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Molecular Phylogeny and Classification of the Lobose Amoebae

Introduction

The lobose amoebae can be defined as presumably asexual amoeboid organisms, producing lobopodia and possessing a simple life cycle, involving only cell division and, in some species, encystment. They move due to the activity of the acto-myosin cytoskeleton; cytoplasmic microtubules, if present, are rare and not organized in bundles (Grebecki 1994; Page 1988, 1991; Rogerson and Patterson 2002; Smirnov and Goodkov 2000).

Amoeboid movement involves the entire cell body, and thus lobose amoebae possess neither a stable cell shape nor differentiated locomotory organelles. As a result, the number of distinctive morphological features in amoebae is very limited, and their morphological identification and classification remain a difficult task. Earlier phylogenetic suggestions based on light-microscopic morphology (e.g. by Schaeffer 1926) or seemingly more basic features, like the nuclear division pattern (Chatton 1953; Singh 1955) were later found to be inadequate. In an attempt to reach a more natural grouping of amoeboid taxa, T. Jahn and E. Bovee created a system based on the pattern of cytoplasmic flow (Jahn and Bovee 1965; Jahn et al. 1974). However, this attempt did not result in a practical system either, mainly because of the difficulties in defining genera and distinguishing species. The line drawings, which were the only available illustrative documentation on lobose amoebae until the early 1960s, were rather “author-specific” and often did not allow a reliable recognition of the species by other investigators. The growing number of species that were difficult to recognize (e.g. Bovee 1953; Lepşi 1960; Sawyer 1975a, b) emphasized the need to clarify the criteria of species identification among amoebae prior to any attempt to create a comprehensive system.

The involvement of microphotography and electron microscopy in the routine practice of protozoology led to the discovery of specific ultrastructural features in many amoeboid protists. It resulted in the re-description of many already known amoeboid organisms and description of numerous new species of amoebae, and allowed investigators to establish the relationships between and within some amoeboid taxa (Flickinger 1974; Page 1978, 1980a, 1985, 1986; Page and Blanton 1985). A new synthetic system of amoeboid organisms, combining light- and electron-microscopical features together with some aspects of their biology and physiology was developed (Page 1987, 1988, 1991). All naked lobose amoebae were grouped in the class Lobosea, subclass Gymnamoebia, with four orders (Euamoebida, Leptomyxida, Acanthopodida and Loboreticulatida), while the testate lobose amoebae (order Arcellinida) were placed in the subclass Testacealobosia. This system was mainly aimed at resolving the low-level relationships among amoebae and provided practical tools for species identification. However, higher-level phylogenetic relationships within amoebae remained “unrecoverable from morphology” (Page 1987), and further development of Page’s system (see Rogerson and Patterson 2002) did not improve the situation.

Molecular Phylogeny of Lobose Amoebae

Early molecular studies based on small-subunit ribosomal RNA (SSU rRNA) gene sequences suggested independent origins for different lineages of lobose amoebae (Cavalier-Smith 2000; Hinkle et al. 1994; Silberman et al. 1999;

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Sims et al. 1999). However, as the number of available sequences increased, and with the use of more accurate methods of phylogenetic analyses, it became evident that the independent branching of lobose amoebae in early phylogenies was an artifact of phylogenetic reconstructions related to the long branch attraction phenomenon. Several recent phylogenetic analyses, including a large taxon sampling of eukaryotes, and using methods correcting for among-site rate heterogeneity, showed that all sequenced lobose amoebae form a clade that generally also includes mycetozoans (Bolivar et al. 2001; Cavalier-Smith et al. 2004; Fahrni et al. 2003; Milyutina et al. 2001). Although usually weakly supported, the monophyly of lobose amoebae and mycetozoans was confirmed by phylogenetic analysis of actin (Fahrni et al. 2003), and multigene analyses of nuclear (Baldauf et al. 2000) and mitochondrial (Forget et al. 2002) protein sequences, as well as the analysis of 123 genes obtained from EST libraries of *Entamoeba*, *Mastigamoeba*, and *Dictyostelium* (Bapteste et al. 2002). However, none of these multigene studies contained representatives of all lineages of amoebae simultaneously, nor included a typical free-living lobose amoeba. In the high-level classification of Protozoa, all naked and testate lobose amoebae, together with entamoebids, pelobionts, and mycetozoans, were grouped in an emended phylum Amoebozoa (Cavalier-Smith 1998, 2002, 2003; Cavalier-Smith et al. 2004).

While several molecular markers now support a common origin of all amoebozoans, evolutionary relationships within lobose amoebae are also becoming clearer. Thanks to a rapidly increasing sampling of species, the SSU rRNA-based phylogeny of Amoebozoa allows the distinction of a few well-defined clades. Bolivar et al. (2001) demonstrated the existence of the "Gymnamoebia *sensu stricto*" containing the families Amoebidae and Hartmannellidae and the order Leptomyxida. This clade was also recovered in an analysis of actin (Fahrni et al. 2003) and in a further SSU rRNA-based study (Cavalier-Smith et al. 2004). A recent study revealed that the testate lobose amoebae of the order Arcellinida also belong to this group (Nikolaev et al. 2005). Among other well-defined clades of lobose amoebae, there is good support for the grouping of *Acanthamoeba* and *Balamuthia*, as first shown by Amaral Zettler et al. (2000) and confirmed by subsequent studies (Bolivar et al. 2001; Cavalier-Smith et al. 2004; Fahrni et al. 2003). Peglar et al. (2003) established the existence of two additional lineages, the first corresponding to the family Vannellidae, and the second comprising members of the families Paramoebidae and Vexilliferidae. There is also good evidence from SSU rRNA- and protein-based studies that all amitochondriate amoebae (entamoebids, mastigamoebids, and *Pelomyxa*) form a monophyletic group of Archamoebae (Arisue et al. 2002; Edgcomb et al. 2002; Fahrni et al. 2003; Milyutina et al. 2001). Multigene analyses of protein data strongly support the relationship between Archamoebae and Mycetozoa (Bapteste et al. 2002), classified together in the subphylum Conosa (Cavalier-Smith 1998). However, because of the fast evolving SSU rRNA gene sequences of most Archamoebae and Mycetozoa, their grouping does not appear in most phylogenies based on this gene (Cavalier-Smith et al. 2004; Fahrni et al. 2003). Finally, there is also some evidence that the free-living amoeboid protists such as *Gephyramoeba* and *Filamoeba* (Bolivar et al. 2001) or the flagellated *Phalansterium solitarium* (Cavalier-Smith et al. 2004) might be more closely related to Mycetozoa and/or Archamoebae than to other lobose amoebae.

Molecular Phylogeny versus Morphological Classification

The molecular phylogeny of lobose amoebae is broadly congruent with the morphological system at the level of genera and some families, but strongly disagrees with it starting at the level of orders and subclasses. The first attempts to find a correlation between the molecular phylogeny and the morphological characters of amoebae at higher taxonomic level generally failed (Amaral Zettler et al. 2000; Bolivar et al. 2001; Peglar et al. 2003). Only two orders of the Gymnamoebia sensu Page (1987), Acanthopodida and Leptomyxida, seem to be supported by molecular data. The other typical naked lobose amoebae (Euamoebida sensu Page 1987) are split into several lineages, one of which includes the Leptomyxida and the Arcellinida.

In a revised classification of the phylum Amoebozoa based on the SSU rRNA phylogeny, Cavalier-Smith et al. (2004) attempted to produce a system congruent with both molecular and morphological data. The authors grouped the majority of typical naked lobose amoebae in two classes: the Lobosea, with "ancestrally cylindrical and non-eruptive" pseudopods, but "sometimes flattened or eruptive", and "lacking glycostyles or scales" (e.g. the families Amoebidae and Hartmannellidae, plus the orders Leptomyxida and Arcellinida); and the Discosea, defined as "flattened amoebae with leading lamellipodium" and "usually with glycostyles or organic scales" (e.g. the families Vannellidae, Paramoebidae, Vexilliferidae, and Thecamoebidae). A third class, Variosea, was created to accommodate the Acanthopodida and the genera *Filamoeba*, *Gephyramoeba*, and *Phalansterium*. The shape of the cell and the pattern of pseudopods seem to be reasonable features to distinguish the first two classes. However, the evolutionary origin and phylogenetic significance of the various surface structures in amoebae are unclear. For example, glycostyle-like structures recognized as "sucker-like elements" (Page 1985) or pentagonal "cup-shaped elements" are present on the cell surface of *Hartmannella* and *Saccamoeba* (Anderson et al. 1997; Page 1988), which belong to Lobosea (sensu Cavalier-Smith et al. 2004), while glycostyle-like elements are completely absent in the cell coat of *Thecamoeba* and *Dermamoeba* (Page and Blakey 1979), which belong to Discosea. The wide diversity of surface structures among gymnamoebae and the fact that most of these surface structures are genus-specific suggest that they may be independent acquirements within low-level taxa. The descriptive diagnoses of taxa used by Cavalier-Smith et al. (2004) in fact are compilative listings of features that characterize lower-level taxa. They contain numerous reservations to accommodate groups that do not fit the general diagnosis and often list both of the alternative conditions for a character. This may be evidence that the basic characters congruent with the branching of molecular lineages remain non-recovered. Finally, this system contains a number of groups that appear paraphyletic or are extrapolated from the morphological evidence in the absence of molecular data.

Morphological Features of the Major Phylogenetic Lineages of Lobose Amoebae

In the present study, we revise the classification of lobose amoebae by defining the morphological characters of the phylogenetic lineages that are congruent with available molecular data. As a framework for a new system, we use a comprehensive SSU rRNA phylogeny of 52 amoebozoans, including all sequenced genera of lobose amoebae and selected sequences of Conosa (Fig. 1). We focus our study on two lineages, called here Tubulinea and Flabellinea, containing the majority of the Gymnamoebia sensu Page 1987 and the testate lobose amoebae of the order Arcellinida. We analyze the systematic composition and morphological characters of every well-defined clade and compare them with both modern and ancient systems of amoebae in order to recover the shared morphological features of each molecular lineage and to evaluate their potential phylogenetic importance.

Tubulinea (Amoebidae, Hartmannellidae, Leptomyxida, Arcellinida, and *Echinamoeba*)

The present analysis confirms once more the existence of this highly supported clade, first revealed by Bolivar et al. (2001). Four well-defined lineages can be outlined within the Tubulinea. The first one includes the families Amoebidae and Hartmannellidae; the second one corresponds to the order Arcellinida (represented in the present tree by *Heleopera sphagni* and *Centropyxis laevigata*); the third one consists of the leptomyxids; and the fourth one includes two species of *Echinamoeba* and *Hartmannella vermiformis* (Figs 2,3). The order Arcellinida appear as the sister-group to the Amoebidae+Hartmannellidae clade, while the "*Echinamoeba* and *H. vermiformis*" lineage is basal to all others.

Even a quick look at the content of the Tubulinea shows that neither the cell surface structures nor other routine morphological features can be regarded as synapomorphies of the clade, since the organisms included in it are very different with respect to their organization, morphology, and ultrastructure (Page 1980a, 1985, 1986, 1991; Rogerson and Patterson 2002). Naked amoebae belonging to this clade exhibit a variety of cell surface structures (Table 1). Within the genera *Amoeba* and *Chaos*, the glycocalyx is filamentous or amorphous (Page 1986; Smirnov and Goodkov 1997). In *Hartmannella*, the glycocalyx is amorphous, while in *Saccamoeba* it is either amorphous or contains fine hexagonal cup-shaped elements (Anderson et al. 1997; Page 1985). It should be noted that these elements can be easily destroyed during EM fixation and thus might have been overlooked in some species. Cell surface structures among Leptomyxida consist of amorphous glycocalyx of different thickness. Finally, members of the order Arcellinida

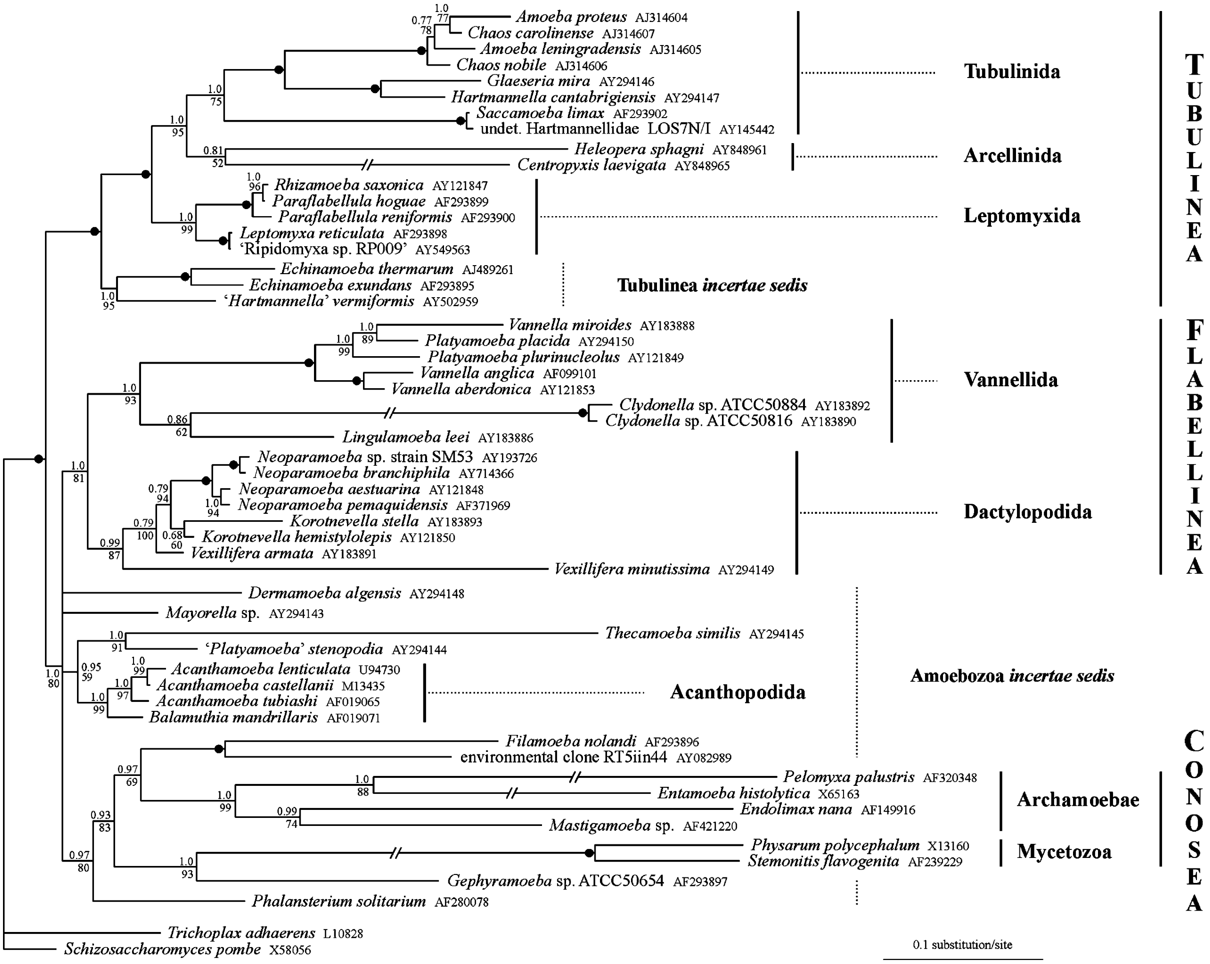

Figure 1. Molecular phylogeny of lobose amoebae inferred from a Bayesian analysis of 54 small-subunit ribosomal RNA sequences, including *Schizosaccharomyces pombe* and *Trichoplax adhaerens* as outgroup. The tree is presented in an unrooted format, with a basal trifurcation. The Bayesian analysis was conducted with MrBayes (Huelsenbeck and Ronquist 2001), using the GTR model of substitution (Lanave et al. 1984; Rodriguez et al. 1990), taking into account a proportion of invariable sites and a gamma-shaped distribution of the rates of substitution among variable sites, with eight rate categories. Four simultaneous chains were run for 1,750,000 generations and 17,500 trees were sampled, the first 1,500 of which were discarded as the burn-in. A total of 1380 unambiguously aligned positions were used in the phylogenetic analyses, and all necessary parameters were inferred from the data set. Numbers at nodes represent the posterior probabilities of the Bayesian analysis (upper values) and the bootstrap percentages (100 replicates) of a maximum likelihood analysis of the same data set with PhyML (Guindon and Gascuel 2003), using the same model (lower values). Black dots indicate the nodes supported by values of 1.0/100% with both methods; branches that were supported by values under 0.5/50% in one or both methods were collapsed in a polytomy. All branches are drawn to scale, except the branches leading to *Centropyxis laevigata*, the two *Clydonella* sequences, *Pelomyxa palustris*, *Entamoeba histolytica*, and the two mycetozoan sequences, which were reduced by half for enhanced clarity.

have an extracellular cell coat — the test. Among naked amoebae, every type of differentiated cell coat is evidently restricted to an internal lineage, such as a genus or a group of related genera (Table 1).

In contrast, observation of the locomotive patterns in these amoebae leads to interesting conclusions. Members of the families Amoebidae and Hartmannellidae have a clear monoaxial streaming of the cytoplasm in

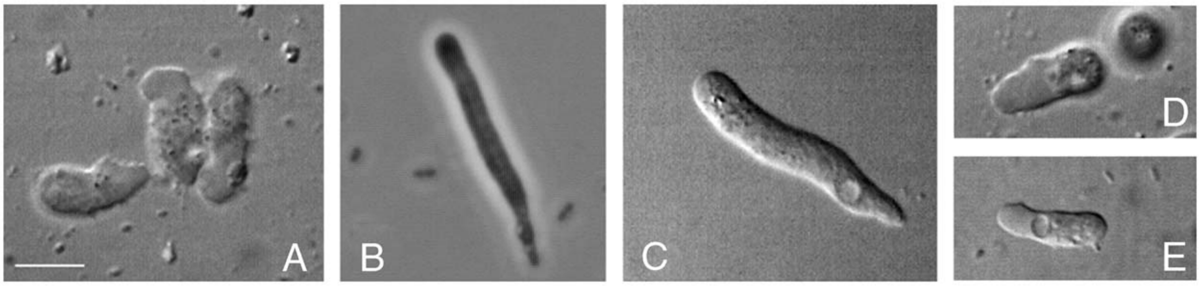

Figure 2. (*A*) *Echinamoeba exundans* CCAP 1534/4. ***B.*** *Hartmannella vermiformis* (unnamed CCAP strain). ***C.*** *Hartmannella cantabrigiensis* CCAP 1534/11. ***D***. Limax-like form of *Echinamoeba exundans*. ***E***. Limax-like form of *Echinamoeba silvestris* CCAP 1519/1. Scale bar: 10 μm. Images A and C—E are DIC, B is phase contrast.

locomotory pseudopodia. The entire cell body (in monopodial cells) or each pseudopodium (in polypodial ones) is tubular, circular, or semicircular in cross-section. Interestingly, amoebae of the genera *Leptomyxa* and *Paraflabellula* (as well as other leptomyxids) are capable, under certain conditions, to alter their locomotive form from a flattened, expanded one to a subcylindrical, monopodial one (Page 1971, 1972; Page and Willumsen 1983; Pussard and Pons 1976a, b; Smirnov 1999). When monopodial, they also demonstrate a monoaxial streaming of the cytoplasm; in some cases, with occasional eruptions at the frontal end of the cell. Moving specimens of *Echinamoeba* are flattened, have a pronounced anterior hyaline zone, and produce short, spineolate subpseudopodia ("echinopodia"); however, they can alter their locomotive morphology and assume a "limax-like", monopodial form under specific conditions, such as a deficit of oxygen (Baumgartner et al. 2003; Page 1967; our observations) (Fig. 2). Finally, the pseudopodia of testate lobose amoebae are tubular, circular in cross-section (Meisterfeld 2002). The above data lead to the suggestion that the ability to form tubular pseudopodia and to demonstrate monoaxial cytoplasmic flow in the entire cell (in monopodial naked amoebae) or in every pseudopodium (in polypodial and testate amoebae) is a synapomorphy of all mentioned lineages.

The close relationship between *H. vermiformis* and *Echinamoeba* might appear surprising, given their different locomotive morphology under normal conditions. However, *H. vermiformis* differs from the "true" Hartmannellidae by having a largely, worm-shaped form and a very well-pronounced hyaline cap which is much more stable than in other *Hartmannella* species (Fig. 2). These morphological features, as well as amphizoic tendencies (ability to live both in the environment and in animals and human), and thermotolerance in *H. vermiformis* (see Dykova et al. 1997; Kadlec 1978; Simitzis et al. 1979) suggest that its similarity to other hartmannellids may be due to morphological convergence. The diagnostic features of the genus *Hartmannella* are so broad (Page 1988) that they allow inclusion of any monopodial amoeba with a pronounced hyaline cap and no nuclear division in cysts (the latter is used to distinguish it from the genus *Glaeseria*). In view of the present analysis and previous molecular studies, we can suggest that *H. vermiformis* represents a separate, yet monotypic group of amoebae phylogenetically related to echinamoebians, and in the future, should be excluded from the genus *Hartmannella*.

Flabellinea (Vannellidae, Paramoebidae, and Vexilliferidae)

Peglar et al. (2003) first revealed the existence of two well-supported lineages of lobose amoebae; the first one comprising the family Vannellidae; and the second one containing members of the families Paramoebidae and Vexilliferidae. The present analysis, including the additional sequences of *Vexillifera minutissima* and *Platyamoeba placida* (Fahrni et al. 2003), confirms the existence of these two lineages and shows that they are sister-groups.

The family Vannellidae includes the flattened, fan-shaped, or spatulate amoebae of the genera *Vannella*, *Platyamoeba*, and *Clydonella*, together with *Lingulamoeba leei*, which is lingulate rather than fan-shaped (Fig. 3). Relationships within the

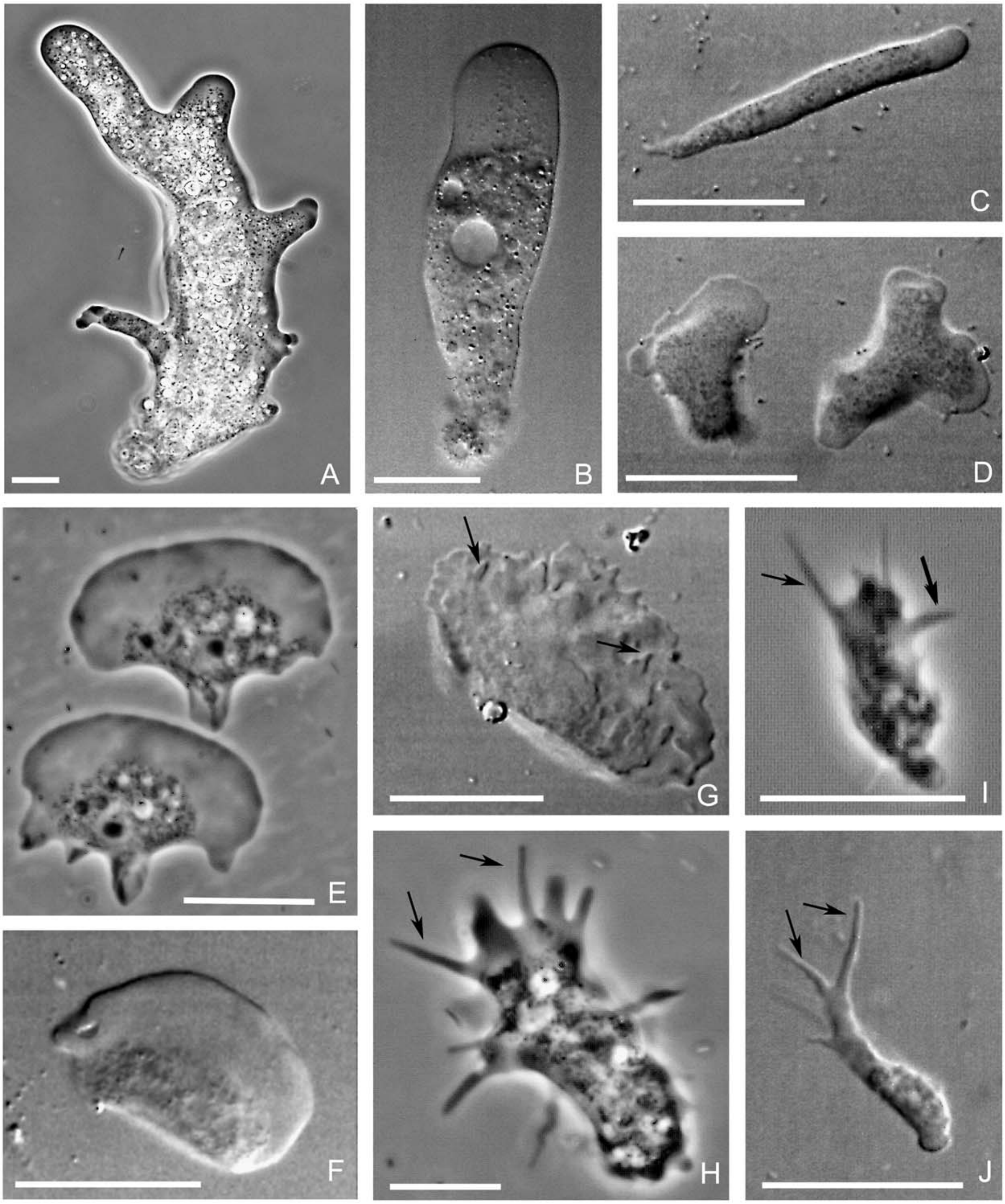

Figure 3. Representatives of the major phylogenetic lineages of naked amoebae. ***A–D.*** Tubulinea. (*A*) *Amoeba leningradensis* CCAP 1503/6 (family Amoebidae), (*B*) *Saccamoeba limax* CCAP 1534/6 (family Hartmannellidae), (*C*, *D*) two alternative forms of *Rhizamoeba saxonica* CCAP 1570/2 (family Leptomyxidae). ***E, F.*** Flabellinea, Vannellida. (*E*) *Vannella simplex* (isolated in Switzerland from a freshwater pond), (*F*) *Vannella devonica* CCAP 1589/5. ***G–J.*** Flabellinea, Dactylopodida. (*G*) *Neoparamoeba pemaquidensis* CCAP 1560/4, (*H*) *Korotnevella stella* CCAP 1547/6, (*I*) *Vexillifera bacillipedes* CCAP 1590/1, (*J*) *Vexillifera minutissima* CCAP 1590/3. Dactylopodia are denoted by arrows. Scale bar: 25 μm.

Table 1. Patterns of locomotion and cell surface structures of the naked lobose amoebae comprising the two largest monophyletic clades, Tubulinea and Flabellinea. Note the diversity of cell surface structures within each lineage, in contrast with the good homogeneity in the locomotive morphology features.

Class	Order	Genus	Cell surface structures	Pattern of locomotion
Tubulinea	Tubulinida	*Amoeba*	Filamentous or amorphous	The entire body (in monopodial species) or every pseudopodium (in polypodial ones) is subcylindrical; monoaxial flow in the entire cell (when monopodial) or in every pseudopodium (when polypodial)
		Chaos		
		Glaeseria	Amorphous	
		Hartmannella	Cup-shaped hexagonal structures or amorphous	
		Saccamoeba		
	Leptomyxida	*Rhizamoeba*	Amorphous	Same as above; some species can alter the locomotive form to a flattened one
		Leptomyxa		Normally reticulate, but can temporarily assume a monopodial locomotive form
		Paraflabellula		Normally flattened, but can temporarily assume a monopodial locomotive form
	incertae sedis	*Echinamoeba*		Flattened, but can assume a monopodial locomotive form
Flabellinea	Vannellida	*Vannella*	Pentagonal glycostyles and simple filaments	The entire body is flattened and fan-shaped; rolling movement of the cell surface; cytoplasmic flow polyaxial or without a pronounced general axis
		Platyamoeba	Short hexagonal prismatic structures	
		Clydonella	Short prismatic glycostyles	
		Lingulamoeba		
	Dactylopodida	*Korotnevella*	Boat-shaped scales	Flattened amoebae with dactylopodia of different length, formed from the frontal hyaline margin or frontal hyaline area; cytoplasmic flow polyaxial or without a pronounced general axis
		Neoparamoeba	Amorphous or pentagonal glycostyles or short hexagonal cups	
		Vexillifera	Short column-like hexagonal glycostyles	

family are well resolved, the genera *Lingulamoeba* and *Clydonella* forming the sister-group to a clade comprising *Vannella* and *Platyamoeba*. All these amoebae have a specific mode of locomotion. The flow of cytoplasm is polyaxial or has no pronounced axis and, where studied, is accompanied by a caterpillar-like rotation of the cell membrane (Hülsmann and Haberey 1973). The cell surface coat of vannellids is highly differentiated and varies among the genera. It may be composed of pentagonal glycostyles, sometimes with long hair-like filaments among them (*Vannella*), of a layer of relatively short prismatic structures (*Clydonella*, *Lingulamoeba*) (Peglar et al. 2003), or of a tightly packed short prismatic hexagonal arrangements, as in *Platyamoeba* (Page 1980b, 1983; Page and Blakey 1979) (Table 1). Interestingly, *Vannella miroides* tends to group with *Platyamoeba spp*. rather than with other vannellas, suggesting that the cell coat may not be an ultimate generic criterion for the family Vannellidae.

Resolution among the members of the genera *Korotnevella* (family Paramoebidae), *Neoparamoeba*, and *Vexillifera* (family Vexilliferidae) is poor. All *Neoparamoeba* species strongly group together, but the branching order between the different species of *Vexillifera* and *Korotnevella* is not clear. This is probably due to the very divergent sequence of *V. minutissima*, which branches at the base of the lineage in some analyses (Fig. 1) or next to *Korotnevella stella* in others (data not shown). However, this lineage makes morphological sense, as it unites flattened amoebae with a polyaxial cytoplasmic flow (the cytoplasm streams in several, more or less pronounced axes), and producing non-furcating, more of less finger-shaped subpseudopodia (dactylopodia) (Fig. 3; see also Dyková et al. 2000; Page 1979, 1981, 1983, 1988; Smirnov 1997, 1999 for illustrations). *Vexillifera* and *Korotnevella* have similar axial cores of microfilaments in their pseudopodia (Page 1987), which may indicate a general similarity of the mechanisms of pseudopodial formation in these genera. The genus *Neoparamoeba* differs from the two others by the presence of a parasome, which was however shown to be the remnant of a symbiont rather than a specific type of organelle (Dyková et al. 2003; Perkins and Castagna 1971).

In contrast with Tubulinea, the synapomorphic characters of the Flabellinea may thus be the flattened shape of the cells, the absence of tubular pseudopodia, and the polyaxial cytoplasmic flow. Again, most genera of this clade have a distinct type of cell surface structure (Table 1).

Other Lineages of Lobose Amoebae

The most consistent grouping among the remaining amoebae species in our tree is the *Acanthamoeba+Balamuthia* clade. The homogeneity of the genus *Acanthamoeba* and its close relationship with *Balamuthia mandrillaris* had already been shown and discussed (Amaral Zettler et al. 2000; Stothard et al. 1998), and our analysis further confirms this clade. Members of the Acanthopodida differ from other amoebae in possessing trilaminate cytoplamic MTOCs, and in the case of the genera *Acanthamoeba* and *Protacanthamoeba*, a specific pseudopodial pattern, the acanthopodia. Perhaps the differences in the morphology of the genus *Balamuthia* — that initially led to its classification as a leptomyxid (Visvesvara et al. 1993) — are due to the specificity of its biology (Deol et al. 2000).

As previously suggested (Smirnov and Goodkov 1994, 1999), the family Thecamoebidae does not appear to be monophyletic, because *Thecamoeba similis* and *Dermamoeba algensis* branch separately in our trees. While *D. algensis* occupies an independent position among lobose amoebae, *T. similis* groups with the sequence of a *Platyamoeba stenopodia*. It was noted long ago that *P. stenopodia* resembles thecamoebids in the locomotive morphology and some other features, prompting its transfer to the genus *Thecamoeba* (Page and Blakey 1979; Smirnov and Goodkov 1999). SSU rRNA data confirm this point of view and show that the morphological similarity between *P. stenopodia* and the vannellid *Lingulamoeba leei* may be a case of homoplasy. The exact status of the family Thecamoebidae will become clearer when more sequences of the key genera become available.

According to morphological systematics, the genus *Mayorella* belongs to the family Paramoebidae (Page 1987). However, the only species of *Mayorella* that we were able to sequence (Fahrni et al. 2003) does not branch with the *Korotnevella+Neoparamoeba+Vexillifera* clade, but represents an independent lineage. We cannot exclude that the presence of short dactylopodia in some of cuticle-bearing *Mayorella* species is due to convergence; in many other features, such as ultrastructure, locomotive and floating forms, *Mayorella* is very different from *Korotnevella*, *Vexillifera*, and *Neoparamoeba* (see Page 1981, 1983).

In our analysis, *Filamoeba nolandi* and *Gephyramoeba* sp. appear to be related to members of the Conosa, but show no close relatedness to each other; this is in contrast with the analyses by Amaral Zettler et al. (2000), Fahrni et al. (2003), and Cavalier-Smith et al. (2004). One or both of these amoebae might indeed be among the closest relatives of mycetozoans (in particular myxogastrids), in agreement with features of the plasmodial stage of *Gephyramoeba* (Visvesvara et al. 1993), a genus initially seen as a possible bridge between lobose amoebae and mycetozoans (Goodey 1915). In any case, it is interesting to note that despite the clear morphological similarity between *Filamoeba* and *Echinamoeba*, evidently these two genera do not group together, demonstrating that the family Echinamoebidae *sensu* Page 1975 is an artificial assemblage.

Higher-level Relationships within Lobose Amoebae

The relationships between the two main clades and independent lineages are poorly resolved. There is moderate support for a link between the Acanthopodida and the *Thecamoeba* lineage (which contradicts the morphological evidence), but more sequences of thecamoebids are needed before any conclusion might be reached. Likewise, the position of *Dermamoeba* and *Mayorella* is as yet completely unresolved. Interestingly, our analysis indicates that *Phalansterium solitarium* may be related to the clade comprising *Filamoeba* and *Gephyramoeba*, as suggested by some of the trees presented by Cavalier-Smith et al. (2004). Together with *Multicilia* (Nikolaev et al. unpublished data), these organisms might thus be more closely related to Archamoebae and Mycetozoa than they are to any other lineage of lobose amoebae.

In our analysis, the Tubulinea branches as a sister-clade to all other lineages of Amoebozoa. Such a scheme was suggested by Cavalier-Smith et al. (2004) because it is in accordance with the supposed absence of gene fusion between cytochrome oxidase 1 and 2 in this clade; a feature that is also shared by *Dictyostelium* and *Acanthamoeba* (Gray et al. 1998). A basal position of the Tubulinea is also supported by actin data (Fahrni et al. 2003; Nikolaev et al. 2005). However, the branching order between the main lineages of Amoebozoa in SSU rRNA analyses is not at all consistent, as shown by the different trees presented by Cavalier-Smith et al. (2004). Hence, further evidence is needed to ascertain the higher-level relationships within the phylum.

Towards a New System of Amoebozoa

The morphological analysis of the main lineages revealed by our phylogenetic analysis of SSU rRNA sequences of lobose amoebae allows us (1) to re-evaluate the importance of ultrastructural and morphological characters used in amoebae systematics and (2) to propose a new high-level system of lobose amoebae, where the taxa are based primarily on locomotive patterns (Table 2). This classification system is based on the assumption that the locomotive pattern of an amoeba is a compound character reflecting the organization of its cytoskeleton, cell coat, and perhaps a number of other basic features (Smirnov and Brown 2004; Smirnov and Goodkov 1999).

Because we found no support for the division of the phylum Amoebozoa in two monophyletic subphyla, Lobosa and Conosa, we propose to abandon these taxa. In the new system, we divide Amoebozoa into three classes. The majority of lobose amoebae belong to one of two classes: Tubulinea and Flabellinea, while Archamoebae and Mycetozoa are placed in the third class Conosea. The Tubulinea comprises amoebae that always produce or can temporarily form tubular pseudopodia with a monoaxial cytoplasmic flow, while the Flabellinea comprises flattened amoebae that never produce tubular pseudopodia and never show a monoaxial cytoplasmic flow. In the systematic content, these classes resemble Lobosea and Discosea sensu Cavalier-Smith et al. (2004), but we believe that proposed new names better reflect the major dichotomy between these taxa and prevent confusion related with the extensive use of the names “lobosa” and “lobosea” at different taxonomic ranks. In accordance with available molecular data, three orders can be distinguished among Tubulinea (Tubulinida, Leptomyxida and Arcellinida), while two orders are proposed within the Flabellinea (Dactylopodida and Vannellida). The diagnoses of these new classes and orders are provided in Table 2. A number of morphological families of amoebae sensu Page 1987 appear to be monophyletic (e.g. Amoebidae and Vannellidae), while some are paraphyletic or polyphyletic (e.g. Hartmannellidae and Vexilliferidae). A larger taxon sampling is thus required for a proper revision of existing amoebae families; however, this is outside the scopes of the present paper. To outline the systematic composition of orders but to avoid artificial groupings, all genera that do not clearly branch within the above-mentioned orders and do not have clear morphological relationships with their members are left *incertae sedis* within the appropriate higher-level taxon, as well as lineages for which no molecular data are available yet. It concerns in particular the family Thecamoebidae, which appears as a polyphyletic assemblage, and is thus not retained in the current version of our system. Genera not yet sequenced but evidently morphlogically related to those already presented in the tree are listed within the respective families as “probably belonging there”.

At the level of classes and orders, the system presented in this study shows some similarities with the system of gymnamoebae based on the pattern of amoeboid movement, which was subsequently developed by Jahn and Bovee (1965), Jahn et al. (1974) and Bovee (1985). The class Tubulinea partly corresponds to Bovee’s suborder Tubulina, which was basically defined as follows: “…body cylindroid, branched or unbranched, pseudopods granular, clear capped…’’ (Bovee 1985). The order Vannellida partly corresponds to Bovee’s suborder Thecina, but there is no full correspondence, because Bovee (1985) grouped together thecamoebids, vannellids, and some poorly known groups of amoebae possessing similar morphology. The Dactylopodida corresponds in part to Bovee’s suborder Conopodina that he defined as follows: “…body more or less triangular to spatulate, tapered, round-tipped pseudopods of determinate length formed from clear margin…” (Bovee 1985). The recent finding that all testate lobose amoebae sequenced so far are closely related to the families Amoebidae and Hartmannellidae (Nikolaev et al. 2005) show that Jahn and Bovee (1965), Jahn et al. (1974) and Bovee (1985) were right in placing testate lobose amoebae in the suborder Tubulina Bovee and Jahn, 1965. In light of this finding, other classifications splitting naked and testate lobose amoebae, such as those by Deflandre (1953), Loeblich and Tappan (1961) and Page (1987), were artificial,

Table 2. New high-rank phylogenetic classification of Amoebozoa. The genera for which no sequence data are available, but that are morphologically evidently related to sequenced species, are listed as "probably belonging" to the appropriate taxa.

Phylum Amoebozoa (Lühe, 1913) Cavalier-Smith, 1998

Class Tubulinea classis nov.
Naked or testate amoebae producing tubular, subcylindrical pseudopodia or capable of altering the locomotive form from a flattened, expanded one to a subcylindrical one. Monoaxial flow of the cytoplasm in every pseudopodium or in the entire cell. No cytoplasmic MTOCs, no flagellate stage in the life cycle.

Order Tubulinida ord. nov.
Naked amoebae producing subcylindrical pseudopodia in locomotion (or the entire cell is monopodial and subcylindrical). No alteration of the locomotive form to a flattened expanded and branched one. No adhesive uroidal structures.
Families **Amoebidae** (Ehrenberg, 1838) Page, 1987 and **Hartmannellidae** (Volkonsky, 1931) Page, 1974; genera *Amoeba, Chaos*, *Glaeseria*, *Hartmannella*, *Saccamoeba* (and probably *Cashia*, *Deuteramoeba*, *Hydramoeba*, *Nolandella*, *Parachaos*, *Polychaos*, *Trichamoeba*).

Order Leptomyxida (Pussard and Pons, 1976) Page, 1987
Naked amoebae with a locomotive form altering from a flattened, expanded, and branched one to a subcylindrical, monopodial one. Uroidal structures of adhesive type.
Families **Leptomyxidae** (Pussard and Pons, 1976) Page, 1987 and **Flabellulidae** (Bovee, 1970) Page, 1987; genera *Leptomyxa*, *Rhizamoeba*, *Paraflabellula* (and probably *Flabellula*).

Order Arcellinida (Kent, 1880)
Testate amoebae with the cell enclosed into an organic or mineral test with a single main opening (e.g. genera *Arcella*, *Centropyxis*, *Heleopera*). Systematic composition according to Meisterfeld (2002).

Tubulinea *incertae sedis*:
Echinamoeba, "*Hartmannella*" *vermiformis*

Class Flabellinea classis nov.
Flattened naked amoebae, never producing tubular, subcylindrical pseudopodia and never altering the locomotive form. Cytoplasmic flow polyaxial or without a pronounced axis. No cytoplasmic MTOCs, no flagellate stage in the life cycle.

Order Dactylopodida ord. nov.
Naked amoebae capable of producing hyaline finger-shaped subpseudopodia (dactylopodia) formed from the frontal hyaline margin or frontal hyaline area.
Families **Paramoebidae** (Poche, 1913) Page, 1987 and **Vexilliferidae** Page, 1987; genera *Korotnevella*, *Neoparamoeba*, *Vexillifera* (and probably *Paramoeba*, *Pseudoparamoeba*).

Order Vannellida ord. nov.
Naked, flattened amoebae, fan-shaped, spatulate or lingulate; the frontal zone of the hyaloplasm occupies about half of the cell, and never forms an antero-lateral crescent; in the locomotive cell, the front edge is always smooth; they do not produce discrete pseudopodia or subpseudopodia.
Family **Vannellidae** Bovee, 1979; genera: *Clydonella*, *Lingulamoeba*, *Platyamoeba*, *Vannella* (and probably *Pessonella*).

Class Conosea Cavalier-Smith, 1998 stat nov.
Diagnosis and composition according to Cavalier-Smith et al. (2004). Rank of the Cavalier-Smith's infraphylums must be decreased to infraclasses; rank of classes — to subclasses; lower level system without changes.

Amoebozoa *incertae sedis*:

Order **Acanthopodida** Page, 1976 (family Acanthamoebidae Sawyer and Griffin, 1975; genera *Acanthamoeba*, *Protacanthamoeba*, *Balamuthia*, *Comandonia*)

Families **Stereomyxidae** Grell, 1966, **Corallomyxidae** Page, 1987

Genera: *Dermamoeba, Filamoeba*, *Gephyramoeba*, *Mayorella*, *Phalansterium*, *Thecamoeba*, *"Platyamoeba" stenopodia*, *Cochliopodium*, *Flamella*, *Gocevia*, *Janickia*, *Malamoeba*, *Malpighamoeba*, *Paradermamoeba*, *Paragocevia*, *Parvamoeba*, *Pellita*, *Pseudothecamoeba*, *Sappinia*, *Stygamoeba*, *Thecochaos*, *Trichosphaerium.*

although more convenient for routine use and for constructing keys and guides.

The present analysis makes it evident that members of each major phylogenetic lineage of lobose amoebae present various cell surface structures, which probably evolved independently within low-level lineages of amoebae, approximately equal to the existing genera (Table 2). Although attractive to taxonomists because of their diversity and complex organization, cell surface structures have no phylogenetic significance at a higher level and cannot be used to reconstruct the relationships among genera and families. This explains the problems that we experienced when trying to compare the phylogenetic system of amoebae with the morphological classification by Page (1987).

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