Zoological Journal of the Linnean Society, 2009, **157**, 451–469. With 10 figures

Three new species of deep-sea *Gromia* (Protista, Rhizaria) from the bathyal and abyssal Weddell Sea, Antarctica

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Received 4 August 2008; accepted for publication 28 October 2008

We describe three new species of the genus *Gromia* from bathyal and abyssal depths in the Weddell Sea. The new species are characterized by a combination of morphological and molecular criteria. All three species possess a distinct oral capsule and a layer of 'honeycomb membranes', which form the inner part of the organic test wall. Both these features are typical of gromiids. Their identification as gromiids is confirmed by analyses of partial small subunit ribosomal DNA (SSU rDNA) gene sequences. ***Gromia marmorea* sp. nov.** is a rounded species with a prominent oral capsule and a characteristically mottled appearance. In ***Gromia melinus* sp. nov.**, the test surface exhibits a polygonal pattern of ridges, with a layer of clay particles coating the surface between the ridges. ***Gromia winnetoui* sp. nov.** represents an elongate morphotype in which the organic test is enclosed within an agglutinated case, a feature previously unknown in gromiids. Phylogenetic analysis using the maximum-likelihood method revealed that all three species form distinct clades, reflecting the morphological differences among Weddell Sea species, as well as between deep-water Southern Ocean *Gromia* and previously described gromiids.

doi: 10.1111/j.1096-3642.2009.00540.x

ADDITIONAL KEYWORDS: ANDEEP – biogeography – secondary agglutinated structure – small subunit ribosomal DNA – taxonomy.

INTRODUCTION

Gromiids are large amoeboid protists, with a monothalamous (single-chambered) proteinaceous test and filose pseudopodia. They are currently accommodated within a single genus, *Gromia*. Although ubiquitously distributed in environments ranging from polar seas to tropical coral reefs, and in areas including the Mediterranean, the coasts of North America, northwestern Europe, New Zealand, and Antarctica, the bathymetric range of *Gromia* was considered for many years to be restricted to intertidal and sublittoral waters, down to a maximum depth of 270 m off South Georgia (Arnold, 1951; Hedley & Bertaud, 1962; Arnold, 1972; Bowser, Marko & Bernhard, 1996; Gooday, Bowser & Bernhard, 1996; Gooday *et al.*, 2005). Most records are assigned to the type species *Gromia oviformis* Dujardin, 1835, which was first described from samples collected on the French Mediterranean coast.

The only convincing early record of *Gromia* from deeper water is that of Schulze (1875), who described a *Gromia sp.* from depths of between 185 and 675 m in Bukenfjord and Korsfjord, Norway. Schulze's species displays the characteristic mound-like oral capsule, with a central canal, and contains stercomata within the cell. This species was named *Gromia schultzei* by Norman (1892). However, it was not until 1994 that the first truly deep-sea specimens of *Gromia* were discovered at bathyal depths on the

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Oman margin of the Arabian Sea (Gooday *et al.*, 2000). Later, in 2003, gromiids were found at similar depths on the opposite side of the Arabian Sea, off the coast of Pakistan (Aranda da Silva, 2005; Aranda da Silva & Gooday, 2009). Two deep-sea species from the Arabian Sea, *Gromia sphaerica* (Gooday *et al.*, 2000) and *Gromia pyriformis* (Gooday & Bowser, 2005), have now been formally described. Although presently assigned to the same genus, both display clear morphological differences from *G. oviformis*. Based on partial small subunit ribosomal DNA (SSU rDNA) gene sequences, at least seven additional undescribed species, ranging in morphology from sausage-shaped to grape-shaped to spherical, have been identified from the Arabian Sea samples (Aranda da Silva, Pawlowski & Gooday, 2006). Other gromiid-like protists have been found at bathyal depths in polar regions, namely between 246 and 2000 m in the deep fjords of Svalbard in the European Arctic (Gooday *et al.*, 2005), and in sediments sampled from around 900 m in depth beneath the Ross Ice Shelf (Pawlowski *et al.*, 2005).

During the 2005 ANDEEP-III Expedition, large numbers of gromiids were collected from bathyal and abyssal sites in the Weddell Sea and adjacent areas of the Southern Ocean (Gooday, Cedhagen & Cornelius, 2006). Previously, gromiids were unknown from this region. Here, we describe three new gromiid species that were selected from the morphotypes present in the ANDEEP material, because of their distinctive and contrasting characteristics. The descriptions are based on a combination of morphological and molecular characteristics.

THE PHYLOGENETIC POSITION OF *GROMIA*

The classification of *Gromia* is still in a state of flux despite a growing body of information regarding the phylogenetic position of these organisms. Early classifications were based on morphological characteristics. The most important of these was identified by Rhumbler (1904), who described a system of filose pseudopodia that differentiate *Gromia* from the Foraminifera, which have granuloreticulose pseudopodia. The nature of the pseudopodia was later used to resolve the confusion between the foraminiferan *Allogromia ovoidea* Rhumbler, 1903 and *G. oviformis* (Hedley, 1958). Based on their pseudopodial morphology, gromiids were initially placed in the class Filosea (Hedley, 1958; Ogden & Hedley, 1980; Bovee, 1985). In their revised classification of the Protozoa, Levine *et al.* (1980) placed gromiids in the phylum Sarcomastigophora, subphylum Sarcodina, superclass Rhizopoda, class Filosea, order Gromiida, describing them as organisms with a body enclosed by a test or rigid external membrane, and possessing a distinct aperture. However, uncertainties remained, and the ambiguity surrounding the relationship of gromiids to other protists was demonstrated by Patterson, Simpson & Rogerson (2000), who referred to them as 'amoebae of uncertain affinities', most likely closely related to the Foraminifera and testate filose amoebae.

The first molecular analysis of *Gromia*, based on partial sequences of the gene coding for large subunit (LSU) rDNA, placed *G. oviformis* within the phylum Rhizopoda at the 'crown' of the ribosomal eukaryotic tree (Pawlowski *et al.*, 1994). Further analysis of SSU rDNA sequences of *G. oviformis* from five localities, including Réunion, Tunisia, Madeira, Guam, and Antarctica, placed *Gromia* in the Cercozoa (Cavalier-Smith, 1998), with all five sequences forming a distinct, independent lineage within this phylum (Burki, Berney & Pawlowski, 2002). Based on their analyses, Burki *et al.* (2002) suggested a revision of the Filosea, and assigned *Gromia* to a separate taxon, the Gromiida. Cavalier-Smith and Chao (2003) also classified *Gromia* in a distinct taxon (class Gromiidea) within the Cercozoa. Although included within the Cercozoa by these authors, *G. oviformis* does not branch with any of the other filosean protists with which it had been linked in morphology-based classifications (Burki *et al.*, 2002).

Since the work of Cavalier-Smith (1998), the phylum Cercozoa has undergone several other revisions and additions. Analysis of actin-based phylogeny by Keeling (2001) demonstrated the close relationship between the Cercozoa and the Foraminifera, leading to the proposition that they belong to a new protistan infrakingdom Rhizaria (Cavalier-Smith, 2002). Originally, the Rhizaria included the Cercozoa and Retaria (Foraminifera + Radiolaria), in addition to some other protists (Cavalier-Smith, 2002). The gromiids were placed together with parasitic Haplosporidia and Phytomyxa in the cercozoan subphylum Endomyxea (Cavalier-Smith, 2002). In an alternative scheme, based on revised analyses of SSU rDNA, actin, and the LSU of the RNA polymerase II (RPB1), gromiids were considered to be a sister group to the Foraminifera and/or the Haplosporidia (Berney & Pawlowski, 2003; Longet *et al.*, 2004; Nikolaev *et al.*, 2004). A phylogenetic analysis of partial SSU rDNA sequences of 23 deep-sea gromiids (including *G. sphaerica*) likewise grouped *Gromia* within the Rhizaria, as a sister group to the Haplosporidia (Aranda da Silva *et al.*, 2006). In a recent study based on four concatenated genes (SSU rDNA, actin, α- and β-tubulin), Tekle *et al.* (2007) also recognized the close relationship between Foraminifera, Haplosporidia, and *Gromia*, and suggested that an amoeboid protist

with anastomosing pseudopodia, *Corallomyxa tenera* Tekle et al., 2007, recently placed in a new genus *Filoreta* (Bass *et al.*, 2008), belongs to the same clade within the Rhizaria. According to their phylogenetic hypothesis, *Corallomyxa* is the closest extant relative of *Gromia*.

In summary, gromiids can be assigned to the Rhizaria, regarded by Adl *et al.* (2005) as one of six 'supergroups' in their new classification of eukaryotes (Adl *et al.*, 2005). Within the Rhizaria, they are most closely related to the Foraminifera, the Haplosporidia, and *Filoreta*. However, there is some disagreement regarding whether gromiids belong within or outside the phylum Cercozoa (Bass *et al.*, 2008; Pawlowski, 2008). Molecular evidence indicates that they are not related to testate filose amoebae, as suggested in earlier, morphology-based classifications.

MATERIAL AND METHODS

Sampling area

The ANDEEP project encompassed three cruises on-board the RV *Polarstern*. The samples used in this study originate from the third cruise (ANDEEP III, RV *Polarstern* cruise ANT XXII/3, from 21st January to 6th April 2005; Fig. 1). RV *Polarstern* sailed from Cape Town towards Neumayer Station along the Greenwich Meridian. After completing a down-slope transect off Kapp Norvegia, the ship proceeded in a north-westerly direction across the Weddell Sea towards the tip of the Antarctic Peninsula, and then southwards towards Rothera Point, before steaming back across the Drake Passage to Punta Arenas (Fahrbach, 2006).

Sampling

The material investigated was recovered with either an epibenthic sledge (EBS) or an Agassiz trawl (AGT). The EBS is equipped with a 1-m-wide lower epibenthic net (500-µm mesh size) and a 1-m-wide upper suprabenthic net (300-µm mesh size). The AGT is 3-m wide and equipped with a cod end-mesh size of 500 µm, except for stations 74#7, 78#11, and 81#9, where a cod end with a 10-mm mesh size was used. Both the EBS and the AGT were trawled

Figure 1. Stations sampled in the Weddell Sea during the RV *Polarstern* cruise, leg ANT-XXII/3, from 22nd January to 6th April, 2006. Filled circles indicate sample stations for ***Gromia marmorea* sp. nov.** (133#2) at 1584-m depth, ***Gromia winnetoui* sp. nov.** (121#7) at ~2600-m depth, and ***Gromia melinus* sp. nov.** (81#8 and 80#9) at 3101- and 4392-m depth, respectively.

across the seafloor for 10 min with a mean velocity of 1 knot (Fahrbach, 2006). Once on-board the ship, samples were kept submerged in chilled water, and were sieved in a cool room on one or more sieves of mesh sizes 500, 300, 125, and 63 μm. The unfixed sediment residues were sorted under a Leica binocular microscope for foraminifera and gromiids as soon as possible after collection. During this process, the residues were kept cool by placing the sorting dish in another dish filled with ice. Photographs of picked specimens were taken using a Nikon CoolPix 4500 digital camera attached to the microscope. Gromiids were fixed in formalin for subsequent morphological analysis, or were frozen in liquid nitrogen for molecular analysis (Gooday *et al.*, 2006).

Morphological descriptions

Gromiids were grouped into preliminary morphotypes based on test characteristics and general appearance during the shipboard sorting (Gooday *et al.*, 2006). On return to the laboratory in Southampton, specimens of each morphotype were counted, measured (test width and length, apertural width and height), and described using light microscopy. Specimens were documented using an SLR digital camera (Canon EOS 350D, DS126071). General views of the test were obtained using a Leica binocular microscope, with the specimens immersed in water in a petri dish. Detailed views of the aperture were taken using an Olympus BH-2 compound microscope, with the specimen placed in water in a glass cavity slide. The subsequent comparison of wall and apertural structures in each individual resulted in the regrouping of specimens based on morphological features. To examine the wall and apertural structures in greater detail, one or more specimens from each group were critical point dried and sputter coated with gold for examination in an LEO 1450VP scanning electron microscope (SEM). X-ray microanalysis in the SEM was used to determine the composition of the agglutinated particles observed on the test surfaces of selected individuals.

Ultrastructural analysis

Resin-block preparation

Formalin-fixed gromiids were washed in 0.1 M Piperazine-1,4-bis(2 ethanesulfonic acid) (PIPES) buffer containing 1% sodium chloride, post-fixed in 1% buffered osmium tetroxide for 1 h, and again rinsed in buffer. The specimens were block stained in 1% aqueous uranyl acetate for 20 min, and were then dehydrated in ethanol (30, 50, 70, 95%, and absolute alcohol) and embedded in spurr resin.

Histological verification and transmission electron microscopy (TEM)

Blocks were sectioned using glass knives on a Leica OMU3 microtome. Sections (0.5-μm thick) were taken for light microscopy and stained in 1% toluidine blue in order to locate sites of interest. Silver sections (70-nm thick) were then cut from these areas, stained with Reynolds lead stain, and viewed on a Hitachi H7000 transmission electron microscope. Sections were often disrupted by silica grains inside the cell body, making it difficult to identify cell structures.

DNA extraction, amplification, cloning, and sequencing

Frozen specimens for molecular analysis were thawed and photographed. For DNA extraction, the DNeasy Plant Mini Kit (Qiagen, Basel, Switzerland) was used, followed by polymerase chain reaction (PCR). Each PCR reaction was performed using 2 μL of DNA extract, resulting in an overall volume of 52 μL of PCR solution being used for amplification. The amplification profile consisted of 40 cycles with 30 s at 94 °C, 30 s at 50 °C, 2 min at 72 °C, and 5 min at 72 °C for the final extension. The re-amplification consisted of 25 cycles at an annealing temperature of either 52 or 55 °C. The re-amplification products were purified using the High Pure PCR Purification Kit (Roche, Rotkreuz, Switzerland). When possible, products were sequenced directly using the Big Dye Terminator Cycle Sequencing Kit and the ABI-3100 DNA sequences (Applied Biosystems, Rotkreuz, Switzerland) for analysis. In most cases, however, the products needed to be ligated into the pGEM-T Vector System (Promega, Wallisellen, Switzerland) before being cloned in XL-2 Ultracompetent Cells (Stratagene, Basel, Switzerland), and were then sequenced as described above, all according to the manufacturers' instructions.

For amplification, the universal primer pairs S12.2 (5′-GATYAGATACCGTCGTAGTC-3′) and SB (5′-TGA TCCTTCTGCAGGTTCACCTAC-3′), as well as S12.2 and GRSSU1 (3′-TCCAAAGTTTTCACCGGATC-5′) were used. Amplified PCR products were re-amplified using the following primer pair combinations: S13_Gro (5′-CTGTGGATAGGACTCGYTCAG-3′) and SB, or S13_Gro and S20R (3′-GACGGGCGGTGTGTA CAA-5′), as well as S13.2R_Gro (5′-GGGCGACGTT GGATAGGACTCGCT-3′) paired with GRSSU1.

Phylogenetic analysis

Using SEAVIEW software (Galtier, Gouy & Gautier, 1996), the partial SSU rDNA sequences from six isolates were manually aligned with *G. oviformis* sequences from Burki *et al.* (2002), and Arabian Sea

gromiid sequences from Aranda da Silva *et al.* (2006). The alignment comprised 45 *Gromia* sequences. The phylogenetic tree was inferred using the maximum-likelihood (ML) method devised by Felsenstein (1981). The ML analysis was performed with the software PhyML v2.4, using the HKY model of substitution (Guindon & Gascuel, 2003). In order to evaluate the reliability of the internal branches, the data were randomized through resampling using the boostrap method (Felsenstein, 1981) with 100 replicates.

SYSTEMATICS

We follow Adl *et al.* (2005) in placing the genus *Gromia* in the supergroup Rhizaria. The identification of these three new species as gromiids is based on their test morphology, the presence of a distinct oral capsule, as well as the internal wall structure that is characterized by a layer of the 'honeycomb membranes', as described by Hedley & Wakefield (1969). The identification is confirmed by molecular analyses.

SUPERGROUP RHIZARIA CAVALIER-SMITH, 2002

GENUS *GROMIA* DUJARDIN, 1835

***GROMIA MARMOREA* SP. NOV.** (FIGS 2–4)

Diagnosis: species of *Gromia* with a rounded test, which is spherical, to droplet-shaped, to ovoid in shape; diameter 1.0–3.4 mm, length : width ratio 0.6–1.9. Overall colour in fresh specimens, greenish with silvery patches, giving marble-like mottling of wall; preserved specimens, brown. Single, prominent, mound-like oral capsule.

Type material and locality: The holotype and paratypes are from an EBS deployment at RV *Polarstern* station 133#2, 62°46.95′S, 53°1.72′W, 1584 m water depth, 16th March 2005 (Table 1). They are deposited at the Research Institute and Natural History Museum Senckenberg, Frankfurt am Main. The holotype is catalogued under reg. no. SMF XXVII 7398. The paratypes are catalogued under reg. no. SMF XXVII 7399. The type specimens were extracted from > 300-μm residue and are preserved in 4% formaldehyde solution buffered with borax.

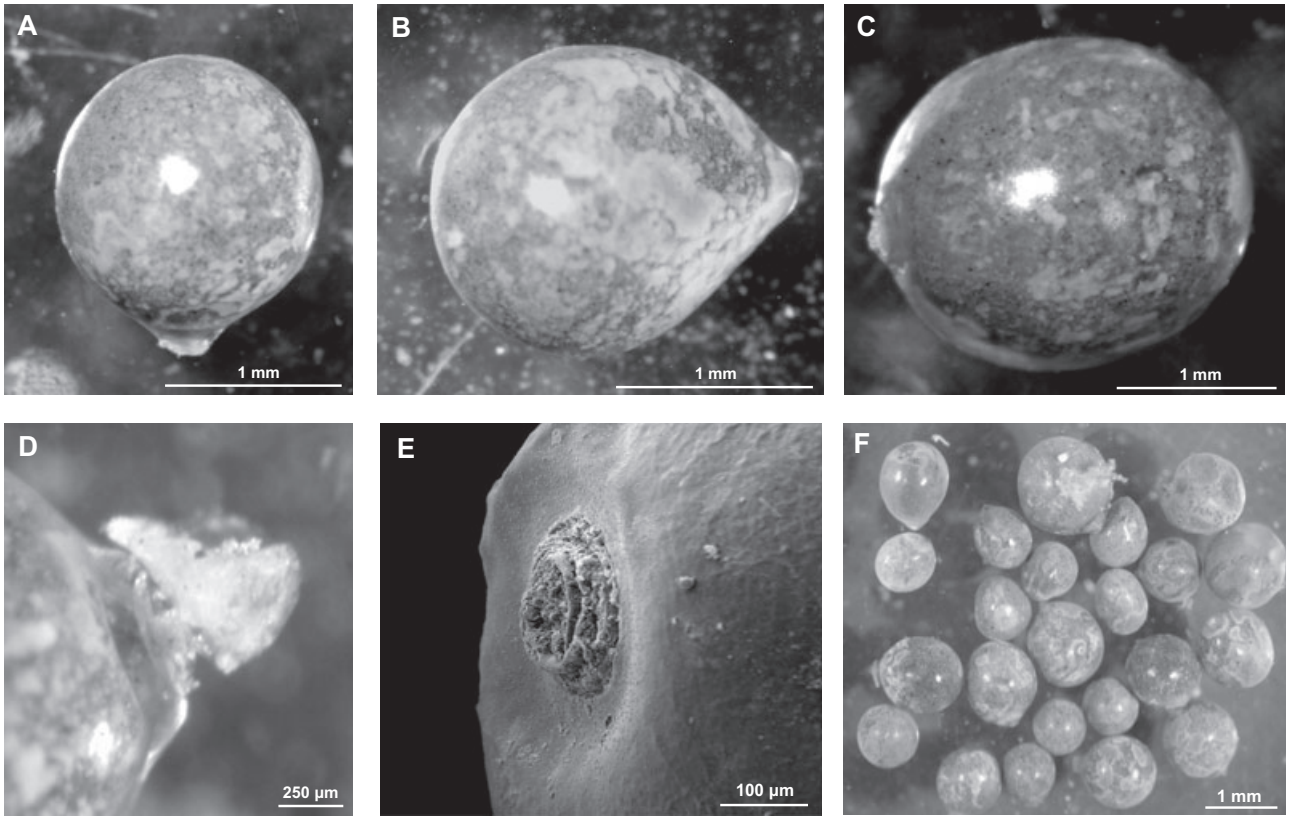

Figure 2. *Gromia marmorea* sp. nov. A–D, reflected-light photographs of preserved specimens, from station 133#2, 1584-m depth. Photographed in water. A, holotype, reg. no. SMF XXVII 7398, spherical morphotype. B, paratype, reg. no. SMF XXVII 7399, droplet-shaped morphotype. C, paratype, reg. no. SMF XXVII 7399, oval morphotype. D, detail of oral capsule. E, scanning electron microscope (SEM) photograph of oral capsule. F, unfixed specimens.

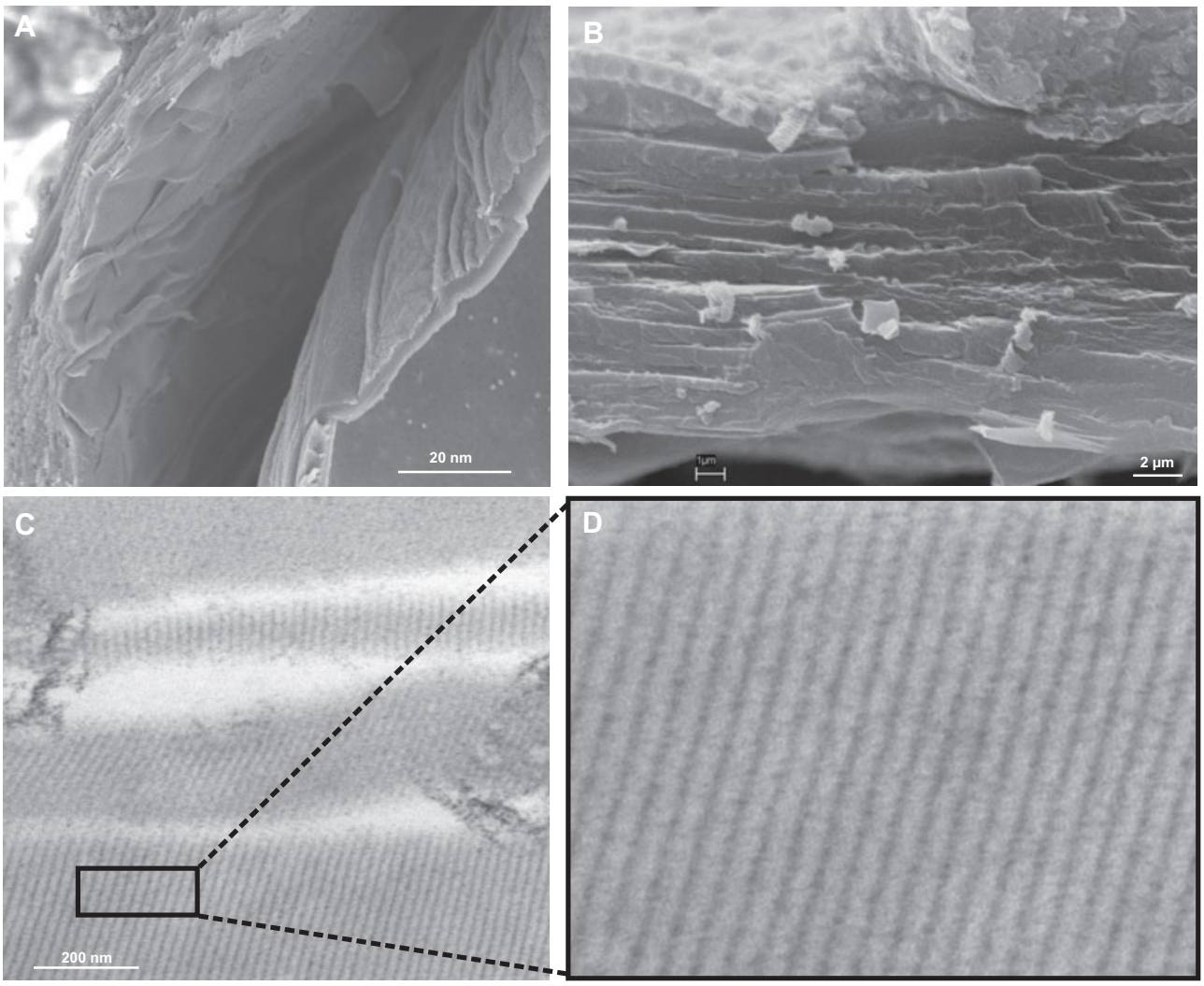

Figure 3. ***Gromia marmorea*** **sp. nov.** A, B, scanning electron microscope (SEM) photographs of wall consisting of multiple layers. C, transmission electron microscope (TEM) photograph of 'honeycomb membrane' layer. D, detail of (C).

Additional material: Station 133#2: approximately 130 specimens.

Derivation of name: From the Latin *marmoreus*, meaning 'like marble', alluding to the marbled pattern of the test surface.

Overall appearance: The 130 specimens range in length from 1.0 to 3.4 mm (mean 1.9 ± 0.4 mm), and range from 0.8 to 3.7 mm in width (mean 1.7 ± 0.4 mm). The length : width ratio varies from 0.6 to 1.9 (mean 1.1 ± 1.0). They vary from nearly spherical, to droplet-shaped, to ovoid in lateral outline. Ovoid specimens are widest behind the midpoint, with a rounded posterior end, and a narrower anterior end terminating in the oral capsule. The degree to which the test narrows towards the aperture varies between specimens, giving rise to the different morphologies. Freshly collected, unfixed specimens were greenish-grey, sometimes with silvery, shiny patches giving the test a mottled appearance. After formalin fixation, specimens were predominantly brown in colour, although the mottling persisted (Fig. 2).

Oral capsule: The single oral capsule is a very distinct, golden brownish-coloured structure, and is roughly circular in plan view (Figs 2E, 4A). In lateral view it appears as a broad, mound-like structure, and is penetrated by a central canal (Fig. 2D). In the ovoid- and droplet-shaped specimens, the oral capsule is located at the narrower end of the test (Fig. 2B).

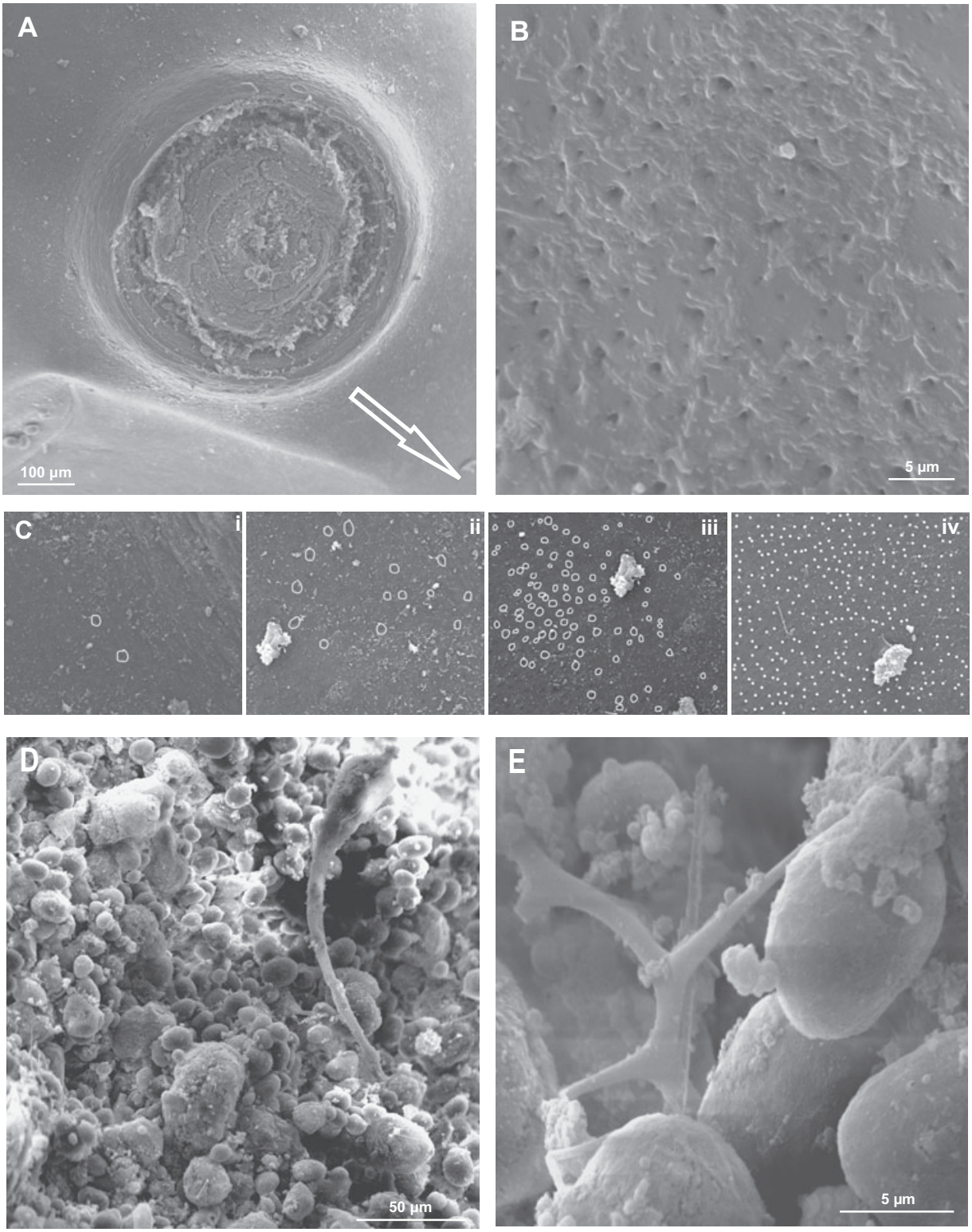

Figure 4. ***Gromia marmorea*** **sp. nov.** scanning electron microscope (SEM) photographs. A, oral capsule (the arrow indicates the direction of the photograph sequence shown in panels Ci–Civ). B, perforations of the test surface. Ci–Civ, sequence of photographs over a distance of 250 µm showing test pores (each pore is highlighted by a white circle), with the number of pores increasing with increasing distance from the aperture (from left to right); 4000× magnification. D, E, interior of broken specimen showing stercomata and other structures.

Table 1. Weddell Sea sample stations, gear, depth, number of examined specimens, position, and environmental data

Station No.	Sample gear	Depth (m)	Number of specimens per species	°S	°W	Approx. bottom temperature (°C)	Bottom salinity	Sediment type
133#2	EBS	1584	~700 *G. marmorea*	69°46.49′	53°3.50′	0.00	34.67	Greenish grey silty sand, moderate bioturbation, scattered Mn-coated dropstones, poorly sorted clay
80#9	EBS	3103	15 *G. melinus*	70°39.07′	14°43.36′	–0.50	34.66	Olive-grey sandy mud, hemipelagite, intensely bioturbated, foraminiferal-rich
81#8	EBS	4392	1 *G. melinus*	70°32.02′	14°35.05′	–0.70	34.66	Olive-grey sandy mud, very poorly sorted, abundant Mn-coated clasts, hemipelagite, and contourites
121#7	EBS, AGT	~2600	36 *G. winnetoui*	63°37.43′	50°45.11′	0.00	34.67	Greenish grey silty sand, moderate bioturbation, scattered Mn-coated dropstones, poorly sorted clay

Sediment data from Howe (2006), Howe, Shimmield & Diaz (2004), and Diaz (2004).

The height of the oral capsule (i.e. the distance it protrudes from the test surface) ranges from 100 to 600 μm (mean 140 ± 100 μm, $N = 129$), and the diameter ranges from 200 to 800 μm (mean 400 ± 100 μm, $N = 129$). In 18 specimens, a flaccid extension (up to a maximum length of 200 μm), composed of organic material, protrudes from the central canal through the aperture opening (Fig. 2D).

Test wall: The test is delicate and tears easily. The organic wall appears featureless and smooth when viewed under a binocular microscope. It is usually partially translucent, sometimes with a milky appearance, and displays a characteristic marble-like pattern (Fig. 2A–C). Viewed using SEM, the wall is composed of multiple layers, and each layer is a fraction of a micron thick (Fig. 3A, B); TEM revealed the existence of an inner layer of honeycomb membranes (Fig. 3C, D). In cross section, the membranes appear as a series of very regular lines. The pores form openings on the test surface, ranging in diameter from ~0.3 to 3.1 μm ($N = 14$; only clearly defined pores were measured; Fig. 4B). They occur across the entire surface, but are usually less frequent around the aperture. In the illustrated specimen, their density increases from about one pore per 10 μm^2, within 40 μm of the aperture, to about 12 pores per 10 μm^2, at ~250 μm from the aperture (Fig. 4C, i–iv). Concurrently, the pore diameter tends to increase from ~0.3 μm close to the oral capsule to about 3.1 μm at a distance of ~250 μm from the capsule. However, the density of pores also varies between specimens. In one of the four individuals investigated by SEM, the pores were smaller and less frequent than in the other specimens, and did not display the decrease in size described above.

Test contents: The test contents are visible through the translucent test wall, and consist mainly of a densely packed mass of small, oval, and brownish stercomata (Fig. 4D). These range from 6 to 22 μm ($N = 140$) in length, and from 3.7 to 20 μm in width ($N = 140$), and are characterized by a very smooth surface. Additional particles visible in SEM include possible mineral grains and sponge spicules (Fig. 4E).

Distribution: Powell Basin, east of the tip of the Antarctic Peninsula, 1584-m depth (Fig. 1).

Remarks: Gromia marmorea sp. nov. is by far the most abundant gromiid in the ANDEEP-III material. The new species encompasses a range of shapes, from spherical, to ovoid, to droplet-shaped, which are also exhibited by *G. oviformis* (Jepps, 1926). However, it differs from the latter in the mottled appearance of the test wall and the dark-greenish, rather than light-brownish, colour of fresh, live specimens. In addition, the organic test wall of *G. marmorea* sp. nov. is very delicate, and tears easily. *Gromia marmorea* sp. nov. ranges in length from 1.0 to 3.4 mm, and is therefore larger than *G. pyriformis* (< 1-mm long; Gooday &

Bowser, 2005) and smaller than *G. schulzei* (8–9-mm long; Schulze, 1875), as well as being smaller than *G. sphaerica* (maximum length 38 mm; Gooday *et al.*, 2000). Like most other gromiids, this new species has a single, large oral capsule rather than many small capsules scattered across the test, as in *G. sphaerica* (Gooday *et al.*, 2000). The capsule is a prominent, relatively low, broad, mound-like structure, and is larger (200–800 μm in diameter) than in other Weddell Sea species. The test is perforated by numerous pores that have a minimum diameter of 0.3 μm (300 nm), compared with 73 nm in *G. pyriformis* (Gooday & Bowser, 2005).

GROMIA MELINUS SP. NOV. (FIGS 5, 6)

Diagnosis: Small species of *Gromia* with a fairly robust test, which is spherical, droplet-shaped, subtriangular, or asymmetrically irregular in shape; length 0.6–1.5 mm; length : width ratio 1.0–1.5. One, occasionally two, small oral capsules. Test wall fairly stiff, with distinctive network of fine ridges and surface coating of clay particles.

Type material and locality: The holotype and paratypes are from an EBS deployment at station 80#9, 70°39.07′S, 14°43.36′W, 3108-m depth, collected on 23rd February 2005 (Table 1). They are deposited at the Research Institute and Natural History Museum Senckenberg, Frankfurt am Main. The holotype is catalogued under reg. no. SMF XXVII 7400. The paratype is catalogued under reg. no. SMF XXVII 7401. The type specimens were extracted from the > 500-μm sediment residue, and are preserved in buffered 4% formaldehyde solution.

Additional material: Stations 80#9 and 81#8; 16 specimens.

Derivation of name: From the Latin *melinus*, meaning 'honey', alluding to the honeycomb pattern of the test wall.

Overall appearance: The 16 specimens range in length from 0.6 to 1.5 mm (mean 0.9 ± 0.2 mm), and in width from 0.4 to 1.4 mm (mean 0.8 ± 0.2 mm). The length : width ratio varies from 1.0 to 1.5 (mean 1.2 ± 0.1). The test morphology varies from subtriangular, to droplet-shaped, to spherical, or asymmetrically irregular (Figs 5A–D, 6A). Specimens are brown to yellowish in colour after formalin fixation.

Oral capsule: Most specimens have a single oral capsule (Fig. 5A–C), but two are present in one individual (Fig. 5D). The oral capsule is roughly circular in plan view, and is a distinct feature. In some specimens it protrudes slightly from the test surface and gives rise to a long, flaccid, translucent extension, composed of organic material (Fig. 5B). In other individuals it is much flatter, circular, and hardly protrudes from the surface (Fig. 5A, D).

Test wall: The wall is fairly rigid and characterized by a very distinctive pattern of ridges, which are clearly visible under the binocular microscope. The ridges form a network, the meshes of which vary in shape but tend to be more or less polygonal (Fig. 5E). The diameter of the polygonal elements ranges from ~30 to ~50 μm. The surface of the test between the ridges is covered with tiny, plate-like particles (Fig. 5F), which were identified as clay minerals, based on X-ray elemental microanalyses that indicated the presence of K, Mg, and Fe, in addition to Al and Si. The inner side of the wall is composed of multiple honeycomb membranes, which appear as a series of regular lines in the TEM images (Fig. 6B–D).

Distribution: Off Kapp Norvegia, eastern Weddell Sea, 3103- and 4392-m depth (Fig. 1).

Remarks: Gromia melinus sp. nov. is distinguished from most other *Gromia* species by the prominent, polygonal pattern of ridges raised from the general test surface. They are not to be confused with the layer of 'honeycomb' membranes described by Hedley & Wakefield (1969) and Bowser *et al.* (1996). This unusual feature has also been described by Aranda da Silva (2005) in *Gromia sp.* 2 from the Oman margin of the Arabian Sea. In both cases, the polygons that make up the pattern are formed from the outermost test layer. In *G. melinus* sp. nov. the relationship of the polygonal ridges to the inner layers of the wall is obscured by the layer of clay particles, which is not present in the Arabian Sea species. The Arabian Sea species also has a more consistent shape (Aranda da Silva, 2005) than *G. melinus* sp. nov. The two species are clearly separated by the molecular analysis (see below).

Like *G. oviformis*, *G. melinus* sp. nov. displays a variety of morphologies. It is a small species, being approximately 0.8-mm wide and 0.9-mm long, and is therefore similar in size to *G. pyriformis* (< 1-mm long), but is smaller than *G. oviformis* (up to 5-mm long), *G. sphaerica* (up to 38 mm), and *G. schulzei* (8–9-mm long) (Schulze, 1875; Hedley & Bertaud, 1962; Gooday *et al.*, 2000; Gooday & Bowser, 2005). Whereas *G. melinus* sp. nov. typically has one oral capsule, like most other gromiids, one spherical specimen had two capsules. According to Jepps (1926), occasional specimens of *G. oviformis* also have more than one aperture. In contrast to the distinct mound-like oral capsule in *G. marmorea* sp. nov., which

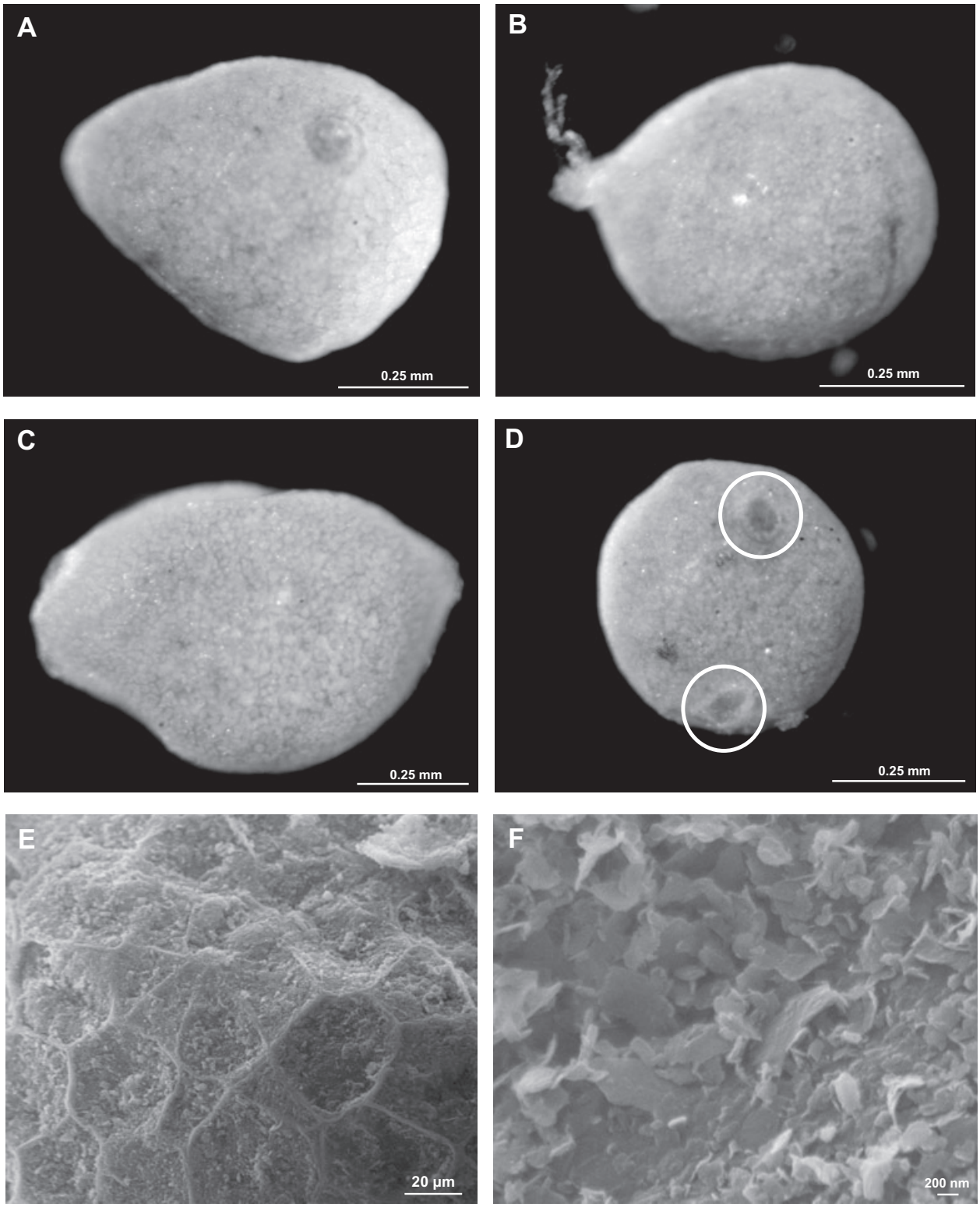

Figure 5. Reflected-light photographs of ***Gromia melinus* sp. nov.** from station 80#9, at 3103-m depth (A–C), and station 81#9, at 4392-m depth (D); photographed in water. A, paratype, reg. no. SMF XXVII 7401, subtriangular morphotype. B, holotype, reg. no. SMF XXVII 7400, droplet-shaped morphotype. C, asymmetrically irregular morphotype. D, spherical morphotype with two oral capsules. E, scanning electron microscope (SEM) photograph of the polygonal pattern on the wall surface. F, SEM photograph of agglutinated clay particles on the surface of the specimen.

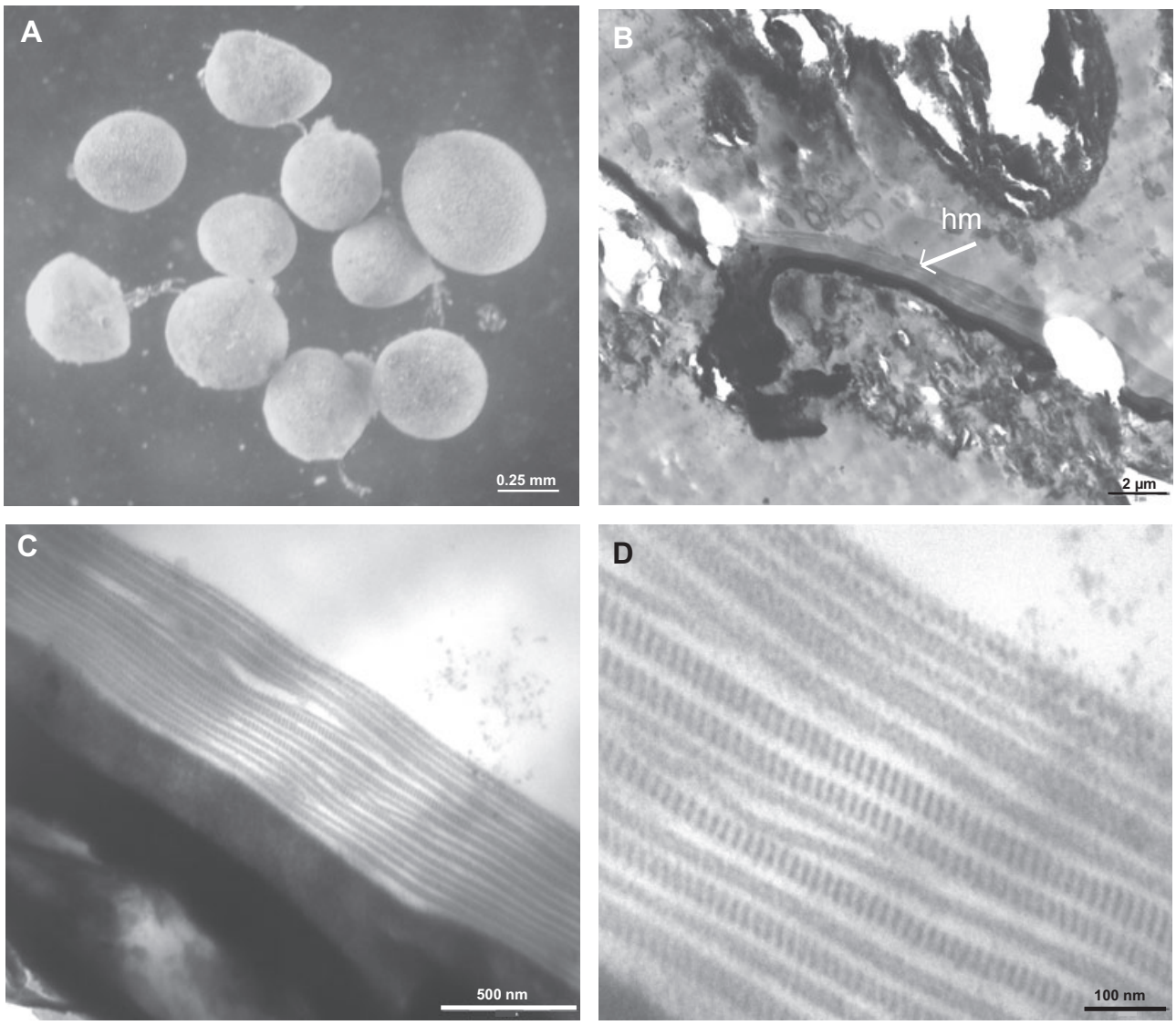

Figure 6. ***Gromia melinus*** **sp. nov.** A, reflected light photograph, station 80#9, at 3103-m depth. B, transmission electron microscope (TEM) photograph of 'honeycomb membrane' layer at a 5000× magnification. C, TEM photograph at a 50 000× magnification. D, TEM photograph at a 100 000× magnification.

clearly projects from the test surface, *G. melinus* sp. nov. has a relatively flat capsule, which hardly protrudes. The long organic extension that arises from the central canal of the oral capsule in some specimens of *G. melinus* sp. nov. also occurs in *G. marmorea* sp. nov., as well as in *G. oviformis* (Jepps, 1926).

GROMIA WINNETOUI SP. NOV. (FIGS 7–9)

Diagnosis: Species of *Gromia* varying from sausage-shaped, to elongate oval, or more irregular in shape. Length 1.0–2.8 mm; width 0.3–0.8 mm; length : width ratio 1.8–8.9. Single, conical oral capsule. Test typically enclosed completely or partially in a coarsely agglutinated case.

Type material and locality: The holotype and paratypes are from an AGT deployment at station 121#7, 63°34.92′S, 50°41.97′W, 2630-m depth, 14th March 2005. They are deposited at the Research Institute and Natural History Museum Senckenberg, Frankfurt am Main. The holotype is catalogued under reg. no. SMF XXVII 7402. The paratypes are catalogued under reg. no. SMF XXVII 7403. The type specimens are preserved in 4% buffered formaldehyde solution.

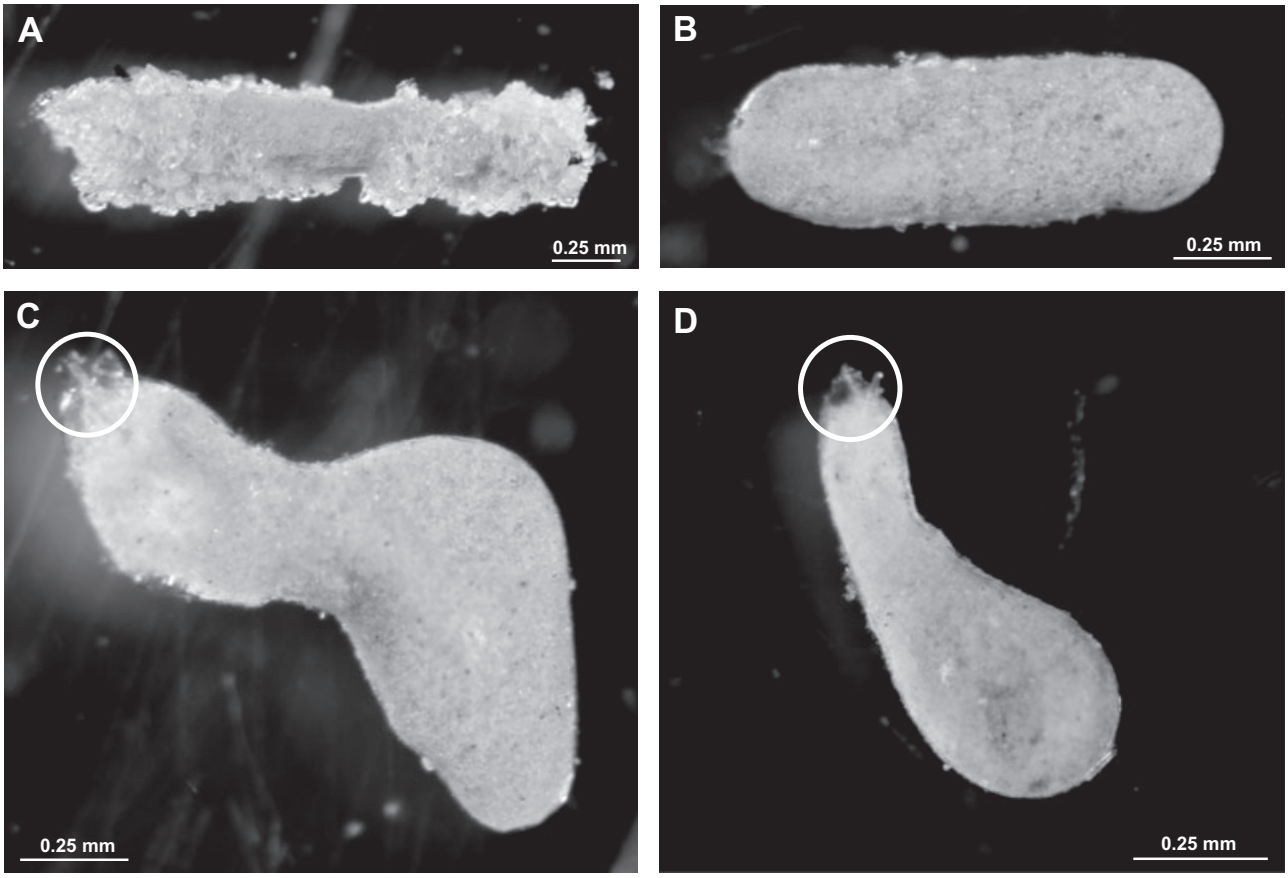

Figure 7. ***Gromia winnetoui*** **sp. nov.** A–D, reflected-light photographs, station 121#7, ~2600-m depth. A, holotype, reg. no. SMF XXVII 7402, agglutinated specimen. B, paratype, reg. no. SMF XXVII 7403, elongate oval specimen (the circle indicates the oral capsule). C, D, irregularly shaped specimen (the circles indicate the oral capsules).

Additional material: Station 121#7; 36 specimens.

Derivation of name: From Winnetou, the Native American hero of several novels written by Karl May, and a personal hero of two of the authors.

Overall appearance: The organic test varies from sausage-shaped, to elongate oval, to irregularly shaped (Fig. 7A–D), and is dark to light brown in colour. specimens range in length from 1.0 to 2.8 mm (mean 1.9 ± 0.5 mm), and in width from 0.3 to 0.8 mm (mean 0.5 ± 0.1 mm). The length : width ratio varies from 1.8 to 8.9 (mean 3.9 ± 1.6). Many specimens are encased completely or partially in a coarsely agglutinated case, which is whitish or light brown in colour (Fig. 7A). The case is composed of fairly large, loosely cemented mineral particles, mainly quartz, but also including some dark grains, giving it a speckled appearance (Figs 7A, 8A). Fine clay particles are also present, particularly on the inside of the case, where they form a cushion between the quartz grains and the organic test wall (Fig. 8B–D).

Oral capsule: The single oral capsule is relatively small, and is often obscured by the agglutinated test (Fig. 7A). Where visible, it protrudes as a conical structure in lateral view, and ranges in height from 80 to 120 μm ($N = 4$), and in width from 40 to 80 μm ($N = 4$) (Fig. 7C, D). The central canal is sometimes visible.

Test wall: Where the wall is visible, it appears dented as a result of pressure from the overlying agglutinated grains. SEM photographs reveal pores ranging in diameter from 10 to 50 nm, scattered across the test surface (Fig. 8D). Clay particles (~200 nm in diameter), presumably derived from the outer case, are partially embedded in the wall surface (Fig. 8D). TEM images revealed multiple layers of honeycomb membranes constituting the inner part of the wall. They appear as regular lines (Fig. 9A–D).

Test contents: The test contents consist of a more or less densely packed mass of light-brown stercomata.

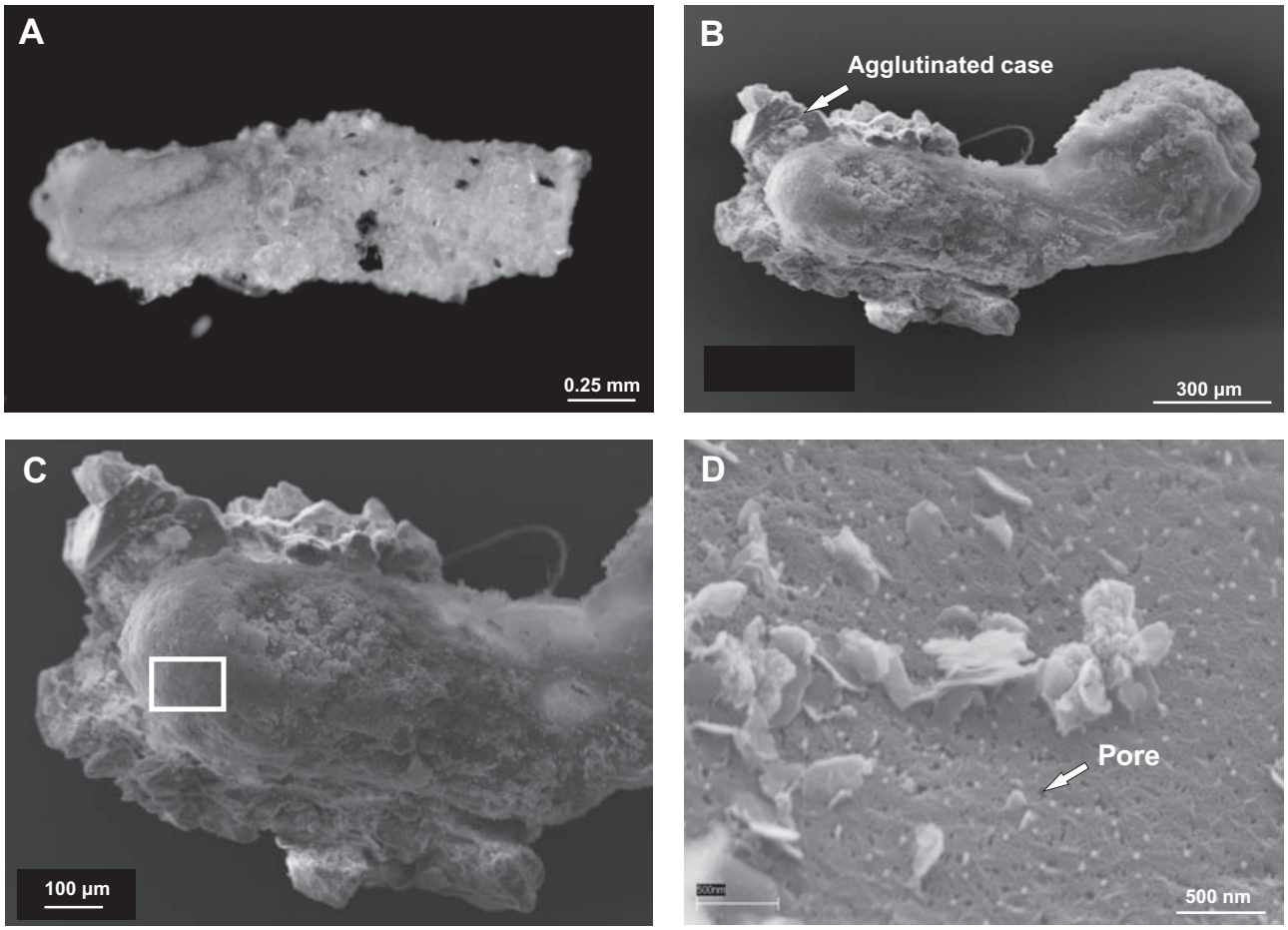

Figure 8. *Gromia winnetoui* sp. nov. A, reflected-light photograph, station 121#7, ~2600-m depth; photographed in water. B, C, scanning electron microscope (SEM) photographs of the agglutinated wall (the rectangle in C indicates the area shown in D). D, SEM photograph of the perforations on the test surface.

Distribution: Powell Basin, east of the tip of the Antarctic Peninsula, ~2600-m depth (Fig. 1).

Remarks: Gromia winnetoui sp. nov. can be distinguished from all previously described gromiids by the agglutinated case that encloses the organic test. This structure resembles the agglutinated capsule that surrounds some allogromiid-like organisms from near-shore habitats in the Antarctic and the Arctic (Gooday *et al.*, 1996, 2005; Gooday, 2002). It also differs from both *G. marmorea* sp. nov. and *G. melinus* sp. nov. in having a more elongate shape.

MOLECULAR CHARACTERISTICS

Overall, 13 SSU rDNA sequences were obtained from the three deep Weddell Sea species: *G. marmorea* sp. nov. (four isolates), *G. melinus* sp. nov. (one isolate), and *G. winnetoui* sp. nov. (one isolate). These confirmed that the organisms are gromiids. The sequences ranged in length between 547 and 766 nucleotides, thereby constituting about one third of the whole SSU rDNA. The GC base composition varied between 47.3 and 49.4%. The Weddell Sea sequences were compared with 26 previously published *Gromia* sequences from the Arabian Sea (Aranda da Silva *et al.*, 2006), and with six *G. oviformis* sequences from Guam, Tunisia, Madeira, Réunion, and Antarctica, published by Burki *et al.* (2002) (Table 2). For the Weddell Sea gromiids, a pair-wise comparison of each sequence with every other sequence revealed a 93.8% similarity, indicating a relatively low level of genetic variation between species. A comparison between Weddell Sea and Arabian Sea gromiids, as well as between Weddell Sea and *G. oviformis* sequences, revealed a higher genetic pair-wise similarity between the latter pair (90.5% versus 85.5%). Intra-individual variation between Weddell Sea gromiids was observed to be less than 1%.

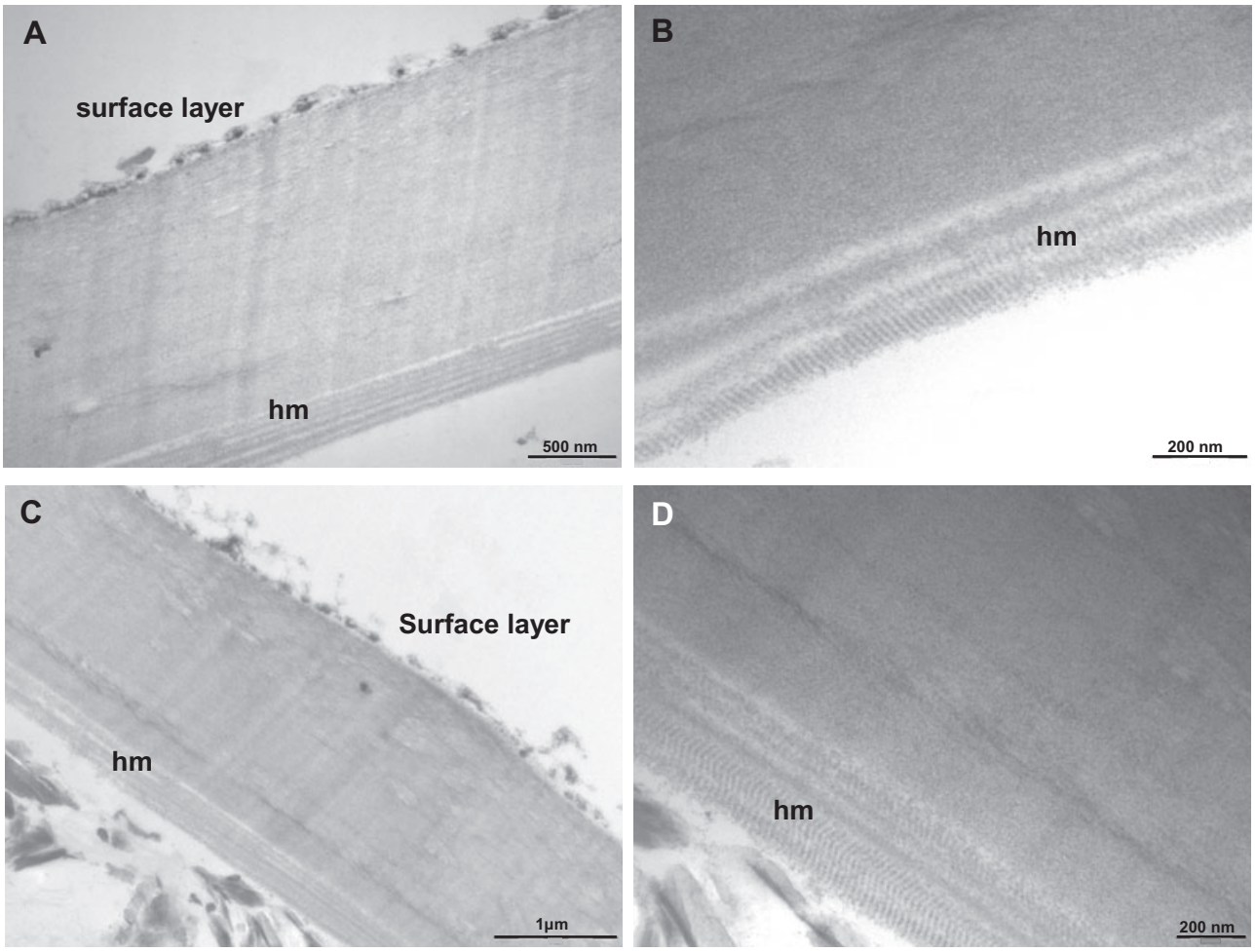

Figure 9. *Gromia winnetoui* sp. nov. A–D, transmission electron microscope (TEM) photographs of the test wall, including the 'honeycomb membrane' layer (hm), station 121#7, ~2600-m depth.

Table 2. List of previously published small subunit ribosomal DNA (SSU rDNA) sequences of *Gromia* used in this study

Taxonomic position	Species name	Accession number
Gromia	*Gromia oviformis* (Madeira)	AJ457811
	Gromia oviformis (Réunion)	AJ457812
	Gromia oviformis (McMurdo)	AJ457813
	Gromia oviformis (Guam)	AJ457814
	Gromia oviformis (Tunisia)	AJ457815

The ML method, which estimates the branch lengths that make the observed differences between sequences most likely, yielded the phylogenetic tree shown in Figure 10. *Gromia marmorea* sp. nov. forms clade A, which is represented by four isolates comprising seven sequences. Clade A has 86% bootstrap support, and reveals a sequence divergence between isolates of 1%. Clade B consists of *G. melinus* sp. nov., represented by four sequences from one isolate with a sequence divergence of 0.4%. It is supported by a 78% bootstrap value. *Gromia winnetoui* sp. nov. is represented by only two sequences from one isolate, forming clade C with *Gromia* sp. 2 from the Arabian Sea, with a bootstrap support of 99%. Bearing in mind the limitations of the data set, the Weddell Sea gromiids fell into three distinct clades, each of which formed a monophyletic group derived from the same ancestor. *Gromia* sp. 1 from the Arabian Sea was sufficiently distant from the rest of the species to form the out-group.

DISCUSSION

This is the first report of SSU rDNA sequences of *Gromia* from the deep Southern Ocean. The only

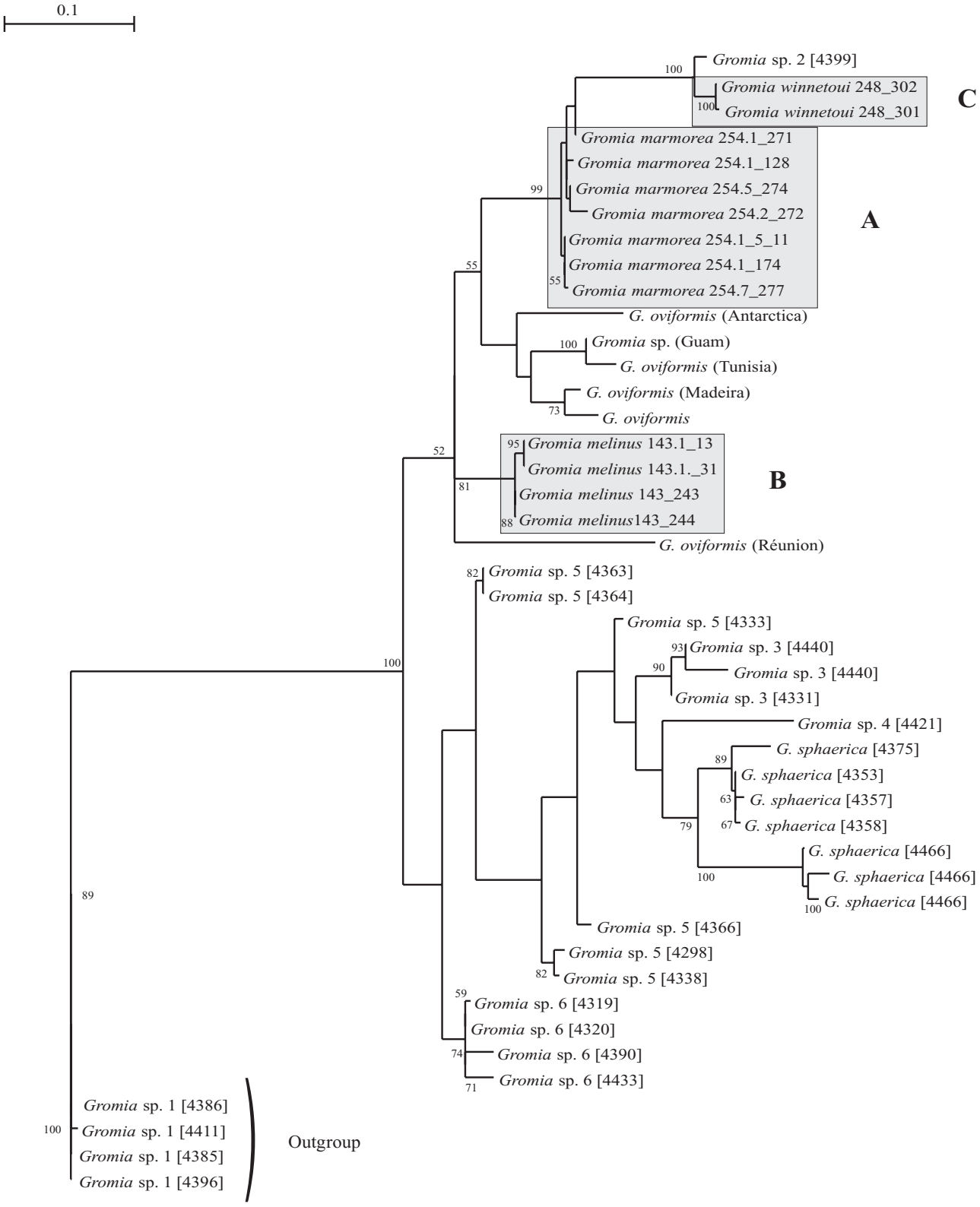

Figure 10. Maximum-likelihood tree of Weddell Sea gromiids (shaded boxes), Arabian Sea gromiids (Aranda da Silva *et al.*, 2006), and *Gromia oviformis* from shallow-water localities (Burki *et al.*, 2002), based on partial small subunit ribosomal DNA (SSU rDNA) sequences. The numbers at the nodes represent the percentage of bootstrap support, > 50%. Weddell Sea gromiids are grouped into three distinct clades (A, B, and C).

other molecular study of deep-sea gromiids was based on material from the Arabian Sea, in which seven distinct lineages of *Gromia* were identified (Aranda da Silva *et al.*, 2006). In the present study, it proved more difficult to obtain unambiguous results, probably reflecting the method of sample collection. The Arabian Sea gromiids were picked directly from the surfaces of core samples that were raised quickly from the ocean floor. Our samples, however, were collected using towed gear: either an EBS or AGT. They therefore took much longer to reach the ship deck, allowing more time for the DNA to deteriorate. Sample sieving and picking introduced a further delay. As a result of these problems, only a small number of sequences could be obtained from each species.

Despite these difficulties, our molecular results and morphological observations confirm that the three new species are gromiids. They corroborate the findings of previous studies indicating that *Gromia* is a diverse taxon that is ubiquitously distributed around the world, in both shallow- and deep-water environments. Our *Gromia* species occurred at bathyal and abyssal depths along the continental margin of the Weddell Sea. *Gromia marmorea* sp. nov. and *G. winnetoui* sp. nov. were sampled at bathyal depths (1584 and 2600 m) on the continental slope of the Powell Basin, on the western boundary of the Weddell Sea. specimens of *Gromia melinus* sp. nov. were collected at depths of 3103 and 4392 m on the eastern side of the Weddell Sea, off Kapp Norvegia. The latter record represents the deepest known occurrence of any confirmed gromiid. Additional *Gromia*-like morphotypes collected from the Weddell Sea during ANDEEP III were found within a similar depth range along the continental margin (Gooday *et al.*, 2006). Based on these and other records, we predict that gromiids are widely distributed on continental margins down to abyssal depths.

Molecular diversity

Two of our three new species, *G. melinus* sp. nov. and *G. marmorea* sp. nov., form two distinct clades, clearly separated from the shallow-water *G. oviformis* (Burki *et al.*, 2002), as well as from the Arabian Sea gromiids (Aranda da Silva *et al.*, 2006). *Gromia winnetoui* sp. nov. forms another clade, but branches closely with *Gromia* sp. 2 from the Arabian Sea despite clear differences in morphology. *Gromia* sp. 2 is a droplet-shaped gromiid with a characteristic reticulated test pattern (Aranda da Silva *et al.*, 2006), similar to that of *G. melinus* sp. nov. In contrast, *G. winnetoui* sp. nov. varies from sausage-shaped to elongate oval in shape, and the test is partially or completely enclosed in a coarsely agglutinated case. The molecular results are therefore inconsistent with the morphological characteristics of these species.

In other respects, the separation into three distinct clades (A, B, and C) appears to reflect the morphological differences among the Weddell Sea species, as well as between these species and those from the deep Arabian Sea and shallow-water sites. However, the ML tree and pair-wise percentage similarity between sequences shows that clades A, B, and C branch more closely to *G. oviformis* from shallow waters than with the deep Arabian Sea gromiids, all of which, except for species 2, form a distinct group of their own. The close branching between *Gromia* from shallow and deep Antarctic waters suggests a possible shallow-water origin for Antarctic deep-sea gromiids. The more or less isothermal water column in the Southern Ocean is believed to make it easier for shallow-water species to migrate to bathyal and abyssal depths (Brey *et al.*, 1996; Tyler, Young & Clarke, 2000; Brandt *et al.*, 2007). Pawlowski, Bowser & Gooday (2007) reported that the calcareous foraminiferan *Epistominella vitrea* (Weltner, 1913) which is usually confined to shallow water, was genetically almost identical across a bathymetric range of 1000 m in the Weddell Sea.

Genetic variation between the Weddell Sea clades A, B, and C was low, despite distinct morphological differences between the species. A much greater degree of morphological homogeneity was observed in the Arabian Sea gromiids. This disparity possibly reflects the greater degree of environmental heterogeneity across our sampling area in the Southern Ocean, compared with the Arabian Sea, where gromiids were more similar within a much smaller geographical area. The intraspecific variation observed in the different clones from each of our isolates was also relatively low.

The secondary agglutnated structure in *G. winnetoui* sp. nov.

The coarsely agglutinated case enclosing the test in *G. winnetoui* sp. nov. represents the first report of a secondary agglutinated structure in *Gromia*. Various calcareous, agglutinated, and organic-walled foraminiferan species construct agglutinated cysts (Heinz, Geslin & Hemleben, 2005): examples include *Elphidium incertum* (*Williamson*, 1858) (Linke & Lutze, 1993) and *Nonionellina labradorica* (Dawson, 1860) (Cedhagen, 1996). However, foraminiferal cysts are often rather diffuse structures, composed of fine sediment particles and detritus. Moreover, as Heinz *et al.* (2005) note, the cyst is often separated from the test by an open space. The structure enclosing *G. winnetoui* sp. nov. appears to most closely resemble the agglutinated casing observed in Saccamminid sp.

1 from the continental slope (850-m depth) off Cape Hatteras, North Carolina (Gooday, Hughes & Levin, 2001; A.J. Gooday, pers. observ.). As in *G. winnetoui* sp. nov., it is composed of loosely cemented quartz grains, which press closely against the test and leave a clear imprint on the flexible underlying test surface.

The function of the agglutinated case in *G. winnetoui* sp. nov. is not clear. In the case of foraminifera several authors have distinguished between reproductive cysts, as observed in *Patellina corrugata Williamson*, 1858 by Meyer (1935), feeding cysts, as described by Jepps (1942) in *Elphidium crispum* (Linnaeus, 1767), and growth cysts that are formed at the onset of chamber formation (Gross, 2002). It has also been observed that cysts serve as adaptations to changes in the environment, by providing protection against mechanical and chemical disturbances (Linke & Lutze, 1993), as well as by disguising the organism from predators (Cedhagen, 1996). In the case of *G. winnetoui* sp. nov., the latter two functions seem the most plausible.

Concluding remarks

Until recently, *Gromia* was only known from shallow waters, and most gromiids were assigned to a single species, *G. oviformis* (Burki *et al.*, 2002). Since the discovery of deep-sea gromiids on the Omani margin of the Arabian Sea in 1994 (Gooday *et al.*, 2000), an increasing number of deep-water species have been identified and described, based on both morphological and molecular characteristics (Gooday & Bowser, 2005; Aranda da Silva, 2005; Aranda da Silva *et al.*, 2006). Ongoing and future work from different parts of the world will increase the number of species further, and will reveal novel morphotypes that might soon warrant a subdivision of the genus *Gromia*.

ACKNOWLEDGEMENTS

We would like to thank Prof. Angelika Brandt for inviting two of us (AJG and TC) to participate in the ANDEEP-III expedition, and for making available material from the EBS and the AGT, Dr Eberhard Fahrbach for the efficient running of the RV *Polarstern* ANT XXII/3 cruise, and the Captain, officers, and crew of the RV *Polarstern* for their help during the cruise. The work of NR at the University of Geneva, Switzerland, was funded by a taxonomic grant from the Census of Abyssal Marine Life (CeDAMar), an award by the United Kingdom Challenger Society for Marine Science, the Kristin Bruhn award, and by the Swiss National Science Foundation (grant number 3100A0-11264 to JP). We are also very grateful to Ana Aranda da Silva, Béatrice Lecroq, Fabien Burki, Jacqueline Guiard, and Mike Thurston for their invaluable help and advice. This is ANDEEP publication number 123.

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