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ACTIN PHYLOGENY OF FORAMINIFERA

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ABSTRACT

Molecular phylogenies of foraminifera are commonly inferred from the small subunit rRNA (SSU) genes, which can easily be obtained from single cells isolated from environmental samples. The SSU phylogenies, however, are often biased by heterogeneity of substitution rates, and their resolution of higher level relationships is often very low. The sequences of protein-coding genes provide an important alternative source of phylogenetic information, yet their availability from foraminifera has been limited until now. Here, we report the first extensive protein sequence data for foraminifera, which comprises 90 actin sequences for 27 species representing five major foraminiferan groups. Our analysis enables grouping foraminiferan actins into two main paralogs, ACT1 (actin type 1) and ACT2 (actin type 2), and several actin-deviating proteins. Phylogenetic analyses of ACT1 and ACT2 confirm the general structure of foraminiferan phylogenies inferred from SSU rDNA sequences. In particular, actin phylogenies support (1) the paraphyly of monothalamous foraminifera, including the allogromiids, astrorhizids and athalamids; (2) the independent divergence of miliolids and their close relationship to *Miliammina*; (3) the monophyly of rotalids; and (4) the rotaliid ancestry of globigerinids. Some foraminiferan taxa can be distinguished in actin sequences by the presence of group-specific introns (rotaliids, allogromiids) or absence of any introns (soritids ACT1).

INTRODUCTION

Foraminifera are one of the most diverse groups of protists, comprising about 5000 living species and tens of thousands of fossil taxa. Yet, less than 20 foraminiferan species have been successfully maintained and reproduced in laboratory culture, and no axenic cultures are available. The lack of suitable laboratory cultures has limited our ability to obtain large monospecific foraminiferan DNA samples, making the amplification of their protein-coding genes extremely difficult. Until now, only three foraminiferan proteins have been sequenced: actin (Pawlowski and others, 1999; Keeling 2001), RNA polymerase II largest subunit (Longet and others, 2003) and ubiquitin (Archibald and others, 2003). Because the aim of most of these studies was to establish the phylogenetic position of foraminifera, the number of examined species (maximum three) was limited and no phylogenetic relations among foraminifera were inferred from protein sequences. All previous broad taxon molecular phylogenies of foraminifera were based on small subunit (SSU) rRNA and large subunit (LSU) rRNA gene sequences (Darling and others, 1996, 1997; Wade and others, 1996; de Vargas and others, 1997; Pawlowski and others, 1997, 2002, 2003; Pawlowski, 2000; Holzmann and others, 2001; Pawlowski and Holzmann, 2002; Holzmann and others, 2003). The advantage of using rRNA genes is that they can be easily amplified from single cell DNA extracts (Holzmann and Pawlowski, 1996). On the other hand, their disadvantage is that they are often biased by heterogeneity of substitution rates (Philippe and Adoutte, 1998; Philippe, 2000). The foraminiferan rRNA genes are particularly well known for extreme variations of substitution rates at different taxonomic levels (Pawlowski and others, 1997). The acceleration of the foraminiferan stem lineage impeded their correct placement in eukaryote phylogeny (Berney and Pawlowski, 2003). The extremely high substitution rates in Globigerinida and unusual base composition in Miliolida obstructed the inference of their phylogenetic position (Pawlowski and others, 1997).

To test the higher-level foraminiferan phylogenies inferred from SSU rRNA, we have analyzed 90 actin sequences obtained from 27 species representing five major taxonomic groups. Two main types of actin (ACT1 and ACT2) have been identified. Phylogenetic relationships within foraminifera were inferred for each type separately. The position of actin introns was correlated with the phylogenetic groupings.

MATERIAL AND METHODS

RNA AND DNA EXTRACTION

Among 27 species of foraminifera examined in this study, two species (*Reticulomyxa filosa* and *Allogromia sp.* A) were from laboratory cultures. The remaining species were collected in various localities (Table 1). Specimens were individually cleaned with a paintbrush and rinsed in several baths of sterile seawater prior to extraction. Total RNA was extracted as described by Bolivar and others (2001). DNA was extracted either using a guanidinium buffer (Chomczynski and Sacchi, 1987) or the DNeasy Plant MiniKit (Qiagen). Each DNA extraction contained from 50–100 specimens of the same morphospecies.

PCR AMPLIFICATION, CLONING AND SEQUENCING

PCR amplifications, cloning and sequencing were done as described by Fahrni and others (2003). The actin gene of *Toxisarcon alba* was amplified by reverse transcriptase PCR with primers ActF1 and 1354R ; the N-terminal part of the molecule was obtained by 5′-RACE with primers TSR1 (5′-CCA CGC TCA TTC AA ATC TTC T -3′) and TSR2 (5′-CCG GTA GTT CGA CCA CTC GCA TA -3′). A list of all other primers used in our study is given in Table 2. All sequences were analyzed using BLAST 2.0 (Altschul and others, 1997) to search for sequences of non-foraminiferans that could be present in DNA extracts. The 88 new sequences of actin reported in this paper were deposited in the

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TABLE 1. List of species and morphotypes examined in this study, including their taxonomic position, collection site, and number of DNA isolate in the foraminifera DNA Collection at University of Geneva.

Species	Order	Locality	DNA isolate #
Allogromia sp. A	Monothalames	Culture	607, 2573
Ammonia sp.	Rotaliida	Le Boucanet – France	2306
Amphisorus hemprichii	Miliolida	RII60 – Reunion Island	x-944
Bathysiphon cf. *B. flexilis*	Monothalames	Dunstaffnage, Scotland	3983
Bathysiphon sp.	Monothalames	Dunstaffnage, Scotland	3987
Bolivina sp.	Rotaliida	Mediterranean – France	615
Bulimina marginata	Rotaliida	Oslofjord – Norway	3599
Edaphoallogromia australica	Monothalames	Queensland – Australia	2252
Elphidium cf. *E. williamsoni*	Rotaliida	Le Boucanet – France	3960
Globigerinella siphonifera	Globigerinida	Villefranche – France	4037
Globobulimina turgida	Rotaliida	Oslofjord – Norway	3601
Haynesina germanica	Rotaliida	Westthoek – Holland	1497
Hyalinea balthica	Rotaliida	Oslofjord – Norway	3604
Marginopora kudakajimaensis	Miliolida	Western Pacific, Guam	x-5240
Marginopora vertebralis	Miliolida	Western Pacific, Guam	1645
Miliammina fusca	Textulariida	Golf du Morbihan – France	4058
Nonionella labradorica	Rotaliida	Oslofjord – Norway	3600
Reophax sp.	Textulariida	Oslofjord – Norway	3598
Reophax sp.	Textulariida	Skagerrak – Sweden	3969
Reticulomyxa filosa	Monothalames	Culture	613
Rosalina sp.	Rotaliida	Mediterranean – France	3675
Sorites sp.	Miliolida	Caribbean Sea, Panama (P244)	x-1646
Sorites sp.	Miliolida	Caribbean Sea, Panama (P251)	x-1653
Stainforthia fusiformis	Rotaliida	Skagerrak – Sweden	3965
Toxisarcon alba	Monothalames	Dunstaffnage, Scotland	–
Tretomphalus sp.	Rotaliida	Bermuda	B123
Trochammina sp.	Textulariida	Golf du Morbihan – France	4057
Unidentified miliolid	Miliolida	Mediterranean – France	3955
Unidentified rotaliid	Rotaliida	Mediterranean – France	3953

GenBank/EMBL database under accession numbers (AY763936–AY764025 and AY766188).

PHYLOGENETIC ANALYSIS

Evolutionary trees were inferred using the maximum likelihood (ML) (Felsenstein, 1981) and neighbor-joining (NJ) methods (Saitou and Nei, 1987). Foraminiferan actin sequences and their eukaryotic homologs were aligned using Clustal X (Thompson and others, 1994), and further adjusted by eye with GeneDoc (Nicholas and others, 2000). A total alignment comprises 88 sequences and up to 344 amino acids. We did not include actin deviating proteins in our present analyses (Flakowski and others, unpublished), and we did not use some previously published sequences, either because they comprise deletions (*Ammonia* sp. ACT2, AJ132373; *Allogromia* sp. ACT1, AJ132370, and ACT2, AJ132371), or because of their deviant character (*Ammonia* sp. ACT1, AJ132372). The paralogous sequences were identified according to their phylogenetic position, i.e., the sequences that grouped with the Actin 1 gene were called ACT1 and the sequences that grouped with Actin 2 gene were called ACT2.

TABLE 2. List of the primers used in this study for amplification with their specificity, sequence and position (relative to the sequence of *Reticulomyxa filosa* AJ132375).

Forward Primer	Specificity	Sequence	Position
Act73D	All Foraminifera	GGT GAY GAY GCN CCA MGA GC	73
Mon85	All Foraminifera	CCR MGA GCH GTM KTY CAA	85
2100gc_D	Specific Rotaliida	CCS TCH GTT GTD GRH CGA	100
113cg_D	Specific Rotaliida	GGW CGA CCW MGA CAY YAA	113
130_Int	All Foraminifera	GGW GTW ATG GTT GGW ATG GGW	130
130_Mil	Specific Miliolida	GGW GTA ATG GTT GGW ATG	130
130_Sor	Specific Soritinae	GGT GTA ATG GTT GGA ATG	130
166_Int	All Foraminifera	GGW GAT GAA GCW CAA GC	166
ActN2	All Foraminifera	AAC TGG GAY GAY ATG GA	238
ActF1	All Foraminifera	CNG ARG CDC CAT TRA AYC	323
Reverse Primer	**Specificity**	**Sequence**	**Position**
1024R	All Foraminifera	ATD SWD CCD CCR ATC CA	1024
1350_R	All Foraminifera	TGA ACA TYT CHT SAA ANG TD	1102
1354R	All Foraminifera	GGA CCA GAT TCA TCA TAY TC	1106
1354rCe	All Foraminifera	GGW CCD GAT TCA TCR TAY TC	1106

To infer the phylogenetic position of foraminifera among eukaryotes, we used an alignment of 63 actin sequences, comprising 10 sequences of foraminifera, and the recently published sequences of the Polycystinea (Nikolaev and others, 2004), Plasmophorida (Archibald and Keeling, 2004) and *Gromia* (Longet and others, 2004). We analyzed a fragment of 240 amino acids, corresponding to about 2/3 of the total length of actin. This is due to the fact that many Cercozoa have been only partially sequenced and their sequences lack about 127 amino acids at the 5′ end of the molecule (Keeling, 2001).

The ACT1 alignment (20 sequences + outgroup) consists of 9 taxa and 299 amino acid positions, the ACT2 alignment (68 sequences + outgroup) consists of 24 taxa and 299 amino acid positions, and the concatenated sequence of both paralogs (2x10 sequences) is based on 8 taxa and 565 amino acid positions. Because the gaps containing sites were not included in analyses, the number of amino acid positions varied depending on the length of the sequences. In the case of concatenated analysis, to increase taxon sampling, we used a short sequence of *Ammonia* sp. ACT1 (AM509_7), which reduced our ACT1 alignment to 266 amino acids. We added also a slightly deviant sequence of *Reophax* sp. (ReoA1057_16) for the concatenated analysis only. We did a homogeneity partition test (Farris and others, 1995) for 1000 replicates with PAUP* (Swofford, 1998) to confirm that the data (ACT1+ACT2) were concatenable and found a P = 0.967, which indicates that there is no significant difference in phylogenetic signal between both paralogs.

To accommodate rate variations among sites, the distances were computed under the JTT substitution model, assuming a gamma distribution with eight rate categories plus invariable sites. We used TREE-PUZZLE 5 (Strimmer and von Haeseler, 1996) to correct the distance for the neighbor-joining analysis, followed with NEIGHBOR 3.6a3 (Felsenstein, 1993) to obtain the NJ tree. The maximum likelihood approach was achieved with PhyML v2.1b1 (Guindon and Gascuel, 2003). The proportion of invariable sites and the shape of the gamma distribution are adjusted to maximize the likelihood of the phylogeny. The reliability of internal branches was assessed using the bootstrap method (Felsenstein, 1985) with 100 bootstrap replicates generated with SEQBOOT 3.6a3 (Felsenstein, 1993). We calculated the NJ bootstraps with NEIGHBOR 3.6a3 (Felsenstein, 1993) under the JTT substitution matrix, with the alpha parameter and proportion of invariable sites calculated by TREE-PUZZLE 5, and the ML bootstraps with PhyML v2.1b1. The consensus trees were obtained with Consens 3.6a3 (Felsenstein, 1993) using the option majority rule (extended).

RESULTS

Phylogenetic Position of Foraminifera

The tree of eukaryotes, including foraminifera, is illustrated in Figure 1. The root of the tree is positioned between the Opisthokonts + Amoebozoa and bikonts, following the recent hypothesis of Stechmann and Cavalier-Smith (2002). The monophyly of the Opisthokonts (animals, fungi and choanoflagellates) and Amoebozoa is supported by NJ/ML bootstrap values of 80/88. The foraminifera branch among the bikonts, which together with *Gromia*, Polycystinea, and Plasmodiophorida, form a sister group to core Cercozoa. Each foraminiferan species possesses two types of actin: ACT1 and ACT2 (Fig. 1). Both types have high NJ/ML support (ACT1 97/98; ACT2 96/97) and we can observe a similar topology within each type. In the NJ tree (Fig. 1), two types of foraminiferan actin branch together, but their grouping is weakly supported. In the ML tree (not shown), one of two actin types (ACT1) branches with *Gromia*, while the second type (ACT2) branches with Polycystinea. The grouping of foraminifera, Polycystinea, Plasmodiophorida, Cercomonads and Chlorarachniophyta is weakly supported. The tree topology for other eukaryotic groups is similar to previously published phylogenies (Nikolaev and others, 2004).

ACT1 Phylogeny

Phylogenetic analysis of ACT1 from nine species representing four foraminiferan orders is illustrated in Figure 2. The tree is rooted with the sequence of *Reticulomyxa filosa* ACT2, to ensure the largest number of unambiguously aligned sites. Two allogromiids, *Allogromia* sp. A and *Edaphoallogromia australica*, form a clade supported by NJ/ML bootstrap values of 50/63%. They branch at the base of the tree, followed by a clade formed by the miliolid *Sorites* sp. and the athalamid *R. filosa*. The crown of the tree is supported by NJ/ML bootstrap values of 53/68% and is formed by the sequences of rotaliids—*Elphidium* cf. *E. williamsoni*, *Haynesina germanica* and *Tretomphalus* sp.—preceded by divergence of two textulariids: *Trochammina* sp. and *Reophax* sp.

ACT2 Phylogeny

Phylogenetic analysis of ACT2 from 24 species is illustrated in Figure 3. We rooted this tree with a sequence of *Reticulomyxa filosa* ACT1. The general topology of the tree is very similar to that of ACT1 (Fig. 2). The monothalamous species *Allogromia* sp. A, *Edaphoallogromia australica* and *Bathysiphon* sp. form a clade at the base of the tree supported by NJ/ML bootstrap values of 48/46%. This basal clade is followed by a clade of miliolids (supported by NJ/ML bootstrap values of 52/59%) which is comprised of an unidentified miliolid and four species of soritids. *Miliammina fusca* appears at the base of the miliolid clade, preceded by the divergence of the athalamid *R. filosa*, which branches as sister to all miliolids and *Miliammina*. The sequence of *Toxisarcon alba* (obtained from RNA) branches with *Bathysiphon flexilis* in weakly supported clade branching next to *R. filosa* and miliolids. At a lower taxonomic level, our data support the distinction of the subfamily Soritinae (*Amphisorus hemprichii*, *Marginopora kudakajimaensis*, *M. vertebralis* and *Sorites* sp.) with high NJ/ML bootstrap support of 97/98%, and the family of Rosalinidae (*Rosalina* sp. + *Tretomphalus* sp.) supported by an NJ/ML bootstrap of 68/68%. However, the relations between other species, especially within the clade of Rotaliida, are not well resolved.

ACT1 + ACT2 Phylogeny

The relations between eight species, for which both paraloguous genes are available, were inferred from concate-

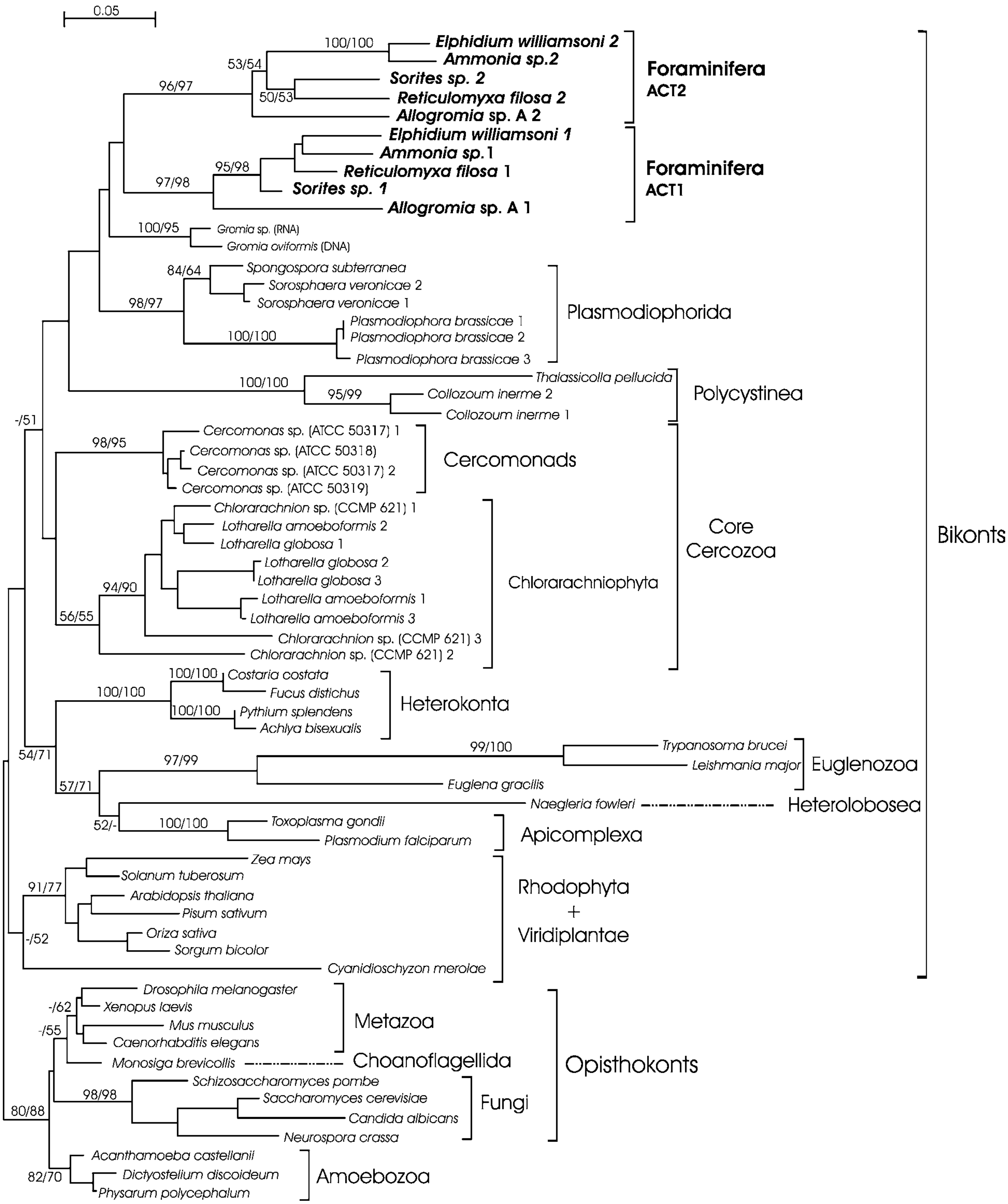

FIGURE 1. Neighbor-joining tree of eukaryotes based on partial (240 sites) actin sequences, showing the phylogenetic position of foraminifera. The two types of foraminiferan actin branch together, but their grouping is weakly supported. The numbers of internal branches are bootstrap values of NJ and ML analyses, higher than 50%.

nated sequences (Fig. 4). The tree has a general structure similar to that of ACT1 and ACT2 analyzed separately (Figs. 2, 3). There is good support for the clade of Rotaliida (NJ/ML bootstrap of 99/97%) and the clade formed by *Allogromia* sp. A and *Edaphoallogromia australica* (NJ/ML bootstrap of 95/99%). *Reophax* sp. clearly branches as a sister group to the Rotaliida. *Reticulomyxa filosa* and *Sorites* sp. branch together, as seen in the other trees.

IDENTIFICATION OF ACTIN INTRONS

Twenty-two foraminiferan actin introns have been identified and will be described elsewhere (Flakowski, unpub-

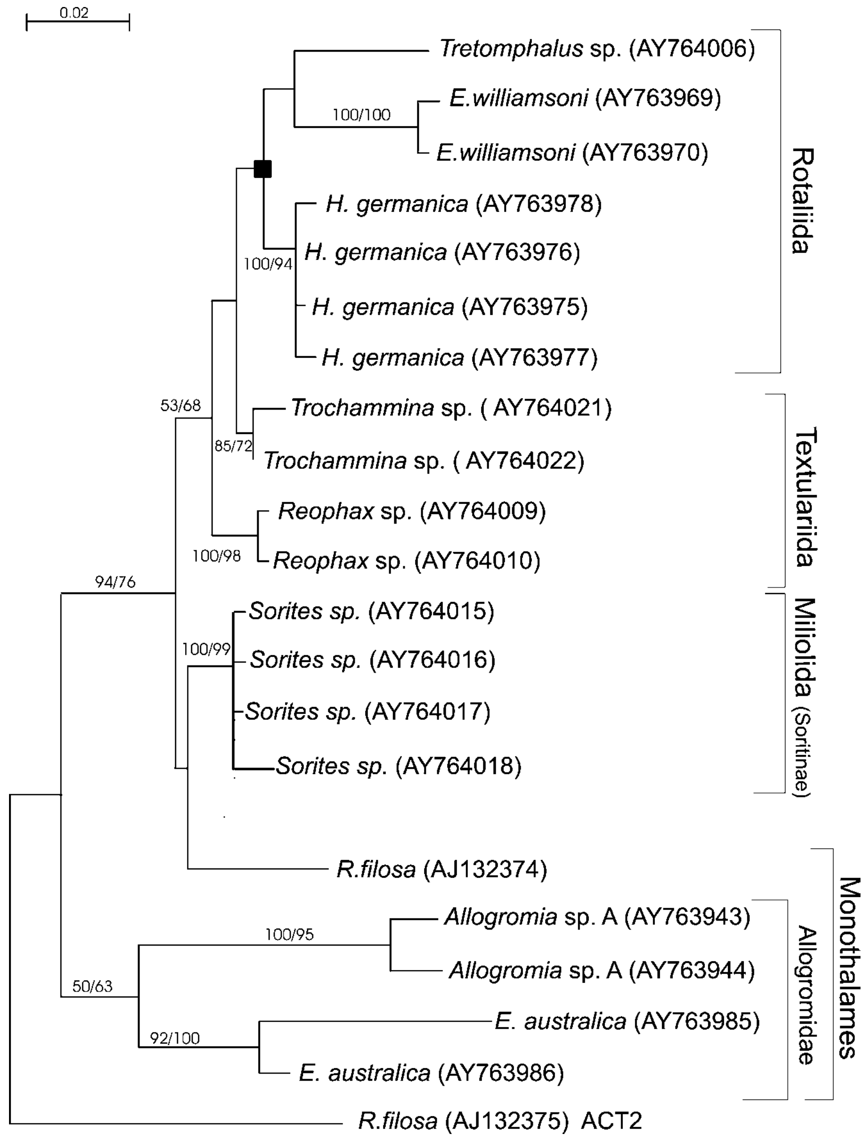

FIGURE 2. Maximum likelihood tree of the actin paralog ACT1 (298 sites) showing the phylogenetic relationship among foraminifera. The tree is rooted at a sequence of *Reticulomyxa filosa* ACT2. The numbers of internal branches are bootstrap values of NJ and ML analyses, higher than 50%. The clades supported by phylogenetically stable introns are indicated by black squares.

lished). Three of them can be used as phylogenetic markers. The intron [137:1] in ACT1 and the intron [84:3] in ACT2 are characteristic for the clade Rotaliida. The intron [64:2] is conserved in the case of the clade containing *Allogromia* sp. A, *Edaphoallogromia australica,* and *Bathysiphon* sp. The intron [248:3] is common to both types of *Allogromia* sp. A and to other eukaryotes (green algae, glaucophytes, metazoans). The other introns are specific for single species. We observed that the majority of Miliolida lack introns.

DISCUSSION

PHYLOGENETIC POSITION OF FORAMINIFERA

The first molecular studies based on LSU (Pawlowski and others, 1994) and SSU (Pawlowski and others, 1996) rRNA gene sequences suggested that foraminifera are an independent lineage branching at the base of eukaryotic tree. This hypothesis was contradicted by a phylogenetic study of actin sequence data, showing the close relationship between foraminifera and Cercozoa (Keeling, 2001). Further studies of protein-coding genes, in particular those of RNA polymerase II (Longet and others, 2003) and ubiquitin (Archibald and others, 2003), as well as the revised analysis of an SSU rRNA gene (Berney and Pawlowski, 2003), confirmed the grouping of foraminifera and Cercozoa. Yet, the branching order between Cercozoa, foraminifera and other related groups remains controversial. In some SSU rDNA trees, the foraminifera and some radiolarians form a sister group to Cercozoa (Cavalier-Smith and Chao, 2003; Nikolaev and others, 2004). In the absence of radiolaria, foraminifera form a sister group to *Gromia oviformis* (Berney and Pawlowski, 2003). In one analysis of actin sequences, it was shown that *G. oviformis* branch among foraminifera, at the base of ACT1 type (Longet and others, 2004).

In the present study, the position of foraminifera changes depending on the method of phylogenetic analysis. In the

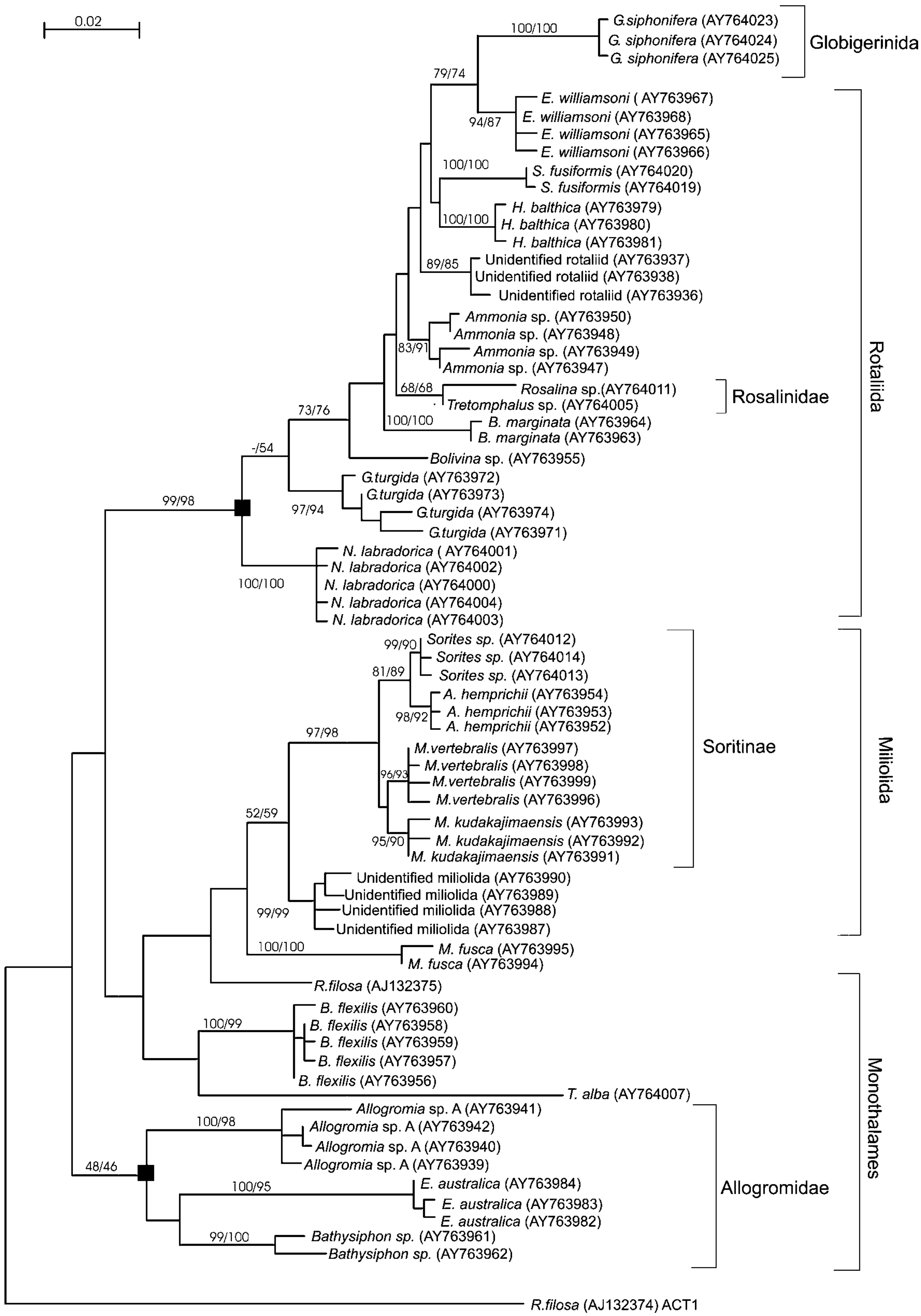

FIGURE 3. Maximum likelihood tree of the actin paralog ACT2 (278 sites), showing the phylogenetic relationship among foraminifera. The tree is rooted at a sequence of *Reticulomyxa filosa* ACT1. The numbers of internal branches are bootstrap values of NJ and ML analyses, higher than 50%, except for the clade of monothalamous taxa formed by *Allogromia* sp. A, *Edaphoalogromia australica* and *Bathysiphon* sp. The clades supported by phylogenetically stable introns are indicated by black squares.

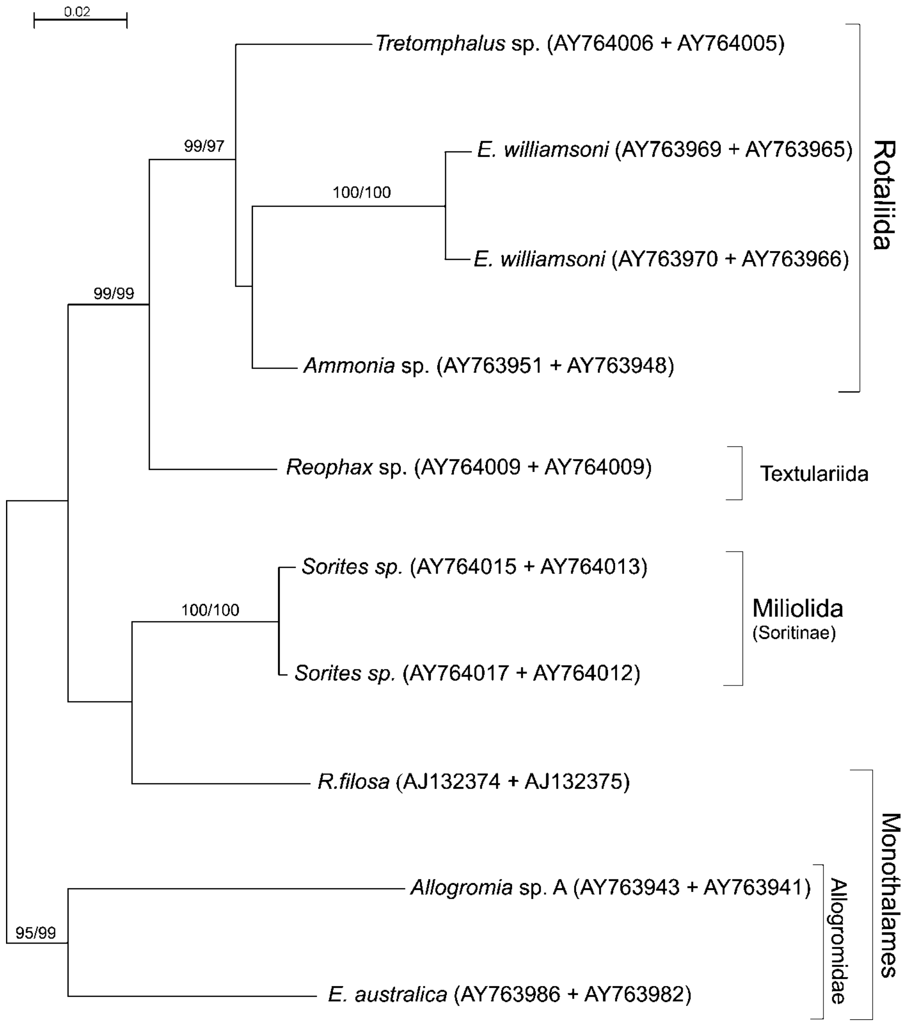

FIGURE 4. Maximum likelihood tree inferred from concatenated sequences of ACT1 and ACT 2 (565 sites), showing the phylogenetic relationship among foraminifera. The tree is unrooted. The numbers of internal branches are bootstrap values of NJ and ML analyses, based on 100 replicates. Only values higher than 50% are noted.

NJ tree, two types of foraminiferan actin branch together, preceded by divergence of *Gromia* and the clade Plasmodiophorida + Polycystinea; however, their relationships are not well supported (Fig. 1). In the ML tree (not shown), *Gromia* branches at the base of ACT1, while ACT2 form a sister group to Polycystinea, as found by Longet and others (2004). The latter topology is almost certainly artifactual, probably due to the difference of rates of substitution in foraminiferan actin paralogs, and perhaps because the sequences are too short (240 amino acids). ACT2 seems to evolve faster than ACT1, and therefore it has a greater tendency to branch with the fast-evolving Polycystinea. Interestingly, in this particular case, the NJ method, which is usually more sensitive to rate variations than ML, seems to be more accurate in inferring the true phylogeny.

Higher-level Phylogeny of Foraminifera

Our analyses of foraminiferan actin sequences support the following four phylogenetic hypotheses:

1) Monothalamous foraminifera are a paraphyletic grouping of allogromiids, astrorhizids and athalamids. Traditionally, the Allogromida, monothalamous (single-chambered) species with an organic test, were considered to be the most primitive foraminiferans, while the Astrorhizida, monothalamous agglutinated species, were either included in the order Textulariida (Loeblich and Tappan, 1988) or considered to be a separate order (Loeblich and Tappan, 1989). This traditional point of view was contradicted by SSU phylogenies, which consistently show allogromiids and astrorhizids grouping together in several lineages at the base of foraminiferal tree (Pawlowski, 2000; Pawlowski and others, 2002, 2003), in agreement with morphological, cytological, and behavioral observations (Bowser and others, 1995). The radiation of monothalamous foraminifera in SSU trees also includes *Reticulomyxa filosa*, a naked freshwater rhizopod considered in the past to be either a giant amoeba or a representative of Athalamida—a separate order of the phylum Granuloreticulosea (Lee and others, 1985).

Our study confirms the position of allogromiids, astrorhizids and athalamids at the base of foraminiferan tree (Figs. 2–4). In spite of limited taxonomic sampling of monothalamous species in our study, the close relations between agglutinated *Bathysiphon* sp. and organic-walled *Allogromia* sp. A observed in the ACT2 tree (Fig. 3), support the grouping of allogromids and astrorhizids. This clade also includes another organic-walled species, *Edaphoallogromia australica,* as suggested by the presence of a common intron in all three species. The grouping of these three species was also observed in the SSU trees (Pawlowski and others, 2002). Another clade of monothalamous foraminifera in actin trees (Fig. 3) comprises *Bathysiphon flexilis* and *Toxisarcon alba.* Neither species group together in SSU trees, and it is highly probable that this association will disappear with broader taxon sampling. Finally, our study confirmed that *Reticulomyxa filosa* branches within the radiation of monothalamous foraminifera, as suggested previously by SSU rDNA studies (Pawlowski and others, 2002, 2003). The position of *R. filosa* as a sister group to Miliolida in actin trees is difficult to explain in view of our general knowledge regarding the biology of these taxa, and must be confirmed by analyses of actin sequence data from a larger taxon sampling of foraminifera. Because most of the species belonging to the radiation of monothalamous foraminiferans possess an organic or agglutinated test, the position of *R. filosa* among them strongly suggests that this naked species has lost its test, probably as an adaptation to the freshwater environment (Pawlowski and others, 1999).

2) Miliolida diverged within the monothalamid radiation. In the early SSU studies, the order Miliolida was placed at the base of the foraminiferan tree (Pawlowski and others, 1997; Pawlowski, 2000). However, such an early origin for this group contradicted the fossil record, which reports the first miliolids in the Carboniferous (Ross and Ross, 1991). Because of the particular character of miliolid rRNA genes and their unusually low GC content, the reliability of the phylogenetic position of Miliolida in the early SSU trees was uncertain. Indeed, a revised analysis of SSU data, including much larger taxonomic sampling and more reliable methods of phylogenetic analysis, showed that the Miliolida cluster with the calcareous Spirillinida and agglutinated *Ammodiscus* in an independent, fast-evolving lineage branching within the radiation of monothalamous species (Pawlowski and others, 2003). This hypothesis was congruent with morphological observations suggesting that miliolids evolved directly from some allogromiid-like ancestor (Arnold, 1978a, b).

Our miliolid protein sequence data confirm the revised analyses of the SSU sequences, indicating the divergence of Miliolida within the radiation of early foraminifera (Fig. 2). Although a previous immunoblotting study suggested that Miliolida possessed only one type of actin (Fahrni and Pawlowski, 1995), our molecular sequence data demonstrate both ACT1 and ACT2 in this group. The phylogenetic analysis of both paralogues gives congruent results, but the exact phylogenetic position of Miliolida remains uncertain. Its establishment in SSU trees was difficult because of the strongly biased base composition in the miliolid stem lineage. Except for a lack of introns and a lower GC content, the actin of miliolids does not show any unusual features, and their evolutionary rates are not accelerated compared to other foraminifera. With larger taxonomic sampling of monothalamous foraminifera it will be possible to establish the phylogenetic position of this group with more reliability.

Our data indicate that *Miliammina* is a sister taxon to Miliolida. The evolutionary position of this genus has always been controversial, because *Miliammina* combines the morphological characteristics of two foraminiferal orders, the Textulariida (agglutinated wall) and the Miliolida (quinqueloculine arrangement of chambers). Depending on whether wall type or chamber arrangement has been considered more important taxonomically, *Miliammina* was classified among either the Textulariida (Loeblich and Tappan, 1988) or the Miliolida (Haynes, 1981). The first molecular data did little to resolve this controversy, because in SSU trees, *M. fusca* appeared as an independent lineage branching among early foraminifera (Pawlowski, 2000). The hypothesis of a miliolid origin of *Miliammina* was supported by immunoblotting data, which showed that *Miliammina* possesses one actin band similar to that of Miliolida, and different from the two actin bands observed in other foraminiferans, including Textulariida and Rotaliida (Fahrni and others, 1997). By showing that *M. fusca* branches at the base of Miliolida in the ACT2 tree, our analysis supports the close relationships between these two taxa. This hypothesis is further reinforced by the lack of introns in *Miliammina* actin, as seen in most miliolids.

3) The Rotaliida are monophyletic. The suborder Rotaliida comprises the calcareous benthic foraminifera having perforate hyaline walls, which appeared in the fossil record during the Jurassic (Loeblich and Tappan, 1988). Most probably they have evolved from agglutinated polythalamous species belonging to the Textulariida. Indeed, the Rotaliida and Textulariida are very closely related as shown by several SSU studies (Pawlowski, 2000; Pawlowski and others, 2003). However, the SSU sequences are not sufficiently variable to resolve their phylogenetic relations. The present analysis of actin sequences provides two arguments in favor of the monophyly of Rotaliida. Firstly, all rotaliids branch together in both ACT1 and ACT2 trees. Secondly, the majority of rotaliid species in ACT1 (except *Elphidium* cf. *E. williamsoni*) and in ACT2 (except *Globobulimina turgida* and *Bulimina marginata*) share an intron in the same position, [137:1] for ACT1 and [84:3] for ACT2. However, given the limited number of textulariid actins in our database, the hypothesis of the monophyly of rotaliids requires further testing.

4) Globigerinida branch among Rotaliida. The Globigerinida are traditionally considered as a separate order comprising all planktonic foraminifera (Loeblich and Tappan, 1988). This taxonomy was based exclusively on their distinctive mode of life, the calcareous tests of Globigerinida being similar to that of rotaliids. The first rRNA-based studies did little to establish the phylogenetic position of Globigerinida because their ribosomal genes evolved 50–100 times faster than those of benthic foraminifera (Pawlowski and others, 1997). In the SSU rDNA trees, the Globigerinida branched in two independent clusters dispersed among Rotaliida and Textulariida (Darling and others, 1997; de Vargas and others, 1997).

The first actin sequences of a globigerinid, *Globigerinella siphonifera,* reported in this study show that it branches with one rotaliid, *E.* cf. *E. williamsoni.* Its position among Rotaliida is strengthened by the presence in *G. siphonifera* of an intron in the ACT2 paralog, in the same position as in all other rotaliids. However, actin of other planktonic foraminifera must be examined before the position of this group among Rotaliida will be established.

CONCLUSIONS AND PERSPECTIVES

Our study represents an initial attempt to infer foraminiferan phylogeny from protein-coding genes. The general structure of the foraminiferan tree as inferred from actin sequence data are in broad agreement with previously published SSU phylogenies (Pawlowski and others, 1997, 2002, 2003; Pawlowski, 2000). Actin appears to be a useful phylogenetic marker of higher level relationships in foraminifera, both in analyzing its sequence data and in using the position of its introns to define some particular phylogenetic groupings. There are several advantages of using actin as a phylogenetic marker, such as (1) the ease of amplifying actin genes, even with a relatively limited amount of DNA; (2) the ability to design specific PCR primers that allow amplification of nearly the entire gene; and (3) the relative constancy of substitution rates compared to the SSU rRNA genes. The main disadvantages of actin are the presence of two paralogous genes and a large number of actin deviating types that must be identified and removed before phylogenetic analysis. Because of the relatively conserved character of its sequence and the limited number of sites, actin phylogenies are also relatively weakly supported.

The actin-based phylogeny inferred in this study must now be confirmed by further analyses of protein sequence data. The phylogenetic studies of the sequences of RNA polymerase II (Longet, unpublished), and α- and β-tubulin (Habura and others, unpublished) will soon be available. Hopefully, other useful protein markers will be defined by analysis of a foraminiferan EST project (in progress in our laboratory). There is also an urgent need to increase the taxon sampling in foraminiferal protein phylogenies, requiring either access to DNA extracts containing a larger number of specimens, or the development of a method to increase the amount or quality of the PCR template. With better taxon sampling, the multigene phylogenies will provide new insights into the evolutionary relationships among foraminifera and an indispensable tool to revise their classification.

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