Early Origin of Foraminifera Suggested by SSU rRNA Gene Sequences

Jan Pawlowski, Ignacio Bolivar,* José F. Fahrni,* Thomas Cavalier-Smith,† and Manolo Gouy‡*

*Département de Zoologie et Biologie Animale, Université de Genève, Switzerland; †Department of Botany, University of British Columbia, Vancouver; and ‡Laboratoire de Biométrie, Génétique et Biologie des populations, Université Claude Bernard, France

Foraminifera are one of the largest groups of unicellular eukaryotes with probably the best known fossil record. However, the origin of foraminifera and their phylogenetic relationships with other eukaryotes are not well established. In particular, two recent reports, based on ribosomal RNA gene sequences, have reached strikingly different conclusions about foraminifera's evolutionary position within eukaryotes. Here, we present the complete small subunit (SSU) rRNA gene sequences of three species of foraminifera. Phylogenetic analysis of these sequences indicates that they branch very deeply in the eukaryotic evolutionary tree: later than those of the amitochondrial Archezoa, but earlier than those of the Euglenozoa and other mitochondria-bearing phyla. Foraminifera are clearly among the earliest eukaryotes with mitochondria, but because of the peculiar nature of their SSU genes we cannot be certain that they diverged first, as our data suggest.

Introduction

The foraminifera and the radiolaria are the last major taxonomic groups whose phylogenetic position among the unicellular eukaryotes has not been investigated by molecular methods. Traditional systematicians include foraminifera in the class Granuloreticulosea, which belongs to the assemblage of Rhizopoda (Lee, Hutner, and Bovee 1985), or classify them separately in a phylum, the Granuloreticulosa (Margulis et al. 1989) or the Reticulosa (Cavalier-Smith 1993*a*). However, the classical assemblage of Rhizopoda may be polyphyletic as suggested by several authors (Clark and Cross 1988; Cavalier-Smith 1993*a*).

Recent attempts to investigate the origin of foraminifera based on molecular data gave conflicting results. Phylogenetic analysis of partial sequences of the large subunit ribosomal DNA (LSU rDNA) have shown (Pawlowski et al. 1994*b*) that, in the eukaryotic tree, the foraminifera branch close to *Entamoeba* and slime molds (*Dictyostelium* and *Physarum*). However, on the basis of one full and one partial small subunit (SSU) rDNA sequences, Wray et al. (1995) placed the foraminifera within the assemblage of Alveolata, as a sister group to the ciliates. Since the respective positions of alveolates and slime molds are well conserved in both SSU and LSU rDNA trees, it must be concluded that either LSU and SSU rDNA have had different evolutionary histories in foraminifera, or that in one of the cases, polymerase chain reaction (PCR)-amplified sequences have been erroneously attributed to the foraminifera. The latter hypothesis is the most probable owing to the difficulties of isolating pure foraminiferal DNA (Langer, Lipps, and Piller 1993; Wray, Lee, and DeSalle 1993).

To settle the question, we have sequenced the SSU rDNA genes of three species of foraminifera (*Ammonia beccarii, Trochammina* sp., and *Allogromia* sp.). Our work relied on the LSU rDNA sequences previously obtained in our laboratory (based themselves on rRNA sequencing; Pawlowski et al. 1994*b*) and was, at each step, confirmed by northern blot hybridization. Phylogenetic analysis of these data shows that the foraminifera branch at the base of the eukaryotic tree, even earlier than suggested by our previous work. These results suggest that the sequences presented by Wray et al. (1995) have been erroneously attributed to foraminiferan rDNA.

Materials and Methods

Cell Collection and Culture

The specimens used in this study were collected along the Mediterranean coast in France, at Le Boucanet salt marsh, near La Grande Motte (*A. beccarii*), and at St. Cyr near Toulon (*Trochammina* sp.), and in Turkey, at Antalya (*Allogromia* sp.). *Trochammina* sp. and *Allogromia* sp. were maintained in laboratory culture for the last 3 years, fed with diatoms and heat-killed *Dunaliella salina.*

DNA Extraction

DNA was obtained from preparations containing one foraminiferal cell as described elsewhere (Pawlowski et al. 1994*b*). For *Allogromia* sp., an additional DNA purification by CTAB precipitation (Clark 1992) was necessary to achieve amplification.

Abbreviations: SSU, small subunit; LSU, large subunit.

Key words: Foraminifera, evolution, molecular phylogeny, SSU rRNA, mitochondria, protists.

Address for correspondence and reprints: Jan Pawlowski, Département de Zoologie et Biologie Animale, Université de Genève, 1224 Chêne-Bougeries, Switzerland. E-mail: pawlow@sc2a.unige.ch.

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Table 1
List of the Amplification and Sequencing Primers

Primer	Sequence	Orientation	Specificity	Position
SSU				
sA	ggttgat(ct)ctgccaga	Forward	Broad	6–21 (M)
s2	atcactta(ct)gcaactgc	Forward	Forams	152–168 (1)
s6	c(ct)gcggtaattccagctc	Forward	Broad	622–639 (M)
s9a	ctcgcaagtctgctcagc	Reverse	*Allogromia*	546–563 (G)
s10	cactgtgaacaaatcag	Forward	Forams	1,060–1,076 (A)
s11	ttacagcttgtcactgc	Reverse	Forams	718–734 (A)
s12	ctaccaaaagcgaaagc	Forward	Forams	1,250–1,266 (A)
s13	gcaacaatgattgtataggc	Reverse	Forams	957–976 (A)
s14	acttaaag(a/g)aattgacgg	Forward	Broad	1,191–1,209 (M)
s14rf	ccttcaagtttcacacttgc	Reverse	Forams	1,809–1,828 (A)
s15	(a/g)a(a/g)cg(a/g)ccatgcac(c/t)ac	Reverse	Broad	1,324–1,342 (M)
s15r......	gtggtgcatggccgt	Forward	Broad	2,002–2,016 (A)
s17	cggtcacgttcgttgc	Reverse	Forams	2,159–2,174 (A)
s20	ttgtacacaccgcccgtc	Forward	Broad	1,691–1,709 (M)
s20r......	gacgggcggtgtgtacaa	Reverse	Broad	1,709–1,691 (M)
s221f.....	aggttcacctaccgatgc	Reverse	Forams	2,847–2,864 (A)
s31	agaatttcacctctgac	Reverse	Alveolates	821–837 (W)
LSU				
2taic	ctc act cga gct gat gtg	Reverse	Forams	1–18 (A)
1f	act ctc tct ttc act cc	Reverse	Forams	610–127 (A)

NOTE.—EMBL/GenBank accession numbers of sequences used as references for primer positions: M—X00686 (mouse), A—X86094 (*Ammonia*), G—X86093 (*Allogromia*), W—U07937 (Wray et al. 1995).

RNA Hybridizations

Northern blots were prepared and hybridized according to Khandjian (1986). Hybridization and wash stringencies were adapted to the T_m value of each probe.

PCR Amplification

The PCRs were performed in a total volume of 50 μL consisting of 1× TAQ buffer, 100 pM of dNTPs, 50 pM each of the two primers, 2.5 U Taq DNA polymerase (Boehringer), and 1 μL of the DNA extract. Special PCR tubes (Sarstedt) with reduced volume were used. The amplification profile consisted of 40 cycles of 35 sec at 93.5°C, 35 sec at 50–52°C and 120 sec at 72°C, followed by 30 min at 72°C for final extension. The amplified PCR product was purified using Spin-Bind DNA extraction units (FMC). Primers sequences are given in Table 1.

DNA Cloning and Sequencing

The amplified products were ligated in the pGEM-T Vector System (Promega), cloned in supercompetent XL2-blue cells (Stratagene) and sequenced with the *fmol* DNA Sequencing System (Promega), all according to the instructions of the manufacturers. Both strands of the inserts were sequenced.

Sequence Analysis

The SSU rDNA sequences reported here were manually added to the multiple alignment of eukaryotic SSU rRNAs compiled by Larsen et al. (1993) under the MASE multiple alignment sequence editor (Faulkner and Jurka 1988). The resulting alignment was checked with reference to the universal secondary structure model of SSU rRNAs (Neefs et al. 1990). Evolutionary trees were built using the neighbor-joining (NJ) method (Saitou and Nei 1987) applied to distances corrected for multiple hits and unequal transition and transversion rates following Kimura's 2-parameter model (Kimura 1980), and using program fastDNAml implementing the maximum likelihood method with the global search option activated (Olsen et al. 1994). All analyses were based on unambiguously aligned sites selected according to Hinkle and Sogin (1993) with some modifications resulting from presence of foraminiferal sequences. Furthermore, all gap-containing sites were excluded. The reliability of internal branches in the NJ tree was assessed using the bootstrap method (Felsenstein 1988) with 1,000 replicates. The ClustalW program (Thompson, Higgins, and Gibson 1994) was used for distance computations, tree building, and bootstrapping. Program njplot (M.G., unpublished) was used for tree plotting.

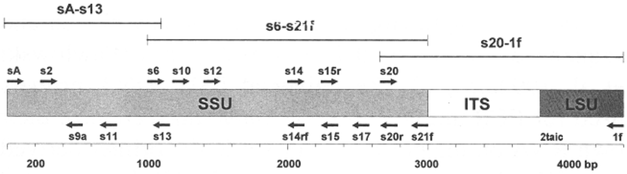

FIG. 1.—Diagram of the cloned fragments of rRNA gene of *Ammonia* with the approximate position of amplification and sequencing primers.

Results

To circumvent the problems of contamination, we have amplified foraminiferal DNA with two kinds of oligonucleotides: (1) a specific foraminiferal primer (1f) designed by reverse transcriptase sequencing of the 5′ end of the LSU rRNA; (2) a very broad specificity primer (s20) designed for the 3′ region of the SSU rDNA (fig. 1, table 1). The resulting fragments contained the 3′ terminal region of the SSU gene, a spacer of about 850 bp, and the expected 5′ sequence of the LSU gene. Then, by constructing 3′ specific primers (s21f, s13) and 5′ universal primers (s6, sA), the whole SSU rDNA genes of three foraminiferal species (*A. beccarii, Trochammina* sp., and *Allogromia* sp.) were amplified, cloned in three overlapping fragments, and sequenced (fig. 1).

For testing the authenticity of amplified sequences we have used our preparations of foraminiferal RNA, which are not contaminated, at a detectable level, by RNAs of non-foraminiferal origin as shown in Pawlowski et al. (1994*b*). Northern blots, containing total RNA from foraminifera and other unicellular eukaryotes, were labeled with three kinds of probes: (1) universal probes designed to hybridize to all SSU rRNAs on the blot; (2) specific probes designed to hybridize to all species but the foraminifera; (3) specific probes designed to hybridize to the foraminifera but not to the other species. All behaved as expected from the sequence data (fig. 2). In particular, primer s31 (fig. 2*B*, table 1) designed to hybridize to Wray's sequence as well as to *Tetrahymena thermophila* and *Paramecium caudatum* (both alveolates) did not label the foraminiferal RNA.

The SSU ribosomal genes of foraminifera display several unusual characteristics. These genes, ranging from 2,800 to 3,300 bp, are among the longest SSU rDNAs described so far. The great length of these sequences results from several long insertions occurring in variable regions of the molecule and, most remarkably, from insertions located where no other known eukaryotic rRNA sequence significantly varies in length. The G+C content ranges from 32% to 46% in whole genes but is about 48% in the aligned regions, a value close to the average of other rRNAs. As deduced from northern blots, the SSU rRNAs of *Ammonia* and *Quinqueloculina* (another foraminifer) are about 1,900 bases in length. In *Ammonia,* therefore, approximately 1,000 bases are eliminated during the processing of the molecule. In *Ammonia* and *Quinqueloculina,* the molecule is cleaved into a 5′ fragment of about 450 bases (not shown) and a 3′ fragment of about 1,450 bases shown in figure 2 (very small fragments would not have been detected).

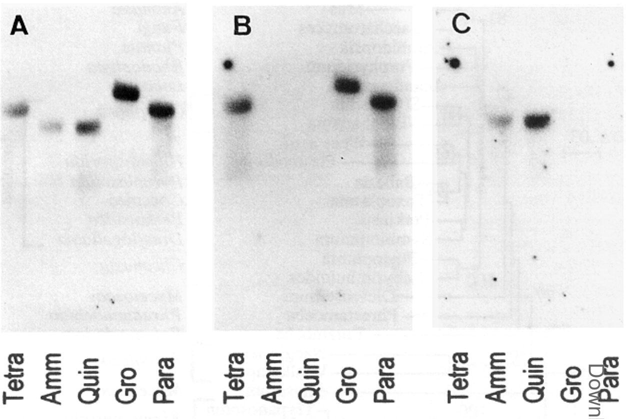

FIG. 2.—Northern blots of total RNA from the ciliate *Tetrahymena thermophila* (Tetra), the calcareous perforate foraminifer *Ammonia beccarii* s.l. (Amm), the porcelaneous foraminifer *Quinqueloculina* sp. (Quin), the filopod *Gromia oviformis* (Gro), and the ciliate *Paramecium tetraurelia* (Para). Probes were: (*A*) universal primer s15; (*B*) primer s31 designed to recognize *T. thermophila, P. tetraurelia,* and Wray's "*Ammonia*" sequence; (*C*) primer s14rf specific for foraminifera.

The phylogenetic position of foraminifera relative to other eukaryotic phyla was inferred using the NJ method. The three SSU rDNA sequences were compared to 25 other complete eukaryotic SSU rDNA sequences chosen to sample all known evolutionary diversity within eukaryotes, and to the SSU rDNA sequence attributed to *A. beccarii* by Wray et al. (1995). Our three foraminiferal sequences are grouped in a clearly monophyletic cluster, which branches very deep in the eukaryotic phylogenetic tree (fig. 3), deeper than all other known mitochondria-bearing organisms (all organisms of fig. 3 but diplomonads and trichomonads). Bootstrap analysis of this tree supports with an 85% score the branch that places foraminifera deeper than other mitochondria-bearing organisms. If sequences of Euglenozoa and *Physarum* are omitted, the branch separating foraminifera from the upper part of the tree is associated with a bootstrap score of 100%. Thus the NJ method locates the evolutionary origin of foraminifera very early in the history of eukaryotes, at a date similar to or possibly earlier than that of Euglenozoa and plasmodial slime molds. Analysis of the same data by the maximum likelihood method yields a tree that differs

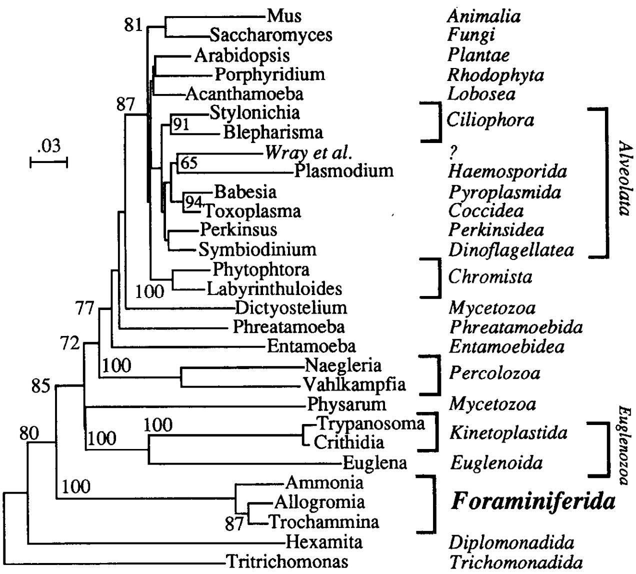

FIG. 3.—Eukaryotic phylogeny inferred from 29 SSU rDNA sequences. The tree is rooted in the *Tritrichomonas* lineage following results of analyses of the evolutionary origins of diplomonads, trichomonads, and microsporidia (Leipe et al. 1993). Horizontal distances are proportional to inferred evolutionary distances according to a scale given in substitutions per site. Bootstrap scores expressed in percentage out of 1,000 replicates are given next to each internal branch; scores <50% were omitted for clarity. Species used [GenBank/EMBL accession nos.]: *Mus musculus* [X00686], *Saccharomyces cerevisiae* [J01353], *Arabidopsis thaliana* [X16077], *Porphyridium aerugineum* [L27635], *Acanthamoeba palestinensis* [L09599], *Stylonychia pustulata* [M14600], *Blepharisma americanum* [M97909], *Ammonia beccarii* (Wray et al. 1995) [U07937], *Plasmodium gallinaceum* [M61723], *Babesia equi* [Z15105], *Toxoplasma gondii* [U03070], *Perkinsus* sp. [L07375], *Symbiodinium microadriaticum* [M88521], *Phytophthora megasperma* [M54938], *Labyrinthuloides minuta* [L27634], *Dictyostelium discoideum* [K02641], *Phreatamoeba balamuthi* [L23799], *Entamoeba histolytica* [X56991], *Naegleria gruberi* [M18732], *Vahlkampfia lobospinosa* [M98052], *Physarum polycephalum* [X13160], *Trypanosoma brucei* [M12676], *Crithidia fasciculata* [X03450], *Euglena gracilis* [M12677], *Ammonia beccarii* [X86094], *Allogromia* sp. [X86093], *Trochammina* sp. [X86095], *Hexamita inflata* [L07836], *Tritrichomonas foetus* [M81842].

from that of figure 3 by the position of *Dictyostelium* and by some details within the large evolutionary radiation at the top of the tree, but places foraminifera below Euglenozoa and *Physarum,* as in figure 3 (data not shown).

Discussion

Faced with the discrepancy between our sequences and those published by Wray et al. (1995) it is necessary to ascertain that the analyzed sequences are of foraminiferal origin and not of any other contaminating microorganisms. We believe that data presented in this paper are of genuine foraminiferal origin for the following reasons: (1) the sequenced SSU rDNA fragments are physically connected by an internal spacer of about 800 nucleotides in length (fig. 1) to the previously cloned and sequenced foraminiferal LSU rDNA genes (Pawlowski et al. 1994*a,b*); (2) the universal SSU rDNA primers used for PCR amplification of *Ammonia* recognize their own RNA on the northern blot while the primer derived from Wray's sequence does not (fig. 2); (3) the presented sequences are homologous to other partial SSU rDNA sequences obtained for a dozen of species representing major taxonomic groups of foraminifera, including planktonic and deep-sea agglutinated forms not presented in this paper (Pawlowski, in preparation); (4) all LSU rDNA sequences, obtained from more than 50 foraminiferal species collected in different localities, form a monophyletic group and their phylogenetic relationships are in good agreement with the morphological data (Pawlowski et al. 1994*a,b,* in press); this would be unlikely if our sequences were not of foraminiferal origin.

There are several reasons to doubt the reliability of Wray's data, consisting only of one complete and one partial sequence attributed to two species of the genus *Ammonia.* The specific SSU rDNA probe designed according to Wray's "*Ammonia*" sequence does not recognize the RNA of *Ammonia* on our northern blots. The labeling shown in the in situ hybridization, which constitutes the unique evidence of the authenticity of Wray's sequences, is ambiguous because the localization and structure of *Ammonia* nuclei are not cytologically demonstrated in the corresponding experimental conditions and we cannot rule out the possibility that what was stained was an endosymbiont, parasite, or food organism. The arguments used by Wray et al. (1995) to justify the position of foraminifera are questionable. A branching of foraminifera within the alveolates would be surprising in view of the fact that cortical alveoli have never been observed in foraminifera (Anderson and Lee 1991). The nuclear dimorphism proposed as a shared character between heterokaryotic foraminifera and karyorelictid ciliates is considered as having originated independently (Raikov 1982); the majority of foraminifera are not heterokaryotic at all, and there is no reason to think that their immediate common ancestor was heterokaryotic. Moreover, among the ciliates, the peculiar nuclear dimorphism of the karyorelictids has been shown recently to be a derived character (Hirt et al. 1995). The phylogenetic position of planktonic foraminifera inferred from LSU and SSU genes (Merle et al. 1994; Darling, personal communication) do not fit with Wray's tree but are similar to our data.

We can only speculate on the origin of Wray's "*Ammonia*" sequences. Theoretically, foraminiferal DNA can be contaminated by DNA originating from food vacuoles, endosymbiotic algae, intracellular parasites, or epiphytic microorganisms living on the surface of foraminiferal tests. As Wray's sequences branch within the alveolates clade, close to the apicomplexan group of *Plasmodium* (fig. 3), they may originate from an apicomplexan parasite similar to the *Trophosphaera* found in the foraminifer *Planorbulina mediterranensis* (cited in Lee, Hutner, and Bovee 1985, p. 372).

Phylogenetic analysis of partial LSU rDNA sequences (Pawlowski et al. 1994*b*) located the origin of foraminifera at a position close to that of *Physarum* and *Entamoeba,* that is, apparently later in the history of eukaryotes than what is deduced here from complete SSU rDNA sequences. These data, however, were too limited (610 homologous sites used) to resolve the branching pattern of all studied phyla and allowed only to place the origin of foraminifera earlier than that of alveolates with statistical significance (see fig. 6 of Pawlowski et al. 1994*b*). The longer sequences studied here (973 homologous sites used) combined with the larger number of eukaryotic phyla available for SSU rDNA analysis allow a more accurate positioning of foraminifera (compare the bootstrap scores of fig. 3 and fig. 5 of Pawlowski et al. 1994*b*). Therefore consideration of the limited degree of resolution of the partial LSU rDNA tree indicates that the SSU and LSU trees concur in revealing an early evolutionary origin of foraminifera.

According to the molecular data, the foraminifera, or their ancestors, may have diverged much earlier than suggested by the fossil record. The oldest described foraminifera, which have an agglutinated wall similar to *Trochammina,* date from the Early Cambrian, about 560 Myr ago (Culver 1991). They are supposed to have evolved from some ancestral forms with organic membraneous tests, similar to those of recent *Allogromia* (Tappan and Loeblich 1988). The oldest calcareous foraminifera have been found in Ordovician, but calcareous tests, as those of *Ammonia,* were not abundant until the Devonian, 400 Myr ago. Our data suggest either that some unfossilized membraneous-walled foraminifera have existed long before the earliest testate forms appeared or that the very early branching of the foraminifera on our tree is exaggerated by exceptionally rapid rRNA evolution. The respective positions of the foraminiferal species on the SSU rDNA tree, and especially the divergence of the calcareous *Ammonia* before the separation of the membraneous-walled *Allogromia* and agglutinated *Trochammina,* suggest that the agglutinated and calcareous forms evolved independently from the common ancestor.

Figure 3 also raises questions about the evolution of mitochondria. Indeed, both Percolozoa and Euglenozoa have mitochondria with discoid cristae, whereas they are tubular in foraminifera (Anderson and Lee 1991) and in most higher Protozoa. The position of foraminifera in the phylogenetic tree would imply that discoid cristae were not the ancestral state, contrary to earlier evidence (Cavalier-Smith 1993*a*), but evolved secondarily from tubular ones. However, it is particularly hard to determine the correct branching order on SSU trees in the region between the divergence points of *Dictyostelium* and foraminifera, as expressed by the low values of bootstrap scores in this region. There is therefore no reason to be confident that the relative branching order between foraminifera, Euglenozoa, Percolozoa, and *Physarum* presented in figure 3 is correct. A foraminiferal divergence between the Euglenozoa and the Mycetozoa would best reconcile our rDNA trees and the prevalent view on mitochondrial evolution based on ultrastructural data. Alternatively, the use of the form of mitochondrial cristae as a taxonomic character of high significance might need reevaluation.

In any case, the foraminifera are the earliest known eukaryotes possessing mitochondria with tubular cristae. They could be the earliest neokaryotes as defined by Cavalier-Smith (1993*b*). Because of the important differences between neokaryotes and earlier eukaryotes (such as Archezoa and Euglenozoa) with respect to genomic organization of both nuclei and mitochondria (Cavalier-Smith 1993*b*), the early origin of foraminifera makes them of pivotal importance for molecular evolutionists. In particular, a study of their mitochondrial DNA might shed much light on the origin of mitochondria.

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