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Phylogenetic Revision of the Order Entactinaria—Paleozoic Relict Radiolaria (Rhizaria, SAR)

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Entactinaria, an order of Radiolaria, are defined by a specific skeletal structure called "initial spicular system (ISS)". The oldest entactinarians appeared in the Ordovician period, and the extant species are thought to have survived until today. However, the morphological observation revealed that the ISSs of entactinarian families are highly variable, and the validity of this characteristic is questionable. This is supported by the results of 18S and 28S rRNA molecular phylogenetic analysis that suggested the polyphyly of the four families analyzed in this study. Orosphaeridae, Rhizosphaeridae, Hexalonchidae and Hexastylidae should be excluded from the order Entactinaria. Considering the present results and the diversity in the ISS, it is also possible that "living entactinarians" are polyphyletic.

Key words: Cercozoa; Entactinaria; Rhizaria; Orosphaeridae; Phaeodaria; unicellular zooplankton.

Introduction

Radiolarians have great importance in the ecosystems and biogeochemical cycles in the ocean, and their accurate identification is indispensable for clarifying the structure of recent and paleomarine environments (Biard et al. 2016; Suzuki and Not 2015). The phylogenetic relationships within Radiolaria are being revealed by recent morphomolecular studies (Biard et al. 2015; Decelle et al. 2012; Ishitani et al. 2012), and the following five extant orders are widely accepted: Spumellaria, Nassellaria, Collodaria, Acantharia and Taxopodia (the last two orders are hardly preserved as microfossils). However, the classification of a large radiolarian order is still controversial. The order Entactinaria Kozur and Mostler, 1982 was initially established based on a Paleozoic family, Entactiniidae (Supplementary Material Fig. S1, Table S1), whose type species is *Entactinia herculea* Foreman, 1963. Typical entactinarians appeared in the Ordovician period (Noble et al. 2017), and descendant species are thought to still exist today

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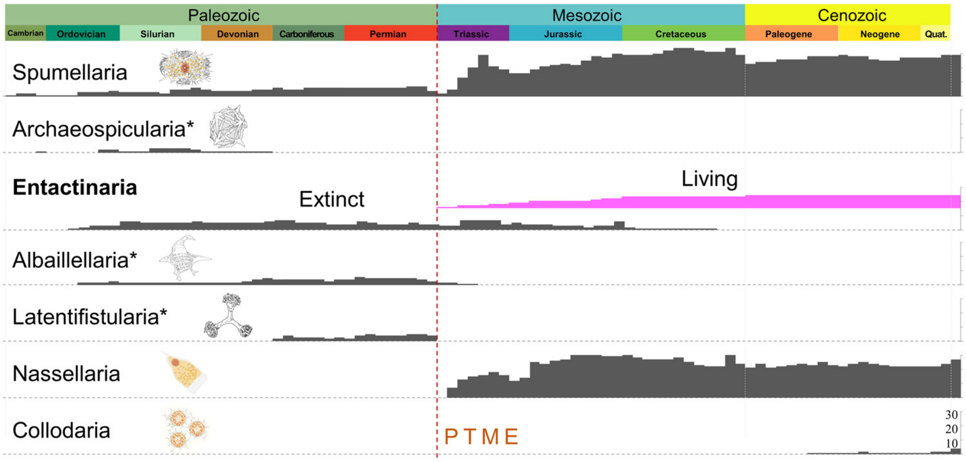

Figure 1. Geologic range of seven radiolarian orders which can be found as microfossils. The vertical axis indicates the number of families derived from a taxonomic database created for this study (data not shown). Note that the remaining two orders (Acantharia and Taxopodia) are not found as microfossils, and their ranges are not shown here.
*: extinct orders

("living entactinarians") (Fig. 1). The morphology of entactinarians was comprehensively reviewed by De Wever et al. (2001) who defined that this order commonly has the "initial spicular system" (ISS, hereafter), a complex of rods arising from the divergent center (median bar). This skeletal structure is hypothesized to be a conservative characteristic evolutionarily inherited from the Ordovician ancestors, generating the idea that entactinarians are survivors of the most severe extinction event in the Earth, the "Permian-Triassic Mass Extinction" (PTME, hereafter), where more than 90% of marine species disappeared (Benton 2003).

The hypothesis concerning the ISS, however, contains some points to be carefully considered. The ISSs of extinct and living entactinarian families are apparently highly variable (De Wever et al. 2001), so that the hypothesis should be revised. The classification of Entactinaria is one of the fundamental issues in radiolarian studies because it influences the taxonomy of Radiolaria from the Paleozoic to the present (Fig. 1) (Kozur and Mostler 2006; Matsuzaki et al. 2015). Indeed, several different classification systems are constructed because of discrepant opinions: 19 orders were proposed by Afanasieva and Amon (2003), whereas 9 orders (including extinct ones) were widely accepted (De Wever et al. 2001; Suzuki and Aita 2011).

As it is impossible to solve this problem only with fossil records, integrative studies on skeletal morphology and molecular phylogeny are indispensable. This study re-examines the evolutionary conservativeness of the ISS and provides essential information to disentangle the issue about the living entactinarians.

Results

Ecological and Morphological Characteristics

A total of 13 living entactinarians belonging to 5 families were obtained through our five-year investigation (Supplementary Material Table S2). In general, living entactinarians were rarely collected compared to other radiolarian orders. Rhizaria sp. was frequently collected in summer time in the area affected by Kuroshio warm current (Supplementary Material Fig. S2, Table S2), and this species was collected also in 2018 and 2019 in the same sea area (data not shown). We could not identify Rhizaria sp. by light microscopy, but its skeleton is composed of several curved spines extended from a center, and this morphological characteristic resembles that of *Thalassothamnus* (belonging to the order Entactinaria). It was possible that this species is a living entactinarian, and therefore, its skeleton and rDNA sequences were further analyzed in detail. *Orodendrum regale* was collected in two different sea areas far from each other (Stas. 5A, OS12102, and OS12103, Supplementary Material Fig. S2).

The detailed microscopic observation of skeletal morphology revealed that all the specimens have the ISSs (Fig. 2B, E, H, K, M). The transverse section of the skeleton was clearly different

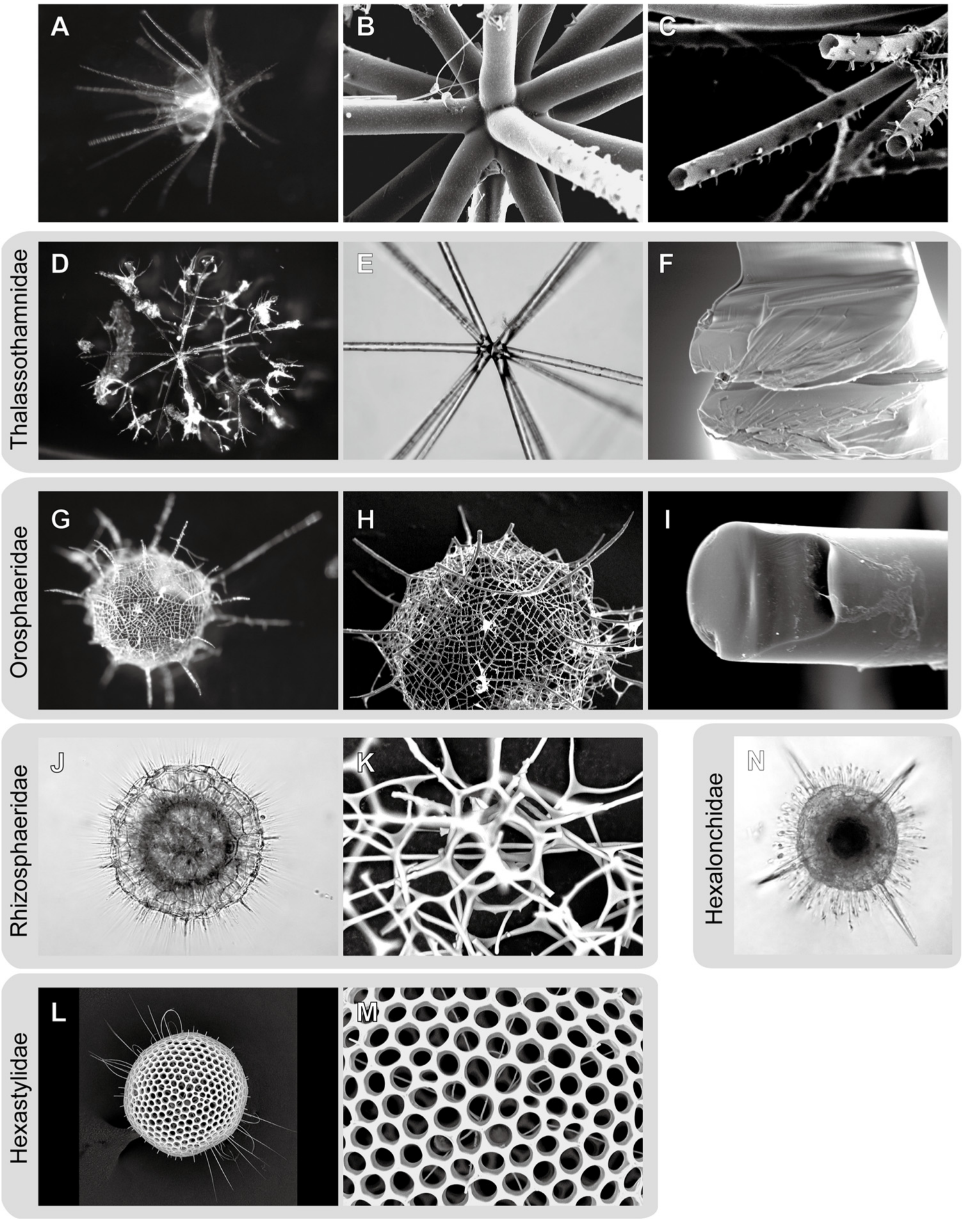

Figure 2. Light and electron microscopic images of living entactinarians: whole cell (A, D, G and I), "initial spicular system" (B, E, H, J and L) and the transverse section of the skeleton (C and F). **A–C**: Rhizaria sp., **D–F**: *Cytocladus tricladus* (Thalassothamnidae), **G–I**: *Orodendrum regale* (Orosphaeridae), **J–K**: *Haliommilla capillacea* (Rhizosphaeridae), **L–M**: *Hollandosphaera hexagonia* (Hexastylidae), N: *Hexacontium* sp. 1 (Hexalonchidae).

among the species: the skeleton of Rhizaria sp. is tubular (Fig. 2C), whereas those of *Cytocladus tricladus* (belonging to Thalassothamnidae) and other species do not have any space inside of the skeleton (Fig. 2F, I). The documentary survey also revealed that the ISSs of entactinarian families show high variability (Supplementary Material Fig. S3), and some living families have ISSs largely different from those of Paleozoic families (e.g., no. 1a–e vs. no. 31).

Molecular Phylogeny

The specimens morphologically identified to belong to the order Entactinaria (Fig. 2; Supplementary Material Table S2) did not form a single clade in the phylogenetic trees, whereas all the other liv-

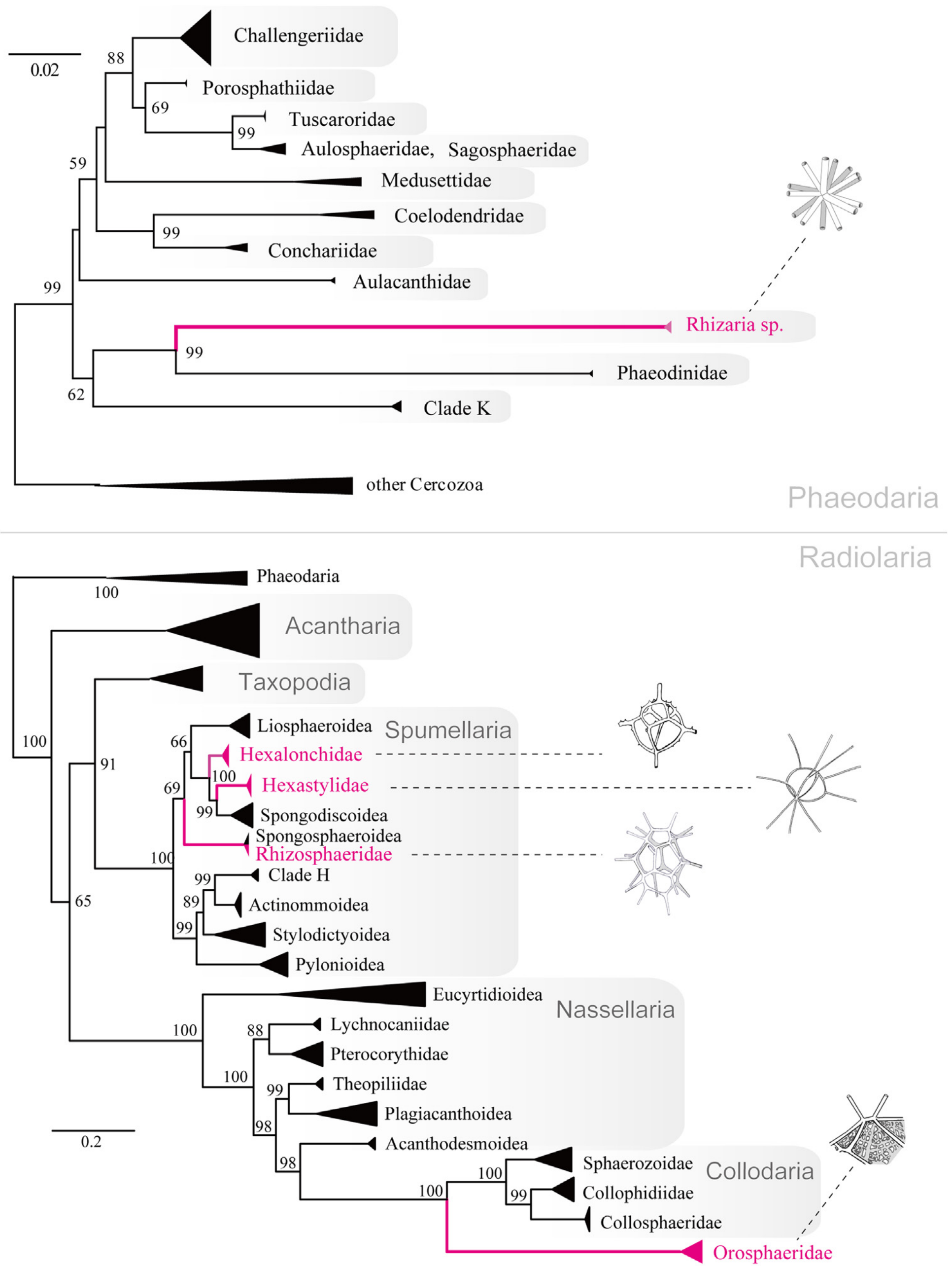

Figure 3. Maximum-likelihood (ML) phylogenetic trees of Phaeodaria and Radiolaria including entactinarian-like rhizarians. The phaeodarian tree was constructed based on 18S rDNA, and the radiolarian tree was derived from concatenated alignments of 18S and 28S rDNA. Numbers at nodes indicate 1,000 bootstrap support values of the ML method. Only bootstrap values higher than 50% are shown. Entactinarians were shown in pink and with the initial specular systems (schematic illustration). Clade K in the phaeodarian tree is referred from Nakamura et al. (2015).

ing radiolarian orders (i.e., Acantharia, Taxopodia, Spumellaria, Nassellaria and Collodaria) formed monophyletic or paraphyletic clades (Fig. 3). The living entactinarians appeared in different clades of Rhizaria: within the phaeodarian clade (Rhizaria sp.), inside of the spumellarian clade (Hexalonchidae, Hexastylidae and Rhizosphaeridae) and next to the collodarian clade (Orosphaeridae) (Fig. 3). Unfortunately, the DNA sequence of *C. tricladus* (Fig. 2D–F) could not be obtained. The DNA of this specimen was probably decomposed before the sampling.

Discussion

Possible Polyphyly of Living Entactinaria

Molecular studies suggest that the morphology of each radiolarian species well reflects family- and genus-level phylogeny (e.g., Ishitani et al. 2012), and consequently each living entactinarian analyzed in this study can be treated as the representative of each family. Our observation of skeleton (Fig. 2) and documentary survey (Supplementary Material Fig. S3) revealed that the ISSs of entactinarian families are highly variable. The validity of this characteristic is therefore questionable, and the monophyly based on morphological characteristics is doubtful for the order Entactinaria. This is supported by the results of the molecular phylogenetic analysis, which supported the polyphyly of analyzed specimens (Fig. 3). The ISSs of analyzed species are clearly different from those of Entactiniidae (Supplementary Material Fig. S3), and therefore, the four families analyzed in this study (Orosphaeridae, Rhizosphaeridae, Hexalonchidae and Hexastylidae) (Supplementary Material Figs S1, S3) should be excluded from the order Entactinaria.

Considering the present results and the diversity in the ISS (Supplementary Material Fig. S3), it is also possible that “living entactinarians” are polyphyletic, and some of them are perhaps members of other orders. Despite our efforts in field sampling, the rest of the living families (Axoprunidae, Excentroconchidae, Quinquecapsulariidae, Saturnalidae and Centrocubidae)(Supplementary Material Figs S1, S3) could unfortunately not be collected in this study. Future studies should examine the morphology and DNA sequences of these families to further clarify the radiolarian phylogeny.

Classification of Living Entactinaria

The family Thalassothamnidae was defined by “a large initial spicule with spines divergent from an almost indistinct median bar”, and this characteristic apparently correspond to that of Rhizaria sp. The ISSs of this species was, however, tubular (porous) (Fig. 2A–C), while radiolarians have generally no large spaces within their skeletons (Bernstein et al. 1990; Nakamura et al. 2018; Takahashi et al. 1983; Takahashi and Hurd 2007). Considering the skeletal structure and the position in the phylogenetic tree, Rhizaria sp. presumably belongs to the family Astracanthidae Haecker, 1906 of Phaeodaria. Since the morphology of this species does not totally correspond to those of hitherto-described species, this species is possibly an undescribed one.

Cytocladus tricladus, classified in the family Thalassothamnidae, would belong to Radiolaria judging from its solid transverse section (Fig. 2F) and the absence of phaeodarian central capsule, which is an important cytological characteristic of Phaeodaria (Nakamura and Suzuki 2015). Before the analysis, the body of this species was covered with the spherical extracapsular cytoplasm, which is a characteristic of the order Collodaria. The spicule-combined skeleton of this species (Fig. 2D–E) is also similar to those of some collodarians (e.g., Sphaerozoidae), and consequently, *C. tricladus* is possibly related to Collodaria. Indeed, some studies regarded the genus *Cytocladus* as a member of Collodaria (Cachon and Cachon 1985; Petrushevskaya 1984).

Despite our five-year efforts in sampling, only three specimens were collected for the family Orosphaeridae (Supplementary Material Table S2), suggesting that abundance of this family is relatively low compared with other abundant Radiolaria (e.g., Acantharia and Collodaria, Biard et al. 2016). *Orodendrum regale* possibly has scattered but wide distribution, considering the fact that specimens were collected in areas geographically separated from each other (Supplementary Material Table S2).

The family Orosphaeridae has a spicular system on the surface (partially in the inside) of its skeleton (Fig. 2H), and this structure apparently corresponds to the definition of the entactinarian ISS (Supplementary Material Fig. S3). The homology in spicules among Orosphaeridae and Entactiniidae is, however, not phylogenetically supported, and orosphaerids should be excluded from the order Entactinaria. Our molecular analysis suggests that orosphaerids are closely related to Collodaria, and this result corresponds to the classification of some previous studies, in which orosphaerids were treated as members of Collodaria (Haecker 1906, 1908; Petrushevskaya 1984). Yet, the morphology of these two groups is remarkably different: Orosphaeridae has fully-covered shell (Fig. 2G), while the soft part is partly or not covered with the skeleton in Collodaria. The living orosphaerids analyzed in this study were solitary and distributed in deep waters (below 200 m, data not shown), unlike collodarians which can be colonial and generally appear in shallow waters. No algal symbionts were observed in our orosphaerid specimens, whereas collodarians are often associated with symbiotic algae (Biard et al. 2015; Suzuki and Not 2015; Zhang et al. 2018). Considering the morphological and ecological difference among these two groups, Orosphaeridae can be categorized as a single order separated from Collodaria.

The molecular analysis in this study indicates that the families Hexalonchidae, Hexastylidae and Rhizosphaeridae are members of Spumellaria (Fig. 3), and their ISSs could not be a synapomorphy. Hexalonchidae and Hexastylidae (as well as Spongodiscoidea) could be classified into the same superfamily. It was already suggested that Hexalonchidae is a member of Spumellaria by a molecular study (Yuasa et al. 2009). Hexalonchidae and Hexastylidae were moved to Spumellaria on the basis of skeletal structure (Matsuzaki et al. 2015), and this opinion was supported by the present study.

Our study suggests that Radiolaria in today's ocean contain only five orders: Acantharia, Taxopodia, Spumellaria, Nassellaria and Collodaria. However, it is also possible that the family Orosphaeridae becomes classified as a single order separated from Collodaria in future.

Methods

Sampling and treatment: Plankton sampling was conducted in 2012–2016 at 10 stations located in 5 sea areas of the Northern Hemisphere: the western and eastern North Pacific Ocean, the East China Sea, the Philippine Sea and the Mediterranean Sea (Supplementary Material Fig. S2, Table S2). After the sampling, all of rhizarians which have entactinarian-like morphologies were manually picked up from the samples under a stereomicroscope or inverted microscope. Isolated individuals were put into wells of cell culture plates and incubated for 1–4 hours. The individuals were then photographed with a digital camera (e.g., Nikon 1 V3, Nikon, Japan) attached on the inverted microscope (e.g., TMS, Nikon, Japan) and individually preserved in tubes filled with approximately 2.0 mL of 99.9% ethanol. The tubes containing the specimens were stored at 4 °C.

Morphological observation and documentary survey: After the morphological observation by an inverted microscope, most of the specimens were coated with platinum and carefully observed with a Scanning Electron Microscope (SEM) (JSM-6390LV with LaB6 gun, JEOL, Japan) to examine their skeletal morphology in detail. The ISS of each specimen was photographed and illustrated. The conditions and parameters were the same as those in Nakamura et al. (2016) and Sandin et al. (2019). The information concerning the ISS was carefully extracted by checking all descriptions of the entactinarian families. The geologic range of each family was thoroughly reviewed, and their ISSs were illustrated.

Molecular analysis: The DNA extraction, amplification and sequencing were conducted according to Nakamura et al. (2015) for Rhizaria sp., *Cytocladus tricladus* and Orosphaeridae, and to Sandin et al. (2019) for other families. A Maximun Likelihood (ML) phylogenetic tree was reconstructed with 1,000 bootstrap replicates (Felsenstein 1985) for the obtained sequences and other rhizarian sequences registered in the GenBank database (Supplementary Material Table S3). A ML tree was constructed based on the 18S rDNA sequences of Phaeodaria. For Radiolaria, the ML tree was derived from concatenated alignments of 18S and 28S rDNA.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors.

Conflict of interest

The authors declare that they have no conflict of interests.

CRediT authorship contribution statement

Yasuhide Nakamura: Conceptualization, Investigation, Resources, Formal analysis, Methodology, Data curation, Writing - original draft, Writing - review & editing. **Miguel M. Sandin:** Investigation, Resources, Formal analysis, Data curation. **Noritoshi Suzuki:** Supervision. **Akihiro Tuji:** Formal analysis, Supervision. **Fabrice Not:** Investigation, Methodology, Supervision.

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Appendix A. Supplementary Data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.protis.2019.125712.

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