

## 論文の内容の要旨

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### 論文題目

Taxonomic study on unarmored dinoflagellates in the family Kareniaceae from Asian coasts

(アジア沿岸域に出現するカレニア科無殻渦鞭毛藻の分類学的研究)

Marine unarmored dinoflagellates in the family Kareniaceae have caused harmful algal blooms (HABs) responsible for economic damages on coastal fisheries. Over the years, a number of karenian dinoflagellates has been reported since *Karenia mikimotoi*, one of the most noxious HAB species, was described after causing mass mortality of farmed pearl oysters in Mie Prefecture in 1935. Since the establishments of the three major genera in the family, *Karenia* and *Karlodinium* in 2000 and *Takayama* in 2003, karenian dinoflagellates have been distinguished from other unarmored dinoflagellates based on their phylogenetic position and shape of apical structure complex (ASC). In Southeast Asian coasts, however, HABs caused by small karenian species associated with fish mass mortalities have become a new emergent issue. Because most members of the Kareniaceae, *Karlodinium* and *Takayama* species in particular, are relatively small and difficult to identify precisely, their occurrence and distribution have remained unclear in the region. In the present study, a taxonomic study on unarmored dinoflagellates in the Kareniaceae was carried out with following objectives; (1) to recognize the taxonomic positions and morphological characters of karenian dinoflagellates occurring in Asian coasts, with new description of undescribed taxa, (2) to elucidate the phylogenetic relationship of species and their chloroplast, (3) to understand the photosynthetic pigments common in the family, and specific in the genus and species, (4) to evaluate characters in terms of the evolutionary stability/variability and taxonomic significance reliable for identification, (5) to understand the impacts of karenian dinoflagellates on other marine phytoplankton and zooplankton, and (6) to facilitate species identification by providing morphological characters distinct from other species and species distribution in Asian coasts.

Unialgal cultures of the Kareniaceae were established from seawater samples in Asian coasts, mainly in Japan and the Philippines, by the use of a modified sampling method to collect small (<20 µm) species. Using these cultures, morphological characters were examined with light and fluorescent microscopy, and scanning and transmission electron microscopy. The phylogenetic relationship was inferred from nuclear-encoded LSU rDNA and ITS region, and the chloroplast phylogeny was inferred from the chloroplast-encoded 16S rDNA and *psbA* (photosystem II protein D1 gene) sequences. The photosynthetic pigment composition was determined by HPLC analysis. Mixotrophy of kareniacean species was observed by feeding experiment on phytoplankton and zooplankton, and the lethality to brine shrimp was estimated after the exposure of kareniacean cultures.

#### [Taxonomic positions and morphological characters]

A total of 98 unialgal culture strains of the Kareniaceae was obtained from Asian coasts, which included 20 species belonging to five genera, i.e., one *Asterodinium* (*A. gracile*), one *Gertia* (*G. stigmatica*), five *Karenia* (*K. asterichroma*, *K. longicanalis*, *K. mikimotoi*, *K. papilionacea* and *K. selliformis*), seven *Karlodinium* (*K. australe*, *K. azanzae*, *K. ballantinum*, *K. decipiens*, *K. gentienii*, *K. veneficum* and *K. zhouanum*), and six *Takayama* species (*T. helix*, *T. tasmanica*, *T. tuberculata*, *T. xiamenensis*, *Takayama* sp.1 and *Takayama* sp.2). Among the species identified by morphological characters, *Karlodinium azanzae* was the newly described species and two undescribed *Takayama* species were also identified. Morphological characters of 20 species were described based on light and electron microscopic observation, of which, e.g., the lack of pyrenoid in *A. gracile*, presence of ventral pore in *K. ballantinum*, and presence of ventral connective in *K. azanzae* were new findings in this family.

#### [Phylogenetic relationship]

The host phylogeny inferred from LSU rDNA and ITS region revealed the clades in kareniacean genera, i.e., *Karenia* separating into six clades, *Karlodinium* into three clades and *Takayama* into three clades. Both *Karenia* and *Takayama* were monophyletic, while *Karlodinium* was paraphyletic, of which *Karlodinium* clade 3 (*K. decipiens* and *K. antarcticum*) was closely related to the *Takayama* clade rather than the two other *Karlodinium* clades. *Gertia stigmatica*, a kareniacean dinoflagellate having a peridinin-type chloroplast, was positioned in a clade with the environmental sequence from the South China Sea and the kleptoplastidic species (Ross Sea dinoflagellate), which was related to the clade composed of *Karlodinium* and *Takayama*. These clades were used for the evaluation of morphological characters in terms of evolutionary stability. The phylogenetic position of an oceanic species *Asterodinium gracile*, which showed a close affinity to *K. papilionacea* in *Karenia*, was newly revealed from established culture. Moreover, the phylogenetic positions of two undescribed species not belonging to any currently recognized kareniacean genera, i.e., a species from Shimizu, Shizuoka sister to *Karenia* and the other from Asamushi, Aomori related to *Gertia*, were determined from sequences obtained by single-cell PCR, although their cultures were not available.

The chloroplast phylogeny was inferred from chloroplast-encoded 16S rDNA and *psbA* sequences. Majority of kareniacean dinoflagellates are possessing fucoxanthin-type chloroplast derived from the haptophyte, which is unique in dinoflagellates, because the peridinin-type chloroplast derived from a red alga is common in photosynthetic dinoflagellates. After several attempts of amplification, 16S

rDNA sequences from 14 cultures of 14 species and *psbA* sequences from 22 cultures belonging to 15 species, were successfully determined, despite the difficulty of chloroplast gene amplification due to their high substitution rate. Phylogenetic analysis showed that the majority of the kareniacean chloroplasts formed a clade in the haptophyte chloroplasts, except for the three species, which did not cluster within the kareniacean clade. In the 16S rDNA tree, the chloroplast of *Ptychodiscus* sp. was related to that from *Dinophysis mitra*, which has the kleptochloroplast from haptophytes, and that of *Karlodinium gentienii* was related to *Chrysochromulina apheles*. In the *psbA* tree, two cultures of *Karlodinium ballantinum* were related to *Emiliania huxleyi*, *K. gentienii* to *Chrysochromulina* spp., and *T. helix* to *Phaeocystis* sp. These phylogenetic positions may suggest that the chloroplasts of *K. ballantinum*, *K. gentienii* and *T. helix* are further replaced by haptophyte chloroplasts of different origin.

#### [Photosynthetic pigment composition]

The fucoxanthin-type chloroplasts of the Kareniaceae are characterized by the presence of fucoxanthin (Fuco) and its derivatives. To understand the photosynthetic pigments common in the genus and species, the pigment compositions of one *Asterodinium* (one culture), four *Karenia* (six cultures), seven *Karlodinium* (14 cultures) and six *Takayama* species (nine cultures) were analyzed. In *Karlodinium*, all the analyzed strains contained Fuco and 19'-butanoyloxyfucoxanthin (But-fuco) and 19'-hexanoyloxyfucoxanthin (Hex-fuco). In *Karenia*, four keto-forms of Fuco were detected in addition to those three pigments. In *Takayama*, Fuco and But-fuco were detected from all cultures, but Hex-fuco was not confirmed from most cultures. On the other hand, Fuco, But-fuco and Hex-fuco were confirmed from *T. helix*, similar to the genus *Karlodinium*. Based on the composition of fucoxanthins and their analogs, the three genera, *Karenia*, *Karlodinium* and *Takayama*, in the family could be discriminated. Within the genus, gyroxanthin diester 1 was detected in some *Karenia* and *Takayama* species, while gyroxanthin diester 2 was detected in many *Karenia* species and *Karlodinium veneficum*. The pigment composition of *K. ballantinum* and *K. gentienii*, having unique chloroplast replacements as suggested by chloroplast phylogeny, did not show significant difference from other *Karlodinium* species, although they have the "Unidentified  $\lambda_{\max}$ 440 peak" not present in *K. azanzae*, *K. australe*, *K. veneficum* and *K. decipiens*. Despite the phylogenetic position in *Karenia*, *Asterodinium gracile* only contained Fuco and Hex-fuco, and other derivatives (But-fuco and four keto-forms) common in *Karenia* were not found. Since it is an oceanic species and also reported from the deep sea, this unique pigment composition probably is acquired for environmental adaptation in the *Karenia* clade.

#### [Taxonomic significance of morphological characters]

The taxonomic reliability of the morphological traits was evaluated by comparing the observed traits with their phylogenetic position, based on their evolutionary stability in the Kareniaceae. Characters evaluated mainly focused on cell size, nuclear shape and position, granular structure on amphiesmal vesicles, apical structure complex (ASC), ventral pore, vertical ridge structure (narrow tube and peduncle), accumulation bodies, chloroplast shape, chloroplast envelope and pyrenoid. Among them, the following characters showed stability in each phylogenetic group. (1) Granular structures on amphiesmal vesicles are widely distributed on the cells of *Karlodinium*, often found in specific vesicles arranged in curved lines in *Takayama*, but unclear in *Karenia*. (2) The ASC consists of a row of knob vesicles located on the left side

of the cell, a row of furrowed vesicles forming the central groove, and a ridge located on the right side. This ridge does not correspond to the row of vesicles. The shape of ASC is stable in genus; straight in *Asterodinium*, *Karenia* and *Karlodinium*, while sigmoidal in *Takayama*. (3) Ventral pore is found in *Karlodinium* and *Takayama*, except for two *Karlodinium* species (*K. digitatum* and *K. zhouanum*). The position of ventral pore is stable in species, which is applicable for identification of some *Karlodinium* species. (4) Ventral ridge is longer in *Takayama* and *Karlodinium* than those in *Karenia*. The peduncle is connected at the upper end of ventral ridge in *Karlodinium* and *Takayama*, whereas it is not present in *Karenia*. (5) Starch-like accumulation bodies are present in *Karlodinium* and *Takayama*, but are absent in *Karenia*. (6) Multiple chloroplasts are present in all species possessing the fucoxanthin-type, and usually surrounded by two membranes. (7) The shape of pyrenoid differs among genera and species. The pyrenoid is laterally positioned in chloroplast in *Karenia*, while it is embedded inside the chloroplast in *Karlodinium*. On the other hand, three types exist in *Takayama* corresponding to its three clades. In the *Takayama* clade 1, one spherical large pyrenoid is usually present, but several small spherical pyrenoids are sometimes present. In the *Takayama* clade 2, a compound central pyrenoid is present. In *Takayama* clade 3, represented only by *T. helix*, an embedded pyrenoid is present similar to the chloroplast of *Karlodinium*, which also contains Hex-fuco.

#### [Mixotrophy and harmful effects]

Some karenian dinoflagellates are known to be mixotrophic, feeding by engulfing small organisms such as phytoplankton (phagocytosis), or by sucking cell contents of large organisms such as zooplankton using the peduncle (myzocytosis). To clarify their feeding abilities, by phagocytosis or myzocytosis, karenian cultures were exposed to various phytoplankton and zooplankton. Phagotrophy on microalgal cells was observed from five *Karlodinium* species (*K. australe*, *K. azanzae*, *K. decipiens*, *K. veneficum* and *K. zhouanum*), but not observed from *Karenia* and *Takayama* species. Peduncle feeding on large dinoflagellates and zooplankton was observed only from *K. azanzae*. Also, lethality to *Artemia* of five *Karlodinium* (*K. australe*, *K. azanzae*, *K. decipiens*, *K. gentienii* and *K. veneficum*) and two *Takayama* species (*Takayama* sp.1 and *Takayama* sp.2) was observed; but it was not observed in any *Karenia* species tested.

For accurate and immediate HAB species identification of the Kareniaceae, morphological, ultrastructural and pigment characters useful for species identification were summarized, and species distribution was compiled with previous reports in the Asian Pacific. Karenian dinoflagellates identified in this study include six newly detected species (*K. papilionacea*, *K. azanzae*, *K. australe*, *K. ballantinum*, *K. zhouanum* and *Takayama* sp.1) in the Philippines and eleven species (*K. asterichroma*, *K. selliformis*, *K. australe*, *K. decipiens*, *K. ballantinum*, *K. gentienii*, *K. zhouanum*, *T. tuberculata*, *T. xiamenensis*, *Takayama* sp.1 and *Takayama* sp.2) in Japan. In the present study, a part of biodiversity in the family was unveiled, and further investigations on the harmful dinoflagellates, e.g., two undescribed *Takayama* species and two undescribed karenian genera found in this study, species and population distribution in this region, and life history in relation to red tide forming mechanisms, would provide better scientific knowledge applicable to mitigation of negative impacts caused by harmful dinoflagellates in the family Kareniaceae.