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Phylogeny and Ultrastructure of *Miliammina fusca*: Evidence for Secondary Loss of Calcification in a Miliolid Foraminifer

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ABSTRACT. The classification of the Foraminifera, a widely distributed group of largely marine protists, has traditionally been based on morphological characters. The most important of these are the composition and structure of the shell or ''test.'' Here, we use both phylogenetic analysis of the genes for small subunit rRNA and β-tubulin and ultrastructural analysis to document a reversion in wall type from more derived calcareous tests to an agglutinated test. These data indicate that the genus *Miliammina*, and possibly other members of the Rzehakinidae, should be placed in the Order Miliolida as opposed to their current assignment in Order Textulariida. We also address the effects this reversion may have had on the ability of rzehakinacids to effectively colonize marginal marine environments. Finally, the hypothesis that some multilocular agglutinated foraminiferans descended from calcareous lineages has implications for interpretation of the foraminiferal fossil record.

Key Words. Calcification, Foraminifera, fossil record, morphology, phylogeny, SSU, test, β-tubulin.

THE Foraminifera are best known for the elaborate hard shells or ''tests'' produced by many species (Lee et al. 2000). Calcareous Foraminifera secrete calcium carbonate, primarily as calcite, on or into an organic matrix to form the test wall. Agglutinated taxa gather materials, such as sand or sponge spicules, from the environment and cement them together, usually in conjunction with one or more organic linings. Some species construct less elaborate organic-walled tests, and the so-called ''atestate'' species, such as *Reticulomyxa filosa*, have only a mucoid outer covering. The morphological synapomorphy for the group is an anastomosing network of pseudopods called reticulopodia, which Foraminifera use for feeding, motility, protection, test construction, and possibly respiration (Bowser and Travis 2002).

Mineralized tests fossilize well, and most of what is known about foraminiferal evolution is based on the fossil tests distributed throughout Phanerozoic marine strata. Test characteristics have been used for approximately 150 years as the main criterion for classification of both fossil and modern representatives of the group (Cifelli 1990; Sen Gupta 1999a). The consensus view has been that the organic-walled and atestate forms are primitive, with agglutination, polythalamy (i.e. the ability to make multiple chambers), and calcification representing successive innovations within the lineage (Tappan and Loeblich 1988). Several orders are defined based primarily on the precise nature of the test wall: species whose non-perforate, porcelain-like wall is made of randomly oriented calcite grains are included among the Miliolida, whereas the various families of the order Rotaliida are partly distinguished by the optical characteristics of their glassy, perforate, ''hyaline'' tests. Additional information for classification derives from the number, shape, and arrangement of the test's chambers, the overall shape of the test, and features of the aperture. Although this classification system has proved relatively robust, the relationships among different orders, and the proper classification of morphologically ambiguous species, have proven problematic.

For example, the correct placement of *Miliammina*, a widespread genus that often dominates brackish-water assemblages, has been the subject of intense controversy (Cifelli 1990). Galloway (1933) placed this genus within the Miliolida on the basis of its milioline chamber arrangement, whereas Cushman (1948) explicitly excluded it from that group on the basis of the agglutinated nature of the test wall. It is currently included, following Cushman's assignment, within the Rzehakinacea, a super family of agglutinated multilocular taxa (Order Textulariina) that have chamber arrangements reminiscent of those found in the Miliolida. Because they do not have the characteristic calcareous imperforate wall of the miliolines, this family has not been considered to be related to the miliolid species that they otherwise resemble, although several recent authors (e.g. Fahrni et al. 1997; Flakowski et al. 2005; Tyszka 1997) have noted the gross morphological similarities.

Recently, DNA-based studies of Foraminifera have provided important new information about the history of the group. Ribosomal small subunit gene (SSU rDNA) phylogenies (Pawlowski et al. 2003) show the primary calcareous taxa, the miliolids and the rotaliids, as robust monophyletic lineages emerging from a radiation of single-chambered allogromiid forms. The rotaliids also show a strong relationship with the polythalamous, hard-walled agglutinated Textulariida. Many textulariid taxa exhibit chamber arrangements that are much like those found in the rotaliids, suggesting that this suite of morphological patterns has remained evolutionarily stable over a very long period of time, perhaps 400 Myr. The miliolids, in contrast, do not show a consistent relationship with any other higher taxon in these analyses, and their morphological antecedents are also obscure. Tappan and Loeblich (1988) postulated that the miliolids descended from the fusulinids, a group of calcareous microgranular foraminiferans that did not survive the end-Permian extinction. Alternatively, Arnold (1978, 1979) proposed that the miliolids evolved from an allogromiid ancestor.

The Foraminifera have been very well sampled with respect to SSU rDNA, with sequences for 252 species reported in GenBank as of November 2005, and the traditional morphological criteria for classification have probably provided as much phylogenetic information as they can. Clearly, there is a need to bring additional lines of evidence, such as finer morphological details and evidence from additional genes, to bear on these problems. Fahrni et al. (1997) demonstrated commonality between the actin isoforms of *Miliammina* and some miliolid species. A phylogenetic analysis of actin isoforms also places *Miliammina* as a possible sister group to the miliolids (Flakowski et al. 2005). However, no clear morphological feature allying *Miliammina* with the miliolids has been identified.

Tubulin genes have often been used in protist phylogenetic studies (e.g. Edgcomb et al. 2001), but few data on these genes have been available for the Foraminifera. A previous study on the α- and β-tubulins of the ''naked'' foraminiferan *R. filosa* (Linder, Schliwa, and Kube-Granderath 1997) identified two isoforms of

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each gene. Although the two α-tubulin isoforms were relatively conventional and very similar in sequence to one another, one of the β-tubulins (''β2'') was highly divergent. We have obtained the β-tubulin sequences of a taxonomically diverse set of eight species of foraminiferans, and demonstrated that the Type 2 sequence is, in fact, the typical foraminiferal β-tubulin, whereas the Type 1 sequence may be from another eukaryote. The structural and functional implications of these unusual sequences are described elsewhere (Habura et al. 2005). Here, we use these sequences in conjunction with SSU rDNA and ultrastructural data to test hypotheses about miliolid evolution, and to help resolve the correct placement of *Miliammina*. We present molecular and morphological evidence that this agglutinated genus, true to its form, did in fact arise from a calcareous miliolid ancestor.

MATERIALS AND METHODS

Genomic DNA. *Reticulomyxa filosa* and *Allogromia* sp. strain NF were maintained in culture as reported by Habura et al. (2005). All other species were harvested from marine sediments by serial sieving and isolation of individual cells. *Miliammina fusca* were collected on Sapelo Island, Georgia, USA (N 31°30′12″, W81°14′9″. *Astrammina rara*, *Crithionina delacai*, *Cornuspira antarctica*, *Pyrgo peruviana*, and *Rhabdammina cornuta* were collected at Explorers Cove, Antarctica (S 77°34′35″, E 163° 31′39″). After morphological identification, individuals were cleaned with a fine artist's brush and allowed to purge in sterile seawater for >24 h. After purging, 2–100 cells (depending on size) were used to obtain genomic DNA as described (Habura et al. 2004a).

Cloning and sequencing of SSU rRNA and β-tubulin genes. A ~880-bp region of the SSU rDNA of *M. fusca* was amplified by nested PCR with primers s14F3a, B, s14F1, and s20r (Pawlowski 2000). PCR reactions were performed on a Techne Genius thermocycler using TaKaRa proofreading ExTaq premix with cycle parameters as described in Habura et al. (2004a). Products were analyzed by gel electrophoresis, cloned into pGEM-T Easy vectors (Promega, Madison, WI), and replicated in *Escherichia coli* strain JM109. Individual clones were purified with the Spin-Prep mini kit (Qiagen, Valencia, CA), and multiple clones for each product were sequenced in both directions using primers M13 and M13 reverse, with a PE-Biosystems ABI PRISM 377XL automated DNA sequencer. This sequence has been deposited in GenBank as DQ291136.

Sequences from the β-tubulin genes of eight foraminiferal species (*Astrammina rara*, *Allogromia* sp., *Cornuspira antarctica*, *Crithionina delacai*, *Miliammina fusca*, *Pyrgo peruviana*, *Reticulomyxa filosa*, and *Rhabdammina cornuta*) were amplified with primers BTub1F (5′-CAATGTGGTAACCAAATTGG-3′) or BTub2F (5′-AATTGGGCAAAAGGACATTA-3′) and BTub1R (5′-CATCTTGTTTGTCTTGATATTCAGT-3′) (Habura et al. 2005). The amplimers were then cloned and sequenced as described. These sequences have been deposited in GenBank as AY818714–AY818726.

Sequences were aligned using CLUSTAL W (Thompson, Higgins, and Gibson 1994). Alignments were adjusted manually in SEAVIEW (Galtier, Gouy, and Gautier 1996) to accommodate introns, insertions, and other regions of variable length in the β-tubulin and SSU rRNA consensus structures (for reviews, see Burns and Surridge 1994, and Habura, Rosen, and Bowser 2004b, respectively). All unambiguously aligned positions were retained for phylogenetic analysis. Alignments are available for viewing at www.bowserlab.org/supplemental.

Phylogenetic analyses. For alignments of DNA sequences, the appropriate model of nucleotide substitution was selected via likelihood ratio test, as implemented in Modeltest 3.06 (Posada and Crandall 1998). For both SSU rRNA alignments, a TVM+I+Γ model was selected as best for the datasets. The indicated substitution models were used to obtain maximum likelihood (ML) trees using the method implemented in PAUP* 4b10 (Swofford 2003), with tree bisection and reconnection, 100 replicates. Maximum parsimony (MP) analyses for both sequence types were also performed using PAUP*, with tree bisection and reconnection, 1,000 replicates.

For alignments of protein sequences, analyses were performed using the ML distance methods implemented in TREE-PUZZLE 5.2 (Schmidt et al. 2002). Bootstrap analyses and phylogeny construction were performed using SEQBOOT, NEIGHBOR, and CONSENSE (Felsenstein 1989), in conjunction with PUZZLE BOOT (Holder and Roger 1999).

For all datasets, Bayesian analysis was conducted using Mr Bayes, version 3.1 (program release May 2005) (Huelsenbeck and Ronquist 2001). We selected a GTR+I+G (for SSU rRNA data) and a GTR (for β-tubulin data) substitution model with six rate categories as being an analog to the model of substitution calculated by Modeltest. Markov Chain Monte Carlo (MCMC) simulations were then run with two sets of four chains using the default (random tree) option, 1,000,000 generations, with trees sampled every 100 generations. The first 75,000 generations were discarded as burn-in for the β-tubulin data, and the first 100,000 were discarded for the SSU rRNA analyses. The remaining trees were used to generate a consensus tree.

Ultrastructural methods. Viable *M. fusca* cells were identified by observation of life processes, such as active gathering of detrital material for feeding. Individual specimens were prepared for TEM using high-pressure freezing followed by freeze substitution (HPF/FS) according to the protocols of Goldstein and Richardson (2002). Specimens were frozen in a Balzers HPM 010 High Pressure Freezing Machine (Furstentum, Liechtenstein), embedded in Araldite Embed 812 (Epon 812) following FS, sectioned with a diamond knife, and examined in a Zeiss EM 902A TEM (Oberkochen, Germany). The freezing machine and the TEM are housed in the Department of Plant Biology at the University of Georgia.

RESULTS

β-tubulin phylogeny of Foraminifera. The β-tubulin sequences obtained from an individual species were very similar, and likely to be simple isoforms of one another. The inclusion of *Miliammina* within a clade of miliolid foraminiferans was unexpected, as this genus is not classified as a miliolid based on traditional morphological criteria (Fig. 1).

SSU rDNA phylogeny of *Miliammina fusca*. In order to test the suggested phylogenetic placement of *M. fusca,* we amplified a diagnostic ~880-bp region of the SSU rRNA and aligned the sequence with a taxonomically diverse group of 43 other foraminiferal sequences. Previously identified groups of Foraminifera were recovered in this analysis, and the association between textulariids and rotaliids appeared as well (Fig. 2). *Miliammina* grouped weakly as a basal taxon to the known miliolids. The analysis rejected the traditional placement of *Miliammina* within the Textulariida.

Miliolid SSU rRNA sequences show several unusual sequence features relative to other foraminiferal SSU rRNAs, often causing their placement within a taxonomically broad tree to be unstable. To eliminate possible distorting effects from other foraminiferal taxa, we constructed an additional alignment of 27 miliolid sequences as a means of improving resolution (Fig. 3). The morphologically assigned families were recovered in this analysis, with the exception that the genus *Laevipeneroplis* appeared in the Soritidae (but see Holzmann et al. 2001). The morphologically simple genus *Cornuspira* appeared as the basal taxon, as it did in

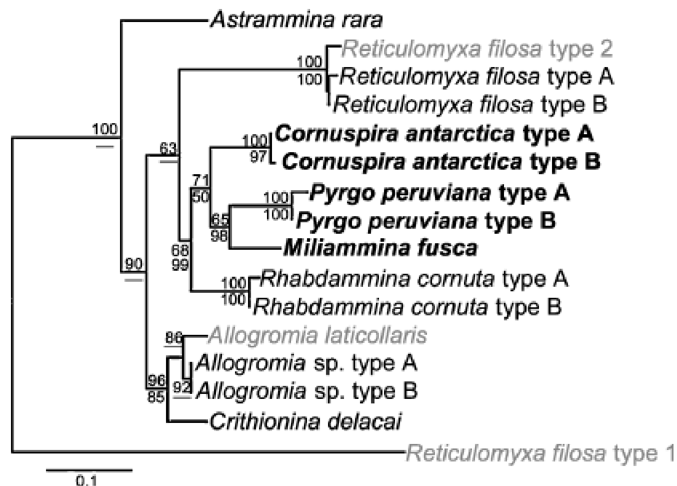

Fig. **1.** Protein maximum likelihood phylogeny of foraminiferal β-tubulin genes. Bootstrap values from PUZZLEBOOT and posterior probabilities for MrBayes (multiplied by 100) are shown for nodes for which the value is >50. A dash represents nodes that differ in the maximum likelihood and Bayesian phylogenies. Previously reported sequences are shown in gray; miliolid and *Miliammina* sequences are indicated in bold.

the β-tubulin analysis. In this analysis, *Miliammina* appeared as a sister taxon to an unusual sequence reported for a member of the calcareous miliolid genus *Quinqueloculina*.

Fine structure of the *Miliammina fusca* test. The overall appearance of the test of *M. fusca* is shown in Fig. 4. Viewed in cross-section (Fig. 5), the test wall was characterized by two prominent layers: an inner organic lining (IOL) that measured from 2.5 to 3.5 μm thick, and an agglutinated layer of variable but slightly greater thickness. In these preparations, the IOL appeared featureless and was in direct contact with the cell membrane. The cytoplasm directly beneath this lacked the numerous vesicles found in many allogromiids (see Goldstein and Richardson 2002), which probably reflects the very different modes of test morphogenesis in these two disparate clades.

The agglutinated layer was separated from the IOL by a thin, opaque organic layer that appears to surround agglutinated grains, which are predominantly quartz. (Conchoidal fracture, particularly evident in Fig. 6, is a distinctive physical property of quartz and various glasses; see also Bender 1989). This layer also covered the outer surface of the test (Fig. 5), although the test otherwise lacked an outer organic lining (OOL) comparable to the IOL in thickness and character. The quartz grains, which were prominent and tightly packed in the agglutinated layer, commonly fractured and pulled out of the sample during sectioning. Nonetheless, these HPF/FS preparations for TEM revealed the fine strand-like appearance of the bioadhesive that interconnected individual sediment grains (Fig. 6). In interior chambers, the IOL separated the lumen (and cytoplasm) of each chamber from the tightly packed agglutinated layer, which was centrally located between chambers (Fig. 7).

The fine structure of the terminal chamber wall and apertural tip differed significantly from that of the older chambers of the test. The bioadhesive within the wall near the apertural tip was thick and more strongly fibrous than anywhere else within the test wall (Fig. 8). The bioadhesive became less fibrous and more massive toward the base of the chamber, where it resembled the bioadhesive found elsewhere within the test.

DISCUSSION

The analyses presented here provide new information about the evolution of foraminiferal test morphology. Taxonomically, the presence of an agglutinated, as opposed to a mineralized, test wall appears to be less phylogenetically significant than does the overall arrangement of test chambers. This suggests that chamber arrangement is a fundamental developmental character in at least some polythalamous clades, and that the composition of the test can be altered over time, possibly reflecting adaptation to changing environmental conditions.

The basal position of *Cornuspira* in our analyses agrees with its earlier, morphologically based identification as one of the earliest miliolids (Tappan and Loeblich 1988). This calcareous genus with a relatively simple coiled-tube morphology appears first in Carboniferous strata; the other miliolids in our analysis appeared no earlier than the Jurassic. The evolutionary sequence in this group, in which calcification apparently preceded the development of more complex morphology, is the opposite of that postulated for the Textulariida/Rotaliida group. In that clade, polythalamous forms substantially pre-date calcitic tests, both in the fossil record (Loeblich and Tappan 1988) and in molecular phylogenies (Pawlowski et al. 2003). The very different methods of calcification used by members of the two clades (Loeblich and Tappan 1988) support the view that calcification was acquired independently in the two lineages.

If *Cornuspira* is indeed basal to *Miliammina*, the most parsimonious explanation is that this agglutinated genus descended from a calcareous ancestor with a ''quinqueloculine'' (i.e. lengthwise coiling with 5-fold symmetry) chamber arrangement, as has also been suggested by other researchers (e.g. Fahrni et al. 1997; Tyszka 1997). In this model, the coiling pattern was retained, but calcification of the test wall was replaced by agglutination, reversing the conventionally accepted evolutionary progression. Given that several hauerinid miliolids, such as the quinqueloculine *Rudoloculina hooperi* (Guilbault and Patterson 1998), cement substantial amounts of exogenous particles on the outer surfaces of their calcareous walls, a behavioral mechanism that would permit secondary agglutination would presumably have been available. (The apparent splitting of the genus *Quinqueloculina* in our SSU rDNA analyses may simply reflect the common occurrence of quinqueloculine chamber arrangements in hauerinid foraminiferans.) Lastly, the appearance of the Alveolinidae as a derived group within the Hauerinidae has been proposed earlier, on morphological grounds (Tappan and Loeblich 1988).

Although *M. fusca* has an agglutinated test, a feature that has supported its inclusion within the Textulariina (Loeblich and Tappan 1987), the fine structure is not that of a typical textulariid. As shown by Bender (1995), textulariid tests typically have a multilayered IOL as well as an OOL, features that are lacking in *M. fusca*. We find that the IOL of *M. fusca* consists of a single, thick organic layer that underlies the agglutinated layer. Sand grains in the agglutinated layer are interconnected by fine strands of bioadhesive. Thicker, strand-like features are also evident in Bender's (1989) SEM illustrations of the wall of *M. fusca*, although the difference in methodologies employed make further comparisons difficult.

Furthermore, the fine structure of the terminal chamber suggests a mode of chamber formation analogous to that found in the miliolid *Spiroloculina hyalina* (Angell 1980) in which new chambers calcify progressively from the abapertural tip toward the aperture of the new chamber. The bioadhesive structure in the terminal chamber of *M. fusca* is thicker and more fibrous near the aperture, but resembles that in the remainder of the test near the base. This suggests a bioadhesive maturation process that begins at the base of the terminal chamber and progresses toward the aperture. Bender (1989) also reported differences between the cement of the terminal chamber and that of older ones, suggesting that this most likely reflected the process of chamber formation. In addition, older chambers in *S. hyalina* are progressively thicker

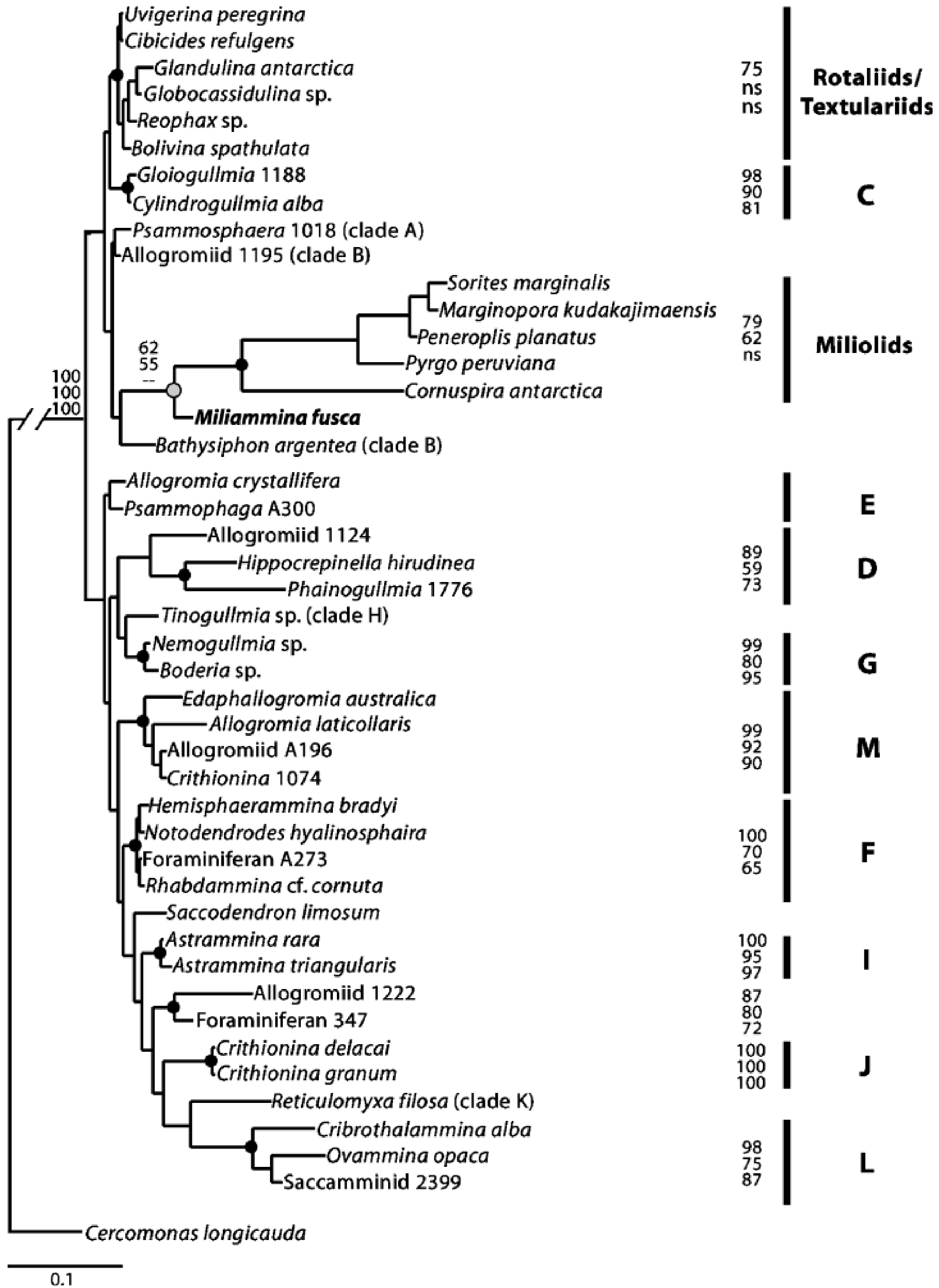

Fig. 2. Bayesian phylogeny of partial foraminiferal small subunit (SSU) rRNA genes. Support values for nodes marked with black dots are given to the right of the tree (in the order Bayesian posterior probabilities ×100/Maximum Likelihood/Maximum Parsimony) Support values <50 are labeled as ''ns.'' Previously identified foraminiferal phylogenetic groups (i.e. the polythalamous rotaliids, textulariids, and miliolids, and several allogromiid clades; Pawlowski 2000) are indicated with labeled black bars. The branch joining the foraminiferans to the outgroup taxon (*Cercomonas longicauda*) is shown at 1/10 actual length. New sequences are indicated in bold.

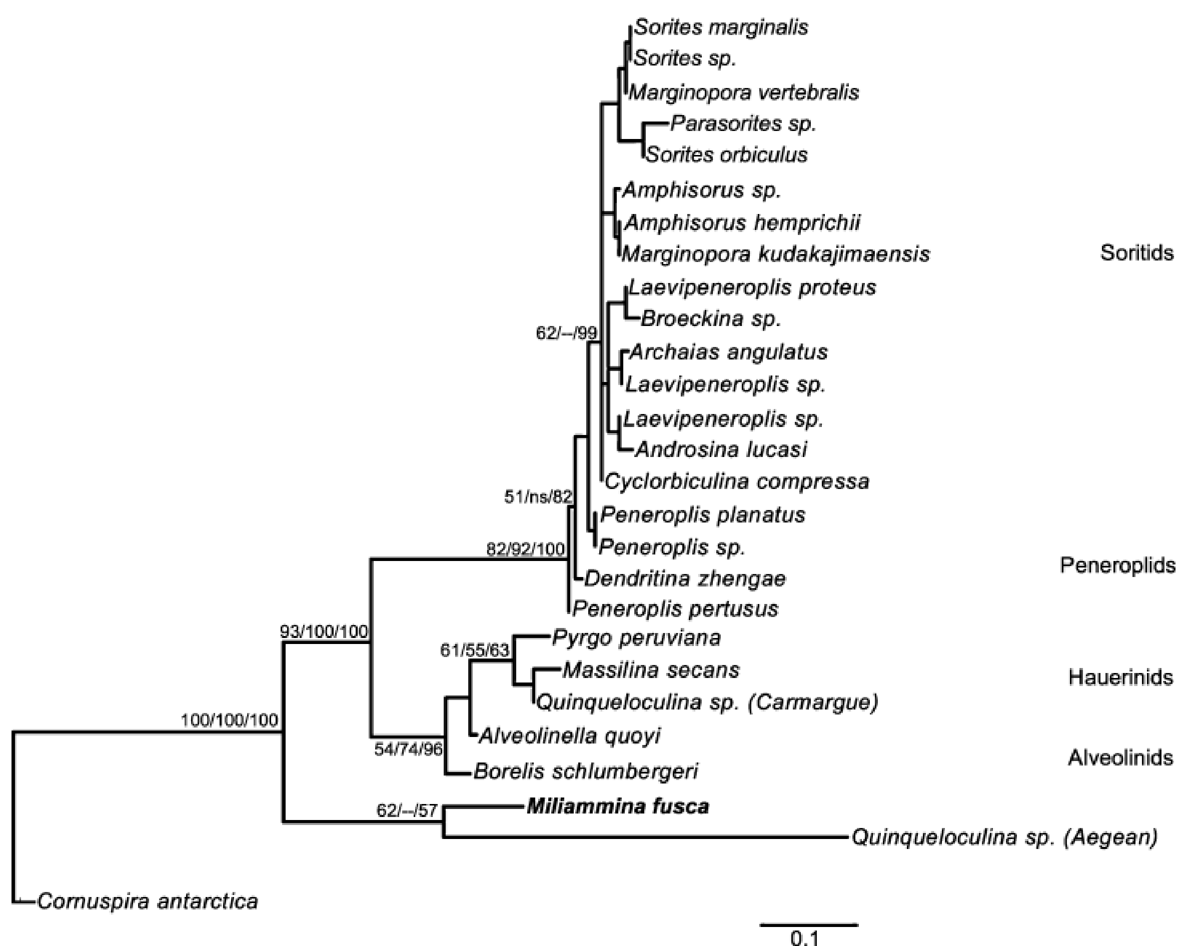

Fig. **3.** Maximum likelihood (ML) phylogeny of partial miliolid small subunit (SSU) rRNA genes. Support values are given in the order ML/ Maximum Parsimony/Bayesian posterior probability, and are labeled as described in Fig. 1. Morphological classification by family is indicated by bars to the right of the tree. New sequences are indicated in bold.

(Angell 1980), and in *M. fusca*, the IOL of interior chambers is considerably thicker than that of the terminal (and youngest) chamber although the agglutinated layer remains of fairly constant thickness.

The evolutionary advantage of constructing an agglutinated test rather than a mineralized one is not immediately apparent. Based on physical manipulation, *Miliammina*'s agglutinated test appears to be structurally weaker, for its mass, than are those of its calcareous relatives (Habura A., pers. observ.). The answer may lie in the observed geographical distribution of both modern and fossil rzehakinacids. *Miliammina* is renowned for its dominance of mesohaline and dysoxic habitats, such as low salt marsh and mangrove swamp, and oligohaline estuarine environments (Sen Gupta 1999b; Tyszka 1997). Indeed, *M. fusca* is often the last identifiable foraminiferal species to survive in marine basins that become cut off from the sea (Lloyd and Evans 2002). Marginal marine environments in general are dominated by agglutinated taxa (Sen Gupta 1999b); only a few calcareous taxa, such as the euryhaline elphidiid rotaliids, appear to be able to survive there (e.g. Boudreau et al. 2001). It is likely that the relatively low pH and oxygen levels and reduced salinity of these environments (e.g. Lloyd and Evans 2002; Sen Gupta 1999b) disfavor calcification in foraminiferans; speed of calcification in these organisms is dependent on the local carbonate concentration (Lea et al. 1995), which decreases in environments that are acidic, dysoxic, low-salinity or unusually cold. Most calcareous foraminiferans also experience substantial decalcification and test deformation in conditions more acid ($pH < 7.8$) than is typical for seawater (LeCadre, Debenay, and Lesourd 2003).

Modern calcareous foraminifera occurring high in estuaries or in other low-carbonate environments are often found in a so-called ''chitinous'' form, in which only the organic lining of the test is present. A possible evolutionary pathway for the rzehakinacids is an invasion of calcareous miliolids into regions in which calcification processes fail due to low carbonate concentration. Today, quinqueloculine miliolids sometimes invade the marginal environments that *Miliammina* dominates, if conditions change so as to become slightly more marine (e.g. Swallow 2000). Decalcification would leave a ''chitinous'' test, which could be overlain by a secondary agglutinated layer, such as that seen in *Miliammina* tests.

The data we report points to a ''reversion'' from calcareous to agglutinated test formation in at least one major lineage of foraminiferan protists, and suggests that the genus *Miliammina*, and possibly other members of its family, should be reassigned to the Order Miliolida. Because of the widespread use of test composition as an important taxonomic character for foraminiferans as a whole, the possibility of reversion may also have implications for other proposed relationships within the Foraminifera.

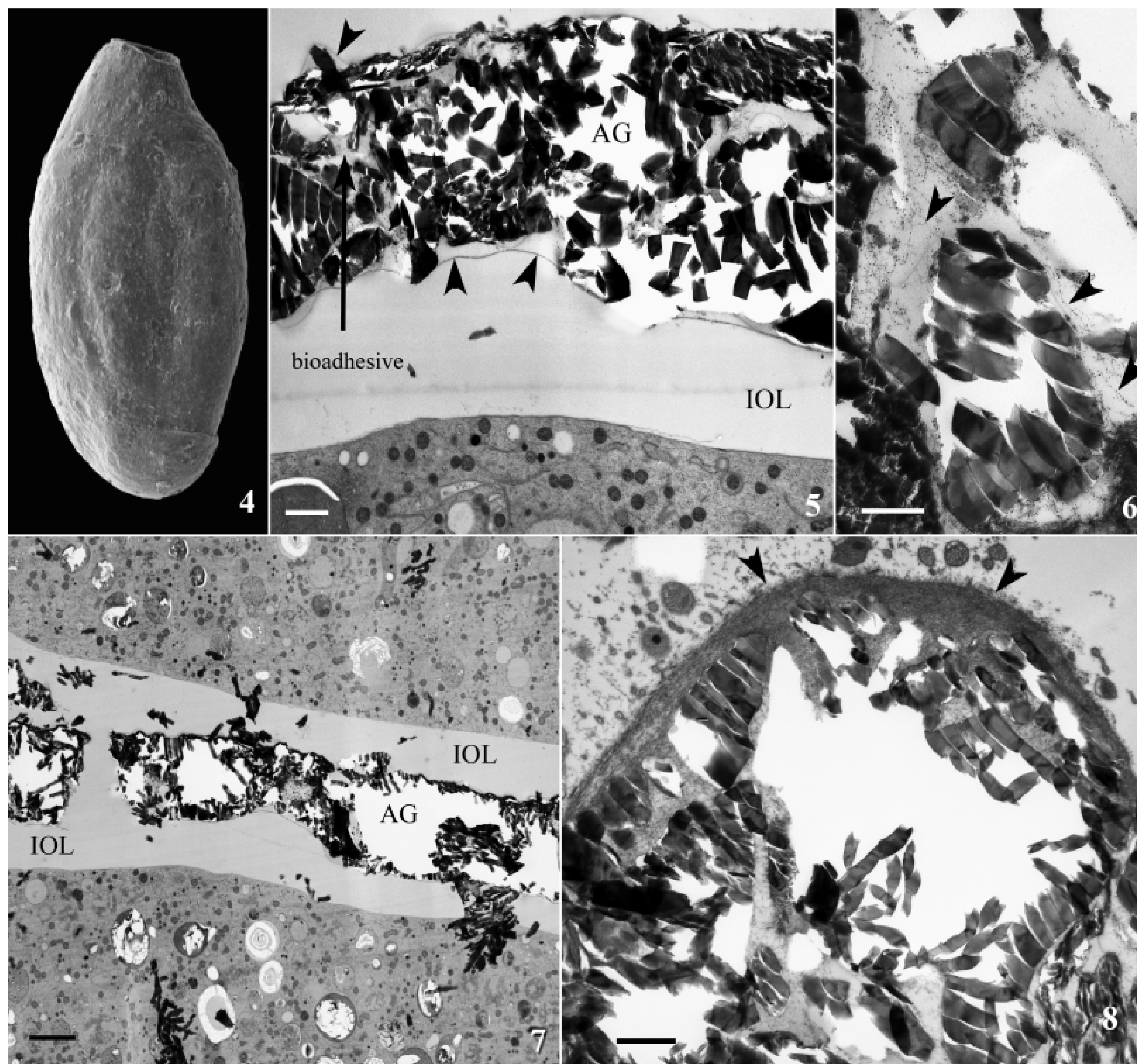

Fig. **4–8.** Fine structure of the test of *Miliammina fusca*. **4.** SEM of the test of *M. fusca*. Test is 438-μm long. **5.** Cross-section (TEM) through the outer wall of the penultimate chamber. A prominent inner organic lining (IOL) separates the agglutinated layer (AG) from the cell membrane and underlying cytoplasm. Quartz grains within the agglutinated layer are held in place by a bioadhesive (long arrow). A thin opaque layer (arrowheads) occurs both between the IOL and AG and along the outer surface of the AG. Scale bar = 1 μm. **6.** Magnified view (TEM) of the bioadhesive between adjacent quartz grains illustrating the finely fibrous component (arrowheads). Conchoidal fracture is evident in the agglutinated quartz grain. Scale bar = 0.5 μm. **7.** Cross-section (TEM) illustrating the test wall between adjacent interior chambers. An IOL separates the lumen and cytoplasm of chambers from a central agglutinated layer. Scale bar = 2 μm. **8.** TEM of a section through the apertural tip of the terminal chamber wall illustrating a prominent, fibrous organic layer (arrowheads) that covers the outer surface of the chamber. Scale bar = 1 μm.

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