Eukaryotic Microbiology
Published by the International Society of Protistologists

Journal of Eukaryotic Microbiology ISSN 1066-5234

ORIGINAL ARTICLE

The Morphology, Ultrastructure and SSU rRNA Gene Sequence of a New Freshwater Flagellate, *Neobodo borokensis* n. sp. (Kinetoplastea, Excavata)

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Keywords
18S rRNA; heterotrophic flagellates; Neobodonida.

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Received: 18 February 2015; revised 16 September 2015; accepted September 16, 2015.

doi:10.1111/jeu.12271

ABSTRACT

A small free-living freshwater bacteriotrophic flagellate *Neobodo borokensis* n. sp. was investigated by electron microscopy and analysis of its SSU ribosomal RNA gene. This protist has paraxonemal rods of typical bodonid structure in the flagella, mastigonemes on the proximal part of the posterior flagellum, two nearly parallel basal bodies, a compact kinetoplast, and discoid mitochondrial cristae. The flagellar pocket is supported by three microtubular roots (R1, R2 and R3) originating from the kinetosome. The cytopharynx is supported by the root R2, a microtubular prism, cytopharynx associated additional microtubules (CMT) and cytostome associated microtubules (FAS) bands. Symbiotic bacteria and small glycosomes were found in the cytoplasm. Cysts have not been found. The flagellate prefers freshwater habitats, but tolerates salinity up to 3–4‰. The overall morphological and ultrastructural features confirm that *N. borokensis* represents a new species of the genus *Neobodo*. Phylogenetic analysis of SSU rRNA genes is congruent with the ultrastructure and strongly supports the close relationship of *N. borokensis* to *Neobodo saliens*, *N. designis*, *Actuariola*, and a misidentified sequence of "*Bodo curvifilus*" within the class Kinetoplastea.

ENVIRONMENTAL sequencing has revealed a great number of undescribed protist species in marine, freshwater, and soil ecosystems, but the morphology, ultrastructure, ecology, and physiology of most of these organisms remain little known and studied (López-García et al. 2001; Šlapeta et al. 2005). Kinetoplastids represent one of the pools of undiscovered protist diversity (von der Heyden and Cavalier-Smith 2005; Moreira et al. 2004; Stoeck et al. 2005).

The class Kinetoplastea Honigberg 1963 is traditionally subdivided into two groups – the free-living Bodonina and parasitic Trypanosomatina (Kivic and Walne 1984; Vickerman 1978; Vickerman and Preston 1976). Because of their importance to human and animal health, trypanosomatids have been studied much more intensively than bodonids, and the diversity of bodonids has started to be truly recognized only with molecular studies (Dolezel et al. 2000; Simpson et al. 2002). An updated recent classification divided Kinetoplastea into two new subclasses, the early-branching Prokinetoplastina containing the bodonid *Ichtyobodo* and *Perkinsiella* symbiont of amoebae, and Metakinetoplastina containing the parasitic Trypanosomatida together with three newly established orders of free-living bodonids: Eubodonida, Parabodonida, and Neobodonida (Moreira et al. 2004). Multiprotein phylogenies later confirmed that bodonids represent a large paraphyletic stem leading to trypanosomatid parasites (Deschamps et al. 2011; Simpson et al. 2004).

From an ecological perspective, free-living bodonids are a major stable component of aquatic ecosystems and have a great significance in food webs as part of microbial loop (see Arndt et al. 2000). In soil, they play an important role in the detrital food web, because they consume bacteria and serve as food for larger organisms such as amoebae, ciliates, and nematodes (Ekelund and Ronn

1994). In spite of their widespread and abundant presence, the diversity and taxonomy of bodonids are still poorly understood. Morphology-based distribution studies have demonstrated that certain species of bodonids inhabit a wide variety of marine, freshwater, and soil ecosystems of different geographical zones from the tropics to the polar regions (Al-Qassab et al. 2002; Ekelund and Patterson 1997; Larsen and Paterson 1990; Patterson and Simpson 1996; Tikhonenkov et al. 2006; Vørs 1992, 1993). This places bodonids as one of the most cosmopolitan and ubiquitous groups of protists, with four species (*Bodo saltans*, *Neobodo designis*, *N. saliens,* and *Rhynchomonas nasuta*) being among the 20 most commonly observed heterotrophic flagellates in natural samples in the world (Patterson and Lee 2000). Based on the SSU rDNA sequence data, however, some morphospecies of bodonids exhibit extensive genetic diversity, particularly when freshwater and marine strains are compared (von der Heyden et al. 2004), and probably represent a vast complex of morphologically indistinguishable but genetically diverse "species" (von der Heyden and Cavalier-Smith 2005). For example, physiological and phylogenetic data indicate that *N. designis* strains fall into several independent groups, which are in part determined by the environment (von der Heyden et al. 2004; Koch and Ekelund 2005). At the same time, some sequences of freshwater *N. designis* cluster in predominantly "marine" clades, whereas a marine strain of *B. saltans* clusters with freshwater and soil isolates, and marine isolates of *Procryptobia sorokini* and *R. nasuta* cluster together with freshwater isolates (Scheckenbach et al. 2006).

Altogether, the current data show the difficulty of accurately determining bodonid species by gross morphology alone, and suggest that morphological characters may be insufficient for a valid taxonomic classification. Many well-known species and genera require reinvestigation by DNA sequence analysis. This is especially true of doubtful genera like *Cruzella*, *Phanerobia*, *Parabodo*, *Procryptobia*, whose representatives have been poorly described even by light microscopy (see for Patterson and Zolffel 1991).

In this study, we combined culturing, morphological, ultrastructural, and molecular approaches to describe a new species, *Neobodo borokensis* n. sp., isolated from a freshwater pond.

MATERIALS AND METHODS

A single cell of a new species of *Neobodo* (clone B-74) was isolated by a micropipette from the sample of littoral detritus from the small pond "Barskyi" near the settlement Borok, Yaroslavskaya oblast, Russia (58.061365 N, 38.245730 E) on August 26 1999. Water conductivity of the sample was 167 μS.

The culture was propagated and maintained in Petri dishes filled with Pratt medium (0.1 g/L KNO_3, 0.01 g/L $MgSO_4 \cdot ^x7\ H_2O$, 0.01 g/L $K_2HPO_4 \cdot ^x3\ H_2O$, 0.001 g/L $FeCl_3 \cdot ^x6\ H_2O$, pH = 6.5–7.5) with addition of *Pseudomonas fluorescens* Migula 1895 bacteria as a food. The strain B-74 is stored in the collection of live protozoan cultures at Institute for Biology of Inland Waters, Russian Academy of Sciences.

Light microscopic observations were made with AxioScope A1 (Carl Zeiss, Oberkochen, Germany) using the phase and interference contrast, and 40X and 70X water immersion objectives. The images were taken with the MC-1009/S video camera (AVT Horn, Aalen, Germany) and directly digitized by using the Behold TV 409 FM tuner.

For scanning electron microscopy (SEM), cells from exponential growth phase were fixed with 2% glutaraldehyde (final concentration) prepared using 0.1 M cacodylate buffer (pH 7.2) for 10 min at 22 °C and gently drawn onto a polycarbonate filter (24 mm, pores 0.8 μm). Following the filtration, the specimens were taken through a graded ethanol dehydration and acetone, and finally put into a chamber of a critical point device for drying. The dry filters were mounted on aluminium stubs, coated with gold-palladium, and observed with a JSM-6510LV (JEOL, Tokyo, Japan).

For transmission electron microscopy (TEM), cells were centrifuged, fixed in a cocktail of 0.6% glutaraldehyde and 2% OsO_4 (final concentration) prepared using a 0.1 M cacodylate buffer (pH 7.2) at 1 °C for 30–60 min, and dehydrated in an alcohol and acetone series (30%, 50%, 70%, 96%, and 100%; 20 min in each step). Finally, cells were embedded in a mixture of araldite and epon (Luft 1961). Ultrathin sections were obtained with the LKB ultramicrotome. For whole-mount preparations, cells were fixed in 2% OsO_4 vapour, air-dried, and shadowed according Moestrup and Thomsen (1980). All TEM observations were done by using the JEM-1011 (JEOL) electron microscope.

Salinity tolerance of B-74 was examined by increasing salinity in Petri dishes by 0.5‰ per day (35‰ artificial marine water was used). Salinity was measured by using the Krüss S-10 salinometer (KRÜSS GmbH, Hamburg, Germany).

For SSU rDNA analysis, cells were collected by centrifugation (10,000 *g* for 10 min, room temperature), and genomic DNA was extracted by using the Epicentre DNA extraction kit (cat. no. MC85200). A near-complete 18S rRNA gene was amplified by general eukaryotic primers (18SFU: ATGCTTGTCTCAAAGGRYTAAGCCATGC and 18SRU: CWGGTTCACCWACGGAAACCTTGTTACG), cloned, and a single clone was sequenced by Sanger dideoxy sequencing. Two 18S rDNA data sets were created, one containing *Diplonema* and Protokinetoplastina (*Ichthyobodo* and *Perkinsiella*) as the outgroup (96 sequences in total), and another limited to Metakinetoplastina (93 sequences). Sequences were aligned by using the local-pair algorithm in MAFFT 6.857b (Katoh et al. 2005) and trimmed in Gblocks 0.91b (Castresana 2000) by using b1 = 50% + 1, b2 = 50% + 1, b3 = 12, b4 = 4, b5 = h′ parameters.

The resulting phylogenetic matrices (1,414 and 1,653 sites respectively) were visually inspected and subjected to maximum likelihood (ML) and Bayesian analyses. ML trees were inferred using RAxML 8.1.20 (Stamatakis 2006) based on 20 inferences from different randomized starting trees under the GTR+GAMMA4 + I model. Standard

bootstrap analysis (1,000 replicates) was used to assess branch support. A second ML analysis was computed in PhyML 3.0.1 (Guindon et al. 2010) by using the GTR+GAMMA4 + I model, 10 random starts, best NNI+SPR inference, and SH-like aLRT branch supports. The Bayesian analysis was run in MrBayes 3.2.0 (Ronquist and Huelsenbeck 2003) by using the GTR+GAMMA4 + I model, 2 chains, and 25% burnin after 1,750,000 generations.

The SSU rDNA sequence of the clone B-74 was deposited in GenBank under accession number KT223311.

RESULTS

The cell shape B-74 is elliptical (ellipsoid) (Fig. 1A–C), 7–12 μm in length (mean 10.2 ± 0.2 μm, $n = 50$), and 3–6 μm in width (mean 3.9 ± 0.1 μm, $n = 50$). Starved cells were two-thirds as wide (Fig. 1B). Two heterodynamic flagella insert subapically below a small rostrum which bears a cytostome on its end (Fig. 1D–F). The anterior flagellum is 8–14 μm long, makes flapping movements, and forms a ventrally curved hook when the cell jumps along the substratum, the most common type of movement (Fig. 1A, C). However, in a fast-jumping cell the flagellar hook curves dorsally, as known for *Neobodo saliens* (Larsen and Paterson 1990) (Fig. 1B). The cell can temporarily attach to the substratum by the acroneme of the trailing flagellum, which is 22–33 μm in length. The length of the acroneme is 0.5 μm, and 5–14 μm in the anterior and posterior flagellum respectively (Fig. 1A, C, G). The cell may also jerk backwards by using the posterior flagellum. The anterior flagellum does not wrap around the anterior part of the body and the cell does not tilt to press the rostrum on the substratum as in *N. designis* (Larsen and Paterson 1990).

Occasionally, cells were observed to swim close to bottom of Petri dishes by fast, dart-like movement in direct lines. During swimming, each cell rotates rapidly around its axis.

The cells multiply by longitudinal binary fission in a moving state. Cysts were not found.

The cell surface is covered only with a plasmalemma. The flagella arise from the flagellar pocket (Fig. 1D, E, H, J, 2A). Both flagella have typical paraxonemal rods (Fig. 2B), which are absent in their distal sections (not shown). The axonemes have the typical "9 + 2" arrangement. The transitional plate is 0.20–0.25 μm above the bottom of the flagellar pocket (Fig. 1I, J). The proximal part of the posterior flagellum (partly within the flagellar pocket) is covered by simple, thin mastigonemes (Fig. 1I, 2A), which are invisible in Fig. 1D–G. The kinetosome 1 sits against a thin electron-dense plate, which lies near the kinetoplast part of the mitochondrion (Fig. 1J).

The kinetosomes lie at an acute angle or almost parallel to each other and are connected by 2–3 fibrils (Fig. 1K). The root R3 starts from the kinetosome 2 and consists of a three-microtubule band and an attached electron-dense plate (Fig. 1K, M). The root R2 begins from the kinetosome 1 and contains six microtubules (Fig. 1K, 2A). The root R1 consists of 2–3 microtubules and is positioned in between the kinetosomes (Fig. 1K–M). This root continues along the wall of the flagellar pocket (Fig. 2A).

The root R3 gives rise to a wide dorsal band of microtubules which goes beneath the cell surface. It partly wraps around the anterior cell end and descends towards the posterior cell end (Fig. 1H–J, 2B). The microtubules of this band are connected to each other by slightly visible bridges. The distance between microtubules is 50–60 nm. The root R1 goes ventrally along the wall of the flagellar pocket to the posterior part of the cell (Fig. 2B) (not shown in detail). The root R2 extends to the anterior cell end where it goes along the margin of the cytostome and continues along cytopharynx (Fig. 2B–E, G).

The cytostome is positioned in the apex of the cell and contains a C-shaped clamp or spur which serves as a microtubular organizing centre for structures such as the FAS band (cytostome associated microtubules), the CMT (cytopharynx associated additional microtubules) band, and the microtubular prism (nemadesm), in addition to the root R2 (Fig. 2C–G).

The root R2 extends anteriorly out of the flagellar pocket before making a hairpin turn to travel back into the cell as part of the support for the cytopharynx. In the place of its turn, the root R2 is covered by tomentum (fringe of thin hairs) (Fig. 2E). The root R2 is connected to the wall of cytopharynx by electron-dense bridges (Fig. 2D, E). Three to four microtubules of the CMT band lie close to the root R2 (Fig. 2B, D). The microtubular prism is trapezoid in profile and consists of four rows of microtubules of the 6 + 5 + 4 + 3 or 5 + 4 + 3 + 2 arrangement (Fig. 1I, 2B, D, F). The microtubules of the prism are surrounded by amorphous material and bridges between the microtubules have not been found. The FAS band initially extends along the cytopharynx and further down the cell, merges with the dorsal band (Fig. 2C, D, F, G, 3D). The submembrane corset of microtubules thus consists of the dorsal and FAS bands (Fig. 3H). The path of the root R1 beneath the plasmalemma towards the cell end was not determined. The microtubular corset is absent in the posterior part of the cell (not shown).

The arrangement of cellular structures in the anterior cell end was reconstructed using a sequence of serial sections (Fig. 3A–L). The FAS band is inserted from the clamp and curves towards the dorsal cell side (Fig. 3A–D). On oblique sections, it is visible that dorsal microtubules form a wide band which probably merges with the FAS band (Fig. 3C, D, E–H). The latter emerges from the clamp and wraps around the cytopharynx (Fig. 3E–H). The similar pattern of bands is seen on four other sections where a microtubular prism is attached to a fibril (Fig. 3I–L), which is most likely an extension of the clamps. The root R2 is clearly visible and readily identified on all section as a group of 5 microtubules (Fig. 3A–L).

The nucleus with a central nucleolus is situated at the level of kinetosomes or below (Fig. 2B, 3B, 4A). The Golgi apparatus is situated in the anterior end of the cell and consists of several flattened vacuoles (cisternae)

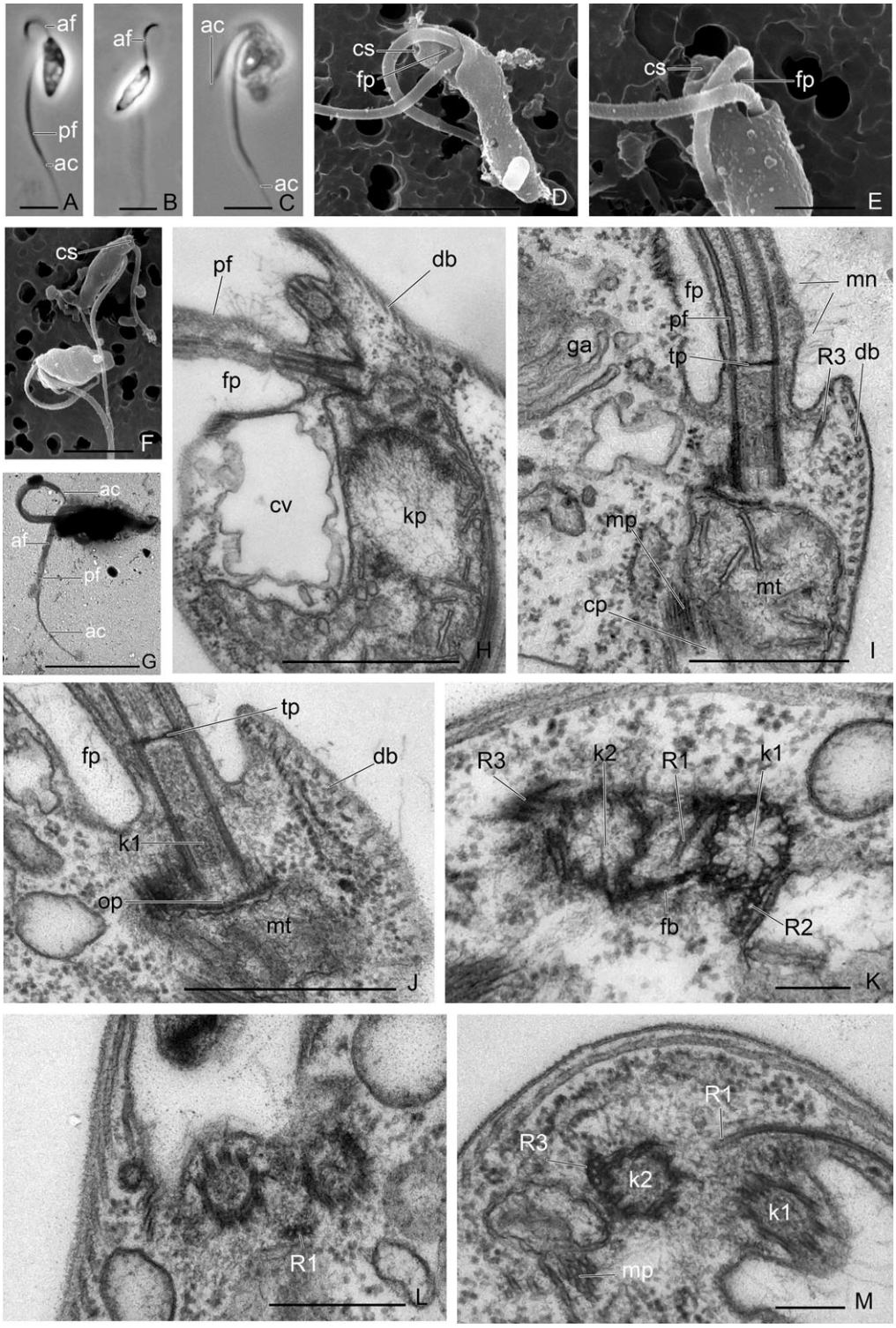

Figure 1 The external morphology and structure of the anterior cell end. A–C. Single cells, light microscopy, phase contrast. D–F. Dried cells, SEM. G–M. TEM (G – whole-mount preparation of the cell, H–J – longitudinal sections of the anterior cell end, K–M – arrangement of the kinetosomes). ac = acroneme of the flagellum, af = anterior flagellum, cp = cytopharynx, cs = cytostome, cv = contractile vacuole, db = dorsal band, fb = fibril connecting kinetosomes, fp = flagellar pocket, ga = Golgi apparatus, k1 = kinetosome of the posterior flagellum, k2 = kinetosome of the anterior flagellum, kp = kinetoplast, mn = mastigonemes, mp = microtubular prism, mt = mitochondrion, op = osmiophilis plate, pf = posterior flagellum, R1 = root R1, R2 = root R2, R3 = root R3, tp = transverse plate. Scale bar: (G) 10 μm, (A–D, F) 5 μm, (E) 2 μm, (H, J) 1 μm, (L) 0.5 μm, (K, M) 0.2 μm.

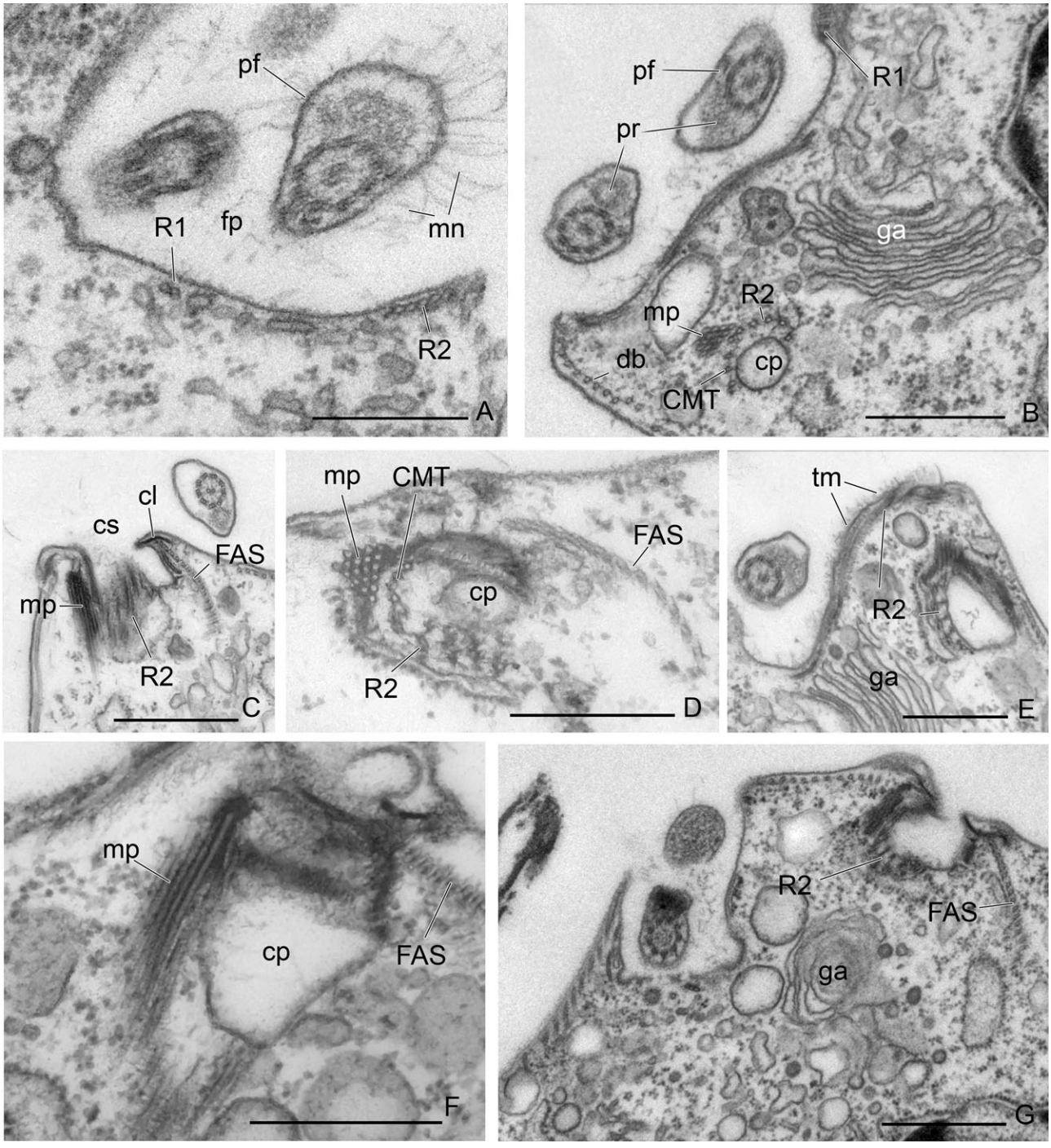

Figure 2 The structures of the anterior cell end. A. Cross-section of the flagellar pocket. B. Relative arrangement of the cytopharynx, Golgi apparatus and nucleus. C–G. Relative arrangement of the flagellar pocket, cytopharynx and surrounding structures. cl = clamp, CMT = cytopharynx associated additional microtubules band, FAS = microtubular band, associate with cytopharynx, n = nucleus, pr = paraxonemal rod, tm = tomentum, for other symbols, see Fig. 1. Scale bar: (C) 1 μm, (G) 0.8 μm, (A, B, D, E) 0.5 μm, (F) 0.4 μm, (E) 0.3 μm.

(Fig. 1I, 2B, C, E, G). The mitochondria were observed to have discoid cristae (Fig. 1I, J, 4A, B, F). The kinetoplast (as part of the mitochondrion) is compact and lies near the kinetosomes in the anterior end of the cell (Fig. 1H). The food vacuole contains engulfed bacteria (Fig. 4A, E). The contractile vacuole lies close to the wall of the flagellar pocket and is surrounded by small vesicles (Fig. 1H, 4C). Lipid droplets are found in the cytoplasm (Fig. 4C). Several glycosomes of irregular shape are scattered in the cytoplasm (Fig. 4D). Symbiotic bacteria have been

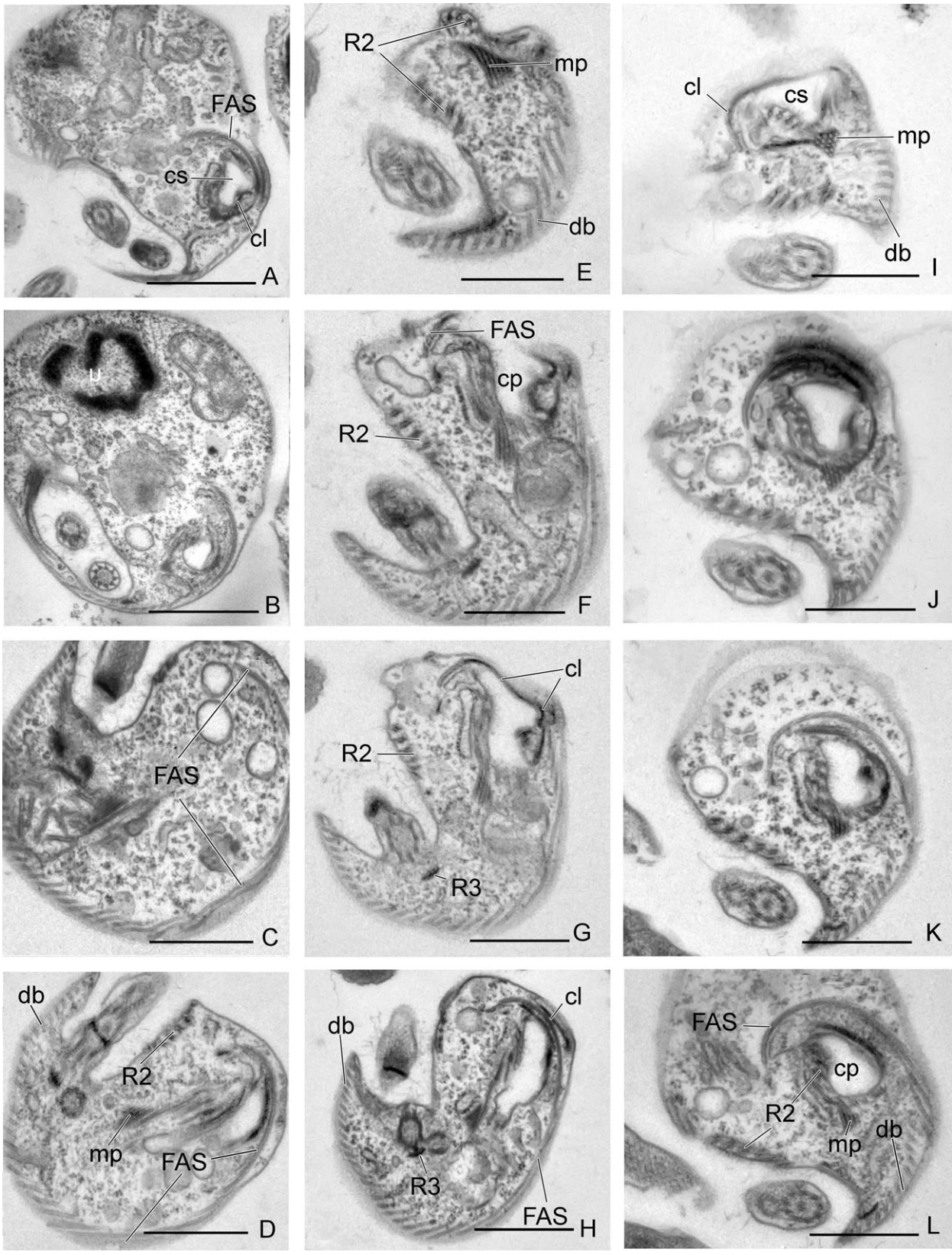

Figure 3 A series of selected cross-sections of the anterior cell end of four specimens. A, B; C, D; E–H and I–L. From the front tip to down of the cell. For symbols see Fig. 1, 2. Scale bar: (A–H) 1 μm, (I–L) 0.5 μm.

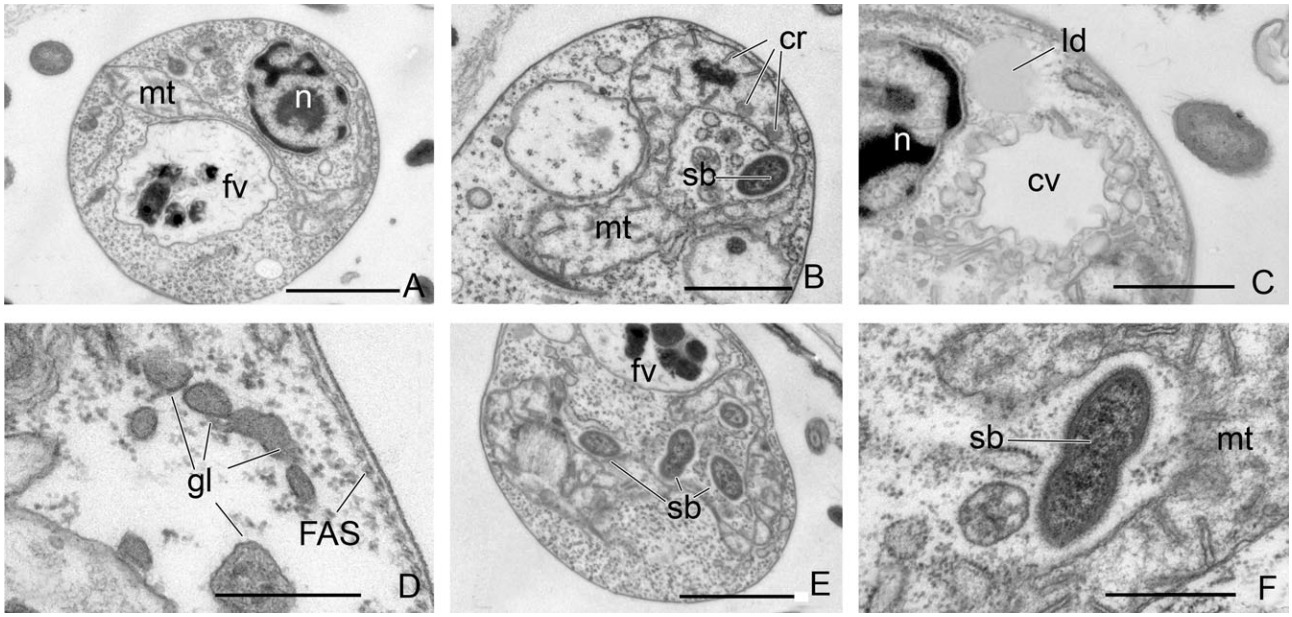

Figure 4 Cross-sections of the cell. A. Nucleus and food vacuole. B. Mitochondrion. Discoid cristae are visible. C. Lipid droplet and contractile vacuole. D. Glycosomes. E, F. Symbiotic bacteria. Bacterial fission is visible. fv = food vacuole, gl = glycosome, ld = lipid droplet, sb = symbiotic bacteria. For other symbols see Fig. 1, 2. Scale bar: (A, B, E) 1 μm, (C, D, F) 0.5 μm.

found in the posterior cell part (Fig. 4E, F). Some bacteria were in fissile phase. Extrusive organelles have not been found.

Salinity tolerance experiment executed in triplicates revealed that B-74 can sustain salinities from 0.11‰ to 3–4‰, suggesting it cannot occur in marine habitats.

Phylogenetic analyses with or without the *Ichtyobodo* and *Perkinsiella* outgroup (Fig. 5) congruently placed *N. borokensis* within the order Neobodonina, in a strongly supported clade consisting of *Actuariola framvarensis*, *N. saliens*, *Cryptaulaxoides*-like, "*N. curvifilus,*" and two *N. designis* sequences, which altogether likely represent a new family within the order Neobodonida. This clade corresponds to the strongly supported "*Actuariola* clade" (bootstrap 97%) from Stoeck et al. (2005).

Within this clade, *N. borokensis* was specifically related to a "*Neobodo curvifilus*" sequence (GI no. 77994639) and three environmental sequences (GI nos. 59891971, 59891964, 59891965). The "*Neobodo curvifilus*" sequence was derived from a bodonid strain isolated by one of us (A.P. Mylnikov) from freshwater sediments of the mesotrophic Lake Schoehsee (Ploen, Germany; Scheckenbach et al. 2006). This strain had been roughly identified as "*Bodo curvifilus*" according to the morphological description of *B. curvifilus* given by Burzell (1975). This morphology, however, does not correspond to the original description given by Griessmann (1913), suggesting that the assignment to *B. curvifilus* was erroneous. The "*Neobodo curvifilus*" sequence (GI no. 77994639) is 98.4% similar to that of B-74 and we thus conclude that this species was misidentified, and in fact represents *N. borokensis*. The three environmental sequences came from a study by von der Heyden and Cavalier-Smith (2005) and originated from freshwater sediments of the River Thames (Oxford, UK), Bejuco River (Panama), and Lake Camino de Cruces (Panama) respectively. These sequences were referred to as *Neobodo* sp. in the publication (von der Heyden and Cavalier-Smith 2005).

Another sequence confusingly named *B. curvifilus* in GenBank (GI 41400279) was generated by von der Heyden et al. (2004). This strain was isolated from Red Deer faeces, Glasgow, UK and referred to as *Parabodo caudatus*-like strain RDF in the manuscript (von der Heyden et al. 2004), an affiliation confirmed by our molecular phylogeny (Fig. 5). Indeed, the definition of *B. curvifilus* is highly problematic (see Discussion), but no morphological or molecular evidence available suggests that this *B. curvifilus* sequence (GI 41400279) is related to B-74.

DISCUSSION

Several kinetoplastid flagellates, both new species and older well-known ones, have been investigated or reinvestigated by electron microscopy (Brugerolle 1985; Brugerolle et al. 1979; Elbrächter et al. 1996; Frolov and Karpov 1995; Frolov et al. 1996, 1997, 2001; Myl'nikov 1986a,b; Myl'nikov et al. 1998; Simpson 1997; Stoeck et al. 2005). As a result, the ultrastructure of clone B-74 can be compared to a diversity of other kinetoplastids.

The ultrastructure of clone B-74 is similar to that of other bodonids and cryptobiids (Breunig et al. 1993; Brugerolle et al. 1979; Frolov et al. 1997; Hitchen 1974; Vickerman 1978; Vickerman and Preston 1976) in the following characteristics: three flagellar roots, paraxonemal rods in the flagella, kinetoplast, cytostome/cytopharynx complex. The following ultrastructure of B-74 cells is

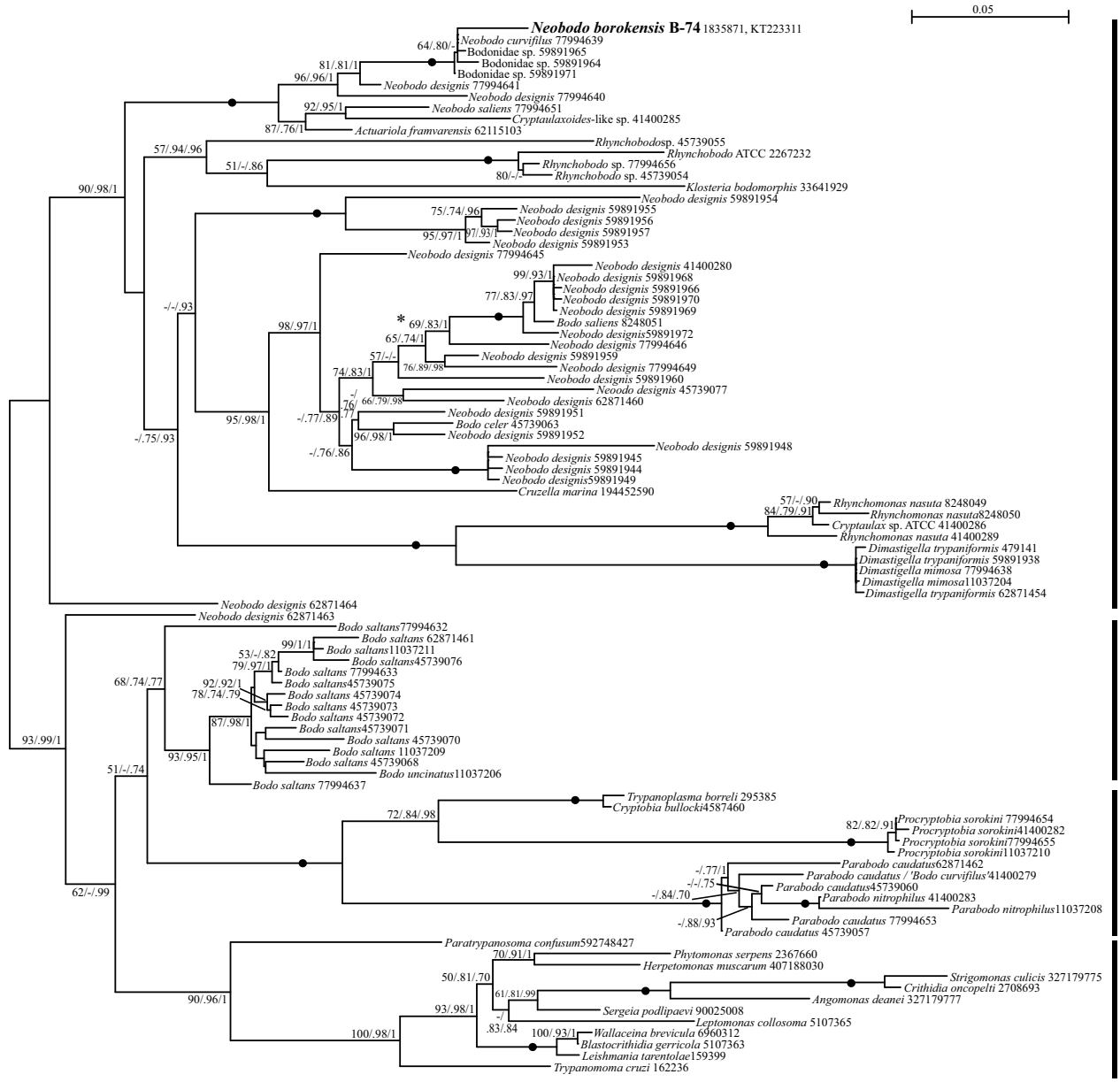

Figure 5 The phylogenetic position of *Neobodo borokensis* (in bold) within Metakinetoplastina. The best RAxML tree (GTR + GAMMA4 + I model) based on the 18S rDNA data set is shown. RAxML standard bootstraps, PhyML aLRT supports, and MrBayes posterior probability branch supports are shown at branches (> 50/> 0.7/> 0.8 were shown as significant; dashes indicate insignificant support). Black dots indicate complete support (100/1/1). Species names correspond to sequence names in GenBank followed by GI accession numbers. The position of the *Diplonema*/*Ichthyobodo*/*Perkinsiella* outgroup in the analysis of the extended data set is indicated by the asterisk.

consistent with the assignment to the genus *Neobodo*: microtubular prism, FAS band, CMT band, root R2 (MTR sensu Brugerolle et al. 1979), acroneme of the posterior flagellum, compact kinetoplast. The genus *Neobodo* was described by Vickerman in Moreira et al. (2004) as solitary phagotrophic flagellate with a single discrete eukinetoplast, apical cytostome and cytopharynx supported by a prismatic rod of microtubules, and free recurrent flagellum used as a skid when not free-swimming. The type species of *Neobodo* is *N. designis* (Skuja) Vickerman 2004, but its phylogenetic position within the bodonids cannot be determined: different sequences of *N. designis* fall in different positions in the phylogenetic tree. The type strain and/or type sequence of *N. designis* was not specified by the authors of genus *Neobodo*.

Parallel or acute-angled orientation of kinetosomes and presence of tomentum on the bent portion of the root R2 are known in B-74, *Hemistasia amylophagus*, *H. phaecysticola*, *Pseudophyllomitus apiculatus,* and *Rhynchobodo armata* (Brugerolle 1985; Myl'nikov 1986a,b; Myl'nikov

et al. 1998; Nikolaev et al. 2003). The margin of the B-74 cytostome contains a clamp, similar to that of *Klosteria bodomorphis*, *P. sorokini*, *H. amylophagus*, *Pseudopyllomitus apiculatus,* and *R. armata* (Frolov and Karpov 1995; Frolov et al. 2001). This clamp, probably, takes part in the formation of the microtubular prism, FAS and CMT bands. The gap between the dorsal and FAS band which is absent in *N. borokensis* is known in *P. caudatus*, *P. nitrophilus*, *B. saltans,* and *P. sorokini* (Brooker 1971; Frolov et al. 2001; Myl'nikov 1986b); however, the FAS band is absent in some other bodonids, such as *K. bodomorphis* (Nikolaev et al. 2003).

Clone B-74 contains prokinetoplast DNA (pro-kDNA) located near the basal body of the flagellum, as described in the eubodonid *B. saltans*, and the neobodonids *Rhynchomonas* and *Neobodo* (Burzell 1975; Eyden 1977; Lukeš et al. 2002).

Some features (microtubular prism, FAS band) link B-74 with *R. armata*, *H. amylophagus,* and *N. designis* (Elbrächter et al. 1996). Interestingly, thin mastigonemes on the anterior flagellum are known in *B. saltans* and *P. apiculatus* (Brooker 1971; Myl'nikov 1986a). However, thin mastigonemes on the proximal part of the posterior flagellum have only been found in B-74 and *A. framvarensis* (Stoeck et al. 2005).

Furthermore, B-74, *Actuariola*, and *N. saliens* are all able to turn the anterior flagellum to the dorsal cell side. This behaviour has served as an identifying characteristic for *N. saliens*, but may in fact be ancestral to the B-74 – *Actuariola* – *N. saliens* clade. B-74 has a very long acroneme of the posterior flagellum which is longer than in *N. designis*. Overall, B-74 appears to be most similar to *N. designis*, *N. saliens*, *N. curvifilus* and *A. framvarensis* (Burzell 1975; Eyden 1977; Larsen and Paterson 1990; Stoeck et al. 2005).

Actuariola framvarensis is marine and has symbiotic bacteria, glycosomes, acronemes of 3–5 μm, oval cysts in the life cycle, mastigonemes on the proximal part of the posterior flagellum, a trapeziform in cross-section microtubular prism, and an elongated cell shape (Stoeck et al. 2005).

The cell shape and size of B-74 resembles the cosmopolitan flagellate *N. designis* (Al-Qassab et al. 2002; Larsen and Paterson 1990; Patterson and Simpson 1996; Vørs 1992). Both species have an ellipsoid body and similar flagella with a relatively long acroneme of the posterior flagellum. The latter is longer in B-74. In contrast to *N. designis*, B-74 cannot coil its anterior flagellum around the anterior cell end and rotate after contact with large particles or the substratum. The flagellar hooks of these two species are similar to one another, but in actively moving cells of B-74, this hook faces towards the dorsal cell side, similarly to *N. saliens*. B-74 and *N. saliens* also share the presence of a rapid, straight, dart-like movement. B-74 is sensitive to increased salinity and died at salinity levels above 4‰, which distinguishes it from marine strains of *N. designis* and *N. saliens*.

Neobodo designis has symbiotic bacteria and microtubular prism of 15 (rarely 10 or 21) microtubules with the 5 + 4 + 3 + 2 + 1 arrangement and a triangular shape on the cross-section. It does not have mastigonemes on both flagella, and does not have glycosomes in the cytoplasm. Its mitochondria contain dense granules. The root R2 consists of five microtubules and the CMT band consists of three microtubules (Eyden 1977; Larsen and Paterson 1990). The acronemes of the posterior flagellum are no longer than 5 μm, and the body size is 6–12 μm (Al-Qassab et al. 2002; Larsen and Paterson 1990; Patterson and Simpson 1996; Vørs 1992).

Neobodo saliens has not been investigated by electron microscopy, but Larsen and Paterson (1990) proposed that this flagellate was probably studied by Burzell (1975) under the name "*Bodo curvifilus.*" This freshwater "*Bodo curvifilus*" has acronemes of 10% of the flagellar length, and a triangular (in profile) microtubular prism is made up of 15 microtubules (5 + 4 + 3 + 2 + 1 arrangement). Gram-negative symbiotic bacteria are present.

The original description of *Neobodo* (*Bodo*) *curvifilus* was from marine waters (Griessmann 1913). Together with *N. saliens*, *N. designis,* and *B. saltans,* as well as *R. nasuta,* this species appears to be among the most widely distributed kinetoplastids in fresh and marine waters (Patterson and Lee 2000). The type of *N. curvifilus* is an oval or bean-shaped, flattened, gliding, and metabolic flagellate (4–7 μm long) with a very thick anterior flagellum (Griessmann 1913). In our opinion, the drawings by Griessmann (Fig. 6A) are similar to some extent by external morphology to another well-known species – *P. caudatus*. *P. caudatus* was originally described as *Amphimonas caudata* by Felix Dujardin (1841) as a very metabolic, usually flattened, and tuberiform organism, convexed from one side and angular from the other. However, this organism was described from fresh water (Northern Europe) and characterized by bigger size – 12–20 μm long (Fig. 6B). *Amphimonas caudata* was later emended as *Bodo caudatus* by Stein (Stein 1878; note that drawings by Stein (Fig. 6C) are confusing and while some do look like *B. caudatus*, other are reminiscent of the colpodellid *Alphamonas edax* Aléxéieff 1924). Griessmann did not cite Dujardin's and Stein's papers and only compared his *B. curvifilus* with *B. saltans* Ehrenberg 1832.

In modern descriptions, the body size of *P. caudatus* varies from 6 to 21 μm (most are under 15 μm) (Lee et al. 2005). The average size of the *P. caudatus* clone BAS-1 (in our collection of living cultures of protists IBIW RAS) is 6–9 μm (our observations); however, average size of 15–18 μm (and sometimes more) was described for cells isolated from hypertrophic waters of treatment plants (Myl'nikov 1977). This species usually inhabits freshwater sites, but it has also been reported from marine and hypersaline environments (Al-Qassab et al. 2002; Post et al. 1983; Ruinen 1938).

In 1975, Boris F. Zhukov described a new marine organism, *Bodo sorokini* from a sample collected from Lake Faro (Sicily) near Messina. He noted that *B. sorokini* is reminiscent of Griessmann's *B. curvifilus* with the exception of a single feature – the adherence of its recurrent

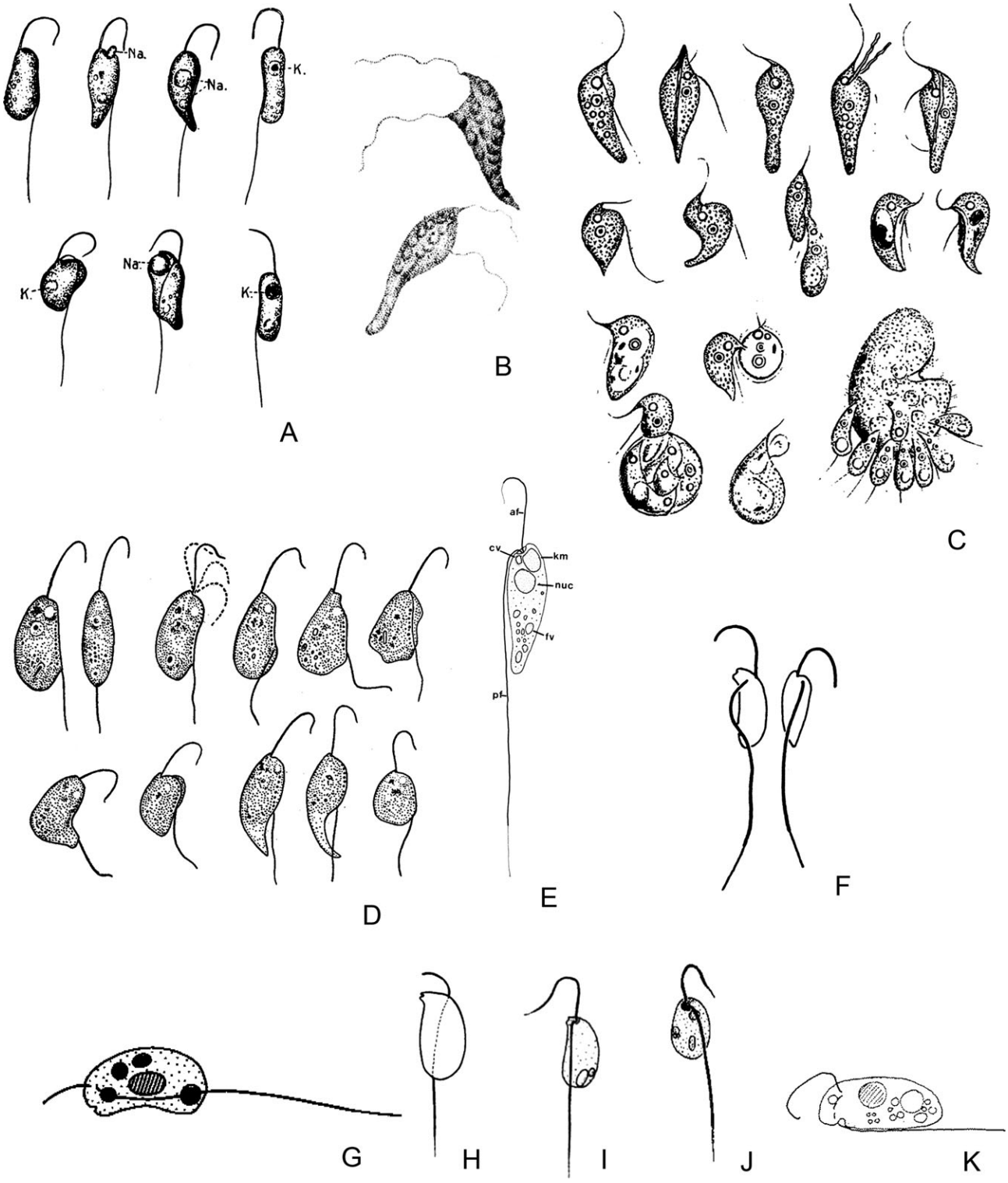

Figure 6 Drawings of selected bodonid species. A. *Bodo curvifilus* from Griessmann (1913). B. *Amphimonas caudata* from Dujardin (1841). C. *Bodo caudatus* from Stein (1878). D. *Bodo sorokini* from Zhukov (1975). E. *Bodo curvifilus* from Burzell (1975). F. *Bodo curvifilus* from Patterson et al. (1993). G. *Bodo curvifilus* from Tong (1997). H. *Bodo curvifilus* from Tong et al. (1997). I. *Bodo curvifilus* from Lee et al. (2003). J. *Bodo curvifilus* from Lee and Patterson (2000). K. *Bodo curvifilus* from Vørs (1992). af = anterior flagellum, cv = contractile vacuole, fv = food vacuole, K = nucleus, km = kinetoplast-mitochondrion, Na. = food intake, nuc = nucleus, pf = posterior flagellum.

flagellum to the ventral cell surface (Fig. 6D) (Zhukov 1975). However, this may not really be an exception, because the posterior flagellum of *B. curvifilus* also appears to be adhered to the cell, according to Griessmann's description (Griessmann 1913). The ultrastructure of *B. sorokini* was later studied and the species was transferred to the genus *Procryptobia* because it is clearly distinguishable from *Bodo* spp. by its more flattened body and the attachment of its recurrent flagellum along the cell surface (Frolov et al. 2001). The latter characteristic was not noted in the original diagnosis of the genus *Bodo* (Vickerman 1976).

The ultrastructural study of an organism identified as *B. curvifilus* was done by Burzell (1975). Due to the presence of a microtubular prism (Burzell 1975), it was proposed that *B. curvifilus* probably belongs to the genus *Neobodo* (Moreira et al. 2004). But Burzell's specimen was not bean-shaped, not flattened, and not metabolic (Fig. 6E). We agree with Larsen and Paterson (1990), who proposed that this flagellate was probably misidentified. Burzell's drawing is more reminiscent of the recently described *N. saliens* and our B-74 (*N. borokensis*) than it is of *B. curvifilus* as described by Griessmann.

An organism identified as *B. curvifilus* was also investigated using TEM by Frolov et al. (1996), who were specifically interested in the ultrastructure of mitosis. Unfortunately, the light microscopy appearance of the organism was not documented, and some details of the general cell ultrastructure were not shown, consequently its identity is unclear. It is unlikely to be the same organism examined by Burzell (1975; see above); however, for instance, no microtubular prism is visible near the cytopharynx in fig. 18 of Frolov et al. (1996).

More recent observations of *B. curvifilus* were made by Lee and Patterson (2000), Lee et al. (2003), Patterson et al. (1993), Throndsen (1969), Turley and Carstens (1991), Tong (1997), Tong et al. (1997), Vørs (1992, 1993). The photos and drawings by these authors (if present; Fig. 6F–K) are almost indistinguishable from *P. sorokini*.

Hence, *Bodo* (or *Neobodo*) *curvifilus* remains a confusing and doubtful species. Perhaps, recent findings of this organism represent the same morphospecies as *P. sorokini* or different-sized marine strains of *P. caudatus*. However, if *B. curvifilus* is a valid species, we propose that it is classified as a parabodonid due to the great similarity with *P. sorokini* and *P. caudatus*.

Phylogenetic analysis of SSU rRNA (Fig. 5) shows that some species of free-living bodonids are characterized by a significant genetic heterogeneity. Sequences of the same morphospecies attributed to *N. designis*, *N. saliens*, and *B. saltans* represent different, and often distantly related clades on the tree. In the case of *N. saliens* this is probably caused by an inconsistent identification of species. At the same time, strains of these and other bodonid species that are closely related in the SSU rRNA tree may inhabit different marine and freshwater habitats in different geographical zones, all of which support the idea that morphospecies of heterotrophic flagellates can have a cosmopolitan and ubiquitous distribution and are often able to tolerate a wide range of salinities.

TAXONOMIC SUMMARY

The data presented above allow us to classify the clone B-74 to the genus *Neobodo* as new species.

The taxonomy is based on the classification by Adl et al. (2012)

Excavata Cavalier-Smith 2002
Discoba Simpson 2009
Discicristata Cavalier-Smith 1998
Euglenozoa Cavalier-Smith 1981
Kinetoplastea Honigberg 1963
Metakinetoplastina Vickerman 2004
Neobodonida Vickerman 2004
Neobodo Vickerman 2004

Neobodo borokensis n. sp

Diagnosis. Elliptical neobodonid kinetoplastid 7–12 μm long, 3–6 μm width, with short inflexible rostrum, eukinetoplast, and trapeziform nemadesm; anterior flagellum 8–14 μm long, makes flapping movements, and forms hook that is curved to the ventral cell side when cell jumps along substratum, but in fast moving cells, organism curves this hook to dorsal cell side; posterior flagellum with long acroneme (5–14 μm) and fine hairs in proximal part; cell rotates rapidly around its axis during swimming and can temporary attach to substratum by acroneme; cysts unknown; freshwater, cannot live at salinity above 4‰.

Type material. A block of chemically fixed resin-embedded cells of the type strain, B-74, is deposited in Marine Invertebrate Collection, Beaty Biodiversity Museum, University of British Columbia as MI-PR204. This constitutes the name-bearing type of the new species (a hapantotype). Figure 1A illustrates a live cell of strain B-74.

Type locality. freshwater pond "Barsky," Borok, Russia (58.061365 N, 38.245730 E).

Etymology. Name is based on the original locality of finding (the settlement Borok in Russian Federation).

Gene sequence. The 18S rRNA gene sequence has the GenBank Accession Number KT223311.

Zoobank Registration. urn:lsid:zoobank.org:act: 4B231C5F-490D-41FA-8936-FC226C088C64.

Comparison. *Neobodo borokensis* differs from *N. curvifilus* by the movement of the anterior flagellum, the shape of the cell, and the type of movement; from *N. saliens* by the capability to form the hook of flagellum turned to ventral cell side; from *N. designis* by the capability to form the hook of flagellum turned to dorsal cell side and by the presence of mastigonemes on the posterior flagellum. *Neobodo borokensis* lacks curling of anterior flagellum around the anterior cell end, the attachment of a cell tip to particles, or electron-dense inclusions in mitochondria, as are found in *N. designis*. *Neobodo borokensis* has a trapeziform profile of its microtubular prism, in contrast to a triangular one as in *N. designis*. Moreover, the posterior flagellum of *N. borokensis* bears a very long acroneme (1/

3 of the flagellum length), which does not occur in other members of the genus *Neobodo*. *Neobodo borokensis* is similar to *A. framvarensis* in cell shape, but differs from it by the inability to live in salt water, an absence of cysts in its life cycle, prismatic (not linear) arrangement of microtubules of the nemadesm, and a longer acroneme of the posterior flagellum. The new species could not live in salinities above 4‰ unlike all marine strains of *N. designis* and *N. curvifilus*.

ACKNOWLEDGMENTS

This work was supported by the Russian Foundation for Basic Research (no. 11-04-00077, 15-04-05841, 15-34-20065, 14-04-00554), the grant of President of Russian Federation MK-7436.2015.4, a grant from the Natural History and Engineering Research Council of Canada to PJK (no. 227301). The work of DVT and phylogenetic analysis was supported by the Russian Science Foundation (no. 14-14-00515). We thank two anonymous reviewers for their constructive suggestions that helped us improve this manuscript.

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