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 amoeboflagellates 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; Hinkle 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 of primitive eukaryotes (Bakker-Grunwald and Wöstmann 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; Hinkle et al. 1994; Sims, Rogerson, and Aitken 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 (Baldauf 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.

Key words: actin, Amoebozoa, Lobosea, molecular phylogeny, small-subunit ribosomal RNA.

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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′-GGACCAGATTCATCATAYTC-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′-CYGGTYGATCCTGCCAGT-3′) – s14r (5′-AAGTTTCAGCCTTGCGACCA-3′) and s12.2 (5′-GATYAGATACCGTCGTAGTC-3′) – sB (5′-TGATCCTTCTGCAGGTTCACCTAC-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 Tree-Puzzle version 5.0 (Strimmer and von Haeseler 1996), and the tree topology was constructed with the ProML program of the PHYLIP version 3.6a3 package (Felsenstein 2002), using the -R option with 10 input order jumbles and global rearrangements. The reliability of internal branches was assessed using the bootstrap method (Felsenstein 1985), with 100 replicates, based on a distance analysis using the program Fitch of PHYLIP. For each data resampling, JTT + G + I corrected distances were calculated by Tree-Puzzle with the utility PuzzleBoot, using the parameters estimated above. ML analyses were also carried out with the ProtML program of the Molphy version 2.3 package (Adachi and Hasegawa 1996). Trees were inferred using the local rearrangement search option, starting from a distance topology obtained with the NJdist program included in the same package. Bootstrap 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).

Results

Sequence Data

Ten new actin sequences of *Amoeba proteus*, *Chaos carolinense*, *Dermamoeba algensis*, *Glaeseria mira*, *Hartmannella cantabrigiensis*, *Mayorella* sp., *Pelomyxa palustris*, *Platyamoeba placida*, *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. cantabrigiensis*, *Mayorella* sp., *P. placida*, *Platyamoeba stenopodia*, *T. similis*, and *Vexillifera minutissima*) were obtained. The size of these sequences varies from 1,893 base pairs in *P. placida* 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

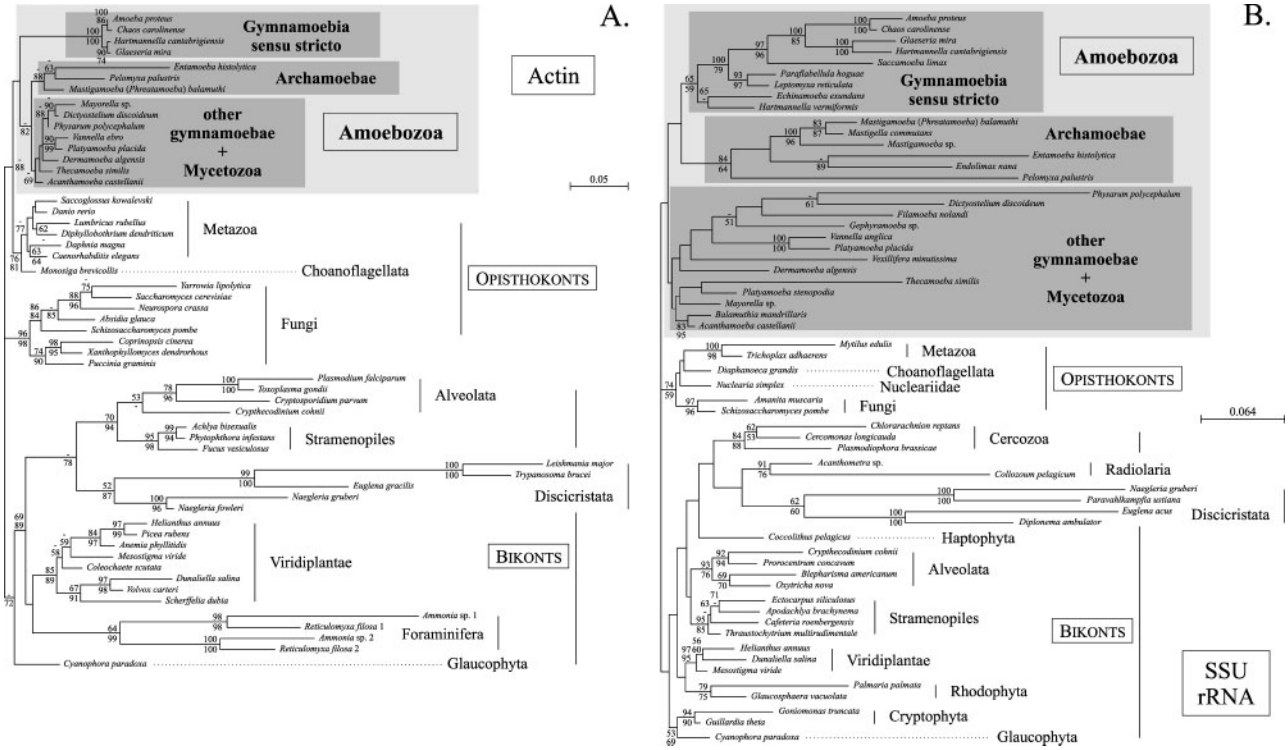

FIG. 1.—Phylogenetic position of lobose amoebae and relationships among Amoebozoa, as inferred from actin (*A*) and SSU rRNA (*B*) sequences. Both phylogenies show that all lobose amoebae cluster together, and they distinguish two well-defined clades of Amoebozoa, the "Gymnamoebia sensu stricto" and the Archamoebae (pelobionts + entamoebids), and one weakly supported and ill-resolved group comprising some naked, lobose amoebae and the Mycetozoa. Because the position of the root of the eukaryotic tree is subject to debate, both trees are presented in an unrooted format, with a basal trifurcation. In both trees, branches are drawn to scale. (*A*) Actin tree inferred from a ML analysis of 55 amino acid sequences of eukaryotes with ProML, using the JTT + G + I model of substitution (see *Materials and Methods*). Numbers at nodes indicate bootstrap support values for 100 replicates of a Fitch-Margoliash analysis using ML-corrected distances (upper) and bootstrap support values estimated through the RELL method with ProtML (lower). Values under 50% were omitted. (*B*) SSU rRNA tree inferred from a ML analysis of 60 sequences of eukaryotes with PAUP*, using the GTR + G + I model of substitution (see *Materials and Methods*). Numbers at nodes indicate bootstrap support values of ML (upper) and NJ (lower) analyses, using the same model, after 100 and 1,000 replicates, respectively. Values under 50% were omitted.

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 1*A* 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 1*B* 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 Cavalier-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. similis* 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 amoebae 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. limax*)—as well as members of the order Leptomyxida (*Leptomyxa reticulata* + *Paraflabellula hoguae*). 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. 1*B*). 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 invertens* [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 *Mastigamoeba balamuthi* was inferred from a combined analysis of rRNA 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 (Vannellidae, Thecamoebidae, Paramoebidae, and Vexilliferidae). The grouping of *Acanthamoeba* 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 Amoebozoa. The Vannellidae (*P. placida*, *V. ebro*, and *V. anglica*) and Thecamoebidae (*T. similis*) 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 "Gymnamoebia sensu stricto" and/or the Archamoebae.

Our data are in opposition to the division of Amoebozoa 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 insufficient taxonomic sampling and, more particularly, the lack of protein data for the lobose amoebae, which, together 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. 1*A*), the Amoebozoa are even included in the opisthokonts and appear as a sister-group to the Metazoa + Choanoflagellata 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 1*A* 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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