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# Allogromiid foraminifera and gromiids from under the Ross Ice Shelf: morphological and molecular diversity

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**Abstract** Allogromiid foraminifera and gromiids constitute an important but poorly known component of the meiobenthos in high-latitude marine settings. Here, we present a first report on the diversity of these protists, together with an account of co-occurring invertebrate taxa, in a sample of sea floor sediment from a water depth of 923 m under the Ross Ice Shelf. Light microscopy of sieved sediment yielded 14 allogromiid foraminifera and three *Gromia* spp. morphotypes. We also obtained 36 partial small subunit rDNA sequences of allogromiids, including 19 sequences of individual isolates and 17 sequences from environmental DNA samples. Phylogenetic analyses of the obtained sequences identified 18 phylotypes, of which seven were closely related to previously sequenced allogromiids, while 11 represented new lineages. Our results demonstrate that a rich meiofaunal assemblage is present under the Ross Ice Shelf, 12 km from the open ocean. Molecular phylogenetic analyses of these allogromiid foraminifera point to the endemic character of the Ross Ice Shelf assemblage; however, more extensive sampling of the molecular diversity of this group of foraminifera in the Antarctic deep sea is necessary to confirm this conclusion.

## Introduction

In 1976, American scientists fashioned a hole through 420 m of ice on the Ross Ice Shelf, at a site known as J9 (82 22.5′S, 168 37.5′W), approximately 450 km from the open sea (Clough and Hansen 1979). In addition to the various geological and oceanographic questions addressed by that project, biologists were eager to learn whether or not life could tolerate the extreme conditions thought to exist there, such as the coldest, densest water on Earth, and absence of photoproduction. To their surprise, their cameras recorded the presence of various fish and invertebrates inhabiting the sub-shelf benthos (Lipps et al. 1979; Bruchhausen et al. 1979). No living benthic meiofauna were reported, although sparse populations of zooplankton were encountered in the water column (Azam et al. 1979).

The biological findings of the Ross Ice Shelf Project (RISP) helped in spawning further national and international efforts to study life at the extremes of earthly conditions (e.g., the U.S. National Science Foundation's LExEn research program established in 1997), and to foster the nascent science of astrobiology. Although such programs have led to an explosion of knowledge regarding microbial extremophiles, the organisms inhabiting highly oligotrophic regions under thick ice cover have not been investigated since that original study, nearly 30 years ago. In particular, the curious absence of meiofauna at the RISP site raises questions about the functioning of deep-water subshelf ecosystems.

Meiofauna play important intermediary roles in the cycling of nutrients in deep-sea settings (e.g., Soltwedel 2000; Gooday 2002a). In such regions, the meiofauna often includes a diverse assemblage of morphologically

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simple, usually single-chambered, foraminiferan protists with either a delicate organic theca or an agglutinated test (Cedhagen et al. 2002), as well as gromiids, a group of testate protists possessing filose pseudopodia (Arnold 1972; Burki et al. 2002). The ecological role of these protists is poorly known but, given their abundance, they are probably involved in the cycling and decomposition of organic matter on the seafloor (Gooday et al. 2000; Gooday 2002a, 2002b). Allogromiids and gromiids are particularly abundant and diverse in polar environments (e.g., Gooday et al. 1996; submitted). Along the western Eurasian Arctic shelf, small allogromiids dominate the foraminiferal assemblage in stressed marginal environments, while large agglutinated ones are the most important contributors to foraminiferal biomass in shelf muds (Korsun 2002).

Despite their abundance and probable ecological importance, the total diversity of allogromiids and gromiids is poorly known, in large part due to the difficulties in their isolation and morphological identification. In particular, these two major protistan taxa possess few structural features that can be used to distinguish them unambiguously. Morphologically, allogromiids are identified mainly by shell (test) features, such as wall composition and structure and number of apertures (Gooday 2002b). Gromiid morphotypes differ principally by body size and shape, number and placement of apertures, and form of the theca. The morphological distinction between gromiids and allogromiids is not always clear; indeed, some gromiids have been mistaken for allogromiids (e.g., *Allogromia marina*; Nyholm and Gertz 1973). Traditional morphology-based, suprageneric classification of foraminifera separates organic-walled allogromiids sensu stricto from agglutinated single-chambered astrorhizids (Loeblich and Tappan 1987).

In the past few years, molecular approaches have been used to study the phylogenetic relationships among foraminifera, and to revise their classification (reviewed in Pawlowski 2000). Molecular data have also been used to examine species diversity in selected groups of benthic and planktonic foraminifera (Hayward et al. 2004; De Vargas et al. 2002; Kucera and Darling 2002). In the case of allogromiid foraminifera, molecular phylogenetic studies have demonstrated that allogromiids sensu stricto are closely related to astrorhizids (Pawlowski et al. 2002b, c; 2003). Based on analyses of molecular data, 13 lineages of allogromiids have been distinguished (Pawlowski et al. 2002b, c). Each lineage is composed of many phylotypes, whose number exceeds by far the number of known morphospecies. For example, 52 molecular phylotypes were identified among allogromiids from Explorers Cove, Antarctica (Pawlowski et al. 2002b), and even more were recognized in environmental DNA screens (Habura et al. 2004). Reports of new allogromiid species, as well as species redescriptions, now routinely include both morphological and molecular data (Bowser et al. 2002; Cedhagen and Pawlowski 2002; Wilding 2002; Gooday and Pawlowski 2004; Gooday et al. 2004b, submitted; Sabbatini et al. 2004). Molecular data have also been used to establish the phylogenetic position of *Gromia* among cercozoan protists (Burki et al. 2002) and to infer its close relationship to the Foraminifera (Berney and Pawlowski 2003; Longet et al. 2004).

In the present study, we use morphological and molecular data to examine the diversity of allogromiids and gromiids in deep-sea sediment under the Ross Ice Shelf. This study is the first report on living benthic protists recovered from under an ice shelf. Several unknown morphotypes and molecular types have been identified, revealing a surprising diversity of these organisms in such a seemingly inhospitable environment.

## Material and methods

A sample of about 1 l of sea-floor surface sediment was collected from a water depth of 923 m bsl under the Ross Ice Shelf at Windless Bight, 12 km east of the ice edge (ANDRILL site HWD-2; 77°50.111′S, 167°20.209′E). Further information on the site and sampling procedures has been published elsewhere (Barrett et al. 2004).

The sediment sample was sieved through serial 1-, 0.5-, and 0.125-mm meshes using filter-sterilized sea water, and the residues were stored in a refrigerator at 0°C. Protists (foraminifera and gromiids) and invertebrates were placed in a Petri dish of seawater kept cool in a dish of ice, and were then picked individually with the aid of a binocular microscope. Specimens of foraminifera and gromiids were photographed with a Nikon Coolpix 900 digital camera mounted on a Wild stereomicroscope. Individual specimens were transferred to microtubes each containing 60 μl of guanidine DNA extraction buffer, or were frozen in $LN_2$, for subsequent molecular analyses (Pawlowski 2000). Other isolated specimens were fixed in 3% glutaraldehyde buffered (pH 7.2) with 0.1 M sodium cacodylate for scanning electron microscopy (SEM), using routine methods (Bowser and Travis 2002). Several 5-ml samples of unsieved sediment, as well as an unsorted sample of the 125–500 μm residue, were frozen in $LN_2$ for extraction of environmental DNA (Habura et al. 2004). Invertebrates were identified to the lowest taxon possible.

A fragment of the foraminiferal small ribosomal subunit (SSU) rRNA gene was amplified by PCR with the primer pair s14F3 (5′ACG CA(AC) GTG TGA AAC TTG) and sB (5′TGTCC TTC TGC AGG TTC ACC TAC), as described by Pawlowski (2000). Some PCR products were re-amplified using nested primers s14F1 (5′AAG GGC ACC ACA AGA ACG C) and/or s20r (5′GAC GGG CGG TGT GTA CAA). PCR products from single-cell extracts were purified using a High Pure PCR Purification Kit (Roche Diagnostics), and were then either sequenced directly or ligated into the pGEM-T Vector system (Promega) and cloned in XL-2 Ultracompetent Cells (Stratagene). PCR products from environmental extracts were cloned, purified, and sequenced as described previously (Habura et al. 2004).

Sequencing reactions were prepared using the ABI-PRISM Big Dye Terminator Cycle Sequencing Kit, and were analyzed with an ABI-377 DNA sequencer (Perkin-Elmer), all according to the manufacturer's instructions.

Sequences were aligned manually to the large database of foraminiferan sequences, using SEAVIEW software (Galtier et al. 1996). Five hundred and three sites were selected for analysis, including 218 variable and 156 informative sites. Phylogenetic analyses were performed with the neighbor joining (NJ) and the maximum likelihood (ML) methods, using the FASTDNAML tree-building algorithm (Olsen et al. 1994). The reliability of internal branches was assessed by bootstrapping (Felsenstein 1985), with 1,000 re-samplings for the NJ and 100 re-samplings for the ML trees. The Phylo_win program (Galtier et al. 1996) was used in distance computations for various models, NJ and ML tree-building, and bootstrapping.

**Table 1** Benthic invertebrate taxa recovered from sediments under the Ross Ice Shelf (ANDRILL HWD-2 site)

| Higher taxon | | # specimens |
|---|---|---|
| Polychaeta | Glyceridae: *Hemipodus* sp.? | 4 |
| | Paraonidae: *Paraonis* sp.? | 2 |
| | Spionidae (juveniles) | 4 |
| | Capitellidae (juvenile) | 1 |
| | Ampharetidae (juvenile) | 1 |
| | *Apharete* sp.? (adult) | 1 |
| | Archiannelida? (*Polygordius*?) | 2 |
| | Oweniidae: *Galathowenia*? | 13 |
| | Opheliidae: *Ophelina*? | 1 |
| Oligochaeta | Oligochaeta? | 1 |
| Nematoda | Nematoda | 6 |
| Sipuncula | Sipuncula | 1 |
| Mollusca | Thyasiridae: *Genaxinus debilis*? Juveniles | 4 |
| | Sareptidae: *Yoldiella* sp.? | 1 |
| Crustacea | Cumacea: *Eudorella* sp.? (juvenile) | 1 |
| | unknown cumacean (juvenile) | 1 |

## Results

### Microscopic observations

The recovered sediment sample (~1 l) consisted of silty mud, laced with sponge spicules. The fractions (>1 mm, 0.5–1 mm, 0.125–0.5 mm) obtained by sieving the sediment sample contained a relatively small volume of material. The largest size fraction (>1 mm) was sparsely populated and contained only a few invertebrates, large gromiids, and algal debris. The 0.5- to 1-mm fraction was more important quantitatively, and contained some algal debris, a few living polychaetes, nematodes, and bivalves. Several large allogromiid foraminifera and gromiids, as well as a few living *Reophax* (a multi-chambered, agglutinated foraminiferan), were found in this fraction. The 0.125-mm fraction contained a large number of empty and/or fragmented multi-chambered agglutinated foraminiferal (textulariid) tests, as well as some living allogromiids, gromiids, nematodes, and algal debris. Metazoans recovered from the total sieved residues included 29 specimens of polychaetes (nine taxa), one oligochaete, six nematodes, one sipunculid, five molluscs (two taxa), and two cumacean crustaceans (Table 1). Many of these specimens were juveniles.

In total, 13 morphotypes of living allogromiid foraminifera and three *Gromia*-like morphotypes were found under the Ross Ice Shelf. The allogromiid and gromiid morphotypes encountered were considered alive, based on the presence of protoplasm inside the test and/or our success in obtaining SSU rDNA sequence data. The morphological characteristics of the isolated allogromiids and gromiids are given in Table 2, and selected morphotypes are illustrated in Figs. 1 and 2.

### Molecular data

We obtained 36 partial SSU rDNA sequences of allogromiid foraminifera, including 19 sequences from DNA extracts of individual isolates and 17 sequences from environmental DNA samples. Additionally, seven sequences were obtained for three different morphospecies of the genus *Reophax*: *R. spiculifer* (RIS3895), *R. guttifera* (RIS3883), and *Reophax* sp. (RIS3884). Some sequences of individual isolates were obtained by direct sequencing. When cloning was necessary, one to three clones were sequenced, but the variability between clones did not exceed 1%. In two cases, different sequence types were obtained. DNA isolate 3923, derived from a komokiacean-like mudball, yielded two different sequence types, thus further illustrating that large foraminifera may serve as a habitat for other "squatting" species (e.g., Moodley 1990). Allogromiid morphotype 5, which was unlikely to harbor squatters, yielded three distinct sequences; this finding may further illustrate convergent evolution among unilocular foraminifera (e.g., Pawlowski et al. 2002c).

In our analysis of environmental DNA, foraminiferan sequences were obtained from only one unsieved sample (SB2) and from two samples of the 0.125- to 0.5-mm sediment fraction (RIS4259 and RIS4260). All sequences from the SB2 sample were identical, and were close to the sequences obtained from isolate 3923_Q, derived from a komokiacean-like mudball. Seven sequences obtained from the two sieved samples were similar, and formed a separate clade branching among the allogromiids (see below). One environmental sequence obtained from isolate 4260 was a match for *Bathysiphon argenteus.*

After removal of identical sequences, 18 sequences were included in our phylogenetic analyses, together with 42 sequences derived from representative allogromiid foraminifera in McMurdo Sound and elsewhere. The phylogenetic positions of all of these sequences are illustrated in Fig. 3. Among the 18 phylotypes, seven were closely related (<1.5% sequence divergence) to those obtained from other regions, while 11 diverged by more than 5% from other sequences. The

**Table 2** Morphological description of live allogromiid foraminifera and gromiids recovered from sediment under the Ross Ice Shelf (ANDRILL HWD-2 site)

| Morphotype | Brief description | Phylotype Number | Sieve fraction (mm) |
|---|---|---|---|
| *Hippocrepinella alba* | Large, white, elongate form; theca with distinct aperture at one end and pinched posterior region; max. length 1 mm (4 specimens) | RIS3906 | 0.5–1 |
| *Bathysiphon argenteus* | Long, thin test, slightly tapering towards one end; white-silver appearance typical for this species; sometimes with debris attached to the aperture (12 specimens) | RIS3888 | 0.125–0.5 |
| *Notodendrodes hyalinosphaira* | Large, spherical, coarsely agglutinated test filled with yellow protoplasm (1 specimen) | RIS3919 | 0.5–1 |
| *Psammosphaera* sp. | Spherical, coarsely agglutinated test, often attached to a long sponge spicule (5 specimens) | Not sequenced | 0.125–0.5 |
| Silver saccammina 1 | Small, ovoid test, silver in appearance; aperture protruding with broad rim (2 specimens) | RIS3894RIS3913 | 0.125–0.5 |
| Silver saccammina 2 | Small, elongate, finely agglutinated silver-white test with apertures at both ends (1 specimen) | RIS3907 | 0.125–0.5 |
| Unidentified allogromiid 1 | *Vellaria*-like, transparent theca; single aperture with long neck and irregular periphery; orange-yellow protoplasm (2 specimens) | Not sequenced | 0.125–0.5 |
| Unidentified allogromiid 2 | Small, ovoid, with transparent theca and single aperture; filled with white protoplasm; nucleus visible (1 specimen) | Not sequenced | 0.125–0.5 |
| Unidentified allogromiid 3 | Long and thin, with transparent theca, *Nemogullmia*-like, with white protoplasm (3 specimens) | RIS3887 | 0.125–0.5 |
| Unidentified allogromiid 4 | Small and fusiform, constricted centrally; theca with aperture at one end (1 specimen) | Not sequenced | 0.125–0.5 |
| Unidentified allogromiid 5 | Elongate and slightly curved, with one end larger than the other; aperture not observed; white granular protoplasm (10 specimens) | RIS3909RIS4026RIS3901 | 0.125–0.5 |
| Unidentified allogromiid 6 | Large, elongate, *Pelosina*-like test with a few arborescent filaments at both ends (1 specimen) | RIS3920 | 0.5–1.0 |
| Unidentified allogromiid 7 | Komoki-like mudball with clumps of filamentous peripheral extensions (4 specimens) | RIS3923 | 0.5–1.0 |
| Unidentified allogromiid 8 | Elongate, transparent theca, partially filled with protoplasm (1 specimen) | RIS3891 | 0.125–0.5 |
| Environmental DNA | Allogromiid phylotype from environmental sample | SB2_1 | Unsieved |
| Environmental DNA | Allogromiid phylotype from environmental sample | RIS4259RIS4260 | 0.125–0.5 |
| *Gromia* sp. 1 | Large, elongate forms with light brown protoplasm densely filling a transparent theca; aperture not observed; max. length 5 mm (4 specimens) | Not sequenced | 0.5–1 |
| *Gromia* sp. 2 | Elongate, straight or slightly curved form; transparent theca, often empty in posterior part; aperture distinctive; max length 2–3 mm (13 specimens) | Not sequenced | 0.5–1 0.125–0.5 |
| *Gromia* sp. 3 | Long, arched form; transparent theca with distinct aperture; protoplasm pocked with large voids; max length 3 mm (4 specimens) | Not sequenced | 0.5–1 |

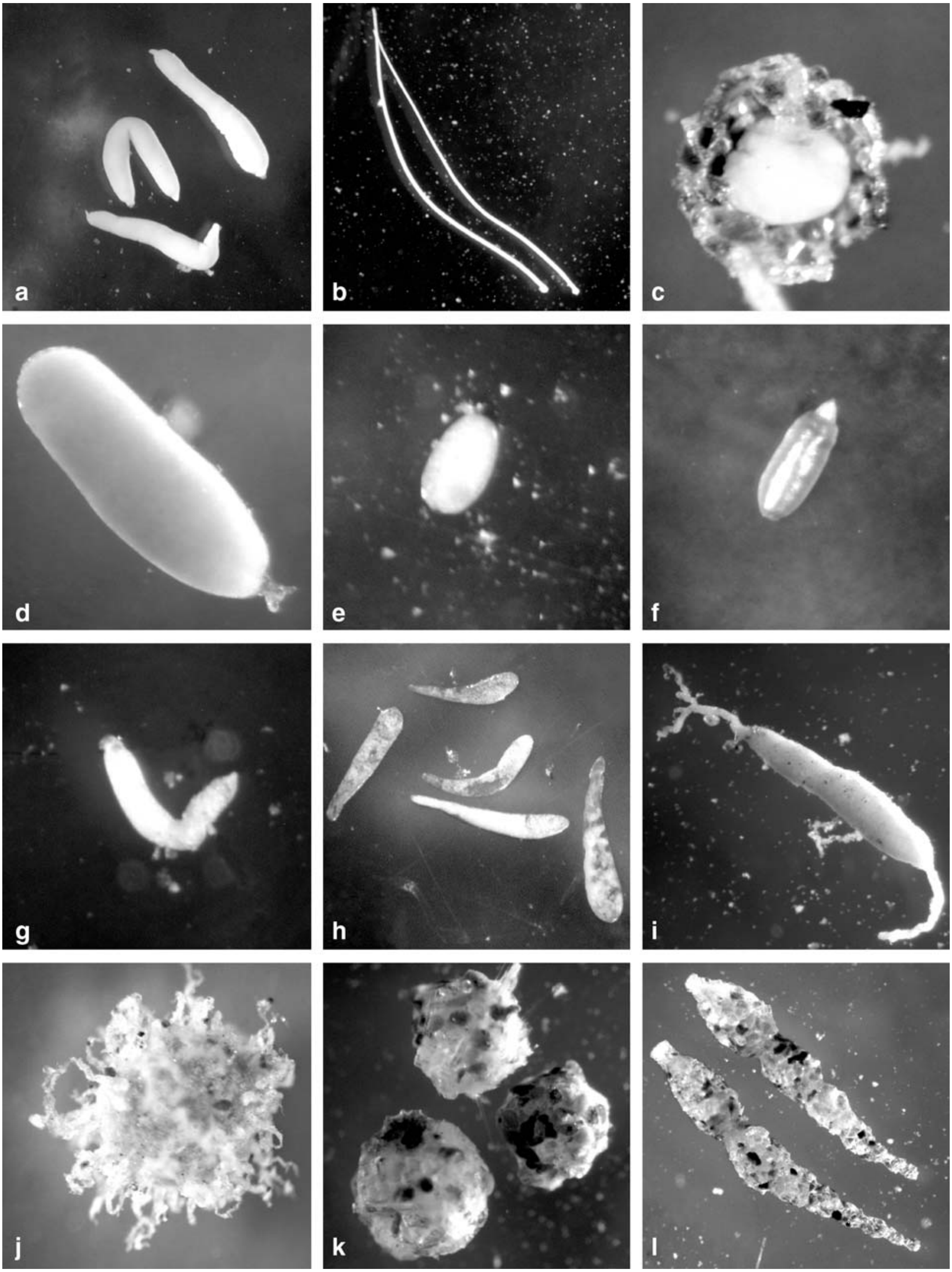

**Fig. 1** Light micrographs of representative foraminiferan protists from sample collected under the Ross Ice Shelf. **a** *Hippocrepinella* sp. (RIS3906), **b** *Bathysiphon argenteus* (RIS3888), **c** *Notodendrodes hyalinosphaira* (RIS3919), **d** Unidentified allogromiid 1 (not sequenced), **e** Unidentified allogromiid 2 (not sequenced), **f** Silver saccamminid 1 (RIS3894), **g** Silver saccamminid 2 (RIS3907), **h** Unidentified allogromiid 5 (RIS3901), **i** Unidentified allogromiid 6 (RIS3920), **j** Komoki-like allogromiid 7 (RIS3923), **k** *Psammosphaera* sp. (not sequenced), **l** *Rheophax* sp. (RIS3884)

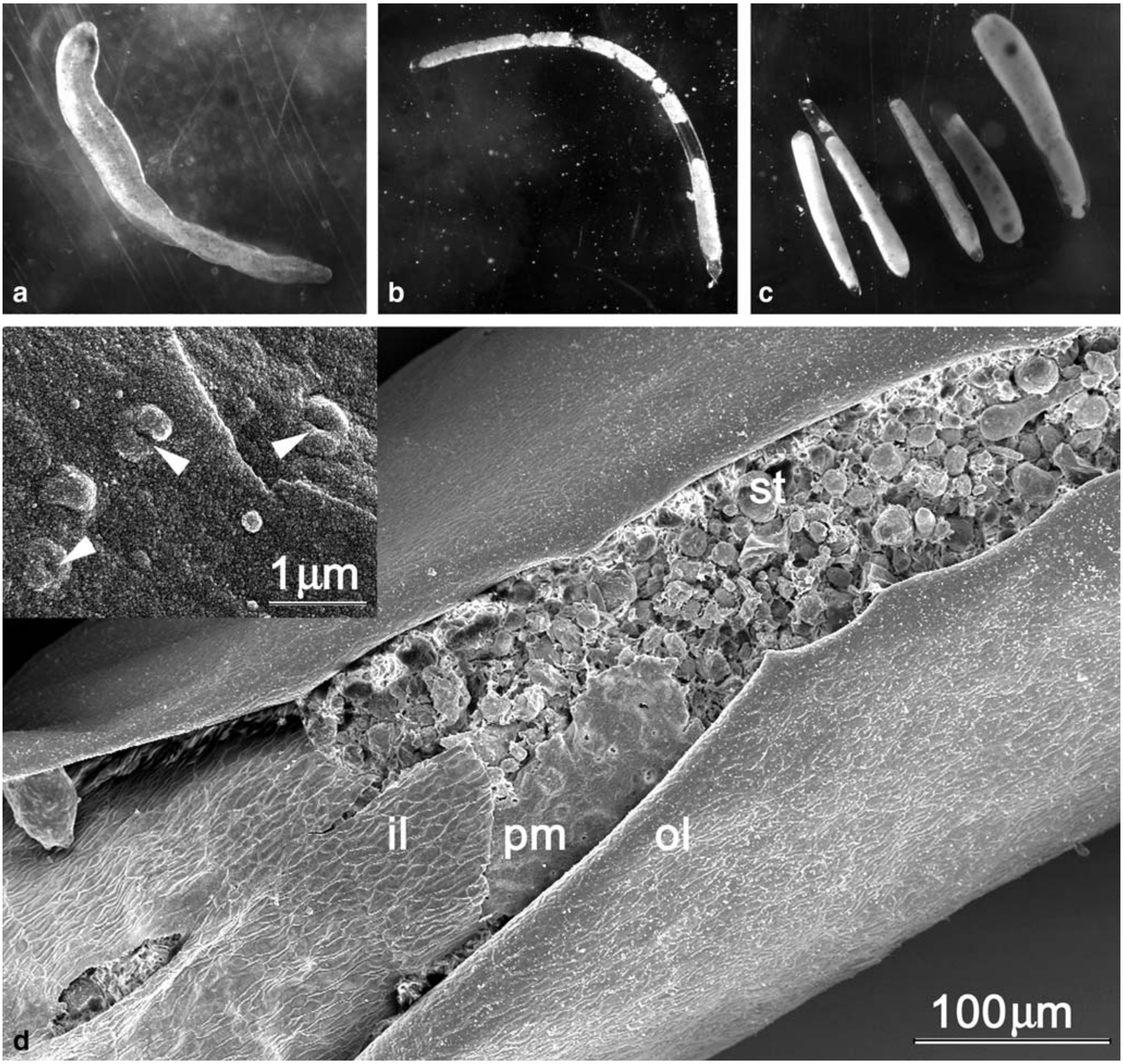

**Fig. 2** Living gromiid morphotypes from under the Ross Ice Shelf. **a** *Gromia* sp.1, **b** *Gromia* sp.2, **c** *Gromia* sp. 3. **d** Scanning electron micrographs of *Gromia* sp. 1, fractured to show outer (*ol*) and inner (*il*) layers of wall, plasma membrane (*pm*), and internal stercomata (*st*). *Inset* shows pores (*arrows*) on outer-layer surface

Ross Ice Shelf sequences were seen to be widely distributed over the tree, branching within 11 distinct lineages. The relationships among these lineages are not well resolved and they differ slightly between trees obtained with the ML method and those obtained with the NJ method. An example is the position of the environmental clade 4259 + 4260, which branches either as a sister to the *Psammosphaera* Reophax clade (Fig. 3) or next to *Bathysiphon argenteus* in the NJ tree (data not shown). However, the positions of other Ross Ice Shelf sequences remain relatively stable within the lineage.

## Discussion

The grab sample made available to us was not amenable to quantitative assessment of faunal abundances. Nevertheless, our results demonstrate that the under-shelf benthos at ANDRILL HWD-2 site, 12 km from the shelf edge, includes diverse meio- and macrofaunal protists and metazoans. This finding contrasts with the results of the earlier sampling at the J9 site, where only the skeletons of such organisms were encountered (Lipps et al. 1979). The disparity is likely the result of differences in advected phytodetritus. Current measurements at HWD-2 indicate an average net flow eastward from McMurdo Sound of around 7 cm/s (Barrett et al. 2004), which undoubtedly advects biogenic particulate matter, either as individual particles or pellets, to reach the sub-

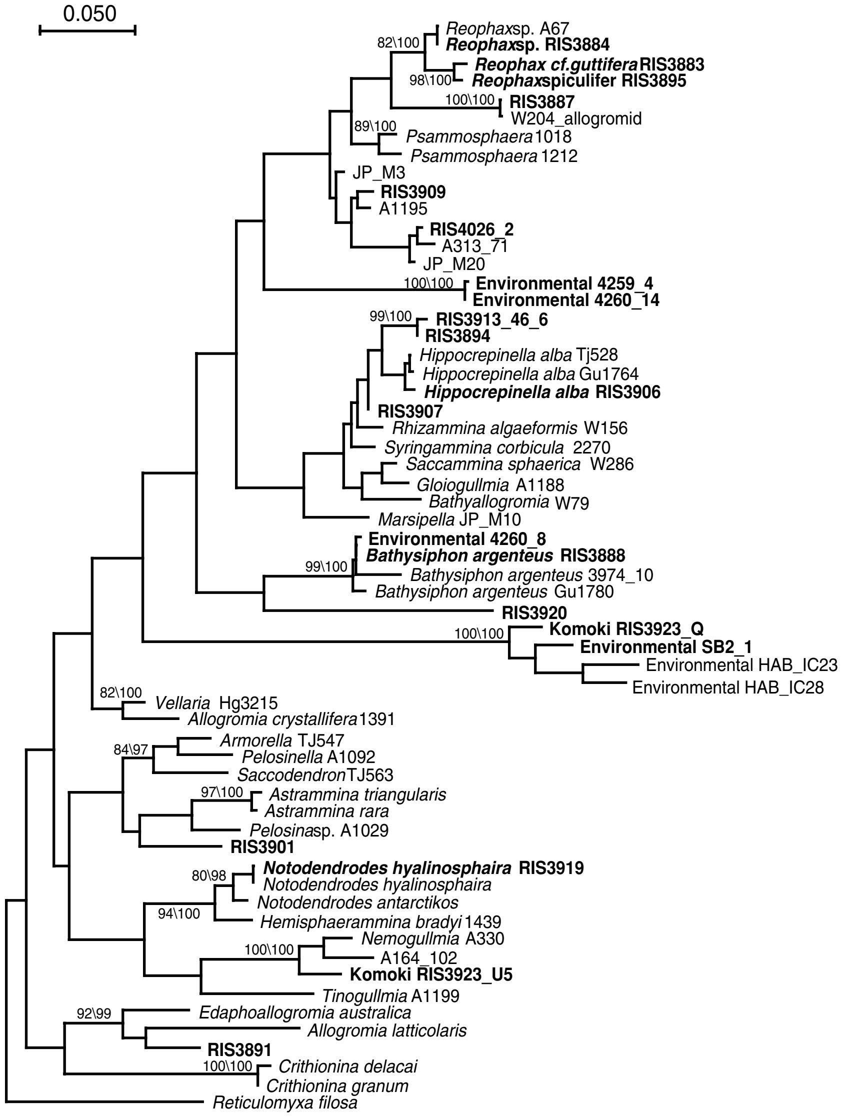

**Fig. 3** Maximum likelihood (*ML*) phylogenetic tree of foraminiferan phylotypes detected under the Ross Ice Shelf. Allogromiid phylotypes are in *boldface*. Bootstrap values for ML and neighbor joining (*NJ*) analyses higher than 80% are given above the nodes as ML value/NJ value

ice shelf site (Dunbar et al. 1998). Nevertheless, the average rate of sediment accumulation determined from $^{14}$C ages on a 60-cm core is rather low, ~0.06 mm/year (Barrett et al. 2004).

Allogromiids and gromiid protists constitute one of the dominant components of benthic biota at the HWD-2 site. Large, elongate gromiids were a common macrofaunal component in the >1-mm-size fraction, whereas foraminifera dominated in the 0.125- to 0.5-mm-size fraction. Although many polythalamous agglutinated foraminiferal (i.e., textulariid) tests were found in this fraction, none of the organisms appeared to be alive, except for a few specimens of *Reophax* sp. By contrast, living unilocular foraminifera were well represented in the sample.

The diversity of allogromiids and gromiids from the Ross Ice Shelf benthos is comparable to that of other polar regions that we have investigated. Several morphotypes, for example, psammosphaerids and silver saccamminids, are similar to those found in western McMurdo Sound (Gooday et al. 1996) and the Weddell Sea (Pawlowski et al. 2003). The genus *Hippocrepinella* was reported from both the Antarctic Peninsula and Scandinavian fjords. Specimens of *Notodendrodes hyalinosphaira* from the Ross Ice Shelf are genetically identical to those living in Explorers Cove (Pawlowski et al. 2002a). (A more detailed account of the distribution of notodendrodids in McMurdo Sound will be reported elsewhere; Habura et al. in preparation.) A sequence obtained from DNA isolate 3887 was similar to the sequences of unidentified allogromiids from 4,000 m depth in the Weddell Sea. Other species that we encountered, e.g., *Bathysiphon argenteus*, are more cosmopolitan in the deep sea. In view of these results, the Ross Ice Shelf assemblage of allogromiids appears to constitute a mixture of shallow- and deep-Antarctic species.

On the other hand, Ross Ice Shelf gromiids are morphologically distinct from those observed in our previous studies of Antarctic areas. Gromiids are abundant in shallow waters of McMurdo Sound, particularly along the eastern coast of Ross Island near McMurdo Station, and in Explorers Cove on the western coast of the continent, but they are represented in these regions by the ovoid morphospecies *Gromia oviformis* (Bowser et al. 1996; Gooday et al. 1996). Elongate gromiids similar to those found under the Ross Ice Shelf have been collected in the Greenland Sea, along the east coast of Svalbard (A.J. Gooday et al., submitted). However, the phylogenetic relationships between the Ross Ice Shelf specimens and Arctic gromiids are not yet established (work in progress).

Screens of whole-sediment environmental DNA conducted in this study revealed only three phylotypes, two of which did not correspond to identified morphotypes. Previous analyses (e.g., Habura et al. 2004) also indicated the presence of cryptic members of the foraminiferal assemblage. We speculate that these phylotypes belong to foraminifera that are fragile or small and therefore vulnerable to loss during sieving and manipulation. Our findings reinforce the contention that the most effective method for making total biodiversity estimates of benthic foraminifera is to use a combination of morphological and molecular approaches.

When compared to the material collected previously in McMurdo Sound, the Weddell Sea, and the Arctic Ocean, some Ross Ice Shelf gromiids and allogromiids appear to be new. Indeed, we found that a high proportion (~50%) of the allogromiid phylotypes are not related to any previously obtained sequences. These data could be interpreted as an indication of a high level of endemism in the Ross Ice Shelf biota. However, the molecular diversity of Antarctic allogromiids has been sampled in only a few localities (Pawlowski et al. 2002b, c, 2003; Habura et al. 2004). It is therefore possible that, with increasing sampling, at least some of the Ross Ice Shelf phylotypes will be identified in other areas.

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