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ORIGINAL PAPER

Morphological and Molecular Characterization of a New Terrestrial Allogromiid Species: *Edaphoallogromia australica* gen. et spec. nov. (Foraminifera) from Northern Queensland (Australia)

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The foraminiferal family Allogromiidae occurs mainly in marine environments, although some genera are described from brackish and freshwater habitats. We report here the occurrence of a terrestrial allogromiid foraminiferan. Phylogenetic relationships were investigated by sequencing part of the SSU rDNA. DNA sequence analysis confirms a close relationship of the new species to the genus *Allogromia*. Morphological studies corroborate the affiliation to the family Allogromiidae but the lack of an entosolenian tube and an internal septum as well as the different ecology do not allow a classification into a known genus of this family. Taking the molecular, morphological and ecological differences into account, a new genus *Edaphoallogromia* with the type species *E. australica* is erected.

Introduction

The foraminiferal family Allogromiidae (Rhumbler 1904) is widely distributed in various marine habitats, ranging from nearshore regions to deep sea trenches and from low to high latitudes (Arnold 1948; Brönnimann et al. 1979; Gooday 1990; Schaudinn 1896). Allogromiid foraminiferans are characterized by a membraneous or pseudochitinious test, which in some cases may have agglutinated foreign matter (Loeblich and Tappan 1964). Although most representatives of this family occur in marine or brackish water habitats, several genera have been described from freshwater environments (Blanc 1876; Claparède and Lachmann 1859; Penard 1899). Recently, the allogromiid foraminiferans have been detected in freshwater sediments by use of molecular methods (Holzmann et al. in prep.)

Until now, very few foraminiferans have been described from terrestrial habitats. In the present study, we report a terrestrial foraminiferan, isolated from soil samples of a forest in Northern Queensland, Australia. The new species was investigated by using morphological and molecular data. We compare and discuss the different data sets, which

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confirm the relationship of the terrestrial foraminiferan to the family Allogromiidae. Based on these data, a new allogromiid genus is proposed.

Results

• ***Edaphoallogromia australica* nov. gen. et nov. spec. (Figs. 1–8)**

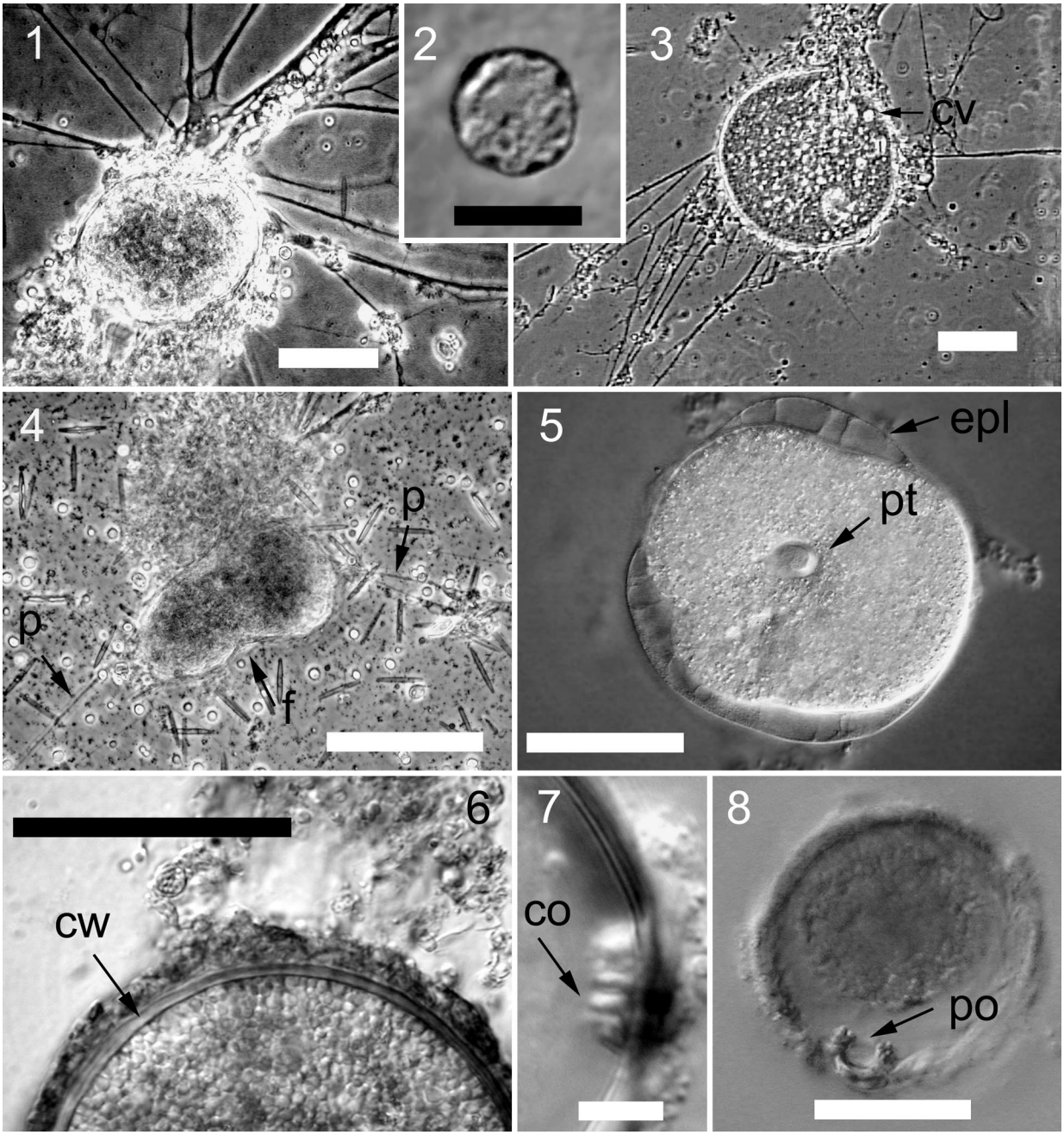
Etymology: The prefix of the genus name refers to the soil habitat.

Type species: *Edaphoallogromia australica*

Diagnosis: Multinucleate allogromiid foraminiferan with a flexible test, single aperture without internal septum and entosolenian tube.

Type location: Forest humus under Eucalyptus, Millstream Falls, Northern Queensland, Australia.

Type material: Two syntype slides fixed with sublimate and mounted in EUPARAL (CHROMA) have been deposited in the Oberösterreichische Landesmuseum Linz, Austria (accession numbers: 2001/183 & 2001/184). Specimens are marked with a diamond on the cover glass.



Description

Vegetative stage (Figs. 1–5)

Test membranous, flexible, ovoid or spherical. Diameter of the cell usually 70–150 µm (mean 122.7 µm, std. dev. 20.4, n = 20), with extremes between 48–230 µm (Fig. 6). One aperture, which is obscure and therefore very difficult to see, often located in a depression of the test. One to four contractile vacuoles usually close to the opening. Cytoplasm granular with numerous food vacuoles. About 30 small nuclei 5.0–6.8 µm each (mean = 5.7 µm, std. dev. = 0.9, n = 30), with chromatin (nucleoli?) concentrated underneath the nuclear membrane (Fig. 2).

Pseudopodia emerge from a very short, often indistinct peduncle or pseudopodial trunk (Fig. 5). A septum or an entosolenian tube (tube-like internal structure extending from the aperture in proximal direction) is never present. The pseudopodia show typical bi-directional granular streaming, forming a pseudopodial network that covers an area of more than 500 µm in diameter. The tectum of the cell is often enveloped by a layer of vacuolated plasma from which pseudopodia are formed (Fig. 5). Actively feeding animals are decorated by food and detritus particles (Fig. 1). Under the culture conditions used cells mainly multiply during the first two weeks, after this period almost all individuals transform to resting stages. Therefore in older cultures only few cells are active.

Resting stages (Figs. 6–8) have a 2.0 to 3.5 µm thick cyst wall of brownish color that darkens with age. Their diameter varies between 55–120 µm (mean = 75.2, std. dev. = 16.1, n = 30). The surface of older cysts is covered with a thick biofilm that mainly consists of bacteria and detritus (Fig. 6). In some of the fixed material, a pore-like structure can be seen which possesses an internal thickening made of small spherical or cone-like organic material (Figs. 7, 8). The inner rim of this ring-like structure is uneven. The plasma contains spherical grana (2.0–2.5 µm each) which are usually homogeneous but in one preparation small inclusion can be seen. Nuclei could not be stained. Refractive crystals are common.

Reproduction and Life Cycle

Although we could not observe the complete cell cycle, dumbbell shaped vegetative stages (Fig. 4) with pseudopodia at opposite sides indicate a pathway for horizontal binary fission where both daughter cells get one half of the tectum. There is no evidence for a complex life cycle with alternating sexual and vegetative reproduction or schizogony with multiple fissions like in *Allogromia laticollaris*.

Sequence Data

Partial SSU rDNA sequences were obtained for 16 foraminiferal species. The sequenced fragment is situated downstream near the 3' terminal end of the SSU rDNA, corresponding to positions 1181–1871 in *Rattus norvegicus* (K-01593). A striking character of foraminiferal sequences is their unusual length. The examined fragment ranges from 964 to 1444 bp, which is about twice as much than in most other eukaryotes. The unusual length results from inser-

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Figure 1. Normal vegetative form with extended pseudopodial network (Phase Contrast).The tectum is covered with food particles. Live cell, scale bar = 50 µm.

Figure 2. Nucleus with peripheral chromatin. Fixed and stained (Interference Contrast). Scale bar = 5 µm.

Figure 3. Large vegetative form (Phase Contrast) with food and contractile vacuoles (cv). Live cell, scale bar = 100 µm.

Figure 4. Dumbbell shaped stage indicating beginning binary fission (f). There are already two openings at opposite poles with independent bundles of pseudopodia (p). Live cell, scale bar = 100 µm.

Figure 5. Apertural view of a slightly pressed specimen showing the pseudopodial trunk (pt). The surface of the cell is covered by an external plasma layer (epl) with numerous vacuoles (Interference Contrast). Live cell, scale bar = 50 µm.

Figure 6. Resting stage filled with granular plasma. The cyst wall (cw) is covered with a thick layer of food particles and detritus. Live cell, scale bar = 50 µm.

Figure 7. Optical section through a resting stage showing the cone shaped structures (co) surrounding the pore. Fixed material, plasma shrunk during dehydration, mounting medium EUPARAL, scale bar = 10 µm.

Figure 8. Resting stage with pore (po) and internal thickening. Fixed material, plasma shrunk during dehydration, medium EUPARAL, scale bar = 50 µm.

tions, unique to foraminiferans, in conserved regions of the gene. The G+C contents varies from 28.9 to 43.9%. Allogromiida in general are among those foraminiferal groups with the lowest G+C content (~30%), a fact that is due to long series of A+T in expansion segments. The examined fragment includes six variable regions, among them region I corresponds to the universal variable region V6 of the prokaryotic secondary structure model (Neefs et al. 1993).

Phylogenetic Analysis

Analysis of the partial SSU rDNA gene conducted by neighbor joining (NJ) and maximum likelihood (ML) methods results in nearly identical phylogenetic trees. Figure 9 represents the ML tree with Miliolida (*Borelis* sp., *Peneroplis* sp.) used as an outgroup. An athalamid foraminiferan, *Reticulomyxa filosa,* branches as a sister group to all others (bootstrap value 39%). The new allogromiid species branches as a sister group to *Allogromia laticollaris*, *Allogromia* sp._A and *Allogromia* sp._J (bootstrap value of 100%). Allogromiidae form a very well supported (98%) monophyletic clade that branches at the base of Astrorhizina (*Astrorhiza triangularis*, *Astrammina rara*) (41% bootstrap). A second group of Astrorhizina containing *Notodendrodes* sp., *Notodendroides hyalinosphaira*, *Hemispahaerammina bradyi* and *Rhabdammina cornuta* builds a sister clade to rotaliid (*Nummulites venosus*) and textulariid (*Textularia* sp.) foraminiferans (76% bootstrap).

The only differences between the ML tree shown in Figure 9, and the NJ tree using Kimura's two-pa-

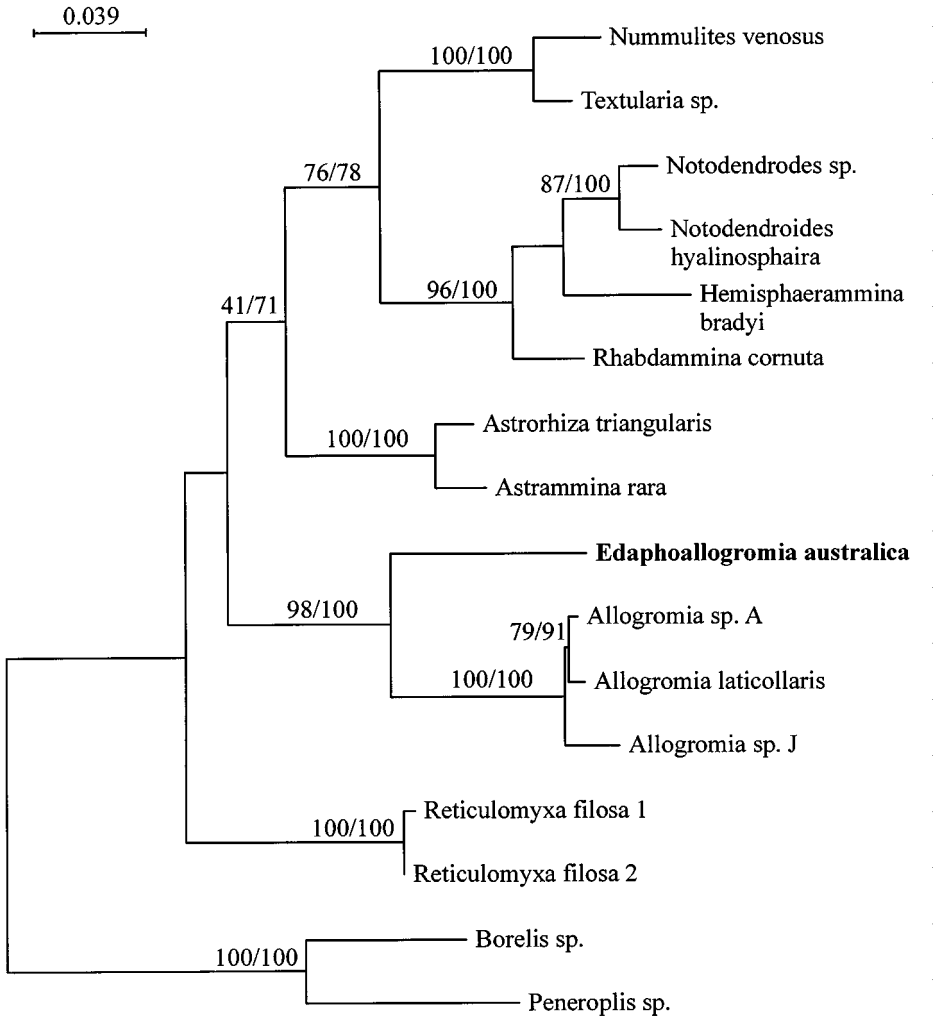

Figure 9. Phylogenetic tree based on partial SSU rDNA sequences of sixteen foraminiferan species using maximum likelihood analysis. The tree is rooted in *Peneroplis* sp. and *Borelis* sp. Bootstrap values are based on 100 resampling for the ML and on 500 resampling for the NJ tree (first and second numbers respectively).

rameter model (Kimura 1980), (data not shown) lies in a switch of the branching position of *H. bradyi* and *R. cornuta*. Compared to the ML tree, *H. bradyi* clusters at the base of the astrorhizine group in the NJ tree. NJ analysis using the Tamura and Nei model (Tamura and Nei 1993) with γ-approximation (Yang 1996), delivered a tree that is nearly identical to the ML tree shown in Figure 9 (data not shown). The only difference concerns a switch in the branching position of *Reticulomyxa filosa* and the *Allogromia* spp./*E. australica* group. Compared to Figure 9, the *Allogromia* spp./*E. australica* group branches as a sister group to all others.

Discussion

The affiliation of the new terrestrial foraminiferan to the family Allogromiidae is unquestionable according to our molecular results (98–100% bootstrap value). Comparison of the sequences shows that the new allogromiid species differs by 19.7–20.8% from the three specimens of *Allogromia* used in our analysis, while sequence variations between the latter three specimens range from 1.5–3.9%. Inter- and intraspecific variations in foraminiferans have been investigated in several studies (de Vargas et al. 1999; Holzmann and Pawlowski 1997, 2000; Holzmann et al. 1996, 1998; Huber et al. 1997) and by comparing the genetic distances within the allogromiid group to the level of divergence in other foraminiferal taxa, the new allogromiid species can be assigned to a different genus.

The classification of the new foraminiferal species into a specific allogromiid genus, however, is a point of discussion as morphological data show some contradictory results. Foraminiferal classification is based exclusively on morphological characters of their tests. Allogromiid foraminiferans are especially an arduous task for taxonomists, as the lack of distinctive morphological characters and poorly defined type descriptions often lead to difficulties in the identification of species.

The new species has several morphological characters in common with *Allogromia*, like the flexible tectum, the symmetrical pseudopodial trunk and the morphology of the nuclei (Table 1). A significant difference is the lack of an entosolenian tube. *Allogromia* Rhumbler, 1904 is mainly reported from marine habitats, but some species have been collected from freshwater or moss environments like *A. ledanteci* De Saedeleer 1934 or *A. oviformis* (Schultze 1854). *A. fluviatilis* (Dujardin 1841) has been reported also from soil (Grandori 1934; Varga 1933), but the respective figures do not show any details of the apertural morphology like an entosolenian tube or a septum. None of these freshwater species has been cultured under controlled conditions. It is not clear whether these species have an alternating life cycle like in *A. laticollaris*. Therefore, their identification and their relation to *A. laticollaris* remains uncertain. On the other hand, some characters of the Australian isolate like the pseudopodial trunk without an entosolenian tube point to a relationship with the genus *Lieberkuehnia*. In *Edaphoallogromia*, however, the trunk is symmetrical whereas in *Lieberkuehnia* it is always asymmetrical and separated by a distinct septum. The size range is comparable to *L. wagneri* Claparède & Lachmann, 1859, a species which has been frequently reported from

Table 1. Synopsis of diagnostic characters of some related allogromid foraminifera. Data were compiled from De Saedeleer (1934); Lee & McEnery (1970) and Penard (1907). In *Allogromia laticollaris* the number of nuclei and the size depends on the stage in the life cycle.

	Pseudo-podial trunc	Sep-tum	Ento-solenian tube	Number of Nuclei	Diameter of the nuclei	Type of nucleus	Size	Sex
Edaphoallo-gromia	sym	–	–	~ 30	6 µm	ovular	50–230 µm	–
Lieberkuehnia wagneri	*as*	+	–	several	6 µm	vesicular	32–200 µm	–
Lieberkuehnia paludosa	as	+	–	1–32	–82 µm	ovular	150–475 µm	–
Allogromia fluviatilis	sym	–	+	1	?	ovular	250 µm	?
Allogromia laticollaris	sym	–	+	1–>100	6–74 µm	ovular	80–500 µm	+

Abbreviations used: as = asymmetrical, sym = symmetrical, sex = sexuality

semiaquatic habitats like mosses, but the vesicular nuclei differentiate *L. wagneri* from our isolate. A similar species but with a much larger nucleus is *L. paludosa* (Cienkowsky, 1876). A non-marine allogromid foraminferan from Australia has been described by Lendenfeld (1885) as *Lieberkuehnia australis*. Averintzew (1906) has placed the latter form in synonomy with *L. wagneri* but the lack of figures and the description of pseudopods as being straight and unbranched make a classification impossible.

A distinct feature of the new species is its resting stage. None of the known allogromiid species has a comparable stage, which is a prerequisite for life in soils with a fluctuating water content.

The chimera morphology of the new allogromiid species does not allow a definite classification into either of the two allogromiid genera. The family Allogromiidae presents in reality a very little known group and inadequate type descriptions exceedingly impede taxonomic work. Our molecular data point to the fact that the newly discovered species is a close relative to *Allogromia*. No *Lieberkuehnia sp.*, however, have so far been genetically analysed, thus there is no possibility to compare the rDNA sequences of the two allogromiid genera. On the other hand, the mixture of morphological characters as well as the particular habitat could also be an indication for a new allogromiid genus. Based on the distinct morphological and genetical characters of the investigated specimens and taking into account the confused taxonomy of the group, we prefer to erect a new genus rather than to press it it arbitrarily into an existing one.

To resolve these problems in a more comprehensive way, additional sampling and morphological as well as molecular characterisation of other allogromiid foraminiferans from different habitats and geographic regions will be necessary.

Methods

Isolation and culture conditions: The new allogromiid species was isolated from a raw culture inoculated with humus material collected in a tropical Eucalyptus forest at Millstream Falls (Ravenshoe, Northern Queensland, Australia). The humus samples were taken in September during the dry season.

Cultures were kept in an artificial freshwater medium which contained the following ingredients per litre distilled water: 11.8 mg $Ca(NO_3)_2$ $4H_2O$, 14.7 mg $CaCl_2$ $2H_2O$, 12 mg KH_2PO_4, 22 mg KNO_3, 41.8 mg $MgCl_2$ $6H_2O$, 25.3 mg $MgSO_4$ $7H_2O$, 9.2 mg Na_2CO_3, 25.3 mg Na_2SiO_3 $9H_2O$, 0.9 mg NH_4Cl, 10 ml trace element solution, 2 mM MES and 100 mg Cerophyll (Sigma) as carbon source. The pH was adjusted with NaOH to a value of 6.5. Different algae (*Chlorella vulgaris*, *Nitzschia palea*), bakers yeast and *Enterobacter aerogens* were added as food components. Culture conditions: 20 °C, dark. Cultures of the protist strain used in this study are available from the authors upon request.

Light microscopy: The morphology of the pseudopodial network and the granular streaming of active specimens were studied in thin plankton chambers with inverted microscopes (LEITZ, OLYMPUS) and phase contrast. For all other details we have used an upright microscope (ZEISS) with Nomarski differential interference contrast (DIC). Dimensions of cells and cysts were measured with an image analysis system LUCIA (NIKON) in manual mode. To study the nuclei, cells were fixed in sublimate (10% saturated solution) and stained with bromophenol blue. After dehydration specimens were mounted in EUPARAL (CHROMA).

DNA extraction, amplification, cloning and sequencing: Living specimens showing extended pseudopodia were identified by use of a stereomicroscope, transferred into an individual receptacle and cleaned by brushing. DNA extractions contain several specimens (5–30) and were performed by using DNeasy Plant Mini Kit (Qiagen).

SSU rDNA was amplified by PCR in a total volume 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. The amplified PCR products were purified using High Pure PCR Purification Kit (Roche Diagnostics), then ligated into pGEM-T Vector system (Promega) and cloned in XL-2 Ultracompetent Cells (Stratagene). Sequencing reactions were prepared by using ABI-PRISM Big Dye Terminator Cycle Sequencing Kit and analysed with an ABI-377 DNA sequencer (Perkin-Elmer), all according to each manufacturer's instructions.

Partial SSU rDNA sequences were obtained by amplification with the primer pair s14F3 (5′ ACGCA(AC)GTGTGAAACTTG; position 1181-1198 in rat) and sB (5′ GTAGGTGAACCTGCAGAAGGATCA; position 1848-1871 in rat). The new sequences reported in this paper have been deposited in the EMBL/GenBank database under the following accession numbers: (AJ311212) *Nummulites venosus*, (AJ311216) *Hemisphaerammina bradyi*, (AJ311215) *Rhabdammina cornuta*, (AJ311214) *Notodendroides hyalinosphaira*, (AJ311213) *Notodendrodes* sp., (AJ311217) *Edaphoallogromia australica*, (AJ311218) *Allogromia laticollaris*, (AJ311219) *Reticulomyxa filosa 2*. The sequence of

Allogromia sp._A *(X86093*) was published by Pawlowski et al. (1996), *Allogromia* sp._J (Z69607), *Astrammina rara* (Z69608), *Astrorhiza triangularis* (Z69609) and *Textularia* sp. (Z69610) sequences were published by Pawlowski et al. (1997), and those of *Reticulomyxa filosa*_1 (AJ132367), *Borelis* sp. (AJ404295) and *Peneroplis* sp. (AJ132368) were published by Pawlowski et al. (1999).

Sequence analysis: Sequences were aligned manually using the GDE 2.2 software (Larsen et al. 1993). Selected sites in homologous regions without gap were retained for phylogenetic analyses. Out of 1693 sites, 247 (33.5%) were informative. The alignment is available from the authors upon request. Phylogenetic analyses were performed with the NJ method (Saitou and Nei 1987), applied to distances corrected for multiple hits and for unequal transition and transversion rates, using Kimura's two-parameter model (Kimura 1980) and the ML method as implemented in the fast DNAml program (Olsen et al. 1994). NJ analysis was additionally tested by applying the Tamura and Nei model (Tamura and Nei 1993) with γ-approximation (Yang 1996) using a shape parameter of 0.2766 (estimated empirically using ML methods). The reliability of internal branches was assessed by bootstrapping (Felsenstein 1988) with 500 resampling for the NJ and 100 resampling for the ML tree respectively. The PHYLO_WIN program (Galtier and Gouy 1996) and PAUP* 4.0b version (Swofford 2000) were used for distance computations, NJ and ML tree-building and bootstrapping.

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Note: *Allogromia* sp._A was sampled in Antalya (Turkey), *Allogromia* sp._J was sampled in Discoverey Bay (Jamaica)

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