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Flexammina islandica gen. nov. sp. nov. and some new phylotypes of monothalamous foraminifera from the coast of Iceland

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Abstract

Monothalamous (single-chambered) foraminifera comprise a poorly known group, the diversity of which is strongly underestimated according to environmental DNA surveys. The gross morphology of monothalamids offers few distinguishing features; their organic-walled or agglutinated tests are often very delicate and make isolation difficult. Here, we use an integrated taxonomic approach, including morphological and molecular analysis, to examine the diversity of monothalamids in a shallow subtidal area on the coast of Iceland. We report nine new phylotypes of single-chambered foraminifera distinguished by SSU rDNA sequences. Among them, we establish a new genus *Flexammina* and a new species *Flexammina islandica*, possessing a very pliable, finely agglutinated test capable of extreme shape transformations. According to molecular data, *F. islandica* belongs to the monothalamid clade M, which also includes the well-known genus *Allogromia*. In addition, we provide brief descriptions and illustrations of nine other monothalamous foraminifera isolated from the same area. Three of them are closely related to some unidentified environmental sequences and serve as the first microscopic documentation of these anonymous lineages.

Key words: marine protists, foraminifera, monothalamids, SSU rDNA, molecular phylogeny, Iceland

Introduction

Shallow-water benthic foraminifera have been the scope of continuous investigation over the last few decades. In comparison to calcareous and agglutinated multi-chambered taxa, single-chambered foraminifera, or 'monothalamids' (Pawlowski *et al*. 2013) only recently were recognized as important taxonomic group, with a number of studies documenting their diversity in shallow-water settings (Altin *et al*. 2009, Goldstein *et al*. 2010, Gooday *et al*. 2011, Majewski *et al*. 2005, Pawlowski & Majewski 2011, Sinniger *et al*. 2008, Voltski *et al*. 2014). In addition to these studies based on morphological and molecular analysis of isolated specimens, recent years have seen the emergence of environmental DNA (eDNA) studies, which enable the assessment of foraminiferal diversity based on metabarcoding approach (Habura *et al*. 2004, 2008, Pawlowski *et al*. 2011, Bernhard *et al*. 2013, Lejzerowicz *et al*. 2013). With the advent of the next-generation sequencing (NGS) technologies, the eDNA studies revealed a huge richness of monothalamous lineages (Lecroq *et al*. 2011, Pawlowski *et al*. 2011, Pawlowski *et al*. 2014). However, this purely molecular approach provides no visual information about organisms living in a given environment, which would allow making the diversity more 'tangible' and available to taxonomic databases. This is especially important in the case of taxonomic groups with the vast proportion of non-described species, such as monothalamids, whose ecology and evolution are extremely poorly understood.

Here, we report the results of a molecular and morphological study of some monothalamous foraminifera isolated from the subtidal zone on the coast of Iceland. One new species has been formally described, leading to the emergence of a new foraminiferal genus. Other new monothalamids comprise a number of small, inconspicuous forms with a subspherical or flask-shaped agglutinated test, designated by informal term 'saccamminids', and delicate organic-walled morphospecies collectively called 'allogromids'. All these forms are illustrated and briefly annotated; however, more material is required for their formal description as a new species.

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Material and methods

Sampling. The sampling was performed in August 2013 near Vogar, the Reykjanes Peninsula, Iceland. The coast in this area has a vast, mostly rocky intertidal zone with occasional small stretches of sandy beach or mudflats in enclosed bays. Further offshore, the bottom is sandy with occasional groups of stones overgrown by *Laminaria hyperborea.*

Samples were collected manually by snorkeling in shallow subtidal area, just in front of a narrow sandy inlet bordered by rocky shores (63°58'25.24" N; 22°24'21.36" W). The sample was taken at ~2.5–3 m water depth in the depression between *Laminaria hyperborea* holdfasts; in this spot, ~2 cm thick upper sediment layer was scooped off the bottom on the area of ~20 cm^2. It consisted of muddy sand with a high proportion of bivalve shell rubble. Upon acquisition, the sediment was immediately brought to the wet lab and sieved through 500-125-64 μm sieves with each fraction placed in an individual container. Large macrofauna (mainly large amphipods and polychaetes) and parts of *Laminaria hyperborea* holdfasts were removed with tweezers. Sediment fractions were stored at the ambient temperature (+8–15 ºC). Shortly afterwards, the samples were transferred to Geneva in a cooler and placed in a cold room at +4 ºC temperature and 18/6 h day/night regime.

DNA extraction, PCR amplification, cloning and sequencing. DNA was extracted from single isolated foraminifera using the guanidine extraction method (Pawlowski 2000). Every DNA extraction was assigned a number in the foraminiferal DNA collection stored at the Department of Genetics and Evolution, University of Geneva. Semi-nested PCR amplification was carried out with the foraminiferal SSU-specific forward primer s14F3 (5' - ACGCAMGTGTGAAACTTG) at the first amplification step, s14F1 (5' - AAGGGCACCACAAGAACGC) for the reamplification, and the NewB eukaryotic SSU reverse primer (5' - TGCCTTGTTCGACTTCTC). PCR parameters were as follows: reaction volume 25 μl; number of cycles 34 at the 1st amplification step, 24 for the reamplification, each cycle consisting of 30 s at 94 °C, 30 s at 50 °C or 52 °C (depending on the melting temperature of the primers used), and 90 s at 72 °C; final elongation for 5 min at 72 °C. Positive PCR products were purified using High Pure PCR Purification Kit (Roche Diagnostics, Basel, Switzerland) and subsequently cloned with TOPO TA Cloning Kit (Invitrogen). Sanger sequencing was performed on ABI 3130XL DNA sequencer using an ABI Prism Big Dye Terminator Cycle Sequencing Kit.

In total 60 sequences were obtained from the isolated single cells with 22 of them belonging to *Flexammina islandica* sp. nov. (EMBL/GenBank accession numbers KM097044–KM097065) and 38 to other new monothalamids (EMBL/GenBank accession numbers KP984698–KP984735).

Phylogenetic analysis. The sequences were compared to foraminiferal SSU rDNA gene sequences available in GenBank. Then they were added to the alignment representing all major groups of monothalamids from our database and some environmental sequences from GenBank. To enable the preliminary positioning of the new phylotypes, the initial automatic profile alignment and raw tree building was performed in Seaview 4.4 (Gouy *et al*. 2010) using ClustalO alignment module and distance tree-building algorithm. After that, the alignment was manually improved, resulting in 2133 sites selected for the final analysis. The maximum likelihood phylogenetic inference was obtained using RAxML (Stamatakis 2006) on a local computing node; GTR+I+G (generalised time-reversible + gamma + proportion of invariant sites) model of nucleotide substitution with 6 substitution rate categories was used after the model selection in Mega 5.2.2 (Tamura *et al*. 2011). The bootstrap support was calculated on the basis of 100 replicates. In addition, the alignment of Clade M monothalamids sequences was performed separately in Seaview 4.4 resulting in 1212 sites selected; the phylogenetic analysis was done in PhyML on the web server (http://www.atgc-montpellier.fr/phyml; Guindon *et al*. 2010) with the same model parameters and 100 bootstrap replicates.

Systematic description

Supergroup Rhizaria Cavalier-Smith 2002

Phylum Foraminifera d'Orbigny 1826

'Monothalamids' Pawlowski *et al*. 2003

***Flexammina* gen. nov.**

Etymology. '*Flex*' from English 'flexible' denoting the plasticity of the test shape; '*ammina*'—from the Greek 'psammon' meaning 'sand'—refers to the agglutinated mineral material covering the test.

Type species. *Flexammina islandica*.

Diagnosis. Test free or attached, monothalamous. The test shape is subspherical or elongated when free, dome-like when attached; aperture subcircular to irregular, surrounded by a short collar; test wall flexible, formed by a thick layer of mineral grains with an underlying layer of organic material.

Phylogenetic position. The type species *F. islandica* branches in the clade M of monothalamous foraminifera, based on the phylogenetic analysis of SSU rDNA gene sequences.

Remarks. There are some monothalamids that morphologically resemble *Flexammina.* Of these, *Leptammina* Cedhagen *et al*. (2009) is a genus from the deep Weddell Sea, with a similar test morphology possessing a flexible spherical test composed of fine mineral particles and a collared aperture. However, there is no evidence that *Leptammina* is able to assume attached dome-shaped form; its test wall agglutination is more uniform and finer. Moreover, this genus is very distant genetically, belonging to the 'clade C' of monothalamids. Some species of *Hemisphaerammina* Loeblich and Tappan (1957) and *Crithionina* Goës (1894) superficially resemble the attached form of *Flexammina* by the test shape and the character of their test wall agglutination. However, their tests lack the aperture on the upper surface and the basal test wall in *Hemisphaerammina* is completely absent. According to genetic data, *Hemisphaerammina* is branching at the base of the 'clade F' of monothalamous foraminifera, while the genus *Crithionina* is polyphyletic.

Finally, some foraminifera reported further in this study, mostly the undetermined saccamminids ICEMON 3 and ICEMON 6 were poorly distinguishable from smaller, free individuals of *F. islandica*. We did not find enough specimens of these saccamminids to provide the full descriptive information and clearly outline the essential morphological differences, but none of them were genetically close to *Flexammina*.

***Flexammina islandica*. sp. nov.**
(Plate 1, figs A–I)

Etymology. The species epithet was given on the basis of the geographical location where the species was encountered for the first time.

Type material. The holotype and 8 paratypes were fixed in formalin and deposited in the collection of the Museum of Natural History in Geneva, Switzerland under accession numbers MHNG-INVE-89244 (holotype) and MHNG-INVE-89245, MHNG-INVE-89246 (paratypes). All specimens were collected from the same sampling locality (see 'Materials and Methods' for details).

Diagnosis. As for genus; this particular species has the ability of attachment to flat surfaces and significant test shape plasticity.

Test morphology. Test monothalamous, flexible in shape and able to change its life mode from free to attached one. Free forms are subspherical, oval, or kidney-shaped in outline; their maximal cross-sectional test diameter varies from 165 to 405 μm (n=17), with the widest portion of the test located approximately at mid-level. Free forms with irregularly shaped tests (e.g. with a completely distorted proximal end or large swellings) also occur. The holotype has a subspherical, slightly elongated test, which was free (unattached) at the moment of collection. Aperture roughly circular, oval or irregularly shaped, located terminally, surrounded by a low agglutinated collar, which is sometimes slightly flared. Underlying organic layer is visible, sometimes projecting forward from the edges of the agglutinated collar and forming an inconspicuous fringe.

Attached individuals are dome-shaped; their edge outline is irregular; those who apparently spent long time on substrate, develop bordering flange around the test. Test diameter of four measured specimens varied from 450 to 1030 μm. The aperture is located on the upper surface of the dome, positioned eccentrically, otherwise looking similarly to the aperture of free individuals. The basal test wall or 'floor' is present or partially present; in detached specimens that seemingly did not possess the complete floor, no organic membrane was observed covering the bottom opening.

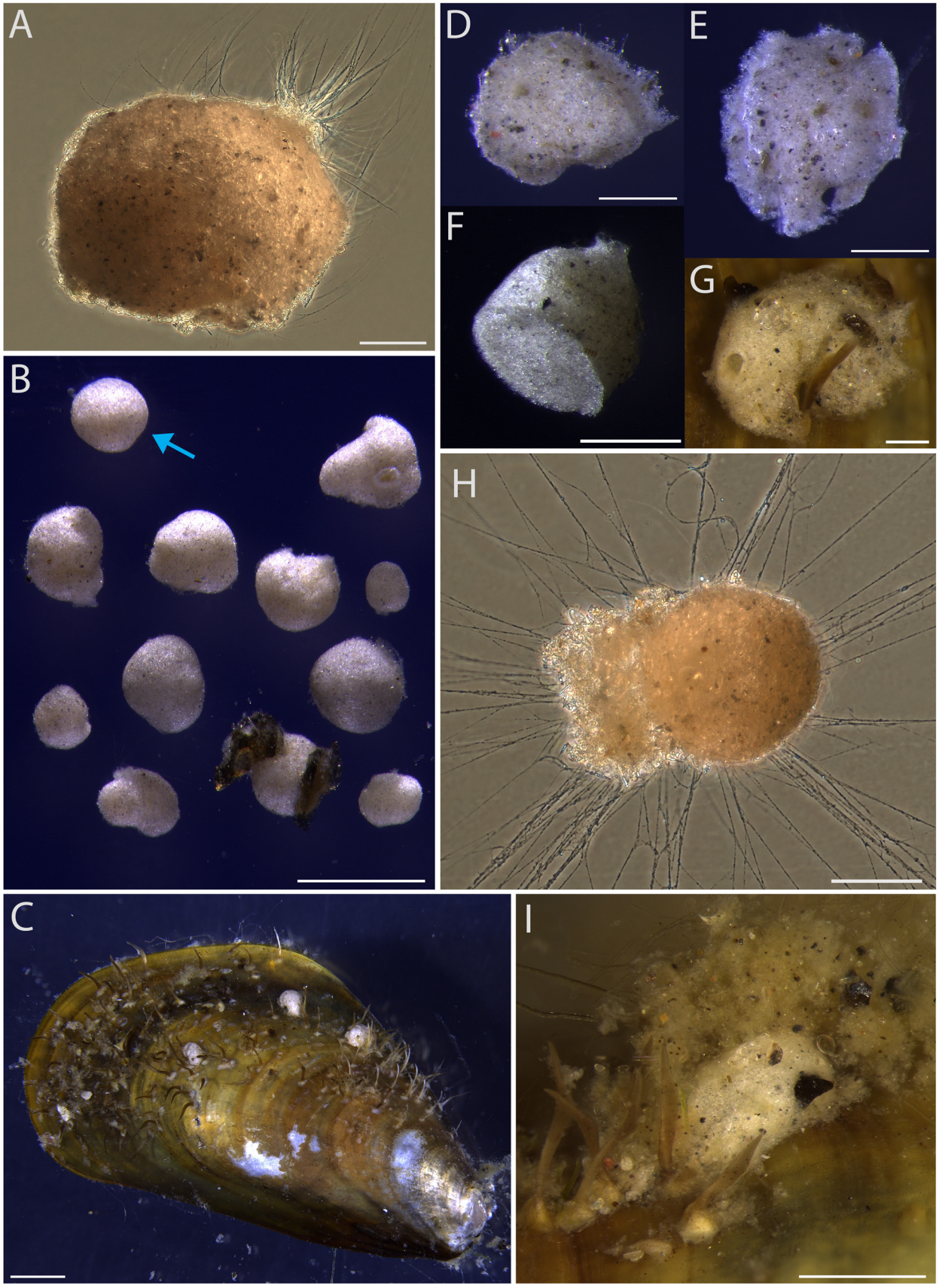
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PLATE 1. *Flexammina islandica* **gen. & sp. nov.** A. Detached dome-shaped individual (DNA #17676) with reticulopodia extended; reticulopodia emanate from the aperture and also raise from the test surface. B. The holotype (arrow) and paratypes #1–8; all are free-living individuals; note that one foraminifer attached two large black sand grains to its test. C. Three attached, dome-shaped individuals on a bivalve shell (DNA #17676, 17677, 17678). D. Dome-shaped individual (detached, view from above, DNA #17677). E. Dome-shaped individual (detached, view from above, DNA #17678). F. Individual with transitional morphology, which became sedentary after two weeks in a separate dish (oblique view). G. Dome-shaped individual on bivalve shell (DNA #17672). H. Free-living individual (DNA #17667) with subspherical test and extended reticulopodia; the cloud of accumulated matter obscures the aperture. I. Dome-shaped individual (DNA #17672) on bivalve shell, partially covered by a layer of phytodetritus, diatoms, sediment and undifferentiated organic matter (side view). Scale bars: 100 μm (A, H); 200 μm (D, E, F, G); 500 μm (B, I); 1000 μm (C).

Wall structure. The test wall is whitish or greyish, opaque, composed of small mineral grains, the majority of which are translucent (presumably quartz), with occasional black grains interspersed between them. The wall of some dome-like attached individuals incorporated very large, disproportionate grains that were transparent or dark. The wall thickness is not uniform, especially in the attached specimens, with some areas (as e.g. the lateral wall parts) being considerably thicker than the basal wall. Direct measurements of the test wall thickness were not possible, as the test was not sectioned. The flexibility of the test wall suggests loose binding between the grains as well as the presence of the underlying organic layer.

Granuloreticulopodia. Once isolated and cleaned of gathered matter, the cell readily deploys extensive granuloreticulopodial network in the surrounding medium (from here on, we will use the term 'reticulopodial' instead). When observed on a planar surface, numerous 'main' branches are visible, radiating from the apertural area. Fully deployed network occupies the area around the cell, with its radius surpassing test diameter at least five times. The number and density of main strands is greater in the prevalent direction of movement, if the cell starts to relocate. Also, the proximal portions of reticulopodial strands frequently fuse into vast, flat cytoplasmic 'veils' with the same radial bidirectional streaming of granules within the veil, as observed in separate reticulopodial strands.

Test interior. Cytoplasm opaque, whitish or with a slight orange tinge, it was visible through the opening in the bottom of some detached dome-shaped tests.

Observations on living individuals. The active, freely moving individuals were discovered in the accumulations of phytodetritus, diatoms and filamentous algae. They invariably gathered a lot of material with vast reticulopodial networks and accumulated it around the aperture (Plate 1, fig. H). The dome-shaped *F. islandica* tests were found on empty *Mytilus* shell fragments, in the coarse fraction of the sample (>500μm); these attached individuals accumulated a lot of matter above themselves resulting in the dome completely covered and hard to reveal (Plate 1, fig. I). Numerous diatoms were frequently found associated with the lower surface of the test floor of attached forms; it was not clear, however, how they became trapped under the basal wall. Transition from the free to attached life mode was documented only once. A free, oval-shaped specimen was isolated and placed in a separate dish. Initially, it wandered on the bottom with reticulopodia extended and forming a large network, but after two weeks it became sedentary and attached itself to the plastic with the aperture turned upwards and a flat rear attachment surface, rendering a bottle-like appearance (Plate 1, fig. F).

Molecular phylogeny. Analysis of partial SSU rDNA sequence data showed that *F. islandica* belongs to the Clade M of monothalamous foraminifera (Fig.1). Its close relative is a saccamminid from the coast of New Zealand, tentatively assigned to the species *Saccammina alba* Hedley (1962). Both species differ by only 6.2% of sequence divergence, but their common branching is not strongly supported (36% BV). The environmental sequence Xeno215.5 branches between the two species. All of them belong to a strongly supported (100% BV) subclade M1, which also includes the genus *Allogromia*, undescribed allogromiid 'twinkle' (GenBank Accession number EU213245) and one environmental sequence (EU213225) (Fig. 2). Other sequences branching at the base of clade M, include various environmental sequences, as well as sequences of specimens which were assigned to genera *Hyperammina* and *Crithionina* on the basis of their morphology (Fig. 2). All these sequences form a morphologically heterogeneous clade M, with the terrestrial species *Edaphoallogromia australica* Meisterfeld *et al*. (2001) at its base.

Remarks. Close relationship of *F. islandica* and *Saccammina alba* raises the question of the generic affinity of both species. The genus *Saccammina* originally described by Sars (1868) has substantial morphological differences when compared to *S. alba* of Hedley (1962) and presently described species. The type species, *Saccammina sphaerica* Sars has a rigid test wall, composed of coarse sand grains (Brady 1881, Heron-Allen & Earland 1913). Although the genetic data are not available for *S. sphaerica* from the original locality in the Norwegian Sea, the sequences of a very similar morphospecies from the Weddell Sea branch within the Clade C monothalamids (Figure 1). Because of its clearly different morphology we think that *S. alba* should be moved to another genus. Yet, its placement in the genus *Flexammina* might be premature as close phylogenetic relations between *F. islandica* and *S. alba* are not supported (Fig. 2). On the other hand, the study of Goldstein (1988) concerned a very similar monothalamid from the coast of California, which was verified by Hedley as belonging to *S. alba*. It exhibits exactly the same behavior as *Flexammina islandica*, being capable of attaching itself to hard surfaces. In his original publication, Hedley does not report any radical transformations of the test shape, as well as attached individuals; however, quite strong variability of the test contour in free individuals is apparent (see Figure 2, Hedley 1962). All sequenced specimens of *S. alba* from the original location were rounded and we did not observe any change of its shape, but the observation time in this case was too brief and attached specimens were possibly overlooked. Taking all the above points into consideration, we think that more genetic data is needed to unambiguously prove the congeneric status of *F. islandica* and similar morphospecies from New Zealand and California.

Other documented Monothalamids

Additional 30 cells were isolated from the examined sample resulting in 8 new molecular phylotypes, randomly dispersed within the radiation of monothalamids (Fig. 1). All of them were given provisional names related to the agglutinated (saccamminid) or organic-walled (allogromiid) nature of the test wall. New phylotypes were provided with a short description and illustration of sequenced individuals (Plate 2). In addition, one potentially new monothalamid was described only morphologically (undetermined allogromiid ICEMON 9). The morphological boundaries between foraminifera belonging to different phylotypes were elusive in several cases. More specimens, as well as comparative light and electron microscopy investigations would be necessary to formally describe the corresponding morphospecies. However, as we were unable to maintain these phylotypes in culture conditions, they will be only briefly annotated here.

Undetermined saccamminid ICEMON 1

(Plate 2, figs A)

Description. Test free, ovoid to fusiform, circular in cross-section. The aperture is single, located terminally. It is surrounded by a funnel with a flared anterior end. The shape of the funnel may be distorted; in one specimen the funnel was turned at approx. 70 degrees relative to the longitudinal axis. The proximal end varies in shape from pointed to rather broadly rounded. The test wall is translucent, whitish in incident light; it is composed of small, transparent mineral grains and occasional rod-shaped elongated objects, which probably represent fragments of sponge spicules. The flexibility of the test suggests the presence of the underlying organic bioadhesive layer, bearing all mentioned particles on its surface. Reticulopodial network is sparse and consists mostly of single, long 'main'/'axial' strands, all originating from the aperture. Sometimes, the dark area of the cytoplasm was visible in the terminal part of the test through the test wall; probably this was the accumulation of recently ingested material. In the 8 examined individuals, test length varied from 45 to 147 μm, test diameter from 26 to 73 μm. The length of the funnel surrounding the aperture measured from 6 to 25 μm.

Observations of living individuals. The species has been found invariably attached to the surface of the mineral grains and organic particles with its flared aperture. Upon detachment most of these foraminifera readily deployed reticulopodia and recovered the initial 'vertical' position, attaching themselves to the flat bottom of the petri dish used for microscopy. Some individuals were highly motile, travelling across the surface.

Molecular phylogeny. On the partial SSU rDNA phylogenetic tree the species formed a sister group to the Undetermined saccamminid ICEMON 2 found in the same sample. Together they branch between the genus *Conqueria,* the Clade E and another undetermined saccamminid ICEMON 5 (Fig 1).

Undetermined saccamminid ICEMON 2
(Plate 2, fig. B)

Description. The test is free, small, pyriform, composed of coarse (relative to the test size) mineral grains and rod-shaped transparent particles with a single terminal aperture located on the narrower end of the test and surrounded by a somewhat elongated, tubular collar. Reticulopodial network was not observed.

Molecular phylogeny. This phylotype branches as sister to the undetermined saccamminid ICEMON 1. As only one individual was found, we cannot confidently compare both of them morphologically.

Undetermined saccamminid ICEMON 3
(Plate 2, figs D, E)

Description. Test is free, oval in profile outline and circular in cross-section, with a single terminal aperture lacking a surrounding collar. The length of the two isolated individuals was 119 and 145 μm, width 70 and 110 μm, respectively. Test wall is thin and composed of small mineral grains. The wall color is brownish-grey in transmitted light. The two cells developed an extensive reticulopodial network with numerous branches radiating from the apertural region, exceeding the test diameter at least five-fold when fully deployed.

Molecular phylogeny. The sequences of the two individuals of this species belong to the Clade O of monothalamids (Fig. 1). The species branches very close to the environmental sequence McM11 (GenBank Accession number AY179178; Habura *et al*. 2004), from which it differs by only 5.5 %. Other species belonging to this clade are *Ovammina opaca* Dahlgren (1962) from the Barents Sea (unpubl.) and *Cedhagenia saltatus* Gooday *et al*. (2011) from the Black Sea. *Cedhagenia* has a characteristic test shape with a pointed proximal end and softer test and therefore differs quite well morphologically, whereas there are virtually no differences with *Ovammina,* apart from the pronounced silvery sheen of the wall surface of the latter. We cannot exclude that undetermined saccamminid ICEMON 3 may represent another species of *Ovammina*, but the genetic distance separating them is quite high (16.1–16.7%).

Undetermined saccamminid ICEMON 4
(Plate 2, figs F, G)

Description. The only examined individual possessed subspherical, slightly elongated test measuring 77 μm in length and 63 μm in diameter; the aperture was circular with a very low collar. The wall was translucent, composed of fine mineral grains. The aboral end was partially obscured by attached organic particles. The cell exhibited a very low reticulopodial activity; only single short branches were occasionally observed emerging from the apertural region.

Molecular phylogeny. The sequences of the unique specimen of this species branch as sister to the clade Y—a chaotic assemblage of diverse organic-walled and agglutinated, morphologically simple monothalamids, including genera *Hippocrepinella*, *Phainogullmia*, as well as some undescribed saccamminids and deep-sea environmental sequences Unc807-18 and Unc810-72 of Tsuchiya (2013). However, its position was poorly supported in the current phylogeny.

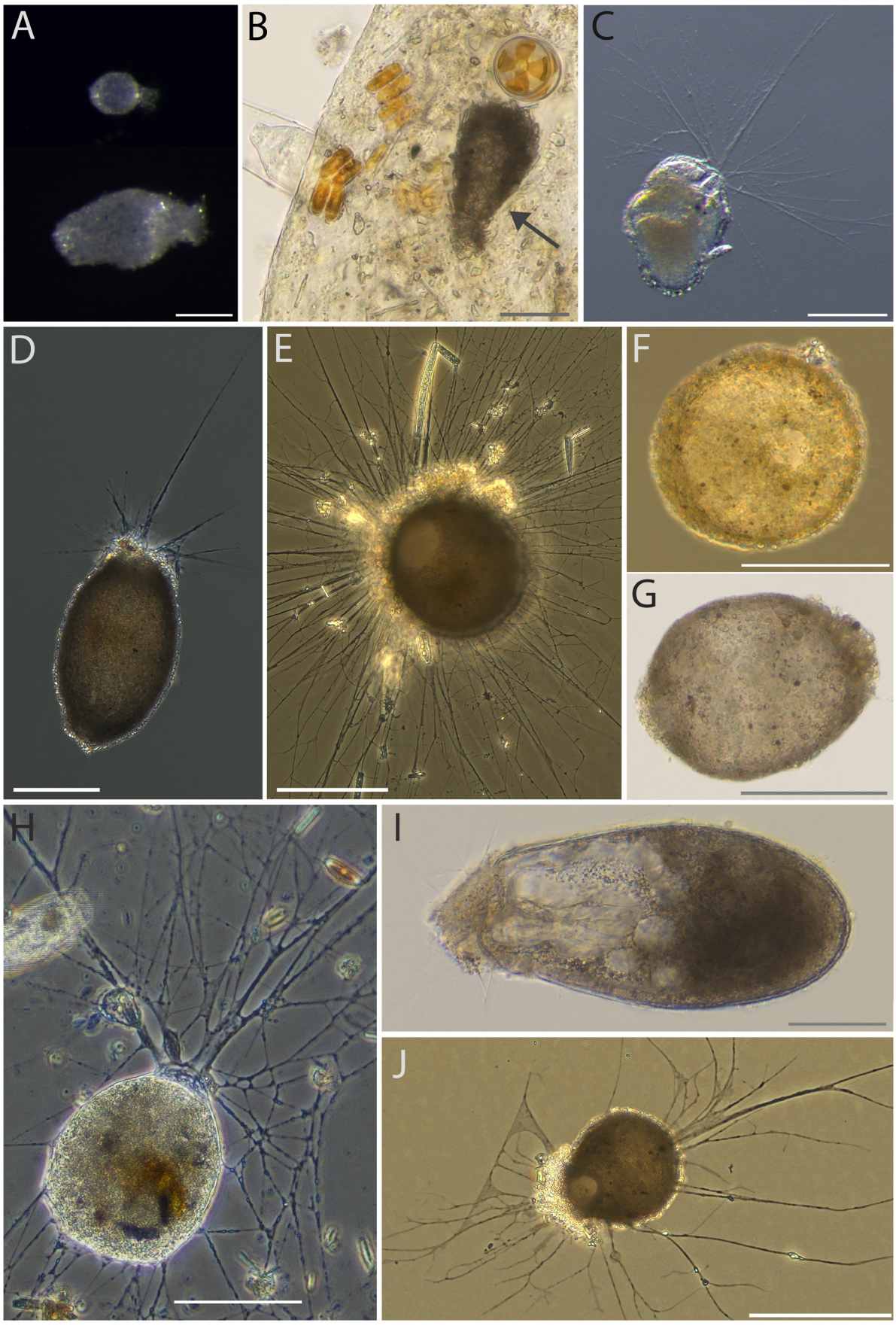
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PLATE 2. Other monothalamous foraminifera isolated from the sample IS1-1_Vogar-1. A. Undetermined saccamminid ICEMON 1, DNA #17959 (lower individual), #17960 (upper individual), live cells. B. Undetermined saccamminid ICEMON 2 (arrow), DNA #17969. C. Undetermined allogromiid ICEMON 7, DNA #17963, active cell with reticulopodia extended; note entosolenian tube and pigmented cytoplasm. D. Undetermined saccamminid ICEMON 3, DNA #17974, active cell with reticulopodia extended. E. Undetermined saccamminid ICEMON 3, DNA #17975, active cell with reticulopodia extended. F–G. Undetermined saccamminid ICEMON 4, DNA #17967 (F—apertural view; G—profile view), live but almost inactive cell, reticulopodia are not visible. H. Undetermined allogromiid ICEMON 9, active cell with reticulopodial network deployed; note diatoms inside and around the cell and also dark mineral grains in the cytoplasm. I. Undetermined allogromiid ICEMON 8, DNA #17668, profile view, active cell. J. Undetermined saccamminid ICEMON 5, DNA #17972, active cell with reticulopodia extended; accumulation of particles around the aperture is visible. Scale bars: 25 μm (B); 50 μm (A, D, F, G, I); 100 μm (C, E, H, J).

Undetermined saccamminid ICEMON 5
(Plate 2, fig. J)

Description. Test is free, subspherical. The aperture is single, terminal, roughly circular, but some flexibility of its shape was observed. A prominent, slightly flared collar surrounds the aperture. The edge of the collar was not clearly defined as many mineral and accumulated particles were loosely attached to it. The test is 103 μm in length and 87 μm in diameter. The wall is quite opaque and consists of coarse mineral grains. The only observed cell was very agile, actively relocating along the bottom; although its reticulopodial network was sparse, consisting of infrequently branching reticulopodial strands and occasional cytoplasmic veils.

Molecular phylogeny. A single sequenced individual is a sister to the clade E, comprising the genera *Vellaria*, *Psammophaga*, *Niveus* and *Nellya*. It branches between the clade E and the two new phylotypes from this study (undetermined saccamminid ICEMON 1 and undetermined saccamminid ICEMON 2), but its position is not well supported (Fig. 1).

Undetermined saccamminid ICEMON 6
(no image provided)

Molecular phylogeny. The morphological appearance of this specimen was indistinguishable from the undetermined saccamminid ICEMON 3. However, the phylogenetic position of both phylotypes is different. The phylotype ICEMON 6 branches within the unresolved radiation of many environmental lineages at the base of the clade Y, while ICEMON 3 is within the clade O. Interestingly, the closest relative to ICEMON 6 is the environmental sequence Unc-Sip27 from the Sippewissett marshes on the US east coast (GenBank Accession number EU213232, Habura *et al*. 2008). Both sequences diverge by only 5.6%.

Undetermined allogromiid ICEMON 7
(Plate 2, fig. C)

Description. The test is free, slightly elongated, with distorted oval outline (the examined individual was slightly deformed upon recovery from the sample); test length is 175 μm; width 127 μm. The test wall is flexible, rather thick (~6 μm), composed of transparent organic material. The outer wall surface is very sticky. The aperture single, terminal, the walls around the aperture sink inside the test, forming a conspicuous entosolenian tube of ~20 μm diameter. The cytoplasm is pigmented, brownish, at least one diatom frustule was observed inside (not distinguishable in the picture). The cell was active and deployed reticulopodial network, exceeding the test diameter at least two-fold.

Molecular phylogeny. This new allogromiid represented by a single specimen was positioned together with the three environmental clones (Unc810-61, Unc810-73, Unc810-78) originating from the deep Western Pacific off the Shimokita Peninsula (Tsuchiya *et al*. 2013). This group of sequences was strongly supported (bootstrap value 94%) and branched within the large radiation of environmental lineages at the base of the clade Y.

Undetermined allogromiid ICEMON 8

(Plate 2, fig. I)

Description. Test free, elongated, pyriform, measuring 190 μm in length and 96 μm in diameter. A single aperture is positioned terminally. Wall thin (~1.5–2 μm), flexible and transparent, composed of organic material. The apertural walls are curved inside the test and form a long entosolenian tube (or 'peduncular sheath'), which may be tracked at least to the mid-level of the test. This structure has a variable width (13–21 μm). The exact length is difficult to estimate as the aboral portion of the test is filled with dense granular cytoplasm. Also, the wall of entosolenian tube and the apertural region seems thicker than that of the main portion of the test. Cytoplasm translucent, with a brownish tinge, contains large vacuoles, concentrated predominantly in the frontal half of the test. Although the cell was apparently active at the moment of collection, it did not deploy any extensive reticulopodial network, showing only separate branches emerging from the pile of material accumulated in the apertural region.

Molecular phylogeny. The sequences obtained from the only examined specimen clustered with those of *Bowseria arctowskii* Sinniger *et al*. (2008), belonging to the monothalamid Clade B. This relationship was supported by 100% BV. Apart from the test size size (all documented *Bowseria* specimens were much bigger), nothing could be confidently stated regarding morphological differences of both species.

Undetermined allogromiid ICEMON 9

(Plate 2, fig.H)

Description. The test is free and very flexible. As the cell crawls along the substratum, the test frequently undergoes stretching by the reticulopodia and becomes elongated with pointed or rounded posterior end; it is more or less spherical when stationary (this equally applies to the test shape mentioned above). Test length is on average 162 μm when stationary and 182 μm when relocating; test diameter varies from 127 μm to 116 μm. Test wall is thin (~1.9 μm), soft and very flexible, and consists of transparent organic material. The aperture is single and also flexible; surrounded by a thin organic collar and frequently obscured by cytoplasmic masses on the test surface and the cell itself; no apparent entosolenian tube present. The cell body is translucent; cytoplasm is granular and colorless. Large pennate diatoms (up to 60 μm in length) along with transparent mineral grains and unidentified dark elongated bodies are present in the cytoplasm. On one occasion, empty diatom frustules were seen being expelled from the test through the aperture. Reticulopodial network extensive, many times exceeding the test diameter. A number of large, frequently branching strands originate from the apertural region, with their proximal parts frequently forming small cytoplasmic veils; smaller network elements cover all area around the cell and are also present on the test surface.

Remarks. It was not possible to obtain reliable molecular data for this specimen.

Discussion

The present study provides the first glimpse into the diversity of Icelandic shallow-water monothalamids. Although we examined only very small amount of collected material, the diversity of tiny monothalamous foraminifera we found there is spectacular. Remarkably, all of the isolated monothalamids turned out to correspond to new molecular phylotypes after comparison with our extensive database and GenBank sequences. Moreover, each of them represents a separate lineage, often branching in a very distant position from others in the phylogenetic tree (Fig. 1). Only two phylotypes (ICEMON 1 & ICEMON 2) form a monophyletic group.

Together with ICEMON 5 they form a sister group to the clade E, members of which commonly occur in shallow-water muddy sediments and received major attention over the past five years (Altin *et al*.2009, Goldstein *et al*. 2010, Gooday *et al*. 2011, Pawlowski and Majewski 2011). There are also three phylotypes (ICEMON 4, 6, 7) that branch closely to the clade Y, but their genetic distance from typical representatives of this clade is very high and there is no support for this grouping.

Our study confirms the rich diversity of monothalamids found in the environmental DNA surveys of shallow-water foraminiferal communities (Habura *et al*. 2004, 2008). Indeed, two phylotypes (ICEMON 3 and ICEMON 6) are genetically similar to the environmental lineages from McMurdo Sound, Antarctica (Habura *et al*. 2004) and Sippewissett marsh, US (Habura *et al*. 2008), respectively. More surprisingly, one of our phylotypes (ICEMON 7) branches closely to the phylotypes originated from the deep Japan Sea locations sampled by Tsuchyia *et al*. (2013). This demonstrates a wide geographic distribution of many low-rank taxonomic units of monothalamids, roughly corresponding to separate genera, as was already shown in Pawlowski & Holzmann (2008) and Pawlowski *et al*. (2008).

By revealing the phylogenetic relationships of our phylotypes we provide the first microscopic documentation of some foraminiferal environmental lineages. In view of their close genetic relation, we can confidently assume that the anonymous phylotypes McM11 and Unc-Sip27 correspond to the morphotypes of ICEMON 3 and ICEMON 6, respectively. It is also possible that the undetermined foraminiferal sequence Xeno215_5 is morphologically similar to the new species described here, although its close distance to '*Saccammina*' *alba* (Hedley 1962) may suggest that its morphology is an intermediary between these two morphospecies.

It is important to notice that the morphological examination using conventional light microscopy is usually insufficient to unambiguously distinguish different genera and species of monothalamous foraminifera. Apart from some particular, easily recognizable morphotypes representing well-known genera (e.g. *Micrometula*, *Pelosina*, *Vanhoeffenella*, *Psammophaga*), in most cases we are dealing with simple monothalamous test designs lacking any conspicuous features. We believe that there must be some underlying, although possibly minor nuances in cellular architecture and behavior useful as distinguishing features / synapomorphies for certain clades of monothalamids. Goldstein and Richardson (2002) examined the test wall ultrastructure of some monothalamids and concluded that it might be feasible to use it as a distinguishing feature at a higher taxonomic level. Indeed, even at low resolution we see that simple organic walls or layers of bioadhesives underlying agglutinated material vary in their mechanical and optical properties among different 'simple' monothalamids. In addition, some clades share common characteristics of reticulopodial pattern, as for example the phylotypes branching at the base of clade E, which all possessed "scarce" (low-branching) networks, or certain gross details of test architecture, as flared apertural end of *Vellaria*. However, these apparently good synapomorphies are only scattered bits of a complex puzzle of monothalamid taxonomy, and there is a long way ahead to resolve it. Besides, it is hard to establish any fast-growing, clean (monoxenic) cultures of monothalamids similar to those of some flagellates and amoebae, as they depend on sediment microenvironment and frequently appear spontaneously in stored samples.

The integrated taxonomic approach adopted here, combining molecular and morphological analysis of single cells, is certainly an important complement to the environmental DNA studies. Yet the standard 'slow' identification pipeline is not very efficient for the comprehensive documentation of protist diversity. This is especially true in the case of 'tough' groups such as the monothalamous foraminifera, when considerable time and effort is spent on the repeated isolation from sample, imaging, DNA extraction and Sanger sequencing of specimens. In the current study, we managed to reveal only a tiny part of the actual diversity in the samples. With the recent developments in the NGS field, allowing us to instantly get vast amounts of sequence data, there is still much more to do for the development of the other, accelerated approach, which would allow rapid isolation and visualization of sediment-dwelling organisms.

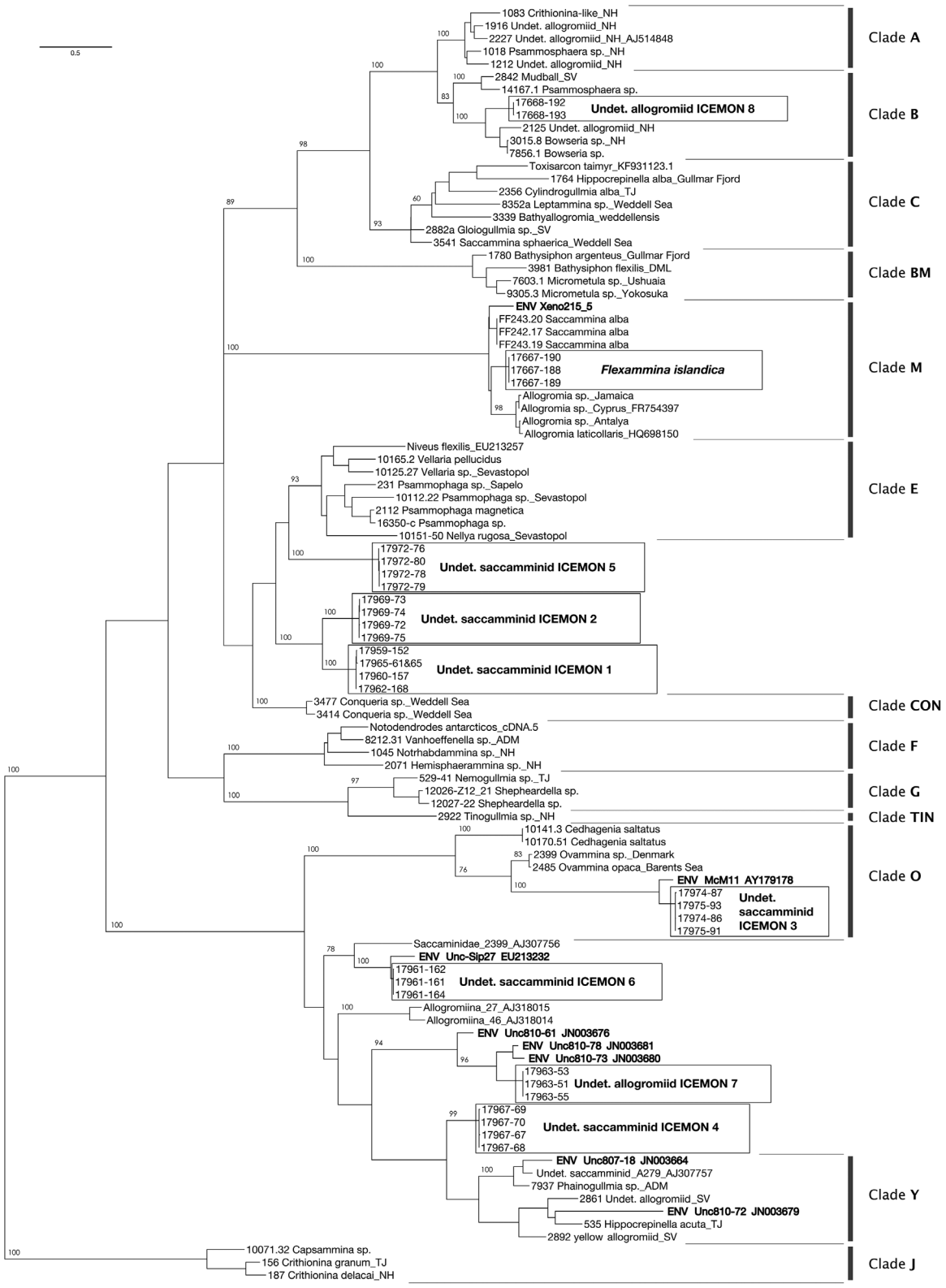

FIGURE 1. Phylogenetic tree of monothalamous foraminifera based on 3' partial SSU rDNA sequences from our database and some GenBank sequences derived from environmental clone libraries (marked by the 'ENV' prefix and highlighted in bold). New sequences obtained in this study are marked by frames with the names of corresponding phylotypes / species given in bold; the name of every new sequence consists of two parts separated by dashes (foraminiferal DNA collection number and clone number). Foraminiferal DNA collection numbers and GenBank accession numbers (if available) are also shown for other sequences. Major monothalamid clades are indicated in accordance with Pawlowski *et al*. (2002, 2011).

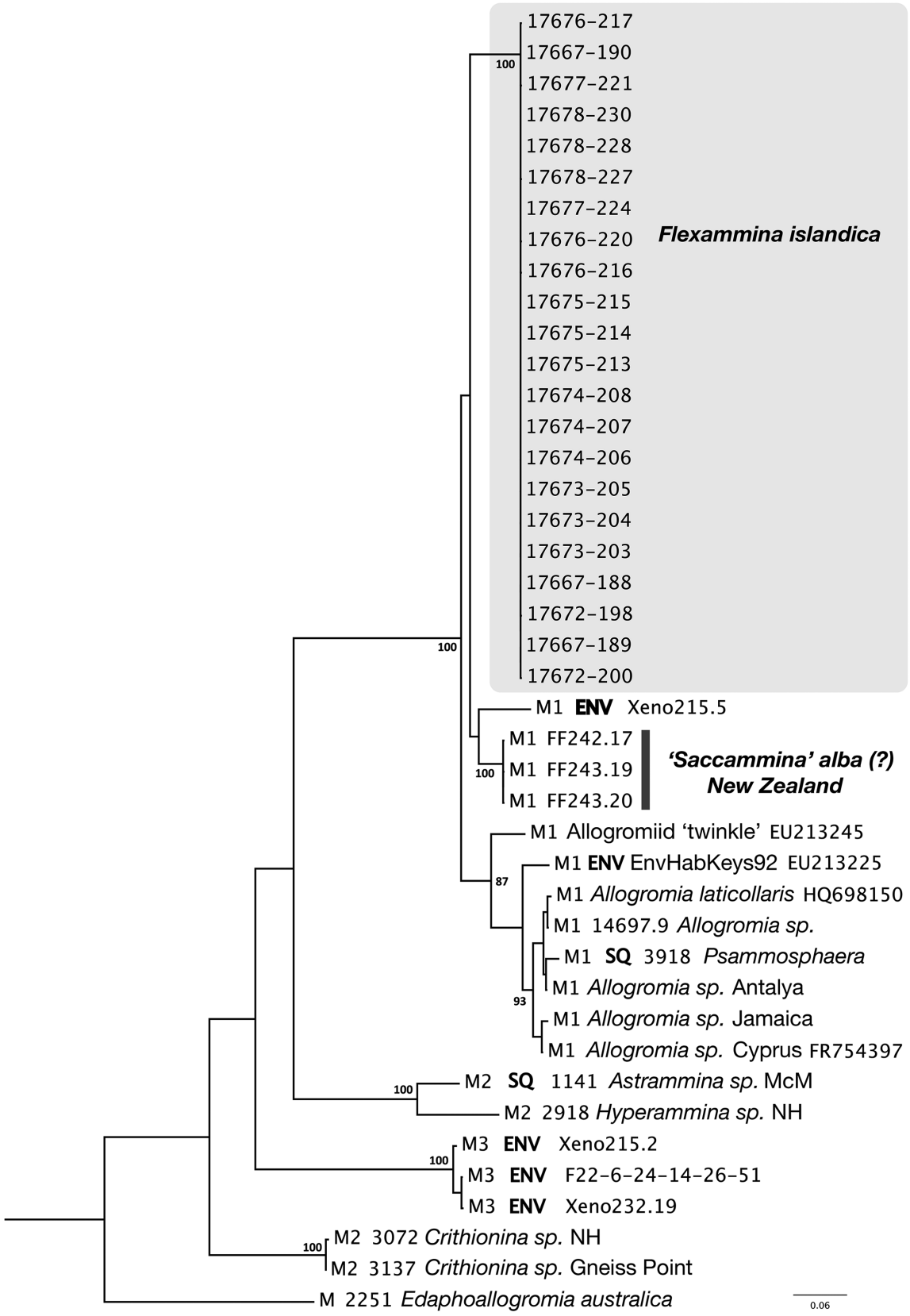

FIGURE 2. Phylogenetic tree of Clade M monothalamous foraminifera illustrating the position of *Flexammina islandica* **sp. nov.** (highlighted) and *Saccammina alba* sequences. Prefixes 'M1-3', 'ENV' and 'SQ' mark three sub-clades, environmental sequences from GenBank and squatter-derived sequences, respectively. Only the bootstrap values exceeding 85% are retained.

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