Survey on diversity of marine/saline anaerobic Heterolobosea (Excavata: Discoba) with description of seven new species

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Diversity of the anaerobic Heterolobosea (Excavata: Discoba) is only poorly understood, especially in marine environments. We have isolated and cultured 16 strains of anaerobic heteroloboseid amoebae and flagellates from brackish, marine and saline anoxic habitats worldwide. Phylogenetic analyses of SSU rDNA sequences and light-microscopic observations showed that all the strains belong to the family Psalteriomonadidae, the main anaerobic lineage of Heterolobosea, and that they represent eight species from the genera Monopylocystis, Harpagon and Pseudoharpagon. Seven species are newly isolated and described here as Monopylocystis minor n. sp., Monopylocystis robusta n. sp., Monopylocystis elegans n. sp., Monopylocystis disparata n. sp., Harpagon salinus n. sp., Pseudoharpagon longus n. sp. and Pseudoharpagon tertius n. sp. Amoebae, cysts and the ultrastructure of the genus Pseudoharpagon are presented for the first time.

Introduction

Heterolobosea is a distinctive lineage of protists belonging to the eukaryotic supergroup Excavata (Adl et al., 2005). The group was coined by Page & Blanton (1985) to acknowledge that shizopyrenid limax amoebae/amoeboid flagellates and acrasid slime moulds together constitute a monophyletic group. Currently, the taxon Heterolobosea encompasses approximately 140 described species (Pánek & Čepička, 2012) and is divided into the subphyla Tetratmitia, which comprises the vast majority of the species, and Pharyngomonada, with only two species of the genus Pharyngomonas (Cavalier-Smith & Nikolaev, 2008; Harding et al., 2013). A potentially separate evolutionary lineage of the Heterolobosea, represented by strain BB2, was discovered recently (Harding et al., 2013). Many heteroloboseid species are true amoeboflagellates, i.e. they switch between an aflagellate amoeba and a flagellate, and vice versa, during the life cycle. Interestingly, the amoebae entirely lack cytoplasmic microtubules. The flagellar apparatus, including basal bodies, is formed de novo during the amoeba–flagellate transformation (Walsh, 2007; Lee, 2010). Several heteroloboseid species have secondarily lost one of the two motile stages and exist as pure amoeba (e.g. species of the genera Vahlkampfia, Neovahlkampfia and Paravahlkampfia) or flagellates (e.g. Percolomonas cosmopolitus, species of the genera Harpagon and Stephanopogon) (see Pánek & Čepička, 2012). In addition, many heteroloboseid species form resistant cysts in harsh conditions.

Heteroloboseid amoebae are usually cylindrical and move with eruptive lobopodia. Amoebae of individual species or even genera are often morphologically virtually indistinguishable, and their morphology cannot be used in taxonomical studies (see e.g. Brown & De Jonckheere, 1999). By contrast, the flagellates are much more variable in their external morphology. They are almost always tetrakont or bikont (i.e. equipped with four or two flagella). A higher number of flagella is known from only a few heteroloboseids: (1) flagellates of Psalteriomonas lanterna, but not that of Psalteriomonas vulgaris, possess four ventral grooves, four nuclei and four tetrakont mastigonts (Broers et al., 1990); (2) members of the genus Stephanopogon are obligate flagellates with several tens of flagella and ciliate-like gross morphology (Yubuki & Leander, 2008); (3) flagellates of the recently discovered Oramoeba fumarolia were described to possess two to ten flagella, but their morphology has not been studied in detail (De Jonckheere et al., 2011a). Phylogenetic analyses.
of SSU rDNA showed that these three lineages are not specifically related (De Jonckheere et al., 2011a). The heteroloboseid flagellates usually possess a conspicuous ventral groove. The groove is considered to be homologous to that of other excavates, although it is supported by partially different cytoskeletal elements (Brugerolle & Simpson, 2004; Park & Simpson, 2011). The groove of some species, e.g. those of the genus Naegleria, has been lost, and the flagellates are short-lived and transient (Fulton, 1993; De Jonckheere, 2002).

Although heteroloboseids are not a species-rich group, they are extremely diverse in their ecology. In particular, a significant number of species have been detected in various extreme habitats, such as thermal springs, Antarctic lakes, and hypersaline or highly acidic habitats (Amaral-Zettler et al., 2002, 2011; Murtagh et al., 2002; Garstecki et al., 2005; Park et al., 2007, 2009; Baumgartner et al., 2009; De Jonckheere et al., 2009, 2011a, b; Park & Simpson, 2011). Ten species of obligately anaerobic/microaerophilic heteroloboseids have been described so far (Perty, 1852; Ruinen, 1938; Broers et al., 1990, 1993; Smirnov & Fenchel, 1996; O’Kelly et al., 2003; Brugerolle & Simpson, 2004; Pánek et al., 2012). It was recently shown that all but one of them belong to a single clade, the Psalteriomonadidae (Pánek et al., 2012). A separate lineage of presumably anaerobic heteroloboseids is represented by the halophilic species Pleurostomum flabellatum (Park et al., 2007). However, its anaerobiosis has yet to be confirmed. The nine species of the family Psalteriomonadidae are currently grouped into five genera: Psalteriomonas, Sawyeria, Monoplylocystis, Harpagon and Pseudoharpagon. All known members of the Psalteriomonadidae possess mitochondrion-related organelles lacking cristae (de Graaf et al., 2009; Barberá et al., 2010; Pánek et al., 2012) and a characteristic mastigont structure of the flagellates (Pánek et al., 2012). The ancestral life cycle (with separate amoeba and flagellate stages) has been retained in some species, such as Psalteriomonas lanterna and Monoplylocystis visvesvarai, but most species seem to have permanently lost one of the stages and are known exclusively as amoebae (e.g. Sawyeria marylandensis) or flagellates (e.g. species of the genus Harpagon, Pseudoharpagon pertyi). Monoplylocystis visvesvarai is the only member of the family Psalteriomonadidae known to be able to produce cysts (O’Kelly et al., 2003). The cysts are unique among the Heterolobosea by having a single pore occluded by a gelatinous plug, hence the name Monoplylocystis (literally ‘cyst with a single pore’).

Members of the family Psalteriomonadidae are predominantly freshwater, and only three species have been found in marine or brackish anoxic sediments: M. visvesvarai, Monoplylocystis anaerobica (formerly Vahlkampfia anaerobica) and Pseudoharpagon pertyi. Moreover, each of them is represented by only one or two strains. To assess the diversity of anaerobic heteroloboseids in brackish/marine habitats and inland salt marshes, we isolated and cultured 16 strains from various localities worldwide. According to light-microscopic morphology and SSU rDNA sequence analysis, our strains are unexpectedly diverse and represent eight distinct species of the family Psalteriomonadidae, seven of which are novel. We also present the ultrastructure of the genus Pseudoharpagon for the first time.

Methods
Organisms. Information on the origin of strains included in the study is summarized in Table 1. Most strains were isolated between 2009 and 2011 from marine or brackish coastal sediments. Strains TSUKIM (Harpagon salinus n. sp.) and TSUKIMV (M. visvesvarai) were obtained in 2012 from an inland salt marsh. Strains FUE3N (Monoplylocystis robustus n. sp.) and FUE4N (Monoplylocystis disparata n. sp.) were isolated in 2011 from saline sediments collected near a salt pan. Strain COORONG of M. disparata n. sp. was isolated in 2010 from a similar environment. All strains were isolated in the seawater-based ATCC medium 1525; approximately 2 ml of the samples was initially inoculated into the medium. The strains were maintained in polyxyenic agnothobioc cultures with unidentified bacteria at room temperature, and they were subcultured once a week. The cultures, except for LAGOS1P (Pseudoharpagon tertius n. sp.), were not monoeukaryotic and contained various other protists besides the heteroloboseids. Strains LAGOS1M (M. disparata n. sp.) and LAGOS1P (Pseudoharpagon tertius n. sp.), and strains EVRO51M (Monoplylocystis elegans n. sp.) and EVRO511 (Pseudoharpagon longus n. sp.), respectively, were originally isolated from a single sample, but were successfully separated and cultured individually after several initial passages. Strains TSUKIMV (M. visvesvarai) and TSUKIM (H. salinus n. sp.) were isolated from a single sample that was inoculated into two tubes containing ATCC medium 1525, and two separate cultures, A and B, were created. Culture A contained a single heterolobosean, M. visvesvarai, whereas culture B contained members of both the genera Monoplylocystis and Harpagon. Culture B was later transferred into the freshwater-based ATCC medium 802 in order to examine the ability of H. salinus n. sp. to grow in freshwater environments.

Light microscopy. Light-microscopic observations were performed using an Olympus Microscope BX51 equipped with an Olympus DP71 camera. Diameters of locomotive amoebae and flagellates were measured in 30 cells. In addition, the morphology of eight strains was examined in protargol-stained preparations. Moist films spread on coverslips were prepared from pelleted cultures obtained by centrifugation at 500 g for 8 min. For better adherence to the coverslip, 1 ml of the sample was mixed with 1 ml egg white diluted to 1:4 with the corresponding cultivation medium prior to the wet smear preparation. The films were then fixed in Bouin-Holland's fluid: cupric sulfate 2.5 g, picric acid 4 g, distilled water 100 mL, 40 % formaldehyde 10 mL, acetic acid 1.5 mL for 5 min and for 30 min at room temperature in 2 ml of a cocktail containing growth medium and 0.16 ml 25 % (w/v) glutaraldehyde (final concentration 2 %). The cells were rinsed three times with the growth medium and 0.16 ml 25 % (w/v) glutaraldehyde (final concentration 2 %). The cells were rinsed three times with the growth medium and were post-fixed for 1 h in 1 ml of a cocktail containing 0.25 ml 4 % (w/v) OsO4 (final concentration 1 %) and 0.75 ml of growth medium. After being rinsed free of post-fixative, the cells were concentrated by centrifugation and were trapped in 2 % (w/v) agarose. The fixed cells were dehydrated in an acetone series and embedded in Epon resin (Poly/Bed 812/Araldite 502, Polysciences). The ultrathin sections were cut using a diamond knife on an Ultracut...
The sequences were aligned using the MAFFT method (Katoh et al., 2002) with the help of the mafft 7 server (http://mafft.cbrc.jp/alignment/server/) with the G-INS-i algorithm at default settings. The first dataset contained 16 newly determined sequences and 12 sequences of the family Psalteriomonadidae retrieved from GenBank. The second dataset was composed of 16 newly determined sequences and 12 sequences of members of the family Psalteriomonadidae retrieved from GenBank. The sequences were aligned using the MAFFT method (Katoh et al., 2002) with the help of the mafft 7 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 datasets contained 1208 and 1768 aligned characters, respectively, and are available from the corresponding author upon request.

Phylogenetic analyses. Two datasets consisting of SSU rDNA sequences were created. The first dataset contained 16 newly determined sequences and 77 sequences representing major lineages of Heterolobosea retrieved from GenBank. The second dataset contained 16 newly determined sequences and 12 sequences of members of the family Psalteriomonadidae retrieved from GenBank. The sequences were aligned using the MAFFT method (Katoh et al., 2002) with the help of the mafft 7 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 datasets contained 1208 and 1768 aligned characters, respectively, and are available from the corresponding author upon request.

Phylogenetic trees were reconstructed 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 datasets. Bayesian analysis was performed using MrBayes 3.2 (Ronquist et al., 2012) under the GTR + I + I- covariation model. Four Markov chain Monte Carlo (MCMC) chains were run for 5.10⁶ and 1.10⁶ generations, respectively, until the mean standard deviation of split frequencies based on last 75% generations was lower than 0.01. The trees were sampled every 500th generation. The first 25% of trees were removed as burn-in.

Results

Light-microscopic observations

Uninucleate flagellates, amoebae and cysts were observed in the cultures. The cells were anaerobic/microaerophilic and were always found at the bottom of the culture tubes. Cell diameters of particular stages are summarized in Table 2.

General morphology of species of the genus *Monopylocystis*. Twelve strains were classified into the genus *Monopylocystis*. The amoebae of species of the genus *Monopylocystis* displayed characteristic features of Heterolobosea; they were monopodial in locomotion and possessed an anterior eruptive hyaline front reaching approximately from 1/5 to 1/3 of the cell length (Fig. 1a–s). The anterior hyaloplasm was clearly separated from the posterior granuloplasm. Floating amoeboid cells of all strains were spherical and did not form pseudopodia (not shown). The nucleus was usually situated in the anterior part of the granuloplasm. It was rounded in some cells, but often strongly deformed during the locomotion. The

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Habitat</th>
<th>Locality</th>
<th>Coordinates</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Monopylocystis visvesvarai</em></td>
<td>GOUVIA</td>
<td>B</td>
<td>Gouvia, Corfu Island, Greece</td>
<td>39° 38’ N 19° 50’ E</td>
</tr>
<tr>
<td></td>
<td>IGO2</td>
<td>B</td>
<td>Iguomenitsa, Greece</td>
<td>39° 31’ N 20° 14’ E</td>
</tr>
<tr>
<td></td>
<td>IGO3</td>
<td>B</td>
<td>Iguomenitsa, Greece</td>
<td>39° 31’ N 20° 14’ E</td>
</tr>
<tr>
<td></td>
<td>KORISSION3</td>
<td>M</td>
<td>Lake Korission, Korfu, Greece</td>
<td>39° 27’ N 19° 52’ E</td>
</tr>
<tr>
<td></td>
<td>TSUKIMV</td>
<td>I</td>
<td>Einot Tsukim Nature Reserve, Israel</td>
<td>31° 42’ N 35° 27’ E</td>
</tr>
<tr>
<td><em>Monopylocystis disparata n. sp.</em></td>
<td>COORONG</td>
<td>S</td>
<td>Coorong, NP, Australia</td>
<td>36° 04’ S 139° 35’ E</td>
</tr>
<tr>
<td></td>
<td>FUEN4</td>
<td>S</td>
<td>Fuencaliente, La Palma island, Spain</td>
<td>28° 27’ N 17° 50’ W</td>
</tr>
<tr>
<td></td>
<td>LAGOS1M</td>
<td>M</td>
<td>Porto Lagos, Greece</td>
<td>41° 00’ N 25° 06’ E</td>
</tr>
<tr>
<td><em>Monopylocystis minor n. sp.</em></td>
<td>AND</td>
<td>M</td>
<td>Elephant beach, Havelock Island, India</td>
<td>12° 00’ N 92° 56’ E</td>
</tr>
<tr>
<td></td>
<td>TIN1</td>
<td>M</td>
<td>Tiwi, Oman</td>
<td>22° 49’ N 59° 15’ E</td>
</tr>
<tr>
<td><em>Monopylocystis elegans n. sp.</em></td>
<td>EVROS1M</td>
<td>B</td>
<td>Evros delta, Greece</td>
<td>40° 48’ N 26° 01’ E</td>
</tr>
<tr>
<td><em>Monopylocystis robusta n. sp.</em></td>
<td>Fuen3</td>
<td>S</td>
<td>Fuencaliente, La Palma island, Spain</td>
<td>28° 27’ N 17° 50’ W</td>
</tr>
<tr>
<td><em>Pseudoharpagon longus n. sp.</em></td>
<td>EVROSI1</td>
<td>B</td>
<td>Evros delta, Greece</td>
<td>40° 48’ N 26° 01’ E</td>
</tr>
<tr>
<td><em>Pseudoharpagon tertius n. sp.</em></td>
<td>LAGOS1P</td>
<td>M</td>
<td>Porto Lago, Greece</td>
<td>41° 00’ N 25° 06’ E</td>
</tr>
<tr>
<td><em>Harpagon salinus n. sp.</em></td>
<td>TSUKIM</td>
<td>I</td>
<td>Einot Tsukim Nature Reserve, Israel</td>
<td>31° 42’ N 35° 27’ E</td>
</tr>
</tbody>
</table>
The flagellates were quadriflagellate and possessed a single prominent pore occluded by a plug (Fig. 6a–h). The cytoplasm within cysts contained several granules protruding to the central area. The peripheral layer was not of uniform thickness and displayed several granules protruding to the central area. The peripheral layer of nucleolar material and homogeneous nucleus of amoebae of all strains possessed a thin peripheral layer of nucleolar material (Fig. 3a, e, h, o). Flagellates of particular species differed in cell diameters.

Cysts were present in many cultures of species of the genus Monopylocystis, where they co-occurred with amoebae. Cysts of species of the genus Monopylocystis were similar to cysts of M. visvesvarai described by O’Kelly et al. (2003), i.e. they possessed a single prominent pore occluded by a plug (Fig. 6a–h). The cytoplasm within cysts contained many tiny globules, and sometimes a single nucleus was observed (Fig. 6h).

Monopylocystis visvesvarai. Amoebae were observed in the cultures of all five strains of M. visvesvarai: GOUVIA, IGO2, IGO3, KORISSON3 and TSUKIMV (Fig. 1a–g).

Table 2. Dimensions of living specimens of heterolobosean strains

Mean ± SD of 30 specimens (smallest–largest value). A, amoeba; F, flagellate; NA, not available.

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Stage</th>
<th>Cell length</th>
<th>Cell width</th>
<th>LW ratio</th>
<th>Cyst diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monopylocystis minor n. sp.</td>
<td>AND</td>
<td>A</td>
<td>18.2 ± 5.0</td>
<td>5.9 ± 0.8</td>
<td>3.1 ± 0.8</td>
<td>8.7 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td></td>
<td>11.0 ± 1.0</td>
<td>2.2 ± 0.5</td>
<td>n.a.</td>
<td>NA</td>
</tr>
<tr>
<td>Monopylocystis elegans n. sp.</td>
<td>TINI</td>
<td>A</td>
<td>16.0 ± 3.4</td>
<td>4.9 ± 1.0</td>
<td>3.4 ± 0.6</td>
<td>8.7 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td></td>
<td>16.0 ± 5.6</td>
<td>1.9 ± 0.5</td>
<td>n.a.</td>
<td>NA</td>
</tr>
<tr>
<td>Monopylocystis robusta n. sp.</td>
<td>EVROS1M</td>
<td>A</td>
<td>23.3 ± 5.5</td>
<td>8.0 ± 1.6</td>
<td>3.0 ± 0.6</td>
<td>9.0 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td></td>
<td>18.1 ± 3.1</td>
<td>1.9 ± 0.5</td>
<td>n.a.</td>
<td>NA</td>
</tr>
<tr>
<td>Pseudoharpagon longus n. sp.</td>
<td>EVROS1I</td>
<td>A</td>
<td>38.5 ± 6.8</td>
<td>12.6 ± 2.7</td>
<td>3.1 ± 0.5</td>
<td>19.0 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td></td>
<td>20.4 ± 4.1</td>
<td>2.1 ± 3.8</td>
<td>n.a.</td>
<td>NA</td>
</tr>
<tr>
<td>Pseudoharpagon tertius n. sp.</td>
<td>LAGOS1P</td>
<td>F</td>
<td>15.5 ± 3.3</td>
<td>n.a.</td>
<td>n.a.</td>
<td>NA</td>
</tr>
<tr>
<td>Harpagon salinus n. sp.</td>
<td>TSUKIM</td>
<td>F</td>
<td>13.7 ± 1.4</td>
<td>6.0 ± 0.7</td>
<td>1.9 ± 0.2</td>
<td>8.1 ± 1.4</td>
</tr>
</tbody>
</table>

Diversity of marine/saline anaerobic Heterolobosea
The amoebae were variable in size, and the mean length of locomotive specimens differed by 7 μm between strain GOUVIA with the smallest cells and strain IGO2 with the largest cells (Table 2). The nucleus was located at the anterior end of the granuloplasm during rapid locomotion. Most locomotive cells formed a bulbous or villous-bulbous uroid, which occasionally bore trailing filaments (Fig. 1a, c, g). Flagellates were observed only in the culture of strain KORISSION3 (Figs. 3a–c); they were morphologically identical to flagellates of strain PC4BIC described by Pa´nek et al. (2012). Cysts with typical morphology of Monopylocystis were present in the culture of strains IGO3, KORISSION3 and TSUKIMV (Fig. 6a–c).

Monopylocystis minor n. sp. Both strains of M. minor n. sp., AND and TINI, consisted of amoebae, cysts and flagellates in early passages of the culture; the flagellates of strain TINI, however, disappeared after a few tens of passages. The amoebae of M. minor n. sp. (Fig. 1h–k) were slightly smaller than those of M. visvesvarai (Table 2). The nucleus was located at the anterior end of the granuloplasm in locomotive amoebae and was always strongly deformed. The cells usually formed a bulbous uroid, sometimes with a single short filament (Fig. 1h, j). Flagellates of both strains were smaller than flagellates of M. visvesvarai (Figs 3d–g, 5a, b). The cysts displayed typical morphology of the genus Monopylocystis (Fig. 6d, e).

**Fig. 1.** Locomotive amoebae of *M. visvesvarai* strains IGO2 (a, b), KORISSION3 (c, d), GOUVIA (e), IGO3 (f) and TSUKIMV (g), *Monopylocystis minor* n. sp. strains AND (h, i) and TINI (j, k), *M. dispersata* n. sp. strain FUEN4 (l–n), *M. elegans* n. sp. strain EVROS1M (o, p), *M. robusta* n. sp. strain FUEN3 (q–s) and *Pseudoharpagon longus* n. sp. strain EVROS11 (t–v). Differential interference contrast (DIC). Bar, 10 μm.
**Monopylocystis disparata n. sp.** Amoebae and cysts were observed in the culture of strain FUEN4; only flagellates were observed in the culture of strain LAGOS1M. Strain COORONG was lost before its morphology could be examined. The amoebae of strain FUEN4 were small in comparison with those of the other *Monopylocystis* strains (Fig. 1l–n, Table 2). The nucleus of locomotive amoebae was located at the anterior end of the granuloplasm. Most cells possessed a well-differentiated villous-bulbous uroid, often with several long filaments. Cysts of strain FUEN4 were smaller than the cysts of the other *Monopylocystis* strains and displayed typical morphology of the genus (Fig. 6f). Flagellates of *M. disparata* n. sp. strain LAGOS1M were the largest among members of the genus *Monopylocystis* (Fig. 3h–k, Table 2).

**Monopylocystis elegans n. sp.** Amoebae, flagellates and cysts were observed in the culture of strain EVROS1M. The nucleus of locomotive amoebae was located deeper in the granuloplasm and was not deformed during the locomotion (Fig. 1o, p); in protargol-stained cells, the shape of the nucleus was more regular than in cells of the other species (Fig. 2k, l). The cells usually formed a uroid, sometimes with short filaments. Flagellates of strain EVROS1M were
similar to that of *M. visvesvarai* (Figs 3l–o, 5c, d); typically, they were slightly larger and more variable in length (Table 2). The cysts displayed typical morphology of *Monopylocystis* (Fig. 6g).

**Monopylocystis robusta** *n. sp.* Amoebae and cysts were present in the culture of strain FUEN3 of *M. robusta* *n. sp.* The amoebae were the largest among *Monopylocystis* species (Fig. 1q–s). The cells appeared more vacuolated than the amoebae of the other strains; the vacuolization was also apparent in protargol-stained preparations (Fig. 2m, n). The nucleus of locomotive amoebae was located anteriorly in the granuloplasm during locomotion. Some cells possessed a villous-bulbous uroid with multiple short filaments (Fig. 1r, s). Cysts displayed typical morphology of *Monopylocystis* and were bigger than the cysts of the other species (Fig. 6h).

**Fig. 3.** Living flagellates of *M. visvesvarai* strain KORISSION3 (a–c), *M. minor* *n. sp.* strains AND (d–f) and TINi (g), *M. disparata* *n. sp.* strain LAGOS1M (h–k) and *M. elegans* *n. sp.* strain EVROS1M (l–o). DIC. Bar, 10 μm.
Fig. 4. Living flagellates of *Pseudoharpagon longus* n. sp. strain EVROS11 (a–i), *Pseudoharpagon tertius* n. sp. strain LAGOS1P (j–o), and *H. salinus* n. sp. strain TSUKIM (p–u). DIC. Bar, 10 μm.
**Pseudoharpagon longus** n. sp. Amoebae, flagellates and cysts were observed in the culture of strain EVROSI1. The amoebae were larger than the amoebae of most species of the genus *Monopylocystis* (Figs 1t–v, 2o, p, Table 2). The cytoplasm contained multiple tiny granules; the granules were abundant in both hyaloplasm and granuloplasm making the boundary between these two zones less apparent (Fig. 1t–v). The cells occasionally formed a bulbous or bulbous-villous uroid (Fig. 1v). The nuclear morphology was similar to that of species of the genus *Monopylocystis* (Figs 1t–v, 2o, p).

*Fig. 5.* Protargol-stained flagellates of *M. minor* n. sp. strain AND (a, b), *M. elegans* n. sp. strain EVROSI1M (c, d), *Pseudoharpagon longus* n. sp. strain EVROSI1 (e–k), *Pseudoharpagon tertius* n. sp. strain LAGOS1P (l–n) and *H. salinus* n. sp. strain TSUKIM (o, p). Bright field. Bar, 10 µm.
The nucleus did not occupy a stable position in the granuloplasm in locomotive amoebae, similarly to *M. elegans* n. sp., and it was occasionally observed even in the uroid (Fig. 1v); it was not deformed during the cell locomotion.

The flagellates of *Pseudoharpagon longus* n. sp. were larger than that of the other known *Pseudoharpagon* species (Fig. 4a–i, Table 2). The cells were usually elongated, and a large variability in the shape of the posterior end was observed (compare Fig. 4a, d, e). *Pseudoharpagon longus* n. sp. differed from the other species of the family Psalterio-monadidae in the number of flagella. Cells with multiple flagella (Fig. 4f–i) occurred in the culture besides the tetrakont ones. The unusual flagellar number of *Pseudoharpagon longus* n. sp. was apparent in protargol-stained cells (Fig. 5e–k). From 30 randomly selected protargol-stained cells, only 23% were tetrakont, whereas 57% possessed five flagella and 20% possessed more than five flagella. The flagella of the multiflagellate cells seemed to be arranged in at least two separate clusters consisting of approximately five flagella (Figs 4g–i, 5i–k). Most such cells possessed a single nucleus (Fig. 5i, j); occasionally, binucleate, multiflagellate cells were observed (Fig. 5k). The nucleus of living cells contained a thin peripheral layer of an uneven thickness (Fig. 4a–c, e). The structure of the nucleus and adjacent area appeared more complex in protargol-stained flagellates, though a peripheral dense layer was visible (Fig. 5f, h–k). The flagellates possessed a ventral groove that reached approximately one half of the cell (less in longer cells; Fig. 4d); the multiflagellate cells possessed two grooves, each being associated with a cluster of flagella (Fig. 4h, i).

The cysts occurring in the culture of strain EVROS11 were considerably larger than cysts of species of the genus *Monopylocystis*, and their morphology was unusual (Fig. 6i–l). They were rounded and usually produced two (one to four) long projections showing annulation or segmentation. The arrangement of the projections differed among individual cysts. Some projections had an aperture at the distal end (Fig. 6j, k), whereas the others seemed to be terminated by a cyst wall (Fig. 6i, l). The cysts contained a dense mass of relatively large globules.

**Pseudoharpagon tertius** n. sp. Amoebae and flagellates were present in the culture of strain LAGOS1P; strain MURANO3 was lost before its morphology could be examined. The amoebae of LAGOS1P were quite rare in the culture, and their morphology is documented only in protargol-stained preparations (Fig. 2q, r). They displayed numerous thin pseudopodia, and the nuclear structure was similar to that of *Pseudoharpagon longus* n. sp. and species of the genus *Monopylocystis*. The flagellates were always tetrakont (Fig. 5l–n) and possessed a ventral groove reaching one half to two thirds of the cell, rarely almost its posterior end (Fig. 4j–o). They displayed certain variability in the shape of the posterior end of the cell, similarly to the other *Pseudoharpagon* species (compare Fig. 4j, k, l). The nucleus of living flagellates was similar to that of *Pseudoharpagon longus* n. sp. (Fig. 4l, n); protargol-stained nuclei were uniformly dark (Fig. 5l–n).

**Harpagon salinus** n. sp. Flagellates, amoebae and cysts were observed in the culture of the strain TSUKIM. However, the amoebae and cysts were ascribed to *M. visvesvarai*, as they were morphologically consistent with the other isolates of this species (Figs 1g, 6c; see above), and SSU rDNA of this species was recovered from the culture together with SSU rDNA of *H. salinus* n. sp. By contrast, the flagellates were dissimilar to those of species of the genus *Monopylocystis* and...
Fig. 7. Transmission electron micrographs of *Pseudoharpagon tertius* LAGOS1P (a, b) and *Pseudoharpagon pertyi* EVROS2N (c–k). Cell of *Pseudoharpagon tertius* in longitudinal section, lateral view (a); mitochondrion-related organelle of *Pseudoharpagon tertius* (b); cells of *Pseudoharpagon pertyi* in longitudinal sections, ventral view (c, f); detail of the dense body of *Pseudoharpagon pertyi* bounded by single membrane (d); possibly dividing cell of *Pseudoharpagon pertyi* in...
resembled those of species of the genera *Harpagon* or *Pseudoharpagon* by the ventral groove, which reached only slightly past one half of the cell (Fig. 4p–u). The cells were smaller than cells of *Harpagon descissus* and *Harpagon schusteri*, were spindle-shaped, and their posterior end was usually more or less pointed. The nucleus of both living and protargol-stained cells was similar to that of species of the genera *Monopylocystis* and *Pseudoharpagon* (Figs 4p, 5o, p).

Fig. 8. Transmission electron micrographs of the flagellar apparatus of *Pseudoharpagon pertyi* EVROS2N. Anterior half of the cell in longitudinal section, lateral view, showing microfibrillar connection between posterior basal bodies and the nucleus (a); anterior part of the cell in an oblique section showing striated rhizoplast and basal bodies 1 and 2 (b); anterior part of the cell in longitudinal section from the lateral view (c, d). Bars, 1 μm (a), 500 nm (b), 200 nm (c), 500 nm (d). Ax, axoneme of the flagellum; BB, basal body of the flagellum; DF, dorsal fan of microtubules; DS, dense sheet; MB, microfibrillar bundle; N, nucleus; RER, rough endoplasmic reticulum; Rh, rhizoplast; R2, microtubular root 2; arrow, fibrous sheet below the proximal end of basal bodies.
Transmission electron microscopy of members of the genus Pseudoharpagon

We examined the cell structure of Pseudoharpagon tertius n. sp. (strain LAGOS1P) and Pseudoharpagon pertyi (strain EVROS2N). General terminology of ultrastructural elements typical for Excavata was adopted from Yubuki et al. (2013), who revised the universal terminology of the excavate flagellar apparatus (see Moestrup, 2000; Simpson, 2003). Terms of elements specific for Heterolobosea (microfibrillar bundle, rhizoplast) and labelling of flagella in the double-bikont flagellar apparatus were adopted from Brugerolle & Simpson (2004).

The terminology of the microtubular root labelled here as the R2 merits a brief comment. In the older literature, the root was named ‘curved microtubule-organizing ribbon’ (MTOR; Broers et al., 1993) or ‘right root’ (e.g. Simpson & Patterson, 2001; Park & Simpson, 2011). Simpson (2003) proposed that the right root of excavates corresponds to root 1 (R1) in Moestrup’s universal system (Moestrup, 2000). Thus, the right root of heterolobosean flagellates was erroneously labelled as root 1 (R1) instead of root 2 (R2) in several studies (Simpson, 2003; Brugerolle & Simpson, 2004; Pánek et al., 2012).

Pseudoharpagon tertius n. sp. Only a few cells of strain LAGOS1P were examined. Preliminary transmission electron microscopy showed that the nucleus was irregular, located apically, and with a thin projection at the anterior part (Fig. 7a). It contained at least two distinct aggregates of dense material (Fig. 7a) and was associated with rough endoplasmic reticulum (data not shown). Mitochondrion-related organelles (MROs) were rounded, 400–800 nm in diameter (Fig. 7b). Some MROs contained internal paracrystalline structures (Fig. 7b), but the quality of the sections was not sufficient to address the presence or absence of internal membranous structures observed in Pseudoharpagon pertyi (see below).

Pseudoharpagon pertyi strain EVROS2N. The nucleus was pear-shaped, located anteriorly, below the basal bodies, and contained a peripheral layer of electron-dense material of uneven thickness. In both longitudinal- and cross-sections of the nucleus, two to four massive aggregations of dense material (parietal nucleoli) were observed (compare Figs 7c, e, 8a, 10c, e). The cells did not possess focused Golgi apparatus represented by stacked membrane-bounded sacs. MROs were in many copies per cell (Fig. 7f); they were rounded (Fig. 7g), sometimes cup-shaped (Fig. 7k), 400–700 nm in diameter, and bounded by a double membrane. Many of them contained a single membranous structure that closely resembled a discoidal crista (Fig. 7h–j). Rough endoplasmic reticulum surrounded the nucleus, but it was not associated with MROs. The cells usually contained food vacuoles with ingested bacteria (Fig. 7c, e). Small dense bodies bounded by a single membrane, 100–250 nm in diameter (Fig. 7d, g), were present under the cell membrane (Fig. 7f). Some cells contained lipid droplets (Fig. 7e, f).

The flagellar apparatus of Pseudoharpagon pertyi was located subapically and was closely associated with the nucleus (Fig. 7c). Four basal bodies were arranged in two pairs. The pairs of basal bodies laid in tandem, basal bodies 1 and 4 formed a posterior pair, 2 and 3 formed the anterior one (Fig. 9a, c). The basal bodies were arranged nearly parallel to each other in a pair (Fig. 8a, c, d) with the proximal end lying on a ‘pad’ or fibrous sheet (Fig. 8a); they were 400–450 nm long. In a ventral view, the basal bodies laid rightwards. Axonemes of flagella displayed the typical ‘9 + 2’ arrangement of microtubules and bore no vanes or paraxonemal structures (Figs 9f, 10f).

Microtubular root R2 anchored in the vicinity of basal body 1 (Fig. 9a). It was a curved row of approximately 55 microtubules that split into two unequal parts soon after the origin. The smaller left portion, iR2, consisted of approximately 15 microtubules; the right portion, oR2, consisted of approximately 40 microtubules. oR2 and iR2 supported the edges of the ventral groove (Fig. 10a, c, e, g). The concave side of R2 was supported by ‘1 fibre’, a non-microtubular structure that appeared latticed in transverse sections (Fig. 9a). oR2 was associated with a non-microtubular string (Fig. 10b). The string may be homologous to the ‘microfibrillar string S’ reported from Psalteriononas lanterna and H. descissus, where it acts as a nucleating centre for spaced microtubules reinforcing the floor of the ventral groove (Broers et al., 1990; Brugerolle & Simpson, 2004).

The concave side of R2 was connected with the posterior pair of basal bodies via a thick microfibrillar bundle.
(compare Figs 8a, b, 9a, c, d). A large striated fibre, the rhizoplast, connected the convex side of R2 and basal bodies 1 and 2. The rhizoplast displayed cross striation with periodicity of approximately 35 nm (Figs 8b, 9c, d) and longitudinal striation with periodicity of approximately 10 nm (Fig. 8b). The rhizoplast was associated with a sheet of dense material (Figs 8c, d, 9d), which ran from the dorsal side of the rhizoplast above basal bodies to the anterior tip of the cell (Fig. 9d).

Another microtubular root, composed of two microtubules, originated anteriorly from basal body 1 (Fig. 9c). Two or three microtubular fibres anchored in the vicinity of basal body 2. One of them, composed of two microtubules, was situated anteriorly (Fig. 9a, b); the other one or two, together composed of four or five microtubules, were located posteriorly in the same position as the fibre R2* in H. descissus (Fig. 9e). No microtubule fibre associated with basal bodies 3 or 4 was observed; however, this part of the flagellar apparatus was not examined in detail.

The dorsal and lateral surfaces of the cell were supported by a fan of microtubules (dorsal fan; Figs 8c, 10f, h). The dorsal fan originated apically, above the basal bodies (Fig. 9f). A few microtubules, putatively derived from the dorsal fan, participated in the support of the right side of the feeding groove (Fig. 10c, d). Two groups of microtubules, collectively referred to as ‘X’, were observed in a single cell (Fig. 7e). They originated at basal bodies 1 and 2 and ran posteriorly.

**Phylogenetic analysis of SSU rDNA**

The SSU rDNA sequence of strain AND of M. minor n. sp. (but not strain TINI of the same species) and strain TSUKIM of H. salinus n. sp. included a single group 1 intron. It was 431 bp in length and inserted after position 1207 in AND (GenBank accession no. KF840521), and 853 bp in length after position 1245 in TSUKIM (KF840535). These positions were homologous to those of group 1 introns of the organisms ‘Pseudomastigamoeba longifilum’ (AF011465), Pleurostomum flabellatum (DQ979962), Acrasis helenhennesi (GU437219; its second intron) and Pharyngomonas kirbyi (HQ898857; its first intron). Tetramita-specific helix 17_1 in the secondary structure of the SSU rDNA molecule was found in all newly determined sequences (not shown) except for strain COORONG of M. disparata n. sp., whose determined part of the sequence started in the helix E23_14.

The phylogenetic tree of Heterolobosea as inferred from SSU rDNA sequences is shown in Fig. 11. The overall phylogeny of Heterolobosea was consistent with previous analyses (Park & Simpson, 2011; Brown et al., 2012; Pánek et al., 2012; Park et al., 2012; Harding et al., 2013). Monophyletic clade VI of Tetratmitia as defined by Pánek et al. (2012) was recovered and was statistically highly supported – maximum-likelihood bootstrap support (BS) 91, Bayesian posterior probability (BPP) 1. The family Psalteriomonadidae as defined by Pánek et al. (2012) appeared robustly monophyletic as well (BS 100, BPP 1).

The family Psalteriomonadidae was closely related to a clade formed by the genera Vrihiamoeba, Oramoeba and Stachyamoeba, and by an uncultured heteroloboseid, WIM43; the monophyly of the clade (BS 70, BPP 0.98) as well as its relationship with the family Psalteriomonadidae (BS 52, BPP <0.9) was only poorly supported.

The family Psalteriomonadidae split into three robust clades (monophyly of each clade was supported by BS ≥94, BPP 1), whose interrelationships were not resolved. The first clade was formed by strains of the genus Pseudoharpagon. Pseudoharpagon pertyi was sister to Pseudoharpagon longus n. sp. Genetic distance (p distance) between particular Pseudoharpagon species ranged between 0.228 and 0.269. The intraspecific genetic distance within Pseudoharpagon pertyi and Pseudoharpagon tertius n. sp. was 0.194 and 0.062, respectively. The second clade of the family Psalteriomonadidae was formed by Sawyeria, Psalteriomonas and two environmental sequences. The genera Sawyeria and Psalteriomonas were robustly monophyletic (BS 100, BPP 1) and appeared closely related (BS 91, BPP 1). The third clade of the family Psalteriomonadidae was formed by the genera Harpagon and Monopylocystis. The genus Harpagon was robustly monophyletic (BS 100, BPP 1) and split into three lineages represented by H. descissus, H. schusteri and H. salinus n. sp., respectively; the former two species were closely related with maximum support. The mean genetic distance between H. salinus n. sp. and H. descissus, and H. salinus n. sp. and H. schusteri was 0.218 and 0.215, respectively.

The genus Monopylocystis appeared monophyletic, although its monophyly was unsupported (BS<50, BPP<0.9). The genus Monopylocystis split into six lineages represented by M. visvesvarai, M. minor n. sp., M. disparata n. sp., M. similis n. sp., M. robusta n. sp. and ‘Pseudomastigamoeba longifilum’. The relationships between the particular lineages were largely unresolved, except for the highly supported close relationship between M. robusta n. sp. and ‘Pseudomastigamoeba longifilum’ (BS 100, BPP 1). The genetic distance between particular Monopylocystis species (when introns were removed) ranged between 0.056 (strain LAGOS1M of M. disparata n. sp. and strain EVROS1M of M. similis n. sp.) and 0.192 (strain TINI of M. minor n. sp. and ‘Pseudomastigamoeba longifilum’). Maximum intraspecific genetic distance was within M. visvesvarai (0.026). The genetic distance between M. robusta n. sp. and ‘Pseudomastigamoeba longifilum’ was 0.120.

To examine relationships within the family Psalteriomonadidae more thoroughly, a separate analysis based on the second dataset was carried out (Fig. 12). The genus Pseudoharpagon was robustly monophyletic (BS 100, BPP 1), and the close relationship between Pseudoharpagon pertyi and Pseudoharpagon longus n. sp. was highly supported (BS 95, BPP 1). The clade of Monopylocystis and Harpagon received relatively high support (BS 86, BPP 1). Monophyly of the genus Harpagon and the close relationship between H. descissus and H. schusteri were
recovered with maximum support. Monophyly of the genus *Monopylocystis* remained only weakly supported (BS 62, BPP 0.99). The relationships between particular *Monopylocystis* species were better resolved than in the analysis based on the first dataset. *M. robusta* n. sp. and *Pseudomastigamoeba longifilum* formed a robust clade that was sister to the remaining *Monopylocystis* species. *M. disparata* n. sp. and *M. similis* n. sp. formed a moderately supported clade (BS 79, BPP 0.99). *M. minor* n. sp. was closely related to *M. visvesvarai* (BS 82, BPP 0.99).

**Fig. 10.** Transmission electron micrographs of *Pseudoharpagon pertyi* EVROS2N. Anterior part of the cell in transverse section showing R2 after separation of oR2 and iR2 (a) and detail of putative microfibrillar string S (b); section posterior to (b) showing microtubules of dorsal fan supporting the feeding groove (c); detail of these microtubules (d); section posterior to (c) showing nucleus surrounded by rough endoplasmic reticulum (e); transverse section through the cell below the nucleus showing widened feeding groove and dorsal fan of microtubules (f); detail of some oR2 microtubules (rotated image when compared with f) (g); detail of some microtubules of the dorsal fan (h). Bars, 500 nm (a), 100 nm (b), 500 nm (c), 200 nm (d), 1 μm (e, f), 200 nm (g, h). Ax, axonemes of flagella; S, putative microfibrillar string S; DB, small dense body; FV, food vacuole; iR2, inner part of microtubular root 2; N, nucleus; RER, rough endoplasmic reticulum; oR2, outer part of microtubular root 2; arrows, microtubules of R2; arrowheads, microtubules belonging to dorsal fan; asterisks, mitochondrion-related organelles; double arrowheads, microtubules of dorsal fan supporting the feeding groove.
Fig. 11. Unrooted phylogenetic tree of Heterolobosea based on SSU rDNA sequences. The tree was reconstructed by the maximum-likelihood method in RAxML (GTR+GAMMA1 model). Values at branches represent statistical support as bootstrap values (RAxML)/posterior probabilities (MrBayes). Support values below 50/0.90 are represented by an asterisk (*). Seven main clades of the subphylum Tetramitia are labelled. Newly determined sequences are in bold type. Bar, 0.1 substitutions per nucleotide position.
Species identities of strains

Recent discoveries of multiple heteroloboseid species and genera suggest that the diversity of this group, especially in non-canonic environments, is only poorly known (e.g. De Jonckheere et al., 2011a, b; Brown et al., 2012; Pánek et al., 2012; Park et al., 2012; Harding et al., 2013). Our data indicate that this is also true for anaerobic heteroloboseids. We have isolated and cultured 16 strains from brackish/marine/saline environments. To assess the phylogenetic position of the newly obtained isolates, we determined and analysed their SSU rDNA sequences, currently the only phylogenetic marker with a sufficient sampling of Heterolobosea. Phylogenetic analyses showed that all the strains belong to the family Psalteriomonadidae sensu Pánek et al. (2012). Based on light-microscopic morphology and phylogenetic position, our isolates belong to eight species.

We assign most (twelve) strains to the genus Monopylocystis. O’Kelly et al. (2003) defined the genus Monopylocystis by the nuclear structure of the amoeba and morphology of the cyst. Later, Pánek et al. (2012) discovered the flagellate stage of Monopylocystis. However, only two strains of Monopylocystis amoebae (from which only one formed cysts) and a single strain of flagellates were available until the present study.

Our new data allow a more precise description of all three stages of members of the genus Monopylocystis. The amoeba is taxonomically the least important stage of heteroloboseids (see Pánek & Čepička, 2012). Nevertheless, the amoeba of Monopylocystis is distinct enough to be easily recognized. It differs from amoebae of species of the genus Psalteriomonas and Sawyeria marylandensis in the structure of the nucleus. The nucleolar material of Monopylocystis amoebae is peripheral, distributed in a thin layer beneath the nuclear membrane (Smirnov & Fenchel, 1996; O’Kelly et al., 2003). By contrast, amoebae of Sawyeria marylandensis, Psalteriomonas lanterna and Psalteriomonas magna possess one or two parietal nucleoli in the nucleus (O’Kelly et al., 2003; Pánek et al., 2012). Although amoebae of Pseudoharpagon longus n. sp. possess the same nuclear structure as amoebae of species of the genus Monopylocystis, the latter species is unique by the absence of clear demarcation between hyaloplasm and granuloplasm. The flagellates of species of the genus Monopylocystis possess a single longitudinal feeding groove that occupies almost the whole ventral side. A similar (although somewhat shorter) ventral groove was described in two species of the genus Psalteriomonas, Psalteriomonas lanterna and Psalteriomonas vulgaris (Broers et al., 1990, 1993). However, the genera Monopylocystis and Psalteriomonas are not closely related, and in phylogenetic trees of SSU rDNA they are separated by at least the genus
Harpagenon, whose ventral groove is much shorter. Finally, we describe here cysts of all known Monopylocystis species except for M. anaerobica. All display the same morphology, i.e. a single prominent pore with a plug, and differ only slightly in diameter.

We classify our strains of the genus Monopylocystis into five species. With at least six species in total (we did not isolate M. anaerobica), Monopylocystis becomes the largest genus among the Psalteriomonadidae. Particular Monopylocystis species are morphologically similar to each other, but significant differences can be found. Five strains belong to the type species M. visvesvarai. Amoebae of M. visvesvarai strains are variable in length and width, suggesting that cell diameters alone are not good taxonomic markers. Unlike the type strain, many amoebae of all novel M. visvesvarai strains produce a uroid, sometimes with filaments of various lengths. Presence/absence of the uroid was important for O’Kelly et al. (2003) to distinguish between their newly described species M. visvesvarai and M. anaerobica (then known as Vaikampia anaerobica; Pánek et al., 2012 transferred it to the genus Monopylocystis). The second important characteristic was the shape of the nucleus, which was reported to be more angular in the latter species (O’Kelly et al., 2003). However, we show here that the nucleus of all Monopylocystis species except for M. elegans n. sp. is deformed to some extent during the locomotion. Moreover, the nuclear morphology of M. visvesvarai and M. anaerobica seems, in fact, to be quite similar (compare Figs 1 and 2 in O’Kelly et al., 2003 with Fig. 1–3 in Smirnov & Fenchel, 1996). The presence/absence of the uroid and shape of the nucleus cannot, therefore, be considered important in this case. By contrast, M. anaerobica differs from M. visvesvarai and all other Monopylocystis species by its ability to form rayed floating cells. The rayed floating form thus remains the only feature that defines M. anaerobica. However, if the rayed form is discovered in M. visvesvarai in the future, the two names will be synonymized with M. anaerobica (Smirnov & Fenchel, 1996) having priority.

Strains of M. minor n. sp., AND and TINI, formed a clade that was closely related to M. visvesvarai in the analysis without non-psalteriomonadid outgroups. Their amoebae were very similar to the amoebae of M. visvesvarai, although they were slightly smaller, and the nucleus seemed to be more deformed during the rapid locomotion. Importantly, flagellates of both strains were considerably smaller than those of M. visvesvarai (approx. 11 vs 16 μm). The small flagellate together with the genetic distance of 0.117 from M. visvesvarai is, in our opinion, sufficient to classify strains AND and TINI into a separate species.

M. elegans n. sp. was represented by strain EVROS1M. Its amoeba differs strikingly from all other Monopylocystis species in having the nucleus located deeper in the granuloplasm in locomotive cells. The cells were observed repeatedly and always displayed this behaviour. The nucleus is also much less deformed during the locomotion and stays approximately spherical. Since no transmission electron microscopy data from M. elegans n. sp. are available, it is impossible to decide how the shape of the nucleus is preserved; it may be surrounded by a thicker layer of the endoplasmic reticulum than in the other species. The flagellates of M. elegans n. sp. are typically slightly larger than those of M. visvesvarai, much smaller than those of M. disparata n. sp., and are considerably more variable in size. M. disparata n. sp., represented by strains LAGOS1M and FUEN4, was closely related to M. elegans n. sp., the genetic distance between the two species being 0.057. Unexpectedly, the two strains of M. disparata n. sp. were morphologically inconsistent with each other despite having almost identical SSU rDNA sequences (single nucleotide difference). The culture of FUEN4 contained amoebae and cysts that were the smallest among members of the genus Monopylocystis, whereas the only observed form of LAGOS1M was a flagellate, which, in turn, was the largest one. The culture LAGOS1M was derived from a sample that originally also contained Pseudoharpagon tertius n. sp. besides M. disparata n. sp. Later, we successfully separated the two species, and two cultures were created: LAGOS1M with Monopylocystis, and LAGOS1P with Pseudoharpagon. The culture LAGOS1M was accidentally lost after several tens of passages. Before that, its SSU rDNA had been sequenced twice independently from a purified PCR product; it was also cloned into a vector, and two clones were partially sequenced to confirm the species identity. Similarly, SSU rDNA of the culture of strain FUEN4 was sequenced three times independently during 2012. From our results, we infer that both LAGOS1M and FUEN4 contained only a single species of the family Psalteriomonadidae, M. disparata n. sp. FUEN4 and LAGOS1M are different strains, although with almost identical SSU rDNA sequences. Therefore, we cannot rule out the possibility that a potential undiscovered flagellate of FUEN4 would be small, while a potential amoeba of LAGOS1M would be large. In that case, the size of the cell would not be an appropriate taxonomic marker for M. disparata n. sp. Nevertheless, the amoeba of FUEN4 differs from the other Monopylocystis amoebae not only in size, but also in the form of the uroid, which is large and almost always bears very long, often multiple, filaments. In addition, it differs from the closely related M. elegans n. sp. in the position of the nucleus.

Strain FUEN3 clearly represents a separate species of the family Psalteriomonadidae on the basis of its phylogenetic position. Together with the organism represented by the sequence AF011462 and sometimes referred to as ’Pseudomastigamoeba longifilum’ (Nikolaev et al., 2004), it appeared closely related to a clade formed by the remaining Monopylocystis species, but the relationship remained unsupported. We, in fact, cannot exclude the possibility that M. robusta n. sp. is closely related to the genus Harpagon, or that it represents a sister lineage to Harpagon+Monopylocystis. The amoebae and cysts of strain FUEN3 are morphologically fully consistent with the genus Monopylocystis. It is, in turn, impossible to compare the morphology of FUEN3 with that
of the genus Harpagon. Therefore, we conservatively place the organism into the genus Monoplylocystis and describe it as *M. robusta* n. sp. In addition to the phylogenetic position, it can be differentiated from the other species of the genus by its considerably larger amoebae and cysts. The close relative of *M. robusta* n. sp., ‘Pseudomastigamoeba longifilum’, very likely represents a separate Monoplylocystis species due to its large genetic distance from *M. robusta* n. sp.

The genera Harpagon and Pseudoharpagon were described by Pánek et al. (2012). Members of both genera are known as pure flagellates; neither amoebae nor cysts have been reported previously. Although the two genera are not closely related in SSU rDNA trees, they are morphologically quite similar and not readily distinguishable from each other (Pánek et al., 2012). The slight differences between members of the genera Harpagon and Pseudoharpagon lie in the extent of the ventral groove (up to one half of the cell length in the former vs one half to two thirds in the latter), cell length (approx. 14 vs 17 μm), variability of cell shape (cells of Harpagon are more pleomorphic than those of Pseudoharpagon), and habitat (freshwater vs marine/brackish anoxic sediments).

Strain TSUKIM was isolated from an inland salt marsh and is placed here into the genus Harpagon as a novel species, *H. salinus* n. sp. Although flagellates, amoebae and cysts appeared in the culture, the amoebae and cysts were ascribed to *M. visvesvarai* strain TSUKIMV (see above), and only the flagellates will be further considered. The morphology of strain TSUKIM was consistent with the genus Pseudoharpagon (longer ventral groove, relatively less variable cell shape, and smaller diameter of the cells). This taxonomic assignment was further supported by the habitat from which it was isolated and by the fact that it did not survive in the freshwater-based medium ATCC 802. Surprisingly, TSUKIM formed a robust clade with members of the genus Harpagon instead of those of the genus Pseudoharpagon in SSU rDNA trees. This indicates that the morphological distinction between these two genera proposed by Pánek et al. (2012) is no longer valid, and the genus Harpagon can be currently distinguished from the genus Pseudoharpagon only by phylogenetic position. Besides the aforementioned morphological and ecological distinctiveness of *H. salinus* n. sp. among members of the genus Harpagon, it differs from the other two described Harpagon species by the structure of the nucleus. Although the nuclei of neither *H. descissus* nor *H. schusteri* were studied in detail, they obviously contained several parietal nucleoli (Fig. 2f in Brugerolle & Simpson, 2004; Fig. 6f, 7D in Pánek et al., 2012). By contrast, the nucleus of *H. salinus* n. sp. is morphologically similar to that of species of the genus Monoplylocystis (and amoebae of species of the genus Pseudoharpagon, see below).

The phylogenetic position of the genus Pseudoharpagon remains uncertain. It forms a separate lineage of the family Psalteriomonadidae in SSU rDNA trees and does not seem to be specifically related to the morphologically similar genus Harpagon. Pánek et al. (2012) isolated two strains of heteroloboseid flagellates, which they accommodated in the newly described species *Pseudoharpagon pertyi* despite the large genetic distance between them. We follow Pánek et al. (2012) and consider the two strains conspecific, because they are closely related and seem to be morphologically identical. We isolated three additional strains that cluster with the genus *Pseudoharpagon* in SSU rDNA trees. Strains LAGOS1P and MURANO3 are placed into a novel species, *Pseudoharpagon tertius* n. sp. Although cells of LAGOS1P are morphologically hardly distinguishable from *Pseudoharpagon pertyi*, the two organisms are phylogenetically separated from each other by clearly distinct *Pseudoharpagon longus* n. sp. In addition to the flagellates, amoebae were observed in the culture of LAGOS1P. Although their species identity remains uncertain, they were morphologically similar to amoebae of *Pseudoharpagon longus* n. sp. (see below) and might thus belong to *Pseudoharpagon tertius* n. sp.

Strain EVROS1I displays an unusual morphology and clearly represents a separate species, here described as *Pseudoharpagon longus* n. sp. Amoebae, flagellates and cysts have been observed in the culture, the latter two being reported for the first time for the genus. The amoebae morphologically resemble members of the genus Monoplylocystis, particularly because of the structure of the nucleus. On the other hand, the granulation of the anterior cytoplasm makes them easily recognizable. The flagellates of *Pseudoharpagon longus* n. sp. are considerably longer than those of the other *Pseudoharpagon* species. Their ventral groove often does not extend beyond the half of the cell, which further complicates morphological distinction between members of the genera Harpagon and Pseudoharpagon. The most prominent feature distinguishing *Pseudoharpagon longus* n. sp. from the other members of the family Psalteriomonadidae is the presence of five flagella in the majority of the flagellates; mastigonts of the other psalteriomonadids are invariably tetrakont (the sixteen flagella of *Psalteriononas lanterna* are arranged into four separate mastigonts). The multiflagellate cells of *Pseudoharpagon longus* n. sp. probably represent dividing forms. If true, it means that the flagellar apparatus is restored prior to the nuclear division in this species. This is in contrast with a single known dividing cell of *Pseudoharpagon pertyi* (Fig. 9M in Pánek et al., 2012), in which the nuclear division preceded duplication of flagella.

The cysts present in the culture of EVROS1I displayed a unique morphology, non-comparable to cysts of Monoplylocystis or any other heteroloboseid. Although we have observed neither encystation nor excystation in the culture, we are convinced that the cysts truly belong to the life cycle of *Pseudoharpagon longus* n. sp. They have been constantly present in the culture of EVROS1I for more than a hundred passages. Moreover, the only contaminating eukaryotic organism in the culture was *Andalucia incarcerata*. It has quite small cells measuring about 7 μm (Bernard et al., 2000). Although cysts of *A. incarcerata* are
unknown, we expect them to also be small, because cysts of the closely related species *Andalucia godoyi* are less than 5 μm in diameter (Fig. 4 in Lara et al., 2006).

**Ultrastructure of members of the genus *Pseudoharpagon***

Most heteroloboseid flagellates are tetrakonts with two pairs of flagella in a single mastigon; each pair bears a similar set of microtubular roots (Brugerolle & Simpson, 2004; Park & Simpson, 2011). Cells of *Pharyngomonas kirbyi*, a member of the subphyla Pharyngomonada, possess a plesiomorphic flagellar apparatus with R1/C fibre system, having duplicated R1/C and R2/I systems, and a possible homologue of the B fibre of other excavates (Park & Simpson, 2011). Members of the subphylum Tetramitida have lost the R1/C system. The role of R1, which normally supports the left side of the feeding groove, was assumed by an expanded R2 in the Tetramitida. The flagellar apparatus of the genus *Stephanopagon* has been substantially modified and does not resemble that of other heteroloboseids (Yubuki & Leander, 2008).

The structure of the flagellar apparatus of members of the family Psalteriomonadidae is well documented thanks to the comprehensive studies of *Psalteriomonas lanterna*, *Psalteriomonas vulgaris* and *H. descissus* (Broers et al., 1990, 1993; Brugerolle & Simpson, 2004); limited ultrastructural data are also available from the flagellates of *M. visvesvarai* (Pánek et al., 2012). These species display almost invariable structure of the flagellar apparatus, generally similar to that of other tetramitids. The cells of psalteriomonadids display a characteristic harp-shaped structure constituted by the microfibrillar bundle and R2 (Broers et al., 1990, 1993; Cavalier-Smith, 1993; Pánek et al., 2012). Although the structure was regarded as a unique feature of the Psalteriomonadidae (Pánek et al., 2012), it is also present in almost identical form in *Percolomonas cosmolopolitans* (Fenchel & Patterson, 1986).

*Pseudoharpagon* is the last genus of the family Psalteriomonadidae with known flagellate stage whose ultrastructure has not yet been convincingly reported. Bernard et al. (2000) published a single transmission electron micrograph of a heteroloboseid flagellate, which they identified as *Percalomonas descissus* (now *H. descissus*). Pánek et al. (2012) tentatively affiliated the organism with the genus *Pseudoharpagon* on the basis of its gross morphology and marine origin. However, its true identity is unclear since the diversity of flagellate psalteriomonadids in marine/saline environments is certainly greater than assumed by Pánek et al. (2012) – this is also shown by our discovery of *H. salinus* n. sp. We investigated the ultrastructure of the type strains of *Pseudoharpagon pertyi* and *Pseudoharpagon tertius* n. sp. Although the data obtained from the latter species were too limited to draw any conclusions, the results from *Pseudoharpagon pertyi* allowed comparison of its ultrastructure with the other members of the Psalteriomonadidae. The flagellar apparatus of *Pseudoharpagon pertyi* is very similar to that of other psalteriomonadids in every essential detail, including the presence of the harp-shaped structure. By contrast, *Pseudoharpagon pertyi* displays several uncommon features. Its basal bodies are underlined by a fibrous sheet. A similar sheet was documented in the aforementioned psalteriomonadid (‘electron-dense pad’; Fig. 4F in Bernard et al., 2000) and is likely to be present also in *H. descissus* (Fig. 1I, o in Brugerolle & Simpson, 2004). Interestingly, this structure was proposed as a synapomorphy of the unrelated heteroloboseid lineages Percolatea, which comprises the genera *Percolomonas* and *Stephanopagon* (Yubuki & Leander, 2008). The simultaneous presence of the sheet and harp-like structure in the family Psalteriomonadidae and class Percolatea could mean that the structures originated early in the evolution of the subphylum Tetramitida, or that the two lineages are specifically related.

In a single cell of *Pseudoharpagon pertyi*, we observed two conspicuous groups of microtubules, collectively referred to as the structure ‘X’. Microtubules in a similar position were described from *Psalteriomonas lanterna* (Broers et al., 1990), but the homology of the structures is questionable. The function of ‘X’ in *Pseudoharpagon pertyi* is unknown, but is possibly connected to initial steps of cell division.

All members of the family Psalteriomonadidae are anaerobic and possess acristate MROs that have properties of hydrogenosomes (O’Kelly et al., 2003; Brugerolle & Simpson, 2004; de Graaf et al., 2009; Barberá et al., 2010; Pánek et al., 2012). MROs of the Psalteriomonadidae display considerable variability in size and shape, both within and between species. They are globular (*M. visvesvarai*), cup-shaped (*Sawyeria marylandensis*), or irregular (*Psalteriomonas* spp.). The MROs of three known *Psalteriomonas* species form an aggregate with symbiotic prokaryotes, presumably methanogenic archaea (Broers et al., 1990, 1993; Pánek et al., 2012). Besides the aggregated MROs, *Psalteriomonas lanterna* and *Psalteriomonas magna* also possess individual MROs in the same cell (de Graaf et al., 2009; Pánek et al., 2012). Most MROs of *Pseudoharpagon pertyi* were globular, although a few cup-shaped MROs, resembling those of *Sawyeria marylandensis*, were observed. Interestingly, the MROs of *Pseudoharpagon pertyi* often contained a single structure that closely resembled a discoidal cristae. Discoidal cristae are relatively rare among the eukaryotes and, notably, are a typical feature of heteroloboseids (Page & Blanton, 1985). If the structure is homologous to the mitochondrial cristae of the aerobic Heterolobosea, it might indicate a more complex mitochondrial metabolism of *Pseudoharpagon pertyi* in comparison with other members of the Psalteriomonadidae. However, the possibility of artefacts formed during fixation has to be taken into consideration, and the presence of the cristalike structure within MROs of *Pseudoharpagon pertyi* has yet to be confirmed.

In addition to MROs, smaller, electron-dense bodies bounded by a single membrane were relatively abundant in the peripheral cytoplasm of members of the genus...
**Pseudoharpagon.** Such organelles have not been described in the other members of the Psalteriomonadidae, but similar structures were found in cells of unrelated *Pharyngomonas kirbyi* and *Selenaion koniopes* (Park & Simpson, 2011; Park et al., 2012). In the former species, the bodies were putatively classified as peroxisomes. The dense bodies of *Selenaion koniopes* (‘B bodies’ in Park et al., 2012) were assumed to be distinct from the peroxisomes, and their function is unclear.

### Taxonomic summary

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

Diagnosis: Marine, brackish or living in inland salt marshes. Uninucleate. Flagellate, cyst and amoeba stages ancestrally present, one or two stages unknown in some species. Amoeba with distinctive demarcation between granuloplasm and hyaloplasm. Flagellate tetrakont, with a ventral groove reaching the posterior end of the cell. Nucleolar material peripheral, distributed in a thin layer beneath the nuclear membrane. Cyst possessing single pore plugged with gelatinous material.

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

Included species: *M. anaerobica* (Smirnov & Fenchel, 1996) = *Vahlkampfia anaerobica* Smirnov & Fenchel 1996; *M. visvesvarai* O'Kelly, Silberman, Amaral Zettler, Nerad & Sogin 2003; *M. disparata* n. sp.; *M. elegans* n. sp.; *M. minor* n. sp.; *M. robusta* n. sp.

**Monopylocystis disparata** n. sp.


Diagnosis: *Monopylocystis* with locomotive amoeba smaller than in other species, 9 to 31 (mean 16.4) μm long. Nucleus located in the anterior part of the granuloplasm and deformed during locomotion. Uroid with multiple, long filaments. Floating form globular. Flagellate 19 to 29 (mean 24.2) μm long. Cyst with diameter 6 to 10 (mean 7.8) μm.

Type locality: Fuencaliente, La Palma Island, Canary Islands, Spain. 28° 27’ N 17° 50’ W.

Habitat: Saline and marine sediments.

Syntype: Protargol preparations of the culture FUEN4 with *M. disparata*, *Cyclidium* sp., and *Chilomastix cuspidata*, deposited in the collection of the Department of Parasitology, Charles University in Prague, Czech Republic, catalogue numbers 10/1, 10/2 and 10/14.

Etymology: *disparata* (Latin adj.) separated, divided. The name points out the disproportionality between the flagellate and amoeba stage.

**Monopylocystis robusta** n. sp.


Diagnosis: *Monopylocystis* with locomotive amoeba larger than in other species, 18 to 51 (mean 32.2) μm long. Nucleus located in the anterior part of the granuloplasm and deformed during locomotion. Floating form globular. Flagellate unknown. Cyst with diameter of 9 to 14 (mean 12.2) μm.

Type locality: Fuencaliente, La Palma Island, Canary Islands, Spain. 28° 27’ N 17° 50’ W.

Habitat: Saline sediments.

Syntype: Protargol preparations of the culture FUEN3 with *M. robusta* and an unidentified flagellate, deposited in the collection of the Department of Parasitology, Charles University in Prague, Czech Republic, catalogue numbers 10/3–10/5.

Etymology: *robusta* (Latin adj.) strong, robust (originally oaken). The amoeba of *M. robusta* n. sp. is the largest amoeba in the genus.

**Monopylocystis elegans** n. sp.

urn:lsid:zoobank.org:act:9E805F86-855B-4B91-BE4D-6DF46F19C29B.

Diagnosis: *Monopylocystis* with locomotive amoeba 16 to 51 (mean 23.2) μm long. Unique among *Monopylocystis* by the nucleus, which does not occupy a stable position in the granuloplasm and is not deformed during locomotion. Floating form globular. Flagellate 13 to 25 (mean 18.1) μm long. Cyst with diameter 7 to 11 (mean 9) μm.
Type locality: Evros delta, Greece. 40° 48’ N 26° 01’ W.
Habitat: Brackish sediments.
Syntype: Protargol preparations of the culture EVROS1M with M. elegans and an unidentified flagellate, deposited in the collection of the Department of Parasitology, Charles University in Prague, Czech Republic, catalogue numbers 9/98–9/100.

Etymology: elegans (Latin adj.) elegant, nice.

**Genus Harpagon Pánek, Silberman, Yubuki, Leander & Cepicka 2012**

Diagnosis: Freshwater and living in inland salt marshes. Uninucleate, tetrakont flagellates. Ventral groove of the flagellate up to 2/3 of the cell length. Amoeba and cyst unknown. Flagellates form several distinct morphotypes. Nucleus with several parietal nucleoli or with peripheral nucleolar layer beneath the nuclear membrane. Phylogenetically distinct from *Harpagon* Pánek, Silberman, Yubuki, Leander & Cepicka 2012; *H. salinus* n. sp.

Type species: *Tetramitus descissus* Perty 1852 by original designation.

Included species: *H. descissus* (Perty, 1852) = *Percolomonas descissus* (Perty, 1852) = *Tetramitus descissus* Perty 1852; *H. schusteri* Pánek, Silberman, Yubuki, Leander & Cepicka 2012; *H. salinus* n. sp.

**Harpagon salinus n. sp.**


Diagnosis: *Harpagon* living in inland salt marshes. Flagellates 11 to 17 (mean 13.7) μm long. Ventral groove 1/2 to 2/3 of the cell length. Amoeba and cyst unknown. Nucleolar material peripheral, distributed in a thin layer beneath the nuclear membrane.

Type locality: Einot Tsukim Nature Reserve, Israel. 31° 42’ N 35° 27’ E.
Habitat: Inland salt marsh sediments.

Syntype: Protargol preparations of the culture TSUKIM with *H. salinus* and *Monoplycocystis visvasvarai*, deposited in the collection of the Department of Parasitology, Charles University in Prague, Czech Republic, catalogue numbers 10/9–10/11.

Etymology: salinus (Latin adj.) of or belonging to salt. First member of the genus *Harpagon* isolated from a saline habitat.

**Genus Pseudoharpagon Pánek, Silberman, Yubuki, Leander & Cepicka 2012**

Diagnosis: Marine or brackish. Uninucleate. Flagellate, cyst and amoeba stages ancestrally present, one or two stages unknown in some species. Amoeba without clear demarcation between granuloplasm and hyaloplasm. Flagellate tetrakont to multiflagellate, with a ventral groove reaching up to 2/3 of the cell length. Nucleolar material peripheral, distributed in a thin layer beneath the nuclear membrane. Cysts rounded, with one or more (most often two) relatively long projections. Phylogenetically distinct from *Harpagon* Pánek, Silberman, Yubuki, Leander & Cepicka 2012.

Type species: *Pseudoharpagon pertyi* Pánek, Silberman, Yubuki, Leander & Cepicka 2012 by monotypy.

Included species: *Pseudoharpagon pertyi* Pánek, Silberman, Yubuki, Leander & Cepicka 2012; *Pseudoharpagon longus* n. sp.; *Pseudoharpagon tertius* n. sp.

**Pseudoharpagon longus n. sp.**

urn:lsid:zoobank.org:act:0436A8A6-0506-4618-B7C1-82EC0B859545.

Diagnosis: *Pseudoharpagon* with locomotive amoeba 29 to 56 (mean 38.5) μm long. Flagellate 14 to 35 (mean 20.4) μm long, predominantly with five flagella. Quadriflagellate and multiflagellate cells present. Cyst with diameter 16 to 22 (mean 19) μm.

Type locality: Evros delta, Greece. 40° 48’ N 26° 01’ E.
Habitat: Brackish sediments.

Syntype: Protargol preparations of the culture EVROSII with *Pseudoharpagon longus* and *Andalucia incarcerata*, deposited in the collection of the Department of Parasitology, Charles University in Prague, Czech Republic, catalogue numbers 10/9–10/11.

Etymology: longus (Latin adj.) long. Flagellates of *Pseudoharpagon longus* are longer and more elongated than those of the other *Pseudoharpagon* species.

**Pseudoharpagon tertius n. sp.**


Diagnosis: *Pseudoharpagon* with flagellate 10 to 22 (mean 15.5) μm long. Quadriflagellate. Amoeba stage present. Cysts unknown. Phylogenetically distinct from *Pseudoharpagon pertyi*.

Type locality: Porto Lago, Greece. 41° 00’ N 25° 06’ E.
Habitat: Marine sediments.

Syntype: Protargol preparations of the culture LAGOS1P with *Pseudoharpagon tertius*, deposited in the collection of the Department of Parasitology, Charles University in Prague, Czech Republic, catalogue numbers 8/90–8/92.

Etymology: tertius (Latin adj.) third. *Pseudoharpagon tertius* is the third described species of *Pseudoharpagon*. 
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