Light microscopic morphometrics, ultrastructure, and molecular phylogeny of the putative pycnotrichid Ciliate, *Buxtonella sulcata*

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Abstract

The ciliate, *Buxtonella sulcata*, was isolated from a bull cow near Tišnov, Czech Republic, and fixed for light (LM), scanning electron (SEM) and transmission electron microscopic (TEM) study. Presented here are the basic morphometrics from LM study, and the fine-structure of both somatic and vestibular ciliary, and other structures. While many morphological features are similar to ciliates belonging to the order Vestibuliferida, some differences have been discovered, and are presented here. Especially emphasized are the microtubular and fibrillar components of the basic kinetid structures for both somatic and vestibular regions of these protists. Also observed in both TEM and SEM samples were enigmatic membrane bulges at the base of many somatic cilia. These ciliates are seen to have abundant endocyttoplasmic bacteria, as seen in LM and TEM. This evaluation of the ultrastructural morphology of *B. sulcata* from cattle is accompanied by detailed determination of its small subunit rRNA (SSU rRNA) gene sequence and also of internal transcribed spacers (ITS1-5.8rRNA-ITS2). All of these data will

contribute to unravel the phylogenetic relationships of medically and veterinary important intestinal ciliates.

Keywords: light microscopy; morphometric data; phylogeny; rDNA; scanning electron microscopy; transmission electron microscopy

Abbreviations

BI, Bayesian Inference; CBC, compensatory base change; EDTA, Ethylene Diamino Tetraacetic Acid; kd, kinetodesmal fibril; LM, light microscopy; ME, minimum evolution; ML, maximum likelihood; MP, maximum parsimony; mts, microtubules; NJ, neighbor-joining; PC mts, post-ciliary microtubules; SEM, scanning electron microscopy; SPR, Subtree-Pruning-Regrafting; Tc, tela corticalis; TEM, transmission electron microscopy

Introduction

Buxtonella sulcata is a ciliated protist inhabiting the caecum and large intestines of cows and water buffaloes. Its life cycle involves two stages: an intestinal trophozoite stage and a cyst stage that appears resistant to environmental stresses (Zaman 1978). This organism was first described and named by Jameson in 1926, based in part on its isolation from the caecum of cattle in Cambridge, England. He presented its general form and a few dimensions; albeit, without statistics. He reported: “During progression [swimming] the mouth end is most frequently the posterior end.” Three years later, Becker and Hsiung (1929) gave its length and width and presented figures of the trophozoite and cyst forms. Statistics were given for the cysts only. Rees (1930) published on its “morphology and behavior.” He reported finding *B. sulcata* “in the caecum of eight out of 32 head of cattle slaughtered at Jeanerette, Louisiana.” Curiously, in Rees’ introduction, he first called this ciliate *Balantidium*, but, near the bottom of that same page decided that it was *Buxtonella sulcata*, as described by Jameson. In his figure 2, he illustrated the macronucleus and micronucleus, the vestibule, “oesophagus,” cytostome, and cytophyge. Partially because of its full body length vestibule, it was placed into the ciliate family,

Pycnotrichidae Poche, 1913. Many decades later, that taxonomic position was questioned by Lynn (2008) when he classified it with the notation of *incertae sedis*. At that time, Lynn listed seven genera in that family including *Nicollella*; although that genus' vestibule is slightly less than half the body length (Chatton and Pérard 1919). However, the pycnotrichids seems to be a significantly morphologically diversified group of ciliates, and those genera differ from one another considerably in shape, vestibular length, and host organism. It is difficult to find some morphological characteristics valid for all genera. There is one characteristic; that the vestibule starts at the anterior end and coils slightly, ending at the vestibular overture that is posterior; but even that is not true for all members of the family Pycnotrichidae (Chatton and Pérard 1919; Grim 1988; Poche 1913).

Buxtonella sulcata seems to be a nearly cosmopolitan ciliate, recorded in England (Fox and Jacobs 1986; Jameson 1926), Poland (Tomczuk et al. 2005), Denmark (Henriksen 1977), Turkey (Göz et al. 2006), Iraq (Al-Saffar et al. 2010), Pakistan (Lubinski 1957), Nepal (Adhikari et al. 2013), Thailand (Kaewthamasorn and Wongsamee 2006), Japan (Ito et al. 2014), Korea (Hong and Youn 1995; Kim et al. 1993), Egypt (Sultan et al. 2013), North-America (Becker and Hsiung 1929; Rees 1930; Urman and Kelly 1964), and Costa Rica (Jiménez et al. 2010). So far, the clinical importance of buxtonellosis in cattle is unclear and, thus, controversial views about its pathogenicity still exist. For example, some studies argued for a commensal role of this intestinal ciliate (Lapage 1956), whereas several have linked the occurrence of *Buxtonella* with diarrhea or ulcerative colitis; e.g., (Al-Saffar et al. 2010; Göz et al. 2006; Tomczuk et al. 2005; Urman and Kelly 1964). Despite the fact that *B. sulcata* seems to be a quite common ciliate across cattle and water buffalo populations, it is noteworthy that its molecular genetic taxonomic classification and the detailed ultrastructural morphology of the trophozoite stage have not been published yet. In addition, without this information, it is not possible to characterize the “new” relative ciliates called *Buxtonella*-like as appeared in Pomajbíková et al. (2013). Thus, we performed here the detailed evaluation of the ultrastructural morphology of *B. sulcata* from cattle, which data is accompanied by determination of its small subunit rRNA (SSU rRNA) gene sequence and also of internal transcribed spacers (ITS1-5.8rRNA-ITS2). All

these data will contribute to unravel the deep phylogenetic relationships of medically and veterinary important intestinal ciliates, which may help to fill gaps in their epidemiology.

Material and methods

Sample collection, fixation and preparation microscopic examination

Buxtonella sulcata were isolated by one of us (KJP) from the colon and caecum of an Aberdeen Angus bull after it was slaughtered according to legislative rules of the Czech Republic and European Union in an accredited slaughter-house near the town of Tišnov, Czech Republic. Prior to fixation, ciliates were washed with several suspension/sedimentation cycles in large volumes of phosphate buffered saline solution. Specimens for LM study were fixed in phosphate buffered 4% formaldehyde, and shipped to one of us (JNG); they were post-fixed in Bouin's fixative overnight, and stained with the protargol technique following Grim (1988); however, the sodium thiosulfate soak was for about 20 h, otherwise, the *B. sulcata* tended to be too dark.

Specimens for electron microscopy were fixed in 2.5% glutaraldehyde in phosphate buffer. They were shipped to JNG in that solution and held for weeks to months in a refrigerator prior to further processing. SEM samples were isolated with a micropipette, washed several times in d-H₂O, and then soaked in 1.0% (w/v) EDTA overnight to remove mucous. After further d-H₂O washes, they were post-fixed in 2.0% osmium tetroxide, washed, ultrasonicated for ca. 3 s, and freeze dried (Small and Marszalek 1969). Cells were examined in a LEO 435VP, or LEO Gemini, Supra 40 V. P. SEM (Zeiss, Oberkochen, Germany). Specimens for TEM study were fixed as above, dehydrated to absolute acetone and embedded in EPON 812 "substitute" (EMbed 812) [Electron Microscopy Sciences, Hatfield, Pa., USA] with catalyst. Thin sections containing only one or two cells were examined in a JEOL 1200EX II (Tokyo, Japan). Different cells were cut in long-section or cross-section; none were randomly oriented. All micrographs, SEM and TEM, were digital.

DNA isolation, PCR and cloning

For the genetic analyses, the specimens were fixed in 96% ethanol. A portion of the sample was dried overnight at 37°C. The total DNA was extracted using PSP® Spin Stool DNA kit (Invitrogen) following manufacturer's instructions.

The complete SSU-rRNA and ITS (ITS1, 5.8S-rRNA and ITS2) regions were PCR-amplified and sequenced. The PCR reactions were performed in a Biometra T-personal thermocycler (Biometra – Schoeller, Czech Republic). For the ITS region, the primers and the PCR amplification program were as described by Ponce-Gordo et al. (2008). For the SSU-rRNA gene, two different PCRs were made. In one of them, a partial sequence of the 5' end comprising ca. 1270 bases of the gene was amplified with the forward primer EukA (5'-AACCTGGTTGATCCTGCCAGT) (Medlin et al. 1988) and the reverse primer SSUrBB (5'-AAATACATAGTCCCTCTAAGAAGTC) (Pomajbíková et al. 2013). In the other PCR reaction, the 3' end comprising ca. 440 bases was amplified with the forward primer IntSSUf (5'-CGAGACCTTAACCTGCTAACTAGTC) (designed by us based on the published sequences of *Balantioides coli* [= *Neobalantidium coli*; Chistyakova et al. 2014] and *B. sulcata* available in the GenBank/EMBL/DDBJ databases) and EukB (5'-TGATCCTTCTGCAGG TTCACCTAC) (Medlin et al. 1988). The resulting sequences have an overlapping region of about 70 bp that allow their assembling. In both SSU-rRNA PCRs, the thermocycler was programmed as follows: 10 min at 94°C, 35 cycles of 1 min at 94°C, 1 min 30 s at 50°C, and 1 min 30 s at 72°C, and finally 5 min at 72°C. The presence of PCR products was checked by electrophoresis using 12 µl of PCR reaction loaded into a 1% agarose gel and run using the Gel Documentation System Vilber Lourmat – DOC-Print VX5 (Fischer Scientifics.r.o., Czech Republic); PCR products were visualized by staining with GoodviewTM (Ecoli s.r.o., Slovak Republic). For the ITS region, the amplicons were purified using the MinElute Gel Extraction kit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocol and sequenced. For the PCR reactions for both genes, the amplicons were cloned by using the PCR 2.1®-TOPO®-vector (Invitrogen; Fisher Scientifics.r.o., Czech Republic) and sequenced in both directions according to manufacturer's instructions. The sequencing of both the ITS and SSU-rRNA products was made by Macrogen, Korea. For each gene, the alignment and assembling of the partial sequences were made by using the Chromas Pro software, version 1.7.5 (Technelysium Pty Ltd). The sequences obtained in this study were

compared with those available in the GenBank/EMBL/DDBJ databases by using the blastn algorithm, available in the National Center for Biotechnology Information (NCBI) web site (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Phylogenetic analyses

For the phylogenetic analysis of the SSU-rRNA, other sequences of ciliates (Vestibuliferida, Entodiniomorphida and Haptoria – as an outgroup) were retrieved from the GenBank/EMBL/DDBJ databases. Their alignment was made with the program 4SALE (Seibel et al. 2008) by taking into account both the primary sequence and the secondary structure; for this purpose, the secondary structure model proposed for the sequence KJ124724 of *Balantidium polyvacuolum* (Li et al. 2014) was transferred to the other sequences by homology modeling using the “model” tool available in the ITS2 Database web site (<http://its2.bioapps.biozentrum.uni-wuerzburg.de>) (Koetschan et al. 2010). The ambiguously aligned positions were excluded from the phylogenetic analysis. The MEGA6 computer package (Tamura et al. 2013) was used to identify the nucleotide substitution model that best fit our data. Phylogenetic trees were obtained by the ML, NJ, MP and ME methods (as implemented in MEGA6; Tamura et al. 2013), and by BI analysis (as implemented in MrBayes 3.2.2; Ronquist et al. 2012). The ML analysis was performed using the Tamura 3-parameter model (Tamura 1992) with a proportion of invariable sites and Gamma distribution (T92+G+I). The NJ and ME trees were constructed using the Tamura 3-parameter method (Tamura, 1992); the rate variation among sites was modeled with a gamma distribution (shape parameter = 5). The MP tree was obtained using the SPR algorithm (Nei and Kumar 2000) in which the initial trees were obtained by the random addition of sequences (10 replicates). The data were bootstrap resampled 1000 times to estimate the relative branch support. For the BI analysis, a GTR+G+I model (n=6, rates=invgamma) was used; two parallel runs were performed for 1000000 generations with trees sampled every 10000 generations and an initial relative burn-in of 25% of the trees; priors were left at default settings.

Results

LM – Morphometrics (Table I)

Six parameters were measured in 15 to 30 cells (Table I). The average length and width of this ciliate (103.2 X 66.1 μm) is smaller than that reported by Rees (1930; no means were given by the author), but the size ranges are relatively close to those of the original report by Jameson (1926). In some cells, the macronucleus was oriented at an angle to the field of view, thus, it was not possible to measure those accurately. Because of this, the 'n' for their length is smaller than for their width.

SEM – General form (Fig. 1A-C)

The SEM view (Fig. 1A-C) of our European specimens is very similar to the drawings by Jameson (1926) and Rees (1930). The vestibule extends the full length of the cell and near the posterior bends slightly toward the dorsal body surface. It has a slightly helical path that is clockwise when viewed from the anterior end. The vestibule has numerous cilia on right and left edges, and those on the cell's left are relatively short (Fig. 1B, C). As the vestibule penetrates deep to the surface, cilia partially obstruct the view (Fig. 1C). We designate that boundary as the vestibular overture (Grim and Buonanno 2009). The non-ciliated surface of the vestibule is often lined with a reticulated network of an unknown substance (Fig. 1B).

TEM results (Fig 1D-I, 2 A-I)

Vestibular ciliary structures: Many cilia near the edge of the vestibule appear to be in contact with one-another (Fig. 1D). Slightly further from the vestibule they are still relatively close – less than the diameter of a kinetosome. The kinetosomes in this region possess typical ultrastructural features, such as: internal cart-wheel, and globular appearing material (Fig. 1E). We have observed four kinetid structures associated with these kinetosomes: kinetodesmal fibrils that are laterally directed but bend toward the anterior very near “their” kinetosome, and do not obviously overlap (Fig. 1E, F); Pc microtubules (mts) (Fig. 1F), T1 mts (Fig. 1F), and, above and below the Tc (tela corticalis) network are hexagonally arranged microtubular bundles typical of nematodesmata (Fig. 1G). They originate from the proximal ends of

vestibular kinetosomes. Microtubules composing these bundles have been counted and range from 10 to at least 26.

In the TEM, the non-ciliated dorsal surface (Vd) of the vestibule has two sets of mts ribbons that penetrate into the cytoplasm, typical of diverse ciliates. One (T1) is seen as a ribbon of mts that are cut in near cross-section (Fig. 1H, I) when viewed when the section is a cross-section of the cell's body; they appear to originate as T1 mts from kinetosomes that abut the vestibule. The second set of mts is perpendicular to that (Fig. 1H, I); their origin has not been determined. Both sets of mts appear to terminate at the Tc network. Also, slightly elongate membranous vesicles can be seen near-to or in fusion with the vestibule plasma membrane (Fig. 1I). These were observed in numerous cross-sections of the ciliate's body. Finally, parts of the vestibule's dorsal surface appear to have a thin amorphous coating on it (Fig. 1H).

Somatic cilia: The kinetosomes of somatic cilia are also typical with a basal cart-wheel structure, and granular material in the more distal portion of the lumen (Fig. 2A) Associated with each of these kinetosomes are: a kinetodesmal fibril –laterally directed and not overlapping (Fig. 2A, B, C), 5 or 6 Pc mts, (Fig. 2A, B), T1 mts that extend anteriorly (Fig. 2A, B), two T2 mts that extend left (Fig. 2A, B), and two to no nematodesmal mts (Fig. 2); for example, somatic kinetosomes near the vestibule have been observed with only two of these mts, while more distant kinetosomes have not been observed with any nematodesmal mts. The long axes of T1 mts are oriented at an angle to, are not parallel to, their kinetosome's long axis even at the base of the kinetosome (Fig. 2B, C, E). The pair of T2 mts has been seen, thus far, only in somatic kineties in which kinetosomes are relatively close.

In many cilia their membrane bulges-out just distal to the cell's plasma membrane (Fig. 2F). These swellings contain an amorphous, electron dense substance. The swellings are also often seen in the SEM (Fig. 2G).

Nuclei: The macronucleus and micronucleus are typical of ciliates. They are, as usual, close together and chromosomes are distinct in the micronucleus (Fig. 2H).

Cytoplasmatic bacteria: Based on both LM and TEM examination, the cytoplasm contains an abundance of bacteria. They do not appear to be within a membrane; thus, are

probably not within food vacuoles (Fig. 2I). Curiously, they seem to have unusually thick cell walls.

Analysis of rRNA genes (Fig. 3-5)

Two sequence variants of the SSU-rRNA gene have been obtained (available in the GenBank/EMBL/DDBJ databases under the accession numbers KP016717 and KP016718); both sequences are 1640 bp long with a 42.0% GC content and they differ in four bases located, in the secondary structure, in paired positions in the helices 16, 23/e1 and 43/e1 and in an unpaired position before helix 23/e12 (Fig. 3). The analysis of the CBCs (a change in the two nucleotides of a paired site while the pairing itself is maintained) made with 4SALE showed that the sequence-structure of *B. sulcata* has a few differences with *Balantioides coli* and the Paraisotrichidae while the differences with the other sequences considered in this study are greater (data not shown). These differences are located mainly between positions 590–740 and 1220–1260 of *B. sulcata* KP016717 (these corresponding to the expansion segments ES6 comprising helices 23/e1 to 23/e14, and ES9 comprising helix 43/e1, respectively).

The alignment for the phylogenetic analysis of the SSU-rRNA gene included 1651 positions in the final dataset. The tree topologies constructed by the different methods (Fig. 4) show that within Vestibuliferida, all families appeared monophyletic except the Balantidiidae, which is clearly separated in two different branches, one (the “Balantidiidae group 1” including *Balantidium ctenopharyngodon* and *B. polyvacuolum*) is related to the Amylovoracidae (the “Australian clade”) with high support (100% bootstrap support in ML, NJ, ME and MP; 1.00 posterior probability in BI) and the other (the “Balantidiidae group 2” including *Balantioides coli* and *Balantidium entozoon*) engroups with *B. sulcata* with a moderate support (ML, 36%; NJ, 45%; ME, 48%; BI, 0.84; in MP, they are in different branches). The position of the Paraisotrichidae varied depending on the phylogenetic tree; it is a different group (close to the “Balantidiidae group 2” and *B. sulcata*) in ML, NJ and ME while it is in the same clade as *B. sulcata* in MP and BI.

The ITS region is 363 bp long and with a GC content of 33.1–33.6%. This variability is due to the existence of three sequence variants showing base differences in six positions. We

have en-grouped them into two main variants, A and B; the variant B includes two sequences differing in only one base. They have been deposited in the GenBank/EMBL/DDBJ databases under the accession numbers KP016715 and KP016716. When comparing these sequences and those available in public databases, there are nine base positions showing differences; most of them are located in unpaired regions of the ITS1 and ITS2 (Fig. 5A). The secondary structure follows the model proposed for *Balantioides coli* by Ponce-Gordo et al. (2011); the structure of the basal portion of the ITS2 helix III could not be proposed with certainty, as several configurations are possible (Fig. 5B,C).

Discussion

This is the first detailed report of the statistical morphometrics, ultrastructure and molecular phylogenies of the symbiotic (and often parasitic) cattle and water buffalo ciliate, *Buxtonella sulcata*. Its general form compares well to three key early publications (Jameson 1926; Rees 1930; Lubinski, 1957). However the specimens from the USA studied by Rees (1930) were appreciably larger than in this study and that of Jameson – which was of *B. sulcata* from European cattle.

Regarding the molecular phylogenetic data, relatively little was described in the study by Pomajbíková et al. (2013); and, that paper was without any morphological data. Up to the present time, ultrastructure of only one morphologically related ciliate has been described, namely *Vestibulogum corlissi* (Grim 1988; Grim and Clements 2013). The kinetid components are similar except that the right vestibule of *V. corlissi* clearly has Kd fibrils that overlap; in *B. sulcata*, such overlap has not been observed; albeit, an overlap might be possible according to our data (Fig. 1E, 1F). Therefore, this morphological character may not be appropriate for all pycnotrichid ciliates. Also, the vestibular dorsal walls of both *B. sulcata* and *V. corlissi* contain the same two sets of mt ribbons that are positioned just beneath the plasma membrane; one is from T1 mts, but the origin of the second (perpendicular to those) has not been determined for either ciliate. For a partial review of these ribbons in diverse vestibuliferans, see Grim and Clements (2013).

The elongate vesicles observed close to or often attached to the dorsal vestibular wall of *B. sulcata*, may be either pinocytotic or membrane recycling from the cytoplasm to the vestibule (Allen 1978, see also his Fig. 34). Similar vesicles have not been observed in the vestibule of *V. corlissi*. We speculate that *B. sulcata* might take in (“drink”) food molecules by pinocytosis. Also, it seems there may be a third physiological possibility, that---instead---these vesicles might be secreting toxic molecules and; hence, be part of the intestinal pathology observed in cattle infected with this protist; that is, that resultant toxically damaged intestinal tissues might leak molecules and/or cellular debris that become part of the ciliate’s food. It is noteworthy that the pathogenic ciliate *Balantioides coli* has been reported to secrete a potential enzymatic toxin, hyaluronidase (Templis and Lysenko 1957). For *B. sulcata*, these ideas require further investigation.

Curiously, neither Jameson (1926) nor Rees (1930) observed food vacuole formation in living specimens. Perhaps *B. sulcata* feeds often by pinocytosis. That would explain the abundance of possible pinocytotic vesicles in the current study. Related to this is Jameson’s statement: “The formation of food-vacuoles....was not observed as the protoplasm is so granular that details of the internal anatomy are difficult to observe in living animals.” The granularity, caused in part by endosymbiotic bacteria, is supported by both LM and TEM views in our study. The cytoplasm, mitochondria, and nuclei of ciliates have been observed by many investigators to contain endosymbiotic bacteria; for example, see the reviews by Fokin et al. (2003a,b), and for members of the Vestibuliferida, Grim (1993) and Grim and Clements (2013).

Our molecular-phylogeny showed that the Vestibuliferida is not monophyletic. In our trees it is possible to observe that the vestibuliferid species are engrouped in derived lineages from an evolutionary trend that finally ends up in the Entodiniomorphida. In this evolution, *Buxtonella* is placed in an ancestral derived lineage which also includes part of the Balantidiidae. The analysis of the secondary structure of the SSU-rRNA of *B. sulcata* showed that it follows the general scheme of other ciliates and that it is closely related to *Balantioides coli* and probably also to the Paraisotrichidae; besides, *B. sulcata* is placed in the same evolutionary branch as *Balantioides coli* in all the phylogenetic trees (ML, NJ, ME, MP, BI). However, the exact phylogenetic relationships between all these ciliates could not be clearly

determined as they vary depending on the type of phylogenetic analysis. In some recent studies, *B. sulcata* has been also placed close to *Balantioides coli* (Pomajbíková et al. 2013) or within the Paraisotrichidae (Chistyakova et al. 2014; Ito et al. 2014). As the morphological characteristics of *B. sulcata* indicate, that it is not a typical vestibuliferan ciliate, it would be necessary to analyze sequences from other related ciliates, mainly pycnotrichid ones, to help resolve the phylogenetic position of this family.

Our results clearly indicate the existence of genetic variants in *B. sulcata*. This situation has been observed in *Balantioides coli* (Ponce-Gordo et al. 2011), in which there are two clearly distinct main genetic variants; however, in the present case the differences are of minor importance. Although the number of ITS cloned sequences we analyzed is low (only six sequences), the differences we found between the genetic variants are constant and we have not found hybrid sequences (i.e., a sequence with the ITS1 of one type and the ITS2 of the other type). Such hybrid sequences might be identified when comparing our sequences with those available; i.e., the sequence JQ073387 is highly similar to our variant A in the ITS1 while it is almost identical to the variant B in the ITS2. Several explanations could be given for this circumstance, i.e. the hybrid sequences may have been the result of real hybrid alleles or of PCR errors; even, the interpretation of double peaks in chromatograms obtained after direct sequencing of the PCR amplicon might generate errors leading to hybrid sequences. At this time, the possibility of real sequence hybrids cannot be accepted or rejected, and the analysis of new isolates could help resolve this question.

Independent of the existence of separate or mixed genetic variants in *B. sulcata*, their ITS2 sequences have minor differences (located mostly in unpaired positions) that do not affect their secondary structure. Also as in *Balantioides coli*, different structures could be proposed for the basal region of the ITS2 helix III. Apparently, the structure based on a minimum energy folding (the one obtained by the RNAalifold web server) would be preferable, but in this case, this will validate the *Balantioides coli* structures that have shown a CBC between the genetic variants A and B, which could be a controversial situation as both variants can be present in the genome of single cells (Ponce-Gordo et al. 2011). It would be preferable to leave this region as unresolved until further sequences from other related species are analyzed.

The proper taxonomic determination of symbiotic ciliates requires detailed examination of morphological and molecular-phylogenetic data. Despite the detailed examination of *B. sulcata* using both approaches, we are not able to resolve its exact taxonomic position. Our data calls for further and broader research addressing the morphology, phylogeny and diversity of these ciliates.

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Table 1. Morphological data for *Buxtonella sulcata* under LM^a.

Character	M	SD	Min	Max	CV	SE	n
Body length	103.2	14.5	65.0	132.0	14.0	2.64	30
Body width	66.1	7.8	50.0	84.0	11.7	1.42	30
Macronuclear length ^b	23.3	3.6	17.6	30.0	14.4	0.76	22
Macronuclear width	12.6	1.8	8.3	15.5	14.3	0.36	25
Micronuclear length	4.9	0.4	4.3	5.6	2.3	0.10	17
Micronuclear width ^c	3.8	0.7	3.0	5.4	17.0	0.17	15

^aAll data from fixed and stained specimens, and in μm . CV, coefficient of variation (%); M, arithmetic mean; Max, maximum value; Min, minimum value; n, sample size; SD, Standard deviation; SE, standard error of the mean.

^b The sample size of the macronuclear length is less than that of width because several were tilted out of perpendicular to viewer.

^c The sample size of this feature is slightly low because the micronucleus is often embedded in the macronucleus and unclear.

Figure Legends

Fig. 1. (A-C) SEM views of *Buxtonella sulcata*. (A) Whole organism, ventral view. Vestibule is on right (as viewed) toward the anterior end (upper A) and arches to the posterior-left. Scale bar = 20µm. (B) Vestibule [V] has many close cilia on its edges and reticulated material on its dorsal wall. Scale bar = 6 µm. (C) Vestibule at posterior end of the cell. Vo (arrow-head) shows where the vestibule penetrates into the cytoplasm. Scale bar = 12 µm. (D-I) TEM views of *Buxtonella sulcata*. (D) Vestibular cilia are very dense where the vestibule penetrates into the cytoplasm. Kineties there have ridges between them. S (upper-left), surface of cell. Scale bar = 2 µm. (E) Vestibular cilia and kinetosomes. Ridge (R) between two kinetosomes contains microtubules. There is globular material (G) within a kinetosome. Cart-wheel within a kinetosome (arrow). Scale bar = 2 µm. (F) Vestibular kinety shows kinetid components: post-ciliary microtubules (Pc), several T1 microtubules (T1), and parallel kinetodesmal fibrils = K. Scale bar = 300 nm. (G) A vestibular kinety cut to show nematodesmatal mt bundles (Nd) beneath a kinetosome (Ki). Scale bar = 200 nm. (H) A vestibular kinety adjacent to the vestibule. T1 microtubules (T1) arch over to buttress the dorsal wall (V-d) of the vestibule. Parts of the vestibular membrane are covered with an amorphous coating (arrow). A dense tela corticalis network (T-c) is slightly deep to the kinetosomes and the vestibule. Scale bar = 1.4 µm. (I) Dorsal vestibular wall shows files of T1 microtubules (T1) and other microtubules (arrow) that are perpendicular to those. Also, present are possible pinocytotic vesicles (arrow-head), and the tela corticalis (T-c) network. Scale bar = 500 nm.

Fig. 2. (A-I) TEM and SEM views of *Buxtonella sulcata*. (A-F) TEM views. Three kinetosomes show kinetid structures: post-ciliary microtubules (Pc), T1 microtubules, T2 microtubules in cross-section (arrow), and kinetodesmal fibrils = Kd. Also present is the cart-wheel feature (Cw) of a kinetosome and a portion of two ridges = R. Scale bar = 400 nm. (B) Single kinety better shows details of post-ciliary microtubules (arrow-head), T1 microtubular ribbon, and T2 microtubules. Scale bar = 200 nm. (C) Tiny fibrils (arrows) connect microtubule triplets of kinetosome to electron-opaque material around the post-ciliary microtubules. Scale bar = 200 nm. (D) Post-ciliary microtubules (Pc) penetrate into a ridge as a group – two rows of three mt each. Scale bar = 200nm. (E) Somatic kinetosomes showing nematodesmal microtubules with only two microtubules (arrows) deep to the tela corticalis = t-c. A kinetosomal cart-wheel (Cw) is distinct. Scale bar = 300 nm. (F) Ciliary membranes bulge out (arrows) near the cell surface. Scale bar = 380 nm. (G) SEM view. Bulged-out region (arrow) seen in some somatic cilia. Scale bar = 2.0 µm. (H-I) TEM views. (H) Macro- (Ma) and micro-nucleus (Mi), the latter showing many chromosomes. Scale bar = 1.1 µm. (I) Bacterial endosymbionts with nucleoid (arrow) in its cytoplasm. Scale bar = 500 nm.

Fig. 3. Proposed secondary structure model of the SSU-rRNA of *Buxtonella sulcata*. The fragment corresponding to the variable region V4 / expansion segment ES6 (positions 587-746)

follow Wuyts et al. (2000) (main diagram) and Alkemar and Nygard (2006) (insert). The helices have been identified according to the numbering system of Wuyts et al. (2002); for the V4/ES6 region, the system of Alkemar and Nygard (2006) is also shown. The base differences between the sequences of *B. sulcata* KP016717 (in red) and KP016718 (in blue) are shown.

Fig. 4. Phylogenetic trees obtained by the maximum likelihood (ML), neighbor-joining (NJ), minimum evolution (ME) and maximum parsimony (MP) methods and by Bayesian inference (BI). The trees have been combined according to their similarities. The numbers at the nodes represent the bootstrap support (ML, NJ, ME and MP methods) or the posterior probabilities (BI) respectively. The symbol * (in the BI tree) indicates that the branch is not supported. The sequences of *Buxtonella sulcata* obtained in the present study are in bold type.

Fig. 5. Secondary structure of the ITS1-5.8S-ITS2 region of *Buxtonella sulcata* type A (KP016715). **A)** Diagram of the complete region; the base of the ITS2 helix III is unresolved. The interactions of the 5.8S-rRNA with the B-region of the LSU-rRNA are proposed based on the secondary structure of *Spathidium amphoriforme* AF223570 (unpublished results). The nomenclature of the helices in the 5.8S-rRNA/LSU-rRNA (B-region) follows De Rijk et al. (1999); for the 5.8S-rRNA helices, the names proposed by Vaughn et al. (1984) are also given in parenthesis. Sequence color codes as follows: red, ITS1 sequence; blue, 5.8S-rRNA; pink, ITS2; orange, LSU-rRNA (5' end). Dashes correspond to the putative interaction of the 5.8S-rRNA with the LSU-rRNA. The base differences between the sequences obtained in this study and those available in the GenBank/EMBL/DDBL databases are shown in boxes; the number in parenthesis is the position of the base in its original sequence. **B)** and **C)** Secondary structure of the base of the ITS2 helix III (in red) obtained by homology modeling from the structures of *Balantioides coli* predicted by minimum energy (RNAalifold web server) (B) or by manual annotation (C) (following Ponce-Gordo et al. 2011).

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.