

# SSU rRNA-based Phylogenetic Position of the Genera *Amoeba* and *Chaos* (Lobosea, Gymnamoebia): The Origin of Gymnamoebae Revisited

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Naked lobose amoebae (gymnamoebae) are among the most abundant group of protists present in all aquatic and terrestrial biotopes. Yet, because of lack of informative morphological characters, the origin and evolutionary history of gymnamoebae are poorly known. The first molecular studies revealed multiple origins for the amoeboid lineages and an extraordinary diversity of amoebae species. Molecular data, however, exist only for a few species of the numerous taxa belonging to this group. Here, we present the small-subunit (SSU) rDNA sequences of four species of typical large gymnamoebae: *Amoeba proteus*, *Amoeba leningradensis*, *Chaos nobile*, and *Chaos carolinense*. Sequence analysis suggests that the four species are closely related to the species of genera *Saccamoeba*, *Leptomyxa*, *Rhizamoeba*, *Paraflabellula*, *Hartmannella*, and *Echinamoeba*. All of them form a relatively well-supported clade, which corresponds to the subclass Gymnamoebia, in agreement with morphology-based taxonomy. The other gymnamoebae cluster in small groups or branch separately. Their relationships change depending on the type of analysis and the model of nucleotide substitution. All gymnamoebae branch together in Neighbor-Joining analysis with corrections for among-site rate heterogeneity and proportion of invariable sites. This clade, however, is not statistically supported by SSU rRNA gene sequences and further analysis of protein sequence data will be necessary to test the monophyly of gymnamoebae.

## Introduction

Phylogeny of naked amoebae remains one of the most knotty questions in protistology. So far, various systems of gymnamoebae have been based mostly on the morphological features (Schaeffer 1926; Lepš 1960), sometimes combined with the nuclear division pattern (Singh 1952; Chatton 1953; Page 1976) and the modes of locomotion (Jahn and Bovee 1965; Jahn, Bovee, and Griffith 1974). All these systems could hardly pretend to reflect phylogenetic relationships among the higher taxa. For convenience, all amoeboid protists with lobose pseudopodia were included in the class Lobosea (Levine et al. 1980; Bovee 1985). Based on the ultrastructure and peculiarities of the life cycle, Page and Blanton (1985) suggested a dichotomy of the classes Lobosea and Heterolobosea. Within the class Lobosea, Page (1987, 1991) recognized four orders of naked amoebae (Euamoebida, Leptomyxida, Acanthopodida, and Loboreticulatida) and grouped them in the subclass Gymnamoebia. At least two groups (the class Heterolobosea and the order Euamoebida) were viewed as more or less natural (Page 1987). The phylogenetic relationships within and between these groups, however, remained largely hypothetical because of the low resolution of the morphological taxonomy.

Very little is known about the evolution of amoebae, and despite the rapid development of molecular studies only a few amoeba sequences are available. For over one decade, the naked amoebae were represented in phylogenetic trees only by three medically important genera, *Acanthamoeba*, *Entamoeba* (Gymnamoebae), and *Naegleria* (Heterolobosea). Although this situation is slowly chang-

ing, at least in what concerns the rRNA database (Sims, Rogerson, and Aitken 1999; Amaral Zettler et al. 2000), our knowledge of phylogenetic relationships among amoebae remains largely fragmentary.

One of the most enigmatic points concerns the phylogenetic origin of amoebae. The first molecular data confirmed the polyphyly of amoebae (Clark and Cross 1988), in agreement with the morphology-based distinction between the classes Lobosea and Heterolobosea (Page and Blanton 1985). Comparison of small subunit (SSU) rRNA gene sequences of *Acanthamoeba castellanii* (Lobosea) and *Naegleria gruberi* (Heterolobosea) shows that both species branch separately, the first one emerging within a radiation of eukaryotes (Gunderson and Sogin 1986), the second one branching in the middle part of the SSU tree (Baverstock et al. 1989). The independent origin of *Naegleria* was later confirmed by molecular studies on other Vahlkampfiidae (Hinkle and Sogin 1993). The analysis of SSU rRNA also suggested a lack of any specific relationship between *A. castellanii* and *Entamoeba histolytica* (Sogin 1989), leading to the exclusion of *Entamoeba* from the Lobosea and to the creation of a new parvkingdom of Entamoebia (Cavalier-Smith 1993). Among all examined Gymnamoebia, only *Hartmannella vermiformis* shows a relationship to *A. castellanii* (Gunderson, Goss, and Sogin 1994; Weekers et al. 1994). The position of both species in the upper part (generally referred to as the crown) of the SSU tree was considered as representative for gymnamoebae. However, the SSU rRNA of *Vannella anglica*, another Gymnamoebia, did not associate with those of the two species (Sims, Rogerson, and Aitken 1999).

The SSU rRNA sequence was also used to examine the phylogeny of other amoeboid protists. In particular, these data showed that the euglyphid filose amoebae (Bhattacharya, Helmchen, and Melkonian 1995) and the anaerobic pelobiontid amoeboflagellate *Phreatamoeba balamuthi* (Hinkle et al. 1994) have an independent or-

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igin. According to Simpson and others (Simpson et al. 1997; Walker et al. 2001), *P. balamuthi* should be considered to belong to the genus *Mastigamoeba*; nevertheless, despite their morphological similarity, the SSU sequences of *P. balamuthi* and *Mastigamoeba invertens* do not group together. Later, this discrepancy was interpreted as an artifact because of the heterogenous evolutionary rates (Stiller and Hall 1999). On the other hand, *M. invertens* was shown to branch among the earliest eukaryotes in the tree based on the gene encoding the largest subunit of RNA polymerase II (RPB1) (Stiller, Duffield, and Hall 1998).

In view of these data, based principally on ribosomal DNA sequences, the polyphyly of amoeboid protists seems to be well established. However, the results of some recent reanalyses of SSU rRNA sequences question the solidity of this hypothesis. Maximum likelihood reanalysis of SSU rRNA sequences (Cavalier-Smith and Chao 1996) showed that *E. histolytica*, *P. balamuthi*, *A. castellanii*, and *H. vermiformis* branch together, in opposition to the previous results of the same data set. Moreover, in some maximum likelihood (ML) and maximum parsimony (MP) trees, Lobosea appears as a sister group to Archamoebae and Mycetozoa, supporting the inclusion of all the three groups in the phylum Amoebozoa (Cavalier-Smith 1998a). The close relationships between gymnamoebae were later confirmed by the study of *E. histolytica* and *Endolimax nana* SSU rDNA sequences (Silberman et al. 1996). The lobose amoebae grouped together also in the ML analysis of the SSU rRNA of leptomyxid amoebae (Amaral Zettler et al. 2000), although the limited number of nonamoeboid species used in this study does not allow any general conclusion.

A common origin for Amoebozoa was also proposed based on the analysis of combined protein data (Baldauf et al. 2000). The grouping of *Acanthamoeba* with Mycetozoa (*Dictyostelium* and *Physarum*) is supported by protein sequences, like actin (Bhattacharya and Weber 1997; Philippe and Adoutte 1998) and actin-related proteins ARP2 and ARP3 (Kelleher, Atkinson, and Pollard 1995; Schafer and Schroer 1999), as well as by similarities in genome organization between *Acanthamoeba* and *Dictyostelium* (Iwamoto et al. 1998). However, the position of gymnamoebae in other protein trees is extremely variable. For example, *E. histolytica* branches at the base of the EF-1 $\alpha$  tree (Baldauf and Palmer 1993) and as a sister group to Euglenozoa in the EF-2 tree (Moreira, Le Guyader, and Philippe 2000), whereas *A. castellanii* branches with plants in the RPB1 tree (Stiller and Hall 1997). Given that in most cases, the sequences of both amoebae are not available for the same protein, their mutual relationships cannot be inferred.

Here, we report the first SSU rRNA gene sequences of four typical large lobose amoebae of the family Amoebidae. Among them is the most commonly studied species, *Amoeba proteus*. These sequences were compared to those of other amoebae and various groups of eukaryotes. The phylogenetic relations within and between amoeboid protists were analyzed using different

evolutionary models and methods to test the hypothesis of gymnamoebae monophyly.

## Materials and Methods

### Cell Cultures

*Amoeba proteus* strain Ge (origin of the strain is unknown) is a gift of Dr. R. K. Peck, Laboratory of Protistology, University of Geneva, Switzerland; this strain was maintained with *Tetrahymena* as a food. *Chaos nobile* CCAP#1511/2 and *Amoeba leningradensis* CCAP#1503/6 were obtained from the Culture Collection of Algae and Protozoa, CEH Windermere, United Kingdom. *Chaos carolinense* is a gift of Dr. Y. Deng, Wadsworth Center for Laboratories and Research, Albany; this species is the strain #WW-13-1324 from Carolina Biological Supply Company, Burlington. According to the company's data, this strain was originally field collected in the United States and identified by their technicians.

### Molecular Genetics

For total RNA extraction the cells were lysed in Catrimox (Iowa Biotechnology Corp.), a surfactant that selectively precipitates nucleic acids (Dahle and Macfarlane 1993). Total RNA was then purified from the Catrimox precipitate with Tri-Reagent (Molecular Research Center, Inc.) (Chomczynski and Sacchi 1987). The whole SSU rRNA of *A. proteus* strain Ge was amplified in three overlapping fragments by RT-PCR using universal primers.

The sequences of *A. leningradensis*, *C. nobile*, *C. carolinensis*, and a partial sequence of another *A. proteus*, the Warsaw strain (data not shown), were obtained by amplifying DNA extractions of these cells with universal as well as *A. proteus*-specific primers. The sequence of *A. proteus* strain Ge was also obtained from DNA amplification and found to be identical to the cDNA sequence. DNA extractions, amplifications, cloning, and sequencing were performed as described previously (Pawlowski et al. 1999). The new sequences reported in this paper have been deposited in the EMBL/GenBank database (accession numbers AJ314604, AJ314605, AJ314607, and AJ314606).

### Sequence Analysis

Four sequences obtained in this study and seven sequences of other amoebae were added to the secondary structure-based alignment of the SSU rRNA (Van de Peer et al. 2000). The sequences were aligned manually using SEAVIEW software (Galtier and Gouy 1996). The 19 unambiguously aligned regions selected for phylogenetic analyses are composed of 1,110 sites (excluding gaps), which include 774 variable characters, among which 713 are parsimony informative. The sequences were analyzed using the following methods: the Neighbor-Joining (NJ) method (Saitou and Nei 1987) using K2P, K3P, F84, HKY85, Tamura-Nei, and general time reversible model (GTR) substitution models; the MP method; and the ML method using HKY85 and

**Table 1**  
**List of SSU rDNA Sequences of Gymnamoebae and Pelobiont Amoeboflagellates (\*)**

Species	Length (bp)	GC Content (%)	Accession Number	Reference
<i>Acanthamoeba castellanii</i> . . . . .	2,226	51.8 <sup>a</sup> (44.8 <sup>b</sup> )	M13435	Gunderson and Sogin 1986
<i>Amoeba leningradensis</i> . . . . .	1,902	51.6 (47.8)	AJ314605	This work
<i>Amoeba proteus</i> . . . . .	1,749	53.8 (49.3)	AJ314604	This work
<i>Balamuthia mandrillaris</i> . . . . .	1,942	47.3 (44.1)	AF019071	Stothard et al. 1998
<i>Chaos carolinense</i> . . . . .	1,935	50.0 (46.3)	AJ314607	This work
<i>Chaos nobile</i> . . . . .	2,043	49.6 (46.4)	AJ314606	This work
<i>Echinamoeba exudans</i> . . . . .	1,754	48.1 (46.3)	AF293895	Amaral Zettler et al. 2000
<i>Endolimax nana</i> . . . . .	2,516	47.4 (46.1)	AF149916	Silberman et al. 1999
<i>Entamoeba histolytica</i> . . . . .	1,874	38.2 (41.7)	X61116	Que and Reed 1991
<i>Filamoeba nolandi</i> . . . . .	1,773	50.5 (48.6)	AF293896	Amaral Zettler et al. 2000
<i>Gephyramoeba</i> sp. . . . .	1,793	48.0 (45.8)	AF293897	Amaral Zettler et al. 2000
<i>Hartmannella vermiformis</i> . . . . .	1,765	49.5 (47.0)	M95168	Weekers et al. 1994
<i>Leptomyxa reticulata</i> . . . . .	1,767	41.3 (43.9)	AF293898	Amaral Zettler et al. 2000
<i>Phreatamoeba balamuthi</i> * . . . . .	2,659	47.2 (47.9)	L23799	Hinkle et al. 1994
<i>Mastigamoeba invertens</i> * . . . . .	1,703	45.7 (45.4)	AF153206	Stiller and Hall 1999
<i>Paraflabellula hoguae</i> . . . . .	1,800	40.9 (42.9)	AF293899	Amaral Zettler et al. 2000
<i>Paraflabellula reniformis</i> . . . . .	1,800	40.6 (42.8)	AF293900	Amaral Zettler et al. 2000
<i>Rhizamoeba</i> sp. . . . .	1,800	40.9 (43.0)	AF293901	Amaral Zettler et al. 2000
<i>Saccamoeba limax</i> . . . . .	1,831	37.5 (43.0)	AF293902	Amaral Zettler et al. 2000
<i>Vannella anglica</i> . . . . .	1,890	39.6 (41.6)	AF099101	Sims et al. 1999

<sup>a</sup> GC content in full sequence.

<sup>b</sup> GC content in sites selected for phylogenetic analyses.

GTR models, all as implemented in PAUP 4.0 (Swoford 1993). The NJ and ML analyses were performed with or without gamma distribution (G) with estimated parameter  $\alpha = 0.45$  and six rate categories. The proportion of invariable sites (I) was estimated at 0.04. In the HKY85 model, the transition-transversion ratio was estimated at 1.3. In GTR models, the estimated substitution probabilities were 1.02 (AC), 2.17 (AG), 1.25 (AT), 0.84 (CG), 3.85 (CT), 1 (GT). All model parameters were estimated via ML. Additionally, the ML tree was constructed using fastDNAmI program (Olsen et al. 1994) as implemented in PHYLO\_WIN (Galtier and Gouy 1996). The reliability of internal branches in NJ, MP, and ML trees was assessed, respectively, by 1,000, 1,000, and 100 bootstrap replicates (Felsenstein 1988). The relative rate test was carried out using RRTree program (Robinson et al. 1998).

## Results

### Sequence Data

The sequences of *Amoeba* and *Chaos* are characterized by unusual length and high-GC content compared with the SSU rDNA of other known amoeboid protists (table 1). The lengths of the four sequences obtained in this study range from 1,749 to 2,043 nucleotides (nt), without terminal regions of about 30 nt that have not been sequenced for all species. The *A. proteus* SSU rRNA is the shortest, for it lacks two long insertions (100–200 nt) present in the expansion segment E23 and in the region of helix 43 of *A. leningradensis*, *C. nobile*, and *C. carolinense*. Although the SSU rRNAs of these three species are much longer than those of many other amoebae, they are not as long as the SSU rRNAs of *P. balamuthi* and *E. nana*, whose sizes exceed 2,500 nt. The GC content in the four amoebae sequences ranges from 49.6% to 53.8%, which is above the typical values found for other amoebae (table 1). The mean GC

content in amoebae is 44.5% with the lowest value of 36.9% found in *V. anglica* and values above 50% found only in *Filamoeba nolandi* and *A. castellanii*. The variations of GC content, however, are much smaller in the set of sites selected for phylogenetic analyses.

The alignment of SSU rRNAs of amoebae and other eukaryotes was examined site by site in search for amoebae-specific sites. *Amoeba* and *Chaos* species were found to share patterns of specific nucleotides with *Saccamoeba*, *Paraflabellula*, *Leptomyxa*, *Rhizamoeba*, and *Hartmannella* in helices 29' and 49'. All these species, except *H. vermiformis*, also possess specific single substitutions in helices 24 and 38, compensated in respective helices 24' and 38'. The T → A substitution observed in helix 24/24' is also present in *F. nolandi*, *P. balamuthi*, *E. histolytica*, and *E. nana*. Two substitutions specific for all gymnamoebae have been detected in E-23 (A → G) and in helix 15 (T → A). The first one was found outside gymnamoebae only in *Naegleria* and *Physarum*. The second is present also in Mycetozoa (*Dictyostelium*, *Physarum*), Foraminifera, Euglenozoa, and Diplomonads. Interestingly, the specific substitution in helix 15 of amoebae reinforce the stem CUA:GAA by replacing mispaired A:A by pair U:A.

### Evolutionary Rates

The relative rate test was used to examine the evolutionary rates within gymnamoebae, and between them and other eukaryotes. The test shows that most gymnamoebae and the amoeboflagellate *M. invertens* evolve at rates similar to those of species which branch in the crown of the SSU eukaryotic tree (table 2). Significantly higher rates (at 1% level) are observed only in Entamoebidae (*Entamoeba* + *Endolimax*), as well as in Heterolobosea (*Naegleria* + *Vahlkampfia*) and Mycetozoa (*Dictyostelium* + *Physarum*). Among other gymnamoebae and amoeboflagellates, the most rapidly evolving are

**Table 2**  
**Relative Rates Test**

Lineage 1	Lineage 2	P value
<i>Acanthamoeba</i> + <i>Balamuthia</i> . . . . .	Crown species <sup>a</sup>	0.849
<i>Hartmannella</i> + <i>Echinamoeba</i> . . . . .	Crown species	0.679
<i>Mastigamoeba invertens</i> . . . . .	Crown species	0.947
<i>Amoeba</i> + <i>Chaos</i> . . . . .	Crown species	0.337
<i>Leptomyxa</i> + <i>Paraflabellula</i> + <i>Rhizamoeba</i> . . . . .	Crown species	0.964
<i>Saccamoeba limax</i> . . . . .	Crown species	0.025*
<i>Vannella anglica</i> . . . . .	Crown species	0.471
<i>Gephyramoeba</i> + <i>Filamoeba</i> . . . . .	Crown species	0.013*
<i>Phreatamoeba balamuthi</i> . . . . .	Crown species	0.019*
<i>Entamoeba</i> + <i>Endolimax</i> . . . . .	Crown species	0.00000604**
<i>Naegleria</i> + <i>Vahlkampfia</i> . . . . .	Crown species	0.00000132**
<i>Dictyostelium</i> + <i>Physarum</i> . . . . .	Crown species	0.0000455**

<sup>a</sup> Crown species: 22 species of fungi, animals, plantae, alveolates etc. branching in the upper part of the SSU tree.

\* Significant at 5% level.

\*\* Significant at 1% level.

*P. balamuthi*, *F. nolandi*, *Gephyramoeba* sp., and *Saccamoeba limax*. Their evolutionary rates are significantly different from those of crown species at the 5% level (table 2). Comparison of rates within gymnamoebae shows significant differences (at the 1% level) only in the case of Entamoebidae and *S. limax*.

#### Phylogenetic Analysis

The 18 species of gymnamoebae were compared with 37 species representing all major taxonomic groups of eukaryotes. Figure 1 presents the phylogenetic position of gymnamoebae inferred using the ML method, without allowing for invariant sites and intersite rate variations. All gymnamoebae branch in the middle part of the tree, just below the eukaryotic crown. The gymnamoebae appear in the same phylogenetic position in most other analyses; however, their branching order changes depending on the phylogenetic method and the evolutionary model (table 3). Three groups of gymnamoebae appear with acceptable statistical support in all types of analyses.

The most important is a group here called Gymnamoebia *sensu stricto* (fig. 1), which includes 11 species of the genera *Amoeba*, *Chaos*, *Saccamoeba*, *Paraflabellula*, *Rhizamoeba*, *Leptomyxa*, *Hartmannella*, and *Echinamoeba*. This group appears in all analyses including NJ, MP, and ML, but is well supported only in NJ and MP analyses, respectively, by 89% and 81%

bootstrap values. The relationships within this clade are stable, with good bootstrap support for the groupings: *Amoeba* + *Chaos* (100%), *Amoeba* + *Chaos* + *Saccamoeba* (75%–97%), and *Paraflabellula* + *Rhizamoeba* + *Leptomyxa* (97%–100%). There is no good bootstrap support, however, for the relations within the clade *Amoeba* + *Chaos*, suggesting that the intergeneric relationships are questionable.

Two other well-supported groups of gymnamoebae are *Acanthamoeba* + *Balamuthia* and *Entamoeba* + *Endolimax*. The last one also includes the pelobiont *P. balamuthi*. Both clades appear in all types of analyses supported, respectively, by 97%–100% and 71%–87% bootstrap values (fig. 1). The positions of both clades change depending on the method of analysis (table 3). The clade *Acanthamoeba* + *Balamuthia* branches with Gymnamoebia *sensu stricto* in MP and ML (GTR or F84) analyses, but clusters with Plantae in NJ trees without gamma correction. The grouping of *Entamoeba* + *Endolimax* + *Phreatamoeba* with Gymnamoebia *sensu stricto* (fig. 1) appears only in ML (F84) trees. In other analyses, the clade *Entamoeba* + *Endolimax* + *Phreatamoeba* branches separately.

Three gymnamoebae species *V. anglica*, *Gephyramoeba* sp., and *F. nolandi*, which appear as independent lineages in the ML tree (fig. 1), change their position in some other analyses. *Gephyramoeba* sp. and *F. nolandi* branch together in MP and some ML and NJ trees (table

**Table 3**  
**Phylogenetic Grouping According to Different Methods of Analysis and Evolutionary Models**

	NJ K2P	MP	ML F84	ML GTR	NJ K2P G + I	NJ GTR G + I	ML GTR G + I
Gymnamoebia <i>sensu stricto</i> . . . . .	+	+	+	+	+	+	+
Entamoebidae + <i>Phreatamoeba</i> . . . . .	+	+	+	+	+	+	+
<i>Gephyramoeba</i> + <i>Filamoeba</i> . . . . .	+	+	–	+	+	–	–
Gymnamoebia <i>sensu stricto</i> + Acanthamoebidae . . . . .	–	+	+	+	+	–	–
All gymnamoebae . . . . .	–	–	–	–	+	+	–
All gymnamoebae + Mycetozoa . . . . .	–	–	–	–	–	+	–

NOTE.—NJ, Neighbor Joining; MP, maximum parsimony; ML, maximum likelihood; K2P, Kimura 2 parameters model; GTR, general time reversible model; G, gamma distribution; I, invariable sites proportion.

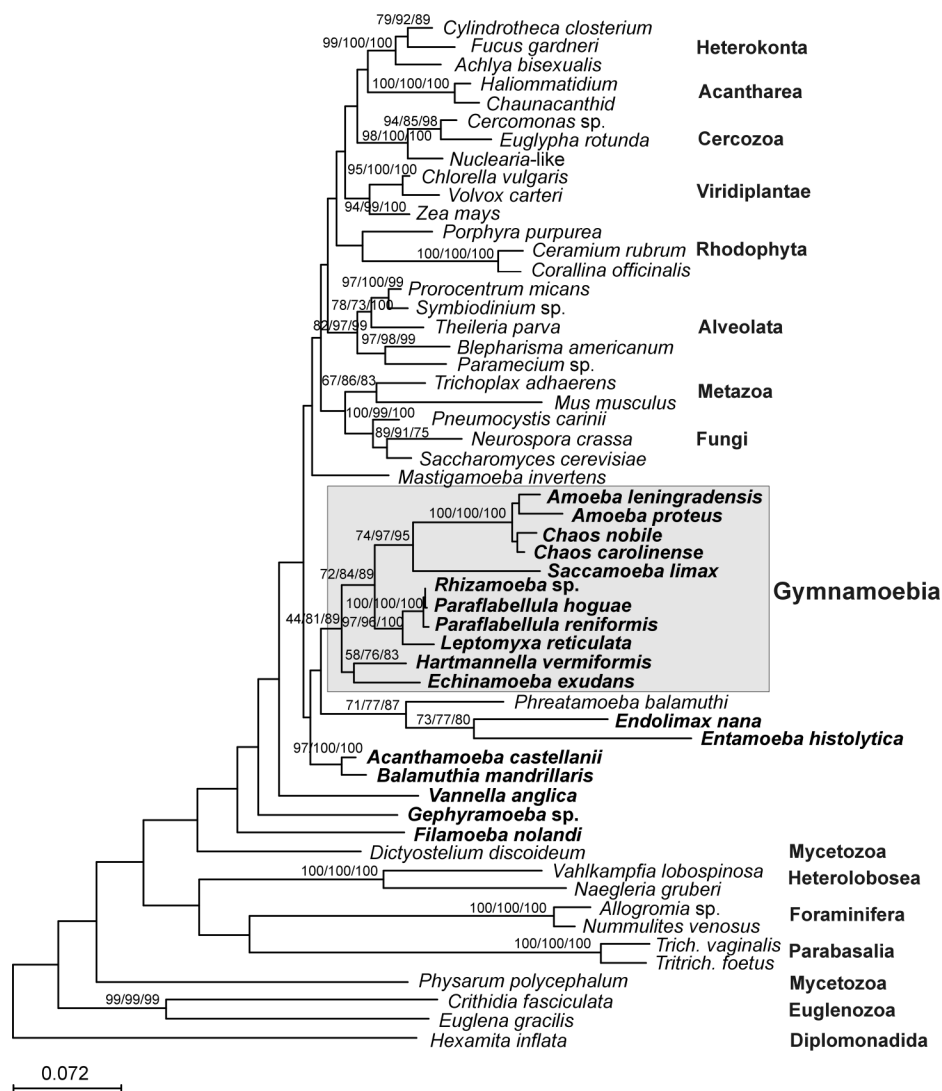


FIG. 1.—Phylogenetic position of gymnamoebae based on SSU rDNA sequences inferred using maximum likelihood (fastDNAMl) method, without allowing for invariant sites and intersite rate variation. The tree is arbitrarily rooted on diplomonadidae. The names of 18 gymnamoebae species are highlighted in bold. The shaded area illustrates the clade of *Gymnamoebia sensu stricto*. The numbers above internal branches represent the values of bootstrap support (higher than 50%) in ML (F84), MP, and NJ (K2P) analyses. The *Nuclearia*-like sequence corresponds to accession number AF289081.

3). Both species branch with Heterolobosea and Mycetozoa (or both) in some analyses (data not shown). Some affinities are also observed between *V. anglica* and the clade *Entamoeba* + *Endolimax* + *Phreatamoeba*. None of these relationships, however, is supported by a bootstrap value higher than 50%.

In the NJ tree with gamma and invariable sites corrections, all gymnamoebae group together (fig. 2). This grouping is not statistically supported, yet it remains stable whatever the model of nucleotide substitution. Depending on the model, the topologies differ in the position of the two mycetozoan species (*Dictyostelium discoideum* and *Physarum polycephalum*). Both species branch within the gymnamoebae when GTR or Tamura-Nei models are used (fig. 2), whereas they appear in the lower part of the tree, close to the Euglenozoa and the Heterolobosea, when K2P or other models are used.

## Discussion

### Phylogenetic Position of Amoebidae

Our data clearly show that the large naked amoebae of the genera *Amoeba* and *Chaos* (family Amoebidae) are closely related to other gymnamoebae belonging to families Hartmannellidae, Flabellulidae, and Leptomyxidae. The representatives of the last three families were already shown to form a monophyletic group (Amaral Zettler et al. 2000). By adding four sequences of Amoebidae, we consolidate this group and confirm that a close relationship exists between the two orders of Gymnamoebia: Euamoebida (Amoebidae, Hartmannellidae) and Leptomyxida (Flabellulidae, Leptomyxidae). The relationships within these orders are in agreement with morphology-based systematics (Page 1987), except for the position of *H. vermiformis*. Traditionally, this species

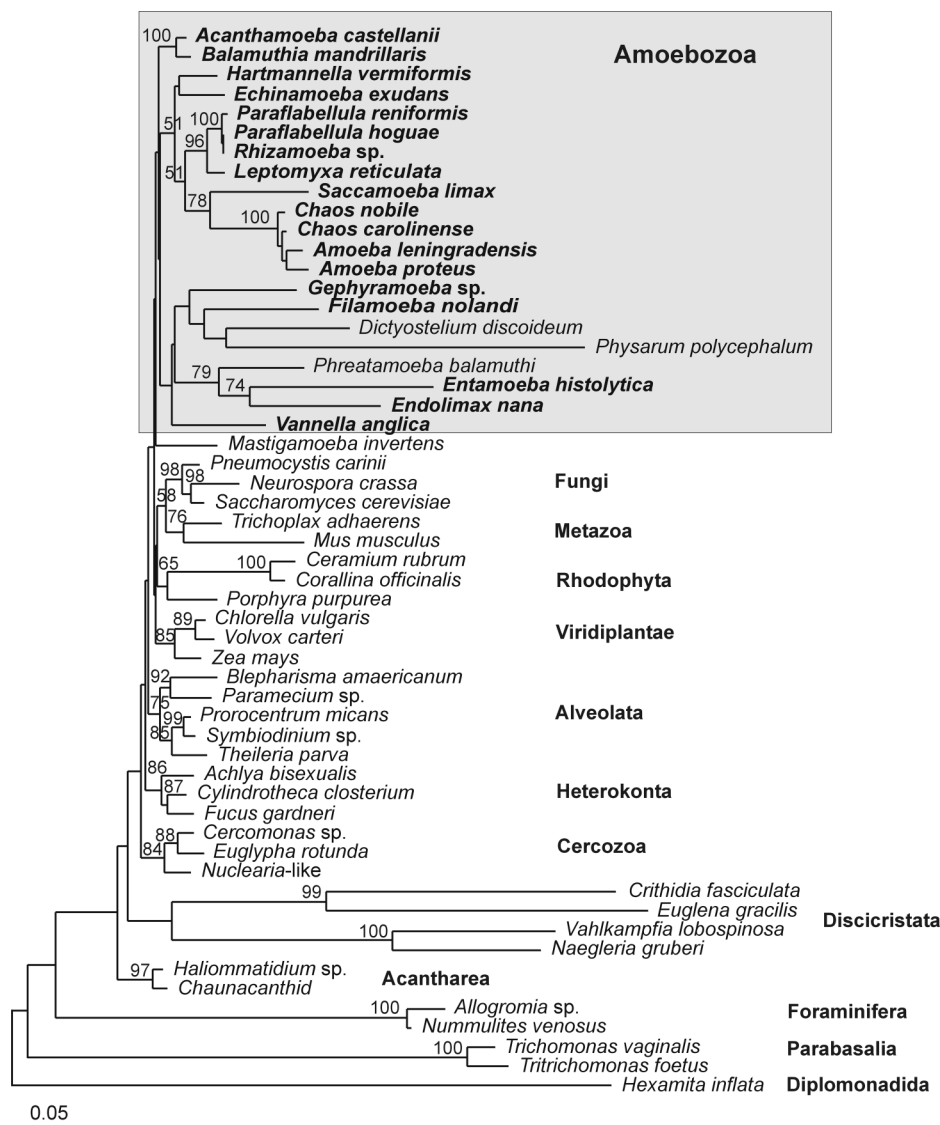


FIG. 2.—Phylogenetic position of gymnamoebae based on SSU rDNA sequences inferred with the Neighbor-Joining method using GTR model and correction for among-sites heterogeneity and invariable sites (NJ + GTR + G + I). The tree is arbitrarily rooted on diplomonadidae. The shaded area illustrates the group of Amoebozoa, including all gymnamoebae and two slime molds, *D. discoideum* and *P. polycephalum*. The numbers above some internal branches represent bootstrap values higher than 50%. The *Nuclearia*-like sequence corresponds to accession number AF289081.

is included in the family Hartmannellidae (Page 1974, 1987). However, in our trees, *S. limax* (another hartmannellid) groups with the Amoebidae, whereas *H. vermiformis* groups with *Echinamoeba exudans*; this latter species belongs to the family Echinamoebidae, which is listed as incertae sedis among Gymnamoebia (Page 1987). No basic morphological characters are shared by *Hartmannella* and *Echinamoeba*. It is interesting to note, however, that although *Echinamoeba* resembles morphologically *Acanthamoeba*, it differs from this genus by lacking microtubule organizing centers (MTOC) (Page 1988). As the same lack is observed also in other gymnamoebae belonging to orders Euamoebida and Leptomyxida (suborder Rhizoflabellina), we speculate that MTOC absence is a characteristic feature for all taxa that cluster with Amoebidae.

The position of Amoebidae and related taxa in the middle part of the eukaryote tree (fig. 1) contrasts with some previous studies that showed gymnamoebae (*A. castellanii* and *H. vermiformis*) branching in the crown of the SSU tree, as a sister group to Plantae (Schlegel 1991) or Metazoa and Fungi (Bhattacharya, Helmchen, and Melkonian 1995; Sogin and Silberman 1998; Sims, Rogerson, and Aitken 1999). Our data show that by adding new taxa, the position of amoebae in the SSU tree changes. It could be argued that this change results from attraction by the most rapidly evolving amoeboid lineages. Several studies demonstrate that the topology of SSU trees, in particular the position of early diverging lineages, is biased by the among-lineages rate variations (Philippe, Germot, and Moreira 2000; Van de Peer et al. 2000). It has also been argued that the position of some

amoebae is biased by their rapid rates of evolution (Stiller and Hall 1999). However, when the rapidly evolving species (*E. histolytica*, *E. nana*, *P. balamuthi*, *Gephyramoeba* sp., and *Filamoeba* sp.) are omitted from the analysis, the other amoeba species remain indeed below the crown. Moreover, the real place for the root of the SSU tree is not known, and therefore one cannot say which taxa diverged early and which late; other possible roots are discussed in Cavalier-Smith (2000a).

#### The System of Gymnamoebia *sensu stricto*

Traditionally, all naked amoebae possessing lobose pseudopodia are included in the subclass Gymnamoebia that comprises four orders and three incertae sedis families (Page 1987). In our analysis, six independent lineages of gymnamoebae appear. The majority of the species (11) branch in a clade grouping mostly species from the orders Euamoebida and Leptomyxida. As these species represent the most typical gymnamoebae, we propose considering this clade as a representative for the subclass Gymnamoebia (fig. 1). Among other amoeba lineages, two are composed of more than one species (*A. castellanii* + *B. mandrillaris* and *E. histolytica* + *E. nana* + *P. balamuthi*), whereas three others are single species lineages (*Gephyramoeba* sp., *F. nolandii*, and *V. anglica*).

The clade *Acanthamoeba* + *Balamuthia* corresponds most probably to the order Acanthopodida, which traditionally includes a single family Acanthamoebidae (Sawyer and Griffin 1975; Page 1987). The close relationship between both genera has been demonstrated using rRNA data (Stothard et al. 1998; Amaral Zettler et al. 2000).

From a morphological point of view their grouping is quite unexpected. *Balamuthia mandrillaris* was initially described as a leptomyxid (Visvesvara et al. 1990). However, more detailed study of *B. mandrillaris* showed that the species differs fundamentally from other leptomyxids (*Gephyramoeba* and *Leptomyxa*) in the morphological, physiological, and antigenic characteristics (Visvesvara, Schuster, and Martinez 1993). At the same time, it has been observed that *B. mandrillaris* possesses a MTOC similar to those seen in *A. castellanii* (Visvesvara, Schuster, and Martinez 1993). The presence of this basic cellular feature in both species reconciles somehow the molecular and morphological data. It will be interesting to compare our data with the sequence of *Stereomyxa*, which also possesses similar MTOC's (Von Benwitz and Grell 1971a, 1971b).

Another well-supported clade of amoeboid protists is composed of *E. histolytica* + *E. nana* + *P. balamuthi*. This clade was shown previously in rRNA trees (Cavalier-Smith and Chao 1997; Cavalier-Smith 1998b, 2000b; Silberman et al. 1999). The clade exists only if the sequence of *E. nana* is included. If *E. nana* is omitted, *E. histolytica* and *P. balamuthi* branch separately. This explains why the relationship between *P. balamuthi* and Entamoebidae has not been suggested by Hinkle et al. (1994). In fact, *P. balamuthi* is a pelobiont, i.e., a free-living amoeboflagellate that lacks mitochondria and

Golgi bodies. It shares with Entamoebidae general features, such as an anaerobic life mode and a remarkably simplified intracellular organization (Silberman et al. 1999), yet no shared derived morphological features have been identified so far.

Among the other three lineages composed of single species (*Gephyramoeba* sp., *F. nolandii*, and *V. anglica*), the first two are quite unusual gymnamoebae. Based on the morphological characteristics, they are classified, respectively, in the suborder Leptoramosina (order Leptomyxida) and in incertae sedis family (Page 1987). In our analyses, both species tend to group together (table 2), but their grouping is weakly supported. The case of *V. anglica* is much more puzzling. Similar to a previous study (Sims, Rogerson, and Aitken 1999), our analysis of the molecular data shows this species as an independent lineage branching in the middle part of the rRNA tree. Yet, the vannellids are quite typical gymnamoebae (Page 1987) and no obvious morphological or ultrastructural feature could distinguish *Vannella* from the other Gymnamoebia. As the unexpected position of *Vannella* in the trees cannot be attributed to a significantly fast rate of evolution (table 2), other explanations will have to be found.

#### Monophyly of Gymnamoebae?

The presence of several independent lineages of gymnamoebae in the SSU tree is in agreement with the hypothesis of a polyphyletic origin of the amoebae. This hypothesis is generally accepted; however, Heterolobosea alone shows an independent origin, based on solid morphological and molecular evidences (Page and Blanton 1985; Page 1987; Clark and Cross 1988; Roger et al. 1996). The polyphyly of other amoebae, based solely on SSU rDNA data, remains disputable. Possible close relationships of gymnamoebae have been discussed already based on some previous analyses of SSU (Cavalier-Smith and Chao 1996; Cavalier-Smith 1998a; Silberman et al. 1999). The present study shows that these relationships appear stronger as the number of examined species increased. In all our analyses, the gymnamoebae branch close to each other; however, they group together only in NJ analysis using gamma correction for among-site rate variations (fig. 2). Although the statistical support for this grouping is very weak, it is possible that by adding more amoebae SSU sequences their relationships will be better resolved.

Most striking is the grouping of Lobosea together with Mycetozoa (represented by *Dictyostelium* and *Physarum*) in the phylum Amoebozoa (Cavalier-Smith 1998a). The same situation is evident in our SSU-tree (fig. 2), in the actin-tree of Bhattacharya and Weber (1997) and in a recently published eukaryote tree based on combined protein data (Baldauf et al. 2000). Interestingly, the two mycetozoan species are closely related to *Gephyramoeba*; the latter was initially seen (Goodey 1914) as a possible bridge between Amoeba and Mycetozoa. Indeed, the plasmodial stages of *Gephyramoeba* clearly evoke those of Mycetozoa (see discussion in Visvesvara, Schuster and Martinez 1993).

Both SSU (this work) and protein trees (Baldauf et al. 2000) also agree in grouping together the Euglenozoa and Heterolobosea in a unit corresponding to the infrakingdom Discicristata (Cavalier-Smith 1993). The major handicap of the protein tree, however, is the very limited number of amoebae protein sequences. In fact, all analyses are based on the sequences of the single species *A. castellanii*. It is therefore difficult to make any conclusion before the number of available protein data on amoebae increases.

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