Introduction to the molecular systematics of foraminifera

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INTRODUCTION

During the past few years there has been a spectacular progress in development of molecular systematics based on analysis of DNA sequences (Hillis et al. 1996). Central to this progress was the development of the applications of polymerase chain reaction (PCR), which allows an exponential amplification of a part of the genome, even from very small amounts of initial material. The common use of the PCR for gene amplification and sequencing produced rapidly increasing data sets of DNA sequences from various types of organisms. Comparison of these sequences allowed the phylogenetic links to be established between organisms at different taxonomic levels. Remarkable progress was achieved in evolutionary studies of unicellular eukaryotes using the ribosomal RNA gene sequences (Sogin 1991, Schlegel 1994, Philippe and Adoutte 1996).

Until recently, interpretations of the taxonomy and evolution of Foraminifera were based exclusively on comparative morphological studies (Loeblich and Tappan 1988). Since over 80% of described foraminiferal species are extinct, morphotaxonomy was the unique tool used for classification. Our knowledge of biology of Foraminifera was too limited to have any impact on the systematics of this group (Lee 1990). Chemotaxonomical studies of planktonic (King and Hare 1972, Robbins and Healy-Williams 1991) and benthic (Haugen et al. 1989) foraminifers were mostly concerned with the amino-acid composition of the fossil foraminiferal tests. Virtually nothing was known about molecular genetics of the Foraminifera.

Application of molecular methods to the study of Foraminifera was delayed because pure DNA could not be obtained and commonly used "universal" PCR primers failed to amplify the foraminiferal DNA. The first attempts to isolate foraminiferal DNA were reported by Langer et al. (1993), Wray et al. (1993) and Stathoplos and Tuross (1994). The first foraminiferal rDNA sequences were published by Pawlowski et al. (1994 a). The sequences obtained to date were used (1) to establish the phylogenetic position of foraminifera among the eukaryotes (Pawlowski et al. 1994a, Pawlowski et al. 1996, Merle et al. 1994, Wray et al. 1995, Darling et al. 1996b, Wade et al. 1996), (2) to examine the higher-level relationships among foraminifera (Pawlowski et al. 1997, de Vargas et al. 1997, Darling et al. 1997), and (3) to identify species in some foraminiferal genera (Pawlowski et al. 1994b, Pawlowski et al. 1995a, Pawlowski et al. 1995b; Holzmann et al. 1996, Holzmann et al. 1997, Holzmann et al. 1998, Huber et al. 1997).

This chapter briefly presents the development of molecular systematics of foraminifera, beginning with the first attempts to obtain foraminiferal DNA sequences and the different methods used to confirm their authenticity. The basic molecular techniques used for isolation and amplification of foraminiferal DNA are then presented, followed by the description of unusual features of foraminiferal ribosomal RNA genes and the survey of the first applications of molecular data to examine the origin, macroevolutionary relationships, evolutionary rates, and species definition in foraminifera. Finally, the future prospects of molecular systematics of foraminifera are discussed.

It is not the intention of this chapter to provide a detailed description of the different techniques and methods used in molecular systematics, but rather to give a general information of what has been achieved in this field concerning the foraminifera during the past five years. In spite of a rapid progress in the field and an avalanche of new DNA sequences, we are still at the stage of rather preliminary conclusions. Some of the hypotheses presented here may not survive by the time this book is published. It is difficult to predict all changes that molecular data can introduce in the present concept of systematics of foraminifera. Such speculations would be outside the scope of this chapter. Its purpose is to introduce the most essential aspects of molecular systematics of foraminifera, and as its major aim to encourage further research in this field.

SEARCHING FOR FORAMINIFERAL DNA

The principal obstacle to rapid application of molecular techniques in the study of foraminifera was the difficulty in obtaining pure foraminiferal DNA. Foraminifera are difficult to maintain and reproduce in laboratory cultures, especially in monoxenic or axenic conditions. Consequently, most of foraminiferal samples contain extraneous DNA originating either from epibiontic microorganisms living on the surface of foraminiferal tests or from algal symbionts or parasitic protists harbored inside foraminiferal cells. As even the smallest amount of DNA can be detected by PCR-based techniques, it is necessary to ascertain that the amplified genomic fragment originated from foraminifera and not from any contaminant.

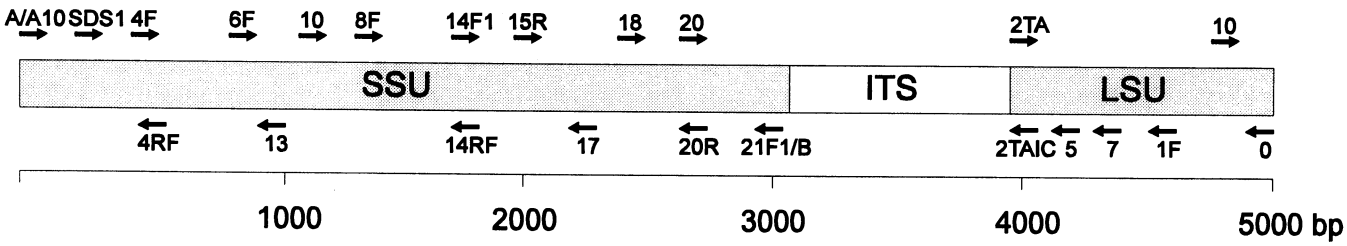
Different approaches have been proposed to circumvent the problem of contamination:

1. Specific foraminiferal primers have been designed based on rRNA sequences obtained from total foraminiferal RNA extracts (Pawlowski et al. 1994a).

2. "Pure" foraminiferal DNA was extracted from the planktonic foraminifera undergoing gametogenesis (Darling et al. 1996a)

3. In situ hybridization using digoxigenin-labeled probes was used to verify putative foraminiferal rDNA sequences (Wray et al. 1995).

The first two approaches tried to minimize the amount of possible contaminants in foraminiferal extracts. The RNA approach was based on presumption that the total RNA extract obtained



TEXT-FIGURE 1
Diagram of the sequenced rRNA genes of *Ammonia* sp. 1 with the approximate position of amplification and sequencing primers.

from thoroughly cleaned, symbiont-free foraminiferal cells, contains predominantly the foraminiferal ribosomal RNA. The fact that the direct RNA sequencing procedure gives the sequence of the major component in the RNA extract reduces considerably the chance of obtaining a contaminant sequence. Moreover, the purity of the RNA extract can be verified by Northern blot hybridization, as demonstrated in Pawlowski et al. (1994a). Once the rRNA sequence is established, it can be used to design specific foraminiferal primers for PCR amplification.

Alternatively, Darling et al. (1996a) used the gametes of planktonic foraminifera as a source of almost pure foraminiferal DNA. This approach was based on observations that the planktonic foraminifera consume or expel most of their symbionts and prey particles prior to gametogenesis (Hemleben et al. 1989). Reduction of the number of associated microorganisms accompanied by production of several hundred thousands of gametes (Spindler et al. 1978) maximizes the ratio of foraminiferal genomic DNA to that of possible contaminants.

Both RNA and gametogenesis-based approaches led independently to similar results. The rDNA sequences obtained by both methods were considered as having originated from foraminifera because they grouped together in the rRNA trees and were different from any other known rRNA sequences. Their authenticity has been reinforced by the addition of several related sequences obtained from different groups of foraminifera, whose phylogenetic relationships showed a good congruence with the morphotaxonomic classification.

On the other hand, the origin of two SSU rDNA sequences obtained from *Ammonia* spp. and considered as authentic on the base of in situ hybridization (Wray et al., 1995) remains enigmatic. These sequences differ substantially from those obtained by RNA and gametogenesis-based approaches. Considering that the similar sequences have not subsequently been reported from Foraminifera, and that the specific probes designed from these sequences did not recognize foraminiferal RNA, indicates that they belong to some extraneous DNA (Pawlowski et al. 1996). Similarity between Wray's sequences and those of some parasitic protists from the group of Apicomplexa would suggest that they may derived from some sporozoan parasites harbored by foraminifera, similar to those described by Le Calvez (1939) or Nyholm (1962).

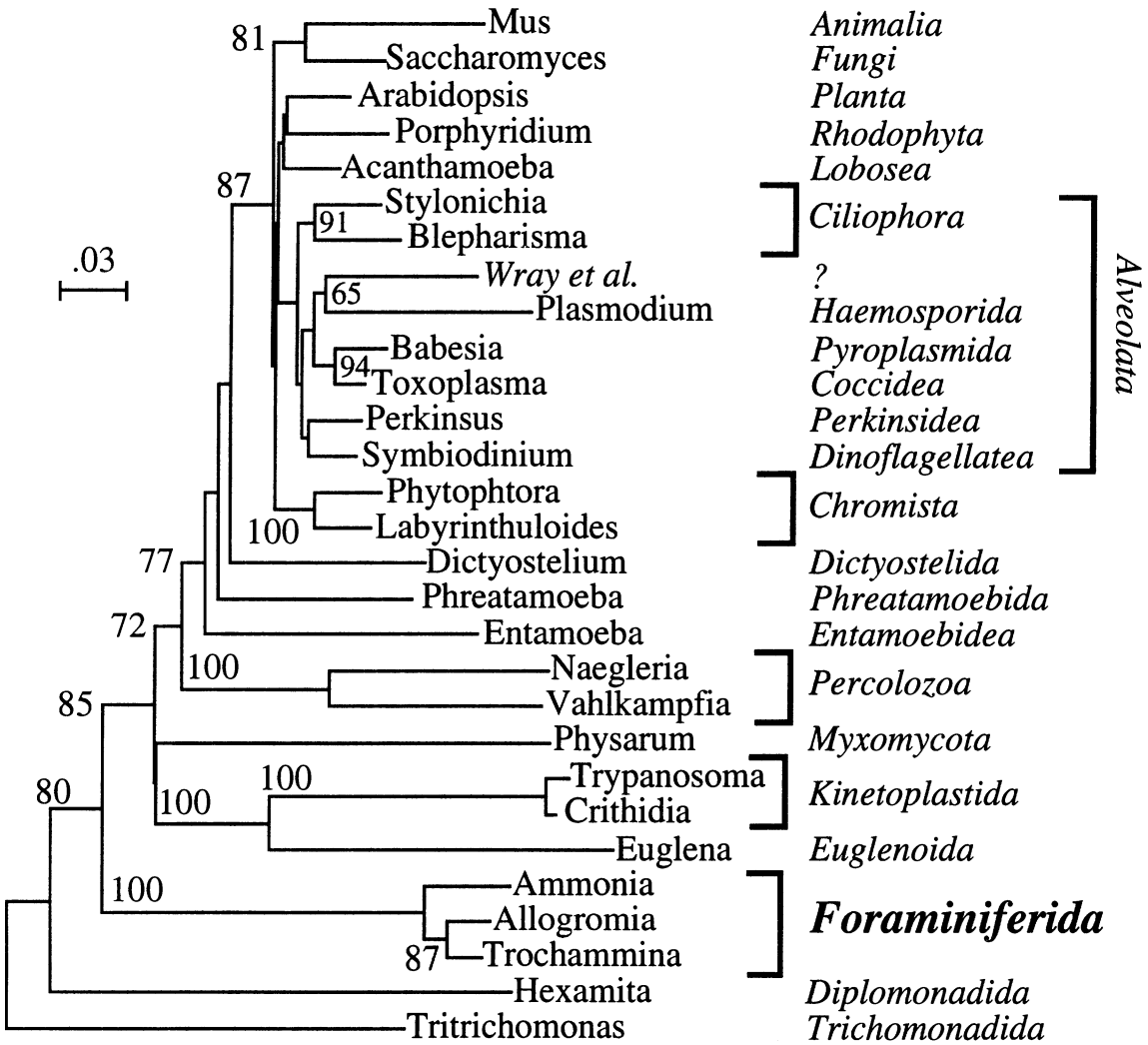
SAMPLING AND MOLECULAR TECHNIQUES

The procedure to obtain DNA sequences from foraminiferal cells consists of the following steps: cell preparation, DNA extraction, PCR amplification, cloning of PCR products (optional), and sequencing. Except for the preliminary steps, all other methods are similar to those applied to any kind of DNA samples. An abundant literature exists on different molecular techniques (Maniatis 1982, Innis et al. 1990), including those particularly applied to molecular systematics studies (Hillis et al. 1996). The attention will be focused here only on the methodological aspects that are specific to Foraminifera and can facilitate their molecular study.

Preparation and storage of foraminiferal cells.

The first step in preparation of Foraminifera for DNA study is to ascertain that the specimens designated for DNA extraction are alive. This may seem trivial, but the distinction between living and dead foraminiferal cells is not always evident. The presence of cytoplasm inside the foraminiferal test is not always a sufficient criterion. It occurs frequently, especially in material isolated from sediment samples, that not all specimens containing cytoplasm give positive results in PCR examination. It seems that the cytoplasm can remain intact inside the foraminiferal tests for long periods of time, while the DNA is degraded more rapidly. The best criterion for identification of living specimens is the observation of extended pseudopodes or the detection of cytoplasmic movement inside the tests. In some agglutinated species, having no translucent tests and very short and fine pseudopodes, the distinction of living specimen is really difficult. Therefore, it is sometimes necessary to maintain foraminifera designated for molecular study in culture dishes and to observe their behavior for a few days.

In case it is impossible to rapidly extract DNA or to maintain Foraminifera in culture, the collected specimens must be stored for some time under conditions that prevent DNA damage. Several different preservation methods were tested using *Ammonia* specimens (Holzmann and Pawlowski 1996). The best results were obtained with frozen samples stored at -20°C. Positive results were obtained also with air-dried samples stored at the room temperature for few weeks, up to 3 years, however longer storage under these conditions provokes degradation of the DNA and, in consequence, allows amplification of shorter DNA fragments only. On the other hand, chemical fixatives, including ethanol and formaldehyde, do not seem to be suitable for preservation of foraminiferal DNA.



TEXT-FIGURE 2
SSU rDNA tree of eukaryotes showing the phylogenetic position of the foraminifera. The tree was inferred by neighbor-joining method. Horizontal distances are proportional to inferred evolutionary distances according to a scale given in substitutions per site. Bootstrap percentage values greater than 50% (out of 1,000 replicates) are given next to each internal branch. The position of the sequence attributed to *Ammonia beccarii* by Wray et al. (1995) is indicated.

DNA extraction

Among different extraction methods tested with foraminifera, the best results have been obtained using sodium deoxycholate - DOC (Pawlowski et al. 1994), guanidine (de Vargas, in prep.), and cetyltrimethylammonium bromide - CTAB (Clark 1992). Detailed protocols of these methods are given in appendix 1. DOC and guanidine-based methods are particularly convenient for field extractions. These methods are simple, rapid, and do not necessitate any sophisticated equipment. The CTAB method is much more time consuming and demands an access to a high speed centrifuge and -20°C freezer. CTAB method, however, is the only one that prove to be efficient for DNA extraction from allogromiids and larger calcareous foraminifera (Pawlowski et al. 1996). Guanidine gives very good results in case of planktonic foraminifera (de Vargas et al. 1997), while the DOC buffer is suitable for DNA extraction from small calcareous and agglutinated species (Pawlowski et al. 1996).

Prior to DNA extraction, specimens should be individually cleaned by brushing them under a dissecting microscope in filtered sea water. This will eliminate most of the associated microorganisms and, what is even more important, will ensure that no other tiny or juvenile foraminifer, which could be recognized by specific primers, remains attached to the test. Although the use of specific foraminiferal primers a priori excludes the possibility of amplifying extraneous DNA, the specificity of primers may not be perfect. Depending on the PCR conditions some primers can match and amplify unwanted DNA. Therefore, careful handling of specimens being prepared for DNA extraction is the best way to avoid contamination.

Usually, a single foraminiferal specimen ground in 50µl of extraction buffer (DOC, guanidine) provides a sufficient amount of genomic DNA for PCR amplification of rRNA genes. Most of the studies, especially those on intraspecific variations, are based on single cell extractions (Holzmann et al. 1996). However, when the specimens are smaller than 100 microns, it may be better to prepare extracts from several specimens. On the other hand, extraction of larger foraminifera should be carried out in larger volumes of extraction buffer, up to 500µl. As the ef-

TABLE 1
List of amplification and sequencing primers.

Primer	Sequence	Orient.	Specificity	Position
SSU				
SA	ggt tga t(ct)c tgc cag a	Forward	Universal	6-21 (Mus)
SA10	ctc aaa gat taa gcc atg caa gtg g	Forward	Forams	35-59 (Amm)
SDS1	gtt tgg cta ata cgt acg	Forward	Forams	264-277 (Amm)
S4F	tct aag gaa cgc agc agg	Forward	Forams	578-595 (Amm)
S4rf	cgc ctg ctg cgt tcc tta g	Reverse	Forams	579-597 (Amm)
S6f	ccg cgg taa tac cag ctc	Forward	Forams	934-951 (Amm)
S13	gca aca atg att gta tag gc	Reverse	Forams	957-976 (Amm)
S10	cac tgt gaa caa atc ag	Forward	Forams	1060-1076 (Amm)
S8f	tcg atg ggg ata gtt gg	Forward	Forams	1183-1199 (Amm)
S14rf	cct tca agt ttc aca ctt gc	Reverse	Forams	1809-1828 (Amm)
S14F1	aag ggc acc aca aga acg c	Forward	Forams	1838-1856 (Amm)
S15r	gtg gtg cat ggc cgt	Forward	Forams	2002-2016 (Amm)
S17	cgg tca cgt tcg ttg c	Reverse	Forams	2159-2174 (Amm)
S18	taa cag gtc tgt gat gcc	Forward	Universal	1485-1502 (Mus)
S20	ttg tac aca ccg ccc gtc	Forward	Universal	1691-1709 (Mus)
S20r	gac ggg cgg tgt gta caa	Reverse	Universal	1709-1691 (Mus)
S21F1	cct tgt tac gac ttc tc	Reverse	Forams	2831-2847 (Amm)
Rib B	tga tcc ttc tgc agg ttc acc tac	Reverse	Universal	1843-1866 (Mus)
LSU				
2TA	cac atc agc tcg agt gag	Forward	Forams	1-18 (Amm)
2TAIC	ctc act cga gct gat gtg	Reverse	Forams	1-18 (Amm)
L5	ttc (ag)ct cgc c(ag)t tac t	Reverse	Universal	84-99 (Mus)
L7	gat g(at)g tca tta cca cc	Forward	Forams	451-467 (Amm)
L1F	act ctc tct ttc act cc	Forward	Forams	612-628 (Amm)
L10	ctg acg tgc aaa tcg tt	Forward	Universal	1406-1422 (Mus)
LO	gct atc ctg ag(ag) gaa act tcg	Reverse	Universal	1482-1498 (Mus)

Note: Position of primers is given according to the sequences of mouse or *Ammonia*

ficiency of PCR reaction depends on both the amount of DNA and the inhibitory effect of the extraction by-products, it is recommended to prepare several extractions with different number of specimens, at least when studying new species.

PCR amplification, cloning and sequencing

The conditions of PCR amplification, cloning and sequencing used for DNA study of foraminifera are similar to those described for other organisms (reviewed by Hillis et al. 1996). Choice of primers is crucial for the efficiency of PCR reaction. To avoid the problem of contamination, it is important always to use at least one foraminiferal specific primer. A list of primers suitable for study of foraminiferal rDNA, including foraminiferal specific and universal eukaryotic primers, is presented in Table 1. Their approximate position is illustrated in text-figure 1.

After purification, the PCR products can be sequenced directly or after cloning. Among foraminifera examined so far, direct sequencing has only succeeded for planktonic species. Cloning of PCR products obtained from benthic species has been necessary. All attempts to directly sequence these products gave multiple, unreadable sequences, probably due to high intracellular variability of foraminiferal rRNA genes (see Sequence Variations section).

CHARACTERISTICS OF RIBOSOMAL GENES OF FORAMINIFERA

Molecular systematics of foraminifera is based principally on ribosomal RNA gene sequences. Until now, more than two hundreds sequences of foraminifera have been deposited in the GenBank/EMBL data base. These sequences usually represent a 400 bp fragment situated at the 5' end of the LSU rDNA and a

1000 bp fragment situated at the 3' end of the SSU rDNA. At the moment complete SSU rDNA sequences exist for ten species only. An almost complete LSU rDNA sequence is available for *Rotaliella elatiana* only.

Compared to other unicellular eukaryotes, the ribosomal genes of foraminifera display several peculiar features: (1) unusual length, (2) numerous insertions, (3) frequent substitutions in conserved regions, (4) unequal base composition, and (5) intracellular sequence variations. The length and G+C content of the SSU rDNA of 10 species representing the major groups of foraminifera are presented in table 2.

Structure and length of rRNA genes

Like other eukaryotes, Foraminifera possess a typical nuclear rRNA genes (rDNA) structure comprising a repeated tandem unit composed of SSU and LSU rRNA separated by an internal transcribed spacer (ITS). Both SSU and LSU genes are a mosaic of highly conserved "core" segments and hypervariable expansion segments (called also "variable regions"). The ITS region in Foraminifera was amplified using primers situated at the 3' terminal region of the SSU rDNA and in the 5' end of the LSU rDNA (Pawlowski et al. 1996). However, ITS region has not been sequenced yet and it is unknown whether the typical eukaryotic structure of this region, including the ITS1 - 5.8S gene - ITS2, is present in foraminifera.

The most distinctive character of foraminiferal rRNA genes is their unusual length. The SSU and LSU rRNA genes of benthic and planktonic foraminifera are the longest ribosomal genes described so far. The average length of the SSU rDNA in benthic foraminifera ranges from 2200 to 3500 nucleotides (nt), while the typical eukaryotic SSU gene does not exceed 2000 nt. At the moment, the longest known SSU rRNA gene (more than 5500 nt !) was found in the planktonic foraminifer *Orbulina universa* (unpublished data). The LSU rRNA gene of *Rotaliella elatiana*, which length exceeds 5000 nt, is among the longest known LSU rRNA genes.

The great length of foraminiferal rDNA sequences results from several long insertions in variable regions of the molecule. Remarkably, some insertions may form new expansion segments that are unique to foraminifera. For example, three such segments have been found in the 3' region of the SSU rDNA of planktonic foraminifera (de Vargas et al. 1997). The origin of these new expansion segments, however, is unknown. A link between some short expansion segments and the presence of a group I intron was proposed from sequence analysis of the LSU rRNA gene of *Rotaliella elatiana* (I.Bolivar, pers. comm.). In this species, a group I intron was identified in the conserved region C9, at the homing site for most LSU rRNA introns. Surprisingly, *R. elatiana* gene also contains two 50-70 nt inserts close to the intron splicing site. Both inserts have been found in the mature RNA of this species and other benthic foraminifera. As these inserts display significant homologies with the *Rotaliella* intron, they may derive from it. Indeed, intron-derived sequences may play an important role in formation of the expansion segments of the rRNA genes in foraminifera.

Nucleotide composition

The foraminiferal rRNA genes show relatively low G+C content compared to other eukaryotes. The nucleotide composition of ribosomal genes, however, varies between different groups of foraminifera (Table 2). The lowest G+C content was observed in the Miliolida (29-32%) and Astrorhizida (30-31%), while in the Globigerinida, it averages 45%. Although the expansion segments contain usually more A+T-rich fragments than the conserved regions, there is no direct relation between the G+C content and the size of the ribosomal genes. The lowest G+C content is observed in the Miliolida that have the shortest rDNA sequences among foraminifera. This group seems to have a very strong bias towards A+T substitutions. A similar bias is observed in 3' terminal region of the SSU in *Ammonia* + *Elphidium* group.

TABLE 2
Length and GC content of SSU rDNA of foraminifera.

Order	Species	Length (nt)	GC content
Allogromiida	*Allogromia sp.*	3043	33%
Astrorhizida	*Astrorhiza triangularis*	4067	29%
Miliolida	*Borelis schlumbergeri*	2866	27%
	Peneroplis pertusus	2464	29%
	Sorites orbiculus	2264	30%
Textulariida	*Trochammina sp.*	3341	37%
	Eggerelloides scabrum	3536	36%
Rotaliida	*Bolivina spathulata*	3111	41%
	Nummulites venosus	3366	42%
	Ammonia sp. 1	2864	46%
Globigerinida	*Orbulina universa*	3985*	48%
	G.truncatulinoides	2989*	44%

Note: (*) corresponds to about 3/4 of the total length

Sequence variations

The mosaic character of rRNA genes, intermingling variable and conserved regions, allows their use for studying phylogenetic relationships at different taxonomic levels. According to the first results, the slowly evolving conserved regions of the SSU rDNA are appropriate to examine the relationships between orders of foraminifera, while the rapidly evolving expansion segments can be used for studying closely related species. The divergent domain D1 of the LSU rDNA has been shown to be appropriate for resolving relationships at the species level (Holzmann et al. 1996). Higher variability is expected also in the ITS region, however, the study of this region is not yet accomplished.

A particular feature of the foraminiferal rRNA genes is the heterogeneity of rRNA copies within a single individual. The intracellular variations of rRNA copies were first observed in the LSU rDNA of *Ammonia* (Holzmann et al. 1996). As suggested by multiple ambiguities in the directly sequenced PCR products, these variations occur also in other foraminifera, except for some planktonic species (the only ones that can be sequenced directly). This heterogeneity contrasts with the generally accepted theory that the multiple copies of the ribosomal genes of a single cell should be identical (Lewin 1994). The factors responsible for these variations are unclear. It was proposed that the ribosomal sequence variability appears in the multinuclear agamonts as a result of some unknown process of somatic nucleus differentiation (Holzmann et al. 1996). This hypothesis, however, needs to be confirmed by study of species in which life-cycle generations can be easily distinguished.

APPLICATIONS OF MOLECULAR DATA

Since the description of the first foraminiferal DNA sequences, different aspects of molecular evolution of Foraminifera have

been examined. The research has been focused on four main topics: (1) origin of Foraminifera, (2) higher-level relationships between major foraminiferal groups, (3) evolutionary rates, and (4) intraspecific variations.

Origin of Foraminifera

The phylogenetic position of foraminifera in the rRNA tree of eukaryotes was the first problem addressed by using molecular data. In spite of a relatively good knowledge of the evolution of foraminifera, the paleontological data yield no evidence concerning the foraminiferal origin. A lack of shared morphologic features between foraminifera and other protists makes phylogenetic placement of this group even more difficult. Classically, foraminifera, together with a few Athalamida and Monothalamida, constitute the class (or phylum) of the Granuloreticulosea, characterized by granular reticulopodes that form anastomosing network with distinctive 2-way streaming (Lee et al. 1985). In recent classifications of Protista, foraminifera are considered as a separate class or phylum (Margulis et al. 1990) or included either in the phylum Rhizopoda (Corliss 1994) or parvkingdom Neosarcodina (Cavalier-Smith 1993).

The first attempts to investigate the origin of foraminifera based on molecular data gave conflicting results. The phylogenetic analysis of partial sequences of the LSU rDNA have shown that foraminifera branch close to slime molds (*Dictyostelium, Physarum*) and *Entamoeba* in the eukaryotic tree (Pawlowski et al. 1994). However, on the basis of two 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 rRNA trees, it was obvious that in one case, PCR-amplified sequences have been erroneously attributed to the foraminifera.

Several complete and partial SSU rDNA sequences of benthic and planktonic foraminifera later confirmed the LSU rDNA tree topology (Pawlowski et al., 1996; Darling et al. 1996, Wade et al. 1996). Phylogenetic analysis of these sequences, compared to those of other unicellular eukaryotes, place the foraminifera in the middle part of the eukaryotic tree, between the amitochondriate Diplomonads and Euglenozoa, i.e., even deeper than in the LSU rDNA tree (text-figure 2). In both LSU and SSU rRNA trees, the branch separating foraminifera from the upper part of the tree is associated with a bootstrap score of 100%, making unlikely their origin within the clade of alveolates as proposed by Wray et al. (1995).

According to ribosomal sequence data, the foraminifera diverged early in the evolution of eukaryotes, among the earliest mitochondriate lineages. Such early origin, however, contrasts with relatively late appearance of the first fossil foraminifera (about 540 millions years ago) and with several highly evolved characters that these organisms possess. It is probable, that foraminifera have evolved from some ancestral protists that did not form tests or from some forms having organic membraneous tests which are poorly preserved in the fossil record. Alternatively, the position of foraminifera in the rRNA trees may be biased by very rapid rates of rRNA evolution in these organisms that produce an artificial grouping of foraminifera with early evolved protists lineages (Sogin 1997).

Macroevolutionary relationships

Foraminifera form a group with probably the best known and the most complete evolutionary series, yet the macroevolutionary relationships between these series remain largely unknown. The distinction of the major groups of fossil and Recent foraminifera relies on the composition and structure of their wall (Loeblich and Tappan 1988). Few existing models of foraminiferal macroevolution imply the progressive transformation of the test, from the primitive membraneous-walled type to the agglutinated one, and finally to the secreted calcareous wall (Hansen 1979). Classically, all lineages of foraminifera were assumed to have originated from some membraneous-walled Allogromiida, that have evolved into unilocular agglutinated Astrorhizida, sometime in the Late Precambrian (Loeblich and Tappan 1974). The Astrorhizida were thought to have given rise to almost all agglutinated and calcareous groups (Grigelis 1978). More recently, Tappan and Loeblich (1988) suggested that the group including the Miliolida, Lagenida and Spirillinida may have evolved independently from some extinct Fusulinida, which derived directly from the Allogromiida.

First attempts to resolve higher-level relationships within foraminifera by using 20 partial SSU rDNA sequences confirmed the monophyletic origin of foraminifera and the distinction of the major morphotaxonomic groups (Pawlowski et al.1997). The phylogenetic analysis, however, was hindered by important differences in rates of substitutions between planktonic and benthic lineages. In order to improve resolution of the phylogeny of benthic foraminifera, some 40 additional sequences have been obtained and analyzed (text-figure 3). At the same time, the phylogenetic analyses of planktonic foraminifera, based on corresponding SSU rDNA sequences have been published (de Vargas et al. 1997, Darling et al. 1997). Analysis of all these data led to the following general conclusions:

1) Miliolida appear as the earliest diverged group of foraminifera. This idea was previously postulated. Based on morphological observations, Arnold (1978, 1979) proposed that the Miliolida arose directly from some allogromiid-like ancestor. An independent origin of Miliolida has been also suggested by Tappan and Loeblich (1988) who proposed that this group evolved from Fusulinida, which in turn evolved from Allogromiida. The molecular data are consistent with these interpretations suggesting that Miliolida evolved independently from a group of naked foraminifera. However, according to ribosomal data the ancestors of Miliolida may be not related to the recent Allogromiida. There are important differences in the structure of rRNA genes between Miliolida and examined Allogromiida. Moreover, it was found by immunoblotting with monoclonal antibodies that Miliolida possess one type of actin of a very high molecular weight, while two actins of lower molecular weight were detected in Allogromiida, Textulariida and Rotaliida (Fahrni and Pawlowski 1995). All these evidences favor the hypothesis that Miliolida form a distinctive group of foraminifera, that diverged long before the first testate foraminifera appeared.

2) Allogromiida and Astrorhizida are closely related. According to the traditional classification, the unilocular agglutinated Astrorhizida differ from unilocular tectinous Allogromiida exclusively by the presence of an agglutinated test (Loeblich and Tappan 1964). However, several authors pointed out that this separation may be artificial (Lipps 1973). Recent morphological, cytological and behavioral observations (Bowser et al. 1995) demonstrated the close affinities between both groups and raised some questions regarding their taxonomic and phylogenetic relationships. SSU rDNA sequence analysis confirms

the close phylogenetic relationships between Allogromiida and Astrorhizida, invalidating their taxonomic separation and demanding a major taxonomic revision of both groups.

3) Globigerinida have polyphyletic origin. This hypothesis contrasts with a generally accepted idea that the planktonic Globigerinida evolved from a single benthic lineage in the Mid-Jurassic (Caron and Homewood 1983) and that the subsequent planktonic radiations following major extinctions evolved from surviving planktonic forms (Tappan and Loeblich 1988). The molecular data confirm the paleontological separation of recent species into three clades: (1) the spinose, globular globigerinids, (2) the non-spinose, more or less flattened and often carenated globorotaliids and (3) the microperforate group, the Candeinidae (de Vargas et al. 1997). The three groups, however, do not cluster together in the SSU rDNA tree (text-figure 4). A single representative of Candeinidae (*Globigerinita glutinata*) is unambiguously placed within the benthic Rotaliida, far from the rest of the planktonic species, suggesting that the microperforate foraminifera diverged independently from a benthic lineage. The globorotaliids branch either as a sister group of the globigerinids or within the Rotaliida. However, this later branching is supported by the existence of several homologous regions in the expansion segments of globorotaliids and rotaliids, regions which are not found in the globigerinids.

All these hypotheses need to be confirmed by further study and analysis of molecular data. More results are necessary also to resolve the relationships between Textulariida and Rotaliida. Both groups seem to be very closely related but it is uncertain to what extent this results from the similarity of their evolutionary rates. Some uncertainty also concerns the position of the *Ammonia-Elphidium* group. In most phylogenetic analyses, this group branches close to the miliolids, in clear opposition with morphological classification. The position of the *Ammonia-Elphidium* group is probably artifactual due to the similar bias in base substitutions as in the Miliolida. The comparison of actin molecular weight shows a clear difference between both groups (Fahrni and Pawlowski 1995). Actin also shows that the genus *Miliammina* is clearly related to the Miliolida (Fahrni et al. 1998), which renders the position of *Miliammina* in the rDNA tree, between Miliolida and Astrorhizida, uncertain.

Rates of evolution

Comparing the number of substitutions with the divergence times inferred from the fossil record allowed estimate of absolute rates of rDNA evolution for major groups of foraminifera (Pawlowski et al. 1997). This study revealed extremely important variations of evolutionary rates between different foraminiferal lineages and particularly between the planktonic and benthic species (Table 3). Some planktonic Globigerinida seem to evolve 50 to 100 times faster than any other foraminiferal lineage. Within the benthic foraminifera, the evolutionary rates vary by factor of 30; the highest values were found in the *Ammonia-Elphidium* group, and the lowest in the clade Rotaliida + Textulariida (0.2 subst./site/10^9 years). Important variations of evolutionary rates were also found between and within different families of planktonic foraminifera. In the Globigerinidae, rates are very high but relatively stable, ranging from 3.2 to 4.7, with a mean value of 4 subst./site/10^9 years. On the other hand, in the Globorotaliidae, three species have a rate of about 1 subst./site/10^9 years, while two species (*G. truncatulinoides, G. menardii*) evolve much faster with rates of more than 7 subst./site/10^9 years (de Vargas and Pawlowski 1998).

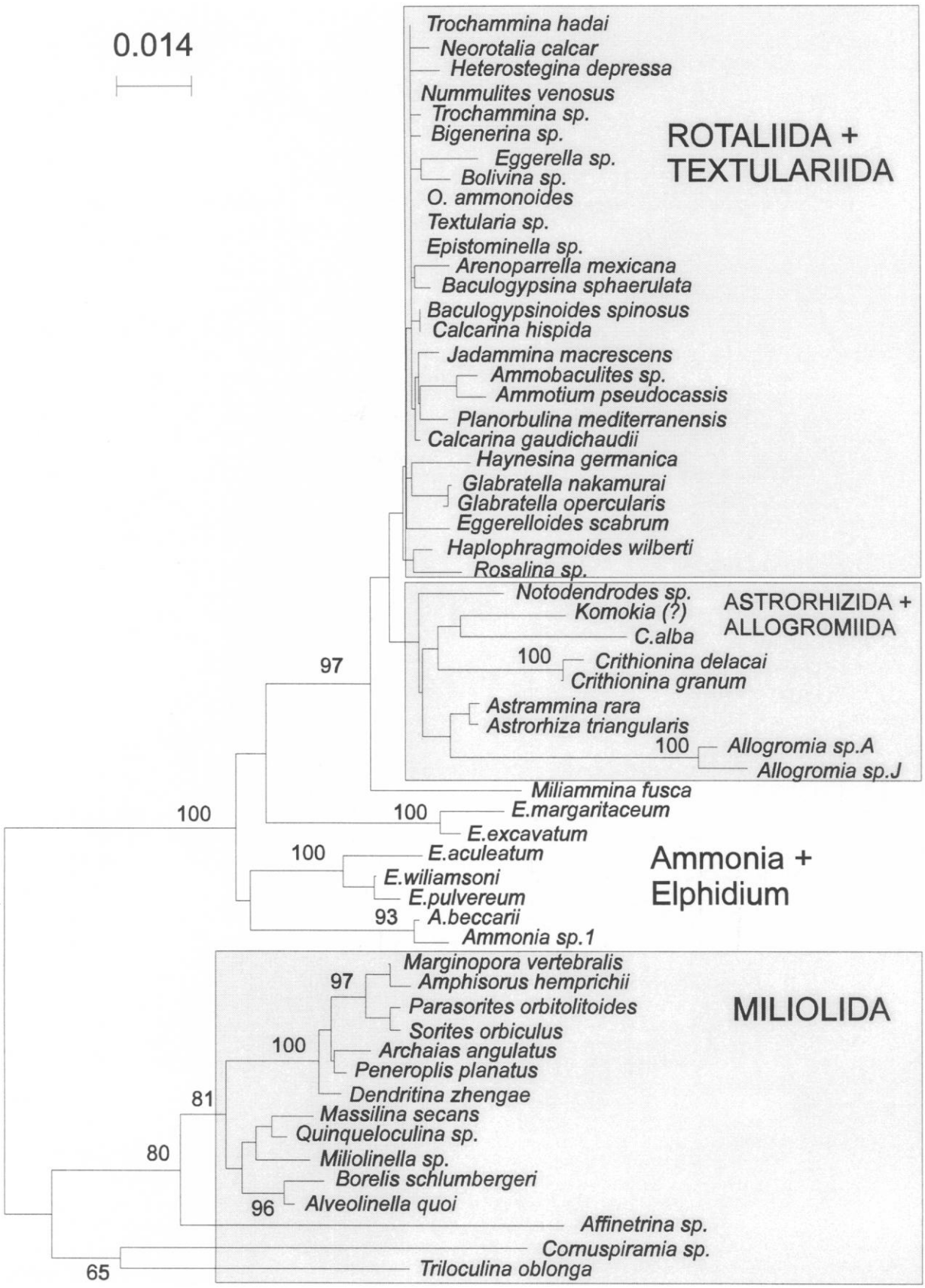
The rates of slowly evolving foraminiferal lineages are close to the estimated rates of vertebrate rRNAs (Hedges et al. 1990). On the other hand, the rates for planktonic foraminifera surpass by far all rates reported until now for SSU rDNA sequences. The mean value of all computed globigerinids rates (3 subst./site/10^9 years) is about 17 times higher than the mean substitution rate proposed for 18S rRNA genes of diatoms, which evolve two to three times faster than Metazoa (Sorhannus 1996).

The reason of such extreme acceleration of rDNA evolution in planktonic foraminifera remains unclear. It has been proposed that the difference in evolutionary rates between planktonic and benthic foraminifera is due to the different mode and tempo of reproduction in both groups (de Vargas et al. 1997). The benthic foraminifera reproduce slowly, occasionally undergoing alternations of asexual and sexual generations (Lee et al. 1991), while in some planktonic foraminifera, the sexual reproduction involves several hundred thousands of gametes released by one individual every two weeks (Hemleben et al. 1989). Similar argumentation was given to explain the differences in rates of evolution between planktonic and benthic diatoms (Kooistra and Medlin 1996). Another factor that may be responsible for the higher mutation rates in planktonic foraminifera is their increased exposure to solar UV radiation compared to benthic species (Pawlowski et al. 1997). Both reproduction and UV radiation, however, can hardly explain the differences of rates observed between closely related species, for example, within Globorotalidae. In this case, some drastic changes in the DNA replication or repair mechanisms could be involved, but the nature of these changes remains enigmatic.

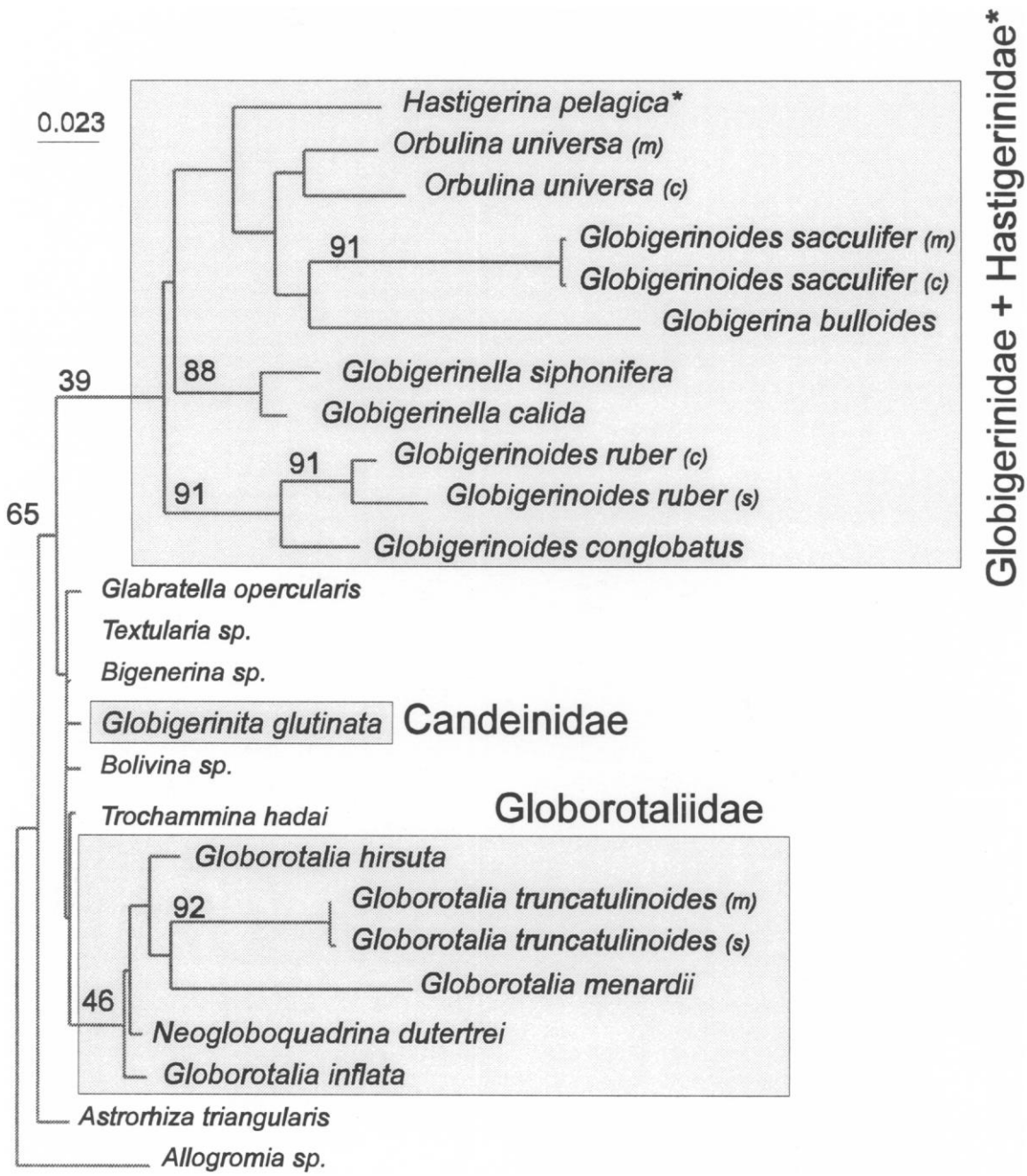
Intraspecific variations

One of the most important application of molecular data is their use as a tool for species determination. Traditionally, foraminiferal species are distinguished on the basis of morphological characters: number and form of the chambers, form of the test periphery and type of ornament (Haynes 1981). Large morphological variability of foraminiferal tests renders species identification particularly difficult. Molecular data provide a new taxonomic criterion, which is independent from the morphological characters of the test.

The first application of rDNA sequence data for taxonomic identification allowed two morphologically similar species of the genus *Glabratella* from the Mediterranean Sea to be distinguished (Pawlowski et al. 1994). A study of the systematics of *Glabratella* from the Japanese Islands is in progress (Tsuchiya 2000, this volume). Several studies are concerned with the species identification in the genus *Ammonia* (Pawlowski et al. 1995; Holzmann et al. 1996; Holzmann and Pawlowski 1997; Holzmann et al. 1998). These studies allowed as many as seven genetically distinctive forms to be recognized. Comparison of genetic, morphologic and ecological characteristics indicates that these forms correspond to seven different species of *Ammonia* (see chapter by Holzmann). In planktonic foraminifera, cryptic species have been identified within *Globigerinella siphonifera* (Huber et al. 1997) and *Orbulina universa* (de Vargas et al. 1999).



TEXT-FIGURE 3
Phylogeny of benthic foraminifera inferred by neighbor joining method from 58 sequences of SSU rDNA. Bootstrap percentage values greater than 50% (out of 1,000 replicates) are indicated along branches.



TEXT-FIGURE 4
Evolutionary relationships between 14 planktonic (shaded rectangles) and 7 benthic foraminifera inferred by maximum likelihood method from partial SSU rDNA sequences. Bootstrap proportions greater than 50% are indicated along branches. Scale is given in substitution/site.

PERSPECTIVES

A considerable effort has been made to develop the molecular systematics of foraminifera. Different methodological approaches were used to confirm the authenticity of the first foraminiferal sequences. More rapid and effective protocols of foraminiferal DNA extraction have been established. The ribosomal genes of major groups of foraminifera are characterized, and specific foraminiferal primers are designated. All these achievements have facilitated access to foraminiferal genome and opened some new perspectives for exploring molecular evolution of foraminifera as well as to compare the interpretation of molecular and micropaleontological data.

The results obtained have allowed some preliminary conclusions that need to be confirmed by further study and analysis of molecular data. Among the questions that remain unanswered is the problem of the origin of foraminifera. Although the analysis of rDNA sequences indicates that foraminifera branch among the earliest mitochondriate eukaryotes, this interpretation may be an artifact of rapid rates of evolution of the ribosomal genes

TABLE 3
Rates of SSU rDNA evolution in different lineages of foraminifera.

Order	Species	Divergence time (MYA)	Subst. per site	Rate of substitution (per site/per 10^9 year)
Miliolida	*Archaias - Peneroplis*	45-55	0.007	0.07
	Marginopora - Amphisorus	22	0.005	0.11
Textulariida	*Bigenerina - Textularia*	55-65	0.002	0.02
Rotaliida	*Bolivina - Glabratella*	> 90	0.02	0.02
Globigerinida	*Orbulina - G. sacculifer*	19	0.17	4.5
	G.ruber - G.conglobatus	9	0.08	4.6
	G. inflata - G. hirsuta	18	0.036	1

in this group. To verify whether the present position of foraminifera in the eukaryotic tree is correct, it will be necessary to obtain and analyze sequences of some protein-coding genes and to compare them with the results of rDNA analysis.

Further studies on macroevolutionary relationships among foraminifera need also to be conducted. An analysis of complete SSU or LSU rDNA sequences is necessary to confirm or revise the conclusions obtained by using partial rDNA sequences only. A study of protein-coding genes sequences may be indispensable to establish the phylogenetic position of Miliolida and to confirm the hypothesis of their early origin as suggested by rDNA sequences. Protein sequences may also be helpful to resolve some controversy about the position of the *Ammonia-Elphidium* group in the rRNA trees. Further research is needed to examine the relationships between different groups of membraneous, agglutinated and calcareous foraminifera, addressing the question of the importance of the wall structure as a higher-rank taxonomic criterion. The phylogenetic position of such groups as Spirilinida and Lagenida, for which DNA sequences are not yet available, remains unknown. There is also very little information about the origin of some primitive membraneous or agglutinated forms, which are traditionally thought to be related to foraminifera, but whose origins remain enigmatic.

There is also a need to further develop studies on intraspecific variations in foraminifera. A study of genetic diversity in the genus *Ammonia* shows that analysis of rDNA sequences combined with morphological, ecological and biogeographical data delineates species boundaries and requires revision of morphotaxonomic characters used for species identification in this genus. Similar studies of other cosmopolitan genera will contribute to a better understanding of the species concept in foraminifera and will help in the interpretation of their morphologic variability. The genetic diversity within some genera of planktonic foraminifera is of particular interest because of their common use in paleoceanographic and paleoecological studies. A preliminary study shows the presence of some cryptic species in this group. However, the use of molecular data to examine a relationship between the species boundaries and water masses distribution remains to be investigated.

Finally, the preliminary attempts to use foraminifera for understanding the mechanisms of molecular evolution are very promising. Because of their excellent fossil record, foraminifera offer a unique possibility to provide a time dimension to the molecular phylogenies and thus to deepen the general knowledge of the tempo and mode of molecular evolution. The results obtained until now indicate that the variations in the rate of rRNA evolution may largely exceed generally accepted values. However, the impact of these variations on the interpretation of protist phylogeny has to be carefully examined.

The use of combined molecular and paleontological data poses several basic questions concerning the rates of evolution. Are the evolutionary rates changing gradually or episodically ? What is the relation between changes of molecular rates and changes in nucleotide composition ? How can we explain the sudden acceleration of rates in some species ? Do local molecular clocks exist in foraminifera ?

The molecular systematics of foraminifera is in its infancy, however, it has already shown extraordinary potential not only to solve some systematic questions, but also to contribute to a better understanding of more basic biological problems.

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APPENDIX 1

Protocols of the DNA extraction from foraminifera (Clark 1992).

Protocol 1. DOC method

Lysis buffer:

TRIS 1M pH 8.5	100µl
EDTA 0.5M (pH 8.0)	8µl
DOC (Na deoxycholate) 10%	100µl
TX-100 10%	20µl
H2O	780µl
Total	1000µl

Procedure:
1. Grind 1 specimen in 50µl of lysis buffer.
2. Incubate at 60°C for 1h.
3. Centrifuge at 10,000 rpm for 5 min (optional).
4. Store at -20°C.

Protocol 2. Guanidine method

Lysis buffer (protocol modified after Maniatis et al. 1982, p. 189):
1. To 100 g guanidinium isothiocyanate add:
   - 100 ml of deionized H2O
   - 10.6 ml 1M Tris/HCl (pH 7.6)
   - 10.6 ml 0.2 M EDTA
2. Stir overnight at room temperature
   (heat to 60-70°C for 10 min to assist dissolution)
3. Add 21.2 ml of 20% Sarkosyl (Na lauryl sarkosinate)
4. Bring the volume to 210 ml with sterile H_2O.
5. Filter through a disposable filter.
6. Add 2.1 ml of ß-mercaptoethanol and mix.
7. Store at 4°C in brown glass bottle.

Procedure:
1. Dissolve specimen in 50-100µl of lysis buffer (grind it if necessary).
2. Centrifuge shortly to discard the remnants of test.
3. Add 1 volume of isopropanol.
4. Allow precipitate the DNA for at least 2 hours at -20°C.
5. Centrifuge at 15,000 rpm for 15 min.
6. Pour off the supernatant.
7. Wash the pellet with 70% ethanol
8. Centrifuge at 15 000 rpm for 5 min.
9. Pour off the supernatant.
10. Dry the pellet and dissolve in 50 l of H_2O.
11. Store at -20°C.

Protocol 3. CTAB method

Reagents needed:
- TE pH 7.6 - 10 mM Tris-HCl (pH 7.6); 1 mM EDTA (pH 8.0)
- Lysis buffer - 0.1 M EDTA (pH 8.0); 0.25% SDS
- CTAB 10% in 0.7 M NaCl
  (heat at 65°C to dissolve and preheat before each extraction)
- 3.5 M NaCl
- Cl - Chloroform/isoamyl alcohol (24/1 v/v)
- PCl - Phenol saturated with 10mM Tris-HCl, pH 8.0
- Proteinase K (10 mg/ml)
- 100% Ethanol

Procedure:
1. Ground specimens in 50µl of TE.
2. Add 250µl of lysis buffer.
3. Add 3µl of Proteinase, vortex.
4. Incubate at 55°C for 1 h.
5. Add 75µl of NaCl 3.5 M, vortex.
6. Add 42µl of CTAB 10%, incubate at 65°C for 15 min.
7. Add 1 volume (420µl) of Cl and mix gently for 5 min.
8. Centrifuge for 10 min at max speed.
9. Take upper (aqueous) phase and add an equal volume of PCl.
10. Centrifuge at 15000 rpm for 10 min.
11. Take upper (aqueous) phase and add 2.5 volumes of 100% ethanol
12. Allow the DNA precipitate at -20°C for overnight.
13. Centrifuge at 15,000 rpm for 10 min.
14. Wash pellet with 70% ethanol.
15. Centrifuge at 15,000 rpm for 10 min.
16. Resuspend the pellet in 50µl H_2O; store at -20°C.