

Diversity of Heterolobosea

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1. Introduction

Heterolobosea is a small group of amoebae, amoebflagellates and flagellates (ca. 140 described species). Since heterolobosean amoebae are highly reminiscent of naked lobose amoebae of Amoebozoa, they were for a long time treated as members of Rhizopoda (Levine, 1980). The class Heterolobosea was established in 1985 by Page and Blanton (Page & Blanton, 1985) by uniting unicellular Schizopyrenida with Acrasida that form multicellular bodies. Later, it was suggested that Heterolobosea might be related to Euglenozoa (e.g., *Trypanosoma*, *Euglena*) instead of other amoebae (Cavalier-Smith, 1998; Patterson, 1988). This assumption based on the cell structure was supported also by early multigene phylogenetic analyses (Baldauf et al., 2000). Currently, the Heterolobosea is nested together with Euglenozoa, Jakobida, Parabasalia, Fornicata, Preaxostyla, *Malawimonas*, and *Tsukubamonas* within the eukaryotic supergroup Excavata (Hampl et al., 2009; Rodríguez-Ezpeleta et al., 2007; Simpson, 2003; Yabuki et al., 2011). The excavate organisms were originally defined on the basis of the structure of flagellar system and ventral feeding groove (Simpson & Patterson, 1999). However, Heterolobosea have lost some of these structures (Simpson, 2003).

The most important heterolobosean taxon is the genus *Naegleria* as *N. fowleri* is a deadly parasite of humans (Visvesvara et al., 2007) and *N. gruberi* is a model organism in the research of assembly of the flagellar apparatus (Lee, 2010). Both the species have been studied in detail for decades and genome sequence of *N. gruberi* was recently published (Fritz-Laylin et al. 2010). On the other hand, the other heteroloboseans are considerably understudied and undescribed despite their enormous ecological and morphological diversity. Many heteroloboseans have adapted to various extreme environments; halophilic, acidophilic, thermophilic, and anaerobic representatives have been described. Few heteroloboseans are facultative endobionts of both vertebrates and invertebrates. *Naegleria fowleri* and *Paravahlkampfia francinae* are even able to parasitize humans (Visvesvara et al., 2007, 2009). The genus *Stephanopogon*, whose members are multiflagellate, was once considered to be a primitive ciliate and was affiliated with Heterolobosea only on the basis of cell structure and phylogenetic position. Finally, acrasids have developed a simple form of aggregative multicellularity and represent the only known multicellular excavates.

2. Morphological diversity

Most heteroloboseans are unicellular and uninucleate, though several species are multinucleate at least in part of their life cycle, e.g., *Stephanopogon* spp., *Gruberella flavescens*,

Pseudovahlkampfia emersoni, *Fumarolamoeba ceborucoi*, *Willertia magna*, and *Psalteriomonas lanterna* (Broers et al., 1990; De Jonckheere et al., 2011b; Page, 1983; Sawyer, 1980; Yubuki & Leander, 2008). All heteroloboseans lack a typical, "stacked" Golgi apparatus. Mitochondria of Heterolobosea are oval, elongated or cup-shaped and possess flattened, often discoidal cristae. Few species are anaerobic and their mitochondria lack cristae. The mitochondrion of Heterolobosea is often closely associated with rough endoplasmic reticulum.

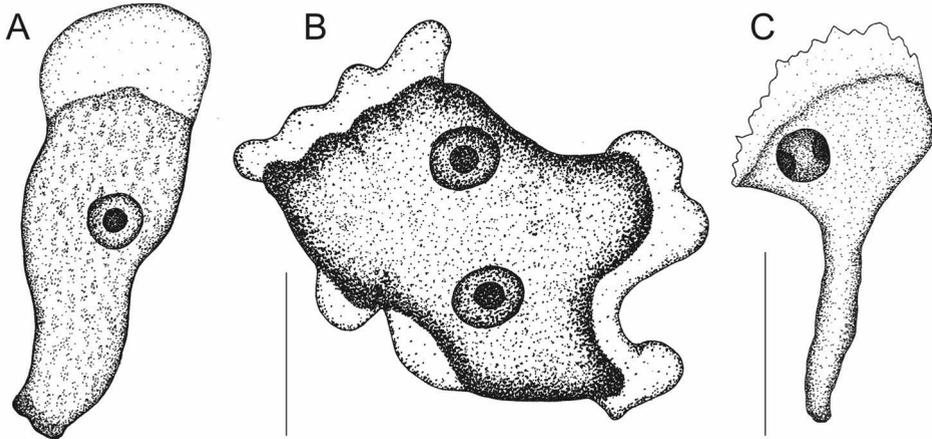


Fig. 1. Amoebae of Heterolobosea. **A**, *Acrasis rosea*; **B**, *Fumarolamoeba ceborucoi*; **C**, flabellate form of *Stachyamoeba lipophora*. Scale bars = 10 μ m. After De Jonckheere et al., 2011b; Page, 1988; Olive & Stoianovitch, 1960).

Typical life-cycle of Heterolobosea consists of amoeboid, flagellate and resting stage (a cyst). However, one or two stages are unknown and presumably have been reduced in many taxa. Heterolobosean amoebae bear no flagella. Interestingly, the flagellar apparatus including basal bodies is assembled *de novo* during the transformation to the flagellate (see below). The amoebae are relatively uniform in shape and size. The locomotive forms are usually of the "limax" type (i.e. cylindrical monopodial amoebae, see fig. 1A) and move rapidly with eruptive lobopodia. Amoebae of some species, e.g., *Fumarolamoeba ceborucoi*, form subpseudopodia in all directions (De Jonckheere et al., 2011b; see fig. 1B). The locomotive form of *Stachyamoeba lipophora* is usually flattened ("flabellate") and its single pseudopodium bears many short subpseudopodia (Page, 1987; see fig. 1C). Many heterolobosean amoebae form a posterior uroid, sometimes with long uroidal filaments. *Vahlkampfia anaerobica* was reported to form a floating form (Smirnov & Fenchel, 1996). The heterolobosean amoebae do not possess any cytoskeleton-underlain cytostomes. However, the amoeba of *Naegleria fowleri* forms so-called amoebastomes, sucker-like surface structures that aid in phagocytosis (Sohn et al., 2010). The amoeboid stage is unknown (and possibly completely lost) in genera *Lyromonas*, *Pharyngomonas*, *Pleurostomum*, *Percolomonas*, and *Stephanopogon*.

Most heterolobosean flagellates have a groove-like cytostome that rises subapically (see fig. 2A-D). On the other hand, cytostomes of genera *Tetramitus*, *Heteramoeba*, and *Trimastigamoeba* open anteriorly (fig. 2E, G). Several heterolobosean flagellates, e.g., *Tetramitus* spp. and *Heteramoeba clara*, have a distinct collar or rim that circumscribes the

anterior end of the cell body (see fig. 2E). In *Tetramitus rostratus* and *Pleurostomum flabellatum* the collar is drawn out into a short rostrum (fig. 2F). The cytostome of *T. rostratus* and *P. flabellatum* has a broad opening and curves into the cell to form a microtubule-supported tubular feeding apparatus (fig. 2F). The cytostome of *Trimastigamoeba phillippinensis* is a gullet-like tube with flagella rising from its bottom (fig. 2G). *Pharyngomonas kirbyi*, the basal-most lineage of Heterolobosea, has a subtle ventral groove and sub-anterior curved cytopharynx (fig. 2A). The cytostome of genera *Naegleria*, *Willaertia* and *Euplaesiobystra* has been reduced (fig. 2H).

Heterolobosean flagellates typically possess two (*Heteramoeba*, *Euplaesiobystra*, *Pleurostomum*, *Pocheina*, most *Naegleria* and some *Tetramitus* species) or four (*Lyromonas*, *Willaertia*, *Percolomonas*, *Pharyngomonas*, *Tetramastigamoeba*, few *Naegleria* and most *Tetramitus* species) flagella which arise at the anterior end of the feeding apparatus. Only few heterolobosean species have a different number of flagella. However, the number of flagella may vary among individuals of a single species. For example, most *Tetramitus jugosus* and *Oramoeba fumarolia* flagellates are biflagellate, but cells with more flagella (up to 10 in *O. fumarolia*) were found as well (Darbyshire et al., 1976; De Jonckheere et al., 2011a). *Psalteriomonas lanterna* has four nuclei, four mastigonts, each with four flagella, and four ventral grooves (fig. 2D). Representatives of genus *Stephanopogon* have over one hundred flagella (fig. 2I).

The flagella are usually equal in length. Alternatively, some flagella may be longer than the other ones (*Percolomonas* spp., *Pharyngomonas kirbyi*). All four flagella of *Percolomonas descissus* beat synchronously and drive water with food particles into the cytostome. *P. cosmopolitus* often attaches to the substrate by the tip of the longest flagellum. Most unattached cells move with a skipping motion across the substrate, as the trailing flagellum repeatedly makes and breaks contact with the surface (Fenchel & Patterson, 1986). Two flagella of *Pharyngomonas kirbyi* are directed anteriorly and actively beat during swimming. The cells can attach to the substrate using these flagella. The remaining two flagella are directed posteriorly and beat slowly. They are used during feeding to drive the water into the cytostome (Park & Simpson, 2011).

In quadriflagellate heteroloboseans, the basal bodies of flagella are arranged into two linked similar dikinetids rather than a single tetrakinetid. Such an unusual organization of the mastigont is called "double bikont". The arrangement of the pairs between each other can be orthogonal (e.g., *Tetramitus rostratus*), in tandem (e.g., *Percolomonas descissus*) or side-by-side (*Pharyngomonas kirbyi*, *Percolomonas sulcatus*). The arrangement of basal bodies in a pair can be orthogonal (*Pharyngomonas kirbyi*), parallel or near parallel (other heteroloboseans) (Brugerolle & Simpson, 2004; Park & Simpson, 2011). The flagellar apparatus of most heterolobosean flagellates possesses only two structures characteristic for Excavata as defined by Simpson (2003). In contrast, the mastigont of *Pharyngomonas kirbyi*, the deepest-branching heterolobosean, is more plesiomorphic and displays additional two or three excavate features (for details see Park & Simpson, 2011). The arrangement of basal bodies within a pair of flagella also seems to be more plesiomorphic in *Pharyngomonas* than that of the other heteroloboseans. In addition, the flagellar apparatus of *Percolomonas sulcatus* seems to be more plesiomorphic as well and is the most obvious example of the double bikont organization (Brugerolle & Simpson, 2004; Park & Simpson, 2011). On the other hand, it lacks the additional excavate features observed in *Ph. kirbyi*.

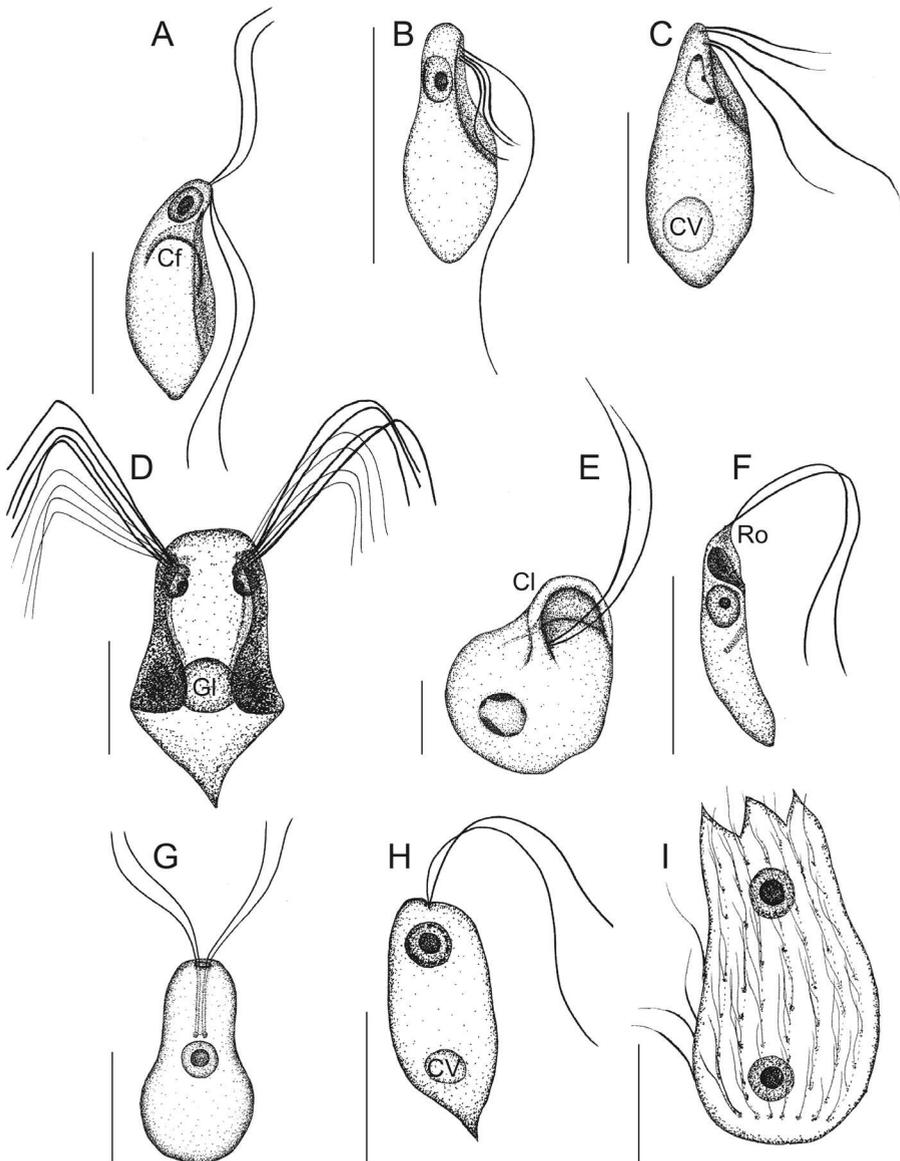


Fig. 2. Heterolobosean flagellates. **A**, *Pharyngomonas kirbyi*; **B**, *Percolomonas cosmopolitus*; **C**, *Percolomonas descissus*; **D**, *Psalteriomonas lanterna*; **E**, *Heteramoeba clara*; **F**, *Pleurostomum flabellatum*; **G**, *Trimastigamoeba philippinensis*; **H**, *Naegleria gruberi* **I**, *Stephanopogon minuta*. Cf - cytopharynx; Cl - collar; CV - contractile vacuole; Gl - globule of hydrogenosomes; Ro - rostrum. Scale bars = 10 μ m. After Broers et al., 1990; Bovee, 1959; Brugerolle & Simpson, 2004; Droop, 1962; Fenchel & Patterson, 1986; Page, 1967, 1988; Park et al., 2007; Park & Simpson, 2011; Yubuki & Leander, 2008.

Members of the eukaryovorous genus *Stephanopogon* are strikingly different from the other heteroloboseans. Their vase-shaped and curved cell bodies possess several longitudinal rows of flagella and two isomorphic nuclei. The cytostome is slit-shaped, dorsally supported by a lip, and accompanied by ventral barbs in most species. The ventral side of the cell bears more than 100 flagella, while only ca. 13 flagella arise from the dorsal side (Yubuki & Leander, 2008).

The cyst is the third heterolobosean life stage. The cyst wall usually consists of two layers, ectocyst and endocyst. They are either closely associated to each other or can be separated and thus easily recognized by light microscope. The surface of the cyst is wrinkled, rough or smooth, and can be sticky (e.g., in *Paravahlkampfia*). Most heterolobosean cysts have no pores and presumably excyst by a wall rupture as in representatives of *Paravahlkampfia* (Visvesvara et al., 2009). The cyst of genera *Tulamoeba* and *Monopylocystis* has a single pore that penetrates the wall and is sealed with a mucoid plug (Park et al., 2009). Members of genera *Willaertia*, *Naegleria*, *Marinamoeba*, *Pernina*, and *Euplaesiobystra* have pores in the cyst wall. The cyst pores of *Naegleria*, *Willaertia* and *Pernina* are similar to each other in that the pores penetrate both the endocyst and ectocyst. In contrast, the pores of *Euplaesiobystra hypersalinica* do not penetrate the endocyst wall (Park et al., 2009). The cyst morphology of the genus *Tetramitus*, including the presence and number of pores, is highly variable. In many heteroloboseans the cyst stage is unknown (*Pleurostomum*, *Neovahlkampfia*, *Sawyeria*, *Psalteriomonas*, *Lyromonas* etc.).

Members of the Acrasidae have developed an additional stage in their life cycle, a simple multicellular fruiting body (sorocarp) formed by an aggregation of amoebae. The Acrasidae is the only known multicellular lineage of Excavata. The cells are in the mature sorocarp differentiated into two types: basal stalk cells and distal spore cells. Unlike in *Dictyostelium* (Amoebozoa: Dictyosteliida), where the stalk-forming cells undergo programmed cell death, the stalk-forming cells of the Acrasidae do not lose their viability. The most studied species of multicellular heteroloboseans is *Acrasis rosea*. Its sorocarps are complex with many branches ("arborescent"; fig. 4). Fruiting bodies of the recently described species *A. helenhemmesae* are simpler, uniseriate, and with only two or three bottle-shaped stalk cells (Brown M.W. et al., 2010). In contrast, the sorocarps of the putative acrasid *Pocheina flagellata* are globular (Olive et al., 1983).

3. Life cycles of Heterolobosea

Present knowledge on the life cycle of most heteroloboseans is fragmentary and it has been studied in detail only in *Naegleria gruberi*. The main active stage of *N. gruberi* is the amoeba which relies on actin-based cytoskeleton (Walsh, 2007) and has no flagella, basal bodies or cytoplasmic microtubules. It normally feeds, moves, and divides. Under certain conditions the amoeba rapidly transforms to the flagellate or the cyst stage (fig. 3). The transformation to the flagellate stage is triggered by various stressors, such as changes in temperature, osmolarity or availability of nutrients. The flagellate of *N. gruberi* is a temporary stage persisting only for several hours. It does not divide and feed, and has no cytostome. This is a typical feature of most *Naegleria* species. In *Naegleria minor* and *Naegleria robinsoni*, however, the juvenile flagellates possess four flagella and divide once to form biflagellate cells similar to flagellates of the other *Naegleria* species (De Jonckheere, 2002). It was reported that the flagellate of *N. gruberi* plays a crucial role in shuttling from the benthos to the water surface

(Preston & King, 2003). Interestingly, the whole microtubule cytoskeleton of the flagellate, including flagella and their basal bodies, is formed *de novo* during the transformation from the amoeba (Fulton, 1977; Fulton & Dingle, 1971; Lee, 2010). The transformation is incredibly fast being completed within ca. two hours.

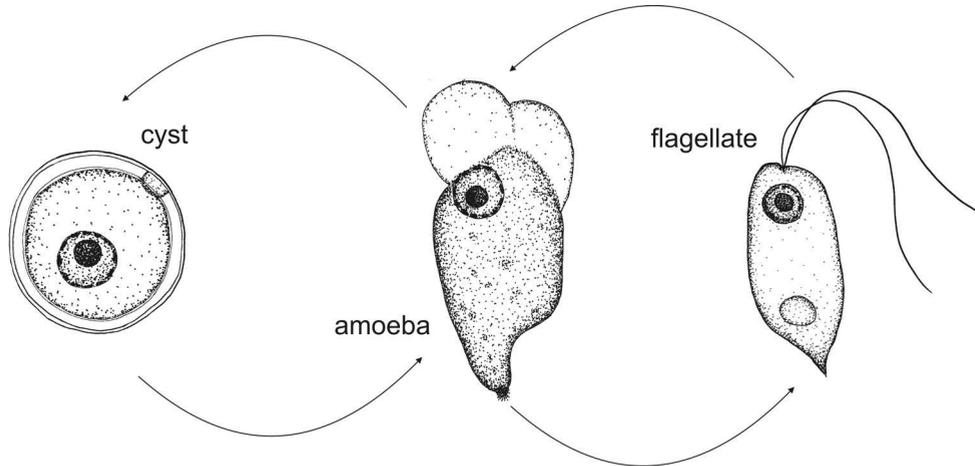


Fig. 3. The life cycle of *Naegleria gruberi*. After Page, 1967.

In contrast to *Naegleria*, flagellates of many other heteroloboseans are able to feed and divide, sometimes for long periods without reverting to the amoeba. In some heteroloboseans the flagellate is the only trophic stage and the ability to form amoebae has been presumably lost. On the other hand, the flagellate is unknown from even more heteroloboseans (see above). The ability to encyst is usually connected with the amoeba stage. The exceptions are *Percolomonas cosmopolitus* and *Stephanopogon* spp. which lack the amoeboid stage and encyst as flagellates (Fenchel & Patterson, 1986; Lwoff, 1936; Raikov 1969).

The amoeba-to-flagellate transformation of many heterolobosean species may be more or less successfully induced *in vitro* (see Page, 1988). In other species, the attempts were unsuccessful. The ability to form flagellates *in vitro* may vary also within the genus. For example, although most *Naegleria* and many *Tetramitus* species are known to produce flagellates, it seems that some of them have lost the ability (e.g., De Jonckheere, 2007; De Jonckheere et al., 2001). However, as culture requirements of almost no heteroloboseans have been studied in detail, some of them may be unable to transform *in vitro* and the observed inability to form certain life stages can be thus artificial. Indeed, strains of particular species known to produce flagellates (*Psalteriomonas lanterna*, *Heteramoeba clara*, *Willaertia*, some *Tetramitus* species) or cysts (*Percolomonas cosmopolitus*) were observed to lose the ability after a prolonged cultivation (Broers et al., 1990; Droop, 1962; Fenchel & Patterson, 1986; Page, 1988). In addition, De Jonckheere et al. (2011a) showed that the number of transformed flagellates of *Oraamoeba fumarolia* depends on the type of bacterial prey.

The life cycle of acrasids is different from those described above and contains an additional multicellular stage, the sorocarp (see above, fig. 4). Individual amoebae of acrasids normally feed on bacteria. When starving, the amoebae aggregate to form sorocarps (Bonner, 2003).

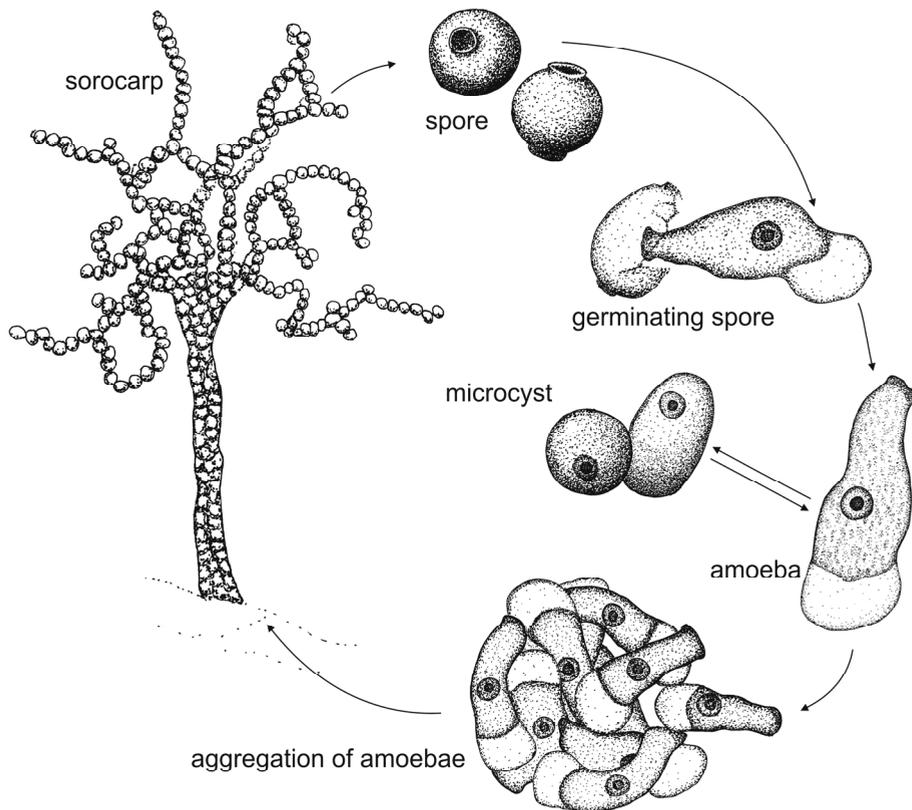


Fig. 4. Life cycle of *Acrasis rosea*. After Olive & Stoianovitch, 1960.

Both slightly different cell types of the fruiting body (basal stalk cells and distal spore cells) are capable of germination through excystment (Bonner, 2003; Brown M.W. et al., 2010). Both cell types of *Pocheina flagellata* are able to produce both amoebae and flagellates (Olive et al., 1983). The formation of multicellular bodies by the aggregation of individual cells is not unique feature of acrasids and has been well documented in several unrelated eukaryotic groups (see Brown M.W. et al., 2011) including the metazoan *Buddenbrockia plumatellae* (Morris & Adams, 2007). Unlike in *Dictyostelium*, the most-studied organism with the aggregative multicellularity, no motile ("slug") stage is formed during the ontogenesis of sorocarps of acrasids and their stalk-forming cells do not undergo the programmed cell death.

The question of the sexuality of Heterolobosea has not been elucidated yet. Although the sexual reproduction of several heterolobosean species has been discussed (e.g., Bunting, 1926; Droop, 1962; Fritz-Laylin et al. 2010; Olive et al., 1961; Olive, 1963; Pernin et al., 1992), no direct evidence has been found and the nature of the putative sexual processes remains unclear. Some authors hypothesize that the amoeba is the diploid stage whereas the flagellate is haploid and represents the gamete (Droop, 1962; Fulton, 1993). In fact, majority of the experiments and observations have to be revised and repeated using modern techniques.

The strongest evidence for sexuality of Heterolobosea has been brought by studies on *Naegleria*. Pernin *et al.* (1992) investigated the genetic structure of a natural population of *N. lovaniensis* by an isoenzyme analysis of 71 strains isolated in France. Analysis of single locus variation revealed that most strains were close to Hardy-Weinberg equilibrium. It indicated segregation and recombination between alleles. Recovery of relatively high number of distinct genotypic associations and the absence of linkage disequilibrium between genotypes at the different loci also supported the existence of recombination. In addition, the level of heterozygosity in *N. gruberi* genome was reported as typical for sexual organism and most of meiotic genes defined by Ramesh (2005) were discovered (Fritz-Laylin *et al.*, 2010). On the other hand, wild populations of *Naegleria gruberi* and *N. australiensis* showed large departures from Hardy-Weinberg equilibrium, low levels of heterozygosity, and strong linkage disequilibrium (Pernin & Cariou, 1997). These findings led to the conclusion that *N. gruberi* and *N. australiensis* have a predominantly clonal genetic structure in the wild. Different species of *Naegleria* thus could have different reproductive strategies.

4. Ecology of Heterolobosea

Heteroloboseans are heterotrophic protists that inhabit a wide range of different habitats worldwide. Most heteroloboseans are bacteriovores, although cannibalism was reported in some species. Members of genus *Stephanopogon* are able to feed on diatoms and other eukaryotes. Most species of Heterolobosea live in soil and freshwater sediments. The number of marine species (30 - 50‰ salinity) is relatively low (e.g., *Neovahlkampfia damariscottae*, *Stephanopogon* spp., *Monopylocystis visvesvarai*, *Pseudovahlkampfia emersoni*). On the other hand, adaptations to various non-canonical environments occurred repeatedly in several heterolobosean lineages.

Heteroloboseans play a very important role in hypersaline habitats. About one third of species of heterotrophic protists recorded from this environment belong to Heterolobosea (e.g., *Pleurostomum* spp., *Pharyngomonas kirbyi*, *Euplaesiobystra hypersalinica*, and *Tulamoeba peronaphora*). However, the halophilic species do not form monophyletic group and differ in the response to various salinity levels. *Tulamoeba peronaphora* grows in the culture at 75‰ - 250‰ salinity, *Pharyngomonas kirbyi* up to 250‰, *Euplaesiobystra hypersalinica* and *Pleurostomum flabellatum* flourish in more than 300‰ salinity. The latter two species are true extremophiles, because they live in nearly salt-saturated solutions (Park *et al.* 2007, 2009; Park & Simpson, 2011). Some other heteroloboseans have adapted to extremely acidic habitats with pH < 3. Sheehan *et al.* (2003) detected DNA of *Naegleria* sp. from a thermal stream with pH 2.7. Amaral Zettler *et al.* (2002) discovered DNA of uncultured *Paravahlkampfia* sp. from the River of Fire (pH of 2.0). Another heterolobosean DNA sequences were reported in a recent study of the River of Fire (Amaral-Zettler *et al.*, 2011). The only cultured acidophilic heterolobosean is *Tetramitus thermacidophilus* isolated from an acidic hot spring. This species flourishes at pH from 1.2 to 6 with the optimal pH of 3.0 (Baumgartner *et al.*, 2009). Particular heterolobosean species differ in the range of temperature at which they are able to grow. Many of them are thermophilic. For example, *T. thermacidophilus* and *Oramoeba fumarolia* grow in temperature up to 54 °C (Baumgartner *et al.*, 2009; De Jonckheere *et al.*, 2011a). *Marinamoeba thermophila*, *Fumarolamoeba ceborucoi* and *Euplaesiobystra hypersalinica* grow up to 50 °C (De Jonckheere *et al.*, 2009, 2011b; Park *et al.*, 2009). Several heteroloboseans, importantly including pathogenic *Naegleria* strains, survive and divide in temperatures around 40 - 45 °C (De Jonckheere 2007; Guzmán-Fierros *et al.*,

2008; Park et al., 2007, 2009). In contrast to thermophilic heteroloboseans, there are also few reports on psychrophilic species adapted to cold environments. The growth optimum of *Vahlkampfia signyensis* is 10 °C and the cells die when the temperature exceeds 20 °C (Garstecki et al., 2005). *Tetramitus vestfoldii* isolated from microbial mat of a brackish Antarctic lake grows at 5 °C (Murtagh et al., 2002).

Representatives of at least two heterolobosean lineages have adapted to the life in anoxic/microoxic habitats (i.e. habitats without oxygen/with low concentration of oxygen). Mitochondria of most of them do not possess cristae. The first lineage is represented by the extreme halophile *Pleurostomum flabellatum*, the second one is more diversified and comprises *Psalteriomonas lanterna*, *Sawyeria marylandensis*, *Monopylocystis visvesvarai*, and most probably also *Percolomonas descissus*, *Lyromonas vulgaris* and *Vahlkampfia anaerobica* (Broers et al., 1990, 1993; O'Kelly et al., 2003; Smirnov & Fenchel, 1996). Mitochondrial derivatives of *Psalteriomonas lanterna* and *Sawyeria marylandensis* were studied in detail and it was shown that they have been transformed to hydrogenosomes (Barberà et al., 2010; de Graaf et al., 2009). Interestingly, presumably aerobic *Naegleria gruberi* recently appeared to be a facultatively anaerobic protist. Its mitochondria possess cristae and a genome, and are probably equipped to function in both aerobic and anaerobic conditions (Fritz-Laylin et al., 2010; Ginger et al., 2010; Opperdoes et al., 2011).

Some heteroloboseans were reported to be endobionts or even pathogens of both invertebrates and vertebrates including humans. *Naegleria fowleri* causes primary amoebic meningoencephalitis (PAM, PAME, see Visvesvara et al., 2007), rare (235 reported cases worldwide, see De Jonckheere, 2011), but rapidly fatal disease of humans and other mammals. The total number of cases has been probably underestimated because *N. fowleri* lives in warm waters and it could be expected that most cases occur in tropical regions where the possibility of diagnosis is limited (De Jonckheere, 2011). Humans are typically infected while recreating in warm fresh water. In contrast to other CNS-infecting amoebae, *N. fowleri* infects primarily healthy individuals. The amoebae entry the central nervous system through the olfactory neuroepithelium and destroy host cells. Without prompt diagnosis and intervention, the patients die usually within two weeks of exposure; about 97 % of patients do not survive the infection. In addition to *N. fowleri*, pathogenicity was suggested also for *N. australiensis* and *N. italica* on the basis of tests on mice (De Jonckheere, 2002). There is a single report on PAM-like disease caused by *Paravahlkampfia francinae* (Visvesvara et al., 2009). In contrast to PAM caused by *N. fowleri*, the affected patient recovered within a few days. Several strains of *Vahlkampfia* sp., *Tetramitus ovis* and *Paravahlkampfia* sp. were isolated from keratitis patients (Aitken et al., 1996; Alexandrakis et al., 1998; De Jonckheere & Brown S., 2005a; Dua et al. 1998; Kinnear, 2003; Ozkoc et al., 2008; Walochnik et al., 2000). However, their importance in pathogenesis is unclear and no direct evidence of their pathogenicity was indicated. Heteroloboseans were found also in the gut of animals (e.g., *Tetramitus* spp., *Paravahlkampfia ustiana*, *Percolomonas sulcatus*) and gills, skin, and internal organs of fish (*Naegleria* spp.) (e.g., Brugerolle & Simpson, 2004; Dyková et al., 2001, 2006; Schuster et al., 2003).

5. Taxonomy of Heterolobosea

The taxon Heterolobosea was created by Page & Blanton (1985) as a class unifying orders Schizopyrenida (limax-type amoebae, often with the flagellate stage) and Acrasida (aggregative amoebae forming multicellular sorocarps) on the basis of the common presence

of limax amoeba with eruptive lobopodia, discoidal mitochondrial cristae, and the absence of a stacked Golgi apparatus. However, it was later shown that several organisms with different morphology are closely related to the Heterolobosea (*Pharyngomonas*) or even form its internal branches (*Percolomonas*, *Stephanopogon*, *Lyromonas*, *Psalteriomonas*, *Pleurostomum*). Currently, two concepts of Heterolobosea, here called Heterolobosea *sensu lato* and Heterolobosea *sensu stricto*, respectively, exist. The concept of Heterolobosea *sensu lato* emphasizes monophyletic taxa and includes all aforementioned genera in Heterolobosea. It means, in fact, that Heterolobosea *sensu lato* is a group containing all descendants of the last common ancestor of *Pharyngomonas* and *Naegleria*. This concept is currently favored by most authors and we follow it as well.

In contrast, some authors emphasize the original definition of Heterolobosea *sensu* Page & Blanton (1985). The absence of mitochondrial cristae and microbodies in *Lyromonas*, *Sawyeria*, and *Monopylocystis*, the presumed absence of the amoeboid stage in *Percolomonas*, *Stephanopogon* and *Pharyngomonas* (but not in *Pleurostomum*), and the different arrangement of flagella of *Pharyngomonas* are considered so important that these genera cannot be members of Heterolobosea. Instead, they are classified in separate classes closely related to the Heterolobosea *sensu stricto*. The taxon corresponding to the Heterolobosea *sensu lato* was named Percolozoa (see Cavalier-Smith, 1991, 1993, 2003). The most recent version of this concept is represented by Cavalier-Smith & Nikolaev (2008). They divide the phylum Percolozoa into four classes: Pharyngomonadea (*Pharyngomonas*), Percolatea (*Percolomonas*, *Stephanopogon*), Lyromonadea (*Lyromonas*, *Psalteriomonas*, *Sawyeria*, *Monopylocystis*), and Heterolobosea (the rest of genera). The latter three classes are united within the subphylum Tetramitia whereas *Pharyngomonas* is the only member of the subphylum Pharyngomonada. Although we do not follow this concept because Heterolobosea *sensu stricto* is highly paraphyletic, we accept the division of Heterolobosea (*sensu lato*) into Pharyngomonada and Tetramitia as it is supported by phylogenetic analyses (see below).

The Pharyngomonada comprises a single family, Pharyngomonadidae, with two species of the genus, *Pharyngomonas*. Pharyngomonads are flagellates with four flagella; amoebae and cysts are unknown. In contrast to Tetramitia, basal bodies within a pair are arranged orthogonally. A large pharynx opens into the anterior end of the longitudinal ventral groove. The mastigont system of *Pharyngomonas* is more plesiomorphic than that of Tetramitia and shows more features of typical excavates (Park & Simpson, 2011).

Synapomorphies of Tetramitia include parallel or nearly parallel basal bodies in a pair and a specific 17-1 helix in the secondary structure of SSU rRNA molecule (Cavalier-Smith & Nikolaev, 2008; Nikolaev et al., 2004). Tetramitia are currently classified into seven families though some authors recognize only three families of Heterolobosea (e.g., Patterson et al., 2002; Smirnov & Brown, 2004). In addition, several heterolobosean genera have not been assigned to any family (e.g., *Oramoeba*, *Fumarolamoeba*, *Pernina*, *Tulamoeba*, *Euplaesiobystra*). The paraphyletic family Vahlkampfiidae contains most heterolobosean genera (e.g., *Allovahlkampfia*, *Fumarolamoeba*, *Marinamoeba*, *Naegleria*, *Neovahlkampfia*, *Paravahlkampfia*, *Pseudovahlkampfia*, *Solunitrus*, *Tetramastigamoeba*, *Tetramitus*, *Vahlkampfia*, and *Willaertia*). It was defined by the presence of amoebae of the limax type and persistence of the nucleolus during mitosis. In addition, the genus *Pleurostomum*, whose amoeboid stage is unknown or has been lost, was placed within Vahlkampfiidae on the basis of its phylogenetic position (Park et al., 2007). In contrast to Vahlkampfiidae, the nucleolus of members of the family

Gruberellidae (genera *Gruberella* and *Stachymoeba*) disintegrates during mitosis. Members of two monotypic families, Lyromonadidae and Psalteriomonadidae, are anaerobic and possess acristate mitochondria. Park et al. (2007) suggested that Lyromonadidae might be a synonym of Psalteriomonadidae. *Lyromonas vulgaris* forms only flagellates with a single kinetid and longitudinal ventral groove, while *Psalteriomonas lanterna* is able to form also amoebae and its flagellates possess four mastigonts, nuclei and longitudinal ventral grooves. The anaerobic genera *Monopylocystis* and *Sawyeria* which were not placed into any family in the original description (O'Kelly et al., 2003) are sometimes placed into family Lyromonadidae or Psalteriomonadidae (Cavalier-Smith, 2003; Cavalier-Smith & Nikolaev, 2008). However, the authors did not specify in which family they classify the genera. The genus *Percolomonas* (family Percolomonadidae) comprises flagellates with four flagella (three in one species) and a longitudinal ventral groove. The amoeba stage is unknown or has been lost. The genus *Percolomonas* is most probably polyphyletic (see later). The peculiar eukaryovorous flagellates of the genus *Stephanopogon* with two nuclei and multiplied flagella comprise the family Stephanopogonidae. Finally, heteroloboseans forming multicellular sorocarps have been accommodated within the family Acrasidae. Cells of their sorocarps are differentiated into morphologically distinct spores and stalk cells.

In addition to the aforementioned families, the family Guttulinopsidae comprising genera *Guttulinopsis* and *Rosculus* was sometimes affiliated with Heterolobosea (e.g., Smirnov & Brown, 2004), though many authors do not consider them as heteroloboseans (Page & Blanton, 1985).

The concept of the genus level of Heterolobosea was historically based on the cyst morphology and the presence/absence of the flagellate stage. All vahlkampfiids which do not form flagellates and whose cysts do not have pores were classified within the genus *Vahlkampfia* (see Brown S. & De Jonckheere, 1999). However, results of phylogenetic analyses showed that *Vahlkampfia* is polyphyletic. Consequently, genera *Paravahlkampfia*, *Neovahlkampfia*, *Allovahlkampfia*, *Fumarolamoeba*, and *Solumitrus* were created to accommodate species of *Vahlkampfia*-like morphology (Anderson et al., 2011; Brown S. & De Jonckheere, 1999; De Jonckheere et al., 2011b; Walochnik & Mulec, 2009). In addition, several former *Vahlkampfia* species were transferred to the genus *Tetramitus* (Brown & De Jonckheere, 1999). Moreover, *Vahlkampfia anaerobica* described by Smirnov & Fenchel (1996) is morphologically almost identical with *Monopylocystis visvesvarai* and is likely congeneric or conspecific with it. Phylogenetic position of several described *Vahlkampfia* species remains unknown. This applies also on the type species, *V. vahlkampfi*. Therefore, Brown & De Jonckheere (1999) nominated *V. avara* as a new type species of *Vahlkampfia*. However, such a change is not possible according to the International Code of Zoological Nomenclature and the current status of the genus *Vahlkampfia* is chaotic.

Although we agree with the classification of organisms of *Vahlkampfia*-like morphology into several independent genera based solely on molecular-phylogenetic analysis, we do not recognize the genus *Solumitrus* as it is closely related to *Allovahlkampfia* with identical morphology. The genetic distance between *S. palustris* and *A. spelaea* SSU rDNA is 93.7 % (Anderson et al., 2011) which is comparable to genetic distances within other heterolobosean genera. The situation is more complicated by the fact that *Allovahlkampfia* itself has been defined solely on the basis its phylogenetic position (Walochnik & Mulec, 2009). The authors did not include the genus *Acrasis* into their analysis and it was showed later that it is the

closest relative of *Allovahlkampfia* (Brown M.W. et al., 2010; present study). The morphology of *Acrasis* and *Allovahlkampfia* amoebae has not been compared and it cannot be ruled out that *Allovahlkampfia spelaea* and *Solunitrus palustris* are, in fact, members of the genus *Acrasis* with unknown or reduced multicellular stage.

The genus *Percolomonas* is even more problematic than *Vahlkampfia*. It was created by Fenchel & Patterson (1986) for the species *P. cosmopolitus* (originally described as *Tetramitus cosmopolitus*) and was later broadened to accommodate flagellates with one long and three shorter flagella inserting at the anterior end of a longitudinal feeding groove (Larsen & Patterson, 1990). In addition, the tritragellate *P. denhami* was described later (Tong, 1997). However, it was soon realized that most *Percolomonas* species were totally unrelated to the type species *P. cosmopolitus*. They were removed from *Percolomonas* and accommodated in genera *Trimastix* (Preaxostyla), *Carpediemonas* (Fornicata), which is, in fact, biflagellate, *Chilomastix* (Fornicata), and *Pharyngomonas* (Heterolobosea) (Bernard et al., 1997, 2000; Cavalier-Smith & Nikolaev, 2008; Ekeboom et al., 1996). Moreover, the mastigont structure of *P. sulcatus* and *P. descissus* is so different from that of *P. cosmopolitus* (Brugerolle & Simpson, 2004) that they should be removed from the genus *Percolomonas* as well. The phylogenetic position of the remaining *Percolomonas* species (*P. denhami*, *P. similis* and *P. spinosus*) is uncertain and no sequence data are currently available. Finally, *P. cosmopolitus*, the only certain member of the genus *Percolomonas*, is possibly paraphyletic (see below) and its true identity is unknown.

6. Species concept of Heterolobosea

Ca. 140 species of Heterolobosea have been described so far. The species were originally distinguished on the basis of light-microscopic morphology. The cyst was the most important life stage as its morphological variability was sufficient to recognize most then-known species (see Page, 1988). Unlike the cysts, amoebae of particular heterolobosean species are usually indistinguishable and have been rarely used for the species identification. Finally, although Page (1988) stressed the importance of heterolobosean flagellates in taxonomy as their morphology is quite diverse (see above), their descriptions were often insufficient and cannot be considered in taxonomical studies. Moreover, the flagellates are unknown and presumably absent in many heteroloboseans.

Since it is often impossible to differentiate between pathogenic and non-pathogenic *Naegleria* strains solely on the basis of their morphology, various biochemical and immunological methods have been applied (see Page, 1988). Later on, several new species of *Naegleria* were described on the basis of molecular markers (see De Jonckheere, 2002, 2011). This continuous process reached a peak in the explicit formulation of a molecular species concept of the genus *Naegleria* based on the ITS region (De Jonckheere, 2004). The concept was soon expanded to cover the whole Vahlkampfiidae (De Jonckheere & Brown S., 2005b). It was shown that vahlkampfiid ITS region is extremely variable. Interestingly, even strains with identical SSU rDNA sequences may slightly differ in the ITS region (Baumgartner et al., 2009; De Jonckheere & Brown S., 2005b). According to De Jonckheere & Brown S. (2005b), almost any two vahlkampfiid strains differing in ITS1, 5.8S rDNA or ITS2 sequences should be classified as different species even when their morphology and ecology are identical. The difference in the ITS region was often used as an accessory criterion in addition to morphological identification. However, several vahlkampfiid species were described solely

on the basis of minute differences in the ITS region without morphology being effectively involved. This was stated e.g. in the diagnosis of *Naegleria canariensis* (De Jonckheere, 2006): "Because of the morphological similarity of the cysts with those of other *Naegleria* spp. molecular identification is required. The species can be identified from the ITS2 sequence, which differs by 2 bp substitutions from that *N. gallica*. The ITS1 and 5.8S rDNA sequence is identical to that of *N. gallica*.". Similarly, *Tetramitus anasazii* and *T. hohokami* were distinguished solely on the basis of few differences in the ITS region (De Jonckheere, 2007).

Although we agree that the ITS region represents a quite effective DNA barcode of the Vahlkampfiidae and can be used for rapid determination of strains belonging to already described species, we are convinced that the concept is misleading when used for new species description. The biggest problem is that it assumes zero level of intraspecific polymorphism within the Vahlkampfiidae. However, the true variability of a vast majority of vahlkampfiid species is virtually unknown. It was convincingly shown that certain closely related *Naegleria* species differ in the ITS region sequence (De Jonckheere, 2004). There is, however, no reason to believe that it is true for the whole genus *Naegleria* or even for the whole Vahlkampfiidae, and, on the other hand, that the ITS region displays no intraspecific polymorphism. Indeed, it is a well-documented fact that there is some degree of ITS polymorphism within certain Vahlkampfiidae species. ITS regions of different strains of *Tetramitus jugosus* differ in a single nucleotide (De Jonckheere et al., 2005). Despite strains of *Naegleria fowleri* are even more variable, they are considered to be conspecific instead of belonging to several different species (De Jonckheere, 2004, 2011). Dyková et al. (2006) even showed that there exists an intragenomic polymorphism in *Naegleria clarki*. All these examples show that the ITS region should be no longer used for new species descriptions until the problem of species concept and intraspecific polymorphism within Heterolobosea is settled.

7. Phylogeny of Heterolobosea

Although many heterolobosean species have been successfully transferred into culture and many strains have been deposited into culture collections, the phylogeny of Heterolobosea has not yet been satisfactorily elucidated. Virtually all molecular-phylogenetic analyses with reasonable taxon sampling are based on a single locus, SSU rDNA (e.g., Brown M.W. et al., 2010; Cavalier-Smith & Nikolaev, 2008; De Jonckheere et al., 2011a, 2011b; Nikolaev et al., 2004; Park & Simpson, 2011; Park et al., 2007, 2009). To evaluate the resolving power of SSU rDNA, we performed a phylogenetic analysis which included members of all heterolobosean genera whose sequences are available, and important environmental sequences affiliated with Heterolobosea. Results of our analysis (fig. 5) were in agreement with previous studies.

Heterolobosea robustly split into two lineages, Pharyngomonada and Tetramitia. We divide here the Tetramitia into six clades whose interrelationships and internal phylogeny remain unresolved. Unfortunately, all the clades but E are currently indefinable on the basis of morphology. The current heterolobosean taxonomy is not consistent with delineation of these six monophyletic groups and has to be revised in the future. The family Vahlkampfiidae is highly paraphyletic.

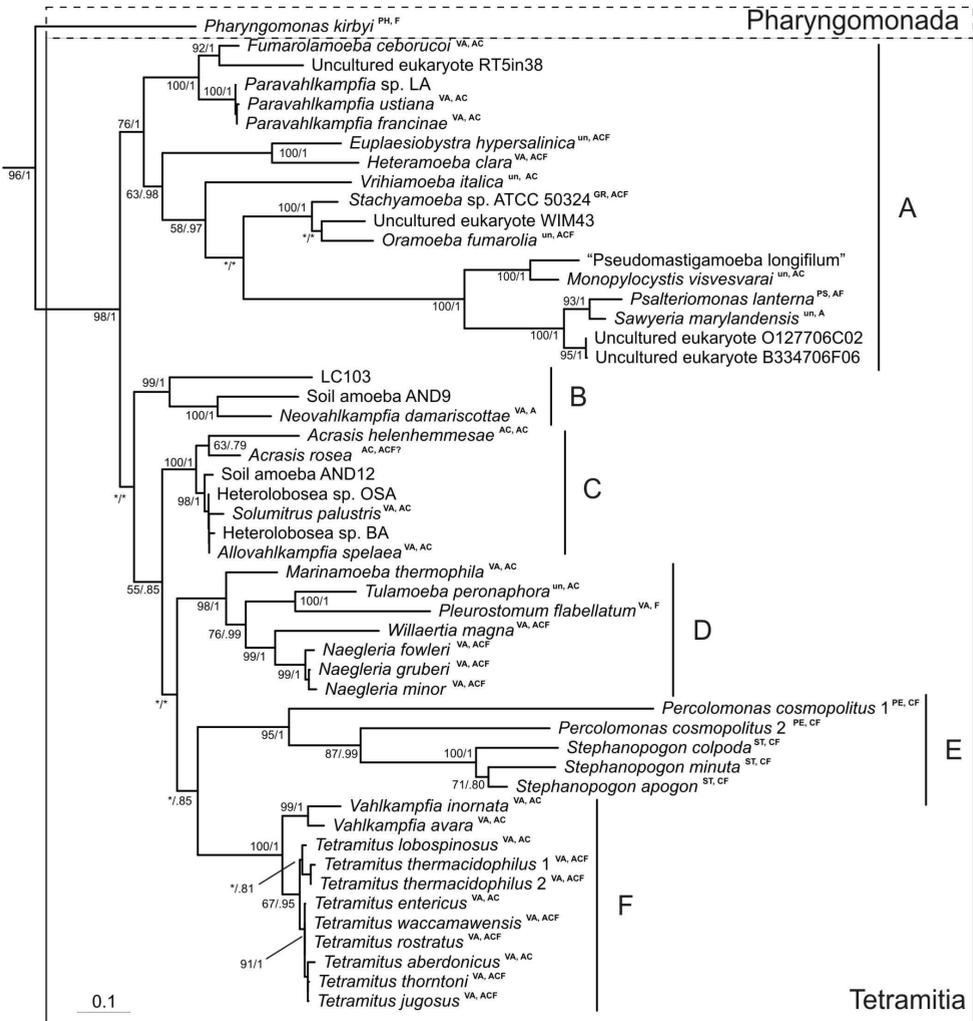


Fig. 5. Phylogenetic tree of Heterolobosea based on SSU rDNA sequences. The tree topology was constructed by the maximum likelihood method (ML) in RAxML 7.2.6 under the GTRGAMMAI model, and by the Bayesian method in MrBayes 3.1.2. under the GTR + Γ + I + covarion model. RAxML 7.2.6 was used for bootstrapping (1000 replicates). The tree was rooted with representatives of other Excavata lineages (outgroups were removed from the tree). The values at the nodes represent statistical support (ML bootstrap values/Bayesian posterior probabilities). Support values below 50%/.50 are represented by asterisks. Heterolobosea are divided into two branches (Pharyngomonada and Tetramitia). Six main clades of Tetramitia are labeled. The classification is indicated: AC - Acrasidae, GR - Gruberellidae, PE - Percolomonadidae, PH - Pharyngomonadidae, PS - Psalteriomonadidae, ST - Stephanopogonidae, un - unassigned, VA - Vahlkampfiidae, The occurrence of particular life stages is indicated: A - amoeba, C - cyst, F - flagellate.

Clade A consists of genera *Paravahlkampfia*, *Funarolamoeba*, *Euplaesiobystra*, *Heteramoeba*, *Stachyamoeba*, *Vrihiamoeba*, *Oramoeba*, *Psalteriomonas*, *Sawyeria*, *Monopylocystis*, and several undetermined heteroloboseans (strains RT5in38, WIM43, and 'Pseudomastigamoeba longifillum', O127706C02, B334706F06). Moreover, morphological data suggest that *Vahlkampfia anaerobica*, *Lyromonas vulgaris*, and *Percolomonas descissus* belong to this clade as well (see above). Many members of the clade A display a unique morphology of the nucleolus. Typical heteroloboseans have nucleus with a single central nucleolus. In contrast, *Heteramoeba*, *Sawyeria*, *Monopylocystis*, *Stachyamoeba*, *Percolomonas descissus*, *Vahlkampfia anaerobica*, and at least some strains of *Psalteriomonas* possess parietal nucleoli or a thin ring of nucleolar material near the nuclear membrane. *Neovahlkampfia damariscottae* and undetermined heteroloboseans AND9 and LC103 constitute the clade B. Although it is quite robust (bootstrap support 99), its position within Tetramitia is uncertain. In some previous analyses the clade formed the basal branch of Tetramitia (Brown M.W. et al., 2010; Park & Simpson, 2011; Park et al. 2007, 2009) while it branched more terminally in the others (Cavalier-Smith & Nikolaev; 2008; De Jonckheere et al., 2011a, 2011b; Nikolaev et al., 2004; this study). *Acrasis*, *Allovahlkampfia*, *Solomitrus* and undetermined heteroloboseans BA, OSA, and AND12 formed tetramitian clade C. All representatives of the clade inhabit freshwater sediments and soil (however, the data are unavailable for strains BA and OSA). Members of the clade D (*Marinamoeba*, *Tulamoeba*, *Pleurostomum*, *Willartaia*, and *Naegleria*) live in wide range of habitats. Interestingly, at least some members of all the genera are able to grow at higher temperatures (40 – 50 °C). The Clade E comprises *Percolomonas cosmopolitus* and the genus *Stephanopogon* (i.e. *Percolatea sensu* Cavalier-Smith & Nikolaev, 2008). Both *Stephanopogon* and *P. cosmopolitus* form long branches in the phylogenetic trees and it cannot be ruled out that their grouping is, in fact, a result of long-branch attraction. However, Yubuki & Leander (2008) identified three morphological features shared by *Stephanopogon* and *P. cosmopolitus* suggesting that the clade E might be monophyletic. In addition, both *Stephanopogon* spp. and *P. cosmopolitus* have lost the amoeba stage and their ability to encyst as flagellates is unique among heteroloboseans. Tetramitian clade F is formed by the remaining genera *Vahlkampfia* and *Tetramitus*. Morphology and ecology of the genus *Tetramitus* is extremely diverse including characteristics used for generic determination (presence and number of cyst pores, number/presence of flagella, marine or freshwater lifestyle, etc.).

Our view on the evolution of Heterolobosea has completely changed after the application of methods of molecular phylogenetics. The analyses of sequence data are currently the only efficient tool for pinpointing relationships between species and genera although it is unable to resolve interrelationships between particular tetramitian clades. The analyses suggested that some morphologically well-defined genera (e.g., *Vahlkampfia* and *Percolomonas*) were polyphyletic. On the other hand, *Tetramitus* spp. is so diverse that it was impossible to group them into a single genus solely on the morphological base. Although it is currently impossible to define morphological synapomorphies of particular tetramitian clades, Heterolobosea itself and both its subphyla, Tetramitia and Pharyngomonada, seem to be well defined on both molecular and morphological level.

8. Conclusion

During the last decade, Heterolobosea have attracted considerable interest because of their extraordinary morphological, ecological and physiological diversity. Members of the most

studied genus *Naegleria* are medicinally important or became model organisms in cell biology. The insight obtained from the genome sequence of *N. gruberi* considerably improved understanding of the early eukaryotic evolution. However, our knowledge about Heterolobosea as a whole is still seriously limited. Since it is currently unclear whether Heterolobosea are sexual or asexual organisms, the biological species concept is not applicable. On the other hand, the current species concept of Heterolobosea based on the ITS region is misleading and most probably considerably overestimates the real number of extant species. Heterolobosean phylogeny is unclear as well. Although the monophyly of Heterolobosea and its split into Pharyngomonada and Tetramitia is strongly supported by both cell structure and molecular-phylogenetic analyses, the internal phylogeny of Tetramitia has not yet been satisfactorily elucidated. Since 18S rDNA has not sufficient resolving power, it is necessary to perform multigene phylogenetic analyses in order to improve the heterolobosean phylogeny. In addition, it is important to obtain sequence data from so-far uncharacterized, potentially important taxa, such as *Percolomonas sulcatus* and *Gruberella flavescens*. There is also a strong possibility that some already-known enigmatic eukaryotes will be shown to belong to Heterolobosea as well. This has already happened in the case of the ciliate-resembling genus *Stephanopogon*. Finally, it is a well-known fact that the current taxonomy of Heterolobosea, particularly the family level, does not reflect the phylogeny and should be changed. This cannot be, however, achieved before the heterolobosean phylogeny is resolved.

9. Acknowledgment

This work was supported by grants from the Czech Ministry of Education, Youth and Sport of the Czech Republic (project MSM0021620828), the Czech Science Foundation (project P506/11/1317) and the Grant Agency of Charles University (project 21610). We would like to thank Pavla Slámová for preparing the line drawings.

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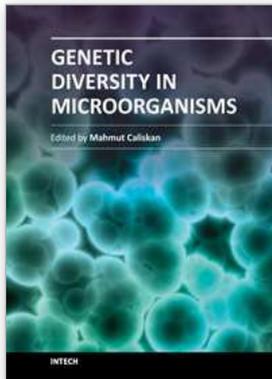
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Genetic Diversity in Microorganisms

Edited by Prof. Mahmut Caliskan

ISBN 978-953-51-0064-5

Hard cover, 382 pages

Publisher InTech

Published online 24, February, 2012

Published in print edition February, 2012

Genetic Diversity in Microorganisms presents chapters revealing the magnitude of genetic diversity of microorganisms living in different environmental conditions. The complexity and diversity of microbial populations is by far the highest among all living organisms. The diversity of microbial communities and their ecologic roles are being explored in soil, water, on plants and in animals, and in extreme environments such as the arctic deep-sea vents or high saline lakes. The increasing availability of PCR-based molecular markers allows the detailed analyses and evaluation of genetic diversity in microorganisms. The purpose of the book is to provide a glimpse into the dynamic process of genetic diversity of microorganisms by presenting the thoughts of scientists who are engaged in the generation of new ideas and techniques employed for the assessment of genetic diversity, often from very different perspectives. The book should prove useful to students, researchers, and experts in the area of microbial phylogeny, genetic diversity, and molecular biology.

How to reference

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Tomáš Pánek and Ivan Čepička (2012). Diversity of Heterolobosea, Genetic Diversity in Microorganisms, Prof. Mahmut Caliskan (Ed.), ISBN: 978-953-51-0064-5, InTech, Available from:
<http://www.intechopen.com/books/genetic-diversity-in-microorganisms/diversity-of-heterolobosea>

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