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A Revised Classification of Naked Lobose Amoebae (Amoebozoa: Lobosa)

Introduction

Molecular evidence and an associated reevaluation of morphology have recently considerably revised our views on relationships among the higher-level groups of amoebae. First of all, establishing the phylum Amoebozoa grouped all lobose amoeboid protists, whether naked or testate, aerobic or anaerobic, with the Mycetozoa and Archamoebea (Cavalier-Smith 1998), and separated them from both the heterolobosean amoebae (Page and Blanton 1985), now belonging in the phylum Percolozoa - Cavalier-Smith and Nikolaev (2008), and the filose amoebae that belong in other phyla (notably Cercozoa: Bass et al. 2009a; Howe et al. 2011).

The phylum Amoebozoa consists of naked and testate lobose amoebae (e.g. *Amoeba, Vannella, Hartmannella, Acanthamoeba, Arcella, Difflugia*), the Variosea – a group unifying aerobic amoebae with pointed branched pseudopods (e.g. *Acramoeba*, *Filamoeba*) and a limited number of flagellates (*Multicilia*, *Phalansterium*), Archamoebea (e.g. *Entamoeba, Mastigamoeba, Pelomyxa*), Mycetozoa (e.g. *Dictyostelium*, *Physarum*, *Protostelium*), and Breviatea (*Breviata*). This review focuses specifically on naked lobose amoebae (gymnamoebae), a group of aerobic amoeboid protists, unified by forming wide, smooth, cytoplasmic projections (lobopodia), driven by an actomyosin cytoskeleton.

Gymnamoebae comprise several distantly related clades in phylogenetic trees. Though formerly known as subclass Gymnamoebia (Page 1987), most are now distributed among two distinctive classes with contrasting pseudopodial morphology: Tubulinea (which comprises both naked and testate lobose amoebae) and Discosea. A few with substantially different pseudopods belong in Variosea (Cavalier-Smith et al. 2004; Smirnov et al. 2005). Tubulinea and Discosea together constitute the amoebozoan subphylum Lobosa, which never have cilia or flagella, whereas Variosea (as here revised) together with Mycetozoa and Archamoebea are now grouped as the subphylum Conosa, whose constituent lineages either have cilia or flagella or have lost them secondarily (Cavalier-Smith 1998, 2009). Figure 1 is a schematic tree showing amoebozoan relationships deduced from both morphology and DNA sequences.

The first attempt to construct a congruent molecular and morphological system of Amoebozoa by Cavalier-Smith et al. (2004) was limited by the lack of molecular data for many amoeboid taxa, which were therefore classified solely on morphological evidence. Smirnov et al. (2005) suggested another system for naked lobose amoebae only; this left taxa with no molecular data incertae sedis, which limited its utility. From the experience of creating these two systems it emerged that (a) there is a clear deficit of sequenced representatives in some amoeba 18S rRNA clades; adding just a few key sequences may considerably improve the phylogenetic tree (especially adding sequences to monospecific branches or more taxa to the most divergent branches without evident relatives in the tree), (b) careful analysis of morphological characters may be highly supportive of sequence trees, and (c) relatively old but prematurely abandoned morphological views on relationships among amoeboid taxa can be congruent with molecular evidence – if both are critically interpreted.

For general reviews of gymnamoeba morphology and biology we refer the reader to Page (1988), Smirnov and Brown (2004), and Smirnov (2008). The primary purpose of the present review is to rationalise the classification of lobose Amoebozoa, unifying the systems we previously proposed (Cavalier-Smith et al. 2004; Smirnov et al. 2005) utilising new molecular and morphological data generated since 2005. We outline key features of
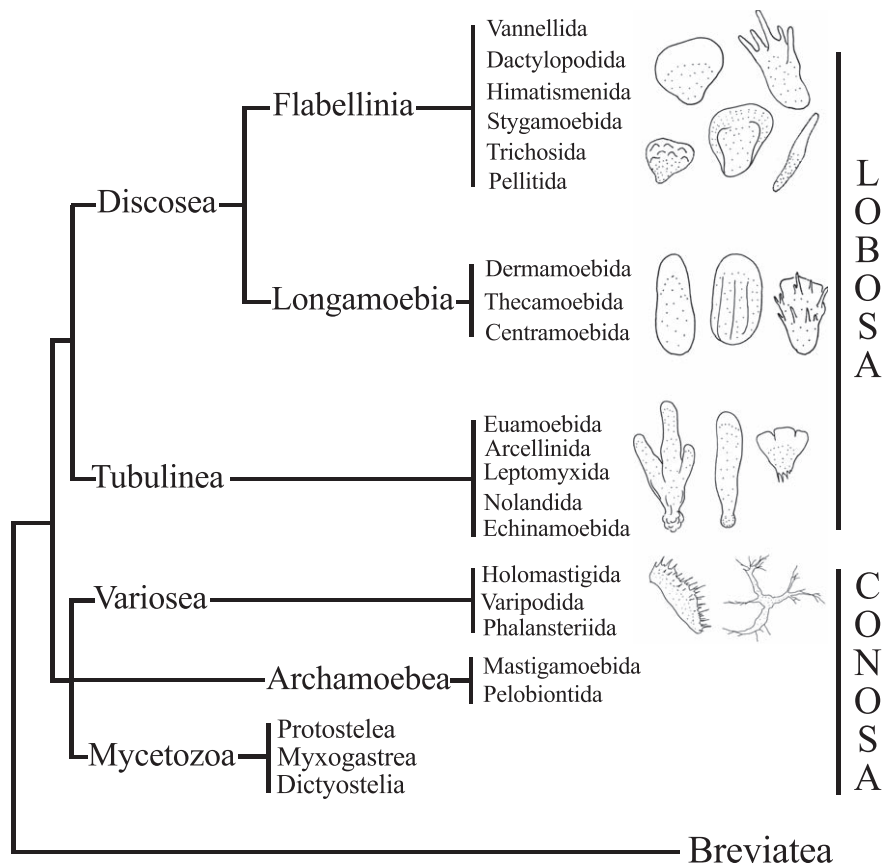
Figure 1. Proposed relationships among the major groups of Amoebozoa. Subphyla Lobosa and Conosa are each shown as holophyletic in conformity with multigene molecular trees and cytological considerations (Cavalier-Smith et al. 2004); however, on 18S rRNA trees either or both may appear paraphyletic or polyphyletic, probably because rapid radiation at the base of the tree makes it hard to resolve amoebozoan basal topology consistently using only relatively short sequences. Although core protostelids appear as four or five separate clades on a recent tree (Shadwick et al. 2009), its resolution does not allow to argue convincingly against their collective holophyly; at least three of them are probably more closely related to Macromycetozoa (Dictyostelea and Myxogastrea: Fiore-Donno et al. 2010) than to other Conosa. Orders shown only for non-Mycetozoa. Pelobiontida includes both Pelomyxidae and Entamoebidae. Mastigamoebida includes both Mastigamoebidae and Endolimacidae.

the history of amoeba systematics to clarify the roots and application of now widely used terms and to draw attention to currently neglected but important and prescient earlier ideas.

The Development of Naked Amoeba Systematics

Amoebae are polymorphic; a single cell can adopt very different shapes, especially when it is stationary or moves in a non-coordinated manner, often changing the direction of locomotion ("non-directed movement"). Most amoeba cells have neither permanently differentiated locomotive organelles (like cilia or flagella) that could be easily described and characterised nor other stable morphological characters. Some earlier authors stated that an amoeba simply has "no shape" (Leidy 1879; Müller 1786). In contrast with many protists, naked amoebae 'preserved' in permanent preparations are usually deformed by fixation and lose many important characters of the live organism. Thus it is very difficult to establish representative "type material"– the background of typological systematics. For over 150 years the only documents on amoeba species were line drawings (sometimes painted; but many colours observed by early authors were artefacts of optical aberrations of their microscopes) and text descriptions, very different

in quality and level of detail from one author to another.

The few morphological characters useful for taxonomy resulted in poor species resolution. Their relative weight was not clear; it was difficult to decide which are species-specific and which useful for creating high-rank taxa. Even whether the type of pseudopodia or the presence/absence of a test is more important was long discussed without ultimate resolution (see Averintzev 1906). None of the numerous early attempts of a convenient classification of amoebae resulted in a long-lived, practical scheme (e.g. Bütschli 1880; Calkins 1901; Delage and Herouard 1896; Hertwig 1879; Lang 1901; Leidy 1879; Rhumbler 1896; West 1903).

Yet it became clear that actively moving amoebae form specific, differentiated structures valuable for species characterisation. Wallich (1863) noted that when moving a posterior end of an amoeba has a specific, remarkable shape useful for taxonomy. Schaeffer (1918), elaborated on this, coining the term "uroid" for this distinctive posterior, including all structures that can be formed there. Greeff (1866, 1874) pointed out the importance of gross nuclear structure in amoeba descriptions; this character was widely applied (Gruber 1881; Penard 1902). Schaeffer (1926 p. 17) noted that an actively moving amoeba, despite minor variations, has a more or less dynamically stable shape (specific general outlines and characters like position of hyaloplasm, dorsal or lateral ridges, flatness) that may be genus- or even species-specific. He introduced the term "locomotive form" to recognize this, arguing that defining this "shape" is the best way to characterize an amoeba; this concept remains the basis of amoeba descriptions. He also noted the importance of nuclear structure and other characters and constructed a synthetic system of amoebae utilising many of the above mentioned characters (Schaeffer 1926). Though criticized by some (Doflein 1929; Calkins 1934; Jepps 1956; Kudo 1954), his wisely multifaceted approach was successfully applied in many studies and became the most frequently cited (Hoogenraad and Groot 1927, 1935; Kufferath 1932; Oye 1933, 1938; Wailes 1927, etc.). Attempts to create an alternative system continued, but none became widely accepted (Hall 1953; Kudo 1939; Raabe 1948; Reichenov 1953, etc.).

Development of amoeba systematics was not gradual, but often sparked by novel laboratory techniques and methods of observation or printing. Goodey (1914) probably first used printed microphotographs to document an amoeba species, *Gephyramoeba delicatula*. The rapid development of histochemistry resulted in attempts to apply a single basic character available from a stained preparation, not living cells, to classify amoebae into higher taxa. The nuclear division pattern was suggested as such a fundamental feature and a number of systems used it (Chatton 1953; Pussard 1973; Singh 1952, 1955; Singh and Das 1970; Singh and Hanumaiah 1979; Singh et al. 1982). This resurrected the approach of Glaeser (1912), who stated that "the most reliable criterion for the classification of amoebae is the division of the nucleus", which despite some support (Calkins 1912) was strongly criticized, e.g. by Schaeffer (1920) who wrote "the classification based on nuclear characters would be a highly artificial system". Subsequent developments have shown that he was correct.

Studies of diverse mechanisms of amoeboid movement stimulated T. Jahn and E. Bovee to use patterns of cytoplasmic flow in pseudopodia (see Rinaldi and Jahn 1963) to group amoeboid protists into higher taxa (Bovee 1954, 1970, 1972; Bovee and Jahn 1960, 1965, 1966; Jahn and Bovee 1965; Jahn et al. 1974); they classified together naked and testate forms of lobose amoebae, as later confirmed by molecular data (Nikolaev et al. 2005). Though widely ignored by other taxonomists, their prescient insights into contrasting pseudopodial patterns yielded a system surprisingly close to the modern molecular phylogeny of lobose amoebae (Smirnov et al. 2005).

In parallel with attempts for a better higher-level grouping of amoebae, much attention was directed to improving microsystematics – i.e. recognition of the borders of amoeba species and establishing more solid genera. Microphotographs, being much less author-specific than line drawings, improved the quality of descriptions - compare, for example, the descriptions by Page (1968) and Page (1977). Microcinematography led to the first movies documenting amoebae species; most were later very helpful for species re-isolation and recognition (e.g. movies from the Institut für den Wissenschaftlichen Film, Göttingen, Germany). Involvement of electron microscopy resulted in the discovery of specific ultrastructural features and clarified relationships between and within some taxa (e.g. Flickinger 1974; Page 1978, 1980a,b, 1985, 1986). However, it became soon clear that electron-microscopy can be helpful at the level of genera but it is usually not useful for species or higher-level taxa, except for the important establishment of the non-amoebozoan class Heterolobosea, separating acrasids and schizopyrenids from naked lobose amoebae (Page and Blanton 1985). That

separation was fully confirmed by molecular data (Clark and Cross 1988); ultrastructural similarities with the non-amoeboid zooflagellates *Stephanopogon* and *Percolomonas* led to Heterolobosea being grouped with them as the phylum Percolozoa (Cavalier-Smith 1991, 1993), also now with strong sequence support (Cavalier-Smith and Nikolaev 2008).

Attempts to create systems of amoebae utilizing more and more new characters never ceased (Delphy 1936; Page 1976; Rainer 1968; Siemensma 1980; Webb and Elgood 1955), but the resolution of light-microscopy methods was exhausted: Bovee (1985) published the last system based solely on light-microscopic morphology. Page (1987) suggested a system utilizing many electron-microscopic findings; his key to gymnamoebae was based on this (Page 1988, 1991). After publication of these books, all other systems of amoebae were virtually abandoned. However, higher-level phylogenetic relationships within amoebae remained "unrecoverable from morphology" (Page 1987); further development of Page's system (see Rogerson and Patterson 2002) did not improve the situation (Smirnov et al. 2005). Even with electron microscopy, morphological data proved insufficient for establishing higher taxa of amoebae and their relationships with other protists.

That higher-level relationships among lobose amoebae needed serious revision was shown by the first molecular study of several naked lobose amoebae (Amaral Zettler et al. 2000), which found that all members of the order Leptomyxida formed a clade that robustly grouped with the non-leptomyxid family Hartmannellidae. By contrast *Echinamoeba* and *Hartmannella vermiformis* formed a sister group to that joint clade. The only apparent exception for leptomyxids was strain ATCC 50654, then named *Gephyramoeba delicatula*, which grouped instead with *Filamoeba nolandi*, making leptomyxids seem polyphyletic. Reinvestigation showed that strain 50654 was misidentified; it is not a *Gephyramoeba* or leptomyxid but a previously unknown member of Variosea, now *Acramoeba dendroida* (Smirnov et al. 2008). Further studies added amoebae of the family Amoebidae to the leptomyxid/hartmannellid clade (Bolivar et al. 2001); and all testate lobose amoebae, as a sister group to Amoebidae + Hartmannellidae (Nikolaev et al. 2005). The above-described grouping is monophyletic and well-supported in all 18S rDNA trees (e.g. Cavalier-Smith et al. 2004; Fahrni et al. 2003; Kudryavtsev et al. 2005, 2009; Tekle et al. 2008; Shadwick et al. 2009; Smirnov et al. 2005, 2008). It was independently named Lobosea sensu stricto

by Cavalier-Smith et al. (2004) and Tubulinea by Smirnov et al. (2005). We use Tubulinea here to allow retention of Lobosa for a more inclusive group.

The phylogeny of other lobose amoebae was more difficult to establish. In trees without Mycetozoa or Archamoebea, they all form a single clade that also includes the multiciliate organism *Multicilia marina* (Nikolaev et al. 2006). But when these two amoebozoan groups were included some lobose amoebae were more closely related to them than to other gymnamoebae (Fahrni et al. 2003; Peglar et al. 2003); those apparently most closely related to Mycetozoa and Archamoebea, were segregated by Cavalier-Smith et al. (2004) as the class Variosea, which included flagellated Amoebozoa, namely - *Multicilia* and *Phalansterium*, as well as some amoeboid organisms without cilia - *Filamoeba* and later added *Flamella, Acramoeba* (Kudryavtsev et al. 2009; Smirnov et al. 2008) and *Grellamoeba* (Dyková et al. 2010a).

The first attempt to make the amoebozoan morphological system congruent with sequence trees was by Cavalier-Smith et al. (2004). Smirnov et al. (2005) suggested an alternative system, focusing on the lobose amoebae and further developed by Smirnov in Adl et al. (2005). Both systems made a clear division of lobose amoebae into two large groups: those with tubular pseudopodia (or able to form them under certain circumstances) - Lobosea sensu stricto of Cavalier-Smith or Tubulinea of Smirnov - and those generally with a flattened body. The latter were initially subdivided somewhat differently, despite both systems agreeing that Vannellida and Dactylopodida are related and should be grouped together. They were treated as Glycostylida within a class Discosea by Cavalier-Smith, containing orders Glycostylida, Dermamoebida and Himatismenida; and as class Flabellinea in Smirnov et al. (2005), initially containing orders Vannellida and Dactylopodida, but later broadened by adding Thecamoebida (Smirnov in Adl et al. 2005). Discosea of Cavalier-Smith et al. (2004) was modified by excluding *Multicilia*, which is phylogenetically closer to Varipodida and Conosa (Nikolaev et al. 2006). All existing phylogenies confirm that discosean and variosean amoebae branch separately from Tubulinea.

Two well-supported clades of Discosea - Vannellida and Dactylopodida (Adl et al. 2005), usually group together, thus unifying three of Page's amoeba families: Vannellidae, Paramoebidae and most Vexilliferidae. However, the grouping of amoebae from families Thecamoebidae and Acanthamoebidae was less stable, differing from one tree to another (Brown et al. 2007; Fahrni et al.

2003; Kudryavtsev et al. 2005; Michel et al. 2006; Smirnov et al. 2005, 2008). Finally, some genera formed a long relatively isolated branch, not grouping reliably with any robust clade, e.g. *Cochliopodium* (Kudryavtsev et al. 2005) and *Vermistella* (Moran et al. 2007). Revising the classification of this part of the tree is our key focus here.

The Dichotomy between Tubulinea and Discosea

Smirnov and Goodkov (1999) and Smirnov and Brown (2004) analysed general patterns of morphodynamic organisation in locomotive forms of naked lobose amoebae, splitting their entire diversity into relatively few distinct morphotypes. The definition of morphotype includes such a features of amoeba locomotive morphology as general outline of the moving cell; presence or absence of pseudopodia and subpseudopodia; the organization of the uroid; the shape of an amoeba in cross-section; and the position of the hyaloplasm in the locomotive cell. All these characters reflect the mechanics of amoeboid movement and peculiarities of cell adhesion, indicating how these mechanisms are realised and combined in a particular amoeba. Thus we can consider a morphotype as a synthesis of the special features characterising the particular kind of amoeboid movement exhibited by a cell.

Analysis of morphotypes and of the list of species belonging to each tells us that all lobose amoebae may be split into three basic groups: (A) those where the entire cell is always cylindrical or subcylindrical; (C) those where it is always flattened, being laterally expanded in cross-section, and (B) those able to alter their locomotive form from cylindrical to flattened under certain conditions (Fig. 2). Furthermore, amoeba species showing morphotypes of groups A or B all belong to the Tubulinea clade in molecular trees, while those in group C belong to Discosea (Smirnov et al. 2005). The only exception in this simple scheme is the acanthopodial morphotype (*Echinamoeba* is a tubulinean while *Acanthamoeba*, *Protacanthamoeba* and *Vexillifera* all are discoseans), but this is just the result of the unification of similar, but actually different, amoebae under the same morphotype, done to simplify identification of amoebae morphotypes for non-specialists. For example, *Echinamoeba* has much shorter and more spine-like subpseudopodia than *Acanthamoeba* and, especially, *Vexillifera*, so one could justify two separate morphotypes for amoebae of this type, but that would be hardly prac-

tical, since few non-specialists would be able to make that discrimination correctly.

Differences in body cross-section between these amoeba groups correlate with those in their general pattern of amoeboid movement, which is still far from exhaustively explained; it is relatively well-studied only in some groups. Most data are on *Amoeba proteus* (family Amoebidae) (Grebecki 1982; Stockem and Clopocka 1988); some are available for *Saccamoeba limax* (family Hartmannellidae) (Grebecki 1987, 1988). Locomotion of amoebae of this type is explained by the general cortical contraction model of amoeboid movement (Grebecki 1979, 1982). Briefly, the entire monopodial amoeba, or each pseudopod of a polypodial amoeba, represents a tube of cortical gel-like cytoplasm rich in polymerised acto-myosin filaments, while the axial interior of the tube is liquid sol-like cytoplasm that streams forward to extend the pseudopod (see e.g. Stockem et al. 1981 p. 77). We termed such cytoplasmic flow monoaxial (Smirnov et al. 2005).

Movement of flattened amoebae is much less studied; we still have no satisfactory model explaining it. Some data on the general pattern of cytoplasmic flow are available for *Thecamoeba* spp. (Abe 1963; Allen 1961) and *Vannella simplex* (Huelsmann and Haberey 1973), but they are much less detailed than for normally tubular amoebae. Flattened amoebae never form true pseudopodia - the flattened cell moves as a whole; liquid cytoplasm flows in streams separated by islands of gel-like cytoplasm (Haberey and Huelsmann 1973), as well shown in drawings by Abe (1963). Such cytoplasmic flow is termed polyaxial (Smirnov et al. 2005).

These two types of movement illustrate the basic difference between the concepts of Tubulinea (Fig. 3) and Discosea (Fig. 4). In locomotion, flattened amoebae, unified as Discosea, never form cylindrical or sub-cylindrical pseudopodia or show clear monoaxial flow of cytoplasm, which differentiates them from Tubulinea in a "negative" sense (absence of features). We cannot yet suggest a more precise "positive" synapomorphy for them; from analysis of morphotypes (which are very diverse – three in Tubulinea, nine in Discosea) and of the varied details of amoeboid movement we suspect that Discosea may not be monophyletic. Some molecular data weakly suggest the same (Kudryavtsev et al. 2009; Smirnov et al. 2008; Tekle et al. 2008). The existence of amoebae able to alter their locomotive morphology from flattened, expanded to tubular, subcylindrical in cross-section (i.e. the entire order Leptomyxida
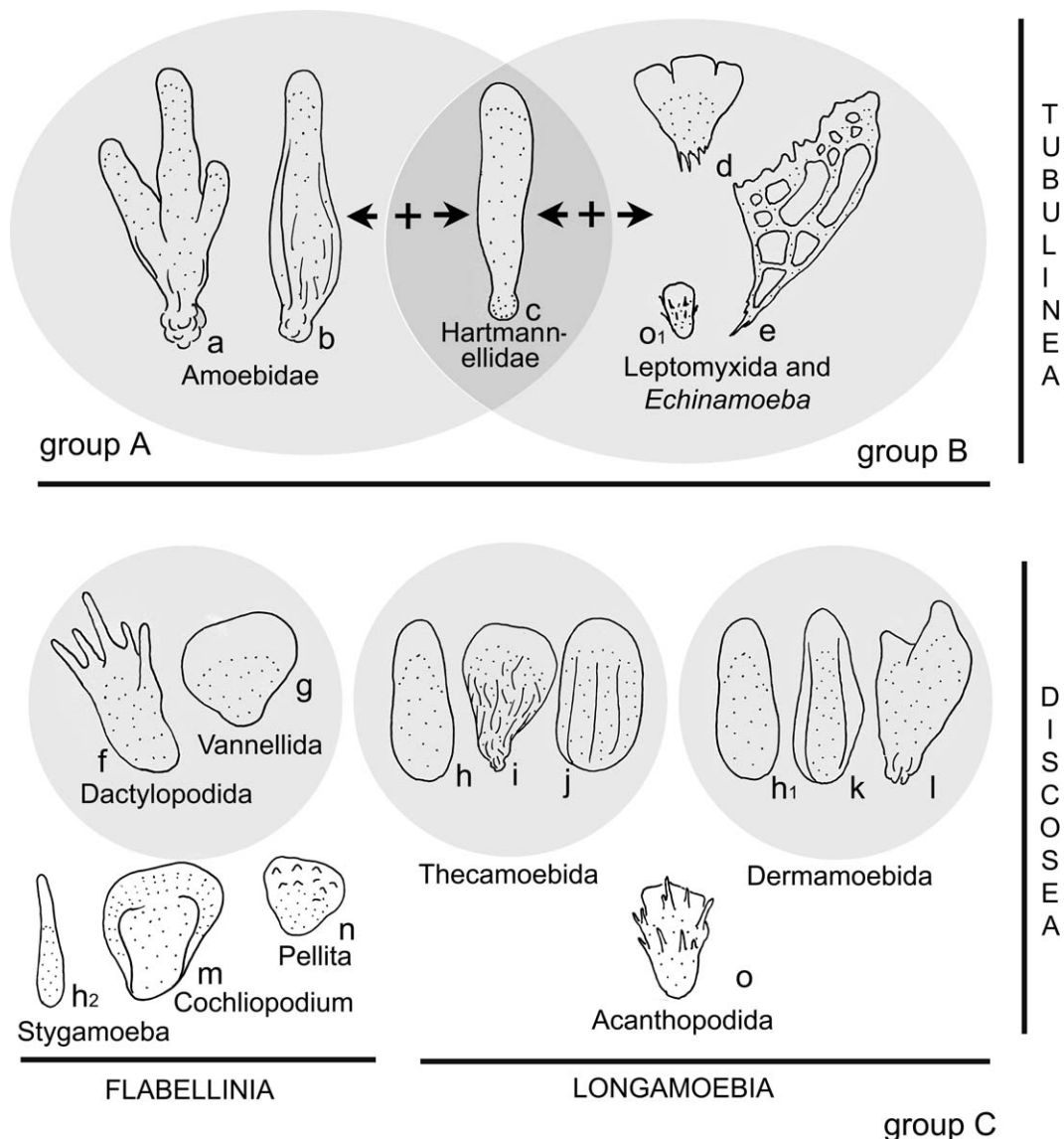
Figure 2. Morphotypes of lobose amoebae grouped according to the main clades which they form in the 18S phylogenetic tree. Clades of lobose amoebae in the phylogenetic tree are shadowed in grey; names of taxa follow our new system (Table 1). Names of morphotypes follow Smirnov and Brown (2004). Morphotypes are labelled with letters; if a morphotype appears in more than one clade it has a numerical index in lowercase (e.g. h_1, h_2). The morphotypes are: a – polytactic; b – orthotactic; c – monotactic; d – flabellate; e – branched; f – dactylopodial; g – fan-shaped; h – lingulate; i – rugose; j – striate; k – lanceolate; l – mayorellian; m – lens-like; n – flamellian; o – acanthopodial.

Group A unifies polytactic, orthotactic and monotactic morphotypes. Group B consists of amoebae that are normally flabellate or branched, but able to adopt a monotactic form. The same is true for *Echinamoeba*, formally belonging to the acanthopodial morphotype. All species of groups A and B belong to the monophyletic clade here recognised as class Tubulinea. Group C unifies all flattened lobose amoebae, never becoming tubular. These species form three recognised clades in the phylogenetic tree (grey shadowing) and a number of independent, single-genus lineages. These organisms are grouped here in the class Discosea, consisting of two subclasses – Flabellinia and Longamoebia, both weakly monophyletic (supplementary tree S1).
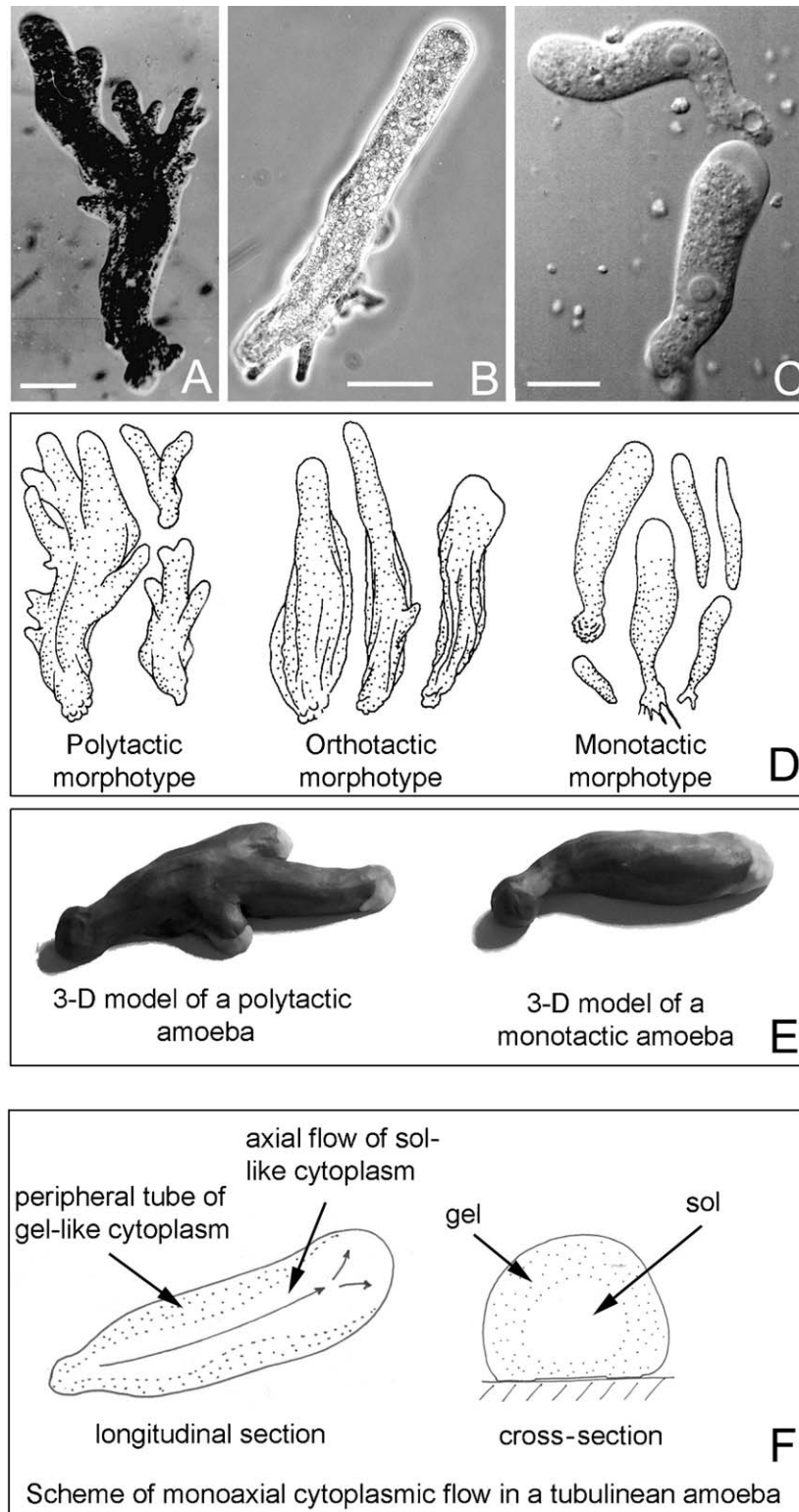
Figure 3. The concept of Tubulinea. **A-C** – sample representatives of the group: **A** – *Chaos glabrum*; **B** – *Polychaos annulatum*; **C** – *Saccamoeba limax*. **D** – schematic drawings of the morphotypes of tubulinean amoeba. **E** – 3-D models of a polytactic and of a monotactic tubulinean amoebae. **F** – scheme of the monoaxial cytoplasmic flow characteristic for all tubulineans.
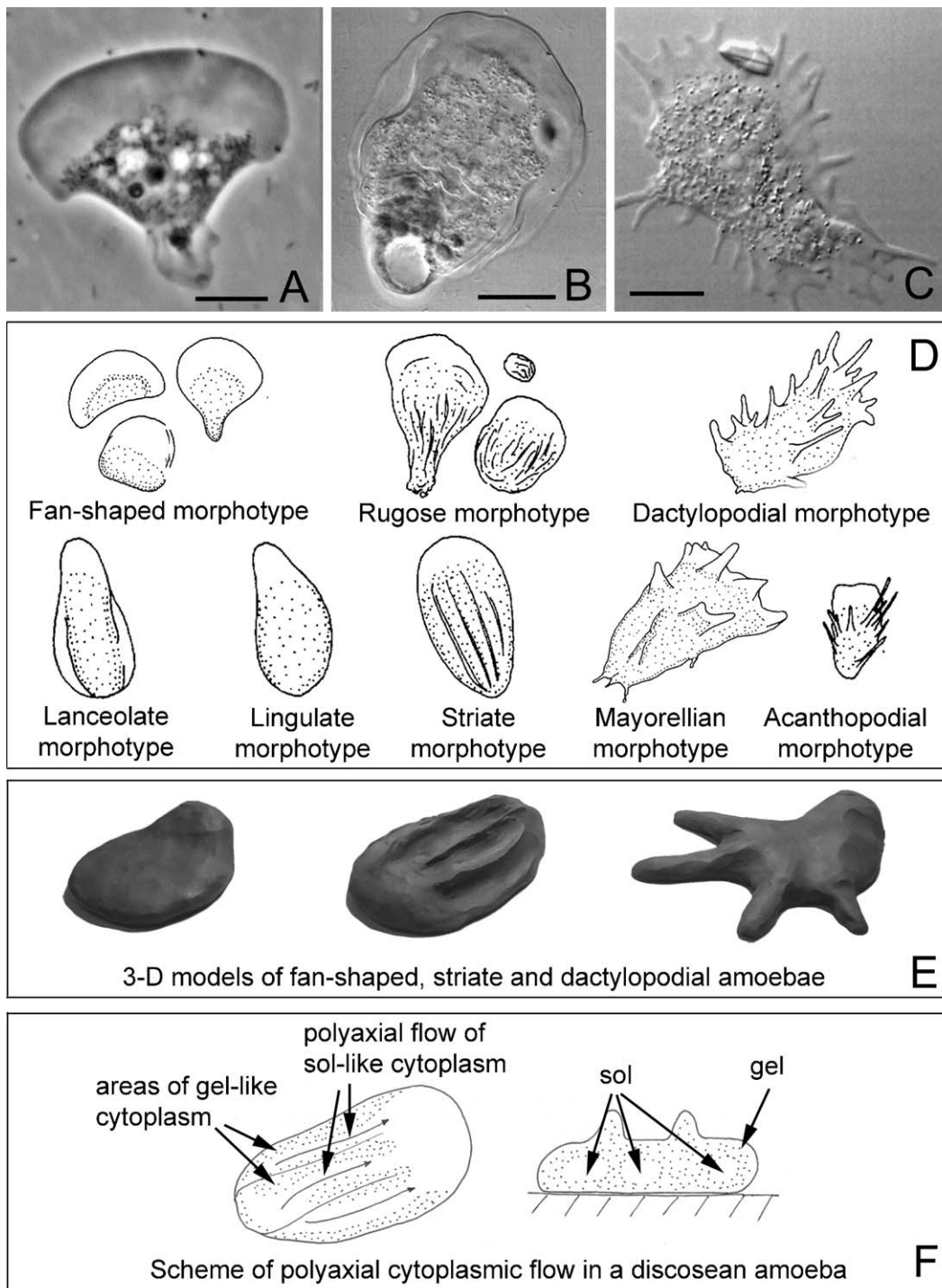
Figure 4. The concept of Discosea. **A-C** sample representatives of the group. **A** – *Vannella simplex*; **B** – *Thecamoeba sphaeronucleolus*; **C** – *Paramoeba eilhardi*. **D** – schematic drawings of the morphotypes of discosean amoebae. **E** – 3-D model of three different discosean amoebae (fan-shaped, striate and dactylopodial morphotypes). **F** - scheme of the polyaxial cytoplasmic flow characteristic for all discoseans.

and the genus *Echinamoeba*: Smirnov et al. 2005) shows that these two types of cell organization are not completely different and can be realized by the cytoskeleton of the same cell.

Testate lobose amoebae also belong within Tubulinea in molecular trees (Nikolaev et al. 2005). Data on their locomotion pattern and pseudopodial structure are so scarce that it is hard to reconstruct a clear picture (e.g. Eckert and McGee-Russell 1973; Mast 1931). LM observations show that they normally or under certain circumstances produce pseudopodia that are basically tubular, cylindrical or subcylindrical in cross-section. Thus, so far, the concept of Tubulinea can accommodate testate lobose amoebae as well.

Limitations of the Amoebozoan rRNA Tree

While the monophyly of Tubulinea was never seriously doubted, the grouping of flattened lobose amoebae in amoebozoan 18S rRNA trees is less solid. Vannellida and Dactylopodida usually group together as a stable clade, which may be strongly or sometimes only weakly supported (Cavalier-Smith et al. 2004; Smirnov et al. 2005; Tekle et al. 2008; Kudryavtsev et al. 2005, 2009) and was named Flabellinea by Smirnov et al. (2005). Pawlowski and Burki (2009) found *Cochliopodium* grouping with the extremely long branch *Clydonella*, forming together with *Vexillifera minutissima* and "*Pessonella* sp." a third sub-clade within Vannellida. But, as they mentioned, that it is probably a long-branch artefact, being contradicted by Kudryavtstev et al. (2005) using more nucleotide positions – and strongly so by their Bayesian tree with covarion correction that should reduce such artefacts and which placed *Cochliopodium* weakly as sister to Flabellinea, not within vannellids.

Members of the family Thecamoebidae were in poorly resolved deep-branching positions on early trees; *Dermamoeba* and *Thecamoeba* failed to group with each other (Fahrni et al. 2003; Kudryavtsev et al. 2005), *Thecamoeba* often being closer to Acanthamoebida. By contrast *Sappinia* is always sister to *Thecamoeba* (Michel et al. 2006; Shadwick et al. 2009; Smirnov et al. 2007; Tekle et al. 2008). All these papers found *Stenamoeba stenopodia* as sister to *Thecamoeba/Sappinia*, whereas in some *Dermamoeba* was sister to *Mayorella* with varying support (e.g. Smirnov et al. 2007; Pawlowski and Burki 2009), but in others these two genera were not grouped together (Tekle et al. 2008). With more sequences, the *The-camoeba/Sappinia/Stenamoeba* clade maintains its tendency to group with *Acanthamoeba*, but the position of this larger clade is unclear (Shadwick et al. 2009; Tekle et al. 2008).

The flagellate amoebozoans *Phalansterium* and *Multicilia* form long, unstable branches in the 18S rRNA tree, but tend to group with *Flamella, Filamoeba* and the purely amoeboid *Acramoeba* (Cavalier-Smith et al. 2004; Nikolaev et al. 2006; Smirnov et al. 2008). This group of flattened amoeboid organisms, possessing pointed subpseudopodia, most similar to those of Mycetozoa, corresponds to the class Variosea (Cavalier-Smith et al. 2004). Pawlowski and Burki (2009) identified an 8-nucleotide 18S rRNA signature that supports the unity of Variosea more strongly than do bootstrap values; however it is absent from *Multicilia* (which might have diverged before other Variosea, and sometimes does not even group with them) and absent or slightly modified in a few others as well as also being present in at least two protostelids, so it is not a totally conservative marker. Several lobose amoebae (*Cochliopodium, Parvamoeba, Vermistella* and *Trichosphaerium*) form independent branches, lacking clear relationships with any major well-defined amoebozoan clade (Cole et al. 2010; Kudryavtsev et al. 2005, 2009; Tekle et al. 2008).

Thus the main limitation of the rRNA tree is not any serious conflict with morphology but simply a general lack of resolution of the deepest branches that makes it hard to decide whether Discosea and Variosea are monophyletic or not or how their orders are related to each other. Another problem is that in some groups, notably myxogastrid Mycetozoa – and *Trichosphaerium*, rRNA evolved so much faster than in others that their placement on the tree is especially problematic. Amoebozoa suffers much more from extremely unequal rates of rRNA evolution than most other protist phyla, as illustrated in the Supplementary Figure S1 (a representative sample of 92 Amoebozoa including three new sequences, *Paradermamoeba levis*, *Thecamoeba aesculea* and *Phalansterium filosum* sp. n.; for Methods, see Supplementary Material).

Revision of Family Thecamoebidae

Prior to the present work, family Thecamoebidae Schaeffer, 1926 comprised eight genera of naked lobose amoebae, unified by an "apparent pellicle-like" cell coat, wrinkled or striated in most genera (Page 1987, 1988), but so diverse in other aspects of cell structure as to raise doubts about whether

they belong to one family. They could be easily split into striate and rugose groups of species; lingulate species and polytactic species. Moreover, this family included the genus *Parvamoeba* with very unusual morphology. Here we review its morphological diversity; exclude *Parvamoeba* from family Thecamoebidae; and subdivide the rest of thecamoebids into two families, arguing from morphological and molecular data that Thecamoebidae was previously polyphyletic.

Striate and rugose species. These are "core" thecamoebids comprising three genera – *Thecamoeba*, *Sappinia* and *Stenamoeba*.

The type genus of Thecamoebidae – *Thecamoeba* Fromentel, 1874 (Fig. 5 A-D, F) unifies 10 marine and freshwater amoebae species of striate and rugose morphotypes (Smirnov and Goodkov 1999), with apparently rigid cell coat and amorphous glycocalyx (Page 1977, 1983; Page and Blakey 1979; Fig. 6 A-B). The most characteristic feature of striate thecamoebians is longitudinal dorsal folds, well-pronounced in species like *Thecamoeba striata*. Rugose amoebae have numerous lateral and dorsal wrinkles, e.g. *Thecamoeba sphaeronucleolus*. Jahn et al. (1974) suggested a separate genus (*Striamoeba*) for striate thecamoebians, but due to weak distinctive characters Page (1987) and others did not accept this; molecular studies confirmed that it was a wrong idea. The cell coat of *Thecamoeba* (Fig. 6 A-B) is mostly amorphous (Page and Blakey 1979). That of *T. sphaeronucleolus* in our TEM images (Fig. 6A) looks bilayered, with traces of vertical structuring between the electron-dense basal and outer layer. This structure was covered with a halo of loose material. In this respect it contrasts with the description by Page and Blakey (1979 p. 120) where it looks amorphous; also they seldom observed filamentous structures in the loose material covering the basal layer.

Sappinia Dangeard, 1896 long contained only *Sappinia diploidea* Dangeard, Hartmann and Naegler, 1908, an amoeba of lingulate morphotype (according to published drawings and images). However its re-investigation (Michel et al. 2006) and study of the redescribed *Sappinia pedata* Dangeard, 1896 (Brown et al. 2007) show that in fresh culture these amoebae may also have lateral wrinkles and thus adopt a rugose morphotype. Our data show that they may even have dorsal folds, becoming clearly striate. Specific diplokaryotic cysts of *Sappinia* and the potential presence of a complex life cycle make it unique among Thecamoebidae. The cell coat of *S. diploidea* was believed to be thin and amorphous (Goodfellow et al. 1974); a recent study of new isolates shows that it may have a complex glycostyle-like layer over the basal layer which appeared to consist of two electron-dense layers, separated by a vertically structured layer (Michel et al. 2006).

The genus *Stenamoeba* Smirnov, Nassonova, Chao and Cavalier-Smith, 2007 (Fig. 5 E) was erected for a single species, *S. stenopodia* (formerly *Platyamoeba stenopodia* Page, 1969). Its lingulate morphotype with occasional striations of the dorsal surface and thin, amorphous glycocalyx (Fig. 6 C), dissimilar from that of other "Platyamoeba", long suggested affinities with Thecamoebidae (Fahrni et al. 2003; Page and Blakey 1979; Smirnov and Goodkov 1999; Smirnov et al. 2005); molecular data supported this transfer (Smirnov et al. 2007). Recently Dyková et al. (2010b) described two more species in this genus, closely related to *S. stenopodia*.

Lingulate species. This group includes genera *Dermamoeba* and *Paradermamoeba*.

Dermamoeba Page and Blakey 1979 (Fig. 5I) comprises two well-documented species of lingulate morphotype, always smooth, with rare exceptions for the uroidal region (Page 1988; Pussard et al. 1979). *Dermamoeba* possess a very thick cell coat (Fig. 6, E), organised in horizontal layers of fibrous material (Page and Blakey 1979). Both species have a complex nuclear structure; often with two spherical, closely apposed endosomes. Only *D. algensis* (Smirnov et al. 2011) is present in SSU rDNA trees (Fahrni et al. 2003). Amoebae of the genus *Paradermamoeba* Smirnov et Goodkov, 1996 (Fig. 5 J-M) resemble *Dermamoeba* in general appearance, but are of lanceolate morphotype, more oblong, with characteristic flatness of the lateral parts of the cell. Both species have a thick cell coat (Fig. 6D) of tightly packed spiral glycostyles with hexagonal cup-like structures on the tips of each (Smirnov and Goodkov 1993, 1994, 2004).

Polytactic species. Two problematic species were assigned to Thecamoebidae in the past. The single species of *Pseudothecamoeba* Page, 1988 - *P. proteoides* (Fig. 5G) - adopts orthotactic or polytactic morphotypes very different from other thecamoebids; its position in this family is doubtful (Page 1988). Its characteristic apparently rigid cell coat and numerous wrinkles of the cell surface, as in rugose thecamoebids, suggested affinities with Thecamoebidae; however a granular nucleus and filamentous glycocalyx may indicate affinities with Amoebidae (Page 1978). Moreover, its cytoplasm is hardly vacuolated, resembling no other lobose amoebae but the "structure
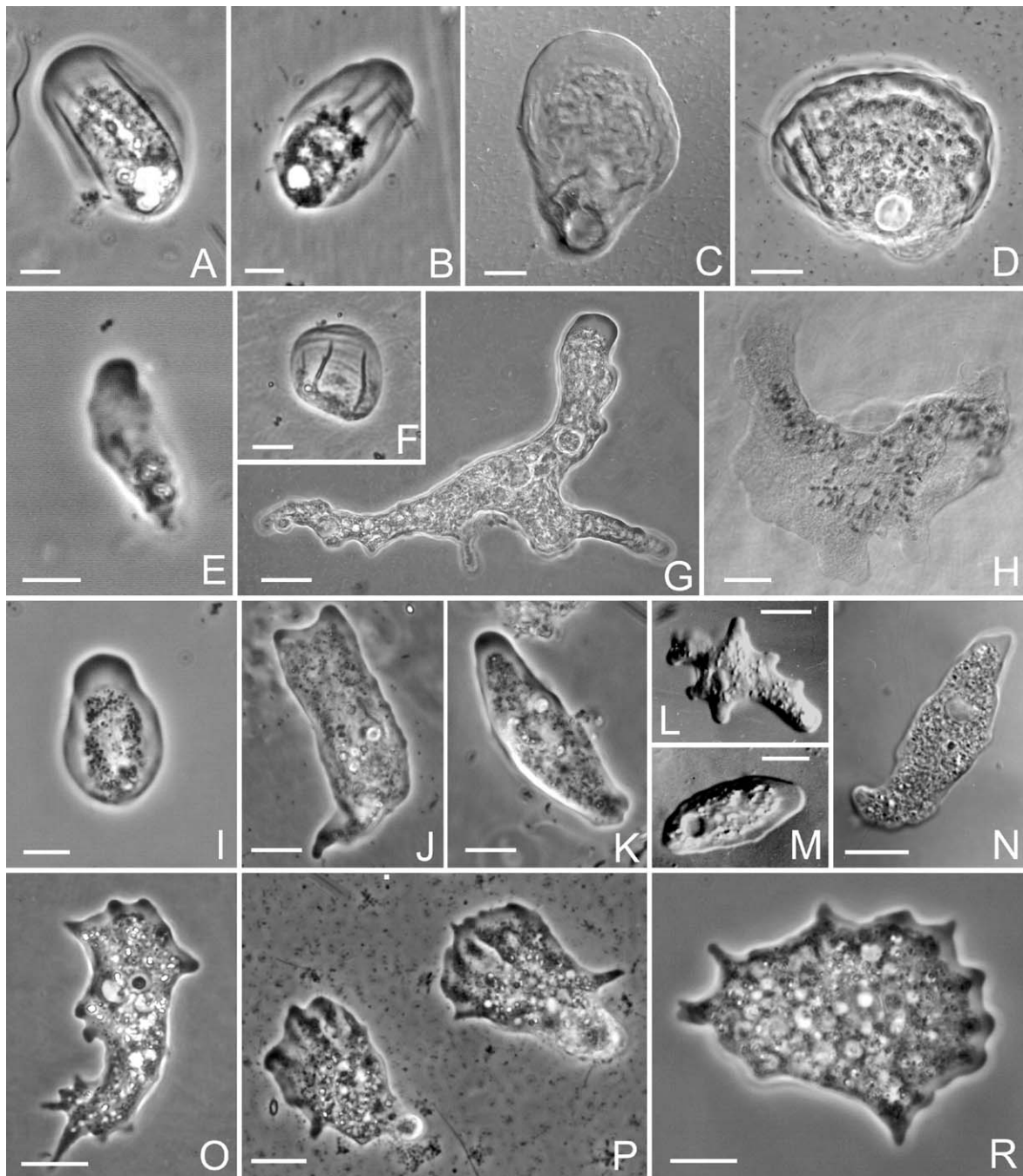
Figure 5. Light microscopic morphology of Thecamoebida (**A-F**) and Dermamoebida (**I – R**). **A** - *Thecamoeba striata* CCAP 1583/4. **B** - *Thecamoeba quadrilineata* Valamo strain (Russia). **C** - *Thecamoeba sphaeronucle-olus* CCAP 1583/3. **D -** *Thecamoeba similis* CCAP 1583/8. **E** - *Stenamoeba stenopodia* CCAP 1565/8. **F** - *Thecamoeba orbis* Nivå Bay Strain (Denmark). **G** - *Thecamoeba* cf. *proteoides* Valamo strain (Russia). **H** - *Thecochaos fibrillosum* (slide by E. Penard, British Museum of Natural History collection). **I** - *Dermamoeba* sp. Geneva strain (Switzerland). **J-K** - *Paradermamoeba valamo* in slow (**J**) and active (**K**) locomotion. Geneva strain (Switzerland). **L-M** - *Paradermamoeba levis* Valamo strain (type strain) resting (**L**) and locomotive form (**M**). **N** - *Mayorella gemmifera* CCAP 1547/8 strain. **O**. *Mayorella* cf. *vespertilioides* Valamo strain (Russia). **P** - *Mayorella* sp. Cam40 strain (Camargue, France). **R** - *Mayorella cantabrigiensis* CCAP 1547/7 strain. Scale bar 10 μm.
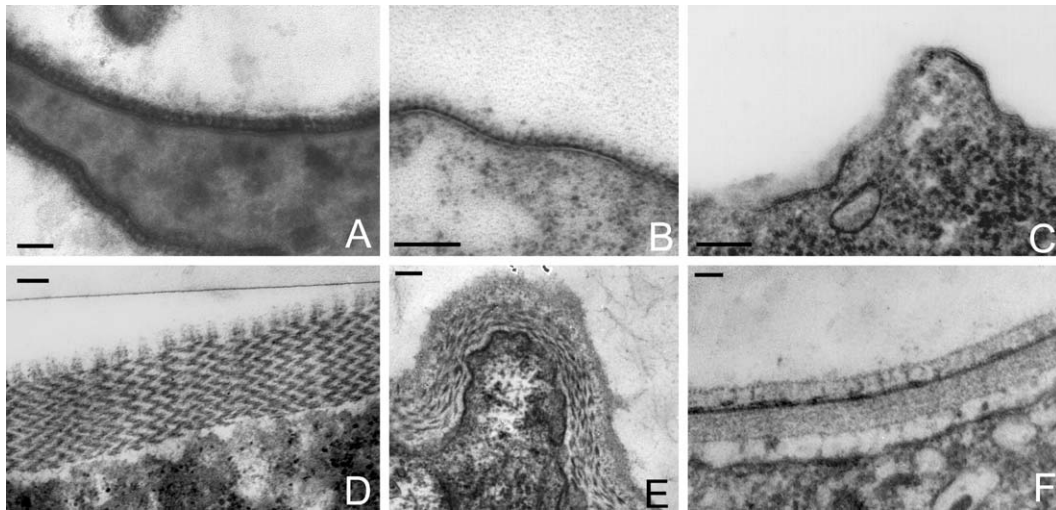
Figure 6. Diversity of cell coats in Thecamoebida (**A-C**) and Dermamoebida (**D-F**). **A** - *Thecamoeba sphaeronucleolus* CCAP 1583/3. **B** - *Thecamoeba striata* Valamo strain (Russia). **C** - *Stenamoeba* cf. *stenopodia* Valamo strain (Russia). **D** - *Paradermamoeba valamo* Valamo strain (Russia, type strain). **E** - *Dermamoeba algensis* (type strain). **F** - *Mayorella* cf. *vespertilioides* Valamo strain (Russia). Scale bar 100 nm.

vacuoles" of the conosan *Pelomyxa* (Goodkov and Seravin 1991). The other doubtful thecamoebid, *Thecochaos* Page, 1981 (Fig. 5H), is known only from a stained preparation by Greef, studied by Page (1981). These amoebae resemble multinucleate *Pseudothecamoeba*; until a representative is isolated and studied, its inclusion in Thecamoebidae is arbitrary.

Parvamoeba. The genus *Parvamoeba* Rogerson, 1993 was erected for the smallest described amoeba, *P. rugata*. This tiny amoeba has an apparently rigid, wrinkled cell surface and amorphous glycocalyx (Rogerson 1993). It is so small that its LM morphology is hard to investigate, making its position in Thecamoebidae somewhat arbitrary. The finding of *P. monoura* – an organism with very unusual morphology, but sequence closely resembling that of *P. rugata*, makes the genus *Parvamoeba* even more mysterious (Cole et al. 2010).

New groupings of thecamoebids. The above review of Thecamoebidae shows it comprised morphologically heterogeneous amoebae. It is therefore not surprising that on our tree (Supplementary Fig. S1) as well as published ones (Michel et al. 2006/7; Kudryavtsev et al. 2009; Pawlowski and Burki 2009) the assemblage of species representing the classical Thecamoebidae is polyphyletic. It forms two clades: one comprises striate/rugose species with a relatively thin, electron-dense cell coat, sometimes with extra structures over the amorphous layer (*Thecamoeba, Sappinia, Stenamoeba*), grouped as the new order Thecamoebida

(Table 1); the other has smooth species with a thick, highly structured cell coat, either cuticle-like or consisting of glycostyle-like structures (*Dermamoeba* and *Paradermamoeba*), here treated as a revised family Dermamoebidae. The divergence of cell coat structure between *Dermamoeba* and *Paradermamoeba* is not as drastic as it first appears, because the conversion of the *Paradermamoeba* glycocalyx into the "cuticle" of *Dermamoeba* by embedding the glycostyles into the matrix and further loss of their regular structure is conceivable.

The weak grouping of *Mayorella* with *Dermamoeba* and *Paradermamoeba* is also not really surprising. The multilayered cell coat of *Mayorella* (Fig. 6F), often termed "cuticle" has much in common with that of *Dermamoeba*. Locomotive morphology of mayorellas (Fig. 5 N-R), especially the smallest species, e.g. *M. dactylifera* (Goodkov and Buryakov 1986), may be similar to that of *Dermamoeba* and especially *Paradermamoeba* (except for the occasional formation of dorsal folds in *Mayorella*; however these are wider and smoother in outline than in *Thecamoeba*). Both species of *Paradermamoeba* may form conical pseudopodia when changing their direction of locomotion, rather similar to those of *Mayorella* (compare Figs 5K and 5N or 5O and 5J). Resting specimens of *P. levis* (Fig. 5L) may form short conical projections or hyaline lobes very similar to those of mayorellas (Smirnov and Goodkov 1994). Such peculiarities of morphology may stem from the organisation of the locomotive mechanism, which depends primarily on the cytoskeleton and cell coat.

As all are of basic importance in amoeba systematics, they reinforce evidence from molecular phylogeny; together they provide a sound rationale for splitting the family Thecamoebidae.

Cavalier-Smith (in Cavalier-Smith et al. 2004) established a new order Dermamoebida to include Thecamoebidae. We now make the thecamoebid genera *Thecamoeba, Sappinia*, and *Stenamoeba* possessing a thin, dense glycocalyx, and showing dorsal folds and/or wrinkles, the core of the refined family Thecamoebidae. Tekle et al. (2008) stated that the grouping of *Stenamoeba* with *Sappinia/Thecamoeba* is spurious, however it is almost as well supported as that between *Sappinia* and *Thecamoeba* (more strongly so in the tree of Shadwick et al. 2009) and is consistently recovered by all published 18S rRNA trees, often with strong support (Fahrni et al. 2003, Kudryavtsev et al. 2005; Michel et al. 2006/7; Smirnov et al. 2005, 2007; Shadwick et al. 2009).

We place *Pseudothecamoeba* and *Thecochaos* incertae sedis until they are re-isolated. The only available data on *Thecochaos* are permanent stained preparations by E. Penard (Page 1981); re-examining them did not clarify the situation because it was not clear if the wrinkled appearance of the cell (Fig. 5H) is natural or a fixation artifact. For *Mayorella* we restore the family Mayorellidae, which Page (1987) abandoned, as trees have repeatedly shown that his including it in Paramoebidae was incorrect; we group Mayorellidae with Dermamoebidae in the order Dermamoebida, which thus unifies amoeba families with a thick, multilayered or highly structured cell coat.

New suborder Parvamoebina. *Parvamoeba* remains a problem: according to published data, two species showing a very close molecular relationship have surprisingly distinct light- and electron-microscopic morphology (Cole et al. 2010; Rogerson 1993). However, light-microscopic data on *P. rugata* are scarce and its re-investigation is desirable. Both species appear to have a similar peculiar locomotion: they move unusually slowly, forming a temporarily projecting single posterior pseudopodium, uniquely in Lobosa. The exact mode and mechanism of movement is unclear. Given the probably unique locomotory mechanism and distinctive morphology of *Parvamoeba*, we remove it from Thecamoebidae, and establish a new family and suborder Parvamoebina for it within Discosea. In a 3-gene tree (18S and 28S rRNA and EF-1α) *P. rugata* robustly grouped with *Cochliopodium* (100% support; Berney, Fiore-Donno, and Cavalier-Smith unpub. observ.) as it does in an actin tree (Kudryavtsev et al. 2011).

Alexander Kudryavtsev (pers. commun.) observed that *P. rugata* forms a small ventral adhesive disk while moving; if true this may explain its relationship with Cochliopodiidae.

Because of the robustness and agreement of the 3-gene and actin trees we place Parvamoebina within Himatismenida and establish a new suborder (Tectiferina) for the previously established himatismenids, which are all characterised by a dorsal tectum, conceptually very different from the parvamoebid surface coat. Within Tectiferina we establish a new family Goceviidae for non-scaly genera, incompletely covered with the fibrous layer and possessing an expanded frontal area of hyaloplasm, unlike *Cochliopodium*, and restrict Cochliopodiidae to *Cochliopodium* and *Ovalopodium*, following Kudryavtsev et al. (2011). Conceivably the ancestral himatismenid had a fibrous dorsal tectum to which scales were later added by *Cochliopodium*, and which probably invested the cell more completely only in the ancestor of *Parvamoeba* when it became miniaturised and evolved the entirely novel posterior pseudopod.

New Data on Morphology and Diversity of Phalansteriida Support Variosea

The discovery that the uniciliate flagellate *Phalansterium solitarium* belonged in Amoebozoa (Cavalier-Smith et al. 2004) was a surprise because the three established species, *P. consociatum* (Cienkowski 1870), *P. digitatum* (Stein 1878), and *P. solitarium* (Sandon 1924), were long considered to be purely zooflagellates without an amoeboid phase. Hence this genus became the first entirely non-amoeboid representative of Amoebozoa. Ekelund (2002) reported that a *P. solitarium*-like flagellate became amoeboid when placed under a coverslip though never did in culture, but he did not describe or figure the temporary amoeboid phase. New observations on the *Phalansterium* aff. *solitarium* ATCC strain sequenced by Cavalier-Smith et al. (2004), but never properly illustrated, are described and illustrated in Supplementary Fig. S2; in our cultures it never showed an amoeboid phase, though slender pseudopodia occur sometimes.

During this study we found another *Phalansterium* described below as *Phalansterium filosum* n. sp. (Fig. 7). It is the first *Phalansterium* documented to form a transitory amoeboid phase with tapering pointed pseudopods that are morphologically similar to those of *Filamoeba*, and to a lesser extent *Acramoeba*. *P. filosum* forms a robust
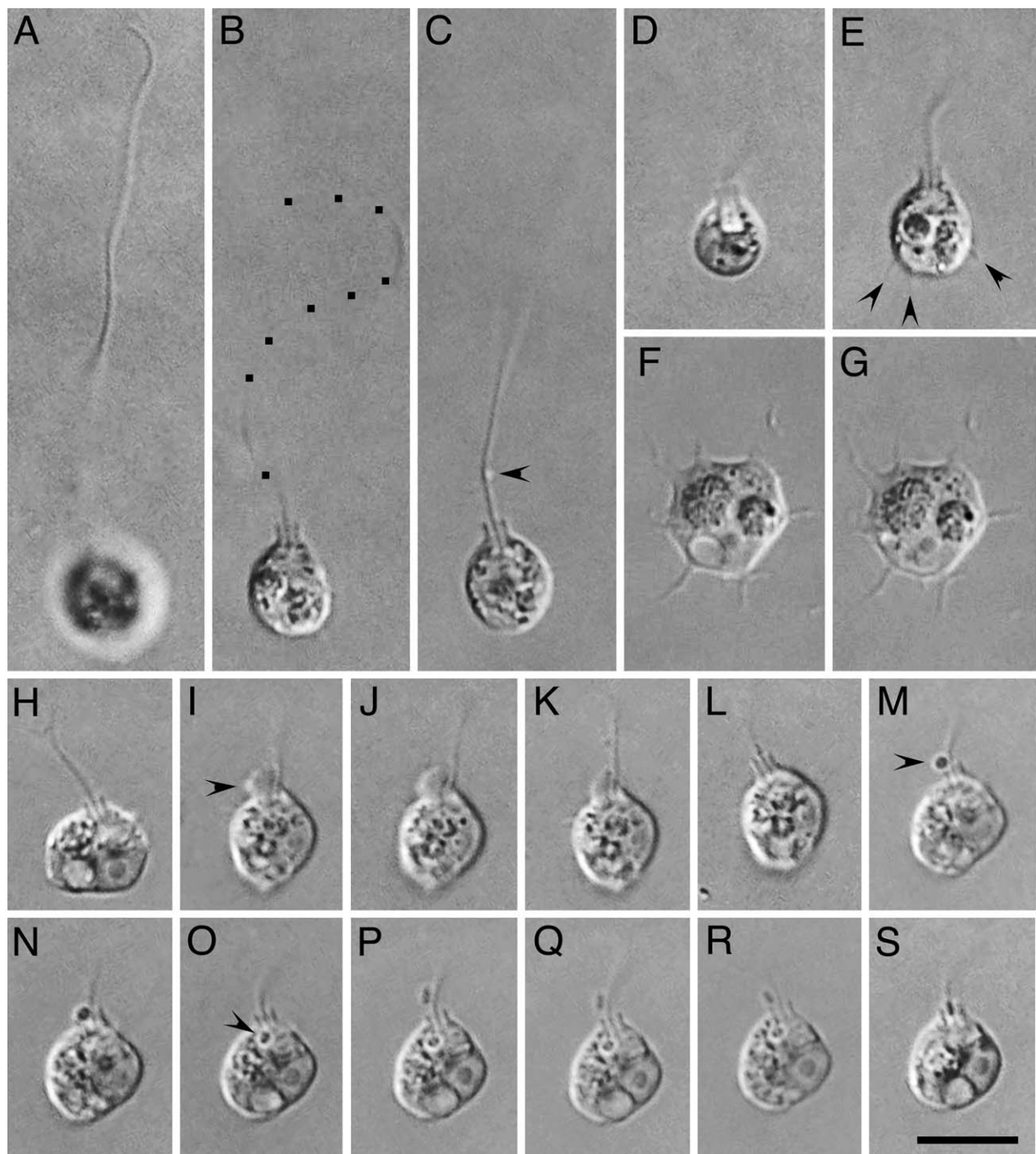
Figure 7. Differential interference contrast micrographs of *Phalansterium filosum* about 1 h after placement in observation chamber. **A-C** - Non-amoeboid flagellate phase, **A** - showing great length of the cilium, **B** - its asymmetric wave (marked by squares), **C** - an attached bacterium (arrow). **D** - ciliary pocket; **E** - cell with a short cilium and threadlike projections that may either be broken attachment stalks or filopodia; **F-G** - the same amoeboid cell with filled (**F**) and a few seconds later contracted (**G**) contractile vacuole; dense nucleolus visible to right of contractile vacuole. **H-S** - successive images of a single feeding flagellate, over 183 s spanning two complete contractile vacuole contraction/growth cycles; **H** - nucleus and nucleolus to right of contractile vacuole, collar normal; **I-J** - collar transiently expands to a lamellipodium; **M** - round bacterium (arrow) trapped

clade with *Phalansterium* aff. *solitarium* reproducibly sister to Varipodida (Supplementary Fig. S1), equally supported by 28S rRNA: Glücksman et al. 2011).

The cilium of *P. filosum* is over five times as long as the cell (Fig. 7A); unlike the strain identified as *P. solitarium* by Ekelund (2002) stated to beat in a sine wave, it beats asymmetrically, the basal region (6 μm approx.) remaining almost straight in cells not engaged in prey ingestion (Fig. 7B-E). In an hour after cells were transferred from old culture dishes in which only flagellate stages were visible, they produced an apparently non-ciliate amoeboid phase forming pointed tapering pseudopods (Fig. 7F-G). Experiments indicated that by five minutes after such transfer up to about half the flagellates may develop extensive pseudopods like those illustrated, mostly without losing their cilia. Two and a half hours later they all had retracted their pseudopodia. In old cultures flagellates are anchored, primarily at the noncilium end by means of one or more short fine stalks, either to the bottom of the culture dish or indirectly to masses of bacteria. Unlike *P. solitarium* (Sandon 1924, 1927), *P. filosum* lacks a granular lorica. We observed and recorded ingestion (Fig. 7H-S) proving phagotrophy for the first time in any *Phalansterium*. Individual bacteria are ingested in the pocket after passing through the periciliary space within the collar; clumps seemed to be rejected after travelling down to the collar. Sometimes the collar extended asymmetrically as a lamellipodium for a few seconds (Fig. 7I). Figure 7H-S documents the growth and contraction of the contractile vacuole, always conspicuous at the hind end of the cell adjacent to the somewhat more anterior nucleus. The strain of Ekelund (2002) resembled *P. filosum* (not *P. solitarium*) in size, but differed from both *P. solitarium* and *P. filosum* in having a non-granular gelatinous sheath and probably represents a third solitary species of *Phalansterium*.

Thus Variosea include both non-ciliate amoebae and flagellates with pointed pseudopods as well as the multiciliated amoeba *Multicilia*. Clearly pointed pseudopodia or subpseudopodia are present in all three groups of Conosa (Fig. 1); moreover cilia have been lost by some lineages within all three conosan groups but retained by others.

Hartmannellidae are Paraphyletic

Amoebae of the family Hartmannellidae Volkonsky, 1931 currently occupy four very different positions in the phylogenetic tree; this family is evidently paraphyletic. The most remarkable case is *Hartmannella vermiformis*, which in all published trees groups with *Echinamoeba* not other hartmannellids. It significantly differs from all other *Hartmannella* spp. in being worm-shaped rather than slightly clavate, with length/breadth ratio usually more than 6, and possessing a strict tendency to branch when changing the direction of locomotion (Fig. 8D-E ; see also Page 1967, 1974). To stress this divergence, we establish a new genus *Vermamoeba* and family Vermamoebidae to accommodate it within the new order Echinamoebida. A body of environmental sequences available in GenBank groups with *V. vermiformis* suggesting that it is not a monospecific lineage (Dyková et al. 2008).

Another separate clade containing a hartmannellid consists of *Nolandella* ATCC50913, *Nolandella* PRA27 strain and the marine "*Hartmannella*" *abertawensis*. The strain ATCC50913 was illustrated by a single photograph in Tekle et al. (2008), showing an amoeba, generally resembling both *Nolandella hibernica* Page, 1980 and *H. abertawensis*. Page (1983 p. 18) mentioned as distinctive characters of *Nolandella* certain eruptive activity and the cell surface coat; however, neither is definitive. Occasional eruptive activity was seen by A.Smirnov during his observations on *H. abertawensis,* type strain CCAP 1534/9; a cell coat ca. 30 nm in thickness, very much resembling that illustrated by Page (1983) was found in a *Saccamoeba* cf. *limax* strain from Valamo Island (North-West Russia) (Fig. 8 I-J). In LM *Nolandella hibernica* and *H. abertawensis* are very similar and differ significantly from all other *Hartmannella* or *Saccamoeba* strains (Fig. 8 A-C; H). Hence we recognise the marine clade containing *Nolandella* and "*Hartmannella*" *abertawensis* as a new order

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at collar, **N** - passing into top of collar space, **O** - in ciliary pocket; **P-Q** - round bacterium visible in ciliary pocket; second, rod bacterium passing down cilium into space inside collar, **S** - round bacterium no longer in ciliary pocket, having passed into the cytoplasm. Scale bar 10 μm. This strain was isolated from a mixed culture from a top cm forest soil sample near a flooded stream in Khao Yai National Park, Thailand, 15 December, 2001 (TCS), serially diluted into soil extract medium on 21 December 2001.
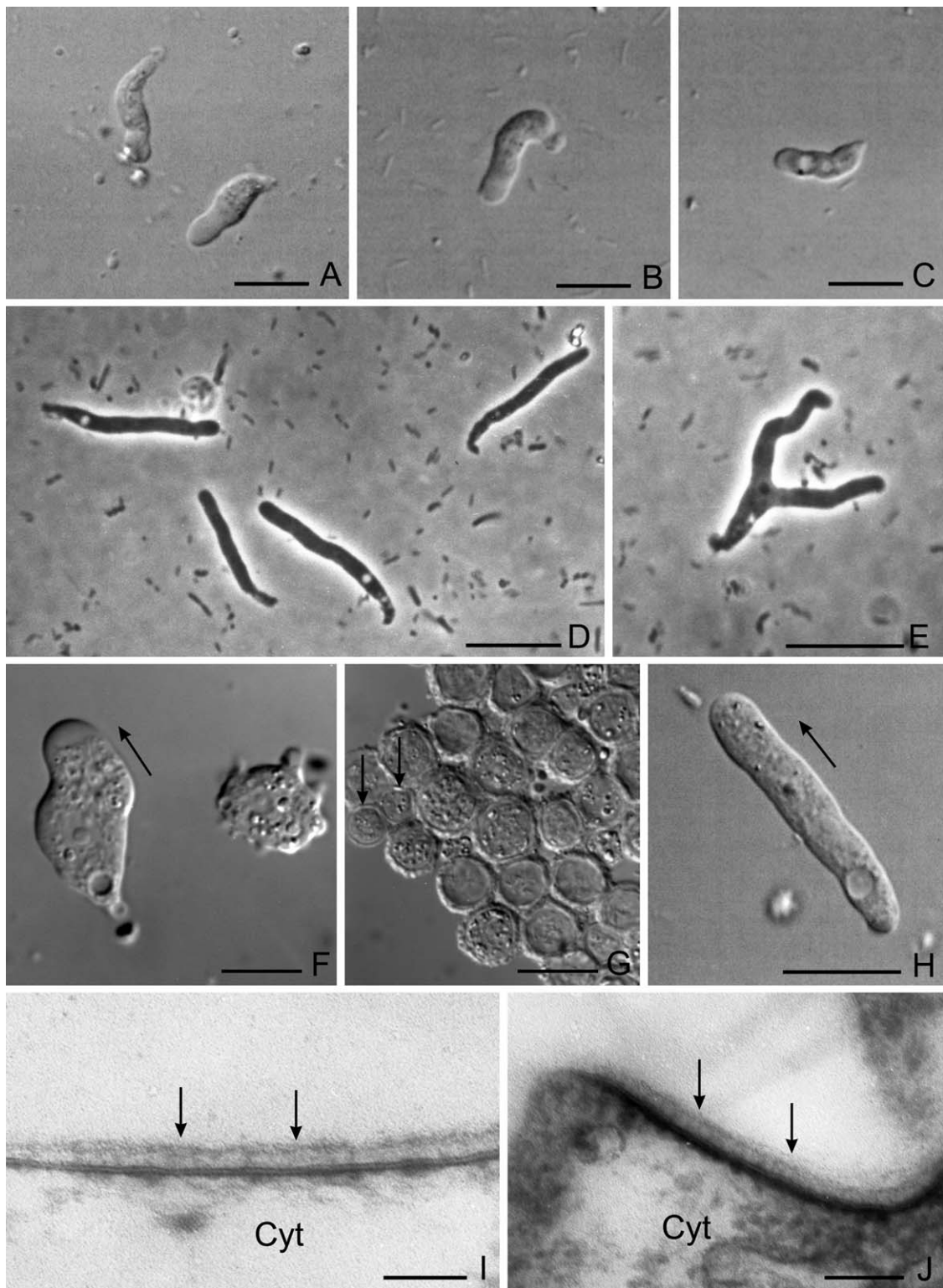
Figure 8. Hartmannellids. **A** - *Nolandella hibernica* CCAP 1534/10 (type strain); **B-C** - *Hartmannella abertawensis* CCAP1534/9 (type strain); **D-E** - *Vermamoeba* (=*Hartmannella*) *vermiformis*, Valamo strain isolated by A.Smirnov. Note characteristic furcation of the cell in **E**; **F-G** - strain 4/3 Da/1D – original photographs from 22.09.2000. Trophozoites and cysts. Data from the record of that time: cells are 16–18 μm

Nolandida with the single family Nolandellidae, renaming *H. abertawensis* Page, 1980 *Nolandella abertawensis* Cavalier-Smith and Smirnov comb. n. Sequencing of the type strain *Nolandella hibernica* CCAP 1534/10 is desirable to clarify the question.

Brown et al. (2011) showed that *H. cantabrigiensis* is closely related to *Copromyxa protea*, considered them congeneric and therefore renamed it *Copromyxa cantabrigiensis* by priority rule, but unlike the present classification retained the name *Hartmannella* for *H. vermiformis* Page, 1967. We accept that *Copromyxa* and *H. cantabrigiensis* must be in the same family; however treating them as one genus may be premature. The life cycle of *Copromyxa* is rather complex and not yet really known; it includes formation of a fruiting body and an incompletely studied part involving formation of sphaerocysts (Brown et al. 2011). These characters are likely of generic level, despite the vegetative morphological and sequence similarity. The latter is not close – the distance between *H. cantabrigiensis* and *C. protea* is comparable with that between *Saccamoeba* and *Glaeseria* (Brown et al. 2011 p. 6). Biological differences of similar level, e.g. nuclear divison in cysts are used to separate *Glaeseria* from other hartmannellids (Page 1974, 1988). We therefore keep the genus *Hartmannella* with *H. cantabrigiensis* the core species; this will be preferable if future work shows that all its relatives closer than *Copromyxa protea* form solitary cysts not fruiting bodies; only if it were shown that the *H. cantabrigensis*/*Copromyxa* clade ancestrally had fruiting bodies would a change to *Copromyxa* be reasonable. We retain the older family Hartmannellidae for this clade plus *Saccamoeba, Cashia* and *Glaeseria* and place the morphologically very similar but not yet sequenced *Copromyxella* in it. The family Hartmannellidae in this revised sense remains paraphyletic (but much less deeply and multiply as before) and seems to be ancestral to Amoebidae (e.g. Cole et al. 2010; Corsaro et al. 2010; Tekle et al. 2008). This means that the monotactic limax morphotype characteristic of Hartmannellidae was ancestral to the polytactic one shared by *Amoeba* and *Chaos*. Such an ability to deduce the ancestral morphotype (often not possible for two holophyletic sister groups) is a neglected phylogenetic advantage of paraphyletic or ancestral taxa, as explained elsewhere (Cavalier-Smith 2010). Strain *Hartmannella* 4/3 Da/10 (originally "4/3 Da/1D"), sequenced by Kudryavtsev et al. (2005) and very closely related to *Copromyxa protea* (Brown et al. 2011) was isolated by Susan Brown from Sourhope soil site (Brown and Smirnov 2004) but never illustrated; we therefore include photographs of it in Figure 8 (F-G). This strain in our culture, maintained on non-nutrient agar without overlay formed solitary cysts, sometimes arranged in clusters; we never observed anything resembling fruiting bodies of *Copromyxa protea*.

Relationship between Centramoebida, Thecamoebida and Dermamoebida

The taxon Centramoebida was created by Rogerson and Patterson (2002) to group *Acanthamoeba*, *Protacanthamoeba* and *Balamuthia*; the name was introduced by Patterson (1994) without proper diagnosis and emended by Cavalier-Smith et al. (2004). If we accept the suggestion that *Comandonia operculata* is a *Flamella* (Kudryavtsev et al. 2009), then all Centramoebida possesses cytoplasmic centrosomes that nucleate microtubules and are distinct from Thecamoebida or Dermamoebida both in this character and in locomotive morphology. However, the fact that *Balamuthia mandrillaris* in morphology resembles leptomyxids (where it was initially classified) not *Acanthamoeba*, but has similar cytoplasmic centrosomes that nucleate microtubules and robustly groups with *Acanthamoeba* in phylogenetic trees indicates that fundamentally related amoebae can diverge substantially in pseudopodial morphology. Thus the persistent tendency of Thecamoebida to group with moderate support with Centramoebida but not with Dermamoebida in our phylogenetic

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in length and 6–8 μm in breadth; vesicular nucleus ca 3 μm in diameter, single central nucleolus ca1.5 μm. Bulbous uroid in some cells, hyaline cap always pronounced. Occasional eruptions of the hyaloplasm were noted. No crystals. Cysts form irregular aggangements on the agar or may be single. Note that some of the cysts (arrowed) are considerably smaller and have finer wall than others. **H** – *Hartmannella cantabrigiensis* CCAP 1534/11 strain. Note very different appearance of this species from both *Vermamoeba vermiformis* and *Nolandella hibernica/H. abertawenis*. **I-J** - cell coat of a strain originating from Valamo island and identified by A.Smirnov as *Saccamoeba* cf *limax*. Note characteristic glycocalix (arrowed), which was noted only in some of the embeddings (not nessesarily in the best fixation). Cyt – cytoplasm. Scale bar is 10 μm in **A-H** and 100 nm in **I-J**.

Table 1. Revised classification of aerobic, non-fruiting, naked amoebae of phylum Amoebozoa.

Subphylum Lobosa Carpenter, 1861, em. Cavalier-Smith, 2009

Class Tubulinea Smirnov et al., 2005 em. (=Lobosea Cavalier-Smith, 2004)
    Order Euamoebida Lepşi 1960 em.
        Family Amoebidae (Ehrenberg, 1838) Page, 1987. *Amoeba, Chaos, Polychaos, Parachaos, Trichamoeba, Deuteramoeba, Hydramoeba*
        Family Hartmannellidae Volkonsky, 1931 em. *Cashia, Copromyxa, Copromyxella, Glaeseria, Hartmannella, Saccamoeba*
    Order Arcellinida Kent, 1880 18 families, not listed
    Order Leptomyxida (Pussard and Pons, 1976) Page, 1987
        Family Leptomyxidae (Pussard and Pons, 1976) Page, 1987. *Leptomyxa, Rhizamoeba*
        Family Flabellulidae Bovee, 1970 em. Page, 1987. *Flabellula, Paraflabellula*
        Family Gephyramoebidae Pussard and Pons, 1976. *Gephyramoeba*
    Order Nolandida Cavalier-Smith ord. n.
        Family Nolandellidae Cavalier-Smith fam. n. *Nolandella*
    Order Echinamoebida Cavalier-Smith, 2004 em. stat. n.
        Family Echinamoebidae Page, 1975 em. *Echinamoeba*
        Family Vermamoebidae Cavalier-Smith and Smirnov fam. n. *Vermamoeba*
Class Discosea Cavalier-Smith in Cavalier-Smith et al. (2004) em.
  Subclass Flabellinia Smirnov et al., 2005 stat. n., em.
    Order Dactylopodida Smirnov et al., 2005
        Family Paramoebidae Poche, 1913 em. Page, 1987; em. *Paramoeba, Korotnevella*
        Family Vexilliferidae Page, 1987. *Vexillifera, Neoparamoeba, Pseudoparamoeba*
    Order Vannellida Smirnov et al., 2005
        Family Vannellidae Bovee, 1979. *Vannella, Clydonella, Lingulamoeba, Pessonella, Ripella*
    Order Himatismenida Page, 1987
     Suborder Tectiferina Cavalier-Smith and Smirnov subord. n.
        Family Cochliopodiidae De Saedeleer, 1934. *Cochliopodium, Ovalopodium*
        Family Goceviidae Cavalier-Smith and Smirnov fam. n. *Gocevia[2], Paragocevia[2]*
     Suborder Parvamoebina Cavalier-Smith and Smirnov subord. n.
        Family Parvamoebidae Cavalier-Smith and Smirnov fam. n. *Parvamoeba*
    Order Stygamoebida Smirnov and Cavalier-Smith ord. n.
        Family Stygamoebidae Smirnov and Cavalier-Smith fam. n. *Stygamoeba, Vermistella*
    Order Pellitida Smirnov and Cavalier-Smith ord. n.
        Family Pellitidae Smirnov and Kudryavtsev, 2005. *Pellita*
    Order Trichosida[1] Moebius, 1889
        Family Trichosidae Moebius, 1889. *Trichosphaerium*
  Subclass Longamoebia Smirnov and Cavalier-Smith subcl. n.
    Order Dermamoebida Cavalier-Smith, 2004 em.
        Family Mayorellidae Schaeffer, 1926 em. *Mayorella*
        Family Dermamoebidae Cavalier-Smith and Smirnov fam. n. *Dermamoeba, Paradermamoeba*
    Order Thecamoebida Smirnov and Cavalier-Smith ord. n.
        Family Thecamoebidae Schaeffer, 1926, em. *Thecamoeba, Sappinia, Stenamoeba*
    Order Centramoebida Rogerson and Patterson, 2002 em. Cavalier-Smith, 2004
        Family Acanthamoebidae Sawyer and Griffin, 1975. *Acanthamoeba, Protacanthamoeba*
        Family Balamuthiidae Cavalier-Smith in Cavalier-Smith et al., 2004. *Balamuthia*

Discosea incertae sedis: Hyalodiscidae Poche, 1913 *Hyalodiscus* Hertwig and Lesser, 1874 (we are uncertain that it belongs in Amoebozoa as its rolling motion is unique; confusingly in botanical nomenclature *Hyalodiscus* Ehrenberg is a diatom)
Lobosa incertae sedis: *Pseudothecamoeba, Thecochaos, Janickia*; Stereomyxidae[4] Grell, 1966 (*Stereomyxa, Corallomyxa*).

Table 1 (*Continued*)

Subphylum Conosa Cavalier-Smith, 1998 em. 2009 (Archamoebea, Mycetozoa omitted)
Class Variosea Cavalier-Smith in Cavalier-Smith et al., 2004 em.
Order Varipodida Cavalier-Smith in Cavalier-Smith et al., 2004
Family Filamoebidae Cavalier-Smith in Cavalier-Smith et al., 2004. *Filamoeba, Flamella*[3]
Family Acramoebidae Smirnov et al., 2008. *Acramoeba, Grellamoeba*
Order Phalansteriida Hibberd, 1983
Family Phalansteriidae Kent, 1880/1. *Phalansterium*
Order Holomastigida Lauterborn, 1895 stat. n. Cavalier-Smith, 1997
Family Multiciliidae Poche, 1913. *Multicilia*

[1] Assignment to Flabellinia needs corroboration

[2] These genera need to be re-isolated and studied to clarify their position

[3] *Comandonia* probably is a junior synonym of *Flamella*, not of *Acanthamoeba* (Kudryavtsev et al. 2009)

[4] Assignment of *Corallomyxa* to Cercozoa (Tekle et al. 2008) was based on misidentification; the strain sequenced belongs instead to a major new endomyxan genus, *Filoreta* distinctly different from all stereomyxids (Bass et al. 2009a)

analyses may reflect a true relationship. The same relationship is found on myosin II trees, which also show Dermamoebida as monophyletic (Berney and Cavalier-Smith unpubl. observ.). We have therefore transferred Centramoebida from Variosea to the class Discosea, which contains both Dermamoebida and Thecamoebida, and established a new subclass Longamoebia for these three orders, which contrasts them with Flabellinia, here treated as a subclass.

Higher-level Groups of Lobose Amoebae

Transfer of Centramoebida to Discosea means that Variosea now include only the orders Phalansteriida, Holomastigida, and Varipodida, the first two of which are vegetatively ciliate, whilst the other has pointed, sometimes branched subpseudopodia unlike any Discosea or Tubulinea. Thus Variosea and Discosea are each now more distinct. Our Bayesian analysis (Supplementary Fig. S1) weakly suggests for the first time that Phalansteriida plus Varipodida may be a distinct clade, whereas *Multicilia* may be less close and possibly sister to the original Conosa (Mycetozoa plus Archamoebae).

Cavalier-Smith (2009) transferred Variosea (in the revised sense of the present paper) to the subphylum Conosa, formally restricting subphylum Lobosa to the classes Tubulinea and Discosea. The thus broadened Conosa is monophyletic and holophyletic on the tree of Shadwick et al. (2009), which has the most comprehensive taxon sampling yet for protostelids, provided that we include only core protostelids (i.e. the first four 'protosteloid' clades

on fig. 3 of Shadwick et al. 2009) within Protostelea and Conosa. We agree that two of the three singleton 'protosteloid' species that branch independently within Lobosa (Shadwick et al. 2009) are best not called protostelids, but treated as convergent origins of stalked cysts within Vannellidae and Acanthamoebidae.

The inclusion of several environmental sequences (Supplementary Fig. S1) makes it clear that Varipodida is a large taxon, more important than hitherto appreciated, containing *Filamoeba*, *Acramoeba, Grellamoeba, Flamella* and also an ATCC 50593 strain labelled '*Arachnula*' (Tekle et al. 2008). However, the single published LM picture shows that this ATCC strain was misidentified, as it does not have expanded reticulose pseudopods as *Arachnula* does (Bass et al. 2009a; Cienkowski 1876). We showed by sequencing a genuine *Arachnula* that it belongs in subphylum Endomyxa of Cercozoa (Bass et al. 2009a).

Revised Classification of Lobose Amoebae (Table 1)

There are three reasons for providing a revised system of lobose Amoebozoa. First, to reconcile and merge the contrasting systems of Cavalier-Smith et al. (2004) and Smirnov et al. (2005). Second, new sequences and an improved phylogeny now allow us to classify many genera left incertae sedis by Smirnov et al. (2005). Finally, morphological studies have improved knowledge on some species not yet sequenced. Table 1 summarizes the classification.

Our revised system retains Tubulinea (Smirnov et al. 2005) slightly expanded to equate it with Lobosea of Cavalier-Smith et al. (2004). To rationalise the non-congruity of sequence trees with both previous higher classifications of Tubulinea and thereby remove the deep paraphyly of Euamoebida (sensu Cavalier-Smith et al. 2004) or the equivalent Tubulinida (Smirnov et al. 2005) we split this assemblage into five orders: Euamoebida, Arcellinida, Nolandida, Leptomyxida and Echinamoebida. We elevate the superfamily Echinamoeboidea Cavalier-Smith, 2004 in rank to order. We retain the older name Euamoebida as a more precisely defined order making it the holophyletic sister to Arcellinida. The clade comprising Arcellinida and the revised Euamoebida is entirely freshwater and with smooth pseudopodia without spines and thus Euamoebida is morphologically more homogenous than before.

We have now sorted the genera simply listed by Smirnov et al. (2005) into families, mostly in line with the morphological system of Page (1987) and Cavalier-Smith et al. (2004). After finding that the ATCC "Gephyramoeba sp." was misidentified, we restored the family Gephyramoebidae Pussard et Pons, 1976 within the order Leptomyxida.

We accept the class Discosea Cavalier-Smith (Cavalier-Smith et al. 2004), while Flabellinea of Smirnov et al. (2005) is reduced in rank as subclass Flabellinia, retaining all subordinate taxa then included. In addition, we add to Flabellinia (1) order Himatismenida, following the findings of Kudryavtsev et al. (2005); (2) order Trichosida (shown to be Amoebozoa by Tekle et al. (2008) but not previously assigned to a class) and (3) a new order Stygamoebida, established for *Stygamoeba* and *Vermistella*. *Vermistella antarctica* (Moran et al. 2007), included in the tree of Tekle et al. (2008), has very specific morphology and ultrastructure (first of all, very characteristic flattened, ribbon-like mitochondrial cristae, combined with the presence of dictyosomes in the cytoplasm), so similar to *S. regulata* Smirnov 1995 that we can reasonably suggest that these two genera are related. So we deduce that Stygamoebida is an independent branch within Flabellinia. We established an order Pellitida to accommodate these unusual flattened, fan-shaped amoebae with extremely thick cell coat and unique mode of adhesion and phagocytosis. We retain Himatismenida Page, 1987 but split it into two suborders, following the dichotomy between *Parvamoeba* and the rest of himatismenids. Within the new suborder Tectiferina we keep the family Cochliopodiidae and make a new family Goceviidae grouping *Gocevia* and *Paragocevia*. We

created suborder Parvamoebina with a single family Parvamoebidae to separate these very unusual organisms from Tectiferina. The emended class Discosea now has a slightly modified diagnosis reflecting that of Flabellinea (Smirnov et al. 2005); subclass Flabellinia, with narrowed diagnosis, now includes essentially all fan-shaped amoebae.

We establish a new family Dermamoebidae for *Dermamoeba* and *Paradermamoeba* and a separate order for Thecamoebidae sensu stricto. We transfer Centramoebida from Variosea to Discosea and group these three orders as new sublass Longamoebia.

The proposed system splits gymnamoebae into 3 classes as in Cavalier-Smith et al. (2004) and (14) orders - more than in previous systems. It reflects the congruence of pseudopodial and cell surface differences with deep branches on the molecular phylogenetic tree. The large number of distinct branches with few genera probably partly stems from currently sparse knowledge of the diversity of these organisms. The total number of known naked amoeba species is only about 200, over 10 times less than even the most modest estimate for ciliate species; virtually any detailed faunistic study of naked amoebae yields many new species (Butler and Rogerson 2000; Finlay and Maberly 2000; Moran et al. 2007; Smirnov and Goodkov 1995). Undoubtedly, many new species still await description. The class Variosea, now consisting of only six genera but many more non-identified environmental sequences, illustrates the potential for such expansion.

Diagnoses of Newly Established and Revised Taxa

Class Discosea Cavalier-Smith 2004 em. Flattened naked amoebae, never producing tubular, subcylindrical pseudopodia and never altering the locomotive form. Cytoplasmic flow polyaxial or without a pronounced axis. No flagellate stage in the life cycle; subpseudopodia, if present, short, never both pointed and branched.

Subclass Flabellinia Smirnov et al. 2005 stat. n., em. Smirnov and Cavalier-Smith. Flattened amoebae, generally fan-shaped, discoid or irregularly triangular, never with pointed subpseudopodia or centrosomes.

Order Stygamoebida Smirnov and Cavalier-Smith ord. n. Flattened, elongate amoebae resembling tooth-pick or splinters, temporarily acquiring forked or branched form. Extended area of anterior hyaloplasm.

Family Stygamoebidae Smirnov and Cavalier-Smith fam. n. with diagnosis of the order. Type genus *Stygamoeba* Smirnov, 1995; other genus *Vermistella*.

Order Pellitida Smirnov and Cavalier-Smith ord. n. Cell coat envelops the entire cell and is integrated with the cell membrane. For locomotion and phagocytosis amoebae produce short subpseudopodia protruding through the cell coat and covered at the distal end solely by the cell membrane.

Order Himatismenida Page 1987 em. Flattened highly mobile amoebae, covered dorsally with a coat independent on the cell membrane or small low mobile globular amoebae with a dorsally irregularly wrinkled and semi-rigid thick cell coat.

Suborder Tectiferina Cavalier-Smith and Smirnov subord. n. Dorsal surface of cell covered with rigid coat with no defined aperture. Ventral surface naked. During cell division dorsal cell coat separates between daughter cells without any specific process of morphogenesis. Etymology: tectum *L.* roof; fero *L.* I bear.

Family Goceviidae Cavalier-Smith and Smirnov fam. n. Flattened, mobile amoebae with expanded crescent-shaped area of frontal hyaloplasm. Dorsal surface of cell covered with layer of fibrous material; frontal hyaloplasm free from this layer; no complete hyaloplasmic veil around locomotive cell. Centrosome may be present. Type genus *Gocevia* Valkanov, 1932. Other genus: *Paragocevia* Page 1987.

Suborder Parvamoebina Cavalier-Smith and Smirnov subord. n. Diagnosis as for sole family **Parvamoebidae** Cavalier-Smith and Smirnov fam. n.: Ovoid cells without flat hyaline margin; scarcely mobile; often stationary. Occasional locomotion very slow by a single, posteriorly projecting, temporary finger-like, filiform or broad pseudopod; irregularly wrinkled thick glycocalyx covers entire cell, except in some species on the pseudopod. Type genus *Parvamoeba* Rogerson, 1993.

Subclass Longamoebia Cavalier-Smith and Smirnov subcl. n. Flattened amoebae, elongated; with pointed subpseudopodia and centrosomes in one order. Etymol: Long- refers to frequent elongation of the cell compared with Flabellinia.

Order Dermamoebida Cavalier-Smith 2004 em. Revised diagnosis: Amoebae with smooth cell surface or with wide ridges, never wrinkled. Cell coat thick, multilayered or consisting of tightly packed helical structures.

Family Mayorellidae Schaeffer 1926 em. Flattened amoebae producing short conical pseudopodia. Cell coat a thick, multilayered "cuticle".

Family Dermamoebidae Cavalier-Smith and Smirnov fam. n. Amoebae with smooth outlines, oblong, lingulate or lancelolate in locomotion. Cell surface never wrinkled. Cell coat thick, multilayered or consisting of tightly packed helical structures. Type genus *Dermamoeba* Page and Blakey, 1979; other genus *Paradermamoeba*.

Order Thecamoebida Smirnov and Cavalier-Smith ord. n. Amoebae with smooth outlines, oblong, striate or rugose, with deep anterolateral hyaline crescent. Cell surface wrinkled, often with longitudinal dorsal folds. Cell coat thin, dense, amorphous or its basal layer is amorphous.

Class Variosea Cavalier-Smith 2004 em. Aerobic ciliated amoebae with conical microtubular cytoskeleton and only temporary pointed pseudopodia or non-ciliate amoebae with long, tapering, usually pointed, often branched subspseudopodia.

Order Echinamoebida Cavalier-Smith 2004 (as superorder) stat. n. em. Flattened limax amoebae with or without spine-like subpseudopodia; if spiny subpseudopodia absent, length/breadth ratio >6. Constituent families Echinamoebidae; Vermamoebidae.

Order Nolandida Cavalier-Smith ord. n. Marine limax amoebae without spiny subpseudopodia; glycocalyx basally of discrete units (truncated pyramids), with (*Nolandella hibernica*) or without (*N. abertawensis*) outer hexagonal layer (surface elements not cup- or sucker-like as in Saccamoebidae and *Vermamoeba*); length/breadth ratio <6. Sole family Nolandellidae fam. n. Cavalier-Smith with the same diagnosis and type genus *Nolandella* Page (1980b).

Genus *Vermamoeba* Cavalier-Smith and Smirnov gen. n. Worm-like amoebae, subcylindrical in cross-section, never clavate; length/breadth ratio >6. Stable anterior hyaline cap, sometimes small bulbous uroid. Often clearly branches when changing direction, temporarily forming two or more pseudopodia. Type species *Vermamoeba* (formerly *Hartmannella*) *vermiformis* (Page 1967) Smirnov and Cavalier-Smith comb. n. Etym: vermis *L.* worm, from its vermiform shape.

Family Vermamoebidae Cavalier-Smith and Smirnov fam. n. Worm-like amoebae, subcylindrical in cross-section, never clavate. Type genus *Vermamoeba.*

Family Hartmannellidae Volkonsky 1931 em. Monotactic amoebae with single vesicular nucleus. No traces of eruptive activity.

Order Euamoebida Lepşi 1960 em. 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.

Phalansterium filosum Cavalier-Smith and Chao sp. n. Diagnosis: A solitary *Phalansterium.* Body length 6.3-8.5 µm (mean 7.3), width 5.8-8.8 µm (mean 7.3). Cilium ~46 µm long, rigid near base, not clearly tapering, beats asymmetrically, catches bacteria and moves them rapidly down to collar and into sub-collar pocket for ingestion; its base surrounded by a collar, 1.2 µm wide and 1.5 µm long, or 1.9 µm when extended during prey uptake (external dimension; on its inner side facing the ciliary pocket it is ~2.4 µm), whose interior opens into a substantial ciliary pocket. Type culture: CCAP 1576/1, contaminated by *Cercomonas nebulosa* (Bass et al. 2009b). Type sequence: GenBank EF143966, 1859 nt of 18S rDNA. Type illustration: Figure 7. Type locality: forest soil near flooded stream, Khao Yai National Park, Thailand; collected by TCS, 15 December, 2001, serially diluted into soil extract medium 21 December 2001, later cultured by EC. Etym: filum *L.* thread, because of its transient filose pseudopodia. Differential diagnosis: differs from *P. solitarium* Sandon, 1924 in seven ways: has transient amoeboid phase with long pointed pseudopods; no obvious lorica; smaller, cell body ~7 µm; slightly oval to almost spherical; contractile vacuole large and conspicuous at cell posterior; cysts much smaller (5 µm, round with smooth undifferentiated wall, non-angular); collar more squat, approximately as wide as long.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.protis.2011.04.004.

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