A New Key to
Freshwater and Soil Gymnamoebae
with instructions for culture

by

F. C. Page
Institute of Terrestrial Ecology

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INTRODUCTION

Despite familiar nomenclature, the taxonomy of gymnamoebae has changed enough in the past decade to make the present key more a successor to than a revision of *An Illustrated Key to Freshwater and Soil Amoebae*. In that time more research reports with taxonomic significance have appeared than in much longer earlier periods.

Light microscopy remains a basic tool, but electron microscopy and non-morphological approaches have radically changed both taxonomy and possible means of identification. Ultrastructural results have affected all levels from class down to genus and in some groups to species. The application of non-morphological methods has unfortunately been much less even. Biochemical and serological procedures have been applied especially to *Naegleria* and *Acanthamoeba* because of the potential pathogenicity of some strains and species of those genera, a motivation tending to obscure their general ecological and scientific importance. Non-morphological methods have also been used to a lesser degree, combined with morphological studies, for some large amoebae of the family Amoebidae, whose size and rôle in cell biological investigations have facilitated use of additional techniques such as nuclear transplantation.

As a consequence, new evidence of relationships has emerged. Electron microscopy has demonstrated the relationship between vahlkampfiid amoebae and acrasid 'slime moulds', which are essentially spore-forming vahlkampfiids; these and related groups are now united in the class Heterolobosea. Most other naked, non-spore-forming, lobose amoebae remain in the subclass Gymnamoebia of the class Lobosea. However, despite dispersal of the former, broader Gymnamoebia amongst three classes (including the Caryoblastea), a common name is still needed for all naked, non-sporulating, lobose amoebae, and that broad sense is retained for the word *gymnamoebae*.

Systematic changes at levels from family upwards have been incorporated into a revised classification of the phylum Rhizopoda, with special attention to naked amoebae, lobose and otherwise (Page, 1987c).

Of greater importance for identification is the firmer basis for familial and particularly generic diagnoses, derived from a correlation of light and electron microscopy. The genus now stands out as the best defined taxonomic unit amongst gymnamoebae, clear support for the claim of Griffiths (1985) that 'the genus represents a significant level of organization in the protozoa'.

Unfortunately, the same degree of clarity has not been achieved at the species level. Some species, of which *Naegleria gruberi* is a notable example, prove almost intolerably heterogeneous on detailed study, while some species distinctions may seem fuzzy. Only a few species have a cell surface structure that sets them apart from others of the same genus. Biochemical and serological work, though it has contributed much to the understanding of intrageneric relationships and diversity, has not settled the question of 'where to draw the line' between species, so that the usefulness and usableness of 'species' are seriously questioned (A. J. Griffiths, personal communication). In the present work the species is regarded, without prejudice on the question of its biological reality, as *a subgroup of a genus sharing characters which make it identifiable and indicate that its members (strains, populations) are significantly more similar to each other than to other subgroups*.

The taxonomic scope of the present work is almost the same as that of the earlier key. The Acrasida have been added because of their close relationship and similarity to other Heterolobosea. The Cochliopodiidae are included, but only those species which are most similar to gymnamoebae.

A word on the practical consequences of recent advances: Despite use of the electron microscope to establish the taxonomic basis, it is almost always possible to make an accurate generic identification with the light microscope, since ultrastructural characters have been correlated with light microscopic ones. However, the electron microscope is useful in confirming some generic identifications and even a few specific identifications, e.g. of *Amoeba leningradensis* or *Pseudothecamoeba proteoides*, though not essential in those cases.

Ecologically also, the past decade has been marked by a great increase in the number and scope of investigations involving gymnamoebae. Again, many have been stimulated by the potential pathogenicity of some *Naegleria* and *Acanthamoeba*. Some reports concentrating on those genera are cited in the respective sections. However, more general ecological studies have also appeared. The sample of recent work in the bibliography includes publications by Baldock & Baker (1980), Bamforth (1980), Bamforth & Stout (1983), Brown *et al.* (1982), Kyle & Noblet (1985, 1986), Old & Chakraborty (1986), Pussard & Delay (1985), Rogerson (1981, 1982), and Rogerson & Berger (1981a, 1981b).

The methods section is more extensive than in the 1976 key, including introductions to axenic culture and to light and electron microscopic preparation. Access to biochemical and serological methods for identification is provided by the references under *Naegleria* and *Acanthamoeba*. The most important category of methods deals with isolation and culture, because both enrichment and clonal isolation remain essential to any serious taxonomic study of the gymnamoebae in the natural environment.

The bibliography, though lengthy, is far from comprehensive. Older works are included only where essential; older references can be found in the recent publications.

A companion key to gymnamoebae in salt water is *Marine Gymnamoebae* (Page, 1983b).

POTENTIAL PATHOGENS

Free-living amoebae pathogenic to human beings and laboratory animals belong to two common but very different genera, *Naegleria* and *Acanthamoeba*. This publication is, however, a general introduction to the identification of the entire range of gymnamoebae in freshwater and terrestrial habitats, not a medical handbook. Furthermore, without minimising the human cost of the sporadic cases of pathogenicity, it must be emphasised that both genera are of far wider ecological and scientific importance.

At the same time, the ecological emphasis of this publication impinges on the public health interest because epidemiology is medical ecology, because anyone investigating gymnamoebae in nature is bound to encounter *Naegleria* and *Acanthamoeba*, and because the literature indicates that medical workers have referred to the earlier key. Initial access to some of the many reports relevant to public health is therefore provided by references. Only a brief summary can be presented here.

Naegleria fowleri, the cause of the rapidly fatal acute primary amoebic meningoencephalitis, has been proved responsible for some human fatalities. It occurs in artificially heated waters such as swimming pools and effluent from industrial cooling processes, as well as in some naturally heated waters. *N. australiensis* is pathogenic to laboratory animals but has not been reported from any human cases.

The taxonomic distribution of pathogenicity amongst species of *Acanthamoeba* is wider, and species distinctions are less precise and less agreed than in *Naegleria*. *A. culbertsoni* is considered the principal but not the only pathogen of the central nervous system. The majority of demonstrated human infections by *Acanthamoeba* have been lesions of the cornea, sometimes resulting in loss of the eye, in which species other than *A. culbertsoni* have been implicated.

It must be recognised that *Acanthamoeba* is very widely distributed in the biosphere, being the most frequently isolated free-living amoeba, possibly the most common free-living protozöon. The worker isolating at 18-22°C, as suggested here, is unlikely to enrich for either *N. fowleri* or *N. australiensis* but is certain to have *Acanthamoeba* spp. frequently in collected material, mixed cultures, and clonal cultures of larger amoebae with unidentified eukaryotic companions. *Acanthamoeba* is also certain to be present in many incompletely purified cultures of other protozoa, algae, and micrometazoans.

No attempt can be made here to give special instructions on handling of potentially pathogenic free-living amoebae, for which a variety of containment procedures have been prescribed. Recommendations for handling '*Acanthamoeba* spp (especially *A. culbertsoni*)' and '*Naegleria* spp (especially *fowleri*)' are included in *Categorisation of pathogens according to hazard and categories of containment* (Advisory Committee on Dangerous Pathogens, 1984), which should be consulted by British workers. Enquiries should be addressed to the Health and Safety Executive at any area office or to the public enquiry point, St. Hugh's House, Trinity Road, Bootle, Merseyside L20 3QY, telephone 051 951 4381. Workers in other countries should consult their national guidelines. Specialists do not all agree on the degree of containment advisable, particularly for strains and species whose pathogenicity is doubtful or not demonstrated.

No report of infection of laboratory staff by *Naegleria* or *Acanthamoeba* is known.

TAXONOMIC INTRODUCTION

Characters for Identification

Since the 1976 key, the number and the reliability of taxonomic characters have increased. In this section the emphasis is practical, using the more accessible characters as far as possible. Taxonomic conclusions are often based on correlation of these more accessible characters with others derived from more difficult techniques. The points at which the serious investigator must employ such methods as electrophoresis for species differentiation (two genera) and where electron microscopy may be desirable for a difficult generic differentiation have been kept to a minimum. The worker without access to such methods should therefore not be discouraged from attempting identifications.

Locomotive form and behaviour. The observation of amoebae moving on a substratum under favourable conditions (cf. Methods of Observation and Study) is the first step and will often suggest a familial and even a generic identification immediately.

A convenient dichotomy to describe general forms is *cylindrical* or subcylindrical versus *compressed* or flattened. The cylindrical form may be branched or *polypodial*, like *Amoeba proteus*, or unbranched or *monopodial*. The distinction is between amoebae advancing in a constant direction; monopodial amoebae may put out a lateral pseudopodium when changing direction. Only consistently monopodial, cylindrical amoebae should be described as 'limax'. (A terminology which is more related to the fundamental differences and distinguishes two functionally different monopodial forms was proposed by Grebecki & Grebecka (1978).)

Whatever the form, the cytoplasm can be divided into *granuloplasm*, the main mass containing visible inclusions, and *hyaloplasm*, more or less clear, which may occupy the anterior half or third, form only a crescent-shaped cap, or even be temporarily absent.

Both limax and flattened amoebae can be divided into those with *eruptive* and those with more steadily flowing locomotive activity. Eruptive limax amoebae commonly advance by means of hemispherical, hyaline bulges at one side or other of the anterior end. In markedly eruptive amoebae of any shape, the hyaloplasm may spill back along one side. The steadier pattern of locomotion also often involves a gentle anterolateral bulging, since it actually proceeds by a succession of pseudopodia, each produced on its predecessor, but hyaloplasm is not reflected along the side. In general, eruptive limax amoebae tend to be thicker (with a lower length : breadth ratio) and to advance more rapidly than more steadily flowing ones, with more marked constrictions of the body.

The outline of an amoeba, seen from above or below, is characteristic. Differences include the *length : breadth* ratio, important in either limax or flattened amoebae, and the diverse shapes of flattened amoebae. Amongst such shapes, some may be more regular and symmetrical, others more irregular or ragged. The former are generally those moving steadily, the latter more eruptive. The reader should not expect that every amoeba of a described species will fit into one of the illustrated forms of that species.

The entire advancing, usually hyaline, anterior end of most amoebae is functionally a single lobose pseudopodium or succession of single pseudopodia, but many more or less compressed amoebae produce, usually from the hyaloplasm, narrow, even fine projections, *subpseudopodia*. Most have no demonstrated function, and they are not correctly called filopodia, no matter how fine. They may be blunt, digitiform or mamilliform, and non-furcate, like the *dactylopodia* of Paramoebidae; slender and non-furcate, either long, as in *Vexillifera*, or short, as in *Paraflabellula*; markedly tapering, blunt or finely tipped, seldom straight, and sometimes furcate, as the *acanthopodia* of the Acanthamoebidae; much finer but otherwise like acanthopodia, as in *Filamoeba*; or short, barely discernible, fine-tipped, sometimes furcate (sometimes called *echinopodia*), as in *Echinamoeba*. It should be noted that furcate subpseudopodia usually branch close to their bases.

Mention must be made of the short, fine projections produced from the underside of *Naegleria* and possibly other Heterolobosea by 'focal contacts' or 'adhesion points' and sometimes called filopodia. They attach during locomotion and appear to be the source of the adhesive uroidal

filaments on such amoebae but are not seen in normal observation until the mass of the amoeba has passed beyond them.

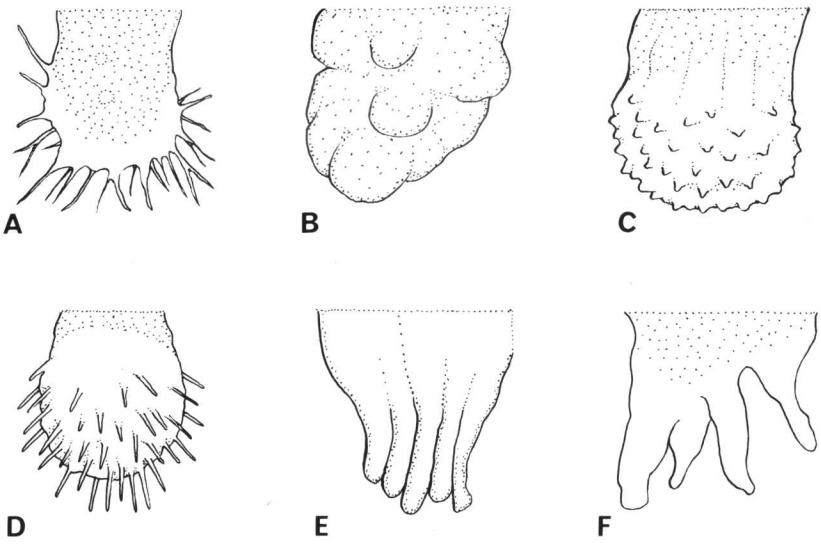

Fig. 1. Kinds of uroids. A, collopodium or mass of adhesive filaments produced by adhesion of posterior end to substratum. B, morulate; C, papillate; D, villous; E, plicate; and F, fasciculate uroids.

The posterior end or *uroid* may be a simple, smooth, round end or be morphologically differentiated. Adhesion to the substratum with subsequent drawing out of adherent points as the amoeba moves on may result in a few trailing adhesive *uroidal filaments* or a *collopodium*, a mass of such filaments (Fig. 1A). Many differentiations (Fig. 1B-F) are of non-adhesive origin, the products of internal processes associated with locomotion, even though such uroids may also be more or less adhesive. A rounded uroid made up of smaller knobs is *morulate.* A *papillate* uroid bears many small papillae, while a *villous* uroid, often bulbous, bears numerous fine, more or less straight, radiating villi, each perhaps 1 or 2 μm long. The surface of a *plicate* uroid is thrown into more or less parallel folds, and a *fasciculate* uroid (e.g., *Vexillifera bacillipedes, Polychaos fasciculatum*) trails remnants of pseudopodia or subpseudopodia.

Adhesive uroidal filaments appear to be produced only on amoebae with a very thin or no discernible glycocalyx, while the differentiations of non-adhesive origin are common on amoebae with thick surface coats.

Measurements. Lengths and breadths of 100 amoebae moving under conditions favouring normal and sustained locomotion are basic. The original measurements of bacteria-feeding amoebae reported here have usually been made on organisms from 6- or 7-day cultures, those of other amoebae from thriving but sometimes older cultures. Some variations occur in a species or even a single strain, the latter probably affected by the point on the growth curve or the vigour of locomotive activity. Some observations suggest differences between dimensions of members of a species in culture and those from the natural habitat. Nuclear and cyst diameters may be more constant.

Nuclear number Unless otherwise indicated, species in the key are uninucleate. Some have a greater tendency to have 1 or more supernumerary nuclei than others. One genus is normally binucleate, with a tendency to extra pairs. Several genera are multinucleate; at least 2 of these are distinguished from uninucleate relatives chiefly by nuclear number.

Nuclear structure. If the key does not specify a nuclear structure, the species has a *vesicular* nucleus (Raikov, 1982) with a central, more or less spherical nucleolus. Some vesicular nuclei have a nucleolus in 2 or more lobes (often but not always joined) in a parietal position, as in *Thecamoeba striata*. The other principal nuclear type is the *ovular* or *granular*, with many nucleoli, usually but

not always in a parietal layer, as in *Amoeba proteus*. Intermediate conditions exist, e.g., with a moderate number of rather small nucleolar bodies, as in *Deuteramoeba algonquinensis*.

The size and shape of the nucleus are important diagnostic characters in the genus *Amoeba*. At least in the Amoebidae, the nuclear diameter is more constant than length of the locomotive form, hence more useful for identification. When measuring amoebae of any strain, the diameters of 25 normally shaped nuclei should also be measured from the same preparation.

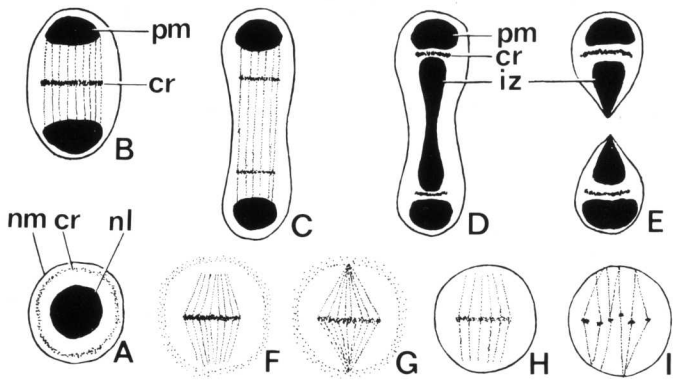

Fig. 2. Nuclear division figures. A, vesicular nucleus with central nucleolus. B, metaphase of promitosis. C, anaphase of promitosis without 'interzonal bodies'. D and E, late stages of promitosis with 'interzonal bodies' (conspicuous spindle remnants). F and G, metaphase of open mitosis (nuclear membrane disintegrated). H and I, metaphase of closed mitosis (nuclear membrane present). *cr,* chromosomal material; *iz,* interzonal body; *nl,* nucleolus; *nm,* nuclear membrane; *pm,* polar mass.

Mitotic pattern. This is an example of a character which may be of value in establishing relationships but is no longer needed for the majority of identifications. In the present work, the principal purpose of examining nuclear division figures is to distinguish vahlkampfiids from other limax amoebae and other eruptive amoebae. (Electron microscopic observations of mitochondria will achieve the same purpose.)

To assist in accurate identifications of limax amoebae (not *Saccamoeba, Trichamoeba*, or *Hydramoeba*, which are usually identifiable *in vivo*), permanent stained preparations should be made, though the *in vivo* technique of Pussard (1973) may also be useful. Such preparations are necessary, for example, to distinguish a *Vahlkampfia* which moves less eruptively than usual (e.g., *V. aberdonica*) from a *Hartmannella*, perhaps to distinguish an especially active *Hartmannella* (e.g., *H. cantabrigiensis*) from a *Vahlkampfia*.

The closed orthomitosis (Raikov, 1982) of vahlkampfiids (Fig. 2) has long been called *promitosis*, though that term no longer implies that it is primitive. Rather than disintegrating, the nucleolar material forms 2 polar masses with the spindle between them, and the nuclear membrane remains intact until separation of the daughter nuclei. A few other members of the Heterolobosea (e.g., *Stachyamoeba*) have closed orthomitosis without persistence and binary division of the nucleolus.

Most Gymnamoebia have various patterns of open or closed acentric mitosis (Fig. 2). Of those included in this publication, only the Acanthamoebidae and *Phreatamoeba* are known to have a centriole-like body.

Cytoplasmic inclusions. At this point we are considering only those discernible with the light microscope.

The most obvious inclusions, along with lipid globules, are crystals. Truncate bipyramidal crystals (triuret) are characteristics of many Amoebidae, *Saccamoeba, Cochliopodium*, and reportedly some other amoebae. Plate-like crystals, also triuret, are also found in some Amoebidae along with the bipyramids. 'Paired bodies', possibly both crystalline but often one appearing less solid, and various

irregular, probably imperfectly crystalline bodies occur in *Mayorella* and in some Amoebidae. Small birefringent inclusions of indistinct form are found in some other gymnamoebae.

Symbiotic bacteria are a constant feature of *Pelomyxa*, which in its most common form also contains mineral grains; the latter are probably the easiest way for the beginner to distinguish this genus. Intracellular bacteria, whether symbiotic or not, occur in *Saccamoeba* but are not necessary for identification.

Zoochlorellae are characteristic of *Mayorella viridis* and *Chaos zoochlorellae*. Both are very similar to species lacking zoochlorellae, and the possibility that they are only strains of those species needs further investigation.

After taxonomic use of the contractile vacuole had been suggested by Patterson (1981), replacement of an emptying vacuole by hyaloplasm in *Mayorella*, as described by Patterson, was found to be one further character for distinguishing that genus from *Dactylamoeba* without using the electron microscope (Fig. 33).

Floating forms. Ways of obtaining these are given under Methods of Observation and Study. Floating forms with long, tapering, radiate pseudopodia are characteristic of a number of unrelated genera and should help to distinguish *Vannella* from *Platyamoeba* and *Dactylamoeba* from *Mayorella*. Bovee's conclusion that *Astramoeba radiosa* is an invalid taxon, probably representing the floating form of one or several genera, is accepted here.

Cysts. The cyst as a source of characters for identification is dealt with at length under *Naegleria* and *Acanthamoeba*. Usually a new finding of *Acanthamoeba* is first recognised by the characteristic cysts. The same may be true of *Naegleria gruberi*, though the recent discovery of *Willaertia,* with similar cysts, introduces some uncertainty. Cyst structure is useful at least as an ancillary character for species determinations in several genera, especially since each cyst has a fixed size and shape.

Flagellate phase. This is necessary to distinguish amongst those genera of Vahlkampfiidae having such a stage. Ways of obtaining it are given in Methods of Observation and Study, and the flagellates are illustrated in connection with the Vahlkampfiidae. Flagellates of *Naegleria gruberi* may be encountered in mixed material, especially infusion cultures. The only non-vahlkampfiids in this key with known flagellate stages are *Pocheina flagellata* and *Phreatamoeba balamuthi*.

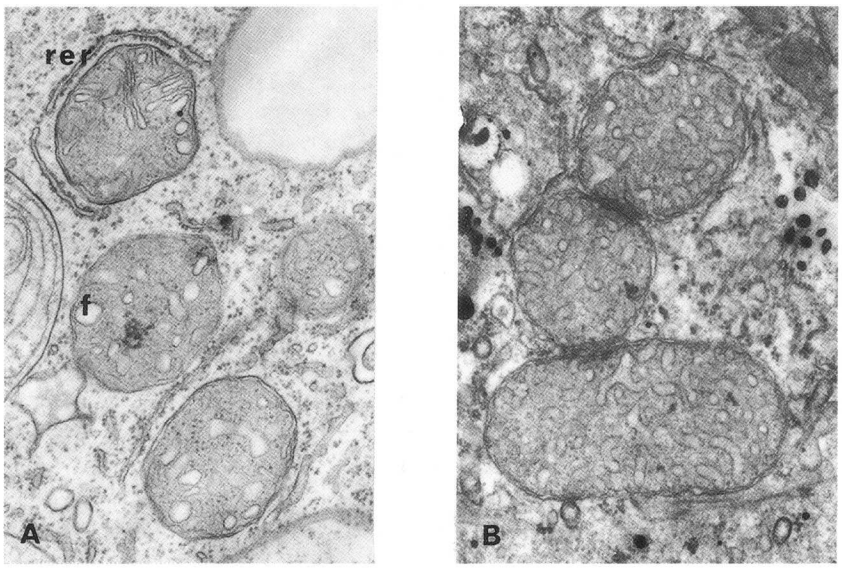

Fig. 3. Two principal kinds of cristae found in the mitochondria of amoebae. A, discoid. Depending on angle of sectioning, the disc-shaped cristae are seen either in side view ('upper' and 'lower' membranes close together) or in 'flat' view (more or less circular space bounded by a membrane), or somewhere between those two views. B, tubular. These cristae are seen in either cross-section (small circles) or longitudinal section. Branching of the cristae can be seen in some longitudinal views. (Both ×25,000). *f*, flat view of discoid crista; *rer*, rough endoplasmic reticulum (often envelops mitochondria with discoid cristae).

Fine structure. The form of the *mitochondrial cristae* (Figure 3) distinguishes Heterolobosea from Gymnamoebia. Electron microscopy can therefore be used to distinguish vahlkampfiids from other

eruptive limax amoebae. The finding of such cristae resulted in the discovery that *Stachyamoeba* is a member of the Heterolobosea.

However, the most widely useful character is cell surface structure, now known for the great majority of species in the dichotomous keys. It is illustrated only where it is one of the distinguishing characters of a genus or species.

Many amoebae have a thin glycocalyx or even none discernible by the usual methods. Some, such as *Thecamoeba*, have a thick, dense glycocalyx with no perceptible morphological differentiation, while in others, such as *Platyamoeba*, the filamentous nature of the glycocalyx is evident and distinctive configurations can be seen in favourable sections. In the Amoebidae, not only is the fibrous nature of the coat evident, but it is often organised into discrete, long filaments. Distinct cup- or sucker-like structures are characteristic of the Hartmannellidae, though sometimes discernible only in the most favourable sections.

The most distinctive structures are *glycostyles* and *microscales*, confused by some authors. *Microscales* are more or less rigid and survive removal from the cell surface. Those of *Dactylamoeba* and *Paramoeba* (the latter marine only) are boat-shaped, those of *Cochliopodium* radially symmetric. A given pattern appears distinctive of each species, but investigation of more species is needed. *Glycostyles* are complex, flexible, radially symmetric structures, each set apart from its neighbour in a distinct pattern and not removable intact from the surface. They may be pentagonally or hexagonally symmetric, and the pattern appears to be usually a generic rather than specific character (but see *Vexillifera*).

A thick *cuticle*, connected to but not following in detail the contour of the cell surface, has been found on the gymnamoebae *Mayorella* and *Dermamoeba*, as well as on *Gocevia* and *Paragocevia*, members of the Cochliopodiidae.

In a few of the lesser known Amoebidae and perhaps in *Pseudothecamoeba*, an examination of the structure of the nuclear envelope may help to confirm the identification of an unfamiliar organism.

Physiological characters. Some of these can already be employed in identification, and more have been used recently in distinguishing species of *Amoeba* but are not necessary for identification.

The *maximum relative locomotive rate* is the maximum ratio of μm per minute to length of amoeba at a temperature of 20-22°C. Usually the rates and lengths of 10 amoebae are measured. In general, the MRLR of vahlkampfiids is higher than that of hartmannellids, but some vahlkampfiids move slowly. This distinction may help with a preliminary identification.

Growth temperature is employed in the keys to species of *Naegleria* and *Acanthamoeba*, usually the maximum or minimum at which a species will grow.

Generation time has been used as a distinction amongst species of *Amoeba*, but its value for identification has not been tested. Other characters of which the same can be said include tolerance of various physical and chemical stresses.

Nucleocytoplasmic compatibility, though genetic rather than physiological, can be considered here. It is determined by transplanting the nucleus of one strain into an amoeba of another strain. It can be used (with awareness of pit-falls) to test conspecificity of strains in laboratories using large amoebae for experimental work. Taxonomic distinctions made with the help of this character and references to the technique will be found in Page & Kalinina (1984) and Kalinina *et al.* (1987).

Biochemical and immunological characters. Application of such characters to species distinctions and references to the methods will be found in the sections on *Naegleria* and *Acanthamoeba*. Amongst the characters used are electrophoretic comparisons of isoenzymes and total proteins, immunoelectrophoretic analysis, the immunofluorescent antibody technique, and tests for the activity of various enzymes. The use of, at least, isoenzyme comparisons is probably essential for some distinctions in the genera *Naegleria* and *Acanthamoeba*.

Summary of Classification

The taxonomic system employed in this key is derived from a newly modified classification of the Rhizopoda (Page, 1987c). Working diagnoses of taxa other than suborders will be found in the keys which follow. Authorships and complete diagnoses of higher taxa will be found in the separate publication. Taxa outside the scope of this key, including marine gymnamoebae, are omitted.

Phylum RHIZOPODA

Heterotrophic protists with lobopodia, filopodia, or reticulopodia.

Class HETEROLOBOSEA
- Order SCHIZOPYRENIDA
  - Family VAHLKAMPFIIDAE
    Adelphamoeba, Naegleria, Paratetramitus, Tetramastigamoeba, Tetramitus, Vahlkampfia, Willaertia
  - Family GRUBERELLIDAE
    Stachyamoeba
- Order ACRASIDA
  - Family ACRASIDAE
    Acrasis, Pocheina
  - Family GUTTULINOPSIDAE
    Guttulinopsis

Class CARYOBLASTEA
- Order PELOBIONTIDA
  - Family PELOMYXIDAE
    Pelomyxa

Class LOBOSEA
- Subclass GYMNAMOEBIA
  - Order EUAMOEBIDA
    - Family AMOEBIDAE
      Amoeba, Chaos, Deuteramoeba, Hydramoeba, Polychaos, Trichamoeba
    - Family THECAMOEBIDAE
      Dermamoeba, Pseudothecamoeba, Sappinia, Thecamoeba, Thecochaos
    - Family HARTMANNELLIDAE
      Cashia, Glaeseria, Hartmannella, Saccamoeba
    - Family VANNELLIDAE
      Platyamoeba, Vannella
    - Family PARAMOEBIDAE
      Dactylamoeba, Mayorella
    - Family VEXILLIFERIDAE
      Vexillifera
  - Order LEPTOMYXIDA
    - Suborder RHIZOFLABELLINA
      - Family FLABELLULIDAE
        Paraflabellula
      - Family LEPTOMYXIDAE
        Leptomyxa, Rhizamoeba
    - Suborder LEPTORAMOSINA
      - Family GEPHYRAMOEBIDAE
        Gephyramoeba
  - Order ACANTHOPODIDA
    - Family ACANTHAMOEBIDAE
      Acanthamoeba, Protacanthamoeba
  - Gymnamoebia *incertae sedis*:
    - Families of uncertain ordinal relationships
      HYALODISCIDAE (*Flamella, Hyalodiscus*)
      ECHINAMOEBIDAE (*Comandonia, Echinamoeba, Filamoeba*)
      ENTAMOEBIDAE (*Entamoeba*)*

* Not included in key, but see note on p. 28.

Genera of uncertain familial relationships
Dinamoeba
Phreatamoeba
Subclass TESTACEALOBOSIA
Order HIMATISMENIDA
Family COCHLIOPODIIDAE
Cochliopodium, Gocevia, Paragocevia

Taxonomic Changes

These have been held to a minimum, since this is a practical publication. Major changes will be found in a recent theoretical publication (Page, 1987c).

The only new taxa in this publication are the genus *Pseudothecamoeba* (p. 67), and the species *Vannella lata* (p. 74). New combinations are *Deuteramoeba mycophaga* (p. 61), *Pseudothecamoeba proteoides* (p. 67), *Dermamoeba minor* (pp. 71, 72), *Vannella cirrifera* (p. 76), *Dactylamoeba stella* (p. 82), *Dactylamoeba bulla* (p. 82), and *Rhizamoeba australiensis* (p. 89). The genus *Thecamoeba* has been emended, with a re-diagnosis (p. 68).

CULTURING GYMNAMOEBAE

Introduction

The purposes of culturing from the environment are enrichment and isolation, both of which result in increased numbers.

By *enrichment*, using conditions favouring the desired organisms, one detects species that would be missed by direct examination of collected material. Such initial cultures are *mixed*, containing diverse organisms. They give a fair idea of the kinds of amoebae present, though similar species may not be distinguishable at this stage. The author has never failed to obtain amoebae by enrichment from water, soil, or leaf litter, especially by inoculation onto agar.

By *isolation* one obtains a culture of a single species, that is, a population. This does not necessarily mean that no other organisms are present, but none of a similar species, likely to be confused with the object of study, are present, a condition usually achieved by cloning. For identification of most gymnamoebae, only cloning will guarantee that only one species is under examination. The eventual aim for further work is to refine the cultures so that only those other organisms or products of organisms needed for food are present, but the degree of refinement achieved is limited by the requirements of the species being cultured and our understanding of those requirements. For example, some *Acanthamoeba* strains have been grown in a *defined* medium containing only those organic compounds which the amoebae cannot synthesise for themselves. On the other hand, *Amoeba proteus* not only needs at least one species of eukaryote as food but has never been cultured in the absence of bacteria.

The lowest degree of refinement is a *polyxenic* culture, with several other species; if their identities are not known it is *agnotoxenic* or *agnotobiotic*. A *monoxenic* culture contains only one other species. This is the maximum refinement achieved for most amoebae, which are often cultured with a single species of bacteria, usually *Escherichia coli*.

For many purposes the ideal culture is *axenic*, with no other species. The organisms are usually absorbing dissolved nutrients, sometimes ingesting inanimate particles. Several axenically cultured strains of *Acanthamoeba* have been used in many biochemical investigations, notably of contractile proteins. The defined media mentioned above are the ultimate refinement, representing a knowledge of the minimum nutritional requirements of a strain.

Besides a suitable medium and food, a few other conditions should be mentioned. All freshwater and soil amoebae except certain potentially pathogenic species can be cultured at room temperature, *c.* 18-22°C, the range recommended except for investigation of potential pathogens. Other than acrasids, which should have a normal day/night photoperiod, amoebae can be grown in either indirect light or in the dark, but cultures in any of the saline solutions or infusions given below should be kept in the dark to prevent algal growth, unless they are feeding on algae.

Culture Media

All media, even saline solutions, should be autoclaved before use. Vessels into which sterile media are poured should also be sterile.

Saline solutions

These very weakly saline solutions are used as non-nutrient media, with regular addition of food organisms; as media with added cereal grains for growth of bacteria and eukaryotic food organisms accompanying the amoebae; as the inorganic component of liquid nutrient media; as the liquid component of agar media; and as transfer liquids for amoebae grown on agar. The use of each is indicated in the appropriate place. Some have actually proved more suitable than others for certain organisms. In other cases, the use of a certain solution for a given purpose is only habitual.

Modified Neff's amoeba saline (AS): Make a separate stock solution of each component by dissolving in 100 ml of glass-distilled water.

NaCl	1.20 g
$MgSO_4 \cdot 7H_2O$	0.04 g
$CaCl_2 \cdot 2H_2O$	0.04 g
Na_2HPO_4	1.42 g
KH_2PO_4	1.36 g

Prepare the final dilution by adding 10 ml of each stock solution to enough glass-distilled water to make 1 litre.

Prescott's & James's solution (PJ): Make up three stock solutions, each with 100 ml of glass-distilled water.

Stock solution A	
$CaCl_2 \cdot 2H_2O$	0.433 g
KCl	0.162 g
Stock solution B	
K_2HPO_4	0.512 g
Stock solution C	
$MgSO_4 \cdot 7H_2O$	0.280 g

Combine 1 ml of each stock solution and 997 ml of distilled water to make 1 litre of the final dilution.

Prescott's & Carrier's solution (PC): Prepare two stock solutions, each with glass-distilled water to make 1 litre.

Stock solution A	
$MgSO_4 \cdot 7H_2O$	0.2 g
KCl	0.5 g
$CaCl_2$	1.0 g
NaCl	1.0 g
Stock solution B	
$CaHPO_4$	0.36 g

Combine 10 ml of each stock solution with 980 ml of distilled water to make 1 litre of the final dilution.

Chapman-Andresen's modified Pringsheim's solution (MP): Make up a separate stock solution of each of the following components in 100 ml of glass-distilled water.

$Ca(NO_3)_2 \cdot 4H_2O$	20.0 g
$MgSO_4 \cdot 7H_2O$	2.0 g
$Na_2HPO_4 \cdot 2H_2O$	2.0 g
KCl	2.6 g
$FeSO_4 \cdot 7H_2O$	0.2 g

The final dilution is made by adding 1 ml of each stock solution to 995 ml of glass-distilled water.

Modified Chalkley's solution (MCh): Prepare a separate stock solution of each of the following components with glass-distilled water to make 100 ml.

NaCl	8.0 g
$NaHCO_3$	0.4 g
KCl	0.4 g
$Ca(H_2PO_4)_2 \cdot H_2O$	0.16 g

Add 1 ml of each stock solution to 996 ml of glass-distilled water to make the final dilution.

Marshall's solution (Ma): Prepare a separate stock solution of each of the following components in 100 ml of glass-distilled water.

$CaCl_2$	5.54 g
$MgSO_4 \cdot 7H_2O$	1.23 g
K_2HPO_4	2.79 g
KH_2PO_4	1.50 g

Add 1 ml of each stock solution to 996 ml of glass-distilled water to make the final dilution.

Liquid media

These may be divided into infusion or soil extract media (1-4) and protein-rich media (5-8), the

latter mainly for axenic cultures. Infusion and soil extract media and saline solutions may be used to a depth of 10-12 mm in culture dishes with a diameter of 50 mm, either plastic or, if glass, sealed with a strip of clingfilm. For *Amoeba* and *Chaos*, somewhat larger dishes (7-9 cm) may be used. Dr Andrew Rogerson, of the Culture Collection of Algae and Protozoa, has found 50 ml Nunc tissue culture flasks, each containing about 25 ml of liquid, convenient for large amoebae. The culture vessels for protein-rich media are usually tubes, though for larger numbers of cells (e.g., *Acanthamoeba* for biochemical studies) flasks are used.

1. ***Cerophyl-Prescott's infusion*** (CP): Cerophyl is a cereal derivative manufactured by Cerophyl Laboratories, Inc., Kansas City, Missouri, USA. In Britain, Sigma Chemicals (Fancy Road, Poole, Dorset) supply a product, C7141 Dehydrated Cereal Leaves, which they state is exactly equivalent to Cerophyl. Boil 0.5-1.0 g of Cerophyl in 1 litre of PJ for 5 minutes, filter out the particles, and restore the volume with distilled water. We have used 1.0 g per litre and diluted the final infusion 1 : 1 with PJ to hold down bacterial growth. Some workers may wish to develop substitutes for the commercial product.

2. ***Grass-seed infusion*** (GS): This may be used instead of CP in the initial stages of isolation but has not been tested with many established strains, such as *Mayorella* spp., usually cultured in dilute CP+rice grains. The use of grains other than lawn grass seed in similar infusions is possible, but these vary in usefulness (Page, 1981c). Boil 2 g lawn grass seeds, untreated with fungicides or pesticides, in 1 litre AS or PJ for 5 minutes. Dispense into culture dishes, with a few seeds per dish; discard excess seeds.

3. ***Liquid with rice*** (r): Either dilute CP or one of the saline solutions may be used with *unboiled* polished rice grains (2 or 3 per 50 mm dish) when food organisms accompany the amoebae, usually *Amoeba* or *Mayorella*. The correct combination for a species is indicated in the dichotomous keys, e.g., PC/r/Chil = PC with rice and *Chilomonas paramecium*, or CP/r, indicating that food organisms accompany the amoebae from nature and are not added.

4. ***Soil extract with salts*** (E+S): An algal medium useful for algivorous amoebae. Into a beaker put enough calcareous garden or agricultural soil (a mixture of 2 or 3 different soils if possible) and natural or tap water so that the supernatant water occupies approximately four-fifths of the depth. Autoclave for 1 hour, then decant or filter the liquid, which is the soil extract. Combine with water and stock solutions of salts.

E (soil extract liquid)	10 ml
K_2HPO_4, 0.1% w/v	2 ml
$MgSO_4 \cdot 7H_2O$, 0.1% w/v	2 ml
KNO_3, 1.0% w/v	2 ml
Glass-distilled water	84 ml

5. ***Proteose peptone glucose*** (PPG): For axenic maintenance of *Acanthamoeba*. 10 g Difco proteose peptone, 18 g glucose, 1 litre AS. Dispense into tubes and autoclave.

6. ***Chang's serum-casein-glucose-yeast extract medium*** (SCGYEM): For axenic culture of *Naegleria* (De Jonckheere, 1977, 1980).

Isoelectric casein	10.0 g
Na_2HPO_4	1.325 g
Glucose	2.5 g
KH_2PO_4	0.8 g
Yeast extract	5.0 g
Foetal calf serum	100 ml
Distilled water	900 ml

If inoculating amoebae from bacterised cultures, add 200 μg/ml each of penicillin and streptomycin. Dispense into tubes.

7. ***Jones's medium***: This can be used for *Entamoeba moshkovskii*, the only free-living *Entamoeba*, as well as for *Phreatamoeba balamuthi*.

(1) Buffered saline (pH 7.2)	
$Na_2HPO_4 \cdot 12H_2O$	2.65 g
KH_2PO_4	0.41 g
NaCl	7.36 g

Glass-distilled water	1 litre
(2) Final medium	
Buffered saline	850 ml
Horse serum	50 ml
1% Marmite solution	100 ml

Filter to sterilise, dispense into sterile tubes, and add to each tube a pinch of rice starch which has been heated in an oven at 150°C for 2 hours.

8. ***Proteose peptone-yeast*** (PY): For axenic cultures of *Tetrahymena pyriformis* to feed *Amoeba proteus* and other large amoebae: 20 g proteose peptone, 2.5 g yeast extract, 1 litre glass-distilled water. Dispense into tubes and autoclave.

Agar media

Except where otherwise indicated, these are made with a non-nutrient base such as Oxoid agar No. 1 or Difco Bacto-Agar, which should be stirred into the liquid and brought to a boil to dissolve. The solution is then autoclaved and poured into sterile, unvented or singly vented petri dishes to a depth of about 5 mm. After inoculation the petri dish should be sealed with clingfilm to retard evaporation, *except for acrasids*, which do not tolerate a humid atmosphere. Agar cultures of amoebae should not be inverted, so that the agar block often used for inoculation will not fall off or the liquid, if amoebae have been inoculated as a suspension, will not run toward the cover. The dishes can be inverted after growth is under way, so that condensed water will not run onto the agar.

Non-nutrient agar (NN): 1 litre of AS and 15 g of non-nutrient agar.

Cerophyl-Prescott's agar (CPA): 1 litre of CP and 15 g of non-nutrient agar.

Grass-seed agar (GSA): May be used instead of CPA when isolating or, if necessary, for established cultures. Its principal disadvantage is uneven distribution of the nutrients. Dissolve 15 g of non-nutrient agar in 1 L GS infusion. After autoclaving, dispense into petri dishes, with a few seeds in each dish, discarding excess seeds.

Stoianovitch's malt extract/yeast extract agar (MYAS): Not listed for any particular species but sometimes useful as a substitute for NN or CPA. However, bacteria often become too abundant; streaking is not necessary, since those accompanying amoebae multiply. 1 litre of AS, 0.1 g malt extract, 0.1 g yeast extract; 15 g non-nutrient agar.

Cornmeal/glucose agar (CMA): For acrasids and the food organism *Rhodotorula mucilaginosa*.

Difco cornmeal agar	17 g
Dextrose	2 g
Yeast extract	1 g
Distilled water	1 litre

Nutrient agar: For growing bacteria, usually *Escherichia coli*, food organism for many species of amoebae growing on agar, the usual commercial preparations from Oxoid or Difco are used as slopes.

Methods of Isolation and Culture

Collection and initial handling

Any of the usual sampling methods can be adapted for collection of gymnamoebae. Collection vessels must always be sterile, with one exception noted below.

Quantitative procedures will be found in the appropriate works, some of which are listed by Finlay (1982). Amongst recent or widely used sources of such methods are, for freshwater gymnamoebae Kyle & Noblet (1986) and O'Dell (1979), and for soil protozoa including gymnamoebae Alabouvette *et al.* (1981), Darbyshire (1973), Singh (1946) and Stout *et al.* (1982).

When the purpose is not quantitative, even sterile jars may be suitable. To avoid stirring up the bottom of bodies of water or to reach less accessible spots, a simple collecting device may be made: a 150 ml Erlenmeyer flask with a bung, through which are inserted one bent glass tube (*c.* 60°) for connecting to a longer straight tube and another bent tube (*c.* 120°) for aspirating into the flask by mouth or with a rubber bulb. A short, flexible plastic tube connects a long straight tube with the

60° bend leading into the flask. The apparatus should be autoclaved (long tube separately) and assembled at the collecting site. The long tube is dipped to the spot from which a sample is to be taken. The flask may then be closed with a sterile bung.

When collecting for Amoebidae only, sterile equipment and precautions are not essential, since these large amoebae will not survive drying out.

Collected material should be handled with sterile precautions until initial cultures have been inoculated, which is best done before examining samples microscopically. Initial observations should be made within a day or two of collecting: drops from bottom and sides of a collecting vessel which has stood an hour or so, then drops taken immediately after thorough mixing.

A fast and easy method of detecting large Amoebidae is suggested by F. J. Siemensma (personal communication). Fill a narrow, tall glass cylinder with collected material, leaving an overlay of several cm of clear water. Soon large amoebae, if present, can be seen moving up the wall of the cylinder. After several hours, the surface layer of the detritus is enriched with many amoebae.

Isolation and purification

Enrichment. Although few amoebae will usually be found in drops of collected material, any larger or medium-sized amoeba can be put into appropriate medium. In general, however, enrichment is necessary. These methods will not necessarily bring out all filose or reticulose amoebae present, particularly in soil. Methods for isolating acrasids are given by Olive (1975).

Depending on the organisms sought, collected material can be inoculated on or into the following media:

(1) CPA or GSA. This will bring out the widest range, all gymnamoebae that will grow on agar, if suitable food organisms accompany them, from the smallest amoebae, encysting or not, and even medium-sized or larger but flattened amoebae including *Thecamoeba, Leptomyxa*, and some but not all *Mayorella* and *Dactylamoeba*.

(2) NNE will give a start to nearly as many: all small and medium-sized encysting amoebae and all smaller non-encysting amoebae.

(3) CPA with an overlay (*c.* 3-5 mm deep) of the collected water or of PJ (not CP). This may encourage some *Mayorella* or *Thecamoeba* and at the same time permit multiplication of flagellates or ciliates serving as food. The same small amoebae which grow out on agar without overlay will be present, possibly in smaller numbers.

(4) CP liquid in a 50 mm dish with 2 or 3 flamed rice grains may provide a start for some of the same organisms found in the preceding sort of culture and may be more favourable for some *Mayorella* spp.

(5) E+S in a 50 mm culture dish, kept in indirect light, is a possible means of isolating algivorous amoebae.

(6) PC, PJ, or MCh with 2 or 3 rice grains or with added *Tetrahymena* or *Colpidium* may yield large amoebae (Amoebidae). However, *Amoeba* or *Chaos* is more often isolated by picking out individual amoebae from slides or small dishes of collected material.

When inoculating agar, 1-2 ml of collected water should be absorbed in a few hours. A sample of any vegetation or other particulate matter should also be added. From terrestrial habitats, soil or leaf litter may be distributed thinly on the agar, leaving free areas for migration of amoebae. A little PJ, AS, or sterile distilled water should be used to moisten the material distributed on the agar.

See p. 16 for conditions of temperature and light.

The initial cultures should be examined about a week after inoculation. Agar plates without liquid overlay are examined by turning the unopened plate over on the microscope stage and focussing through the agar to its free surface with the ×4 objective. Cysts are immediately discernible by their distinct walls and regular shape. If amoebae or cysts appear to be present, some idea of their identity can be obtained by examining them on a slide, making a suspension with a sterile pipette and small quantity of liquid. Sterile precautions must be observed at all stages.

Some medium-sized and larger amoebae may not have multiplied greatly until a fortnight after inoculation. In general, liquid cultures, in which one is seeking medium-sized and larger amoebae, take longer to grow out than agar cultures without liquid overlay. Liquid cultures may, if necessary, be examined in a relatively clean atmosphere with the cover removed, since one is not seeking

small, encysting amoebae in such cultures and cannot expect bacteria-free cultures of most medium-sized and large amoebae. An inverted microscope or a laminar flow cabinet would prevent all airborne contamination.

Clonal isolations should be made as soon as possible to avoid any loss of species from the mixed cultures. The procedures differ for amoebae growing on agar and those in liquid. Furthermore, one more step may be necessary before clonal isolation.

If the initial mixed culture on agar contains much particulate matter from the natural habitat, it is as well to inoculate a second mixed culture on the same medium. Wash the surface of the original culture with 1-2 ml of PJ or AS and transfer the suspension to a fresh surface. Do not discard the first plate, since some larger amoebae may still come up.

Mixed cultures in liquid may also be subcultured for further enrichment, but with liquid cultures there may be more chance of species loss or there may be an unwanted increase in accompanying organisms. Especially if only a few large amoebae are present, it is better to proceed to the next step.

Clonal isolation. When cloning from a mixed culture on agar, wash the surface with about 1 ml of PJ or AS, using a pipette. If the amoebae are encysted, streak some of the resulting suspension on a fresh non-nutrient agar surface with a bacteriological loop. With the petri dish open on the microscope stage, find a cyst *well* separated from others, inspect the adjacent surface carefully to make sure that no active amoebae are present, make a bright spot with the condenser at the location of the cyst, and lower the stage to give working room. Burn off a fine scalpel blade with methylated spirit, and when it has cooled cut out a square of agar bearing the cyst, taking care not to draw in any other cells, and place on fresh agar, with the side of the block bearing the cyst downwards. If the fresh agar is NN, place the block at the end of a streak of *E. coli*; if it is CPA, bacteria accompanying the cyst will multiply and furnish food.

Pussard & Pons (1977) devised a method of cloning cysts which eliminates all risk of air-borne contaminants.

To clone active amoebae of non-encysting species, put 3 drops of suspension from the mixed culture, with a pipette, about 1 cm apart at one side of the agar isolation surface, and immediately tip the plate so that the drops run in parallel paths. Leave the plate overnight for the liquid to soak in and the amoebae to separate by migration. The rest of the procedure is as for cysts, but one must search the surrounding area carefully for other amoebae, either on the surface or burrowed into the agar.

If amoebae are relatively few and medium-sized, clone-founders may be picked out without waiting for migration. Such medium-sized and large amoebae, e.g., *Thecamoeba, Dactylamoeba, Mayorella*, often feed on smaller amoebae, flagellates, or ciliates, so that a clone can be started with an agar block bearing one larger amoeba plus smaller organisms. When the clone is established, any accompanying eukaryotes not serving as food can be eliminated by re-cloning.

Such an agar cloning surface can also be used for amoebae growing in liquid if they will survive a half hour on agar, as some will. The block is then placed into fresh liquid medium. However, most Amoebidae do not last on agar and are cloned by transferring through several sterile drops on slides or watch-glasses with fine-tipped pipettes.

Ord (1978) has devised an elegant capillary technique for cloning large amoebae. It makes unnecessary the frequent transfers of the clone-founder or its immediate progeny to avoid accumulation of excess bacteria in its tiny environment, which may be a watch-glass or a cubicle in a plastic tray.

Cloning from agar surfaces should if possible be carried out in a laminar flow cabinet or similar clean environment, though risk of contamination is not great if cloning onto a medium of low or no nutrient content. Cloning by pipette should be carried out in as clean an environment as possible.

Because many cloning attempts do not succeed, about 10 or 12 should be carried out from a mixed culture believed to contain only one or two species of amoebae. Multiple clones permit intraspecific comparison.

Clones on agar should show easily noticeable growth in a week (*Thecamoeba* and other larger amoebae sometimes more slowly), those in liquid in 2 or 3 weeks, but both should be given at least a week longer. If a clone grows out on a medium that is not optimal, other media and possibly other food organisms should be tested.

Monoxenicisation. Amoebae cloned onto NNE may be almost monoxenic from the start, but bacteriological tests should be carried out. A suggested method of monoxenicisation is a modification of the usual maintenance method. Streak *E. coli* across a NN surface in a 9 cm petri dish, and inoculate a block of agar bearing amoebae at one end of the streak. When the amoebae have almost reached the other end, take a block from that end to start a new culture. Make several serial cultures in rapid succession.

Axenicisation. The recommended medium for axenic *Acanthamoeba* cultures is PPG. For *Naegleria*, SCGYEM has been used. Other media are given in references cited under those genera. Two principal methods of eliminating bacteria have been used: antibiotics and migration.

Amoebae on an agar block from a bacterised culture can be added to medium containing 200 μg/ml each of penicillin and streptomycin, but the bacteriostatic action in the first culture does not guarantee that succeeding cultures will be axenic.

The migration method is based on the work of Neff (1957, 1958). The following modification has been successful with several species of *Acanthamoeba*: At one side of a fresh NN surface, make a crescentic streak of *E. coli* 1.5-2.0 cm in diameter, open toward the side of the dish. Into the area bounded by the crescent place a drop of amoeba suspension. The amoebae will multiply and move outward from the crescent. Examine daily for migration. When the first amoebae appear well clear of any bacteria (*c.* 1 cm from streak, several days after inoculation), cut out blocks bearing a few amoebae each and place into tubes of PPG. As larger numbers of amoebae migrate out, some bacteria are bound to be brought along; it is important to pick out migrating amoebae as soon as possible. Keep in mind that one may not want to re-clone.

Surface sterilisation of cysts is another possible method, but not enough information is available on use of this method with *Naegleria* and *Acanthamoeba* to give detailed procedures. The cyst of *Acanthamoeba* is more resistant to chemical disinfectants than is that of *Naegleria*.

Tubes in which axenicisation has been attempted should be incubated at room temperature and inspected in a few days for gross bacterial contamination. Those remaining clear should be examined for amoebae after a fortnight or more by placing a drop of medium on a slide after agitating to dislodge amoebae from the wall of the tube; *Acanthamoeba* grows mainly toward the top. If visual inspection reveals amoebae but no bacteria, bacteriological tests should be carried out.

Maintenance of established strains

Notes on culture methods for many species accompany the dichotomous key. If further instructions are essential, they should be requested from the Culture Collection of Algae and Protozoa, Freshwater Biological Association, The Ferry House, Ambleside, Cumbria LA22 0LP.

Agar cultures. The normal method of transferring to fresh medium is to cut a block *c.* 2 mm square from the parent culture and place it firmly, face down, on the new surface, near one side of the dish to permit maximum distance for migration. If the strain is on NNE, place the block at one end of a straight bacterial streak extending across the dish. Amoebae which migrate or multiply slowly may be transferred as a suspension washed from the parent culture, added to the fresh surface, and spread by tilting the plate, using as little liquid as possible.

For acrasids grown on CMA, *Rhodotorula mucilaginosa*, a yeast grown on the same medium, is streaked on fresh agar just before addition of a block from the parent acrasid culture.

Liquid cultures. Transfer is always by sterile pipette. Axenic cultures are subcultured by transferring a pipette full after agitation with the pipette to dislodge amoebae. Cultures in dishes are transferred by transferring the entire parent culture into a dish of fresh medium, then pipetting half the mixture back into the old dish. The liquid should be not more than about 1 cm deep.

Frequency of culture. Cyst-forming strains, with a few exceptions, are viable for months on agar at room temperature if the agar does not dry out or become contaminated. Most non-encysting amoebae on agar must be subcultured at intervals of 4-8 weeks; a few which rapidly consume the food organisms and then die out, at 2-3 weeks. Liquid cultures in CP with rice should be transferred monthly. Cultures of Amoebidae in a saline solution with rice grains should also be transferred monthly, but from parent cultures 2 months old or older, since they usually take more than a month to become abundant and remain good several months. Cultures fed with *Tetrahymena* or *Colpidium* can usually be kept for months, but new ones should be started monthly from good parent cultures. An exception is *Chaos carolinense*, which should be transferred more often, even weekly.

Feeding and food organisms. Most Amoebidae, the larger amoebae, require added eukaryotic food organisms, unlike smaller amoebae feeding exclusively on bacteria or accompanied from their natural habitat by eukaryotic prey.

The easiest but not cleanest method for large amoebae is the initial addition of *Chilomonas paramecium* to an amoeba strain in a saline solution such as PC with rice grains. The flagellates accompany the amoebae during subsequent transfers. Such cultures do best when hyphae of a phycomycete such as *Dictyuchus* are growing from the rice grains. The amoebae live and feed amongst the mycelium, though the population on the bottom of the dish later becomes abundant.

The cleanest cultures, used for most research purposes employing *Amoeba*, require the addition, two or three times a week, of washed *Tetrahymena* to cultures in a saline solution. The *Tetrahymena* are grown axenically in PY medium at room temperature. Before being fed to the amoebae they must be washed free of PY by 3 or 4 centrifugations in fresh lots of the saline solution.

Both *Chaos carolinense* and *C. nobile* are fed on *Colpidium*, e.g. *C. striatum*. The medium is an infusion made by boiling several barley or wheat grains in the same liquid in which the amoebae are grown for 5 minutes. When the infusion has cooled, *Colpidium* with accompanying bacteria are added. The cultures take about a fortnight to become abundant. It is recommended that the *Colpidium* be washed free of some of the many bacteria before being fed to the amoebae.

Overfeeding is often fatal to a culture of large amoebae. The quantity should be tested, starting with 1 or 2 drops of ciliate suspension per 50 ml culture dish. Few if any food organisms should be left when the time for the next feeding comes.

METHODS OF OBSERVATION AND STUDY

Light microscopy

Living amoebae. Gymnamoebae should be observed and measured in normal locomotion under conditions of adequate oxygen, minimal evaporation, and freedom from mechanical pressure. Observation chambers can be devised, and an inverted microscope is useful, but for amoebae which adhere reasonably well to glass, hanging drops provide the necessary conditions, with the amoebae moving on the underside of cover glasses.

Prepare a suspension from an agar culture in 0.5-1.0 ml of AS or PJ or concentrate from a liquid culture. Amoebae from a bacterised culture may be washed by centrifugation. Place a drop of suspension on a cover glass and add a concavity slide or a slide with a ring about 2 mm deep cemented to it, sealing the junction with petroleum jelly. Let the preparation stand a few hours or overnight, turn it over carefully, and let it stand a while so that some bacteria sink to the bottom.

The amoebae which adhere well are generally those with a thin or no detectable glycocalyx. Many adhere poorly (e.g., Thecamoebidae, most Paramoebidae), though a few smaller ones with a well developed glycocalyx may adhere better than others in the same genus, e.g., *Vannella*. Slight improvement in adhesion may sometimes be obtained by pre-treating the coverslips in 1% NaOH at 80-90°C for a few minutes, then rinsing thoroughly. Useful observations of large amoebae can be made at low magnifications as they move in a thin layer of liquid, without a cover glass, on a slide or in half a plastic petri dish. Otherwise, medium-sized and larger amoebae may be observed on a flat slide under a cover glass supported on 2 sides by ridges of petroleum jelly, occasionally adding liquid through an open side. At any rate, the amoebae must be given time to take on a normal locomotive form.

Amongst the useful measurements made on moving amoebae are lengths and breadths of 50-100 amoebae, the measurements on a single amoeba being made in rapid succession before change of shape, and locomotive rates (μm/minute) of 10 amoebae, with lengths of the same 10.

To measure nuclei, transfer the hanging drop carefully, dislodging as few amoebae as possible, to a flat slide and observe with phase contrast or differential interference contrast. Nuclei of amoebae still extended on the cover glass are the more easily measured.

The same preparation used to measure nuclei can be used to look for optically active inclusions with a polariser.

Floating forms. These are obtained by washing the agar surface, then putting a drop of the suspension on a slide and viewing immediately. At first the amoebae may be rounded up, with no projections, but in a few minutes long pseudopodia will appear on some amoebae, if the species has a radiate floating form. Amoebae growing in liquid may be agitated by vigorous pipetting, or the entire dish may be agitated and examined under low power for floating forms (probably the best way for *Mayorella* and *Dactylamoeba*).

Cysts. These should be examined and measured with ordinary bright field or differential interference contrast optics, not with phase contrast. Measure the greatest dimension of 100 cysts. Staining procedures are given below.

Excystment. If it is desired to observe excystment rather than simply look for pores, wash the cysts, place them on a fresh NN surface streaked lightly with *E. coli*, and observe at intervals up to 24 hours if necessary. *Naegleria gruberi* excysts in a few hours in a drop of cyst suspension on a slide in a moist chamber; begin observations an hour after making the preparation. Excystment of *N. gruberi* can also be stimulated by adding AS or distilled water to an agar culture containing cysts.

Flagellate transformation. A modification of De Jonckheere's method for *Naegleria* is undoubtedly the easiest: From an area of a 2-day-old culture with many amoebae cut a piece of agar *c.* 1 cm square and place it into 1 ml of distilled water or AS in a tube. One can also add a thin layer of

distilled water or AS (too much to soak in) to the culture itself, or prepare hanging drops. Incubate at the growth temperature of that strain and examine hourly, even to 24 hours for suspected *N. fowleri*. Stimulation of excystment of *Naegleria* (above) usually results in subsequent transformation of some excysted amoebae.

For a possible *Paratetramitus*, the test (at room temperature) should be prolonged until the next day. *Adelphamoeba* produces only a few flagellates.

With *Tetramastigamoeba hoarei* Singh & Hanumaiah obtained good results only if the amoebae were washed free of many bacteria by 3 or 4 slow centrifugations, then incubated at 25°C for 5-8 hours. (Washing is probably advantageous with any vahlkampfiid.) Michel & Raether induced flagellate formation in *Protonaegleria westphali*, possibly a synonym of *Willaertia magna*, by washing amoebae twice in AS, re-suspending in fresh AS, and incubating at 30°C (best results), examining at intervals up to 24 hours.

Obtaining flagellates from *Tetramitus rostratus* amoebae is difficult. Fulton (1970) gives a better and more complicated method, but the following procedure yields a few flagellates from the common laboratory strain: wash the amoebae by centrifugation and concentrate to 0.2 ml liquid. Add 0.2 ml PY medium (p. 19), resuspend the amoebae, prepare hanging drops, and incubate at room temperature. Begin observations 4 hours later, continuing at least 24 hours. If negative, repeat at least twice on separate cultures.

To count flagellar number, a drop of saturated $HgCl_2$ or Lugol's iodine can be added to a drop containing flagellates on a slide.

Mitotic preparations. The method of Pussard (1973) permits observation and photography of the process *in vivo*. However, permanent preparations are sometimes desirable.

The amoebae for whose identification mitotic preparations are desirable (vahlkampfiid-like amoebae; see Characters for identification) adhere well to glass. The preferred method is fixation and staining on 22 mm coverslips, using Columbia staining jars, which have a capacity of 10 ml (Arthur H. Thomas Co., Philadelphia, USA; obtainable through Hospital and Laboratory Supplies Ltd., 12 Charterhouse Square, London EC1). However, the same procedure can be modified for use with slides.

Wash amoebae with approximately 0.5 ml of AS from a 1- to 2-day-old abundant culture, and put 1 drop on each of at least 4 coverslips in a moist chamber made with a petri dish and absorbent paper. Let the drops stand an hour or two.

The primary fixative is Nissenbaum's, which must be prepared fresh:

$HgCl_2$, saturated aqueous solution	10 ml
Glacial acetic acid	2 ml
Formalin (38-40% formaldehyde)	2 ml
Tertiary butyl alcohol (*tert*-butanol)	5 ml

Remove the coverslip from the moist chamber, check microscopically for attachment of amoebae, and put on a half petri dish or other flat surface. Drop a drop of fixative from a pipette on the amoebae from a height of 1 cm. When the waves stop, lower the pipette and fill the coverslip with fixative but do not allow the liquid to run over the sides. After 1-3 minutes, place the coverslip in a staining jar of acidified $HgCl_2$ (10 ml saturated $HgCl_2$, 0.5 ml glacial acetic acid) for 5-10 minutes, then put it through 50% ethanol (4 changes), 35% ethanol, and distilled water, 3-5 minutes each, and begin the staining procedure.

Pussard recommends fixatives containing OsO_4.

Although the Feulgen procedure is advantageous for mitotic studies and makes dividing nuclei conspicuous (interphase nuclei of most amoebae being Feulgen-negative), it is not essential for distinguishing between promitosis and other patterns.

A rapid staining method uses Kernechtrot (Chroma-Gesellschaft, Stuttgart-Untertürkheim, Germany), although care is needed to get the density correct. The preparations can sometimes be examined advantageously with phase contrast. Heidenhain's iron haematoxylin gives slightly less resolution of adjacent structures but usually stains more densely and lends itself to photography.

Kernechtrot is made up by adding 0.1 g of the powder to 100 ml of hot (not boiling) 5% aluminium sulphate solution. Dissolve the dye, cool to room temperature, and filter. The final solution should be a bright cherry red. If it looks thin, with a flocculent precipitate, it should be discarded; usually it is best not to use a solution much more than a month old.

Bring the coverslips bearing fixed amoebae down to distilled water. Leave in Kernechtrot solution 15 minutes (varying if necessary). Pass through 3 distilled water washes, 3 minutes in each, dehydrate, and mount.

When staining with Heidenhain's iron haematoxylin, leave 1-2 hours in 2% ammonium ferric sulphate, rinse quickly in distilled and then tap water, leave 1-2 hours in 0.5% haematoxylin, differentiate in ammonium ferric sulphate (may be finished in 2 minutes but examine sooner), wash gently in tap water 20 minutes, dehydrate, and mount. Details will be found in the usual references.

Procedures for* Acanthamoeba *cysts. These procedures, both involving staining with silver, were originally described by Pussard & Pons (1977), where further details will be found. Silver proteinate can be obtained from the Chroma-Gesellschaft, Stuttgart-Untertürkheim, or Etablissements Roques, Paris.

For both procedures the cysts are mixed with Mayer's albumin (see standard microtechnique references) on a slide and fixed with Clarke's fixative (95% ethanol/glacial acetic acid, 9 : 1). Fixation with formalin is favoured by Pussard & Pons for removing any pseudoreticulation of the cyst surface. Leave 2 hours in Clarke's fixative. When using formalin, fix 15-30 minutes in a pellet, wash, prepare slides, and postfix with Clarke's for 2 hours to ensure adhesion.

1. ***Warm impregnation.*** Solutions required:
   0.5% silver proteinate in doubly distilled water, made by sprinkling the silver proteinate powder on the water, not stirring it in;
   1% hydroquinone in 5% sodium sulphite.

Rinse fixed preparations in distilled water and immerse them in silver proteinate solution at 60°C for 2 hours. Transfer without washing to hydroquinone reducing bath, in which they should be left for periods from a few seconds to 5 minutes; at least 3 preparations, each reduced for a different time, recommended.

This procedure reveals the relief of the outer cyst wall.

2. ***PAT Ag r*** (periodic acid-Thiéry silver, reduced): Stains PAS-positive (polysaccharide-containing) structures, in this case the inner cyst wall, but is of value with some species of *Acanthamoeba* chiefly for empty cyst walls. It can also be used to demonstrate the pores of *Naegleria* cysts.

   1% periodic acid, 10 minutes
   Running water, *c.* 15 minutes
   0.2% thiocarbohydrazide in 20% acetic acid, 1 hour
   20%, 10%, and 5% acetic acid, *c.* 2 minutes each
   Running water, 5 minutes
   Distilled water
   0.5% silver proteinate solution (above), 5-30 minutes at room temperature
   Reducing bath (above), 5 minutes
   Running water, at least 5 minutes
   Dehydrate and mount

Electron microscopy

No one procedure will give satisfactory results for all gymnamoebae. Two which have proved widely applicable are offered as examples.

(1) This procedure was successful with most Amoebidae but also with some much smaller amoebae such as *Vexillifera bacillipedes* and *Rosculus ithacus*. It preserves the cortical filamentous layer in large amoebae.

Solutions: 0.1 M Na cacodylate buffer, pH 7.0; 4% gluteraldehyde in cacodylate buffer; 2% OsO_4 in cacodylate buffer; 1% uranyl acetate in distilled water.

Bring glutaraldehyde to 20°C.

Add amoebae suspended in saline solution (e.g., PC or AS) drop-wise to equal quantity of glutaraldehyde (usually 2 ml of each). Leave 30 minutes at 20-22°C. Centrifuge toward the end of that time.

Substitute 4% glutaraldehyde for the supernatant and leave another 30 minutes at the same temperature.

Transfer to ice bath.
Wash 4X over 20 minutes in buffer.
OsO_4, 60 minutes.
Wash 4X in distilled water over 20 minutes.
Uranyl acetate, 30 minutes.
Wash 2X in distilled water, fairly quickly.
30%, 50%, and 70% ethanol, 10 minutes each.
Remove from ice bath.
95% and 2X 100% ethanol, 10 minutes each.
Epoxy-propane, 2X, 10 minutes each.
Epoxy-propane/Epon (1 : 1), over night.
Embed in Epon.
Stain sections with 2% uranyl acetate and Reynolds's lead citrate.

(2) This procedure has proved useful for membranous organelles in a fair number of limax amoebae, but not all, and is given as an example of the sort of method that can be used, with variations of timing. It is not intended to preserve cytoplasmic filaments and microtubules. The basic reagents are as for the preceding procedure, but the primary fixative is added to a pellet, and fixation is in an ice bath from the start.

Glutaraldehyde/OsO_4 (1 : 1), 15 minutes.
Wash 3X in buffer, quickly.
OsO_4, 10 minutes.
Washing 4X in distilled water, quickly.
Uranyl acetate, 15 minutes.
Wash 2X in distilled water, quickly.
Dehydrate and embed as above.

Biochemical and immunological procedures

These methods, including concanavalin A agglutination, will be found in the references given under the genera *Naegleria* (especially Table 1) and *Acanthamoeba*.

USING THE KEYS

The keys are arranged in four steps: (1) classes and (for the Lobosea) subclasses, (2) orders and families (of Heterolobosea and Gymnamoebia), (3) genera of each family, and (4) species of each genus. The keys to genera and species are arranged under the families.

The keys will not lead to the family Entamoebidae, one member of which, *Entamoeba moshkovskii*, is free-living. Taxonomic information on that species was summarised, with illustrations, in the previous key (Page, 1976). Nor will the keys lead to *Dinamoeba* and *Phreatamoeba*, which cannot be placed with certainty into any existing family. Both have conical, tapering subpseudopodia but are probably not related to any present family with such subpseudopodia. They are described and illustrated under 'Genera *incertae sedis*' (p. 107).

Some space has been saved by usually omitting any formal diagnosis of each family or genus under its name, since such a diagnosis is incorporated in the preceding steps of the key, making room for other introductory material.

The dichotomous keys to species include only those species for which information seems adequate and reliable enough to fulfil the empirical definition of species given in the Introduction. Others whose outlines seem somewhat vaguer are listed in 'other species' and similar categories, but not all species names ever employed for freshwater and soil gymnamoebae are included. Some undoubtedly valid taxa not included in the dichotomous keys need further investigation. The additional information on each species in the dichotomous key, beyond that strictly needed for a logical dichotomy, is deemed necessary for confirming identifications and for distinguishing between the described species and any somewhat similar undescribed amoeba which may be isolated.

The reader who does not find a generic or specific name in the expected place should look in the Index to Genera and Species. A worker who considers an omission unjustifiable should recognise and try to meet the need for an adequate published re-description.

Measurements have in some cases been rounded up or down. A range of means (e.g., $\bar{x}$ 23-28 μm) indicates measurements on different strains or, rarely, on the same strain at different times, but such sets of measurements have been combined in a single mean when such an indication of variation seemed unnecessary. L indicates the length (anterior-posterior) of amoebae in locomotion; B, the breadth; L/B, the length : breadth ratio. The figures on the scale bars of drawings are in micrometres (μm).

The geographic distribution may not always include all reports. In general, it has been given by continents; both the British Isles and the European part of the USSR are included under Europe. The notation 'terr' indicates that a species is known to occur in terrestrial habitats but is omitted if that has already been stated in the introductory material or is true of the entire genus. The great majority of amoebae occurring in terrestrial habitats also live in aquatic habitats, except spore-formers such as acrasids.

Most of the culture methods recommended have been used by the author. Media and food organisms are indicated in the notes on species by the following abbreviations, which are often combined:

AS: modified Neff's amoeba saline
Chil: *Chilomonas paramecium*
CMA: cornmeal/glucose agar
Colp: *Colpidium striatum*
CP: Cerophyl-Prescott's infusion
CPA: Cerophyl-Prescott's agar
CPAE: the same streaked with *E. coli*.
E+S: soil extract with salts
Ma: Marshall's solution
NN: non-nutrient agar
NNE: the same streaked with *E. coli*
PC: Prescott's & Carrier's solution

PJ: Prescott's & James's solution
PPG: proteose peptone glucose
PY: proteose peptone yeast
r: uncooked polished rice grains
Rh: *Rhodotorula mucilaginosa*
SCGYEM: Chang's serum-casein-glucose-yeast extract medium
Tet: *Tetrahymena pyriformis*

It seemed unnecessary, in view of the size of this work, to repeat drawings of 'other species' which had been published in the previous key (Page, 1976), to which the reader is referred if the diagnostic summary suggests the organism under observation.

Definitions of diagnostic characters, such as nuclear or uroidal structure, will be found under 'Characters for identification'.

KEY TO CLASSES AND SUBCLASSES

Phylum RHIZOPODA Von Siebold, 1845

Amoebae commonly cylindrical and monopodial, usually markedly eruptive, with length to 65 μm (rarely more) but usually smaller; rarely flattened, with a few short, fine subpseudopodia; usually uninucleate; flagellate stages in many; cyst-forming, a few spore-forming; mitochondrial cristae discoid; mitosis intranuclear, in most with binary division of nucleolus. ***Heterolobosea***, p. 31

Monopodial, thickly cylindrical or ovoid, sluggish, multinucleate amoebae, up to more than 2 mm long but often much smaller; occasionally binucleate smaller amoebae; feeding on algae and cyanobacteria; all but smallest usually containing mineral particles; no separate flagellate stage but rather sparse, non-motile, flagellum-like structures distributed over much of surface, discernible with electron microscope; no mitochondria; bacterial endosymbionts; mechanism of nuclear division unknown; in oxygen-poor habitats. ***Caryoblastea***, p. 48

Amoebae from a few micrometres to 2 mm or more long, rarely a small plasmodium; various kinds of lobopodia, often with slender, sometimes filiform subpseudopodia produced from broader, hyaline lobopodium; usually uninucleate, rarely binucleate, sometimes multinucleate; no flagellate stages; mitochondrial cristae tubular; cysts formed in many genera, unknown in others; no spores; mitotic patterns diverse; a heterogeneous group, as are its subclasses. ***Lobosea***

1 Without test; any surface covering more or less uniform over entire body, with no openings. ***Gymnamoebia*** p. 50

— Incompletely enclosed in flexible cuticle or tectum of microscales, with irregular and variable opening through which contact is made with substratum. ***Testacealobosia, Himatismenida, Cochliopodiidae***, p. 102

CLASS HETEROLOBOSEA

PAGE & BLANTON, 1985

Key to orders and families

1 No fruiting bodies (SCHIZOPYRENIDA) **2**

— Fruiting bodies formed (ACRASIDA)* **3**

2 Nucleolus dividing to form polar masses in mitosis (promitosis); amoeboid form cylindrical; flagellate stages in most genera ***VAHLKAMPFIIDAE,*** p. 31

— Nucleolus but not nuclear membrane disintegrating in mitosis; amoeboid form of single known freshwater/soil species commonly flattened, with a few short, fine subpseudopodia; no flagellate stage known ***GRUBERELLIDAE,*** p. 44

3 (1) Cells of sorocarp differentiated into morphologically distinct spores and stalk cells; flagellate stage present or absent ***ACRASIDAE,*** p. 45

— Cells throughout sorocarp essentially all alike; no flagellate stages known ***GUTTULINOPSIDAE,**** p. 45

Keys to genera and species

Order SCHIZOPYRENIDA Singh, 1952

Family VAHLKAMPFIIDAE Jollos, 1917

Reference: Daggett & Nerad (1983); Page (1986); Page & Blanton (1985).

In general, any eruptive limax amoeba without an alternate flattened form should be considered a probable vahlkampfiid. Vahlkampfiid amoebae *usually* move more rapidly (maximum relative locomotive rate >4) and are on the average thicker (mean L/B <3) than other small limax amoebae. The amoeboid forms of the different genera are very similar. Those of one or two genera may be more irregular than others; e.g., *Naegleria* amoebae are sometimes temporarily branched when not very active. The amoebae may trail several uroidal filaments formed by adhesion. Species of several genera have a strong tendency to supernumerary nuclei. All known freshwater/soil vahlkampfiids are cyst-forming, though the ability to encyst sometimes declines after years in culture, especially in the genus *Vahlkampfia*.

Either a promitotic division pattern or the presence of discoid mitochondrial cristae is evidence that a non-sporulating limax amoeba is a vahlkampfiid.

All species without known flagellate stages are classified in the genus *Vahlkampfia*. However, some strains of species known to have a flagellate stage have failed to produce flagellates under laboratory conditions, and strains may also suffer reduction or loss of ability to produce flagellates after several months in culture, a loss known especially for *Paratetramitus, Tetramastigamoeba, Tetramitus* and *Willaertia*. Flagellates should therefore be sought as soon as possible after isolation. Procedures for inducing flagellates are given in Methods of Observation and Study (p. 24). Flagellates are illustrated diagrammatically in Fig. 4.

Flagellates are always formed by transformation of amoebae; cysts always give rise to amoebae, as far as known, though these may then transform into flagellates.

The flagellar numbers given in the keys and descriptions are the usual or common ones; variation occurs. A strain which fails to produce flagellates but conforms fully to the other characters of a

* *Rosculus*, in which no fruiting bodies have been found, is provisionally placed into the Guttulinopsidae (Acrasida), q.v.

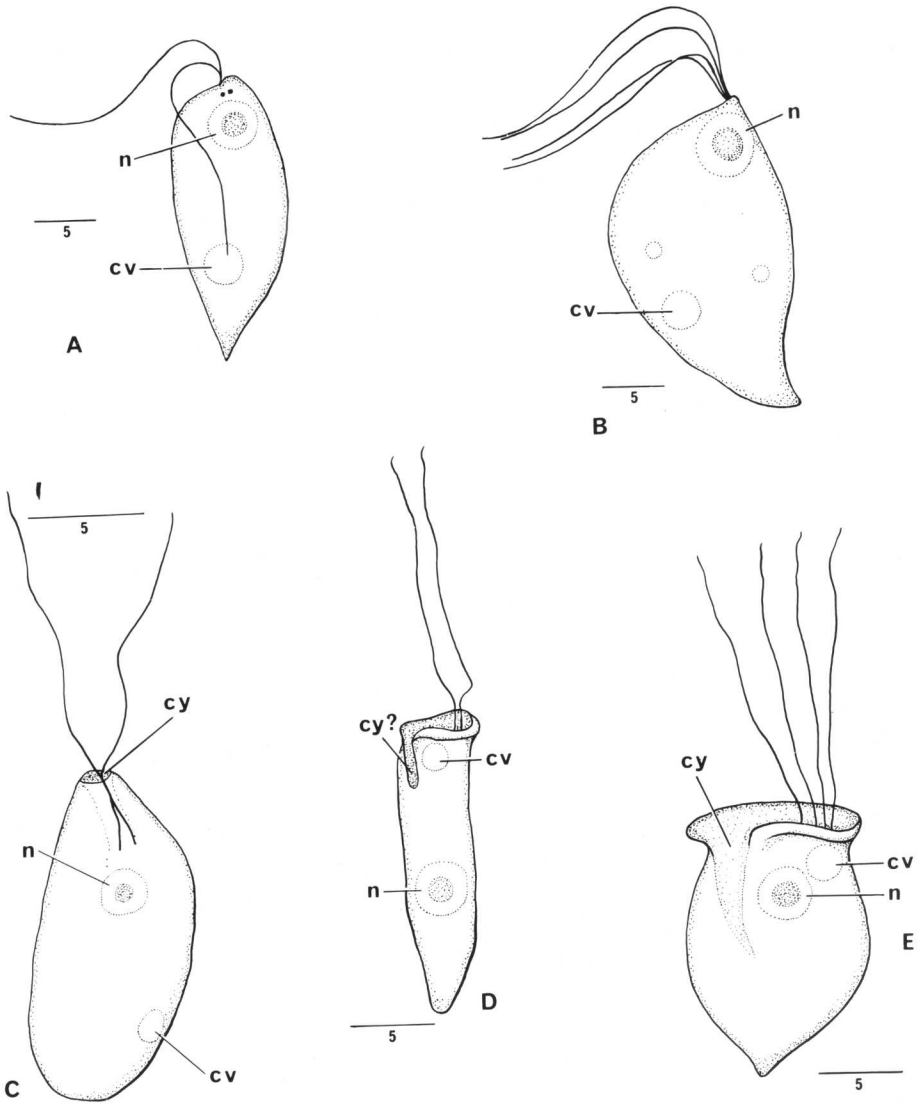

Fig. 4. Flagellate stages of Vahlkampfiidae. A, *Naegleria*. B, *Willaertia* or *Tetramastigamoeba*. C, *Adelphamoeba*. D, *Paratetramitus*. E, *Tetramitus*. *cv*, contractile vacuole; *cy*, cytostome; *n*, nucleus.

well described and distinctive species should be classified, at least provisionally, in that species and genus.

Although differences can, with care and experience, be found amongst amoebae, those differences are seldom distinctive enough for identification. The cyst provides the most useful characters for species identification and in at least two cases is important for generic identification. Attention should be given to any variation in cyst structure within a species. The flagellate is, however, the essential distinction amongst genera having such a stage.

Non-morphological methods of identification have been applied, in this family, principally but not exclusively to the genus *Naegleria*. Because of the difficulty of obtaining flagellates of some genera, the development of such methods is desirable (Daggett & Nerad, 1983).

The names *Vahlkampfia* and Vahlkampfiidae are equivalent to the *Schizopyrenus* and Schizopyrenidae of Singh. The generic name *Protonaegleria* Michel & Raether, 1985, will not be found in the key because it is a junior synonym of *Willaertia*.

1 No flagellate stage ***Vahlkampfia***

— With flagellate stage **2**

2 Cysts with pores; flagellate without cytostome **3**

— Cysts without pores*; flagellate with or without cystostome **4**

3 Biflagellate; division in amoeboid stage only ***Naegleria***

— Quadriflagellate; division in flagellate as well as amoeboid stage ***Willaertia***

4 (2) Biflagellate; with or without division in flagellate as well as amoeboid stage **5**

— Quadriflagellate; division in both amoeboid and flagellate stages **6**

5 Flagellate without anterior rostrum; nucleus anterior, contractile vacuole toward posterior end; no division in flagellate stage reported ***Adelphamoeba***

— Flagellate with anterior rostrum; nucleus not near anterior end, contractile vacuole anterior; division in both amoeboid and flagellate stages ***Paratetramitus***

6 (4) Flagellate with cytostome and rostrum; nucleus and contractile vacuole anterior ***Tetramitus***

— Flagellate without cytostome or rostrum; nucleus anterior, contractile vacuoles elsewhere in cell ***Tetramastigamoeba****

Genus *Vahlkampfia* Chatton & Lalung-Bonnaire, 1912

References: Page (1967a, 1974a); also as for family.

Probably some published identifications of '*Vahlkampfia* sp.' would be better listed as 'vahlkampfiid', and in the older literature the name sometimes has an even vaguer significance. Species identifications are, however, possible. All species in the dichotomous key have been studied with both light- and electron-microscopes. Other species described in recent decades are not included in the dichotomous key because of insufficient information about measurements and cyst structure and will be found under 'Other species'. With the apparent exception of *Schizopyrenus horticolus*, no known *Vahlkampfia* cyst has pre-formed excystment pores.

Taking into account only recent workers using modern procedures, *Vahlkampfia* species have been found in numerous freshwater habitats as well as soil.

1 Cysts with distinct gelatinous coating on smooth wall, shape affected by pressure during encystment, occasionally circular, sometimes with rounded angles, sometimes thickly crescent-shaped, 7-14 μm ($\bar{x}$ 9.7 μm); amoebae 14.5-33 μm long, often with trailing uroidal filaments; nucleus usually 3.4-4.8 μm. (Figs. 5A, B, 6A) ***Vahlkampfia avara*** Page, 1967
(North America. NNE. Cf. *V. atopa.*)

— Cysts without distinct gelatinous coating (may be somewhat sticky) **2**

2 All or nearly all cysts circular or oval in outline **3**

— Cysts circular, oval, reniform, pyriform or irregular in outline **4**

3 Amoebae often elongate and straight, not consistently eruptive, 18-37 μm; nucleus 3.2-4.6 μm; cysts 6.5-13 μm ($\bar{x}$ 9.5 μm), with outer layer partly separated in rare cases. (Figs. 5C, D, 6B) ***V. aberdonica*** Page, 1974
(Europe, terr. NNE.)

— Amoebae consistently eruptive, balling up and changing direction frequently, sometimes with a few trailing filaments, 17-24 μm; nucleus 4.6-8.4 μm; cysts 8.5-17 μm ($\bar{x}$ 10.4 μm), outer wall lifted off one side in a minority, cysts sometimes with vacuolate appearance; multiplies and encysts well at both 23°C and 37°C (Figs. 5E, 6C) ***V. enterica*** Page, 1974
(Europe. Isolated from intestine of turkey but found not pathogenic. NNE.)

4 (2) Amoebae, 30-65 μm, often with prominent collopodium on bulbous posterior end; nucleus 5-6.5 μm; cyst outline circular, oval, reniform, pyriform, usually 12.5-21 μm ($\bar{x}$ 15.4 μm) excluding outer coat, which may be sticky; multinucleate irregularly shaped cysts not uncommon; polar masses with somewhat fragmented appearance at some stages of mitosis. (Figs. 5F, 6D, E) ***V. ustiana*** Page, 1974
(Europe, Antarctica. NNE.)

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* This key is constructed in accordance with the original description of *Tetramastigamoeba* as having 'no obvious pores in the cyst', a report that some workers find questionable.

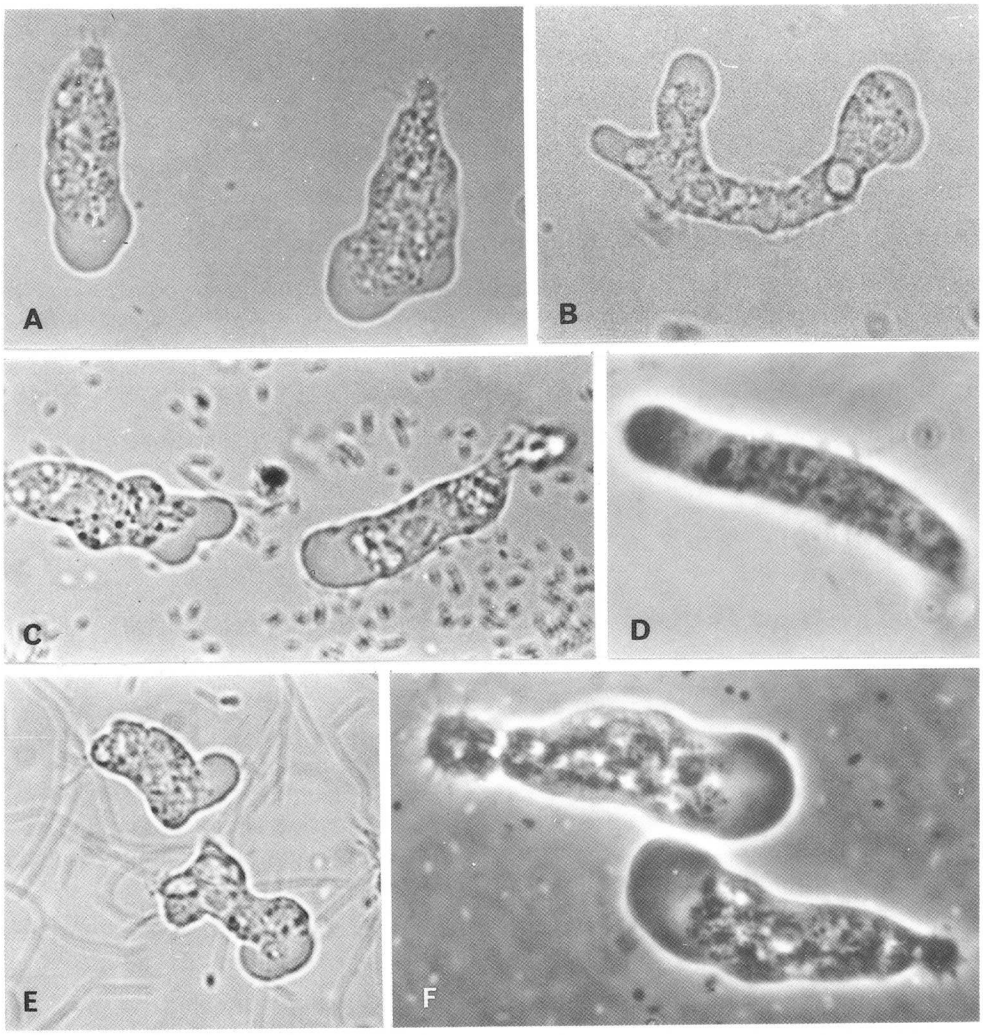

Fig. 5. *Vahlkampfia*, amoebae. A, B, *V. avara*. C, D, *V. aberdonica*. E, *V. enterica*. F, *V. ustiana*, with conspicuous adhesive uroids. (D, which is fixed, ×2,000; all others, ×1,000).

— Cyst outline commonly circular or oval but also reniform, with thin, somewhat sticky outer layer, 8.6-13.2 μm (x̄ 10.9 μm); amoebae commonly with a few uroidal filaments, 18.5-36.5 μm; nucleus 3.4-4.5 μm; polar masses compact, not fragmented. (Fig. 6F)

V. inornata Page, 1967

(North America. NNE)

Other species

Some of these species are probably unrecognisable; others are represented by existing cultures but have not been adequately described.

1. Cyst walls with two distinctly separated layers:

V. vahlkampfi (Chatton, 1910): Type species of the genus but not certainly identifiable, partly because of obvious errors in the original description (as *Amoeba limax*) by Vahlkampf.
Illustration in Page (1976).

V. lobospinosa (Craig, 1912): The original description was based on cultures isolated in the Philippines, possibly containing more than one species. Amoebae attributed to this species have been investigated in the USA, without a re-description of the cyst. The American isolate has a unique isoenzyme pattern. The amoebae appear to be typical vahlkampfiids, often trailing uroidal filaments. The cyst has distinct ectocyst and endocyst, and the ectocyst is thick and scalloped (P.-M. Daggett, personal communication). Measurements of cyst diameter from several sources agree on a mean of 9.4-10 μm (range 7.8-15 μm), but the reported mean lengths of the amoebae vary from

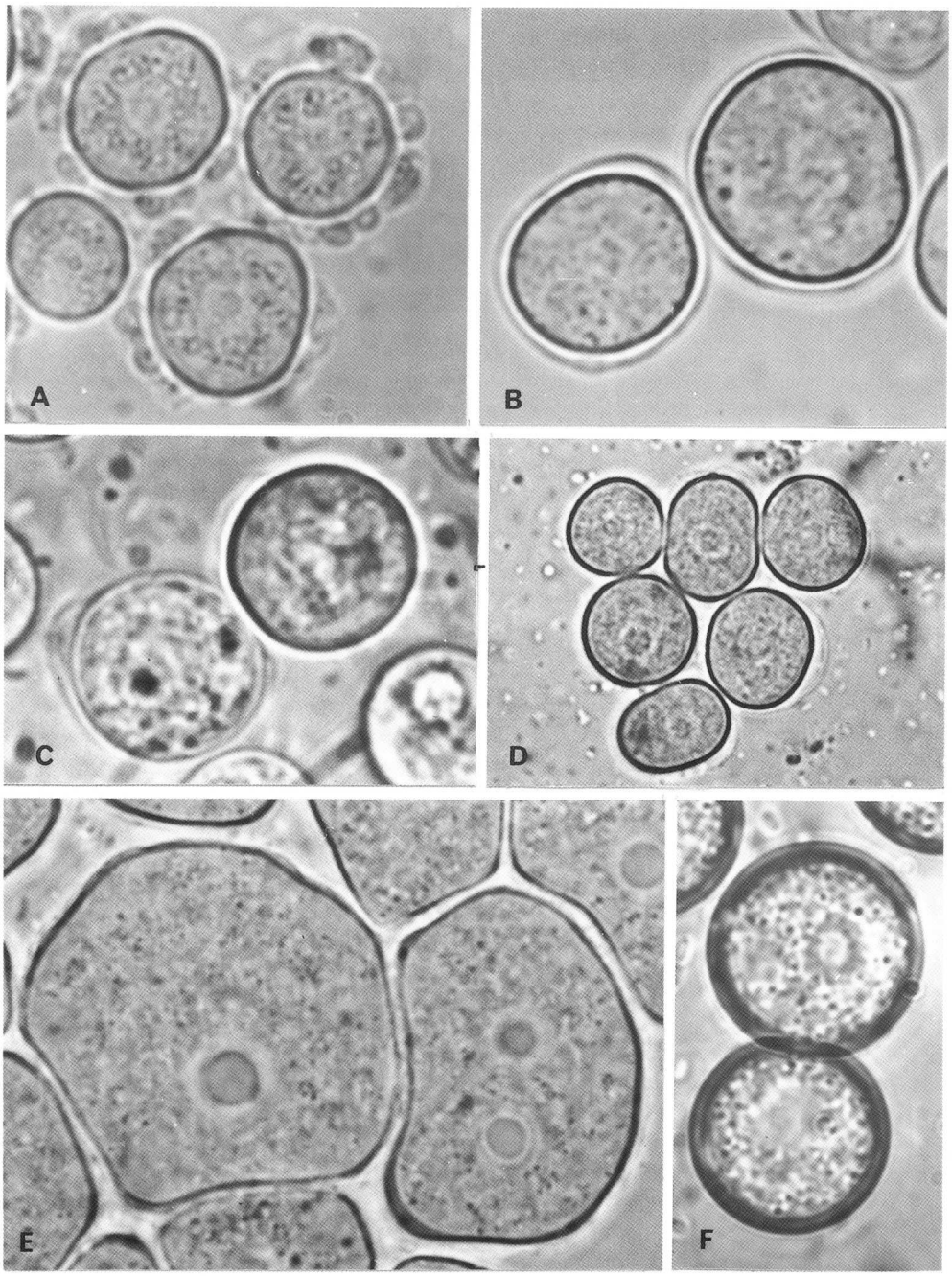

Fig. 6. *Vahlkampfia*, cysts. A, *V. avara*. B, *V. aberdonica*. C, *V. enterica*. D, E, *V. ustiana*. F, *V. inornata*. (D, ×1,000; E, ×2,000; others, ×2,500.)

16-29 μm; nucleus 3.5-5 μm ($\bar{x}$ 4.4 μm). Fizer & Wilhelm investigated extrusible rod-like bodies in their isolate. Grows at 25-45°C; optimal growth at 37°C.

References: Daggett & Nerad (1983); Fizer & Wilhelm (1978); Wilhelm & Anderson (1971).

V. russelli (Singh, 1952): The cyst was originally described and figured (under the name *Schizopyrenus russelli*) as having two concentric walls, with no indication of variation in the outer wall, but Singh & Das reported that the majority of cysts in later cultures had an irregularly wavy outer wall, like that of *Paratetramitus jugosus*. No measurements were reported for the cyst, but illustrations indicate a diameter up to 20 μm; diameter of rounded amoebae 15-23 μm. Anyone isolating an amoeba with such cysts should look for a flagellate stage like that of *P. jugosus*, in view of the possible synonymy. Grows at 37°C or 42°C.

References: Singh (1952); Singh & Das (1970); Singh & Hanumaiah (1979); Page (1986). Illustrations in Page (1976).

V. hartmanni (Nägler, 1911): Cysts often not round, sometimes binucleate (a condition occurring also in other *Vahlkampfia* species), with irregular outer wall, partly separated from inner wall; amoebae rather sluggish, 'size' 10-20 μm.

Illustration in Page (1976)

V. ovis (Schmidt, 1913): Outer cyst wall apparently not wavy but well-separated from inner wall around much of circumference; cyst diameter 8.5-16 μm; 'size' of amoebae 20-35 μm, nucleus 3.5-5 μm.

Illustrations in Page (1976).

Schizopyrenus horticolus Singh & Hanumaiah, 1979: The only *Vahlkampfia* (= *Schizopyrenus*) reported to have an excystment pore. With rare exceptions, a 'hood-like structure plugged with some kind of structureless substance' in outer wall; excystment through a hole at that point. Cyst diameters derived from illustrations *c.* 12-20 μm.

Reference: Singh & Hanumaiah (1979).

2. Cysts with distinct gelatinous covering:

V. froschi (Hartmann, 1907): Cyst spherical, with outer layer of irregular thickness, diameter 7-10 μm; average 'size' of amoebae 8-12 μm.

Illustration in Page (1976).

V. atopa (Singh, 1952): Cyst usually rounded, sometimes irregular, depending on grouping of amoebae during encystment; a single wall covered with a gelatinous layer; cyst diameter to 20 μm; loses ability to encyst after some months in culture; amoebae when rounded up *c.* 15-30 μm; red pigment may be produced in agar. Similar to *V. avara* but reported to be larger. Grows at 37°C but not at 42°C.

Illustration in Page (1976)

3. Cysts without marked separation of wall layers or distinct gelatinous coating:

V. lacustris (Nägler, 1909): Cyst shape irregular, poorly described, 6-8 μm; amoebae seldom extended into elongate form, changing shape rapidly, 'size' 8-15 μm.

Illustration in Page (1976).

V. debilis Jollos, 1917: Cysts spherical, with a single wall, rarely more than 10 μm, often much smaller; amoebae ('diameter' 15-20 μm) advance rapidly.

Illustration in Page (1976).

V. magna Jollos, 1917: Cysts spherical, with a single wall; median diameter 18-20 μm, but cysts of half that size not uncommon; amoebae rather sluggish, 20-40 μm rounded up; grows at 18-20°C.

Illustration in Page (1976)

V. erythaenusa (Singh, 1952): Cyst spherical, with single wall consisting of two layers, the outer rather thick; very variable in size, *c.* 23 μm; amoebae 15-35 μm when rounded up; pink pigment may be produced in agar.

Illustration in Page (1976).

Genus *Naegleria* Alexeieff, 1912; emend. Calkins, 1913

References:

(1) *Culture*: Band & Balamuth (1974); Červa (1977); De Jonckheere (1977); Fulton *et al.* (1984); Haight & John (1980, 1982); Laverde & Brent (1980); Nerad *et al.* (1983).

(2) *Taxonomy*: Daggett & Nerad (1983); De Jonckheere (1981b, 1982, 1987a, 1987b); De Jonckheere & Diericks (1982); De Jonckheere & van de Voorde (1977a); De Jonckheere *et al.* (1974, 1984b); Nerad & Daggett (1979); Page (1975a); Pernin *et al.* (1983, 1985); Pussard & Pons (1979); Stevens *et al.* (1980); Willaert (1976); Willaert & Le Ray (1973).

(3) *Ecology and distribution*: Brown *et al.* (1983); Červa (1978); Červa & Simanov (1983); Červa *et al.* (1980, 1982); De Jonckheere (1979a, 1979b, 1979c); De Jonckheere & van de Voorde (1977b); Delattre & Oger (1981); Derr-Harf & De Jonckheere (1984); Dive *et al.* (1981); Esterman *et al.* (1984a, 1984b); Griffin (1983); John & De Jonckheere (1985); Kadlec *et al.* (1978); Michel & De Jonckheere (1983a, 1983b); Pernin & De Jonckheere (1984); Sykora *et al.* (1983); Tyndall (1985), Umeche (1983).

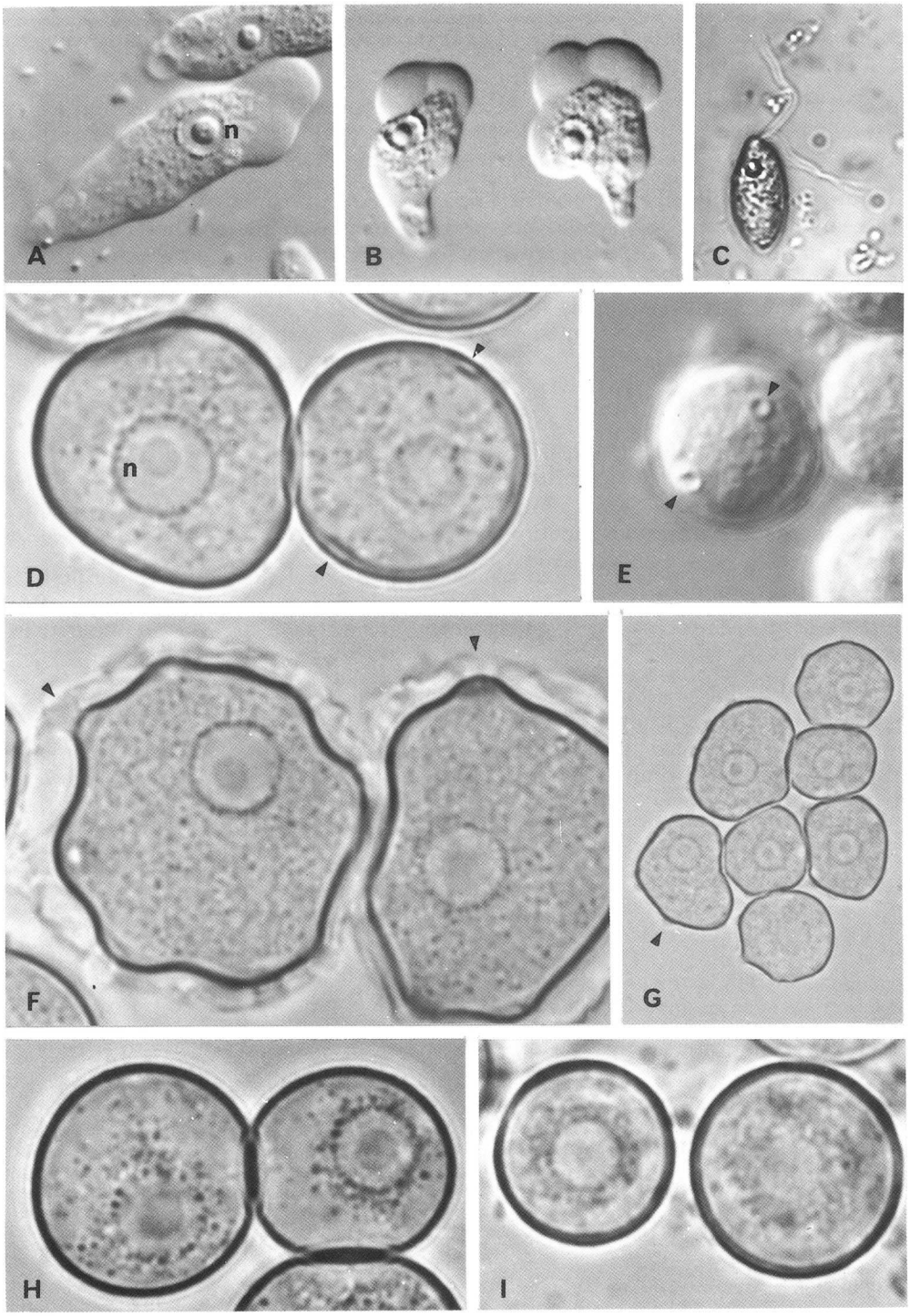

Fig. 7. *Naegleria*. A, B, *N. gruberi*, amoebae. C, *N. gruberi*, flagellate. D, E, *N. gruberi*, smooth cysts; E, surface view with differential interference contrast. F, *N. gruberi*, rough cysts. G, *N. gruberi*, angular cysts with thick plugs. H, *N. jadini*, cysts. I, *N. fowleri*, cysts. (A-C, G, ×1,000; D, F, H, I, ×2,500; E, ×2,000) *n*, nucleus. Arrowheads indicate plugged pores in cyst walls.

(4) *General*: Griffin (1978); John (1982); Schuster (1979).

These are vahlkampfiids whose flagellate stage normally has two flagella, lacks a cytostome, and does not divide. The cysts have plugged pores through which the amoeba excysts.

Naegleria is common in freshwater and terrestrial habitats.

Didascalus thorntoni Singh, 1952, included in this genus by Page (1976), is listed under 'Other

Vahlkampfiidae'. Since 1976 the presence of excystment pores, lacking in *D. thorntoni*, has been recognised as characteristic of the genus.

All species of *Naegleria* can be grown on non-nutrient agar with *E. coli* as well as in axenic culture (references above), at appropriate temperatures (Table 1). However, the cysts of some species (e.g., *N. gruberi*) remain viable much longer than others (e.g., *N. jadini*).

The dichotomous key should be considered no more than a preliminary tool for species identification, though only the first two dichotomies are likely to concern those maintaining their cultures at room temperature (20-22°C). *Any identification below the second dichotomy should not be considered adequate without using tested non-morphological methods* such as those summarised in Table 1 and included in the taxonomic references above. At present, isoenzyme comparisons are the best and most widely applicable method, though newer methods are being developed. Techniques are described in the references for biochemical and immunological methods in Table 1. The review by De Jonckheere (1987a) gives the present taxonomic picture clearly and reproduces zymograms of the species.

In connection with differentiation of the two pathogenic species, *N. fowleri* and *N. australiensis*, De Jonckheere *et al.* (1984b) have cautioned that '. . . species identification of pathogenic *Naegleria* cannot be done solely on the basis of Con A agglutination and/or results of virulence tests. Serological confirmation of species identity is necessary'.

The morphological information on cyst characters in Table 1 does not include pore number. Those who wish to make the plugged pores more easily distinguishable can use the PAT Ag r method of Pussard & Pons (p. 26). Cyst diameters are included in Table 1 rather than in the dichotomous key.

British workers should be aware of the recommendations for handling '*Naegleria* spp' issued by the Advisory Committee on Dangerous Pathogens (ACDP, 1984). Not all British workers agree on the interpretation of those recommendations as they relate to different species of *Naegleria*. Workers in other countries should consult the appropriate authorities on the handling of *N. fowleri* and *N. australiensis*.

1 Grows well at 20°C **2**

— Does not grow well at 20°C; good growth above 40°C **3**

2 Cyst pores with noticeably thickened rim; cyst wall may be smooth, rough, or angular. (Fig. 7A-G) ***Naegleria gruberi*** (Schardinger, 1899)
(Common and widely distributed, even in Antarctica. Morphological and biochemical diversity indicates that this group includes more than one species.)

— Cyst pores without noticeably thickened rim; cyst wall smooth. (Fig. 7H)
N. jadini Willaert & Le Ray, 1973
(Belgium. Not as much studied as other species.)

3 (1) Grows at 45°C; rim of cyst pores not noticeably or only slightly thickened **4**

— Grows at 42°C but not at 45°C; rim of cyst pores noticeably thickened **5**

*4 Agglutinated by concanavalin A; rim of cyst pores often slightly thickened
N. lovaniensis Stevens, De Jonckheere & Willaert, 1980
(Europe, North America.)

— Not agglutinated by concanavalin A; rim of cyst pores not noticeably thickened. (Fig. 7I)
N. fowleri Carter, 1970
(Widely distributed, especially in artificially warmed waters. A lethal pathogen, the cause of acute primary amoebic meningoencephalitis. *N. aerobia* and *N. invades* are junior synonyms.)

5 (3) Agglutinated by concanavalin A ***N. australiensis australiensis*** De Jonckheere, 1981
(Australia, Asia, Europe, North America. Pathogenic to laboratory mice.)

— Not agglutinated by concanavalin A
N. australiensis italica De Jonckheere, Pernin, Scaglia & Michel, 1984
(Europe. More virulent to laboratory mice than is *N.a. australiensis*.)

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* A useful ancillary character for preliminary differentiation of *N. lovaniensis* and *N. fowleri* is that the former grows more vigorously on bacteria at any temperature; on agar plates, the advancing front as the amoebae migrate is more sharply delineated in *N. fowleri* than in *N. lovaniensis* (J. F. De Jonckheere, personal communication).

Table 1. Species of *Naegleria*

+, Positive result; −, Negative result; D, Procedure contributes information of diagnostic value; V, Variable: Procedure gives results which vary from one strain to another of this species or subspecies. The references indicate details of procedures.

Characters	*N. gruberi*	*N. fowleri*	*N. jadini*	*N. lovaniensis*	*N. australiensis australiensis*	*N. australiensis italica*
Growth at: 20°	+		+			
30°	+	+	+	+	+	+
37°	V	+	—	+	+	+
42°	—	+	—	+	+	+
45°	—	+	—	+	—	—
Known pathogenicity: in mice	—	+	—	—	+	+(*)
in humans	—	+	—	—	—	—
Cyst diameter (μm)	Means of strains: 10-16	Range 7-15	x̄13.9	Means of strains: 9.6-13	x̄11.6	
Cyst pores: thickened rim observable with light microscope	+	—	—	Slightly thickened	+	+
Biochemical and immunological methods used to establish taxonomy or to identify:						
Immunofluorescent antibody technique (De Jonckheere *et al.*, 1974; Stevens *et al.*, 1980)	V	D	D	D	**	**
Immunoelectrophoretic analysis (De Jonckheere *et al.*, 1984b; Stevens *et al.*, 1980; Willaert, 1976)		D	D	D	D	D
Isoenzyme comparison (Daggett & Nerad, 1983; De Jonckheere, 1982, 1983; De Jonckheere *et al.*, 1984b)	V	D	D	D	D	D
Total proteins (De Jonckheere *et al.*, 1984b)	V	D		D	V	D
Acid phosphatase and leucine amino peptidase activity (De Jonckheere & Dierickx, 1982)		D				
Agglutination with concanavalin A (De Jonckheere *et al.*, 1984b)	±	—	—	+	+	—
Other characters	Cyst morphology variable (Page, 1975a)			Nucleus enveloped in RER; dictyosome		

* More virulent than *N. a. australiensis*

** Distinguishable from other species but not from the other subspecies of *N. australiensis* by this method.

Genus *Willaertia* De Jonckheere, Dive, Pussard & Vickerman, 1984

References: De Jonckheere *et al.* (1984a); Michel & Raether (1985).

This genus was erected for a species with cysts containing pores like those in the cyst wall of *Naegleria gruberi* but, as then thought, no flagellate stage. Serological and isoenzyme analyses confirmed the distinctiveness of *Willaertia*. A short time later Michel & Raether erected the genus *Protonaegleria* for an organism with similar cysts but a quadriflagellate stage lacking a cytostome and not, as far as their observations showed, dividing in that stage.

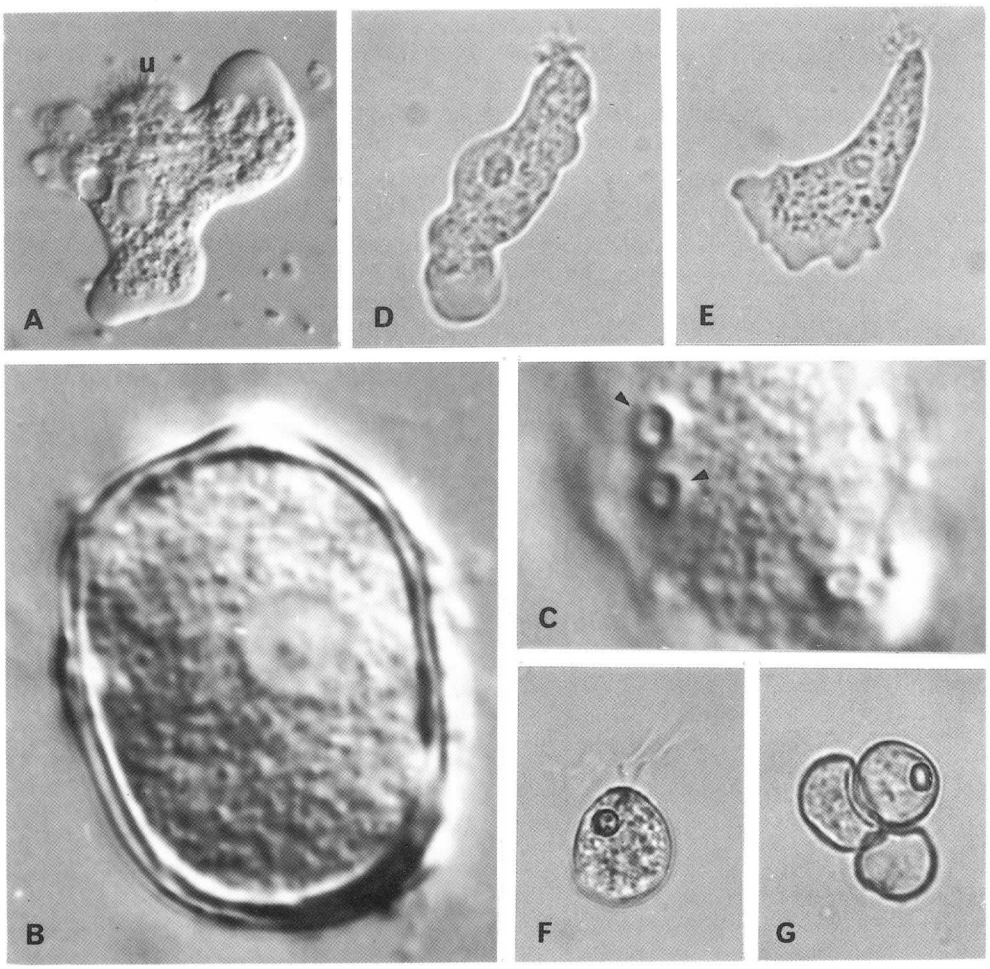

Fig. 8. A-C, *Willaertia magna*; A, amoeba; B, C, cysts, with plugged pores indicated by arrowheads in C. D-G, *Adelphamoeba galeacystis*; D, E, amoebae; F, flagellate; G, cysts. (A, D-G, ×1,000; B, C, ×2,500.) *u*, uroid.

Subsequently, flagellates like those described for *Protonaegleria westphali* have been found in strains of *Willaertia magna*, including one of the original strains. Furthermore, the isoenzymes and restriction enzyme DNA patterns of *P. westphali* and *W. magna* are comparable (De Jonckheere, personal communication). Therefore, though the original diagnosis of *Protonaegleria* was more complete with respect to the flagellate stage than was the original diagnosis of *Willaertia*, the latter name has priority because of its earlier publication date.

There is, however, a possibility that the generic name *Tetramastigamoeba* Singh & Hanumaiah, 1977, is a senior synonym of *Willaertia*. Singh & Hanumaiah found 'no obvious pores' in the cysts of their organism but observed some pore-like structures. A new finding that *W. magna* divides in the flagellate as well as the amoeboid stage (B. Robinson and J. F. De Jonckheere, personal communication) increases the similarity. However, until a strain of *Tetramastigamoeba* can be re-examined and biochemical comparisons with *Willaertia* made, it seems reasonable to recognise both genera.

The descriptions of amoebae and cysts are based on the published reports and on observations of an Australian isolate from Mr Robinson; the photographs were also made from the Australian isolate. The diagram of the flagellate is based on photomicrographs supplied by Dr De Jonckheere.

I am indebted to Dr De Jonckheere for permission to mention the finding of the flagellate stage and the division of that stage, information which had not been published when the present publication was being prepared, in the interest of accuracy of this key. Details will be published by Mr Robinson and Dr de Jonckheere.

In general, *W. magna* strongly resembles a large *N. gruberi*, from which *W. magna* differs in having a quadriflagellate rather than normally biflagellate stage and in undergoing division in the flagellate as well as the amoeboid stage. The pores of the cysts, with a thickened rim, are much like those of *N. gruberi*. There is also a perinuclear layer of globules, as in *N. gruberi*.

W. magna can be grown at temperatures from normal room temperature to 44°C, but both sets of investigators found that it was not pathogenic to mice.

Amoebae up to 100 μm long (possibly with supernumerary nuclei), more often 50 μm or smaller, uroidal filaments prominent; strong tendency to supernumerary nuclei; usually a perinuclear layer of globules; nuclear diameter *c.* 4-7.5 μm; flagellates 19-30 μm long ($\bar{x}$ 22 μm); cysts with distinct, thick inner wall layer, delicate outer layer loosely applied, spherical or polygonal depending on culture conditions, with plugged pores bounded by thickened rim, 18-28 μm ($\bar{x}$ 21-23 μm); specific isoenzyme pattern. (Fig 8A-C)

Willaertia magna De Jonckheere, Dive, Pussard & Vickerman, 1984

(Europe, Asia, Australia. NNE at 20-44°C; some strains adaptable to axenic culture (De Jonckheere *et al.*, 1984).)

Genus *Adelphamoeba* Napolitano, Wall & Ganz, 1970

References: Napolitano *et al.* (1970); as for family.

This genus is morphologically somewhat similar to *Naegleria*. Its amoebae, like those of *Naegleria*, have a tendency to branched, irregular forms when not actively advancing. The flagellate resembles that of *Naegleria* but for possession of a small cytostome (seen with difficulty) and absence of the beak-like anterior projection which often gives *Naegleria* flagellates an asymmetrical appearance. Ultrastructural similarities have also been found. However, there are no pores in the cyst wall, and the amoebae transform to flagellates less readily than do those of *Naegleria*.

Given the proportionately small number of flagellates produced by the one species and the difficulty in discerning the cytostome of the flagellate, a synonymy with *Didascalus* Singh, 1952 (see 'Other Vahlkampfiidae') must be considered possible, but the described species of each genus appear to have very different cysts.

This genus does not appear to have been found in the many samples of freshwater taken over the past 10 to 15 years, but it might be mistaken for a *Naegleria* or, in the absence of flagellates, a *Vahlkampfia*.

One species:

Amoebae 12-30.5 μm, sometimes drawn out and branched, but with more compact monopodial form in active locomotion; flagellate 9-19 μm, oval to pyriform, with nucleus anterior; cysts appear spherical or pushed in on one side, producing helmet-like shape, 8-11 μm (Fig. 8D-G)

Adelphamoeba galeacystis Napolitano, Wall & Ganz, 1970

(North America; terr. NNE.)

Genus *Paratetramitus* Darbyshire, Page & Goodfellow, 1976

References: Darbyshire *et al.* (1976); Read *et al.* (1983); as for family.

The essential morphological difference from *Tetramitus* in flagellate structure is that *Paratetramitus* is normally biflagellate. In addition, the flagellate is more elongate, and the nucleus is far removed from the base of the flagellar apparatus, an unusual character in vahlkampfiid flagellates. The flagellate, like that of *Tetramitus rostratus*, has a conspicuous lip-like rostrum over the flagellar insertion. An extra pair of flagella often has its own rostrum. Whether the flagellate has a cytostome is uncertain; flagellates have not been seen to ingest food.

Division occurs in the flagellate as well as the amoeboid phase. The ability to transform to the flagellate stage is usually lost after some months in culture, and efforts to induce flagellates failed with a few strains which were otherwise indistinguishable from those which produced flagellates.

The cyst wall has no pores; excystment is through a hole digested or burst in the wall.

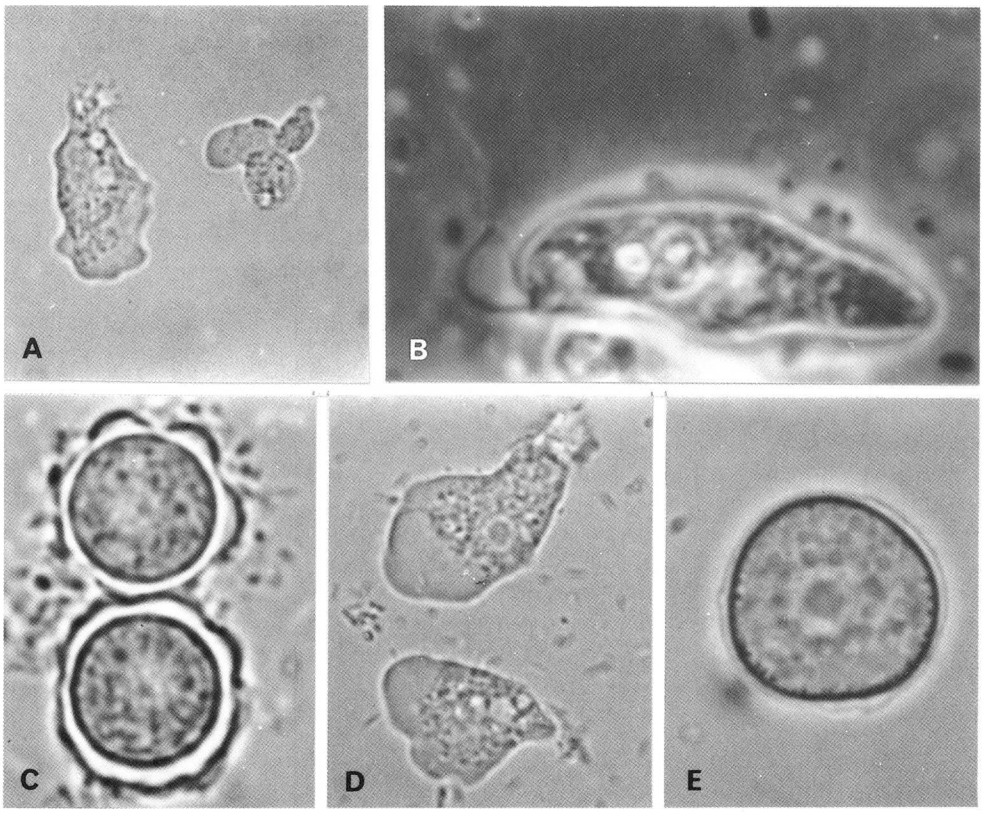

Fig. 9. A-C, *Paratetramitus jugosus*; A, amoebae; B, flagellate, with flagella and presumed cytostome at left; C, cysts. D, E, *Tetramitus rostratus*; D, amoebae; E, cyst. (A, D, ×1,000; B, C, E, ×2,500.)

Daggett & Nerad (1983) found a single zymogram pattern for strains from widely separated sources.

The single species is widely distributed in Europe and North America, in both freshwater and soil. Isolation of this species from Laguna Figueroa, a hypersaline lagoon in Mexico, led to the discovery that strains from freshwater and soil were also euryhaline.

The possible synonymy of this species with *Schizopyrenus russelli* Singh, 1952, has been pointed out (Page, 1986); see also *Vahlkampfia russelli* (p. 35).

Amoebae 13-38 μm long ($\bar{x}$ 22 μm); nuclei 2.8-4.0 μm; cysts with wall often separated into two layers, with outer layer commonly thrown into irregular waves or mounds, often with scalloped appearance, but not wrinkled; mean cyst diameters of many strains 7.5-11.8 μm; flagellates usually 14-19 μm long, with length normally about 4 × breadth; flagella usually equal in length, about as long as normally extended cell (Fig. 9A-C)

Paratetramitus jugosus (Page, 1967)

(NNE)

Genus *Tetramitus* Perty, 1852

References: Balamuth *et al.* (1983); Balamuth & Outka (1962); Fulton (1970); Schuster (1979); as for family.

Despite the great increase in sampling for amoebae and amoeboflagellates, especially vahlkampfiids, over the past two decades, amoebae belonging to this genus are rarely reported. Flagellates probably belonging to this genus are sometimes seen in collected material, and some species may lack an amoeboid phase. One reason for the apparent rarity of isolations may be the relative difficulty in obtaining flagellates from amoeboid cultures, leading to incorrect identifications as '*Vahlkampfia* sp.' Reduction or loss of ability to produce flagellates after culture on agar for a year or more has been reported.

The cyst has no excystment pores.

One non-marine species with amoeboid, flagellate, and encysted stages well described:

Amoebae 16-30 μm long, sometimes spreading anteriorly to a markedly triangular form; flagellate 14-18 μm, with 4 equal flagella and a cytostomal groove which is broad anteriorly, with a distinct lip-like rostrum; cysts 6-18 μm ($\bar{x}$ *c.* 11.5 μm), spherical or ovoid, with a smooth wall from which an outer layer is separated at some points. (Fig. 9D, E)

Tetramitus rostratus Perty, 1852

(Europe, North America, Asia; terr. NNE. Axenic culture: Balamuth & Outka (1962).)

Genus *Tetramastigamoeba* Singh & Hanumaiah, 1977

References: Singh & Hanumaiah (1977, 1979).

The flagellate stage has 4 flagella and no cytostome, with division in the flagellate as well as the amoeboid stage. Successive divisions produce smaller flagellates, which either die or transform to small amoebae. The ability to produce flagellates is greatly reduced after 6-12 months in culture.

Although Singh & Hanumaiah reported, 'There are no obvious pores in the cyst' and 'no preformed pore is present', their description and figures leave some uncertainty. The possibility that *Willaertia* is a junior synonym of *Tetramastigamoeba* has been mentioned under the former genus. The present separate treatment of these genera is based on the assumption, badly in need of further investigation, that *Tetramastigamoeba* does not have excystment pores. The need for biochemical comparison and for careful morphological re-examination of *T. hoarei* is obvious.

Information on the single species attributed to this genus is inadequate for a complete description, since no measurements were reported except for rounded up amoebae. The other measurements given below are derived from published illustrations. These organisms have not been examined with the electron microscope.

Singh & Hanumaiah found indications that this species is mildly pathogenic to mice.

Rounded amoebae *c.* 35-60 μm; some tendency to supernumerary nuclei; flagellate rigidly oval, with anterior nucleus and 2-5 contractile vacuoles; cysts with single wall consisting of 2 layers, mostly rounded to irregular in outline, *c.* 23-32 μm; smaller cysts produced by small amoebae resulting from flagellate division spherical, *c.* 12 or 13 μm

Tetramastigamoeba hoarei Singh & Hanumaiah, 1977

(Asia. NNE at 42°C.)

Other Vahlkampfiidae

Didascalus thorntoni Singh, 1952: In the previous key (Page, 1976), this species was classified as a *Naegleria*, but it is now clear that possession of excystment pores (then uncertain for some species of *Naegleria*) is a generic character of *Naegleria*. *D. thorntoni* produces flagellates sparingly, a few out of hundreds of cells in 20-40 hours at 20-21°C; these are biflagellate cells apparently much like those of *Naegleria*. No perinuclear layer of globules has been reported for *D. thorntoni*. The cysts are smooth-walled (*c.* 11-21 μm), with a fairly thick gelatinous coating. Found in Europe, Asia, possibly North America. Grown on NN with bacteria. One strain grew at 42°C but was found non-pathogenic to mice. Since this species is in culture, a more precise morphological description and biochemical results can be expected to determine the validity and relationships of the genus.

References: Singh (1952); Singh & Das (1970); Singh & Hanumaiah (1979); illustration in Page (1976).

Trimastigamoeba philippinensis Whitmore, 1911: According to the original description of an organism isolated in Manila, the flagellate stage normally had 3 flagella (one figure showing 4), and no indications of multiplication in that stage were seen. The cyst was round or oval, with 2 wall layers, the outer one of which was usually lost in fixation; excystment was through a hole burst in the wall. Whitmore gave the cyst diameters as 8-14 μm, the lengths of the flagellates as 16-22 μm. Bovee, after studying an American isolate which he attributed to this species, reported that the flagellate normally had 2 pairs of flagella, with their bases in a long, tube-like invagination of the anterior end, and that division occurred in the flagellate as well as the amoeboid stage. There is no doubt that the organisms studied by both these workers were vahlkampfiids; Whitmore showed promitosis, and Bovee described typically vahlkampfiid amoebae (30-40 μm long in rapid locomotion).

References: Whitmore (1911); Bovee (1959).

Family GRUBERELLIDAE Page & Blanton, 1985

Reference: Page & Blanton (1985)

The essential difference between Gruberellidae and Vahlkampfiidae is the mitotic pattern. Gruberellidae do not have the classic promitosis because the nucleolus distintegrates in mitosis, which otherwise is like that of vahlkampfiids, with an intranuclear spindle. Only one genus is known from freshwater.

Genus *Stachyamoeba* Page, 1975

References: Page (1975b, 1987b).

Locomotive form usually flattened, expanded, with irregular, sometimes flabellate or spatulate shape, with a few short, fine subpseudopodia produced from a hyaloplasmic lobe; sometimes cylindrical (limax), with no discernible subpseudopodia; eruptive activity in either form. Uninucleate. Cyst wall without pores.

The discovery of discoid mitochondrial cristae gave greater significance to other characters resembling those of the Schizopyrenida. It also demonstrated that fine subpseudopodial projections with a filamentous core can occur in this order.

A single known species, reported so far only from soil in Scotland. The cysts must be distinguished from those of *Paratetramitus* and *Acanthamoeba*.

L of flattened amoebae in locomotion *c.* 15-33 μm ($\bar{x}$ 22 μm); fixed nucleus 2.8-4.6 μm ($\bar{x}$ 3.5 μm), with nucleolar material parietal, in 1-3 lobes; almost always one large lipid droplet, *c.* 4 μm, and smaller ones; cysts usually spherical, sometimes reniform or crescentic from pressure during encystment, with smooth inner wall and outer wall raised into many peaks, without pores or opercula, diameter 8.4-13.0 μm ($\bar{x}$ 9.9 μm). (Fig. 10)

Stachyamoeba lipophora Page, 1975

(Europe; terr. NNE.)

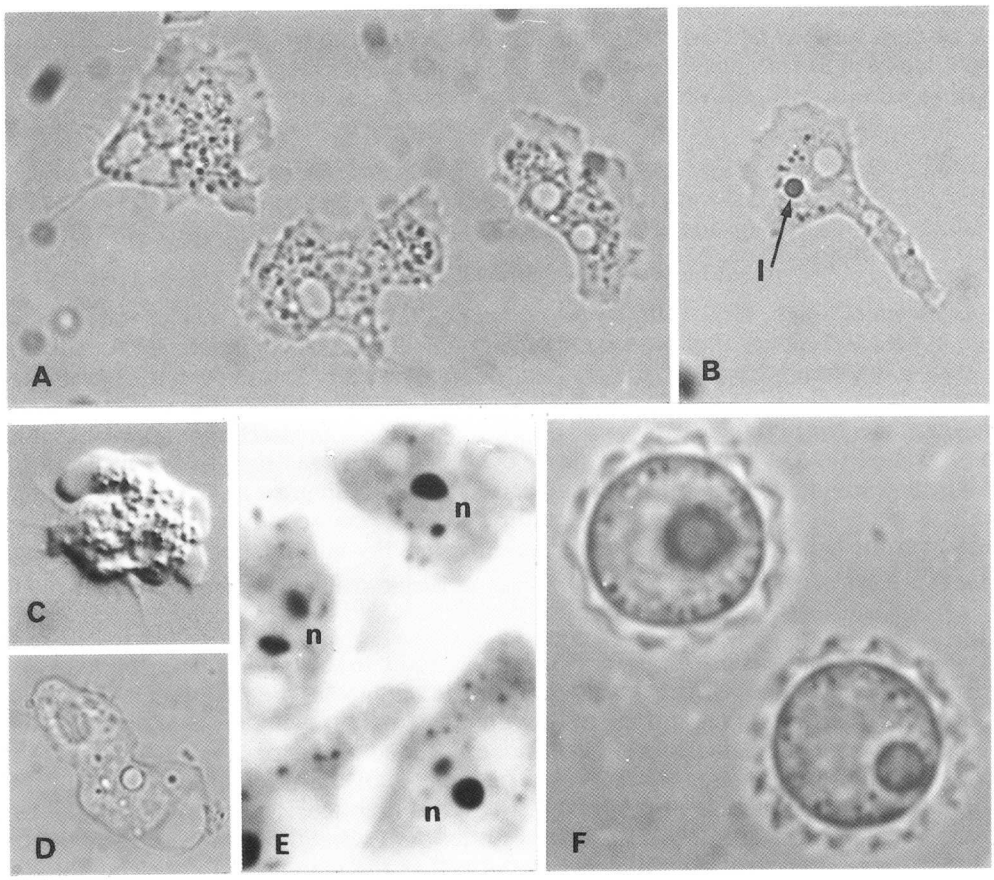

Fig. 10. *Stachyamoeba lipophora*. A-D, amoebae; D, limax form. E, haematoxylin preparation showing three nuclei with parietally arranged nucleolar material (nuclear membranes not distinct). F, cysts. (A-D, ×1,000; E, F, ×2,500.) *l*, lipid globule; *n*, nucleus.

Order ACRASIDA Schröter, 1886; emend. Page & Blanton, 1985

References: Dykstra (1977); Olive (1970, 1975); Page & Blanton (1985).

These are the first 'slime moulds' whose close relatives amongst non-sporulating amoebae were identified. They are true rhizopods, not fungi, though usually collected as fruiting bodies. Unlike the Dictyosteliida, acrasid amoebae do not show co-ordinated streaming before sporulation, nor do the aggregates migrate in a slug-like manner.

The order as here conceived is somewhat narrower than that of Olive, since the Copromyxidae are eliminated by their ultrastructure. The Guttulinopsidae are retained, as indicated by their ultrastructure (discoid mitochondrial cristae), though their position is subject to further investigation (R. L. Blanton, personal communication).

Family ACRASIDAE Van Tieghem, 1880; emend. L. S. Olive, 1970

References; As for order.

This, the better known of the two families, might be considered more advanced, since the sorocarps are more highly differentiated. They are usually collected as fruiting bodies from bark or other dead plant parts which remain attached to the plant. The amoebae are morphologically indistinguishable from those of vahlkampfiids but have a pinkish to orange colour, found also in the cytoplasm of other stages. During mitosis the nucleolus may form polar masses or disintegrate. The flagellates, when present, have 2 flagella of equal length.

Cultures should have a normal day/night photoperiod. Culture dishes should not be closed with clingfilm, because high humidity is detrimental. For the same reason, cultures should not be kept in closed tubes. Strains sometimes lose the ability to sporulate.

1 Spores produced in chains, either simple or branched ***Acrasis***

— Spores produced in a terminal sorus ***Pocheina***

Genus *Acrasis* Van Tieghem, 1880; emend. L. S. Olive, 1970

References: Page (1978a); others as for order.

One well-described species, collected from dead plant parts:

Spores spherical, with smooth walls and spore connection scars, in simple or branched chains; amoebae 25-65 μm long ($\bar{x}$ 43 μm); microcysts mostly spherical, usually 9.5-20 μm, sometimes larger. (Fig. 11A-C) ***Acrasis rosea*** Olive & Stoianovitch, 1960

(Cosmopolitan. CMA/Rh.)

Other species:

Acrasis granulata Van Tieghem, 1880: Although this is the type species, it has not been reported since the original finding on beer yeast. The spores, brownish violet, with minutely punctate walls, were produced in simple chains. The form of the amoebae is not known.

Genus *Pocheina* Loeblich & Tappan, 1961

References: Olive *et al.* (1983); others as for order.

Both species have been found on bark. Culture methods are given by Olive *et al.*

1 Spores produce only amoeboid cells on germination; polar masses in mitosis. (Fig. 11D, E) ***Pocheina rosea*** (Cienkowsky, 1873)

(Europe, North America.)

— Spores typically produce biflagellate cells (2 per spore), as well as some amoebae; no polar masses in mitosis

P. flagellata Stoianovitch, Olive & Bennett, 1983

(North America. The only 'cellular slime mould' known to have flagellate cells; flagellates transform to amoebae after *c.* 24 hours.)

Family GUTTULINOPSIDAE L. S. Olive, 1970

References: Olive (1965); Raper et al. (1977); others as for order.

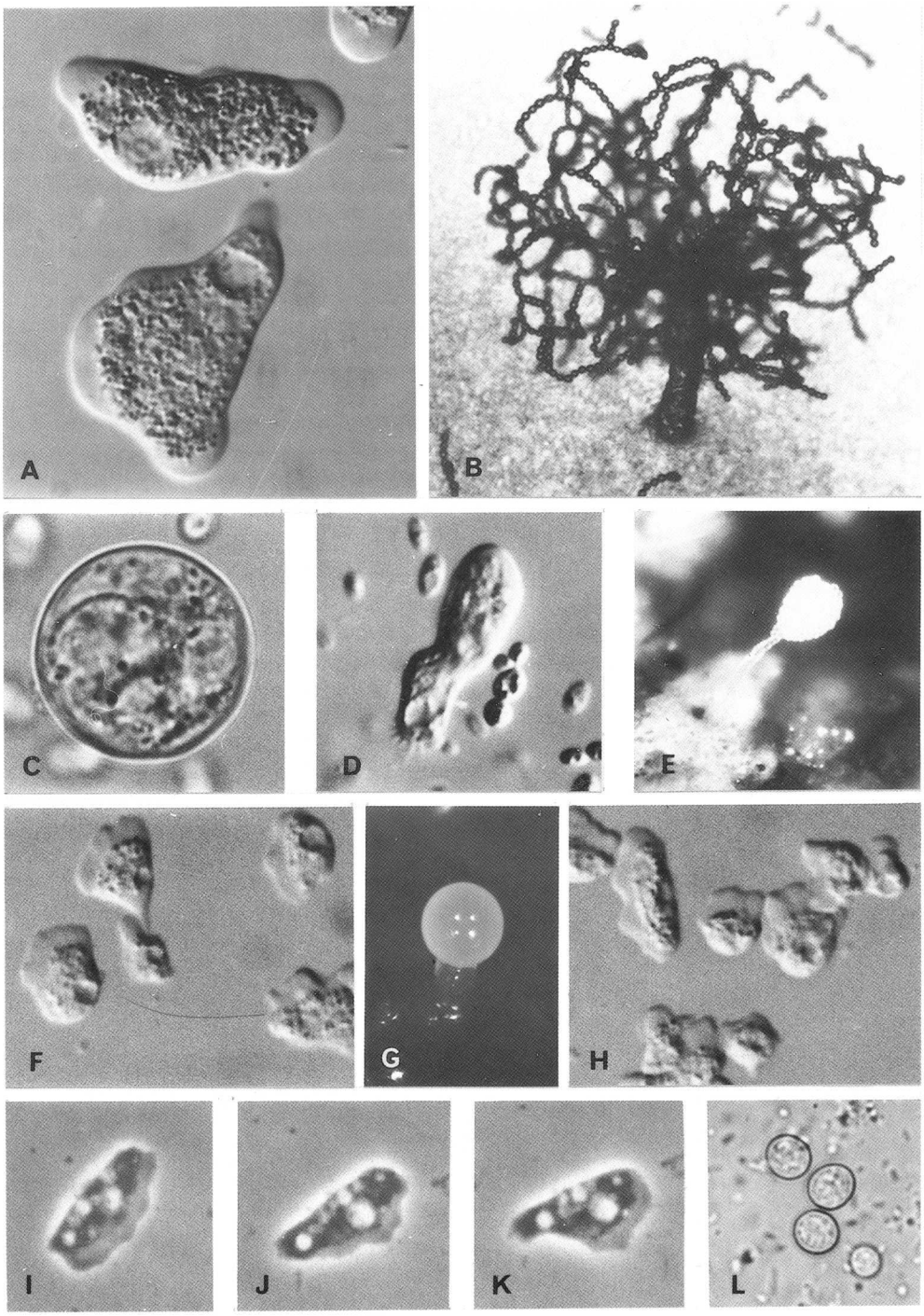

Fig. 11. Acrasida. A-C, *Acrasis rosea*; A, amoebae; B, fruiting body (sorocarp); C, cyst. D, E, *Pocheina rosea*; D, amoeba; E, sorocarp, F, G, *Guttulinopsis vulgaris*; F, amoebae; G, sorocarp. H. *Guttulinopsis nivea*, amoebae. I-L, *Rosculus ithacus*; I-K, change of form of a single amoeba; L, cysts. (A, D, F, H-L, ×1,000; B, E, G, ×105; C, ×1,575.) B, D-H by Dr R. L. Blanton.

Unlike Vahlkampfiidae and Acrasidae, the amoebae of this family are generally flattened and expanded, rather than limax, with rapid and frequent changes of shape. They may be triangular in outline, or broader and somewhat flabellate, or asymmetrical, always with an anterior hyaloplasm which may become temporarily divided into two lobes.

Only the genus *Guttulinopsis* is known to belong to this family. The position of the genus within the Heterolobosea is established by the mitochondrial structure of *G. vulgaris* and of an 'undescribed acrasid cellular slime mold' (Dykstra, 1977) subsequently identified as *G. nivea* (R. L. Blanton, personal communication). The genus *Rosculus* is also included here, for reasons given below.

Genus *GUTTULINOPSIS* E. W. Olive, 1901

References: As for family.

1 Sorocarps without constant, distinct stalk; amoebae generally 10-20 μm in greatest dimension; spores often irregular, commonly 5.5-7.5 μm; no microcysts known; consistent fruiting only in presence of dung and mixed bacterial flora. (Fig. 11F, G)
Guttulinopsis vulgaris E. W. Olive, 1901

(Cosmopolitan; on dung, especially of horse and cow. Culture methods: Olive (1975); Raper *et al.* (1977).)

— Sorocarps with definite stalk flared at base; amoebae commonly 8-15 μm in greatest dimension; spores globose to irregular, majority 4.5-6.0 μm; microcysts produced; fruits abundantly on agar with *E. coli* or *Klebsiella pneumoniae*. (Fig. 11H)
G. nivea Raper, Worley & Kessler, 1977

(Central America, Java; dung, forest litter, soil. Culture method: Raper *et al.* (1977).)

Other species:

G. stipitata Olive, 1901, and *G. clavata* Olive, 1901, were described from dog dung but have not been reported since the original descriptions. Their generic positions are therefore uncertain.

Genus *Rosculus* Hawes, 1963

Reference: Page (1974b).

These amoebae are indistinguishable in form and activity from those of *Guttulinopsis* and have discoid mitochondrial cristae (unpublished). However, no fruiting bodies have ever been seen in culture. This genus could therefore be defined as non-fruiting *Guttulinopsis*, but its exact relationship to *Guttulinopsis* is still under investigation (R. L. Blanton, personal communication).

The strains classified in this genus are grown on non-nutrient agar streaked with *E. coli*. The ability to encyst has usually been lost in a few months after isolation. Because the amoebae are voracious and soon consume the bacteria, strains are easily lost.

Originally described from the rectum of *Natrix natrix* in England, the single species has also been found in freshwater and forest litter in England and on maize and in the human throat in North America. The throat isolate was found to be non-pathogenic.

Greatest dimension of locomotive forms usually 5-17 μm, sometimes more when greatly expanded, $\bar{x}$ 7-10 μm in different strains; nucleus, $\bar{x}$ 1.9-2.4 μm; cysts smooth, oval or spherical, 3.5-8.8 μm. (Fig. 11I-L)
Rosculus ithacus Hawes, 1963

(Europe, North America; terr. NNE.)

CLASS CARYOBLASTEA

MARGULIS, 1974

Use of this name is recognition of its chronological priority and does not imply acceptance of the hypothesis that *Pelomyxa* is a relic primary heterotroph or that a mitotic process is lacking.

Order PELOBIONTIDA Page, 1976

With the characters of the class.

Family PELOMYXIDAE Schulze, 1877

With the characters of the order.

Genus *Pelomyxa* Greeff, 1874

References: Andresen (1973); Andresen *et al.* (1968); Chapman-Andresen (1978, 1982); Chapman-Andresen & Hamburger (1981); Griffin (1979); Page (1981a).

It is now fairly certain that this genus contains a single species and that all amoebae attributed to the genus *Pelomyxa* are either (1) forms of *Pelomyxa palustris* or (2) members of other genera. Attribution of *Chaos carolinense* and other species of *Chaos* to the genus *Pelomyxa* has been indefensible for at least 20 years.

Pelomyxa palustris is usually sought and found in polysaprobic habitats such as the bottom mud of ponds and ditches and is considered a micro-aerobic organism, a description that must be somewhat modified, as suggested below.

Amoebae of this species are commonly cylindrical or ovoid and more or less sluggish. They exhibit, at least in the mature stage, a distinct, bidirectional fountain streaming of the cytoplasm

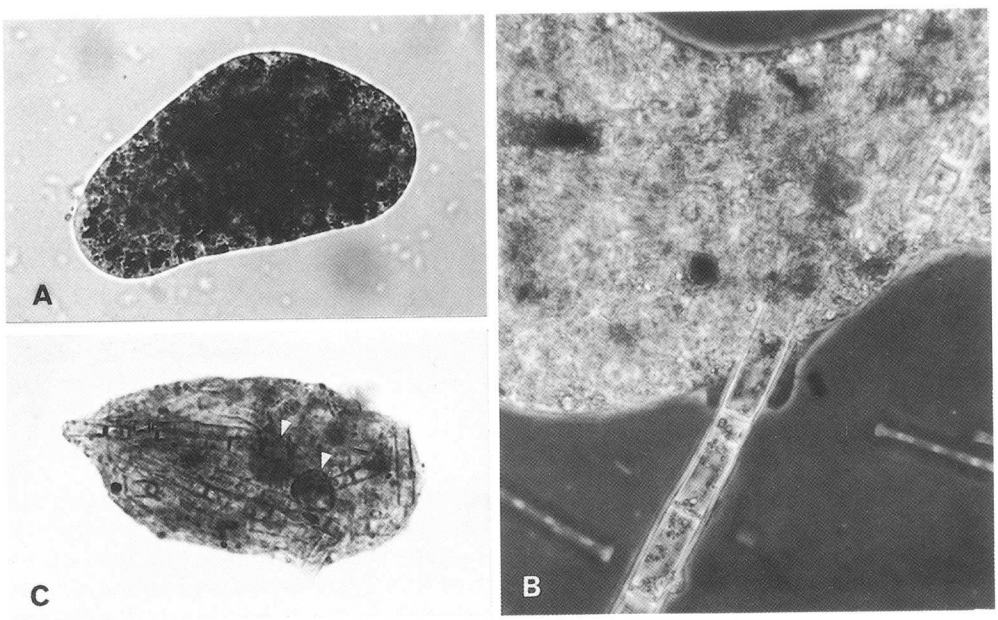

Fig. 12. *Pelomyxa palustris*. A, a rather small but mature multinucleate form; B, ingestion of an algal filament; C, a binucleate form (nuclei indicated by arrowheads), with many ingested algal filaments. (A, C, ×250, B ×300). B by Dr B. J. Finlay. C from preparation by Penard.

and often have short villi or adhesive filaments on a broadly rounded uroid. They sometimes produce over much or part of their periphery short, conical, hyaline subpseudopodia with no known locomotive or feeding function. Their diet is made up of non-motile algae, cyanobacteria, and sometimes other plant matter; they have never been known to ingest motile protozoa.

The glycocalyx appears to be thickest towards the posterior end but does not have the structure seen in the Amoebidae. Scattered over the amoeba are sparse, non-motile, flagellum-like structures, deviating somewhat ultrastructurally from the usual eukaryote flagellum. *Pelomyxa* lacks mitochondria and dictyosomes. It contains 3 species of endosymbiotic bacteria, some at least of which may carry out aerobic metabolism; production of methane has also been attributed to some of them (Van Bruggen *et al.*, 1983). Although contractile vacuoles have been reported in some 'species' of smaller *Pelomyxa*, they do not exist at least in the common large grey type. A few, very rare bipyramidal crystals have been reported in smaller forms, but *Pelomyxa* does not produce triuret crystals as seen in the Amoebidae. On the other hand, it often contains glycogen bodies. The most striking inclusions of the common large grey forms, and one of the characters making rapid identification possible, are the many mineral grains. These are absent from less mature types.

The nuclei vary in size and structure in the various forms of the amoeba and to some degree in the large grey ones but in these latter are usually spherical with many nucleolar pieces. Although the statement that *Pelomyxa* lacks mitosis is often repeated, no other method of nuclear division has been demonstrated.

Pelomyxa has never been brought into such relatively simple, controllable cultures as other amoebae. Much work has been done on organisms from reliable sources such as a pond in Copenhagen.

In using these descriptions, the great variability of multinucleate amoebae in size and nuclear number should be kept in mind. This description of the stages of the single species, *Pelomyxa palustris* Greeff, 1874 (Fig. 12), incorporates the life history described by Chapman-Andresen.

Stages of ***Pelomyxa palustris*** Greeff, 1874:

(1) *Large grey type*: Maximum length in locomotion *c.* 200 μm (sometimes less) to 5000 μm, majority not above 1200 μm. Sluggish; thick, with L/B usually less than 3.0. Nuclei numerous, to about 400; diameter 8-16 μm, commonly *c.* 12 or 13 μm. Many mineral grains, few or no glycogen bodies. Microaerobic; intolerant of oxygen above about 4%, apparently can survive fully anaerobic conditions. A stationary or intermediate phase, which may divide to form cysts or small binucleate amoebae or may return to the growth phase. The form to which the name *P. palustris* is universally applied.

(2) *Black type*: Smaller, probably derived from the large grey type by plasmotomy. Also a form to which the species name has usually been applied.

(3) *Binucleate type* (Fig. 12C): Formed by plasmotomy of large grey type after expulsion of food vacuoles and mineral grains, pear-shaped. Known to occur in the spring. Large oval nuclei, diameter *c.* 50 μm in living amoebae; occasionally uninucleate. No mineral grains or glycogen bodies. The form which Penard investigated under the name *P. binucleata* (Gruber, 1885), for which he reported an average length of 200 μm and a nuclear diameter of 30-33 μm.

(4) *Small light type*: Sometimes apparently the product of growth of the binucleate type, at other times an early stage after excystment. Few nuclei, no mineral grains, variable number of glycogen bodies. Possibly equivalent to *P. belevskii* Penard, 1893, (1-12 nuclei, averaging 25 μm), or *P. caulleryi* Hollande, 1948.

(5) *Large light type*: Derived from above by increased nuclear number and body size. Many glycogen bodies, no mineral grains. The growth stage, more active than the grey type, with lively cytoplasmic streaming and often a pronounced uroidal differentiation. Tolerant to an atmospheric level of oxygen, though it survives longer at lower concentration; does not survive in pure nitrogen. Thus fully aerobic. Apparently passes into large grey type with physiological changes to O_2-intolerant condition and acquisition of mineral grains.

(6) *Cysts*: Formed by expulsion of food vacuoles and mineral grains from large grey type, followed by plasmotomy. Four nuclei, each with diameter *c.* 30 μm. Viable at least 7 years.

Pelomyxa viridis Bourne, 1891, found in India, is of uncertain generic and specific status. Bourne stated that it could spread out flat to as much as one-third of an inch in diameter and that a large individual might contain 10,000 nuclei. He said that the cytoplasm contained chlorophyll-bearing spherules, which were more fluid than are chloroplasts, as well as bacteria. Whether this was a *Pelomyxa* containing zoochlorellae or simply feeding on algae cannot be determined.

CLASS LOBOSEA

CARPENTER, 1861

Subclass GYMNAMOEBIA HAECKEL, 1862

Key to orders and families

1 Locomotive forms cylindrical, subcylindrical, or flattened; if flattened, with a regular outline, not numerous short subpseudopodia; subpseudopodia, if present, not furcate; trailing uroidal filaments absent, with very rare exceptions; not markedly eruptive; complex differentiations of glycocalyx in most (EUAMOEBIDA) **2**

— Locomotive forms usually more or less flattened, sometimes irregular; some also with more active forms subcylindrical, either monopodial or polypodial; frequent changes of shape in most; sometimes eruptive; often with subpseudopodia, which are often furcate; glycocalyx usually thin and amorphous **7**

2 With no subpseudopodia **3**

— With subpseudopodia **6**

3 Cylindrical or subcylindrical **4**

— Flattened **5**

4 Commonly polypodial, except 2 monopodial genera; length usually more than 75 μm, often several 100 μm; floating form usually with similar but thinner pseudopodia; uni- or multinucleate; in majority, nucleolar material in many small pieces; numerous cytoplasmic crystals; glycocalyx usually a thick, distinctly filamentous coat ***AMOEBIDAE***, p. 51

— Monopodial except when changing direction; length usually less than 75 μm, often much less; floating form almost always without pseudopodia; uninucleate; nucleus in all known species with central nucleolus; cytoplasmic crystals in some; glycocalyx thin, with discrete elements difficult to discern but cup-like ***HARTMANNELLIDAE***, p. 62

5 (3) Usually oblong, with hyaloplasm an anterolateral crescent; with apparent pellicle-like layer, which may be distinctly wrinkled; usually uninucleate, but one genus binucleate and one multinucleate; no cytoplasmic crystals; surface usually covered with thick, amorphous glycocalyx, in one genus distinct radiating filaments, in one genus a cuticle ***THECAMOEBIDAE***, p. 66

— Usually more or less flabellate, oval, or spatulate, rarely linguiform, with hyaloplasm occupying up to half of length; floating form usually with either slender, tapering, or blunt, shorter, hyaline pseudopodia; usually a single, central nucleolus, rarely parietal nucleolar lobes; cytoplasmic crystals reported in only one species; glycocalyx differentiated into discrete pentagonal glycostyles or less distinct hexagonal arrangements ***VANNELLIDAE***, p. 72

6 (2) Digitiform or mamilliform, blunt, hyaline subpseudopodia (dactylopodia), usually produced from anteror hyaloplasm; floating form usually with long, fine, or shorter, tapering pseudopodia; uninucleate; nucleolar material in central body; surface covered with either boat-shaped microscales or cuticle ***PARAMOEBIDAE***, p. 78

— A few slender, conical or linear subpseudopodia produced from anterior hyaloplasm or surface of cell; floating form usually with fine, often asymmetrically distributed pseudopodia; uninucleate, usually with central nucleolus; glycocalyx amorphous or differentiated into glycostyles ***VEXILLIFERIDAE***, p. 85

7 (1) Subpseudopodia rare and, when present, never furcate; locomotive form usually flattened, sometimes subcylindrical in most active locomotion, sometimes a thin sheet, sometimes reticulate, sometimes much branched (LEPTOMYXIDA) **8**

— Subpseudopodia usually present, often furcate **10**

8 Uninucleate amoebae usually divided into long, slender, lobose branches, which often rebranch but do not anastomose or form a reticulum ***GEPHYRAMOEBIDAE***, p. 87

— Flattened forms commonly flabellate, spatulate, or reticulate; eruptive activity and uroidal filaments common in some flabellate and subcylindrical forms; uninucleate or multinucleate **9**

9 Normally uninucleate; flabellate or spatulate, anterior margin often irregular, with conical, round-tipped subpseudopodia in one known freshwater/soil species ***FLABELLULIDAE***, p. 88

— Uninucleate amoebae with tendency to supernumerary nuclei, or multinucleate microplasmodia; in some species, most active form cylindrical and monopodial but often becoming flabellate and even multilobed; microplasmodia often highly ramified and reticulate; never any anterior subpseudopodia ***LEPTOMYXIDAE***, p. 89

10 (7) Regularly discoid, flattened ovoid, or fan-shaped, usually with breadth the greatest dimension, with granular hump surrounded anteriorly and laterally, sometimes completely, by flattened hyaline border with slender, short, conical subpseudopodia; locomotion usually rapid ***HYALODISCIDAE***, p. 98

— Not a regular, broad, discoid form **11**

11 Prominent, slender, flexible, occasionally furcate subpseudopodia (acanthopodia) tapering to fine or blunt tip; uninucleate; cysts of most species with pores closed by opercula; trophic amoebae of species without cyst pores identical to others of family; centriole-like body ***(ACANTHOPODIDA) ACANTHAMOEBIDAE***, p. 91

— Several to many fine, sometimes furcate subpseudopodia, finer than on Acanthamoebidae and in 2 genera much shorter; usually uninucleate, tendency to supernumerary nuclei in one genus; cysts with or without pores; no centriole-like body ***ECHINAMOEBIDAE***, p. 99

Keys to genera and species

Order EUAMOEBIDA Lepşi, 1960

Family AMOEBIDAE Ehrenberg, 1838

References: Friz (1979, 1984, 1987); Page (1981a, 1987a); Penard (1902); Schaeffer (1916).

1 Multinucleate ***Chaos***

— Uninucleate **2**

2 Monopodial, except when changing direction **3**

— Commonly or often polypodial **4**

3 Free-living ***Trichamoeba***

— Parasitic on freshwater coelenterates ***Hydramoeba***

4 (2) Several more or less equal pseudopodia produced from common posterior mass; often a posterior cluster of pseudopodial remnants (fasciculate uroid); surface coat consisting of or including discrete, fine, more or less straight filaments perpendicular to surface ***Polychaos***

— Usually one pseudopodium dominant at any one time; no prominent fasciculate uroid **5**

5 Larger amoebae (L>200 μm), with granular nucleus and surface coat of discrete, crinkled filaments rising from amorphous basal layer or, exceptionally, of such basal layer alone ***Amoeba***

— Smaller amoebae (L<200 μm), with nucleolar material in fewer pieces and coat of filaments but these not rising perpendicular to surface ***Deuteramoeba***

NOTE: The genus *Pseudothecamoeba*, classified in the Thecamoebidae (q.v.), strongly resembles some genera of this family.

Genus *Chaos* Linnaeus, 1767

References: Gromov (1986); Page (1981a, 1987a); Willumsen (1982).

At present this genus includes all multinucleate members of the family. However, multinucleate Amoebidae occur more widely than previously thought (Siemensma, unpublished; Willumsen, 1982 and unpublished), and there is no particular reason why all multinucleate Amoebidae should belong to one genus any more than all uninucleate Amoebidae do.

All known species have granular or ovular nuclei (Raikov, 1982), with many or all nucleolar pieces arranged in a parietal layer. All those in the following key are often polypodial, but *C. zoochlorellae* is just as often monopodial and Penard reported that *Amoeba laureata* (see 'Other possible species') is usually monopodial.

All 3 species whose ultrastructure has been reported have surface coats much like that of *A. proteus*, and a loose, honeycomb-like lamina has been found in the nuclei of 2 species, but those characters differ in at least one multinucleate strain (Willumsen, personal communication).

1 Often with zoochlorellae; often monopodial; L 165-400 μm ($\bar{x}$ 275 μm); nucleus spherical, 5.3-12.3 μm ($\bar{x}$ 8.3 μm); 8-50 nuclei per amoeba; crystals plate-like or bipyramidal; no cyst observed
Chaos zoochlorellae Willumsen, 1982

(Europe.)

— Without zoochlorellae; more often polypodial, much like *Amoeba proteus* in general form **2**

2 L *c.* 700 μm to 2 mm in polypodial form, to 3 or rarely 5 mm monopodial; nucleus biconvex disc, 22-31 μm ($\bar{x}$ 27 μm); up to 1,000 or more nuclei per amoeba; most crystals bipyramidal; cyst reported (Fig. 13A-C) ***C. carolinense*** (Wilson, 1900)
(North America. Culture method MP/Colp.)

— Smaller **3**

3 L *c.* 500-800 μm in polypodial form, to 1.5 mm monopodial; nucleus spherical, 14-16 μm; several hundred nuclei per amoeba; crystals bipyramidal and plate-like; cysts common (Fig. 15A)
C. illinoisense (Kudo, 1950)

(North America.)

— L *c.* 240-820 μm ($\bar{x}$ *c.* 450-500 μm); nucleus biconvex disc, 15-23 μm ($\bar{x}$ 18.5 μm); *c.* 6-49 nuclei per amoeba; all or most crystals bipyramidal; no cyst observed (Fig. 13D-F)
C. nobile (Penard, 1902)

(Europe, North America. Culture method MP/Colp.)

Other possible species

Amoeba laureata Penard, 1902: L generally 500-800 μm but can reach 1.4 mm; mostly monopodial, seldom polypodial like *A. proteus*; nuclei spherical, 8-10 μm; sometimes over 1,000 nuclei per amoeba; very small crystals, either bipyramidal or plate-like.
Europe.

Genus *Trichamoeba* Fromentel, 1874

References: Bovee (1972); Schaeffer (1926); Siemensma & Page (1986).

These are the uninucleate, regularly monopodial, free-living Amoebidae. Other members of the family may occasionally become monopodial in rapid locomotion or under special conditions, and some such monopodial forms have pronounced uroids. It was to such a normally polypodial amoeba that Wallich gave the name *Amoeba villosa* (Siemensma, personal communication). But *Trichamoeba* is always monopodial during steady locomotion in one direction, putting out another pseudopodium only for a marked change in direction.

Trichamoeba may be confused with the more common *Saccamoeba*. With the light microscope these are most easily distinguished by the nucleus, which is ovular in all known species of *Trichamoeba* and has a single central nucleolus in known species of *Saccamoeba*. The floating form of most species of *Trichamoeba* has the pseudopodia characteristic of such forms in the Amoebidae, while that of *Saccamoeba* is irregularly rounded without pseudopodia.

1 Average length more than 150 μm **2**

— Average length less than 150 μm **3**

2 Slender, L/B more than 4, usually more than 5; L approximately 125-325 μm, usually about 200 μm or somewhat less; nucleus *c.* 14-27 μm ($\bar{x}$ *c.* 20 μm); usually many bipyramidal crystals; uroid often bulbous, smooth or with short papillae or villi; probable cysts observed. (Figs. 14A-C, 19A) ***Trichamoeba sinuosa*** Siemensma & Page, 1986
(Europe, CP/r/Chil.)

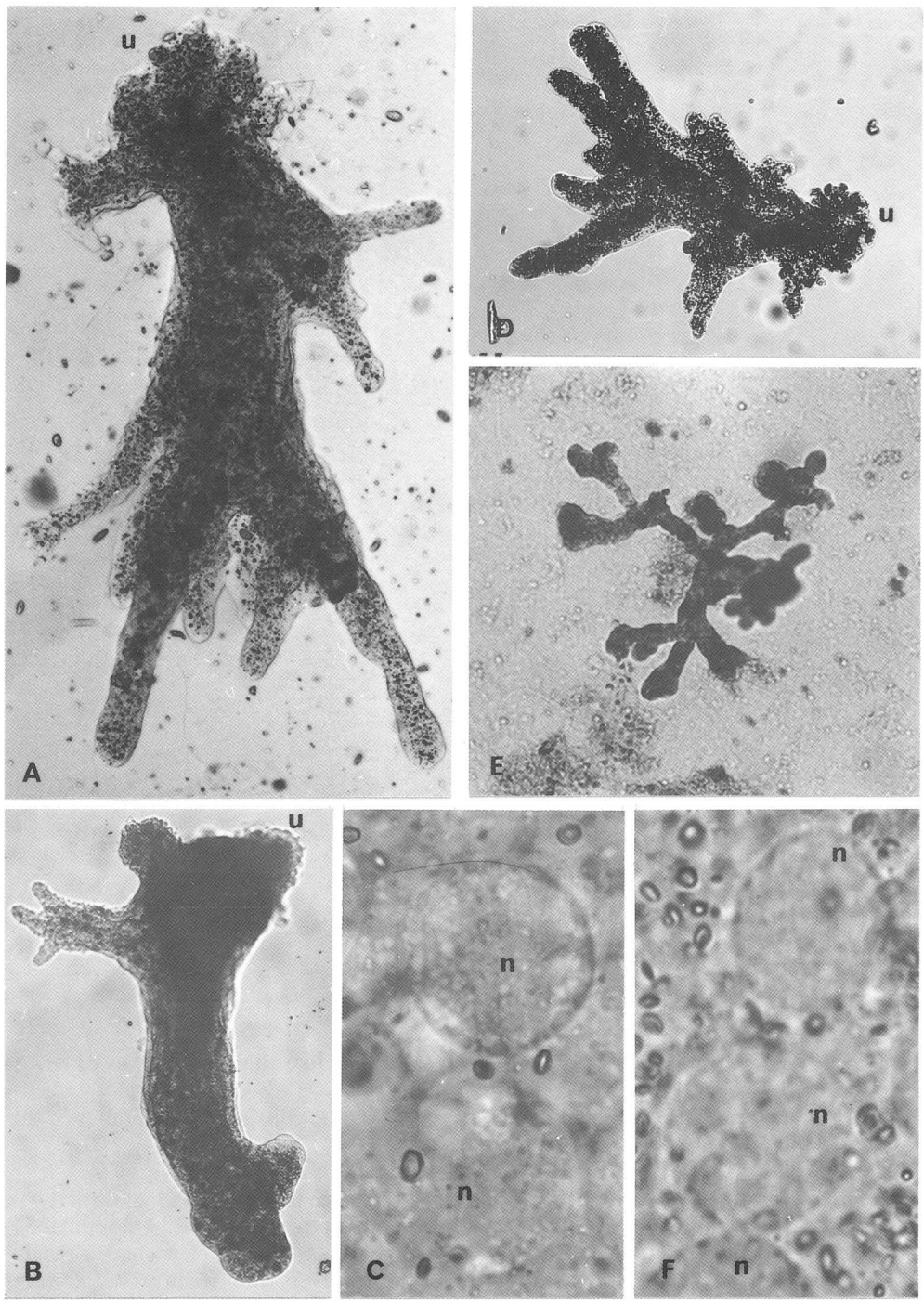

Fig. 13. *Chaos*. A-C, *C. carolinense*; C, nuclei. D-F, *C. nobile*; E, floating form with rebranching pseudopodia; F, nuclei. (A, ×80; B ×50; C, ×1,000; D, ×125; E, ×100; F, ×1,575.) *n*, nucleus; *u*, uroid.

— Thicker, L/B 2.5-3; L 200-300 μm, nucleus 40-60 μm; up to 3 dozen short bipyramidal crystals; uroid a permanent hyaline bulb, which may be papillate; floating form without pseudopodia; no cysts known. (Fig. 15B) ***T. myakka*** Bovee, 1972
(North America.)

3 (1) L/B 3-4 in rapid locomotion, broader in slow movement; L 100-150 μm; nucleus 13-17 μm; many bipyramidal crystals; uroid not bulbous, with many short projections, apparently adhesive; no cysts reported. (Fig. 15C) ***T. osseosaccus*** Schaeffer, 1926
(North America.)

— L/B 2.25-3; L 80-150 μm; nucleus 8-15 μm; up to 30 flattened though essentially bipyramidal

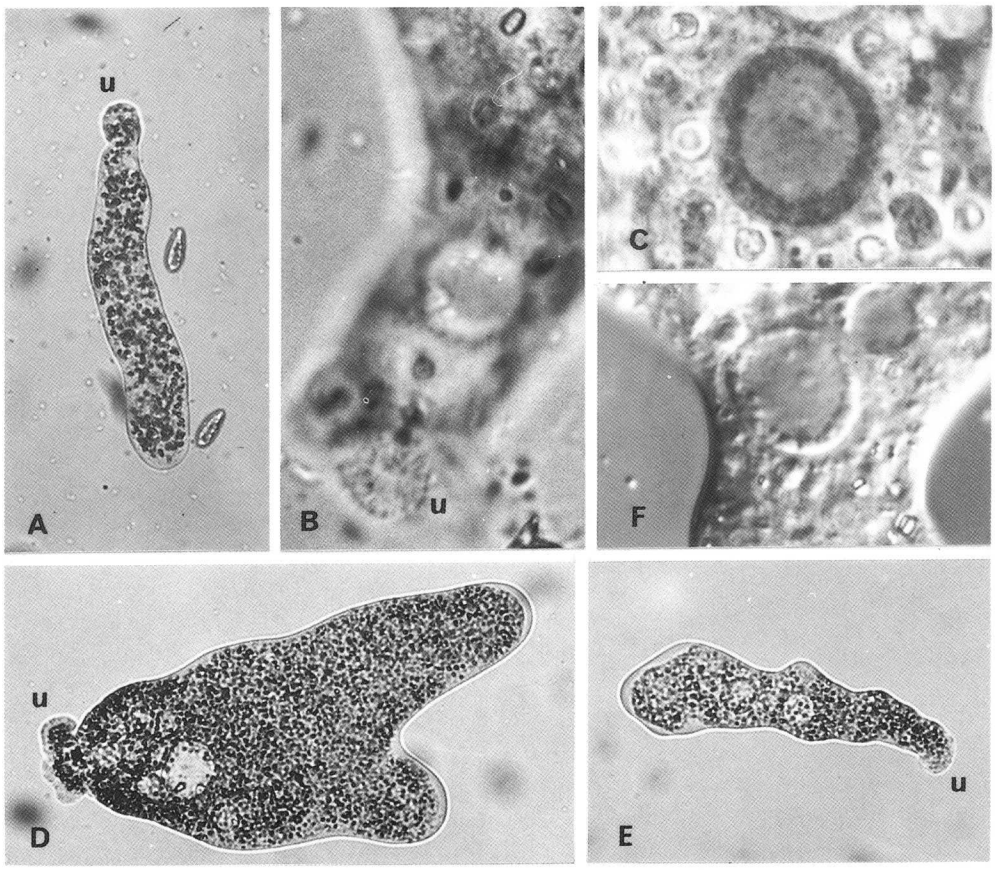

Fig. 14. A-C, *Trichamoeba sinuosa*; B, uroid; C, nucleus. D-F, *Hydramoeba hydroxena*; F, nucleus. (A, D, E, ×250; B, C, F, ×1,000.) *u*, uroid.

crystals and more smaller ones; uroid often bulbous, villous or papillate with a few adhesive filaments; no cysts known. (Fig. 15D) ***T. cloaca*** Bovee, 1972
(North America.)

Other species
Trichamoeba hirta Fromentel, 1874, is the type species but, like the type species of several other genera of gymnamoebae, appears to be unidentifiable. It was about 125 μm long. For reasons explained above, *Amoeba villosa* Wallich, 1863, cannot even be considered a member of this genus; it is probably unidentifiable.

Amoeba gorgonia Penard, 1902, might be a *Trichamoeba*, since it apparently had both the monopodial locomotive form of the genus and the floating form characteristic of the Amoebidae. Penard's description does not make clear whether it contained crystals. The nucleus had a single central nucleolus, but such a nucleus is now known to occur rarely in this family (see *Deuteramoeba mycophaga*).

Likewise, *Metachaos oxyuris* Schaeffer, 1926, described from only two specimens, might belong to this genus.

Genus *Hydramoeba* Reynolds & Looper, 1928

Reference: Page & Robson (1983, with references to earlier works.)

Although the single species appears to be an obligate parasite, it is large and common enough to be expected occasionally in freshwater samples containing hydras. Furthermore, it is so similar to *Trichamoeba*, both light- and electron-microscopically, that only its parasitism justifies a separate genus.

The sizes given are derived from reports of several workers, who have noted a great diversity in a single population. However, little doubt exists that all the amoebae found by these workers belong to the same species.

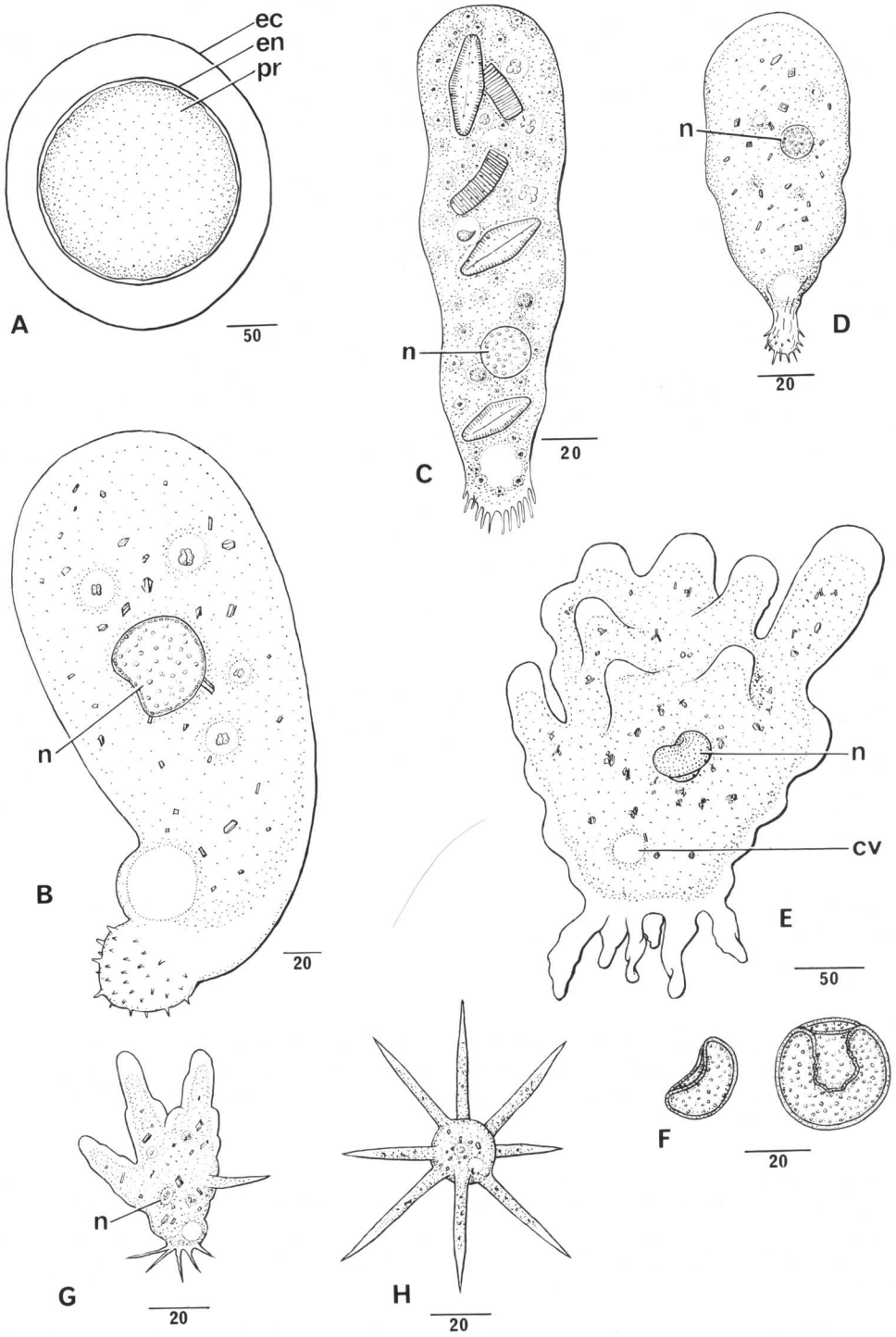

Fig. 15. A, *Chaos illinoisense,* cyst. B, *Trichamoeba myakka.* C, *Trichamoeba osseosaccus.* D, *Trichamoeba cloaca.* E, F, *Polychaos nitidubium*; F, nucleus. G, H, *Polychaos timidum.* (A, after Kudo; B-H, after Bovee.)

The amoebae may be found externally on their hosts or in the coelenteron.

L 100-380 μm, commonly 150-250 μm; nucleus *c.* 10-30 μm, commonly 10-20 μm, with peripheral layer of coarse nucleolar fragments; amoebae with 2 or 3 nuclei fairly common; often many bipyramidal cytoplasmic crystals. No cysts known. (Fig. 14D-F)

Hydramoeba hydroxena (Entz, 1912)

(Widely distributed in the Northern Hemisphere; pathogenic to many species of hydras, also on *Craspedacusta*; not yet cultured apart from hydras.)

Genus *Polychaos* Schaeffer, 1926

References: Page (1987a); Page & Baldock (1980).

As recognised here, the genus is still essentially that proposed by Schaeffer, and the two most studied species are those which he placed into the genus, *P. dubium* and *P. fasciculatum*. Light- and electron-microscopic differences cast doubt on the classification of those species in the same genus, but similarities also exist.

Since several pseudopodia are often extended side by side, they tend to fuse at their bases as the main cell mass advances; they may also flatten out somewhat. Some pass toward the posterior end, narrowing as the solated endoplasm flows out of them, this process giving rise to the fascicle of pseudopodial remnants often found. However, monopodial forms also occur, either in rapid locomotion or (a more club-like form with a prominent bulbous uroid) in lowered oxygen concentration.

1 Nucleus granular **2**

— Other nuclear structure **3**

2 Often several more or less equal pseudopodia projecting in a semi-circle, though amoeba elongates in rapid advance; L or greatest diameter of polypodial form 280-400 μm ($\bar{x}$ *c.* 310 μm); nucleus ovoid or spherical, *c.* 32-45 μm ($\bar{x}$ 38 μm), with nucleolar granules dispersed in nucleus; crystals often irregular, including some paired bodies, some bipyramidal and plate-like; cyst reported (Figs. 16A-D, 19B) ***Polychaos dubium*** (Schaeffer, 1916)
(North America, Europe. PC/Chil or Colp.)

— Pseudopodia tending to be shorter, fewer, and more anteriorly directed than those of *P. dubium*, often fusing into a broad front or a single pseudopodium during rapid advance; polypodial form 220-450 μm, monopodial to 550 μm; nucleus more or less cup-shaped, 27-35 μm, with nucleolar granules parietal; many bipyramidal crystals, some others, often in clusters; ultrastructure unknown. (Fig. 15E, F) ***P. nitidubium*** Bovee, 1970
(North America.)

3 (1) A few short, parallel pseudopodia, sometimes fusing into one; club-shaped monopodial form common in reduced O_2 tension; normal locomotive forms *c.* 65-140 μm ($\bar{x}$ 110 μm); nucleus spherical, 11-23 μm ($\bar{x}$ 15.8 μm), with nucleolar material in large, lobed, parietal band; usually many bipyramidal crystals. (Fig. 16E-J) ***P. fasciculatum*** (Penard, 1902)
(Europe. MP/r/Chil or CPAE with accompanying smaller amoebae.)

— Polypodial form 45-60 μm, monopodial 80-100 μm; uroidal cluster usually spiny, hyaline; floating form with several pointed but granular pseudopodia; nucleus 6.5-7 μm, with central body apparently a cluster of particles; many crystals of various shapes; ultrastructure unknown. (Fig. 15G, H) ***P. timidum*** Bovee, 1972
(North America.)

Other possible species

Amoeba annulata Penard, 1902, somewhat resembles *P. fasciculatum*. However, the nucleolar material resembles that of *Thecamoeba terricola* more than that of *P. fasciculatum*, and Penard gave the average size (omitted in the original description) as 250 μm in a later publication. There is no good reason to consider this species a *Polychaos*.

Metachaos gratum Schaeffer, 1926, is more similar to *P. fasciculatum* and to *Polychaos* in general. Its average length was 100 μm. The nucleus, somewhat like that of *P. fasciculatum*, had an average diameter of 25 μm. The crystalline inclusions were more irregular than those of *P. fasciculatum*.

Genus *Amoeba* Bory de St. Vincent, 1822

References: Kalinina *et al.* (1987); Page & Kalinina (1984).

All three species in the dichotomous key have been studied with the electron microscope and non-morphological procedures. All have a honeycomb-like nuclear lamina. They are incompatible with each other by nuclear transplantation. None is known to form cysts. See 'Other species' for possible synonyms.

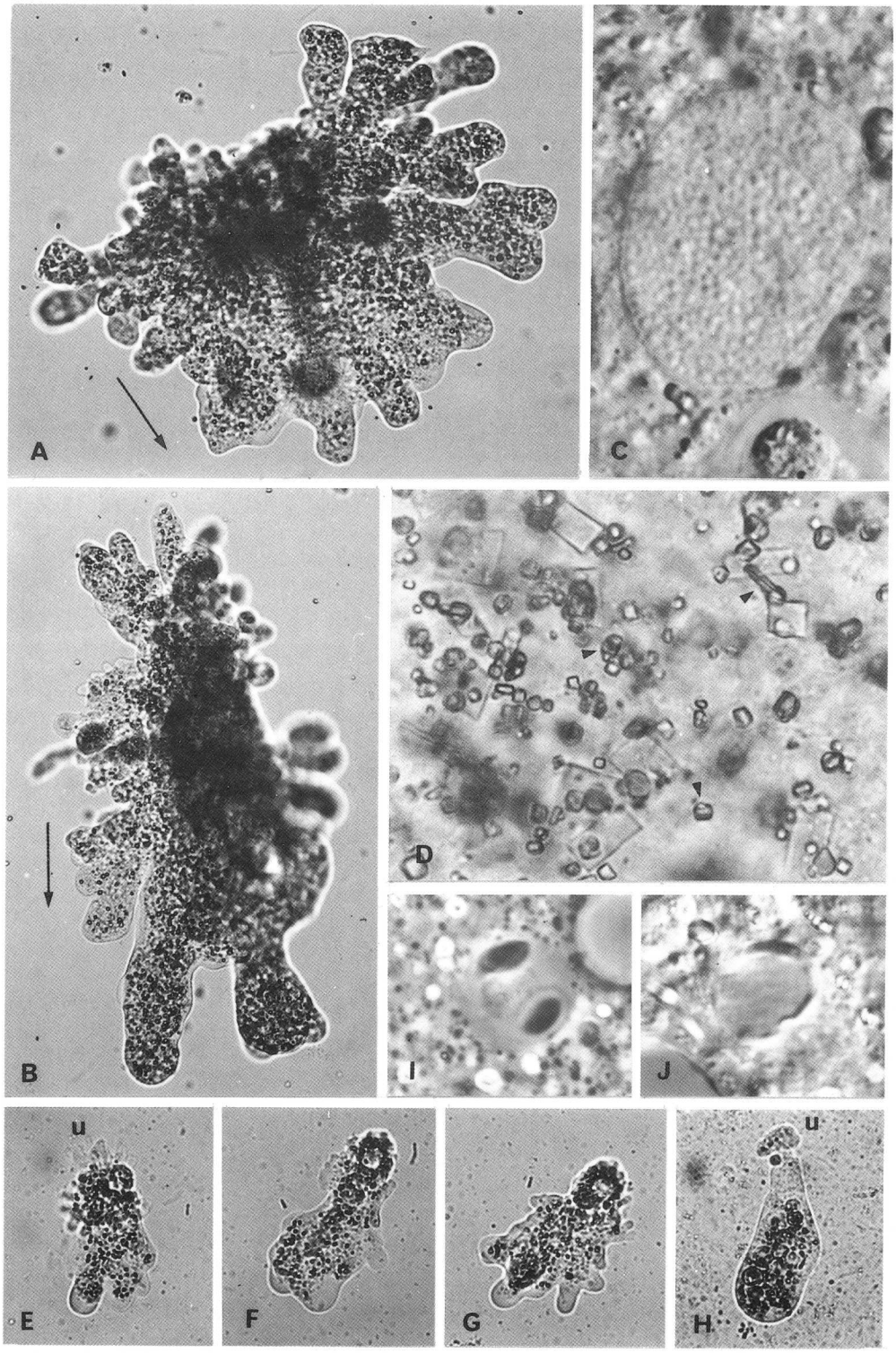

Fig. 16. *Polychaos*. A-D, *P. dubium*; C, nucleus; D, crystals, with some irregular bodies or groups indicated by arrowheads. E-J, *P. fasciculatum*; H, clavate or monopodial form such as seen under conditions of low oxygen; I, J, nuclei. (A, B, ×200; C, D, I, J, ×1,000; E-H, ×250.)

1 Nucleus discoid, often concave, 22-62 μm ($\bar{x}$ 40 μm); L in locomotion *c.* 220-760 μm ($\bar{x}$ *c.* 425 μm); majority usually polypodial under favourable conditions, with small minority monopodial; great majority of crystals truncate bipyramids; surface coat of crinkly filaments up to 230 nm above plasma membrane. (Figs. 17A-F, 19C, H)

Amoeba proteus (Pallas, 1766) Leidy, 1878

(Europe, North America. PC/Tet, PJ/Tet, PC/r/Chil, PJ/r/Chil.)

— Nucleus spherical, ovoid, or compressed, but not with concave surfaces, and smaller than

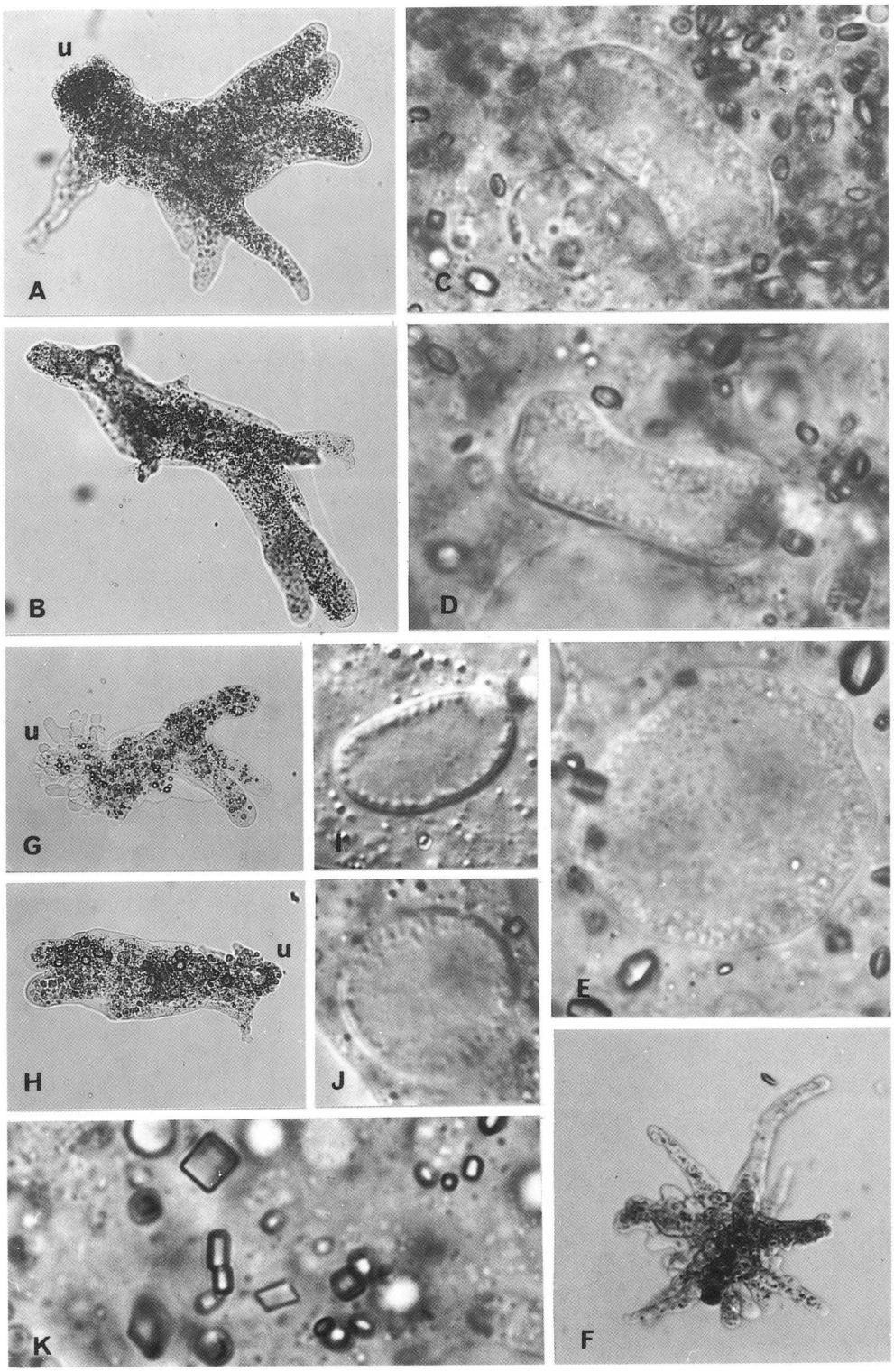

Fig. 17. *Amoeba*. A-F, *A. proteus*; C, side view of nucleus of Soviet strain; D, side view of nucleus of Scottish strain; E, flat view of nucleus of American strain; bipyramidal crystals in all three figures of nuclei, which do not differ morphologically in these strains; F, floating form, similar to that found in all species of genus. G-K, *A. borokensis*; I, J, nuclei; K, crystals. (A, B, G, H, ×125; C-E, I, J, ×1,000; F, ×100; K, ×1575.) *u*, uroid.

nucleus of *A. proteus* **2**

2 Majority of crystals rectangular plates; nucleus a compressed sphere or ovoid, occasionally almost discoid but not concave, usually 23-37 μm (x̄ 29 μm); L *c.* 210-545 μm (x̄ 370 μm); usually majority polypodial; surface coat of filaments somewhat finer than those of *A. proteus*, up to

250 nm above plasma membrane but usually less, with somewhat matted appearance; nuclear DNA content approximately half that of *A. proteus*. (Figs. 17G-K, 19D)

A. borokensis Kalinina, Afon'kin, Gromov, Khrebtukova & Page, 1987

(Europe. PC/Tet, in which generation time may be less than 24 hours; PC/r/Colp; grows poorly with Chil.)

— Cytoplasmic crystals mostly truncate bipyramids, numerous, giving amoebae dark appearance; nucleus spherical, often slightly compressed, thus appearing ovoidal, 21-29 μm ($\bar{x}$ 25 μm); L *c.* 160-550 μm ($\bar{x}$ *c.* 360 μm); often large minority monopodial; surface coat amorphous, less than 20 nm thick, without layer of long filaments. (Figs. 18A-C, 19E)

A. leningradensis Page & Kalinina, 1984

(Europe. PC/Tet, PC/r/Chil.)

Other species

A. amazonas: This name is invalid, because it was given without a description that is taxonomically valid or would permit recognition, though it may represent a real species.

Reference: Flickinger (1974).

A diminutiva Bovee, 1972: This small, polypodial amoeba needs to be examined with the electron microscope before its relationships with and within the Amoebidae can be determined. L 15-20 μm; nucleus discoid, of obscure structure, 3-4 μm; tiny crystals, less than 0.5 μm, of undetermined shape.

Illustrations in Page (1976).

A. discoides Schaeffer, 1916: There are strong indications that the laboratory strain attributed to this species is actually an *A. proteus*, but it may have been misidentified (Jeon & Lorch, 1973), and the question of the validity of Schaeffer's distinction of *A. discoides* remains unsettled. His statements that the endoplasm is crowded with bipyramidal crystals and that this species is less often polypodial than *A. proteus* suggest a similarity with *A. leningradensis*, but the discoidal shape and the size (40 μm) of the nucleus rule out an identity with the latter.

A. indica: This name, applied to a laboratory strain, is invalid for the same reasons cited for *A. amazonas*. This is very similar to *A. proteus*. The nucleus is occasionally discoid, sometimes cup-shaped or infolded, with a diameter of 31-53 μm ($\bar{x}$ 39.0 μm). Friz found indications that this might be a strain of *A. proteus*, but further results suggested otherwise. This strain is still extant and is available from the Culture Collection of Algae and Protozoa, Freshwater Biological Association, Ambleside. Culture methods: MCh/Tet, MCh/r/Chil.

References: Friz (1983, 1987); Rao & Chatterjee (1974).

A. kerrii Taylor, 1947: Apart from the observation 'Longitudinal folds of the ectoplasm are not present', the published description is inadequate to distinguish this species.

Illustration in Page (1976).

A. lescherae Taylor & Hayes, 1944: Observations of strain CCAP 1503/2, originally attributed to this species, support its classification as *A. proteus*. The nucleus is often deformed, a characteristic which remained constant over nearly nine years, but no other distinguishing morphological characteristic has been observed. The life histories reported for *A. lescherae* and *A. kerrii* cannot be credited.

A. navina Shah, 1971: The description of this, 'the largest mononucleate form among Amoebidae known so far', is inadequate. Its length is given as 0.5-1.2 mm; the nucleus is described, without a report of diameter, as 'spherical, oval or elongated polymorphic'; the amoeba is said to lack crystals.

A. nitida Penard, 1902: Undoubtedly a synonym of *A. proteus*.

Genus *Deuteramoeba* Page, 1987

References: Baldock *et al.* (1983); Chakraborty & Old (1986); Old *et al.* (1985); Page (1987a); Pussard *et al.* (1980).

The diagnosis of this genus has been expanded somewhat for this key to accommodate *D. mycophaga*, which has typically bipyramidal crystals rather than the paired inclusions found in the type species, *D. algonquinensis*. These are smaller polypodial Amoebidae with the nucleolar material in fewer pieces than in *Amoeba*, with a fibrous inner nuclear lamina not organised in the honeycomb pattern and with a glycocalyx differing morphologically from that of the larger Amoebidae.

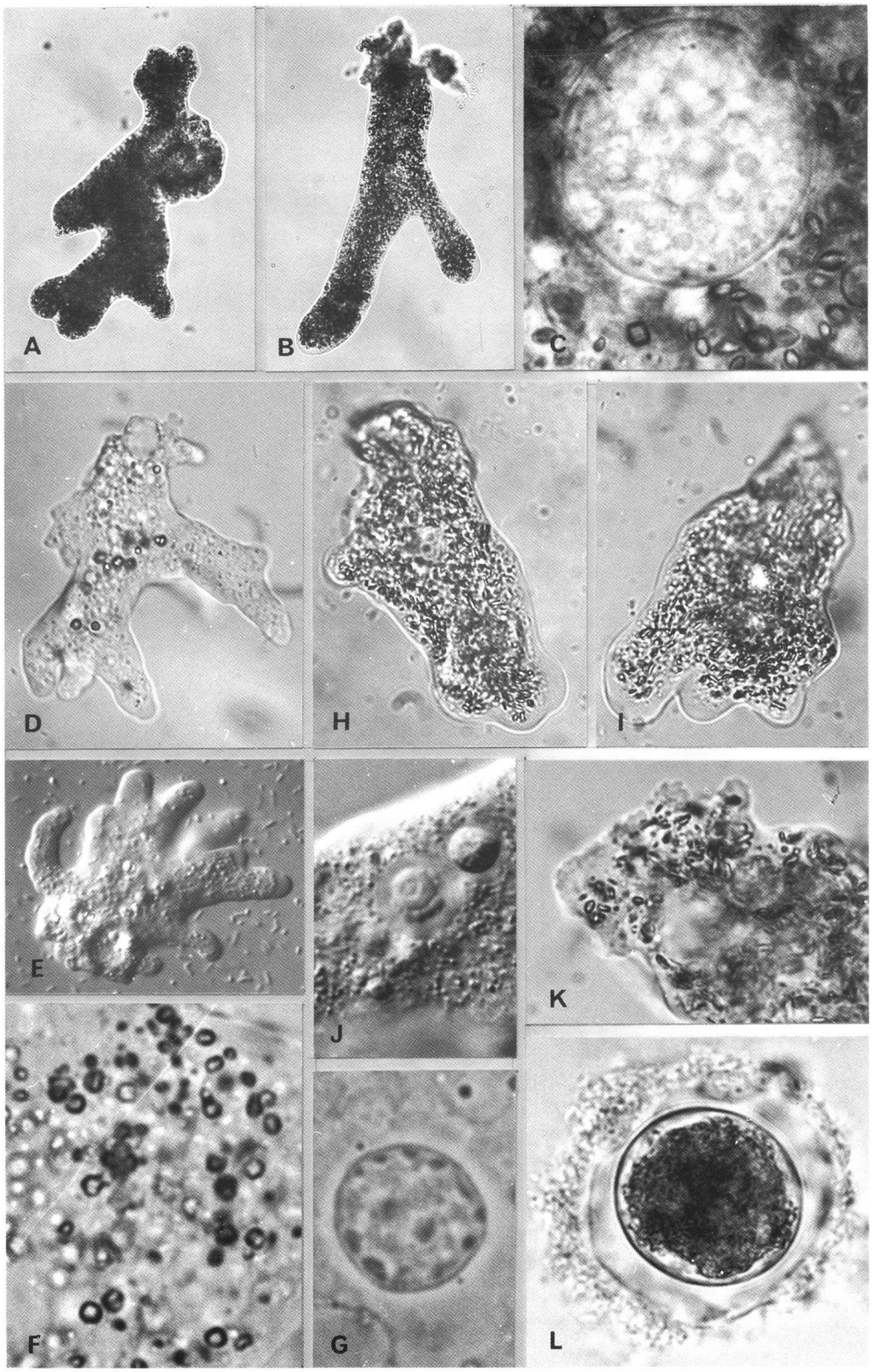

Fig. 18. A-C, *Amoeba leningradensis*; C, nucleus and crystals. D-G, *Deuteramoeba algonquinensis*; F, crystals; G, nucleus. H-L, *Deuteramoeba mycophaga*; J, nucleus; K, uroid; L, cyst. (A, B, ×125; C, J-L, ×1,000; D, E, H, I, ×600; F, ×1,575; G, ×2,500.)

1 Nucleus more or less spherical, *c.* 7.5-11.5 μm ($\bar{x}$ *c.* 9 μm), with nucleolar pieces of varying sizes, most parietal, some in interior of nucleus; locomotive form typically polypodial, L $\bar{x}$ *c.* 80 μm; many paired inclusions, few if any bipyramidal crystals; no cysts observed. (Figs. 18D-G, 19F, I) ***Deuteramoeba algonquinensis*** (Baldock, Rogerson & Berger, 1983)
(North America. CPAE with *Hartmannella vermiformis*; PJ/r/Chil, usually less successful.)

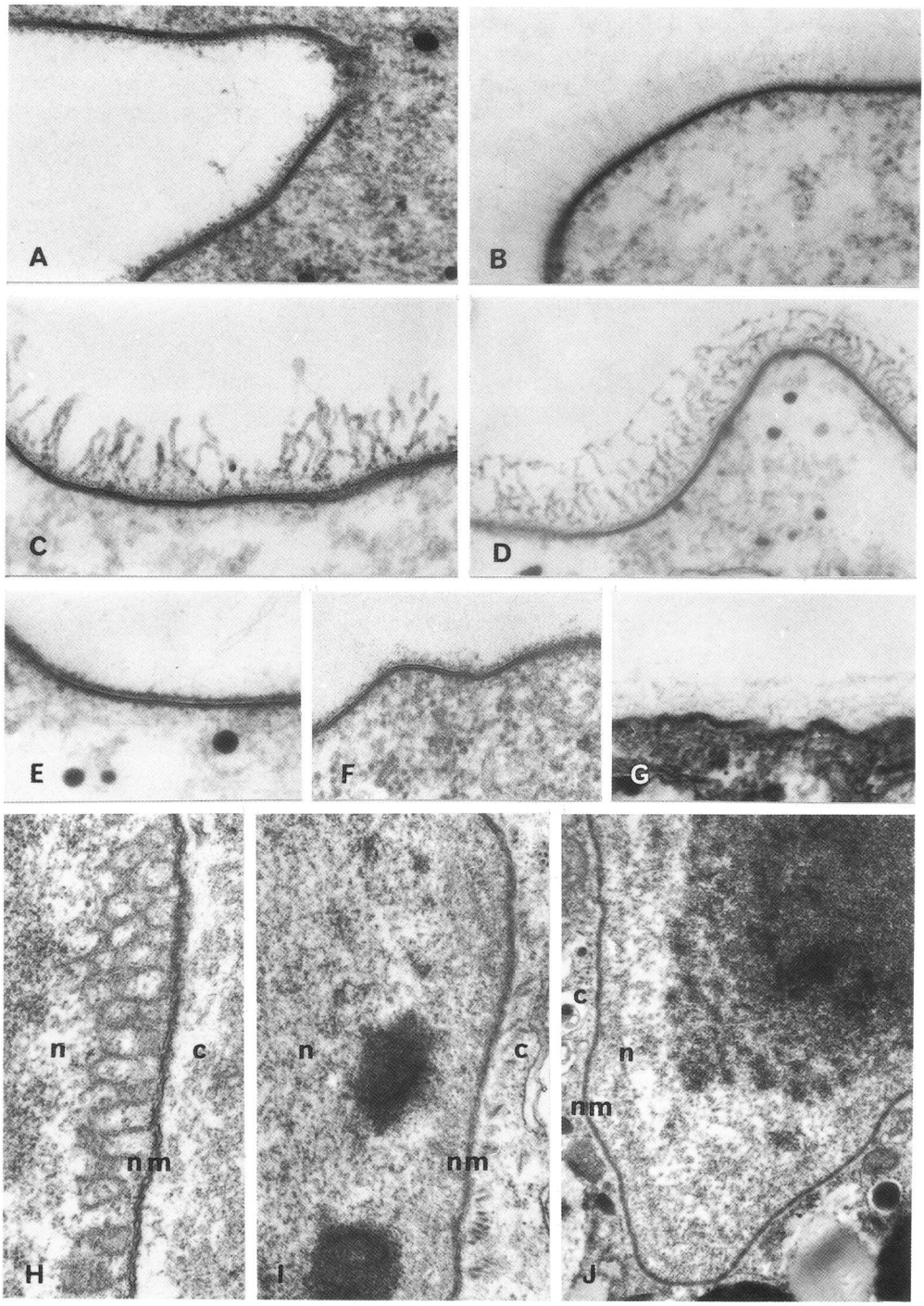

Fig. 19. Amoebidae; ultrastructural details of possible value in identification. A-G, surface structure: A, *Trichamoeba sinuosa*; B, *Polychaos dubium*; C, *Amoeba proteus*; D, *Amoeba borokensis*; E, *Amoeba leningradensis*; F, *Deuteramoeba algonquinensis*; G, *Deuteramoeba mycophaga*. H-J, nuclear envelopes: H, *Amoeba proteus* (similar structure in other species of *Amoeba* and in *Polychaos dubium*); I, *Deuteramoeba algonquinensis*; J, *Deuteramoeba mycophaga*. (A-G, ×50,000; H, I, ×20,000; J, ×10,000.) *c*, cytoplasm; *n*, nucleus; *nm*, nuclear membrane.

— Nucleus more or less spherical, 12-20 μm, with single central nucleolus; locomotive form often broadly monopodial, occasionally polypodial, L *c.* 70-180 μm, $\bar{x}$ *c.* 110 μm; many small bipyramidal crystals; double-walled cysts, with two walls often widely separated. (Figs. 18H-L, 19G, J)

Deuteramoeba mycophaga (Pussard, Alabouvette, Lemaitre & Pons, 1980) n. comb.

(Europe, Australia; terr. PJ/r/Chil; also on agar, with fungi as food, cf. Pussard *et al.*, 1980, and Old *et al.*, 1985. This is the same species designated *Trichamoeba mycophaga* by Chakraborty & Old, 1986.)

Amoeba hugonis Taylor, 1952: With a supposed life cycle which appears to be derived from cultures containing more than one species. Often monopodial, *c.* 100 μm long. Nucleus 8 μm, with central nucleolus, unusual for Amoebidae (cf. *Deuteramoeba mycophaga*), but apparent floating form ('star shaped amoebula') suggests that this species may not be a *Saccamoeba*. Numerous crystals. Scotland.

Amoeba taylorae Hayes, 1955: Generally elongated and monopodial, average L 420 μm, reaching 500 μm, with villous uroid. Biconvex ovular nucleus, *c.* 30 μm. Many small, slender, apparently bipyramidal crystals. Possibly an *Amoeba* or a *Polychaos* observed under conditions favouring monopodial form. Scotland.

Family HARTMANNELLIDAE Volkonsky, 1931; emend. Page, 1974

References: Bovee (1972); Page (1974a, 1986).

These are truly limax amoebae with tubular mitochondrial cristae. They must be distinguished from (1) limax amoebae of the class Heterolobosea, which can usually be distinguished by their eruptive movement and have discoid mitochondrial cristae and intranuclear mitosis, usually promitosis; (2) monopodial members of the Amoebidae (*Trichamoeba, Hydramoeba*), which have a granular nucleus (known species) and a floating form with distinct, largely granular pseudopodia; (3) limax forms of the Leptomyxidae, which are markedly eruptive, often have flabellate or other flattened forms, and have conspicuous adhesion uroids and, sometimes, holdfasts. See also remarks under the genus *Saccamoeba*.

Sometimes during locomotion slight bulges are formed to one side or the other at the anterior end, but these bulges are never markedly eruptive, and the hyaloplasm never runs posteriorly along the side, as in Heterolobosea and at least some Leptomyxidae. *Most* hartmannellids have a maximum relative locomotive rate no greater than 4 and a mean L/B ratio above 4.

A recent ultrastructural study (Page, 1986) showed very delicate sucker- or cup-like structures on all strains of Hartmannellidae examined. They appear to be a distinguishing character of the family, though somewhat similar configurations have been noticed on some other Euamoebida. No such structures are found on Heterolobosea or on any Leptomyxidae studied with the electron microscope.

1 Hyaline cap almost always present in continuous locomotion, usually at least as deep antero-posteriorly as broad; no nuclear division in cysts, if cysts formed ***Hartmannella***

— Hyaline cap often or usually absent in continuing locomotion 2

2 Cysts often binucleate (also uninucleate, rarely trinucleate) as result of mitosis in immature cyst, often containing one, rarely two, densely staining bodies; hyaline cap fairly common in locomotion though often obliterated by intrusion of granuloplasm ***Glaeseria***

— No nuclear division in cysts, if cysts formed; hyaline cap usually present only at initiation of pseudopodium or as shallow crescent **3**

3 Villous or finely papillate uroidal knob often present; conspicuous bipyramidal crystals in some ***Saccamoeba***

— Villous knob never formed; mitochondrial cristae conspicuously helical ***Cashia***

Genus *Hartmannella* Alexeieff, 1912; emend. Page, 1974

References: Page (1967a, 1974a, 1986).

The tendency of some authors to employ this name almost indiscriminately for diverse, unrelated amoebae (including the very different *Acanthamoeba*) has been reduced in the past few years, but care is still necessary to determine the exact sense in which the name is used in any given publication.

The mitochondria of the two freshwater and one marine species studied with the electron microscope are sometimes elongate, in contrast with those of some other hartmannellid species.

1 L/B usually about 4.5, maximum about 6.5; hyaline cap sometimes obliterated; occasionally a few trailing filaments; L 16-33 μm ($\bar{x}$ 23 μm); nucleus 2.5-4.5 μm ($\bar{x}$ 3.4 μm); usually several small (*c.* 1 μm) bipyramidal crystals; floating form with few blunt, hyaline pseudopodia; cysts smooth, spherical or ovoid, with thicker inner layer and thin, closely apposed outer layer,

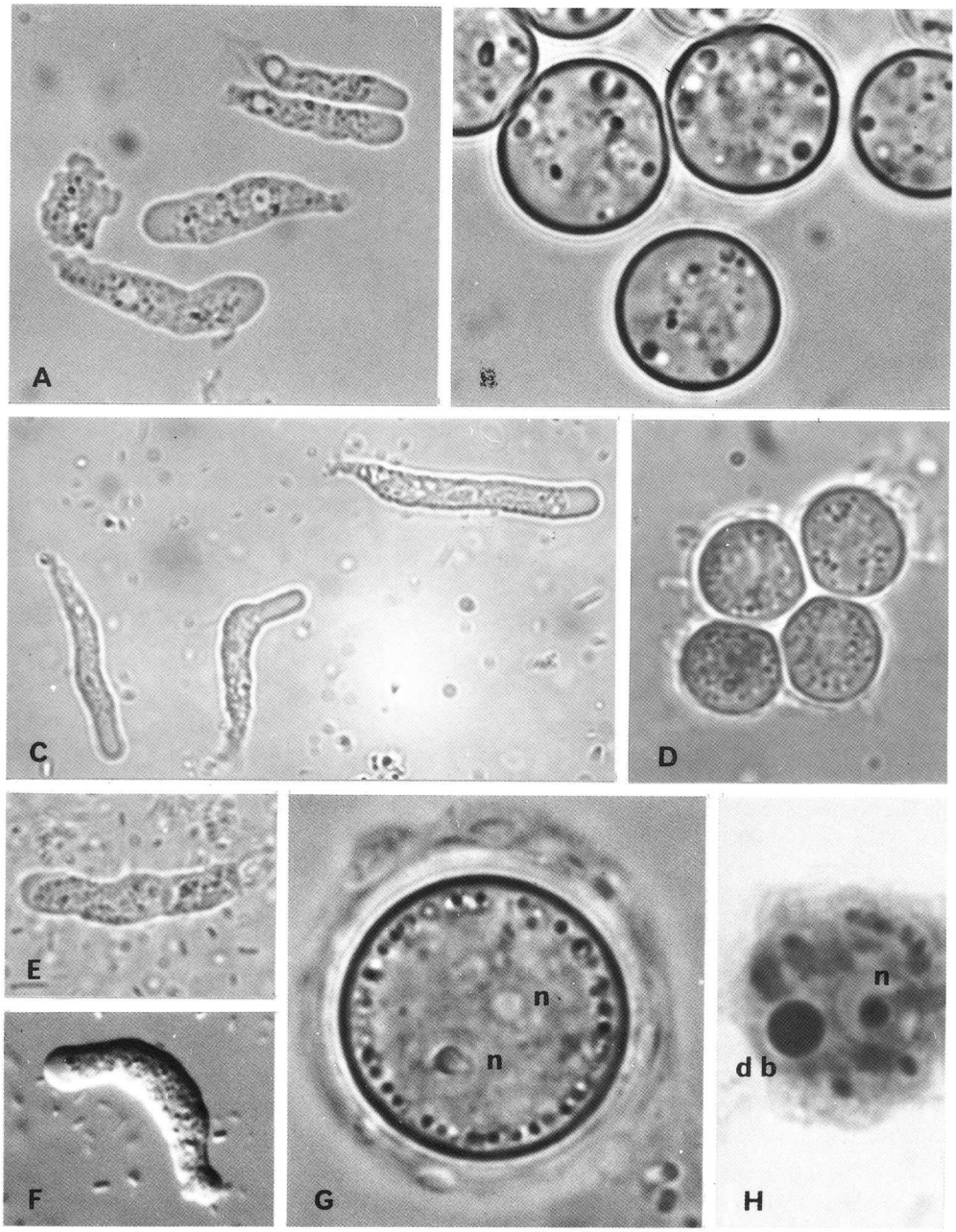

Fig. 20. A, B, *Hartmannella cantabrigiensis*, amoebae and cysts. C, D, *Hartmannella vermiformis*, amoebae and cysts. E-H, *Glaeseria mira*; G, binucleate cyst; H, haematoxylin-stained early cyst stage, with nucleus and densely staining body. (A, C, E, F, ×1,000; B, D, G, H, ×2,500.) *db*, densely staining body; *n*, nucleus.

diameter 7-13 μm ($\bar{x}$ 8.8 μm); cup-like surface structures with diameter *c.* 15-20 nm. (Fig. 20A, B) ***Hartmannella cantabrigiensis*** Page, 1974

(Europe. NNE.)

— L/B usually more than 6, maximum about 8.4; hyaline cap always present, antero-posterior depth to twice breadth; L 12-37 μm; nucleus *c.* 2.1-4.1 μm; no crystals; cysts spherical or slightly ovoid, with outer wall layer of minority somewhat separated, diameter 4.0-9.5 μm ($\bar{x}$ *c.* 7.0 μm); cup-like surface structures with diameter *c.* 12.5 nm. (Fig. 20C, D)

Hartmannella vermiformis Page, 1967

(North America, Europe, Australia; terr. Some variation must be expected in the measurements of this widely distributed species. NNE.)

Other possible species

Of the many species, often poorly described, attributed to this genus, only a few can be mentioned.

Hartmannella hyalina (Dangeard, 1900): The type species by original designation, is unrecognisable.

Hartmannella agricola (Goodey, 1916): It is not certain that Singh, who transferred this species to the genus *Hartmannella*, had the same organism as Goodey.

Reference: Singh (1952).

Hartmannella crumpae Singh & Hanumaiah, 1979: The authors separated this species from *H. vermiformis* by two incorrect distinctions.

Genus *Glaeseria* Volkonsky, 1931

References: Page (1974a, 1986).

The surface structures on the single free-living species are more cup-shaped and larger than those on the *Hartmannella* species. The mitochondria, unlike those of *Hartmannella*, appear never to be elongate (sausage-shaped).

Slender, with L/B averaging more than 4 and sometimes exceeding 6.5; occasionally a few uroidal filaments; L 20-46 μm ($\bar{x}$ 31 μm); nucleus 3.0-6.0 μm ($\bar{x}$ 4.2 μm); no crystals; cyst with smooth inner wall, more or less smooth outer wall, and sticky coating, diameter 7.4-19.0 μm ($\bar{x}$ 12.1 μm); cup-like surface structures with diameter slightly less than 30 nm. (Fig. 20E-H) ***Glaeseria mira*** (Gläser, 1912)

(Europe. NNE.)

Genus *Saccamoeba* Frenzel, 1892; emend. Bovee, 1972

References: Bovee (1972); Page (1969a, 1974a, 1986).

This genus contains the largest hartmannellids and bears the most resemblance to the Amoebidae, especially in the frequent occurrence of crystals and the frequent possession of a villous-knob uroid, which sometimes occurs on some Amoebidae. It is most like *Trichamoeba*, from which it has been distinguished by having a vesicular rather than a granular nucleus. With the finding that at least one member of the Amoebidae has a central nucleolus, this basis of distinction is somewhat weakened. However, no undoubted *Saccamoeba* has a floating form with long, granular pseudopodia. The finding of cup-like surface structures similar to those of other hartmannellids strengthens its place in this family.

The possession of endocytic bacteria may be a generic characteristic; they have been found in two strains of *S. limax* and three strains of *S. stagnicola*. It should be noted that one of the infected strains of *S. limax* was Scottish and one American. Since *Saccamoeba* is aerobic and has mitochondria, the bacteria do not play the same role as in *Pelomyxa*.

It is still a good guide that a medium-sized, not eruptive but active limax amoeba with a vesicular nucleus, cytoplasmic crystals, a villous or papillate uroidal knob, and a bulging contractile vacuole is likely to be a *Saccamoeba*. If it has long floating pseudopodia, however, it is probably a *Trichamoeba*, but electron microscopy may be required to decide. The occasional absence of cytoplasmic crystals must also be kept in mind.

A 'broad' *Saccamoeba* has a mean L/B ratio less than 4; a 'slender' one, a ratio above 4.

1 Cyst-forming; L 30-75 μm ($\bar{x}$ 50 μm), L/B $\bar{x}$ *c.* 4.2; nucleus 4.5-8.5 μm ($\bar{x}$ 6.5 μm); no cytoplasmic crystals; endocytic bacteria in all known strains; cysts with circular or slightly oval outline, usually sticky coat, diameter 12-19 μm ($\bar{x}$ 15 μm); cup-like surface structures *c.* 25 nm in diameter; mitochondria often elongate. (Fig. 21A-C, F) ***Saccamoeba stagnicola*** Page, 1974
(Europe, NNE.)

— No cysts known; distinct cytoplasmic crystals **2**

2 Numerous small (1-3.5 μm) crystals; amoebae broad **3**

— Usually fewer than 30 crystals; amoebae broad or slender **4**

3 L to 175 μm, some not exceeding 130 μm; pseudopodia sometimes flattened in slow locomotion; distinct hyaline cap in rapid advance; wrinkled villous-bulb uroid; nucleus 3.5-6.5 μm; one to several contractile vacuoles. (Fig. 22A) ***S. wakulla*** Bovee, 1972
(North America.)

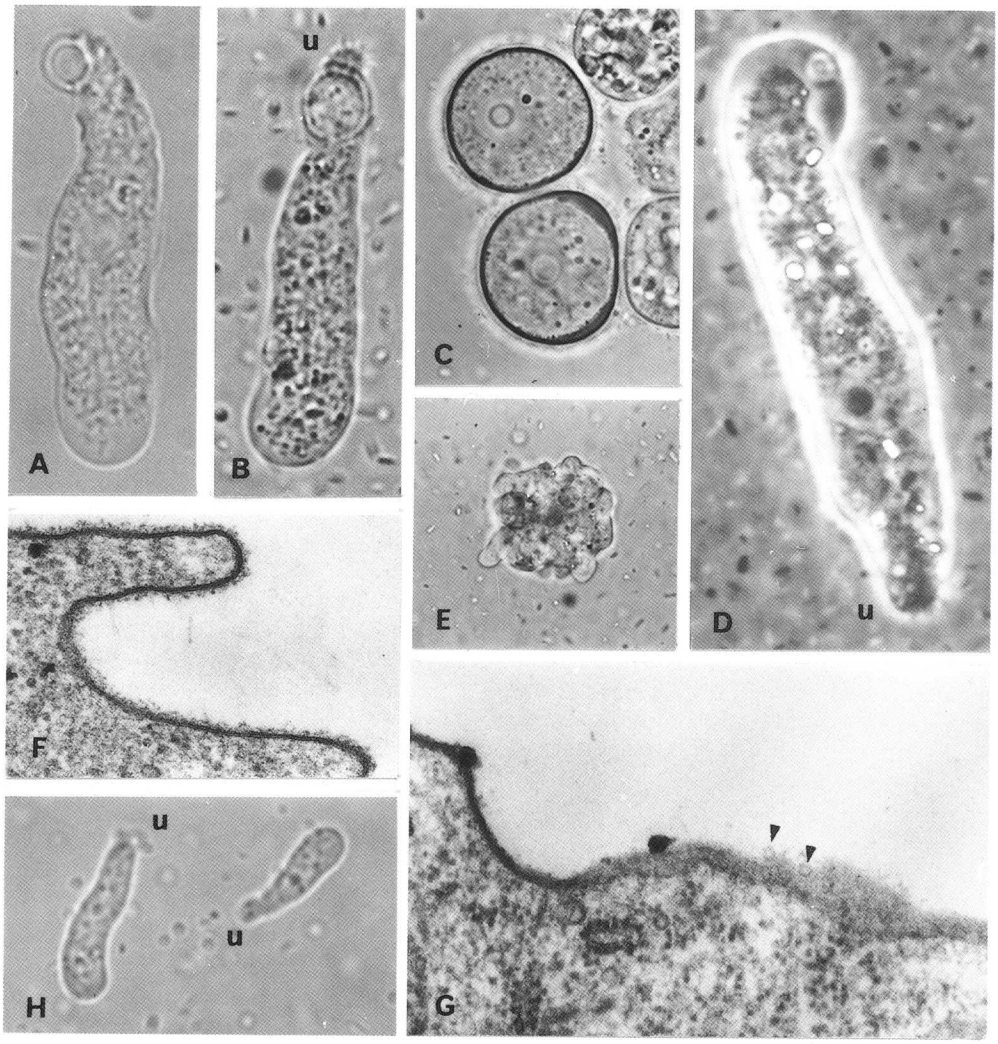

Fig. 21. A-C, *Saccamoeba stagnicola*; C, cysts. D, E, *Saccamoeba limax*; E, floating form. F, G, surface structure of (F) *S. stagnicola* and (G) *S. limax*, with arrowheads indicating outlines of some cup-shaped elements in G. H, *Cashia limacoides*. (A-D, H, ×1,000; E, ×500; F, G, ×50,000.) *u*, uroid.

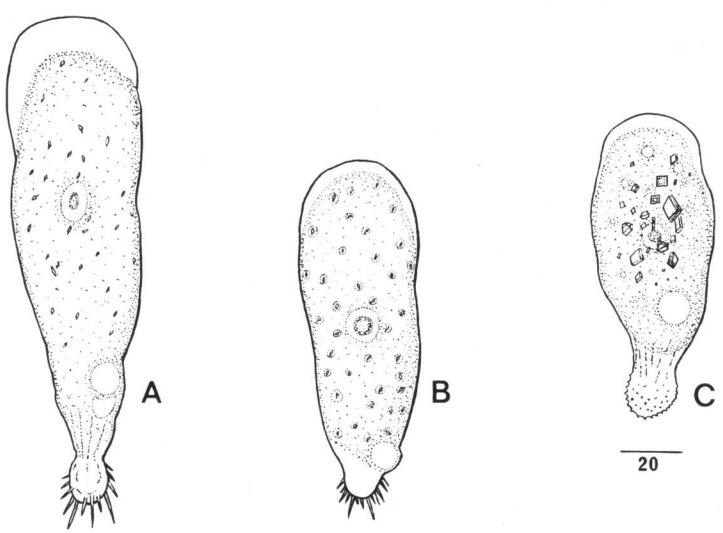

Fig. 22. *Saccamoeba*. A, *S. wakulla*. B, *S. limna*. C, *S. lucens*. (All after Bovee; all to same scale as C.)

— L to 120 μm; hyaline cap reduced in rapid advance; uroidal villi on clear disc or bulb; nucleus 10-15 μm; one contractile vacuole. (Fig. 22B) ***S. limna*** Bovee, 1972
(North America.)

4 (2) Broad, L 70-100 μm; smooth or finely papillate bulbous uroid; nucleus 6-7 μm; usually fewer than 20 crystals, 5-15 μm, often appearing cuboidal or plate-like. (Fig. 22C)
S. lucens Frenzel, 1892, *sensu* Bovee, 1972
(North America, possibly South America and Europe.)

— Slender ($\bar{x}$ L/B *c.* 4.8), L 35-85 μm ($\bar{x}$ 60 μm); villous bulb common; nucleus *c.* 6-11 μm ($\bar{x}$ *c.* 8 μm); bipyramidal crystals to about 3.5 μm, seldom more than 30 present, sometimes lost after years in culture; endocytic bacteria in known strains; cup-like surface structures *c.* 40 nm in diameter; mitochondria never elongate. (Fig. 21D, E, G) ***S. limax*** (Dujardin, 1841)
(Europe, North America. The identity of this widely occurring species with the *Amoeba limax* of Dujardin and Penard is questionable. NNE.)

Other possible species

Hartmannella leptocnemus Singh, 1952: According to Singh, 'There is no clearly defined ectoplasm and endoplasm even when the amoebae move'. This one character does not, of course, make this species a *Saccamoeba*. The cyst has two clearly separated walls.

Saccamoeba angelica Bovee, 1972: In the previous key (Page, 1976) this species was classified as a *Cashia* because it never had a villous uroid. However, it is much larger than the type species of *Cashia* and seems to correspond with *Saccamoeba* in every way except its morulate uroid. Its position cannot be determined with certainty until it has been studied electron microscopically. L 50-100 μm, slender in rapid locomotion; crescentic hyaline cap usually present; morulate uroid common; lateral pseudopodia appearing in slow locomotion; nucleus 5-6 μm; numerous bipyramidal crystals, *c.* 1-2 μm long.
Illustration in Page (1976).

Genus *Cashia* Page, 1974

References: Page (1976a, 1986).

With the finding in *Cashia limacoides* of distinctly helical mitochondrial cristae, that character is added to the diagnosis of the genus. The species included in the earlier key (Page, 1976) as *Cashia angelica* is now listed under *Saccamoeba*, q.v.

L 7.5-25.5 μm (median 14 μm), tapering from rather broad anterior end (L/B $\bar{x}$ 3.3) to narrow posterior end, which may be smooth or bear small bulb; crescentic hyaline cap much reduced or absent; nucleus 2.8-4.1 μm; no crystals; no cysts; sucker-like surface structures 30 nm in diameter; mitochondria never elongate. (Fig. 21H) ***Cashia limacoides*** (Page, 1967)
(North America, Europe. NNE; multiplies slowly.)

Family THECAMOEBIDAE Schaeffer, 1926; emend. Page, 1987

References: Page (1977, 1978b, 1987c); Page & Blakey (1979).

The Thecamoebidae show a diversity of nuclear patterns, some of them similar to those of the Amoebidae. Members of at least three genera are often found in terrestrial habitats.

1 Uninucleate **2**

— Binucleate or multinucleate **4**

2 Elongate (L/B commonly 4 or more), often wrinkled, sometimes branching, with surface coat of discrete radiating filaments ***Pseudothecamoeba*** n. g.

— Generally a flattened ovoid, with outline oblong, oval, or elliptical, L/B not above 2.5, usually less **3**

3 Often with folds or wrinkles; dense glycocalyx *c.* 25-70 nm thick ***Thecamoeba***

— Surface folds lacking, or one on either side only at posterior end; non-mucoid tegument or cuticle, more than 0.5 μm thick ***Dermamoeba***

4 (1) Binucleate, with members of pair closely apposed; sometimes supernumerary pairs; appear-

ance of amoeba much like *Thecamoeba* but usually not wrinkled; possible sexual process in cyst ***Sappinia***

— Multinucleate, otherwise much like *Thecamoeba* ***Thecochaos***

Genus *Pseudothecamoeba* n. g.

References: Page (1976, 1977, 1978b).

Diagnosis; A much flattened cylinder, monopodial or branching, usually with an anterior hyaline cap; length-breadth ratio often 4 or more; surface folds or wrinkles often present. Uninucleate, with granular nucleus in known species. Surface coat of discrete filaments radiating outward from plasma membrane.

Type species: *Pseudothecamoeba proteoides* (Page, 1976) n. comb.

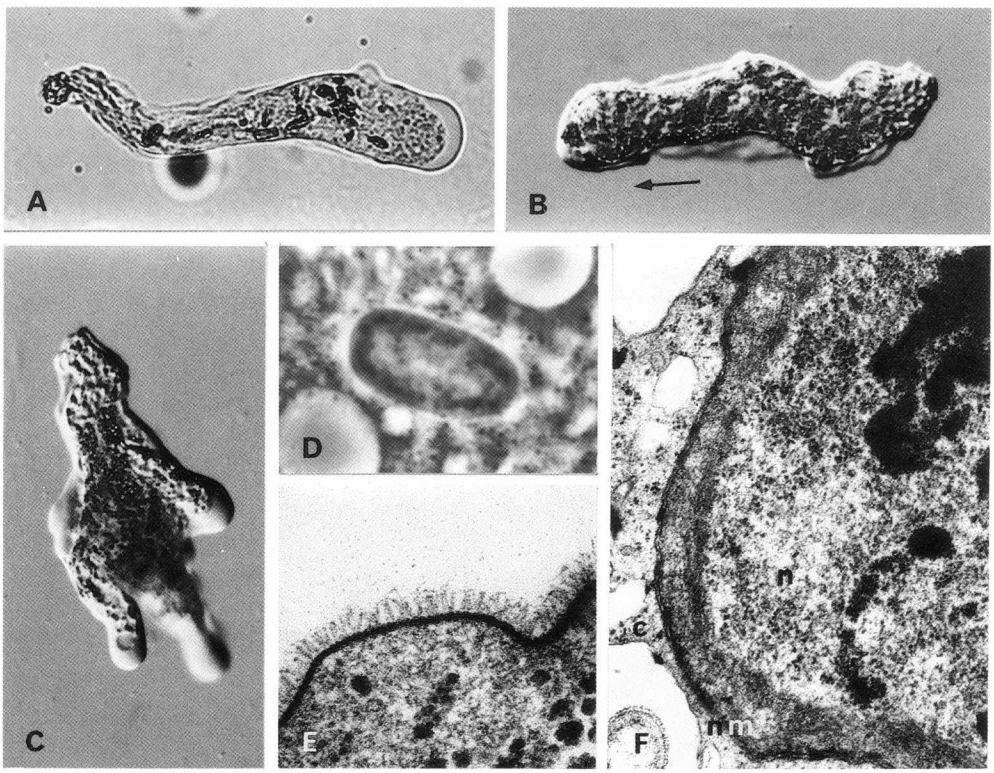

Fig. 23. *Pseudothecamoeba proteoides*. D, nucleus; E, surface structure; F, ultrastructure of nuclear envelope, which shows some faint indications of honeycomb-like inner lamina similar to that illustrated in Fig. 19H. (A-C, ×250; D, ×1,000; E × 50,000; F, ×20,000.) *c*, cytoplasm; *n*, nucleus, *nm*, nuclear membrane.

The only species which can with certainty be attributed to this genus is the type species. It has similarities with the family Amoebidae but is flatter, lacks the crystals which are (with one reported exception) universal in that family, and lacks the usual floating form. It appears more fluid than *Thecamoeba*.

The correct date for the type species is 1976; although it was designated 'sp. nov.' in Page (1977), the name and description were actually published first in Page (1976). It was isolated only once, and the strain has been lost.

Monopodial forms more common but polypodial forms frequent; L 60-280 μm ($\bar{x}$ *c.* 170 μm), L/B of monopodial forms 1.4-10.5 ($\bar{x}$ 4.0); nucleus somewhat elongate, maximum diameter 12-24 μm, with numerous nucleolar fragments distributed throughout nucleus; surface filaments mostly to 80 nm above plasma membrane, some longer; inner nuclear lamina with possible loose honeycomb structure; no cyst known. (Fig. 23)

Pseudothecamoeba proteoides (Page, 1976) n. comb.

(Europe. Ma/r/Chil; E+S with diatoms and other accompanying algae.)

Other species

Amoeba vesiculata Penard, 1902, may well belong to this genus. It is elongate (L/B 3-4), reaching 200 μm. The nuclear structure resembles that of *P. proteoides*, and the cytoplasm is highly alveolar, as is that of *P. proteoides*. However, Penard stated that *A. vesiculata* is never branched.

Illustration in Page (1976).

Genus *Thecamoeba* Fromentel, 1874

References: Singh & Hanumaiah (1979); Singh, Misra & Sharma (1982); otherwise as for family.

Re-diagnosis: A flattened ovoid or oblong, with length usually less than 2.5 times width; usually with longitudinal surface wrinkles, or folds; hyaloplasm an anterior crescent, often with lateral extensions toward posterior end; no branching. Normally uninucleate. A thick, dense, commonly amorphous glycocalyx. No cysts known.

Type species: *Thecamoeba quadripartita* Fromentel, 1874.

Singh & co-workers proposed to subdivide *Thecamoeba* into 8 genera on the basis of nuclear structure and mitotic pattern, classifying 4 genera in the Schizopyrenidae (=Vahlkampfiidae) and 4 in the Hartmannellidae.

These proposals discounted important light microscopic characters as well as what was known of fine structure at the time; subsequent electron microscopic findings further support the rejection of their proposals. At least 4 of the species which they would classify in the Schizopyrenidae have tubular mitochondrial cristae (unpublished), as do all other Thecamoebidae which have been examined with the electron microscope, whilst the Vahlkampfiidae have discoid cristae.

The genus *Thecamoeba*, from which *Dermamoeba* was separated earlier, is emended above to distinguish it from *Pseudothecamoeba* and is retained with full recognition of its nuclear diversity as well as of the characters binding this genus and family together.

1 Outline and general surface fairly smooth, often with several parallel folds extending far anteriorly; normal locomotive form and activity in fresh wet mounts ('smooth' species) **2**

— Surface of stationary amoebae moderately to highly wrinkled, becoming somewhat smoother in locomotion, but most species with rather wrinkled edges and irregular, usually longitudinal dorsal folds; well-extended and active locomotive forms rare on slides under cover glasses ('rugose' species) **3**

2 Nucleus with a single, central, smoothly contoured nucleolus; L usually *c.* 35-100 μm (x̄ 50-70 μm), giant forms in some cultures to 170 μm; x̄ L/B 1.7; nucleus usually *c.* 7.5-11.0 μm (x̄ 9.5 μm). (Fig. 24A, B, G) ***Thecamoeba quadrilineata*** (Carter, 1856)
(Asia, Europe, North America. CPA with accompanying bacteria and *Vexillifera bacillipedes.)*

— Nucleus with 2 or 3 (occasionally more) parietal nucleolar lobes; L 30-80 μm (x̄ 47-54 μm); x̄ L/B 1.7; no gigantism observed; nucleus 6-10 μm (x̄ 7.7 μm); very similar to *T. quadrilineata* except in nuclear structure. (Fig. 24C, D, H) ***T. striata*** (Penard, 1890)
(Europe, North America. CPA with accompanying bacteria and *Naegleria gruberi.*)

3 Nucleolar material in parietal pieces **4**

— Nucleolar material not parietally arranged **5**

4 L usually 60-200 μm (x̄ 110-120 μm), sometimes larger; x̄ L/B *c.* 1.6-1.7; in locomotion, usually wrinkles around periphery and sometimes fine surface wrinkles extending far forward from more or less wrinkled, sometimes plicate uroid; sometimes small hyaline knobs or scallops on anterior edge; nucleus an elongate ellipsoid, with rather larger parietal nucleolar pieces of various sizes, 14-31 μm (x̄ *c.* 21 μm). (Fig. 24E, F, I) ***T. terricola*** (Greeff, 1866)
(Europe, India, terr. Sometimes confused with *T. verrucosa*. CPA with accompanying bacteria and *Acanthamoeba polyphaga.*)

— L 30-80 μm (x̄ 46-57 μm), often broader than most members of genus (L/B 0.8-1.8, x̄ 1.3); surface moderately wrinkled on stationary amoebae, smoother in locomotion, sometimes with a few dorsal folds converging posteriorly; nucleus usually ovoidal or ellipsoidal with numerous small, parietal nucleolar pieces, 7.7-13.9 μm (x̄ 10.0 μm). (Fig. A-D)
T. similis (Greeff, 1891)
(Europe; terr. CPA with accompanying bacteria and *Rosculus ithacus.*)

5 (3) L 80-130 μm (x̄ 98 μm), x̄ L/B 1.4; sometimes with knobby uroid but not strongly tapered

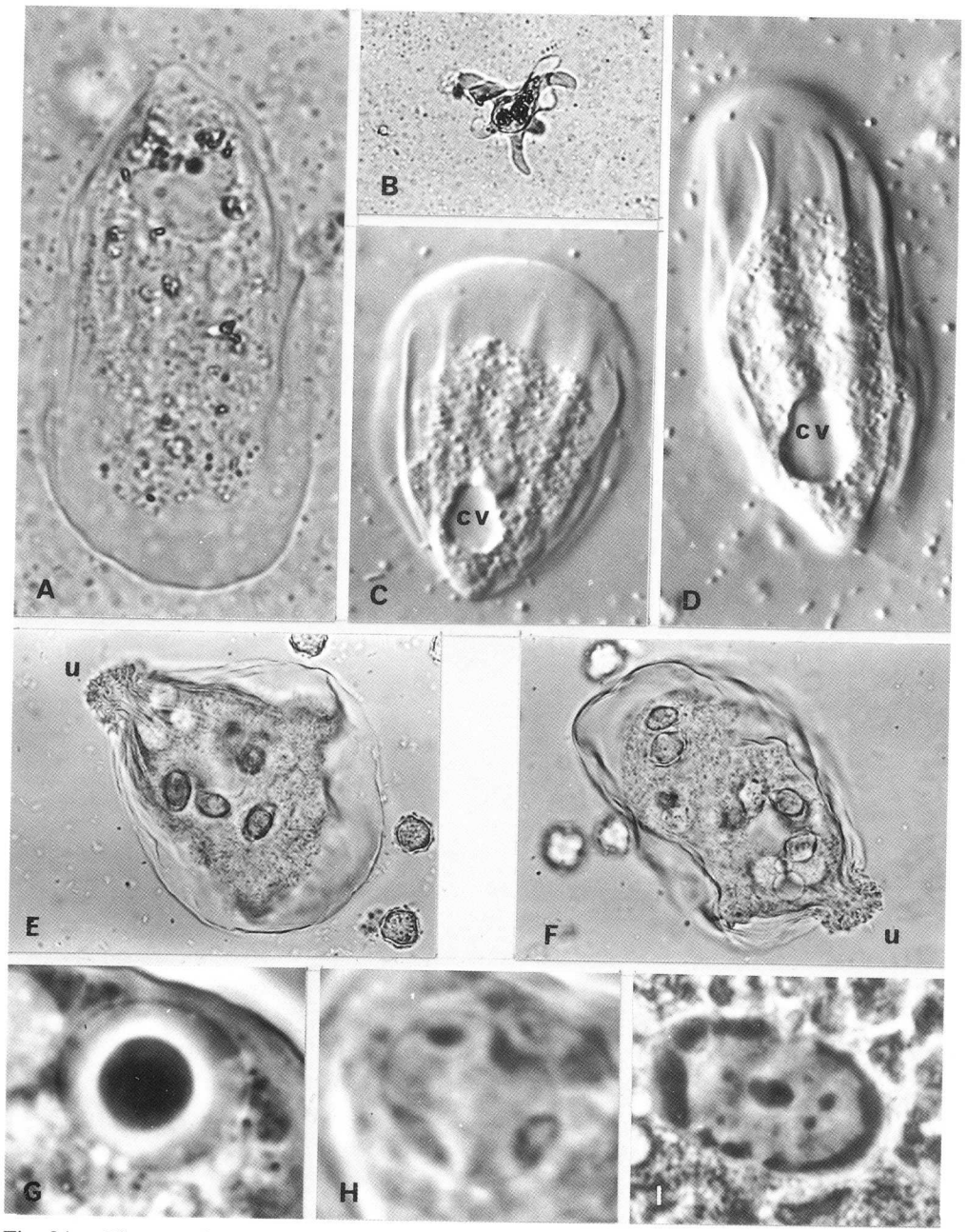

Fig. 24. *Thecamoeba*. A, B, *T. quadrilineata*, locomotive and floating forms. (The crystals in A are from ingested *Vexillifera*.) C, D, *T. striata*. E, F, *T. terricola*. G-I, nuclei: G, *T. quadrilineata*; H, *T. striata*; I, *T. terricola*. (A, C, D, I, ×1,000; B, ×250; E, F, ×500; G, H, ×2,500.) *cv*, contractile vacuole; *u*, uroid.

posteriorly; nucleus spherical or ovoid (11.0-15.5 μm, $\bar{x}$ 12.8 μm), with nucleolar material in coarsely granular central mass, sometimes partly or completely divided into 2 or 3 fragments in centre of nucleus. (Fig. 25E-G) ***T. sphaeronucleolus*** (Greeff, 1891)

(Europe, North America; terr. The strain on which this description is based may not belong to the same species as some used in recent experimental work. CPA with accompanying bacteria and *Vannella* sp.)

— L approximately as above or slightly larger, with more tendency to form shrivelled uroid on markedly tapered posterior end (outline sometimes triangular); two central, closely apposed endosomal structures in nucleus. (Fig. 26)

T. verrucosa (Ehrenberg, 1838) *sensu* Gläser, 1912

(Europe. Sometimes confused with *T. terricola*.)

Other species

Thecamoeba quadripartita Fromentel, 1874: Not certainly identifiable. Smooth, with parallel dorsal

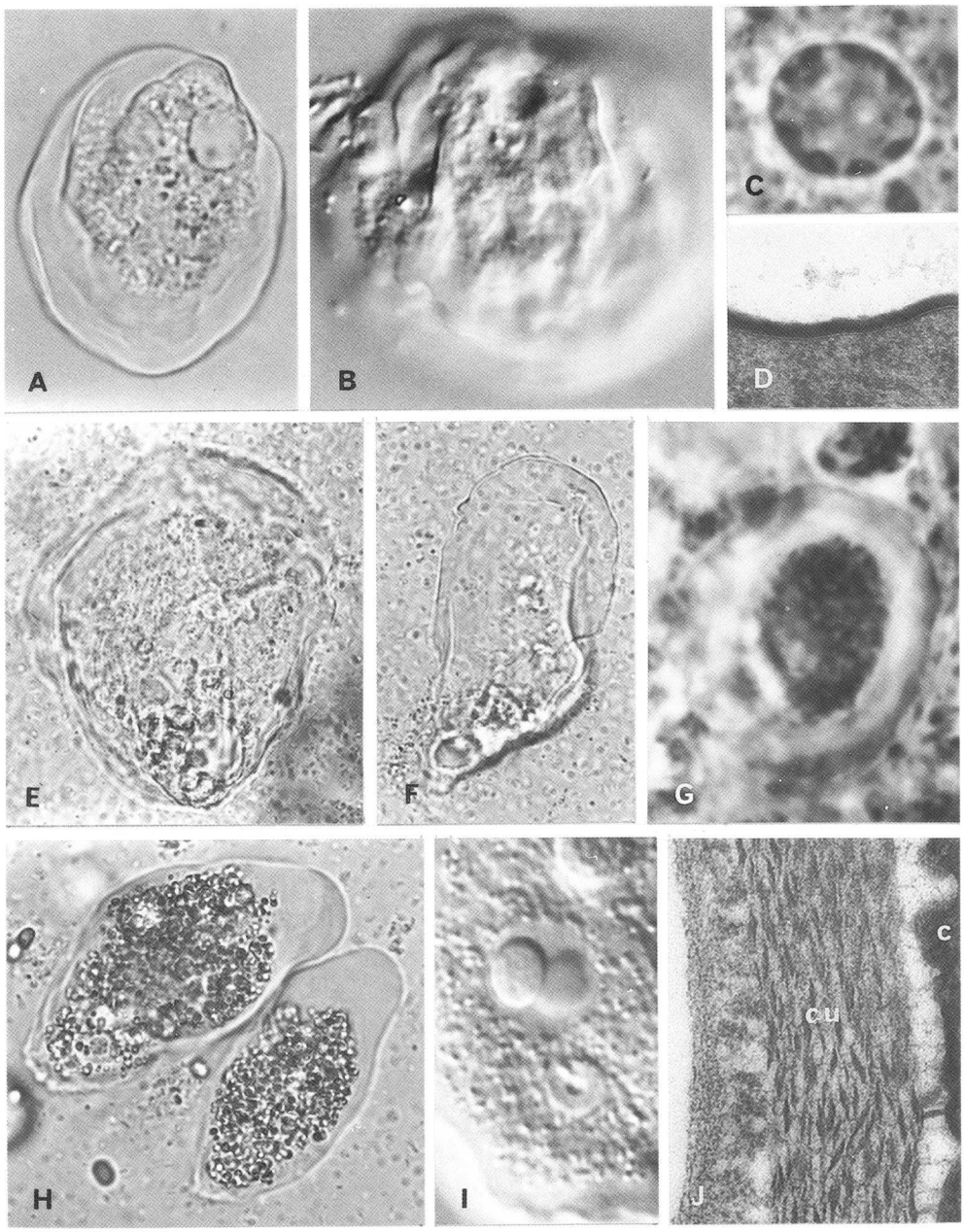

Fig. 25. A-D, *Thecamoeba similis*; B, surface folds shown by differential interference contrast; C, nucleus; D, surface structure, very similar to that of several species of *Thecamoeba* and of *Sappinia diploidea*. E-G, *Thecamoeba sphaeronucleolus*; G, nucleus. H-J, *Dermamoeba granifera*; I, nucleus; J, ultrastructure of cuticle. (A, B, H, ×1,000; C, G, ×2,500; D, J, ×50,000; E, F, ×500; I, ×1,575.) *c*. cytoplasm; *cu*, cuticle.

folds, about 100 μm long. No nucleus mentioned or figured, so probably not with a central nucleolus. Possibly a largish *T. striata*.

T. papyracea (Penard, 1905): Resembles *T. terricola* but differs somewhat in appearance and especially in nuclear size and structure. Nucleus an elongate ovoid or ellipsoid, *c*. 38-72 μm, with many small nucleolar spherules in outer region of nucleus. L usually more than 200 μm.

T. bilzi (Schaeffer, 1926): A smooth *Thecamoeba* 70-90 μm long, with a nucleus like that of *T. striata*.

T. corrugata Bovee, 1953: A smooth *Thecamoeba* 30-40 μm long, about 6 dorsal folds, a nucleus like that of *T. quadrilineata*.

T. ovalis Lepsi, 1960: Broad, *c*. 35-40 μm long, with nucleus like that of *T. quadrilineata*.

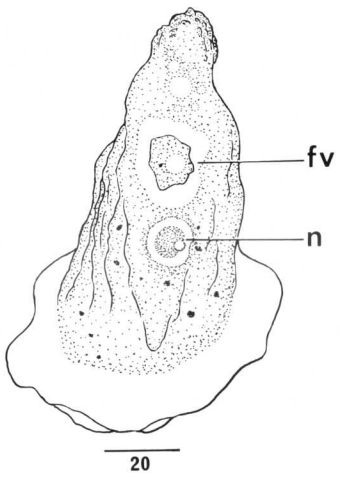

Fig. 26. *Thecamoeba verrucosa* (after Gläser). *fv*. food vacuole; *n*, nucleus.

T. hoffmani Sawyer, Hnath & Conrad, 1974: From sections of trout gills; believed to be involved in pathological condition.

Reference: Sawyer *et al.* (1974).

The following species were described by Singh and colleagues from soil in India, with little information about measurements.

Greeffia soli Singh & Hanumaiah, 1979: Similar to *T. similis*. Diameter 'in rounded condition' *c.* 70-125 μm; according to figures may be 100 μm long in locomotion. Nucleus filled with numerous nucleoli.

Carteria bengaliensis Singh, Misra & Sharma, 1982: A smooth *Thecamoeba* often with 2-4 longitudinal folds; diameter in rounded condition *c.* 30-50 μm; according to figures may be 60 μm long in locomotion. Nucleus with a single central nucleolus.

Sahnium lucknowensis Singh, Misra & Sharma, 1982. Possibly a rugose *Thecamoeba*, judging by some of figures; diameter in rounded condition *c.* 80-120 μm; according to figures may reach 90 μm in locomotion. Hyaloplasm may become divided into two or three parts, but this does not appear to be a true polypodial condition. Nucleus with nucleolar granules parietally arranged.

Genus *Dermamoeba* Page & Blakey, 1979

References: Page (1977); Page & Blakey (1979); Pussard *et al.* (1979)

Outline in locomotion an elongate ellipse or oval, often narrower anteriorly; deep, crescent-shaped anterior hyaloplasm, often with narrow extensions along sides to posterior end; smooth, except often a broad, morulate uroid from which surface folds may extend a short distance anteriorly on either side. Nucleus usually containing two central bodies, one smoother than the other, varying in their relative sizes. Endoplasm usually filled with yellowish or brownish spherules (at least partly lipoidal), *c.* 0.5-3.0 μm in diameter.

The only other amoeba in which a similar nuclear structure in known is *Thecamoeba verrucosa*.

The amoeba which Pussard *et al.* designated *Thecamoeba granifera* ssp. *minor* is here raised to the rank of a species. Although it has not been examined with the electron microscope, it presumably has a cuticle like that of *D. granifera*.

Pussard *et al.* found that *D. minor* could be grown exclusively on fungi as food organisms and suggested that *D. granifera* may also be mycophagous.

1 L *c.* 50-85 μm ($\bar{x}$ 63 μm), $\bar{x}$ L/B 1.8, often narrowing toward anterior end; nucleus 7.7-12.4 μm ($\bar{x}$ 9.4 μm); no cyst known. (Fig. 25H-J) ***Dermamoeba granifera*** (Greeff, 1866)
(Europe; terr. Culture method: CPA with accompanying organisms.)

— L 30-50 μm ($\bar{x}$ 41 μm), $\bar{x}$ L/B 1.6, less narrowed toward anterior end than preceding species; nucleus 8-12 μm; cysts round, 17-27 μm ($\bar{x}$ 21 μm); penetrates walls of fungal structures and

ingests contents ***Dermamoeba minor*** (Pussard, Alabouvette & Pons, 1979) n. comb.
(Europe; terr. Culture method: see Pussard *et al.*, 1979.)

Genus *Sappinia* Dangeard, 1896

Reference: Goodfellow *et al.* (1974).

This amoeba looks much like a smooth *Thecamoeba*, and its thick glycocalyx resembles that of such species as *T. similis*. But by its life cycle, including a probable sexual process in the cyst, and by the presence in the trophic amoeba of a pair of closely apposed nuclei (often 2-4 pairs), it is unique. These amoebae do not extend well on glass and are best observed on the agar surface.

One well described species.

L *c.* 45-85 μm ($\bar{x}$ 63 μm), $\bar{x}$ L/B 1.9; lengths of pairs of closely joined nuclei 9.8-12.2 μm ($\bar{x}$ 10.8 μm); *c.* 35% of trophic amoebae with more than one pair of nuclei; forming cysts which begin as pair of closely apposed binucleate amoebae, the mature cyst unicellular and binucleate, not stalked, diameter 13-37 μm ($\bar{x}$ 22 μm). (Fig. 27A-D)
Sappinia diploidea (Hartmann & Nägler, 1908)
(Europe, Middle East, Japan, North America, West Indies; terr. NNE.)

Other species

Sappinia pedata Dangeard, 1896, is the type species, but most workers have identified their isolates as *S. diploidea*. According to Dangeard, *S. pedata* had a stalked resting stage or cyst, but he did not report an apparent sexual stage or measurements. Possibly *S. diploidea* is a junior synonym. Both species have been reported from mammalian faeces.
Illustration in Page (1976).

Genus *Thecochaos* Page, 1981

References: Page (1981a); Penard (1902).

Often irregularly oval to oblong in outline but sometimes more elongate, branching usually only when changing direction; surface wrinkles; hyaloplasm usually a more or less crescentic cap; many nuclei.

These amoebae are known to the author only from Penard's specimens, nor are reports of any subsequent findings known. They appear to be essentially multinucleate *Thecamoeba*, though *T. fibrillosum* somewhat resembles *Pseudothecamoeba* on the slides.

The measurements are derived principally from a few specimens fixed with absolute ethanol; those from Penard's description are presumably of living specimens. His description of the nucleus of *T. album* was inaccurate, possibly because of lack of an oil-immersion objective.

1 Nuclei often elongate, also ovoid, with single central nucleolus of same shape; nuclear diameter 7.0-10.8 μm ($\bar{x}$ 8.9 μm), *c.* 100 nuclei per amoeba; amoebae broad and flattened or more slender, sometimes with temporary branching, 160-320 μm (Fig. 27E, F)
Thecochaos fibrillosum (Greeff, 1891)
(Europe.)

— Nuclei often elongate ellipsoids or more nearly spherical, with nucleolar material as parietal bands or lobes; elongate nuclei 7.0-12.0 μm ($\bar{x}$ 9.0 μm), more spherical ones 6.5-7.5 μm ($\bar{x}$ 7.0 μm) (Penard; about 10 μm in diameter); *c.* 100-200 nuclei per amoeba (Penard: up to several hundred); amoebae generally broad, L 165-275 μm (Penard: on average about 360 μm in the resting state), L/B 1.1-2.2. (Fig. 27G, H) ***T. album*** (Greeff, 1891)
(Europe.)

Family VANNELLIDAE Bovee, 1979

References: Page (1987c); Page & Blakey (1979).

These genera were previously included in the family Thecamoebidae. The present family is defined as follows:

Locomotive form flattened, usually more or less flabellate, rarely linguiform, with anterior hyaloplasm usually up to one half of length; floating form often with radiating, hyaline pseudopodia.

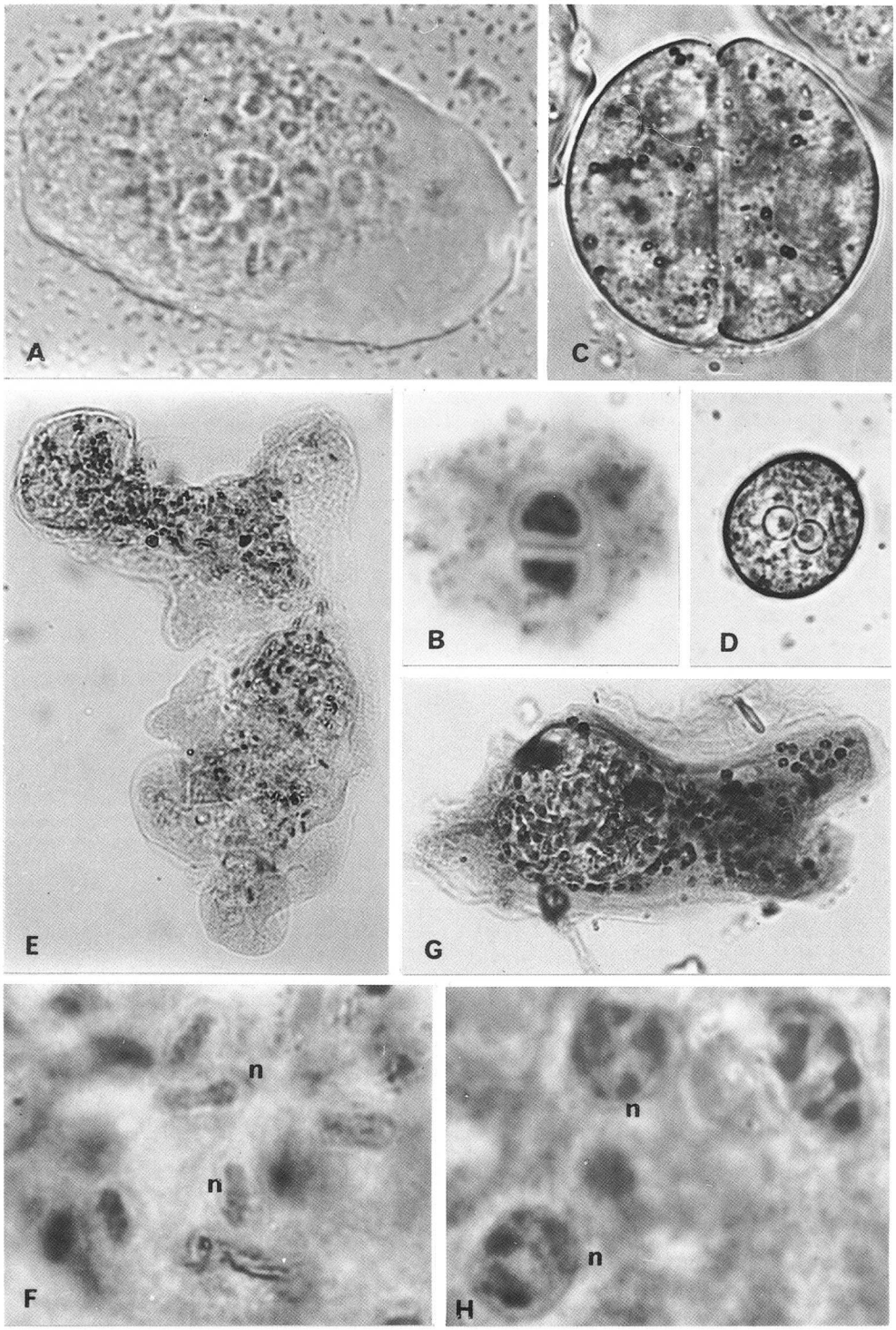

Fig. 27. A-D, *Sappinia diploidea*; A, amoeba moving on agar; B, stained nuclear pair; C, early cyst stage, with two amoebae still separate, secreting cyst wall; D, mature cyst. E, F, *Thecochaos fibrillosum*, in preparation of E. Penard; F, nuclei. G, H, *Thecochaos album*, also in preparation by Penard; H, nuclei. (A, C, D, ×1,000; B, H, ×2,500; E, G, ×250; F, ×1,575.) *n*, nucleus.

Uninucleate; usually a central nucleolus, in a few species parietal lobes. No cytoplasmic crystals (one reported exception). Glycocalyx differentiated into discrete pentagonal glycostyles or less distinct hexagonal arrangements of filamentous material.

1 Locomotive form commonly flabellate, often semi-circular with broad hyaline margin around sides, or in some spatulate with long tail; no longitudinal wrinkles; floating form of freshwater

species with pseudopodia tapering markedly, sometimes pointed, sometimes tightly helical; pentagonal glycostyles slightly more than 100 nm tall ***Vannella***

— Locomotive form flabellate, ovate, or oblong, but not spatulate; sometimes slight longitudinal wrinkles near sides, especially when turning; floating form with few pseudopodia, not tapering much, blunt, often short; known freshwater species cyst-forming; fuzzy glycocalyx, with hexagonal arrangements of filamentous material (not glycostyles) discernible in favourable sections ***Platyamoeba***

Genus *Vannella* Bovee, 1965

References: Bovee (1965); Page (1968, as *Flabellula*); Page & Blakey (1979).

This was the first *genus* clearly associated with a distinctive surface structure, the pentagonal glycostyles (Fig. 28), which have the same structure and almost identical dimensions on freshwater and marine isolates from many parts of the world. Although some marine species lack tapering pseudopodia on the floating form, such pseudopodia occur on all confirmed freshwater *Vannella*. (Radiate floating forms are also found in a few other genera.)

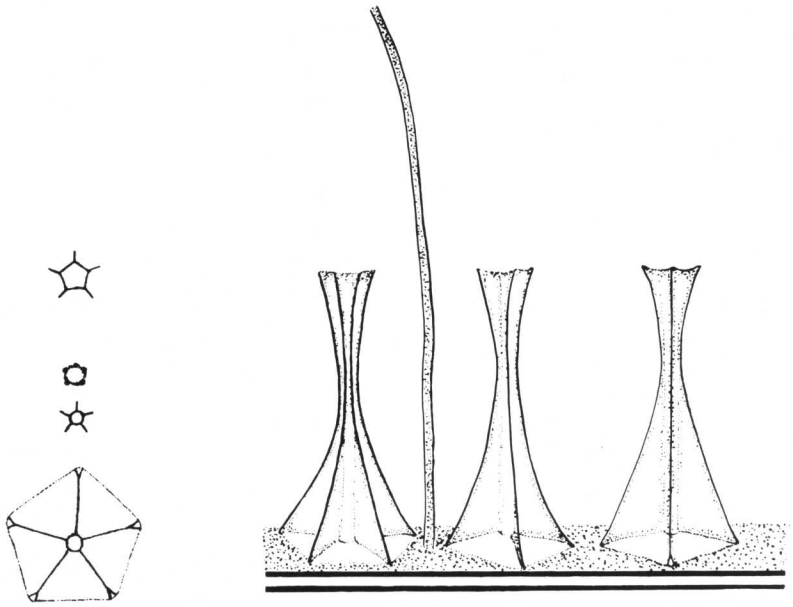

Fig. 28. Surface structure of *Vannella*, showing pentagonal glycostyles found on all freshwater and marine species and simple filament also found on many. Cross-sections of glycostyle at different levels illustrated at left. See actual sections of surfaces of some freshwater species in Fig. 31.

Cysts have not been found in any species classified with certainty in this genus, though *Vannella* has been cultured from leaf litter.

Normally electron microscopic study of the surface should not be required for identification, but it is recommended for isolates which cannot be distinguished with certainty from *Platyamoeba*.

The new combination *Vannella cirrifera* (Frenzel, 1892) is proposed for the freshwater species formerly designated (Page, 1976) as *Vannella mira* (Schaeffer, 1926). Schaeffer's *Flabellula mira* was marine, and long experience has failed to reveal any other gymnamoeba occurring in both fresh and salt water. Furthermore, Frenzel's name has priority, and his figures (Frenzel, 1892) greatly resemble those in this publication.

The species problem seems difficult in *Vannella*. Given its commonness, it may not be possible to assign all isolates to named species.

Vannella lata n. sp.

Diagnosis; Breadth almost always greater than length, never less; 24-46 μm ($\bar{x}$ 33 μm); length/breadth ratio 0.5-1.0 ($\bar{x}$ 0.6), hyaloplasm commonly extending around sides; floating form often with about 8 tapering, pointed pseudopodia, whose length may exceed 3 times the diameter of the

central mass, but many floating cells without pseudopodia; nucleus 3.7-6.5 μm ($\bar{x}$ 5.1 μm); a few simple filaments amongst glycostyles.

Observed habitat: Loch Morar.

Type slides deposited in British Museum (Natural History): Holotype 1986 : 8 : 11 : 1; paratype 1986 : 8 : 11 : 2.

Amoebae of this species adhered well to glass after a few hours.

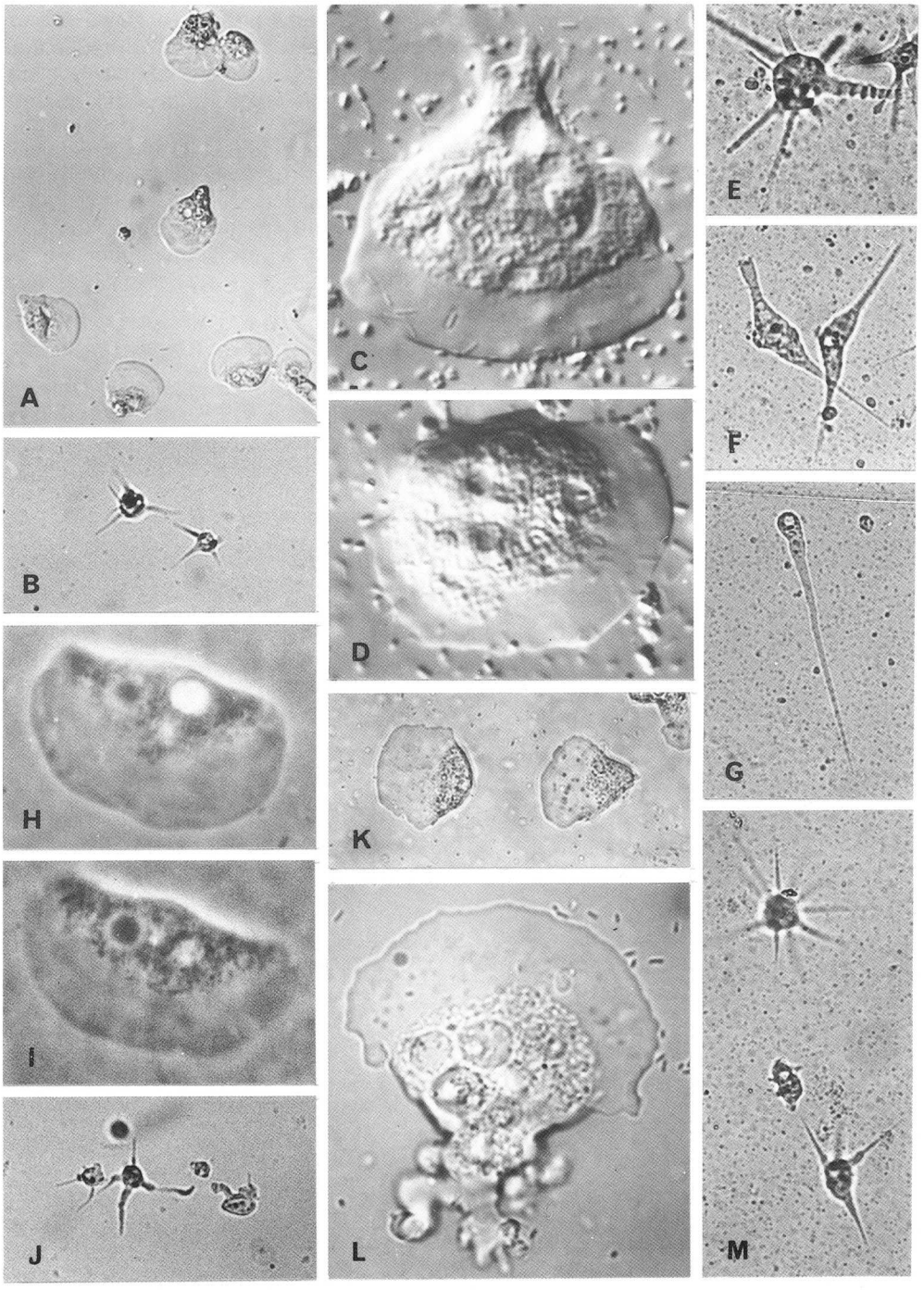

Fig. 29. *Vannella*, A, B, *V. platypodia*, locomotive and floating forms. C-G, *V. simplex*, locomotive (C, D) and floating (E-G) forms. H-J, *V. lata*; J, floating forms; K-M, *V. cirrifera*; L, locomotive forms still trailing remnant of one or more floating pseudopodia; M, floating forms. (A, K ×400; B, E-G, J, M, ×250; C, D, H, I, L, ×1,000.)

1 Locomotive form of large minority, sometimes majority, somewhat longer than broad, sometimes spatulate, prolonged posteriorly; greatest dimension 10-30 μm ($\bar{x}$ 16-21 μm); nucleus 3.4-

5.0 μm; settles from floating form in a few minutes; attaches well to glass; simple filaments amongst glycostyles. (Figs. 29A, B, 31A) ***Vannella platypodia*** (Gläser, 1912)
(Europe, North America. CPAE, NNE.)

— Larger, with breadth commonly the greatest dimension; seldom an elongated posterior end. **2**

2 Breadth greater than length in great majority; greatest dimension 25-80 μm ($\bar{x}$ 42-52 μm), L/B 0.4-1.3 ($\bar{x}$ 0.8); nucleus *c.* 6-11 μm ($\bar{x}$ 7.4-8.6 μm); occasionally 1 or 2 supernumerary nuclei; often slow to settle from locomotive form; usually adheres poorly to glass; some simple filaments amongst glycostyles. (Fig. 29C-G) ***V. simplex*** (Wohlfarth-Bottermann, 1960)
(Europe, North America. CPAE, NNE.)

— Smaller **3**

3 Breadth often nearly twice length, never less than length, with hyaloplasm usually extending around sides; B 25-45 μm ($\bar{x}$ 33 μm), L/B 0.5-1.0 ($\bar{x}$ 0.6); nucleus 3.7-6.5 μm ($\bar{x}$ 5.1 μm); many floating amoebae without radiate pseudopodia, but those with pseudopodia having 3-14, more or less symmetrically distributed; attaches well to glass; a few simple filaments amongst glycostyles. (Figs. 29H-J, 31B) ***V. lata*** n. sp.
(Europe, NNE.)

— Not so broadened **4**

4 Usually flabellate, seldom somewhat spatulate; greatest dimension 25-55 μm; nucleus 4.8-8.3 μm; floating form often symmetrical but sometimes irregular, with much of cytoplasm in 1-3 very long pseudopodia; slow to settle from floating form, attaches poorly; no simple filaments amongst glycostyles (Figs. 29K-M, 31C)
Vannella cirrifera (Frenzel, 1892) n. comb.
(South America, North America, Europe. Culture method: CPAE, NNE.)

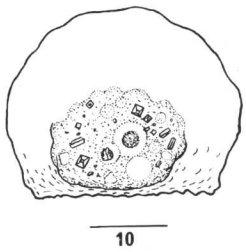

Fig. 30. *Vannella miroides* (after Bovee).

— More or less flabellate, with anterior edge tending to be somewhat wavy; greatest dimension 25-35 μm; nucleus 4-4.5 μm; floating form very regular, with tapered pseudopodia of almost equal lengths; may be slow to settle from floating form; bipyramidal crystals to 1.5 μm reported; fine structure not known. (Fig. 30) ***V. miroides*** Bovee, 1965
(North America.)

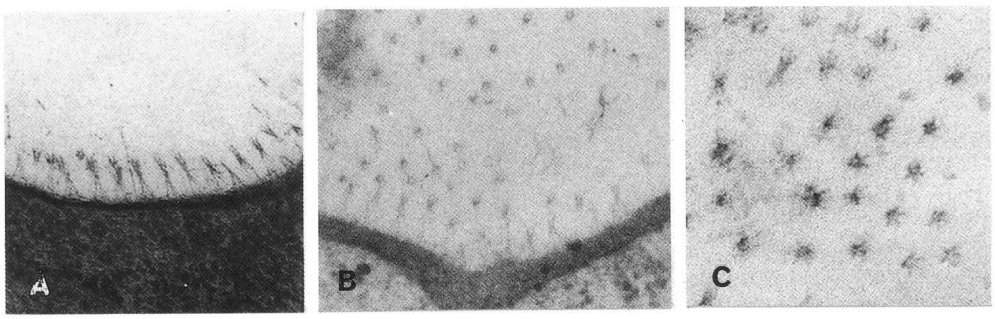

Fig. 31. *Vannella*, surface structure. A, *V. platypodia*, glycostyles and simple filaments; B, *V. lata* n. sp., also with simple filaments and showing some cross-sections of glycostyles. C, *V. cirrifera*, cross-sections of glycostyles. (A, B, ×50,000; C, ×100,000.) See interpretative diagram, Fig. 28.

Other possible species

Amoeba velata Parona, 1883: Although the description might apply to a *Vannella*, the figures do not resemble this genus. The amoeba which Penard (1902) identified as *A. velata* might be a *Vannella*, but he gave no measurements.

Amoeba velifera Maggi, 1888: Possibly a large *Vannella* but might be a broad *Thecamoeba*.

Vannella multimorpha Mote, 1968: The description includes a 'life cycle' with the usual locomotive and radiate forms; a 'cyst' stage representing both inactive and moribund cells but no true cysts; and 'amoeba buds' representing the faecal pellets often found in *Vannella* cultures. A large *Vannella*, with uninucleate fan-shaped locomotive forms often 80 μm wide, radiate forms which could have a diameter including pseudopodia of 175 μm, and a nuclear diameter of 7.7 μm. Binucleate cells common. Some similarity to *V. simplex*.

Vannella cutleri Singh & Hanumaiah, 1979: This amoeba, which had no radiate floating form and produced cysts, was not a *Vannella* but might have been a broad *Platyamoeba* like *P. placida*.

Genus *Platyamoeba* Page, 1969

References: Page (1968 as *Rugipes*, 1969b); Page & Blakey (1979); Singh & Hanumaiah (1979).

Although the genus of gymnamoebae most commonly isolated from seawater, *Platyamoeba* is less common in freshwater and terrestrial habitats.

The genus can usually be distinguished from *Vannella* by the light-microscopic characters on which it was established: (1) occasional inconspicuous folds, (2) difference in floating form, (3) production of cysts by known freshwater and soil *Platyamoeba*. In addition, two named species have a linguiform shape, never found in *Vannella*. The anterior edge is sometimes truncate.

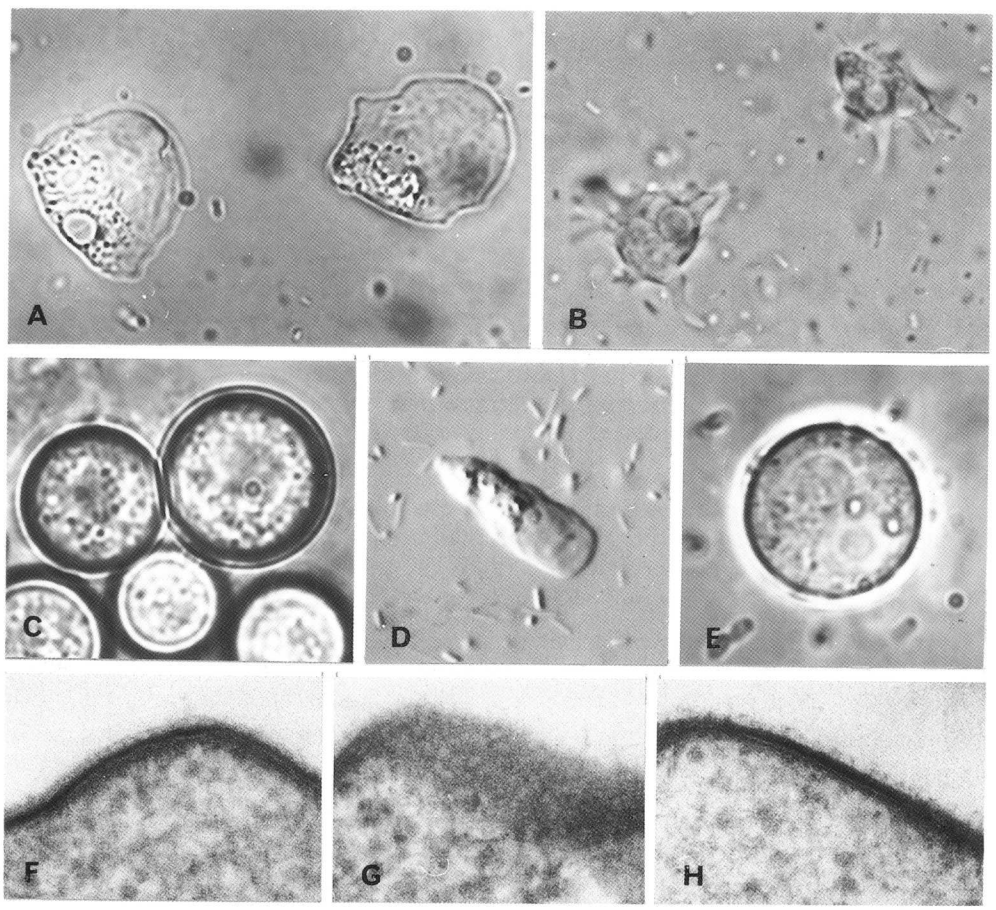

Fig. 32. *Platyamoeba*. A-C, *P. placida*, locomotive forms, floating forms, cysts. D, E, *P. stenopodia*, locomotive form and cyst. F-H, surface structure: F, G, *P. placida*, with hexagonal arrangements of filamentous material (not glycostyles) discernible in tangential section in G; h, *P. stenopodia*. (A, B, D, ×1,000; C, E, ×2,500; F-H, ×100,000.)

Nevertheless, in case of doubt an electron-microscopic examination might be desirable, e.g., if the isolate lacks *Vannella*-like floating forms but fails to produce cysts, or if one suspects that a cyst-forming isolate is, exceptionally, a *Vannella*.

1 Outline usually oval, often broadly elongate, less often flabellate, anterior edge arched or truncate; greatest dimension, usually L, 15-35 μm, L/B 0.6-1.9 ($\bar{x}$ 1.2); nucleus 3.4-5.5 μm; cysts spherical, smooth, with closely apposed outer wall layer thinner than inner wall layer, diameter *c.* 7-10.5 μm (Fig. 32A-C, F, G) ***Platyamoeba placida*** (Page, 1968)
(North America, Europe, NNE.)

— Elongate, linguiform, or an elongate oval, with anterior end often truncate **2**

2 L 15-35 μm ($\bar{x}$ *c.* 24 μm), L/B 1.5-4.5 ($\bar{x}$ *c.* 2.5); nucleus 3-5 μm; cysts spherical, with smooth inner wall layer and thinner, often slightly wrinkled, closely applied outer layer, diameter 7-11.5 μm. (Fig. D, E, H) ***P. stenopodia*** Page, 1969
(North America, Europe; terr. NNE. Loses capacity to encyst after long period in culture.)

— Appearance of amoebae much like that of *P. stenopodia*; cysts with inner wall covered by gelatinous layer; approximate dimensions, derived from illustrations: L of amoeba, 27-29 μm; cyst diameter, 14-19 μm. ***P. schaefferi*** Singh & Hanumaiah, 1979
(Asia; terr. Culture method: NNE.)

Other Vannellidae

Pessonella Pussard, 1973: The single species of this genus. *P. marginata* Pussard, 1973, is flattened, with a flabellate, oval, semi-circular, or elliptical outline; it measures approximately 35×45 μm. Under the conditions of observation, small bosses occur on the hyaloplasm. The floating form has blunt, hyaline, bent pseudopodia. The nucleus, with 2-7 (usually 3-5) parietal nucleolar lobes, measures 4-8 μm. No cyst has been observed. It was isolated from compost in France.
Illustrations in Page (1976).

This could be either a *Vannella* or a *Platyamoeba*. Similar nuclei are found in one marine species of each genus. Its floating form resembles that of *Platyamoeba*; its apparent lack of cysts, *Vannella*. It cannot be placed accurately in either of those genera or in a separate genus until it has been examined with the electron microscope.

Family PARAMOEBIDAE Poche, 1913; emend. Page, 1987

References: Bovee (1970); Page (1972a, 1982, 1983a, 1983b, 1987c).

Digitiform or mamilliform blunt, hyaline, never furcate subpseudopodia (dactylopodia), usually produced from anterior hyaloplasm of locomotive form, which is somewhat compressed and longer than broad; floating form often with hyaline, radiating pseudopodia; no filamentous cores in subpseudopodia or floating pseudopodia. Uninucleate; nucleolar material in central body. Cysts seldom if ever produced. Surface covered with cuticle or boat-shaped microscales.

This is the family also known by the junior synonym Mayorellidae. The recent re-diagnosis eliminates smaller amoebae whose subpseudopodia and floating pseudopodia have filamentous cores, the Vexilliferidae. Two other genera, *Dinamoeba* and *Phreatamoeba*, which have hyaline, conical pseudopodia but differ in other ways from the Paramoebidae and are probably not related to them, are described in Genera *incertae sedis* (p. 107).

The generic nomenclature follows that introduced in a key to marine gymnamoebae (Page, 1983b). Essentially, the genus *Mayorella* includes those species with a cuticle and *Dactylamoeba* those with microscales, i.e., scales discernible only with the electron microscope. *Paramoeba*, which also has microscales, does not occur in freshwater. The name *Hollandella* Page, 1982, is invalid, for reasons discovered in further investigations after it was proposed.

In both genera, the subpseudopodia may be at the anterior ends of cytoplasmic ridges.

Since both cuticle and microscales can be detected only with the electron microscope an attempt has been made to correlate surface structure with light microscopic characters, but it is still impossible to place all described species into one genus or the other, because some descriptions seem to indicate that a single species may have characters attributed to both genera. Species which cannot reasonably be placed into either genus are listed under *Mayorella sensu lato*.

In the clonal strains investigated, the presence of paired, optically active bodies always distinguished Mayorella *from* Dactylamoeba.

These largely medium-sized amoebae, or at least some species of them, feed on both unicellular algae and protozoa.

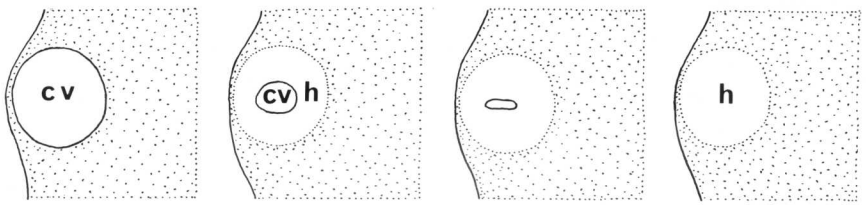

Fig. 33. Replacement of contractile vacuole by hyaloplasm in *Mayorella*. Such a hyaloplasmic replacement has not been observed in *Dactylamoeba* and appears to be one means of distinguishing the two genera without using the electron microscope. *cv*, contractile vacuole; *H*, hyaloplasm.

1 Surface covered with cuticle about 200 nm thick, composed of thin-walled, funnel-shaped elements in fibrillar matrix; dactylopodia often temporarily absent in some species; contractile vacuole replaced after emptying by temporary hyaloplasmic area, where studied (Fig. 33); floating form usually without clear separation into a regularly spherical central mass and pseudopodia, and pseudopodia, if present, often broad at bases; crystalline inclusions, often attached to spherical body or paired ***Mayorella***

— Surface covered with complex, boat-shaped, lattice-work microscales, larger microscales *c.* 500-700 nm long, accompanied by smaller ones *c.* 150 nm long; locomotive form like that of *Mayorella* but less likely to lack dactylopodia; contractile vacuole not replaced by hyaloplasmic area, where studied; floating form more likely to have regularly spherical central mass, with slender radiate pseudopodia; crystalline inclusions absent in known members of genus ***Dactylamoeba***

Genus *Mayorella* Schaeffer, 1926

References: Cann (1981); Schaeffer (1926); others as for family.

This genus appears to contain a fair number of species, several of which are probably comprehended under the older names *Amoeba spumosa* and *Amoeba vespertilio*. The restriction of the dichotomous key largely to species investigated by this author does not discount the value of some earlier observations. However, it seems likely not only that each older name has been applied to several species but also that some descriptions may have been based on representatives of several species.

Possible synonymies of recently proposed names with older ones were discussed by Page (1983a).

Strains of *Mayorella* have only recently been brought into clonal culture; the possibility of size differences in amoebae of the same species from nature and from culture is therefore particularly relevant. Amoebae of this genus may have morulate or, less often, plicate uroids, but these do not yet appear useful for a dichotomous key.

1 With zoochlorellae; L 90-160 μm ($\bar{x}$ 120 μm); mamilliform subpseudopodia often overtaken by advancing hyaloplasm; nucleus *c.* 9-15 μm ($\bar{x}$ 12 μm); cuticle *c.* 200 nm thick. (Fig. 35A)
Mayorella viridis (Leidy, 1874)

(North America, Europe. NN overlaid with PJ, kept in indirect light.)

— Without zoochlorellae **2**

2 Mean L usually 100 μm or more; subpseudopodia almost always present **3**

— Smaller; subpseudopodia sometimes or often absent, with anterior end then broad and truncate; cuticles 180-200 nm thick **4**

3 L 100-300 μm, sometimes greater in older cultures, $\bar{x}$ *c.* 150 μm in younger cultures; fairly broad; floating form with long radiate pseudopodia; nucleus *c.* 12 μm. (Fig. 34)
M. bigemma (Schaeffer, 1918)

(North America, Europe. Type species.)

L 55-180 μm ($\bar{x}$ 101 μm), L/B 1.2-8.6 ($\bar{x}$ 2.8); floating form somewhat rounded, sometimes with short, hyaline pseudopodia but never with long radiate pseudopodia; nucleus 8.4-13.0 μm ($\bar{x}$ 10.1 μm); cuticle usually 200-230 nm thick, sometimes to 280 nm (Fig. 35B-D, J, N)

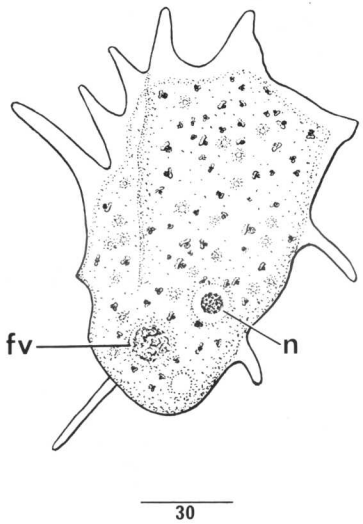

Fig. 34. *Mayorella bigemma* (after Schaeffer). *fv*, food vacuole: *n*, nucleus.

M. cantabrigiensis Page, 1983

(Europe. CP/r with accompanying organisms.)

4 (2) L 35-125 μm ($\bar{x}$ 68 μm), L/B 1.2-6.5 ($\bar{x}$ 2.5), subpseudopodia often lacking; floating form sometimes with radiate pseudopodia tapering from broad base; nucleus 5.6-13.0 μm ($\bar{x}$ 8.5 μm). (Fig. 35E-G, K, O) ***M. penardi*** Page, 1972

(Europe, North America. The amoeba incorrectly designated *Amoeba spumosa* Gruber, 1885, by Penard. CP/r with acompanying organisms; never numerous in culture.)

— L 35-90 μm ($\bar{x}$ 61 μm), L/B 1.2-4.6 ($\bar{x}$ 2.6), subpseudopodia sometimes lacking; floating forms sometimes with radiate pseudopodia; nucleus 5.6-10.0 μm ($\bar{x}$ 7.4 μm). (Fig. 35H, I, L, M, P) ***M. vespertilioides*** Page, 1983

(Europe. CP/r with accompanying organisms; often numerous in culture.)

Other possible species

Amoeba vespertilio Penard, 1902: Original description possibly drawn from several species. The possibility that some English isolates might belong to this species was rejected for reasons given earlier (Page, 1983a). Penard said that the length of amoebae with short pseudopodia was generally about 70 μm; the nucleus in one of his fixed preparations has a diameter of 12.5 μm.

Mayorella augusta Schaeffer, 1926: Described from a single specimen. L 250-350 μm; narrow, with anterior end sometimes dividing into two branches (cf. *M. cantabrigiensis*), subpseudopodia usually present; nucleus *c.* 18 μm; crystals of irregular shape, sometimes in clusters.

Illustrations in Page (1976)

M. godesae De la Arena, 1953: L 200-250 μm; subpseudopodia sometimes reduced; floating form with long, tapering pseudopodia; nucleus 15-20 μm; crystalline inclusions like those in *M. bigemma* and other species.

Illustrations in Page (1976).

Amoeba lyncaea Cigada Leonardi, 1980: Similar to *M. penardi*.

Many of the species described by Bovee probably have a cuticle, but they are treated as a group under *Mayorella sensu lato*, below.

Genus *Dactylamoeba* Korotneff, 1880

References: Korotneff (1880); Page (as for family); Pennick & Goodfellow (1975); Schaeffer (1926).

Isolations of *Mayorella*-like amoebae from freshwater have yielded many more cuticle-bearing than microscale-bearing strains. Since several microscale-bearing strains have been cultured with little difficulty both in liquid and on agar, inappropriate culture methods do not appear to be the reason for the imbalance. These results and the fact that many of Bovee's species (under *Mayorella*

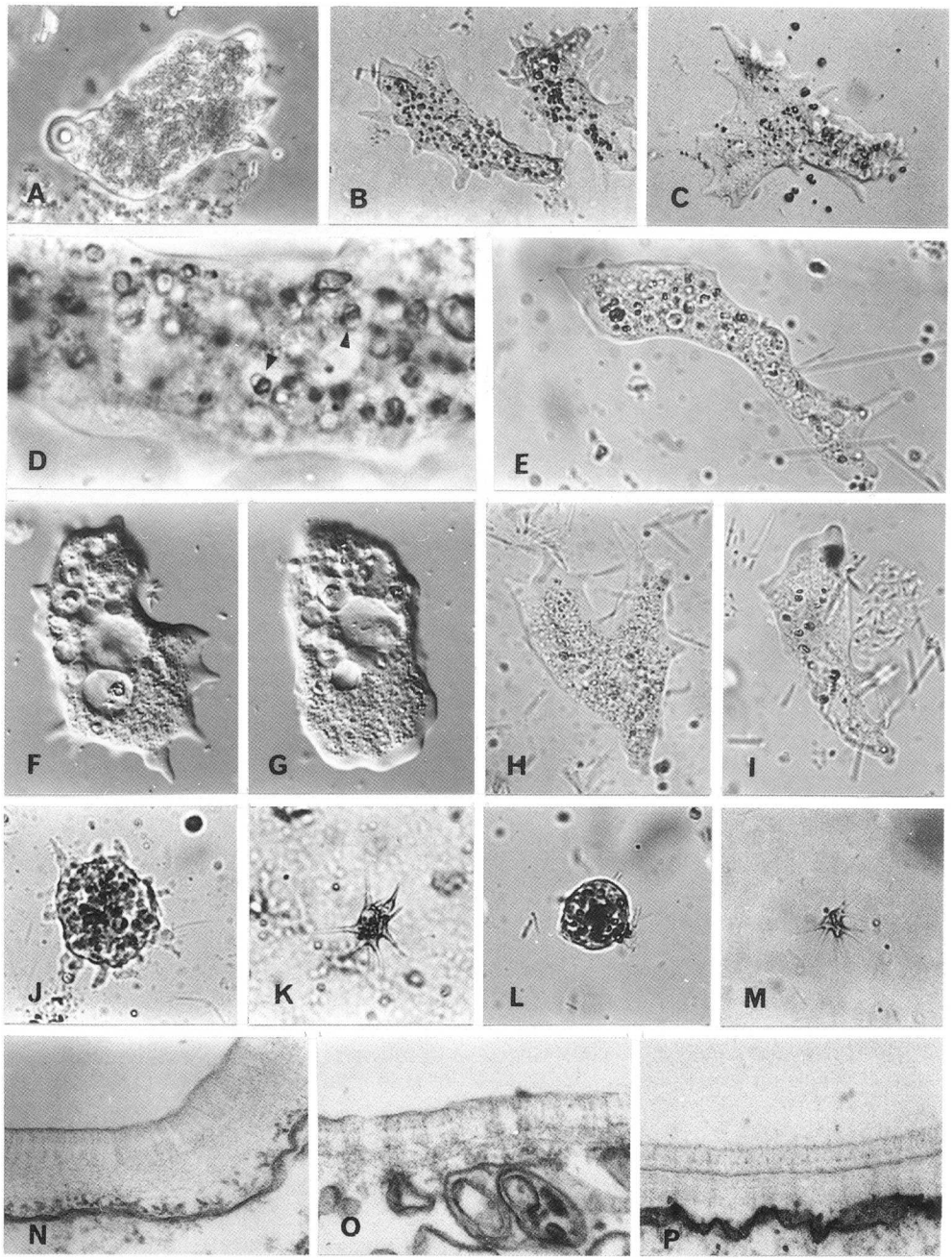

Fig. 35. *Mayorella*. A, *M. viridis*, B, C, locomotive forms of *M. cantabrigiensis*. D, paired inclusions (arrowheads) of *M. cantabrigiensis*, similar to those in all well studied species of *Mayorella* and one means of distinguishing *Mayorella* from *Dactylamoeba*. E-G, *M. penardi*. H, I, *M. vespertilioides*. J-M, floating forms of *M. cantabrigiensis* (J); *M. penardi* (K), in which the floating form often lacks the long pseudopodia shown here; and *M. vespertilioides* (L, M). N-P, cuticles of *M. cantabrigiensis* (N), *M. penardi* (O), and *M. vespertilioides* (P). The cuticle may appear somewhat different by different fixation procedures and should not be used for species identification but is diagnostic of the genus. (A-C, J, L ×250; D, ×1,000; E-I, ×500; K, M, ×100; N-P, ×40,000.)

sensu lato, below) have characters associated with *Mayorella sensu stricto* suggest that microscale-bearing strains, here classified as *Dactylamoeba*, are less common.

The conclusion that *Mayorella stella* (Schaeffer, 1926) Page, 1982 and *M. riparia* Page, 1972, are the same species means that each of the species of *Dactylamoeba* examined with the electron microscope, *D. stella* and *D. bulla*, has its own distinctive microscale pattern, as far as we know.

1 Large microscales with basket-like structure, no terminal spikes (Fig. 36F, G), each *c.* 550 nm long, 270 nm high above cell surface; L of amoebae varying greatly amongst strains, c. 20-

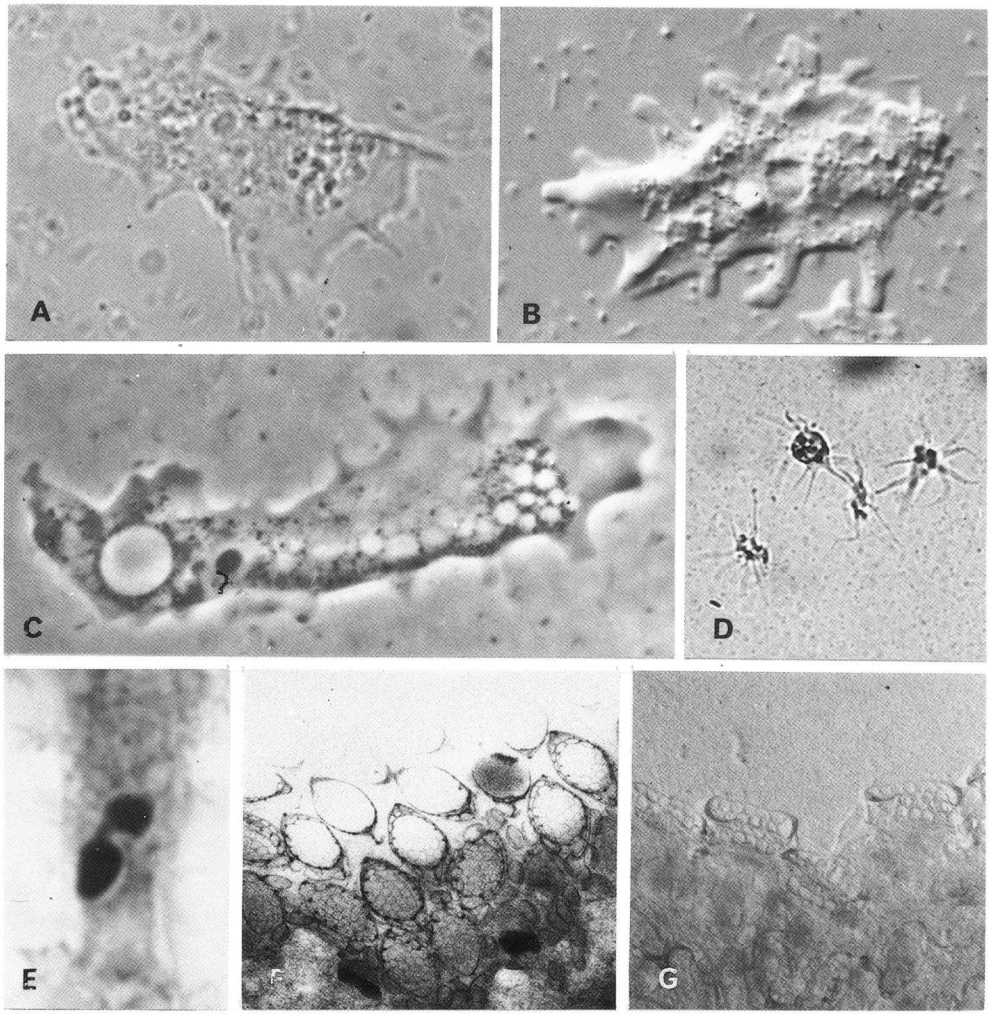

Fig. 36. *Dactylamoeba stella*. A-C, locomotive forms; in C, the angle form which the nucleus is seen makes it appear to have a spherical, central nucleolus. D, floating forms. E, stained nucleus. F, G, microscales; F, section tangential to cell surface, showing smaller microscales amongst the larger ones; G, whole microscales in shadowed preparation (A-C, ×1,000; D, ×250; E, ×2,500; F, G, ×25,000.)

120 μm (x̄ 32-65 μm); x̄ L/B *c.* 2-3, but forms with L/B greater than 4 not rare; floating forms with usually bent pseudopodia whose length may exceed twice diameter of central mass; nucleus 4-13 μm (x̄ 7-9 μm), usually elongate, with nucleolar material in long, lobed, but not parietal body, often giving appearance of binucleate condition (Fig. 36)

Dactylamoeba stella (Schaeffer, 1926) n. comb.

(North America, Europe. Probably the most common *Dactylamoeba*. The strain described as *Mayorella riparia* Page, 1972, was somewhat smaller than other strains studied, but even clones from the same sample differed greatly in size. CPAE; also feeds on smaller protozoa.)

— Large microscales with prominent, prow-like terminal spines (Fig. 37F, G), each microscale *c.* 720 nm long at base, with top of spines *c.* 550 nm above base; L 35-165 μm (x̄ 80 μm), L/B 1.0-4.2 (x̄ 2.0); floating forms with slender, often somewhat sinuous pseudopodia whose length may exceed 4 times diameter of central mass; nucleus 8.5-16.5 μm (x̄ 13 μm), with irregular nucleolar pieces usually in loose clump or ring in centre of nucleus. (Fig. 37)

D. bulla (Schaeffer, 1926) n. comb.

(North America, Europe. MP/Tet.)

Other species

Dactylamoeba elongata Korotneff, 1880: The type-species. Not clearly identifiable, though its length (not exceeding 130 μm) puts it at upper end of range for *D. stella*, which also often has an elongate locomotive form with an extensive hyaloplasm. Korotneff did not find a nucleus.

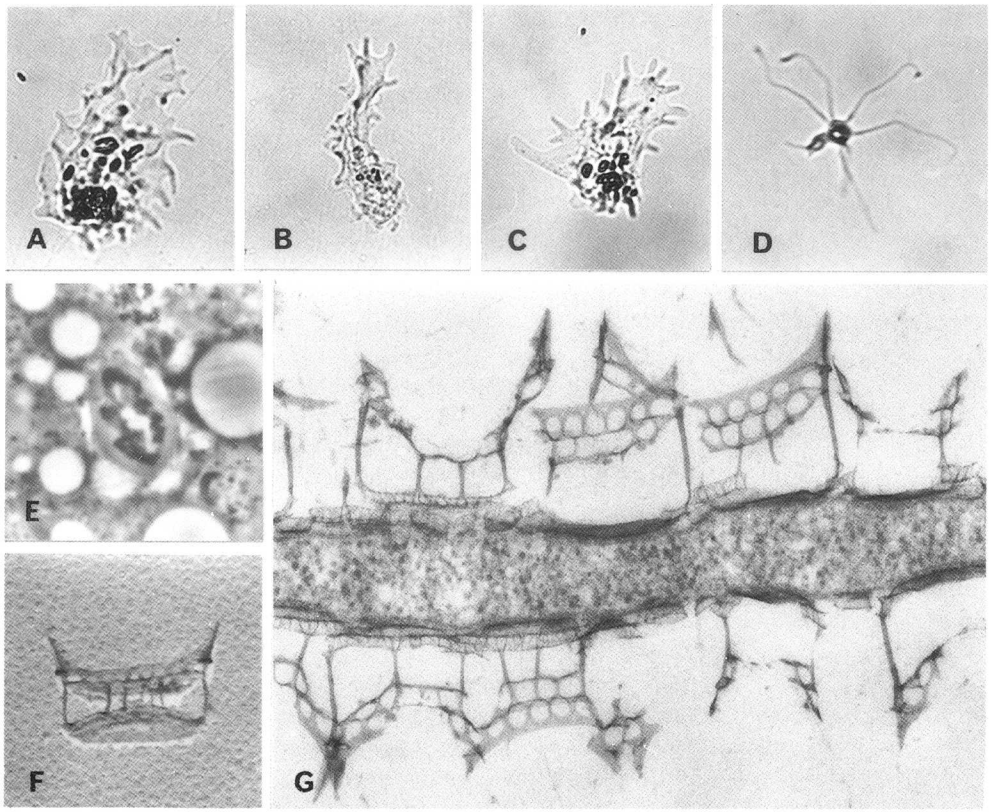

Fig. 37. *Dactylamoeba bulla*. A-C, locomotive forms. D, floating form. E, nucleus. F, single whole, shadowed large microscale. G, section of floating pseudopodium bearing both large and small microscales. (A-C, ×250; D, ×100; E, ×1,000; F, ×25,000; G, ×40,000.)

Astramoeba tatianae De la Arena, 1955: This Cuban species bears some resemblance to *D. bulla*, though the figures show a less regular floating form. No crystals or birefringent inclusions observed; L 80-180 μm (including pseudopodia?); nucleus 10-16 μm.

Mayorella sensu lato

Under this heading are listed those *Mayorella*-like organisms which have not been studied with the electron microscope and cannot on the basis of the light microscopic descriptions be placed confidently into either *Mayorella sensu stricto* or *Dactylamoeba*.

Illustrations of all except *Astramoeba torrei* can be found in Page (1976).

Mayorella clavella Bovee 1951: L 90-140 μm in moderate to rapid locomotion, L/B to 5.6; subpseudopodia absent or obscure in rapid locomotion, when amoeba may appear limax-like; uroidal knob with several short, conical projections; nucleus 8 μm; many bipyramidal crystals, 1-3 μm; floating form irregularly rounded, not radiate.

Astramoeba torrei De la Arena, 1952: L 80-160 μm; numerous subpseudopodia; nucleus 8-10 μm; no crystals seen; floating form with slender pseudopodia; spherical cysts, 40-43 μm.

Mayorella cultura Bovee, 1961: L 20-30 μm; one or more pairs of subpseudopodia; nucleus 3.5-7.5 μm; up to 20 or more crystals, usually less than 1.0 μm, shape obscure; floating form without long radiate pseudopodia; smooth, elliptical cysts, 10-12 μm.

The rest of the species listed were described, or in one case re-described, in a single publication by Bovee (1970). The descriptions, with selected illustrations, were summarised by Page (1976), without information on crystalline inclusions, which now seems more important taxonomically. For ease of reference these species are listed approximately in decreasing order of size.

M. leidyi Bovee, 1970: L 100-200 μm, L/B to 3; except in most rapid locomotion, 2 or more pairs of mamilliform subpseudopodia, often with prolonged distal portions; in rapid locomotion, short

subpseudopodia projecting downward from anterior end, with 3 or 4 longer, more slender ones from upper surface of anterior 1/3; nucleus 11-12 μm; many crystals, of which 10-20 are 3-4 μm long, bipyramidal; no radiate floating form known.

M. hohuensis (Wang, 1932): L 80-160 μm; L/B to 8 in moderate to rapid locomotion, less in slower locomotion; pseudopodia hemispherical to broadly conical; nucleus 6 μm; a few dozen crystals, about 2 μm, many tiny ones; floating form with slender, radiate pseudopodia.

M. lacona Bovee, 1970: L 60-130 μm, L/B to 3 or slightly more; approximately 4 narrowly conical subpseudopodia on pyramidal bases; nucleus 8 μm; many bipyramidal crystals, to 1.5 μm, a few larger ones; floating form with slender pseudopodia.

M. limacis Bovee, 1970: L 60-100 μm in moderate to rapid locomotion, L/B to 4; in limax-like form of rapid locomotion, subpseudopodia formed sequentially at either side of anterior end; uroid morulate or wrinkled; nucleus 8-9 μm; many crystals, 1 μm or less, and 2 or 3 dozen larger bipyramidal ones, to 3 μm; floating form with slender, radiate pseudopodia.

M. clavabellans Bovee, 1970: L 40-100 μm, with L/B to 6.6 in club-shaped form of most rapid locomotion but usually less; commonly 2 bluntly conical subpseudopodia at anterior end, others along sides; nucleus 6-8 μm; many small crystals to 1.5 μm, several dozen 2-4 μm, the latter bipyramidal, sometimes paired; floating form with many short, mamillate projections.

M. inquisita Bovee, 1970: L 50-70 μm, L/B approximately 2; several broadly rounded, nearly hemispherical subpseudopodia formed along anterior edge, not extended from a deep hyaline lobe; nucleus 5-6 μm; many small crystals to 0.6 μm long, a few dozen larger, to 2.0 μm, the latter probably bipyramidal; floating form with slender, radiate pseudopodia.

M. oblonga Bovee, 1970: L 35-60 μm, L/B to 5; oblong, usually 1 or 2 pairs of subpseudopodia at anterior end, one of each pair longer than other, with pair connected by hyaline web so that outlines of individual subpseudopodia are indistinct; nucleus 6-7 μm; many small crystals, less than 1 μm, 20-30 about 2 μm long, apparently bipyramidal; floating form with a few short, blunt pseudopodia.

M. ambulans Bovee, 1970: L 25-70 μm, depending on breadth, becoming linguiform in more rapid locomotion; several long subpseudopodia, with members of pair joined by web-like sheet which in linguiform amoeba extends nearly to tips; also 'ambulatory' rapid locomotion, with amoebae passing over subpseudopodia extended anteriorly and downward; nucleus 3-4 μm; many tiny crystals to 1 μm, several dozen larger, bipyramidal, to 2 μm; floating form with short, stubby pseudopodia.

M. bicornifrons Bovee, 1970: L 20-50 μm; elongate with clear, conical tip in slow locomotion, becoming more triangular in more rapid locomotion, pairs of subpseudopodia from anterior edge, and one pair extending upwards from dorsal surface; nucleus 4-4.5 μm; many tiny crystals to 0.6 μm, about 20 somewhat larger, to 2 μm, probably bipyramids; floating form rounded, with bulges.

M. oclawaha Bovee, 1970: L 30-50 μm, L/B to more than 2; 1-4 pairs of subpseudopodia, which also occur singly; nucleus 3.5 μm; many tiny crystals about 0.5 μm long, 20-30 larger, bipyramidal ones to 1.5 μm; floating form with long, slender, radiate pseudopodia.

M. cypressa Bovee, 1970: L 30-45 μm, maximum L/B less than 4; subpseudopodia usually embedded in hyaloplasm, which often extends posteriorly along sides, or with subpseudopodia absent from periphery during rapid locomotion; nucleus 5-6 μm; several dozen bipyramidal crystals to 1.5 μm; floating form rounded, with no pseudopodia.

M. microeruca Bovee, 1970: L 20-30 μm, more or less elongately cylindrical, often with short, conical subpseudopodia on sides; pairs of subpseudopodia produced in changing direction; nucleus 1.7 μm; many tiny crystalline granules, 0.2 μm or less, and 10-20 irregular crystals to 0.5 μm; floating form sometimes with one or 2 pairs of very short pseudopodia.

M. spatula Bovee, 1970: L 12-20 μm, L/B to 3, often less; 1-3 pairs of subpseudopodia, in rapidly moving spatulate forms often overtaken by hyaline waves; nucleus 1.3-1.4 μm; many crystals less than 0.5 μm, several larger ones to 1 μm, possibly bipyramidal; floating form sometimes with long radiate pseudopodia, more often not.

Other possible Paramoebidae

These genera were assigned by Bovee, to the family Mayorellidae (Paramoebidae) and are therefore listed here, but their status and familial positions in the present system are uncertain.

Flagellipodium Bovee, 1953: 'Like *Mayorella*, but also rapidly extends and re-retracts long thin tapered vibratile pseudopod(s) which may also be held rigidly forward in locomotion' (Bovee, 1970). Type species *Flagellipodium eilhardi* (Frenzel, 1897). Illustrations and description in Bovee (1985a). The length of the locomotive form is given as 35-55 μm, not including a long, vibratile pseudopodium 40-50 μm long.

Oscillosignum Bovee, 1953: 'Like *Mayorella*, but extends one or more long clear tapered pseudopods entered by granules except at conical tip; each waves actively; body may enter and pass through a forward one; radiate pelagic stage also forms them' (Bovee, 1970).

O. proboscidium Bovee, 1953: L 25-60 μm not including long pseudopodium; hyaline web connecting 2 short pseudopodia of pair for less than half their length; nucleus 4-4.5 μm.
Illustrations in Page (1976).

O. dakotaensis Bovee, 1953: L 15-28 μm not including long pseudopodium; hyaline web connecting 2 short pseudopodia of pair for more than half their length; nucleus *c.* 1.8 μm.
Illustrations in Page (1976).

Subulamoeba Bovee, 1953: 'Similar to *Oscillosignum*, but body becomes awl-shaped in rapid locomotion and maintains a clear conical anterior tip' (Bovee, 1970). Bovee designated as type species an amoeba which he identified as *Subulamoeba saphirina* (Penard, 1902). The description of Bovee's amoeba can be summarised as follows: In slow or moderate locomotion 30-65 μm, with anterior pseudopodia sometimes flattened at their tips; in awl-like form of rapid locomotion, 100-130 μm, with posterior breadth 5-7 μm; uninucleate, rarely binucleate. However, Penard's description of *Amoeba saphirina* does not appear to apply to the same species and could refer to a *Rhizamoeba*.
Illustrations of Bovee's organisms in Page (1976).

Triaenamoeba Bovee, 1953, and ***T. bulla*** Bovee, 1953: names published in an abstract. Generic diagnosis (Bovee, 1970): 'Body globular to trapezoidal; pseudopods long, slender, in groups of 2 to 4, usually 3, at anterior end, or as short ones from anterior margin in rapid locomotion.' A description and illustrations of *T. bulla* appear in Bovee (1985a). The size of the rounded amoeba is given as 8-12 μm, the nucleus as 2 μm.

Family VEXILLIFERIDAE Page, 1987

Reference: Page (1987c).

One or more slender, tapering or linear, never furcate subpseudopodia produced from anterior hyaloplasm of locomotive forms, which often have length greater than breadth; floating form usually with fine pseudopodia projecting asymmetrically from central mass; filamentous core in both subpseudopodia and floating pseudopodia. Uninucleate, usually with central nucleolus. Glycocalyx usually differentiated into glycostyles.

This family has been separated from the Paramoebidae chiefly because electron microscopic observations have shown that the subpseudopodia are only superficially similar to those of genera left in the Paramoebidae and that cell surface structure is very different.

The only freshwater genus is *Vexillifera*.

Genus *Vexillifera* Schaeffer, 1926

References: Bovee (1985b); Mascaró *et al.* (1986); Page (1969c, 1979a, 1979b); Sawyer *et al.* (1978).

Because the subpseudopodia are often carried back to the posterior end, these amoebae often look spiny, giving a superficial resemblance to *Acanthamoeba*. Amongst the differences from *Acanthamoeba* are lack of a cyst, lack of bifurcation in the subpseudopodia, and lack of a centriole-like body (microtubular organising centre), this last character recently confirmed by a new electron microscopic examination of *V. bacillipedes*.

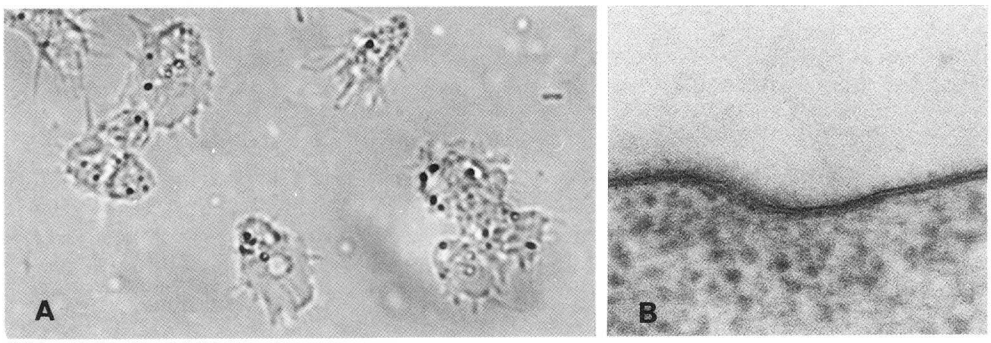

Fig. 38. *Vexillifera bacillipedes*. A, locomotive forms (×1,000). B, surface structure (×100,000), with possible sub-units of glycocalyx barely suggested.

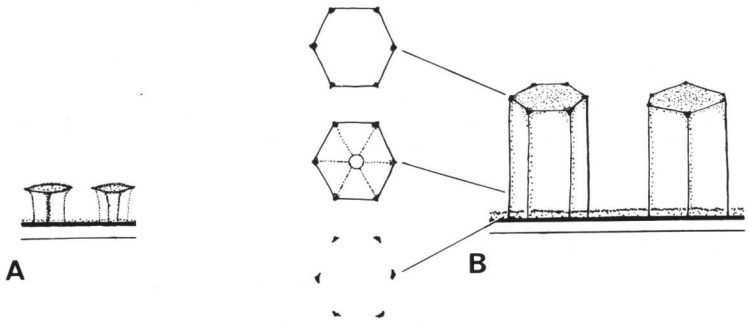

Fig. 39. *Vexillifera*, surface structure, diagrammatic. A, probable form of glycostyles of *V. granatensis* (after Mascaró *et al.*). B, glycostyles of marine species of *Vexillifera*; similar structures have not yet been found on freshwater species.

The two freshwater species studied with the electron microscope do not have glycostyles of the size found in marine species (Fig. 39B). However, *V. granatensis* has distinct glycostyles (Fig. 39A) which are much smaller than those on marine species, and a new fixation of *V. bacillipedes* has given an indication of similar, though in this material less distinct, surface structures. The glycostyles of marine *Vexillifera* are also illustrated in recognition of their possible occurrence on undescribed freshwater organisms. Whether the difference in surface structure will lead to a division of the genus is uncertain.

A few crystals to about 1 μm long are present in the known freshwater species, though their shape is often indistinct.

1 L excluding long subpseudopodia 8-24 μm ($\bar{x}$ 13 μm), L/B 0.9-2.7 ($\bar{x}$ 1.6); outline often triangular, broad anteriorly; 0-6, rarely more, long linear subpseudopodia, extending up to 7 μm or more beyond anterior edge of amoeba; nucleus (stained) 1.5-4.0 μm ($\bar{x}$ 2.4 μm); indistinct cylindrical surface structures approximately to 10 nm above plasma membrane, diameter 10 nm. (Fig. 38) ***Vexillifera bacillipedes*** Page, 1969

(North America, Europe. NNE. The amoebae associated by Sawyer *et al* (1978) with lesions in trout were *Vexillifera*, but the identification as *V. bacillipedes* is not entirely certain.)

— L excluding subpseudopodia 10-15 μm, breadth 4-13 μm; Long, apparently tapering subpseudopodia up to 25 μm long; nucleus (apparently stained) 3-6 μm; glycostyles rising to about 17 nm above plasma membrane, apparently with flared top about 25 nm wide. (Fig. 39A)
V. granatensis Mascaró, Osuna & Mascaró, 1986

(Europe.)

Other species

Vexillifera lemani Page, 1976: Type-species, the species which Penard (1902) identified, from 2 individuals, as the one which he had earlier described from a single specimen as *Amoeba ambulacralis*, an identification which was certainly incorrect. Flattened and broad, *c.* 20-30 μm long without the subpseudopodia, it had a considerable number of very elongate, fine, linear subpseudopodia but no trailing pseudopodial remnants.

Illustration in Page (1976).

The following 8 species were recently described by Bovee (1985b), with illustrations. The lengths do not include the subpseudopodia. Ultrastructural information would be desirable to confirm the generic classification.

Vexillifera telma Bovee, 1985: L 20-32 μm, B 14-26 μm, x̄ 25×19 μm; subpseudopodia extended from anterior end and dorsal surface of body mass, which may have a triangular outline; no uroidal differentiation; nucleus 2-3 μm; floating form spherical, with many short pseudopodia.

V. arionoides Bovee, 1985: Limaciform in steady locomotion, L 22-38 μm, x̄ 29×7 μm, L/B 4.3; a pair of subpseudopodia at anterior end, usually also 2 or 3 from anterior surface of granuloplasm; no uroidal differentiation; nucleus 2.5-3 μm; floating form with 3-6 radiate pseudopodia.

V. filopodia Bovee, 1985: More or less triangular in locomotion; extensive anterior hyaloplasm, 15-18 μm broad, with long, fine subpseudopodia (to 30 μm) extended from anterior edge or main cell mass; nucleus 2.5 μm; floating form with 6-8 long, fine pseudopodia, to 52 μm long.

V. variabilis Bovee, 1985: In locomotion irregular, trapezoidal to elongate, even limaciform, L 20-52 μm, B 6-10 μm, with subpseudopodia from anterior hyaloplasm and from cell surface; no uroidal differentiation except sometimes a temporary bulge from subpseudopodia being resorbed; nucleus 3-3.5 μm; floating form with 6-8 slender radiate pseudopodia.

V. minuta Bovee, 1985: L 13.5-17.8 μm, x̄ 15.3×6.2 μm, L/B 2.5, with subpseudopodia (to 18 μm) anteriorly and from cell surface, arising in pairs; no uroidal differentiation except temporary clear bulb; nucleus 2.3 μm; floating form spherical, with usually 6 slender pseudopodia to 26 μm.

V. subula Bovee, 1985: If spatulate L 14-20 μm, x̄ 17×7.5 μm; if ovoid or awl-shaped, L 18-36 μm, x̄ 26.3×4.1 μm, L/B 6.2; no uroidal differentiation; nucleus 2.2 μm; floating form spherical, with 4 radiate pseudopodia, tapering from relatively broad base.

V. anapes Bovee, 1985: Somewhat resembling duck's foot, spatulate to triangular in outline, with 2 or 3 pairs of subpseudopodia connected by flattened hyaloplasm; L 16-20 μm, B 12-14 μm; retracting pseudopodia at posterior end may briefly resemble uroidal filaments; nucleus 2 μm; floating form with 8 fine radiate pseudopodia, to 25 μm.

V. displacata Bovee, 1985: Granuloplasmic mass ovate, L 7-12 μm, B 6-8 μm, with 2-4 pairs of subpseudopodia from hyaline bulges at advancing end or from body surface; retracting subpseudopodia may trail at posterior end; nucleus 3.3 μm; floating form irregularly radiate.

Order LEPTOMYXIDA Pussard & Pons, 1976; emend. Page, 1987

References: Page (1987c); Pussard & Pons (1976a).

These amoebae present a range from uninucleate to plasmodial, with their cytoplasm commonly flattened and expanded, as a single, fan-shaped body, a thin and sometimes reticulate sheet, or a number of long, flat branches, often re-branching, connected only at their bases. A few take a monopodial limax form in most active advance. Eruptive activity occurs normally and may also be stimulated by bright illumination. Most freshwater and soil members encyst, though the encystment capability is often reduced after years in culture.

This order has been expanded to include the Flabellulidae, and two suborders were defined (Page, 1987c). Employment of the new suborders does not seem essential for this key (cf. Summary of Classification, p. 13).

Family GEPHYRAMOEBIDAE Pussard & Pons, 1976

References: Pussard & Pons (1976a, 1976c).

Although these amoebae are often much branched, they are not plasmodia. They are always

uninucleate and do not anastomose or form a reticulum. Spatulate forms with a long tail sometimes occur. These amoebae do not have the conspicuous collopodial filaments found in the Leptomyxidae.

Genus *Gephyramoeba* Goodey, 1914

References: As for family.

Only one species is known in this genus and family. The fine structure has not been reported.

Amoebae sometimes fan-shaped with a long narrow tail, sometimes a highly ramified ribbon, never anastomosing; 30-300 μm; uninucleate, few with supernumerary nuclei, nuclear diameter 10-16 μm; cysts spherical or ovoid with a single wall, 15-40 μm ($\bar{x}$ 20-25 μm). (Fig. 40A, B)
Gephyramoeba delicatula Goodey, 1914

(Europe; terr. Culture method: Pussard & Pons (1976c).)

Family FLABELLULIDAE Bovee, 1970

References: Page (1980, 1983b).

Uninucleate amoebae, some with a tendency to supernumerary nuclei; often flabellate or spatulate, never multi-lobed except temporary division of hyaloplasm sometimes when changing direction; when somewhat elongate, still flattened rather than limax-like. The hyaloplasm is more extensive than on the flabellate lobes of Leptomyxidae.

Most marine; one species known with certainty from freshwater/terrestrial habitats.

Genus *Paraflabellula* Page & Willumsen, 1983

References: Page (1983b); Page & Willumsen (1983); Singh & Hanumaiah (1979).

Flattened, with extensive hyaloplasm; flabellate, spatulate, often with breadth greater but

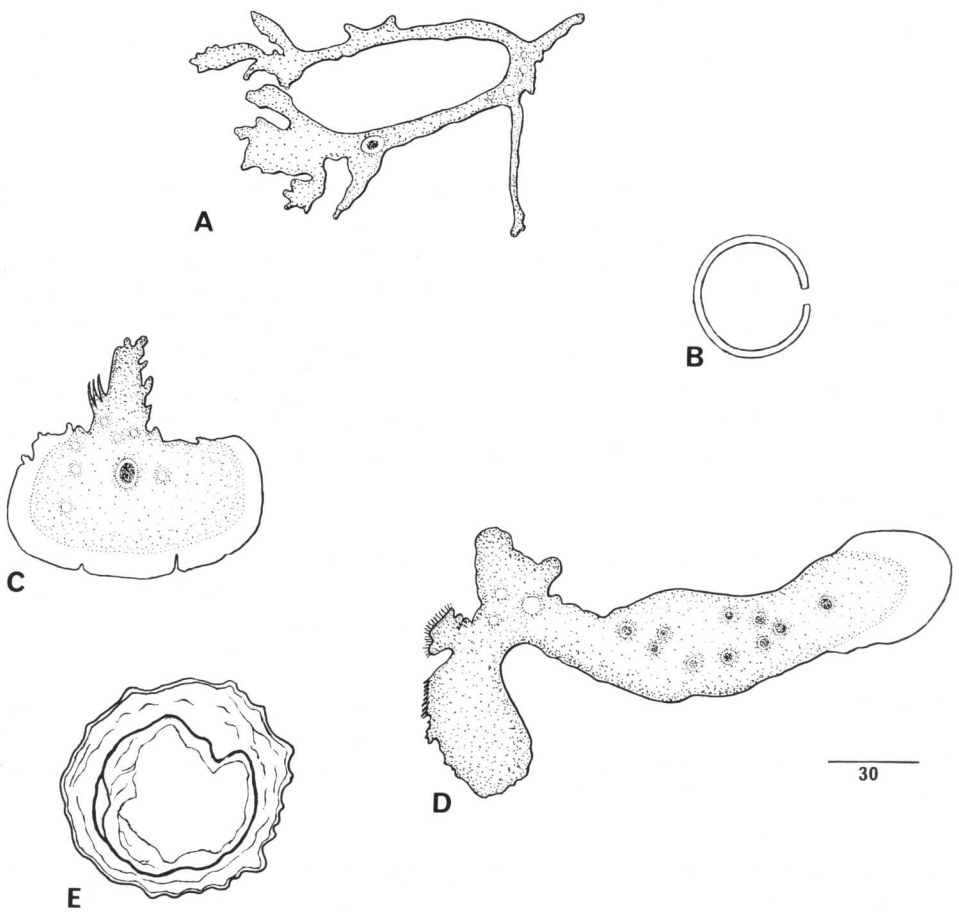

Fig. 40. A, B, *Gephyramoeba delicatula*; B, empty cyst wall. C-E, *Rhizamoeba flabellata*; E, empty cyst wall. (All after Goodey.)

sometimes elongate; form changing rapidly; short, narrow, round-tipped, non-furcate subpseudopodia produced from hyaloplasm.

Although the type-species, *P. reniformis* (Schmoller, 1964) was found in Baltic waters of low salinity, attempts to grow one isolate on freshwater agar were unsuccessful, and cysts were not found. Singh & Hanumaiah described a freshwater, cyst-forming species which seems to have subpseudopodia characteristic of the genus. Unfortunately, it has not been possible to obtain permission to reproduce their figures. Measurements in the present description are derived from those drawings. For illustrations of this and similar organisms, consult the references cited above.

Paraflabellula kudoi (Singh & Hanumaiah, 1979): Usually broadly spatulate or flabellate; often deep rifts in hyaloplasm; conical subpseudopodia of various sizes from hyaloplasm or granuloplasm; usually uninucleate, often binucleate; cysts round or oval, with gelatinous outer layer; amoebae *c.* 26-40 μm; L/B 0.7-1.2; cysts *c.* 16-25 μm; grows at 37°C and 42°C.
Asia.

Another possible member of this genus or at least of this family:

Amoeba cirrifera Penard, 1890: L 25-40 μm; not including uroidal filaments.

Family LEPTOMYXIDAE Pussard & Pons, 1976; emend. Page, 1987

References: As for order.

The familial definition used here (p. 51) differs from the original one chiefly in omission of the mitotic character, since further investigation of that character is needed in the genus *Rhizamoeba*; particularly, the taxonomic significance above the species level needs further study.

1 Rarely or never reticulate; limax-like in most active advance, often flabellate or irregularly expanded in less active state; uninucleate, with a tendency to supernumerary nuclei, or multinucleate, usually with fewer than 20 nuclei ***Rhizamoeba***

— Microplasmodium, which may be reticulate and is always multi-lobed or branched, never limax-like; often hundreds of nuclei ***Leptomyxa***

Genus *Rhizamoeba* Page, 1972

References: Cann (1984); Chakraborty & Pussard (1985); Page (1972b, 1980); Pussard & Pons (1976b); Siemensma (1980).

This genus was originally erected for marine amoebae, but the resemblance to some soil and freshwater species was recognised from the beginning. However, electron microscopic studies of several species are still needed to settle the generic diagnosis. As defined here the genus includes the organism for which Chakraborty & Pussard erected the genus *Ripidomyxa*.

The filaments produced by adhesion to the substratum, which gave this genus its name, are more or less conspicuous at the posterior end of limax forms and often around parts of the periphery of less active amoebae. In the marine *R. saxonica* they were associated ultrastructurally with collosomes, but no collosomes were found in the organism here designated *Rhizamoeba australiensis* (Cann, 1984, as *R. flabellata*).

Eruptive activity is noticeable in limax forms, sometimes with hyaloplasm spilling posteriorly along the side, as well as in more expanded forms; confusion with the Vahlkampfiidae must therefore be avoided.

Little or no anastomosis of pseudopodia occurs.

1 Cysts with two well separated wall layers, *c.* 25-65 μm ($\bar{x}$ 40 μm), multinucleate; amoebae varying in size according to nuclear number, *c.* 50-500 μm, often toward lower end of range, exceptionally larger; only about 4% uninucleate, mean nuclear number 10, rarely more than 50 nuclei; nuclear diameter 14-17 μm. (Fig. 40C-E) ***R. flabellata*** (Goodey, 1914)
(Europe; terr. Culture method: Pussard & Pons, 1976b.)

— Cysts with single wall, *c.* 15-50 μm ($\bar{x}$ 37 μm), almost all uninucleate, rarely up to 4 nuclei; amoebae in limax form 50-180 μm long; more than 95% uninucleate; nuclear diameter *c.* 6.5-13.0 μm ($\bar{x}$ 10 μm). (Fig. 41A-E)
Rhizamoeba australiensis (Chakraborty & Pussard, 1985) n. comb.

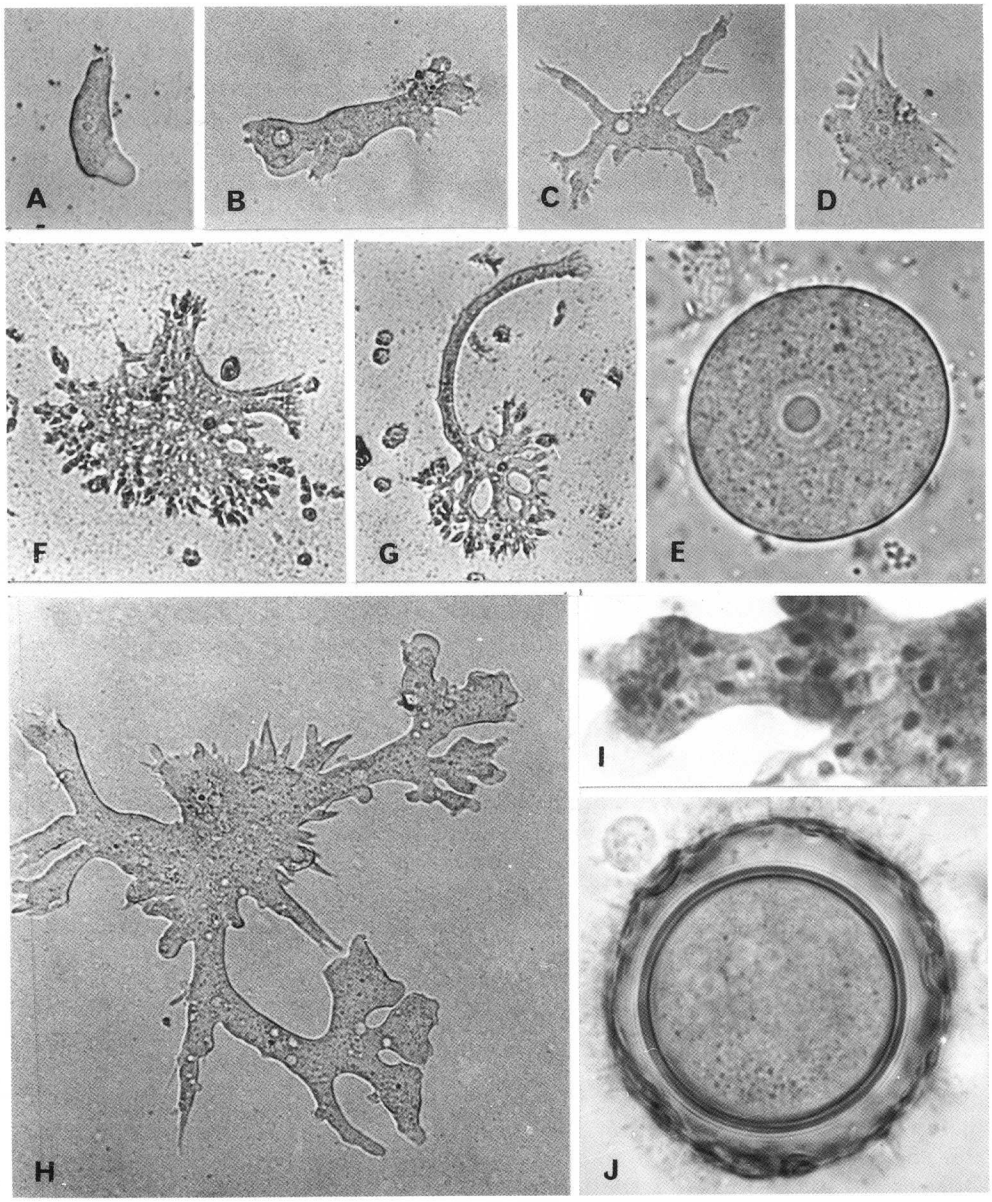

Fig. 41. Leptomyxidae. A-E, *Rhizamoeba australiensis*; A-D, locomotive forms on coverglass in hanging drop; E, cyst. F-J, *Leptomyxa reticulata*; F, G, plasmodia on agar; H, plasmodium on coverglass in hanging drop; I, part of plasmodium stained to show nuclei; J, cyst, with several nuclei discernible (A-D, H, ×250; E, I, J, ×1,000; F, G, ×100).

(Australia, Europe; terr. CPAE, NNE. This diagnosis accepts the identification of the English strain studied by Cann (1984) under the name *R. flabellata* as belonging to this species, despite minor differences.)

Other possible species

Siemensma has classified in this genus the first 2 species listed below, each originally described from a single freshwater amoeba, with which he has identified organisms found in the Netherlands. Since none of the 3 species listed has been cultured on agar, their ability to form cysts and the structure of any cysts are unknown, a lack which prevents judgement on their possible identity with *R. flabellata* or *R. australiensis*.

Rhizamoeba caerulea Schaeffer, 1926: Limax form *c.* 150-250 μm long, with eruptive activity and fine collopodial filaments; flattened form with such filaments around entire periphery; 1-32 nuclei, diameter *c.* 3.5 μm, with central nucleolus; elliptical crystalline inclusions (unknown in any other *Rhizamoeba*); amoeba 'of a beautiful sky-blue colour', according to Schaeffer.

North America, Europe.

Rhizamoeba clavarioides (Penard, 1902): Limax form *c.* 125-150 μm; locomotively less active form with many branches, changing shape rapidly; always uninucleate; in only specimen seen by Penard, nucleolar material in 4 or 5 fragments in central cluster.

Europe.

Trichamoeba clava Schaeffer, 1926, might be a *Rhizamoeba*. L 75-125 μm; eruptive: uroid of fine filaments; uninucleate, nuclear diameter *c.* 14 μm.

North America.

Genus *Leptomyxa* Goodey, 1914

References: Cann (1984); Pussard & Pons (1976a).

This non-sporulating plasmodium is widespread in terrestrial habitats and has been found in freshwater.

Occasional eruptiveness and frequent formation of adhesive filaments are seen in this genus as in other members of the family. Two plasmodia can fuse and unite quickly. In the usual photomicrographs and drawings of plasmodia on agar without liquid overlay the lobose terminations of advancing branches often appear somewhat crenate (Fig. 41F, G). However, the plasmodia attach well to glass; in a hanging drop the advancing hyaloplasm often has a smoother contour, and anastomosis of pseudopodia seems somewhat less frequent (Fig. 41H).

The number and size of plasmodia in a culture are related to quantity of food. After months or years in culture, the ability to encyst is reduced or lost. The cysts appear to be viable only a few months. Normally each cyst contains a single encysted plasmodium; exceptionally, 2 or more separately encysted plasmodia may be found within a single outer wall.

After removal of *Rhizamoeba flabellata*, a single species:

> Plasmodium often approximately 1 mm across under favourable conditions, sometimes smaller (100 μm or less), sometimes larger; occasionally fan-shaped and undivided, often much branched, often reticulate, with reticulation produced by both formation of lacunae and anastomosis of branches; nuclear number from few more than 10 to several hundred; nuclear diameter 3-8 μm; cyst multinucleate, with 2 well-separated wall layers, diameter of cysts containing a single plasmodium 32-65 μm ($\bar{x}$ 45-51 μm). (Fig. 41F-J)
>
> ***Leptomyxa reticulata*** Goodey, 1914
>
> (Europe, Australia; terr. CPAE or NNE with *Vexillifera* sp.)

Another possible species: As this publication was completed, F. J. Siemensma identified '*Pelomyxa*' *fragilis* Penard, 1904, as another species of *Leptomyxa*. Penard described an organism (length 400-450 μm) uncharacteristic of *Pelomyxa* in its changeable, even polypodial form.

Order ACANTHOPODIDA Page, 1976

Family ACANTHAMOEBIDAE Sawyer & Griffin, 1975

Reference: Sawyer & Griffin (1975).

These rather flattened amoebae may be broad and somewhat irregular in outline, though in steady locomotion they sometimes become elongate. Slender, flexible, tapering subpseudopodia (acanthopodia), sometimes furcate near their base, are produced from a broad, hyaline lobopodium. The acanthopodia are often somewhat thicker and less pointed in larger amoebae such as *Acanthamoeba astronyxis*. Although the cytoplasm contains no crystals, small lipid globules are a fairly regular feature. The electron microscope reveals centriole-like bodies (microtubular organising centres) known otherwise amongst gymnamoebae only in the Stereomyxidae, the Corallomyxidae, and *Phreatamoeba*.

The trophic amoebae of the two genera are not distinguishable.

The genus *Comandonia* is here considered a member of the Echinamoebidae.

1 Cyst wall without preformed pores and opercula; excystment through break in wall ***Protacanthamoeba***

— Cyst wall with preformed pores and opercula; excystment through pore ***Acanthamoeba***

Genus *Protacanthamoeba* Page, 1981

References: Page (1981b); Singh & Hanumaiah (1979).

The cysts are circular to oval in outline, with a smooth appearance. In a minority, splitting of the wall may give the appearance of 2 wall layers, and the outer part may then sometimes look slightly wrinkled.

1 Grow at 22°C, not at 37°C; amoebae in locomotion mostly 20-40 μm, mean in rounded condition 19 μm; nucleus 7.0-9.2 μm ($\bar{x}$ 8.3 μm); cysts 11-21 μm ($\bar{x}$ 16.5 μm). (Fig. 42A-C)
Protacanthamoeba caledonica Page, 1981

(Europe, probably elsewhere. NNE; attempted axenicisation unsuccessful. This is probably the species incorrectly designated *Hartmannella glebae* or *Acanthamoeba glebae* by some recent workers, though *A. glebae* has been reported to grow at 37°C but not at 42°C)

— Grow at 37-42°C, pathogenic to mice by intracerebral and possibly by intranasal inoculation; diameter in rounded condition *c.* 18-30 μm; cyst sizes not reported but illustrations suggest 20-27 μm. ***P. invadens*** (Singh & Hanumaiah, 1979)

(Asia. NNE at 42°C.)

Genus *Acanthamoeba* Volkonsky, 1931

References:

(1) *Culture*: Biddick *et al.* (1984); Ingalls & Brent (1983); Neff (1957, 1958).

(2) *Taxonomy: Byers et al.* (1983); Costas *et al.* (1983); Costas & Griffiths (1984a, 1984b, 1985, 1986); De Jonckheere (1980, 1983, 1987a); Pussard & Pons (1977); Willaert (1976).

(3) *Individual new species*: Lewis & Sawyer (1979); Molet & Ermolieff-Braun (1976); Sawyer *et al.* (1977); Willaert *et al.* (1978).

(4) *Ecology*: Bamforth & Stout (1983); Brown *et al.* (1983); Bryant *et al.* (1982); Coleman *et al.* (1978); Daggett *et al.* (1982); De Jonckheere (1981a); Elliott *et al.* (1979); Kyle & Noblet (1985, 1986); O'Dell (1979).

(5) *Medically related topics*: Anand *et al.* (1983); Baron *et al.* (1980); Danes & Červa (1981); De Jonckheere (1980); Gogate *et al.* (1984); Griffin (1980); Grillot & Ambroise-Thomas (1981); Holden *et al.* (1984); Lalitha *et al.* (1985); Pernin & Riany (1980); Rowbotham (1983); Samples *et al.* (1984); Skinner *et al.* (1983); Tyndall & Domingue (1982).

(6) *General*: Schuster (1979).

This is the most frequently isolated and probably the most common genus of gymnamoebae, possibly even the most common free-living protozöon. Anyone collecting from freshwater or soil is certain to collect *Acanthamoeba*, even if taking only a few samples. It has often been isolated from salt water (Page, 1983b) and plant matter.

Some of the scientific interest has arisen from this ubiquity and more from its usefulness, in axenic culture, for biochemical research, but much of the attention is due to sporadic pathogenicity. Although *A. culbertsoni* has been the main suspect as a possible invader of the central nervous system, several other species have been implicated in infections of the human cornea. At least until recently, pathogenicity did not seem to be limited to certain species of *Acanthamoeba*, as of *Naegleria*, though the results of De Jonckheere (1983) suggest that it may be possible to classify most of the pathogens in a few species.

Although this key may provide an introduction for public health or other medical workers, they should consult the appropriate literature in those fields. The new taxonomic survey by De Jonckheere (1987a) especially merits the attention of such workers, who should be warned that much of the earlier medical literature referring to *Acanthamoeba* is taxonomically unreliable; e.g., there is no connection between *Acanthamoeba* and *Hartmannella*, and the latter genus includes no known or suspected pathogens.

As with *Naegleria*, British workers should be aware of the recommendations for handling '*Acanthamoeba* spp (especially *A. culbertsoni*)' issued by the Advisory Committee on Dangerous Pathogens (ACDP, 1984). Workers in other countries should consult their national authorities. All investigators should be aware of the impossibility of avoiding contact with *Acanthamoeba* in natural habitats as well as in any laboratory in which unsterilised water or soil is present.

All species of *Acanthamoeba* can be cultivated on non-nutrient agar with bacteria (e.g., *E. coli*) at the appropriate temperature. All grow at 30°C, but some multiply and encyst more rapidly at higher temperatures. The majority of strains can be cultured axenically in PPG or SCGYEM. See 'Culturing Gymnamoebae' for media and methods of axenicisation.

Although the *Acanthamoeba* cyst immediately identifies the genus, differentiation of species is difficult.

Morphological characters for taxonomy or identification have been drawn entirely from the cyst, but variation even within a clone and the difficulty of expressing the characters objectively are two problems with this approach. The most detailed study of cyst morphology is that of Pussard & Pons (1977). However, they had before them only one strain each of the majority of species investigated, so that interclonal variation could not always be taken into account.

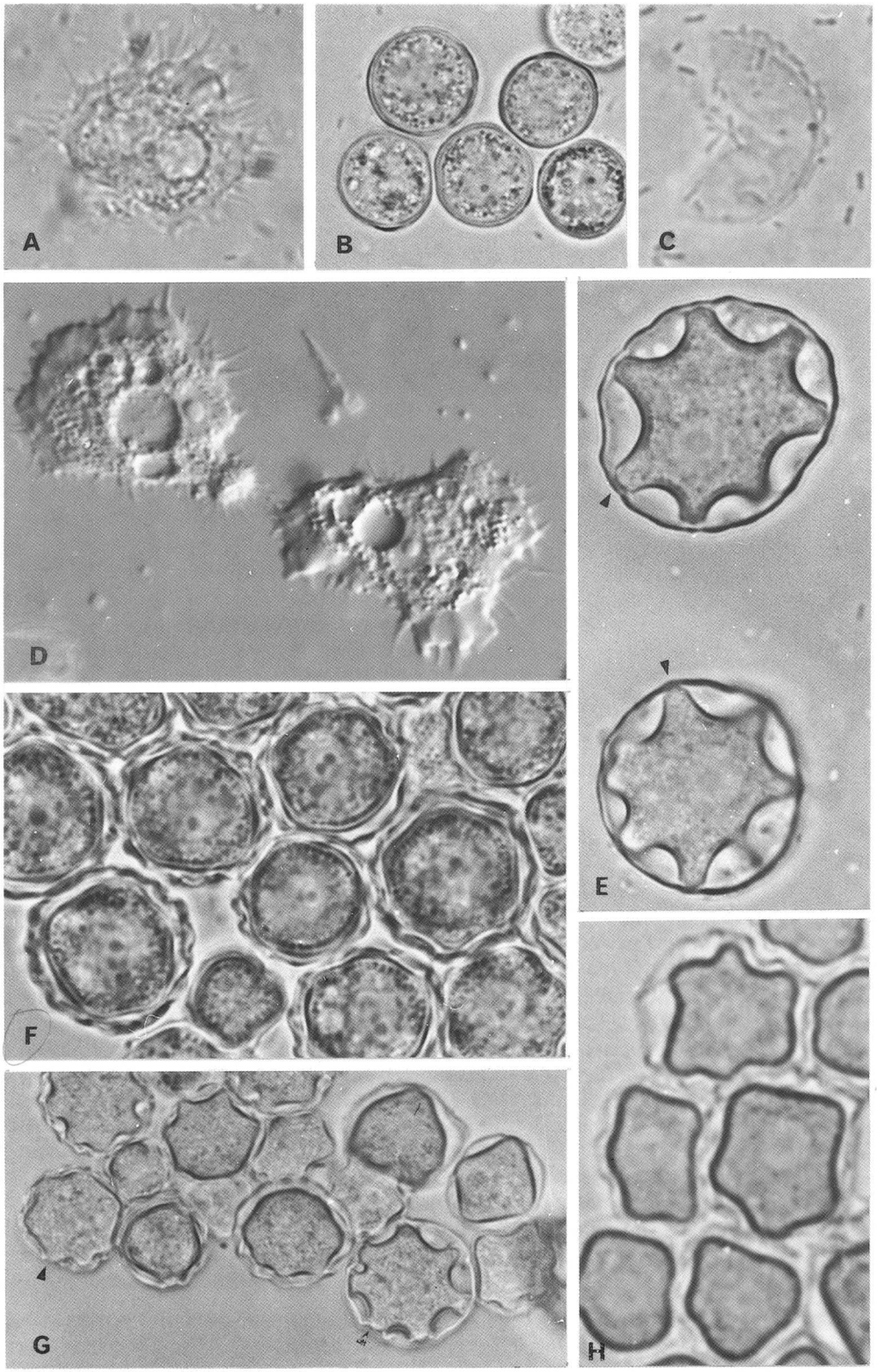

Fig. 42. Acanthamoebidae. A-C, *Protacanthamoeba caledonica*; B, cysts; C, empty cyst wall after excystation. D. *Acanthamoeba castellanii*, amoebae, which with slight variations are similar to those of other species of genus. E-H. cysts, E, *A. astronyxis*; F, *A. castellanii*, Neff strain (CCAP 1505/1a); G, *A. castellanii*, original Castellani strain (CCAP 1501/2a); H, *A. rhysodes*. (A-D, G ×1,000; E, F, H, ×1,575.) Arrowheads indicate pores, closed with opercula.

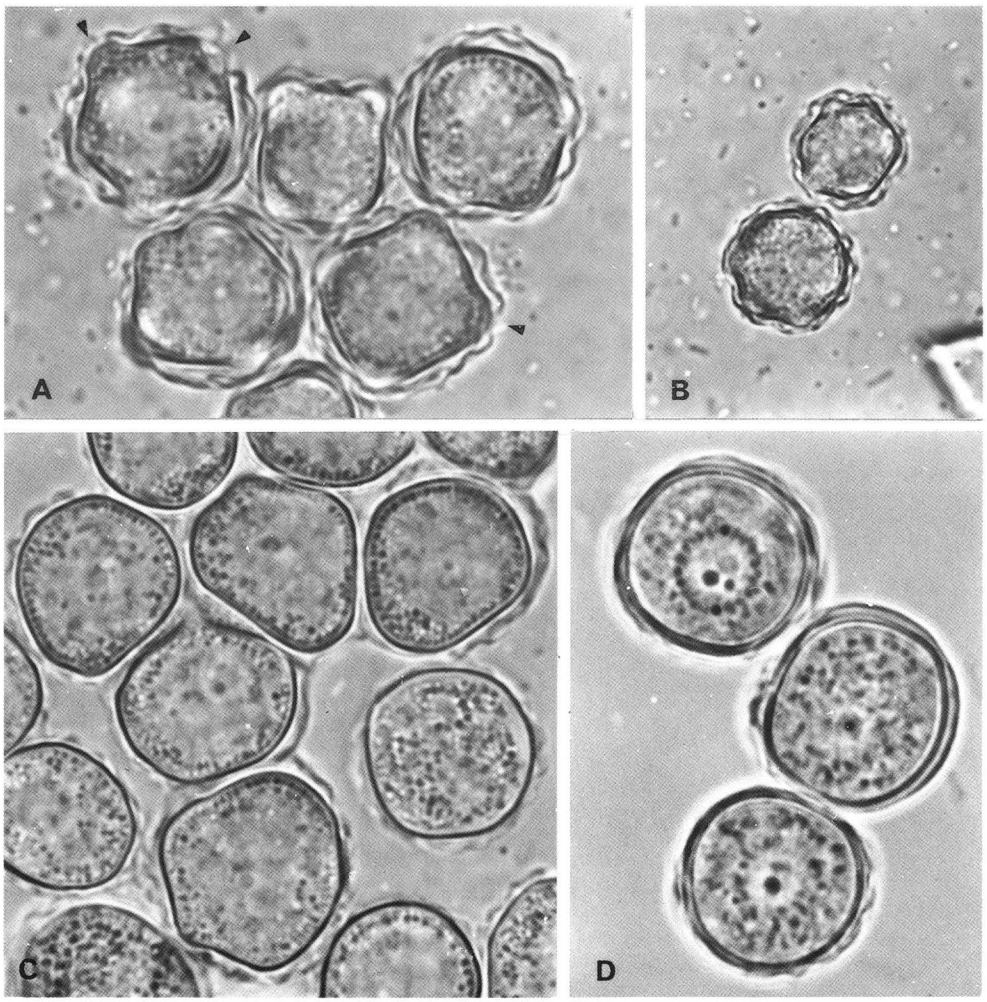

Fig. 43. *Acanthamoeba* (continued). Cysts of: A, *A. polyphaga*; B, *A. griffini*; C, *A. palestinensis*; D, *A. culbertsoni*. (B, ×1,000; others ×1,575.)

The cyst is produced abundantly by most isolates. Its wall consists of two layers, the *ectocyst* or outer wall and the *endocyst* or inner wall. Pussard & Pons preferred the terms *exine* and *intine*, respectively, because endocyst may be taken by extension to include the cell within the inner wall and might therefore be ambiguous. The outer and inner wall layers are most widely separated in Group I, where endocyst meets ectocyst at the ends of *arms* or *rays*. At each point where the two layers meet is a *pore* or potential opening closed by an *operculum*. The number of arms and the number of pores are therefore the same. In Group III the ectocyst is a thin layer, and the more or less rounded endocyst does not produce arms, since the two layers are close together around most of the circumference. Cysts of Group II are somewhat intermediate between the two extremes; the endocyst may be somewhat stellate or may be polygonal, so that the two wall layers meet at a corner rather than an arm of the endocyst, and the endocyst may even be more or less rounded, though not as consistently as in Group III. In Group I the operculum is more or less at the level of the ectocyst. In Group II the operculum is generally in a depression formed by the infolding of the ectocyst, and the term *ostiole* is used principally for such a depression, though strictly it is equivalent to pore.

In a few species of Group II, the ectocyst is thrown into a network of anastomosing veins, about 1-1.5 μm wide, the character designated in the key as *reticulation* (Fig. 44). This reticulation should not be confused with any random folding of the ectocyst or with a pseudo-reticulation which does not in fact produce a reticulum.

Non-morphological approaches have been employed, since the study of Pussard & Pons, by several groups including Costas & Griffiths (references above), Daggett *et al.* (1982), and De

Jonckheere (references above). Although Costas & Griffiths modified their procedures to permit use of monoxenic cultures, most work has involved only those strains which could be axenicised, thus omitting a minority of strains, nor did all groups use the same strains of every 'species', so that their results are not always comparable.

In the key below, a preliminary synthesis of morphological and non-morphological results has been attempted. It must be emphasised that *establishment of clones is essential* before attempting identification by any method or combination of methods. The best procedure would appear to be a preliminary screening on the basis of cyst morphology and growth temperature, followed by application of one or more non-morphological methods. The screening begins with determination of the major morphological group to which an isolate belongs. These groups were employed in initial steps of the earlier key (Page, 1976) and explicitly recognised by Pussard & Pons. In this key they have been arranged in the order of the latter authors, and this key is to a degree a modification of that of Pussard & Pons. Identification of a member of Group I (dichotomies 2,3) to species may be possible by morphological characters alone. Identification of a strain of Group II (dichotomies 5-13) may also be attempted by morphological characters, in combination with growth temperature where the latter is diagnostic. However, the morphological diagnoses of some of these species are somewhat uncertain because of the strains upon which they are based (a weakness not absent from the non-morphological information on one or two species). At any rate, non-morphological procedures (see below for suggestions) are strongly recommended to improve the accuracy of identifications in Group II. In Group III (dichotomies 14-16) *A. palestinensis* (not often reported) can perhaps be identified by a combination of morphological characters and growth temperature. The numerous small pores may identify *A. royreba*, but application of non-morphological methods to this and the other potentially pathogenic species in Group III should be considered obligatory.

Two morphological methods which may be useful, the 'warm impregnation' and the PAT Ag r of Pussard & Pons, are described in the section 'Methods of Observation and Study'. The former shows the relief of the cyst surface, demonstrating reticulation (Fig. 44) and pores. The PAT Ag r procedure is intended to stain polysaccharide-rich structures, including the opercular apparatus. However, it was used especially on empty cysts after induction of mass excystment. If differential interference contrast (Nomarski) optics are available, that method might be tried before resorting to staining procedures, though it is unlikely to be so useful in counting pores.

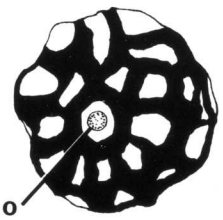

Fig. 44. Cyst of *Acanthamoeba castellanii*, treated by the warm impregnation method of Pussard & Pons, illustrating the 'reticulate' character of a few *Acanthamoeba* cysts. (After Pussard & Pons.) *o*, operculum, or ostiole in which operculum is found.

The most widely employed non-morphological procedure is isoenzyme electrophoresis. Methods and resulting zymograms for comparison will be found in De Jonckheere (1983) and Costas & Griffiths (1984b); isoenzyme patterns are also illustrated by De Jonckheere (1987a). Costas & Griffiths (1985) have also applied the API Zym methods, widely used for bacteria, to *Acanthamoeba*, distinguishing 19 hydrolytic enzymes (API System SA, La Balme, Les Grottes, 38390 Montalieu, Vercieu, France). Some difference of opinion about the usefulness of the API Zym system for *Acanthamoeba* exists. A. J. Griffiths (personal communication) suggests that it may be preferred because isoenzyme comparisons may give too fine a resolution, separating strains which might be considered members of the same 'species'. The only published standard for comparison is the results of Costas & Griffiths (1985). J. F. De Jonckheere (personal communication) found the usefulness of this system doubtful because differences between species were difficult to see and results varied from one experiment to another.

Although in its outlines the present key follows that of Page (1976) and, in more detail, that of Pussard & Pons (1977), it incorporates synonymies suggested by recent work and new species described in the past few years. No indications of geographic distribution are included, because the genus as a whole is so widely distributed and the bases of identification differ from one author to another.

1 Ectocyst smooth or gently wrinkled, at least as thick as inner wall layer, well separated from

endocyst except at points where tips of endocyst arms meet ectocyst; endocyst usually more or less stellate; tips of endocyst arms approximately at level of ectocyst, not in depressions; mean cyst diameter 18 μm or more (Group I) **2**

— Endocysts not predominantly stellate or, if endocysts predominantly stellate, with tips of endocyst arms (opercula) in depressions formed by ectocyst **4**

2 Three to 5 (usually 3 or 4) endocyst arms; mean cyst diameter *c.* 23 μm; grows at temperatures to 38°C, encysts also at 40°C ***Acanthamoeba tubiashi*** Lewis & Sawyer, 1979

— Often more than 5 endocyst arms; no growth at 37°C **3**

3 All or most endocyst arms contact ectocyst in the same plane; up to 9 arms; mean cyst diameter 19.2-22.0 μm (Fig. 42E) ***A. astronyxis*** (Ray & Hayes, 1954)

— Arms contact ectocyst in various planes; up to 14 arms; mean cyst diameter 18.0-25.3 μm ***A. comandoni*** Pussard, 1964

(Not yet axenicised. This diagnosis accepts the synonymy of *A. echinulata* Pussard & Pons, 1977, with *A. comandoni*, as suggested by the results of Costas & Griffiths (1984b).)

4 (1) Endocyst often with pronounced polygonal, stellate, or scalloped shape, though sometimes rather spherical or ovoid; ectocyst distinct, sometimes as thick as endocyst wall; mean cyst diameter usually less than 18 μm (Group II) **5**

— Endocyst nearly rounded or with only slight angles; ectocyst very thin; mean cyst diameter generally less than 19 μm (Group III) **14**

5 Mean number of endocyst arms 7 **6**

— Mean number of endocyst arms 6 or fewer **8**

6 Cyst not reticulate; endocyst typically polyhedral, with tendency to be spherical; any arms broad, not prominent; ectocyst closely applied to endocyst; mean cyst diameter 14 μm; isoenzyme and total protein patterns similar to those of *A. quina*, but this species is pathogenic to mice ***A. lugdunensis*** Pussard & Pons, 1977

(Two isolates from human corneal infections have been placed into this species, and at least one more may belong to it.)

— Ectocyst not closely applied, maintains contour independently of shape of endocyst **7**

7 Cyst reticulate (Fig. 44) more or less spherical endocyst connected to ectocyst by little conical arms; ectocyst thick and very folded; mean cyst diameter 14-16 μm (Figs. 42D, F, G, 44) ***A castellanii*** (Douglas, 1930)

(This diagnosis is based on the Neff strain, which is more widely used than the original Castellani strain and generally assumed to be the same species.)

— Some cysts pseudo-reticulate; endocysts often with conical or somewhat tubular arms; a minority stellate or like cog-wheel; mean number of endocyst arms 7-7.5; mean cyst diameter 16-18 μm (Fig. 42H) ***A. rhysodes*** (Singh, 1952)

8 (5) Mean number of endocyst arms or corners 5-6 **9**

— Mean number of endocyst arms or corners 3 or 4; endocyst usually triangular or quadrangular in outline; endocyst wall between 2 angles often gently concave **13**

9 Cyst reticulate; endocyst spherical or ovoid, rarely polyhedral, with distinct arms rarely formed and then small; ectocyst rather thick, with circular contour, not closely applied to endocyst; mean number of endocyst arms or corners (i.e., pores) 5; mean cyst diameter 14 μm; distinguished from *A. castellanii* by number of endocyst corners and by isoenzyme and total protein patterns ***A. mauritaniensis*** Pussard & Pons, 1977

— Cysts seldom or not reticulate **10**

10 A small proportion of cysts reticulate; endocyst very irregular, practically never stellate; ectocyst less strongly folded than in *A. castellanii* or *A. mauritaniensis*, thin, here and there widely separated from endocyst; mean number of endocyst corners 5-6; mean cyst diameter 14 μm; isoenzyme and total protein patterns distinctive (Fig. 43A)
A. polyphaga (Puschkarew, 1913)

(Diagnosis based on one strain.)

— Cysts never reticulate **11**

11 Does not grow at 37° in bacterised culture; endocyst variable, sometimes spherical or ovoid with or without short arms, sometimes stellate with well-developed arms giving appearance of cog-wheel; ectocyst wavy, applied to endocyst except when latter is stellate; mean number of arms or pores in endocyst 6; mean cyst diameter 14 μm; unique isoenzyme pattern. (Fig. 43B)
A. griffini Sawyer, 1971

— Grows at 37°C in bacterised culture **12**

12 Endocyst spherical, ovoid, sometimes quandrangular, pentagonal, or pyriform; ectocyst thin, closely applied to endocyst; mean number of endocyst arms 4 or 5; mean cyst diameter 12 μm; isoenzyme and total protein patterns similar to those of *A. lugdunensis*
A. quina Pussard & Pons, 1977

— Endocyst spherical or ovoid, often deformed by rather large protuberances; ectocyst often completely smooth, not applied closely to endocyst; mean number of endocyst arms 5-6; mean cyst diameter 13 μm; characteristic isoenzyme and total protein patterns
A. divionensis Pussard & Pons, 1977

(According to De Jonckheere (1983), *A. paradivionensis* Pussard & Pons, 1977, is a synonym of this species.)

13 (8) Grows in bacterised culture at 37°C; mean number of endocyst arms fewer than 4; mean cyst diameter 13 μm; distinguished from next species by isoenzyme pattern; not pathogenic to mice
A. triangularis Pussard & Pons, 1977

(Not yet axenicised.)

— Grows in bacterised culture at 40°C; 3 or 4 endocyst arms; mean cyst diameter 13 μm; distinguish from preceding species by isoenzyme pattern; pathogenic to mice
A. hatchetti Sawyer, Visvesvara & Harke, 1977

14 (4) No growth at 37°C; endocyst spherical or ovoid, rarely with a few conical protuberances; ectocyst fine, more or less wrinkled, usually fairly closely applied to endocyst; pores difficult to discern; mean pore number *c.* 7.5; mean cyst diameter usually 17-18 μm (Fig. 43C)
A. palestinensis (Reich, 1933)

(*A. pustulosa* Pussard & Pons, 1977, is considered by De Jonckheere (1983) a synonym of *A. palestinensis*, though one report (Costas & Griffiths, 1984b) distinguished them. Cf. Pussard & Pons (1977).)

— Growth on agar with bacteria at 40°C and in axenic culture at 37°C; isoenzyme patterns or serology required to distinguish these species clearly; *all 3 pathogenic to mice* **15**

15 Mean number of cyst pores 13-14; pores *c.* 1-2 μm, smaller than in other species of Group III; endocyst circular, oval, sometimes more or less polygonal; ectocyst faintly visible, slightly wavy and closely applied to endocyst; mean diameter of cyst 15 μm
A. royreba Willaert, Stevens & Tyndall, 1978

— Mean number of pores fewer than 6 **16**

16 Endocyst usually regularly rounded, occasionally slightly polygonal; ectocyst thin, undulate or wrinkled, more or less closely applied to endocyst; mean pore number 5-6; mean diameter of cyst 15-18 μm; serologically very distinct. (Fig. 43D) ***A. culbertsoni*** (Singh & Das, 1970)

(Indications that human infections were due to this species have been reported.)

— Endocyst circular; ectocyst sometimes thin and slightly undulate, sometimes thick and folded, forming 'saw-tooth' layer all around cyst; mean pore number 4.5-6.0; mean diameter of cysts 11-13 μm
A. lenticulata Molet & Ermolieff-Braun, 1976

Other species

Acanthamoeba gigantea Schmoller, 1964: Isolated from Baltic waters of low salinity. Cyst diameter 13-22 μm; endocyst with 5-8 corners, usually 6. De Jonckheere (1987a) suggests that this might be synonymous with *A. castellanii*. *Acanthamoeba* (possibly encysted) has often been isolated from sea waters (Page, 1983b).

OTHER FAMILIES OF GYMNAMOEBIA

The families Hyalodiscidae and Echinamoebidae, though apparently Gymnamoebia, cannot be placed into any order of the present classification. The fine structure of *Hyalodiscus* is unknown, and information about both *Hyalodiscus* and *Flamella* (tentatively placed in the Hyalodiscidae) is insufficient to determine their relationships. Although the fine structure of the genera included in the Echinamoebidae is known (largely unpublished), it is not certain that those genera are related, and there is some speculation about relationships with mycetozoans.

Family HYALODISCIDAE Poche, 1913

Many and different and unrelated amoeboid organisms have a granular hump and a flattened hyaloplasm, and some have been incorrectly placed into this family and genus. The scope and relationships of the family can be determined only when the ultrastructure of the type genus is known. Earlier assumptions of a relationship to the Vampyrellidae, based on the algivorous diet and the radiate form sometimes reported, are questionable; the Vampyrellidae are Filosea.

The only genus which can with certainty be classified in this family is the type genus.

Genus *Hyalodiscus* Hertwig & Lesser, 1874

References: Cash & Hopkinson (1905); Page & Willumsen (1980); Penard (1902).

In locomotion discoid, usually with breadth the greatest dimension, consisting of a granuloplasmic hump surrounded anteriorly and laterally, often also posteriorly, by a flattened hyaloplasmic margin; many fine, short, always or usually non-furcate subpseudopodia on anterior hyaloplasm; shape fairly regular and constant during locomotion, which is often rapid and involves rolling movement; no cuticle or microscales known. Algivorous, probably also bacterivorous.

This organism is confused with members of several other genera. However, *Cochliopodium* has microscales which are usually detectable with the light microscope, as well as easily discerned bipyramidal crystals. *Paragocevia* (p. 105), to which *Hyalodiscus* is most similar light microscopically, has a distinct cuticle and moves much more slowly, though the 2 genera are very similar in possession of many fine, short subpseudopodia on the anterior hyaloplasm. The subpseudopodia of *Hyalodiscus* appear to be somewhat finer.

The generic diagnosis will remain incomplete until *Hyalodiscus*, identified with certainty, has been examined with the electron microscope.

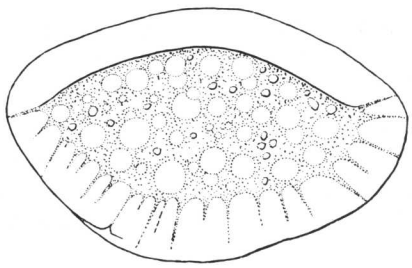

Fig. 45. *Hyalodiscus rubicundus* (after Cash).

One certain species:

Breadth in locomotion *c.* 30-100 μm, commonly 40-60 μm; granuloplasmic inclusions usually giving a reddish, sometimes brownish or greenish colour; floating form with radiate pseudopodia, which may remain temporarily as projections through hyaline margin of transitional form; change from floating to locomotive form rapid; one, sometimes 2 nuclei with central nucleolus. (Fig. 45). ***Hyalodiscus rubicundus*** Hertwig & Lesser, 1874

(Europe, North America; apparently rare.)

Similar organisms:

The amoeba found in Denmark in 1975 by Willumsen (Page & Willumsen, 1980) was almost certainly a *Hyalodiscus* (Fig. 46); its identity as *H. rubicundus* is made uncertain chiefly by its lack

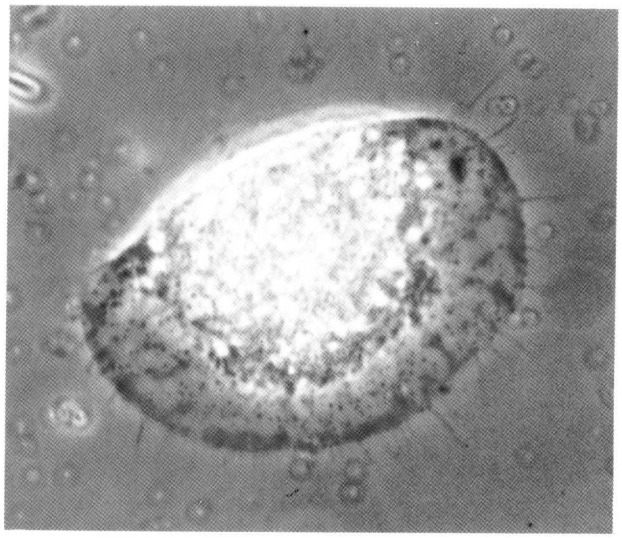

Fig. 46. *Hyalodiscus*, probably *H. rubicundus* (×1,000). Photograph by Dr N. B. S. Willumsen.

of algal inclusions. Willumsen reported that his organism had a breadth of 54.8±7.5 μm and contained a nucleus with a diameter of 5.9±0.7 μm. It advanced at a rate of 250 μm per minute, according with the comments of Hertwig & Lesser and Penard on the rapid locomotion of this species.

The organism used by Haberey & Hülsmann (1973) under the name of *H. rubicundus* agrees well with the present concept of this species, though the illustration indicates that its size is toward the upper end of the range.

Almost since it was first described, *Plakopus ruber* Schulze, 1875, has been considered a member of this genus and a possible synonym of *H. rubicundus*. Schulze gave its size in his text as 0.2-0.6 mm, but the scale of his illustrations indicates that the organisms pictured measured about 75-145 μm, mostly 75-95 μm.

Other possible Hyalodiscidae

Flamella Schaeffer, 1926: A flattened ovoid or fan shape with posterior granular mass, extensive flattened anterior and lateral hyaloplasm, with numerous, usually short, conical subpseudopodia, often in clusters of 2 or 3 (furcate at base); locomotion and change of shape rapid; nucleus obscure.

These amoebae have a much less regular form than *Hyalodiscus*, and the subpseudopodia appear to be more conical and less linear. *Flamella* should not be confused with *Paraflabellula*, which is somewhat eruptive and has less coarse subpseudopodia and a different range of shapes. Lack of ultrastructural information on *Flamella* prevents further comparisons.

F. citrensis Bovee, 1956, is the only species described from freshwater. Subpseudopodia in 2's and 3's from pyramidal bases at anterior edge of granuloplasm; occasionally a few, short, trailing filaments; breadth increasing with rate of locomotion, 35-55 μm; floating form spherical with hyaline papillae; anaerobic. From citrus pulp wastes in freshwater tanks, USA. Illustrations in Page (1976).

Family ECHINAMOEBIDAE Page, 1975

Reference: Page (1975b).

An essential difference between Echinamoebidae and Acanthamoebidae is that all Acanthamoebidae have a centriole-like body, but electron microscopy is not necessary for practical differentiation.

The former distinction in cyst structure, that cysts of Acanthamoebidae have pores and cysts of Echinamoebidae do not, is no longer valid since the the discovery of *Protacanthamoeba*, unquestionably an acanthamoebid, and *Comandonia*, which is better placed into the Echinamoebidae. Nevertheless, the practical likelihood of confusion is small, since the cysts of all species of *Acanthamoeba* have pores, and the amoeboid form of *Protacanthamoeba* (much less common than *Acanthamoeba*) soon betrays its familial relationship.

The 'echinopodia' (Pernin & Pussard, 1979) of *Echinamoeba* and *Comandonia* are much smaller than the acanthopodia of the Acanthamoebidae, and the longer, filiform subpseudopodia of *Filamoeba* are more numerous, have a narrower base than acanthopodia, are less conical and more linear, and never have a blunt tip. But even these long, fine subpseudopodia of *Filamoeba* are not functionally filopodia, because they are produced from a lobopodium and do not themselves advance the amoeba by attaching and shortening, as do the filopodia of *Nuclearia*.

The genus *Stachyamoeba* (p. 44) has been removed from this family. Though it often has a compressed form with spine-like subpseudopodia, its fine structure and other characters place it in the Heterolobosea.

1 Amoebae triangular, elongate, flabellate, or irregular in outline, mean length not above 15 μm, often with a few fine subpseudopodia (echinopodia) about 1-1.5 μm long; cysts spherical, thin-walled, never above 10 μm in diameter and usually much smaller ***Echinamoeba***

— Amoebae and cysts larger; cysts with thick wall **2**

2 Amoebae with filiform subpseudopodia, often numerous, up to 7 μm long; cysts without pores or opercula ***Filamoeba***

— Amoebae often with echinopodia; cyst with pores closed by opercula ***Comandonia***

Genus *Echinamoeba* Page, 1975

Reference: As for family.

These small amoebae resemble eumycetozoan myxamoebae more than do any other Gymnamoebia. Fruiting bodies have never been seen in the cultures, but the possibility that conditions are unsuitable for sporulation has not been eliminated. A study of their fine structure (unpublished) has not settled the problem.

The 2 species are very similar, but the degree of difference, particularly in cyst structure, by which they were distinguished receives some support from the electron microscopic results.

1 Amoebae expanded and fan-like to elongate, almost limax-like, with irregular anterior edge, L 7-22 μm ($\bar{x}$ 14 μm); nucleus 2.1-2.8 μm, with relatively small nucleolus, *c.* 0.5 μm; cyst wall often not separated into 2 layers, cyst diameter 3.8-8.4 μm ($\bar{x}$ 5.4 μm). (Fig. 47A, B)
Echinamoeba exundans (Page, 1967)

(North America, Europe, NNE.)

— Amoebae often with triangular outline, seldom broadened or spatulate, L 6.5-21 μm ($\bar{x}$ 11 μm); nucleus 2-2.8 μm, with nucleolar diameter about half that of nucleus; narrow space between inner and outer layers of cyst wall, cyst diameter 5.6-10 μm ($\bar{x}$ 7.5 μm). (Fig. 47C, D)
E. silvestris Page, 1975

(Europe; terr. NNE. Unlike *E. exundans*, the original strain of this species suffered a marked reduction of encystment ability within a few years of isolation.)

Genus *Filamoeba* Page, 1967

Reference: Page (1967b).

These amoebae spread out much more thinly than *Acanthamoeba* and sometimes have a long, narrow posterior end. They may have 2 or 3 expanded hyaloplasmic lobes in different directions. Unpublished electron micrographs failed to reveal a centriole-like body. A single species.

Outline of amoebae varying from semi-circular to elongately spatulate, sometimes multipolar with 2 or more usually fan-shaped hyaloplasmic lobes, sometimes flattened into thin sheet; greatest dimension of locomotive and extended forms *c.* 15-50 μm; usually uninucleate; nuclear diameter *c.* 3.5-5.5 μm; few to more than 15 contractile vacuoles; cysts smooth, round, irregularly ovoid, or reniform, rather thick-walled, with little or no splitting of wall into separate layers, 8-15 μm ($\bar{x}$ *c.* 10 μm). (Fig. 47E-J) ***Filamoeba nolandi*** Page, 1967

(North America, Europe, NNE.)

Genus *Comandonia* Pernin & Pussard, 1979

Reference: Pernin & Pussard (1979).

The generic characters of *Comandonia*, like those of *Filamoeba*, are derived from a single known

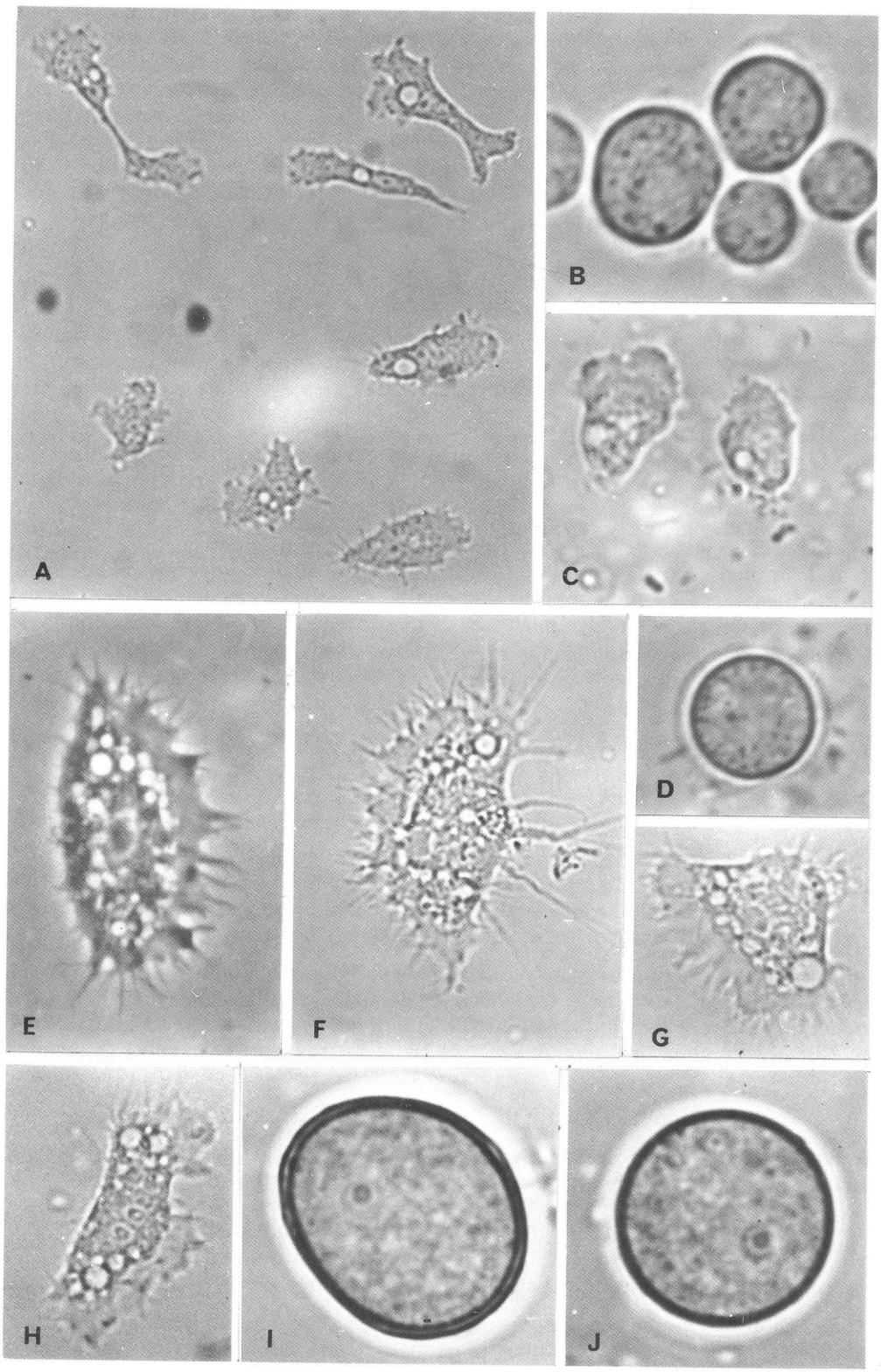

Fig. 47. Echinamoebidae. A, B, *Echinamoeba exundans*, amoebae and cysts. C, D, *Echinamoeba silvestris*, amoebae and cyst. E-J, *Filamoeba nolandi*; H, amoeba with hyaloplasm divided into two lobes (two or more such lobes may be found in more extended amoebae; the binucleate condition of this amoeba is not related to the distribution of hyaloplasm); I, J, cysts (thickenings do not indicate positions of pores, which are not present in this genus). (A, E-H, ×1,000; B, D, I, J, ×2,500; C, ×1,575.)

species. This amoeba often has short, fine subpseudopodia, perhaps somewhat more prominent than those of *Echinamoeba* but still little more than spinous processes, unlike those of *Filamoeba* and *Acanthamoeba*. No centriole-like bodies have been found. The ostioles and opercula of the cyst wall are entirely features of the thick inner wall layer and do not involve the outer layer, as in *Acanthamoeba*.

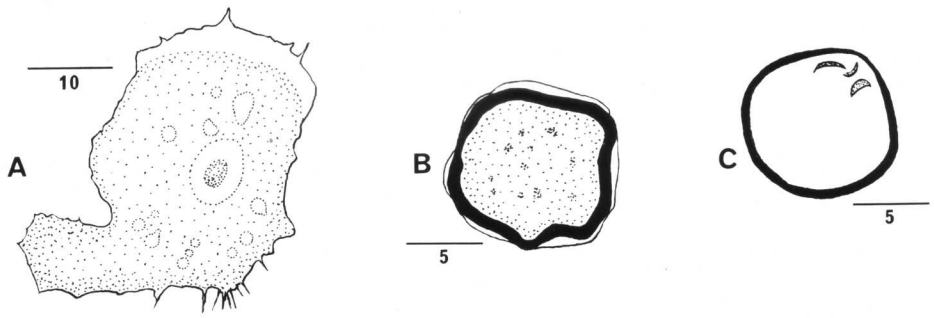

Fig. 48. *Comandonia operculata*. B, cyst; C, empty cyst wall containing detached opercula. (After Pernin & Pussard.)

Amoebae expanded on substratum, with irregular shape, narrow hyaloplasmic border, and somewhat bristly appearance, due in part to short, fine projections (echinopodia); often with adhesive uroidal filaments; mean greatest dimension 40 μm; normally uninucleate, mean nuclear diameter 4.2 μm; numerous contractile vacuoles; cysts irregularly polyhedral, or sometimes mamillate, with thick inner wall layer and fine, barely discernible outer layer, mean cyst diameter 12.7 μm. (Fig. 48) ***Comandonia operculata*** Pernin & Pussard, 1979 (Europe.)

Subclass TESTACEALOBOSIA DE SAEDELEER, 1934

Order HIMATISMENIDA Page, 1987

Reference: Page (1987c).

Discoid or globose amoebae incompletely enclosed in a flexible cuticle or a tectum of intricate microscales, open on the side applied to the substratum but without a well-defined aperture; flattened locomotive forms with hyaline margin, from which short subpseudopodia may be produced, completely or partly surrounding granular hump. Centriole-like body known in some.

This order is included because some of its members are easily taken for Gymnamoebia and can even be cultured by methods used for Gymnamoebia. Bovee (in Jahn *et al.*, 1979) placed the Cochliopodiidae in an order Pharopodida, which, however, included several families of Gymnamoebia. The dichotomous keys give attention to those species most resembling gymnamoebae.

Family COCHLIOPODIIDAE De Saedeleer, 1934

The family is understood here in the sense of recent testacean workers (e.g. Schönborn, 1966) and has the characters of the order.

1 Covered with a tectum of microscales closely applied to cell surface, often appearing punctate to light microscope ***Cochliopodium***

— Covered with a fibrous, spongiose cuticle, often appearing smooth to the light microscope **2**

2 Without subpseudopodia produced from surface of hyaloplasm, though edge of hyaline margin may have irregular, even conspicuous projections; some species carrying foreign particles ***Gocevia***

— With many narrow subpseudopodia on surface (not just edge) of hyaloplasmic margin during locomotion (subpseudopodia usually or always absent from stationary cells) ***Paragocevia***

Genus *Cochliopodium* Hertwig & Lesser, 1874

References: Bark (1973); Nagatani *et al.* (1981); Page (1968, as *Hyalodiscus*; 1976); Penard (1902); Yamaoka *et al.* (1984).

The genus may include at least 2 major groups and several species not assignable to either group.

The ones with which we are principally concerned include *Cochliopodium bilimbosum*, generally considered the type-species. The dichotomous key includes only the few members of that group which have been studied in culture. Characters of the group include: a tectum of microscales usually perceptible light microscopically, with good optical conditions, as a fine, regular punctation on the hyaline margin (also present over the granuloplasm); a globose inactive or floating form, from which radiate pseudopodia may project, sometimes from a limited area; a more or less flattened, limpet-like locomotive form consisting of a thickened granular mass partly or completely surrounded by a hyaline margin; and truncate bipyramidal crystals similar to those of Amoebidae and *Saccamoeba*. These and most other species attributed to the genus have a vesicular nucleus with a central nucleolus.

Unlike the microscales of Paramoebidae, those of *Cochliopodium* are circular rather than boat-shaped, consisting of a grid-like base, a column of 4 or 5 legs, and a capital. Furthermore, they appear distinctive of each species. Unfortunately, with one exception the microscale patterns have not been connected with named species, so that we are deprived of an invaluable taxonomic character. Descriptions and illustrations of scales can be found in Bark (1973), Nagatani *et al.* (1981), and Yamaoka *et al.* (1984).

1 Microscales easily distinguished with the light microscope as a regular punctation; distinct tubular configuration of tectum around pseudopodial bases of floating form; a few finely conical, sometimes furcate subpseudopodia sometimes produced from anterior edge; many cytoplasmic crystals; globose inactive or floating form 32-47 μm ($\bar{x}$ 41 μm); greatest dimensions of locomotive form 49-90 μm ($\bar{x}$ 68 μm); nucleus 11-12.5 μm; no cyst known. (Fig. 49A, B)
Cochliopodium bilimbosum (Auerbach, 1856)
(Europe, North America. Culture method: Bark (1973).)

— Punctation less conspicuous, sometimes imperceptible with light microscope; amoebae generally smaller than above; anterior border seldom or never producing distinct subpseudopodia **2**

2 Locomotive form *c.* 15-80 μm, mean varying between 38 and 61 μm even in one strain, depending on culture conditions and expansion of amoebae; tectum clearly visible as doubly contoured covering on granular hump; subpseudopodia rarely if ever produced by anterior edge; often with many trailing filaments; crystals sometimes numbering 30 or more; nucleus 6.9-13.8 μm ($\bar{x}$ *c.* 10 μm); cyst spherical or oval, with smooth inner wall and thick, irregular outer layer, diameter 18.5-27 μm. (Fig. 49C-G) ***C. actinophorum*** (Auerbach, 1856)
(Europe, North America. NNE. *C. pellucidum* Hertwig & Lesser, 1874, is probably a synonym.)

— Locomotive form 14.5-41 μm, usually 18-38 μm ($\bar{x}$ *c.* 25 μm); short, finely conical subpseudopodia sometimes produced from anterior edge, which may be rather ragged, sometimes, with trailing filaments along posterior edge; crystals numbering to about 10; nucleus 4.8-9.3 μm ($\bar{x}$ *c.* 6-7 μm); cysts produced by some but not all strains, 11.5-21 μm (Fig. 49H)
C. minus Page, 1976
(North America, Europe, NNE.)

Other species
These include both flattened species easily mistaken for gymnamoebae and species which are more habitually globose. Amongst the former may be the organisms originally described as *Amoeba lamellipodia* Gläser, 1912, *A. cucumis* Martin & Lewin, 1914, and *A. gobanniensis* Martin & Lewin, 1914. Such flattened forms also include strains investigated with the electron microscope by Bark, Nagatani *et al.*, and Yamaoka *et al.* but not identified to species.

Genus *Gocevia* Valkanov, 1932

References: Pussard (1965); Pussard *et al.* (1977).

This genus was erected for *Cochliopodium*-like rhizopods with a hyaline envelope covered with foreign bodies. However, one species, *G. fonbrunei*, was not, under laboratory conditions, covered with foreign particles, and it is upon that species that the description of the cuticle is based. The granular mass is globular or flattened and hump-like. Organisms with a similar cuticle but many subpseudopodia have been transferred to the genus *Paragocevia*. Given the nature of the cuticle in both genera, as revealed by electron microscopy, there seems no reason why members of either genus might not have foreign particles adherent to the cuticle.

Apart from *G. fonbrunei*, the species included in the dichotomous key to this genus have not

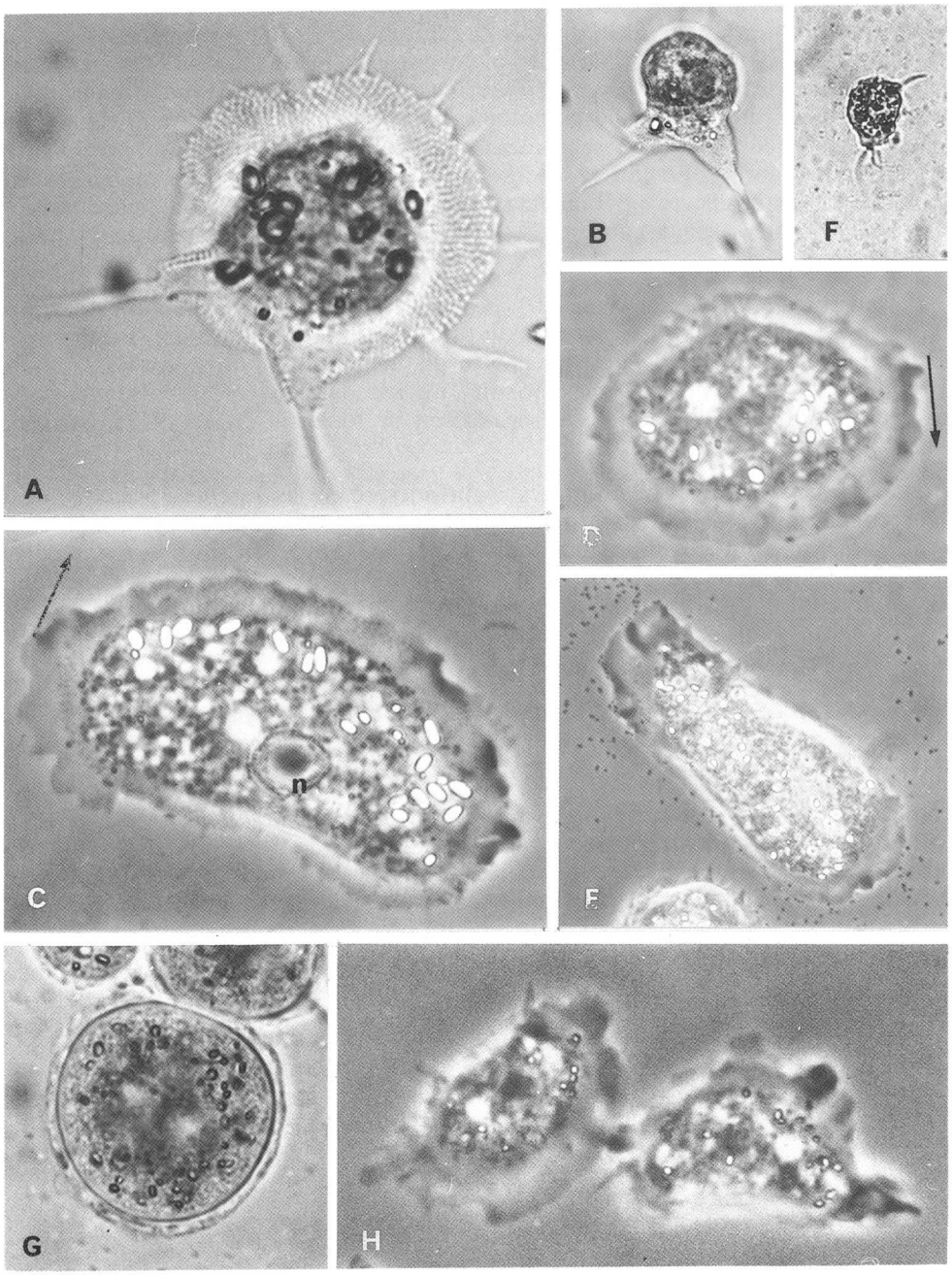

Fig. 49. *Cochliopodium*. A, B, *C. bilimbosum*; B, bell-shaped floating form. C-G, *C. actinophorum*; C, punctation caused by microscales especially distinct on anterior hyaline margin; F, floating form (sometimes with more radiate pseudopodia than here); G, cysts. H, *C. minus*. (B, ×500; F, ×250; all others, ×1,000.) *n*, nucleus.

been investigated sufficiently to permit their inclusion under the usual standard of this key, but they are included to give a picture of the genus.

1 Hyaline cuticle without foreign bodies, distinguished with some difficulty; protoplast filling cuticle completely; uninucleate; unornamented cyst; amoebae generally below 50 μm; cyst 12-16 μm. (Fig. 50A) ***Gocevia fonbrunei*** Pussard, 1965

(Europe. Culture method: NN with bacteria and baker's yeast.)

— Cuticle bearing foreign particles **2**

2 Usually with covering of foreign bodies, though young cells may be nearly free of them; protoplast not filling cuticle completely; usually or always binucleate; 29-30 μm. (Fig. 50B) ***G. binucleata*** De Saedeleer, 1934

(Europe.)

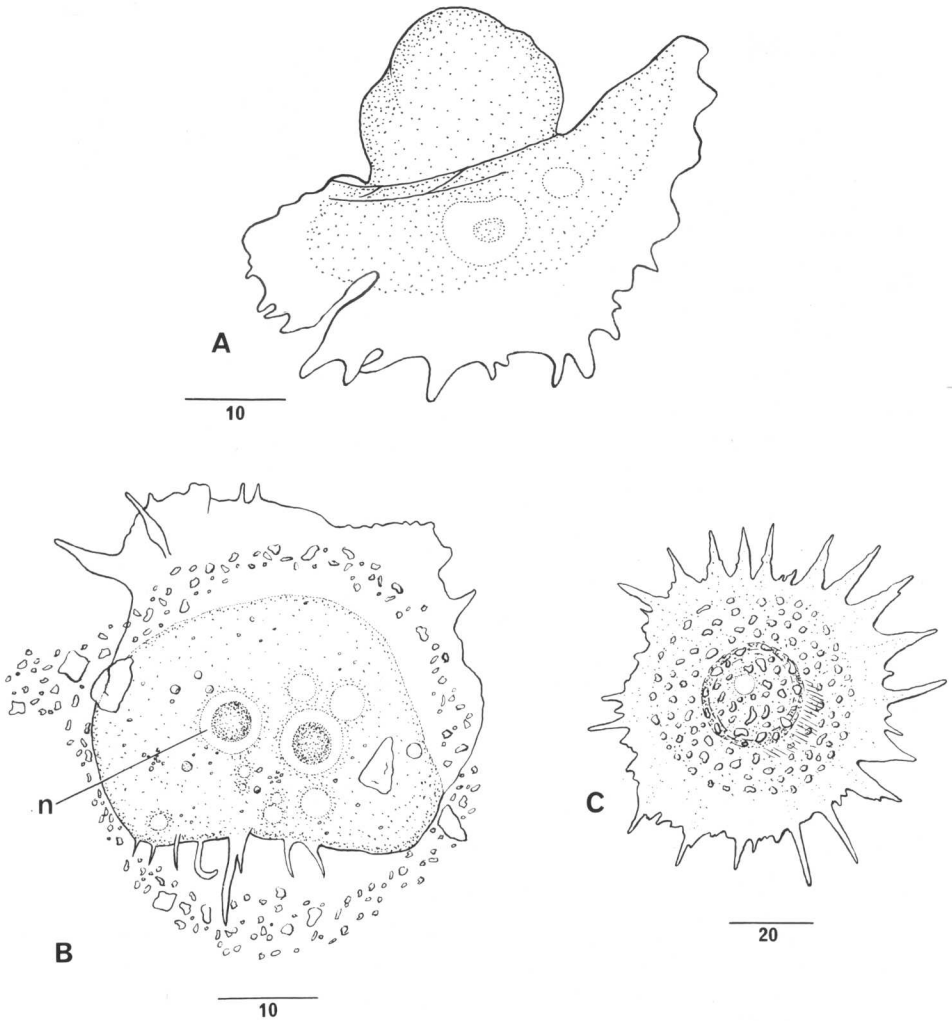

Fig. 50. *Gocevia*. A, *G. fonbrunei* (after Pussard). B, *G. binucleata* (after De Saedeleer). C, *G. obscura* (after Penard).

— Heavy covering of refractile grains, which individually are clear or yellowish but give cuticle a blackish appearance; 25-30 μm rounded, up to 50 μm flattened, not including prominent, somewhat irregularly conical subpseudopodia from edge; uninucleate; nucleus *c.* 10 μm (Fig. 50C) ***G. obscura*** (Penard, 1890)
(Europe.)

Genus *Paragocevia* Page, 1987

References: Page (1987c); Page & Willumsen (1980).

Formally this genus is like *Gocevia* except in the occurrence of many short, digitate subpseudopodia on the flattened hyaline margin in locomotion. Its cuticle somewhat resembles that of *G. fonbrunei* in fine structure. However, the one described species is more commonly flattened and more regular in shape than the species of *Gocevia*.

Paragocevia bears some light microscopic resemblance to species classified as *Hyalodiscus*. The use of the present specific name for the one species is based on its identification with an organism previously considered a *Hyalodiscus*. It seems important for distinguishing the genera that *Paragocevia* moves much more slowly than *Hyalodiscus*, perhaps because of the cuticle.

The cuticle is observed with difficulty on living amoebae. It can often be seen with differential interference contrast on flattened but non-moving cells, extending a few micrometres beyond the hyaloplasm. It is also distinguishable around the hump of somewhat rounded cells fixed for light microscopy.

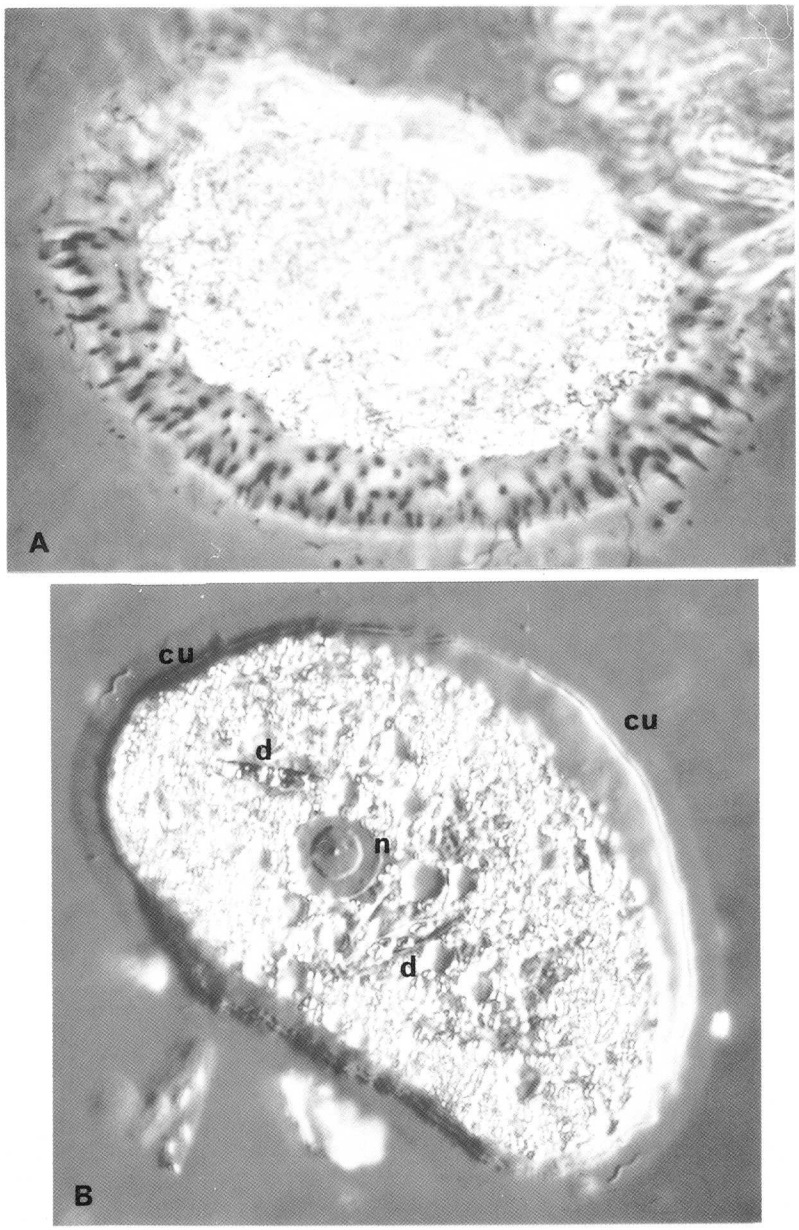

Fig. 51. *Paragocevia placopus*. B, stationary flattened form viewed by differential interference contrast, with cuticle discernible beyond hyaloplasmic margin. (Both ×800.) *cu*, cuticle; *d*, ingested diatom; *n*, nucleus.

Locomotive form flattened, often remaining flattened when stationary, 62-125 μm ($\bar{x}$ 95 μm), L/B 0.6-1.0 ($\bar{x}$ 0.8 μm); flattened hyaloplasmic margin surrounding slightly raised granuloplasm anteriorly and laterally, rarely posteriorly; subpseudopodia *c.* 5-10 μm long, often more than 100 on hyaloplasmic margin in locomotion; protoplast filling cuticle completely; granuloplasm sometimes more rounded up in stationary forms; seldom any conical pseudopodia on floating cells; nucleus 12.4-15.0 μm, commonly 14 μm, with central nucleolus; ingests diatoms, bits of filamentous algae and blue-greens, ciliate protozoa; no cyst known (Figs. 51, 52)

Paragocevia placopus (Hülsmann, 1974)

(Europe. E+S with accompanying eukaryotes.)

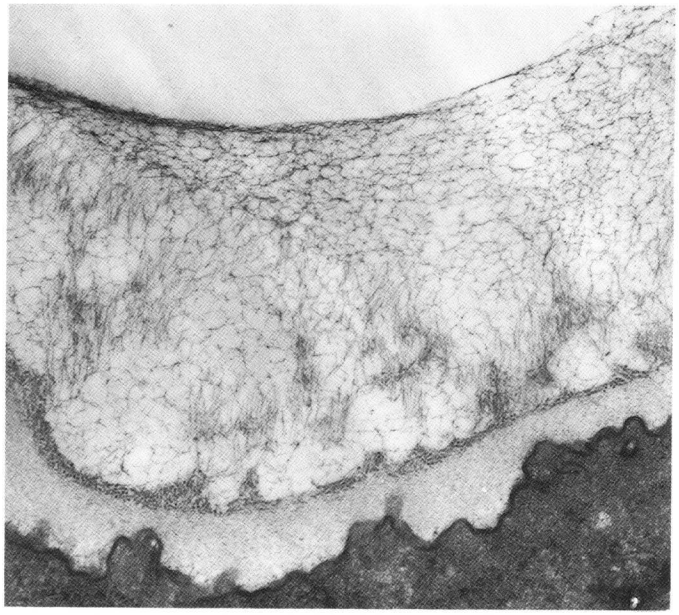

Fig. 52. *Paragocevia placopus*, fine structure of cuticle (×20,000).

Other possible species:
Cochliopodium digitatum (Greeff, 1866), as described by Penard, resembles *P. placopus*.

GENERA *INCERTAE SEDIS*

Genus *Dinamoeba* Leidy, 1874

References: Page (1970), with references to earlier works.

In locomotion pyriform, compressed, with numerous conical, hyaline, flexible, sometimes furcate subpseudopodia, which taper to a blunt or fine tip, originating from both anterior hyaloplasm and granuloplasm, over much of cell. Surface, including subpseudopodia, sometimes covered by gelatinous layer in which many bacterium-like rods are embedded. Sluggish, algivorous. Cyst, possibly short-lived, reported.

These organisms move very slowly, possibly by brief, somewhat eruptive bursts. The subpseudopodia, sometimes put out rapidly and sometimes furcate, appear to play no part in locomotion. In their relative length as well as in being conical, hyaline, and flexible, they resemble those of *Acanthamoeba*, though those on the posterior part of the cell may be longer than those in a corresponding position on *Acanthamoeba*. Ingestion of food apparently occurs at the posterior end.

One well-described species:

L 130-340 μm; anterior end often broader, though posterior end may be; reportedly binucleate, with central nucleolus; cytoplasm usually filled with unicellular or filamentous algae or blue-greens and coloured by digestion products; one or more contractile vacuoles in freshwater. (Fig. 53) ***Dinamoeba mirabilis*** Leidy, 1874

(North America, Europe; fresh and brackish water.)

Several questions about this organism are important for identification as well as their bearing on relationships. These questions have been open for more than a century, because the organism is rare and has never been brought into culture.

The first is the connection if any between *D. mirabilis* and the flagellate *Mastigamoeba aspera* Schulze, 1875. The similarities are striking, and both organisms are algivorous and euryhaline, but

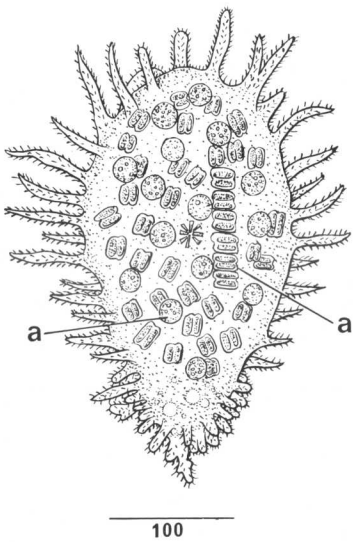

Fig. 53. *Dinamoeba mirabilis* (after Leidy). *a*, ingested alga.

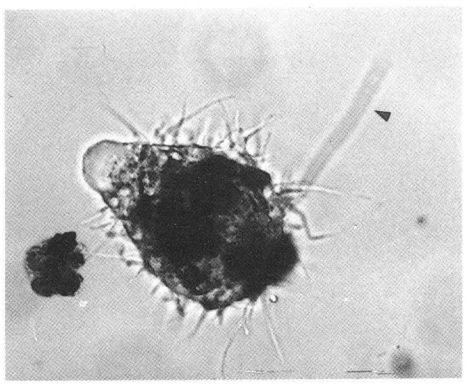

Fig. 54. *Dinamoeba* sp., with no rod-like structures on surface (×250).

the question of a possible identity remains unsettled. An observer should examine every specimen for a flagellum. Penard suggested compression with the coverslip to make the flagellum appear.

The second problem is the nature of the surface covering, which may be present or absent. It consists of two components, a sticky coat and many rod-like structures, which have been called spicules, cils, granulations, or grains. Judging from the descriptions and from observation of a similar covering on *Mastigamoeba aspera*, the rods (which Penard reported seeing divide) may be bacteria living in a slimy covering of the amoeba.

Nuclear number and structure are other unsettled questions. The masses of ingested matter strongly obscure the nucleus (-i). Penard reported that *D. mirabilis* is binucleate, a questionable generalisation.

Schaeffer described a species *D. horrida* on the basis of one amoeba. In the present state of knowledge it seems best to recognise only one species.

Increased knowledge of the family Paramoebidae (=Mayorellidae) and of the essential differences between superficially similar subpseudopodia makes a classification in that family impossible, nor do the somewhat similar subpseudopodia justify classification in the Acanthamoebidae.

Genus *Phreatamoeba* Chávez, Balamuth & Gong, 1986

Reference: Chávez *et al.* (1986).

Dominant phase multinucleate, occasionally uninucleate, amoebae, with few hyaline, conical, sometimes furcate subpseudopodia; temporary uninucleate, uniflagellate phase; cyst. No mitochondria or Golgi bodies found. Nuclear division with distintegration of nucleolus but persistence of nuclear envelope; centriole present.

A single species:

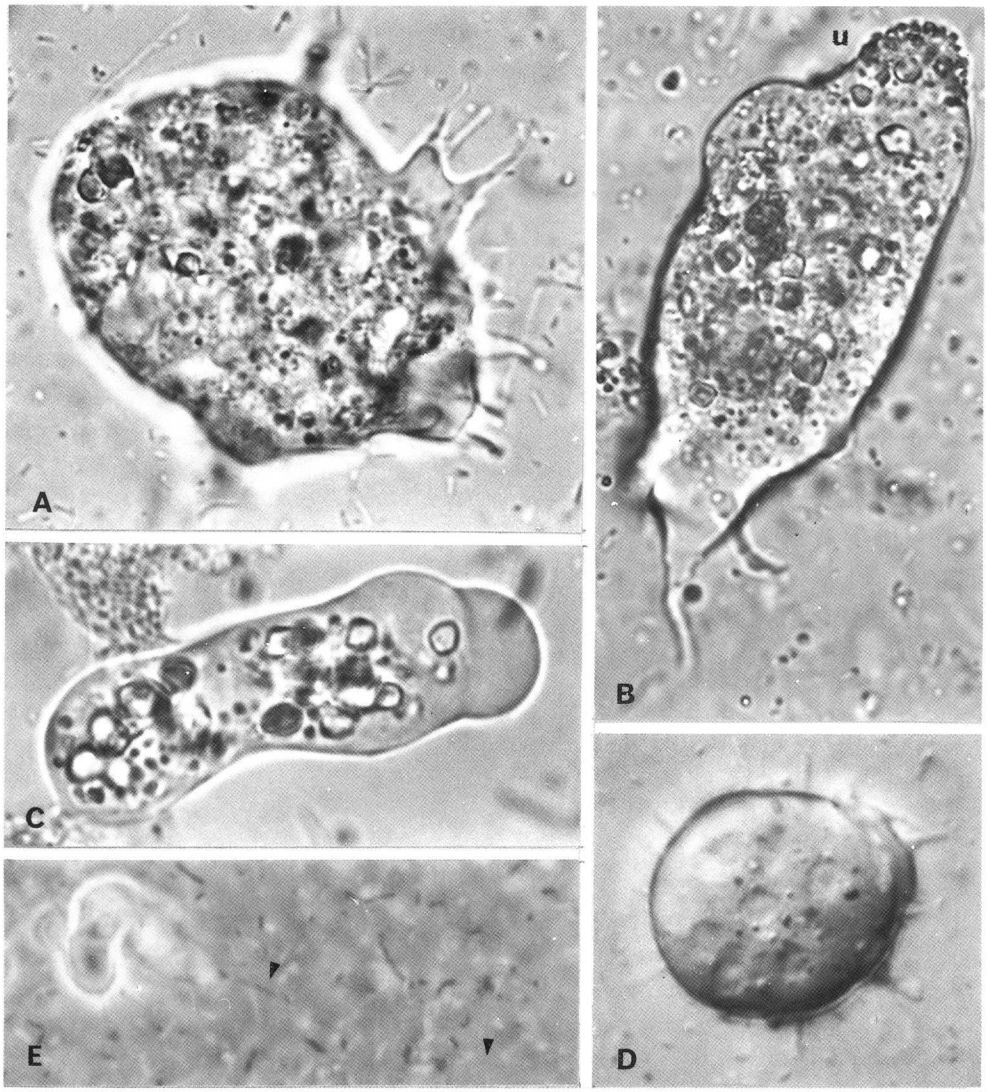

Fig. 55. *Phreatamoeba balamuthi*. A-C, various forms of amoebae. D, amoeba viewed with differential interference contrast, with focussing to show several nuclei. E, flagellate stage, with parts of long flagellum indicated by arrowheads. (All ×1,000.)

Amoebae subcylindrical or flattened, moving sluggishly, with hyaline lobe from which 1 or a few subpseudopodia are often produced, L 11-160 μm, mean diameter in rounded or oval form *c*. 54 μm; nuclear number of amoebae 1-46, $\bar{x}$ 15, nuclear diameter 2.5-4.5 μm, central nucleolus; temporary uniflagellate stage produced by transformation of uninucleate amoeba or bud from multinucleate amoeba, no cytostome, no division in flagellate stage; cyst spherical to oval, 6-8 μm; reproduction by plasmotomy of multinucleate amoebae; loose, amorphous cell coat, 67-182 nm thick, on amoebae from bacterised cultures; micro-aerobic, not tolerating atmospheric level of oxygen; growth at 21-27°C, do not survive at 37°C; survive osmotic concentration above that of freshwater. (Fig. 55)

Phreatamoeba balamuthi Chávez, Balamuth & Gong, 1986

(Africa. Culture methods: See below.)

This unusual organism was isolated from a village well in the Gambia, and all the strains in several laboratories are descended from that isolate. It was maintained at the Culture Centre of Algae and Protozoa for more than 10 years in Jones's medium with accompanying bacteria but has also been grown in proteose peptone with *E. coli* and axenically. *P. balamuthi* is very unusual amongst free-living amoebae in thriving in a medium devised for *Entamoeba*, containing serum and with a salt concentration far above that of freshwater. It requires reducing conditions and is found in large numbers at the bottom of the culture tube, unlike *Acanthamoeba* in proteose peptone. It does not, however, survive for any length of time (days) at mammalian body temperature.

The life history has been verified by repeated cloning. Flagellate cells are often seen in cultures 1-2 weeks old. Transformation to the flagellate stage can be induced by adding rice powder but not purified starch to bacterised cultures; reducing the tonicity does not induce transformation.

In locomotion these sluggish amoebae have a broad, sometimes triangular hyaline lobe, from which conical, hyaline subpseudopodia are put out; such subpseudopodia are also produced from various points on stationary forms, which may be compressed, rounded, or cylindrical. The subpseudopodia are sometimes furcate near the base. Their superficial similarity to the subpseudopodia of Paramoebidae is not in itself indicative of a relationship. Somewhat longer pseudopodia on floating forms are occasionally helical, though these are not regularly radiate floating forms.

A contractile vacuole is seen in amoebae grown in proteose peptone but not in those from Jones's medium.

The mitotic figures as seen with the light microscope and some other characters might be taken to suggest a relationship with the Gruberellidae (class Heterolobosea), which includes a multinucleate marine organism, but the finding of a centriole appears to rule out such a relationship.

Unlike *Pelomyxa, Phreatamoeba* does not have endosymbiotic bacteria, and a relationship with *Pelomyxa* seems unlikely. Although a relationship with some mycetozoans, as suggested by Chávez *et al.*, may be possible, there is yet no evidence for such a relationship. This organism is, therefore, taxonomically isolated amongst gymnamoebae. Its unusual nature and rarity would make confirmed reports of further isolations of general interest.

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INDEX TO GENERA AND SPECIES OF GYMNAMOEBAE, ACRASIDA, AND COCHLIOPODIIDAE

The principal taxonomic references are in **bold-face** type.