

Molecular phylogenetic affinity of *Hayaster cf. perplexus* based on 18S rDNA sequences

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Abstract A partial 18S rDNA sequence of *Hayaster cf. perplexus* (1674 bp) was obtained from an isolated cell from Tosa Bay, off the coast of Japan (NW Pacific Ocean), using the single cell PCR technique. In molecular phylogenetic analyses, the sequence of *H. cf. perplexus* fell in the Coccolithales clade and clustered with the sequences of the Coccolithaceae, Calcidiscaceae, an uncultured clone, and holococcolith-bearing taxa with very high posterior probability for Bayesian Inference analyses and very high bootstrap support for PhyML analyses. However, phylogenetic relationships among the sequences of *H. cf. perplexus*, Coccolithaceae, and Calcidiscaceae are uncertain due to low posterior probability and bootstrap values. These results suggest that the separation of the families Coccolithaceae and Calcidiscaceae may not be justified.

Keywords Coccolithophore, Calcidiscaceae, Coccolithaceae, *Hayaster*, molecular phylogeny

1. Introduction

Coccolithophores are unicellular marine haptophytes characterised by calcified scales called coccoliths. The classification of coccolithophores is based on the morphology of the coccoliths and the crystallographic orientation of the calcite elements that form them (e.g. Young et al., 2003). Molecular phylogenetic studies of coccolithophores mainly based on 18S rDNA sequences from culture strains started in the late 1990s, and, whilst providing much new information on phylogenetic relationships, they largely supported the classical morphological and crystallographic classification (e.g. Medlin et al., 2008; Edvardsen et al., 2011). In addition, at the species level, phylogenetic studies based on other genetic regions or on genomic analyses of culture strains revealed that fine morphological variation of coccoliths within several morpho-species are often the result of pseudo-cryptic speciation (e.g. Sáez et al., 2003; Geisen et al., 2004; Bendif et al., 2023). Molecular data are therefore a powerful tool for understanding the taxonomy, phylogeny, and diversity of coccolithophores, although the

feasibility of analyses fundamentally depends on availability of culture strains. Less than one quarter of the ~300 described species have been cultured and sequenced, so the molecular phylogenetic tree of the coccolithophores lacks most uncultured species.

Molecular data from uncultured phytoplankton can be obtained using the single cell PCR technique (Takano & Horiguchi, 2004), although the sequence regions that can be determined from single cells are limited. Takano et al. (2006) were the first to apply the single cell PCR technique to coccolithophores. They determined nearly complete 18S rDNA sequences of *Braarudosphaera bigelowii* from two cells, and found differences in the size of the pentoliths and in 18S rDNA sequences between the cells. Subsequent studies based on the single cell PCR technique revealed relationships between 18S rDNA genotypes and size morphotypes of *B. bigelowii* (Hagino et al., 2009), the 16S rDNA genotype of the endosymbiont (nitrogen-fixing cyanobacterium UCYN-A) in the *B. bigelowii* 18S genotype III (Hagino et al., 2013), and the phylogenetic positions

of *Gladiolithus* sp. (Young et al., 2014) and of *Tergestia adriatica* (Hagino et al., 2015). Thus, the single-cell PCR technique is useful for the study of selected genetic regions of uncultured coccolithophores.

While studying coccolithophores in seawater from Tosa Bay off the coast of Japan (NW Pacific Ocean), we encountered an unfamiliar coccoid cell using an inverted light microscope. Since the condition of the cell did not appear good enough for culture experiments, we applied the single-cell PCR technique to it. Here we report the results of morphological and molecular phylogenetic studies of the cell.

2. Materials and Methods

A 10 L surface seawater sample was collected for microscopic and molecular studies of coccolithophores from Tosa Bay (33°15'N, 133°28'E) using a bucket from the boat *Neptune* of the Usa Marine Biological Institute, Kochi University (Japan) on 16 October 2014. The sample was pre-filtered through a 50 µm mesh size plankton net (Sefar Inc. NY50-HD), and then concentrated using a 1 µm mesh size plankton net (Sefar Inc. NY1-HD). One of the unidentified coccoid cells, labelled as Tosa-3, was isolated using a capillary tube under an inverted light microscope (LM) (Olympus CKX41). The isolate was placed on a glass slide with a drop of sterile seawater, covered with a glass coverslip, and then photographed under plane- and cross-polarised light using a Nikon DS-Fi2 digital camera equipped on a Nikon E600POL microscope. After photography, the coverslip was removed and the coccosphere was re-isolated using a capillary tube under the inverted LM, then subjected to single cell PCR amplification as outlined in Takano & Horiguchi (2004).

The first round of PCR was performed using external primers (SR1 and SR12) to amplify almost the entire 18S rDNA sequence. The PCR condition for the first round was one initial denaturation at 94°C for 60 s, followed by 35 cycles of denaturation at 94°C, annealing at 50°C for 30 s, and an extension cycle at 72°C for 60 s. The temperature profile was completed by a final extension cycle at 72°C for 4 min. In the second round of PCR, internal short sequences of 18S rDNA region were amplified using the 0.5 µL of the first round PCR product as a DNA template with three pairs of internal primers: SR1c–SR5, SR4–SR9, and SR8tak–SR12b. The sequences of primers used in this study were listed in Takano & Horiguchi

(2004). The PCR condition for the second round was one initial denaturation step at 94°C for 30 s, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and an extension cycle at 72°C for 30 s. The temperature profile was completed by a final extension cycle at 72°C for 4 min. The products of the second round PCR amplification were sequenced directly using the BigDye Terminator Cycle Sequencing Kit ver. 3.1 (ThermoFisher Scientific) with the DNA auto sequencer ABI PRISM 3130 Genetic Analyzer (Applied Biosystem) in Kochi Institute for Core Sample Research, JAMSTEC. The quality of each sequence result was checked and ambiguous results (~10–30-bp at the 5' and 3' ends of each sequence) were removed using the software 4Peaks (Mekentosj). The accuracy of the sequence results was confirmed based on the sequences obtained by forward and reverse primers for the same genetic region being identical on SeaView 5.04 (Manolo Gouy) (Gouy et al., 2009). A partial 18S rDNA sequence of the isolate Tosa-3 (1674bp) (Accession number LC771592) was reconstructed by connecting the confirmed sequences on SeaView 5.04. For phylogenetic analysis, 75 gene sequences of 18S rRNA from haptophytes were obtained from GenBank, including a sequence of the Pavlovophyceae as an outgroup. The sequence of the specimen Tosa-3 was aligned together with the sequences obtained from the GenBank by MAFFT (<https://mafft.cbrc.jp/alignment/server/>).

Phylogenetic trees were constructed based on Bayesian Inference (BI) using Mr. BAYES v3.2.7 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) and on PhyML method using SeaView 5.04 (Guindon et al., 2010). Substitution models were selected using MrModeltest 2.2 (Nylander, 2004) and Modeltest 3.7 (Posada & Crandall, 1998) for BI and PhyML, respectively. For the BI analysis, GTR + I + G model was selected by the MrModeltest, and Markov chain Monte Carlo iterations were carried out until 1 million generations when the average standard deviations of split frequencies were below 0.01, indicating convergence of the iterations. For the PhyML analyses, a likelihood score (–lnL = 10496.6123) was obtained under the GTR + I + G model with the following parameters: assumed nucleotide frequencies A = 0.2318, C = 0.2215, G = 0.2919, T = 0.2548; substitution rate A-C = 1.0902, A-G = 1.4920, A-T = 0.8190, C-G = 0.7768, C-T = 4.3052, G-T = 1.0000; proportion of sites assumed to be invariable = 0.5521; and rates for variable

Plate 1

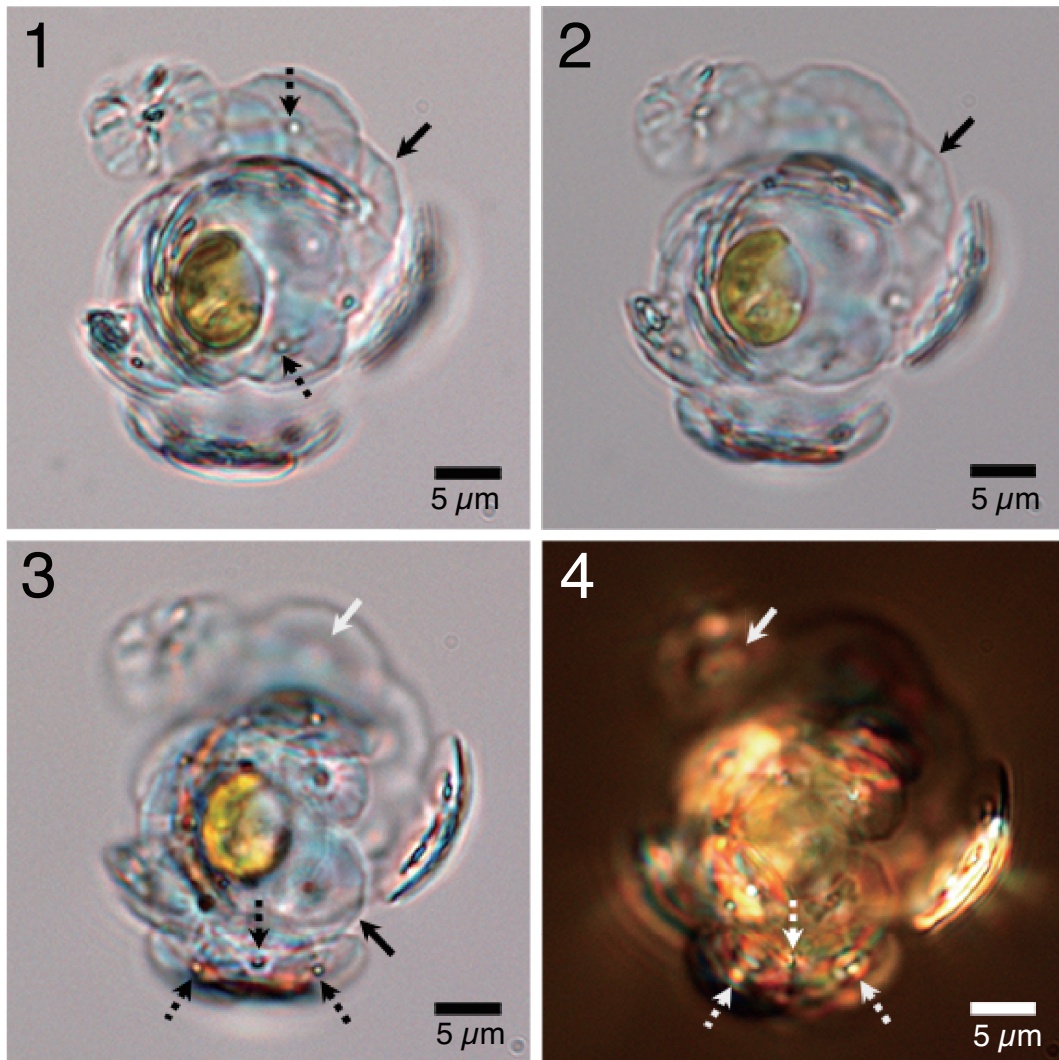


Plate 1: Light microscopic images of the isolate Tosa-3. 1–3. Plane-polarised light view. 4. Cross-polarised light view. Black and white arrows indicate polygonal outline of distal shield and proximal shield, respectively. Dashed arrows show bright spots in central area of distal shield that may correspond to the central process of *Hayaster perplexus* shown in Nishida (1979).

sites assumed to follow a gamma distribution with shape parameter = 0.4539, were estimated by Modeltest 3.7 (Posada & Crandall, 1998). Bootstrap analyses with 1000 replicates for PhyML analysis were applied to examine the robustness and statistical reliability of the topologies (Felsenstein, 1985).

3. Results

3.1. Microscopic Study

The coccosphere of the isolate Tosa-3 was carefully observed under a polarised light microscope (Plate 1) before the coccosphere was crushed for molecular study. It was a partially disintegrated spherical coccosphere approxi-

mately 15 µm in diameter. The cell of the coccolithophore was about 7 µm in diameter and only occupied a part of the internal coccosphere space; it had a prominent, dark yellow chloroplast (Plate 1).

The coccosphere was monomorphic consisting of ≥ 30 large placoliths. The placoliths consisted of a broad (6–12 µm), thin, concavo-convex distal shield and a much smaller proximal shield (white arrows in Plate 1, figures 3 and 4), joined by a narrow tube. The distal shields were circular or subcircular in outline, but with a polygonal rim, and were formed of 10–14 elements (black arrows in Plate 1, figures 1–3). Very small (≤ 0.5 µm) bright spots were observed on the centre of the distal shields in both plane- and

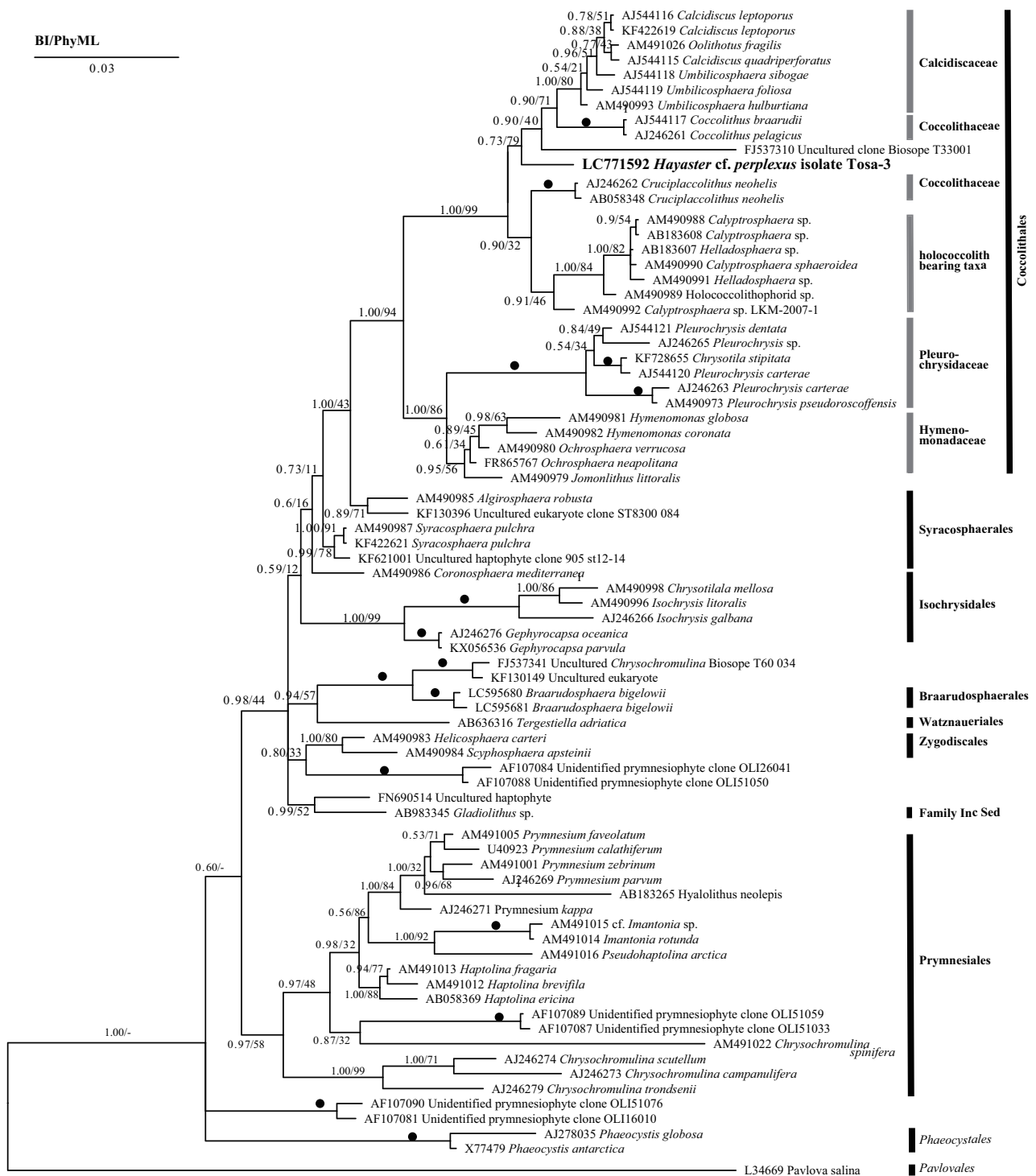


Figure 1: Phylogenetic tree based on 18S rDNA sequences using the Bayesian Inference (BI) method. The number on each node indicates the posterior probability of BI analysis and bootstrap values from PhyML analysis. Solid circles indicate the clades supported by very high posterior probability (1.00) by BI analysis and bootstrap values (100%) by ML analysis.

cross-polarised light microscopy (black and white dotted arrows in Plate 1, figures 1, 3, and 4). The spots occasionally appeared brighter than the surrounding distal shield elements under cross-polarised light (bottom two dotted arrows in Plate 1, figures 1–4), suggesting that the central area was filled with a calcified structure, and that the

orientation of the calcite crystals forming the structures differed from that of the distal shield.

3.1. Molecular phylogenetic study

The topography of the trees obtained by BI and PhyML analyses resemble each other. Figure 1 shows only the BI

tree with posterior probability obtained by BI analysis and bootstrap values from PhyML analysis. The sequence of the specimen Tosa-3 fell in the Coccolithales Clade with very high posterior probability (1.0) and high bootstrap support (94%). In the Coccolithales Clade, the sequence of the isolate Tosa-3 made a subclade with the sequences of Calcidiscaceae, Coccolithaceae, and holococcolith-bearing taxa with very high posterior probability (1.0) and bootstrap support (99%). In the subclade, the isolate Tosa-3 clustered with Calcidiscaceae + *Coccolithus pelagicus* (Coccolithaceae) and an environmental sequence (Biosope T33.001) with low posterior probability (0.73) and low bootstrap support (79%).

4. Discussion

4.1. Identity of the coccolithophore

The observed coccosphere resembles *Hayaster perplexus* (Bramlette & Riedel, 1954) Bukry, 1973 in that it is a monomorphic coccosphere with large, thin, circular placoliths with small proximal shields, and in the polygonal outline of the distal shield. Typical *H. perplexus* coccoliths have a solid tube and only a slight depression on either the proximal or distal surface (e.g. Young et al., 2003). However, an unusual *H. perplexus* specimen, which has a small central process and pore on the polygonal distal shield, was reported from the North Pacific Ocean (Nishida, 1979, pl. 4 fig. 2a; note that this image is also shown in Winter & Siesser, 1994, p. 120 and on the Nannotax3 website [Young et al., 2023]). The bright spots observed in the central area of our isolate may correspond to the central processes observed in *H. perplexus* by Nishida (1979). Culture strains of *H. perplexus* have never been established and there is no information on the cause of the morphological variation in the central structure of coccoliths of *H. perplexus*. Since the distal shields of our isolate are polygonal, we are confident that our specimen belongs to *Hayaster*. However, we have no evidence to determine whether the observed central spots/processes reflect intraspecific morphological variation of *H. perplexus* or pseudocryptic speciation. In current taxonomy, the described species that most closely resembles our specimen is *H. perplexus*; therefore, we refer to our isolate Tosa-3 as *Hayaster cf. perplexus*.

4.2 Molecular phylogenetic placement of *Hayaster cf. perplexus*

The families Coccolithaceae Poche, 1913 emend Young

& Bown, 1997 and Calcidiscaceae Young & Bown, 1997 are both characterised by diploid stages with monomorphic coccospheres of placolith coccoliths and haploid stages with monomorphic coccospheres of holococcoliths. In both, the heterococcoliths have a distal shield formed of V-units (i.e. with sub-vertical crystallographic c-axes), a proximal shield of R-units (i.e. with sub-radial crystallographic c-axes), and the locus of the proto-coccolith ring is embedded within the coccolith (Young et al., 2004). Finally, both families have fossil records extending through the Cenozoic, from the Paleocene to the Recent. They were separated by Young & Bown (1997) on the basis that in the Coccolithaceae, the V- and R-units are intertwined, forming an inner tube with an upper layer of R-units and lower layer of V-units, whilst this configuration is absent in the Calcidiscaceae (see Figure 2). This separation was consistent with a previous molecular phylogenetic study based on the *tufA* region with the Calcidiscaceae sequences forming a discrete clade with very high posterior probabilities for MrBayes (MB = Bayesian inference) and low bootstrap support for Maximum Parsimony (MP) and Neighbor Joining (NJ) analyses (MB/MP/NJ, 100/<50/62), albeit nesting within the Coccolithaceae (Medlin et al., 2008).

In this study, *Calcidiscus*, *Oolithotus*, and *Umbilicosphaera* made the Calcidiscaceae clade with very high

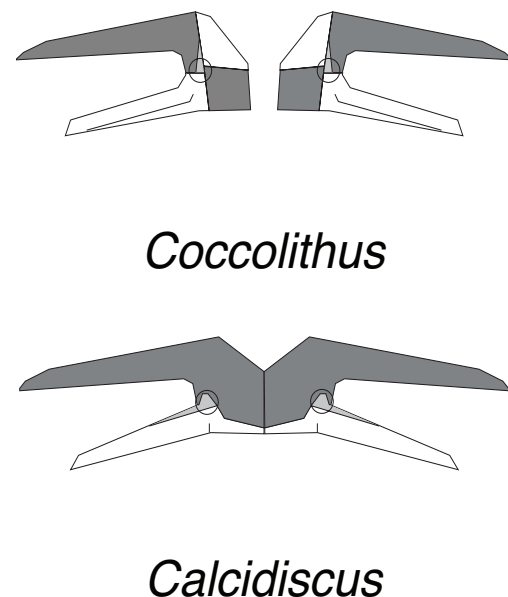


Figure 2: Schematic diagram of a cross section of placoliths of *Coccolithus* and *Calcidiscus* species. Colour indicates difference in orientation of elements: dark grey = V-unit and white = R-unit. Light grey zone shows overlap of the V- and R-units. The open circles indicate the location of the proto-coccolith ring.

posterior probabilities for BI analyses and low bootstrap support for PhyML analyses (BI/PhyML, 1.00/80%) similar to the *tufA* tree shown in Medlin et al. (2008). However, the sequence of *Hayaster* cf. *perplexus* unexpectedly falls outside the Calcidiscaceae clade, together with the sequences of Coccolithaceae, although *Hayaster perplexus* has a Calcidiscaceae-type structure and has been included within the Calcidiscaceae in modern classifications (e.g. Young et al., 2003; Jordan et al., 2004). Our results suggest that the Calcidiscaceae/Coccolithaceae division may prove artificial, in which case the two families should be recombined into a single family (Coccolithaceae), or the definition of the Calcidiscaceae should be emended to exclude *Hayaster*. Our data are not sufficiently robust to support a change of classification at this stage, but they do suggest that further investigation using additional taxa or additional genetic systems would be worthwhile.

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References

- Bendif, E.M., Probert, I., Archontikis, O.A., Young, J.R., Beaufort, L., Rickaby, R.E. & Filatov, D. 2023. Rapid diversification underlying the global dominance of a cosmopolitan phytoplankton. *The ISME Journal*, **17**: 630–640. <https://doi.org/10.1038/s41396-023-01365-5>
- Bramlette, M.N. & Riedel, W.R. 1954. Stratigraphic value of Discoasters and some other microfossils related to Recent coccolithophores. *Journal of Paleontology*, **28**: 385–403.
- Bukry, D. 1973. Phytoplankton stratigraphy, Deep Sea Drilling Project Leg 20, Western Pacific Ocean. In: B.C. Heezen, I.D. MacGregor, et al., *Initial Reports of the Deep Sea Drilling Project*, **20**: 307–317. <https://doi.org/10.2973/dsdp.proc.20.114.1973>
- Edwardsen, B., Eikrem, W., Throndsen, J., Sáez, A.G., Probert, I. & Medlin, L.K. 2011. Ribosomal DNA phylogenies and a morphological revision provide the basis for a revised taxonomy of the Prymnesiales (Haptophyta). *European Journal of Phycology*, **46**(3): 202–228. <https://doi.org/10.1080/09670262.2011.594095>
- Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, **39**: 783–791. <https://doi.org/10.1111/j.1558-5646.1985.tb00420.x>
- Geisen, M., Young, J.R., Probert, I., Sáez, A.G., Baumann, K.-H., Bollmann, J., Cros, L., de Vargas, C., Medlin, L.K., & Sprengel, C. 2004. Species level variation in coccolithophores. In: H.R. Thierstein & J.R. Young (Eds). *Coccolithophores – From molecular processes to global impact*. Springer, Berlin: 327–366.
- Gouy, M., Guindon, S. & Gascuel, O. 2009. SeaView Version 4: A Multiplatform Graphical User Interface for Sequence Alignment and Phylogenetic Tree Building. *Molecular Biology and Evolution*, **27**(1): 221–224. <https://doi.org/10.1093/molbev/msp259>
- Guindon, S., Dufayard, J.-F., Lefort, V., Anisimova, M., Hordijk, W. & Gascuel, O. 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Systematic Biology*, **59**(3): 307–321. <https://doi.org/10.1093/sysbio/syq010>
- Hagino, K., Onuma, R., Kawachi, M. & Horiguchi, T. 2013. Discovery of an endosymbiotic nitrogen-fixing cyanobacterium UCYN-A in *Braarudosphaera bigelowii* (Prymnesiophyceae). *PLoS One*, **8**(12): e81749. <https://doi.org/10.1371/journal.pone.0081749>
- Hagino, K., Takano, Y. & Horiguchi, T. 2009. Pseudo-cryptic speciation in *Braarudosphaera bigelowii* (Gran and Braarud) Deflandre. *Marine Micropaleontology*, **72**: 210–221. <https://doi.org/10.1016/j.marmicro.2009.06.001>
- Hagino, K., Young, J.R., Bown, P.R., Godrijan, J., Kulhanek, D.K., Kogame, K. & Horiguchi, T. 2015. Re-discovery of a “living fossil” coccolithophore from the coastal waters of Japan and Croatia. *Marine Micropaleontology*, **116**: 28–37. <https://doi.org/10.1016/j.marmicro.2015.01.002>
- Huelsenbeck, J.P. & Ronquist, F. 2001. MRBAYES: Bayesian inference of phylogeny. *Bioinformatics*, **17**: 754–755. <https://doi.org/10.1093/bioinformatics/17.8.754>
- Jordan, R.W., Cros, L. & Young, J.R. 2004. A revised classification scheme for living haptophytes. *Micropaleontology*, **50**: 55–79. <https://doi.org/10.2113/50.Suppl.1.55>
- Medlin, L.K., Sáez, A.G. & Young, J.R. 2008. A molecular clock for coccolithophores and implications for selectivity of phytoplankton extinctions across the K/T boundary. *Marine Micropaleontology*, **67**(1–2): 69–86. <https://doi.org/10.1016/j.marmicro.2007.08.007>
- Nishida, S. 1979. Atlas of Pacific Nannoplanktons. *News of Osa-*

- ka Micropaleontologists*, **S3**: 1–31.
- Nylander J.A.A. 2004. MrModeltest 2.2. Computer program distributed by Uppsala University. Evolutionary Biology Centre, Uppsala University.
- Poche, F. 1913. Das System der Protozoa. *Archiv für Protistenkunde*, **30**: 125–321.
- Posada, M.A. & Crandall, K.A. 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics*, **14**: 817–818. <https://doi.org/10.1093/bioinformatics/14.9.817>
- Ronquist, F. & Huelsenbeck, J.P. 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, **19**: 1572–1574. <https://doi.org/10.1093/bioinformatics/btg180>
- Sáez, A.G., Probert, I., Geisen, M., Quinn, P., Young, J.R. & Medlin, L.K. 2003. Pseudo-cryptic speciation in coccolithophores. *Proceedings of the National Academy of Science*, **100**(12): 7163–7168. <https://doi.org/10.1073/pnas.1132069100>
- Takano, Y. & Horiguchi, T. 2004. Surface ultrastructure and molecular phylogenetics of four unarmored heterotrophic dinoflagellates, including the type species of the genus *Gyrodinium* (Dinophyceae). *Phycological Research*, **52**: 107–116. <https://doi.org/10.1111/j.1440-183.2004.00332.x>
- Takano, Y., Hagino, K., Tanaka, Y., Horiguchi, T. & Okada, H. 2006. Phylogenetic affinities of an enigmatic nannoplankton, *Braarudosphaera bigelowii* based on the SSU rDNA sequences. *Marine Micropaleontology*, **60**: 145–156. <https://doi.org/10.1016/j.marmicro.2006.04.002>
- Winter, A. & Siesser, W.G. 1994. Atlas of living coccolithophores. In: A. Winter & W.G. Siesser (Eds). *Coccolithophores*. Cambridge University Press, Cambridge: 107–159.
- Young, J.R. & Bown, P.R. 1997. Cenozoic calcareous nannoplankton classification. *Journal of Nannoplankton Research*, **19**(1): 36–47. <https://doi.org/10.58998/jnr2278>
- Young, J.R., Bown, P.R. & Lees, J.A. 2023. Nannotax3 website. International Nannoplankton Association. Accessed 8 August 2023. URL: <https://www.mikrotax.org/Nannotax3/index.html>
- Young, J.R., Geisen, M., Cros, L., Kleijne, A., Probert, I. & Ostergaard, J.B. 2003. A guide to extant coccolithophore taxonomy. *Journal of Nannoplankton Research*, **S1**: 1–132. <https://doi.org/10.58998/jnr2297>
- Young, J.R., Hagino, K. & Poulton, A. J. 2014. *Gladiolithus adeyi* sp. nov.: a new deep photic coccolithophore species and new molecular genetic and crystallographic observations on *Gladiolithus flabellatus*. *Journal of Nannoplankton Research*, **34**: 61–68. <https://doi.org/10.58998/jnr2300>
- Young, J.R., Henriksen, K. & Probert, I. 2004. Structure and morphogenesis of the coccoliths of the CODENET species. In: H.R. Thierstein & J.R. Young (Eds). *Coccolithophores – From molecular processes to global impact*. Springer, Berlin: 191–216.