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Molecular Phylogeny of the Widely Distributed Marine Protists, Phaeodaria (Rhizaria, Cercozoa)

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Phaeodarians are a group of widely distributed marine cercozoans. These plankton organisms can exhibit a large biomass in the environment and are supposed to play an important role in marine ecosystems and in material cycles in the ocean. Accurate knowledge of phaeodarian classification is thus necessary to better understand marine biology, however, phylogenetic information on Phaeodaria is limited. The present study analyzed 18S rDNA sequences encompassing all existing phaeodarian orders, to clarify their phylogenetic relationships and improve their taxonomic classification. The monophyly of Phaeodaria was confirmed and strongly supported by phylogenetic analysis with a larger data set than in previous studies. The phaeodarian clade contained 11 subclades which generally did not correspond to the families and orders of the current classification system. Two families (Challengeriidae and Aulosphaeridae) and two orders (Phaeogromida and Phaeocalpida) are possibly polyphyletic or paraphyletic, and consequently the classification needs to be revised at both the family and order levels by integrative taxonomy approaches. Two morphological criteria, 1) the scleracoma type and 2) its surface structure, could be useful markers at the family level.

Key words: Phaeodaria; Cercozoa; Rhizaria; 18S rDNA; single-cell PCR.

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Introduction

Phaeodarians are marine unicellular protists. This group is defined by 1) the presence of a "central capsule" containing one or several nuclei, 2) a mass of brown aggregated particles named "phaeodium", and 3) a hollow siliceous skeleton called "scleracoma", in most species (Howe et al. 2011; Nakamura and Suzuki 2015; Takahashi and Anderson 2000). The size of the scleracoma varies with the different families and ranges from several hundred micrometers (e.g. Challengeriidae) to a few millimeters (e.g. Tuscaroridae). The type of the scleracoma and its surface structure are important taxonomic characters to classify these organisms at the order and family levels. Phaeodarians have long been considered as a member of Radiolaria but based on molecular analyses (Nikolaev et al. 2004; Polet et al. 2004; Sierra et al. 2013), they are now classified as a subclass in the class Thecofilosea of the phylum Cercozoa (Adl et al. 2012; Howe et al. 2011).

Phaeodarians are heterotrophic plankton organisms, which are distributed in the world ocean from the surface to the deep sea (Nakamura and Suzuki 2015). Despite their wide range of distribution, this plankton group has attracted little attention from marine biologists (Nakamura et al. 2013). Their abundance has been very likely underestimated, because their scleracoma is fragile and breaks easily when samples are collected by plankton nets. Yet, careful examination of the zooplankton community revealed that these protists can be numerous and their high abundance has occasionally been reported. For instance, the vertical flux of phaeodarians was higher than that of polycystine radiolarians in the Panama Basin (Takahashi and Honjo 1983) and in the California coast (Gowing and Coale 1989). The combined biomass of the Aulosphaeridae, Sagosphaeridae, Aulacanthidae and Coelodendridae was estimated to contribute 2.7–13.7% of the total metazoan biomass in the 150–1000 m layer of the western North Pacific (Steinberg et al. 2008). The proportion of an Aulacanthidae species, *Aulographis japonica*, with respect to the total zooplankton biomass is 22.3% in 250–3000 m layer in the Sea of Japan, and this percentage is the second largest, following that of copepods (Nakamura et al. 2013). Considering that they possess siliceous skeletons and occur occasionally at high biomass, this plankton group can play an important role in ecosystems locally and have a significant impact on the silica cycle of the ocean. Their importance in the carbon cycle is also reported from the Northeast Atlantic (Lampitt et al. 2009). Cercozoans, including phaeodarians, have recently been considered important players in the material cycles and food webs of the ocean (Howe et al. 2011). Research on environmental DNA revealed that Cercozoa represented a high percentage of the 18S rDNA sequences obtained from sea-floor sediments of the Arctic and the Southern Ocean (Pawlowski et al. 2011). Consequently, improving our knowledge on phaeodarians will be important for the entire field of marine biology. Accurate knowledge of phaeodarian classification is critical to better assess their genetic diversity and ecology. In the comprehensive systematics framework established by Haeckel (1887), phaeodarian species were grouped into families and orders according to their morphological similarities. Since then, some authors amended this classification, but the relationship between the families remains uncertain (Cachon and Cachon 1985; Campbell 1954; Kling and Boltovskoy 1999; Takahashi and Anderson 2000) and has never been examined from the molecular point of view. Comprehensive molecular studies on phaeodarians are difficult to conduct. They cannot be cultured, and therefore their DNA has to be extracted from individual specimens collected and sorted from the environment. Some phaeodarian species, however, live in the deep sea, and it is hard to obtain specimens. In addition, efficient phaeodarian-specific primers do not exist, increasing the risks of amplifying and sequencing contaminating organisms attached to or ingested by the Phaeodaria.

Yet, an integrative taxonomy approach merging both morphological and molecular information has recently been applied successively to other unicellular and shell-bearing rhizarian taxa such as Polycystines, Acantharia and Foraminifera (e.g. Decelle et al. 2012; Kunitomo et al. 2006; Pawlowski et al. 2013). Such an approach, highlighting match and mismatch between morphology and phylogenetic analysis, contributed to an overall better understanding of the classification and evolutionary patterns among these groups.

The information on phaeodarian 18S ribosomal DNA currently exists for only 7 out of ca. 200 species, 18 families and 7 orders recognized in the latest phaeodarian classification (Nakamura and Suzuki 2015; Takahashi and Anderson 2000), and discrepancies between morphological classification and analysis of these partial 18S rDNA were pointed out (Nakamura et al. 2013). Here, we designed new phaeodarian-specific primers and analyzed the whole 18S rDNA sequence

of extant single-cell specimens of phaeodarian species, covering all orders recognized in the current classification.

Results

A total of 39 phaeodarian specimens belonging to 26 distinct taxa (essentially distinct species) were collected from 0 m to 3 000 m depth, and their sequences were obtained by single-cell PCR. Detailed information and micrographs of analyzed specimens are shown in Table 1 and Figure 1, respectively.

The sequences from all phaeodarian specimens formed a single clade far separated from other Thecofilosea taxa (Cryomonadida, Ebriacea and *Pseudodifflugia*), and the monophyly of this clade was strongly supported by bootstrap values and BPP (Fig. 2). The phaeodarian clade consisted of

Table 1. List of phaeodarian sequences used for the phylogenetic analysis in this study. the specimens are coded according to the sampling station: the wesern North Pacific (WNP); the eastern north Pacific (ENP); the East China Sea (ECS); the Sea of Japan (SJ); the Mediterranean Sea (MS).

Taxon	Accession no.	Specimen name	Sampling station	Depth range (m)	Sampling date
Protocystis vicina	AB998884	WNP1	WN1	250–500	Aug. 2013
Protocystis vicina	AB998885	WNP2	WN1	250–500	Aug. 2013
Challengeriidae sp. 1	AB998886	WNP3	WN2	100–150	Aug. 2013
Porospathis holostoma	AB998887	WNP4	WN2	150–250	Aug. 2013
Challengeriidae sp. 2	AB998888	WNP5	WN2	500–750	Aug. 2013
Challengeron bethelli	AB998889	WNP6	WN2	500–750	Aug. 2013
Protocystis thomsoni	AB998890	WNP7	WN2	500–750	Aug. 2013
Challengeron tizardi	AB998891	WNP8	WN2	750–1000	Aug. 2013
Challengeron tizardi	AB998892	WNP9	WN2	1500–2000	Aug. 2013
Challengeron tizardi	AB998893	WNP10	WN2	1500–2000	Aug. 2013
Entocannula infundibulum	AB998894	WNP11	WN2	1500–2000	Aug. 2013
Porospathis holostoma	AB998895	WNP12	WN2	1500–2000	Aug. 2013
Protocystis harstoni	AB998896	WNP13	WN2	1500–2000	Aug. 2013
Protocystis murrayi	AB998897	WNP14	WN2	1500–2000	Aug. 2013
Tuscaretta belknapi	AB998898	ENP1	EN1	0–300	Aug. 2012
Coelodendrum furcatissimum	AB998899	ENP2	EN2	0–1000	Aug. 2012
Sagoscena sp. 1	AB998900	ENP3	EN2	0–1000	Aug. 2012
Tuscaretta belknapi	AB998901	ENP4	EN3	0–1000	July 2012
Tuscaretta sp. 1	AB998902	ENP5	EN4	0–1000	July 2012
Auloceros arborescens	AB998903	ENP6	EN5	0–1000	July 2012
Coelodendrum furcatissimum	AB998904	ENP7	EN5	0–1000	July 2012
Conchariidae sp. 1	AB998905	ECS1	E1	0–150	May 2013
Challengeron radians	AB998906	ECS2	E2	200–500	May 2013
Conchariidae sp. 2	AB998907	ECS3	E2	200–500	May 2013
Challengeron diodon	AB998908	ECS4	E3	650–800	May 2012
Challengeron radians	AB998909	ECS5	E3	650–800	May 2012
Challengeron willemoesii	AB998910	ECS6	E3	650–800	May 2012
Challengeron willemoesii	AB998911	ECS7	E3	650–800	May 2012
Protocystis xiphodon	AB998912	ECS8	E4	0–150	May 2012
Protocystis xiphodon	AB998913	ECS9	E4	0–150	May 2012
Aulographis japonica	AB998914	SJ1	S1	250–750	Apr. 2013
Auloscena sp. 1	AB998915	SJ2	S1	250–750	Apr. 2013
Protocystis vicina	AB998916	SJ3	S1	250–750	Apr. 2013
Aulographis japonica	AB998917	SJ4	S2	250–750	Apr. 2012
Auloscena sp. 1	AB998918	SJ5	S3	500–750	Mar. 2013
Aulacantha scolymantha	AB998919	MS1	M1	0–40	Nov. 2012
Medusetta parthenopaea	AB998920	MS2	M1	0–40	Nov. 2012
Phaeodina sp. 1	AB998921	MS3	M1	0–40	Nov. 2012
Phaeodina sp. 1	AB998922	MS4	M1	0–40	Nov. 2012

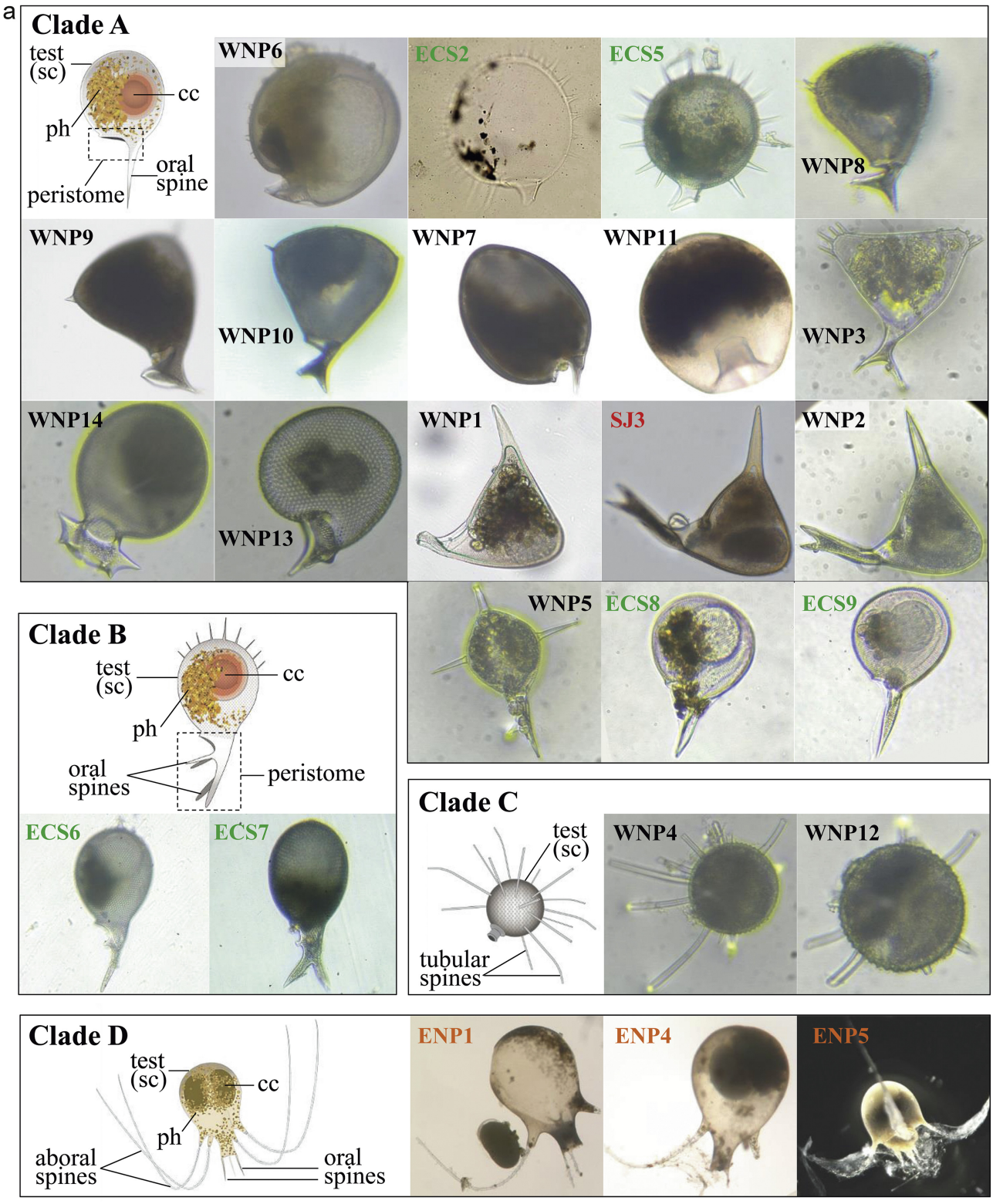


Figure 1. Light micrographs of phaeodarian specimens analyzed in this study. The information on each specimen is presented in Table 1. cc: central capsule; ph: phaeodium; sc: scleracoma.

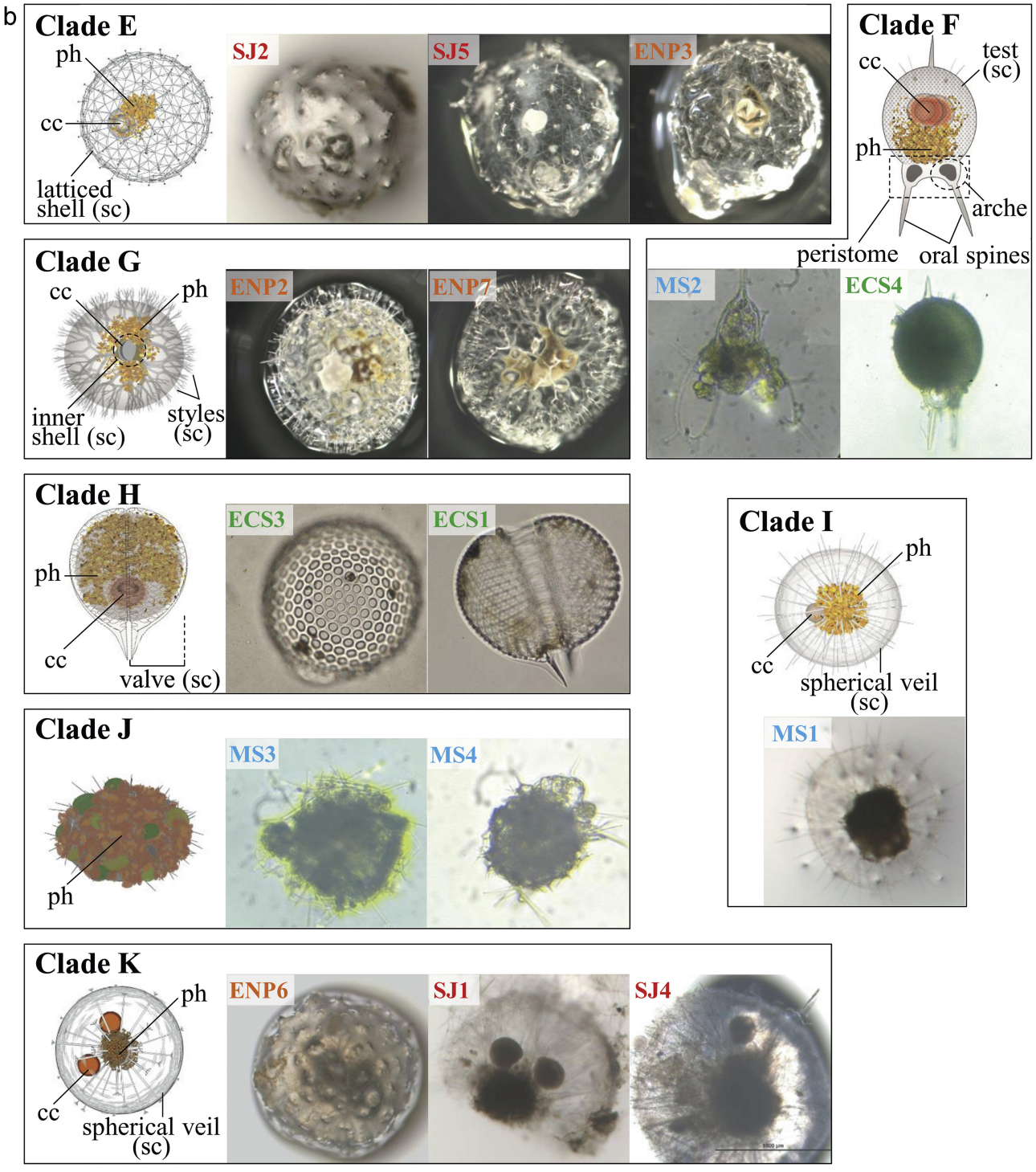


Figure 1. (*Continued*)

11 subclades (A–K) defined based on support values and morphological features (Fig. 3). Subclade A contains the highest number of individuals (n = 18). Subclades A, E, F, G, H and K were composed of sequences from distinct sampling locations. For instance, subclade E contains the specimens from the Mediterranean Sea (MS), the Sea of Japan (SJ) and the eastern North Pacific (ENP).

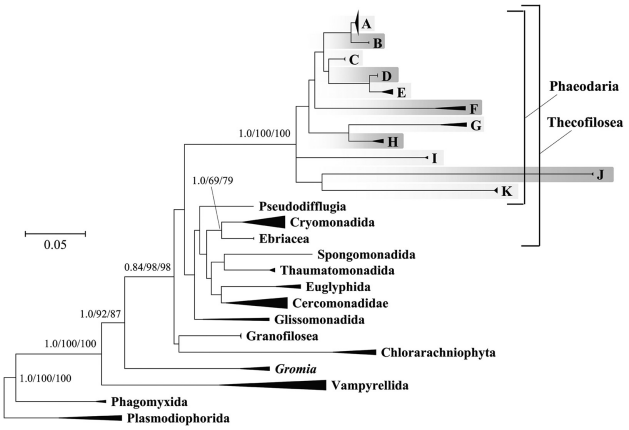


Figure 2. Phylogenetic tree of phaeodarians and other cercozoans based on 18S rDNA alignments and maximum-likelihood (ML) method. Note that the phaeodarian clade is presented with 11 subclades (A–K) detected according to the support values and morphological features. The details of this clade are shown in Figure 3. Numbers at nodes indicate Bayesian posterior probabilities (BPP) and bootstrap support values of neighbor-joining (NJ) and ML methods (BPP/NJ/ML). Only values higher than 0.5 pp and 50% are shown.

The 11 subclades and the families classified by Takahashi and Anderson (2000) generally did not match well, and disagreement was seen for the families Aulosphaeridae, Sagosphaeridae, Challengeriidae and Aulacanthidae (Fig. 3). *Sagoscena* sp. 1 (ENP3) of Sagosphaeridae formed the subclade E along with two Aulosphaeridae species, being nested one into each other. Challengeriidae and Aulacanthidae were scattered across the phylogenetic tree. Most of the challengeriid phaeodarians appeared in subclade A, but two specimens of *Challengeron willemoesii* Haeckel (ECS6 and ECS7) formed the subclade B clearly distinct from subclade A with high support values (1.0/100/100, Fig. 3). The sequences of *C. diodon* (Haeckel) (ECS4 and NA (AB218765)) belonged to subclade H, distant from subclade A, together with the *Medusetta parthenopaea* Borgert (MS2) of Medusettidae (Fig. 3). Two Aulacanthidae species, *Aulographis japonica* Nakamura, Tuji and Suzuki (SJ1, SJ4 and SJ (AB820365)) and *Auloceros arborescens* Haeckel (ENP6) formed a single subclade K, whereas *Aulacantha scolymantha* Haeckel (MS1 and MS (AY266294)), which also belongs to Aulacanthidae, composed subclade I whose exact position compared to other subclades is unclear.

The orders of the current classification did not correspond to the phylogenetic analysis overall (Fig. 3). The order Phaeogromida was scattered across subclades A, B and F, and the order Phaeocystida has representatives in subclades I and K. The order Phaeosphaerida, composed of two families Sagosphaeridae and Aulosphaeridae (subclade E), turned out to be nested within the order Phaeocalpida (subclades C and D).

Discussion

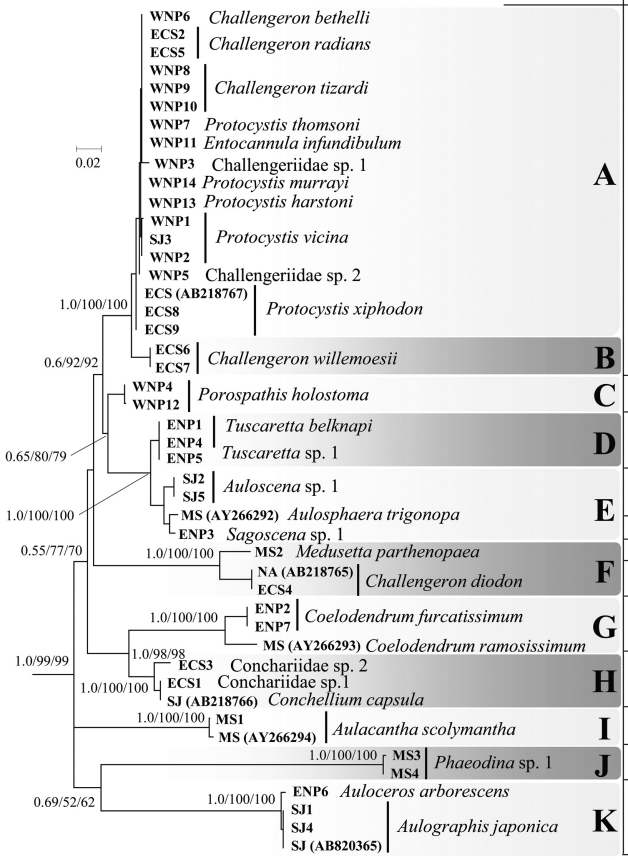
Comparison Between the Phylogenetic Tree and the Current Classification

Previous studies analyzed partial 18S rDNA of phaeodarian species (n=6) belonging to 5 distinct families and orders, and reported that their sequences formed a monophyletic clade outside of Radiolaria but within cercozoans (Polet et al. 2004; Yuasa et al. 2006). In this study, the monophyly of Phaeodaria within Cercozoa is further confirmed based on a larger data set, including 29 distinct species distributed over all 7 orders. Phaeodarians are now classified as a member of the class Thecofilosea, together with Cryomonadida, Ebriacea and *Pseudodifflugia* (Adl et al. 2012; Howe et al. 2011). However, these Thecofilosea taxa did not form a monophyletic group in the 18S rDNA tree (Fig. 2), and their phylogenetic relationship could not be confirmed in the present study. For better clarifying the phylogenetic relationship between phaeodarians and other cercozoans, it would be necessary to add more taxa whose 18S rDNA sequences are not yet available (e.g. Ventricleftida).

The 11 subclades in the 18S rDNA phylogenetic reconstruction did not well correspond to the families and the orders in the current classification system (Fig. 3). The family Challengeriidae is possibly a polyphyletic group, and the family Aulosphaeridae would be a paraphyletic group. As for ordinal level categorization, Phaeogromida turned out to be polyphyletic. The branching pattern of subclades C, D and E suggests that the order Phaeocalpida is a paraphyletic group. Consequently, the current classification system is not supported by the 18S rDNA phylogeny and needs to be reconsidered.

Comparison Between the Phylogenetic Tree and the Morphological Characters

Previous studies classified phaeodarian families according to morphological characters, especially based on 1) the scleracoma type and 2) the surface structure of the test (Cachon and Cachon



Clade	Family	Order	Scleracoma type	Surface of test
A	Challengeriidae	Phaeogromida	cape-shaped or ovate test usually with spines	smooth or with regularly arranged pores
B				
C	Porospathidae	Phaeocalpida	spherical test with tubular spines	trizonal meshwork
D	Tuscaroridae		test with long oral feet	smooth
E	Aulosphaeridae	Phaeosphaerida	lattice-shell	—
	Sagosphaeridae			
F	Medusettidae	Phaeogromida	campanulate test with oral arches	dimpled or alveolated
	Challengeriidae			
G	Coelodendridae	Phaeodendrida	inner shell and styles	—
H	Conchariidae	Phaeoconchida	test composed of two valves	large-pored
I	Aulacanthidae	Phaeocystida	spherical veil	—
J	Phaeodinidae	Phaeogymnocellida	—	—
K	Aulacanthidae	Phaeocystida	spherical veil	—

Figure 3. The phaeodarian clade in the phylogenetic tree inferred by maximum-likelihood (ML) method, based on 46 sequences of 18S rDNA. The outgroups are shown in Figure 2. Note that the 11 subclades (A–K) are detected based on the support values and morphological features. Each specimen is coded according to its sampling station as shown in Table 1, and species retrieved from NCBI database are presented with their accession numbers. Numbers at nodes indicate Bayesian posterior probabilities (BPP) and bootstrap support values of neighbor-joining (NJ) and ML method (BPP/NJ/ML). Only values higher than 0.85 pp and 50% are shown. The families and orders are based on Takahashi and Anderson (2000).

1985; Campbell 1954; Haeckel 1887; Kling and Boltovskoy 1999; Takahashi and Anderson 2000). The 11 subclades were generally congruent with these two morphological criteria in this study (Fig. 3). The scleracoma of subclades A, B, C, D, F and H formed relatively firm "test", but displaying distinction between each other: cape-shaped or ovate test possessing smooth or small-pored surface (A and B), spherical test with "tubular spines" and the surface of "trizonal meshwork" (C), test of smooth surface with short "oral spines" and long "aboral spines" (D), campanulate test possessing developed "peristome" with "arches" (F), and test composed of two "valves" with large-pored surface (H). The scleracoma of subclade E corresponds to a sphere of geometric meshwork called "lattice-shell". The phaeodarians of subclade G possess scleracoma composed of "inner shell" and numerous branch-like structure called "styles". The specimens included in subclades I and K have scleracoma called "spherical veil". Specimens belonging to subclade J do not possess scleracoma (Figs 1, 3).

Thus, the scleracoma type and the surface structure of the test would be valuable characters for the family-level classification. Future studies should rearrange phaeodarian families and orders according to these two criteria and the branching pattern of the phylogenetic tree. The combination of both detailed morphological examination and molecular phylogeny helped identifying new characters for the taxonomic classification of Phaeodaria. Such an

integrative approach has already been proven valuable for a number of taxa, including some among Rhizaria (e.g. Acantharia, Decelle et al. 2012).

Taxonomical Consideration on the Families Challengeriidae and Medusettidae

The subclade A contains 18 specimens belonging to 3 different challengeriid genera: *Challengeron*, *Protocystis* and *Entocannula*. These 3 genera were erected according to the difference in their morphological characteristics. Phaeodarians of these genera, however, did not make subclades clearly distinct from each other, and there is a possibility that the current classification within the family Challengeriidae is artificial. In order to further clarify their intra-family relationship, it is necessary to analyze higher resolution genetic maker regions, such as the D1 and D2 regions of the 28S rDNA or ITS.

The genus *Challengeron* contains ca. 13 species among which 5, including the type species *C. bethelli* (Murray) (WNP6), were analyzed in this study (Campbell 1954). The scleracoma of *C. willemoesii* (ECS6 and ECS7) forms a firm "test", similar to other challengeriid phaeodarians composing subclade A (Fig. 1). This species has a test with the surface made of a regularly arranged amphora-shaped structure, which is common to other challengeriid species, except *C. diodon* (ECS4) (Takahashi 1991). Consequently, the surface structure does not distinguish this species from other challengeriids examined in this study. *Challengeron willemoesii* has a developed "peristome" with four "oral spines" (Fig. 1). This complex peristome structure is not seen in other challengeriids belonging to subclade A, and this character may be a morphological criterion distinguishing this species from other challengeriids.

The fact that *C. diodon* (ECS4) appeared in subclade F together with *Medusetta parthenopaea* (MS2) of the family Medusettidae suggests that the former is phylogenetically closer to the medusettids than other challengeriids. Morphological differences of *C. diodon* from other challenderiids have previously been noticed: 1) a dimpled surface; 2) clusters of small pores associated with individual amphorae (inner shell surface); and 3) thin and delicate amphorae (Takahashi 1991). This author also mentioned that the overall appearance of this species was closer to that of medusettid. The two species composing subclade F have one "aboral spine" and the developed "peristome" with "arches" in common (Fig. 1). These structures could also be important new characteristic features to distinguish this subgroup. Considering the morphological features and the position in the phylogenetic tree, these two species would better to be included in the family Medusettidae.

Wide Distribution of Phaeodarians

Specimens of phaeodarian were collected from both deep sea and surface (e.g. MS1–4, Table 1), emphasizing their wide vertical distribution as previously reported (Nakamura and Suzuki 2015). Based on sampling location, *C. diodon* is likely a cosmopolitan species. The two sequences of *C. diodon* within subclade F (Fig. 3) have been collected at distinct locations, Sogndalsfjord, western Norway (NA [AB218765], Yuasa et al. 2006) and the East China Sea (ECS4, Table 1). This species was also found in the North Sea, the Labrador Sea, the South Atlantic and the Mediterranean Sea (Borgert 1901).

The present study provides a strong morphogenetic framework for phaeodarian classification, and will be a valuable tool for accurate interpretation of ongoing environmental diversity surveys based on DNA sequencing. This morphogenetic approach and the single-cell PCR method can further elucidate the intra- and inter-group relationships of phaeodarians and other protists by combining other biological information such as distribution or physiology.

Methods

Sampling and identification: Plankton samples were collected in 2012 and 2013 from 14 stations located in 5 seas across the northern hemisphere: the western and eastern North Pacific Ocean, the Sea of Japan, the East China Sea and the Mediterranean Sea (Supplementary Material Table 1, Supplementary Material Fig. S1). Phaeodarian individuals were isolated manually from the samples under a stereomicroscope or inverted microscope, as soon as possible after the sampling. Isolated specimens were put into wells of cell culture plates and incubated for several hours to allow self-cleaning. The specimens were carefully identified according to their morphological characters under an inverted stereomicroscope and photographed with a digital camera (DIGITAL SIGHT DS-Ri1, Nikon, Japan). For identification, all the documents concerning phaeodarian taxonomy were examined, and a taxonomic database of synonyms of Phaeodaria was constructed. The specimens were then individually preserved in tubes filled with approximately 2.0 mL of 99.9% ethanol. The tubes containing the specimens were stored at 4 °C. Each specimen was labeled according to its sampling station (Table 1 and Fig. 1).

Single-cell PCR: After confirming that there were no other organisms attached to the cell surface, each specimen was put into 50 μL of guanidine-containing extraction buffer (GITC buffer, Decelle et al. 2012) and preserved in -80 °C overnight. Some large specimens (e.g. Aulosphaeridae) were dissected by a sterilized scalpel, and only their "central capsules"

Table 2. Sequences of the primers for 18S rDNA used in this study.

Primer name	Position*	Sequence 5′-3′	Direction	Specificity	Reference
Medlin A	5′-end	AAC CTG GTT GAT CCT GCC AGT	Forward	Universal	Medlin et al. (1988)
PhaeoF3a	288–319	TCA TTC AAA TTT CTG CCC TAT CAG CTW GAY GG	Forward	Phaeodaria	This study
NS2	546–566	GGC TGC TGG CAC CAG ACT TGC	Reverse	Universal	White et al. (1990)
Phaeo11F	559–583	CAG CAG CCG CKG TAA TTC CAG CTC C	Forward	Phaeodaria	This study
PhaeoF4-2	872–890	AKG GAT AGT TGG GGG TGC T	Forward	Phaeodaria	This study
Phaeo880F	872–893	AKG GAT RGT TGG GGG TGC TAG T	Forward	Phaeodaria	This study
Phaeo R	885–904	GGC CAY TRA ATA CTA GCA CC	Reverse	Phaeodaria	Nakamura et al. (2013)
SR6	1761–1777	TGT TAC GAC TTT TAC TT	Reverse	Universal	designed in Vilgalys lab, Duke University.
Medlin B	3′-end	CCT TCT GCA GGT TCA CCT AC	Reverse	Universal	Medlin et al. (1988)

*Position within 18S rDNA of *Protocystis xiphodon*

(protoplasmic body with nucleus) were transferred into the GITC buffer. The specimens were heated at 70 °C for 20 min to break the wall of their central capsules. The extracted DNA was purified with a chemagic DNA Plant kit (PerkinElmer chemagen, Germany). Single-cell PCR was conducted to amplify 18S rDNA with a total reaction volume of 25 μL. A total of 9 primers were used, including 4 phaeodarian-specific primers designed in this study: Phaeo F3a, Phaeo11F, PhaeoF4-2 and Phaeo880F (Table 2). PCR reactions were performed using the following protocol: initial denaturation at 95 °C for 10 min, 40 cycles at 95 °C for 20 sec, 58 °C for 15 sec and 72 °C for 120 sec with a final extension at 72 °C for 7 min. The amplified PCR products were purified with AMPure XP Kit (Beckman Coulter, USA). Sequencing reactions were conducted using an ABI PRISM 3130xl Genetic Analyzer (ABI, U.S.A.).

Phylogenetic analysis: The sequences obtained were assembled using ChromasPro (Technelysium Pty Ltd, Australia), and the alignments were checked manually. The sequences of 6 phaeodarian species, 57 cercozoans were also retrieved from the Basic Local Alignment Search Tool (BLAST) at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi)(Supplementary Material Table 2), and all the sequences were then aligned using MEGA 5 (Tamura et al. 2011). Phylogenetic trees were inferred by maximum-likelihood (ML), neighbor-joining (NJ) and Bayesian analysis methods. For the ML and NJ trees, the General Time Reversible (GTR) model plus Gamma with the shape parameter for among-site rate variation (G) was selected based on the lowest Bayesian Information Criterion (BIC) score, and the substitution nucleotide matrix parameters were calculated. The Bootstrap values (Felsenstein 1985) were estimated based on 1000 pseudo-replicates. GTR model with invariant sites and the gamma distributed model (IG) were selected for the Bayesian analysis, and the Bayesian posterior probabilities (BPP) were calculated with Mr Bayes version 3.2.2. (Ronquist and Huelsenbeck 2003). The Markov Chain Monte Carlo chains ran for 3.0 x 10^7 generations, sampling every 1000 generations. The first 1.0 x 10^6 generations were discarded as burn-in, checking by a program Tracer version 1.6 (Rambaut and Drummond 2009). The remaining data reaching the steady state were used for building the consensus tree, and the tree was visualized by Fig Tree version 1.4.0. (Rambaut 2012).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.protis.2015.05.004.

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