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Achradina pulchra, a Unique Dinoflagellate (Amphilothesales, Dinophyceae) with a Radiolarian-like Endoskeleton of Celestite (Strontium Sulfate)

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Abstract. We examined the planktonic dinoflagellate *Achradina pulchra* by light and scanning electron microscopies from the South and North Atlantic oceans. The basket-like skeleton has been interpreted as a thick cell covering or pellicle of organic composition, or as a siliceous endoskeleton. The skeleton of *Achradina* is known only from fresh material, being absent in preserved samples, sediments or the fossil record. X-ray microanalysis revealed that the endoskeleton of *Achradina* is composed of celestite (strontium sulfate) with traces of barite (barium sulfate), two minerals that readily dissolve after cell death. To date, Acantharia and polycystine radiolarians (Retaria) were the only known organisms with a skeleton of this composition. We can now add a dinoflagellate to the list of such mineralized skeletons, which influence on the biogeochemical fluxes of strontium and barium in the oceans. Moreover, we provided the first molecular data for a skeleton-bearing dinoflagellate. Molecular phylogeny based on the SSU rRNA gene sequences revealed that *Achradina* and several environmental clones branched as an independent lineage within the short-branching dinokaryotic dinoflagellates. To date, seven clades of dinokaryotic dinoflagellates are known living as symbionts in the endoplasm of Acantharia and polycystine radiolarians. Because celestite built skeletons were unknown outside radiolarians, we suggested that the ancestors of *Achradina* acquired the genes implicated in the deposition of strontium and barium from radiolarian hosts through a horizontal gene transfer event between microbial eukaryotes.

Key words: Acantharea, *Amphilothus*, barite, celestite skeleton, Dinoflagellata, endosymbiosis, gene transfer, plankton symbiosis, Retaria

INTRODUCTION

Dinoflagellates possess several remarkable ultrastructural and genetic features that distinguish them from other eukaryotes; such features include a very particular nucleus with permanently condensed chromatin (the dinokaryon) and a huge genome. Dinoflagellate

biology and genome evolution have been fundamentally influenced by the frequent transfer of individual genes and large-scale transfer of genes (Lin 2011, Wisecaver and Hackett 2011). Only a handful of dinoflagellate species with a mineral skeleton are known: *Actiniscus pentasterias*, *Dicroerisma psilonereia*, *Monaster*, *Amphilothus* and *Achradina* (Sournia 1986, Fensome *et al.* 1993). Among other features, the presence of an endoskeleton was used to suggest a phylogenetical relationship between radiolarians and dinoflagellates (Hollande *et al.* 1962). *Achradina pulchra*,

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known from warm seas, possesses a basket-like skeleton which composition has been matter of speculation for a century. Some authors have considered it as an exoskeleton of organic composition, and an opening or window in the skeleton has even been compared to an archeopyle, or excystment opening (Hernández-Becerril and Bravo-Sierra 2004). Other authors have considered the skeleton to be siliceous (Taylor 1987). However, it is striking that the skeleton of *Achradina* has never been reported from sediments and the fossil record. This suggested that the skeletons are not preserved after the cell's death, and are thus probably not composed of silica. In the present study, we solve the controversy over the composition of the skeleton with the aid of X-ray microanalysis. We provide the first molecular data from a dinoflagellate with a mineral skeleton, and propose a hypothesis for the evolutionary origin of the skeleton.

MATERIALS AND METHODS

Sampling and microscopy

Sampling was carried out in April 2013 in the São Sebastião Channel (23°50'4.05"S, 45°24'28.82"W, 40 m depth). Cells were collected using a phytoplankton net (20 µm mesh size) in surface waters. The living, concentrated samples were examined in Utermöhl chambers at a magnification of $\times 200$ with an inverted microscope [Diaphot-300 (Nikon Inc., Tokyo, Japan)], and photographed with a digital camera (Cyber-shot DSC-W300; Sony, Tokyo, Japan) mounted on the microscope's eyepiece.

Scanning electron microscopy (SEM) and X-ray energy dispersive spectroscopy (EDS)

The cells were obtained during a biogeochemical study of particulate organic matter in the water column over two seamounts in the northeastern Atlantic Ocean (Seine Seamount; 33°46'N, 14°21'W and Sedlo Seamount; 40°19'N, 26°40'W; see also Kiriakoulakis *et al.* 2009). We illustrated some of the cells from selected samples collected at 50 m depth in November 2003 (FS Meteor cruise #60/1), March 2004 (FS Poseidon cruise #309) and July 2004 (Discovery cruise #282). Suspended particulate material was collected on large (290 mm diameter) glass-fibre filters (GF/F, Whatman, Brentford, UK) using large-volume *in situ* pumps. The filters were initially stored at -80°C and then freeze-dried until further analysis in the lab could be carried out. Punched circles ($\sim 133\text{ mm}^2$) of selected filters were glued to stubs, sputter-coated with 60% gold/palladium and viewed using a scanning electron microscope (Philips XL30, Eindhoven, The Netherlands) equipped with an energy-dispersive X-ray spectrometer (Voyager, Noran Instruments, Middleton, WI, USA) operated at 15 kV. X-ray energy dispersive spectroscopy (EDS) was used for qualitative chemical analyses of the skeletons.

PCR amplification, cloning and DNA sequencing

Cells of *Achradina pulchra* for molecular analysis were collected from surface waters in the middle of the São Sebastião Channel in April 22, 2013. Each cell was micropipetted with a fine capillary into a clean chamber and washed several times in serial drops of 0.2 µm filtered and sterilized sea water. Finally, five cells were deposited in a 0.2 ml Eppendorf tube filled with several drops of absolute ethanol. The sample was kept at room temperature and in darkness until the molecular analysis could be performed. The sample was centrifuged for 5 min at 504 g. The ethanol was then evaporated in a vacuum desiccator and the cells were resuspended directly in 25 µl of GoTaq PCR buffer (Promega, Lyon, France). PCRs were done in a reaction mix containing the eukaryotic-specific SSU rDNA primers EK-42F (5'-CTCAARGAYTAAGCCATGCA-3') and EK-1520R (5'-CYGCAGGTTACCTAC-3') (López-García *et al.* 2001). PCRs were performed under the following conditions: 3 min denaturation at 95°C; then 45 cycles with denaturation at 95°C for 15 s, annealing at 50°C for 30 s and extension at 72°C for 2 min; final extension at 72°C for 7 min. A nested PCR was then carried out using 2 µL of the first PCR products in a GoTaq polymerase reaction mix containing the eukaryotic-specific primers EK-82F (5'-GAAACTGC-GAATGGCTC-3') and EK-1498R (5'-CACCTACGGAAACCTTGTTA-3') (López-García *et al.* 2001, Lara *et al.* 2011) and similar PCR conditions as described above, except for the annealing temperature of 55°C (instead of 50°C) and 40 cycles (instead of 45 cycles). Amplicons of the expected size (~ 1700 base pairs) were cloned using a PCR2.1 Topo TA Cloning system (Invitrogen, Life Technologies, Saint Aubin, France) following the instructions provided by the manufacturer. Cloned products were sequenced using primers situated on the vector. Amplicons of the expected size were fully sequenced using a BigDye197 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) and analysed with an ABI-3130XL DNA sequencer (Applied Biosystems).

Phylogenetic analyses

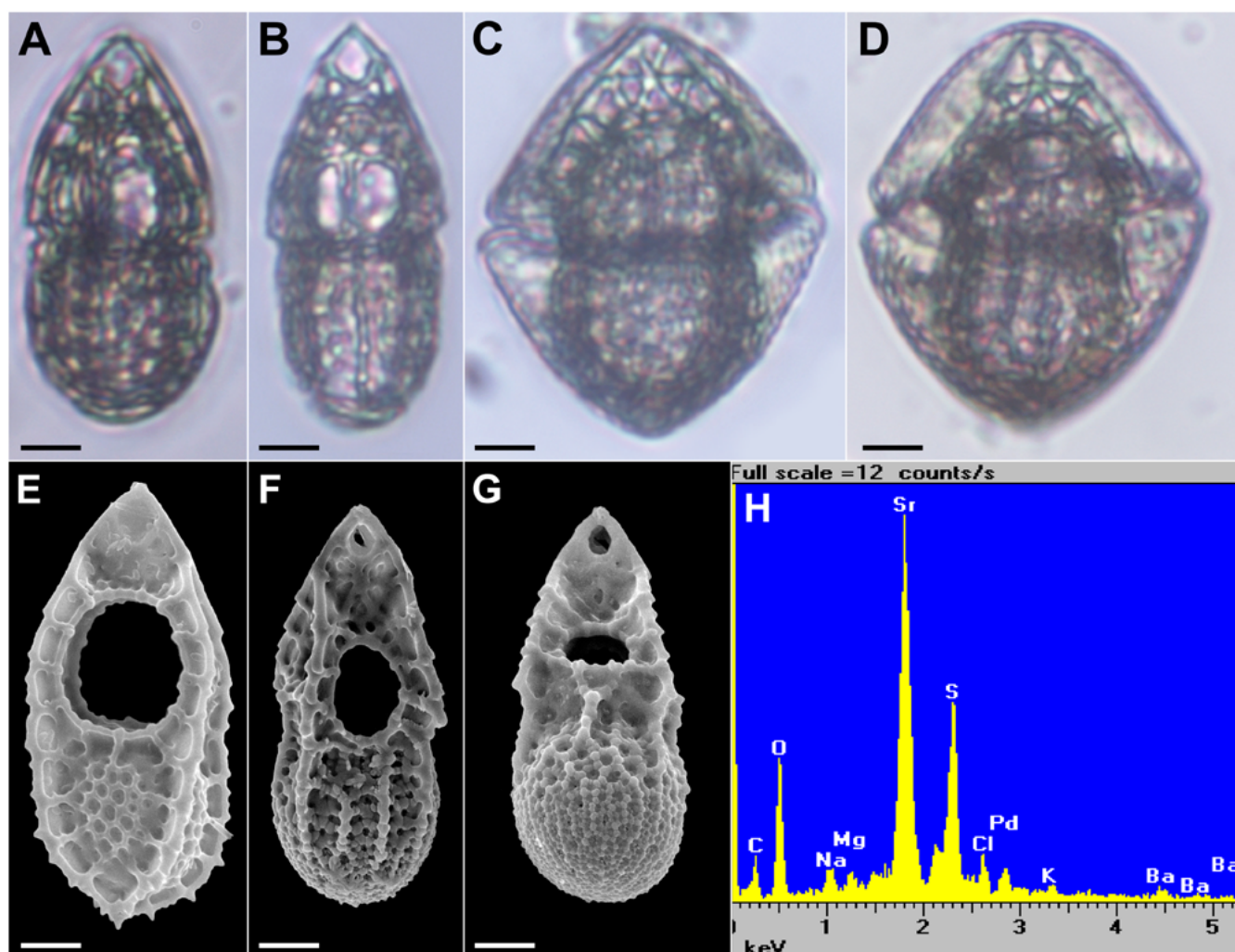
The three new SSU rDNA sequences were aligned to a multiple sequence alignment containing a diverse set of 58 dinokaryotic dinoflagellate sequences using the profile alignment option of MUSCLE 3.7 (Edgar 2004), with the aim of including species of all mutualist symbiotic dinokaryotes in polycystine radiolarians for which sequences were available (Gast and Caron 1996, 2001; Dolven *et al.* 2007, Yuasa *et al.* 2016). Decelle *et al.* (2012) reported sequences of dinokaryotic dinoflagellates isolated from an acantharian species, which branched into the clades of *Azadinium*, *Heterocapsa*, *Scrippsiella* and *Pelagodinium*. Their sequences were too short to be included in our alignment. However, these clades were presented with other complete sequences of these four genera. The sequences of the syndinean dinoflagellates *Hematodinium* (EF065717) and *Syndinium* (DQ146404), as well as one related environmental clone (KJ761049), were used as outgroups. The resulting alignment was manually inspected using the program BioEdit 7.1.11 (Hall 1999). Ambiguously aligned regions and gaps were excluded in phylogenetic analyses. A phylogenetic tree was constructed using a RAxML model where nodes were evaluated by rapid bootstrapping (Stamatakis *et al.* 2008) as computed in the CIPRES phylogenetic portal (Miller *et al.* 2010). The proportion of invariable sites was estimated. The newly-generated sequences were deposited in DDBJ/EMBL/GenBank under accession numbers MF543363–MF543365.

RESULTS

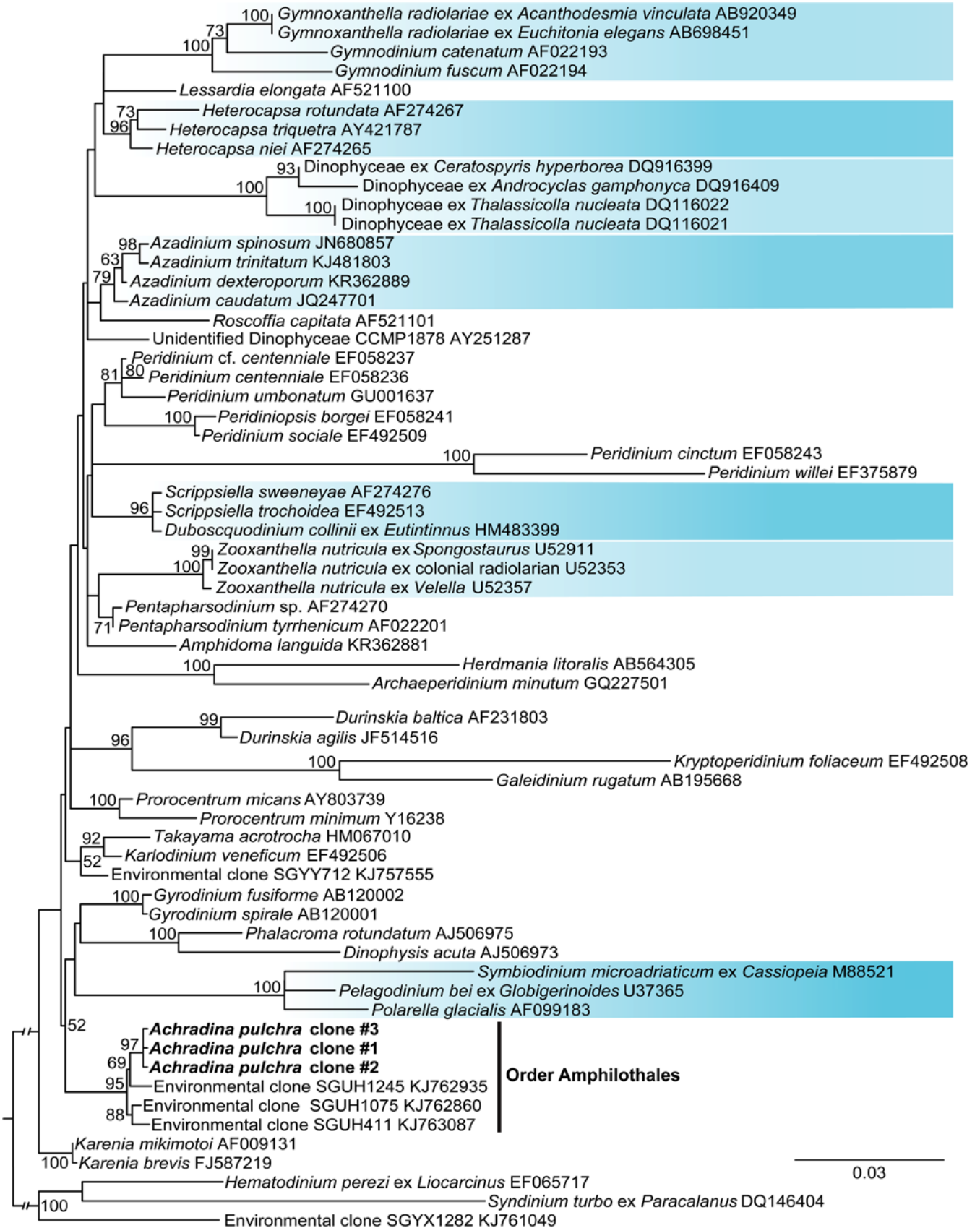
We obtained the SSU rDNA sequences of several clones from a sample with five cells of *Achradina pulchra* isolated on April 22, 2013 at São Sebastião Channel in Brazil (Figs 1A–B). The cell covering easily lysed due to the manipulation. The skeleton was an internal structure (Figs 1C–D). In the observations by scanning electron microscopy, the cells were devoid of cell covering and cytoplasm, and thus only the endoskeleton remained (Figs. 1E–G). The endoskeleton was a basket-like structure with a tessellation of irregular polygons. We examined the chemical composition of the endoskeleton using X-ray energy dispersive spectroscopy (EDS).

The X-ray EDS spectrum showed a strong peak of strontium, and two secondary peaks of sulfur and oxygen (Fig. 1H). There were several consecutive small peaks of barium. Other peaks corresponded to chloride, sodium, magnesium and potassium, major constituents of sea water salts. Consequently, the major composition of endoskeleton is a mineral composed of strontium, sulfur and oxygen, plus small fractions of barium (Fig. 1H). These elements corresponded to the composition of celestite (strontium sulfate, SrSO_4) with traces of barite (barium sulfate, BaSO_4).

We examined the phylogenetic position of *Achradina* with respect to a variety of dinoflagellate SSU rRNA gene sequences, with an especial focus on available



Figs 1A–H. *Achradina pulchra*. **A–B** – Light micrographs of the isolated cells of *Achradina pulchra* for PCR analysis from the SW Atlantic (São Sebastião Channel). **C–D** – Other cells from the same sample. Note that the skeleton is internal. **E–G** – Scanning electron micrographs of the skeleton from the NE Atlantic (Seine and Sedlo Seamounds). **H** – X-ray energy dispersive spectroscopy (EDS) spectrum of the endoskeleton. Scale bars: 5 μm .



sequences of symbiotic dinoflagellates and sequences of dinoflagellates retrieved from PCR amplifications of acantharian and polycystine radiolarian cells (Fig. 2). The clade formed by *Achradina* and three environmental clones is robustly supported (bootstrap value of 95%). It constituted a novel clade that branched within the large lineage comprising Gymnodiniales, Peridinales, Dinophysales and Prorocentrales but with poor support, making it difficult to infer its affinity with known dinoflagellates. The dinokaryotic dinoflagellates isolated from acantharian and polycystine radiolarian hosts branched into seven different clades, which were not whatsoever related to *Achradina* (Fig. 2).

DISCUSSION

To date, only spicules of Acantharia, central capsules of vegetative adults of polycystine radiolarians, and the membrane-bound vesicles of their bi-flagellated swimmers were known to be built with celestite and barite (Anderson *et al.* 1990). Like the skeleton-bearing dinoflagellates, acantharians, which are extremely abundant in the plankton, are absent from sediments and the fossil record because their skeletons rapidly dissolve upon cell death (Bernstein *et al.* 1987). Our study reveals that the Acantharia and the polycystine radiolarians are not the only organisms that control the fluxes of strontium and barium in the world's oceans.

There are two possible scenarios for the acquisition of a celestite skeleton by *Achradina*: (1) an ancestral trait that has been developed independently sometime in the evolution of dinoflagellates, and subsequently lost in all other lineages, and (2) a novel trait that has been acquired, most probably because of a symbiotic relationship with Radiozoa. The first hypothesis implies many function losses, and appears therefore most unlikely. Conversely, the physical proximity of Radiozoa and dinoflagellates may well have favored the second one. Indeed, since the earlier plankton studies, the Radiozoa (Acantharia and Polycystinea) were known to harbor microalgal symbionts, including dinoflagellates

(Anderson 2014). Few of these symbionts have been characterized by morphological and molecular methods (Probert *et al.* 2014, Yuasa *et al.* 2016). However, molecular surveys revealed a high diversity of these symbionts (Gast 2006; Gast and Caron 1996, 2001; Dolven *et al.* 2007). To date, seven clades of dinokaryotic dinoflagellates have been reported as symbionts of acantharians and polycystine radiolarians (Dolven *et al.* 2007, Decelle *et al.* 2012, Fig. 2). Undoubtedly more examples will appear in the future. Dinoflagellates have an extraordinary capability to acquire organelles and whole gene clusters from other groups of organisms, and these transfers are even more common when organisms develop a symbiotic relationship (Lin 2011, Wisecaver and Hackett 2011). We suggest that the ancestors of *Achradina* have developed a symbiotic relationship with some Radiozoan species, and subsequently acquired the genes necessary for Sr and Ba deposition.

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Fig. 2. Bayesian phylogenetic tree of dinoflagellate SSU rDNA sequences, based on 1,610 aligned positions. Names in bold represent sequences obtained in this study. The clades containing sequences of the symbionts of acantharians and polycystine radiolarians are highlighted in shaded boxes. Numbers at nodes are bootstrap values (values < 50 are omitted). The scale bar represents the number of substitutions for a unit branch length.

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