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Freshwater Foraminiferans Revealed by Analysis of Environmental DNA Samples

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ABSTRACT. Sediment-dwelling protists are among the most abundant meiobenthic organisms, ubiquitous in all types of aquatic ecosystems. Yet, because their isolation and identification are difficult, their diversity remains largely unknown. In the present work, we applied molecular methods to examine the diversity of freshwater Foraminifera, a group of granuloreticulosan protists largely neglected until now. By using specific PCR primers, we detected the presence of Foraminifera in all sediment samples examined. Phylogenetic analysis of amplified SSU rDNA sequences revealed two distinct groups of freshwater foraminiferans. All obtained sequences branched within monothalamous (single-chambered), marine Foraminifera, suggesting a repeated colonization of freshwater environments. The results of our study challenge the traditional view of Foraminifera as essentially marine organisms, and provide a conceptual framework for charting the molecular diversity of freshwater granuloreticulosan protists.
Key Words. Diversity, Foraminifera, phylogeny, ribosomal RNA, SSU.

BENTHIC meiofaunal communities serve as an interface between the microscopic world of bacteria and the more noticeable macrofauna, like fishes and molluscs. Protists are some of the most active and conspicuous consumers among the meiofauna, yet they are much less well known than are macrofaunal organisms with respect to their life cycles, environmental impact, and taxonomy (Gooday et al. 1992; Lee and Patterson 1998; May 1990). Recent molecular studies revealed a surprisingly high diversity in several groups of marine planktonic and benthic bacteria and unicellular eukaryotes (Lopez-Garcia et al. 2001; Massana et al. 1997; Moon-van der Staay et al. 2001; Nubel et al. 2000; Vetriani et al. 1999). Only a few studies, however, have addressed the molecular diversity of freshwater sediment-dwelling eukaryotes (Smirnov et al. 2002).

Freshwater Foraminifera are one of the most enigmatic protistan groups. Foraminifera are typically defined as marine organisms characterized by the presence of granuloreticulopodia and the possession of a membranous, agglutinated, or calcareous test, which is either monothalamous (single-chambered) or polythalamous (multi-chambered) (Loeblich and Tappan 1988). A few foraminiferal genera were described from freshwater environments during the 19th century (Archer 1877; Blanc 1888; Cienkowski 1876; Claparède and Lachmann 1859; Leidy, 1879; Penard 1899, 1905). Most of these forms were grouped by later authors in the orders Athalamida and Monothalamida, which are sister groups to the Foraminifera (Bovee 1985). This traditional classification was only recently challenged by the results of molecular phylogenetic studies. For example, a freshwater athalamid (*Reticulomyxa filosa*) from laboratory cultures (Pawlowski et al. 1999a, b), and a new monothalamid species (*Edaphoallogromia australica*) from a tropical Australian rain forest (Meisterfeld et al. 2001) branch within the foraminiferal clade.

To examine more precisely the origin and diversity of freshwater Foraminifera, we searched for foraminiferal sequences in sediment collected from two lakes, a wetland habitat, and a river in Europe, as well as three bodies of water in North America. Total DNA was purified from each sample, and was subjected to PCR using foraminiferal-specific primers for the ribosomal small subunit DNA (SSU rDNA). All investigated freshwater sites were found to contain foraminiferal DNA.

MATERIALS AND METHODS

Sampling and subsequent treatment of sediment samples was independently carried out in two different laboratories, one in North America (Albany, NY, USA) and the other in Europe (Geneva, Switzerland).

Sampling. European sediment samples were taken from a small river (Seymaz, Geneva, Switzerland), two different Swiss lakes (Lake Geneva, Lake Neuchâtel), and a nature reserve (Grande Caricaie) that encompasses the southern border of Lake Neuchâtel. North American samples were collected from a small urban lake (Washington Park Lake, Albany, NY), a rural pond (Smith Pond, Chatham, NY), and the upper reaches of the estuarine part of the Hudson River. An overview of the sampling localities is given in Table 1.

DNA extraction. For European material, total DNA was extracted from untreated sediment samples, following modified protocol from Zhou et al. (1996) and Clark (1992), as detailed in Smirnov et al. (2002). The sediment samples (5 g) were mixed with 9 ml of extraction buffer (100 mM Tris, 100 mM EDTA, 100 mM sodium phosphate, 1.5 M NaCl, 1% CTAB) and 100 μl Proteinase K (10 mg/ml), for 30 min at 37 °C. After mixing the samples on a rotatory platform at 225 rpm for 30 min at 37 °C, 1.5 ml SDS (20%) was added and the samples were incubated at 65 °C for 2 h. Each sediment sample was re-extracted two times and the combined supernatants were transferred to 50-ml tubes, mixed with an equal vol. of chloroform-isoamylalcohol (24:1), and centrifuged at 16,000 *g* for 20 min. The aqueous phase of each sample was recovered and mixed with an equal vol. of ether to remove traces of chloroform. The DNA was then precipitated by adding 2 vol. of 100% ethanol and leaving the samples overnight at −20 °C. After centrifugation (16,000 *g*/20 min), the resulting pellet was rinsed with 70% ethanol and dried at 37 °C for 30 min.

North American samples were extracted using the same protocol, with two modifications: samples were ground for 1 min in a mortar and pestle before incubation in sample buffer, and cetyltrimethylammonium bromide (CTAB) was not added until after the first hour of incubation. Mortars and pestles were cleaned of any prior DNA contamination by soaking in 1 M HCl followed by 1 M NaOH. The pH was neutralized with Tris (pH 8), and the implements were given several water rinses, followed by UV irradiation and autoclaving. The crude DNA extract was purified using the DNeasy Plant Mini Kit (Qiagen, Basel, Switzerland), or from the gel using the glass milk method of Vogelstein and Gillespie (1979).

The quality of the total DNA pool extracted from freshwater sediment samples was assessed by gel electrophoresis. DNA extractions from positive control sediments (marine + freshwater) were then used as templates for specific amplification, employing conditions as described below and yielding PCR products of the expected size.

DNA amplification, cloning and sequencing. SSU rDNA

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Table **1**. Sampling localities and number of obtained sequences.

Sample	Locality	Coordinates	Collection date	Remarks	Number of sequences s14F1-sB	Number of sequences s14F1-s17	Lineages
6	Lake Geneva (Ouchy)	N46°30′23″E6°38′08″	May 2000	30 m	1		L
26	Lake Geneva (Ouchy)	N46°29′58″E6°37′69″	May 2000	200 m		1	L
27	Lake Geneva (Ouchy)	N46°28′72″E6°37′27″	May 2000	300 m	2	1	L
33	Seymaz River (Geneva)	N46°12′28″E6°12′06″	June 2000	surface sediment		1	L
46	Grande Caricaie (Yverdon)	N46°49′40″E6°48′04″	June 2000	surface sediment	2	4	L
50	Grande Caricaie (Yverdon)	N46°50′22″E6°48′50″	June 2000	surface sediment		1	L
51	Neuchâtel Lake (Yverdon)	N46°50′22″E6°48′41″	June 2000	surface sediment		1	N
52	Seymaz River (Geneva)	N46°11′14″E6°11′02″	June 2000	surface sediment		2	N
5A	Smith Pond (Chatham, N.Y.)	N42°21′50″W73°36′27″	October 2000	surface sediment	2	1	N, O
SaB	Hudson River (N.Y.)	N42°39′33″W73°43′07″	September 2000	surface sediment	1		N
LaB	Hudson River (N.Y.)	N42°39′29″W73°43′12″	September 2000	surface sediment	2		N
NB6	Washington Park Lake (Albany, N.Y.)	N42°39′04″W73°46′57″	July 2000	surface sediment	1	2	N

was amplified by PCR in a total vol. of 50 μl. The thermal cycle parameters consisted of 40 cycles of 30 s at 94 °C, 30 s at 50 °C, and 120 s at 72 °C, followed by 5 min at 72 °C for final extension. Partial SSU rDNA sequences were obtained by amplification with the primer pairs s14F3-s17 and s14F3-sB (Holzmann et al. 2001; Pawlowski 2000). The PCR products were used for reamplification with the primer pairs s14F1–s17 and s14F1–sB (Pawlowski 2000), using the same thermal cycle parameters during 25 cycles. Primers sequences are: s14F1 (5′AAG GGC ACC ACA AGA ACG C), s14F3 (5′ACG CA(AC) GTG TGA AAC TTG), s17 (5′CGG TCA CGT TCG TTG C), sB (5′TGA TCC TTC TGC AGG TTC ACC TAC). Positive reamplification products were purified using a High Pure PCR Purification Kit (Roche Diagnostics, Rotkreuz, Switzerland), ligated into pGEM-T Vector system (Promega, Wallisellen, Switzerland), and cloned in XL-2 Ultracompetent Cells (Stratagene, Basel, Switzerland). Both strands of the amplified and cloned PCR products were sequenced for each re-amplified PCR product from one to six clones. Sequencing reactions were prepared by using ABI-PRISM Big Dye Terminator Cycle Sequencing Kit and analyzed with an ABI-377 DNA sequencer (Perkin-Elmer, Rotkreuz, Switzerland), all according to the manufacturer's instructions. The freshwater foraminiferal sequences reported in this paper have been deposited in the EMBL/GenBank database under the following accession numbers: AF381179-AF381180, AF474143, AF381182-AF381183, AJ3180009, AJ318011, and AJ318013-AJ318016.

Prevention of contamination. Several measures were taken to limit possible contamination. Sampling equipment was thoroughly cleaned between every use. Untreated surface sediment from each sampling site was immediately deposited in sterile Falcon tubes and stored in a cool box until arrival in the laboratory. In both laboratories, DNA extraction, amplification and cloning were conducted in different rooms, using different pipette sets for each step and sterile filter tips (Biosphere, SARSTEDT, Sevelen, Switzerland). Negative controls, using the extraction buffer and PCR reaction products without adding the DNA template, were carried out during DNA isolation and amplification.

Sequence analysis. The sequences obtained from freshwater sediment samples were compared to SSU rDNA sequences of other eukaryotic organisms by using BLAST 2.2.3 (Altschul et al. 1997) and yielded significant alignments with foraminiferal sequences only. Sequences were carefully examined to identify and eliminate PCR-induced artifacts and chimeras. The obtained sequences were aligned using GDE 2.2 software (Larsen et al. 1993). A copy of the alignment is available from the corresponding author. A chi-square test was applied to all aligned sequences to test the homogeneity of base frequencies across taxa using PAUP* 4.0b (Swofford 2000), resulting in chi-square = 18.981, df = 159 and $p = 1.00$. According to the results of the chi-square test, which indicated an unbiased base composition, we chose Kimura's two-parameter model (K2P), applied to distances corrected for multiple hits and for unequal transition and transversion rates (Kimura 1980).

Phylogenetic analyses were conducted using the neighbor joining (NJ) method (Saitou and Nei 1987) and the maximum likelihood (ML) method, employing the fast DNAml program (Olsen et al. 1994). The illustrated tree (Fig. 1) was based on NJ and ML methods using K2P model. The reliability of internal branches was assessed by bootstrapping (Felsenstein 1988), with 1000 re-samplings for the NJ and 100 re-samplings for the ML trees. The PHYLO_WIN program (Galtier and Gouy 1996) was used for distance computations, NJ and ML tree building, and bootstrapping.

Additionally, phylogenetic analyses were conducted using NJ method with Tajima and Nei's (T & N) six-parameter model (Tajima and Nei 1984) and ML method with the General Time Reversible (GTR) model (Lanave et al. 1984), as implemented in the PAUP* 4.0b (Swofford 2000). Parameters for ML (GTR) analysis were estimated separately by using Modeltest (Posada and Crandall 1998), with a shape parameter $\alpha = 0.729772$ and among site rate variation pI = 0.306605.

RESULTS

Sequence data. Two primer pairs (s14F1-s17, s14F1-sB) that amplify a region near the 3′ end of the SSU rDNA were selected for phylogenetic analysis because there are many corresponding sequences available for this region in the GenBank database. The region s14F1-sB corresponds to positions 1212–1871 in *Rattus norvegicus* (K-01593), but in Foraminifera it ranges in size between 877 and 1444 bp, which is about twice the length of that region in most other eukaryotes. The unusual length results from insertions, unique to foraminiferans, in conserved regions of the SSU rRNA genes. The region s14F1-s17 is a fragment of the s14F1-sB region, corresponding to the positions 1212–1395 in the rat sequence (K-01593). Its length in Foraminifera ranges between 217 and 510 bp.

Phylogenetic analysis. Eleven sequences of the larger SSU rDNA fragment (s14F1-sB) were amplified from total DNA extracted from sediment samples. These sequences were compared to 42 sequences from Foraminifera, including six poly-

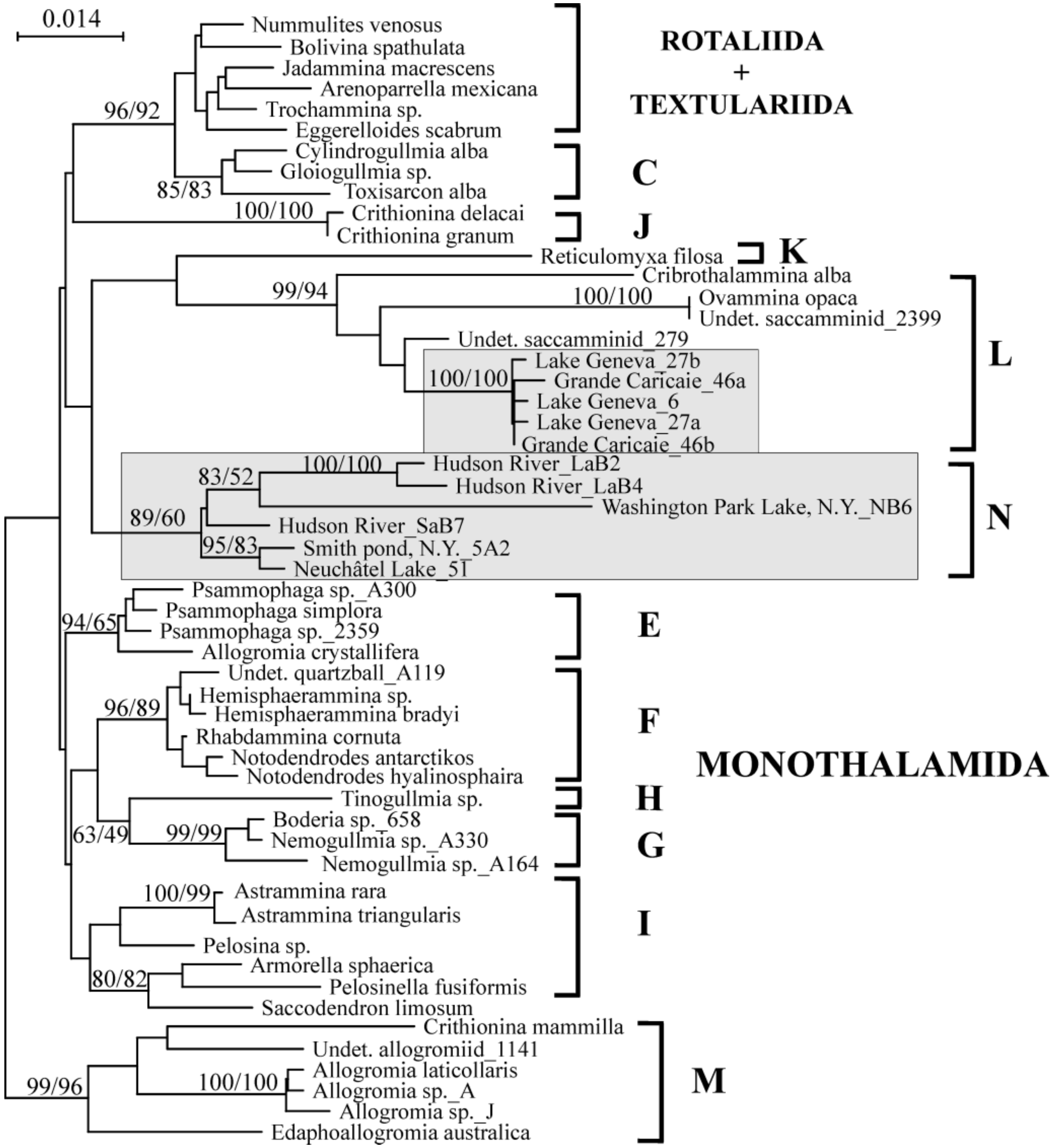

Fig. **1**. Phylogenetic tree, based on 53 partial foraminiferal small subunit (SSU) rDNA sequences using NJ (K2P) analysis, shows two clades of freshwater foraminiferal sequences related to the monothalamous forms. The tree was rooted in *Allogromia* spp. Bootstrap values are based on 1,000 re-samplings for the NJ (K2P) tree and on 100 re-samplings for the ML (K2P) tree (first and second numbers on branch, respectively). Note: letters a-b indicate different clones of the same PCR product.

thalamous (i.e. two calacareous rotaliids and four agglutinated textulariids), an athalamid, and 35 monothalamous (i.e. 19 astrorhizids and 16 membranous allogromiid) species.

The *Allogromia* clade was chosen as an outgroup following previous analyses (Pawlowski et al. 2002). Both models applied for NJ analysis (K2P, T&N) yielded an identical tree (Fig. 1). The athalamid and monothalamid sequences cluster in ten different lineages (C–M), which were previously defined as groups supported by bootstrap values higher than 50% in both NJ and ML analyses (Pawlowski et al. 2002). Phylogenetic analyses of the obtained sequences show that all freshwater species branch among monothalamous Foraminifera. A clade consisting of five

North American sequences (Smith Pond, N.Y._5A2, Hudson River_SaB7, Hudson River_LaB2 and LaB4, Washington Park Lake, N.Y_NB6) and one European sequence (Neuchâtel Lake_51) branches as a sister group to the clades K and L. The clade is supported by a bootstrap value of 89% (Fig. 1) and represents a new lineage, here termed clade N. A second clade consisting of five European sequences (Lake Geneva_6, 27 a, 27b, 46a, 46b) branches within foraminiferal lineage L as a sister group to an undetermined marine saccamminid _279 (bootstrap value of 100%). Lineage L also includes the marine species *Ovammina opaca*, the undetermined saccamminid_2399, and *Cribrothalammina alba* (Pawlowski et al. 2002).

Maximum likelihood analysis confirms two clades of freshwater foraminiferans, but their branching order differs from that observed in the NJ tree (data not shown). In the ML (K2P) tree, the freshwater foraminiferal lineage N branched at the base of clades I + G + H + F, while lineages L + K remain as sister groups to the clade Rotaliida + Textulariida + C. The ML (GTR) analysis showed that both freshwater foraminiferal lineages L + N are well supported (87% and 89% bootstrap respectively). The main difference between both ML analyses concerned lineage I, which in ML (GTR) splits into two subgroups (43% bootstrap), one containing *Saccodendron limosum*, *Pelosinella fusiformis*, and *Armorella sphaerica*, and the other consisting of *Pelosina* sp., *Astrammina triangularis*, and *Astrammina rara* (data not shown).

Phylogenetic analysis was additionally carried out for 14 freshwater foraminiferal sequences lying between the primers s14F1 and s17 (Table 1). Analysis of this shorter fragment was conducted by the NJ method and confirmed the division of freshwater foraminiferans into two lineages (L, N). One of the sequences obtained from Neuchâtel Lake (sample 51) was closely related to the sequence of *Reticulomyxa filosa* (lineage K), obtained previously (Pawlowski et al. 1999a, b) (data not shown).

DISCUSSION

The freshwater bodies sampled for this study are geographically widespread and diverse in their hydrology. Three of them are still-water environments, ranging from a large (580 km^2) natural mountain lake to a small (2,500 m^2) lake in an urban park. One of the rivers is estuarine, while the other represents a small lake effluent.

The results of our study show that Foraminifera are widespread in freshwater environments, as all of our samples, from whatever source, contain foraminiferal sequences. Foraminifera have been identified consistently only in marine sediments and in saline areas like estuaries and coastal marshes in the past (Loeblich and Tappan 1988). The presence of foraminifera-like rhizopodial protists in freshwater habitats was reported by several authors (for reviews see De Saedeler 1934, Loeblich and Tappan 1964, 1988), but they were generally considered to be uncommon. Together with recent findings (Meisterfeld et al. 2001; Pawlowski et al. 1999a, b), our results reveal a previously unknown diversity of these organisms.

The sequences derived from the environmental DNA samples fall into two groups, L and N, one of which is European, while the other contains mostly sequences of North American origin. The members of each group are closely related to some marine Foraminifera. This suggests that there are multiple lineages of freshwater Foraminifera, perhaps derived from different populations of marine species at different times. It is also possible that one or more lineages of marine Foraminifera had a freshwater ancestor. Because marine Foraminifera are already known to be able to inhabit oligohyaline environments, it is possible that one or more groups completed the transition and moved into more conventional riverine or lacustrine habitats.

Microscopic observation of the investigated sediment samples failed to reveal foraminiferal tests. The described agglutinated freshwater foraminiferans are rather large, up to 1 mm in length (Penard 1899, 1905), so their detection should not be hampered by size. It is possible that our sequences represent some minute athalamid foraminiferans, which cannot be isolated by conventional sieving or observed by means of a stereomicroscope, and which might also not thrive under laboratory conditions. The question remains whether most of the obtained sequences were derived from previously described freshwater protists, or whether they represent novel species. Only the single sequence branching with *R. filosa* can be assigned to a known foraminiferal genus. The group of freshwater foraminiferal sequences clustering within clade L could represent one of the protists described by Penard (1899, 1905) from Lake Geneva. The agglutinated tests of Penard's species resemble the marine saccamminids to which these sequences are closely related. This assumption, however, could only be confirmed by isolation of living specimens of the respective species, which has not been possible. Fluorescently labeled, specific oligonucleotide probes allow detection of individual cells in environmental samples (reviewed in Amann et al. 1995). This approach could be used in further studies to identify and assign foraminiferal organisms to the obtained sequences.

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LITERATURE CITED

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhanh, Z., Miller, W. & Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucl. Acids Res.*, **25**:3389–3402.

Amann, I. R., Ludwig, W. & Schleifer, K. H. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microb. Rev.*, **59**:143–169.

Archer, W. 1877. Résumé of recent contributions to our knowledge of ''Freshwater Rhizopoda.'' Part IV, *Quart. J. Microsc. Sci., New Ser.*, **17**:107–124.

Blanc, H. 1888. La *Gromia brunnerii* un nouveau foraminifère. *Rec. Zool. Suisse*, **4**:498–513.

Bovee, E. C. 1985. Class Granuloreticulosea De Saedeleer. 1934. *In*: Lee, J. J., Hunter, S. H. & Bovee, E. C. (ed.), An Illustrated Guide to the Protozoa. Society of Protozoologists, Lawrence, KS p. 246–252.

Cienkowski, L. 1876. Über einige Rhizopoden und verwandte Organismen. *Arch. Mikrosk. Anat.*, **12**:15–50.

Claparède, E. & Lachmann, J. 1859. Etude sur les infusoires et les rhizopodes. *Mém. Inst. Genèv.*, **6**:261–482.

Clark, C. G. 1992. DNA purification from polysaccharide-rich cells. P.D-3.1.2. *In*: Lee, J. J. & Solido, A. T. (ed.), Protocols in Protozoology, Vol. 1. Society of Protozoologists, Lawrence, KS.

De Saedeleer, H. 1934. Beitrag zur Kenntnis der Rhizopoden: morphologische und systematische Untersuchungen und ein Klassifikationsversuch. *Mém. Mus. Roy. Hist. Nat. Belgique*, **60**:1–112.

Felsenstein, J. 1988. Phylogenies from molecular sequences: inference and reliability. *Ann. Rev. Genet.*, **22**:521–565.

Galtier, N. & Gouy, M. 1996. SEAVIEW and PHYLO_WIN: two graphic tools for sequence alignment and molecular phylogeny. *Comput. Appl. Biosci.*, **12**:543–548.

Gooday, A., Levin, L. A., Linke, P. & Heeger, T. 1992. The role of benthic Foraminifera in deep-sea food webs and carbon cycling. *In*: Rowe, G. T. & Pariente, V. (ed.), Deep-sea Food Chains and the Global Carbon Cycle. Kluwer Academic Publ., Dordrecht. p. 63–91.

Holzmann, M., Hohenegger, J., Hallock, P., Piller, W. E. & Pawlowski, J. 2001. Molecular phylogeny of large miliolid Foraminifera (Soritacea Ehrenberg 1839). *Mar. Micropal.*, **43**:57–74.

Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.*, **16**:111–120.

Lanave, C., Preparata, G., Saccone, C. & Serio, G. 1984. A new method for calculating evolutinary substitution rates. *J. Mol. Evol.*, **20**:86–93.

Larsen, N., Olsen, G. J., Maidak, B. L., McCaughey, M. J., Overbeek, R., Macke, T. J., Marsh, T. L. & Woese, C. R. 1993. The ribosomal database project. *Nucl. Acids Res.*, **21**:3021–3023.

Lee, W. J. & Patterson, D. J. 1998. Diversity and geographic distribution of free-living heterotrophic flagellates-analysis by PRIMER. *Protist*, **149**:229–244.

Leidy, J. 1879. Fresh-water rhizopods of North America. *U.S. Geol. Surv. Terr. Rep.*, **12**:277–287.

Loeblich, A. J. R. & Tappan, H. 1964. Sarcodina chiefly ''Thecamoebians'' and Foraminiferida. *In*: Moore, R. C. (ed.), Treatise on Invertebrate Paleontology, part C, Vol. 1–2. Geol. Soc. of America and Univ. of Kansas Press, Lawrence, KS. p. 900.

Loeblich, A. J. R. & Tappan, H. 1988. Foraminiferal Genera and their Classification, Vol. 1–2, Van Nostrand Reinhold, N.Y.

Lopez-Garcia, P., Rodriguez-Valera, F., Pedros-Alio, C. & Moreira, D. 2001. Unexpected diversity of small eukaryotes in deep-sea Antarctic plankton. *Nature*, **409**:603–607.

Massana, R., Murray, A. E., Preston, C. M. & DeLong, E. F. 1997. Vertical distribution and phylogenetic characterization of marine planktonic Archea in the Santa Barbara Channel. *Appl. Environ. Microbiol.*, **63**:50–56.

May, R. M. 1990. How many species? *Phil. Trans. Roy. Soc. London*, **B 330**:293–304.

Meisterfeld, R., Holzmann, M. & Pawlowski, J. 2001. Morphological and molecular characterization of a new terrestrial allogromiid species: *Edaphoallogromia australica* gen. et spec. nov., (Foraminifera) from Northern Queensland (Australia). *Protist*, **152**:185–192.

Moon-van der Staay, S. Y., De Wachter, R. & Vaulot, D. 2001. Oceanic 18S rDNA sequences from picoplankton reveal unsuspected eukaryotic diversity. *Nature*, **409**:607–610.

Nubel, U., Garcia-Pichel, F., Clavero, E. & Muyzer, G. 2000. Matching molecular diversity and ecophysiology of benthic cyanobacteria and diatoms in communities along a salinity gradient. *Environ. Microbiol.*, **2**:217–226.

Olsen, G. J., Matsuda, H., Hagstrom, R. & Overbeek, R. 1994. FastDNAml: a tool for construction of phylogenetic trees of DNA sequences using maximum likelihood. *Comp. Appl. Biosci.*, **10**:41–48.

Pawlowski, J. 2000. Introduction to the molecular systematics of foraminifera. *Micropaleontology*, **46**(Suppl. 1):1–112.

Pawlowski, J., Bolivar, I., Fahrni, J. F., de Vargas, C. & Bowser, S. S. 1999a. Molecular evidence that *Reticulomyxa filosa* is a freshwater naked foraminifer. *J. Eukaryot. Microbiol.*, **46**:612–617.

Pawlowski, J., Bolivar, I., Fahrni, J. F., de Vargas, C. & Bowser, S. S. 1999b. Naked foraminiferans revealed. *Nature*, **399**:27.

Pawlowski, J., Holzmann, M., Fahrni, J. F., Cedhagen, T. & Bowser, S. S. 2002. Phylogenetic position and diversity of allogromiid Foraminifera inferred from rRNA gene sequences. *J. Foram. Res.*, **32**: 334–343.

Penard, E. 1899. Les rhizopodes de faune profonde dans le lac Léman. *Rev. Suisse de Zool.*, **7**:83–99.

Penard, E. 1905. Les sarcodines des grands lacs. H. Kuendig Publ., Genève. p. 68–81.

Posada, D. & Crandall, K. A. 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics*, **14**:817–818.

Saitou, N. & Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.*, **4**:406–425.

Smirnov, A., Nassonova, E., Holzmann, M. & Pawlowski, J. 2002. Morphological, ecological and molecular studies of *Vannella simplex* Wohlfarth-Bottermann 1960 (Lobosea, Gymnamoebia), with a new diagnosis of this species. *Protist*, **153**:367–377.

Swofford, D. L. 2000. PAUP* Phylogenetic analysis using parsimony (* and other methods). Version 4. Sinauer Assoc., Sunderland, MA.

Tajima, F. & Nei, M. 1984. Estimation of evolutionary distance between nucleotide sequences. *Mol. Biol. Evol.*, **3**:269–285.

Vetriani, C., Jannasch, H. W., MacGregor, B. J., Stahl, D. A. & Reysenbach, A. L. 1999. Population structure and phylogenetic characterization of marine benthic Archea in deep-sea sediments. *Appl. Environ. Microbiol.*, **65**:4375–4384.

Vogelstein, B. & Gillespie, D. 1979. Preparative and analytical purification of DNA from agarose. *Proc. Natl. Acad. Sci.*, **76**:615–619.

Yang, Z., Goldman, N. & Friday, A. 1994. Comparison of models for nucleotide substitution used in maximum-likelihood phylogenetic estimation. *Mol. Biol. Evol.*, **11**:316–324.

Zhou, J., Bruns, A. M. & Tiedje, J. M. 1996. DNA recovery from soils of diverse composition. *Appl. Environ. Microbiol.*, **62**:316–322.

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