Phylogeny of Lobose Amoebae Based on Actin and Small-Subunit Ribosomal RNA Genes

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Lobose amoebae are abundant free-living protists and important pathogenic agents, yet their evolutionary history and position in the universal tree of life are poorly known. Molecular data for lobose amoebae are limited to a few species, and all phylogenetic studies published so far lacked representatives of many of their taxonomic groups. Here we analyze actin and small-subunit ribosomal RNA (SSU rRNA) gene sequences of a broad taxon sampling of naked, lobose amoebae. Our results support the existence of a monophyletic Amoebozoa clade, which comprises all lobose amoebae examined so far, the amitochondriate pelobionts and entamoebids, and the slime molds. Both actin and SSU rRNA phylogenies distinguish two well-defined clades of amoebae, the “Gymnamoebia sensu stricto” and the Archamoebae (pelobionts + entamoebids), and one weakly supported and ill-resolved group comprising some naked, lobose amoebae and the Mycetozoa.

Introduction

Despite their obvious ecological and medical importance (Anderson 1997; Szenasi et al. 1998; Butler and Rogerson 2000; Finlay et al. 2000), the origin and evolutionary history of lobose amoebae remain enigmatic. For convenience, earlier protist classifications placed all amoebae possessing lobose pseudopodia in the class Lobosea, belonging to the superclass or phylum Rhizopoda (Levine et al. 1980; Bovee 1985). However, based on ultrastructure and life cycle studies, the amoebae and amoebalflagellates with discoidal mitochondrial cristae and without typical dictyosomes were excluded from the Lobosea and placed in the class Heterolobosea (Page and Blanton 1985; Page 1987). The distinction of both classes was confirmed later by analysis of small-subunit ribosomal RNA (SSU rRNA) sequences (Clark and Cross 1988; Hinkley and Sogin 1993). Furthermore, the position of pelobionts—the free-living amitochondriate amoebae—is debated. For example, this group was considered either as a separate phylum (Margulis 1974; Margulis et al. 1990), as a separate class within the Rhizopoda (Page 1987), as an order within the Lobosea (Bovee 1985), or placed in the phylum Archamoebae, among early diverging amitochondriate eukaryotes (Cavalier-Smith 1987, 1993; Corliss 1994). Another group of amitochondriate amoebae, the entamoebids, viewed by some as the model for primitive eukaryotes (Bakker-Grunwald and Wüstmann 1993) was transferred from Lobosea to the Archamoebae (Cavalier-Smith 1987) or later placed in a separate phylum, the Entamoebia (Cavalier-Smith 1993). Early SSU rRNA-based phylogenies suggested independent origins for pelobionts, entamoebids, and other lobose amoebae (Sogin 1991; Hinkley et al. 1994; Sims, Rogerson, and Atkin 1999), supporting their separation into different classes or phyla. Based on ultrastructural data and following ribosomal RNA phylogenies, recent protist classifications widely accept the polyphyly of lobose amoebae, splitting them into at least three taxonomic groups (Hausmann and Hülsmann 1996; Lee, Leedale, and Bradbury 2000).

A recent opposite view proposes that all lobose amoebae, with the exception of Heterolobosea, are monophyletic (Cavalier-Smith 1998). This view is based on molecular evidence that the pelobionts and entamoebids have lost their mitochondria secondarily (Clark and Roger 1995) and that they group together with lobose amoebae in some revised ribosomal RNA phylogenies (discussed in Cavalier-Smith and Chao 1996, and Cavalier-Smith 2000, and demonstrated later by Bolivar et al. 2001, and Milyutina et al. 2001). The phylum Amoebozoa Lühe, 1913 was emended to group together the naked and testate lobose amoebae, the pelobionts, the entamoebids, and the Mycetozoa (Cavalier-Smith 1998). The latter group was included into Amoebozoa based on analysis of actin and actin-related proteins (Kelleher, Atkinson, and Pollard 1995; Bhattacharya and Weber 1997; Schafer and Schroer 1999). This was recently confirmed by the combined analysis of nuclear (Baldaufl et al. 2000) and mitochondrial (Forget et al. 2002) protein sequences of the lobosean Acanthamoeba and the slime molds Dictyostelium and Physarum. A common origin for Entamoeba, Mastigamoeba, and Dictyostelium was also inferred from combined analysis of EF-1a and EF-2 sequences (Arisue et al. 2002) and is strongly supported by the analysis of 123 genes obtained from EST libraries (Bapteste et al. 2002). However, none of these studies includes representatives of typical free-living, lobose amoebae (order Euamoebida).

To test further the relationships among Amoebozoa, we obtained 10 new actin sequences and eight new SSU rRNA sequences of naked, lobose amoebae. Phylogenetic analyses using several evolutionary models support the hypothesis that all lobose amoebae are closely related and reveal the existence of two well-defined clades within Amoebozoa.
Materials and Methods
Cultures and Sequencing

Most amoebae strains were obtained from the Culture Collection of Algae and Protozoa (Windermere, United Kingdom). The strain numbers, food, and culture mediums for each species are available in table 1 of the Supplementary Material online (www.molbiolevol.org). The DNA was extracted either with guanidinium buffer (Chomczynski and Sacchi 1987) or with NaOH (Wang, Qi, and Cutler 1993). Total RNA was extracted as described previously (Bolivar et al. 2001). The actin gene was amplified by RT-PCR with primers ActN2 (5'-AACTGGGAYGAYATGGA3') and 1354R (5'-GGAC-CAGATTCCATCATAYTC-3'); the N-terminal part of the molecule was obtained by 5'-RACE. The SSU rRNA gene was amplified by PCR or RT-PCR in two overlapping fragments, using primer pairs sA (5'-CYGGTYGATC-CTGCAGT-3') – s14r (5'-AAGTTTCAGCCTGGCA-CCA-3') and s12.2 (5'-GATYAGATACCCTGTAATC-3') – sB (5'-GTAGCCTGTCAAGTCCACTAC-3'). Amplification, purification, cloning, and sequencing were performed as described previously (Pawlowski et al. 1999). The new sequences reported in this paper were deposited in the GenBank/EMBL database under accession numbers AY294143 to AY294160 (see table 2 in the Supplementary Material online for the species names, taxonomic position, and accession numbers of all actin and SSU rRNA sequences used in our analyses).

Actin Analysis

The actin protein sequences were manually aligned using the Genetic Data Environment (GDE) software (Larsen et al. 1993). Only complete sequences were selected among already available data, and a total of 364 amino acid positions were used in the phylogenetic analyses. An evolutionary tree of actin was inferred from the amino acid sequences with the maximum-likelihood (ML) method (Felsenstein 1981) using the JTT substitution matrix (Jones, Taylor, and Thornton 1992) and taking into account a proportion of invariable sites and a gamma-shaped distribution of the rates of substitution among variable sites with eight rate categories. All necessary parameters were estimated from the data using the GDE software, as above, following secondary structure models (Neefs et al. 1993; Wuyts et al. 2000). Already available sequences were selected so that most major taxonomic groups of eukaryotes were represented, and the sampling more or less matched the one for actin; highly diverging lineages such as Foraminifera and Microsporidia were omitted. A total of 1,150 unambiguously aligned positions were used in the phylogenetic analyses. Evolutionary trees were inferred utilizing the ML method, the neighbor-joining (NJ) method (Saitou and Nei 1987), and the maximum-parsimony (MP) method, using PAUP* (Swofford 1998). The reliability of internal branches was assessed with 100, 1,000, and 500 bootstrap replicates for ML, NJ, and MP analyses, respectively. ML analyses were performed with the GTR model of substitution (Lanave et al. 1984; Rodriguez et al. 1990), taking into account a proportion of invariable sites and a gamma-shaped distribution of the rates of substitution among variable sites, with eight rate categories. All necessary parameters were estimated from the data using ModelTest (Posada and Crandall 1998). Starting trees of ML searches were obtained via NJ and swapped with the tree-bisection-reconnection algorithm. NJ analyses were performed with ML-corrected distances using the same parameters. The most parsimonious trees for each MP bootstrap replicate were determined using a heuristic search procedure with 10 random-addition-sequence replicates and tree-bisection-reconnection branch-swapping. The transversions cost was set to twice the transitions cost. ML and NJ analyses using simpler models (see table 3 in Supplementary Material online) were performed with Phylo_win (Galtier, Gouy, and Gautier 1996).

Results
Sequence Data

Ten new actin sequences of Amoeba proteus, Chaos carolinense, Dermamoeba algensis, Glaeseria mira, Hartmannella cantabriiensis, Mayorella sp., Pelomyxa palustris, Platymoeba placidia, Thecamoeba similis, and Vannella ebro were obtained. No introns or peculiar structural patterns were found.

New SSU rRNA sequences of eight species of gymnamoebae (D. algensis, G. mira, H. cantabriiensis, Mayorella sp., P. placidia, Platymoeba stenopodia, T. similis, and Vexillifera minutissima) were obtained. The size of these sequences varies from 1,893 base pairs in P. placidia to 2,409 base pairs in T. similis. Size variations occur mainly in the 5' part of variable region V4, but expansions were observed in the variable region V2 for Mayorella sp. and in the variable regions V4, V7, and V8 for T. similis. The sequences also vary importantly in GC probabilities were estimated with the RELL method (Kishino, Miyata, and Hasegawa 1990; Hasegawa and Kishino 1994). In addition, a quartet-puzzling tree was obtained with Tree-Puzzle (using the JTT + G + I model with the parameters estimated above).

SSU rRNA Analysis

The SSU rRNA sequences were manually aligned using the GDE software, as above, following secondary structure models (Neefs et al. 1993; Wuyts et al. 2000). Already available sequences were selected so that most major taxonomic groups of eukaryotes were represented, and the sampling more or less matched the one for actin; highly diverging lineages such as Foraminifera and Microsporidia were omitted. A total of 1,150 unambiguously aligned positions were used in the phylogenetic analyses. Evolutionary trees were inferred utilizing the ML method, the neighbor-joining (NJ) method (Saitou and Nei 1987), and the maximum-parsimony (MP) method, using PAUP* (Swofford 1998). The reliability of internal branches was assessed with 100, 1,000, and 500 bootstrap replicates for ML, NJ, and MP analyses, respectively. ML analyses were performed with the GTR model of substitution (Lanave et al. 1984; Rodriguez et al. 1990), taking into account a proportion of invariable sites and a gamma-shaped distribution of the rates of substitution among variable sites, with eight rate categories. All necessary parameters were estimated from the data using ModelTest (Posada and Crandall 1998). Starting trees of ML searches were obtained via NJ and swapped with the tree-bisection-reconnection algorithm. NJ analyses were performed with ML-corrected distances using the same parameters. The most parsimonious trees for each MP bootstrap replicate were determined using a heuristic search procedure with 10 random-addition-sequence replicates and tree-bisection-reconnection branch-swapping. The transversions cost was set to twice the transitions cost. ML and NJ analyses using simpler models (see table 3 in Supplementary Material online) were performed with Phylo_win (Galtier, Gouy, and Gautier 1996).
content, with a mean value of 42%, ranging from 33.5% in *V. minutissima* to 52.8% in *P. stenopodia*. However, these variations in the GC content occur mainly in the variable regions of the molecule and are smaller in the set of sites selected for phylogenetic analyses (42.5% to 47.9%, with a mean value of 45%).

**Phylogenetic Analyses**

Figure 1A shows the result of a ML analysis of 55 actin sequences of eukaryotes, including 13 lobose amoebae. The topology shown was obtained using the JTT substitution matrix, taking into account a proportion of invariable sites (10%) and a gamma-shaped distribution of the rates of substitution among variable sites, with eight rate categories (alpha = 0.55). Figure 1B shows the result of a ML analysis of 60 SSU rRNA sequences of eukaryotes, including 26 lobose amoebae. The topology shown was obtained using the GTR model of substitution, taking into account a proportion of invariable sites (7.92%) and a gamma-shaped distribution of the rates of substitution among variable sites, with eight rate categories (alpha = 0.42). Because the position of the root of the eukaryotic tree is still subject to debate (see e.g., Stechmann and Cavalieri-Smith 2002), the trees are presented in an unrooted format, with a basal trifurcation. The general topology of both trees is congruent with previous large-scale actin and SSU rRNA phylogenies of eukaryotes, and all well-recognized high-level taxa are recovered with good statistical support. In both trees, all lobose amoebae and Mycetozoa cluster together at the exclusion of any other eukaryote in a clade called Amoebozoa. Although this group is not supported by bootstrap analysis, in either actin or SSU rRNA trees, it is recovered by several methods of tree reconstruction and models of substitution (see table 3 in Supplementary Material online). Moreover, sites 289, 321, 385, 515, 777, 1010, and 1051 of the SSU rRNA gene show an Amoebozoa-specific character configuration that was not found in any other eukaryote (see table 4 in Supplementary Material online), and sequences of the members of Amoebozoa are characterized by an insertion of one nucleotide in the short loop between stems 30 and 28 (sites 1060 to 1064 [see table 4 in Supplementary Material online]).

Interestingly, both trees congruently show a division of the Amoebozoa in the same three groups. The first one contains most of the well-known typical gymnamoebae such as *A. proteus*, and corresponds to the “Gymnamoebia sensu stricto,” as defined by Bolivar et al. (2001). The second one comprises all amitochondriate amoebae—that
is, the pelobionts and the entamoebids—and corresponds to
the Archamoebae, as defined by Cavalier-Smith (1998). The
third group represents a very weakly supported and ill-
resolved clustering of various other lobose amoebae plus the
Mycetozoa. Table 3 in the Supplementary Material online
indicates the support for these groups according to different
methods of tree reconstruction and models of substitution.

Discussion

Monophyly of Amoebozoa

Our analyses based on actin and SSU rRNA sequences
show that lobose amoebae are all closely
related to each other and support the idea that they
constitute, together with pelobionts, entamoebids, and
Mycetozoa, the phylum Amoebozoa (fig. 1). Although the
existence of this phylum was already assessed, our
results—including the first well-sampled protein data on
lobose amoebae—significantly widen our knowledge
about which organisms actually belong to the group.
According to our data, the Amoebozoa clade includes all
amoebae belonging to the subclass Gymnamoebia (Page
1987) plus the pelobionts, the entamoebids, and the slime
molds. It could also include the testate lobose amoebae of
the subclass Testacealobosia (Page 1987); however, no
molecular data for this group exist at the moment.

Although the Amoebozoa clade is not supported by
bootstrap analysis in either actin or SSU rRNA trees (fig.
1; see table 3 in Supplementary Material online), it is
recovered in all ML analyses and also in SSU rRNA
distance trees if a gamma correction is used and the
divergent sequence of T. simulis is excluded. Furthermore,
eight diagnostic positions were found in the SSU rRNA
alignment (see table 4 in Supplementary Material online).
The lack of bootstrap support for the Amoebozoa in SSU
rRNA phylogenies is probably due to a very low number of
changes during the stem evolution of the group, coupled
with the fact that there are many different rates of
evolution among the different extant amoebozoan species.
The lack of bootstrap support for the Amoebozoa in the
actin phylogeny might be due to the apparently slow rate
of actin evolution in amoebae and animals. In view of our
analyses, previous SSU rRNA studies suggesting the
polyphyly of lobose amoebae and/or an independent origin
for pelobionts, entamoebids, and Mycetozoa (e.g., Sogin
1991; Hinkle et al. 1994; Cavalier-Smith 2000), were
probably biased by the limited number of available
amoeba sequences, coupled with the wide range of
divergence between them (Bolivar et al. 2001).

High-level Relationships Among Amoebozoa

Among the three major groups distinguished within
Amoebozoa in figure 1, two (the “Gymnamoebia sensu
stricto” and the Archamoebae) are well supported by
molecular, morphological, and ultrastructural data.

The “Gymnamoebia sensu stricto” comprise two
families of the order Euamoebida—Amoebidae (A. proteus +
C. carolinense) and Hartmannellidae (H. cantabrigiensis +
G. mira + S. linax)—as well as members of the order
Leptomyxida (Leptomyxa reticulata + Parafflabellula
hoguei). The closest relatives to these taxa are
Echinamoeba exundans and Hartmannella vermiformis (the
generic status of the latter species is uncertain, as it is not
closely related to other Hartmannellidae), but their relations
to other “Gymnamoebia sensu stricto” are not well
supported (fig. 1B). The close relationship between
Leptomyxida and the clade E. exundans + H. vermiformis
was already demonstrated by Amaral Zettler et al. (2000).
Here, we confirm their relationship to the Amoebidae, as
suggested in our previous study (Bolivar et al. 2001), and
show a highly supported relationship (bootstrap values of
96% to 100%) between Amoebidae and Hartmannellidae.

The Archamoebae comprise all amitochondriate
amoebae, including entamoebids, mastigamoebids, and
Pelomyxa. The relationship between entamoebids and the
pelobiont genus Mastigamoeba (but not Mastigamoeba
inverted [e.g., Edgcomb et al. 2002]) was already
suggested by SSU rRNA-based studies (Silberman et al.
1999; Edgcomb et al. 2002), and a strong support for the
relationship between Entamoeba histolytica and Mastig-
amoeba balamuthi was inferred from a combined analysis
of rDNA and protein data (Arisue et al. 2002; Bapteste et al.
2002). The classic pelobiont Pelomyxa was recently added
to this group (Milyutina et al. 2001). The actin sequence of
Pelomyxa presented in this study confirms that this genus
belongs to Archamoebae, although its relationship to other
pelobionts and entamoebids is not well resolved.

The third group is a very weakly supported and ill-
resolved clustering, composed of morphologically different
amoeboid lineages. In addition to Mycetozoa, it also
comprises amoebae belonging to the order Acanthopodida
and various families of the order Euamoebida (Vannelli-
dae, Thecamoebidae, Paramamoebidae, and Vexilliferidae).
The grouping of Acanthamoebida and Mycetozoa was
considered as evidence for a common origin of all amoebae
(Baldauf et al. 2000), but in view of our data, it represents
only part of the Amoeboza. The Vannellidae (P. placida,
V. ebro, and V. anglica) and Thecamoebidae (T. simulis)
share some common morphological features (Smirnov
2001), but the relationships between other families are
unclear. However, given the weak support for this cluster
(see table 3 in Supplementary Material online), it is
probable that with an increasing number of taxa, this group
will prove paraphyletic and disappear, replaced by a series
of independent lineages that might include the “Gymna-
amoebia sensu stricto” and/or the Archamoebae.

Our data are in opposition to the division of
Amoeboza into two groups: Lobosa and Conosa
(Cavalier-Smith 1998). The independent branching of
Mycetozoa and Archamoebae within loboseans in both
actin and SSU rRNA trees makes the Lobosa paraphyletic
and refutes the holophyly of Conosa (Archamoebae +
Mycetozoa) suggested by Bapteste et al. (2002). Their
analysis was apparently misleading because of an in-
sufficient taxonomic sampling and, more particularly,
the lack of protein data for the lobose amoebae, which,
gether with Mycetozoa, form the third group in our
analyses. Although the relationships within this group are
not well established, there is no indication of closeness
between Mycetozoa and Archamoebae in either actin or
SSU rRNA sequences.
Phylogenetic Position of Amoebozoa

The phylogenetic position of Amoebozoa is of crucial importance for inferring early events in eukaryotic evolution. Following a recent hypothesis (Stechmann and Cavalier-Smith 2002), the root of the eukaryotic tree lies between opisthokonts and bikonts, but the position of Amoebozoa in this work is still unclear. Our results are congruent with the idea that Amoebozoa are branching between opisthokonts and bikonts. In the actin tree (fig. 1A), the Amoebozoa are even included in the opisthokonts and appear as a sister-group to the Metazoa + Choano-flagellata clade. However, such a position is very suspicious, because several independent lines of evidence clearly support the monophyly of the opisthokonts at the exclusion of Amoebozoa (e.g., Baldauf and Palmer 1993). Thus, the topology shown in figure 1A might rather reflect slightly higher rates of actin evolution in fungi as compared with animals and Amoebozoa.

Given the available data, the possibility that the root of the eukaryotic tree lies within Amoebozoa, and that all other extant eukaryotes derive from an amoebozoan ancestor, cannot be excluded. A larger sampling of genes will be needed to test further both the holophyly of Amoebozoa and the position of the phylum in relation to opisthokonts and bikonts.

Supplementary Material

Table 1 in the online Supplementary Material contains strains data. Table 2 includes taxonomic position, species names, and GenBank accession numbers of the actin and SSU rRNA sequences used in this study. Table 3 shows phylogenetic groupings according to different methods of analysis and evolutionary models. Table 4 lists the selected sites in the SSU rRNA alignment defining the Amoebozoa and their position among other eukaryotes.

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Literature Cited


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