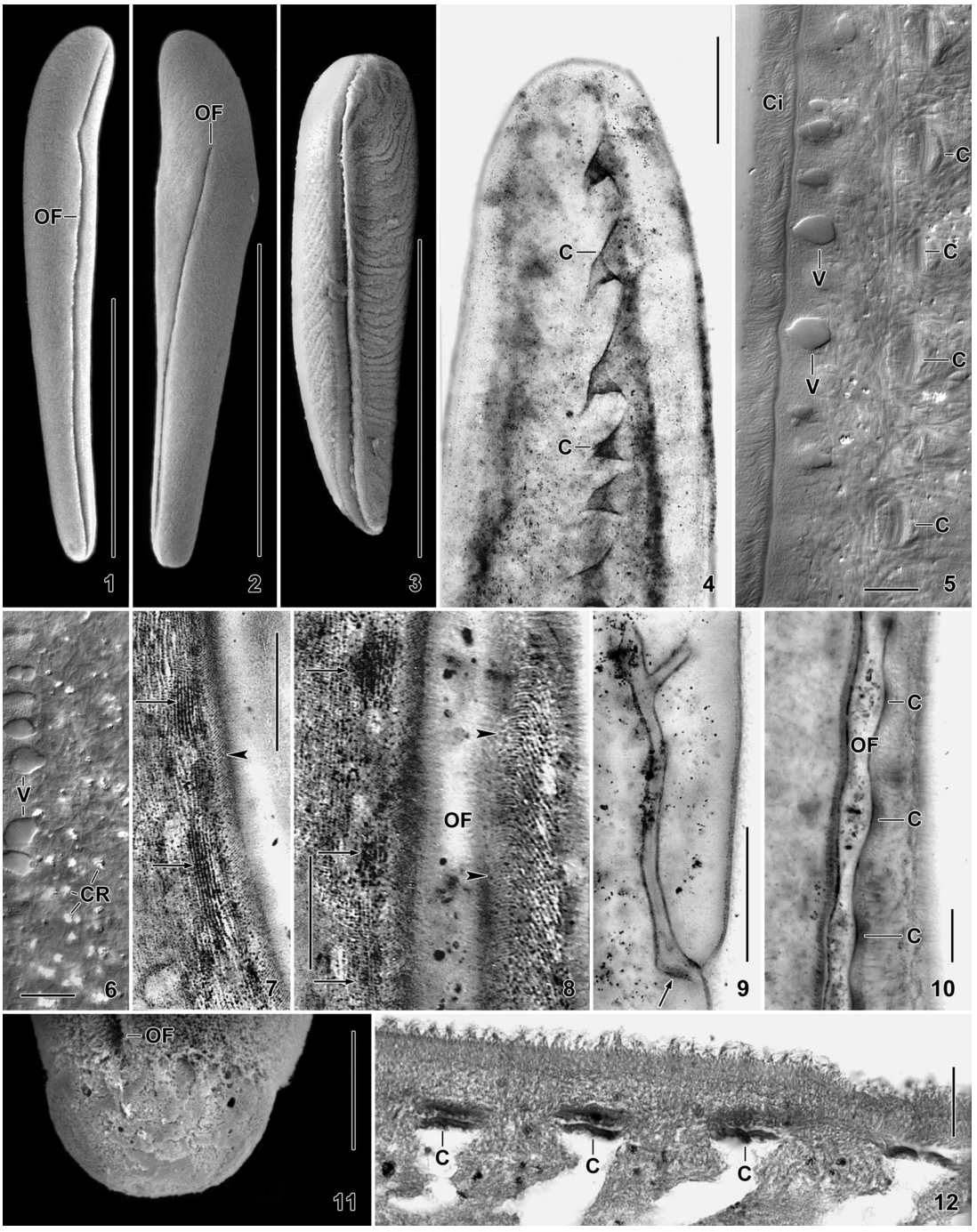
rotifers; they occur in soil, fresh-water and marine environments and are top unicellular predators in microbial food webs and several genera are indicators of water quality (Foissner et al., 1995, 1999). Endosymbiotic litostomes occur in the digestive tract of vertebrates and usually feed on bacteria and plant material. Among the endosymbiotic trichostomes, we distinguish forms occurring in the foregut (e.g. rumen ciliates) and forms inhabiting the hindgut of a large variety of



vertebrates – mammals, birds, amphibia, and fish. In fact, the only known pathogenic ciliate for humans is a member of this group – *Balantidium coli* (synonyms *Balantioides coli, Neobalantidium coli;* based on molecular gene analyses, Pomajbiková et al. (2013) proposed to reclassify the species as *Neobalantidium coli*. Chistyakova et al. (2014) considered this name a junior synonym of *Balantioides coli*, which was proposed by Alexeief (1931). Because the nomenclature is under revision and to avoid confusion, we use the name that remains accepted and commonly used - *Balantidium coli*).

Within the Trichostomatia we can distinguish two general morphotypes: holotrichously ciliated forms with a prominent and densely ciliated vestibulum surrounding the cytostome, and sparsely ciliated forms with the ciliature arranged in bands, tufts, or girdles. Holotrichously ciliated taxa of the Trichostomatia have historically been grouped together in the order Vestibuliferida although recent molecular studies show that this order is clearly non-monophyletic (Vd'ačný, 2018).

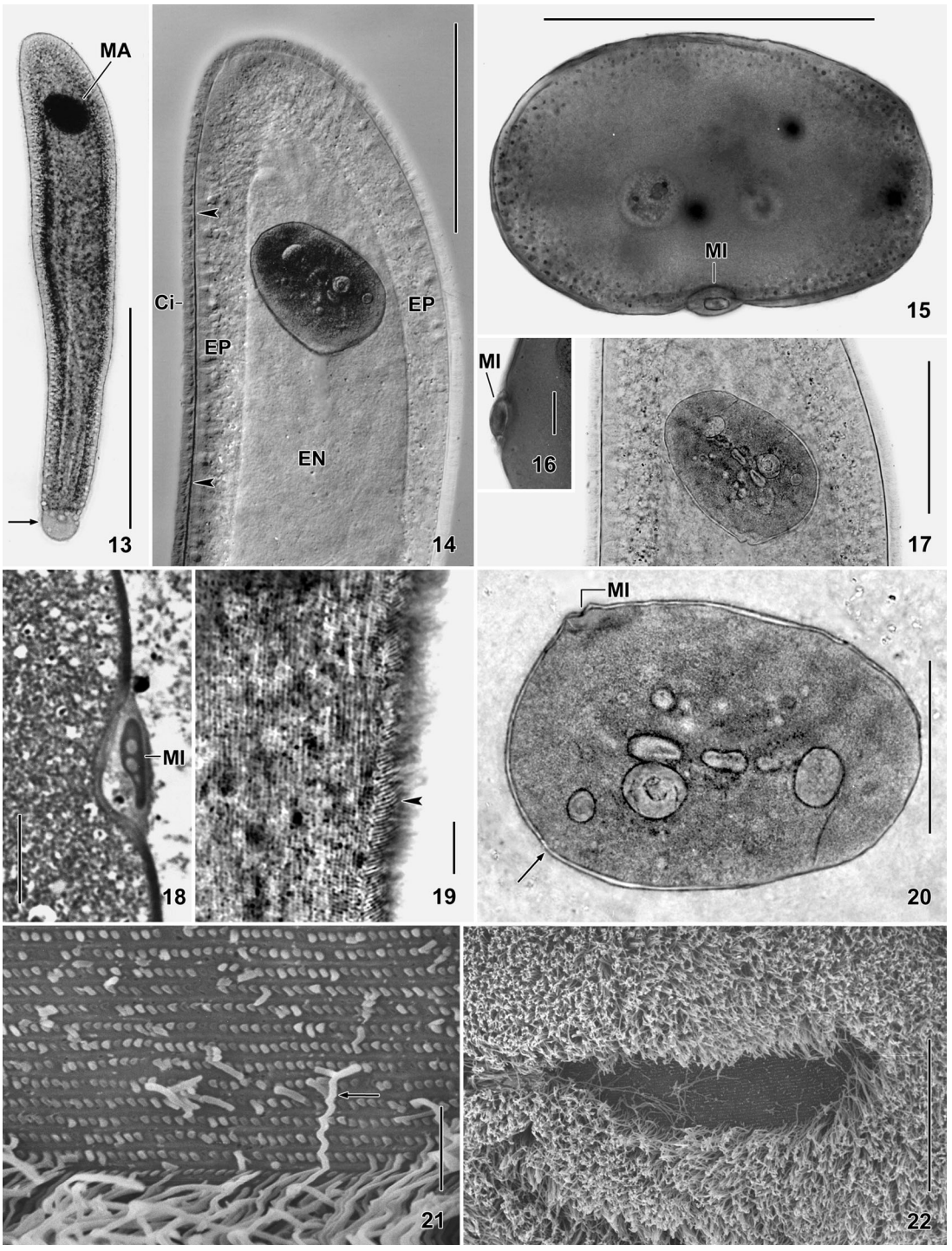
Pycnothrix monocystoides Schubotz, 1908 is an extremely large ciliate and was described by Schubotz (1908) based on formalin-fixed material collected by Prof. Leonhard Schultze from the gut contents of the Rock Hyrax, *Procavia capensis* (Pallas, 1766). *Procavia capensis* is native to South Africa and the Middle East and a member of the order Hyracoidea, a group related to the Proboscidea (e.g. elephants) and Sirenia (e.g. manatees, dugongs). Previously, the species had been found in material collected by Brumpt in 1901 from *Procavia brucei* in Harar, Ethiopia (described in Chatton and Pérard, 1921). To our knowledge, *P. monocystoides* has not been found since. The species displays morphological features typical of vestibuliferid ciliates like the holotrichous ciliation and a densely ciliated oral furrow (Lynn, 2008). However, at the same time we find unique characteristics, e.g. the large body size (~2.2-3.3 mm long), the full body-length vestibulum, the strongly developed cortex, and the excretory canal, which led Poche (1913) to the creation of the family, Pycnotrichidae, and even order, Pycnotrichidea. Later on, Corliss (1979) included other genera within the Pycnotrichidae: *Buxtonella* Jameson, 1926; *Collinina* Chatton and Pérard, 1924; *Infundibulorium* Bozhenko, 1925; *Muniziella* da Fonseca, 1939; *Nicollella* Chatton and Pérard, 1919; *Taliaferria* Hegner & Rees, 1933; and Lynn (2008) added *Vestibulongum* Grim, 1988 but regarded *Buxtonella* as *incertae sedis* in the Pycnotrichidae. These genera seem morphologically significantly diversified (Grim et al., 2015) and thus the question arises whether the family Pycnotrichidae is monophyletic. *Buxtonella sulcata* was redescribed by Grim et al. (2015) in detail. They could not determine its exact phylogenetic position, neither morphologically nor by molecular markers.

This study had been initiated many years ago and it was on Denis Lynn's list of collaborative projects to finish and publish. His untimely passing impeded our plans and, therefore, this Tribute Issue seems the adequate venue to publish our results, even though the species is endosymbiotic in a land mammal. However, given the overall importance of litostome ciliates in aquatic food webs, we hope our study is of value and helps to shed light onto the complex relationships within this taxon.

One goal of our study was to test the taxonomic affiliation based on morphology and gene

Figure 1–12. *Pycnothrix monocystoides* in the scanning electron microscope (1–3, 11), in protargol preparations (4, 7–10; 4, from a wet, pressed specimen), in Bouin's-fixed specimens, interference contrast (5, 6), and in the transmission electron microscope (12). **1–3:** Overviews, showing the slightly spiral oral furrow (OF) moderately shortened anteriorly on left side (2), and metachronal ciliary waves (3). **4**, **5**, **12:** Anterior (4) and mid-portion (5, 12), showing some of many conical cytostomes (C) appearing as ellipsoid structures in surface view (5, C), or as paired, deeply stained lines in transverse section (12, C). Note the very dense ciliature (5, Ci) and many small, ovoid vacuoles in the ectoplasm, very likely non-contractile because no pores could be recognized (5, V). **6:** Surface view, showing highly refractive crystal vacuoles (CR) and some small, very likely non-contractile vacuoles (V; see above). **7, 8:** Oral furrow, showing a membranellar band each on right and left wall. Both are produced by the curved anterior region of the very narrowly spaced somatic kineties and extend obliquely from right to left (arrowheads). At the right side of the oral furrow are deeply impregnated special ciliary fields (arrows). **9:** Distal portion of canal system. Arrow marks basal bodies of cilia. **10:** Surface view of a slightly opened oral furrow (OF), showing some cytostomes (C). **11:** Posterior end of a specimen with an unciliated bulge. Scale bars 10 μm (10, 12), 20 μm (4–9), 50 μm (11), 500 μm (3), and 1000 μm (1, 2).

sequences, especially with regards to *Buxtonella* and the order Vestibulifera. The second goal was to present micrographs (Figure 1–30) from new structures and from the structures described and drawn so painstakingly by Schubotz (1908) and Chatton and Pérard (1921).



Methodology

Specimen collection and deposition of materials

We found masses of *P. monocystoides* Schubotz, 1908 in the intestine of one male *Procavia capensis*. Despite further efforts, we were not able to find *Pycnothrix* in other examined Rock Hyraxes. This animal was from the Abha mountains in the Asir region, in the southwest of Saudi Arabia. The specimens were fixed by the huntsman immediately with ethanol (96%), formalin (4%), and Bouin's solution, as described in Foissner (1991).

Schubotz (1908) used *P. monocystoides* collected and fixed with formalin by Prof. Dr. Leonard Schultze in southern Africa. Schultze wrote a preface to the article of Schubotz (1908) and informed us that he stored the material in three, half-filled tubes "not sufficient to clarify all secrets of *Pycnothrix*" (Schubotz, 1908). Schultze stored all his materials at the University of Berlin (now Humboldt University) where Schubotz was assistant in the Zoological Institute; his remark indicates that Schubotz used the complete material. Unfortunately, Schubotz (1908) did not provide any information about the locality where he deposited the slides with sectioned and stained specimens; perhaps they are still in Berlin. In case that they cannot be found our material can be used as neotype. We stored all materials in a single box deposited in the Biology Centre of the Oberösterreichische Landesmuseum in Linz (LI). Well impregnated and/or sectioned specimens are marked on the coverslip. The TEM sections are in a grid-box.

Morphology and cytology

We employed various histological staining methods for the light microscopical investigations: hematoxylin, Feulgen, toluidine-blue, methyl green-pyronin, following the staining protocols as described in Adam and Czihak (1964) and Foissner (1991). Protargol staining proved to be difficult and showed some success only if protargol protocol A from Foissner (1991) was applied in specimen smaller than 800 µm. Silver carbonate staining failed entirely.

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were performed with Bouin's-fixed specimens, using the protocols of Foissner (1991, SEM) and Foissner and Foissner (1985, TEM). While SEM produced good results, TEM was very mediocre due to the poor fixation. Nonetheless, some details could be better seen than with other methods.

More details on fixation, staining procedures and problems can be found in the supplementary material.

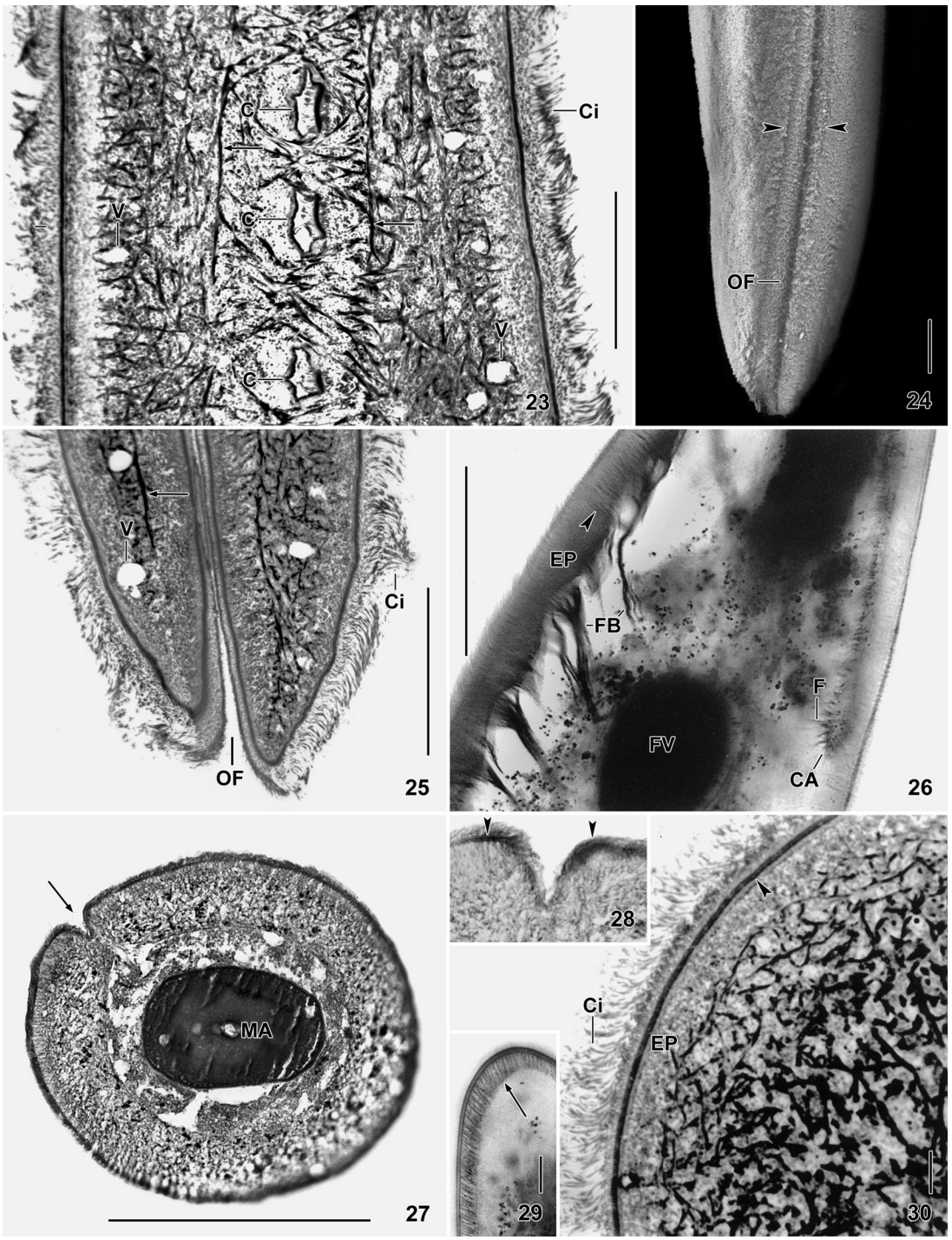
Molecular methods

DNA was successfully extracted from the ethanol-fixed cells, amplified, purified, and sequenced following standard procedures described in a previous study (Strüder-Kypke et al., 2007). Details are described in the supplementary material.

The SSU rRNA gene sequence fragments of *P. monocystoides* were imported into Sequencher ver. 4.0.5 (Gene Codes Corp., Ann Arbor, MI, USA), trimmed at the ends, assembled into contigs, and checked for sequencing ambiguities. Sequences of all available litostome taxa were downloaded from GenBank. The final dataset was aligned via the MAFFT version 7 server (Katoh et al., 2019), the alignment was imported into G-Blocks ver. 0.91b (Castresana, 2000) and poorly aligned, hypervariable regions were removed. Distance data were inferred from complete sequence alignments with only the ends trimmed (100 taxa and 1,626

Figure 13–22. *Pycnothrix monocystoides* in a methyl green-pyronin stain (13), in semithin sections and interference contrast (14, 17, 20), in protargol preparations (15, 16, 19), in the transmission electron microscope (18), and in the scanning electron microscope (21, 22). **13:** Overview, showing the location of the macronucleus and a damaged (?) posterior body end (arrow). **14:** Anterior portion, showing the broadly ellipsoid macronucleus, the thick ectoplasm (EP), the smooth endoplasm (EN), and the dense ciliature (Ci). The arrowheads mark a dark line very likely produced by the basal bodies of the cilia. **15–18, 20:** Nuclear apparatus. Both, macronucleus and micronucleus (15, 17) contain many inclusions, possibly parasites. The macronucleus is surrounded by two membranes (20, arrow). The micronucleus (18, MI) has an ordinary membrane and is covered by a thick membrane at the endoplasmic side (15, 16, 18, 20). **19, 21:** Showing the extreme densely spaced ciliary rows and basal bodies within the rows. The arrow in (21) marks a spirochaete. **22:** A nude, subterminal site, showing the fur-like ciliature. Scale bars 1 µm (19), 2 µm (21), 5 µm (16, 18), 20 µm (22), 50 µm (15, 20), 100 µm (17), 150 µm (14), and 500 µm (13).

positions). Pairwise distances were calculated with Mega7 (Kumar et al., 2016), based on the Kimura-2-Parameter model (Kimura, 1980). The final alignment for phylogenetic analyses comprised 100 taxa and 1,601 nucleotides (93.68% of original alignment). For the SSU rRNA gene sequences,



four different phylogenetic analyses were performed: Maximum Likelihood (ML; Stamatakis et al., 2008); Bayesian Inference (BI; Ronquist et al., 2012); Maximum Parsimony (MP; Swofford, 2002), and Neighbor Joining (NJ; Saitou and Nei, 1987). Detailed parameter settings of the alignment and phylogenetic analyses are described in the supplementary material.

Results

Nobody has ever seen live *Pycnothrix* specimens. Thus, some open questions remain: e.g. regarding feeding. Sizes are prone to shrinkage due to fixation and staining. The TEM micrographs have poor quality due to insufficient fixation; nonetheless, they show some features better than the light microscope.

Morphometry

All data are from chemically fixed specimens, some additionally influenced by various histological methods. Basically, the sizes match those given by Schubotz (1908), and most characteristics have a small or moderate variability, i.e. less than 15% (Table S1). However, some show high variability, e.g. the root fibres and the length of the special ciliary fields.

The protargol-impregnated specimens are much smaller than the remainder of the cells because we selected for specimens smaller than 800 µm (details, see method section). This distinctly reduces the values of the data, but at least it is a first approximation.

Photographical presentation of new structures and of structures shown by line drawings in the publications of Schubotz (1908) and of Chatton and Pérard (1921)

1. The average size of ethanol and Bouin's-fixed specimens is 1,905 × 363 µm (Table S1): some cells reach 3,000 µm or 3,700 µm which matches the measurements of Schubotz (1908). Size and shape are maintained in SEM and TEM preparations.

2. Body shape is very elongate cuneate to almost cylindroid (Figure 1–3, 13), not spindle-like as stated by Schubotz (1908). Usually, the cells are slightly curved, especially the anterior fifth, and slightly flattened laterally (Figure 27). The posterior body end looks damaged in about 15% of specimens (Figure 11, 13), suggesting that the cells were attached to the intestine of the host. However, we could not find any anchoring structures in specimens looking undamaged.

3. *Pycnothrix monocystoides* has two oral furrows in the ectoplasm, according to Schubotz (1908). However, our observations show a single, V-shaped furrow extending over the posterior body end (Figure 1–3, 24, 25). The furrow is about 15 µm deep, and roughly 35 µm wide when the slightly bulged walls, which bear the beginning of the adoral membranelles, are included (Figure 1, 3, 8, 10, 24, 27, 28).

4. Usually, the ellipsoid to broadly ellipsoid macronucleus is in the endoplasm of the anterior fifth, rarely in posterior half (Figure 13–17, 20, 27; Table S1); the nucleus is globular in the populations studied by Schubotz (1908) and Chatton and Pérard (1921). It is surrounded by two distinct membranes (Figure 20) and contains

Figure 23–30. *Pycnothrix monocystoides* in the transmission electron microscope (23, 25, 27, 28), in the scanning electron microscope (24), in protargol preparations (26, 29), and in a semithin section (30). **23, 25:** Subsurface sections in mid-body and in posterior end, showing three out of many cytostomes (C) in the oral furrow (OF), and small, non-contractile vacuoles (V). The roots of the basal bodies form the wall of the cytostomes and make a chaotic net in the ectoplasm except of the ecto-endoplasm boundary (arrows). **24, 28:** The margins of the oral furrow are slightly dome-shaped because occupied by the distal half of the adoral membranelles (arrowheads). **26:** Posterior quarter of body, showing the distal portion of the canal system (CA) anchored to the ectoplasm by short fibres (F); up to 50 µm-sized food particles (FV) in the endoplasm; and conspicuous, up to 50 µm long fibre bundles (FB) produced by converging roots of the somatic basal bodies (arrowhead). **27, 28:** Transverse section in anterior quarter of body, showing the right branch of the oral furrow (arrow) and the macronucleus (MA). **29:** Distal portion of the basal body roots. **30:** Subapical transverse section, showing the dense ciliation; the thick, dark layer produced by the basal bodies of the cilia; and the intense netting of the basal body roots. Scale bars 10 µm (29), 20 µm (30), 40 µm (26), 50 µm (23–25), and 200 µm (27).

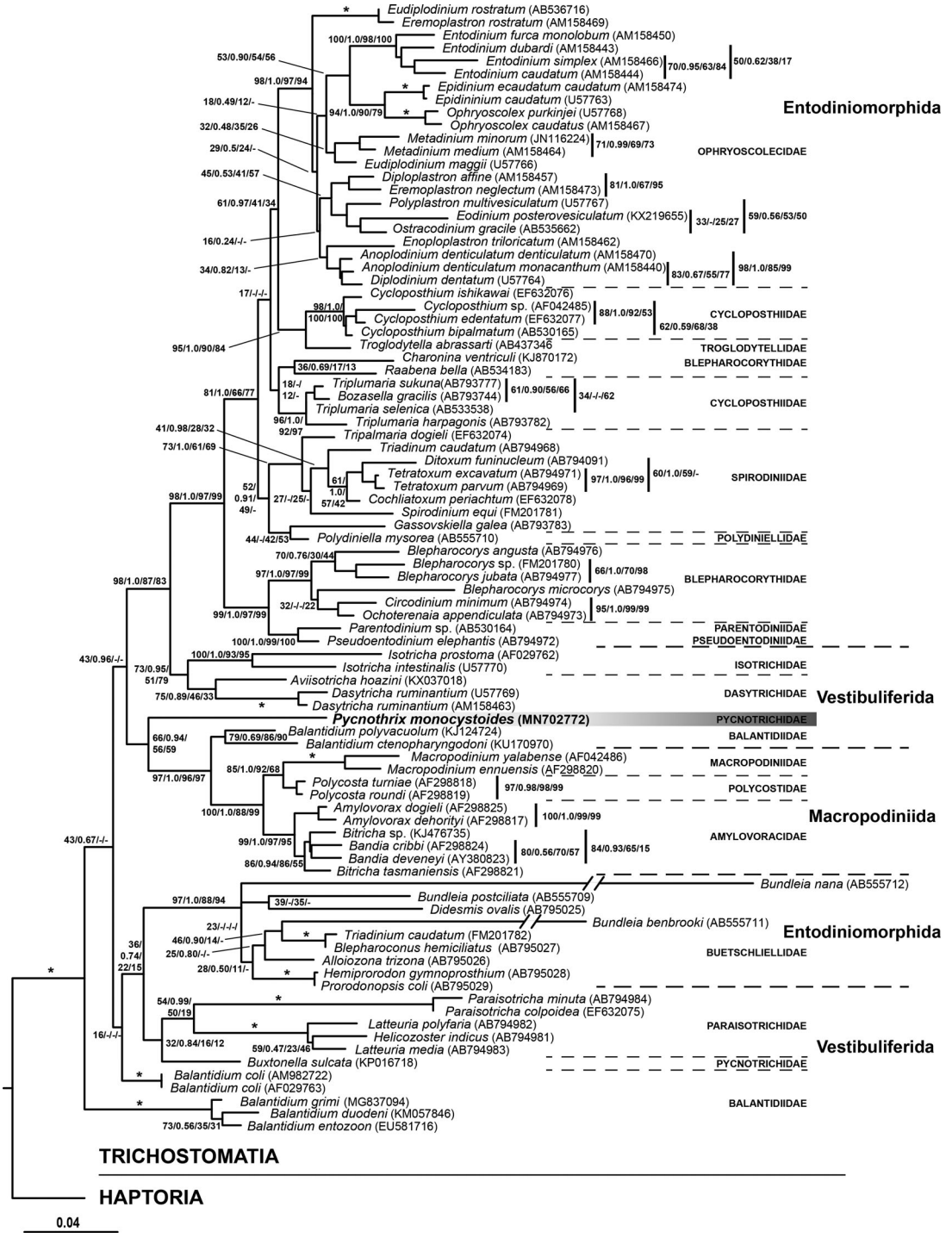


Figure 31. Maximum likelihood tree computed with RAxML (Stamatakis et al. 2008), based on the General Time-reversible (GTR + I+Γ) model. The first numbers at the nodes represent the bootstrap support of the ML analysis, followed by the BI posterior probabilities and support values for MP (Swofford 2002) and NJ (Saitou and Nei 1987), respectively. An asterisk indicates full support in all analyses while dashes indicate support below 10%. The scale bar represents 4 changes per 100 positions. The new sequence appears in bold face.

numerous small and rather large inclusions also described by Schubotz (1908), which might be bacteria or small protists (Figure 14, 15, 17, 20). Very rarely occur two small, narrowly spaced, globular macronuclear nodules.

5. There is a single, ellipsoid micronucleus attached to a concavity of the macronucleus; two micronuclei occur in one out of 30 specimens investigated. The micronucleus also contains various inclusions (Figure 15, 18) and is surrounded by a membrane. At the endoplasmic side it is additionally covered by a thick membrane possibly confluent with the external macronuclear membrane (Figure 18; Table S1); possibly, this is a kind of perinuclear space as, e.g. in some colpodids (Foissner, 1993).

6. The endoplasm contains countless, minute food particles and up to 100 µm-sized food vacuoles (Figure 9, 14, 26; Table S1). Further, there is a branched, lacunar canal system with an opening about 30% distant from body end; there is no pore but the distal canal region is covered by the somatic cortex, including the cilia, as already described by Schubotz (1908). The distal portion is anchored to the ectoplasm with short fibres (Figure 9, 26; Table S1). We agree with Schubotz (1908) that this is an excretory system.

7. Cortex and ectoplasm are about 40 µm thick and contain many yellowish, 3–5 µm-sized crystal vacuoles (Figure 6) and rows of ovoid vacuoles 14.4×9.2 µm in size and without pore (Figure 5, 6, 23, 25; Table S1), matching the observations of Schubotz (1908). The distal portion of the ectoplasm appears finely striated by the roots of the ciliary basal bodies (Figure 26, 29). The roots are up to 50 µm long and form a plate-like layer when approaching the ecto-endoplasm boundary where they become a chaotic net misinterpreted by Schubotz (1908) as myonemes (Figure 23, 25, 26, 30). However, there is no indication of a myoneme system because *Pycnothrix* does not contract when fixed for preparations.

8. The body is covered by 12–15 µm long, very narrowly spaced cilia, forming nice metachronal waves (Figure 3, 5, 19, 21, 22). In one specimen, we observed a nude region in the posterior fifth indicating that this is associated with the excretory canal (Figure 22). The ciliary rows on the left wall of the oral furrow drastically decrease in length from the anterior to the posterior end of the cell because their anterior end curves to the right to become the adoral zone of membranelles (Figure 8). On the right wall of the oral furrow the membranelles originate in the same way but the length of the somatic kineties increases from anterior to posterior (Figure 7, 19).

According to four partially deciliated cells (Figure 21) and the average size of ethanol-fixed specimens (Table S1), we calculated 2,600 very narrowly spaced, longitudinal ciliary rows each with 7,620 cilia, resulting in about two million cilia/cell. The much smaller protargol-impregnated specimens have only 300–600 ciliary rows and thus have a much lower total (~45,000 cilia/cell). Six to eight ciliary rows to the right of the oral furrow show patches with increased affinity to protargol (Figure 7, 8); the length of these areas decreases from about 50 µm anteriorly to 5 µm near the posterior body end (Table S1). Very likely, these patches consist mainly of dikinetids or of extremely narrowly spaced basal bodies with a special chemistry. Unfortunately, we could not find these areas in the SEM either because they are ordinarily ciliated or have shortened cilia hidden by the extremely dense ciliation. Schubotz (1908) and Chatton and Pérard (1921) did not describe this important peculiarity (see Discussion).

9. The inactive oral furrow sensu stricto is about 15 µm wide and contains the proximal half of the adoral membranelles and the distal margin of the cytostomes (Figure 1, 2, 4, 5, 12, 23, 27, 28). The membranelles begin on slight elevations of the furrow walls adding about 10–15 µm each to the left and right wall edge. The cilia of the membranelles are even more narrowly spaced than those of the somatic kineties (Figure 7, 19). When the furrow is slightly open, the membranelles extend obliquely from right to left and the furrow becomes wavy (Figure 7, 8, 10). Usually, the oral furrow is covered by small particles producing small heaps over the cytostomes; these particles are very similar to those found in the endoplasm, indicating that the whole furrow collects food. In the small, protargol-impregnated cells, there is an average of 18 cytostomes in the right branch of the furrow and of 11 in the left branch (Table S1). The conical cytostomes are supported by the roots of the membranellar ciliary basal bodies in the left wall of the oral furrow. The cytostomes decrease in size from

anterior to posterior and have a diameter of 10–20 µm and a depth of 20–50 µm. Most of these observations are new because Schubotz (1908) and Chatton and Pérard (1921) did not have the advantage of silver impregnation and electron microscopy.

Genetic data and phylogenetic analyses

The almost complete sequence of the SSU rRNA gene of *Pycnothrix monocystoides* was deposited in GenBank under the accession number MN702772. Consistent with other litostome sequences, it is 1,563 nucleotides long and has a GC content of 42.2%.

A preliminary analysis including specimens from all ciliate classes assigned *Pycnothrix* to the class Litostomatea (data not shown). Due to the long-branch separating the litostomes from other ciliate taxa, the analyses included in this study focus on the in-group taxa only. The calculated distance values depict the species *Balantidium coli* with 5.1% divergence and *Buxtonella sulcata* with 6.0% divergence, *Balantidium polyvacuolum* and *B. ctenopharyngodoni* with 6.7% and 6.1%, as well as species in the families Amylovoracidae and Polycostidae, order Macropidiniida (divergence 5.8-6.3%) as closest relatives to *Pycnothrix*.

In all phylogenetic analyses, *P. monocystoides* grouped unambiguously within the subclass Trichostomatia (Figure 31). The ML tree clustered *P. monocystoides* with the Australian clade of trichostomes (order Macropodiniida) and two *Balantidium* species albeit with low bootstrap support of 66% (Figure 31). The other analyses supported this placement, but also with low support (0.94 BI, 56% MP, 59% NJ). None of the inferred topologies grouped *Pycnothrix* with *Buxtonella sulcata* and the *Buxtonella*-like isolates. Instead, *Buxtonella* grouped with the family Paraisotrichidae (represented by *Paraisotricha*, *Latteuria*, and *Helicozoster*) and the entodiniomorphid family Buetschliellidae in ML and BI analyses (32% ML, 0.84 BI), as sister to the Trichostomatia in an unresolved polytomy in the MP analysis, and as sister to *B. coli* in the NJ analysis (43% support). As shown in previous studies, the vestibuliferids are not monophyletic. The remaining vestibuliferid genera – *Dasytricha, Isotricha,* and *Aviisotricha* – are placed as sister group to the remaining families of the order Entodiniomorphida (98% ML, 1.0 BI, 87% MP, 83% NJ); and the *Balantidium* species group either as sister to the entire Trichostomatia clade (*B. duodeni, B. entozoon, B. grimi*) or as sister to the Macropodiniida clade (*B. ctenopharyngodoni* and *B. polyvacuolum,* 97% ML, 1.0 BI, 96% MP, 97% NJ).

Discussion

Morphology and phylogeny of *Pycnothrix*

Our morphological investigations support the excellent study by Schubotz (1908) in many respects, especially we can confirm that *Pycnothrix* is the first and only ciliate with many serially arranged cytostomes, a feature supporting the family classification by Poche (1913). The possession of many mouths (= polypharyngy) is also very rare in metazoans. It occurs in some specific populations/species of triclads and some other flatworms (Remane, 1952). Neither the early generalists (Dogiel, 1929; Sewertzoff, 1931; Gelei, 1950) nor recent ciliate textbooks (Corliss, 1979; Lynn, 2008) have credited Schubotz (1908) for his discovery.

Kaur and Oberoi (1987) briefly described the only other *Pycnothrix* species so far, *P. patialaensis* from the intestine of the lizard *Varanus monitor*. It differs from *P. monocystoides* by the host and by body size, viz., about 270 µm vs. usually 2,000-3,000 µm.

An oral furrow comparable to that of *Pycnothrix* is present in only a few other intestinal ciliates, such as *Vestibulongum* and *Buxtonella* (Grim, 1988, 2015), *Collinella* (Chatton and Pérard, 1921), and in *Muniziella* (Batisse, 1965). The oral furrow of these and possibly some other ciliates (Corliss, 1979) has membranelle-like ciliary polymerizations on both walls of the furrow which is thus considered as a vestibulum (Corliss, 1979; Grim, 1988; Lynn, 2008; Jankowski, 2007). The membranelle-like kineties and their great number are produced during division by bifurcation of the anterior end of the somatic kineties (Fauré-Fremiet, 1955). The next character "ciliary patches" suggests that *Balantidium* also belongs to the "furrow group" because its slit-like mouth opening, though not extending beyond mid-body, has polymerized "adoral membranelles" on both sides of the mouth

entrance (Guinea et al., 1992; Grim, 1993). Remember that in ciliates the ciliature in the right side of an oral furrow is usually composed of a monokinetal or dikinetal ciliary row called "paroral or undulating membrane" while the "adoral membranelles" on the left wall are composed of several ciliary rows (see ciliate textbooks, e.g. Lynn, 2008).

A rather unexpected feature of *Pycnothrix* are the small special ciliary fields right of the oral furrow and the cytostomes. Such fields are highly specific and diverse in free-living and intestinal Litostomatea (Foissner et al., 2002; Jankowski, 2007; Lynn, 2008; Vd'ačný and Foissner, 2012; Foissner, 2016). In *Pycnothrix*, the ciliary fields are highly similar to those of balantidiids, both in location and structure. However, this field has not (yet?) been described in many intestinal litostomateans because it becomes visible only in silver preparations and, occasionally, in the electron microscope. Further, it has been described under various names hiding the homology, e.g. "Villeneuve-Brachon field" or "dextr-oral field" in *Balantidium* by Guinea et al. (1992) and Grim (1993), "crete aboral" in *Isotricha* by Grain (1966), "undulating membrane" in *Paraisotricha* and *Rhizotricha* by Wolska (1964), and e.g. "paralabial organelle" in *Ophryoscolex* by Schrenk and Bardele (1987). In the free-living litostomateans such fields are now widely called "dorsal brush" (Foissner et al., 2002; Vdačný and Foissner, 2012; Foissner, 2016). The detailed investigations of this specialization greatly promoted generic and species taxonomy in the free-living Vestibulifera and Trichostomatia. We propose that this will happen also in the intestinal ciliates because such fields are very ancestral structures also present in endemic intestinal ciliates from Australia, e.g. in the genus *Bandia* ("clavate groove cilia", Fig. 52 in Cameron and O'Donoghue, 2002).

Molecular phylogeny

The length of 1,583 nucleotides corresponds to the typical length for litostome SSU rRNA gene sequences and is a result of several deletions across the SSU rRNA in the V4 region (Leipe et al., 1994; Strüder-Kypke et al., 2006; Wright and Lynn, 1997a, 1997b; Wright et al., 1997).

In the phylogenetic trees, *Pycnothrix* is not closely related to the only other represented member of the Pycnotrichidae, *Buxtonella sulcata*. In fact, *P. monocystoides* is separated by a long branch from any other taxon. The family Pycnotrichidae comprises highly diverse genera in a large variety of hosts and it is questionable whether this taxon is actually monophyletic (Grim et al., 2015). A recent study of endosymbiontic ciliates in the rodent *Hydrochoerus hydrochaeris* (Capybara) in South America included another supposed pycnotrichid species, viz., *Muniziella cunhai* but was not (yet) accompanied by detailed morphological data or gene sequences (Cedrola et al., 2018). Likewise, we have detailed morphological descriptions of *Vestibulongum corlissi* without molecular data (Grim, 1988; Grim and Clements, 2013).

The divergence values of *Pycnothrix* to other trichostome taxa all range between 5.1–8.6%.

While the lowest divergences are between *Pycnothrix* and other vestibuliferids (*Buxtonella, B. coli, Balantidium* spp.) and some macropodiniids (*Amylovorax, Polycosta, Bitricha*), the divergence between *P. monocystoides* and several free-living Haptoria (mainly *Spathidium* spp.) is on average 8% (this data set). This high divergence among the trichostome taxa is partially a result of the high nucleotide substitution rate that was estimated for this group (av. 3.06×10^{-4} per 1 My; Vd'ačný, 2018), which is almost twice as high as the one calculated for free-living litostomes (av. 1.76×10^{-4} per 1 My; Vd'ačný, 2018).

The general topology of the tree confirms recent studies on trichostome ciliates (Strüder-Kypke et al., 2006, 2007; Ito et al., 2014; Grim et al., 2015; Kittelman et al., 2015; Bardele et al., 2017; Vd'ačný, 2018). The order Vestibuliferida is a paraphyletic group and morphologically mainly defined by the endosymbiontic life style, the holotrichous ciliation and a densely ciliated vestibulum (Lynn, 2008). Likewise, our data on sequenced vestibuliferids and their hosts show repeated and independent invasion of various hosts (not shown). Strüder-Kypke et al. (2007) suggested that the vestibuliferids represent the oldest lineage within the trichostome ciliates and – due to the large variety of hosts they inhibit and a fast evolutionary rate – have highly divergent SSU rRNA gene sequences and thus a recovery as monophyletic taxon is not likely. However, a second explanation might be that this group is morphologically only defined by plesiomorphic characters and, therefore, truly is closer

related to other trichostomes. Interestingly, we find vestibuliferid taxa as sister-group of (a) the entire trichostome clade (some *Balantidium* spp.), (b) the Buetschliellidae (*Buxtonella*, *Paraisotricha*, *Latteuria*, *Helicozoster*, and *B. coli*), (c) the Entodiniomorphida (*Isotricha*, *Dasytricha*, *Aviistotricha*), and (d) the Macropodiniida (*Pycnothrix*, some *Balantidium* spp.). Unfortunately, most of these nodes are only weakly supported (except Macropidiniida-*Balantidium* spp., and Entodiniomorphida-Isotrichidae), likely the result of undersampling and long-branch-attraction. Only 4 of the 6 families within the order Vestibuliferida are represented by gene sequences, and only 8 of the 30 genera listed in Lynn (2008).

The order Macropodiniida was established by Lynn (2008) as so-called ribo-order, since no morphological synapomorphies are apparent, yet, molecular phylogenetic support for this clade is strong. The macropodiniid families Amylovoracidae and Polycostidae show typical vestibuliferid features like holotrichous ciliation and a vestibulum (Cameron and O'Donoghue, 2003). Hence, the low divergence and the clustering of *P. monocystoides, B. polyvacuolum* and *B. ctenopharyngodoni* with these two families may not be such a surprise. The family Macropodiniidae, on the other side, reveals typical entodiniomorphid characteristics (Cameron et al., 2001).

Conclusions

We were able to add one more sequence to the data set and with that, to confirm the placement of *Pycnothrix monocystoides* within the class Litostomatea, subclass Trichostomatia. Contrary to the morphological characteristics, its placement within the order Vestibuliferida could not be fully validated by gene sequence data, since this taxon is non-monophyletic. The genetic distances do not support a close relationship of *Pycnothrix monocystoides* and *Buxtonella sulcata*, and the removal of the latter from the Family Pycnotrichidae seems justified.

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Supplemental material

Supplementary material for this article can be accessed on-line at the publisher's website.

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