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Diversity, Evolution and Molecular Systematics of the Psalteriomonadidae, the Main Lineage of Anaerobic/Microaerophilic Heteroloboseans (Excavata: Discoba)

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We isolated and cultivated 31 strains of free-living heterolobosean flagellates and amoebae from freshwater, brackish, and marine sediments with low concentrations of oxygen. Phylogenetic analysis of small subunit (SSU) rDNA showed that the strains constitute a single clade, the Psalteriomonadidae. According to combined light-microscopic morphology plus molecular phylogeny, our isolates belong to seven species and five genera, from which three species and two genera are new. In addition, previously described anaerobic species *Percolomonas descissus* and *Vahlkampfia anaerobica* are transferred to the Psalteriomonadidae. We identified a flagellate stage of *Monopylocystis visvesvarai* which was reported to produce only amoebae. Two environmental sequences previously obtained from acidic environments belong to the Psalteriomonadidae as well, suggesting a broad ecological importance of the Psalteriomonadidae. The ultrastructure of two psalteriomonadid species was also studied. Unifying features of the Psalteriomonadidae are acristate mitochondrial derivates, flagellates with a ventral groove and four flagella, and a harp-like structure in the mastigont. A new overall classification of the Psalteriomonadidae is proposed. Our data show that the Psalteriomonadidae are much more diverse than previously thought and constitute the main anaerobic lineage within the Heterolobosea.

Key words: Heterolobosea; Psalteriomonadidae; evolution; taxonomy; new species; anaerobiosis.

Introduction

The Heterolobosea is a small group of amoeboflagellates, belonging to the eukaryotic supergroup Excavata. Typical morphological features of heteroloboseans include intranuclear orthomitosis; flattened, often discoidal, mitochondrial cristae; a close association of rough endoplasmic reticulum with mitochondria; absence of stacked Golgi complexes; and movement by eruptive lobopodia in the amoeboid stage (Page and Blanton 1985). Heterolobosean flagellates have retained some distinctive excavate features (e.g. the ventral feeding groove) (Brugerolle and Simpson 2004; Park and Simpson 2011; Simpson 2003), although they

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have been reduced in some genera. In addition to morphological characteristics, all heterolobosean, except for the early-branching *Pharyngomonas kirbyi*, share a novel helix 17-1 in the secondary structure of the SSU rRNA molecule (Nikolaev et al. 2004; Park and Simpson 2011; Park et al. 2007, 2009; Wuyts et al. 2001; Yubuki and Leander 2008).

Phylogenomic analyses showed that the Heterolobosea are closely related to the Euglenozoa and the Jakobida, together forming a more inclusive clade called the Discoba or JEH (Burki et al. 2008; Hampl et al. 2009; Rodríguez-Ezpeleta et al. 2007). *Tsukubamonas globosa* was recently described as a new discobid lineage, although its precise phylogenetic position within the Discoba remains unresolved (Yabuki et al. 2011). In the last decade, the root of the eukaryotic phylogenetic tree was hypothesized to lie between the so-called "Unikonta" and "Bikonta" (Richards and Cavalier-Smith 2005; Stechmann and Cavalier-Smith 2002, 2003); however, the unikont/bikont hypothesis has fallen into disfavor (see Kim et al. 2006; Roger and Simpson 2009) and several new hypotheses regarding the position of the root of the eukaryotic phylogenetic tree were formulated. Some of them place the root specifically within the Discoba (Cavalier-Smith 2010; Gray et al. 2004; Rodríguez-Ezpeleta et al. 2007).

Despite the fact that the Heterolobosea is a relatively species-poor group (approximately 140 described species), members of the group are immensely diverse at both morphological and ecological levels. Some heteroloboseans, such as *Naegleria*, *Willaertia*, *Tetramitus, Heteramoeba*, *Euplaesiobystra*, and *Psalteriomonas*, are true amoeboflagellates that alternate between amoeboid and flagellated stages during their life cycle. The transition from the amoeba to the flagellate is incredibly fast in some lineages; for instance, this transformation in *Naegleria* takes ca. 2 hours and involves de novo formation of basal bodies with the accompanying cytoskeleton (Fulton 1993; Lee 2010). On the other hand, some members of the Heterolobosea are known to produce only flagellates or amoebae. *Acrasis* and a few of its putative relatives are sorocarpic amoebae that produce multicellular fruiting bodies (see Brown et al. 2010; Olive et al. 1983). *Naegleria fowleri* and, possibly, *Paravahlkampfia francinae* are deadly facultative parasites of humans (Visvesvara et al. 2007, 2009). *Stephanopogon* is a peculiar organism reminiscent of ciliates whose affiliation to the Heterolobosea was revealed only by detailed ultrastructure and molecular phylogenetic analyses (Cavalier-Smith and Nikolaev 2008; Lipscomb and Corliss 1982; Parfrey et al. 2010; Patterson and Brugerolle 1988; Yubuki and Leander 2008). Although most heteroloboseans live in sediments and feed on bacteria, the ecological diversity of the group is vast as well. In comparison with the other eukaryotic groups, many members of the Heterolobosea flourish in extreme environments such as acidic, hot, and hypersaline conditions (Amaral Zettler et al. 2002; Baumgartner et al. 2009; De Jonckheere et al. 2009; Guzmán-Fierros et al. 2008; Park et al. 2007, 2009; Park and Simpson 2011; Sheehan et al. 2003).

Anaerobic/microaerophilic heteroloboseans constitute another such ecological group living under harsh conditions. At least two independent lineages have been discovered. The first one is represented by the extreme halophile *Pleurostomum flabellatum* which grows at low oxygen concentrations and appears to lack mitochondrial cristae (Park et al. 2007). The second lineage is more diverse and includes *Monopylocystis visvesvarai*, *Psalteriomonas lanterna*, and *Sawyeria marylandensis* (Broers et al. 1990; Nikolaev et al. 2004; O'Kelly et al. 2003). Mitochondria of *Sawyeria* and *Psalteriomonas* have been reduced to hydrogenosomes (Barberà et al. 2010; de Graaf et al. 2009). In addition, *Vahlkampfia anaerobica*, *Lyromonas vulgaris*, and *Percolomonas descissus* have been described from habitats with low oxygen concentrations (Bernard et al. 2000; Broers et al. 1993; Brugerolle and Simpson 2004; Smirnov and Fenchel 1996). However, their phylogenetic position is unclear due to the lack of DNA sequence data. The general metabolic potential of heteroloboseans has become complicated by the recently published genome project of *Naegleria gruberi* (Fritz-Laylin et al. 2010). It appears that this presumably bona fide aerobe with typical mitochondrial morphology may be capable switching to anaerobic metabolism under hypoxia. Therefore, anaerobic life styles (or facultatively anaerobic life styles) may be more common in heteroloboseans than expected.

The systematics of heteroloboseans is chaotic due to (1) absence of reliable morphological features, (2) unclear internal phylogeny, and (3) non-unified concepts for the main heterolobosean subgroups. Even the definition of the Heterolobosea itself is confusing and two different taxonomic concepts exist. The first concept, emphasized by Park and Simpson (2011), considers the Heterolobosea as a monophyletic taxon containing organisms with trophic life stages as amoebae-only, amoeboflagellates, and flagellates-only and includes diverse taxa such as *Acrasis*, *Naegleria*, *Neovahlkampfia*, *Tetramitus*,

Stephanopogon, *Pharyngomonas*, and *Psalteriomonas*. In contrast, Cavalier-Smith (1993b) and Cavalier-Smith and Nikolaev (2008) refer to this inclusive group as the "Percolozoa" and consider the 'Heterolobosea' as a paraphyletic subgroup of taxa that were historically classified as amoebae. We concur with Nikolaev et al. (2004) who suggested that "the term Percolozoa should probably become the junior synonym of the term Heterolobosea" and recognize the broader and monophyletic concept of the Heterolobosea in this paper (Park and Simpson 2011).

Heterolobosean genera have been classified into 8 families: Acrasidae Van Tieghem, 1880, Vahlkampfiidae Jollos, 1917, Stephanopogonidae Corliss, 1961, Gruberellidae Page and Blanton, 1985, Psalteriomonadidae Cavalier-Smith, 1993, Lyromonadidae Cavalier-Smith, 1993, Percolomonadidae Cavalier-Smith and Nikolaev, 2008, and Pharyngomonadidae Cavalier-Smith and Nikolaev, 2008. The largest family Vahlkampfiidae Jollos, 1917 is paraphyletic and the other families, except for the Pharyngomonadidae, branch from within it. Although reclassification of the Vahlkampfiidae by splitting it into more monophyletic groups is necessary, the data for doing so are currently equivocal.

Although heteroloboseans are highly important from evolutionary and ecological points of view, their diversity has been considerably understudied. The aim of the present study is to thoroughly examine the diversity of anaerobic heteroloboseans. We have isolated 31 heterolobosean strains from freshwater, brackish-water and marine anoxic/microoxic habitats, and studied their light-microscopic morphology and phylogenetic position as inferred from the analysis of SSU rDNA sequences. We also examined the cell ultrastructure of two isolates. We recognized each species based on either a distinct morphology, placement in the SSU rDNA tree or a combination of both criteria. We showed that the diversity of anaerobic heteroloboseans is richer than previously expected and that non-halophilic anaerobic heteroloboseans, such as *Percolomonas descissus* and *Vahlkampfia anaerobica*, constitute a single clade, the Psalteriomonadidae. In this paper, we described three new species and two new genera, and revised the taxonomy of the Psalteriomonadidae.

Results

General Observations

Information on the origin of strains included in the present study is summarized in Table 1. Cells of all strains were found at the bottom of the culture tubes in the presence of bacteria and slowly died when exposed to oxygen near the upper part of the tubes, suggesting that the cells were anaerobic or microaerophilic. Only amoebae (*Psalteriomonas* spp., *Sawyeria marylandensis*) or flagellates (*Monopylocystis visvesvarai*, *Harpagon* spp., *Pseudoharpagon pertyi*) were present in the cultures; no sign of transformation of flagellates into amoebae or vice versa, and no cysts were observed during ca. 200 passages of the cultures. The morphology of amoebae was characteristic for the Heterolobosea, i.e. they were of the "limax" type, with an anterior, eruptive hyaline front, and locomotive forms were monopodial. No floating forms were observed. All amoebae were isolated from freshwater sediments. The amoebae of each species differed in size and in the presence/absence of hydrogenosomal aggregates. Flagellates were isolated from freshwater, brackish and marine sediments. They were always quadriflagellated with acronematic flagella and possessed a conspicuous ventral feeding groove. The flagella inserted subapically at the top of the groove, parallel to each other, and were directed ventroposteriorly. They beat together, close to the groove. Cells rotated when swimming. The nucleus was subapical. The flagellates of the particular species differed in the size and shape of the ventral groove, relative lengths of flagella, and degree of pleomorphism.

Light-microscopic Species Observations

Psalteriomonas lanterna

The amoebal stage of freshwater strains CIZOV2, VIT3, VIT5, VIT9, and VT2 corresponded to the original description by Broers et al. (1990), except for the structure of nucleus (Fig. 1). Unlike the type strain of *P. lanterna* whose amoebae were reported to possess a single central nucleolus, the cells of our strains possessed one or two parietal nucleoli located opposite each other in the nucleus. A hydrogenosomal aggregate was present close to the nucleus (Figs 1, 4E, F). The uroid was smooth, without filaments. Cells of the strain VIT3 were 47.5 ± 7.1 (32.1 – 63.7) μm long and 12.2 ± 2.2 (7.2 – 18.0) μm wide, with the mean length/width ratio 4.1. Cells of the strain VIT5 were 44.3 ± 6.7 (31.4 – 60.2) μm long and 10.1 ± 1.9 (6.8 – 14.1) μm wide, with the mean length/width ratio 4.5. Cells of the strain VT2 were 47.5 ± 8.1 (28.9 – 63.1) μm long and 10.8 ± 2.0 (7.1 – 15.1) μm wide, with the mean length/width ratio 4.5. The mean cell dimensions of the three strains of *P. lanterna* were 46.4 ± 7.4

Table 1. List of strains included in the study. [1]same isolate as in Kolisko et al. (2008) and Cepicka et al. (2010).

Species	Isolate	Locality	Coordinates	Habitat	GenBank acc. no.
Harpagon descissus	AMT	Tambopata NNR, Peru	12°49′S, 69°16′W	Fresh-water sediment	JN606327
	BAUM	Baum pond, Fayetteville, AR, USA	36°05′N, 94°18′W	Fresh-water sediment	JN606328
	IND2	Bhangarh, India	27°05′N, 76°17′E	Fresh-water sediment	JN606329
	KG3N	valley close to Engilchek village, Kyrgyzstan	41°57′N, 79°18′E	Fresh-water sediment	JN606330
	KOMPKOJ[1]	Kojčice, Czech Republic	49°28′N, 15°15′E	Compost pile	JN606331
	PANT1	Pantanal NP, Brazil	16°37′S, 56°44′W	Fresh-water sediment	JN606332
	SOOS1	Soos NNR, Czech Republic	50°08′N, 12°24′E	Fresh-water sediment	JN606333
	TEXEL	Texel island, Netherlands	53°01′N, 04°44′E	Fresh-water discharge ditch	JN606334
	TOCOV	Former village Tocov, Czech Republic	50°18′N, 13°05′E	Fresh-water sediment	JN606335
	VT1	Velký Tisý pond, Czech Republic	49°03′N, 14°43′E	Fresh-water sediment	JN606336
	WS	Lake Wilson stream, Fayetteville AR, USA	36°01′N, 94°14′W	Fresh-water sediment	JN606337
Harpagon schusteri	CIZOV1	Čížov, Czech Republic	48°52′N, 15°52′E	Fresh-water sediment	JN606338
	IND8	Bhangarh, India	27°05′N, 76°17′E	Fresh-water brook	JN606339
	INDSIP	Boroda dam, India	27°02′N, 76°15′E	Fresh-water sediment	JN606340
	OLB4	Olbasee lake, Germany	51°16′N, 14°35′E	Fresh-water sediment	JN606341
	TUN2	Chomutov, Czech Republic	50°27′N, 13°21′E	Fresh-water sediment	JN606342
	VITSED	Kamenice, Czech Republic	49°54′N, 14°35′E	Fresh-water sediment	JN606343
Monopylocystis visvesvarai	PC4BIC	Peggy's Cove, Canada	44°29′N, 63°55′W	Marine sediment	JN606344
Psalteriomonas lanterna	CIZOV2	Čížov, Czech Republic	48°52′N, 15°52′E	Fresh-water brook	JN606345
	VIT3	Kamenice, Czech Republic	49°54′N, 14°35′E	Fresh-water sediment	JN606346
	VIT5	Kamenice, Czech Republic	49°54′N, 14°35′E	Fresh-water sediment	JN606347
	VIT9	Kamenice, Czech Republic	49°54′N, 14°35′E	Fresh-water sediment	JN606348
	VT2	Velký Tisý pond, Czech Republic	49°03′N, 14°43′E	Fresh-water sediment	JN606349
Psalteriomonas magna	CERETE	Cereté, Columbia	08°53′N, 75°47′W	Fresh-water sediment	JN606350
	IND7	Bhangarh, India	27°05′N, 76°17′E	Fresh-water brook	JN606351
	KIZILLAR	Kizillar, Turkey	37°31′N, 35°42′E	Fresh-water sediment	JN606352
Sawyeria marylandensis	ITZAVL	Italy	n.a.	Fresh-water irrigation canal	JN606353
	LUH2	Alluvial plain of Vltava river, Czech Republic	48°48′N, 13°57′E	Fresh-water sediment	JN606354
	LUH3[1]	Alluvial plain of Vltava river, Czech Republic	48°48′N, 13°57′E	Fresh-water sediment	JN606355
Pseudoharpagon pertyi	EVROS2	Evros delta, Greece	40°48′N, 26°01′E	Brackish sediment	JN606356
	NY0199	Bamfield, Canada	48°82′N, 125°12′W	Marine sediment	JN606357

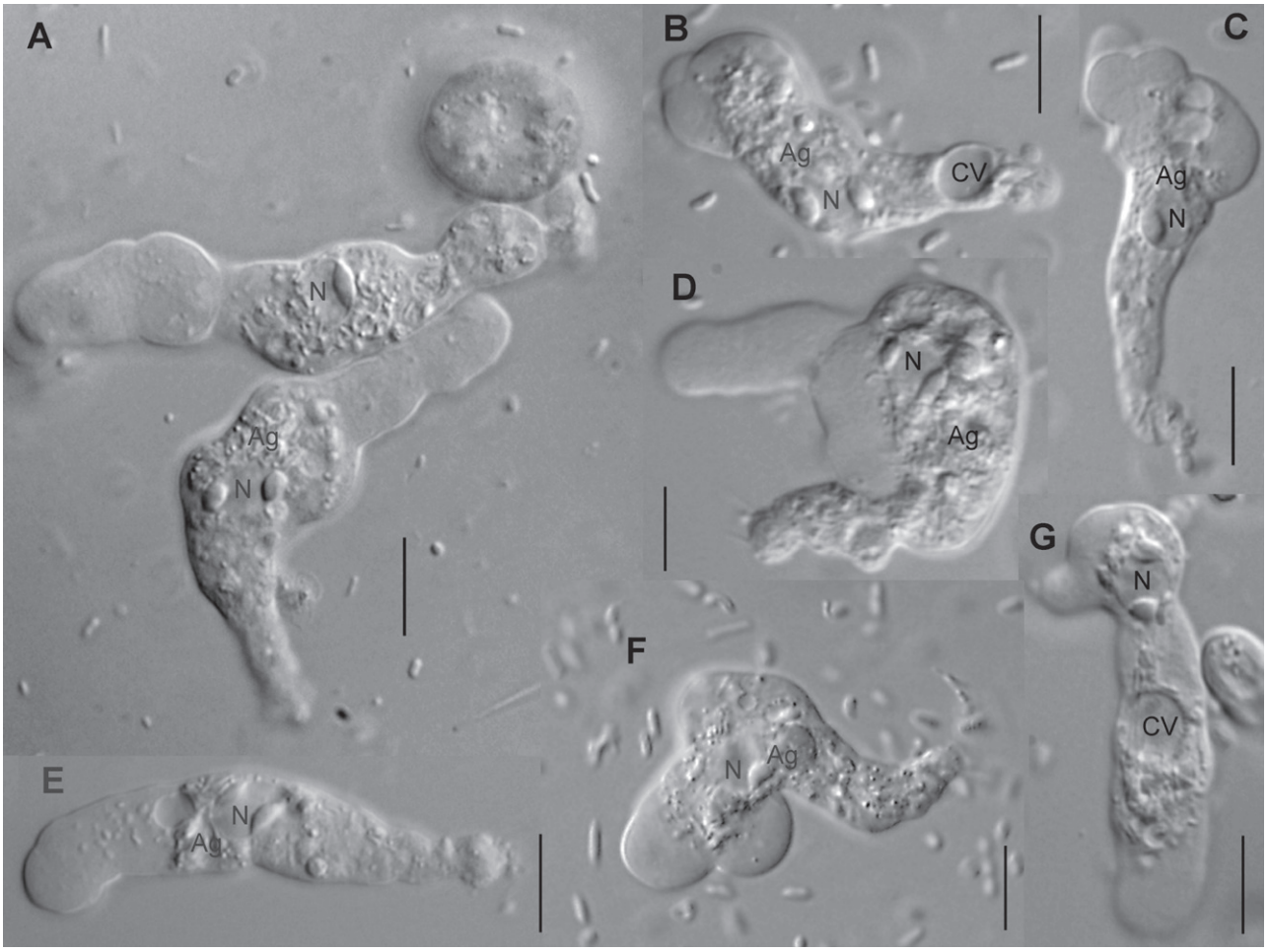

Figure 1. Living cells of *Psalteriomonas lanterna* strains VIT3 (**A, E**) and VT2 (**B – D, F – G**). Ag – aggregate of hydrogenosomes; CV – contractile vacuole; N – nucleus. Bar = 10 μm.

(28.9 – 63.7) μm × 11 ± 2.2 (6.8 – 18.0) μm, with the mean length/width ratio 4.4. Flagellate and cyst forms were not observed.

Psalteriomonas magna sp. nov.

The amoebal stage of freshwater strains KIZILLAR, CERETE, and IND7 was bigger than that of *P. lanterna* (Fig. 2). Cells of the strain KIZILLAR were 72.2 ± 11.8 (49.9 – 102.3) μm long and 16.4 ± 4.0 (8.1 – 30.0) μm wide, with the mean length/width ratio 4.6. Cells of the strain CERETE were 65 ± 9.1 (50.1– 90.8) μm long and 14.7 ± 2.3 (10.2 – 19.0) μm wide, with the mean length/width ratio 4.5. Cells of the strain IND7 were 66.2 ± 8.6 (40.1 – 82.9) μm long and 14.9 ± 2.4 (10.0 – 23.1) μm wide, with the mean length/width ratio 4.6. The mean cell dimensions of the three strains of *P. magna* were 67.8 ± 10.4 (40.1 – 102.3) × 15.3 ± 3.1 (8.1 - 30.0) μm, with the mean length/width ratio 4.5. An aggregate of hydrogenosomes was situated close to the nucleus (Figs 2, 4). The nucleus contained one or two parietal nucleoli located opposite each other in the nucleus (Fig. 2). Long uroidal filaments were observed in some cells (Fig. 2C, E, H). Flagellate and cyst forms were not observed.

Sawyeria marylandensis

The amoebal stage of freshwater strains ITZAVL, LUH2, and LUH3 corresponded to the original description of *Sawyeria marylandensis* (O'Kelly et al. 2003), (Fig. 3). Cells of the strain ITZAVL were 41.1 ± 11.9 (17.1 – 69.8) μm long and 9.5 ± 2.0 (5.9 – 14.3) μm wide, with the mean length/width ratio 4.4. Cells of the strain LUH3 were 34.0 ± 6.3 (22.0 – 51.5) μm long and 9.6 ± 2.5 (5.4 – 16.7) μm wide, with the mean length/width ratio 3.7. The mean cell dimensions of the two strains of *S. marylandensis* were 37.6 ± 10.1 (17.1 – 69.8) × 9.5 ± 2.3 (5.4 – 16.7) μm, with the mean cell length/width ratio 4.0. The nucleus of strain ITZAVL contained

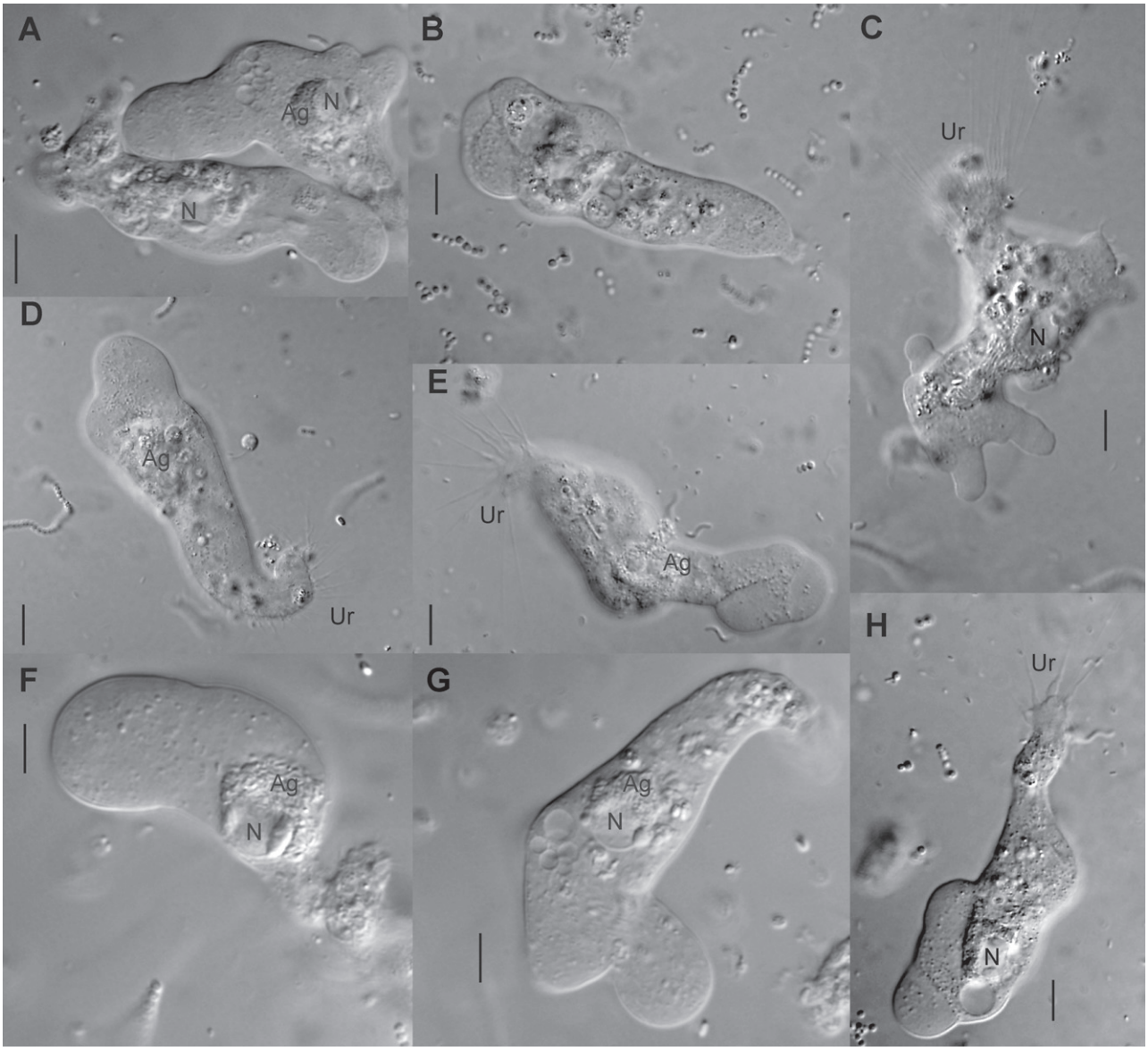

Figure 2. Living cells of *Psalteriomonas magna* strains KIZILLAR (**A – D, F, G**) and IND 7 (**E, H**). Ag – aggregate of hydrogenosomes; N – nucleus; Ur – uroidal filaments. Bar = 10 μm.

two parietal nucleoli situated opposite each other (Fig. 3B, C, D), whereas strain LUH3 possessed several parietal nucleoli (Fig. 3E – G). No aggregate of hydrogenosomes was observed (Fig. 4G, H). Flagellate and cyst forms were not observed.

The Flagellate Stage of *Monopylocystis visvesvarai*

The flagellate stage of the marine strain PC4BIC was elongated and relatively uniform in shape and size (Figs 5, 9F – J). The posterior end of the cell was either rounded or pointed. The cell length was 15.7 ± 1.7 (11.1 – 19.9) μm. A slightly spiral prominent ventral groove started anteriorly and extended almost to the posterior end of the cell (Figs 5C, D, E, G, 9H). The four flagella were unequal; the longest flagellum was long up to 1.5 times body length, two other flagella were more or less shorter than the longest flagellum and the fourth flagellum was the shortest one. Many cells adhered to the substrate by the distal ends of their four flagella. Dividing protargol-stained cell with two nuclei and two pairs of flagella was observed (Fig. 9I). Amoeboid and cyst stages were not observed.

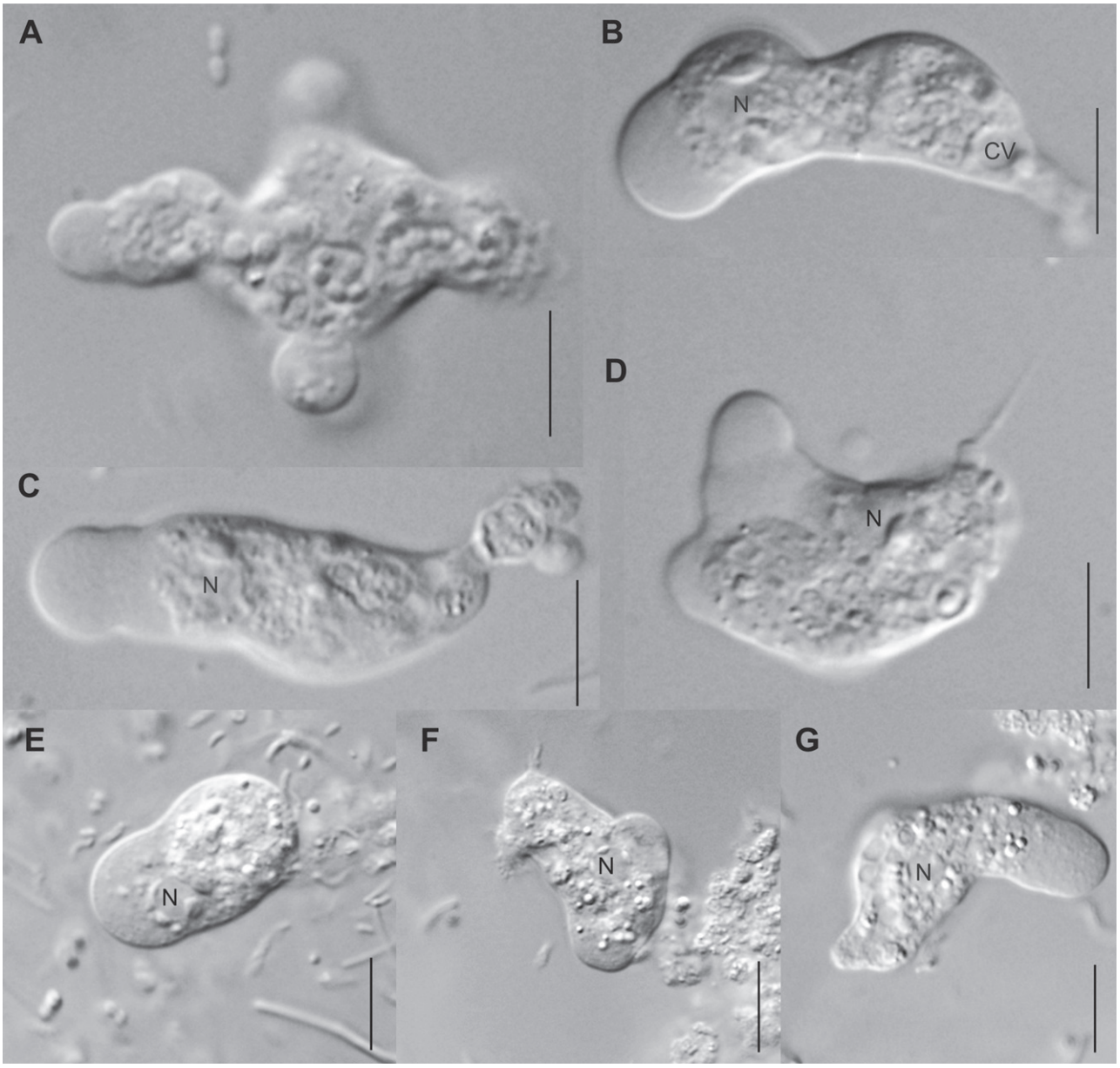

Figure 3. Living cells of *Sawyeria marylandensis* strains ITZAVL (**A – D**) and LUH3 (**E – G**). CV – contractile vacuole; N – nucleus. Bar = 10 μm.

Harpagon descissus comb. nov.

The flagellate stage of *H. descissus* freshwater strains corresponded to the previous observations of *Percolomonas descissus* (Brugerolle and Simpson 2004; Klug 1936). The cells were elongated (Figs 6, 9D, E). The cell length of four *H. descissus* strains was: PANT1S – 16.8 ± 3.3 (10.5 – 24.9) μm, SOOS1 – 17.8 ± 3.8 (10.2 – 25.0) μm, TEXEL – 18.2 ± 3.6 (12.7 – 29.7) μm, and VT1 – 18.1 ± 4.9 (12.0 – 31.4) μm. The mean cell length of the four strains of *H. descissus* was 17.0 ± 4.0 (10.2 – 31.4) μm. The cells were highly variable in shape, in part associated with the nutritional status and age of the culture, and four distinct morphotypes were determined: spindle-shaped cell with a short pointed posterior end (Fig. 6D), spindle-shaped cell with elongated pointed posterior end (Fig. 6A, I), slender elongated cell (Fig. 6H, L), and pear-shaped short cell with rounded posterior end (Fig. 6B, C, E, F, K). Cells which were morphologically intermediate between particular morphotypes were also observed. Relative abundance of the

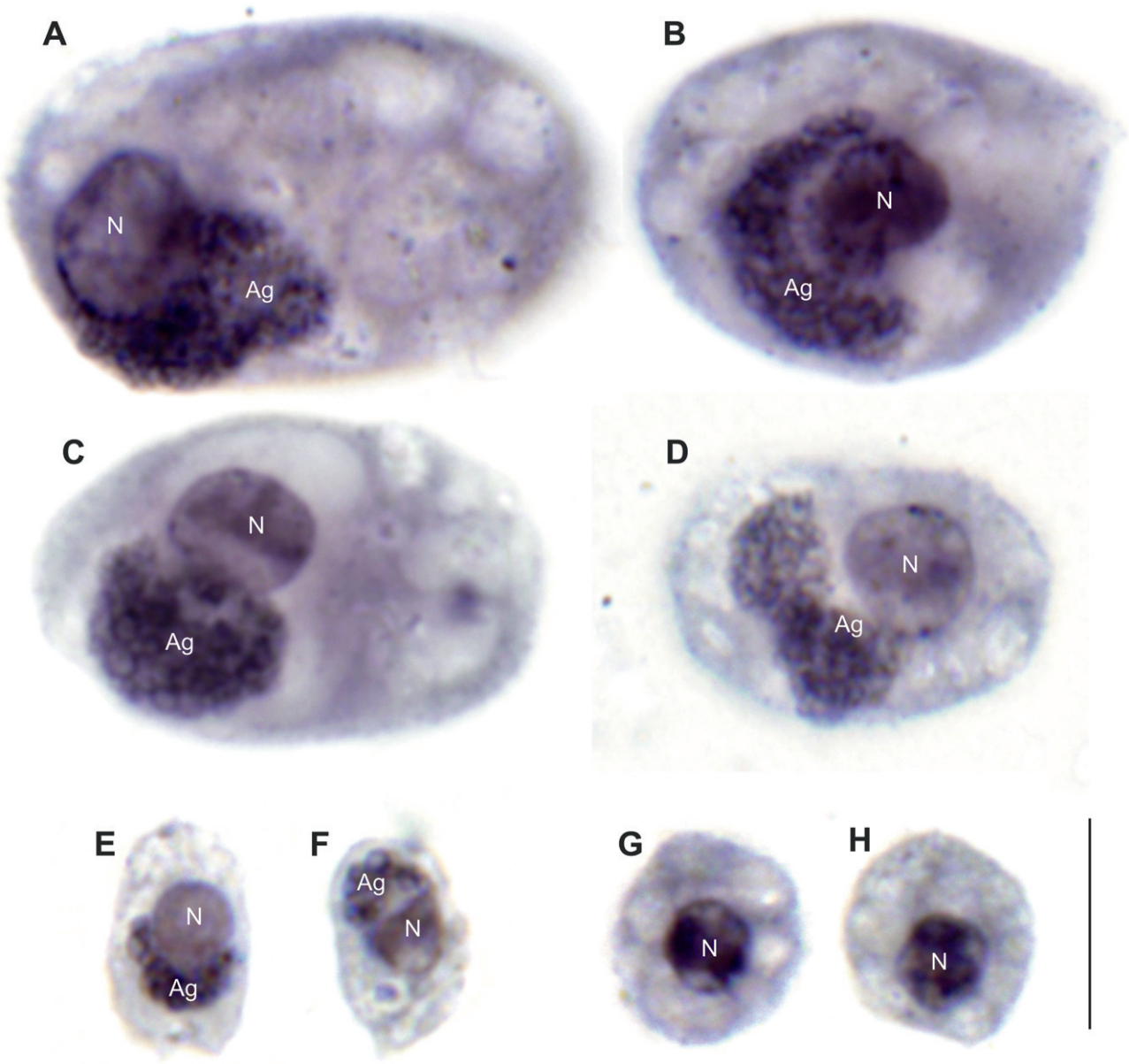

Figure 4. Protargol-stained specimens of *Psalteriomonas magna* sp. nov. strain KIZILLAR (**A – D**), *P. lanterna* strain VIT5 (**E, F**), and *Sawyeria marylandensis* strain ITZAVL (**G, H**). Ag – aggregate of hydrogenosomes; N – nucleus. Bar = 10 μm.

morphotypes varied within strains and observations, but all four morphotypes were observed in all examined strains. The ventral groove was less conspicuous than in *Monopylocystis visvesvarai* and extended to ½ of the length of the cell body in the spindle-shaped morphotype. The four flagella were unequal (Fig. 9D, E). The longest flagellum was long, at 1 – 2 times body length. The second flagellum was shorter, about 3/5 length of the longest flagellum. The remaining two flagella were equal in length and slightly shorter than the second flagellum. Some cells of *H. descissus* sp. nov. adhered to the substrate by the posterior end of the cell. A dividing cell with six flagella was observed (Fig. 6I, J). Amoebae and cysts were not observed.

Harpagon schusteri sp. nov.

The flagellate stage of *H. schusteri* sp. nov. freshwater strains were in most essential details, such as cell and flagella lengths, identical to those of

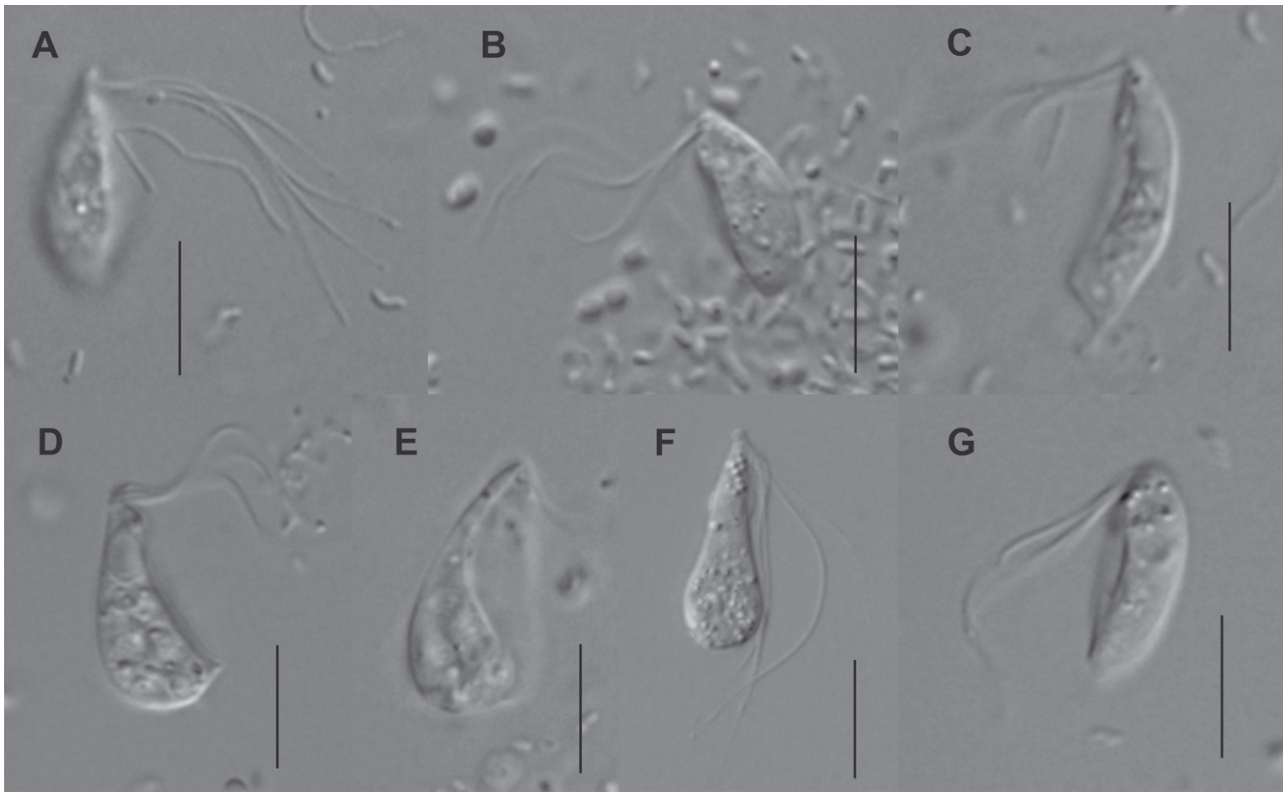

Figure 5. Living cells of *Monopylocystis visvesvarai* strain PC4BIC. Bar = 10 μm.

H. descissus (Figs 7, 9A – C). The cell length of two strains was: IND8 – 18.0 ± 3.6 (13.0 – 34.2) μm, INDSIP – 16.3 ± 3.2 (10.8 – 25.2) μm. The mean cell length of the two strains of *H. schusteri* was 17.1 ± 3.5 (10.8 – 34.2) μm. Three distinct morphotypes, the same as in *H. descissus* comb. nov., could be identified in the culture: spindle-shaped cell with a short pointed posterior end (Fig. 7G, H), slender elongated cell (Fig. 7B), and pear-shaped short cell with rounded posterior end (Fig. 7C, D, E, F). Cells with an elongated pointed posterior end were not observed in any strain. Amoebae and cysts were not observed.

Pseudoharpagon pertyi sp. nov.

The morphology of the flagellate stage from brackish and marine strains EVROS2 and NY0199 was similar to *Harpagon* spp., though the cells were slightly smaller. They were elongated and relatively uniform in shape and size (Figs 8, 9K – O). The posterior end was either rounded or pointed. The cell length of the two strains was: EVROS2 – 13.7 ± 3.5 (8.5 – 21.3) μm, NY0199 – 14.0 ± 2.0 (11.5 – 19.7) μm. The prominent ventral groove extends to ½ - 2/3 of body length. The four flagella were unequal in length. The longest flagellum was up to 1.5 times body length. The remaining three flagella were approximately equal and cell-body in length. Some cells of *P. pertyi* sp. nov. adhered to the substrate by a cytoplasmic bridge formed from the posterior end of the cell (Fig. 8B, F). A dividing protargol-stained cell with two nuclei and two pairs of flagella was observed (Fig. 9M). Amoebae and cysts were not observed. The nucleolar morphology could not be determined from our light-microscopic pictures.

Ultrastructure Observations

Flagellate Stage of *Monopylocystis visvesvarai*

The structure of the flagellar apparatus of *Monopylocystis visvesvarai* was very similar to that of *Harpagon descissus* comb. nov., *Psalteriomonas lanterna*, and *P. vulgaris* (Fig. 10A, see Broers et al. 1990, 1993; Brugerolle and Simpson 2004). All four basal bodies possessed flagella and were arranged in two pairs, #1 and #4 forming the posterior pair, and #2 and #3 forming the anterior one. There were two straight rhizoplasts (Rh_1 and Rh_2) that originated anteriorly and were closely associated. The periodicity of cross striation of the rhizoplasts was about 38 nm. The rhizoplasts connected to basal

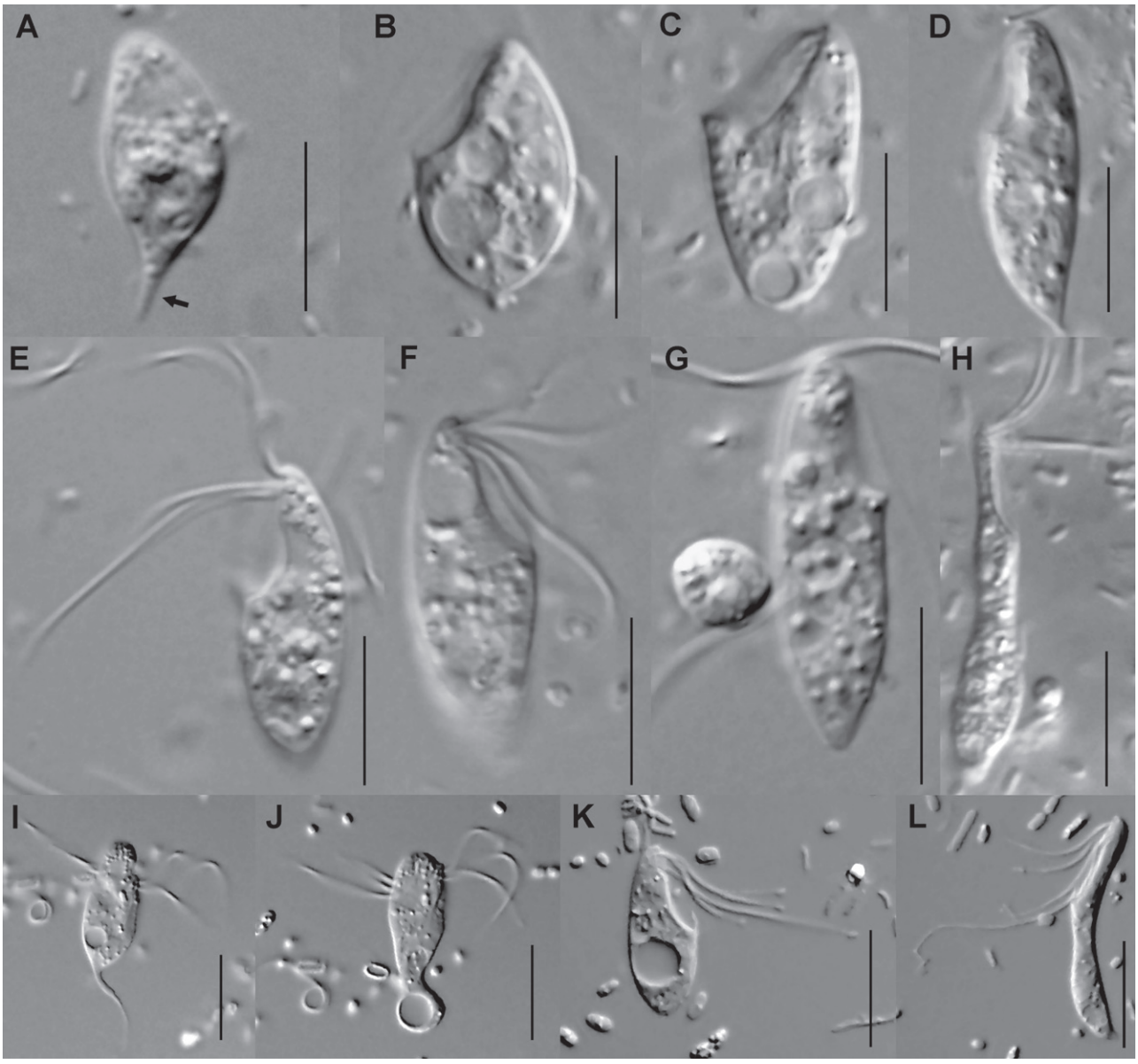

Figure 6. Living cells of *Harpagon descissus* comb. nov. strains TOCOV (**A**), TEXEL (**B – G**), VT1 (**H**), and PANT1 (**I – L**). Arrow – long spike at the posterior end. Bar = 10 μm.

bodies #2 (Rh_1) and #1 (Rh_2), respectively. Both the rhizoplasts continued posteriorly and connected to microtubular ribbon R_1. The ribbon R_1 was a prominent structure and consisted of a curved row of thick microtubules originating near basal body #1. The basal bodies were interconnected by fibres (Fi). A bundle of microfilaments (MB) connected the posterior pair of basal bodies with the concave side of R_1. MB with R_1 constituted characteristic harp-like structure.

The nucleus (N) was surrounded by rough endoplasmic reticulum (RER) and nucleolar material (Nu) formed a thin ring close to the nuclear membrane (Fig. 10B). Certain parts of the ring were thickened. No stacked Golgi apparatus was observed. Mitochondrion-related organelles (MRO) were acristate, rounded, with diameter approximately 700 – 800 nm (Fig. 10C). They were not surrounded by rough endoplasmic reticulum, but were sometimes situated close to it.

Psalteriomonas magna sp. nov.

Preliminary transmission electron microscopy of *P. magna* is presented in Figure 11. There were no traces of a flagellar apparatus in the amoeba of

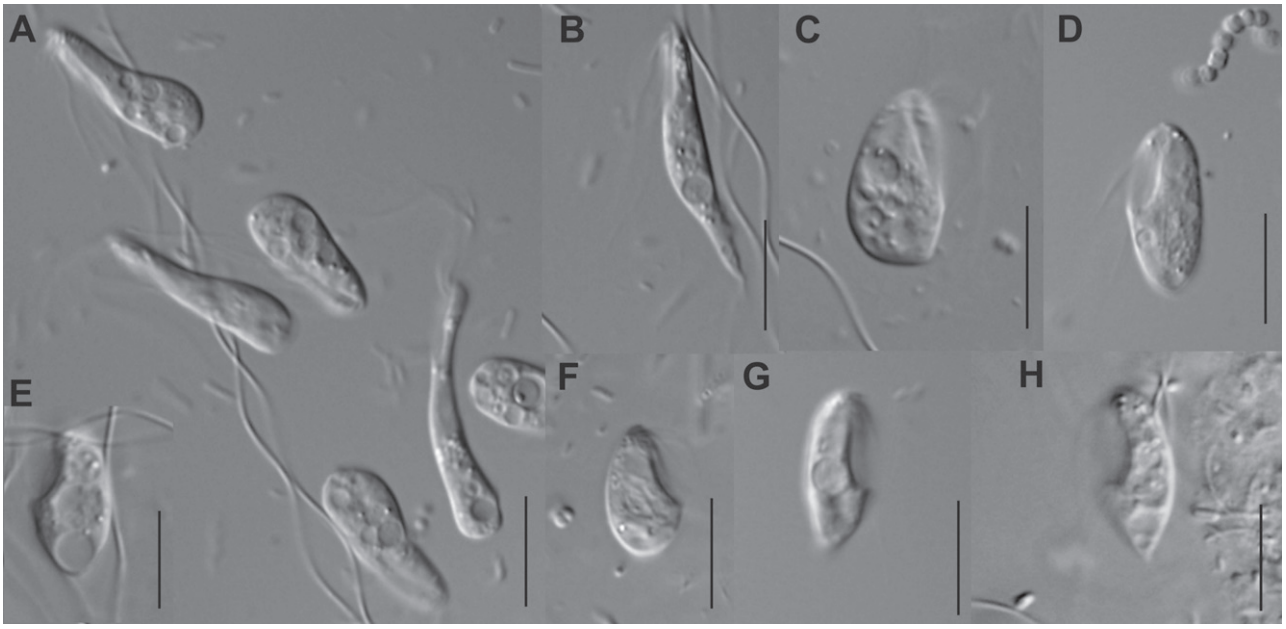

Figure 7. Living cells of *Harpagon schusteri* sp. nov. strain INDSIP. Bar = 10 μm.

the strain KIZILLAR. The nucleus (N) contained one or two parietal nucleoli (Nu) situated in nuclear lobes (Fig. 11A, C, D). No stacked Golgi apparatus was observed. Mitochondrion-related organelles (hydrogenosomes, H) were irregular in shape with a maximal diameter about 2000 nm (Fig. 11B). They were aggregated to form a cup-shaped complex that was interlaced with bacilliform bacteria (Ba; Fig. 11B, C). The prominent hydrogenosomal aggregate was situated close to the nucleus and was not surrounded by rough endoplasmic reticulum (RER). In addition to the aggregate, individual hydrogenosomes were observed in the cytoplasm of *P. magna* (IH; Fig. 11C). Although the individual hydrogenosomes seemed not to be surrounded by endoplasmic reticulum, we cannot rule out the possibility that fixation artifacts occurred.

Molecular Phylogenetic Analyses

The phylogenetic tree of the Heterolobosea as inferred from SSU rDNA is shown in Figure 12. A monophyletic group of heteroloboseans was recovered by both maximum likelihood and Bayesian methods with strong statistical support. The earliest diverging lineage of the Heterolobosea was formed by *Pharyngomonas kirbyi*. The rest of the Heterolobosea (Tetramitia sensu Cavalier-Smith and Nikolaev 2008) was split into six robust lineages whose interrelationships were unresolved: I. Genus *Neovahlkampfia* and related undetermined heteroloboseans LC103 and AND9. II. Genera *Acrasis*, *Solumitrus* and *Allovahlkampfia*, and undetermined heteroloboseans BA, OSA and AND12. Since the genetic distance between *Solumitrus* and *Allovahlkampfia* was relatively low and the observed morphological differences were minute (see Anderson et al. 2011), the genus *Solumitrus* should be synonymized with *Allovahlkampfia* in the future. III. Genera *Naegleria*, *Pleurostomum*, *Tulamoeba*, *Willaertia*, and *Marinamoeba*. IV. Genera *Percolomonas* and *Stephanopogon*. V. Genera *Vahlkampfia* and *Tetramitus*. VI. Genera *Paravahlkampfia*, *Heteramoeba*, *Euplaesiobystra*, *Vrihiamoeba*, *Oramoeba*, *Stachymoeba*, undetermined heteroloboseans RT5in38 and WIM43, and the Psalteriomonadidae. A clade formed by *Vrihiamoeba*, *Oramoeba*, *Stachyamoeba*, and the environmental sequence WIM43 was sister to Psalteriomonadidae, though without statistical support. The Psalteriomonadidae was monophyletic with high support and split into three robust subclades that were largely unresolved with respect to each other. The first subclade was formed by the genus *Pseudoharpagon* gen. nov. Genetic distance (uncorrected p-distance) between strains EVROS2 and NY0199 of *P. pertyi* sp. nov. was 0.189. The second subclade was formed by genera *Sawyeria* and *Psalteriomonas*, and two environmental sequences FN865111 and FN865530. The relationships within the second subclade were well resolved. The mean genetic distance between the two *Psalteriomonas* species was 0.049. The intraspecific genetic distance within

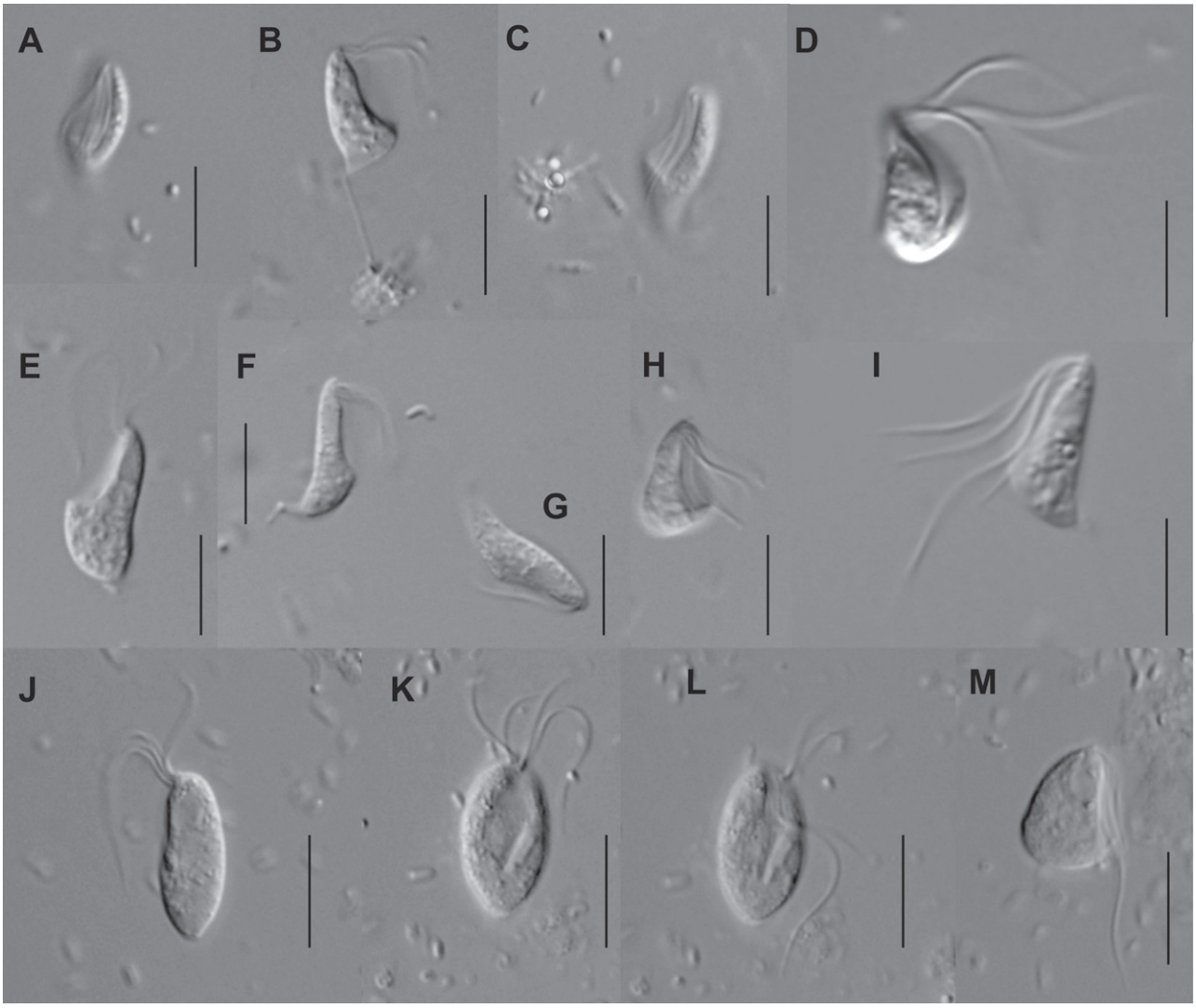

Figure 8. Living cells of *Pseudoharpagon pertyi* sp. nov. strain EVROS2 (**A – I**) and NY0199 (**J – M**). Bar = 10 μm.

Psalteriomonas lanterna reached 0.015. The intraspecific distances within *P. magna* sp. nov. and within *Sawyeria marylandensis* were negligible reaching less than 0.001. The third subclade of Psalteriomonadidae was formed by genera *Monopylocystis* and *Harpagon* gen. nov., and by the environmental sequence AF011462. The mean genetic distance between *Harpagon descissus* comb. nov. and *H. schusteri* sp. nov. was 0.127 (235 bp). Intraspecific genetic distances within *Harpagon descissus* comb. nov. and *H. schusteri* sp. nov. reached 0.036 (67 bp) and 0.024 (45 bp), respectively. The genetic distance between the type strain of *Monopylocystis visvesvarai* and strain PC4BIC was 0.004.

Discussion

Species Identity of the Isolates

The Heterolobosea is a very diverse group of protists, and different stages in the life cycle do not have the same significance in species determination. Diversity in cyst morphology, when known, may be most important in the discrimination of morphospecies. By contrast, the amoebae of many heteroloboseans share the same gross morphology and are of limited value in species identification. Due to the lack of morphological variability in heterolobosean amoebae, unrelated genera with the same cyst morphology

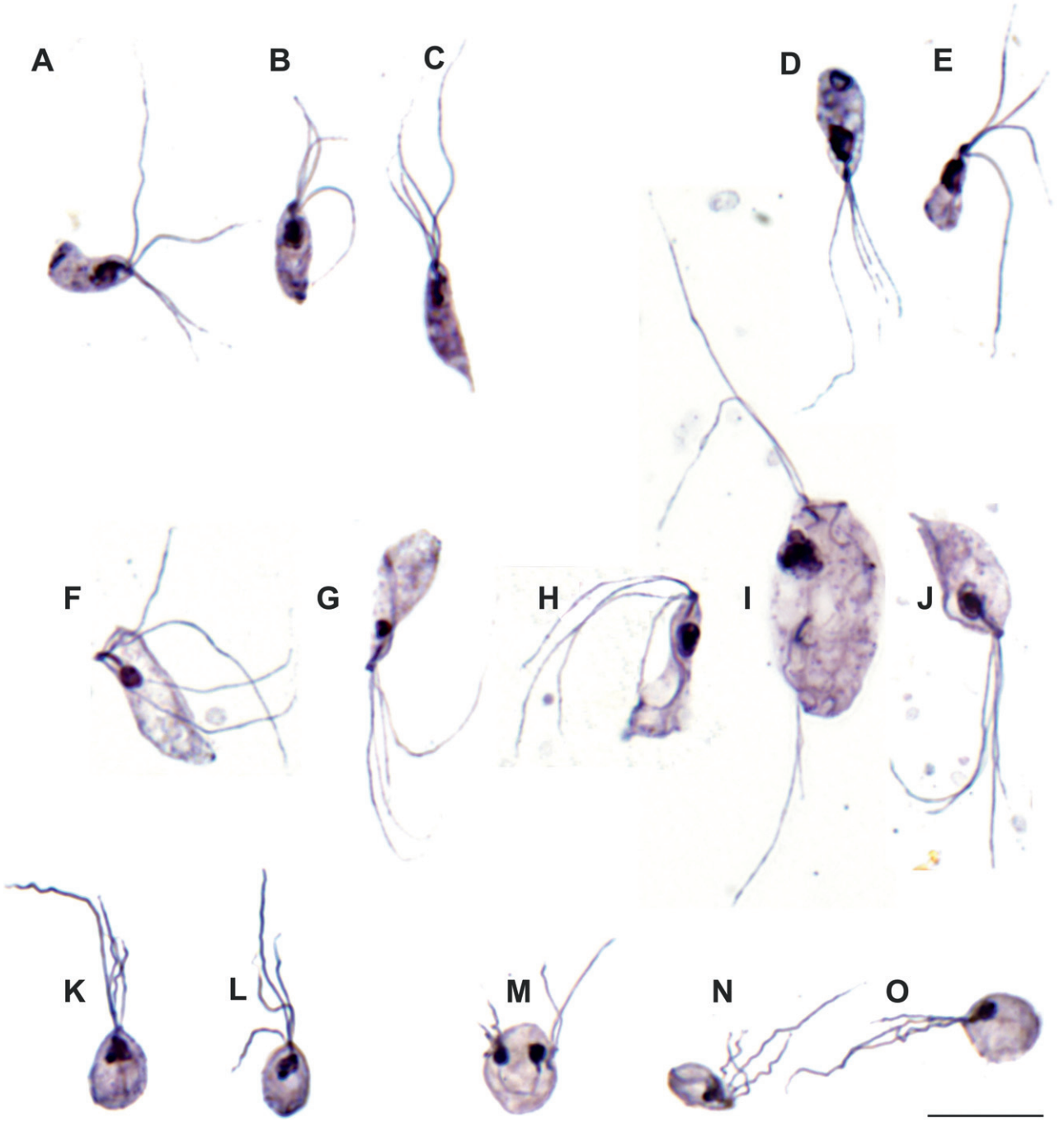

Figure 9. Protargol-stained specimens of *Harpagon schusteri* sp. nov. strain INDSIP (**A – C**), *H. descissus* comb.nov. strain TEXEL (**D, E**), *Monopylocystis visvesvarai* strain PC4BIC (**F – J**; dividing cell in **I**), and *Pseudoharpagon pertyi* sp. nov. strain EVROS2 (**K – O**; dividing cell in **M**). Bar = 10 μm.

(e.g. *Vahlkampfia*, *Neovahlkampfia*, *Allovahlkampfia*, and *Paravahlkampfia*) must be distinguished solely on the basis of their molecular-phylogenetic distances. Heterolobosean flagellates are more diverse than the amoebae. Although ultrastructural studies have shown that the mastigont of flagellates bear many morphological features important for taxonomy, most heterolobosean flagellates are considerably understudied and their ultrastructure is unknown. The number of flagella is variable at both the species and genus levels (Darbyshire et al. 1976), but most often the cells are

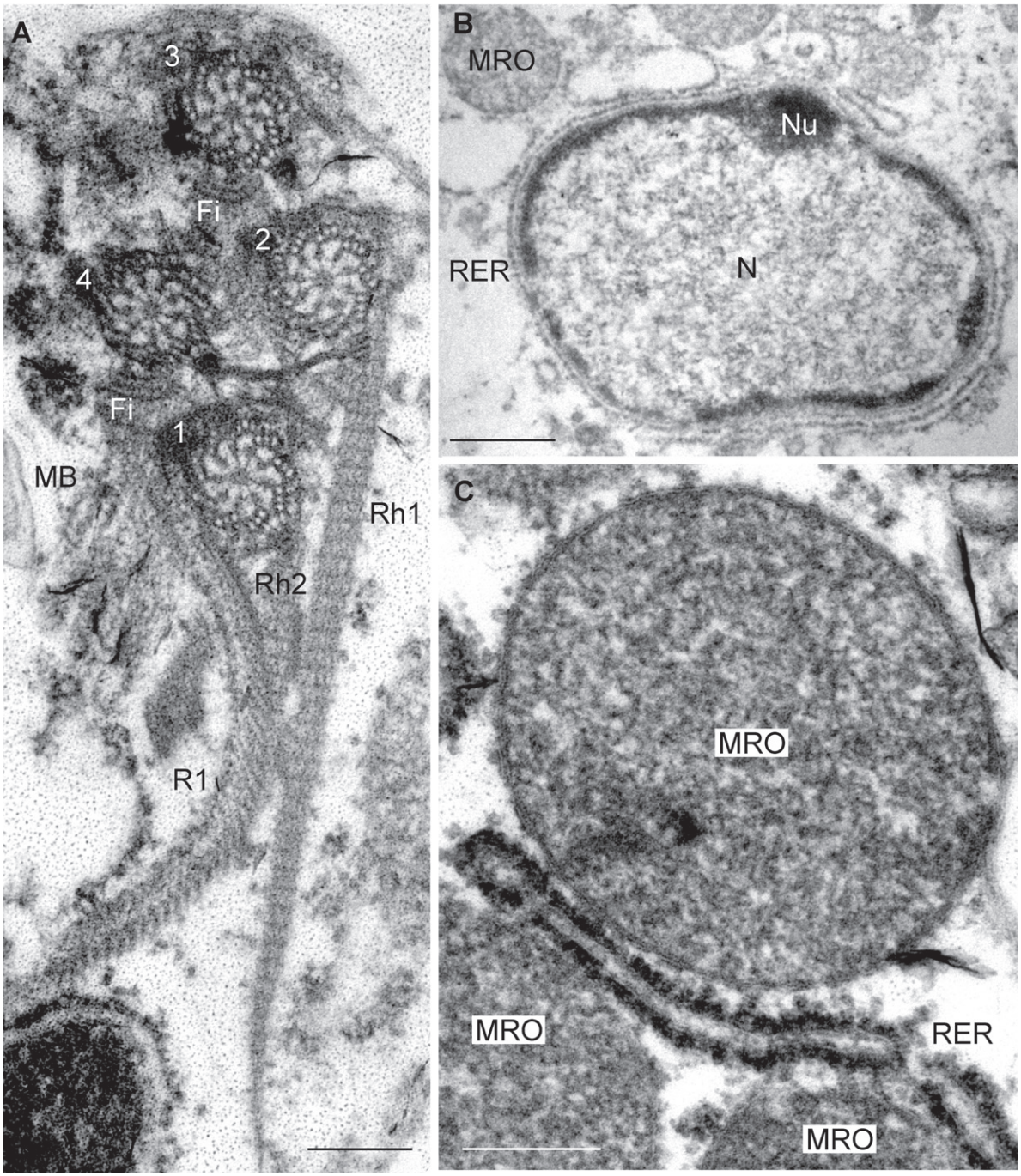

Figure 10. Ultrastructure of the flagellate of *Monopylocystis visvesvarai* strain PC4BIC. **A**, mastigont; **B**, nucleus surrounded by rough endoplasmic reticulum; **C**, mitochondrion-related organelle. 1, 2, 3, 4 – basal bodies; Fi – Fibres interconnecting the basal bodies; MB – bundle of microfilaments; MRO – mitochondrion-related organelle; N – nucleus; Nu – nucleolus; R1 – microtubular ribbon R_1; RER – rough endoplasmic reticulum; Rh1, Rh2 – rhizoplasts. Bar = 200 nm for A, C, 500 nm for B.

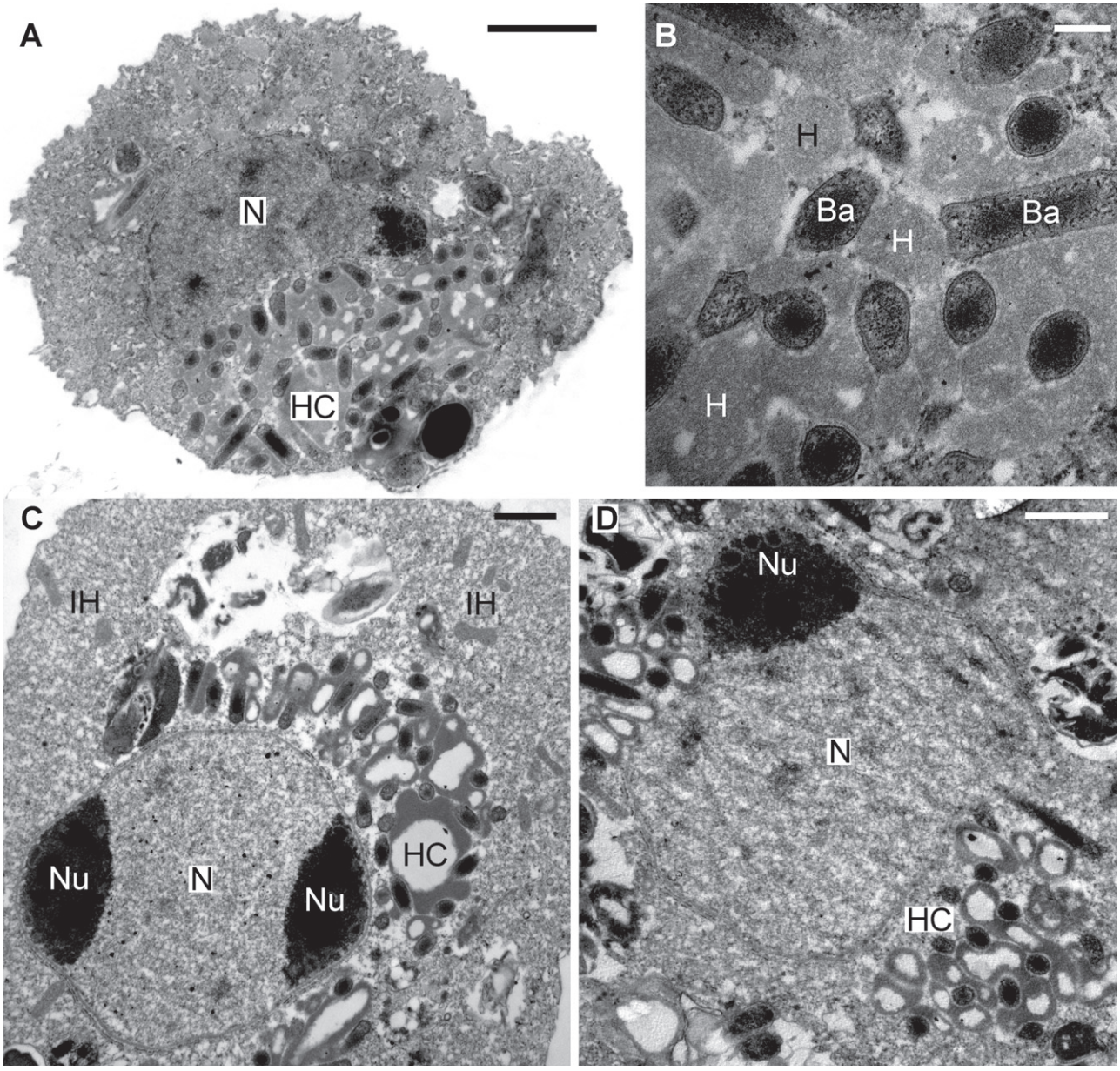

Figure 11. Ultrastructure of *Psalteriomonas magna* sp. nov. strain KIZILLAR. **A**, whole cell; **B**, detail of hydrogenosomal complex with associated bacteria; **C**, detail of the nucleus with two parietal nucleoli, hydrogenosomal complex and individual hydrogenosomes in the cytoplasm; **D** – detail of the nucleus surrounded by the hydrogenosomal complex. Ba – endosymbiotic bacterium; H – hydrogenosomes in the hydrogenosomal complex; HC – hydrogenosomal complex; IH – individual hydrogenosomes; N – nucleus; Nu – nucleolus. Bar = 2 μm for A, 250 nm for B, 1 μm for C and D.

biflagellate or quadriflagellate. The morphology of the cytostome is also variable. *Tetramitus rostratus* and *Pleurostomum flabellatum* flagellates possess a conspicuous rostrum and a tubular cytostome (Park et al. 2007; Patterson et al. 2000) while flagellates of some other species have a broad longitudinal feeding groove and lack a rostrum. In addition, the cytostome is reduced in some genera, e.g. in *Naegleria* and *Willaertia* (Page 1988; Robinson et al. 1989). Because the life cycle of many species has not been thoroughly examined, it is theoretically possible to inadvertently describe two life stages of one organism as different species.

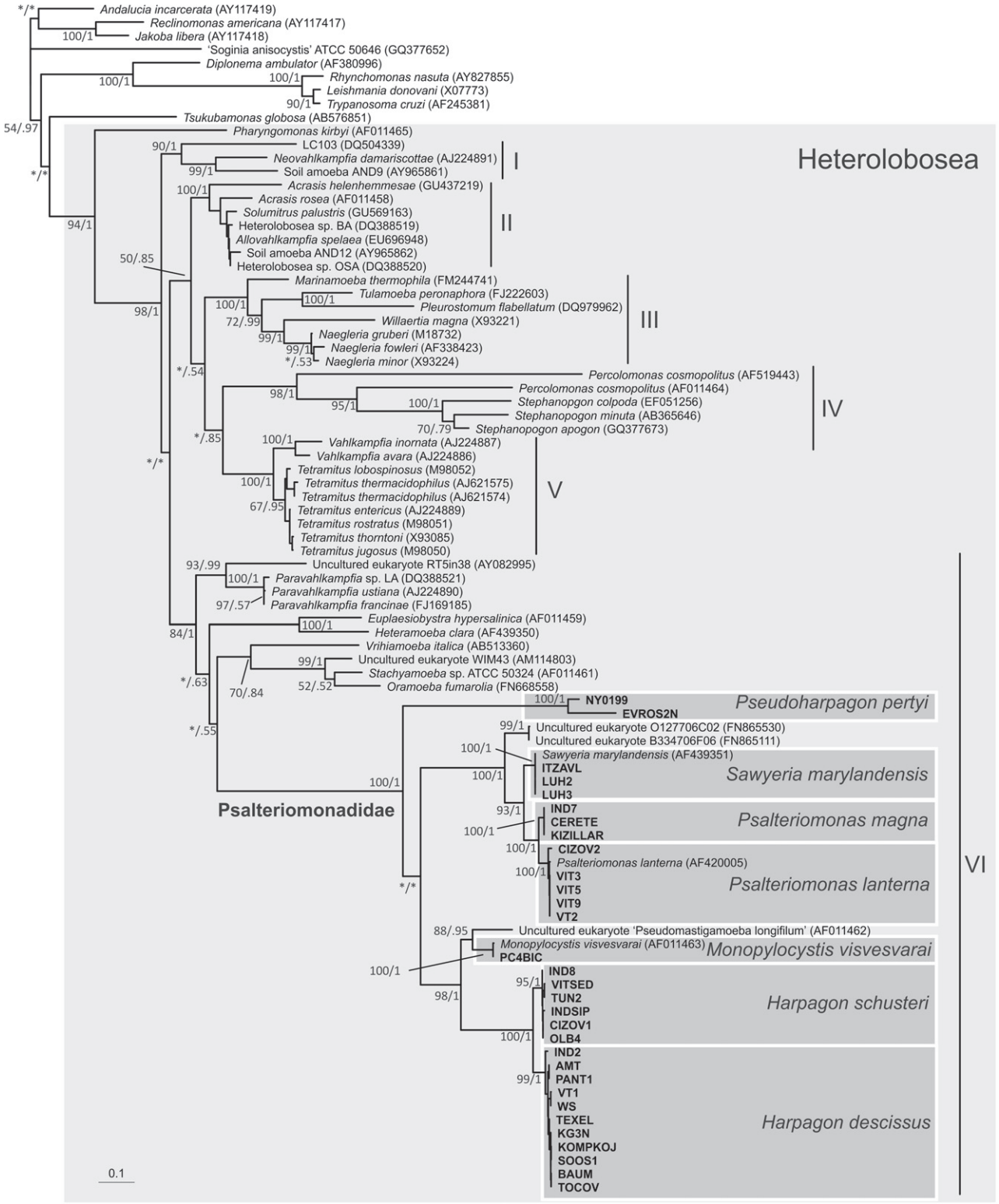

Figure 12. Phylogenetic tree of Heterolobosea based on SSU rDNA. The tree was constructed by the maximum likelihood method in RAxML (GTRGAMMAI model) and was rooted with representatives of other Discoba lineages. The values at the nodes represent statistical support in bootstrap values (RAxML)/posterior probabilities (MrBayes). Support values below 50%/.50 are represented by an asterisk (*). Six main clades of Tetramitia are labeled.

A molecular species concept based on the ITS region of the ribosomal operon was proposed by De Jonckheere (2004) for the genus *Naegleria* that was later extended to the Vahlkampfiidae, the most species-rich family within the Heterolobosea (De Jonckheere and Brown 2005). They proposed that unique ITS sequences define species while each genus forms a distinct clade in 5.8S trees. For example, *Naegleria canariensis* was distinguished from *N. gallica* by two single-nucleotide substitutions in the ITS2 sequence (De Jonckheere 2006). Sequences of SSU rDNA, ITS1, 5.8 rDNA, and morphology of the two species are, however, identical. Although many species have been described on the basis of the ITS approach, it could be problematic for several reasons: (1) It is not universal. For example, this concept cannot be used for *Naegleria fowleri* because of its high ITS variability (De Jonckheere 1998, 2004). In addition, little is known about ITS region variability outside the genus *Naegleria*. (2) It was shown that intragenomic polymorphism of the ITS region exists at least in some heteroloboseans. Such taxa could be classified into different species (Dyková et al. 2006). (3) The boundary between intra- and interspecies polymorphism is unknown in most heteroloboseans. This concept assumes the same, almost zero, level of intraspecific variability in all (or almost all) vahlkampfiid species. However, outside the Vahlkampfiidae, e.g. in Psalteriomonadidae or Acrasidae, the molecular polymorphism could be significantly higher, invalidating the concept.

In this study, we decided to identify previously described species and to establish new ones initially on morphological grounds; molecular polymorphisms and phylogeny were used as an accessory criterion. We are aware that our approach, albeit suitable for the Psalteriomonadidae, would be problematic when used for the Vahlkampfiidae as it would considerably underestimate the real number of species. On the other hand, the ITS-based approach clearly overestimates the species number and should be modified to take intraspecies polymorphism into account. Particular species within the Psalteriomonadidae differ in the shape and length of cells, relative flagellar length, nuclear structure, shape of mitochondrial derivates, and lifestyle (freshwater/marine). Our data showed that these characteristics are sufficiently stable among different strains of a single species. Likewise, molecular phylogeny and assessment of SSU rDNA sequence divergence allowed us to identify a flagellated stage of *Monopylocysis visvesvarai* where only the amoebal stage was previously described and allowed confirmation of species identification when we isolated an amoebal-stage (e.g. *P. lanterna*). Our strains belong to at least seven species from five genera, including three new species and two new genera.

Members of the genus *Psalteriomonas* possess a hydrogenosomal complex close to the nucleus. The nucleolus of *Psalteriomonas lanterna* was originally described as central in both flagellate and amoeba stages (Broers et al. 1990). In contrast, we have never seen a centrally located nucleolus in any of our strains of *Psalteriomonas lanterna* and *P. magna* sp. nov. Instead, all the strains possessed one or two parietal nucleoli. Moreover, Prof. J. H. P. Hackstein observed a prominent bright-fluorescing structure within the nucleus, at its periphery, after staining the type strain of *P. lanterna* with ethidium bromide (J. H. P. Hackstein, pers. comm.). This most likely indicates the presence of a parietal nucleolus rather than a central one in the cells of the type strain. If so, the number and position of nucleoli among *P. lanterna* strains seems to be variable.

Psalteriomonas magna sp. nov. differs from *P. lanterna* in the median cell size. With its approximately 70 μm in length, the locomotive amoeba of *P. magna* is about 25 μm longer than the amoeba of *P. lanterna*. We transfer *Lyromonas vulgaris* back to the genus *Psalteriomonas* as originally described by Broers et al. (1993) because of its phylogenetic position and because its cells contain the hydrogenosomal complex (characteristic of the genus). Unfortunately, careful morphological comparison of *P. magna* sp. nov. and *P. vulgaris* comb. nov. is currently not possible as only the amoeba stage is known from the former species and only the flagellated stage is known from the latter. It is worth noting that the amoeba/flagellate length ratio is similar among *Psalteriomonas* species and that the amoeba of *P. magna* sp. nov. is about 40% longer than that of *P. lanterna*, and the *P. vulgaris* flagellate is about 50% smaller than the flagellate of *P. lanterna*. Moreover, *P. magna* amoebae differ from *P. vulgaris* in the presence of individual hydrogenosomes in the cytoplasm. Although we cannot fully exclude the possibility that *P. magna* sp. nov. represents the amoeba stage of *P. vulgaris* comb. nov., we are convinced that they belong to separate species.

The amoebae of our strains of *Sawyeria* are morphologically similar to *Psalteriomonas* strains, but have no hydrogenosomal complex. *Sawyeria marylandensis* is well studied, but the flagellate stage is unknown and is possibly missing altogether. In contrast, at least two species of *Psalteriomonas* are able to form flagellates. One or two parietal

nucleoli located opposite each other in the nucleus were described in the type culture of *S. marylandensis* (O'Kelly et al. 2003). It is consistent with our observation of the strain ITZAVL. However, cells of the strain LUH3 possess more than two parietal nucleoli. We consider this difference to reflect intraspecific variability within *S. marylandensis*, similarly to that of *Psalteriomonas lanterna*.

The genus *Monopylocystis* differs from other psalteriomonadids by a peripheral distribution of nucleolar material (nucleolar ring). Its marine life style is also uncommon in the Psalteriomonadidae, being shared with *Pseudoharpagon pertyi* sp. nov. and an undetermined organism with SSU rDNA sequence AF011464. Only a single species, *M. visvesvarai*, has been described (O'Kelly et al. 2003). We transfer *Vahlkampfia anaerobica* described by Smirnov and Fenchel (1996), which possesses a nucleolar ring and lives in marine environments, to the genus *Monopylocystis*. *Monopylocystis anaerobica* comb. nov. also possesses mitochondrion-related organelles without cristae ("microbody-like structures" in the original description of Smirnov and Fenchel, 1996), a typical feature of the Psalteriomonadidae. *Monopylocystis anaerobica* comb. nov. differs from *M. visvesvarai* in the presence of uroidal filaments and a floating form. The cyst stage has not been found in *M. anaerobica*. Unlike the type cultures of the two species, our *Monopylocystis* strain PC4BIC forms exclusively flagellates under our culturing conditions and neither amoebae nor cysts have been observed. The genetic distance between PC4BIC and the type strain of *M. visvesvarai* was considerably lower than intraspecific molecular polymorphism within related *Harpagon descissus* comb. nov. or *H. schusteri* sp nov. As PC4BIC represents the first case of the flagellate stage in the genus *Monopylocystis* and sequence data of *M. anaerobica* are lacking, at the present time, the problem of species identity of PC4BIC cannot be definitively settled. We provisionally consider the PC4BIC strain to belong to *Monopylocystis visvesvarai* due to the low genetic distance of PC4BIC and the type culture.

Our phylogenetic analysis convincingly showed that *Harpagon descissus* (formerly *Percolomonas descissus*) is unrelated to the presumably closely related *Percolomonas cosmopolitus*. The two species differ considerably in the structure of the mastigont and in their feeding habit. While *H. descissus* feeds by a synchronous action of all four flagella, *P. cosmopolitus* uses the longest flagellum for attaching to the substrate and the remaining three shorter flagella are used to generate a water current for feeding (Fenchel and Patterson 1986). In contrast to *P. cosmopolitus* that possesses discoidal mitochondrial cristae (Fenchel and Patterson 1986), the mitochondrion-related organelle of *H. descissus* is acristate (Brugerolle and Simpson 2004), like in other psalteriomonadids. Therefore, we remove former *P. descissus* from the genus *Percolomonas* and accommodate it in the new genus *Harpagon*. We also describe a second species of the genus *Harpagon*, *H. schusteri* sp. nov. *H. descissus* comb. nov. and *H. schusteri* sp. nov. are morphologically quite similar and the only difference we could detect is the absence of the form with long spike-shaped posterior end in *H. schusteri* sp. nov. This morphologic difference was completely consistent with the molecular phylogenetic analyses that place all isolates of each particular species in a distinct clade that are sister to each other. The genus *Harpagon* gen. nov. contains flagellates with highly polymorphic cells. They differ from members of the genus *Monopylocystis* in the nucleolar structure (several independent parietal nucleoli in *Harpagon* gen. nov., see Brugerolle and Simpson 2004; nucleolar ring in *Monopylocystis*, see O'Kelly et al. 2003, this study), freshwater lifestyle, and shape of the ventral groove.

Pseudoharpagon pertyi gen. nov., sp. nov. is similar to *Harpagon* spp., but differs in several aspects. Its ventral groove is longer and more conspicuous than that of *Harpagon* spp. The cell shape of *P. pertyi* sp. nov. is more stable and cells are about 3 μm shorter than cells of *Harpagon* spp. The two strains of *P. pertyi* sp. nov. were isolated from marine or brackish sediments, whereas members of *Harpagon* are exclusively freshwater. Our data suggest that cells of *Percolomonas descissus* depicted in fig. 3F–J of Bernard et al. (2000) represent, in fact, *P. pertyi* sp. nov.

Phylogeny of the Heterolobosea

The molecular phylogeny of the Heterolobosea is currently inferred almost exclusively from analyses of the SSU rRNA gene (Cavalier-Smith and Nikolaev 2008; De Jonckheere et al. 2009; Nikolaev et al. 2004; O'Kelly et al. 2003; Park and Simpson 2011; Park et al. 2007, 2009; Weekers et al. 1997; Yubuki and Leander 2008). The basal branch of Heterolobosea is in all analyses formed by the sequence designed '*Macropharyngomonas halophila*' which was recently formally described and assigned to the taxon *Pharyngomonas kirbyi* (Park and Simpson 2011). The relationships within the rest of Heterolobosea, Tetramitia *sensu* Cavalier-Smith and Nikolaev (2008), are generally

unresolved in SSU rDNA analyses. Based on the results of previous studies and our analysis, we divide Tetramitia into six robust clades (Fig. 12). To better resolve the phylogeny of Heterolobosea, increased taxon sampling and analyses based on more genes are required. There are still numerous heteroloboseans and organisms tentatively classified within the Heterolobosea that do not have publically available DNA sequence data (e.g. *Gruberella flavescens*, *Percolomonas sulcatus*, *Pernina chaumonti*, *Pseudovahlkampfia emersoni*, *Pocheina* spp., *Tetramastigamoeba hoarei*, *Trimastigamoeba philippinensis*).

Our analysis groups Psalteriomonadidae with genera *Paravahlkampfia*, *Heteramoeba*, *Euplaesiobystra*, *Vrihiamoeba*, *Oramoeba*, and *Stachymoeba* forming together the heterolobosean clade VI. All of these genera except those in Psalteriomonadidae are aerobic suggesting that obligate anaerobiosis appeared relatively recently within the Heterolobosea. Although *Heteramoeba clara, Euplaesiobystra hypersalinica, Stachyamoeba* sp. ATCC 50423 and *Oramoeba fumarolia* are known to form flagellates (De Jonckheere et al. 2011; Droop 1962; Murase et al. 2010; Park et al. 2009), their ultrastructure has yet to be studied in detail, so their relationship with the Psalteriomonadidae cannot be corroborated using morphology alone.

Diversity of the Psalteriomonadidae

The Psalteriomonadidae flourish in sediments with low oxygen concentrations and are found worldwide. *Monopylocystis visvesvarai* and *M. anaerobica* comb. nov. are marine heteroloboseans, *Pseudoharpagon pertyi* sp. nov. was isolated from brackish and marine sediments, and *Psalteriomonas* spp., *Harpagon* spp., and *Sawyeria marylandensis* are inhabitants of freshwater sediments. Cavalier-Smith and Nikolaev (2008) assumed that the immediate ancestor of Psalteriomonadidae was a marine protist and fresh water was invaded only once, by a common ancestor of *Psalteriomonas* and *Sawyeria*. In contrast, our data indicate that marine/freshwater or freshwater/marine transitions were more common than previously hypothesized. At least two such events took place in Psalteriomonadidae. However, available data are not sufficient for pinpointing the directions of these transitions and it is impossible to decide whether the last common ancestor of Psalteriomonadidae was a marine or fresh-water organism. In addition, we showed that environmental sequences FN865111 and FN865530 from an extremely acidic habitat (Amaral-Zettler et al. 2011) represent undescribed psalteriomonadids closely related to the freshwater genera *Psalteriomonas* and *Sawyeria*. Invasions of habitats of high salinity or acidity are not unusual in Heterolobosea and the Psalteriomonadidae is no exception. On the species and generic level, the ecological characteristics seem to be stable in psalteriomonadids.

There is quite a bit of variation in mitochondrial morphology within the Psalteriomonadidae. The mitochondrion of psalteriomonadids does not possess cristae and has been reduced to a hydrogenosome (Barberà et al. 2010; de Graaf et al. 2009). Acristate mitochondrion was also reported from the unrelated species *Pleurostomum flabellatum* (Park et al. 2007), but not in its close relative *Tulamoeba peronaphora* (Park et al. 2009). *Sawyeria marylandensis* has cup-shaped hydrogenosomes that are not surrounded by rough endoplasmic reticulum (Barberà et al. 2010). In *Psalteriomonas vulgaris*, the hydrogenosomes are associated with bacteria and are clustered to form a spherical aggregate situated close to the nucleus (Broers et al. 1993). Interestingly, *P. lanterna* and *P. magna* were reported to possess two hydrogenosomal morphs (Broers et al. 1990; de Graaf et al. 2009; this study). Similar to the two aforementioned species, a huge aggregate of sausage shaped and dumb-bell shaped hydrogenosomes is present in *P. lanterna*. In addition, individual hydrogenosomes surrounded by rough endoplasmic reticulum are also present. The shape of the individual hydrogenosomes is similar to the aggregated ones, or they are bean-shaped. Our TEM study showed that *Monopylocystis visvesvarai* strain PC4BIC possesses a novel morphology of psalteriomonadid mitochondrial derivates. Its mitochondrial derivates are spherical and are not surrounded by rough endoplasmic reticulum. Although mitochondria were not reported in the description of *M. anaerobica* comb. nov. (Smirnov and Fenchel 1996), the 'microbody-like structures' are morphologically identical with the mitochondrion-related organelle in *M. visvesvarai*, except for the diameter, which is smaller in the latter species.

The majority of described heteroloboseans possess a single central nucleolus in the nucleus during interphase (Page and Blanton 1985). In contrast, members of the Psalteriomonadidae possess a number of different nucleolar morphologies. A nucleus with one or a few parietal nucleoli is typical for genera *Psalteriomonas*, *Sawyeria*, and *Harpagon* (Barberà et al. 2010; Brugerolle and Simpson 2004; O'Kelly et al. 2003; this study). The nucleolar material of *Monopylocystis* spp. is

distributed in a thin ring near the nuclear membrane (O'Kelly et al. 2003; Smirnov and Fenchel 1996; this study). The nucleolar morphology of *Pseudoharpagon pertyi* sp. nov. could not be determined.

The mastigont of the psalteriomonadid genera *Psalteriomonas*, *Harpagon* and *Monopylocystis*, is almost identical in fine structure (Broers et al. 1990, 1993; Brugerolle and Simpson 2004; this study). They share the same number and arrangement of flagella, basal bodies, rhizoplasts and their connection to microtubular ribbon, R_1. The ribbon R_1 is the most prominent structure of the mastigont of Psalteriomonadidae and consists of a curved row of thick microtubules originating near the basal body #1. The microtubular fibres that originate in the vicinity of the other three basal bodies consist of only a few microtubules and are not as conspicuous as R_1. One of these poorly developed fibres is an immature R1′ (after Brugerolle and Simpson 2004), which is conspicuous in some other heteroloboseans (e.g. *Tetramitus rostratus*, *Pharyngomonas kirbyi*, and *Percolomonas sulcatus*, see Brugerolle and Simpson 2004; Park and Simpson 2011). The bundle of microfilaments – MB (after Broers et al. 1990; Brugerolle and Simpson 2004) that connects the concave side of R_1 with basal bodies #1 and #4 forms a harp-like structure that represents a synapomorphy of psalteriomonadid flagellates.

The heterolobosean life cycle is variable too. In *Naegleria* and *Willaertia*, the amoeba represents the main trophic stage and the flagellate stage is reduced and does not feed. Flagellates of these two genera are formed under unfavorable conditions and are used for transport (Preston and King 2003). Some authors, however, argued that flagellates of *Naegleria* are gametes (Fulton 1993), but meiosis has been never observed. Flagellates of some heterolobosean genera have not been found at all (e.g. *Vahlkampfia*, *Sawyeria*, *Tulamoeba*, *Neovahlkampfia*, *Paravahlkampfia*). Other heteroloboseans are able to normally feed in both amoeba and flagellate stages (e.g. *Psalteriomonas lanterna*, *Tetramitus* spp.). Finally, representatives of several genera are pure flagellates and the amoeba stage is unknown (e.g. *Stephanopogon*, *Percolomonas*, and *Pleurostomum*). One of the supposed benefits of the phenotypic plasticity of trophozoites is the ability to produce a better phenotype-environment match across more environments than would be possible by producing a single phenotype in all environments. The transformation of the amoeba to flagellate can be induced in vitro in some heterolobosean species (e.g. Fulton 1970; Page 1988; Robinson et al. 1989), while in others attempts were unsuccessful. However, it is highly probable that some heteroloboseans described only as amoebae are able to form flagellates under certain conditions and vice versa. Our inability to induce the transformation in the culture is most likely explained by our limited knowledge of culture demands of many heteroloboseans (e.g. the size, type, and amount of prey in the culture). However, we cannot exclude the possibility that the transformation is connected with meiosis in some species. There is also a possibility that the ability to form flagellates may be blocked by a spontaneous mutation in a gene important for formation of flagella. Importantly, some strains belonging to the genera *Tetramitus*, *Tetramastigamoeba*, *Heteramoeba*, and *Willaertia* were reported to have lost the ability to form the flagellate in the culture (Droop 1962; Page 1988).

Cells of the two strains of *Monopylocystis visvesvarai* appear either as flagellates (strain PC4BIC) or as amoebae and cysts (the type strain) and no sign of the transformation between amoebae and flagellates, or vice versa, has been observed. The strains were cultivated in the same medium. Broers et al. (1990) reported that a minority of *Psalteriomonas lanterna* flagellates could be transformed to amoebae in the culture by an increase in the concentration of oxygen. However, the conversion of the amoeba back to the flagellate was completely unsuccessful. We tried to expose cells of the strain PC4BIC of *Monopylocystis visvesvarai* to oxygen. However, the cells slowly died and no sign of their transformation to amoebae was observed.

The life cycle of the last common ancestor of Psalteriomonadidae was most probably complex and contained three stages: amoeba with eruptive lobopodia, cyst as resting stage, and flagellate with four flagella and ventral feeding groove. The simultaneous presence of two morphological characters can be considered synapomorphies of Psalteriomonadidae: mitochondria without cristae and harp-like structure in the mastigont.

Taxonomic Summary

Family Psalteriomonadidae Cavalier-Smith, 1993

Synonym: Lyromonadidae Cavalier-Smith, 1993.

Diagnosis: Microaerophilic/anaerobic, living in marine, freshwater or brackish sediments. Mitochondrial cristae absent. Ancestrally with amoeba, flagellate, and cyst stage. One or two of the stages lost/unknown in most genera. Flagellates quadriflagellate. Flagellar basal bodies arranged in two lateral pairs, one posterior and one anterior. Basal bodies parallel in a pair. One branching or two associated rhizoplasts, connected to basal bodies #1 and #2. Ribbon R_1 prominent,

R_1' immature. Harp-like structure present in mastigont. Ventral feeding groove present in flagellates. Nucleolus usually not central. Five genera.

Type genus: *Psalteriomonas* Broers, Stumm, Vogels & Brugerolle, 1990.

Other genera: *Monopylocystis* O'Kelly, Silberman, Amaral-Zettler, Nerad & Sogin, 2003; *Sawyeria* O'Kelly, Silberman, Amaral-Zettler, Nerad & Sogin, 2003; *Harpagon* gen. nov.; *Pseudoharpagon* gen. nov.

Remark: Lyromonadidae Cavalier-Smith, 1993 becomes a younger synonym of Psalteriomonadidae Cavalier-Smith, 1993. Although both names were created in the same year, Psalteriomonadidae is older. When Cavalier-Smith created the family Lyromonadidae (Cavalier-Smith 1993b), the name Psalteriomonadidae was already published (Cavalier-Smith 1993a) and was properly cited in Cavalier-Smith (1993b).

Genus *Psalteriomonas* Broers, Stumm, Vogels & Brugerolle, 1990

Diagnosis: Freshwater. Uninucleate to quadrinucleate. Ancestrally with both amoeboid and flagellate stage. Cyst unknown. Cells contain aggregate of hydrogenosomes. Nucleolus parietal, possibly central. Three species.

Type species: *Psalteriomonas lanterna* Broers, Stumm, Vogels & Brugerolle, 1990.

Other species: *P. vulgaris* Broers, Meijers, Symens, Stumm & Vogels, 1993; *P. magna* sp. nov.

Psalteriomonas lanterna Broers, Stumm, Vogels & Brugerolle, 1990

Diagnosis: Both amoeboid and flagellate stage present. Flagellate stage quadrinucleate, with four mastigonts and four feeding grooves. Flagellate ca. 25 μm long. Four equal flagella per mastigont, ca. 1.5 times the cell length. Ventral groove about 2/3 of the cell length. Locomotive amoeba ca. 45 μm long.

Psalteriomonas vulgaris Broers, Meijers, Symens, Stumm & Vogels, 1993

Synonym: *Lyromonas vulgaris* (Broers, Meijers, Symens, Stumm & Vogels, 1993).

Diagnosis: Amoeboid stage unknown. Flagellate stage ca. 12 μm long with single nucleus, mastigont, and ventral groove. Four equal flagella, ca. 1.5 times the cell length. Ventral groove about 2/3 of the cell length.

Psalteriomonas magna sp. nov.

Diagnosis: Flagellate stage unknown. Locomotive amoebae uninucleate, ca. 70 μm long. Long uroidal filaments present in some cells.

Type locality: Kizillar, Turkey. 37°31′N, 35°42′E.

Syntype slides: protargol preparations of the strain KIZILLAR, deposited in the collection of the Department of Parasitology, Faculty of Science, Charles University, Prague, Czech Republic, catalogue numbers 7/40 – 7/43.

Habitat: Freshwater sediments.

Type culture: strain KIZILLAR, deposited in the culture collection of the Department of Parasitology, Charles University in Prague, Czech Republic.

Etymology: *magna* [Latin] – great, large. The amoebae of *P. magna* are considerably bigger than that of *P. lanterna*.

Genus *Sawyeria* O'Kelly, Silberman, Amaral-Zettler, Nerad & Sogin, 2003

Diagnosis: Freshwater. Uninucleate amoebae. Flagellate and cyst stage unknown. Hydrogenosomes individual, cup-shaped, not surrounded by rough endoplasmic reticulum. Nucleus with one to several parietal nucleoli. Single species.

Type species: *Sawyeria marylandensis* O'Kelly, Silberman, Amaral-Zettler, Nerad & Sogin, 2003.

Sawyeria marylandensis O'Kelly, Silberman, Amaral-Zettler, Nerad & Sogin, 2003

Diagnosis: As for the genus. Locomotive amoeba ca. 35 μm long.

Genus *Monopylocystis* O'Kelly, Silberman, Amaral-Zettler, Nerad & Sogin, 2003

Diagnosis: Marine. Uninucleate. Flagellate, cyst and amoeba stage ancestrally present, flagellate and cyst unknown in one species. Nucleolar material peripheral, distributed in a thin ring beneath the nuclear membrane. Cyst possessing single pore plugged with gelatinous material. Two species.

Type species: *Monopylocystis visvesvarai* O'Kelly, Silberman, Amaral-Zettler, Nerad & Sogin, 2003.

Other species: *M. anaerobica* (Smirnov & Fenchel, 1996) comb. nov.

Monopylocystis visvesvarai O'Kelly, Silberman, Amaral-Zettler, Nerad & Sogin, 2003

Diagnosis: Locomotive amoebae ca. 22 μm long. Floating form unknown. Uroidal filaments absent. Cyst present, with diameter ca. 11 μm. Flagellate stage ca. 16 μm long. Four unequal flagella, one longest, two shorter and equal, one shortest. The longest flagellum up to 1.5 times the cell length. Ventral groove almost reaches the posterior end of the cell.

Monopylocystis anaerobica (Smirnov & Fenchel, 1996) comb. nov.

Synonym: *Vahlkampfia anaerobica* Smirnov & Fenchel, 1996.

Diagnosis: Flagellate unknown. Locomotive amoeba ca. 23 μm long. Uroidal filaments present. Floating form present.

Genus *Harpagon* gen. nov.

Diagnosis: Freshwater. Uninucleate flagellates. Four flagella. Ventral groove up to ½ of the cell length. Amoeba and cyst unknown. Flagellates form several distinct morphotypes. Nucleus with several parietal nucleoli. Two species.

Type species: *Harpagon descissus* (Perty, 1852) comb. nov.

Other species: *Harpagon schusteri* sp. nov.

Etymology: Named after Harpagon, the main character of the Molière's comedy The Miser. The flagellar beating of *Harpagon* spp. and the other members of Psalteriomonadidae is reminiscent of a hand grabbing money.

Harpagon descissus (Perty, 1852) comb. nov.

Synonyms: *Tetramitus descissus* Perty, 1852; *Percolomonas descissus* (Perty, 1852).

Diagnosis: Flagellate ca. 17 μm long. Four unequal flagella, one longest, one shorter, two shortest are equal. Four distinct morphotypes: spindle-shaped cell with a short pointed posterior end, spindle-shaped cell with elongated pointed posterior end, slender elongated cell, pear-shaped short cell with rounded posterior end.

Harpagon schusteri sp. nov.

Diagnosis: Flagellate ca. 17 μm long. Four unequal flagella, one longest, shorter, two shortest are equal. Three distinct morphotypes: spindle-shaped cell with a short pointed posterior end, slender elongated cell, pear-shaped short cell with rounded posterior end. The morphotype with a long pointed posterior end is absent. Differs from *H. descissus* comb. nov. in more than 10% of nucleotides in SSU rDNA.

Type locality: Boroda dam, India. 27°02′N, 76°15′E.

Syntype slides: protargol preparations of the strain INDSIP, deposited in the collection of the Department of Parasitology, Charles University in Prague, Czech Republic, catalogue numbers 5/82 – 5/84.

Habitat: Freshwater sediments.

Type culture: strain INDSIP, deposited in the collection of the Department of Parasitology, Charles University in Prague, Czech Republic.

Etymology: Dedicated to F. L. Schuster, who passed away in 2009. He was an authority in the field of research and cultivation of pathogenic and opportunistic free-living amoebae. He authored or co-authored many publications on *Naegleria fowleri* and *N. gruberi*.

Pseudoharpagon gen. nov.

Diagnosis: Brackish and marine. Uninucleate flagellates. Ventral groove ½ – 2/3 of the cell length. Amoeba and cyst unknown. Single species.

Type species: *Pseudoharpagon pertyi* sp. nov.

Etymology: *pseudo-* [Greek] – false; in scientific use, denoting close resemblance to the following element. The genus is morphologically similar to *Harpagon* gen. nov.

Pseudoharpagon pertyi sp. nov.

Diagnosis: As for the genus. Flagellate ca. 14 μm long. Four unequal flagella, one longest, three shorter. The longest flagellum up to 1.5 times the cell length.

Type locality: Evros delta, Greece, 40°48′N, 26°01′E.

Habitat: Brackish and marine sediments.

Syntype: Protargol preparations of the isolate EVROS2, deposited in the collection of the Department of Parasitology, Charles University in Prague, Czech Republic, catalogue numbers 6/64 and 6/65.

Type culture: EVROS2, deposited in the collection of the Department of Parasitology, Charles University in Prague, Czech Republic.

Etymology: Named after outstanding protozoologist M. Perty, who described *Tetramitus descissus*, now *Harpagon descissus*.

Methods

Organisms: The strains were isolated from fresh-water, brackish or marine sediments. Freshwater strains were isolated in Sonneborn's *Paramecium* medium (ATCC medium 802); marine and brackish strains PC4BIC and EVROS2 were isolated in seawater 802 medium (ATCC medium 1525) and marine strain NY0199 was isolated in 5% TYGM-9 medium (ATCC medium 1171) prepared with sterilized seawater. Approximately 2 ml of the samples were initially inoculated into the medium. The cultures were maintained in polyxenic agnothobiotic cultures at room temperature and were subcultured once a week. Most cultures were not monoeukaryotic and contained various other protists besides the heteroloboseans. All strains are available upon request from the collection of the Department of Parasitology, Charles University in Prague, Czech Republic. Monoeukaryotic culture of *Pseudoharpagon pertyi* NY0199 has been deposited at the ATCC, Manassas, VA, USA (accession number PRA-359).

Light microscopy: Light-microscopic observations were performed using an Olympus Microscope BX51 (Olympus Corporation, Tokyo, Japan) equipped with camera Olympus DP70 or a Zeiss Axioplan 2 imaging microscope (Carl Zeiss, Oberkochen, Germany) equipped with Leica DC500 digital camera (Leica Microsystems, Wetzlar, Germany). Diameters of

locomotive amoebae and flagellates were measured in 50 cells (30 in NY0199). The morphology of strains EVROS2, INDSIP, ITZAVL, KIZILLAR, PC4BIC, TEXEL, TOCOV, and VIT5 was also examined in protargol-stained preparations. Moist films spread on cover slips were prepared from pelleted cultures obtained by centrifugation at 500 g for 8 minutes. For better adherence to the cover slip, 1 μl of the sample was mixed with 1 μl of egg white diluted to 1:5 with the corresponding cultivation medium prior to the wet smear preparation. The films were then fixed in Bouin-Hollande's fluid for ca. 5 hours, were washed with 70% ethanol, and were stained with 1% protargol (Bayer, I. G. Farbenindustrie Actinengesellschaft) following the Nie's (1950) protocol.

Transmission electron microscopy: Cells of strains PC4BIC and KIZILLAR were pelleted by centrifugation at 500 g for 8 minutes, resuspended in a solution containing 2.5% glutaraldehyde (Polysciences) and 5 mM $CaCl_2$ in 0.1 M cacodylate buffer (pH 7.2), and fixed at room temperature for 4 hours. After washing in 0.1 M cacodylate buffer (three times per 15 minutes), the cells were postfixed with 2% OsO_4 in 0.1 M cacodylate buffer for 3 hours. After washing with an excess volume of 0.1 M cacodylate buffer (three times per 15 minutes) the fixed cells were dehydrated in an acetone series and embedded in Epon resin (Poly/Bed 812, Polysciences). The ultrathin sections were stained with lead citrate and uranyl acetate (2 – 3%) and examined using a TEM JEOL 1011 transmission electron microscope.

DNA isolation, amplification, cloning and sequencing: Genomic DNA was isolated using the DNeasy Blood & Tissue Kit (Qiagen), PureGen Tissue DNA isolate kit (Qiagen), or MasterPure Complete DNA and RNA purification Kit (Epicentre, WI, USA) according to the manufacturer's instructions. Almost complete SSU rDNA of most strains was amplified using primers MedlinA (CTGGTTGATCCTGCCAG) and MedlinB (TGATCCTTCTGCAGGTTCACCTAC) (Medlin et al. 1988) with an annealing temperature of 50 °C, except for *H. descissus* strains BAUM and WS, which utilized 42 °C. SSU rDNA of the strain NY0199 was amplified using NPF1 (TGCGCTACCTGGTTGATCC) and R4 (GATCCTTCTGCAGGTTCACCTAC) with an annealing temperature of 49 °C. SSU rDNA of strains CERETE, CIZOV1, IND7, KIZILLAR, LUH2, LUH3, VIT5, and VIT9 was amplified using primers HETERF1 (GCTTATTTCRAAGATTAAGCCATGYAAA) and HETER1K (AAYTCAGGGACGTAATCATT), which were newly designed on the basis of available SSU rDNA sequences of freshwater Psalteriomonadidae (genera *Psalteriomonas*, *Sawyeria*, and *Harpagon*), with an annealing temperature of 60 °C. The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen), Zymoclean™ GEL DNA Recovery Kit (Zymo Research) or UltraClean 15 DNA Purification Kit (MO BIO) and were either directly sequenced or were cloned into either the TOPO T/A vector pCR4 or the pGEM®-T EASY vector using the pGEM®-T EASY VECTOR SYSTEM I (Promega) and at least two clones were sequenced. Sequence data reported in this paper are available in GenBank under accession numbers JN606327 – JN606357.

Phylogenetic analyses: A data sets containing 31 newly determined SSU rDNA sequences, 48 sequences of Heterolobosea retrieved from GenBank, and 9 sequences of other Discoba (Jakobida, Euglenozoa, *Tsukubamonas*, 'Soginia') used as outgroups, was created. The sequences were aligned using the MAFFT method (Katoh et al. 2002) with the help of the MAFFT 6 server http://mafft.cbrc.jp/alignment/server/ with the G-INS-i algorithm at default settings. The alignment was manually edited in BioEdit 7.0.4.1 (Hall 1999). The final data set contained 1246 aligned characters and is available from the corresponding author upon request. Phylogenetic trees were constructed by maximum likelihood and Bayesian methods. Maximum likelihood analysis was performed in RAxML 7.0.3 (Stamatakis 2006) under the GTRGAMMAI model. Bootstrap support values were generated in RAxML from 1000 pseudoreplicate data sets. Bayesian analysis was performed using MrBayes 3.1.2. (Ronquist and Huelsenbeck 2003) under the GTR + I + Γ + covarion model. Four MCMCs were run for $4{\cdot}10^6$ generations, until the mean standard deviation of split frequencies based on last 75% generations was lower than 0.01. The trees were sampled every 100th generation. The first 25% of trees were removed as burn-in.

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