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**Pseudo-cryptic speciation in *Braarudosphaera bigelowii* (Gran and Braarud)
Deflandre**

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Abstract

Partial SSU rDNA sequences were obtained from 13 naturally collected cells of *Braarudosphaera bigelowii* obtained from various parts of seas surrounding Japan. Together with the two previously reported sequences, 15 specimens were classified into five SSU rDNA Genotypes I-V. Based on the side length of the pentaliths forming the coccosphere, these specimens were also classified into three size-morphotypes; Intermediate form-A, Intermediate form-B, and Large form. Genotypes of *B. bigelowii* were well correlated with size-morphotypes but not with sampling area. This result indicates that size differences in *B. bigelowii* are the results of speciation and not of intra-population variety. Therefore, Genotypes I and II (Intermediate form-A) and Genotypes IV and V (Large form) are regarded as pseudo-cryptic sibling species of typical *B. bigelowii* (Genotype III, Intermediate form-B), which corresponds to the original description of the species. From the SSU rDNA sequences, it is evident that Genotype V arose from Genotype IV, and Genotype IV originated from Genotype III. The specimens of Genotypes III-V showed size increase of pentaliths in accordance

with their branching order. The consistency in the relationship between genotypes and size-morphotypes of living *B. bigelowii*-complex observed in this study suggests that inconsistency of size range of '*B. bigelowii*' pentoliths among different geological ages reported by palaeontological studies stem from additional pseudo-cryptic speciation in the lineage of '*B. bigelowii*' in the geological past.

1. Introduction

The family Braarudosphaeraceae Deflandre (1947) is one of the oldest extant coccolithophore families, it is distinguished by having a coccosphere formed of five-fold symmetric calcareous scales called pentoliths. The family has a long fossil record extending back to the Early Cretaceous (ca. 140Ma, Bown et al., 1998). In the geological record, they show greater morphological variation in their pentoliths than in modern populations. Based on the morphology of the five segments forming a pentolith, six genera have been recognized in the Braarudosphaeraceae; *Braarudosphaera*,

Bukryaster, *Micrantholithus*, *Pemma*, *Pentaster* and *Trapezopentus* (Perch-Nielsen, 1985a and b). In the early Cretaceous, *Micrantholithus*, whose pentoliths consist of triangular segments, was the most common genus, and *Braarudosphaera* distinguished by trapezoidal segments was the second major genus in the family. *Braarudosphaera* replaced *Micrantholithus* as most common genus in the middle Cretaceous, and has remained so to the present (Bown, 2005). All genera of Braarudosphaeraceae except *Braarudosphaera* and many species of *Braarudosphaera* became extinct in the Mesozoic and Cenozoic. In the present ocean, only two species of *Braarudosphaera*; *Braarudosphaera bigelowii* (Gran and Braarud, 1935) Deflandre (1947) and *Braarudosphaera magnei* Lefort (1972) are extant. *B. bigelowii*, the type species of *Braarudosphaera*, is characterized by regular dodecahedral coccospheres consisting of twelve regular-pentagonal pentoliths. Distribution of this species has been restricted to nearshore and hemipelagic waters almost through their history from the late Cretaceous to the present, although they occasionally became abundant in the open-ocean realm during the low biodiversity events, notably in the Early Danian immediately after the K/T mass extinction and in the Oligocene Diversity Minimum (Paleo-Alampay et al.,

1999; Bown et al., 2004). The second living species *B. magnei* has never been reported since its original description, and fossils of this species are unknown (Young et al., 2003). *B. magnei* differs from other members of *Braarudosphaera* in the structure of the coccosphere. *B. magnei* has a spherical coccosphere consisting of many rounded pentoliths, whereas the other living and extinct species of *Braarudosphaera* have dodecahedral coccospheres consisting of twelve pentoliths (e.g., Lambert, 1987; Mai et al., 1997). Currently *B. magnei* is included in the genus *Braarudosphaera*, however, its phylogenetic affinity to *B. bigelowii* has not yet been genetically verified.

Notable size variations have been reported in living and fossil populations of *B. bigelowii*. However, size has not been used in the classification of *B. bigelowii*, because the factor controlling the size of *B. bigelowii* was unknown. In the current classification scheme, therefore, all *Braarudosphaera* pentoliths with simple pentagonal form are lumped into a single species name, *B. bigelowii* regardless of occurrence age and of size variation. Hagino (1997) reported unusually small specimens of living *B. bigelowii* from the Tsugaru Warm Current regime. She segregated ‘small specimens’ with cell diameter <5.5 μm from ‘normal-sized specimens’ with cell diameter >8.5 μm .

In subsequent studies, size of pentoliths instead of diameter of cell was used for classification of morphotypes to allow more accurate size measurements. Hagino et al (2005) revised the size range of 'small' and 'normal' specimens to 1.6-2.4 μ m and 4.0-7.2 μ m in side length of pentolith, respectively. Takano et al (2006) examined the size-frequency distribution of living *B. bigelowii* pentoliths, and further divided the 'normal sized specimens' of Hagino et al (2005) into two size categories; Intermediate forms –A and –B. In the study, they obtained SSU rDNA sequences of two cells of *B. bigelowii* picked from sea water samples, using a single cell PCR (polymerase chain reaction) technique, and found 16-bp differences including indels in the SSU rDNA sequences of between the cell belonging to Intermediate form-A and the cell belonging to Intermediate form-B. Their results suggested that the *B. bigelowii* is likely to be a species complex composed of multiple genetically distinct species, but that this might be an example of pseudo-cryptic speciation, with size variation allowing discrimination of the species. However, the taxonomic position of the forms was not emended since the size range of these two forms overlapped, and only one cell was sequenced from each form. Recently, Konno et al (2007) reported additional variation in the surface

morphology of Intermediate forms -A and -B from Bering Sea. They classified their specimens into two morphological groups; flat and concave forms, respectively. The cause of the morphological variation found by Konno et al (2007) is unknown.

To study genetic distances between the morphotypes of *B. bigelowii*, we determined SSU rDNA sequences of cells of *B. bigelowii* based on the single cell PCR technique. Single cell PCR is an effective technique that enables molecular phylogenetic studies of protists such as *B. bigelowii*, which have never been successfully cultured. The technique has clarified the molecular phylogenetic positions of various non-culturable dinoflagellates (e.g., Edvardsen et al., 2003; Hansen and Daugbjerg, 2004; Takano and Horiguchi, 2004; Yamaguchi and Horiguchi, 2005) and of *B. bigelowii*, which was proven to be a coccolithophore (Takano et al, 2006). In the usual process of this technique, morphological observation prior to the genetic experiments is limited to light microscopy. Takano and Horiguchi (2006) did succeed in undertaking SEM observation of dinophyte isolates prior to the genetic studies, however this is not practical for the very small cell of *Braarudosphaera*. Fine morphological variation in pentaliths shown by Konno et al. (2007) is only visible under an SEM, and so cannot

practically be examined in the process of the single cell PCR technique. To estimate morphological characters of *B. bigelowii* sequenced, the morphology of *B. bigelowii* coccospheres was also studied under an SEM using the samples collected at the same sampling stations from which the cells used for genetic experiments were collected.

2. Materials and Method

2-1. Phylogenic studies of living *Braarudosphaera bigelowii*

Living cells of *Braarudosphaera bigelowii* were isolated from sea surface water samples collected using a bucket or a plankton-net from various parts of seas surrounding Japan (Table 1, Fig. 1). Prior to the isolation of cells, water samples collected by bucket were concentrated using a plankton-net with 1µm openings in the laboratory. Concentrated water samples were examined under an inverted light microscope (Olympus CKX41), and living cells of *B. bigelowii* encountered were isolated using a pipette. Each isolate was washed in sterilized seawater. For

measurements of size of pentoliths, the cell was photographed at x1000 magnification under a light microscope (Olympus CX50), following Horiguchi et al (2000). Side length of pentolith in each isolate was measured electronically using the software program Canvas 3.5.6 (Deneva). After measurement of the side length of pentoliths, the cell was subjected to single cell PCR amplification as outlined in Takano and Horiguchi (2004).

The first-round of PCR was performed using external primers, SR1 and LSU R2, to amplify almost the entire SSU rDNA, ITS, and partial LSU rDNA. In this study, however, only SSU rDNA sequences were used for analysis, because SSU rDNA data of *B. bigelowii* are available for comparison from previous work (Takano et al. 2006). The PCR condition for the first round was one initial denaturation at 94°C for 60s, followed by 35 cycles of denaturation at 94°C, annealing at 50°C for 30s and extension cycle at 72°C for 60s. 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 SSU rDNA region were amplified using the 0.5µL of the first round PCR product as DNA template with three or four pairs of primers; SR1c-SR3, SR1c-SR5, SR2TAK-SR7TAK, SR4-SR9,

SR8-SR12 and/or SR8TAK-SR12b. The sequences of primers used in this study were listed in Takano and Horiguchi (2004, 2006). The PCR condition for the second round was one initial denaturation step at 94°C for 30s, followed by 35 cycles of denaturation at 94°C for 30s, annealing at 55°C for 30s and extension cycle at 72°C for 30s. The temperature profile was completed by a final extension cycle at 72°C for 4 min. The products of second round PCR amplification were sequenced directly using the ABI PRISM BigDye Terminator Cycle Sequencing Kit (Perkin-Elmer, Foster City, CA, USA) and DNA auto sequencer ABI PRISM 3130 Genetic Analyzer (Perkin-Elmer). The results were confirmed by sequencing both forward and reverse strands.

Table 2 lists the species used for the analysis and their GenBank accession numbers. The SSU rRNA gene sequences were aligned manually for phylogenetic analysis, incorporating information on the secondary structure of the SSU rRNA molecule from the European ribosomal RNA database (Wuyts et al. 2004). The aligned sequences were examined using Maximum Likelihood (ML), Neighbor Joining (NJ), and Maximum Parsimony (MP) methods, using PAUP version 4.0b10 (Swofford, 2002). To decide which evolutionary model for ML fit the data best, the program Modeltest

3.06 (Posada and Crandali, 1998) was used. The models selected by the hierarchical likelihood ratio tests (hLRTs) and by the Akaike information criterion (AIC) for all data sets were the same: the Tamura and Nei (1993) model (TrN) with the proportion of invariable sites (I) and the variable sites (G); gamma distribution shape parameter for among-site rate variation, as calculated from the data (TrN+I+G; base frequencies and substitution parameters were estimated by Modeltest). ML was performed using the heuristic search option with a branch-swapping algorithm (Tree bisection-reconnection; TBR). Starting trees were obtained by stepwise random addition of sequences (10 replicates). The distance matrix was calculated using Kimura two parameter distances (Kimura, 1980), and the distance tree was constructed using the NJ method (Saitou and Nei, 1987). MP was performed using the heuristic search option with random addition of sequences (1000 replicates) and a branch-swapping algorithm (TBR). All characters were weighted equally and gaps were treated as missing. Bootstrap analyses with 100 replicates for ML analyses, and 1000 replicates for NJ and MP were applied to examine the robustness and statistical reliability of the topologies (Felsenstein, 1985). For ML bootstrapping, heuristic search option with a branch swapping algorithm, nearest

neighbor interchange (NNI) were employed.

2-2. Morphological studies of extant *Braarudosphaera bigelowii*

Scanning electron microscopic observations of *B. bigelowii* were made only if excess cells were available after genetic studies, and the preparation method of the SEM samples differed among the sampling stations depending on the kinds of sample available. Sufficient seawater was available from the samples from Shukutsu Harbor, and 1 liter of fresh seawater sample was filtered using type HA Millipore membrane filters. For morphological study of *B. bigelowii* from Tosa Bay, the concentrated seawater sample using plankton net was filtered again by membrane filter (type HA, Millipore) after 14 living cells were picked for genetic studies. The samples from Furue and Usuka Bays were originally collected for other biological studies, and were not available for filtering. For the morphological studies of *B. bigelowii* in these samples, cells of *B. bigelowii* were isolated using a pipette and then placed onto small pieces of membrane filter. All samples on membrane filters were air-dried. A portion of filter (ca. 5 x 5 mm) was mounted on an aluminum SEM stub using double sided tape and then

sputter-coated with platinum using an ion sputterer (E-1020; Hitachi Corp., Hitachinaka, Japan) for SEM observation. SEM images of *B. bigelowii* were obtained (Hitachi S-3000H and JEOL JSM-6390LAKII) and stored in a computer database.

3. Results.

3-1. Morphometric measurements of living cells used for single cell PCR

Prior to the genetic study, the side length of pentoliths of each isolate was measured on a light microscopic image, and the measured specimens were classified into size-morphotypes based on the size categories of Takano et al (2006) (Table 1). One specimen (Yatsushiro-1) from Yatsushiro Sea and two specimens (Furue-15 and Furue-16) from Furue Bay were extraordinarily large; c.a. 9.0 μm , 8.0 μm and 8.1 μm , respectively (Table 1, Plate 1). This is the first report of living specimens >7.5 μm in side length of pentoliths, and no morphotype name was available for this size range. Here we refer to the specimens >7.5 μm in side length of pentolith as Large form (Table

1).

3-2. Molecular phylogenetic studies

A total of 33 specimens of *B. bigelowii* were subjected to single cell PCR technique, and the almost complete SSU rDNA sequences were obtained from 13 cells in this study. Together with molecular data of two cells from Takano et al. (2006), *B. bigelowii* cells were classified into five Genotypes I-V (Tables 1, 3). The numbers of substitutions between each genotype are listed in Table 3. Genotype II had 2-bp degenerate sites; R (A and G) corresponding to A and G site of all other genotypes, respectively. For Maximum Likelihood (ML), Neighbor Joining (NJ) and Maximum Parsimony (MP) analyses, a total of 67 operational taxonomic units (OTUs) including four members of the Pavlovophyceae as an outgroup, were used. A likelihood score ($-\ln L = 8268.1113$) was obtained under the TrN+I+G model with the following parameters: assumed nucleotide frequencies A= 0.2260, C= 0.2229, G= 0.2897, and T= 0.2615; substitution-rate AC= 1, AG= 1.6126, AT= 1, CG= 1, CT= 4.4159, GT= 1;

proportion of sites assumed to be invariable = 0.6101; rates for variable sites assumed to follow a gamma distribution with shape parameter = 0.5149, and number of rate categories = 4, estimated by Modeltest 3.06. Parsimony analysis of the ca. 1540 bp of SSU rDNA sequences resulted in one most parsimonious tree of 1105 steps (CI = 0.498, RI = 0.810).

The ML, NJ, and MP analyses resulted in similar trees those obtained by previous studies (e.g., Edvardsen et al., 2000; Nakayama et al., 2000; Takano et al., 2006). Here we show only ML tree with bootstrap consensus values obtained from ML, NJ, and MP analyses (Fig. 2). Five genotypes of *B. bigelowii* made a clade with very high bootstrap supports (99/100/100%; ML/NJ/MP). This clade was included in Clade C⁺ of Takano et al. (2006), consisting of Coccolithales, Isochrisidales, Chrysoculterceae, Pontosphaeraceae, Rhabdosphaeraceae, Syracosphaeraceae, and *Coronosphaera*, with quite low bootstrap values (< 50%) (Fig. 2). Due to low bootstrap support, branching order of *Braarudosphaera* clade in the Clade C⁺ is unknown. Branching order of the genotypes in the *B. bigelowii* clade; Genotype ((I, II) (III (IV, V))) was consistent in the ML, NJ, and MP trees, and all subclades of genotypes were supported by high bootstrap

values in all analyses (Fig. 2).

3-3. Scanning Electron Microscopic studies of natural *B. bigelowii* population

In this study, a total of 34 specimens of *B. bigelowii* were measured under an SEM. The morphometric data of *B. bigelowii* reported by Takano et al (2006) were incorporated in this study, and the number of available size data increased to 181 (Fig. 3). The morphometric data shows a multi-modal distribution pattern basically similar to the results shown by Takano et al (2006), although extraordinarily large specimens >7.5 μm in side length of pentoliths (Large form) were found from Furue and Usuka Bays in this SEM study (Fig. 3). The morphotype composition of *B. bigelowii* was different among the sampling stations. *B. bigelowii* population in Shukutsu harbor, Tosa Bay, and Furue Bay were dominated by Intermediate form-A, Intermediate form-B, and Large form, respectively. In Usuka Bay, Intermediate form-B was most abundant, but one Intermediate form-A and one Large form cells were also found.

SEM observations showed fine morphological variations of surface nature of pentoliths within/among size-morphotypes. In the eight Intermediate form-A specimens

from Shukutsu-Harbor, four specimens had flat pentaliths (Plate 2, Fig. 1), and remaining four specimens had concaved pentaliths (Plate 2, Fig. 2). All specimens from Shukutsu-Harbor had some particles, look like overgrowth of calcite, on their distal surface. The number and size of the particles differed greatly between the specimens. Typically, small particles occurred sporadically at and around the contact surface of adjacent pentaliths (Plate 2, Figs. 1-2), however, those particles occasionally developed well and covered the distal surface of pentaliths (Plate 2, Fig. 3). Regardless of variation in the surface nature of pentaliths, the corners of pentaliths of the specimens from Shukutsu Harbor were always sharp, and the pentaliths butted closely on the coccosphere, without spaces on the distal surface (Plates 2, Figs. 1-3).

The Intermediate form-B specimens from Tosa and Usuka Bays and Large form specimens from Furue Bay resembled each other, but were slightly different from the Intermediate form-B specimens from Tsugaru Warm Current regime, which were photographed for morphometric studies of previous studies (Hagino, 1997; Hagino et al., 2005; Takano et al., 2006). The Intermediate form-B specimens from the Tsugaru Warm Current regime had dodecahedral coccospheres without apparent spaces at the contact

surface between adjacent pentaliths (Plate 3, Fig. 1). Those closely-attached contact surfaces stem from gradual size increase of tightly piled up laminae (Plate 3, Fig. 2). Corners of several distal laminae are slightly rounded, and result in a very small space at the joining point of corners of three pentaliths (Plate 3, Fig. 1). The Intermediate form-B specimens from Tosa and Usuka Bays and Large form specimens from Furue Bay had spaces at the contact surface between the adjacent pentaliths at the distal surface level of the coccosphere (Plate 2, Figs. 4-6; Plate 3, Figs. 3-6). Those spaces between the adjacent pentaliths stem from concave laminae near distal surface (Plate 3, Figs. 5-6). The distal surface laminae often fall off the pentaliths, then the surface nature of inner laminae is exposed (Plate 2, Figs. 5-6; Plate 3, Figs. 4-5). Whereas the distal and proximal surface laminae have smooth surfaces, the inner laminae have fine grooves parallel to the c-axis crystallographic direction of the calcite of each segment (Plate 2, Fig 6; Plate 3, Fig. 4). Tightly piled up proximal side laminae have sharp corners, while, concave distal side laminae often had rounded corners (Plate 2, Fig. 6; Plate 3, Fig. 6). Roundness of corners of concave laminae increases toward the distal side (Plate 2, Fig. 6).

4. Discussion

4-1. Pseudo-cryptic speciation of *Braarudosphaera bigelowii*

The five recognized SSU rDNA genotypes showed a clear relationship with the morphotypes based on side length of pentaliths, but not with sampling area (Table 1, Fig. 3). All five specimens of both Genotypes I and II were included in the Intermediate form-A in size range. The seven specimens belonging to Genotype III all correlated with Intermediate form-B in size. The three specimens of Genotypes VI and V had large pentaliths > 7.5 μm in side length, and can be assigned to Large form. Since Genotype III (Intermediate form-B) coexisted with Genotype I (Intermediate form-A) in the Tsugaru Strait, and with Genotype IV (Large form) in the northwest coast of Hirado Island (Furue and Usuka Bays), it appears very likely that the genetic variations between Genotypes I, III, and IV are results of speciation, not merely an intra-populational variation. Only one substitution separates genotypes I from II, and

both are included in Intermediate form-A in size category. The Funahama-T3 specimen is the only example of Genotype II, and so the size range of Genotype II is unknown. For further discussion of relationships between Genotype I and II, additional size information of this genotype is required. The Genotype V specimen (Yatsushiro-1) was larger than the specimens of Genotype IV (Furue 15 and 16), and two substitutions exist between the Genotypes V and IV (Table 3, Fig. 3). These results suggest speciation between the Genotypes IV and V is taking place, and is expressed as the size variations. However, there is insufficient molecular and morphometric data to allow definite conclusions on the relationship of size and genetic differences between Genotypes IV and V (Table 1, Fig. 1).

Morphological variations in the surface nature of pentoliths were observed across size-morphotype separations, even though size-morphotypes can be correlated with genotypes. Specimens with flat surface and without spaces at the contact surface between the adjacent pentoliths were found from both Intermediate form-A and Intermediate form-B populations (Hagino et al., 2005; Konno et al., 2007). Concave specimens with sharp corners of pentolith and without spaces at contact surface between

adjacent pentoliths were only found in Intermediate form-A population in this study, but have been reported from an Intermediate form-B population from the Bering Sea (Konno et al., 2007). The specimens characterized by rounded corners of pentoliths and by the spaces at the contact surface between the adjacent pentoliths were observed from both Intermediate form-B and Large form populations. The causes of morphological variations in the surface nature of pentoliths are unknown, however, morphological variations found across the different size-morphotypes appears likely to be a result of intra-specific variation rather than inter-specific difference, since relationships between genotypes and size-morphotypes is generally congruent.

Of the morphological features examined in this study, only pentolith size can be correlated with genotypes of *B. bigelowii*. In their original description, Gran and Braarud (1935) gave the cell diameter of *B. bigelowii* (as *Pontosphaera bigelowii*) as 16µm, and this is equivalent to a side length of its pentolith of ca. 6µm. So, of the size-morphotypes observed in this study, Genotype III corresponds to the original description of *B. bigelowii* by Gran and Braarud (1935) in size. SSU rDNA is a conservative gene, and substitutions found in this genetic region have been interpreted

as indicative of speciation (e.g., Saéz et al., 2003). Therefore, Genotypes I, II, IV, and V can be considered as pseudo-cryptic species of typical *B. bigelowii* (Genotype III).

4.2. Size variation of *Braarudosphaera bigelowii* with genetic diversification

Nine common substitutions as well as six insertion/deletions differentiate Genotypes III-V from Genotypes I and II, and an additional three common substitutions separate Genotypes IV and V from Genotype III (Table 3). Therefore, it is evident that the common ancestor of Genotypes IV and V was separated from Genotype III after the separation of Genotype III from the common ancestor of Genotypes I and II, as the phylogenetic tree indicate (Fig. 2). Furthermore, two characteristic substitutions found in the specimen Yatsushiro-1 (Genotype V) reveal that Genotypes V was separated from Genotype IV after the common ancestor of Genotypes IV and V was separated from Genotype III (Table 3, Fig. 2). Interestingly, specimens of Genotypes IV were larger than the specimens of Genotype III, and the specimen Yatsushiro-1 (Genotype V) was larger than two specimens of Genotype IV (Fig. 3), although it is still uncertain whether the extraordinarily large size of the specimen Yatsushiro-1 (Genotype V) can be

correlated with genetic separation from Genotype IV or not. Comparison of divergence order and size range of genotypes, however, suggests gradual size enlargement of pentalith along with genetic diversification from Genotypes III-V. Does this size-increasing trend stem from some evolutionary trend, or is it just an accidental result without evolutionary trend? At this moment, we do not have enough evidence to answer this question, but the geological history of other coccolithophore lineages gave us some sort of clue for this question.

It is known that common coccolithophore lineages with continuous fossil records show time-progressive size increase episodes repeatedly in their history, although the causes of these size enlargements have not been determined. Typically, new genera start their history with relatively small-sized species, and then show gradual size increase at the some point of their history. Those size enlargement events are often terminated by the abrupt extinction of the enlarged population, and surviving small population then becomes the source of the next diversification of the genus with size variation (enlargement) in the following ages. This type of size-increase events and the subsequent extinction events of the enlarged population have been studied in detail for

many lineages of placolith-bearing genera, notably including *Gephyrocapsa* (Matsuoka and Okada 1989, 1990; de Kaenel et al., 1999), *Reticulofenestra* (Young, 1990, 1998), *Pseudoemiliana* (Young 1990, de Kaenel et al., 1999), and *Calcidiscus* (Knappertsbush, 2000). Genetic evidence to explain speciation with size variation of coccolithophores is limited, due to shortage of culture strains. Modern *Coccolithus* consists of two species differentiated from each other in size range of coccoliths and in fine morphological features; *Coccolithus pelagicus* (6-10µm in coccolith length with closed central area) and *Coccolithus braarudii* (9-15 µm in coccolith length with open central area). These two species are identical in SSU rDNA sequences but are different from each other in morphology of holococcoliths produced in haploid phase, and in ITS and *tufA* sequences (Sáez et al., 2003, Geisen et al. 2004). Due to the lack of genetic and morphological data of their common ancestor, process of their diversification with size change was unknown. Living *Calcidiscus* consists of three size-differentiated taxa; *Calcidiscus quadriperforatus* (7-11µm in coccolith diameter, obscured sutures around the crest of tube), *Calcidiscus leptoporus* (5-8 µm in coccolith diameter, distal shield sutures smoothly curved), and *Calcidiscus leptoporus* small type (3-5 µm in coccolith

diameter, distal shield sutures angular and serrated) (Kleijne 1993; Geisen et al., 2002; Geisen et al., 2004). Detailed biostratigraphic studies by Knappertsbush (2000) suggest that *C. quadriperforatus* (his morphotype L) separated from *C. leptoporus* (his morphotype I) in the late Miocene (c.a. 9.8 Ma), *C. leptoporus* separated from *C. leptoporus* small type (his morphotype S) in the early Miocene (20.1 Ma), and *C. leptoporus* small (his morphotype S) appeared from unknown ancestor in the Early Miocene or earlier. Currently, culture strains and genetic data are only available for *C. quadriperforatus* and *C. leptoporus*, and the divergence age between *C. quadriperforatus* and *C. leptoporus* was estimated to 11.57 ± 1.61 Ma based on *tufA* sequence (Sáez et al., 2003). This divergence age based on *tufA* sequence is almost consistent to their fossil records (Knappertsbush, 2000). Unfortunately, *C. leptoporus* small type has not yet been cultured nor sequenced. If genetic data of *C. leptoporus* small type is obtained, evolutionary process of *Calcidiscus* with size enlargement will be revealed both fossil and genetic evidences.

At the moment, the genetic mechanism controlling the size of *B. bigelowii* is unknown, however, it is certain that the size of *B. bigelowii* pentoliths is controlled

genetically (i.e. not environmentally affected), and is almost certainly an important criterion for the classification of *Braarudosphaera*. When did the lineage of *B. bigelowii* start diversification with size variation in their history? Unlike other coccolithophore lineages, information on size of fossil *Braarudosphaera* is very limited, because *B. bigelowii* is not useful for biostratigraphic datum and occurs only sporadically in open ocean sediments. Only Svábenická (1999) reported size variation of fossil *B. bigelowii* from Bohemian Cretaceous Basin (Turonian), and showed that *B. bigelowii* population from Turonian consists of two size-groups; small (5-8 μm) and normal-sized (12-14 μm) specimens. Svábenická (1999) did not describe her measurement method in detail, but it is safe to assume that she measured the maximum length of pentaliths not of side length of pentaliths. Hence, the side lengths of her small and normal morphotypes are calculated to 3.2-5.3 and 7.8-9.1 μm , respectively. Her observation indicates that lineage of *B. bigelowii* has already diversified greatly in size in Turonian; within 10-my of their appearance in the Cenomanian or Turonian (Perch-Nielsen, 1985a). Interestingly, the small morphotype of Svábenická (1999) does not overlap with size range of living population of *Braarudosphaera*. No morphometric data is available for the lineage of *B.*

bigelowii from the Coniacian through Holocene (89.3Ma-present), however, photographs of *B. bigelowii* pentoliths shown in several papers indicate that *B. bigelowii* had variation in their size in the Early Danian, immediately after the K/T mass extinction. From the Early Danian deposits of various geological settings, very small specimens (c.a. 2.5µm in side length), intermediate-sized specimens (4-6 µm in side length), and very large specimens exceeding 10 µm in side length of the pentoliths were reported (e.g., Okada and Thierstein, 1979; Mai et al., 1997; Tantaway, 2003; Lamolda et al., 2005). These results indicate that *B. bigelowii* populations in Early Danian had unusually large specimens that have never been reported from Turonian and living populations. Since the biostratigraphic record of *B. bigelowii* is discontinuous, the evolutionary history of size variation in the lineage of *B. bigelowii* is unknown. However, inconsistency of size range of pentoliths among Turonian, Early Danian, and Present indicates that the lineage of *B. bigelowii* had size variation events multiple times in their history.

The lineage of *B. bigelowii* was surprisingly stable in their general morphology of pentoliths through their >90 my long history. Since SSU rDNA

genotypes and size-morphotypes of living *B.bigelowii* show good correspondence, it is almost safe to say that the genetic diversification of this lineage can be reflected to the size diversification of pentaliths also in geological ages. In turn, diversification in size of *B. bigelowii* pentaliths in geological ages could be indicative of genetic diversification in the past. Inconsistency of size range of pentaliths among different geological ages suggests that the lineage of *B. bigelowii* had experienced pseudo-cryptic speciation (or genetic diversification) with size changes multiple times in their history, similar to the other lineages of coccolithophores.

5. Summary

1. Partial SSU rDNA sequences were determined for thirteen isolates of *Braarudosphaera bigelowii* independently using the single cell PCR technique. Based on the SSU rDNA sequences, sequenced specimens were classified into five Genotypes, I-V. These SSU rDNA genotypes showed consistent relationships with

morphotypes of *B. bigelowii* based on size, but not with sampling area. From these facts, the genetic differences among genotypes of *B. bigelowii* can be regarded as results of speciation, not of geographic isolation.

2. The surface nature of living *B. bigelowii* specimens was investigated under an SEM using natural samples. Similar morphological variations were observed across size-morphotypes, which were genetically distinct from each other. Therefore, fine morphological variations observed across the size-morphotypes were regarded as intra-specific variations.

3. In the morphological factors examined in this study, only pentolith size can be correlated with genotypes of *B. bigelowii*. On the basis of size, Genotype III is comparable with the holotype of *B. bigelowii* as described by Gran and Braarud (1935). As a consequence, other four genotypes are considered as pseudo-cryptic sibling species of *B. bigelowii*.

4. The genetic variation observed among size-morphotypes of living *Braarudosphaera*, together with size variation in fossil populations of various geological ages, suggest that the so-called *B. bigelowii* is a species complex of many biologically distinct

species, and that the lineage of *B. bigelowii* have experienced pseudo-cryptic speciation with size variation repeatedly in their >90 my history.

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Plate Captions

Plate 1. Light micrographs of the specimens used for the molecular phylogenic studies in this study. Fig. 1: Funahama-T3, Fig. 2: Shukutsu-19, Fig. 3: Shukutsu-22, Fig. 4: Shukutsu-27, Fig. 5: Usuka-15.5.6, Fig. 6: Tosa-2, Fig. 7: Tosa-5, Fig. 8: Tosa-6. Fig. 9: Tosa-8, Fig. 10: Tosa-14, Fig. 11: Yatsushiro-1, Fig. 12: Furue-15, and Fig. 13: Furue-16.

Plate 2. Scanning micrographs of Intermediate form-A and Large form. Fig. 1: Intermediate form-A specimen with flat pentoliths from Shukutsu Harbor. Note small particles at and around the contact surface of adjacent pentoliths (arrows), Fig. 2: Intermediate form-A specimen with concave pentoliths from Shukutsu Harbor. Note small particles at and around contact surface of adjacent pentoliths (arrows), Fig. 3: Intermediate form-A specimen with developed attached particles from Shukutsu Harbor, Fig. 4: Large form specimen from Furue Bay, Fig. 5: Large form specimen from Furue Bay. Note distal surface layers of pentoliths fall off from the pentoliths (arrows), Fig. 6: High-magnification image of the specimen of Fig. 5. Note-1. Fine grooves run along the c-axis of crystal of each segment. Note-2. Roundness of corners of

pentaliths increases toward distal surface.

Plate 3. Scanning micrographs of Intermediate form-B. Fig. 1: Intermediate form-B specimen from Tsugaru strait, Fig. 2: Collapsed coccosphere of Intermediate form-B from Tsugaru strait, Fig. 3: Intermediate form-B specimen from Tosa Bay, Fig. 4: Intermediate form-B specimen from Tosa Bay. Note distal surface layers have partially fallen off the pentaliths, and inner layers are exposed. Fine grooves run along the c-axis of calcite crystal of each segment. Fig. 5: Collapsed specimen of Intermediate form-B from Tosa Bay, Fig. 6: High magnification image of Fig. 5. Note proximal-side layers have sharp corners.

Table Captions

Table 1. Sampling date, location, in situ sea surface water temperature (SST), sampling method, and morphometric and genetic information of each isolate used for molecular phylogenetic studies of *Braarudosphaera bigelowii*.

Table 2. List of Haptophyte algae included in the phylogenetic study.

Table 3. Number of observed substitutions between SSU rDNA sequences of examined specimens. Insertions or deletions are given in parentheses. Also right column, genotype assignments based on SSU rDNA sequences

Figure Captions

Figure 1. Distribution of surface currents around the Japanese islands (Tomczak and Godfrey, 1994) and location of samples used in this study. (a) Arrows with solid and dotted lines indicates warm and cold currents, respectively. Solid circles in (b)-(d) represent the locations of samples.

Figure 2. Phylogenetic tree based on the SSU rDNA sequences using the Maximum Likelihood Method (ML) ($-\ln L = 8268.1113$). Representatives of the Pavlovophyceae were used as an outgroup. Asterisks refer to the clade names of Edvardsen et al. (2000). The numbers on each node indicate the bootstrap values from ML, NJ, and MP analyses. New SSU rDNA sequences obtained

for this study are indicated in boldface.

Figure 3. Frequency distribution for side length of pentalith of *Braarudosphaera bigelowii*, size range of morphotypes, and relationships between size distribution and SSU rDNA Genotypes. Numbers in parentheses indicate the number of measured specimens in each sample. Morphometric data from stations 1 and 3 of KT90-9 and from st. 13 of KR03-06 were from those of Takano et al (2006).

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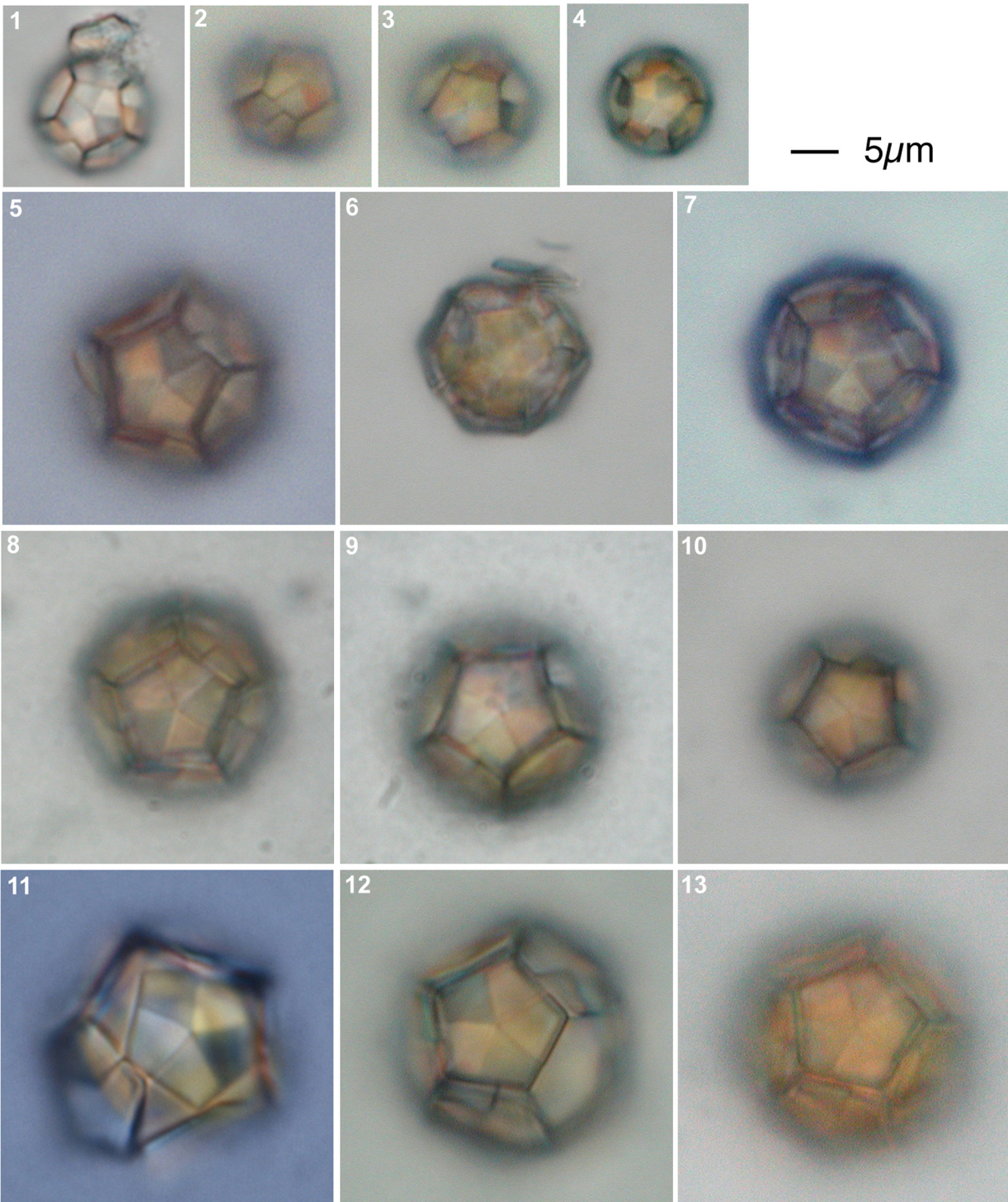
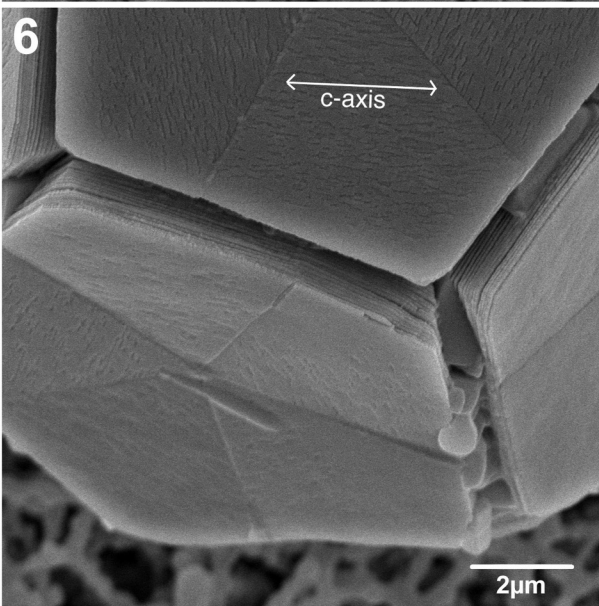
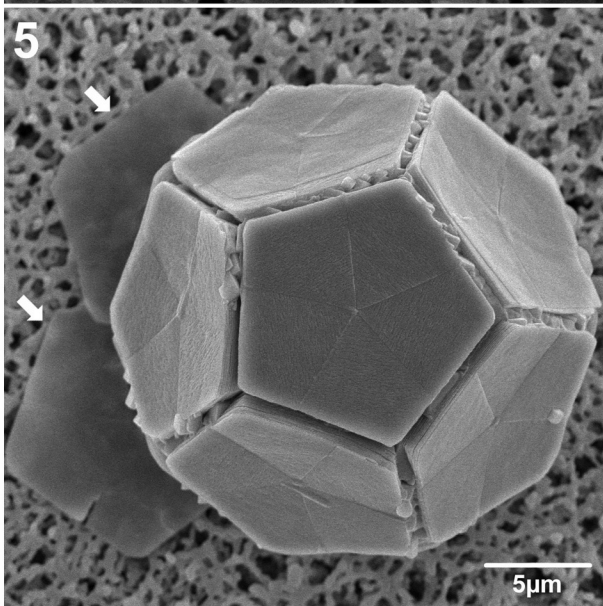
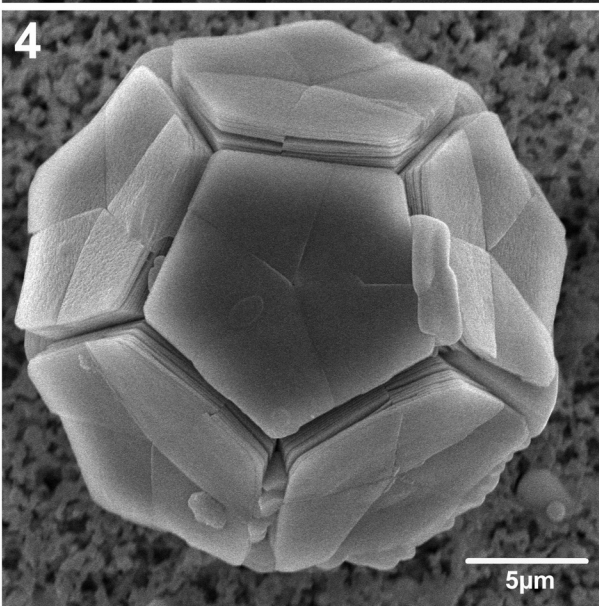
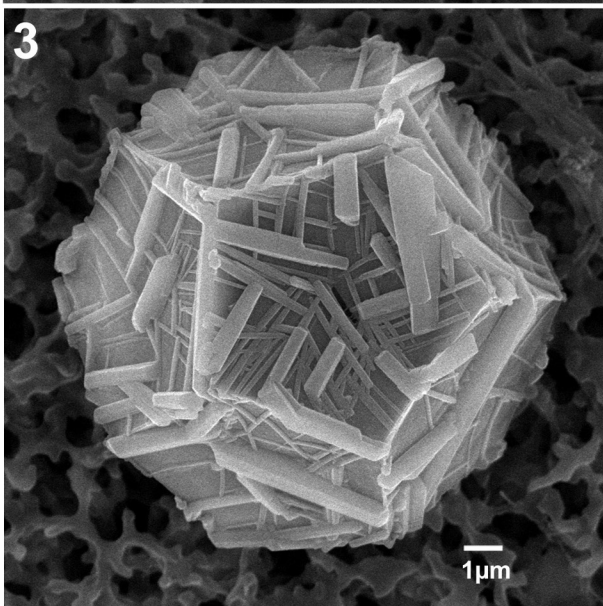
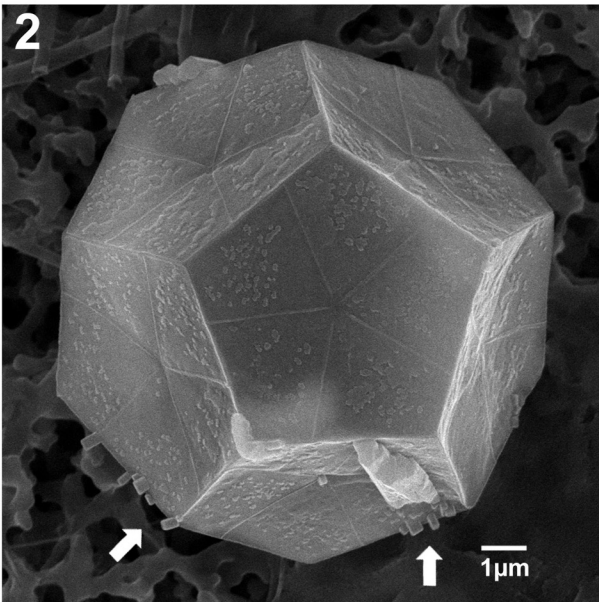
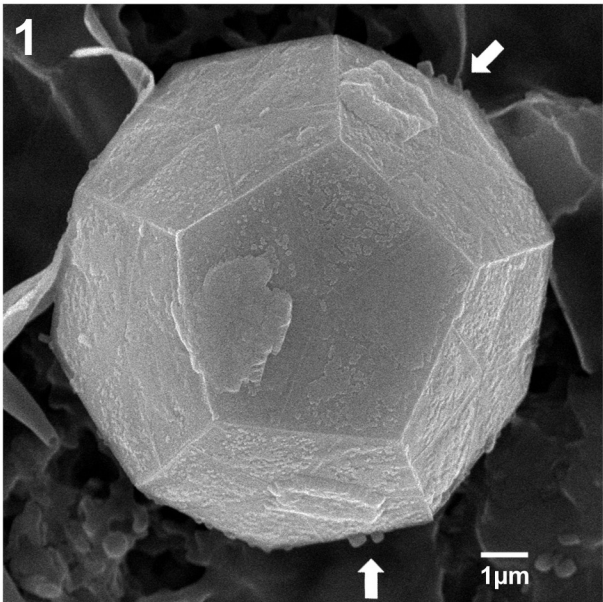


Plate 1, Hagino et al



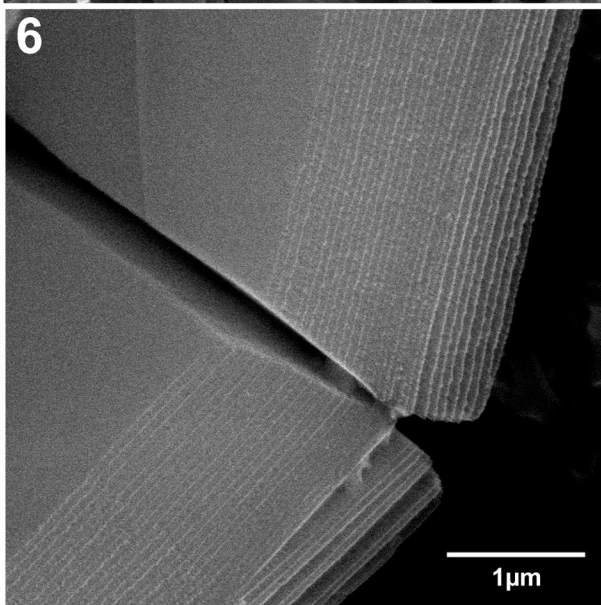
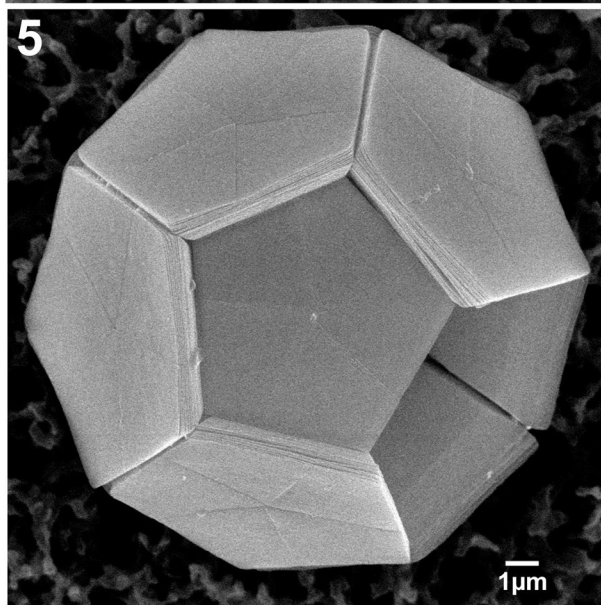
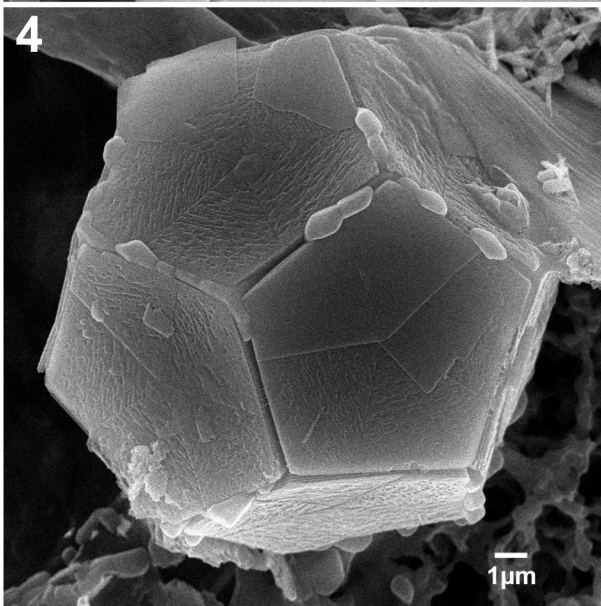
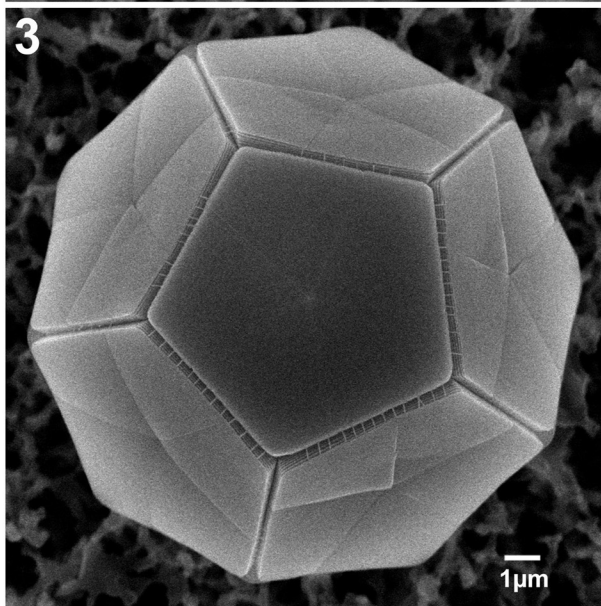
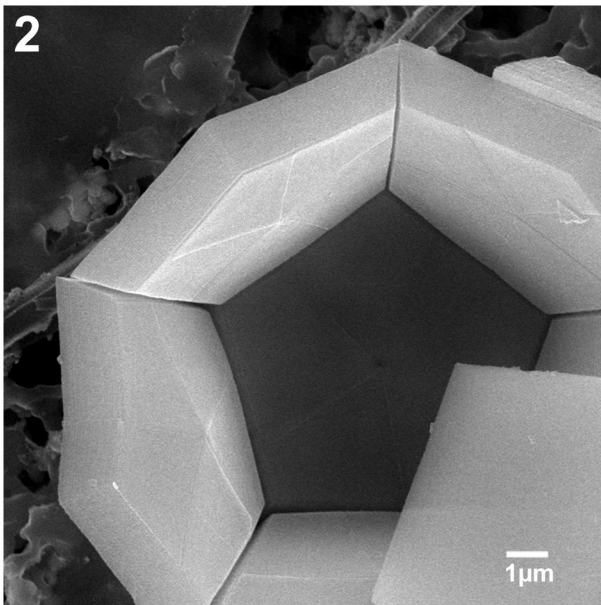
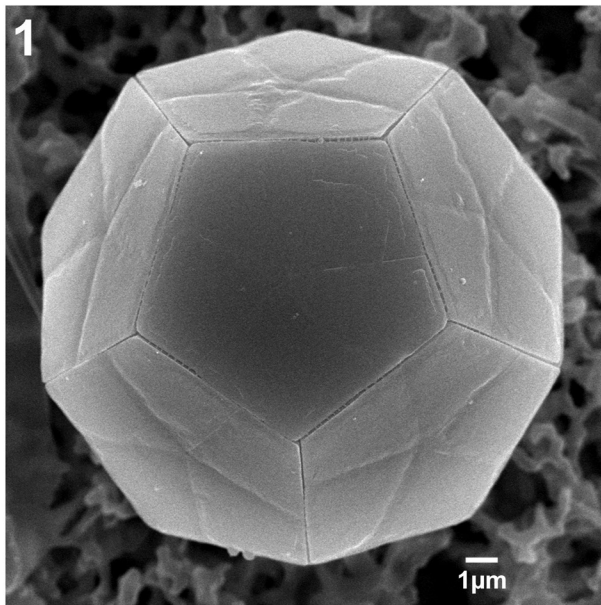


Table 1. Sampling date, location, in situ sea surface water temperature (SST), sampling method, and morphometric and genetic information of each isolate used for molecular phylogenetic studies of *Braarudosphaera bigelowii*.

Specimen number	Sampling date	Location	Latitude	Longitude	in situ SST	Sampling tool	Side length of pentolith (μm)	morphotype	SSU rDNA genotype	reference
TP05-6-a	2005.6.21	Tsugaru Strait, Aomori, st. 5	41°29.0N	141°14.2E	15.3	bucket	6.0	Intermediate form-B	III	Takano et al, 2006
TP05-6-b	2005.6.21	Tsugaru Strait, Aomori, st. 9	41°32.6N	141°17.4E	15.1	bucket	4.0	Intermediate form-A	I	Takano et al, 2006
Yatsushiro-1	2006.6.22	Sea of Yatsushiro, Kumamoto	32°21.5N	130°15.4E	-	plankton net	c.a. 9.0	Large form	V	this study
Funahama-T3	2006.7.2	Funahama coast, Hokkaido	43°10.9N	141°02.3E	19.5	bucket	4.7	Intermediate form-A	II	this study
Usuka-15.2.6	2007.5.23	Usuka Bay, Hirado Isl. Nagasaki	33°22.1N	129°31.4E	n.a.	bucket	6.8	Intermediate form-B	III	this study
Tosa-2	2007.6.1	Tosa Bay, Kochi	33°38.3N	133°53.6E	21.2	bucket	5.6	Intermediate form-B	III	this study
Tosa-5	2007.6.1	Tosa Bay, Kochi	33°38.3N	133°53.6E	21.2	bucket	6.7	Intermediate form-B	III	this study
Tosa-6	2007.6.1	Tosa Bay, Kochi	33°38.3N	133°53.6E	21.2	bucket	7.0	Intermediate form-B	III	this study
Tosa-8	2007.6.1	Tosa Bay, Kochi	33°38.3N	133°53.6E	21.2	bucket	6.1	Intermediate form-B	III	this study
Tosa-14	2007.6.1	Tosa Bay, Kochi	33°38.3N	133°53.6E	21.2	bucket	6.1	Intermediate form-B	III	this study
Furue-15	2007.6.3	Furue Bay, Hirado Isl. Nagasaki	33°22'0N	129°31.1E	21.3	bucket	8.1	Large form	IV	this study
Furue-16	2007.6.3	Furue Bay, Hirado Isl. Nagasaki	33°22'0N	129°31.1E	21.3	bucket	8.0	Large form	IV	this study
Shukutsu-19	2007.6.17	Shukutsu Harbor, Hokkaido	43°14.0'N	141°1.1'E	17.0	bucket	4.6	Intermediate form-A	I	this study
Shukutsu-22	2007.6.17	Shukutsu Harbor, Hokkaido	43°14.0'N	141°1.1'E	17.0	bucket	4.9	Intermediate form-A	I	this study
Shukutsu-27	2007.7.3	Shukutsu Harbor, Hokkaido	43°14.0'N	141°1.1'E	18.0	bucket	4.1	Intermediate form-A	I	this study

Table 2. List of Haptophyte included in the phylogenetic study.

Species	GenBank accession numbers
<i>Algirospira robusta</i> (Lohmann) Norris	AM490985
<i>Braarudosphaera bigelowii</i> (Gran & Braarud) Deflandre, TP05-6A	AB250784
<i>Braarudosphaera bigelowii</i> (Gran & Braarud) Deflandre, Usuka-15.2.6	= AB250784
<i>Braarudosphaera bigelowii</i> (Gran & Braarud) Deflandre, Tosa-2	= AB250784
<i>Braarudosphaera bigelowii</i> (Gran & Braarud) Deflandre, Tosa-5	= AB250784
<i>Braarudosphaera bigelowii</i> (Gran & Braarud) Deflandre, Tosa-6	= AB250784
<i>Braarudosphaera bigelowii</i> (Gran & Braarud) Deflandre, Tosa-8	= AB250784
<i>Braarudosphaera bigelowii</i> (Gran & Braarud) Deflandre, Tosa-12	= AB250784
<i>Braarudosphaera bigelowii</i> (Gran & Braarud) Deflandre, TP05-6B	AB250785
<i>Braarudosphaera bigelowii</i> (Gran & Braarud) Deflandre, Shukutsu-19	= AB250785
<i>Braarudosphaera bigelowii</i> (Gran & Braarud) Deflandre, Shukutsu-22	= AB250785
<i>Braarudosphaera bigelowii</i> (Gran & Braarud) Deflandre, Shukutsu-27	= AB250785
<i>Braarudosphaera bigelowii</i> (Gran & Braarud) Deflandre, Funahama-T3	AB478412
<i>Braarudosphaera bigelowii</i> (Gran & Braarud) Deflandre, Furue-15	AB478413
<i>Braarudosphaera bigelowii</i> (Gran & Braarud) Deflandre, Furue-16	= AB478413
<i>Braarudosphaera bigelowii</i> (Gran & Braarud) Deflandre, Yatsushiro-1	AB478414
<i>Calcidiscus leptoporus</i> (Murray & Blackman) Leblach & Tappan	AJ544116
<i>Calcidiscus quadriperforatus</i> (Kamptner) Quinn & Geisen	AJ544115
<i>Calyptrosphaera radiata</i> Sym & Kawachi	AM491024
<i>Calyptrosphaera</i> sp.	AB183608
<i>Calyptrosphaera</i> sp.	AM490988
<i>Calyptrosphaera</i> sp.	AM490992
<i>Calyptrosphaera sphaeroidea</i> Schiller	AM490990
<i>Chrysochromulina acantha</i> Leadbeater & Manton	AJ246278
<i>Chrysochromulina campanulifera</i> Manton & Leadbeater	AJ246273
<i>Chrysochromulina hirta</i> Manton	AJ246272
<i>Chrysochromulina kappa</i> Parke & Manton	AJ246271
<i>Chrysochromulina polylepis</i> Manton & Parke	AJ004866
<i>Chrysochromulina scutellum</i> Eikrem & Moestrup	AJ246274
<i>Chrysochromulina thronsdonii</i> Eikrem	AJ246277
<i>Crysoculter rhomboidea</i> Nakayama, Yoshida, Noël, Kawachi & Inouye	AB158370
<i>Chrysotila lamellosa</i> Anand	AM490998
Coccolith haptophyte	U40925
Coccolith haptophyte	U40924
<i>Coccolithus braarudii</i> (Gaarder) Geisen, Billard, Broerse, Cros, Probert, Young	AJ5441171
<i>Coccolithus pelagicus</i> (Wallich) Schiller	AJ246261
<i>Coronosphaera mediterranea</i> (Kamptner) Heimdal	AM490986
<i>Cruciplacolithus neohelis</i> (McIntyre & Bé) Reinhardt	AJ246262
<i>Emiliana huxleyi</i> (Lohmann) Hay & Mohler	L04957
<i>Emiliana huxleyi</i> (Lohmann) Hay & Mohler	M87327
<i>Gephyrocapsa oceanica</i> Kamptner	AJ246276
<i>Helicosphaera carteri</i> (Wallich) Kamptner	AM490983
<i>Helladosphaera</i> sp.	AB183607
<i>Helladosphaera</i> sp.	AM490991
<i>Holococcolithophorid</i> sp.	AM490989
<i>Hymenomonas coronata</i> Mills	AM490982
<i>Hymenomonas globosa</i> (Magne) Gayral & Fresnal Hap	AM490981
<i>Imantonia rotunda</i> Reynolds emend. Green & Pienaar	AJ246267
<i>Imantonia rotunda</i> Reynolds emend. Green & Pienaar	AM491014
<i>Imantonia</i> sp.	AM491015
<i>Isochrysis galbana</i> Parke emend. Green & Pienaar	AJ246266
<i>Isochrysis litoralis</i> Billard & Gayral	AM490996
<i>Jomonolithus litoralis</i> Inoue & Chihara	AM490979
<i>Ochrosphaera verrucosa</i> Schussnig	AM490980
<i>Oolithus fragilis</i> (Lohmann) Martini & Müller	AM491026
<i>Pavlova gyrans</i> Butecher emend. Green & Manton	U40922
<i>Pavlova salina</i> (Carter) Green	L34669
<i>Pavlova</i> sp.	AJ243369
<i>Phaeocystis antarctica</i> Karsten	X77481
<i>Phaeocystis antarctica</i> Karsten	X77479
<i>Phaeocystis cordata</i> Zingone & Chrétiennot-Dinet	AF163147
<i>Phaeocystis globosa</i> Scherffel	X77476
<i>Phaeocystis jahnii</i> Zingone	AF163148
<i>Phaeocystis pouchetii</i> (Hariot) Lagerheim	X77475
<i>Pleurochrysis carterae</i> (Braarud & Fagerland) Christensen	AJ246263
<i>Pleurochrysis elongata</i> (Droop) Jordans	AJ246264
<i>Pleurochrysis</i> sp.	AJ246265
<i>Prymnesium calathiferum</i> Chang & Ryan	U40923
<i>Prymnesium nemamethecum</i> Pienaar & Birkhead	AJ246268
<i>Prymnesium parvum</i> Carter emend. Green, Hibberd & Pienaar	AJ246269
<i>Prymnesium patelliferum</i> Green, Hibberd & Pienaar	L34670
<i>Reticulosphaera socialis</i> Grell	X90992
<i>Scyphosphaera apsteinii</i> Lohmann	AM490984
<i>Syracosphaera pulcha</i> Lohmann	AM490987
<i>Umbilicosphaera foliosa</i> (Kamptner) Geisen	AJ544119
<i>Umbilicosphaera hultburtiana</i> Gaarder	AM490993
<i>Umbilicosphaera sibogae</i> (Weber-van Bosse) Gaarder	AJ544118

Table 3. Number of observed substitutions among SSU rDNA sequences of examined specimens, and genotypes identified based on SSU rDNA sequences. Insertions or deletions are given in parentheses.

specimen number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	SSU rDNA genotype
1 TP056-B	-															I
2 Shukutsu-19	0	-														
3 Shukutsu-22	0	0	-													
4 Shukutsu-27	0	0	0	-												
5 OCH06-T3	1	1	1	1	-											II
6 TP0506-A	10 (6)	10 (6)	10 (6)	10 (6)	11 (6)	-										III
7 Usuka-15.2.6.5	10 (6)	10 (6)	10 (6)	10 (6)	11 (6)	0	-									
8 Tosa-2	10 (6)	10 (6)	10 (6)	10 (6)	11 (6)	0	0	-								
9 Tosa-5	10 (6)	10 (6)	10 (6)	10 (6)	11 (6)	0	0	0	-							
10 Tosa-6	10 (6)	10 (6)	10 (6)	10 (6)	11 (6)	0	0	0	0	-						
11 Tosa-8	10 (6)	10 (6)	10 (6)	10 (6)	11 (6)	0	0	0	0	0	-					
12 Tosa-14	10 (6)	10 (6)	10 (6)	10 (6)	11 (6)	0	0	0	0	0	0	-				IV
13 Furue-15	12 (6)	12 (6)	12 (6)	12 (6)	13 (6)	4	4	4	4	4	4	4	-			
14 Furue-16	12 (6)	12 (6)	12 (6)	12 (6)	13 (6)	4	4	4	4	4	4	4	0	-		
15 Yatsushiro-1	14 (6)	14 (6)	14 (6)	14 (6)	15 (6)	6	6	6	6	6	6	6	2	2	-	V

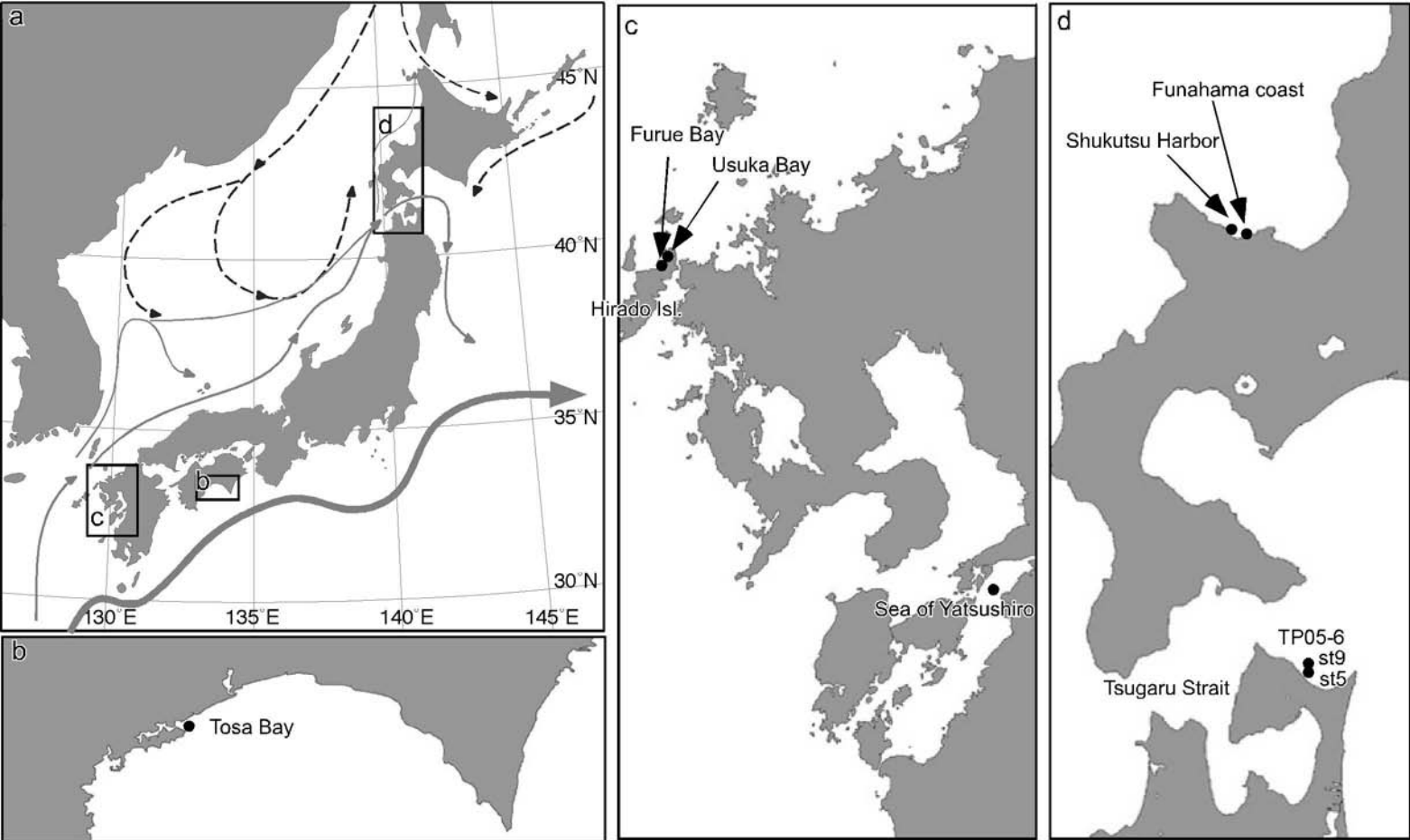


Fig. 1, Hagino et al.

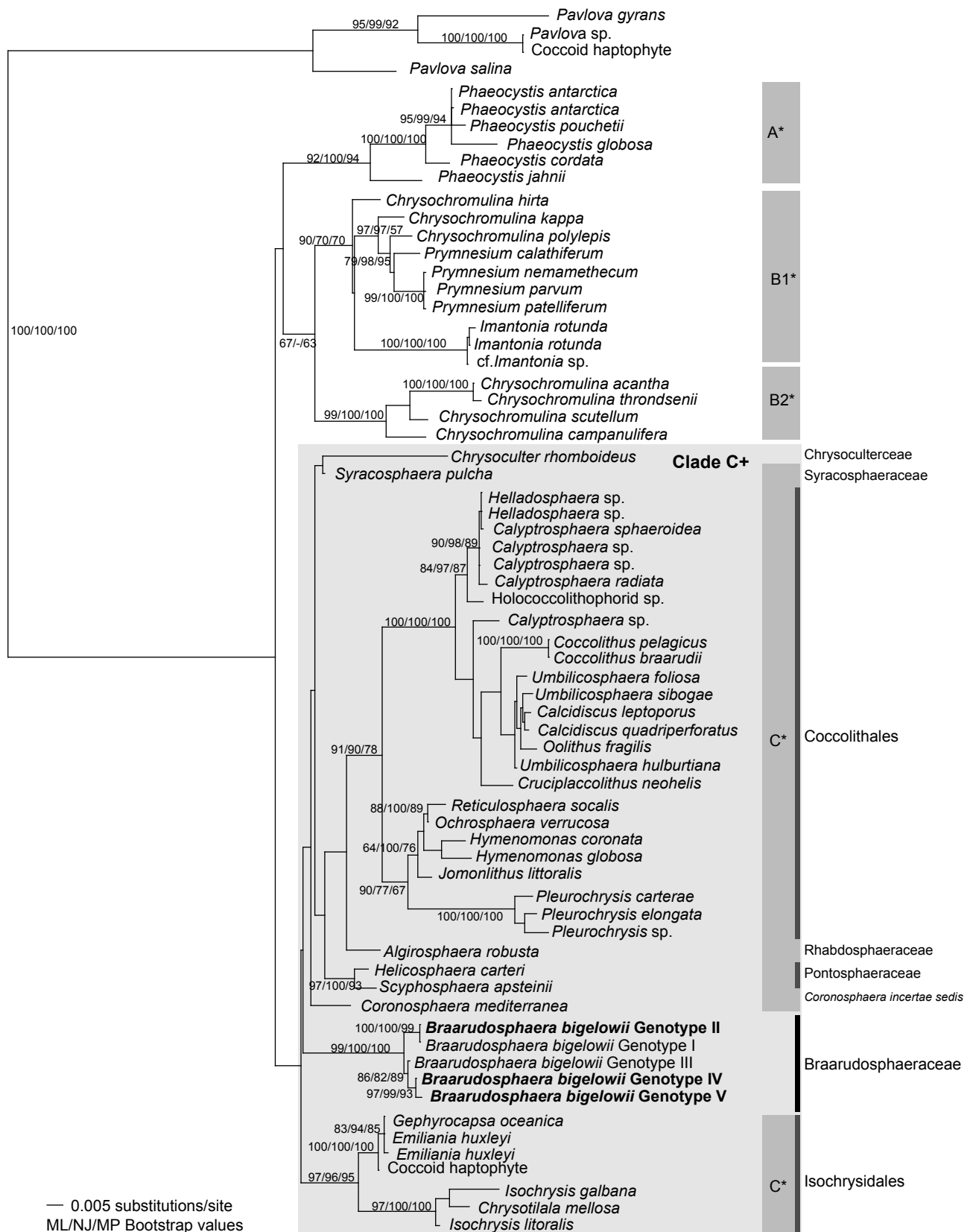


Fig. 2, Hagino et al.

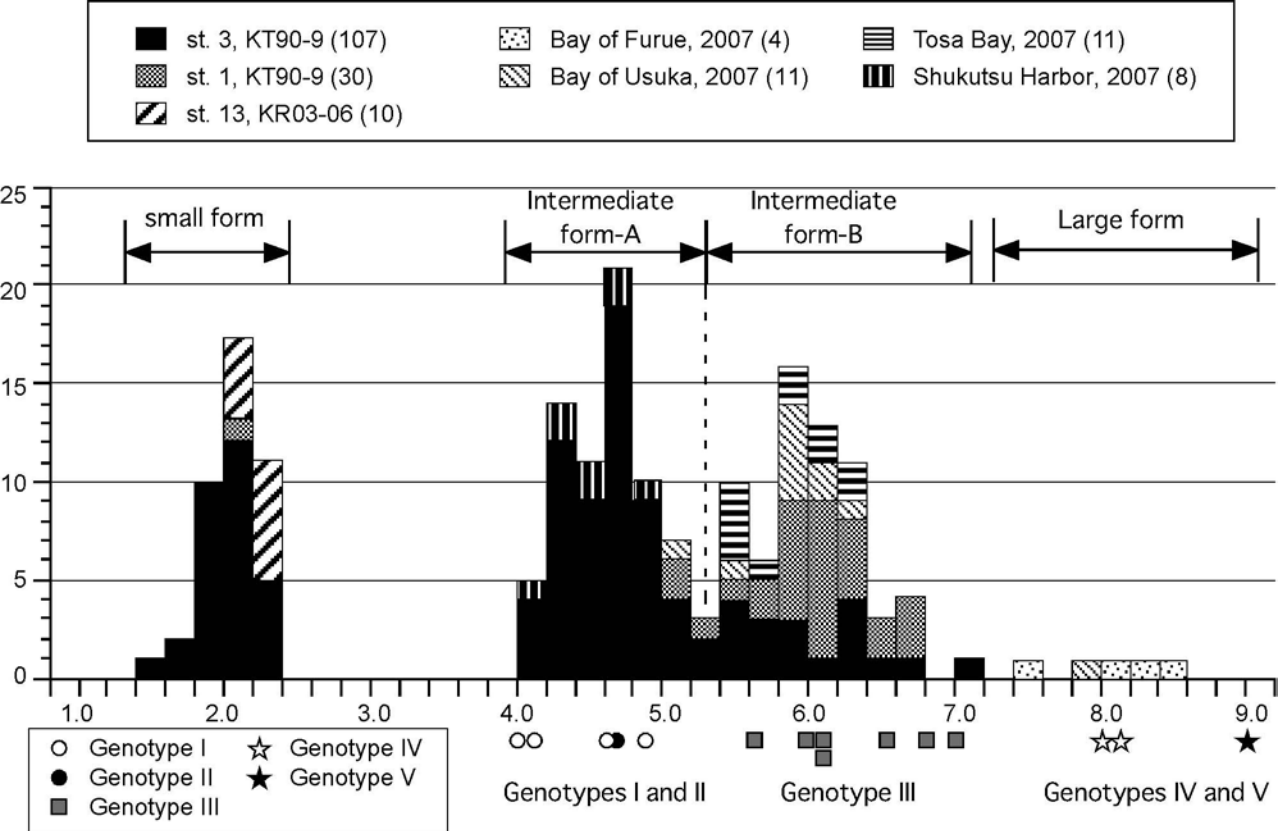


Fig. 3, Hagino et al