

These organisms are no more "one-celled animals and one-celled plants" than people are shell-less multicellular amebas.
Lynn Margulis, 1990

CHAPTER 1

GENERAL INTRODUCTION

1.1. What is a foraminifer?

Foraminifers (often abbreviated to 'forams') are unicellular organisms distributed worldwide. Traditionally, foraminifers were studied by paleontologists and for that reason they are mainly known as organisms bearing a shell (called a test) and living in marine environments. However, recent publications showed that naked (without a test) and/or fresh water protists such as *Reticulomyxa filosa* (Pawlowski et al., 1999a; 1999b), *Toxissarcon synsuicidica* (Cedhagen & Pawlowski, 2002; Wilding, 2002) or the terrestrial *Edaphoallogromia australica* (Meisterfeld et al., 2001) are also foraminifers. These results demonstrate that the definition of foraminifers has to be based rather on other features, such as the nature of the pseudopodia than on the occurrence in marine environments or the presence of a shell (Pawlowski et al., 1999a; 1999b; Pawlowski & Holzmann, 2002). Foraminifers have rather thin pseudopodia, which are called granuloreticulopodia, because they contain granules and form a network. Because of their reticulopods, foraminifers were traditionally placed in the class Granuloreticulosea and grouped with lobose and filose amoebae in the superclass Rhizopoda, subphylum Sarcodina (Lee et al. 1985). However, the first molecular data, mainly ribosomal DNA sequences, challenged the monophyly of Rhizopoda (Clark & Cross, 1988; Cavalier-Smith, 1993, 1998). New classifications, based on molecular phylogenies of several genes show that the foraminifers are closely related to the Cercozoa (Keeling, 2001; Simpson & Roger, 2002; Archibald et al., 2003; Baldauf, 2003; Berney & Pawlowski, 2003; Longet et al., 2003), a heterogeneous group recognized only by molecular techniques and including chlorarachnean algae, euglyphid filose testate amoebae, some zooflagellates and plasmodiophorid plant pathogens (Cavalier-Smith, 1998; Cavalier-Smith & Chao, 2003). These taxa together with Radiolaria are currently included in the supergroup of Rhizaria, one of the six major groups of eukaryotes (Cavalier-Smith, 2002; Nikolaev et al., 2004; Simpson and Roger, 2004; Adl et al., 2005).

Contrary to many other protists, foraminifers have a particularly complex reproduction cycle, with alternating sexual and asexual generations (e.g. Lee et al., 1991; Goldstein, 2002 for details). The alternation of generations may be facultative or even disappear in some taxa, whereas others practice self-fertilization (Goldstein, 2002). The life cycle may also vary within one species according to the environmental conditions (Lee et al., 1991; Gooday & Alve, 2001). Foraminifers may have a benthic or planktonic mode of life. Benthic foraminifers live on or in the sea floor sediments and represent the vast majority of foraminiferal species: approximately 99.5% of the extant species recognized are benthic (data from Sen Gupta, 2002). Planktic species originated from the benthic ones during the middle Jurassic (Culver, 1993) and inhabit the water column.

Among the shelled foraminifers, ones with an organic, agglutinated and calcareous test are distinguished. The last group is separated in three subgroups: microgranular (fusulinids, extinct at the Permian-Triassic boundary), porcellaneous (miliolids) and hyaline (rotaliids and several related orders). The genera studied here belong to the rotaliids.

1.2. Species concept

The species is the fundamental concept in systematics, and it is the only one supposed to be clearly defined. However, several definitions of the species coexist, depending on available information.

The biological species concept

The biological species concept is the following: all the individuals that can interbreed are considered as belonging to the same species. This definition was defended by several founders of the modern synthetic theory of evolution as Dobzhansky, Mayr or Huxley, but this concept goes back prior to Darwin's time (Ridley, 1996). Problems in studying species appear when no interbreeding can be observed, for instance when species are extinct or reproduce asexually. Both cases may

concern the study of foraminifers. Because the knowledge of foraminifers is principally based on fossils, the main species concept used to classify them is morphological.

The morphospecies concept

The morphology remains the main feature to study extinct organisms¹. Therefore, the species concept traditionally used in paleontology is the typological definition of the species or the morphospecies concept. In this case, the species is defined by a type, generally represented by the holotype, sometimes accompanied by one or more paratypes. This species concept can be quite rigid and the specimens deviating from the type morphology will be given a new species name and a new type. To smooth this typological species concept and decrease the number of newly described species, some authors working on foraminifers have introduced the assemblage concept, where the species is defined by a homogeneous group of individuals, considered more representative of the population (e.g. Zachariasse, 1975; Van der Zwaan, 1982). In the assemblage concept the morphological range covered by one species may be considerable, although it has to remain gradual and easily distinguishable from other species units (Van der Zwaan, 1982). Therefore, a species can house several morphotypes connected by morphological intermediates.

The phylogenetic species concept

A third definition of the species, used in phylogenetic analyses is the phylogenetic or genealogical species concept (Freeman & Herron, 2004). The species as well as higher taxa are defined as monophyletic groups, which include all the descendants of a common ancestor. The recognition of groups implies their genetic isolation and consecutive divergence. Unlike the biological species concept, the phylogenetic concept can also apply to extinct or asexually reproducing species.

The molecular or genetic species concept

Within the molecular phylogenetic analyses, monophyletic groups representing species may be detected with the help of clones² or defined through a sequence divergence threshold (e.g. 5% in Pawlowski et al., 2002b). For the time being, this concept is not well established.

1.3. Selection of *Cibicides* and *Uvigerina*

This work focuses on the evolution, phylogeny and microhabitat occupation of two rotaliid genera, *Cibicides* and *Uvigerina*. As seen before, the order Rotaliida includes benthic hyaline calcareous foraminifers. Because of their good fossil record and their sensitivity to environmental factors rotaliids are important tools for the reconstruction of paleoenvironments and paleoclimates. Representatives of *Cibicides* and *Uvigerina* are and have been important elements of the marine meiofaunal community and are employed in, for instance, micropaleontological and stable isotope studies to reconstruct past environmental change, despite the fact that there is little knowledge on their evolution. A better insight in their evolutionary history will certainly help to understand their (paleo)ecological functioning and thus improve their proxy value in paleoecological and paleoclimatological studies. Even more important is the fact that the success or failure in using them as proxies rests on the assumption that taxa can be properly distinguished on morphological grounds.

1) In exceptional cases DNA is still available from Quaternary remains, but no DNA older than 50,000-100,000 years has been found until now (Lindahl, 1993; Austin et al., 1997).

2) Clones allow for investigation of the intra-individual variations and therefore for the exploration of the limits of populations.

1.4. Selection of bioprovince and time slice

The most recent of the three major Cenozoic turnovers affecting benthic foraminifers occurred during the middle Miocene. Earlier episodes were the Paleocene-Eocene boundary, characterized by an extinction of benthic foraminifers (BEE, benthic extinction event, e.g. Speijer, 1994; Schmitz et al., 1996; Alegret et al., 2005), and the late middle Eocene-earliest Oligocene (e.g. Miller et al., 1992; Zachos et al., 2001). The research reported here focuses on benthic foraminiferal evolution since the middle Miocene cooling. This is the time when modern oceanic conditions originated, and the present water mass circulation took shape with prevailing cool bottom waters (Douglas & Woodruff, 1981). A large part of the extant deep-sea rotaliid species arose around the middle Miocene (Douglas & Woodruff, 1981; Miller et al., 1992), which reduces taxonomical bias that is introduced by differing nomenclatures for different time slices. Moreover, many of the taxa that evolved since the middle Miocene are alive today.

At the same time, the proto-Mediterranean was subject to important tectonic events, such as the closure of the connection between the Tethys and the Indian Ocean by the northward movement of the African plate. These changes transformed the well-ventilated Tethys into a poorly ventilated and even periodically stagnating Mediterranean basin since 14 Ma (Chamley et al., 1986; Seidenkrantz et al., 2000 and references herein), ultimately leading to the Messinian salinity crisis. Extensive studies in the area have led to the development of an extremely detailed and well-constrained time frame (e.g. Krijgsman et al., 1999; Abels et al., 2005). Next to a detailed time scale research in the Mediterranean area has focused on paleoenvironmental reconstruction, including anoxic and dysoxic environments. This has led to rather good insight in the relation between benthic foraminifers and specific environments. Moreover, the taxonomy of Mediterranean-Atlantic benthic assemblages is rather well constrained, although there are minor differences between schools. This allows minimizing taxonomical problems such as encountered when different bioprovinces are compared (see for instance Chapter 4: *Uvigerina akitaensis* and *U. peregrina* are the same species when molecular phylogeny is considered).

1.5 Molecular tools provide a new perspective in the phylogeny of foraminifers

Until now, all foraminiferan classifications (Haynes, 1981; Loeblich & Tappan, 1988, 1992; Sen Gupta, 2002) are based on morphological criteria of the test only. One of the problems encountered is, whether the criteria used at different taxonomic levels are relevant or not. The choice of the best characteristics has long been under debate (e.g. Towe & Cifelli, 1967; Hansen, 1979; Cifelli & Richardson, 1990; Haynes, 1990; Sen Gupta, 2002), and the different classifications have placed emphasis on such different criteria as the composition of the wall, its crystallographic nature through polarized light, the shape of the aperture, and the number or the arrangement of chambers (d'Orbigny, 1826; Williamson, 1858; Cushman, 1928; Galloway, 1933; Hofker, 1951; Loeblich & Tappan, 1964, 1988, 1992; Haynes, 1981; Mikhalevitch & Debenay, 2001). These classifications, however, are mainly typological and do not always represent relations between living organisms. A better understanding of the living species through genetic data would improve the phylogenetic background knowledge of foraminifers.

The ribosomal RNA (rRNA) genes have the advantage of being present in several hundreds of copies in each cell. For this reason, it is possible to amplify ribosomal DNA (rDNA) from one single foraminifer specimen. However, rRNA gene phylogenies are often biased by heterogeneity of substitution rates (Pawlowski et al. 1997; Philippe, 2000) and they give a low resolution of higher-level relationships (Flakowski et al., 2005). The study of other genes was restrained by the difficulty to cultivate foraminifers, because many more specimens (at least 50-100) are needed for amplification. For a limited number of species four foraminiferal proteins have been obtained: actin (Pawlowski et al., 1999a; Keeling, 2001; Flakowski et al., 2005), RNA polymerase II largest subunit (Longet et al., 2003), ubiquitin (Archibald et al., 2003) and tubulin (Linder et al., 1997; Habura et al., 2005). Revised analysis of the SSU (small subunit) rDNA omitting long-branching lineages confirmed the results found with these other genes (see above) and showed that SSU

rDNA data remained a valuable source of information for phylogenetic purposes (Berney & Pawlowski, 2003).

These rDNA studies (SSU and LSU (large subunit) have focused on the position of foraminifers in the tree of life (Pawlowski et al., 1994, 1996, 1999a, 1999b; Wade et al., 1996), links between the foraminiferal orders (Darling et al., 1997; Pawlowski et al., 1997, 2002a; Flakowski et al., 2005), and on species concepts in planktonic foraminifers (Darling et al., 1996, 1999, 2000; de Vargas et al., 1997, 1999, 2001, 2002; Huber et al., 1997; Stewart et al., 2001) and the benthic foraminifer *Ammonia* (Pawlowski et al., 1995; Holzmann et al., 1996; Holzmann & Pawlowski, 1997, 2000; Holzmann, 2000; Hayward et al., 2004). Several studies have concerned benthic taxa in general (Ertan et al., 2004) or have focused on specific groups, such as large foraminifers (Holzmann et al., 2001, 2003) and Glabratellidae (Tsuchiya et al., 2000, 2003). The low number of papers having the DNA of deeper-water benthic foraminifers as a subject can be explained by the difficulties encountered in obtaining living material from these locations.

1.6. Obtaining DNA

Obtaining DNA from benthic foraminifers is not an easy task. The specimens have to be alive at the moment they are grinded for DNA extraction. It is not yet known how long exactly after death the DNA is destroyed; however, this happens probably within hours or days. For this reason, the Rose Bengal staining method is not precise enough to indicate whether a specimen is dead or alive. The method we used to isolate live individuals was the direct observation of the specimens in sea water, under a dissection microscope and without any staining. Most of the collected specimens came from fully marine, relatively deep-water (>200m) environments and no pseudopodial activity was observed under the microscope. The color of the protoplasm, a good condition of the test (not damaged or broken), and detritus near the aperture were positive signs of life.

One of the main limiting factors to keep foraminifers alive as long as possible, particularly the deep-sea specimens, is temperature (Lutze & Altenbach, 1988; Altenbach et al., 2003). The cold chain has to be maintained from the sampling point until the moment the foraminiferan is dried or grinded for DNA extraction. There is no possibility to interact during the return of the boxcore or multicore, which can take a few hours, depending of the sampling depth³. This time interval can be rather critical, particularly if the sample is derived from deep waters and if the temperature difference between the sea floor and the sea surface is high (up to 10-15°C). Consequently, sampling for live specimens is generally much more successful at high latitudes or during mid-latitude winters, than at low latitudes or during summer in mid-latitudes. From this point of view, perfect places to sample deep-sea species are the Scandinavian fjords where these species are found at shallow depths. When the sample is on board, it is important to sieve the sediment immediately, if possible with bottom water at ambient temperature, but at least with cold sea water. Afterwards, the sieved sample will be stored in the refrigerator, and kept under the (preferentially cold) light of the microscope for the shortest possible time, and on ice or in a cold room.

An additional problem may be the huge pressure difference experienced by the specimens collected at deep-sea locations. Decompression may not be a great problem for foraminifers sampled at 1000-2000m water depth (Altenbach et al., 1992). Nevertheless, the pressure difference between deep-water and surface-water environments becomes critical below 2200m and appears to be lethal for most deep-sea foraminifers (Kitazato, 1994). Deep-sea specimens are also more difficult to sample because the total number of foraminifers decreases with the increase of depth, perhaps due to a diminution of the amount of food (Corliss, 1991).

Even though drying is inevitable to obtain SEM pictures, it considerably reduces the quality of DNA. Once the specimen is dried, the delay before DNA extraction is also critical. Two examples can illustrate this. In 2002, we sampled in the Oslo Fjord and found promising material; within one month (a maximum of 23 days), the specimens were picked, dried, SEM pictured and DNA

3) The hauling speed is about 1m/s. It will take around half an hour to obtain a multicore from 2000m.

was extracted. The percentages of positive results were excellent (62%, 37 positive out of 60 extractions). One year later, in the same season, we collected samples on the west coast of Sweden, not far away from the Oslo Fjord and under the same conditions. The delay between drying and extraction of the material was longer (31 to 43 days, depending when it was sampled during the cruise). The percentage of positive results decreased dramatically (12%, 7 positive out of 60 extractions). The second example comes from Mediterranean samples. A few specimens of *Cibicides* were collected near Marseille. Four living individuals were immediately extracted after cleaning and picking and all of them gave DNA; four other specimens were dried, SEM pictured and DNA extracted two months later. Only one individual gave a positive result but the quality of its DNA was much worse than for the freshly extracted specimens.

1.7. This study

The aim of this study is to compare classical phylogenies of *Cibicides* and *Uvigerina* based on morphology and the fossil record with the new ones derived from molecular analyses. Synthesis of these two approaches may lead to new insights in the evolutionary history of the two genera. We hope to connect steps in this evolutionary history with large scale changes in the paleoenvironmental or paleoceanographic setting of the Mediterranean area. Specifically, we hope to connect the evolutionary history with the known microhabitat preferences of the various species. Although research over the past decades has brought together many data on microhabitat occupation and regulation, it is virtually unknown why and when taxa started to inhabit them.

In the three following chapters the molecular results are presented: the phylogeny of the rotaliids based on the complete SSU rDNA (Chapter 2), the phylogeny of *Cibicides* based on two fragments, representing 2/3 of the SSU rDNA (Chapter 3), and the phylogeny of *Uvigerina* based on the 3' end fragment of the SSU rDNA (Chapter 4). The subsequent chapters concern the classification, taxonomy, morphology and the microhabitats of *Cibicides* (Chapter 5) and *Uvigerina* (Chapter 6), respectively. In these chapters we also compare the molecular and morphological phylogenies and build new ones. The final chapter discusses the main findings and compares the phylogenies and evolutionary histories of both genera (Chapter 7).