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Guide to the methods of study and identification of soil gymnamoebae

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Summary

The present guide is an attempt to build a "bridge" between the general textbooks on protozoa and the guides to amoebae intended for specialists. We try to outline the subset of freshwater amoebae species that may be found in the soil and list them in the text. The extended introduction section provides detailed descriptions of the methods and shortcomings of amoeba investigation and gives one some ideas on the peculiarities, biology and ecology of soil amoebae. Special section guides the reader through the identification process to prevent him from potential errors. From our experience, dichotomous keys to amoebae are rather artificial and difficult in use, so this guide is based on a classification system of amoeba morphotypes, i.e. on the classification of the generalized shapes of the locomotive form of an amoeba. It allows easy and fast initial classification of an amoeba into one of 16 groups of species containing from two to twenty-five species. The section dedicated to each morphotype contains the sample plate of photographs and the list of relevant literature for further identification of species of the chosen morphotype.

Key words: gymnamoebae, amoeba, systematics, identification, methods, guide

Foreword

Amoeboid protists are among the most common and abundant microbes in all types of soil habitats. They can be isolated easily using relatively simple methods and most soil samples yield dozens of amoeba species. However, identification of most species is a difficult and challenging task and, in many cases, cannot be conclusive. Identification of amoebae (based on morphological and ultrastructural data) requires establishment of cultures and both light (LM) and electron (EM) microscopy examination, and is highly dependent on the carefulness and experience of the investigator. Many species are poorly or insufficiently described and correct identification of such amoebae requires detailed analysis of the literature, including older reports. Consequently, amoebae remain beyond the scope of attention of most soil biologists.

There are several comprehensive keys to marine, freshwater and soil amoebae, by Bovee and Sawyer (1979), Bovee (1985) and Page (1976a, 1983, 1988, 1991). They are intended principally for researchers who are already familiar with amoeboid protists and with LM and EM methods. Usage of these keys by a beginner results in numerous errors and misidentifications. On the other hand, textbooks on protozoa do not contain sufficiently detailed information on amoeboid protists to enable a reader to become familiar with this group. The section dedicated to Gymnamoebia published in the new Illustrated Guide to the Protozoa (Rogerson and Patterson, 2000) is a very useful source of data and amoebae images, but it does not represent a detailed key for the practical identification of species. There is an urgent need for an "intermediate" guide, and the present text is an attempt to build a "bridge" between the textbooks on protozoa and the guides to amoebae by E.C. Bovee, T.K. Sawyer and F.C. Page.

Soil amoebae have never been made the subject of separate guide or key. F.C. Page reasonably considers most of them to form a subset of the freshwater amoebae fauna (Page, 1976a, 1988, 1991). In this publication we try to outline this subset of amoebae more clearly. The present text has been designed to provide the reader with basic knowledge of the methods and shortcomings of amoeba investigation, together with some ideas on the peculiarities, biology and the ecology of soil amoebae, and to guide him though the identification process so that he will be better able to understand the keys and other relevant literature.

The greatest difficulty for beginners concerns the generic identification of amoebae and the use of dichotomous keys, which are rather artificial in the case of these organisms. To avoid this problem, this guide is based on a classification system of amoeba morphotypes (Smirnov and Goodkov, 1999a) based on the shapes of the locomotive form. We hope that this morphotype system represents a more recognisable and clearer structuring of amoebae diversity than a dichotomous key.

1. Introduction

1.1. What are "naked amoebae"? The scope of this guide

Within the field of protozoan ecology naked amoebae (or "gymnamoebae") are generally considered as a single "functional group", despite the fact that they are highly diverse and systematically heterogeneous. In this guide we decided to restrict the terms "naked amoebae" (and "gymnamoebae") to two groups of protists that are isolated from the environment using a common set of methods and which require similar approaches to their observation and study. These are the lobose gymnamoebae (class Lobosea, subclass Gymnamoebia), and the class Heterolobosea, which includes the schizopirenids and acrasids, in accordance with the classification scheme of Page (1987). We also include the order Himatismenida – a group of lobose amoebae of unclear systematic affinity that are very common in soils and frequently isolated together with gymnamoebae. All other naked amoeboid protists are beyond the scope of this guide, although the reader may find them occasionally in his soil samples.

1.2. Systematics of naked amoebae

There is a general consensus that the term "amoeba" embodies a type of organisation of the cell rather than a solid taxon or monophyletic group of organisms (e.g. Schaeffer, 1926; Page, 1987). Attempts to construct morphology-based systematics of amoebae using LM data (Schaeffer, 1926; Jahn and Bovee, 1965; Bovee and Jahn, 1965; 1966; Jahn et al., 1974; Page, 1976a) lead to the conclusion that morphology presents insufficient features for reliable species differentiation and recognition. Use of other characteristics, like the nuclear division patterns (Singh, 1955a; Singh et al., 1982) also generated more theoretical, rather than practical, schemes. The implementation of EM techniques allowed resolution of ultrastructural details which, when combined with morphological and biological characteristics, lead to the development of a more elaborate system for gymnamoebae (Page, 1987), and enabled species to be identified with a higher degree of confidence (Page, 1988, 1991). More recently, from the fruits of molecular biology research, it has been concluded that in some taxa even the resolution provided by Page's system is insufficient (e.g. De Jonckheere, 1998; Brown and De Jonckheere, 1999), and non-morphological data are required to distinguish isolates, especially among heteroloboseans. Other molecular studies have confirmed many of Page's groupings of amoeboid taxa and cast doubt on others; we now know for certain that gymnamoebae do not form a single, monophyletic group, as in trees based on both ribosomal DNA and actin sequence comparisons, naked amoebae are divided between at least three large clades (as well as several independent lineages) that are not reflected in the higher levels of the morphology-based classification of amoebae (Amaral-Zettler et al., 2000; Bolivar et al., 2001; Peglar et al., 2003). However, most of the morphologically defined lower level taxa from Page's system, like genera and even some families of amoebae, can be recognised as clusters in the molecular trees (Fahrni et al., 2003).

It is evident that a comprehensive, natural system of amoeboid protists is goal for the future. However, Page's system of gymnamoebae is still the most popular and useful for practical studies. It is a "classification" (an artificial system allowing the construction of keys and species identification, but not promising to reflect the phylogenetic relationships between all included taxa), designed for wide application across the field of protozoan research and both to facilitate the study of gymnamoebae morphological diversity and to differentiate between species. Consequently, the classification of gymnamoebae used in this guide (listed below) is based on that of Page (1987). All free-living genera, including marine, are listed here, as there is a need for an up-to-date checklist of taxa which includes "post-Page" modifications and additions.

The present checklist lists only "well-recognised" genera, which we define as follows:

a) The genus was correctly established (or revised) with an appropriate diagnosis following the basic rules of taxonomical nomenclature listed in the current edition of the International Code of Zoological Nomenclature.

b) The diagnosis of the genus and the descriptions of the species within the genus are sufficient for reliable re-isolation of these species. The distinctive features characterising the genus cannot be discounted by factors such as polymorphism, artefacts of observation, and culture conditions.

In this guide we do not follow some of the taxonomic innovations in the system of gymnamoebae offered by Rogerson and Patterson (2000). In particular, we prefer to maintain the traditional taxon Gymnamoebia instead of Ramicristate amoebae, and do not recognise the taxon Centramoebida which lacks a formal diagnosis (and which is, in our opinion, insufficiently justified). We omit some genera of vexilliferid amoebae, such as *Boveella*, *Striolatus*, and *Triaenamoeba*. Members of these genera are mostly known only from single isolates. Consequently, they are poorly documented and require more extensive photographic documentation, EM study and re-description to clarify their taxonomic status. Currently, due to the relatively high level of polymorphism of vexilliferids and paramoebids it is barely possible to confidently assign an isolate to any of these genera. For similar reasons we do not include the genus *Metachaos* Schaeffer, 1926 (see Smirnov and Goodkov, 1998 for details). In this chapter, to complement F.C. Page's keys, we prefer to accept the classification of the Heterolobosea by Page and Blanton (1985) and Page (1987) but to add all the well-described genera established since 1987. To simplify identification and comparison, we have kept the taxonomic range of groups of amoebae as listed by Page (1987). The taxa including (or consisting of) freshwater and/or soil species are in bold.

Class Lobosea Carpenter 1861
Subclass Gymnamoebia Haeckel 1866
Order Euamoebida Lepsi 1960
1. Family Amoebidae (Ehrenberg 1838) Page 1987
Genera: ***Amoeba, Chaos, Polychaos, Parachaos, Trichamoeba, Hydramoeba, Deuteramoeba***
2. Family Thecamoebidae (Schaeffer 1926) Smirnov and Goodkov 1994
Genera: ***Thecamoeba***, ***Sappinia, Dermamoeba, Paradermamoeba, Pseudothecamoeba,*** *Parvamoeba,* ***Thecochaos***
3. Family Hartmannellidae (Volkonsky 1931) Page 1974
Genera: ***Hartmannella, Saccamoeba, Cashia, Glaeseria***, *Nolandella*
4. Family Paramoebidae (Poche 1913) Page 1987
Genera: ***Mayorella, Korotnevella***, *Paramoeba*
5. Family Vexilliferidae (Page 1987)
Genera: ***Vexillifera***, *Pseudoparamoeba, Neoparamoeba*
6. Family Vannellidae (Bovee 1970) Page 1987
Genera: ***Vannella, Platyamoeba, Pessonella,*** *Clydonella, Lingulamoeba*
Order Acanthopodida Page 1976
1. Family Acanthamoebidae Sawyer and Griffin 1975
Genera: ***Acanthamoeba, Protacanthamoeba***
Order Leptomyxida (Pussard and Pons 1976) Page 1987
Suborder Rhizoflabellina Page 1987
1. Family Flabellulidae (Bovee 1970)
Genera: *Flabellula,* ***Paraflabellula, Flamella***
2. Family Leptomyxidae (Pussard and Pons, 1976) Page 1987
Genera: ***Rhizamoeba, Leptomyxa***
Suborder Leptoramosina Page 1987
1. Family Gephyramoebidae Pussard and Pons 1976
Genus: ***Gephyramoeba***
2. Family Stereomyxidae (Grell 1966)
Genus: *Stereomyxa*
Order Loboreticulatida Page 1987
1. Family Corallomyxidae Page 1987
Genus: *Corallomyxa*

Class Lobosea incertae sedis:
Family Echinamoebida Page 1975
Genera: ***Echinamoeba, Filamoeba, Comandonia***

Class Heterolobosea Page and Blanton 1985
Order Schizopyrenida Singh 1952
1. Family Vahkampfiidae Jollos 1917
Genera: ***Vahlkampfia***, ***Paravahlkampfia***, *Neovahlkampfia, Heteramoeba,* ***Naegleria***, ***Willaertia, Tetramitus, Tetramastigamoeba***, *Pernina,* ***Learamoeba,***

Singhamoeba, *Monopylocystis,* ***Sawyeria***, ***Psalteriomonas***

Order Acrasida (Schroeter 1886) Page and Blanton 1985

1. Family Acrasidae (Van Tieghem 1880) Olive 1970
Genera: ***Acrasis, Pochenia***
2. Family Guttulinopsidae Olive 1970
Genera: ***Guttulinopsis, Rosculus***
3. Family Gruberellidae Page and Blanton 1985
Genera: *Gruberella,* ***Stachyamoeba***

Class Heterolobosea incertae sedis:
Euhyperamoeba fallax Seravin and Goodkov 1982
Order Himatismenida Page 1987
1. Family Cochliopodidae De Saedeleer 1934
Genera: ***Cochliopodium, Gocevia, Paragocevia,*** *Ovalopodium*

(The order Himatismenida we list as "incertae sedis", because of the uncertain taxonomic position of this taxon; see: Rogerson and Patterson, 2000).

1.3. Habitats of amoebae and the definition of "soil species"

Defining amoebae by their habitat is a problem, and perhaps lacks meaning. In fact, there can be no real distinction between "freshwater" and "soil" species (Page, 1988). Basically, all amoebae live in water; nominal "soil species" actually inhabit the layer of capillary water surrounding soil particles and filling the pores between them (Elliot et al., 1980; Foissner, 1987; Ronn et al., 1995). Thus, soil amoebae are aquatic species that have invaded (and continue to invade) terrestrial habitats (Stout, 1963; Old and Chakraborty, 1986) with differing degrees of success, depending on their ability to adapt to the different aspects of soil as a habitat.

Soil is a complex, highly structured habitat. Any soil is a system, which, in addition to the mineral compounds of the soil itself, includes numerous and diverse organisms - bacteria, protists, fungi, plants and animals, comprising several functional groups (Coleman, 1976). Due to their feeding activity, amoebae play an important role as grazers of bacteria (Coleman et al., 1978; Anderson et al., 1979) and have been recognised as one of the main controllers of bacterial populations because of their fast response to increases in bacterial numbers (Elliot et al., 1979; Clarholm, 1981; Pussard and Rouelle, 1986). Foster and Dormann (1991) demonstrated that soil amoebae produce pseudopods that can penetrate even into tiny micropores of soil aggregates in order to engulf bacteria. They suggest that this partly explains why bacteria are generally confined to the interior of soil macroaggregates, where they are unavailable to amoebae. Soil amoebae can feed on diatoms, nematodes, particles of organic matter, fungi and protozoa (Weber et al., 1952; Page, 1976a, 1988; Old and Chakraborty, 1986). Thus they enhance the nutrient cycling in the soil (Clarholm, 1984), and together with other protozoa they stimulate carbon and nitrogen cycling (Ekelund and Ronn, 1994; Alphei et al., 1996; Finlay et al, 2000). Amoebae themselves are an important source of food for soil nematodes, that prefer (when possible) to feed on them rather than on bacteria (Coleman et al., 1977) and, perhaps, for other soil-inhabiting metazoans (Anderson and Bohlen, 1998). Other important roles of amoebae in the soil include decomposition of organic matter and chemical substances, chemical influences in the plant rhizosphere (Geltzer, 1962) and transport of viruses (Old and Chakraborty, 1986).

Acting together, many abiotic and biotic factors in the soil create numerous and diverse microhabitats so that soil can harbour virtually any amoeba species which is tolerant to the relatively low salinity of the soil capillary water. Perhaps that is why most freshwater species may be found in soil habitats, while nominal "marine" species are not detected in soil (at least not in active populations). Generally, most of the soil-inhabiting amoebae species are small or medium-sized organisms which are able to fit into the small pore spaces in the soil and are capable of surviving temporary drying of the soil. A relatively small size seems to be an important characteristic of soil-inhabiting species, because it allows them to explore the smaller pores and capillary spaces unavailable to nematodes (Elliot et al., 1980). Also, the largest pores empty first when soil dries which may limit the distribution of larger amoebae in soils – and give smaller species, which are able to persist in the remaining, smaller, water-filled pores, an advantage (Bamforth, 1963). However, size itself does not play a decisive role; there are large specialised reticulate amoeboid species in soil, e.g. those belonging to the order Leptomyxida, which may reach several millimetres in length. From examination of existing species data, it appears that the only group which does not exist in most types of soil are the largest, non-encysting, "proteus-like", amoebae of the genera like *Amoeba* and *Chaos* (Clarholm, 1981; Bischoff and Anderson, 1998). Probably, these characteristics mean that they cannot survive in a thin water film or fit into the small pores between soil particles (this and related problems were extensively reviewed by Ekelund and Ronn, 1994). Non-encysting species occur much more rarely in soil than encysting species, and recent data (Smirnov and Brown, 2000; Smirnov et al., 2001) indicate that our understanding of the capacities for encystment in most amoebae is incomplete. In this chapter we accept that "soil amoebae", in terms of species diversity, is just a subset of "freshwater" species,

together with a small number of species which have only ever been isolated from soil.

2. Organisation of the cell and the biology of amoebae

2.1. Morphology and cell structure (light-microscopy)

Amoeboid protists most often isolated from soil can be divided into three types which are easily differentiated by their pseudopodial pattern. These are **lobose**, **filose** and, more rarely, **reticulose** amoebae (Fig. 1). Lobose amoebae are the most common. Reticulose and filose species are only isolated occasionally using the methods described below and are beyond the scope of this guide.

When you observe a lobose amoeba under the light microscope for sufficient time several remarkable features can be seen. The cell may exhibit many different conformations, especially when it is stationary ("**resting**") (Fig. 2 A). When a cell starts to move, it changes shape rapidly, and it is hard (perhaps impossible) to describe a typical form of an amoeba during **non-directed** movement (Fig. 2 B). However, when the cell adopts continuous, directional **locomotion** (Fig. 2 C) it becomes more stable. The shape of such a cell still undergoes minor changes, but it maintains the same type of organisation until it either stops moving or changes direction. The shape of an actively, continuously moving amoeba is called the **locomotive form**, as first recognised by Schaeffer (1926, p. 17) and established as a term by F.C. Page in the 1970's.

The cytoplasm of the locomotive form is clearly subdivided into two types. At the leading edge of a cell (especially in fan-shaped amoebae) you may see the transparent **hyaloplasm**, which does not contain any optically visible inclusions. The hyaloplasm is usually situated anteriorly and forms a **frontal hyaline area**, **antero-lateral hyaline crescent** or **anterior hyaline cap** (Fig. 3). However it should be stressed that these terms are partly artificial, as they are normally applied to the cell viewed from the top, and do not take into account the fact that, for example in *Thecamoeba* spp., dorsal ridges also consist of hyaloplasm. The remainder of the cytoplasm is filled with various granules, crystals and other inclusions and is called the **granuloplasm**. Here we should stress that the terms "hyaloplasm" and "granuloplasm" are not equivalent to the widely used terms "ectoplasm" and "endoplasm". The latter refer to the "gel" and "zol" viscosity (respectively) of the cytoplasm described in models of amoeboid movement (Mast, 1926; Allen, 1962; Grebecki, 1982).

Many amoebae produce **pseudopodia**. These are variable cell projections, which participate in movement and include both granuloplasm and hyaloplasm. However, most small and medium-sized amoebae move "as a whole", without forming distinct pseudopodia. In addition, most amoebae may form **subpseudopodia** which are small hyaline projections of different forms which do not take part in the movement of the cell.

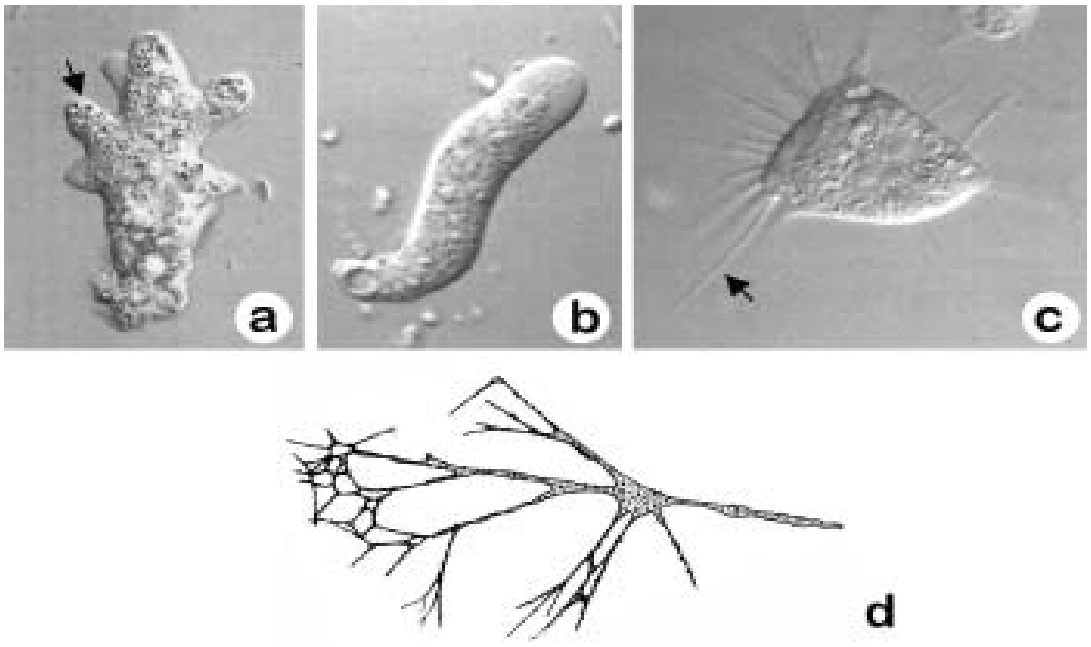

Fig. 1. Basic pseudopodial patterns in amoebae. a-b - lobose amoebae (*Deuteramoeba algonquinensis* and *Saccamoeba stagnicola)*, lobopodium is arrowed in a; c - filose amoeba (*Nuclearia simplex*), filopodium is arrowed; d - reticulopodial amoeba (*Biomyxa vagans*, after Bovee, 1985, modified). Not to scale.

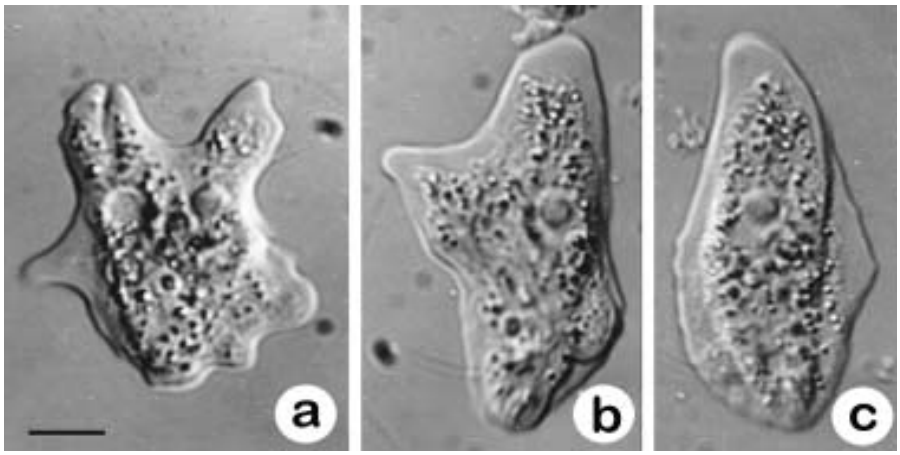

Fig. 2. Basic forms of an amoeba. *Paradermamoeba valamo*. a - stationary; b - during non-directed movement; c - locomotive. Scale bar: 10 μm.

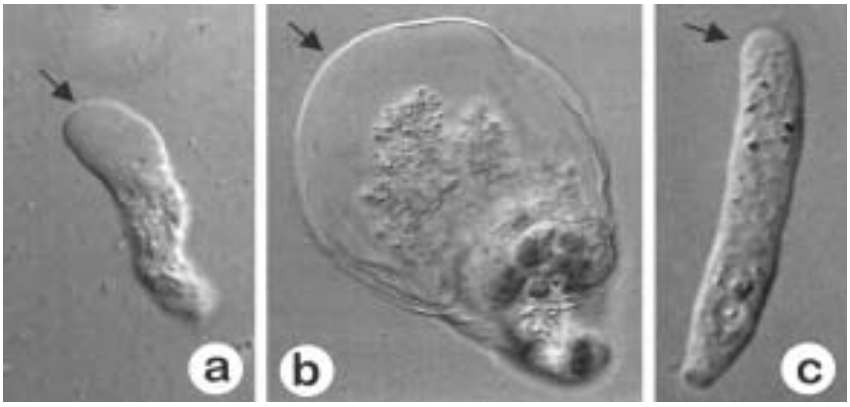

Fig. 3. The hyaloplasm in amoebae. a - frontal hyaline area (arrowed) in *Platyamoeba stenopodia*; b - antero-lateral hyaline crescent (arrowed) in *Thecamoeba sphaeronucleolus*; c - anterior hyaline cap (arrowed) in *Hartmannella cantabrigiensis*. Not to scale.

The posterior end of the locomotive form - **uroid** - has a distinct characteristic appearance in many amoebae species. Posterior formations are called **uroidal structures**. They have been recognised for a long time; it was Wallich (1863a, 1863b) who first decided that the appearance of the posterior part of an amoeba might be taxonomically important. Schaeffer (1918) seems to be the first who used the term "uroid". Later, Page (1972, 1974a, 1988) defined this term and classified uroidal structures into several types (Fig. 4).

Some amoebae have **folds** or **wrinkles** on the dorsal surface of the locomotive form. Wrinkles may appear also on the lateral surface of the cell (Fig. 5).

If you observe amoebae in a freshly made slide preparation or in liquid culture using an inverted microscope (see section 3.6) you may see cells floating in the liquid. These so-called **floating forms** (as with locomotive form, the term is applicable to both the conformation and to the cell itself) usually have several pseudopodia radiating from the central body mass (Fig. 6). All amoebae are able to float; consequently it is impossible to subdivide amoebae in water habitats into "benthic" and "planktonic" species. In any single location the same amoebae may be found in, and isolated from, both the water column and the benthos. However, numerical distribution of species may differ between the two habitats.

Observation of the locomotive forms of several amoeba species will soon reveal that there are clear differences between species in the characteristics of cytoplasmic flow during locomotion. In some amoebae cytoplasmic flows are steady, continuous, sometimes even hardly visible (Fig. 7 A). In other species, mostly in small "**limax**" (monopodial and cylindrical) amoebae, cytoplasmic flow is eruptive; short periods of rapid formation of the leading pseudopodium are followed by short breaks when an amoeba does not show any activity. This is called **eruptive movement** and is a characteristic feature of heteroloboseans (Fig. 7 B). It is important to note that some monopodial lobose amoebae may also display single cytoplasmic eruptions (Fig. 8). For example, it is a characteristic of the leptomyxids, but they never move in this way continuously.

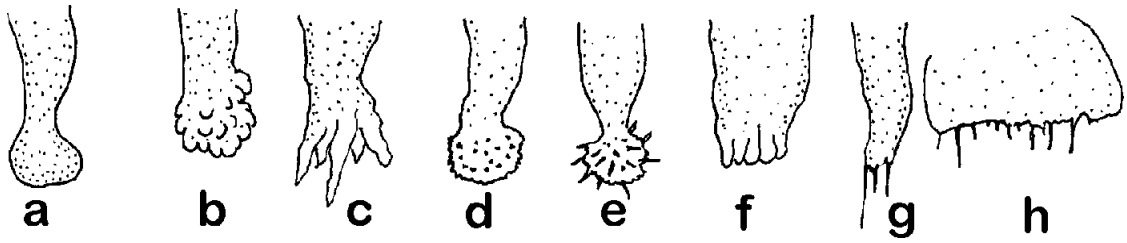

Fig. 4. Basic types of uroidal structures in amoebae. a - bulbous; b - morulate; c - fasciculate; d - spineolate; e - villous-bulbous; f - plicate; g-h - adhesive uroidal filaments. Not to scale.

If you are equipped with either phase contrast or differential interference contrast (DIC) optics you are usually able to see the nucleus of the cell. Inside the optically 'empty' nucleus there is a large dense patch (or many small dense patches) called the **endosome**, a term which may be used for any dense body within the nucleus. It is important to note that only a dense body confirmed (by EM or cytochemistry) to be an aggregation of ribonucleoproteins (RNP) may be termed "nucleolus". Unfortunately, in the literature these terms are usually used synonymously. The position of the nucleolus and number of nucleoli differ between species; they determine the type of the nucleus (Fig. 9) and require TEM for more detailed analysis (see below).

In most amoebae you may see one or several **contractile vacuoles**. The remaining notable structures visible by LM are **crystals** and other **cytoplasmic inclusions**. These are usually very characteristic, however, their appearance and shape are influenced by culture conditions and type of prey. It is believed that in most cases crystals are excretion products, and most other visible refractive inclusions are either lipid globules or endobionts. Mitochondria and other organelles may appear as dark spots, but they are not identifiable at LM level and require EM investigation.

Members of the order Himatismenida are rounded, lens-like amoebae, with a specific structure called a **tectum** covering the dorsal surface (Fig. 10). The tectum consists of a monolayer of scales, as in the genus *Cochliopodium*, or of a fibrose "cuticle", as in *Gocevia* and *Paragocevia*. The tectum is usually visible by LM as a fine punctuation of the amoeba periphery or as a "double-wall" of the granuloplasmic "hump" of the cell. Sometimes it is possible to see hyaline subpseudopodia, which the amoeba puts out from under the tectum.

2.2. Ultrastructure (electron microscopy)

Electron microscopy revolutionised our visualisation of the organisation of amoebae cells and approach to amoebae systematics. First applied to selected genera (Faure-Fremiet and Andre, 1968; Bark, 1973; Flickinger, 1974) and then systematically (Page, 1978, and further), EM revealed a variety of structures which are now routinely exploited in gymnamoebae systematics.

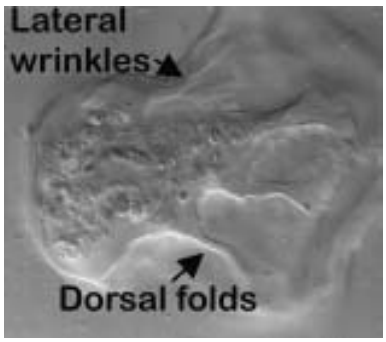

Fig. 5. Folds and wrinkles in amoebae. Sample: *Thecamoeba sphaeronucleolus.*

The cell coat of amoebae consists of a highly differentiated **glycocalyx**. It forms a variety of structures, and the various types (Fig. 11) can be described as follows:

a) **Amorphous glycocalyx**. This may be of different thicknesses and is most apparent in amoebae of the genus

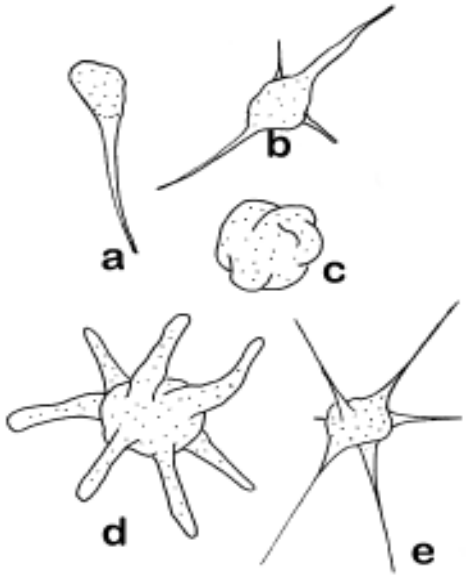

Fig. 6. Floating forms of amoebae. a-b and d-e - with radiating pseudopodia; c - without defined pseudopodia. Not to scale.

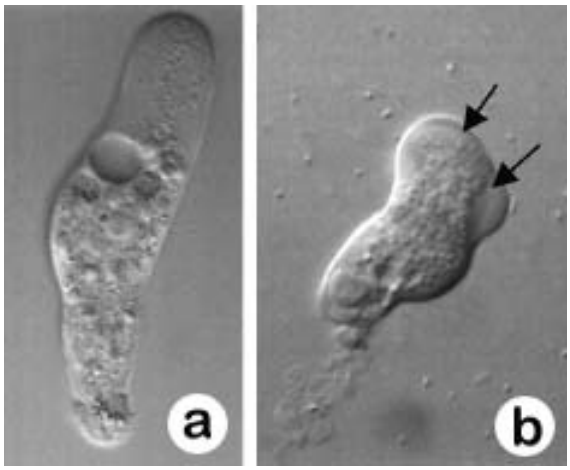

Fig. 7. a - *Saccamoeba limax*, moving by steady flow of the cytoplasm; b - *Acrasis rosea* showing eruptive movement (eruptions of the frontal hyaloplasm are arrowed). Not to scale.

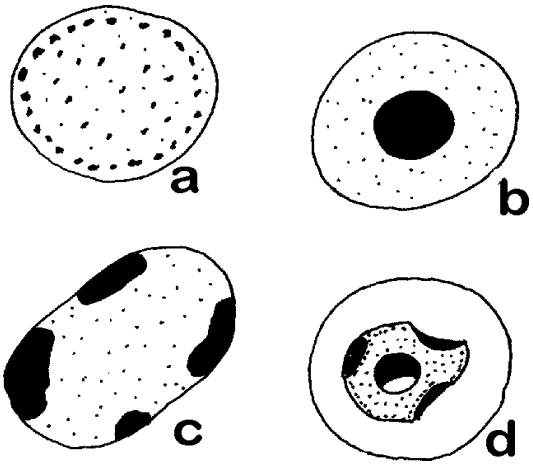

Fig. 9. Basic types of nuclear structure in amoebae. a - granular nucleus; b - vesicular nucleus; c - nucleus with peripheral nucleoli; d - nucleus with complex nucleolus. Not to scale.

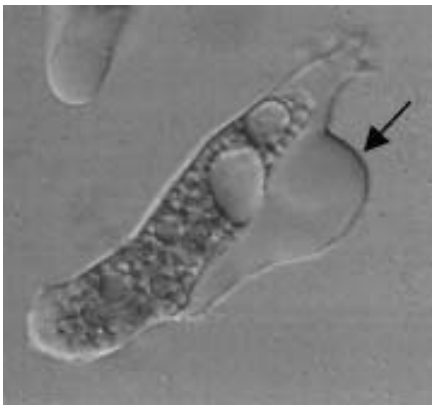

Fig. 8. Single cytoplasmic eruption in *Rhizamoeba flabellata*.

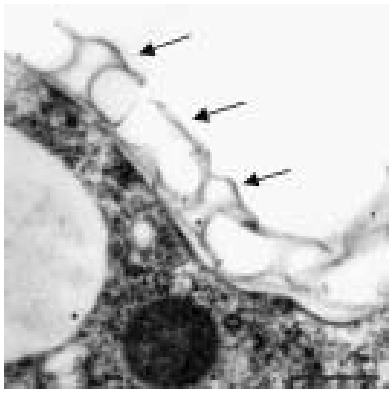

Fig. 10. TEM photographs of the tectum of *Cochliopodium*. The monolayer of scales is arrowed. Scale bar: 1 μm.

Thecamoeba, for example. It should be remembered that any glycocalyx will appear amorphous if it has been destroyed by suboptimal fixation. In some species of the genus *Saccamoeba* the glycocalyx usually appears to be amorphous, however, if fixation is optimised then it is possible to distinguish some cup-like or prismatic structures.

b) **Filamentous glycocalyx**. This consists of a layer of radiating filaments over a thin amorphous layer. The filaments may be rather thick, as in *Amoeba proteus* and *Chaos carolinense*, or very fine and hardly discernible, as in *Polychaos dubium* and *Polychaos annulatum*.

c) **Glycostyles**. The variety of these structures is extensive; they may be pentagonal, as in *Vannella* spp. (and in some species there are also long simple **filaments** among the glycostyles), hexagonal, as in *Vexillifera* spp., spiral, as in *Paradermamoeba* spp., or may form short, irregular hexagonal pyramids, as in *Pseudoparamoeba pagei*. Illustrations can be found in Page (1983, 1988).

d) "**Cuticle**". Although it is widely used in the literature, this term is not very suitable for a type of glycocalyx as it is has another meaning. Used in this context it indicates a thick cell coat consisting of several layers. The layers differ in structure, as in *Mayorella* spp. and *Dermamoeba* spp. For the latter Page and Willumsen (1979) also apply the term "tegument". In members of the genera *Gocevia* and *Paragocevia* only the dorsal surface of the cell is covered with a filamentous "cuticle".

e) **Scales**. Some amoebae, namely members of the genera *Korotnevella* and *Paramoeba*, are covered with a layer of scales. The chemical composition of scales is unknown, so we hesitate to classify them as a modification of the glycocalyx. Scales may be of different shapes and cover the entire cell of amoebae of both these genera.

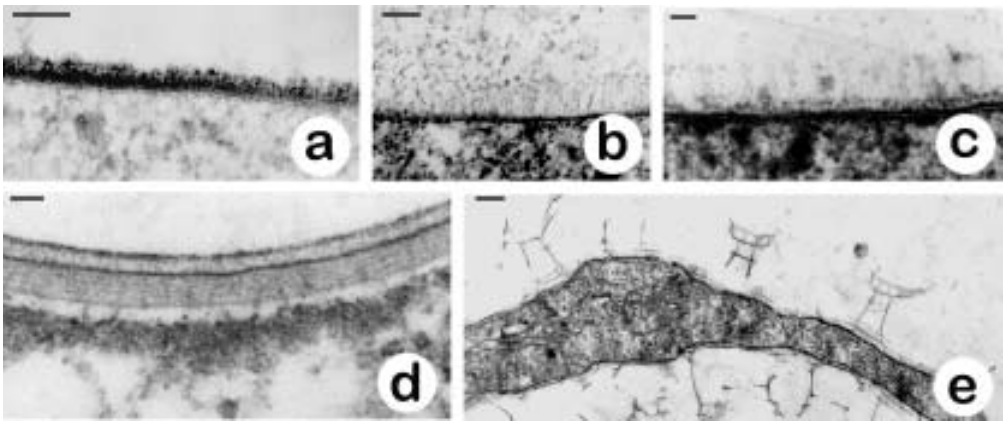

Fig. 11. Cell coat of various amoebae species. a - amorphous cell coat of *Chaos glabrum*; b - filamentous cell coat of *Polychaos annulatum*; c - glycostyles of *Vannella*; d - thick, multilayered cell coat "cutuicle" of *Mayorella*; e - scales of *Korotnevella bulla*. Scale bar: 100 nm.

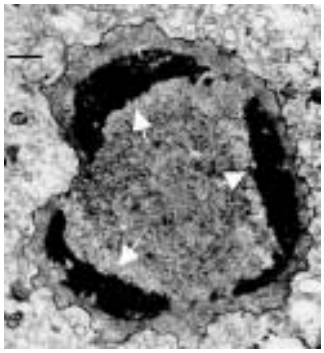

Fig. 12. Complex nucleus of *Polychaos annulatum*. Nucleoli are arrowed. Scale bar: 100 nm.

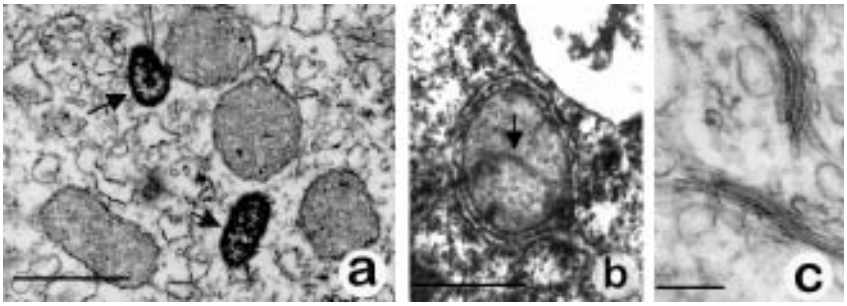

Fig. 13. Mitochondria, endobionts and dictyosomes in amoebae. a - mitochondria with tubular cristae and endobionts (arrowed) in the cytoplasm of *Polychaos annulatum*; b - flattened mitochondrial cristae (arrowed) in *Euhyperamoeba fallax*; c - dictyosomes in *Polychaos annulatum*. Scale bar: 0.5 μm.

In contrast, the tectum of *Cochliopodium*, which also consists of a layer of scales, covers only the dorsal surface of the locomotive form.

One of the most remarkable and characteristic structures in the nucleus is the nucleolus (or nucleoli). According to the classification by Raikov (1982) we can distinguish between a **vesicular** nucleus with a single central nucleolus and a **granular** nucleus with many small nucleoli. There are also intermediate types with several **large peripheral nucleoli**, as in *Thecamoeba striata*, or with a very complicated nucleolus structure, as exemplified by *Polychaos fasciculatum* (see Baldock and Baker, 1980) and *Polychaos annulatum* (Fig. 12).

Another recognisable structure, which can be observed in some nuclei, is the internal **nuclear lamina**. This term refers to layers of hexagonal, honeycomb-like structures (as in *Amoeba proteus* and *Thecamoeba sphaeronucleolus*) or to layers of fine filaments (as in *Saccamoeba limax*). The functional role of this layer is unclear and speculation about its mechanical properties appears unfounded. More probably the honeycomb-like layer fulfils some regulatory role in the exchange of material between nucleus and cytoplasm.

Mitochondria of amoebae are of two different types (Figs 13 A, B), those with **tubular** cristae and those with flattened, **discoid** cristae. If the shape of cristae is investigated more closely (Seravin, 1993), it becomes clear that it is more correct to say "cristae of tubular type", as many variations are possible within this type. The same is true for flattened cristae which are usually discoid, resembling a plate growing on a thin leg, but which may have other 3d appearances.

The Golgi complex of an amoeba may be organised either as **dictyosomes** (Fig. 13 C), which are stacks of flattened saccules well-visible in EM sections (and are characteristic of the class Lobosea) or as a set of small vesicles (in the Heterolobosea), which are indistinguishable in EM sections and only discernible using cytochemical stains.

Together, the type of mitochondrial cristae, the characteristics of movement and the organisation of the Golgi complex clearly differentiate schizopyrenids, acrasids and other members of the class Heterolobosea from members of the class Lobosea.

Among the other cytoplasmic inclusions, crystals

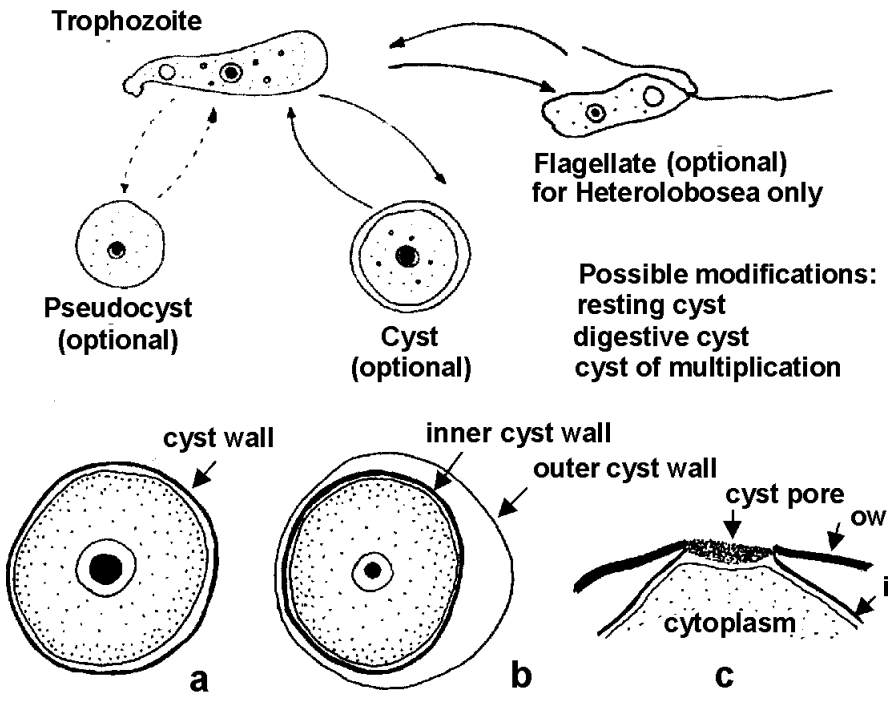

Fig. 14. Life cycle of an amoeba (scheme) and cyst structure. a - single-walled cyst; b - double-walled cyst; c - cyst pore (scheme). *Abbreviations*: ow - outer cyst wall, iw – inner cyst wall. Not to scale.

are usually washed out during the treatment of the cell (only the empty spaces created can be observed), but lipid globules are usually numerous and well visible. Endobionts are present in most amoebae (Smirnov and Ossipov, 1995; Ossipov et al., 1996), and are evident in EM sections (Fig. 13 A). However, it is sometimes difficult to differentiate endobiotic bacteria from engulfed food bacteria.

2.3. Life cycles, encystment and cyst structure

Life cycles of amoebae vary between systematic groups and even between related species. Variations are found in every stage of amoeba life cycles, as illustrated in Fig. 14.

Basically, the life cycle consists of two stages, **trophozoite** (any active amoeba, either locomotive, resting or floating) and **cyst**. Not all amoebae species are known to produce cysts in culture, however, we can never be sure that the ability to encyst was not lost during adaptation to laboratory culture conditions. Multiplication of the trophozoite is by either binary division or by the fragmentation of the plasmodium (the latter mode is characteristic of the order Leptomyxida).

Cyst formation is a complicated process, which has been studied in detail in some amoebae. Briefly, prior to encystment an amoeba stops feeding, decreases in cell volume by reducing the amount of the water in the cytoplasm, rounds up and then secretes a cyst wall. Some species stop at this point - they have **single-walled cysts** (Fig. 14 A). In other species the cell continues to reduce its volume and size and, after some time, secretes a second cyst wall, forming a **double-walled** cyst (Fig. 14 B). At the sites of contact of the two cyst walls **cyst pores** (Fig. 14 C) appear. The pore marks the place where an amoeba can destroy the cyst wall when it excysts. Some amoeba species do not form cysts in culture, but become rounded and survive in this state for some time without any evidence of activity. This stage is called a **pseudocyst** and differs from the true cyst by the absence of any cyst wall and by the consequent reduction in survival capacity, for whilst the true cyst remains viable after drying, a pseudocyst soon dies.

Life cycles of amoebae of the orders Euamoebida and Acanthopodida are very similar and relatively simple. They include only the trophozoite stage, or alternation between cyst and trophozoite. Both other orders of the subclass Gymnamoebia (Leptomyxida and Loboreticulatida) have more complicated life cycles. For leptomyxids a number of peculiarities characterise different species. The trophozoites of some leptomyxid species (i.e. *Flabellula baltica* and *Flamella lacustris*) are capable of agamic fusion (Smirnov, 1999a; Michel and Smirnov, 1999), and members of several taxa exist only as multinucleate plasmodia (with a few or upto several hundreds of nuclei). Fewer nuclei and smaller sized plasmodia are

characteristic of *Rhizamoeba* spp., whilst the largest plasmodia are formed by *Leptomyxa* spp. Members of the genera *Leptomyxa* and *Gephyramoeba* have acquired several specific adaptations, including diversification of cysts into several functional groups (digestive, multiplication and resting) which may be formed consecutively in the life cycle of the same organism (Pussard and Pons, 1976). This adaptation may be related to the relatively large cell size and the network structure of the plasmodium of these organisms.

The order Loboreticulatida stands alone in terms of life cycles, and the complex life cycles of corallomyxids have been summarised by K. Grell (1966). The life cycles of schizopyrenids usually include a flagellate stage, which is of varying importance and may occupy different parts of the life cycle (see Page, 1988). Some marine amoebae, like *Euhyperamoeba fallax* are capable of various types of agamic fusions (Seravin and Goodkov, 1987, 1999, 2003).

2.4. Distribution patterns

Difficulties in species identification, loosely defined "borders" between taxonomic groups and other essential and historical reasons have resulted in a paucity of published data on gymnamoebae distribution (Arndt, 1993). We still have no clear understanding of the rules which determine species distribution across different habitats, at either global or local scales. Relative to other groups of protists, amoebae have been poorly sampled across the different geographical regions of the world. Almost all published data concerns the amoebae faunas of Europe, North America and Antarctica, and there is little reliable data on amoebae populating such regions as South America, the Far East and the Middle East, Oceania and the Indo-Pacific area. Based on the available data we can only state that some species may occur in widely spaced habitats (even on different continents) (e.g. Page, 1976b; Smirnov, 1999a, 2001; Goodkov et al., 1999; Smirnov et al., 2001; Smirnov, 2003). This leads us to conclude that some amoeba species have a worldwide distribution, as demonstrated for many other protists (Fenchel et al., 1997; Finlay and Fenchel, 1999; Finlay 2002). However, many other amoebae species have been detected in only a single location, which supports the opposite point of view (see Bovee, 1985; Page, 1988; Smirnov and Fenchel, 1996; Smirnov, 1999a). It is impossible to decide if this is the consequence of global undersampling of amoebae or is actually caused by the restricted distribution of certain species.

A remarkable aspect of amoeba distribution at a local scale is the variation in temporal and spatial heterogeneity of species composition even within small habitats. If the comprehensive papers on amoebae biodiversity in various habitats are compared it becomes apparent that long-term studies involving repetitive sampling of a habitat (e.g. Sawyer, 1980; Sawyer and Bodammer, 1983; Butler and Rogerson, 1995, 2000; Smirnov and Goodkov, 1995; Smirnov, 1999a, 2001) always recover many more species than sampling on a single or few occasions (e.g. O'Dell, 1979). Amoebae show no pronounced short-term or long-term dynamics, however, certain seasonal variations in general abundance and diversity have been observed (Smirnov and Goodkov, 1995; Anderson, 2000). It is known that the distribution of amoebae at the scale of millimetres and centimetres can be highly heterogeneous, with pronounced 'patchiness' exhibited by some species (Bischoff and Anderson, 1998; Anderson, 2002; Smirnov, 2001; Smirnov and Thar, 2003). These and other similar facts (recognised not only for amoebae but also for various other protozoan species) concur with Bamforth (1963), when he proposed the existence of microhabitats in the environment, selectively populated by numerous and diverse protozoan species. Bischoff and Anderson (1998) and Anderson (2002) suggested that the soil fauna might experience wide variations in resources and prey communities within a distance of several millimetres or less, forming soil eukaryotic microbiogeocoenoses.

To encompass these observations in a single model, Smirnov (1999a, 1999b, 2001, 2002, 2003) and Smirnov and Thar (2003) have developed the concept of a "hidden community". They suggest that each local habitat (ecotope) harbours a large diversity of "microhabitats", which may provide suitable conditions for amoebae and in which populations of different species may develop. The microhabitat composition of an ecotope is dynamic in space and time – a microhabitat of a certain type may disappear and reappear in another location, depending on many factors, like the presence of local edificators (objects that determine the environmental conditions in their close surrounding) and the bulk environmental conditions, which cause changes in the ecotope. Amoebae species that appear in the ecotope by a variety of different means may either find an appropriate microhabitat and start to multiply and form active populations or, instead, form resting stages and persist for years without multiplication. Alternatively, they may gradually die off. Thus, any ecotope contains a pool of numerous species, of which only a minor fraction is abundant and actively participating in mass and energy flows at any one point in time. The remainder merely persist in the habitat (with varying viability over time) awaiting the chance occurence of environmental conditions which provide their particular growth requirements. According to the estimates by Umeche (1983) and Anderson (2000) the fraction of inactive species in the soil may constitute up to 80 % of the

observed abundance of amoebae under low (10% v/w) moisture conditions but decrease to 20-30% if the moisture content increases to 25%. Their results indicate that amoebae can react very rapidly to changes in environmental conditions (which create or destroy suitable microhabitats) by excysting or encysting. Species will not only differ in the type, but also in the range, of microhabitats in which they can multiply and some species will be more tolerant of changes in their environment than others. This will also affect the species composition in the habitat.

Smirnov (1999b) proposed to term all species that actually appear in the habitat (both resting and active stages) the "hidden" community. The populations of amoebae species which constitute the hidden community together represent a kind of "seedbank" of species, and any species in this seedbank is able to increase its numbers rapidly following a change to "favourable environmental conditions". The last definition actually means an increase in the number of suitable microhabitats for a species. An important characteristic of the hidden community is that the seedbank appears to be dispersed relatively randomly throughout the whole ecotope. Thus the formation of suitable microhabitats, whenever it takes place, results in the immediate activation of the hidden species located in that part of the ecotope. There is virtually no delay required for immigration into, or dispersal of species throughout, the newly formed microhabitats and this is the reason for the experimentally observed rapid reaction of protozoan communities to changing environmental conditions (Cook et al., 1974; Clarholm, 1981; Pussard and Delay, 1985; Pussard and Rouelle, 1986; Anderson, 2000).

Because enrichment cultivation (see section 3.4) remains the only viable method for isolating amoebae from the environment, we recover only the species which can find suitable microhabitats in our culture vessels and which were sufficiently numerous in the habitat for individuals to have been included in the small volume of sample material actually inoculated (Smirnov, 2003). The community of amoebae that we can isolate using current methods has been termed the "observed community" (Smirnov, 1999b), and will vary between samples, sampling occasions and even between different inoculated cultures. It will not necessarily consist only of the most abundant species, because the culture conditions may stimulate the multiplication of species that were numerically rare in the sampled ecotope but were "lucky" both to be sampled and to find suitable microhabitats in culture. Moreover, among the species isolated may appear those that were present as a consequence of random dispersal, but for which a suitable microhabitat would be most unlikely to form in the soil environment. This accounts for the isolation of amoeba and other protozoan species from totally unsuitable environments (Fenchel and Finlay, 1995; Butler and Rogerson, 1995; Smirnov, 1999a, 2000, 2001; Smirnov and Thar, 2003). Therefore, as there are many factors affecting the nature and variety of microhabitats, it is impossible to predict accurately the short-term dynamics of amoeba diversity in any habitat. However, there are also global factors (temperature, lighting, etc.), which influence almost all habitats, and can stimulate a cascade of changes. This explains the existence of unresolved, but recognisable, seasonal and other repetitive changes in observed communities.

From the hidden community model we deduce that it is virtually impossible to recover the full extent of amoeba diversity from any habitat. The observed community will represent only a fraction of the species inhabiting an ecotope, because we cannot reproduce in the laboratory the full range of conditions existing in the microhabitats comprising the ecotope. Moreover, only a subset of the species recovered from a habitat actually participates in mass and energy flows, and even very abundant species that may be calculated to contribute significantly to the energy balance in the ecosystem, in reality may be resting at the time of sampling and have no influence at all. Some of the recovered species may not be part of the characteristic "population" of a habitat, but are occasional immigrants that would die off under normal conditions.

Microhabitats are, to some extent, independent of the global location of the ecotope. Similar microhabitats may appear, for example, in garden soil or freshwater ponds all over the world. Consequently, the amoebae species populating such microhabitats may be found almost everywhere. That is why some amoeba species appear to have a worldwide distribution. But other species may be very intolerant of changes in their environment and/or populate rarely occuring microhabitats and thus have restricted geographic distribution.

These factors complicate the analysis and understanding of the diversity of amoebae and their functional role in natural habitats, but they should not be ignored. Furthermore, it is clear that to obtain meaningful data, repetitive, long-term (at least all-season) sampling of a habitat is essential. Another essential requirement is the detailed characterisation of the ecotope being sampled that may allow, to some extent, comparison of the nature and diversity of microhabitats between different ecotopes.

2.5. Food spectra

Food spectra of amoebae are poorly understood, and most relevant data are either old or fragmentary (Ekelund and Ronn, 1994). Evidently, the primary food

source for the soil amoebae is bacteria (Pussard and Rouelle, 1986; Foster and Dormaar, 1991). There appear to be many specialised fungi-feeding amoebae species in soil (Chakraborty et al., 1983). But most amoebae species are polyphagous and in culture are able to feed on bacteria or other protists of suitable size (Kalinina and Page, 1991). Small amoebae are unable to engulf anything larger than bacteria, and dissolved organic substances may play an important role in their nutrition (Rogerson, 1993). Some species prefer to feed on algae. However, these are only single examples. The food spectra of amoebae in natural habitats may differ from those in culture and require further detailed study.

2.6. Facultative pathogenic (amphizoic) species

It is widely known that some normally free-living species of the genera *Acanthamoeba* and *Naegleria* which can be isolated from the environment globally can also cause fatal infections in animals and humans. The human pathogen, *Balamuthia mandrillaris* (which was first isolated from the brain of a Mandrill baboon), has now been isolated from soil, despite being difficult to culture on agar, and may prove to have a global distribution (Schuster et al., 2003). Some *Acanthamoeba* species are able to multiply on the surface of the eye causing amoebic keratitis (e.g. Mathers et al., 1996). *Vahlkampfia*, *Vannella* and *Hartmannella* species have also been isolated from the eye surface but, like many other amoeba species which have been found in the tissues and organs of various animals, their pathogenicity is unproven (e.g. Aitken et al., 1996; Dykova et al., 1998). *Rosculus ithacus* is one of several species which were initially found in animals and only later discovered to be free-living (Hawes, 1963; Page, 1974b). No cases of infection of researchers by environmental isolates have been reported (Page, 1988) but workers should be aware that these organisms are common in soil, and if cultures of *Acanthamoeba* (for example) are generated then the appropriate health and safety regulations should be observed. Furthermore, some amoebae (for example, *Acanthamoeba* and *Hartmannella* species) may act as vectors of various pathogenic organisms (e.g. Danes and Cerva, 1981; Anand et al., 1983; Kilvington and Price, 1990).

3. Basic methods for recovery, study and identification of gymnamoebae

3.1. General approach

With rare exceptions, amoebae in fresh samples are almost invisible, being mostly rather flattened and attached to particulate matter (Singh, 1955b). By direct microscopical examination Foissner (1987) recovered only 2% of individuals added to a soil suspension in an experiment. Therefore, amoebae must first be isolated from their environment. This can be achieved either by placing a glass slide (or other suitable substratum) into the soil, sediment or water column, allowing time for the amoebae to colonise the object before removal and examination (Darbyshire et al., 1974), or using enrichment cultivation methods (Cutler, 1920). The aim of enrichment cultivation is to create suitable, selective conditions which will encourage one group of protists to multiply and to give them an advantage (in growth rate, or by absence of predation, for example) over other protists.

3.2. Media

The following list of media is similar to the one compiled by Page (1988) for freshwater and soil amoebae. From his list we have selected only the media which seem to be most appropriate for soil amoebae. Note that many of Page's media have too high a concentration of organic nutrients which results in rapid fungal growth, especially in initial cultures, and these media may require dilution. Each medium should be autoclaved at 1.5 psi for 15 minutes after preparation.

Saline solutions:

AS (modified Neff's amoeba saline) Source: Page (1988).

Prepare the five stock solutions. To make AS, combine 10 ml of each stock solution with 950 ml of distilled water.

Stock solution 1: NaCl - 1.20 g / 100 ml H_2O
Stock solution 2: $MgSO_4 \cdot 7H_2O$ - 0.04 g / 100 ml H_2O
Stock solution 3: $CaCl_2 \cdot 2H_2O$ - 0.04 g / 100 ml H_2O
Stock solution 4: Na_2HPO_4 - 1.42 g / 100 ml H_2O
Stock solution 5: KH_2PO_4 - 1.36 g / 100 ml H_2O

PJ (Prescott's and James's solution). Source: Prescott and James (1955), protocol adopted from Page (1988).

Prepare the three stock solutions. To make PJ, combine 1 ml of each stock solution with 1 litre of distilled water. This solution is easier to prepare than AS and is a suitable substitute.

Stock solution 1:
$CaCl_2 \cdot 2H_2O$ - 0.433 g / 100 ml H_2O
KCl - 0.162 g / 100 ml H_2O
Stock solution 2:
K_2HPO_4 - 0.512 g / 100 ml H_2O
Stock solution 3:
$MgSO_4 \cdot 7H_2O$ - 0.280 g / 100 ml H_2O

Other liquid media:

SES (Soil Extract with Salts). Source: Page (1988).

Into a beaker put untreated garden or agricultural soil and tap water so that the overlying water occupies approximately four-fifths of the total depth. Autoclave for one hour, then decant and filter the supernatant through Whatman No. 1 filter paper. Combine with water and stock solutions of salts.

Soil extract	100 ml
K_2HPO_4 0.1%w/v	20 ml
$MgSO_4 \cdot 7H_2O$ 0.1%w/v	20 ml
KNO_3 0.1% w/v	20 ml
Distilled water	840 ml

CP (Cerophyl-Prescott infusion). Source: Page (1988), modified.

Cerophyl is a cereal derivative which can be obtained (with the product name Cereal Grass Media) from Ward's Natural Science Est. (www.wardsci.com).

Boil 0.5 g of cereal leaves in 1 litre of PJ for 5 minutes, filter and restore the volume with PJ. The resultant medium is rather rich in organic nutrients, thus additional dilution with PJ may be required to avoid excessive bacterial growth in cultures. From our experience, the dilution of the initial suspension to a final concentration of 0.05-0.1% is appropriate for most cultures.

Agar media:

NNA (Non-Nutrient agar). Source: Page (1988).

Add 15 g of *non-nutrient* agar to 1 litre of AS or PJ solution.

CPA (Cerophyl-Prescott agar). Source: Page (1988).

Add 15 g of *non-nutrient* agar to 1 litre of CP infusion.

3.3. Sampling, inoculation and examination of initial cultures

The soil sample, collected using clean (preferably sterile) instruments, should be diluted first with PJ or AS; for example 15 g of soil/1 litre of liquid. The degree of dilution should be adapted so that there is sufficient free space between soil particles for amoebae to be observed on the agar or plastic surface of a dish after inoculation.

The following set of media are suggested for initial inoculation:

- 100 mm Petri dishes with PJ and two wheat (or rice) grains in each dish
- 100 mm Petri dishes with SES and two wheat (or rice) grains in each dish
- 60 or 90 mm Petri dishes with NNA
- 60 or 90 mm Petri dishes with CPA and an overlay of PJ

Each dish should be inoculated with approximately 1-2 ml of diluted sample. It is important to have several soil particles in each dish. In dishes with liquid media (both agar and non-agar dishes), the "spot" of particles should be small and placed preferably near the edge of the dish to leave enough space for observation of amoebae. In dishes without an overlay, 1-2 ml of diluted sample should be dropped near the edge of the dish, then the dish should be held vertically to allow the drop to flow down, forming a path across the dish. Dishes without overlay should be tightly sealed with Parafilm (or similar laboratory film) to avoid drying. Cultures should be incubated under normal room conditions of lighting and temperature. Temperature-controlled rooms, with the temperature close to that of initial habitat, do not seem to improve the success of species isolation.

Initial cultures should be examined several times, as there will be a rapid and significant succession of amoebae species during incubation (Smirnov, 2003). Liquid cultures should be examined 5-6, 10-11, 17-20 and 30 days after inoculation. For agar cultures the 30th day examination may be omitted. In case of a shortage of time, the most important examinations appear to be after 10-11 and 17-20 days of incubation (Smirnov, 2003).

Liquid cultures should be examined using first a dissection microscope and then an inverted microscope (for large amoebae). Agar with a liquid overlay is easy to examine using water-immersion phase-contrast optics, at 200× -400× magnification. Normally, trophozoites of small heterolobosean species and the smallest vannellids appear after 5-6 days and disappear (by death, encystment or enflagellation) after 10-11 days. The maximum development of populations of medium-sized amoeba is at around 10-11 and 17-20 days. Larger species may appear in cultures after a month or even more. Water-immersion optics are strongly recommended for agar cultures with a liquid overlay, as in these cultures there is no other way to see the surface of the agar clearly.

Cultures without overlay usually show more rapid growth of amoebae, but only small and medium-sized species multiply in these cultures. They should be examined, without opening, under a dissection microscope at 40x magnification (or higher). It is easy to locate amoebae as, in contrast with other protists, they have a strong tendency to migrate across the agar surface beyond the initial 'path' of the inoculate, forming a set of small clumps with narrow tracks of

liquid below them. However, some species do prefer to stay within the initial paths. Location of amoebae on the agar surface is followed by cloning (see below). For the final examination it may be useful to cover the dish with a layer of PJ medium, leave it overnight (but not more, to avoid overgrowth of bacteria and fungi) and to examine it using water-immersion objective lenses of 20x – 40x magnification.

3.4. Enumeration methods

A variety of methods have been developed for enumerating amoebae. The general consensus is that any form of direct counting allows only a small fraction of species diversity to be recovered from a sample (reviewed by Foissner, 1987; Ronn et al., 1995). This leaves only indirect (culture) methods. Historically, the first to be developed was the "most probable number" (MPN) technique that originates from the dilution methods commonly used in bacteriology and was first adopted for enumeration of protozoa (including amoebae) by Killer (1913), Schermann (1914) and several other authors. These investigators used bacteriological media, like bloodmeal and hay infusion, that were common at that time. Microscopic examination of the culture medium from each dilution allowed the development of protozoan populations to be recorded and their abundance calculated using standard statistical methods. Cutler (1920) modified this method; he used agar media and spread 1 ml of liquid from each dilution over the agar surface. Protozoa have low mobility on the agar and, multiplying on its surface, they form kind of "negative" colonies in the layer of bacteria that grow on the agar. Counting these colonies was much easier than microscopical examination of liquid cultures and made possible the estimation of the number of protozoa in the initial dilution using statistical tables, like those by McCrady (1918).

The major shortcoming of this approach was the use of relatively nutrient-rich medium for enrichment cultivation. Intensively growing bacteria often suppress the growth of amoebae and other protozoa (Smirnov, 2003). Severtzoff (1922) proposed the use of non-nutrient agar as the most appropriate medium for amoebae and offered a further modification to the dilution method - gradually increasing the dilution of the sample (i.e. 1:100 – 1:1000 – 1:2000 – 1:5000 – 1:10000, etc.) with the subsequent common statistical estimation of the results. Non-nutrient agar supports only limited growth of bacteria and results in the multiplication of many more amoebae species; this medium remains the most commonly used.

The dilution method was developed to its "classical" form by (Singh, 1946) and then further modified over the years (Darbyshire et al., 1974; Clarholm, 1981; Griffiths and Ritz, 1988; etc.). Menapace et al. (1975) tried to improve this approach by using agar with a semi-liquid overlay for enrichment cultivation of serial dilutions of a soil sample. This allowed the development of more species than on dry agar but prevented active migration of amoebae and resulted in the formation of relatively well-outlined colonies of amoebae. However, the essence of the technique remains the same - the serial dilution of the sample with subsequent incubation of dilutions in sets of dishes with an enrichment medium, including dilutions that contain no amoebae. The total number of amoebae may be rapidly estimated from the total number of cultures that do not show any growth (negative cultures) in the whole set of dilutions, using a relatively simple table (Singh, 1946) or routine statistical methods.

The protocol developed by Singh (1946) can be summarized as follows:

1. Prepare a serial dilution of the sample. For example, take 10g of soil and mix it with 50 ml of water (in the original protocol) or any mineral medium for amoebae, like PJ, AS, SE, etc (see above for recipes). Shake well, allow particles to settle for about 30 sec. This is the first dilution (1:5). Mix 5 ml of this suspension with 5 ml of medium. Shake, allow to settle (as above). This is the second dilution (1:10). Repeat until the 15th dilution (1: 81920).

2. Inoculate one drop of medium from each dilution into eight small (40-60 mm diameter) Petri dishes filled with non-nutrient agar. Add *E. coli* as a food source. Originally (because there was no cheap disposable plastic dishes at that time), for each dilution, Singh used one 110 mm glass Petri dish containing eight glass rings, 2 cm in diameter, filled with a 2 mm layer of agar (equivalent to eight separate dishes). He inoculated the agar with *E.coli* two days before the inoculation of the sample to allow the bacteria to grow, which remains a reasonable approach.

3. After the incubation (about three days in the original protocol) all dishes (or rings) are examined under the microscope. For each dilution the number of negative (amoeba-free) dishes (or rings) is recorded. The MPN of amoebae can be estimated from the total number of negative cultures using the table published by Singh (1946).

At the time of its development the method by Singh was evidently progressive. However, over the years many criticisms of this method have arisen. Firstly, the method is very much dependent on the ability of amoebae to grow on the chosen medium (it still remains true for all enrichment cultivation methods). Secondary, the statistical approach used in the "classical" Singh method (Fischer and Yates, 1943) probably overestimates amoebae numbers. Lepinis (1970) analysed Singh's and

other methods of amoebae enumeration in detail and demonstrated that, depending on the medium used and the statistical treatment of the results, the estimated number of protozoa in the same sample may differ by two orders of magnitude. Using a single medium and food organism evidently will select for only a proportion of amoebae species, and will lead to an underestimation of amoeba diversity in the sample (Ronn et al., 1995). Studies by Singh (1941, 1942, 1945), Heal (1963) and others indicated the high selectivity of amoebae in predating different bacteria. The dependence of the result of MPN enumeration on the type of food bacteria used was further highlighted by Ekelund and Ronn (1994). Finally, the Singh's dilution method requires the examination of a relatively large number of dishes (or rings) for each sample (120); it is laborious and time-consuming work.

The shortcomings and technical difficulties of classical MPN methods have stimulated the development of alternative approaches, also based on enrichment cultivation. Perhaps the most promising one is based on a single dilution of the sample and differentiated count of amoebae morphotypes (or even species, if the experience of investigator allows clear distinction and differentiation of amoebae) (Anderson and Rogerson, 1995). This approach assumes that after the appropriate degree of dilution of the sample each resultant culture of an amoeba species is initiated by a single cell (i.e. represents a clone). If the abundance of each species under the chosen dilution is relatively low, the number of dishes populated by each recorded species corresponds to its initial number in the sample that was diluted. The essence of the approach is that the abundance of each particular species is normally just a minor fraction of the total amoebae abundance (with rare exceptions). Thus, almost all dishes will be populated by amoebae, but each species will occur in only a fraction of the dishes. Then we assume that all the cultures obtained are clonal and count the number of dishes containing each species. Totalling these numbers we obtain the total number of amoebae in the sample. Based on this approach, several modifications have been developed (Butler and Rogerson, 1995; Anderson, 1997; Smirnov et al., 1998), but the essential differences are only in the type and number of dishes used for inoculation (Petri dishes or 24-well plates) and in the enrichment medium used.

The shortcoming of this approach is the need to differentiate amoebae by eye during the screening of cultures. This may be difficult, especially for the beginner. However, the essence of the approach is that amoebae must be differentiated, but not necessarily identified. One may differentiate amoebae, say, to morphotypes but should remember that a decrease in resolution in amoebae differentiation requires a respective increase in the dilution of the sample (otherwise each morphotype will appear in virtually all dishes, making the enumeration meaningless). This, in its turn, warrants a decrease in the amount of sample actually inoculated and thus reduces the accuracy of the count. With increasing experience, an investigator will be able to distinguish individual species and to name them as "species 1", "species 2", etc. During further studies perhaps it will be possible for him to perform the systematic identification of these species and to assign taxonomic names to them. Usually the quantification of amoebae using the single-dilution method is preceded by a faunistic survey, which allows exploration of the basic amoebae diversity in the studied habitat and reliable differentiation of species during the further enumeration studies.

Application of the single-dilution method drastically decreases the number of cultures to be examined, but requires the reliable differentiation of amoebae species. Otherwise serious numerical errors are likely. Often, different species vary greatly in abundance and some may appear in almost every other dish, while most occur in only 5-15% of dishes. Evidently, the estimation of the number of the latter is reliable while the number of the former could be underestimated. That is why Smirnov (2002) proposes to term the abundance obtained with this method "minimal possible abundance" (MPA). To solve this problem, Garstecki and Arndt (2000) proposed that applying the Poisson series would generate a more accurate estimation of MPN. Their approach improves the accuracy of enumeration of the most abundant species by taking into account the probability of inoculating one dish (or well) with several specimens of one species. Normally, this correction results in a significant increment in the most probable number of the abundant species compared with the MPA estimates (Smirnov, 2002; Smirnov and Thar, 2003). Details of this statistical treatment are provided by Garstezki and Arndt (2000).

The modification of Anderson's and Rogerson's (1995) single-dilution method as used by Smirnov et al. (1998) is as follows:

1. Dilute the sample. The degree of dilution required will vary, depending on the estimated number amoebae in the sample. Sometimes a preliminary test is required to establish the appropriate dilution for a novel type of sample; 1:1000 seems to be suitable for many soil and sediment samples. Shake well. Note that both the dilution and the actual amount of inoculated material are relevant to the enumeration of amoebae. The dilution determines the accuracy of the count. Ideally each species should occur in not more than 13% of inoculated dishes. In this case the MPA estimate and MPN estimate will correspond, the probability of statistical artefacts is minimal and the count is most precise. The larger the volume of

sample actually inoculated, the lower is the probability of serious errors due to occasional patchiness (or absence) of amoebae in the inoculated material. However, an increase in the volume of inoculated material warrants an increase in the number of inoculated dishes and makes the examination longer and more laborious. So each investigator needs to adopt his own balance of these factors.

2. Distribute a measured volume of the diluted sample into culture dishes. The volume depends on the culture dishes used. For example, 200 ml of the dilution may be distributed (without the remainder) into thirty 40 mm Petri dishes. Then, calculate the total amount of material actually inoculated. It is very important to keep the diluted sample homogeneous, preventing the precipitation of particles with adhered amoebae by constant gentle shaking. If the number of different samples is small, 40-60 mm Petri dishes are ideal for inoculation because they allow easy and detailed examination under an inverted microscope. In the case of numerous samples, 24-well culture plates are more appropriate. Among the variety of media tested, the best seem to be non-nutrient agar without an overlay (for soil); with an overlay (for freshwater samples) and artificial seawater with one drop of 1% cerophyl infusion (for marine samples) (Smirnov, 2002; 2003). The number of dishes inoculated with aliquots of each diluted sample should be in the range 24-72 (smaller number results in large errors, and larger numbers normally do not further improve the accuracy of the results). Incubate under normal room conditions.

3. Examine dishes at least twice, after 9-12 and 21–22 days. If multiwell plates are used, examination should be performed earlier, e.g. after 7-9 and 14-15 days. Numerous examinations are essential due to the rapid succession of amoebae species in the cultures (Smirnov, 2003). Record the occurrence of each species.

4. Note the number of the number of dishes (or wells) in which species was observed. This represents the number of amoebae in the total volume of diluted sample which was inoculated.

The enumeration method described below has been adopted for soil samples and is also based on that of Anderson and Rogerson (1995). Soil samples are air-dried and sieved soon after collection. Nutrient enrichment is used in the enumeration stage (steps 3-5) to reveal the abundance of the population of naked amoebae that develop in response to rain water alone in the preceding incubation stage (steps 1-2). The method of Anderson and Rogerson (1995) does not include a flooded soil incubation stage prior to the enumeration stage. The aim is to assess the potential of soil to support a community of naked amoebae in the presence of other types of soil microorganisms (Finlay et al., 2000). Note that this method estimates the total number of trophozoites and cysts of naked amoebae present in the flooded soil at the end of the incubation stage. No attempt is made to determine the relative numbers of trophozoites and cysts.

1. A 5 g sample of the sieved soil is weighed out into a small Petri dish. Sufficient filtered (0.2mm) rainwater is added to just "flood" the soil, i.e. a small excess of water can be seen when the dish is tilted. The water and soil are mixed well with a small spatula. The amount of water (in g) added to the soil sample is recorded.

2. The dish is incubated in a moist container (sandwich box + wet paper towels + small beaker of water), in the dark, at 15°C, for four weeks. Each week, the soil sample is stirred thoroughly. If necessary, after one day and after one week, more water is added to re-flood the soil sample if it has absorbed the excess water. The weight of the extra water added is noted.

3. The moisture content of the flooded soil sample is calculated, as a percentage of the total weight of the flooded soil = $5x + 100y / 5 + y$, where x = the % moisture content of the sieved soil and y = the weight of water added to 5 g of sieved soil.

4. After four weeks incubation, the flooded soil sample is mixed well and 1 g sub-sample is removed and added to 10 ml of AS in a plastic test tube. The tube is shaken vigorously for 30 seconds and the soil is allowed to settle for two minutes. Next, 1 ml of the uppermost supernatant is transferred to 9 ml AS in a plastic test tube, and mixed well by rolling. Then, 10 ml sub-samples are aliquoted from the latter into a total of 48 wells (two multiwell plates), each containing 2 ml of AS plus a 2 mm cube of nutrient agar.

5. Multiwell plates are incubated in moist containers, in the dark, at 15°C. After two and five weeks incubation each well is examined with an inverted microscope (400×, phase) and scored "+" or "-" for the presence of naked amoebae.

6. The density of amoebae in the sub-sample is calculated from the proportion of positive wells. The presence of amoebae in a well indicates that at least one individual of that species was present in the 10 µl aliquot. Based on the proportion of wells containing amoebae, the density of amoebae is calculated and expressed as number per gram of flooded soil, using the following equation: $N = (n \times 10^6) / (V \times F)$, where N = number of naked amoebae per gram of flooded soil; n = number of wells containing naked amoebae; V = total number of µl of inocula deposited in the culture wells (480); 10^6 = conversion factor for µl to litre; F = dilution factor (10; 1 g sub sample was mixed with the equivalent of 0.1 litre AS).

For the volumes and dilutions indicated in step 4, this equation simplifies to: N = number of positive wells x 208.33.

Therefore, the "detection range" at the soil dilution indicated in step 4 is 208 to10,000 per g of flooded soil. The detection range can be altered by increasing or decreasing the dilution in step 4.

7. Subsequently, the number of amoebae per g dry soil can be calculated = $100x/y$, where x = the number of amoebae per g flooded soil and y = the percentage of dry matter in the flooded soil.

The detection range at the soil dilution indicated in step 4 depends on the water content of the flooded soil, which varies between samples; for example, if the water content of the flooded soil is 80%, the detection range is 1,040 to 50,000 per g of dry soil.

In conclusion, the single dilution method with the Poisson series treatment of the results seems to be the best available so far. However, as with the original MPN method, the cysts and trophozoites are enumerated together. Attempts to find a reliable way to segregate numbers of "active" and "passive" forms have varied in success (Bodenheimer and Reich, 1933; Pussard and Delay, 1985; Ekelund and Ronn, 1995; Anderson, 2000) and have never been completely successful.

3.5 Cloning techniques

In terms of Page's systematics of amoebae, identification of a species requires the establishment of an amoeba in culture, i.e. it is the identification of a clone. Thus, after recognising an amoeba morphotype in an initial culture, the amoeba should be cloned before further investigation in order to avoid errors which would arise if the culture consists of a mixture of similar amoebae species.

A variety of methods for cloning amoebae have been suggested (see Page, 1988). However, their levels of success (% of clones obtained) are very similar. We suggest trying three basic methods; the reader is referred to the cited literature for further techniques. The proportion of attempts resulting in cultures is rather low, and normally does not exceed 10%.

Migration method. This method is appropriate for amoebae growing on agar (with or without liquid overlay). When you observe the initial culture of amoebae on agar (without an overlay) under a dissection microscope, you need to find areas where amoebae have migrated away from the initial path of the inoculate and are not too abundant. If cells are very abundant and/or different morphotypes are mixed in one area then this may mean that the soil sample should have been diluted more initially. With a scalpel (or needle with flattened end) cut out one amoeba or one cyst on a small block of agar. The block should be transferred to a fresh Petri dish with the same agar medium, and the cell should be washed off from the block by the addition of a drop of the liquid used for the initial dilution.

Pipette technique. This is intended for amoebae growing with a liquid overlay. The method is simple in concept but requires practice. After heating in a Bunsen flame, pull out the end of a Pasteur pipette into a fine capillary tube and try to capture one cell (or cyst) from the culture. The resolution of this method is much lower than for the migration method - more mixed cultures are obtained. However, it is the only practical method for cultures growing in liquid media.

Dilution technique. Wash all the amoebae (and other material) from the surface of a dish using fresh media, and dilute the supernatant 1:100, 1:1000, etc. Inoculate 1ml of each dilution into individual new dishes. The intention is that one of the dilutions should contain only one (or very few) cells. If during regular examinations you only see one morphotype in a dish then it is possibly a clone. If no amoebae, or many types, are observed in all the dishes then the experiment should be repeated with different dilutions.

For all three techniques it is preferable to repeat the cloning process. Only sub-clones are reliable for further study. Possible sub-clones must be monitored for about a month to check that no other amoebae (and preferably no other protists of similar size) are present.

3.6 Identification

Identification of amoebae remains a very difficult problem, and much has been written about this (Bovee, 1953; Ishii, 1985; Smirnov and Goodkov, 1999a). For correct generic and specific identification EM is obligatory in most cases. Another problem is our relatively low level of knowledge about global amoeba biodiversity; the probability of finding new species in any habitat is very high. For example, a detailed faunistic survey of a freshwater lake revealed 32 Gymnamoebia species, of which 15 were found to be new to science (Smirnov and Goodkov 1995; 1999b). Therefore, you should always be prepared to discover unknown species in your cultures.

In order to identify an amoeba you need first to decide which level of identification is appropriate for your current investigation. If your are satisfied with the level of morphotype you have no need to clone the amoebae and observation of initial cultures will be sufficient. However, if you wish to identify amoebae to the level of genus or species then you must obtain a comprehensive set of data as described in, for example, Page's key (1988). Unfortunately, this may be laborious and time-consuming. But if you do not fulfil all the requirements of systematic identification then it is possible that your conclusions about the genus/species identities of your strains may be incorrect.

Identification itself consists of several distinct steps. You are welcome to stop either at the first step, if you are

going to classify only to level of morphotype, or to follow them all (sequentially) for systematic identification, using methods from the relevant literature as required.

If your aim is systematic identification of amoebae then first consult F.C. Page's keys (1988 - in English, 1991 - in German; the latter is more comprehensive). They provide a great deal of information and also the basic steps for systematic identification. They may be sufficient for species identification, but when you have determined the species always check the original description in the literature and also check later papers describing this species (if availiable). In particular, review the literature concerning your species (and related taxa) which was published after 1991 and contain recent revisions and descriptions of "post-Page" genera and species.

Locomotive form.

The morphology of an amoeba in continuous, directed movement is very informative. If you are working with water-immersion or inverted optics, find locomotive amoeba on a clean area of surface, free of bacteria and detritus (the presence of material on the bottom of the dish may affect the locomotive form). If you are working with an agar culture, wash amoebae from the agar with a drop of medium, then place this drop on a glass slide and cover with a coverslip. The drop should be of a size that the coverslip does not squash the cells. If you are working with large amoebae, scrape a piece of wax (or petroleum jelly) under each corner of the coverslip prior to application. This will allow more space under the coverslip. It is very important to avoid squashing the amoebae as this would affect the locomotive form and may result in misidentification. It may require some time for amoebae to start moving and to adopt true locomotive forms (as opposed to non-directed movement), thus it is better to place prepared slides in a moist chamber for two-three hours before observation.

Observe actively moving cells and note their shape and characteristics (uroid, hyaloplasm, ridges, lateral flatness, shape of subpseudopodia, lobes and wrinkles, if present). Sketches, videoprints or photographs of moving amoeba can be very useful. Measure the locomotive forms using a micrometer. Preferably several amoebae should be measured, but make sure that they all belong to the same species! If your aim is to classify to the level of species you should work with clones, and measure no fewer than 30 amoebae to obtain an average. Note the nuclear size and structure, shape and size of crystals (if present) and the typical (if any) position of the contractile vacuole.

If the amoebae were maintained on agar without an overlay, it may be extremely difficult to observe moving cells in subsequent preparations. It seems that strains partly lose their locomotive capabilities when cultivated under these conditions for several generations. To avoid this problem, prior to removing cells for observation, cover the agar with an overlay of medium and leave for two-three days. In most cases this is sufficient to restore normal locomotion. If it fails, try adding a liquid overlay to successive sub-cultures. The latter is especially appropriate for amoebae strains that were obtained from culture collections. These strains, if cultured on agar medium without overlay, may not produce the typical locomotive form on the slides at all. Several sub-cultures in liquid media (or with overlay) are required to restore their locomotive capability.

Floating form.

The floating form is also very important for species identification. Floating forms from clonal cultures should be observed, unless there is sufficient size difference between the amoeba you are interested in and other amoebae in co-culture. In some liquid cultures, or in agar cultures with a liquid overlay, you may be able to observe floating forms at any time under a dissection microscope. If not, shake the culture carefully and observe the floating forms as they develop. Not all amoebae form them readily, and you need to watch the floating amoebae as they change in shape over time to ensure that you have seen fully developed floating forms. For smaller amoebae, and for amoebae which are maintained on agar without overlay, wash cells from the dish with a drop of medium, place the drop on a slide, cover with a coverslip and observe immediately. Sometimes it is possible to see floating forms and, in turn, locomotive forms on the same glass slide.

Note the appearance of the floating form, the shape and number (min/max) of pseudopodia, the composition of the pseudopodia (hyaloplasm only, or with granuloplasm), the shape of the ends of pseudopodia, and their thickness. Note if the amoebae have the tendency to form coiled or spiral pseudopodia. Measure the length of pseudopodia in comparison with the size of the central mass of cytoplasm in floating forms with radiating pseudopodia. Some amoebae species have a tendency towards gradual modification of the floating form with increasing time in culture, thus the floating form of fresh isolates may differ slightly from the floating form of the same species maintained by culture collections.

Some amoeba species merely retain their typical locomotive morphology whilst floating. However, it is important to make repeated and careful observations before concluding that an amoeba does not have a differentiated floating form.

Nuclear structure and crystals.

Nuclei and crystals are clearly visible using oil immersion optics (DIC or phase contrast) and a x100 objective lense. For these observations amoeba should

be slightly squashed by the coverslip in order to make the nucleus and crystals more visible. Note the nuclear structure, number and position of nucleoli, shape and size of crystals, and estimate the number of crystals. However, you should not measure nucleus diameter under these conditions!

Cysts.

Cyst formation and cyst structure are very important criteria in amoebae systematics. However, not all species form cysts in culture. To observe cysts you need to have a clonal culture. Cysts may be found after 7-15 days in agar cultures and after longer periods (up to a month) in liquid cultures. Some species lose the capacity to encyst after some time in laboratory culture, and some form cysts only in cultures with a liquid overlay. Different conditions should be tested and cultures should be observed for at least a month before conclusions are made about cyst formation. Cysts should be observed with LM under x100 oil immersion; shape, size, structure and number of cyst walls, presence, distribution and structure of cyst pores should be noted. Cysts should also be the subject of EM examination.

TEM studies.

TEM studies are obligatory if you would like to identify an amoeba to the level of genus or species as the microsystematics of gymnamoebae are based on EM features. This has the great disadvantages of requiring time and specialist facilities but there is no other way of distinguishing most species with a sufficient level of confidence. We do not attempt to describe all EM protocols here, only the most important details are listed.

To prepare amoebae for EM, wash them from the agar surface with a drop of medium or carefully shake a liquid culture. Concentrate the cells by gentle centrifugation. We do not advise embedding amoebae in agar blocks after fixation, as it seems to damage the cell coat. If amoebae are firmly adhered to the agar, it may be easier to cut out small (max 2*x*2*x*1 mm) blocks of agar with adherent amoebae and transfer them to glass wells for further treatment. For amoebae which are not numerous in culture the following approach may be useful (Smirnov and Goodkov, 1994):

Prepare a layer of polymerised resin in 40 mm Petri dishes. Store them opened for 2-3 days after polymerisation. Place several drops containing amoebae on the resin and mark the position of the drops by scraping the resin around the drops with a needle. Leave them in a moist chamber for 30-60 min to allow the amoebae to adhere to the resin. Fix the amoebae and treat them the same as in glass wells. The only difference is that you should not use acetone or any other substance which may dissolve the plastic during the embedding process (we use 100% ethanol to dilute the resin during the final embedding steps). Cover the amoebae in the dish with a thin layer of the same resin. Blocks should be sectioned so that the border of the resin layers is either parallel or perpendicular to the knife.

This method is suitable for very small amoebae, and even for single cells, as it is possible to examine the embedded cell(s) under the microscope (the markings of the initial position of drops with amoebae being highly useful here) to ensure that you really have the organism of interest in the preparation and to know its exact position in the block. This method, which fixes the locomotive form, often gives better results than those which involve centrifugation.

Fixation. This is the most crucial part of TEM preparation, and only properly fixed specimens should be selected for observation. Numerous artefacts are possible, notably the elimination or coagulation of the filamentous cell coat as a consequence of unsuitable fixation (Smirnov and Goodkov, 1998; Smirnov, 1999a). Any of three basic protocols may give satisfactory results, depending on the species. There are many other methods and the reader is referred to Page (1983; 1988) and other relevant literature for more information. We suggest that all the fixatives mentioned in the following protocols should be prepared in 0.1M phosphate buffer (PBS), pH 7.4 or in 0.05M sodium cacodylate buffer, pH 7.0 - in most cases there is no noticeable difference between these in the quality of fixation.

Procedure 1.
- add 4% glutaraldehyde and incubate for 30 min.
- wash three times in buffer for 5 min.
- replace with osmium tetroxide 1% and incubate for 30-60 min.
- wash three times in buffer for 10 min.

Procedure 2.
- osmium tetroxide 0.1% - 5 min.
- osmium tetroxide 1% - 30-60 min.
- wash 3 x 10 min in buffer

Procedure 3.
- osmium tetroxide 0.1% - 5 min.
- wash 3 x 5 min. in buffer
- 4% glutaraldehyde - 30 min.
- wash 3 x 5 min. in buffer
- osmium tetroxide 1% - 30-60 min.
- wash 3 x 10 min in buffer

The last protocol is the most complicated, however, sometimes it gives perfect results. For example, it was the only method which preserved the filaments in the cell coat of *Polychaos annulatum* (Smirnov and Goodkov, 1998) rather than resulting in their collapse.

Fixation is followed by standard protocols for dehydration, embedding in resin, sectioning, staining

of sections with uranil acetate and Reynold's lead citrate, and TEM examination.

Some amoebae have glycostyles or scales on their surfaces. This is characteristic for amoebae of fan-shaped and dactylopodial morphotypes. These structures are poorly visible in TEM, but chromium shadowing is a simple and highly useful technique which may preserve them. In fan-shaped amoebae *only* chromium shadowing can reliably confirm the absence of glycostyles. The following protocol was developed by Ken Clarke (CEH Windermere, UK):

For shadowing, concentrate amoebae from liquid cultures or wash them off an agar surface. In most cases, cells in cultures are densely covered with adherent bacteria (this is especially true for agar cultures). Try to wash off the bacteria by placing a drop of cell supension in a glass well, dilute with distilled water and repeatedly pipette the mixture. The amoebae should then be re-collected with a pipette. Significant loss of cells is possible at this stage. Floating forms are preferable, thus wait until the cells adopt them (if they do). Place drops with washed amoebae onto formvar-coated grids in a Petri dish, add several drops of osmium tetroxide, cover with the lid dish cover and leave for 2 min. Allow the drops to dry on the grids. Remove the grids and transfer them to a vacuum coater for shadow-casting. The 'grazed lighting' effects produced by this process will enhance the visibility of fine structure such as glycostyles and scales. Use gold-palladium (medium resolution, high contrast shadows), platinum (high resolution, low contrast shadows) or chromium (recommended for routine use) as a shadow source. Shadow at 30° - 40° to the horizontal for the examination of general cell shape, or 20° - 30° to the horizontal for viewing surface filaments, scales, glycostyles, etc. Examine each grid by TEM. As shadowed cells on the formvar grid-coating may shrink in size when exposed to the electron beam, be sure not to confuse shrinkage artifacts with fine surface structure.

Special techniques.

Together, the five steps above provide sufficient data for the identification of most gymnamoebae. However, some groups may require special techniques. Identification of *Acanthamoeba* species, for example, requires physiological tests, impregnation of cysts and biochemical tests, as described in the relevant literature (see keys for references). Most heterolobosean amoebae can be identified and differentiated only to the level of genus using morphological and ultrastructural data and even this requires further analysis to determine whether they can tranform into flagellates and, if so, to describe the characteristics of this additional stage in the life cycle. For most of these eruptive, limax, amoebae reliable identification of species is only possible using molecular methods, principally because they are very homogenous in morphology.

4. A guide to soil naked amoebae

This "guide" to soil gymnamoebae is not a "key" in the traditional sense. Its purpose is to help those investigating amoebae in environmental samples to recognise non-systematic groups of amoebae - **morphotypes**, and to enable the recognition of a morphotype from observing the locomotive form of a few individuals or even a single amoeba. It is not intended for identification to the level of genus or species (which requires much more detailed study and analysis) but does offer advice on this subject and provides some key references from the sea of literature relating to naked amoeba systematics.

The guide is based on a set of morphotypes of gymnamoebae (Smirnov and Goodkov, 1999a) - a system for recognising the "shapes" of amoebae (Figs. 15-16). Each morphotype is represented by a drawing with a description of its most important features, stressing those which are unique and distinctive. Only morphotypes that include freshwater and soil species are listed in this section. In contrast with the initial system of morphotypes (op. cit.), "Palmate" and "vexilliferian" amoebae are no longer considered to be specific morphotypes and the system has been expanded to include "eruptive" and "lens-like" morphotypes. These changes are the result of increasing experience of using the system.

We suggest that the guide is used in the following way:

Step 1: Observe amoebae that are active and "healthy" (i.e. do not contain numerous food vacuoles or food vacuoles containing large objects like a big diatom, are not highly vacuolarised, have normal cytoplasmic flows, are well-adhered to the substratum and not depressed by the coverslip) during normal locomotion (i.e. continuous, directional movement). The correct observation and interpretation of the locomotive form is critical for the recognition of the morphotype. Whenever possible try to observe several different cells of the same species. Observe the variety of shapes of the locomotive form and note the characteristic morphological details i.e. position and shape of the hyaloplasm, uroidal structures, presence of dorsal or lateral folds or wrinkles. Do not rely on the shape of an amoeba that does not move actively or which changes its direction of movement too often. If you observe this – then it is non-directed movement of an amoeba.

Step 2: From the drawings and accompanying descriptions below, decide which morphotype most resembles your specimen.

Step 3: Check the choice of morphotype by comparison with the plates of representative amoeba species and against the accompanying notes. For each morphotype only a few representatives have been selected to display the characteristic appearance (and range of appearances) of amoebae of each morphotype and to give you an impression of the reliability of your choice. Check several similar morphotypes before making a decision. Note that a single species may belong to more than one morphotype.

If you do not intend to continue with a more detailed classification, and to establish the amoeba in culture, then stop here. If published, your identification of the morphotype, especially if accompanied by size measurements and a description of distinctive features, will allow an amoeba systematicist to suggest a list of possible species to which the isolate may belong. Hence, you will have made a valid contribution without the risk of making an incorrect species identification. However, if you have the need and the facilities to make a systematic identification then continue as follows.

Step 4: Consult the literature, starting with the basic keys and monographs listed after this paragraph and then the papers listed for each morphotype. Note that to identify an amoeba to the level of species requires expertise, and identification is reliable only if your strain matches exactly the original detailed description (or later re-descriptions) of the species, not just the brief description provided in keys. In most cases you will need to establish the strain in culture (preferably, a clonal culture), because the mean size measurements must be obtained from a set of specimens that definitely belong to the same species. Most of the special techniques, described in the introduction section, also require cultures rather than just a few individuals.

Note that there are many little known or dubious species that are not listed here, but which are described in the older literature. These descriptions are usually incomplete and do not include details of many of the features required for the reliable re-isolation of a species. However, there is a tendency to identify isolates that resemble such poorly-described species on the basis of their "general similarity" but without proper re-description. In our opinion, this practice is totally wrong. For example, many amoebae species described before the application of EM techniques cannot be re-isolated reliably because many genera have since been differentiated on the basis of the ultrastructure of the cell coat (for example, it would be hard to distinguish *Vannella* from *Platyamoeba*, or some species of *Mayorella* from some species of *Korotnevella* without EM) The only appropriate solution in such situations, in our opinion, is the exhaustive study and re-description of the little-known species according to the normal practice of zoological taxonomy (i.e. with revised diagnosis and correct differential diagnosis) and the establishment of the neotype or paratype (if the holotype is still available). Preferably, type material should be a culture or permanently stained preparations, optionally accompanied with TEM embeddings (but not only the embeddings!), deposited with one of the recognized culture collections (for example, the Culture Collection of Algae and Protozoa (CCAP), UK or the American Type Culture Collection (ATCC), USA) or collections of type preparations (for example, the collection of the National History Museum (London, UK), currently preserving most of the existing type preparations of naked amoebae).

Only in a few cases is reliable re-isolation or recognition of a poorly-described species possible (e.g. Smirnov and Goodkov, 1998; Michel and Smirnov, 1999; Smirnov, 1999a), and in most cases we have to conclude that the species cannot be reliably re-isolated and should be declared "nomina dubia", or that the specific name should no longer be used (e.g. Goodkov, 1988; Smirnov, 1997).

Finally, note that the biodiversity of amoebae remains poorly explored. Detailed faunistic studies of marine and freshwater habitats always reveal many new species (up to half of the total discovered) (Smirnov and Goodkov, 1995; Smirnov 1999a, 2001). The probability of encountering a new species is high and this possibility should be always be considered when identifying an isolate.

Fundamental papers and monographs for amoebae identification:

Bovee E.C. 1985. Class Lobosea Carpenter, 1861. In: An Illustrated Guide to the Protozoa. (Eds. Lee J.J., Hutner S.H., Bovee E.C.). Allen Univ. Press, Kansas. pp. 158-211.

Page F.C. 1976. An illustrated key to freshwater and soil amoebae. Freshwater Biol. Ass. Ambleside.

Page F.C. 1983. Marine gymnamoebae. Inst. Terr. Ecol.

Page F.C. 1988. A new key to freshwater and soil gymnamoebae. Freshwater Biol. Ass. Ambleside.

Page F.C. 1991. Nackte Rhizopoda. In: Nackte Rhizopoda Und Heliozoea (Protozoenfauna, Band 2). Gustav Fisher Verlag, Stuttgart, New York. pp. 3-170.

Page F.C. 1987. The classification of 'naked' amoebae (Phylum Rhizopoda). Arch. Protistenk. 133, 199-217.

Rogerson A. and Patterson D.J. 2000. The naked ramicristate amoebae (Gymnamoebae) In: An illustrated guide to the Protozoa, 2nd ed. (Eds. Lee J.J., Leedale G.F. and Bradbury P.). Society of Protozoologists, Kansas. pp. 1023–1053.

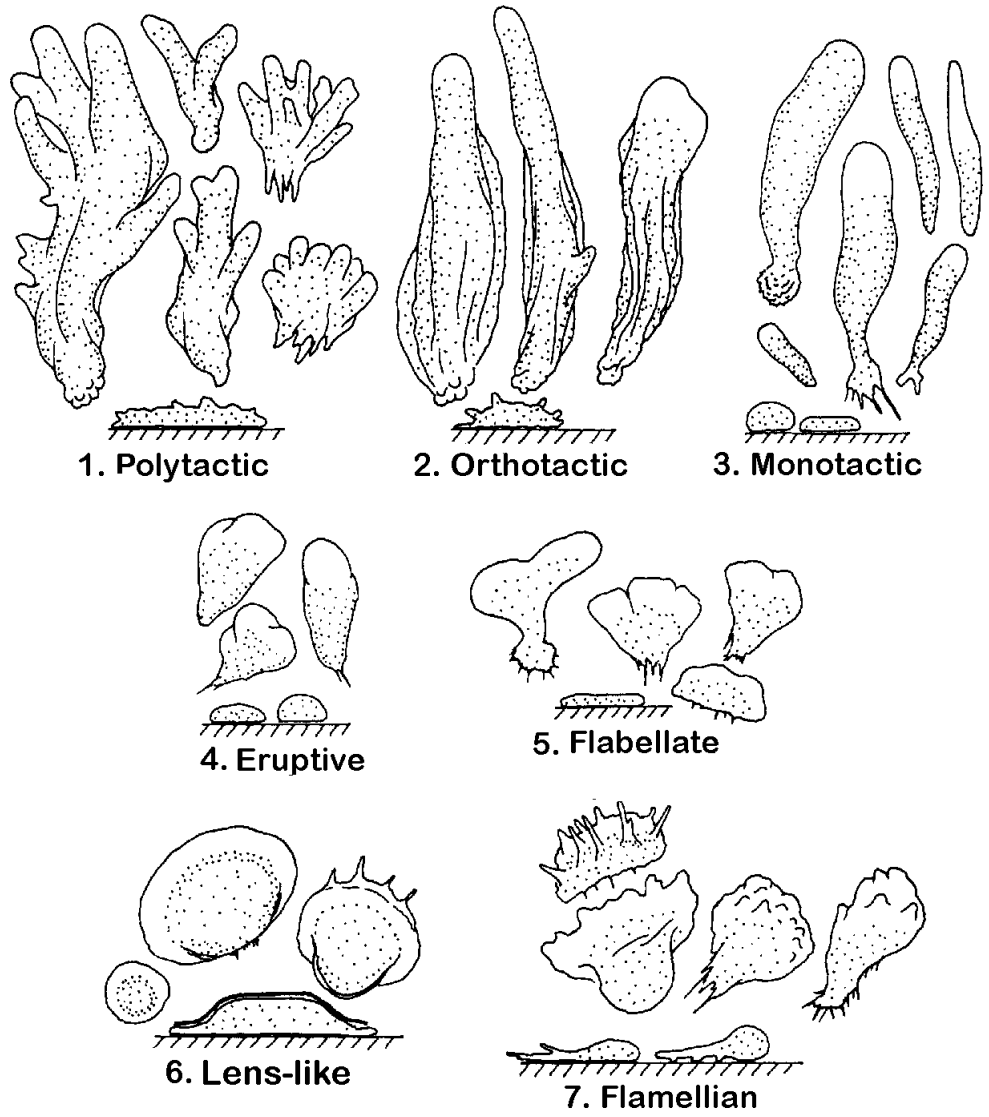

Fig. 15. Morphotypes of amoebae. Schematic drawings (part 1). See text for descriptions.

Morphotypes of amoebae

Polytactic morphotype: polypodial amoebae with large, distinctive pseudopodia. Pseudopodia consist both of the granuloplasm and the hyaloplasm.

Amoebae of polytactic morphotype (Fig. 17) are easily recognisable as they are the only ones that form well-pronounced pseudopodia. In polytactic amoebae pseudopodia actively participate in the movement of the cell. Some cells form numerous pseudopodia, others form only two or three. Most polytactic amoebae have an alternative orthotactic locomotive form and so belong to the orthotactic morphotype as well. Some polytactic species also may temporarily become monotactic, especially under unfavourable conditions. Only two species of this morphotype (members of the genus *Deuteramoeba*) appear to be true soil species, but other species may appear in water-saturated soil and marshes.

Polytactic morphotype: list of freshwater/soil species

Amoeba proteus (Pallas 1766) Leidy 1878

Amoeba borokensis Kalinina, Afon'kin, Gromov, Khrebtukova et Page 1987

Amoeba leningradensis Page et Kalinina 1984

Amoeba amazonas Flickinger 1974

Chaos carolinense (Wilson 1900) King et Jahn 1948

Chaos illinoisense (Kudo 1950) Goodkov, Smirnov et Skovorodkin 1999

Chaos nobile (Penard 1902) Bovee et Jahn 1973

Chaos glabrum Smirnov et Goodkov 1997

Deuteramoeba algonquinensis (Baldock, Rogerson et Berger 1983) Page 1987

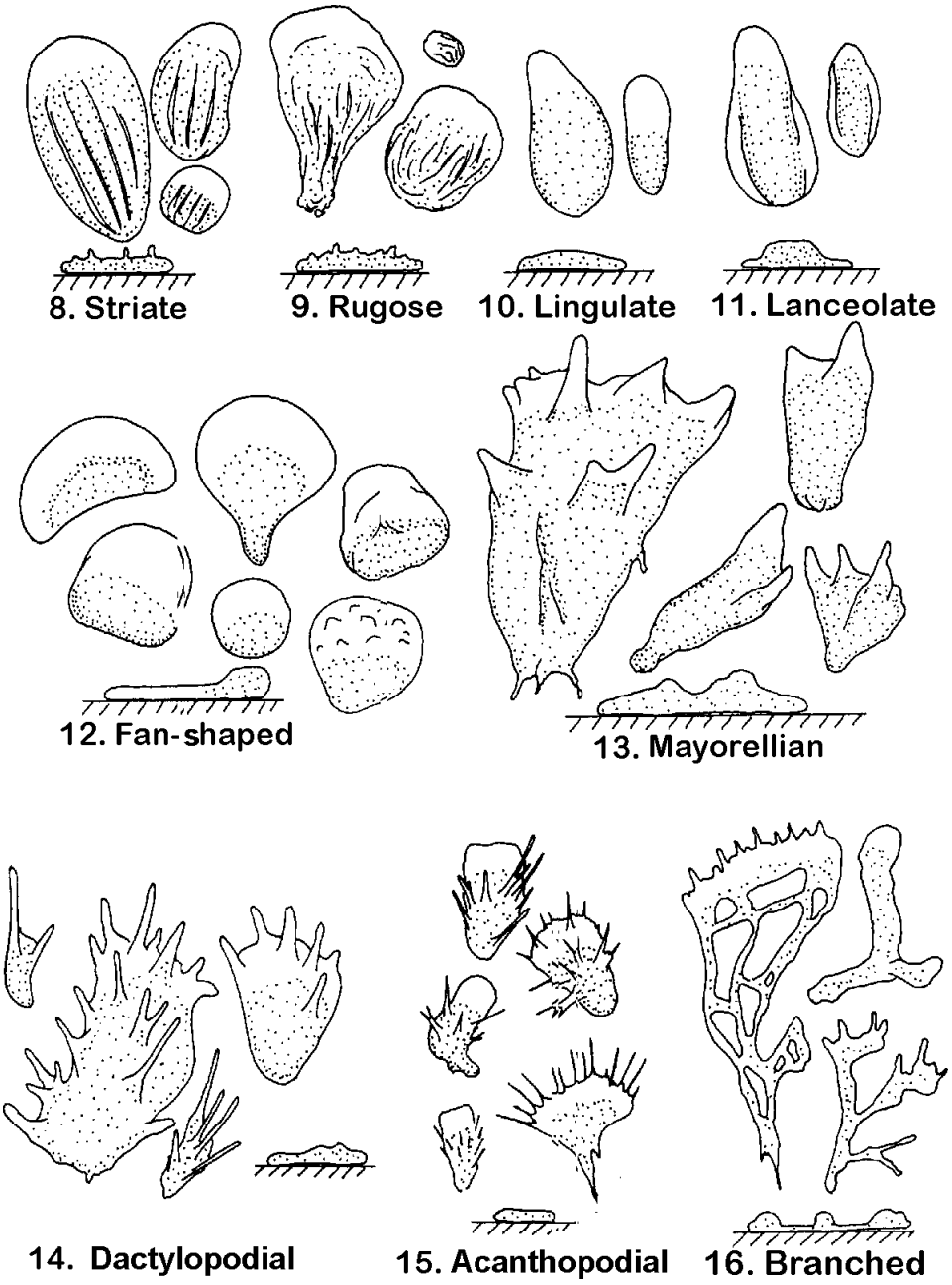

Fig. 16. Morphotypes of amoebae. Schematic drawings (part 2). See text for descriptions.

Deuteramoeba mycophaga (Pussard, Alabouvette, Lemaitre et Pons 1980) Page 1987

Polychaos fasciculatum (Penard 1902) Schaeffer 1926

Polychaos annulatum (Penard 1902) Smirnov et Goodkov 1998

Polychaos dubium (Schaeffer 1916) Schaeffer 1926

Polychaos nitidubium Bovee 1970

Pseudothecamoeba proteoides (Page 1976) Page 1988

Thecochaos album (Greef 1891) Page 1981

Thecochaos fibrillosum (Greef 1891) Page 1981

Some references for identification of well-known polytactic species:

Baldock B.M., Rogerson A. and Berger J. 1983. A new species of freshwater amoeba: *Amoeba algonquinensis* n. sp. (Gymnamoebia, Amoebidae). Trans. Amer. Microsc. Soc. 102, 113-121.

Baldock B.M. and Baker J.H. 1980. An occurence and growth rates of *Polychaos fasciculatum* - a rediscovered amoeba. Protistologica. 16, 79-83.

Bovee E.C. and Jahn T.L. 1973. Taxonomy and phylogeny. In: The Biology of Amoeba. Acad. Press.,

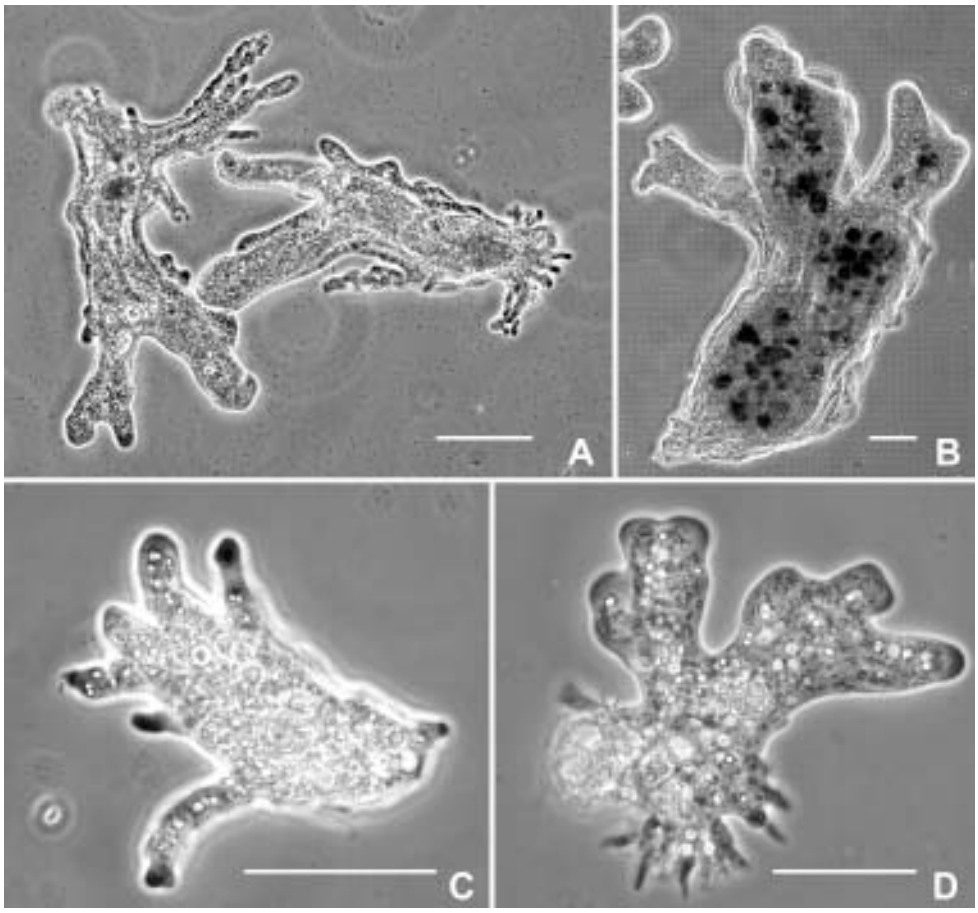

Fig. 17. Representatives of amoeba of polytactic morphotype. a - *Amoeba proteus* CCAP 1503/9 (phase contrast); b - *Chaos carolinense* Carolina Biol. Suppl.WW13-1324 (phase contrast); c - *Deuteramoeba algonquinensis* CCAP 1503/5 (phase contrast); d - *Polychaos fasciculatum* CCAP 1564/1 (phase contrast). Scale bars: a-b - 100 μm, c-d - 50 μm.

New York. pp. 38-82.

Flickinger C.J. 1974. The fine structure of four "species" of *Amoeba*. J. Protozool. 21, 59-68.

Goodkov A.V., Smirnov A.V. and Skovorodkin I.N. 1999. Study of a rediscovered large freshwater multinucleate amoeba *Chaos illinoisense* (Kudo 1950). Protistology. 1, 55-61.

Kudo R.R. 1951. Observations on *Pelomyxa illinoisensis*. J. Morphol. 88, 145-173.

Page F.C 1981. Eugene Penard's slides of Gymnamoebia: re-examination and taxonomic evaluation. Bull. Br. Mus. Nat. Hist. (Zool.). 40, 1-32.

Page F.C. 1986. The genera and possible relationships of the family Amoebidae, with special attention to comparative ultrastructure. Protistologica. 22, 301-316.

Page F.C. and Kalinina L.V. 1984. *Amoeba leningradensis* n. sp. (Amoebidae): a taxonomic study incorporating morphological and physiological aspects. Arch. Protistenk. 128, 37-53.

Smirnov A.V. and Goodkov A.V. 1997. Description of the large multinucleate lobose amoeba *Chaos glabrum* sp. n. (Lobosea, Amoebidae), with notes on the diagnosis of the genus *Chaos*. Acta Protozool. 36, 227-233.

Smirnov A.V. and Goodkov A.V. 1998. Study of *Polychaos annulatum* Penard, 1902 comb. nov. (Gymnamoebia, Amoebidae) with taxonomical analysis of *Polychaos fasciculatum*-like species. Europ. J. Protistol. 34, 1-9.

Orthotactic morphotype: monopodial amoebae, subcylindrical in cross-section, always with lateral and/or dorsal wrinkles. Cells may retain trailing remnants of small lateral pseudopodia, which do not participate in locomotion.

The orthotactic morphotype (Fig. 18) is an alternative locomotive form for some amoebae which belong to the polytactic morphotype. Its most characteristic feature, which also differentiates these amoebae from those of monotactic morphotype, is the presence of wrinkles on the lateral or dorsal surface of the amoeba. Most polytactic amoebae only adopt the orthotactic locomotive form temporarily, during

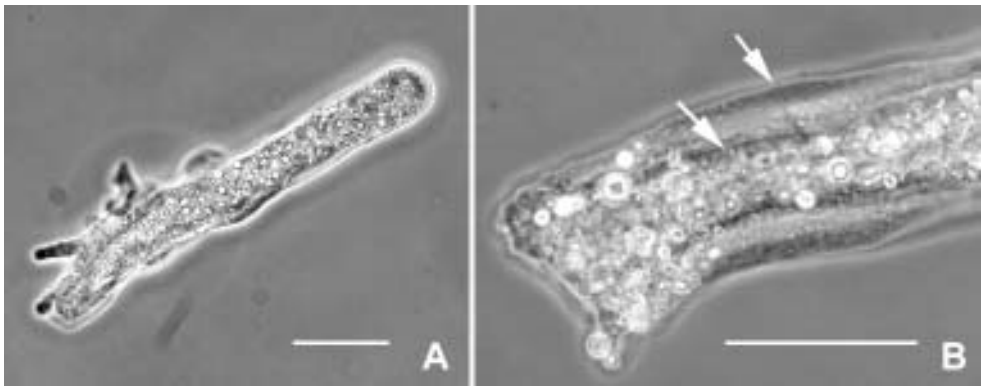

Fig. 18. Representative of amoeba of orthotactic morphotype - *Amoeba borokensis* CCAP 1503/7. a - locomotive cell (phase contrast); b - dorso-lateral ridges located in the posterior part of locomotive cell (arrowed) (phase contrast). Scale bar: 50 μm.

active, fast, movement. However, there are species for which the orthotactic form is the norm during locomotion.

Orthotactic morphotype: list of freshwater species

Amoeba proteus (Pallas 1766) Leidy 1878
Amoeba borokensis Kalinina, Afon'kin, Gromov, Khrebtukova et Page 1987
Amoeba leningradensis Page et Kalinina 1984
Amoeba amazonas Flickinger 1974
Chaos illinoisense (Kudo 1950) Bovee et Jahn 1973
Chaos carolinense (Kudo 1950) Bovee et Jahn 1973
Chaos nobile (Penard 1902) Bovee et Jahn 1973
Chaos glabrum Smirnov et Goodkov 1997
Polychaos annulatum (Penard 1902) Smirnov et Goodkov 1998
Pseudothecamoeba proteoides (Page 1976) Page 1988

References and notes for identification of orthotactic species are the same as listed for the polytactic morphotype.

Monotactic morphotype: monopodial amoebae, subcylindrical in cross-section, with steady cytoplasmic flow. Cells always smooth, without lateral and/or dorsal wrinkles.

Amoebae of monotactic morphotype (Fig. 19) are elongate, monopodial and subcylindrical in cross-section, with a smooth dorsal surface. They are easily differentiated from amoebae of orthotactic morphotype as they never have lateral or dorsal wrinkles. Furthermore, they are smaller than most orthotactic species and much more uniform in locomotion. Some orthotactic and polytactic amoebae become monotactic during active locomotion or under unfavourable conditions, while for other species only the monotactic morphotype is observed.

Monotactic morphotype: list of freshwater/soil species

Amoeba leningradensis Page et Kalinina 1984
Amoeba proteus (Pallas 1766) Leidy 1878
Cashia limacoides (Page 1967) Page 1974
Deuteramoeba algonquinensis (Baldock, Rogerson et Berger 1983) Page 1987
Deuteramoeba mycophaga (Pussard, Alabouvette, Lemaitre et Pons 1980) Page 1987
Glaeseria mira (Glaeser 1912) Volkonsky 1931
Hartmannella cantabrigiensis Page 1974
Hartmannella vermiformis Page 1967
Hydramoeba hydroxena (Entz 1912) Reynolds et Looper 1928
Trichamoeba sinuosa Siemensma et Page 1986
Trichamoeba myakka Bovee 1972
Trichamoeba osseosaccus Schaeffer 1926
Trichamoeba cloaca Bovee 1972
Parachaos zoochlorellae (Willumsen 1982) Willumsen, Siemensma et Suhr-Jessen 1987
Polychaos annulatum (Penard 1902) Smirnov et Goodkov 1998
Polychaos fasciculatum (Penard 1902) Schaeffer 1926
Saccamoeba stagnicola Page 1974
Saccamoeba limax (Dujardin 1841) Page 1974
Saccamoeba wakulla (Bovee 1972)
Saccamoeba limna (Bovee 1972)
Saccamoeba lucens (Frenzel 1892) Bovee 1972
Saccamoeba wellneri Siemensma 1987
Saccamoeba angelica Bovee 1972
Rhizamoeba australiensis (Chakraborty et Pussard 1985) Page 1988
Rhizamoeba flabellata (Goodey 1914) Cann 1984

Identification of these amoebae is difficult. Ultrastructure is not informative, although it is essential for distinguishing monotactic amoebae from non-

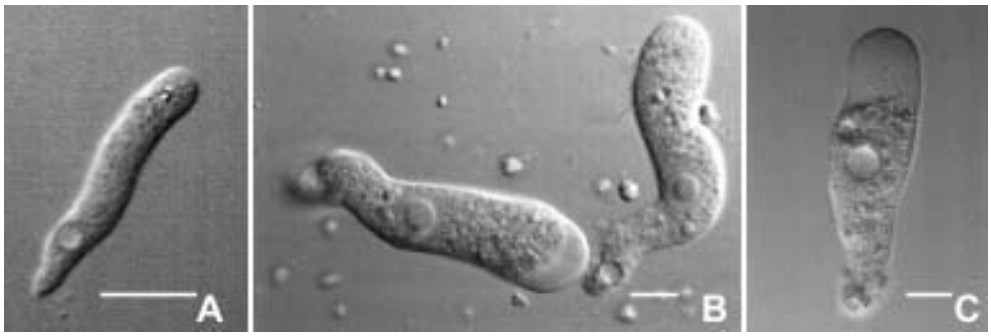

Fig. 19. Representatives of amoeba of monotactic morphotype. a - *Hartmannella cantabrigiensis* CCAP 1534/11 (DIC); b - *Saccamoeba stagnicola* CCAP 1572/2 (DIC); c - *Saccamoeba limax* CCAP 1534/6 (DIC). Scale bars: 10 μm.

markedly eruptive amoebae of the class Heterolobosea. Distinctive features are the number of nuclei, the nuclear structure and uroidal structures and cell coat composition. A mononucleate limax amoeba with a vesicular nucleus, with or without a clear, well-visible hyaline cap is most probably a member of the family Hartmannellidae. If an amoeba has distinct adhesive uroidal filaments, and exhibits occasional eruption of the frontal hyaloplasm in a direction opposite to the direction of movement, then it is most probably a species of *Rhizamoeba*. Please, consult references for polytactic species as well, if you suspect that an amoeba is a monotactic form of *Amoeba*, *Deuteramoeba* or *Polychaos*. Perhaps, for the inexperienced observer, there may be some confusion in the differentiation of monotactic amoebae from amoebae of the lingulate morphotype, but note that lingulate amoebae are always flattened in cross section and normally demonstrate polyaxial cytoplasmic flow.

Some references for identification of monotactic species:

Bovee E.C. 1972. The lobose amebas IV. A key to the order Granulopodida Bovee and Jahn, 1966, and description of some new and little-known species in this order. Arch. Protistenk. 114, 371-403.

Cann J.P. 1984. The ultrastructure of *Rhizamoeba flabellata* (Goodey) comb. nov. and *Leptomyxa reticulata* Goodey (Acarpomyxea: Leptomyxida). Arch. Protistenk. 128, 13-23.

Chakraborty S. and Old K.M. 1986. Ultrastructure and description of a fungus-feeding amoeba, *Trichamoeba mycophaga* n. sp. (Amoebidea, Amoebae), from Australia. J. Protozool. 33, 564-569.

Page F.C. 1967. Taxonomic criteria for limax amoebae, with descriptions of 3 new species of *Hartmannella* and 3 of *Vahlkampfia*. J. Protozool. 14, 499-521.

Page F.C. 1972. *Rhizamoeba polyura* n. g., n. sp., and uroidal structures as a taxonomic criterion for amoebae. Trans. Amer. Microsc. Soc. 91, 502-513.

Page F.C. 1974. A further study of taxonomic criteria for limax amoebae, with description of new species and a key to genera. Arch. Protistenk. 116, 149-184.

Page F.C. 1980. A light and electron microscopical comparison of limax and flabellate marine amoebae belonging to four genera. Protistologica. 16, 57-78.

Pussard M., Alabouvette C., Lemaitre I. and Pons R. 1980. Une nouvelle amibe mycophage endogee *Cashia mycophaga* n. sp. (Hartmannellidae, Amoebida). Protistologica. 16, 443-451.

Siemensma F.J. and Page F.C. 1986. A light- and electron-microscopical study of *Trichamoeba sinuosa* n. sp. (Amoebida) with a re-diagnosis of the genus. Protistologica. 22, 117-125.

Siemensma F.J. 1987. De nederlandse naaktamoeben (Rhizopoda, Gymnamoebia). Hoogwoud. Koninklijke Nederlandse Natuurhistorische Vereniging.

Willumsen N.B.S. 1982. *Chaos zoochlorellae* nov. sp. (Gymnamoebia, Amoebidae) from a Danish freshwater pond. J. Nat. Hist. 16, 803-813.

Eruptive morphotype: monopodial amoebae, markedly eruptive in locomotion.

Amoebae of eruptive morphotype (Fig. 20) differ from monotactic amoebae by their characteristic, eruptive, cytoplasmic flows. Eruptions may be more or less pronounced (it may require some experience to recognise them), but are always visible. These amoebae have variable, but generally monopodial shape but are never as flattened as amoebae of flabellate morphotype.

Eruptive morphotype: list of freshwater/soil species

(Due to the large number of species displaying this morphotype only the genus name is listed if all members of a genus belong to this morphotype).

genus *Vahlkampfia* Chatton et Lalung-Bonnaire 1912

genus *Naegleria* (Alexieff 1912) Calkins 1913
genus *Tetramitus* Perty 1852
Paravahlkampfia ustiana (Page 1974) Brown et De Jonckheere 1999
Willaertia magna De Jonckheere, Dive, Pussard et Vickerman 1984
Tetramastigamoeba hoarei Singh et Hanumaiah 1977
Stachyamoeba lipophora Page 1975
Acrasis rosea Olive et Stoianovich 1960
Pochenia rosea Cienkowsky 1873
Pochenia flagellata Stoianovich, Olive et Bennet 1983
Learamoeba waccamawensis Sawyer, Nerad, Cahoon et Nearhoff 1998
Singhamoeba horticola Sawyer, Nerad et Munson 1992
Monopylocystis visversvarai O'Kelly, Silberman, Zettler, Nerad et Sogin 2003
Sawyeria marylandensis O'Kelly, Silberman, Zettler, Nerad et Sogin 2003
Psalteriomonas lanterna Broers, Stumm, Vogels et Brugerolle 1990

To assign an isolate to a described species requires detailed comparison of the morphology and ultrastrucure of trophozoites, cysts and flagellates (if formed) with original species descriptions. However, more recently, new species have been defined and assigned to genera using ribosomal DNA sequence information and without detailed phenotypic analyses (De Jonckheere and Brown, 1997). This is because phenotypic features have been proven to be unreliable for differentiating between genera (Brown and De Jonckheere, 1999). Therefore, ribosomal DNA sequence information may be necessary for assigning your isolate to a known (or new) species.

Some references for identification of eruptive species:

Alexieff A. 1912. Sur les characteres et la systematique des Amibe de groupe limax (*Naegleria* nov. gen. et *Hartmannia* nov. gen.) et des Amibes parasites des vertebres (*Protamoeba* nov. gen.). Bull. Soc. Zool. France. 37, 55-74.

Balamuth W., Bradbury P.C. and Schuster F.L. 1983. Ultrastructure of the amoeboflagellate *Tetramitus rostratus*. J. Protozool. 30, 445-455.

Blanton R.L. 1990. Phylum Acrasea. In: Handbook of Protoctista. (Eds. Margulis L., Corliss J.O., Melkonian M. and Chapman D.J.). Jones and Bartlett Publishers, Boston. pp. 75-87.

Brown S. and De Jonckheere J.F. 1999. A reevaluation of the amoeba genus *Vahlkampfia* based on SSUrDNA sequences. Europ. J. Protistol. 35, 49-54.

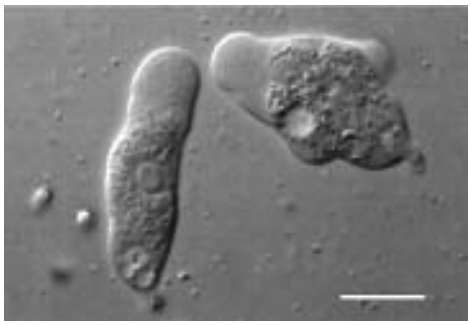

Fig. 20. Representative of amoeba of eruptive morphotype. *Acrasis rosea* CCAP 1508/2 (DIC). Scale bar: 10 μm.

Darbyshire J.F., Page F.C. and Goodfellow L.P. 1976. *Paratetramitus jugosus*, an amoebo-flagellate of soils and freshwater, type-species of *Paratetramitus* nov. gen. Protistologica. 12, 375-387.

De Jonckheere J.F. 1998. Relationships between amoeboflagellates. In: Evolutionary Relationships Among Protozoa. (Eds. Coombs G.H., Vickerman K., Sleigh M.A. and Warren A). Chapman and Hall, London. pp. 181-194.

De Jonckheere J.F., Dive D.G., Pussard M. and Vickerman K. 1984. *Willaertia magna* gen. nov., sp. nov. (Vahlkampfiidae), a thermophilic amoeba found in different habitats. Protistologica. 20, 5-13.

De Jonckheere J.F., Brown S. and Robinson B.S. 1997. On the identity of the amoeboflagellates *Didascalus thorntoni* and *Adelphamoeba galeacystis*. J. Euk. Microbiol. 44, 52-54.

De Jonckheere J.F. 2002. A century of research on the amoeboflagellate genus Naegleria. Acta Protozool. 41, 309-342.

Dyer B.D. 1990. Phylum Zoomastigina. Class Amoebomastigota. In: Handbook of Protoctista. (Eds. Margulis L., Corliss J.O., Melkonian M. and Chapman D.J.) Jones and Bartlett Publishers, Boston. pp. 186-190.

O'Kelly C.J., Silberman J.D., Zettler L.A.A., Nerad T.A. and Sogin M.L. 2003. *Monophylocystis visvesvarai* n. gen., n. sp. and *Sawyeria marylandensis* n. gen., n. sp.: two new heterolobosean amoebae from anoxic environments. Protist. 154, 281-290.

Page F.C. 1967. Taxonomic criteria for limax amoebae, with descriptions of 3 new species of *Hartmannella* and 3 of *Vahlkampfia*. J. Protozool. 14, 499-521.

Page F.C. 1974. A further study of taxonomic criteria for limax amoebae, with description of new species and a key to genera. Arch. Protistenk. 116, 149-184.

Page F.C. 1978. *Acrasis rosea* and the possible relionship between Acrasida and Schizopyrenida. Arch. Protistenk. 120, 169-181.

Page F.C. 1985. The limax amoebae: comparative fine structure of the Hartmannellidae (Lobosea) and

further comparisons eith the Vahlkampfiidae (Heterolobosea). Protistologica. 21, 361-383.

Page F.C. and Blanton L. 1985. The Heterolobosea (Sarcodina: Rhizopoda), a new class uniting the Schizopyrenida and the Acrasidae (Acrasida). Protistologica. 21, 121-132.

Robinson B.S., Christy P.E. and De Jonckheere J.F. 1989. A temporary flagellate (mastigote) stage in the vahlkampfiid amoeba *Willaertia magna* and its possible evolutionary significance. BioSystems. 23, 75-86.

Sawyer T.K., Nerad T.A. and Munson, D.A. 1992. *Singhamoeba horticola* (Singh and Hanumaiah, 1979) n. comb., type species of *Singhamoeba* n. g. J. Protozool. 39, 107-109.

Sawyer T.K., Nerad T.A. Cahoon, L.B.and Nearhoof J.E. 1998. *Learamoeba waccamawensis*, n. g., n. sp. (Heterolobosea: Vahlkampfiidae), a new temperature-tolerant cyst-forming soil amoeba. J. Euk. Microbiol. 45, 260-264.

Flabellate morphotype: flattened, usually irregular, triangular cells with prominent anterior hyaloplasm and uneven frontal edge. Cell never forms distinct subpseudopodia from the frontal hyaloplasm, though it may be divided into distinct lobes with deep clefts.

Amoebae of flabellate morphotype (Fig. 21) are always very flattened and expanded, and change shape rapidly. The frontal hyaloplasm may be uneven, but these amoebae never have subpseudopodia, distinct lobes or other projections on either the lateral or the dorsal surface of the hyaloplasm, in contrast with amoebae of flamellian morphotype. Some species have distinct adhesive uroidal filaments in locomotion. There are few freshwater/soil species of this morphotype.

Flabellate morphotype: list of freshwater/soil species

Stachyamoeba lipophora Page 1975
Rosculus ithacus Hawes 1963
Guttulinopsis vulgaris E.W. Olive 1901
Guttulinopsis nivea Raper, Worley et Kessler 1977

These amoebae are rather small (5-25 μm) and difficult to observe. Adhesive uroidal filaments are common in some species, but may be hardly visible. All these amoebae are capable of very rapid changes in conformation during locomotion, and occasional eruptive behaviour is observed in some species. Note: in addition, there are at least several undescribed freshwater species of the marine genus *Flabellula* (Smirnov, unpubl) and a poorly described *Paraflabellula* species, *P. kudoi* (Singh and Hanumaiah 1979) Page 1983. The latter may also exhibit flamellian morphotype and are listed there as well (see below).

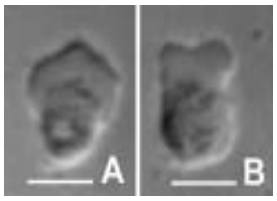

Fig. 21. Representative of amoeba of flabellate morphotype. a-b - *Rosculus ithacus* (Sourhope isolate) (DIC). Scale bar: 10 μm.

Some references for identification of flabellate species:

Hawes R.S.J. 1963. On *Rosculus ithacus* gen. n. sp. n. (Protozoa, Amoebina), with special reference to its mitosis and phylogenetic relation. J. Morph. 113, 139-150.

Olive E.W. 1901. Preliminary enumeration of the Sorophoraceae. Proc. Am. Acad. Arts. Sci. 37, 333-334.

Olive L.S. 1965. A developmental study of *Guttulinopsis vulgaris* (Acrasiales). Am. J. Bot. 52, 513-519.

Olive L.S. 1975. The Mycetozoans. Acad. Press, New-York, San-Francisco and London.

Page F.C. 1974. *Rosculus ithacus* Hawes, 1963 (Amoebida, Flabellulidae), and the amphyzoic tendency in amoebae. Acta Protozool. 13, 143-154.

Page F.C. 1975. A new family of amoebae with fine pseudopodia. Zool. J. Linn. Soc. 58, 61-77.

Flamellian morphotype: either all the cell is flattened and expanded, or only the frontal part of the cell is flattened. Subpseudopodia, lobes and waves are formed from the frontal hyaloplasm.

The flamellian morphotype includes flattened amoebae (Fig. 22) that may resemble amoebae of the flabellate morphotype but produce distinct subpseudopodia and lobes from the frontal hyaloplasm or from the lateral or ventral surfaces. The body shape in locomotion may vary from elongate to semicircular or crescent-shape. Some species have a cuticle, covering the dorsal surface only, and belong also to the lens-like morphotype. Interestingly, alternative locomotive forms of at least two *Rhizamoeba* species belong to this morphotype.

Flamellian morphotype: list of freshwater species

Flamella citrensis Bovee 1956
Flamella tiara Fishbeck et Bovee, 1993
Flamella aegyptia Michel et Smirnov 1999
Flamella lacustris Michel et Smirnov 1999
Gocevia fonbrunei Pussard 1965

Some references for identification of flamellian species:

Bovee E.C. 1956. Some observations on the morphology and activities of a new amoeba from citrus wastes, *Flamella citriensis* n. sp. J. Protozool. 3, 151-153.

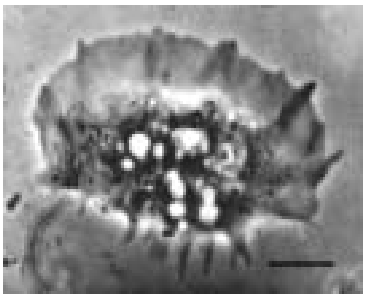

Fig. 22. Representative of amoeba of flamellian morphotype. *Flamella lacustris* (Valamo Island, North-Western Russia) (phase contrast). Scale bar: 10 μm.

Fig. 23. Representative of amoeba of lens-like morphotype. *Cochliopodium* sp. (Ebro Delta, Spain) (DIC). Scale bar: 10 μm.

Fishbeck D.W. and Bovee E.C. 1993. Two new amoebae, *Striamoeba sparolata* n. sp., and *Flamella tiara* n. sp., from freshwater. Ohio J. Sci. 93, 134-139.

Michel R. and Smirnov A.V. 1999. The genus *Flamella* Schaeffer 1926 (Lobosea, Gymnamoebia), with description of two new species. Europ. J. Protistol. 35, 403-410.

Chakraborty S. and Pussard M. 1985. *Ripidomyxa australiensis* nov. gen. nov. sp. , a mycophagous amoeba from Australian soil. Protistologica. 21, 133-140.

Pussard M. and Pons R. 1976. Etudes de genres *Leptomyxa* et *Gephyramoeba* (Protozoa, Sarcodina). II. *Leptomyxa flabellata* Goodey, 1914. Protistologica. 12, 307-319.

Lens-like morphotype: cell has a lens-like profile and its dorsal surface is covered by a rigid envelope. It may produce hyaline subpseudopodia from under this envelope (or through the envelope) or have numerous spineolate subpseudopodia on the ventral surface.

Amoebae of lens-like morphotype (Fig. 23) are covered on the dorsal surface only with either a "tectum" or a "cuticle". From under this cover they may produce hyaline subpseudopodia. Cells are usually lens-like in crosssection and have a granuloplasm in a hump which is located centrally or (in rapid locomotion) posteriorly and which is surrounded by a hyaloplasmic border, if the cell is viewed from above. The tectum is easily recognisable (as a punctuation of the hyaloplasm) in larger species using LM examination, but is invisible in smaller species. The cuticle is more difficult to see; it appears as a mucose layer under DIC optics, but is clearly visible on wrinkled areas of the cell where it is perpen-dicular to the focal plane.

Lens-like morphotype: list of freshwater/soil species

Cochliopodium bilimbosum (Auerbach 1856) Leidy 1879

Cochliopodium actinophorum (Auerbach 1856) Page 1976

Cochliopodium minus Page 1976

Cochliopodium larifeili Kudryavtsev 1999

Gocevia fonbrunei Pussard 1965

Paragocevia placopus (Huelsmann 1974) Page et Willumsen 1980

Amoebae of this morphotype are poorly studied, and only the few well-known freshwater/soil species are listed here. However, the potential diversity of these amoebae is extensive. In particular, there is strong evidence (A.A. Kudryavtsev, pers. com.) for the existence of many more species of the genus *Cochliopodium*. Many species of this morphotype are described in older literature, and have not been found more recently. Identification requires analysis of the surface structure with TEM or, for scale-bearing species, after shadowing of whole-cell mounts.

Some references for identification of lens-like species:

Bark A.W. 1973. A study of the genus *Cochliopodium* Hertwig and Lesser 1874. Protistologica. 9, 119-138.

Dykova I., Lom J. and Machaekova B. 1998. *Cochliopodium minus* - a scale-bearing amoeba isolated from orgens of perch Perca fluviatilis. Dis. Aquat. Org. 34, 205-210.

Kudryavtsev A.A. 1999. Description of *Cochliopodium larifeili* n. sp. (Lobosea, Himatismenida), an amoeba with peculiar scale structure, and notes on the diagnosis of the genus *Cochliopodium* (Hertwig and Lesser, 1874) Bark, 1973. Protistology 1, 66-71.

Page F.C. and Willumsen N.B.S. 1980. Some observations on *Gocevia placopus* (Huelsmann, 1974), an amoeba with a flexible test, and on Gocevia-like organisms from Denmark, with comments on the genera *Gocevia* and *Hyalodiscus*. J. Nat. Hist. 14, 413-431.

Pussard M., Senaud J. and Pons R. 1977. Observations ultrastructurales sur *Gocevia fonbrunei* Pussard

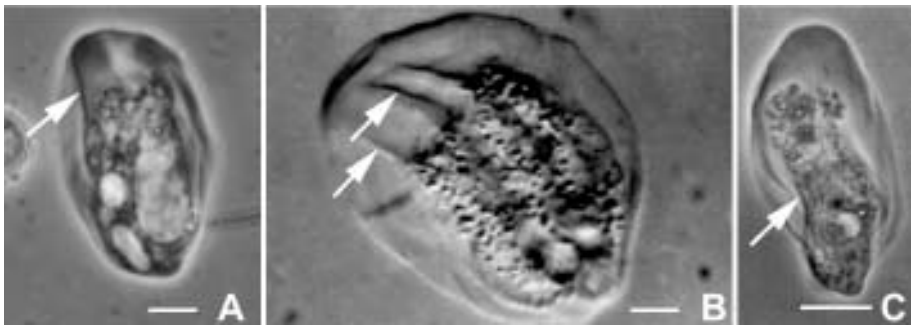

Fig. 24. Representatives of amoeba of striate morphotype. a - *Thecamoeba striata* CCAP 1583/4 (phase contrast); b - *Thecamoeba striata* (Valamo Island, North-Western Russia) (DIC), note pronounced dorsal ridges; c - *Thecamoeba quadrilineata* (Valamo Island, North-Western Russia) (phase contrast). Longitudinal dorsal ridges are arrowed. Scale bars: 10 μm.

1965 (Protozoa, Rhizopodea). Protistologica. 13, 265-285.

The following references contain the descriptions of little-known *Cochliopodium* species and should be checked (as well as references therein) if an isolate appears to be a new species.

Archer W. 1877. Resume of recent contribution to our knowledge of "fresh-water Rhizopoda". Quart. J. Microsc. Soc. 17, 330-353.

Leidy J. 1879. Freshwater Rhizopods of North America. Rep. U.S. geol. Surv. 12.

Penard E.C. 1902. Faune Rhizopodique du la bassin de Leman. Henry Kuendig, Geneva.

West K. 1901. On some British freshwater Rhizopods and Heliozoa. J. Linn. Soc. (Zool.). 28, 309-342.

Striate morphotype: flattened, oblong or rounded amoebae, with several nearly parallel dorsal folds.

Amoebae of striate morphotype (Fig. 24) always display regular, parallel, longitudinal dorsal folds during locomotion. Less regular wrinkles may also appear but are never numerous, and longitudinal ridges always dominate during locomotion. Only one representative species has been found in soil to date. However, it seems likely that all species of this morphotype are able to grow in wet soil habitats.

Striate morphotype: list of freshwater species

Thecamoeba quadrilineata (Carter 1856) Page 1977
Thecamoeba striata (Penrad 1890) Page 1977
Thecamoeba similis (Greef 1891) Page 1977
Thecamoeba sparolata Fishbeck and Bovee 1993 (as *Striamoeba)*

Striate *Thecamoeba* are among the few species for which the identification is based mainly on LM features. Most important are the nuclear structure, size and outline of the locomotive form, position and typical number of dorsal folds. *T. similis* may resemble rugose species, but it always has prominent longitudinal ridges during locomotion.

Some references for identification of striate species:

Fishbeck D.W. and Bovee E.C. 1993. Two new amoebae, *Striamoeba sparolata* n. sp., and *Flamella tiara* n. sp., from freshwater. Ohio J. Sci. 93, 134-139.

Page F.C. 1977. The genus *Thecamoeba* (Protozoa, Gymnamoebia). Species distinction, locomotive morphology and protozoan prey. J. Nat. Hist. 11, 25-63.

Page F.C. and Blakey S.M. 1979. Cell surface structure as a taxonomic character in the Thecamoebidae (Protozoa, Gymnamoebia). Zool. J. Linn. Soc. 66, 113-135.

Rugose morphotype: flattened, oblong or rounded amoebae, with numerous irregular dorsal wrinkles.

Amoebae of rugose morphotype (Fig. 25) always have many irregular dorso-lateral wrinkles. Dorsal folds, if present, are irregular and not parallel in arrangement. In contrast with most other amoebae, all species of this morphotype have only ever been isolated from soil habitats and so may be considered to be 'real' soil amoebae. There are many poorly described verrucosid amoebae in older literature, thus it is likely that only a minor part of the diversity of these amoebae has been properly described.

Rugose morphotype: list of soil/freshwater species

Thecamoeba sphaeronucleolus (Greeff 1891) Page 1977
Thecamoeba verrucosa (Ehrenberg 1838) Glaeser 1912
Thecamoeba similis (Greeff 1891) Page 1977
Thecamoeba terricola (Greeff 1866) Page 1977

Rugose *Thecamoeba* can also be identified from LM observations. As with cells of striate morphotype, the

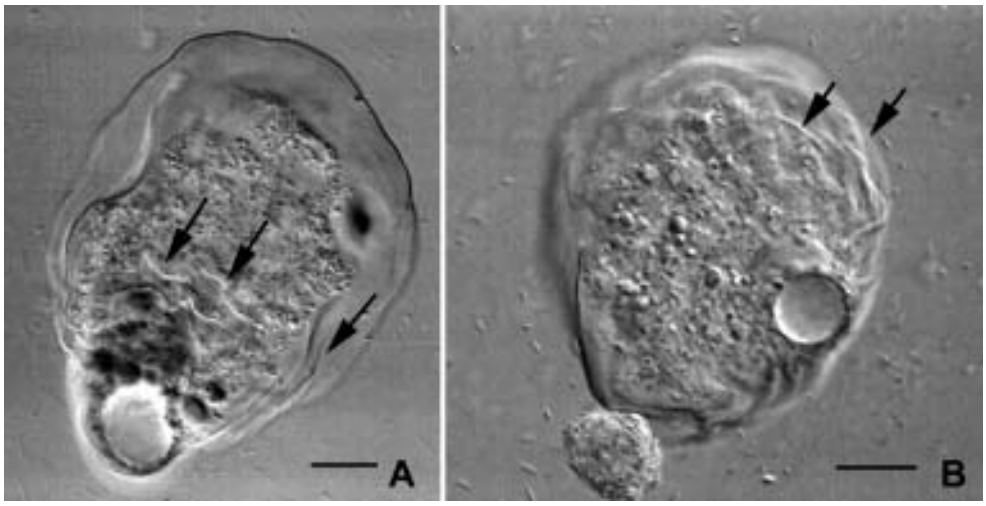

Fig. 25. Representatives of amoeba of rugose morphotype. a - *Thecamoeba sphaeronucleolus* CCAP 1583/3 (DIC); b - *Thecamoeba similis* CCAP 1583/8 (DIC). Irregular longitudinal and transverse ridges are arrowed. Scale bars: 10 μm.

most important characters are nuclear structure, size and outline of the locomotive form. Some rugose *Thecamoeba* move very slowly, and it may take a long period of time to observe the locomotive form.

Some references for identification of rugose species:

Bovee E.C. 1960. Studies of feeding behaviour of amebas. I. Ingestion of thecate rhizopods and flagellates by verrucosid amebas, particularly *Thecamoeba sphaeronucleolus*. J. Protozool. 7, 55-60.

Houssay D. and Prenant M. 1970. *Thecamoeba sphaeronucleolus*. Donnees physiologiques et etude morphologique en microscopie optique et electronique. Arch. Protistenk. 112, 228-51.

Mattes O. 1924. Ueber Lebensweise. Morphologie und Physiologie von *Amoeba sphaeronucleolus* Greef und *Amoeba terricola* Greeff. Arch. Protistenk. 47, 386-412.

Page F.C. 1977. The genus *Thecamoeba* (Protozoa, Gymnamoebia). Species distinction, locomotive morphology and protozoan prey. J. Nat. Hist. 11, 25-63.

Page F.C. and Blakey S.M. 1979. Cell surface structure as a taxonomic character in the Thecamoebidae (Protozoa, Gymnamoebia). Zool. J. Linn. Soc. 66, 113-135.

Penard E.C. 1902. Faune Rhizopodique du bassin du Leman. Geneve: Henry Kundig.

Penard E.C. 1905. Observations sur les amibes a pellicule. Arch. Protistenk. 6, 175-206.

Lingulate morphotype: flattened, oblong, smooth amoebae, without any folds or wrinkles.

Amoebae of lingulate morphotype (Fig. 26) never have dorsal folds or wrinkles but one or two small lateral wrinkles may appear temporarily. They are always flattened in cross-section. In contrast with species of lanceolate morphotype they do not display lateral flatness of the cell.

Lingulate morphotype: list of freshwater/soil species

Dermamoeba granifera (Greeff 1866) Page et Blakey 1979

Dermamoeba minor (Pussard, Allabovette et Pons 1979) Page 1988

Sappinia diploidea (Hartmann et Naegler 1908) Alexeieff 1912

Platyamoeba stenopodia Page 1969

Platyamoeba schaefferi Singh et Hanumaiah 1979

Some references for identification of lingulate species:

Goodfellow L.P., Belcher J.H. and Page F.C. 1974. A light- and electron-microscopical study of *Sappinia diploidea*, a sexual amoeba. Protistologica. 10, 207-216.

Page F.C. 1969. *Platyamoeba stenopodia* n. g. n. sp., a freshwater amoeba. J. Protozool. 16, 437-441.

Page F.C. 1977. The genus *Thecamoeba* (Protozoa, Gymnamoebia). Species distinctions, locomotive morphology and protozoan prey. J. Nat. Hist. 11, 25-63.

Page F.C.and Blakey S.M. 1979. Cell surface structure as a taxonomic character in the Thecamoebidae (Protozoa, Gymnamoebia). Zool. J. Linn. Soc. 66, 113-135.

Pussard M., Alabouvette C. and Pons R. 1979. Etude preliminaire d'une amibe mycophage *Thecamoeba granifera* ssp. *minor* (Thecamoebidae, Amoebida). Protistologica. 15, 139-149.

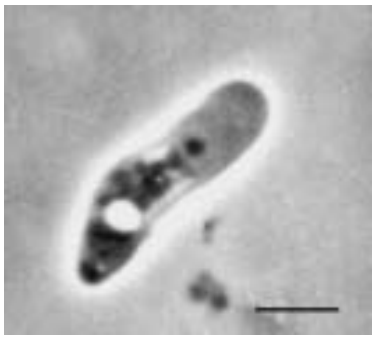

Fig. 26. Representative of amoeba of lingulate morphotype. *Platyamoeba stenopodia* (Valamo Island, North-Western Russia) (phase contrast). Scale bar: 10 μm.

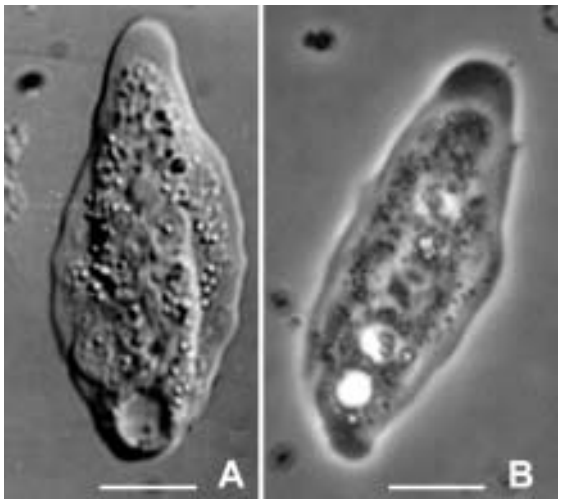

Fig. 27. Representatives of amoeba of lanceolate morphotype. a - *Paradermamoeba valamo* (Valamo Island, North-Western Russia) (DIC); b - *Paradermamoeba valamo* (Geneva, Switzerland) (phase contrast). Scale bars: 10 μm.

Lanceolate morphotype: lancet-like cells, with lateral flatness, amoebae have neither folds nor wrinkles.

Amoebae of lanceolate morphotype (Fig. 27) never have dorsal or lateral folds or wrinkles. However, they do display a distinctive lateral flatness, which makes them appear "hat-like" in cross-section. Both known species have only been isolated from, but may not be restricted to, freshwater habitats.

Lanceolate morphotype: list of freshwater species

Paradermamoeba valamo Smirnov et Goodkov 1993

Paradermamoeba levis Smirnov et Goodkov 1994

Some references for identification of lanceolate species:

Smirnov A.V. and Goodkov A.V. 1993. *Paradermamoeba valamo* n. g, n. sp. (Gymnamoebia, Thecamoebidae) - freshwater amoeba from the bottom sediments. Zool. Zh. 72, 5-11 (in Russian with English summary).

Smirnov A.V. and Goodkov A.V. 1994. Freshwater Gymnamoebae with a new type of surface structure - *Paradermamoeba valamo* and *P. levis* n. sp. (Thecamoebidae), and notes on the diagnosis of the family. Acta Protozool. 33, 109-115.

Fan-shaped morphotype: flattened, fan-shaped, semi-circular or spatulate amoebae, without subpseudopodia. All cells of this morphotype always have a wide frontal hyaline zone, which is normally smooth or forms small dorsal lobes, waves or/and temporary wrinkles.

Amoebae of fan-shaped morphotype (Fig. 28) are flattened and their general outlines are variations and derivates of a semi-circle. The frontal hyaloplasm may have lobes or a few wrinkles but never produces subpseudopodia and the edge is always smooth or slightly waved, but never lobate. Amoebae of this morphotype never have adhesive uroidal structures.

Fan-shaped morphotype: list of freshwater/soil species

Vannella simplex (Wohlfarth-Bottermann 1960) Bovee 1965
Vannella platypodia (Glaeser 1912) Page 1976
Vannella lata Page 1988
Vannella cirrifera (Frenzel 1892) Page 1988,
Vannella miroides Bovee 1965
Vannella persistens Smirnov et Brown 2000
Platyamoeba placida (Page 1968) Page 1969
Pessonella marginata Pussard 1973

Most of the species listed above normally have a smooth frontal hyaline area but, depending on the culture conditions, some species may form a few irregular ridges or folds on the dorsal surface. *Pessonella marginata* normally forms many lobes on the frontal hyaloplasm and (in culture) some species of *Vannella* may temporarily do the same. For generic identification EM investigation of the cell coat (preferably using chromium-shadowed preparations) is obligatory, whilst species identification is based mostly on LM features and is often a very difficult task. Species of this morphotype are amongst the most common in all habitats and it is likely that many remain undescribed. Therefore, the probability of isolating a new species is high.

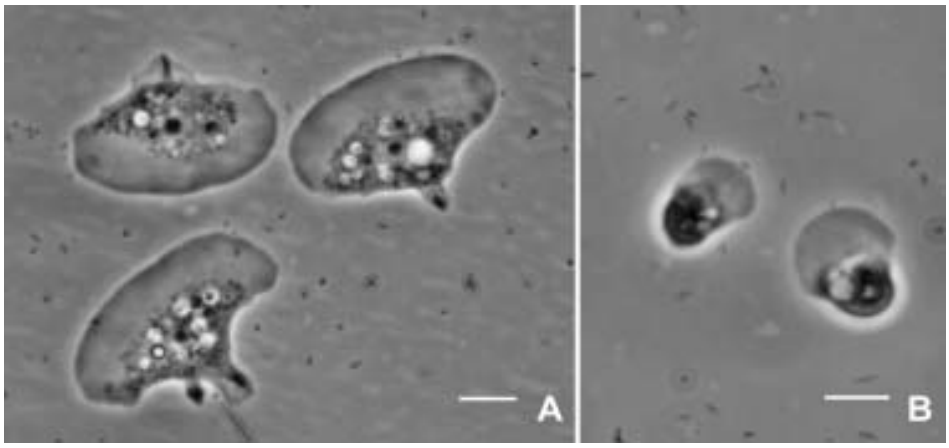

Fig. 28. Representatives of amoeba of fan-shaped morphotype. a - *Vannella simplex* (Geneva, Switzerland) (phase contrast); b - *Vannella platypodia* (Geneva, Switzerland) (phase contrast). Scale bars: 10 μm.

Some references for identification of fan-shaped species:

Ariza C., Guevara D.C. Libeda J.M. and Cutillas C. 1989. Description of four species of the genus *Vannella* isolated from freshwater. Microbiologia. 5, 25-33.

Bovee E.C. 1965. An emendation of the amoeba genus *Flabellula* and a descriprtion of *Vannella* gen. nov. Trans. Am. Microsc. Soc. 84, 217-227.

Pussard M. 1973. Description d'une amibe de type flabellulien: *Pessonella marginata* n. g. n. sp. (Mayorellidae, Amoebaea). Protistologica. 9, 175-185.

Smirnov A.V. and Brown S. 2000. First isolation of a cyst-forming *Vannella* species, from soil - *Vannella persistens* n. sp. (Gymnamoebia, Vannellidae). Protistology. 1, 120-123.

Wohlfarth-Botterman K. 1960. Protistenstudien. X. Licht- und electronenmikroskopische Untersuchungen an der amobe *Hyalodiscus simplex* n. sp. Protoplasma. 52, 58-107.

Mayorellian morphotype: elongate or irregularly triangular cells, with distinct narrow antero-lateral border of hyaloplasm. Cells form blunt conical or mamilliform subpseudopodia. Dactylopodia, if present (rarely), are small.

Amoebae of mayorellian morphotype (Fig. 29) always produce characteristic conical or mamilliform pseudopodia, but some may form a few dactylopodia. They may be distinguished from typical amoebae of dactylopodial morphotype (see below) by the presence of at least some conical or mamilliform pseudopodia and by their much less developed frontal hyaline zone. During locomotion amoebae of mayorellian morphotype are less flattened and many of them have a tendency to form longitudinal dorsal ridges. Most species adopt an oblong shape during rapid locomotion and move "as a whole", without distinct pseudopodia and subpseudopodia.

Mayorellian morphotype: list of freshwater/soil species

Mayorella viridis (Leidy 1874) Harnisch 1968
Mayorella augusta Schaeffer 1926
Mayorella bigemma (Schaeffer 1918) Schaeffer 1926
Mayorella cantabrigiensis Page 1972
Mayorella penardi Page 1972
Mayorella vespertilioides Page 1983

It is clear that the diversity of amoebae of this morphotype has not been fully explored. Only accurately described species, for which the ultrastructure of the cell coat is known, are listed above. However, Page (1991) lists an additional 15 poorly described species and four putative genera of *Mayorella*-like amoebae. These must be checked before an isolate is described as a new species.

Some references for identification of mayorellian species:

Bovee E.C. 1970. The lobose amebas. I. A key to the suborder Conopodina Bovee and Jahn, 1966 and descriptions of thirteen new and little known *Mayorella* species. Arch. Protistenk. 112, 178-227.

Cann J.P. 1981. An ultrastructural study of *Mayorella viridis* (Leidy) (Amoebida: Paramoebidae): a rhizopod containing zoochlorellae. Arch. Protistenk. 124, 235-238.

Page F.C. 1972. A study of two *Mayorella* species and proposed union of the families Mayorellidae and

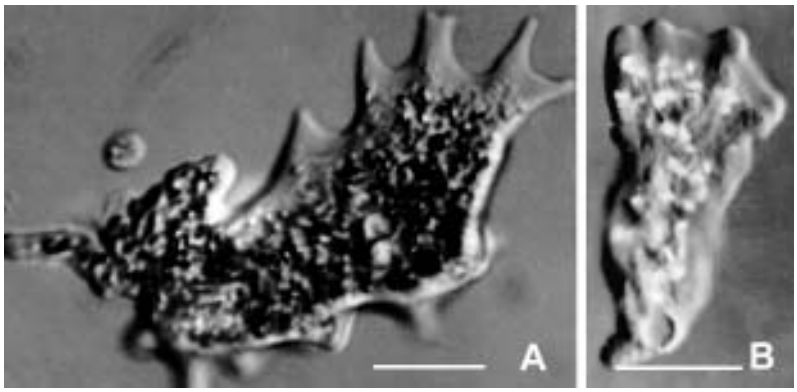

Fig. 29. Representatives of amoeba of mayorellian morphotype. a - *Mayorella cantabrigiensis* (Valamo Island, North-Western Russia) (DIC); b - *Mayorella vespertilioides* (Valamo Island, North-Western Russia) (DIC).

Paramoebidae (Rhizopodea, Amoebida). Arch. Protistenk. 114, 404-420.

Page F.C. 1981. *Mayorella* Schaeffer, 1926, and *Hollandella* n. g. (Gymnamoebia), distinguished by surface structures, with comparisons of three species. Protistologica. 17, 543-562.

Page F.C. 1983. Three freshwater species of *Mayorella* (Amoebida) with cuticle. Arch. Protistenk. 127, 201-221.

Siemensma F.J. 1987. De nederlandse naaktamoeben (Rhizopoda, Gymnamoebia). Hoogwoud. Koninklijke Nederlandse Natuurhistorische Vereniging.

Dactylopodial morphotype: cells usually irregularly triangular, with distinct wide anterior hyaline border or frontal hyaline zone. They form dactylopodia of different length, mostly from the frontal hyaloplasm. Conical and mammiliform subpseudopodia are very rare, and may be formed only temporarily.

Amoebae of dactylopodial morphotype (Fig. 30) produce characteristic finger-shaped hyaline subpseudopodia (dactylopodia) and only rarely (and temporarily) form conical or mamilliform pseudopodia. In locomotion they have an expanded frontal hyaline zone. Large specimens have a strong tendency to produce dactylopodia from the dorsal surface of the cell also.

Dactylopodial morphotype: list of freshwater/soil species

Korotnevella bulla (Schaeffer 1926) Goodkov 1988
Korotnevella stella (Schaeffer 1926) Goodkov 1988
Korotnevella diskophora Smirnov 1999

The generic name *Korotnevella* is applied here instead of *Dactylamoeba*, which was used by F.C. Page (1988, 1991). The latter was proven to be taxonomically incorrect and should not be used (Goodkov, 1988; see also Smirnov, 1997). For identification, EM analysis of the scales covering the cell surface of these amoebae is obligatory. Either chromium-shadowing (preferably) or TEM are appropriate. The probability of finding new species of this morphotype is very high, but not only the shape of the scales, but all LM and EM features must be compared with published species descriptions. Size polymorphism may be very high in some species, so you should not rely on cell size even for an initial classification.

Note that some species of the genus *Vexillifera* that we list under acanthopodial morphotype may resemble amoebae of dactylopodial morphotype. In case of doubt, especially if you find small (15-30 μm) amoeba with long, thin subpseudopodia, consider the acanthopodial morphotype as well (below).

Some references for identification of dactylopodial species:

Goodkov A.V. 1988. *Korotnevella* nom. n. (Gymnamoebia, Paramoebidae) - a new generic name for *Mayorella*-like amoebae with cell surface bearing scales. Zool. Zh. 67, 1728-1730.

Page F.C. 1981. *Mayorella* Schaeffer, 1926, and *Hollandella* n. g. (Gymnamoebia), distinguished by surface structures, with comparisons of three species. Protistologica. 17, 543-562.

Pennick N. and Goodfellow L. 1975. Some observations on the cell surface structures of species of *Mayorella* and *Paramoeba*. Arch. Protistenk. 117, 41-46.

Smirnov A.V. 1999a. *Korotnevella diskophora* n. sp. (Gymnamoebia, Paramoebidae) - small freshwater amoeba with peculiar scales. Protistology. 1, 30-33.

Acanthopodial morphotype: cells are usually irregularly triangular, with short, sharp, tapering, pointed, sometimes furcate, hyaline subpseudopodia,

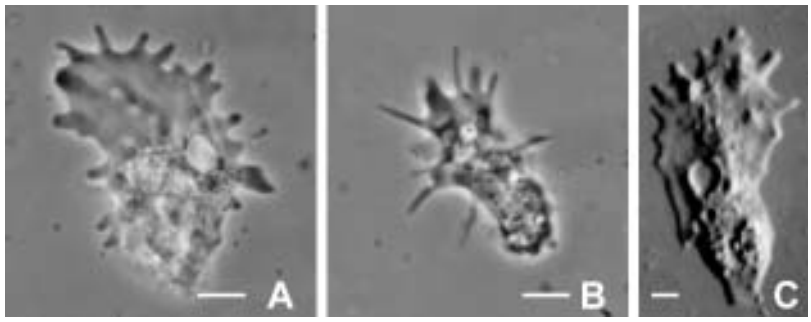

Fig. 30. Representatives of amoeba of dactylopodial morphotype. a-b - *Korotnevella stella* CCAP 1547/6 (phase contrast); c - *Korotnevella bulla* (Valamo Island, North-Western Russia) (DIC). Scale bars: 10 μm.

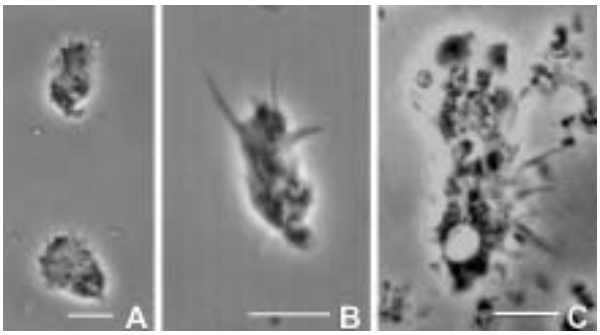

Fig. 31. Representatives of amoeba of acanthopodial morphotype. a-b - *Vexillifera bacillipedes* CCAP 1590/1 (phase contrast); c - *Acanthamoeba sp.* (Valamo Island, North-Western Russia) (phase contrast). Scale bars: 10 μm.

formed both from the anterior hyaloplasm and from the lateral and dorsal parts of the cell.

Acanthopodial morphotype (Fig. 31) unifies all amoebae with short, spineolate (but not filose) subpseudopodia. Many species, differing greatly in cytology and biology, belong to this morphotype. They can be sub-divided into groups that are easily distinguishable but it may be difficult to assign an amoeba to a group if there are only a few specimens in a mixed culture.

The first group is represented by members of the genus *Vexillifera*. These amoebae are relatively easy to recognise because their subpseudopodia are never branched and never noticeably pointed. They produce subpseudopodia mostly from the frontal hyaline area. They do not form cysts. They are poorly understood despite the large volume of literature relating to this genus; many species descriptions are inadequate. The genus is not homogeneous in cell coat structure (Page, 1988).

The second group includes the genera *Acanthamoeba* and *Protacanthamoeba*. Amoebae assigned to these genera usually have branched subpseudopodia (acanthopodia) which may have pointed tips. All members of these genera readily form cysts. *Protacanthamoeba* species do not have cyst pores, in contrast with *Acanthamoeba* species. Physiological and biochemical tests are obligatory if an *Acanthamoeba* isolate is to be assigned to a species, and speciation within this genus is controversial. The acanthamoebae are very common in soil habitats.

The third group includes the poorly defined genera *Echinamoeba*, *Filamoeba* and *Comandonia*. Members of these genera produce many very fine, spine-like subpseudopodia and may even resemble filose amoebae. They produce characteristic cysts. All these genera remain poorly characterized.

Acanthopodial morphotype: list of fresh-water/soil species and genera

Vexilifera bacillipedes Page 1969
Vexillifera granatensis Mascaro, Osuna et Mascaro 1986
Vexillifera lemani Page 1976
Vexillifera expectata Dykova, Lom, Machakova et Peckova 1998
genus ***Acanthamoeba*** Sawyer et Griffin 1975
genus ***Protacanthamoeba*** Page 1981
genus ***Filamoeba*** Page 1967
genus ***Echinamoeba*** Page 1975
Comandonia operculata Pernin et Pussard 1979

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The genus *Vexillifera*:

Bovee E.C. 1951. A proposed dichotomy of the genus *Vexillifera* (Schaeffer, 1926) on types of waving pseudopods. Proc. Am. Soc. Protozool. 2, 4-5.

Bovee E.C. 1985. The lobose amebas III. Description of nine new conopodous amoebae of the genus *Vexillifera* Schaeffer, 1926 emend. Bovee, 1951, 1970 with comments on the genus. Arch. Protistenk. 129, 101-118.

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encysting amoebae isolated from organs of freshwater fish. Folia Parasitol. 45, 17-26.

Page F.C. 1979. The fine structure of *Vexillifera bacillipedes* (Amoebida, Paramoebidae). Arch. Protistenk. 122, 9-19.

The genera *Acanthamoeba* and *Protacanthamoeba*:

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Page F.C. 1967. Re-definition of the genus *Acanthamoeba* with descriptions of three species. J. Protozool. 14, 709-724.

Page F.C. 1981. A light- and electron-microscopical study of *Protacanthamoeba caledonica* n. sp., type-species of *Protacanthamoeba* n. g. (Amoebida, Acanthamoebidae). J. Protozool. 28, 70-78.

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Pussard M. 1966. Le genre *Acanthamoeba* Volkonsky, 1931 (Hartmannellidae, Amoebida). Protistologica. 2, 71-93.

Pussard M. 1972. Comparison morphologique de 4 souches d' *Acanthamoeba* du groupe astronyxis-comandoni. J. Protozool. 19, 557-563.

Pussard M. and Pons R. 1977. Morphologie de la paroi kystique et taxonomie du genre *Acanthamoeba* (Protozoa, Amoebida). Protistologica. 13, 557-598.

Pussard M. and Pons R. 1978. Etude de la 'centrosphere' d' *Acanthamoeba echinulata* Pussard et Pons, 1978. Protistologica. 14, 247-251.

Ray D.L. and Hayers R.E. 1954. *Hartmannella astronyxis*; a new species of free-living amoeba. Cytology and life cycle. J. Morphol. 95, 159-188.

Sawyer T.K. and Griffin J.L. 1971. *Acanthamoeba comandoni* and *A. astronyxis:* taxonomic characteristics of mitotic nuclei, "centrosomes" and cysts. J. Protozool. 18, 382-388.

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Willaert E. 1976. Etude immunotaxonomique des genres *Naegleria* et *Acanthamoeba.* (Protozoa, Amoebida). Acta Zool. Path. Antwerp. 65, 1-239.

Willaert E., Stevens A.R. and Tyndall R.L. 1978. *Acanthamoeba royreba* sp. n. from a human tumor cell culture. J. Protozool. 25, 1-14.

Willaert E., Stevens A.R. and Tyndall R.L. 1978. Identification of *Acantamoeba culbertsoni* from cultured tumor cells. Protistologica. 14, 319-336.

The genera *Filamoeba, Echinamoeba* and *Comandonia*:

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Pernin M. and Pussard M. 1979. Etude en microscopie photonique et electronique d'une amibe voisine du genre *Acanthamoeba*: *Comandonia operculata* n. gen., n. sp. (Amoebida, Acanthamoebida). Protistologica. 15, 87-102.

Branched morphotype: flattened, branched amoeboid organisms with anastomosing lobose pseudopodia (loboreticulopodia) and poorly coordinated movement. Pseudopodia never contain granules and never form an expanded network. Cytoplasmic flows never distinct or shuttle-like.

Branched morphotype (Fig. 31) includes amoeboid organisms with an extensive, branched body, but without the fast, axial cytoplasmic streaming which is charac-teristic of the large plasmodia of slime molds from the subclass Myxogastria. Amoebae of this morphotype never produce an anastomosing network of pseudopodia nor do they form filopodia. However, the reader should be aware that *Leptomyxa reticulata*, for example, contracts very quickly when exposed to the light, and the consequent trailing remnants of the cytoplasm may resemble short, spineolate pseudopodia in photographs, as illustrated above. Apparently, all amoebae of this morphotype, if disturbed, may adopt a monotactic-like form for a short time (in contrast with the plasmodium of slime molds), but (with the exception of *Rhizamoeba* spp.) they do not maintain this form during locomotion.

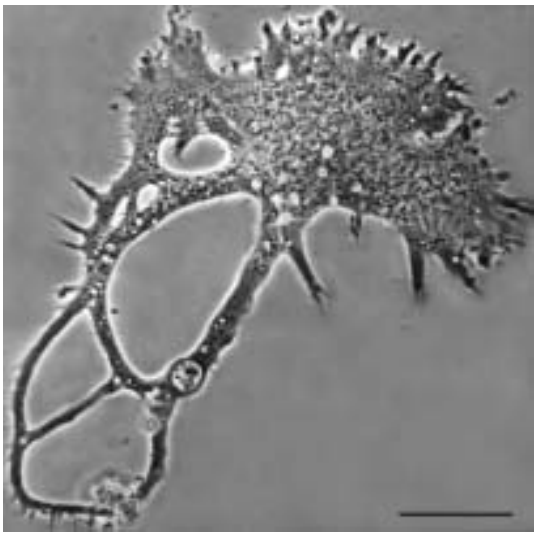

Fig. 32. Representative of amoeba of branched morphotype. Large specimen of *Leptomyxa reticulata* (Valamo Island, North-Western Russia), slowly contracting under the light of the microscope lamp (phase contrast). Scale bar: 1 mm.

Branched morphotype: list of freshwater/soil species

Rhizamoeba australiensis (Chakraborty et Pussard 1985) Page 1988
Rhizamoeba flabellata (Goodey 1914) Cann 1984
Leptomyxa reticulata Goodey 1914
Leptomyxa fragilis (Penard 1904) Siemensma 1987
Gephyramoeba delicatula Goodey 1914

All these species are described best in Page's key (1991). Bovee (1985) does not include freshwater *Rhizamoeba* (as they where described more recently) and *Leptomyxa* and *Gephyramoeba* are listed in the class Acarpomyxea Page, 1976 (which was later revised and rejected by Page and Blanton, 1985). Note that in Bovee (1985) there is an error in the figure legend; Fig. 5 on page 213 represents *Gephyramoeba delicatula* but is called "*Gephyramoeba reticulata*" (this species does not exist). In the same book there is an excellent section dedicated to Mycetozoea by Hutner and Olive (1985), which contains numerous illustrations including the plasmodia of Myxogastria. For the latter group, Olive (1975) is recommended as well.

Some references for identification of branched species:

Cann J.P. 1984. The ultrastructure of *Rhizamoeba flabellata* (Goodey) comb. nov. and *Leptomyxa reticulata* Goodey (Acarpomyxea: Leptomyxida). Arch. Protistenk. 128, 13-23.

Chakraborty S. and Pussrad M. 1985. *Ripidomyxa australiensis* nov. gen. nov. sp., a mycophagous amoeba from Australian soils. Protistologica. 21, 133-140.

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