

Title: Re-discovery of living fossil coccolithophores from coastal water of Japan and of Croatia

Kyoko Hagino^{1*}, Jeremy R. Young², Paul R. Bown², Jelena Godrijan³, Denise K. Kulhanek⁴, Kazuhiro Kogame⁵, and Takeo Horiguchi⁵

¹*Faculty of Science, Department of Natural Science, Kochi University, Akebono-cho 2-5-1, Kochi-city, Kochi Prefecture, 780-8520 Japan*

²*Dept. of Earth Sciences, University College London, Gower St., London WC1E 6BT, UK.*

³*Centre for Marine Research, Ruđer Bošković Institute, 52210 Rovinj, Croatia.*

⁴*Integrated Ocean Drilling Program, Texas A&M University, College Station, TX 77845 USA.*

⁵*Department of Natural History Sciences, Faculty of Science, Hokkaido University, N10W8, Sapporo, 060-0810, Japan.*

Corresponding Author: Kyoko Hagino,*

Phone; +81-(0)858-43-3827,

E-mail addresses; kyokohagino@frontier.hokudai.ac.jp

key words; calcareous nannofossil, coccolithophore, *Cyclagelosphaera*, *Tergestiella adriatica*, Watznaueriaceae

Abstract

The extant coccolithophore *Tergestiella adriatica* Kamptner, which has never been reported since its original description, was re-discovered from coastal-nearshore waters of Tomari, Tottori, Japan and offshore Rovinj, Croatia. Morphological and molecular phylogenetic studies of *T. adriatica* revealed that *Cyclagelosphaera* is a synonym of *Tergestiella*, and that extant *T. adriatica* is a direct descendent of the Mesozoic fossil genus *Cyclagelosphaera* (Watznaueriaceae), which had been thought to go extinct in the Paleogene (54Ma). It is thus a remarkable example of a living fossil. Coastal patchy distribution of living *T. adriatica* and restricted occurrence of Miocene-Oligocene fossil *Cyclagelosphaera* in shallow waters, which was recently reported from New Jersey shelf, suggest that *Tergestiella/Cyclagelosphaera* was constrained to a nearshore environment in the Cenozoic, and this restricted distribution caused the lack of fossil records from oceanic sediments in the last 54 Ma.

Floristic study of coccolithophores in coastal and offshore waters of the Tomari area over a six-year period showed that *T. adriatica* and *B. bigelowii* occur predictably and synchronously every June. The environmental factors that induced the co-occurrence of these two taxa are unknown, and *T. adriatica* did not co-occur with *B. bigelowii* at other sites. Regular occurrence of middle-lower photic zone coccolithophore taxa in Tomari area every autumn suggests the presence of seasonal mixing of the water column in this period.

1. Introduction

Coccolithophores are marine unicellular haptophytes characterized by calcareous scales called coccoliths. Fossil coccoliths and other calcareous microfossils of uncertain affinities are collectively referred to as calcareous nannofossils. This group has an abundant fossil record and is of great value for biostratigraphy and palaeoceanography. As a result, the evolutionary history of calcareous nannofossils has been extensively documented from their first occurrence in the late Triassic (~225 Ma) to the present day (Bown, 1998). Calcareous nannofossils progressively diversified through the Jurassic and Cretaceous periods despite some extinction and turnover events. The highest diversity of calcareous nannofossils was recorded in Campanian and Maastrichtian (80-68Ma). However, 85 % of genera and 93 % of species went extinct at the K/Pg (Cretaceous/Paleogene) mass extinction event at 65.5 Ma (Bown et al., 2004), which was almost certainly triggered by the Chicxulub asteroid impact (Schulte et al., 2010). The mechanism of extinction of coccolithophores at the event is still under debate, however, recent studies have proposed the hypothesis that rapid ocean acidification caused by sulphuric acid produced by the impact caused the extinction of marine calcareous planktons including coccolithophores (e.g. Alegret et al., 2012; Ohno et al., 2014). Immediately after the extinction, a low diversity assemblage of survivor species flourished before giving rise to an evolutionary radiation of new Cenozoic taxa (e.g. MacLeod et al., 1997). Diversity fluctuated through the rest of the Cenozoic, with notable declines associated with intensification of glaciation in the Early Oligocene and Late Pliocene, however, there was no further mass extinction. Consequently the K/Pg

boundary is by far the largest event in nannofossil evolutionary history and many important Mesozoic nannofossil groups have no known living ancestors.

One of us (KH) has been carrying out an extended study of coccolithophores and especially *Braarudosphaera* in the coastal-offshore waters of Tomari Port, Tottori, Japan (western Japan Sea) (Hagino et al., 2013). During this study we encountered coccolithophore specimens with distinctive circular coccoliths, which were unlike any we had previously seen in the plankton but which were essentially identical to the supposedly extinct Mesozoic genus *Cyclagelosphaera*. This suggested the possibility that it might be a remarkable new example of a living fossil. Indeed Since *Cyclagelosphaera* first occurs in the Middle Jurassic, ca 170Ma (Bown and Cooper, 1998) this would make it the oldest known coccolithophore genus and probably the oldest known microplankton genus. Subsequently, we found similar specimens in offshore seawater samples from Rovinj, Croatia (north Adriatic Sea). As described below, re-examination of the classic literature revealed that this species was described as *Tergestiella adriatica* by Kamptner (1940) from north Adriatic Sea, although it had not been reported in the 70 years of subsequent studies, and was not known to recent researchers.

In order to determine the phylogenetic affinities of *Tergestiella*, we conducted detailed morphological studies of the specimens from both Tomari and Rovinj areas using SEM, to test whether the apparent similarity to *Cyclagelosphaera* was likely to be significant. We also carried out molecular phylogenetic studies based on 18S rDNA sequences obtained from the specimens from Tomari port to test whether *Tergestiella* was closely

related to any of the well-known extant coccolithophore groups, or deeply diverged from them as would be expected if it is a descendent of the Mesozoic *Cyclagelosphaera*. Furthermore, we studied seasonal succession in abundance of living coccolithophores including *T. adriatica* in coastal and offshore waters of Tomari area in order to determine their ecological preferences.

Our findings raised two other questions. First, why extant *T. adriatica* had not been reported since its description over 70 years ago despite having large, distinctive coccoliths. Second, why there is no record of fossil *Cyclagelosphaera* though most of the Cenozoic if it is present in the modern plankton. *Cyclagelosphaera* is well-documented as a K/Pg survivor taxon (e.g. Bown, 2005) but was thought to have gone extinct in the Early Eocene (ca. 54ma) (need Reference). In this paper, we will discuss the possible causes of lack of their records in modern and paleontological studies based on floristic study of living coccolithophores including in Tomari, and re-examine the possible records of fossil *Cyclagelosphaera* from the Oligocene-Miocene sediments from the New Jersey margin (Mountain et al., 2010).

2. Materials and Methods

2.1. Sampling

Sea-surface water samples were collected for floristic and culture studies of coccolithophores in the Tomari Port and offshore Tomari, Tottori Prefecture, Japan, and offshore Rovinji, Croatia (Fig. 1). In Tomari area, a total of 157 samples were collected from the quay or breakwaters of Tomari Port (stations Tomari 1, A, B, C, D, and/or E) and from the breakwater of Ishiwaki (station Ishiwaki) from July 2008 to May

2014 (Fig. 1, Appendix 1). Additionally, 65 sea surface water samples offshore Tomari were collected and provided by Mr. Takayuki Tanaka. The offshore samples were collected using a 10L plastic bottle from a fishing boat *Ryoto-Maru*, owned by Mrs. Takayuki and Tsutomu Tanaka, from July 2007 to July 2013 (Appendix 1). The precise localities of the offshore sampling were not always recorded by GPS. The water depth at the sampling locality of the offshore samples, as measured by a fish-detector equipped to the *Ryoto-Maru*, ranged from 20-50m.

The interval of sampling was adjusted according to the season from July 2008 to March 2012. Sampling was conducted more than twice a week in June for isolation of *B. bigelowii*, about once a month from mid March to May and from mid September to early December for isolation of other coccolithophore species, and less than once a month in coccolithophore-scarce periods, from mid July - mid September and from mid December to early March (Appendix 1). From April 2012 through May 2014, sea surface water samples were collected at least once a month throughout the year, in order to characterize the seasonal succession in coccolithophores in the studied area.

Samples for coccolithophores from offshore Rovinj, Croatia, were collected as a supplement to a regular time-series sampling programme conducted by the Centre for Marine Research (e.g. Maric et al., 2012). The samples used in this study were taken from a regular sampling station RV001, nautical mile 1852 (m) from the coast in water 27 m deep once or twice a month from August 2009 to 2010 (Fig. 1; Appendix 2).

2.2 Floristic, morphological, and culture studies.

For floristic study, sea surface water samples from Tomari-area were pre-filtered through a 50- μm plankton net (Sefar Inc. DIN-110) in order to remove other large planktons. One liter of pre-filtered water was filtered onto Millipore HAWP04700 or Whatman 7060-4710 membranes. The filters were then air-dried and were stored in plastic Petri-dishes. An elongate strip running from the center to the rim of the filter was cut out, and rendered transparent and fixed with Norland Optical Adhesive 74 in order to study the cell densities and species composition under the light microscope. The major coccolithophore taxa were counted and identified on > 300 fields of view, corresponding to >5ml of seawater, using a cross-polarized light microscope (Nikon E600POL) at 1500x magnification. Samples prepared from various sites in the seas around NE Japan and in the North Sea and in the Bay of Biscay, which were known to contain *B. bigelowii* from previous studies (Hagino et al., 2009b; Young et al. 2014), were re-examined using the same method described in above (Fig. 1; Appendix 3). Seawater samples from Rovinj were collected using Niskin bottles. For estimation of cell density, subsamples of 50 ml fixed with neutralized formaldehyde (2% final concentration) were sedimented. Cells were counted on a half transect at 400x or 200x magnification using an inverted light microscope (Zeiss Axiovert 200). For further study, 50 ml of sample was filtered (Whatman 110612), and a piece of filter was mounted on microscope slide, fixed with Norland Optical Adhesive 74 or Canadian balsam, for cross-polarized light microscopic studies (Zeiss Opton Invertoskop D or Zeiss Axioplan).

Scanning Electron Microscope (SEM) observation was used for morphological studies of selected species and to confirm identifications, but not for quantitative studies of coccolithophores, since the abundant diatoms and organic matter often covered small coccolithophores, and made the quantitative study of small species difficult under the SEM. Segments of selected filter samples from Tomari and Rovinj were mounted on aluminium stubs and sputter coated with gold-palladium (Cressington Scientific 208HR) or gold (Sanyu SC701 MKII) for observations under SEM (Phillips XL30 FEG SEM or JEOL JSM 7001F). Taxonomic classification of the coccolithophores followed Young et al. (2003), and of other members of non-calcifying Haptophytes followed Edvardsen et al. (2011). The descriptive terminology of coccoliths followed Young et al. (1997).

In order to establish culture strains of coccolithophores, 10 liter of seawater sample from Tomari Port was pre-filtered through a 50- μm plankton net. The pre-filtered samples were then concentrated using a piece of 1 μm mesh-size plankton net (Sefar Inc. NY1-HD). Living cells of major coccolithophore taxa were isolated from concentrated seawater sample for culture experiments using micropipette under Inverted light microscope, placed on 24-well culture well with MNK medium (Noël et al., 2004), and stored in 20°C incubator in a 18:6 light:dark regime.

2.3 Molecular phylogenetic studies

The single cell PCR method of Takano and Horiguchi (2004) was applied to *T. adriatica* specimens, in order to determine their molecular phylogenetic position based on 18S rDNA sequence. For this, 10 liter samples from Tomari collected on 21st and 23rd

June 2010 were pre-filtered through a 25- μ m plankton net (Sefar Inc. P-25). The pre-filtered samples were then concentrated using a piece of 1 μ m mesh-size plankton net (Sefar Inc. NY1-HD) placed on a kitchen sieve. Six *T. adriatica* cells were isolated using a micropipette under an inverted light microscope (Olympus CKX41), and labeled as T1-T6. Each cell was photographed using a Nikon E600POL microscope, and then subjected to single cell PCR amplification. The primers used for PCR amplification, PCR condition, procedure of sequence determination and the sequencers were the same as in Hagino et al. (2009). The results were confirmed by sequencing both forward and reverse strands. Partial 18S rDNA sequences (1721bp) were successfully obtained from two specimens, T5 (Figs 2a and 2b) and T6 (Figs 2c and 2d), independently from each other.

For the phylogenetic analysis of the 18S rRNA gene, a total of 52 18S rRNA gene sequences of the Division Haptophyta including a sequence of the Pavlovophyceae as an out-group were obtained from GenBank. Since 18S rDNA sequences of the specimens T5 (GenBank Accession number AB636316) and T6 (GenBank Accession number AB636317) were identical to each other, we used the sequence of T5 as representative of them. The sequences were aligned together with the sequences obtained in this study using Clustal W (<http://www.genome.jp/tools/clustalw/>).

Phylogenetic trees were constructed based on Maximum Likelihood (ML) method and Bayesian inference (BI) using PAUP version 4.0b10 (Swofford, 2002) and Mr. BAYES v3.1.2 (Ronquist and Huelsenbeck, 2003), respectively.

For ML analysis, a likelihood score ($-\ln L = 9909.2666$) was obtained under the TrN+I+G model with the following parameters: assumed nucleotide frequencies A= 0.2347, C= 0.2277, G= 0.2876, and T=0.2560; substitution-rate AC= 1, AG= 1.5584, AT= 1, CG= 1, CT= 4.6681, GT= 1; proportion of sites assumed to be invariable = 0.5926; and rates for variable sites assumed to follow a gamma distribution with shape parameter = 0.5012, were estimated by Modeltest 3.7. The ML analysis was performed using the heuristic search option with a branch swapping algorithm Tree bisection-reconnection (TBR) with the NJ tree as a starting tree. Bootstrap analyses with 1000 replicates for the ML analysis were applied to examine the robustness and statistical reliability of the topologies (Felsenstein, 1985). For the BI analysis, GTR+I+G model was selected by the MrModeltest. Markov chain Monte Carlo iterations were carried out until 6 million generations, when the average standard deviations of split frequencies were below 0.01, indicating convergence of the iterations.

2.4. Hydrography

2.4.1 Sea surface temperature of Tomari area

In situ sea surface water temperature (SST) of almost all coastal samples and some offshore samples were measured using a bar thermometer in the sampling from July 2008 through February 2014. SST of Tomari Port and offshore Tomari changed seasonally. SST ranged from 27.0 to 30.5 °C in summer (mid July - September), and from 10 to 12 °C in winter (January - February) (Figs. 6a and 6b, Appendix 1).

2.4.2 Hydrographic condition offshore Rovinj

In situ seawater temperature and salinity was measured with a CTD equipped to Niskin bottles. On all sampling occasions (18th and 28th August 2009, and 19th August 2010), the temperature was relatively high (> 25 °C) at the surface (≥ 10 m), and dropped to 15-16 °C at 27m depth. Salinity was relatively low (34.5-36.1) in the surface water (≥ 10 m), and relatively high (37.4-37.8) in deeper water (≥ 20 m) (Table 1).

2.5. Oligocene-Miocene fossil record

One of us (DK, in Mountain et al. (2010)) reported fossils of '*Cyclagelosphaera* sp.?' without further interpretation from Oligocene and Miocene sediments deposited on the New Jersey shelf, which were cored during Integrated Ocean Drilling Program (IODP) Expedition 313. For this study, we re-examined selected samples used in Mountain et al. (2010) using cross-polarized light microscopy to assess whether these are in-situ or reworked. We also compared the distribution of the '*Cyclagelosphaera* sp.?' specimens with paleo-water depth interpretations of the sites based of benthic foraminifer faunal analysis (Mountain et al., 2010).

3. Results

3.1. Morphological observations

We found unusual coccolithophore specimens with distinctive circular coccoliths, unlike any well-known extant species, from the shallow marine waters of around Tomari Port, Tottori, Japan (Figs. 2a-2e). Subsequently, similar specimens were found in samples collected offshore Rovinj, Croatia (Fig. 2f). The specimens from Tomari (Japan Sea) and Rovinj (Adriatic Sea) are indistinguishable from each other in

morphology in both cross-polarized light microscope (Fig. 2e and 2f) and SEM (Fig. 3a and 3i).

The specimens form spherical coccospheres composed of around 12-15 circular coccoliths. The coccoliths are circular placoliths with closed central area (Figs. 2a and 2c). In cross-polarized light, the coccoliths show high birefringence of the proximal shield, distal shield and inner tube with sub-radial extinction lines indicating that their elements have sub-radial crystallographic c-axis orientation (R-units). There is also a narrow median cycle showing low birefringence, indicating sub-vertical crystallographic c-axis (V-units) (Fig. 2e and 2f).

The diameter of the coccoliths was measured on SEM images of well-formed specimens, it ranged from 4.3 to 6.4 μm , mean 5.6 μm (58 coccoliths) in Tomari, and from 4.5 to 5.7 μm , mean 5.2 μm (18 coccoliths) in Rovinj. In the SEM in distal view three cycles of elements are visible: an outermost cycle with sinistral obliquity (distal shield elements); a median cycle with dextral obliquity; and a central cone with anticlockwise imbrication (Figs 3a, 3j, and 4). The size and intensity of calcification of the median cycle elements varied between specimens. Pores were observed between the median cycle and outer cycle (distal shield), and at the apex of the central cone (Figs 3a, 3j, and 4). In proximal view, two cycles of elements are visible: an outer cycle with nearly radial sutures forming the proximal shield, and an inner cycle with more irregular radial elements in the central area (Fig. 3b-d). There is a narrow zone with complex sutures between the inner and outer cycles (Fig. 3c). Incomplete coccoliths at various growth stages were observed from the samples collected in June

2013, indicating that it produces coccoliths intracellularly (Figs. 3e-f). The median cycle is consistently present on the specimens even at very early growth stages indicating that it extends downward into the coccolith, as indicated on Fig. 4.

3.3. Taxonomic identification

Erwin Kamptner, a distinguished pioneer of coccolithophore research, carried out an extensive study of the coccolithophores of the Northern Adriatic from the Rovinj marine station. Re-examination of his monograph, Kamptner (1940) revealed that he had described a species *Tergestiella adriatica*, which is clearly similar to our specimens. Figs. 2g and 2h shows images of the holotype of *T. adriatica*, which were reproduced from Kamptner (1940) with the permission of publisher. The light micrographs of the holotype of *T. adriatica* are rather indistinct (Figs. 2g and 2h) but are comparable to our specimens (Figs. 2a and c). More details are visible on his line drawings (Fig. 2i); this is often the case with Kamptner and indeed other workers of the time, reflecting the limitations of contemporary photomicroscopy. His drawings of other coccolithophores have proven reliable, so we can expect the drawings of *T. adriatica* to be an accurate reflection of what he observed. The most prominent feature on the diagrams is a median cycle of elements on the distal surface (Fig. 2i), which is also very obvious on our specimens (Figs 3a, 3g and 3j), and which is not seen on any other modern coccolithophores. He indicates a coccolith diameter of 5.7 μm , without stating whether this was an average or maximum value. This is consistent with the size range of coccoliths of our specimens (4.3-6.4 μm , see above). Kamptner's specimens were collected from the same locality and at the same time of year as our

Croatian specimens, clearly the specimens we observed were the same species as that of Kamptner and so should be referred to the species *Tergestiella adriatica*.

3.4. Observation of living cells

We examined the condition of the cells of major taxa during isolation of cells for culture study under inverted light microscope. All *T. adriatica* cells observed were non-motile, and flagella and haptonema were not observed. Due to the robust coccoliths the cell contents of *T. adriatica* were poorly visible but incomplete coccoliths were observed intracellularly. Flagella and haptonema were not observed on the following non-motile species; *B. bigelowii*, *E. huxleyi*, *G. oceanica* and *U. sibogae*.

Algirosphaera robusta, *Discosphaera tubifera*, *Calciopappus rigidus* *Oolithotus antillarum*, *Helicosphaera wallichii*, *Rhabdosphaera clavigera*, and *Syracosphaera pulchra* appeared to motile in the concentrated seawater sample, they possessed two flagella and a haptonema.

We established multiple clonal culture strains of *E. huxleyi*, *G. oceanica*, and *S. pulchra* from coastal and offshore water of Tomari (unpublished data of KH), but could not grow *T. adriatica* or *B. bigelowii* in culture despite repeatedly isolating single cells into growth media.

3.5. Molecular phylogenetic analyses

We have obtained partial SSU rDNA sequences (1721bp) from two cells, T5 (Figs. 2a-2b, AB636316) and T6 (Figs. 2c-2d, AB636317), picked from field samples from Tomari. These two sequences are identical to each other, and differed from any other known haptophyte sequences. They were compared to a database of sequences from 24

coccolithophores, 20 other haptophytes and 7 unidentified sequences, and ML and BI analyses were undertaken to determine phylogenetic affinity (Fig. 5). The ML and BI analyses resulted in similar trees. Here we show ML tree with bootstrap consensus values obtained from ML analysis and posterior probabilities obtained from BI analysis (Fig. 5). The topology of the trees resembles those of previous studies (e.g. Hagino et al., 2013) except for the position of the clade that included *B. bigelowii* and *T. adriatica*. In both ML and BI analyses, *T. adriatica* made a clade with the clade containing *B. bigelowii* and unidentified *Prymnesiaceae* specimens (AB058358 and FJ537341), but without bootstrap support and with low posterior probability (0.70). The Clade *T. adriatica* + *B. bigelowii* + unidentified *Prymnesiaceae* clustered with the Clade C⁺ (Takano et al., 2006), which contains all the other calcifying (coccolithophore) genera including some non-calcifying genera (*Isochrysis*, *Reticulosphaera*, and *Chrysoculter*) and two environmental sequences (OL51050 and OLI26041), without bootstrap support and with low posterior probability (0.78).

3.6. Floristic studies

3.6.1. Seasonal succession in standing crop in Tomari

Coastal and offshore samples showed a similar seasonal succession in standing crop of coccolithophores, although the standing crop in the offshore samples is usually higher than in the coastal samples (Figs. 6c and 6d, Appendix 1). Standing crop of coccolithophores in coastal stations was usually <4000 cell/liter in winter (late December – February). Abundance of coccolithophores in offshore stations was unknown due to lack of offshore samples in winter. The standing crop increased to

5,000-10,000 cells/liter in coastal stations, and to >10,000 cells/liter in offshore stations, from March to early May. SST gradually increased from 13-16 °C in this period. The standing crop, however, dropped to < 1,000 cells or absent in both coastal and offshore stations from mid May through early June. In this period, SST ranges from ca. 16-19°C. In this period, nearshore water along the coast of Tottori Prefecture have blooms of the bioluminescent dinoflagellate *Noctiluca*. While *Noctiluca* appeared in the port or neighboring area, samples from Tomari were barren of coccolithophores. After disappearance of *Noctiluca*, coccolithophores recover in abundance, together with abundant diatoms (e.g. *Chaetoceros* spp.). The cell density of coccolithophores is usually >10,000 cells/liter in coastal stations and >20,000 cells/liter in offshore stations from mid June to mid July. In this period, SST increases from 19 to 27 °C. The cell density dropped to < 1,000 cells/liter again in both coastal and offshore stations from mid July through mid September, when the SST exceeds 28°C. Standing crop increased from late September through to early December, typically to >3,000 cells/liter in coastal stations and > 50,000 cells/liter in offshore stations (Figs. 6c-6d) In this period, SST gradually decrease from 26°C to 13°C.

3.6.2. Seasonal changes in major taxa in Tomari

Figs. 6e-7 shows the seasonal succession in cell density of major taxa whose cell density exceeded 2000 cells/l at least once in the study period (July 2008-May 2014). *T. adriatica* regularly occurred from mid June to early July (Figs. 6e-f) in synchronization with the occurrence of *B. bigelowii* (Figs. 6g-h). SST increases from 19-26 °C in this period. *T. adriatica* never occurred from mid July through to the next early June. They

were found from the coastal stations every June, and in offshore stations in June of 2009 and of 2011-2013, but were not found in offshore samples of June 2012. Cell density of *T. adriatica* was lower than 2,000 cells/liter in June of 2009-2012, but exceeded 15,000 cells/litter in June 2013 (Figs. 6e-f, Appendix 1). *B. bigelowii* regularly occurred from mid June to early July in both coastal and offshore stations (Figs. 6g-h). They were usually absent from mid July through the next early June, although a single cell, the specimen TMR-scBb1 reported by Hagino et al. (2013), was found in 22nd November 2012. The highest cell density of *B. bigelowii*, 6,600 cells/liter, was observed in 21st June 2009 (Figs. 6g-h, Appendix 1).

Algirosphaera robusta occurred from October to end of November regularly (Figs. 7a-b). SST gradually decreases from 24 to 16°C in this period. The cell density of *A. robusta* was higher in offshore samples than in the coastal samples. *Florisphaera profunda*, *Helicosphaera walichii*, *Michaelsarsia* sp., *Oolithotus* spp., *Ophiaster* sp., *Umbilicosphaera foliosa* and/or *Umbilicosphaera sibogae* occurred in autumn at low abundance (< 2000 cells/L) together with *A. robusta* (Appendix 1).

Calciopappus rigidus occasionally occurred from coastal or offshore samples typically at low abundance (< 600 cells/liter), although at the end of June 2011 they bloomed in Tomari Prot reaching a cell density of ca. 48,000 cells/liter (Figs. 7c-d).

E. huxleyi, *G. oceanica*, and *G. ericsonii*, members of the Family Noëlaerhabdaceae, occurred in both coastal and offshore stations almost through the year although they became very rare (<200 cell/L) or absent during the occurrence period of *Noctiluca* (May) and midsummer when SST exceeded 28°C (August). The cell density of these

species is higher in spring and autumn than in summer and winter, and higher in offshore stations than in the coastal stations (Figs. 7e-j).

Syracosphaera spp. (mainly *Syracosphaera pulchra*) occurred from late June or early July, as decline of number of *B. bigelowii* and *T. adriatica* (Appendix 1). They increased in abundance mainly in early summer (mid June - early July) and in autumn (late September - November), but did not occur in mid-summer (August) and in spring (March-May) (Figs. 7k-l).

3.6.3. Re-examination of filter samples used in previous studies

We re-examined by cross-polarized light microscopy 9 samples, which were known to contain *B. bigelowii* from previous studies conducted in other parts of the seas of Japan (Fig. 1a, Table 2). *T. adriatica* forms relatively large coccoliths and they are very distinctive in cross-polarised light so it was possible to rapidly scan a large area of filter for all these samples. The re-examined samples did not have even any fragments of *T. adriatica* (Table 2).

Subsequent to the discovery of *Tergestiella* one of us (JRY) participated in a research cruise, D366, around the NW European Continental shelf (Young et al. in press) in June/July 2011 (Fig. 1a). During this cruise samples were taken from 74 CTD station depth profiles and 200 additional surface water underway samples. During light microscope counting of these particular attention was applied to *Braarudosphaera* and *Tergestiella/Cyclagelosphaera*.

Braarudosphaera was found in 12 out of the 600 samples examined but no specimens of *Tergestiella* were observed, even as loose coccoliths.

3.7. Occurrence of *T. adriatica* from Rovinj

In the samples collected from the station RV001 from January 2009 to September 2010, coccospheres and free coccoliths of *T. adriatica* occurred very rarely in samples collected in late August 2009 and 2010, respectively (Table 2). *B. bigelowii* and members of *Gephyrocapsa*, which were common in the Tomari samples containing *T. adriatica*, were not found from Rovinj samples.

3.8. New Jersey Margin Fossil Material

In Mountain et al. (2010), one of us (DK) reported ‘*Cyclagelosphaera?* sp.’ specimens from Oligocene-Miocene sediments deposited on New Jersey shallow shelf, which were cored with IODP Exp. 313. We re-examined the ‘*Cyclagelosphaera?* sp.’ specimens from Miocene New Jersey shelf sediments in the cross-polarized light microscope (Fig. 2i). These Miocene specimens (Fig. 2j) closely resembled those from extant *T. adriatica* (Figs. 2e and 2f).

We reevaluated the data presented in Mountain et al. (2010) in order to consider whether the *Cyclagelosphaera?* sp. specimens are part of biocoenosis or result of reworking. All three sites M0027-29 included calcareous nannofossil species typical of mid-Eocene to Oligocene assemblages (e.g., *Reticulofenestra umbilicus*, *R. samodurovii*, *R. reticulata*, *R. lockeri*, *Transversopontis* spp.), *Cruciplacolithus* spp. and *Cyclagelosphaera?* sp. Mountain et al. (2010) interpreted *Cruciplacolithus* spp. as reworked specimens, but there is a possibility that it was a part of neashore biocoenosis not reworked, since extant *Cruciplacolithus neohelis* show affinity to neashore waters such as tidal pool and lagoon (e.g. Kawachi et al., 1991; Konno and

Jordan, 2006). *Cyclagelosphaera?* sp. occurred nearly consistently from early-middle Miocene (NN4-NN5) sediments from the most distal site, M0029, without any specific reworked taxa, if *Cruciplacolithus* spp. are not reworked, and in many samples *Cyclagelosphaera* is present without any recorded Cretaceous or Paleogene reworking. We observed coccospheres of *Cyclagelosphaera* sp. from multiple samples of middle Miocene of the site M0029 (unpublished data, DK). These results strongly suggest that Miocene *Cyclagelosphaera* specimens from the New Jersey Shelf were part of nearshore biocoenosis not reworked.

Paleo-water depths of the sites M0027-29, which yielded *Cyclagelosphaera*, were estimated on the basis of benthic foraminifer faunal analyses (Mountain et al., 2010). *Cyclagelosphaera* was consistently present in rare to few numbers in middle Miocene sediments, of nannofossil zones NN4-6/7 (Martini, 1971), at the sites M0027-29. It was also found sporadically in rare to few numbers in Oligocene and lower Miocene (NP22 to NN2) sediments from Site M0027 and more consistently at rare to few numbers in lower Miocene sediments (NN2) of Site M0028. It was not reported from lower Miocene sediments of the most distal site (M0029). During the middle Miocene *Cyclagelosphaera* typically occurred in sediments interpreted as having been deposited in depths of 25-75 m at Sites M0027 and M0028, although they may have been somewhat deeper (up to 100 m) for short intervals at the latter site. At Site M0029, the most distal site, paleo-water depth estimates are somewhat deeper, generally ranging from 75-100 m, with estimates during some intervals exceeding 100 m. Paleo-water depths from Oligocene and lower Miocene sections are generally

deeper, but more varied on the New Jersey shelf. At the most proximal site (M0027) depths are usually greater than 100 m, although during several distinct shallowing intervals, as inferred from sedimentology, *Cyclagelosphaera* sp. is present. At Site M0028, early Miocene water depths were on the order of 50-75 m. At Site M0029, where this taxon is not recorded in lower Miocene sediments, water depths are typically 100+ m (Mountain et al., 2010).

4. Discussion

4.1. Comparison of *Tergestiella* and *Cyclagelosphaera*

Tergestiella adriatica is evidently the correct identification of our specimens, as explained in the results, however, they also very closely resemble the common and well-known extinct Mesozoic genus *Cyclagelosphaera*, a member of the family Watznaueriaceae. The Watznaueriaceae form robust placolith coccoliths which frequently dominate Mesozoic nannofossil assemblages from the Early Jurassic to the end Cretaceous (need reference). If *T. adriatica* is a direct descendant of *Cyclagelosphaera* and so a member of the Watznaueriaceae this is of considerable palaeobiological interest, so the hypothesis deserves investigating in detail. The structure of Watznaueriaceae coccoliths was described in detail by Young and Bown (1991), based on *Watznaueria fossacincta*. *Cyclagelosphaera* coccoliths differ from those of *Watznaueria* in being circular with a closed central area, but they have essentially identical structure (Bown and Young, 1997).

Both *Cyclagelosphaera* and *Tergestiella* have circular coccoliths, with the placolith morphology of two shields separated by a tube, and have closed central areas (NB

terminology follows Young et al. (1997), and is summarized in Fig. 4). In distal view, three cycles of elements are visible: an outermost cycle with sinistral obliquity; a median cycle with irregular sutures; and a central cone of small elements. In proximal view, two cycles of elements are visible: an outer cycle with nearly radial sutures forming the proximal shield, and an inner cycle with more irregular radial elements in the central area. Between these two cycles is a narrow zone with complex sutures (arrow on Fig. 3c).

Young and Bown (1991) showed from study of growth sequences and broken specimens that the zone of complex sutures on the proximal surface corresponds to the proto-coccolith ring, i.e., the site of nucleation of the coccolith crystals, from which they grow during coccolith formation. In suitably preserved specimens it consists of a cycle of peg-like tube-core elements alternating with thin elements connecting the main cycles of the proximal shield. They also showed that the distal shield, proximal shield and inner tube elements were interconnected and formed of crystal-units with sub-radial orientation of the crystallographic c-axis (R-units). By contrast the medial tube-core elements visible on the distal side were connected to the peg-like elements on the proximal surface forming a cycle of wedge-shaped tube-core elements, and these elements had sub-vertical orientation of the crystallographic c-axis (V-units). In our *T. adriatica* specimens the peg-like tube-core elements (V-units) are not clearly visible on the proximal surface, but the inner tube cycle protrudes slightly from the proximal surface so there is a step at the junction between the two cycles and complex suture patterns are visible in this zone (Figs. 3c-d). On etched specimens the base of

the tube-core elements are often dissolved leaving a row of holes, marking the position of the proto- coccolith ring, (Figs 3d), as is often the case with etched *Watznaueria* and *Cyclagelosphaera* specimens. These etched specimens also show that the cycles of elements in the proximal shield and central area interconnect (i.e., they are formed from a single crystal unit). Further the coccolith specimens at intermediate growth stages we observed in this study are directly comparable to the fossil specimens illustrated by Young and Bown (1991) confirming that the development sequence is the same in modern *Tergestiella* as in fossil Watznaueriaceae.

In cross-polarized light our *T. adriatica* specimens produce very similar interference figures to *Watznaueria* and *Cyclagelosphaera*, showing that they have similar crystallographic orientation. In particular the high birefringence of the proximal shield, distal shield and inner tube with sub-radial isogyres indicates that they have sub-radial crystallographic c-axis orientation (i.e., they are R-units), whilst the tube core elements show low birefringence indicating that the crystallographic c-axis is sub-vertical (i.e., they are V- units) (Fig. 1c).

So, not only the general morphology but also the detailed structure, and crystallography of *Tergestiella adriatica* are identical to that of *Cyclagelosphaera*.

There are other groups of coccolithophores, which form coccoliths similar in shape to those of *T. adriatica* but they have distinctly different structures; in the *Calcidisceae* and *Coccolithaceae* the distal shield is formed from V-units, whilst in the Noëlaerhabdaceae the V-units are vestigial. So, the morphological similarity of

Cyclagelosphaera and *Tergestiella* is significant and strongly, suggests that *T.*

adriatica is a direct descendant of *Cyclagelosphaera*.

4.2. Molecular phylogenetic study

The phylogenetic relationship of *T. adriatica* with other coccolithophores was tested based on 18 rDNA sequences. The fossil record shows that coccolithophores evolved in the Late Triassic and underwent an evolutionary radiation in the early Jurassic (ca. 190Ma) (Bown, 1987). A major group, the Watznaueriales, which included *Cyclagelosphaera*, diverged during this radiation and no extant descendant taxa have previously been identified, even tentatively. The basal divergence seen in molecular phylogenies of coccolithophores is thought to correspond to this early Jurassic radiation (Medlin et al., 2008), hence we would predict a basal position for *T. adriatica* in the coccolithophore clade if it is a direct descendant of *Cyclagelosphaera*.

In both ML and BI analyses, two extant species with Mesozoic origins *T. adriatica* and *B. bigelowii* occur near the base of the coccolithophore clade C⁺, although the precise relationship among *T. adriatica*, *B. bigelowii* and the other coccolithophores cannot be determined from the analyses due to low bootstrap support and posterior probability (Fig. 5). The phylogenetic positions of *T. adriatica* in 18 SrDNA trees, however, suggests that they separated from the common ancestor of other coccolithophore species in the Early Jurassic radiation, and supports the hypothesis that *Tergestiella* is a direct descendent of *Cyclagelosphaera*.

4.3. Fossil record of *Tergestiella/Cyclagelosphaera*

In the Mesozoic, *Cyclagelosphaera* appears to have had a broad distribution but it was more abundant in neritic environments (Bown, 2005). In the Late Jurassic in addition to typical marine occurrences *Cyclagelosphaera* formed lagoonal sediments such as the Late Jurassic Solnhofen limestone (Keupp, 1977) and lagoonal deposits of the French Southern Jura mountains (Tribovillard et al., 1992). This is an ecological preference that is very unusual in coccolithophores and makes it a key component of the Late Jurassic lithographic limestone facies, which includes some of the most important lagerstätten known. Through the Cretaceous *Cyclagelosphaera* becomes progressively less abundant and although it frequently occurs at very low abundances in open marine sediments it is only abundant in near shore sediments. The most prominent exception to this pattern of neritic affinity is seen immediately after the K-Pg extinctions where *Cyclagelosphaera* is a common component of mid-latitude oceanic assemblages in the early Danian recovery assemblages (Pospichal, 1996; Bown, 2005). Subsequently it declined and was thought to have become extinct in the early Eocene (54 m.y. ago: Bybell and Self-Trail, 1994).

Our observations of fossil *Cyclagelosphaera* from inner shelf deposits off New Jersey and of living *Tergestiella* in nearshore environments off Japan and Croatia suggest an alternative hypothesis: that *Cyclagelosphaera* did not become extinct in the Early Eocene but was confined to nearshore environments. Such environments are not usually suitable for coccolith preservation and the absence of records may also have been due to *Cyclagelosphaera/Tergestiella* having a patchy distribution and to specimens being misinterpreted as reworked. Finally, sporadic occurrences of *Cyclagelosphaera* in

Cenozoic studies have likely been interpreted as reworking of Mesozoic *Cyclagelosphaera*. We suspect that, if more well-preserved nannofossil assemblages from warm-water shallow shelf sites are studied carefully, then the apparent gap in the fossil record of these taxa between the Paleocene and the modern will prove illusory.

4.4. Taxonomic notes

Two fossil species of *Cyclagelosphaera* are commonly recorded in sediments of Late Maastrichtian and Early Paleocene age (Bown, 1998): *Cyclagelosphaera margerelii* Noël (Noël, 1965) and *Cyclagelosphaera reinhardtii* (Perch-Nielsen) Romein (Romein, 1979). Of these *C. margerelii* has a weakly developed cone in the central area, whereas *C. reinhardtii* has a prominent cone (Figs. 3k and 3l). Our *T. adriatica* specimens are intermediate in cone development between these two species and so cannot easily be assigned to either. In addition our specimens are distinguished by having a cycle of pores developed between the tube core (median cycle) and distal shield elements, as well as a pore at the apex of the central cone (Figs. 3a, g and j). *C. reinhardtii* often shows an apical pore and sometimes pores between the cone and the tube-core, but never between the tube core (median cycle) and the distal shield. A very well preserved specimen of *Cyclagelosphaera* with a moderately developed cone as well as a flat cycle with irregular sutures between the central cone and distal shield was reported as *Cyclagelosphaera* sp. cf. *reinhardtii* from post K-Pg deposits from the Netherlands (Mai et al., 1997). Our specimens resembles it in the general morphology of the median tube core cycle, but differ from it in having a cycle of

pores between the flat cycle and distal shield elements. So it is clearly sensible to regard the modern species *T. adriatica* as distinct from the fossil species.

In terms of conventional coccolith morphotaxonomy it would be logical to regard the genera *Tergestiella* Kamptner (1940) and *Cyclagelosphaera* (Nöel 1965) as synonyms, even though they were described respectively from the modern and the Upper Jurassic.

Tergestiella has priority over *Cyclagelosphaera*, and indeed the combinations *Tergestiella margerelii* and *Tergestiella reinhardtii* were previously proposed by Reinhardt (1971) and Shumenko (1969), respectively. Hence we recommend usage of *Tergestiella* for both living and fossil specimens, although we note that this is a subjective recommendation. NB The generic name *Tergestiella* was derived from the Latin name *Tergestum* for the port of Trieste which is 60km NNE of the type locality.

4-5. Floristic studies

Coccolithophores occurred commonly in Tomari from mid June - early July and from September through April with seasonal abundance changes, but were very rare or absent in mid summer (mid June through mid September) and in late spring (mid May through early June). The scarce occurrence of coccolithophores in mid summer is probably due to high water temperature ($> 28^{\circ}\text{C}$). Our observation is consistent with the result of Hagino et al. (2000); they reported that coccolithophores become rare at the sea surface level in the Western Pacific Warm Pool where the SST exceeds 28°C . However, scarce occurrence of coccolithophores from mid May to early June is unusual. Temperature of this period should be suitable for growth of coccolithophores, since coccolithophores were common before May and after mid June. Coccolithophores was completely absent

in Tomari Port during a bloom of the bioluminescent dinoflagellate *Noctiluca*, although it is uncertain whether *Noctiluca* is a direct cause of the absence/low abundance of coccolithophores.

The members of the Noëlaerhabdaceae occurred almost through the year with seasonal abundance fluctuations, even though they occasionally become nearly absent in May and in August. Other coccolithophore taxa, however, only occurred in specific seasons. *T. adriatica* and *B. bigelowii* occurred very regularly in mid June to early July in the Tomari-area (Figs. 6e-6h). Their simultaneous occurrences lead us to speculate that they have similar environmental preferences. However, the re-examination of samples known to contain *B. bigelowii* from previous studies conducted in other parts of the seas of Japan and of North Sea revealed that these samples did not contain any *T. adriatica* (Table 2). The restricted occurrence of *T. adriatica* with *B. bigelowii* in Tomari is curious, since sampling localities in Tomari have direct surface water exchange with that of the Japan Sea (Fig. 1), and *B. bigelowii* occurs in many parts of the Japan Sea and its related water masses in late June (e.g. Hagino et al. 2009), at the same time that *T. adriatica* and *B. bigelowii* co-occur in Tomari. There is nothing obviously distinctive about the environmental setting of Tomari. The absence of *T. adriatica* in samples with *B. bigelowii* from other parts of the Japan seas indicates that *T. adriatica* have coastal patchy distribution.

B. bigelowii and members of *Gephyrocapsa*, which were common in the Tomari samples containing *T. adriatica*, were not found in the offshore Rovinj samples that yielded *T. adriatica* in this study (Table 1). The type specimen of *T. adriatica* was

collected from Rovinj in September 1934 (Kamptner 1940), and our *T. adriatica* specimens were found from August samples. These results indicate that *T. adriatica* is a late summer/early autumn species at Rovinj. The cause of the difference in occurrence season of *T. adriatica* between Tomari and Rovinj is unknown.

Algirosphaera robusta is known as a middle-lower photic zone dweller (e.g. Andrulleit et al., 2003; Hagino et al., 2000). *A. robusta* co-occurred with other middle to lower photic zone species such as *Michaelsarsia* spp., *Oolithotus antillarum*, *Ophiaster* spp., and *Florisphaera profunda* in the surface water of the Tomari-area in autumn (Appendix 1). Co-occurrence of middle-lower photic zone dwellers in surface level suggests they were transported from deeper water by mixing of the water column, although no hydrographic data that supports the mixing of water column is available. In autumn, *U. sibogae* and *U. foliosa* co-occurred with *A. robusta*, *O. antillarum*, *F. profunda*. *U. sibogae* and *U. foliosa*. prefer moderately eutrophic shallow water, and increase in abundance in the infra-marginal waters of upwelling areas (Hagino and Okada, 2004; 2006). The occurrence of *U. sibogae* and *U. foliosa* also suggests the presence of upwelling near Tomari area in autumn.

Floristic studies in Tomari showed that *T. adriatica* and *B. bigelowii* differ from other coccolithophore taxa in distribution and occurrence period. *T. adriatica* and *B. bigelowii* occurred in both coastal and offshore stations in mid-late June, although they were occasionally rare or absent in offshore stations, indicating their affinity to coastal environment. Their occurrence period ranged between years from 5 to 15 days, but did not exceed 20 days. On the other hand, other coccolithophore taxa were usually more

abundant in offshore stations than in coastal stations, suggesting their affinity to oceanic environment. Their occurrence period continued for 1.5-2 months or longer. In the literature, occurrence records of coastal species are rare in comparison to records of oceanic species. We suspect that strong seasonality of coastal species is one of the causes of their lack of record. In this study, we found *T. adriatica* from two widely separated sites, offshore Rovinj (N Adriatic Sea) and from coastal-offshore waters of Tomari (W Japan Sea). Therefore it is thought that *T. adriatica* will be found from other coastal waters if the researches of coccolithophores are conducted in various coastal conditions successively with short sampling interval (≤ 1 week, ideally). The occurrence records in the Adriatic Sea and the western Sea of Japan and absence in an extensive survey of the NW European Shelf suggest that *Tergestiella* may be confined to sub-tropical environments and be absent from temperate waters.

4-6. Living fossil coccolithophores survived the K/Pg event

The coccoliths of *Tergestiella* and *Cyclagelosphaera* are virtually indistinguishable and genetic data support a direct line of descent. *Cyclagelosphaera* is a well-defined evolutionary lineage, a representative of the dominant Watznaueriales Mesozoic coccolithophore group and first appeared in the Middle Jurassic (Perch-Nielsen, 1985). The extension of its range to the present day gives it a longevity of 175My, making it by far the longest lived of any coccolithophore genus and a remarkable example of a living fossil. They survived K/Pg event and multiple turn over events. Thus, information of ecology and environmental preference/tolerance of living *T. adriatica* may help the

understanding of Jurassic and Cretaceous coccolithophores and environmental change that caused extinction events.

In this context it is also noteworthy that there are three other extant coccolithophore genera that appear to have survived the K/Pg extinctions, to have persisted relatively unchanged in morphology to the present day. These are *Braarudosphaera*, *Cruciplacolithus* and *Calciosolenia*. *Braarudosphaera* is the best known of these since it forms uniquely distinctive pentagonal plates with a laminated ultrastructure (Hagino et al. 2009), it is virtually impossible to misidentify and has a continuous fossil record back to the Aptian, mid Cretaceous (Bown 1998). *Calciosolenia* is also highly distinctive, forming rhombic muraliths with an unusual central area structure of offset laths (Malinverno 2004, Young et al in press). Again it has an unambiguous, continuous record, from Hauterivian, Early Cretaceous (Bown, 1998) to the present.

Cruciplacolithus is a slightly more obscure example, it is predominantly a Paleogene genus and indeed a very characteristic component of the post K/Pg recovery assemblages, especially the species *C. primus*. It has recently been shown that it also occurs in shelf sediments of Late Maastrichtian age and so should be considered a survivor species (Mai et al., 2003). Like *Cyclagelosphaera* it has often been regarded as an extinct genus but one species *C. neohelis* occurs sporadically in coastal-neritic environments (e.g. Kawachi and Inouye, 1994; Konno and Jordan, 2006). Recently Medlin et al. (2008) used molecular genetics to show that it probably diverged from *Coccolithus* ca 60-70Ma and so was quite likely to be a direct descendant of Palaeogene *Cruciplacolithus*. Subsequently Bown (200x) showed from study of exceptionally

preserved specimens of *C. primus* from Tanzania that it has identical central area lath structure to modern *C. neohelis*, again supporting the idea that they were directly related.

These four genera have very different morphologies and belong to four different families or even orders of coccolithophores but as well as being survivors from the Mesozoic. *B. bigelowii*, *T. adriatica* and *C. neohelis* share a common affinity for neritic environments, unlike the majority of extant coccolithophores which are predominantly oceanic. *Calciosolenia* has a much broader distribution (Tanaka, 1991) but a distinct neritic affinity was suggested by (Andruleit and Rogalla, 2002). So three of four living fossil survivors of the K/Pg extinctions share the unusual trait for coccolithophores of being predominantly neritic, at least in their calcifying phase, this supports the inference of Bown (2005) and Medlin et al. (2008) that neritic habit may have been a key factor in allowing survival of the K/Pg extinctions. There are at least two possible reasons why neritic taxa survived the K/Pg extinctions better than oceanic ones. First, the neritic environment is inherently unstable and so taxa adapted to it are likely to have broad tolerances for temperature, salinity, pH and other physicochemical variables.

Alternatively neritic survivorship may have been because these taxa had a dormant benthic stage in their life cycles, similar to dinoflagellate cysts, which would have been capable of major surviving ecological shock. At our present state of knowledge of the life cycles and ecology of these taxa it is not possible to determine which is more likely but the palaeobiological significance of these taxa clearly makes more biological research worthwhile.

5. Summary

1. *Tergestiella adriatica* Kamptner was re-discovered from coastal and offshore waters of Tomari, Tottori Prefecture, Japan and from offshore Rovinj, Croatia. Cross-polarized light and SEM observations revealed that extant *Tergestiella* is extremely similar to the fossil genus *Cyclagelosphaera* in both morphology and crystallography. Their similarity suggests that *Cyclagelosphaera* is a synonym of *Tergestiella*, and *T. adriatica* is a direct descendent of *Cyclagelosphaera*.
2. Molecular phylogenetic analyses based on 18S rDNA sequences showed separation of *Tergestiella* from the base of the clade of other coccolithophores, suggesting their Jurassic separation. This result was predicted by the hypothesis that *Tergestiella* is a direct descendent of fossil genus *Cyclagelosphaera*.
3. Oligocene-Miocene examples of fossil *Cyclagelosphaera* were recently reported from New Jersey shelf. Re-assessment of these occurrences indicates that they were part of the biocoenosis at this locality, not reworked specimens. Together with the patchy nearshore distribution of extant *T. adriatica* observed in this study, restricted occurrences of Oligocene-Miocene fossil *Cyclagelosphaera* in shallower sites suggest that *Cyclagelosphaera/Tergestiella* was restricted to nearshore environments in most of the Cenozoic.
4. Successive floristic study of coccolithophores showed that two Mesozoic species *T. adriatica* and *B. bigelowii* synchronously occur in coastal and nearshore waters of Tomari area every June, although the environmental factor inducing the co-occurrence of these two Mesozoic taxa are still unknown.

5. Regular occurrence of middle-lower photic zone coccolithophore species in Tomari area in every autumn suggests the presence of seasonal mixing of water column in autumn.
6. The hypothesis that neritic adaptation was a significant factor in survivorship of the K/Pg extinctions for calcareous nannofossil is supported by our observations.

Acknowledgement

We thank Natsuko Tomioka, Takayuki and Kayo Tanaka for help with sampling in Tomari-area, Tottori Japan. We also thank Y. Hanahusa for suggestions on molecular experiments. Miocene shelf sediments used for Fig. 2j was provided by the International Ocean Drilling Program, the International Continental Scientific Drilling Program and the Tanzania Drilling Project. This research was supported by Grant in Aid for environmental study of art and sciences by Tottori Prefecture, Japan, Grant-in-aid for Scientific Research from the Japan Society for the Promotion of Sciences (No. 20740296), the Croatian Ministry of Science, Education and Sports (No. 098-0982705-2731) and by the European Community Research Infrastructure Action under the FP7 "Capacities" Program (SYNTHEsys, Project GB-TAF-132).

Figure Captions

Fig. 1. Location of samples used in this study: (a) Double circles shows the bases used for repeated sampling in this study, and solid circles show the locality of samples

containing *Braarudosphaera* which were re-examined in this study. (b) Locality of samples offshore Rovinj. (c) Locality of samples of coastal and offshore Tomari Port.

Fig. 2. Light microscopic images of modern *Tergestiella adriatica* and fossil *Cyclagelosphaera* (a) Specimens T5 used for molecular study in plain polarised light. (b) Specimen T5 in cross-polarized light. (c) Specimen T6 used for molecular study in plain polarized light. (d) Specimen T6 in cross-polarized light. (e) Collapsed coccosphere of *T. adriatica* from Tomari. (f) Collapsed coccosphere of *T. adriatica* from Rovinj. (g and h) light microscopic images of *T. adriatica*, reproduced from original description by Kamptner (1940). (i) Line drawings of *T. adriatica* reproduced from Kamptner (1940). (j) *Cyclagelosphaera* sp. from Miocene shelf sediments; ODP Sample 313-M0028A-30R-1, 24 cm (Mountain et al., 2010). (k) *Cyclagelosphaera margerelii* from xxxxxx. (l) *Cyclagelosphaera reinhardtii* from xxxx. All scale bars are 1 μ m.

Fig. 3. SEM images of modern *Tergestiella adriatica* and fossil *Cyclagelosphaera*; (a) Modern *T. adriatica* from Tomari (st. 3, 27/6/2009). (b) Proximal side of a coccolith of *T. adriatica* (st. 3, 27/6/2009). (c) close-up view of (b) showing narrow zone with complex sutures between outer and inner cycles. (d) Slightly etched specimen from Rovinj, Croatia (RV001, 18/8/2009). (e) Very early growth stage of coccolith of *T. adriatica* seen in distal view, inside a collapsed *T. adriatica* coccosphere (st. 1, 18/6/2013). (f) Close-up view of the very early growth stage specimen shown in (e). (g) Early-growth stage coccolith seen in

distal view, on collapsed coccosphere of *T. adriatica* (st. 1, 18/6/2013) (h) close-up view of early growth stage specimen shown in (g). (i) incomplete coccoliths of *T. adriatica* (st. 1, 18/6/2013), (j) Modern *T. adriatica* from Rovinj (28/8/09). (k) *Cyclagelosphaera margerelii* from the N Atlantic Ocean (DSDP Site 105). (l) Early Paleocene *Cyclagelosphaera reinhardtii* from Shatsky Rise, NW Pacific Ocean (ODP Hole 1209C). All scale bars 1 μm .

Fig. 4. Schematic diagram of coccolith of *Tergestiella/Cyclagelosphaera*. Based in part on a diagram in Young & Bown (1991) but reconstruction of the V-unit shape in cross-section verified by observations on early growth stage and incomplete coccoliths from this study.

Fig. 5. Phylogenetic tree based on 18S rDNA sequences using the Maximum Likelihood method. A representative of the Pavlovophyceae (*Pavlova gyrans*) was used as an out-group. The numbers on each node indicate the bootstrap values from ML analysis and posterior probability of BI analysis. Solid circles indicate the clades supported by very high bootstrap values (100%) by ML and posterior probability (1.00) by BI analysis. Clade C⁺ shaded gray includes all calcifying haptophytes. (Takano et al., 2006). Note the deep divergence of *T. adriatica* and *B. bigelowii* from other coccolithophores, as predicted from the interpretation of it as a living fossil representative of a major Mesozoic clade.

Fig. 6. Succession in hydrographic data and abundance of *T. adriatica* and *B. bigelowii* in coastal and offshore stations of Tomari area. (a) SST in coastal samples. (b) SST in offshore samples. (c) standing crop of coccolithophores in coastal samples.

(d) standing crop of coccolithophores in offshore samples. (e) cell density of *T. adriatica* in coastal samples. (f) cell density of *T. adriatica* in offshore samples. (g) cell density of *B. bigelowii* in coastal samples. (h) cell density of *B. bigelowii* in offshore samples.

Fig. 7. Succession in abundance of major coccolithophore taxa. (a) cell density of *A. oryza* in coastal samples. (b) cell density of *A. oryza* in offshore samples. (c) cell density of *C. rigidus* in coastal samples. (d) cell density of *C. rigidus* in offshore samples. (e) cell density of *E. huxleyi* in coastal samples. (f) cell density of *E. huxleyi* in offshore samples. (g) cell density of *G. ericsonii* in coastal samples. (h) cell density of *G. ericsonii* in offshore samples. (i) cell density of *G. oceanica* in coastal samples. (j) cell density of *G. oceanica* in offshore samples. (k) cell density of *Syracosphaera* spp. in coastal samples. (l) cell density of *Syracosphaera* spp. in offshore samples.

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