

# Re-discovery of a “living fossil” coccolithophore from the coastal waters of Japan and Croatia

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## Abstract

The extant coccolithophore *Tergestiella adriatica* Kamptner, which had not been reported since its original description in 1940, was recently re-discovered in coastal-nearshore waters at Tomari, Tottori (Japan) and offshore Rovinj (Croatia). Morphological analysis shows that extant *Tergestiella* and the Mesozoic genus *Cyclagelosphaera* (Watznaueriaceae), thought to have been extinct since the early Eocene (~54 Ma), are virtually identical. Molecular phylogenetic study supports the inference that *T. adriatica* is a direct descendent of *Cyclagelosphaera*. It is therefore a remarkable example of a living fossil. Our documentation of patchy coastal distribution in living *T. adriatica* and records of rare occurrences of fossil *Cyclagelosphaera* in Oligocene-Miocene shallow water sediments, from the New Jersey shelf, suggest that *Tergestiella/Cyclagelosphaera* was restricted to nearshore environments during much of the Cenozoic. This restricted ecology explains the lack of fossil *Tergestiella/Cyclagelosphaera* recorded in open ocean sediments deposited during the last 54 myr.

Floristic study of coccolithophores in the coastal and offshore waters of Tomari over a six-year period, show that *T. adriatica* occurs synchronously with the unusual neritic species, *Braarudosphaera bigelowii*, in mid-June. The environmental factors that induce the co-occurrence of these two taxa are uncertain, and *T. adriatica* did not co-occur with *B. bigelowii* at any other sites.

## 1. Introduction

Coccolithophores are marine unicellular haptophytes characterized by calcareous scales called coccoliths (e.g., Young et al., 2003). Fossil coccoliths and other calcareous microfossils of uncertain affinities are collectively referred to as calcareous nannofossils (Bown, 1998). Calcareous nannofossils, which appeared in the late Triassic (~225 Ma), progressively diversified through the Jurassic and Cretaceous periods, despite some extinction and turnover events. The highest diversity of calcareous nannofossils is recorded in the Campanian and Maastrichtian (80-68 Ma); however, 85 % of genera and 93 % of species went extinct at the K/Pg (Cretaceous/Paleogene) mass extinction event at 66 Ma (Bown et al., 2004). Immediately after the extinction, a low diversity assemblage of coccolithophore survivor species flourished, followed by a rapid evolutionary radiation of new Cenozoic taxa (e.g., MacLeod et al., 1997). Most K/Pg survivors went extinct in the Cenozoic; however, three K/Pg survivor taxa are known to be extant: *Braarudosphaera bigelowii*, *Calciosolenia* spp., and *Cruciplacolithus neohelis* (Bown, 2005; Young et al., 2003).

During studies of living *Braarudosphaera* in the coastal-offshore waters of Tomari Port, Tottori, Japan (western Sea of Japan) (Hagino et al., 2013), 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, as *Cyclagelosphaera* first occurred in the Middle Jurassic (ca. 170 Ma; Bown and Cooper, 1998) this would make it the oldest extant coccolithophore 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 Kamptner (1940) described this species as *Tergestiella adriatica* from the northern Adriatic Sea, although it has not been reported in 70 years of subsequent studies, and was not known to current researchers.

To determine the phylogenetic affinities of *Tergestiella* to *Cyclagelosphaera*, we conducted detailed morphological studies of the specimens from both Tomari and Rovinj using the scanning electron microscope (SEM) and cross-polarized light microscope (LM). We also carried out molecular phylogenetic studies based on 18S rDNA sequences obtained from the specimens from Tomari 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*. Also, we studied the seasonal succession of living coccolithophores, including *T. adriatica*, from the coastal and offshore waters of Tomari in order to determine their ecological preferences. Furthermore, we

re-examined fossil specimens of '*Cyclagelosphaera?*' reported from Oligocene-Miocene sediments of the New Jersey margin (Mountain et al., 2010) under the LM.

## **2. Materials and Methods**

### *2.1. Sampling*

Sea-surface water samples (10-12 liters) were collected by bucket 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) on 167 occasions from July 2008 to June 2014 (Fig. 1, Appendix 1). 65 sea-surface water samples from offshore Tomari were collected from the fishing boat (*Ryoto-Maru*) using a 10L plastic bottle, from July 2007 to July 2013 (Appendix 1). The offshore sampling water depth was measured by a fish-detector and ranged from 20-50m. The sampling interval was adjusted according to the season, from July 2008 to March 2012 (Supplementary Materials).

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

The in situ sea-surface temperature (SST) of almost all the coastal samples and some offshore samples of Tomari were measured using a bar thermometer (Appendix 1). In situ seawater temperature and salinity of the samples offshore Rovinj was measured with a SBE 25 Sealogger CTD probe (Sea Bird Electronics Inc., Bellevue, Washington, SAD) (Appendix 2).

### *2.2 Floristic and morphological studies.*

The water samples from Tomari were pre-filtered through a 50- $\mu$ m plankton net (Sefar Inc. DIN-110) to remove large plankton, and then one or two liters were filtered onto Millipore HAWP04700 or Whatman 7060-4710 membrane filters. The filters were then air-dried and stored in plastic Petri dishes. A portion of the filter was mounted on a glass slide with Norland Optical Adhesive 74 for LM study. Coccolithophore taxa were identified on  $\geq 300$  fields of view, corresponding to  $\geq 5$ ml of seawater, using a Nikon E600POL cross-polarized LM at 1500x magnification. Samples prepared from various sites around NE Japan, as well as the North Sea and Bay of Biscay, which were known to contain *B. bigelowii* (Hagino et al., 2009; Young et al. 2014), were re-examined using the method described above (Fig. 1a; Appendix 3).

Seawater samples from Rovinj were collected using Niskin bottles (Appendix 2). Subsamples of 50 ml fixed with neutralized formaldehyde (2% final concentration) were sedimented

and cells counted on a half transect at 400x or 200x magnification using an inverted LM (Zeiss Axiovert 200) following Utermöhl (1958). Subsequently, the sample used for LM study was filtered (Whatman 110612), and a piece of filter mounted on a microscope slide and fixed with Norland Optical Adhesive 74 or Canada balsam, for additional cross-polarized LM studies (Zeiss Opton Invertoskop D or Zeiss Axioplan).

For morphological studies of selected species, segments of selected filter samples from Tomari and Rovinj were mounted on aluminum stubs and sputter coated with gold-palladium (Cressington Scientific 208HR) or gold (Sanyu SC701 MKII) for the SEM (Phillips XL30 FEG SEM or JEOL JSM 7001F). Taxonomic classification of the coccolithophores follows Young et al. (2003); members of other non-calcifying haptophytes follow Edvardsen et al. (2011). The descriptive terminology of coccoliths follows Young et al. (1997).

### 2.3 Molecular phylogenetic studies

The single cell Polymerase Chain Reaction (PCR) method (Takano and Horiguchi, 2004) was applied to *T. adriatica* specimens, in order to determine their molecular phylogenetic position based on 18S rDNA sequences. For this, 10 liter samples collected from Tomari on the 21 and 23 June 2010 were pre-filtered through a 25- $\mu$ m plankton net (Sefar Inc. P-25), and then concentrated using 1  $\mu$ m mesh-size plankton net fabric (Sefar Inc. NY1-HD). Six *T. adriatica* cells (T1-T6) were isolated with a micropipette under an inverted LM (Olympus CKX41). Each cell was photographed using a Nikon E600POL microscope, and then subjected to single cell PCR amplification. The primers used for PCR amplification, PCR conditions, procedure for sequence determination and the sequencers, were as described in Hagino et al. (2009). Results were checked by sequencing both forward and reverse strands. Identical 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 - GenBank Accession numbers of T5 and T6 are AB636316 and AB636317, respectively.

For phylogenetic analysis, 52 gene sequences of 18S rRNA from haptophytes, were obtained from GenBank, including a sequence of the Pavlovophyceae as an out-group. The sequences were aligned using Clustal W (<http://www.genome.jp/tools/clustalw/>). Phylogenetic trees were constructed using Maximum Likelihood (ML) with PAUP version 4.0b10 (Swofford, 2002) and using Bayesian inference (BI) with Mr. BAYES v3.1.2 (Ronquist and Huelsenbeck, 2003).

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 Bayesian (BI) analysis, the GTR+I+G model was selected by the MrModeltest. Markov chain Monte Carlo iterations were carried out to 6 million generations, when the average standard deviations of split frequencies were below 0.01, indicating convergence of the iterations.

### **3. Results**

#### *3.1. Morphological observations*

The unusual coccolithophore specimens with distinctive circular coccoliths unlike any well-known extant species were initially found from shallow marine waters around Tomari Port in June 2009 (Figs 2a-2e, Appendix 1). Subsequently, similar specimens were observed in samples from offshore Rovinj in August 2009 (Fig2f, Appendix 2). Specimens from the two localities are indistinguishable from each other in morphology under cross-polarized LM (Figs 2e and 2f) and SEM (Figs 3a and 3j).

The coccospheres are spherical with 12-15 circular placolith coccoliths with a 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) (Fig. 2e and 2f). There is also a narrow median cycle showing low birefringence, indicating sub-vertical crystallographic c-axes (V-units) (Fig. 2e and 2f).

The diameter of the coccoliths was measured on SEM images of well-formed specimens; it ranges from 4.3 to 6.4  $\mu\text{m}$  (mean 5.6  $\mu\text{m}$ , sdev 0.4  $\mu\text{m}$  [58 coccoliths]) in Tomari, and from 4.5 to 5.7  $\mu\text{m}$  (mean 5.2  $\mu\text{m}$ , sdev 0.31  $\mu\text{m}$  [18 coccoliths]) in Rovinj. In the SEM, the distal surface shows three cycles of elements: 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 degree 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 and 3j). 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 (Figs 3b-3d). There is a narrow zone with complex sutures between the inner and outer cycles (arrow on Fig. 3c). Incomplete coccoliths at various growth stages were observed on the inside of collapsed coccospheres, indicating that it produces coccoliths intracellularly (Figs 3e-f). The median cycle is

consistently present on the specimens even at very early growth stages (arrows on Figs 3h and 3i) indicating that it extends downward into the coccolith, as indicated on Fig. 4.

### 3.2. Taxonomic identification

Our specimens resemble *Tergestiella adriatica*, which was described from the north Adriatic Sea off Rovinj Marine Station (Kamptner, 1940), but has never subsequently been reported. The light micrographs of the holotype of *T. adriatica* are rather indistinct (Fig 2g-h), but more details of *T. adriatica* are visible on his line drawings (Fig. 2i). 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 is not seen on any other modern coccolithophores. Kamptner (1940) indicates a coccolith diameter of 5.7  $\mu\text{m}$ , without stating whether this was an average or maximum value. This is consistent with the size range of the coccoliths we observed (4.3-6.4 $\mu\text{m}$ , see above). Kamptner's specimens were collected from the same locality and at the same time of year as our Croatian specimens, and clearly were the same species. So our specimens should be referred to the species *Tergestiella adriatica*.

Our specimens also closely resemble the fossil genus *Cyclagelosphaera*, both in cross-polarized light and in the scanning electron microscope (Figs 2 and 3). 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 medial cycle (tube core elements) show low birefringence indicating that the crystallographic c-axis is sub-vertical (i.e., they are V-units) (compare Figs 2e-f with Figs 2k-l). Detailed analysis of the structure and crystallography of *T. adriatica* confirms that it has all the characteristic features of coccoliths of the Watznaueriaceae (Supplementary Material). The coccoliths of *T. adriatica* differ in minor features from any described species of *Cyclagelosphaera*, but not by enough to allow generic separation. Thus, *Tergestiella* (Kamptner, 1940) and *Cyclagelosphaera* (Nöel, 1965) are synonyms. Since *Tergestiella* has priority over *Cyclagelosphaera*, we refer to our specimens as *T. adriatica* (Supplementary Material).

### 3.3. Molecular phylogenetic analyses

We could not grow *T. adriatica* in culture, despite many attempts (Hagino, personal observation), and so determined partial SSU rDNA sequences of *T. adriatica* picked from the Tomari field samples using the single cell PCR method. The ML and BI analyses resulted in similar trees (Fig. 5), and the topology of the trees resembles those of previous studies (e.g., Hagino et al., 2013) except for the position of the clade that includes *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<sup>+</sup> (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).

#### 3.4. Occurrence of *Tergestiella adriatica* in Tomari

The coccolithophore floras at Tomari were monitored for 6 years (2008-2013) and a total of 20 coccolithophore taxa were recorded using cross-polarized light microscopy (Appendix 1). *T. adriatica* and *B. bigelowii* show similar distribution patterns, occurring only from mid-June to early July, as SSTs increased from 19 to 26 °C (Fig. 6; Supplementary Material). This occurrence period ranged from 5-15 days, and is markedly shorter than the period of any other coccolithophores ( $\geq 1.5$  months, Appendix 1). *T. adriatica* and *B. bigelowii* did not occur from mid-July through to the next early June, except for a single cell of *B. bigelowii* found in 22 November 2012 (Hagino et al., 2013). *Emiliana huxleyi*, *Gephyrocapsa ericsonii* and *G. oceanica* co-occurred with *T. adriatica* and *B. bigelowii* in mid-late June, but also had a much broader distribution, occurring in July, and from September to April (Appendix 1).

*T. adriatica* was found at the coastal stations every June, and at the offshore stations in June 2009 and 2011-2013, but not in June 2010, suggesting its affinity to the coastal environment. *B. bigelowii* occurred in both coastal and offshore stations every June from 2009 to 2013 (Figs 6a-b, Appendix 1). Samples collected from early-mid June 2014 did not contain cells of *T. adriatica* or *B. bigelowii*; however, loose coccoliths of *T. adriatica* and loose pentoliths of *B. bigelowii* were present, except in the final sample collected on 25 June 2014. These results suggest that *T. adriatica* and *B. bigelowii* were present in mid-June 2014 but at very low abundance.

#### 3.5. Re-examination of filter samples used in previous studies

In order to determine if *T. adriatica* has similar ecological preferences to *B. bigelowii* in other regions, we re-examined filter samples known to contain *B. bigelowii* from previous studies conducted in other parts of the seas of Japan and around the NW European continental shelf, using cross-polarized LM. (Fig 1a). The re-examined samples did not contain even any fragments of *T. adriatica* (Appendix 3).

#### 3.6. Occurrence of *T. adriatica* from Rovinj

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

### 3.7. New Jersey Margin Fossil Material

We re-examined selected smear slides prepared from Oligocene and Miocene sediments deposited on the New Jersey shelf, which were known to contain '*Cyclagelosphaera?* sp.' (Mountain et al., 2010), using cross-polarized LM. We confirmed that the sample contains both loose coccoliths and multiple coccospheres of *Cyclagelosphaera* (Fig. 2j) and that these specimens are indistinguishable from modern *T. adriatica*. The paleo-water depth of the samples yielded '*Cyclagelosphaera?* sp.' was inferred from benthic foraminiferal assemblages to be shallower than 120 m (Appendix 4, Supplementary Material).

## 4. Discussion

### 4.1. *Tergestiella adriatica*, a living fossil coccolithophore

The fossil record shows that coccolithophores evolved in the Late Triassic and underwent an evolutionary radiation in the Early Jurassic (ca. 190 Ma) (Bown, 1987). A major group, the Watznaueriales, which includes *Tergestiella/Cyclagelosphaera*, diverged during this radiation and no extant descendant taxa had previously been identified. The basal divergence seen in molecular phylogenies of coccolithophores is thought to correspond to this Early Jurassic radiation (Medlin et al., 2008). In our molecular phylogenetic analysis, two extant species with Mesozoic origins (*T. adriatica* and *B. bigelowii*) occur near the base of the coccolithophore clade C<sup>+</sup>, although the precise relationship among *T. adriatica*, *B. bigelowii* and the other coccolithophores cannot be determined from the analyses due to low bootstrap support values and low posterior probabilities (Fig. 5). The phylogenetic positions of *T. adriatica* in 18S rDNA trees, however, suggests that they diverged 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*.

*Cyclagelosphaera* is a well-defined evolutionary lineage, a representative of the dominant Watznaueriales Mesozoic coccolithophore group that first appeared in the Middle Jurassic (Bown and Cooper, 1998). The extension of their range to the present day gives them a longevity of 175 myr, making it by far the longest lived of any coccolithophore genus and a remarkable example of a living fossil that survived the K/Pg event and multiple evolutionary turnover events.



#### 4.2. Floristic studies

Floristic studies at Tomari show that *T. adriatica* and *B. bigelowii* differ from other coccolithophore taxa in both distribution and occurrence period. They occur at both coastal and offshore stations in mid-late June, although they were occasionally rare or absent in offshore samples, indicating their affinity for coastal environments. In detail, their occurrence period varied from year to year, ranging from 5 to 15 days, but never exceeding 20 days. By contrast, other coccolithophore taxa were usually more abundant offshore, indicating their affinity for oceanic environments, and they were present for longer, typically 1.5-2 months or longer (Appendix 1). Records of occurrences of *T. adriatica* and *B. bigelowii* are rare in the literature and we suspect that strong seasonality and a short occurrence period are the causes of this. Here, we found *T. adriatica* at two widely separated sites for very short intervals. Therefore, we suspect that *T. adriatica* will be found in other coastal waters if sampling is conducted in various coastal conditions successively with a short sampling interval (ideally  $\leq 1$  week).

Simultaneous occurrences of *T. adriatica* and *B. bigelowii* in Tomari lead us to speculate that they have similar environmental preferences. However, re-examination of samples known to contain *B. bigelowii* from previous studies conducted in other parts of the seas of Japan, North Sea, and the Bay of Biscay revealed that these samples did not contain any *T. adriatica* (Appendix 3). The restricted occurrence of *T. adriatica* with *B. bigelowii* in Tomari is curious as it is directly connected to the Sea of Japan (Fig. 1), where *B. bigelowii* was reported in late June (e.g., Hagino et al., 2005; Hagino et al., 2009). There is nothing obviously distinctive about the environmental setting of Tomari, so the absence of *T. adriatica* from other parts of the seas surrounding Japan suggests that *T. adriatica* has patchy coastal distribution.

#### 4.3. The fossil record of *Tergestiella/Cyclagelosphaera*

In the Mesozoic, *Cyclagelosphaera* appears to have had a broad distribution but was more abundant in neritic environments (Bown, 2005; Lees et al., 2004). In the Upper Jurassic, in addition to typical marine occurrences, *Cyclagelosphaera* was abundant in lagoonal sediments such as the Upper 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 unusual in coccolithophores and makes it a key component of the Upper Jurassic lithographic limestone facies, which includes some of the most important lagerstätten known. Through the Cretaceous, *Cyclagelosphaera* became progressively less abundant, and although it occurred at low abundances in open marine sediments, it was only common in shelf sediments. The most prominent exception to this pattern of neritic affinity occurred immediately after the K/Pg extinctions, where *Cyclagelosphaera* was a common component of mid-latitude oceanic

assemblages in the early Danian recovery assemblages (Bown, 2005; Pospichal, 1996). Subsequently, it declined and was thought to have become extinct in the early Eocene (~54 Ma; Bybell and Self-Trail, 1994).

As noted above, *Cyclagelosphaera* is generally regarded as having its last occurrence in the earliest Eocene; however, Mountain et al. (2010) reported fossils of ‘*Cyclagelosphaera?* sp.’, from Oligocene and Miocene sediments deposited in shallow water on the New Jersey shelf. The samples also contain diverse nannofossil assemblages, which allowed zonation of the cores, as well as rare reworked Eocene taxa (e.g., *Reticulofenestra umbilicus*, *Transversopontis* sp.), a small *Cruciplacolithus* species and *Braarudosphaera bigelowii*. These records are summarised in our Supplementary Material and in Appendix 4 along with paleodepth estimates derived from benthic foraminifera (Katz et al., 2013). *Cyclagelosphaera?* and *Cruciplacolithus* generally co-occur with *Braarudosphaera*, but do not correlate with the obviously reworked Eocene specimens, which are confined to the lower part of the section (Tables T3 and T4 of (Mountain et al., 2010). Coccospheres of *Cyclagelosphaera?* sp. were noted in Site M0029 (Fig. 2j) and in all sites preservation of *Cyclagelosphaera* and *Cruciplacolithus* was similar to that of the in situ Neogene assemblage. Hence it seems reasonable to interpret the specimens as in situ and therefore a Neogene record of these taxa.

Our observation of living *Tergestiella* in nearshore environments off Japan and Croatia and fossil *Cyclagelosphaera* from inner shelf Miocene deposits off New Jersey (Mountain et al., 2010) suggests that *Cyclagelosphaera* did not become extinct in the early Eocene but was restricted to nearshore environments. Such environments are not usually suitable for coccolith preservation and the absence of records may also be due to *Cyclagelosphaera/Tergestiella* having a patchy coastal distribution. Finally, sporadic occurrences of *Cyclagelosphaera* found in previous studies of other late Paleogene to Recent sections have likely been interpreted as reworked Mesozoic to early Paleogene *Cyclagelosphaera*. We suspect that if more well-preserved nannofossil assemblages from warm-water shallow shelf sites are studied carefully, the apparent gap in the fossil record of these taxa between the lowermost Eocene and the modern will prove illusory.

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

It is noteworthy that there are three other extant coccolithophore genera that appear to have survived the K/Pg extinctions, and then 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 laminated ultrastructure (e.g., Hagino et al., 2009), 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 muroliths with an unusual central area structure of offset laths (Malinverno, 2004). Again it has an unambiguous, continuous record, from the Hauterivian (Early Cretaceous) (Bown, 1998) to the present. *Cruciplacolithus* is a slightly more obscure example, as it is a predominantly Paleogene genus and indeed a very characteristic component of the post K/Pg recovery assemblages. Especially the species *C. primus*. argued that it 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 today (e.g., Kawachi and Inouye, 1994; Konno and Jordan, 2006). Medlin et al. (2008) used molecular genetics to show that *C. neohelis* probably diverged from *Coccolithus* at around 60-70 Ma and so was quite likely to be a direct descendant of Paleogene *Cruciplacolithus*. Subsequently, Bown (2010) showed from study of exceptionally preserved specimens of Eocene-Miocene *C. primus* from Tanzania that it has identical central area lath structure to modern *C. neohelis*, again supporting the idea that they are directly related.

These four genera have very different morphologies and belong to four different families or even orders of coccolithophores, However, ecologically *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 Andruleit and Rogalla (2002) suggested a distinct neritic affinity. So, three of the 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, which supports the inference of Bown (2005) and Medlin et al. (2008) that neritic habit may have been a key factor in facilitating survival of the K/Pg extinctions.

Selective survivorship of neritic taxa during the K/Pg extinctions is also reported from other calcareous plankton. In particular, planktonic foraminifera are a predominantly oceanic group and were nearly eliminated at the K/Pg event, with repopulation occurring from a few neritic taxa (Berggren and Norris, 1997; Liu and Olsson, 1992). Calcareous dinoflagellates by contrast are a primarily neritic group and were much less affected by the extinctions; indeed they flourished in the recovery interval, except for the outer-shelf adapted pithonellids, which became extinct (Wendler and Willems, 2002). This pattern of selective extinction of oceanic taxa, has only been noted in calcareous plankton and not in groups such as radiolaria, diatoms and organic-walled dinoflagellates, which are characterized by siliceous scales/frustules or organic walls (de Wever et al., 2003; Hollis, 2003; MacLeod et al., 1997; Sims et al., 2006). However, the records of these groups are not as complete as those of the calcareous plankton and at least for dinoflagellates the lack of extinctions may reflect a predominantly neritic habitat. It therefore

remains uncertain whether oceanic plankton in general were especially affected by the K/Pg extinctions, or only calcareous oceanic plankton.

One mechanism that could cause selective extinction of calcareous oceanic plankton is short-lived ocean acidification, which might have occurred after the K/Pg impact event as a result of shock heating of the atmosphere (Kring, 2007) or vaporization of CaSO<sub>4</sub> in target rocks (D'Hondt et al., 1994). If significant rapid ocean acidification did occur, then oceanic calcareous plankton, which are adapted to a narrow range of pH conditions, may have gone extinct synchronously. Conversely, the far greater range of pH and salinity conditions that neritic taxa routinely experience may have facilitated their survivorship. The possibility of ocean acidification during the K/Pg event has been argued from patterns of extinction seen in corals (Veron, 2008) and foraminifera (Alegret et al., 2012).

In this study, we re-discovered extant *Tergestiella/Cyclagelosphaera* from coastal waters of Japan and of Croatia. Our findings support the hypothesis of selective survivorship of coastal coccolithophores in particular and of coastal calcareous plankton in general, possibly as a result of ocean acidification during the K/Pg event. However, our current state of knowledge of the ecology of coastal survivors does not allow us to determine the cause(s) of differential survival rates between coastal and oceanic calcareous plankton. More biological research of coastal taxa is worthwhile in order to test hypotheses of survivorship, which could ultimately help us better understand potential consequences of future ocean acidification due to climate change.

## 5. Summary

1. *Tergestiella adriatica* Kamptner was re-discovered from coastal and offshore waters of Tomari (Japan) and from offshore Rovinj (Croatia). Cross-polarized LM and SEM observations show that extant *Tergestiella* is extremely similar to the fossil genus *Cyclagelosphaera* in both morphology and crystallography. Their similarity suggests that *T. adriatica* is a direct descendent of *Cyclagelosphaera*.
2. Molecular phylogenetic analyses based on 18S rDNA sequences show separation of *Tergestiella* from the base of the clade of other coccolithophores, suggesting a divergence time during the Early Jurassic radiation. This result supports the hypothesis that *Tergestiella* is a direct descendent of the fossil genus *Cyclagelosphaera*.
3. Patchy nearshore distribution of extant *T. adriatica* and restricted occurrences of Oligocene-Miocene fossil *Cyclagelosphaera* in shallow water sites suggest that *Cyclagelosphaera/Tergestiella* was restricted to nearshore environments for most of the Cenozoic.

4. Floristic studies show that *T. adriatica* and *B. bigelowii* occur synchronously every June in the coastal and nearshore waters of the Tomari area. The environmental factor(s) inducing the co-occurrence of these taxa are uncertain.
5. The hypothesis that neritic adaptation was a significant factor in survivorship of the K/Pg extinctions for calcareous nannofossils is supported by our observations.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.marmicro.2015.01.002>.

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## Figure Captions

Fig. 1. Location of samples used in this study: (a) Double white circles shows the bases used for repeated sampling in this study, and solid black circles show the locality of samples containing *Braarudosphaera* that 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 plane-polarized light. (b) Specimen T5 in cross-polarized light. (c) Specimen T6 used for molecular study in plane-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, IODP Expedition 313; coccosphere from Sample 313-M0029A-92R-1, 21 cm and single coccolith (inset) from Sample 313-M0028A-30R-1, 24 cm (Mountain et al., 2010). (k) Jurassic (Oxfordian) *Cyclagelosphaera margerelii* from Cleveland Farm Pit, Wiltshire, UK. (l) Earliest Danian *Cyclagelosphaera reinhardtii* from Shatsky Rise, NW Pacific Ocean (ODP Site 1210A). All scale bars are 1  $\mu\text{m}$ .

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) Upper Jurassic (Oxfordian) *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  $\mu\text{m}$ .

Fig. 4. Schematic diagram of coccolith of *Tergestiella/Cyclagelosphaera*, based in part on a diagram in Young & Bown (1991). The reconstruction of the V-unit shape in cross-section was verified by SEM observations on early growth stage and incomplete coccoliths and by LM observations of coccoliths in side view.

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<sup>+</sup> (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 abundance of *T. adriatica* and *B. bigelowii* (a) Cell density of *T. adriatica* in coastal samples. (b) Cell density of *T. adriatica* in offshore samples. (c) Cell density of *B. bigelowii* in coastal samples. (d) Cell density of *B. bigelowii* in offshore samples.

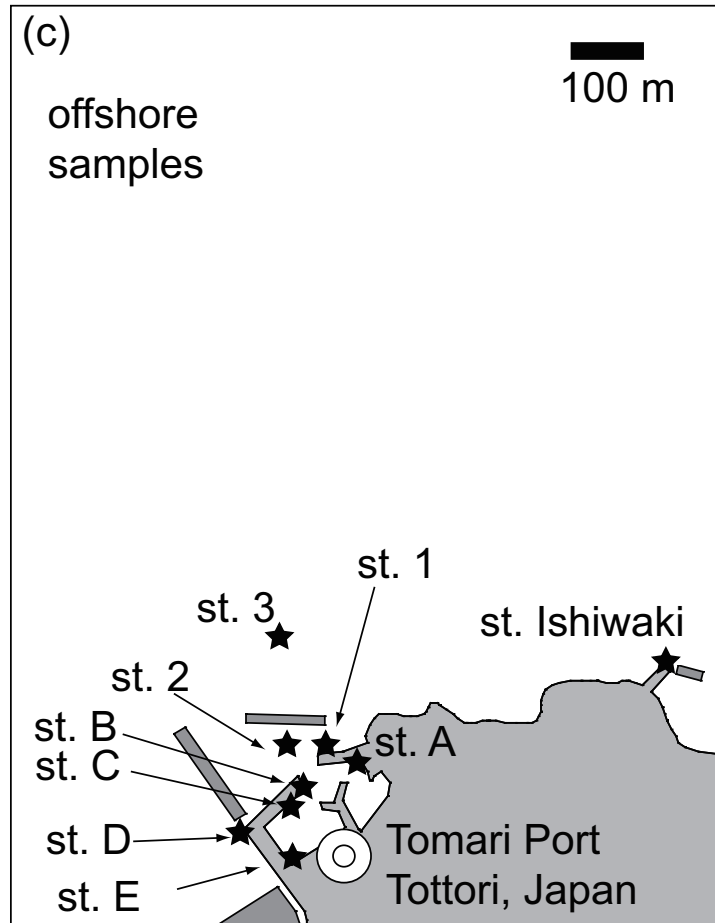
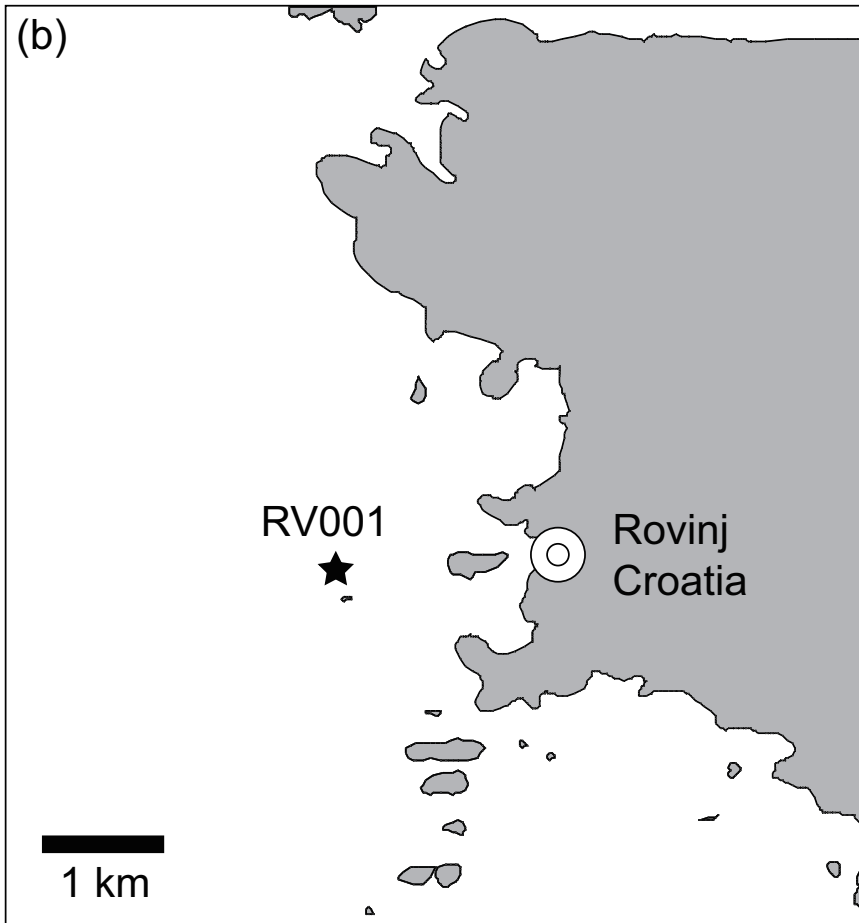


Fig. 1 Hagino et al.

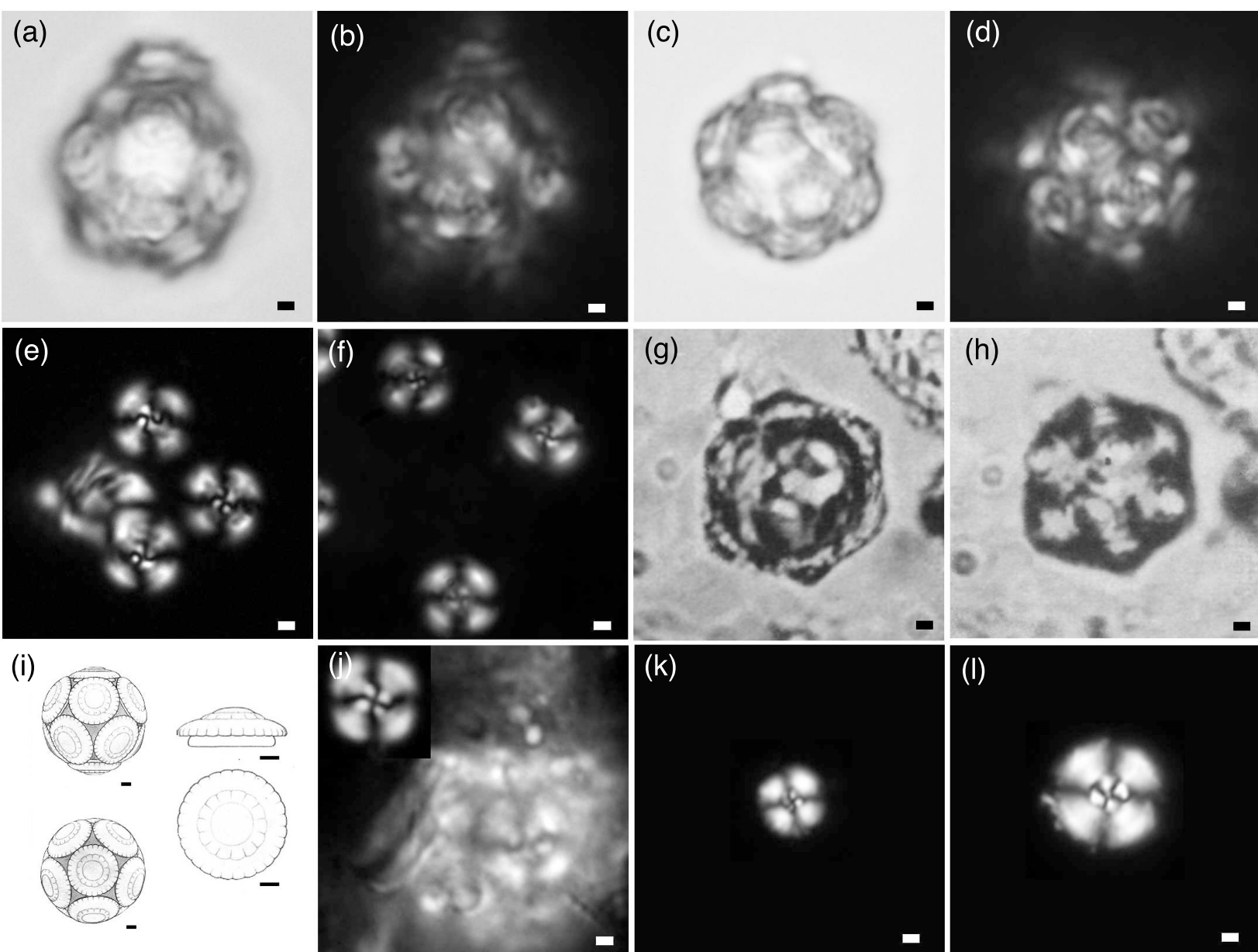


Fig. 2

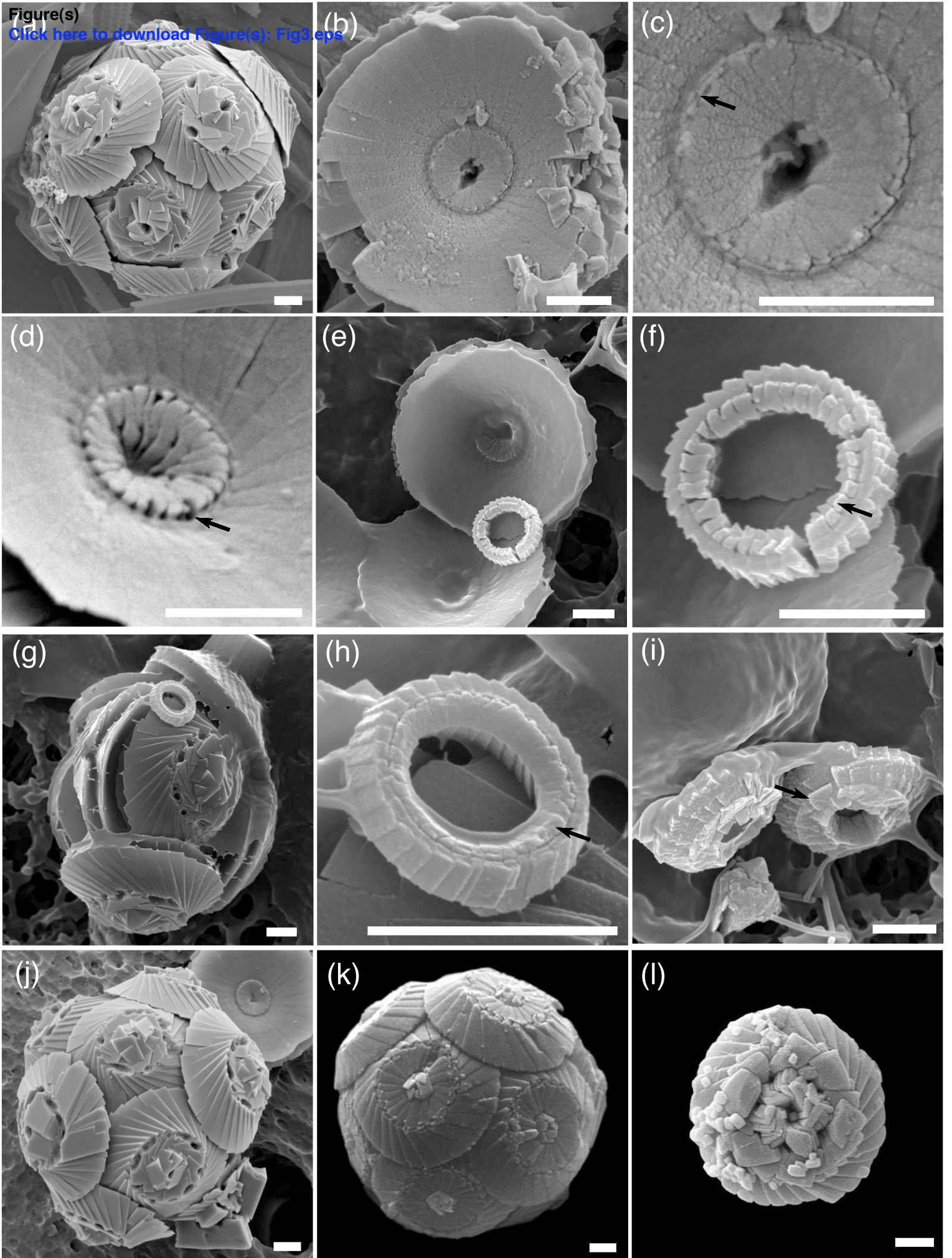


Fig. 3

Figure(s)

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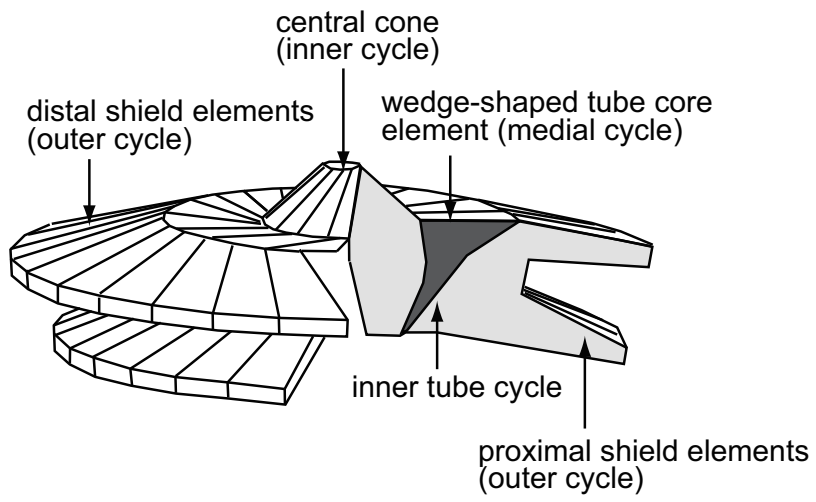
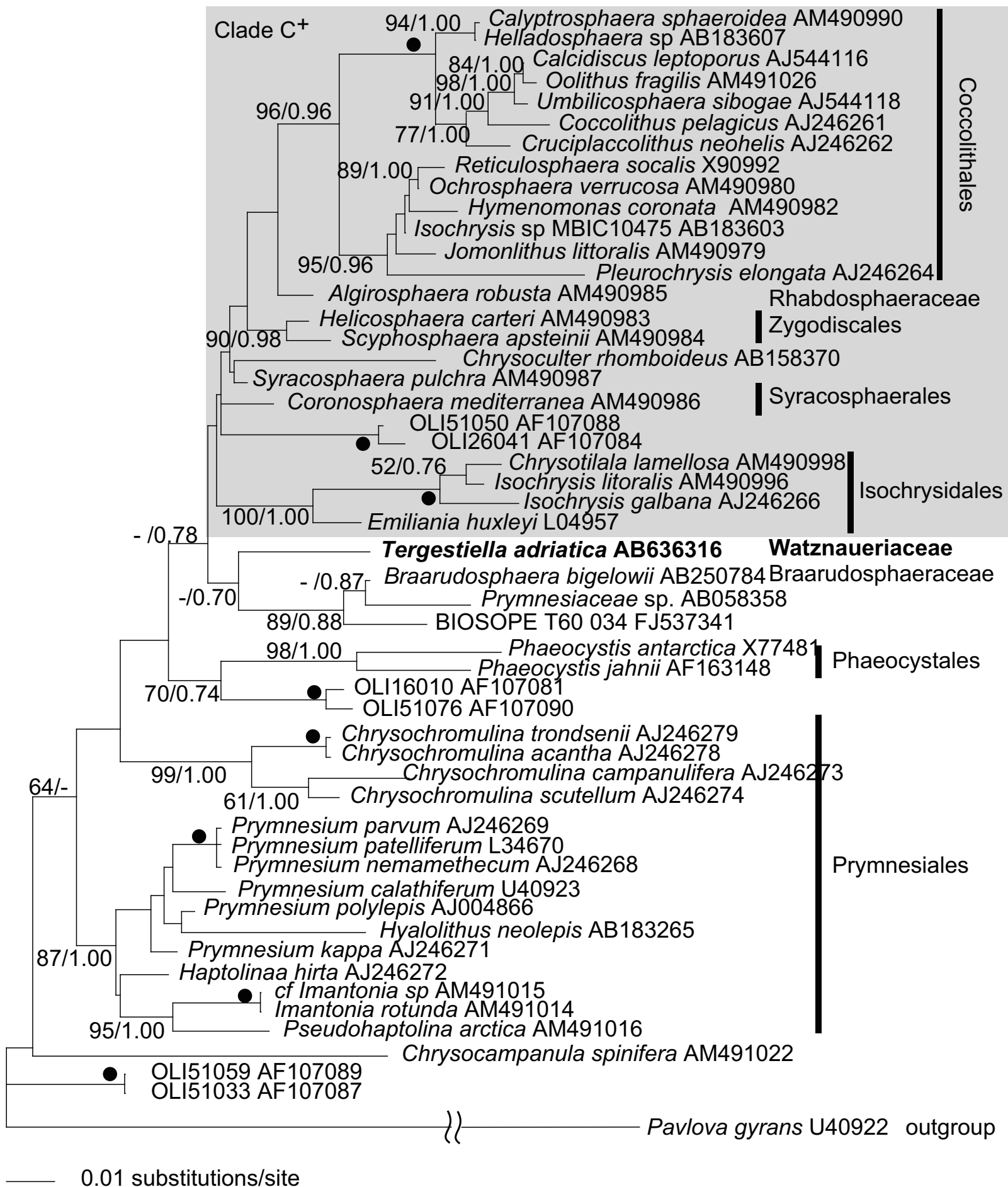


Fig4. Hagino et al.



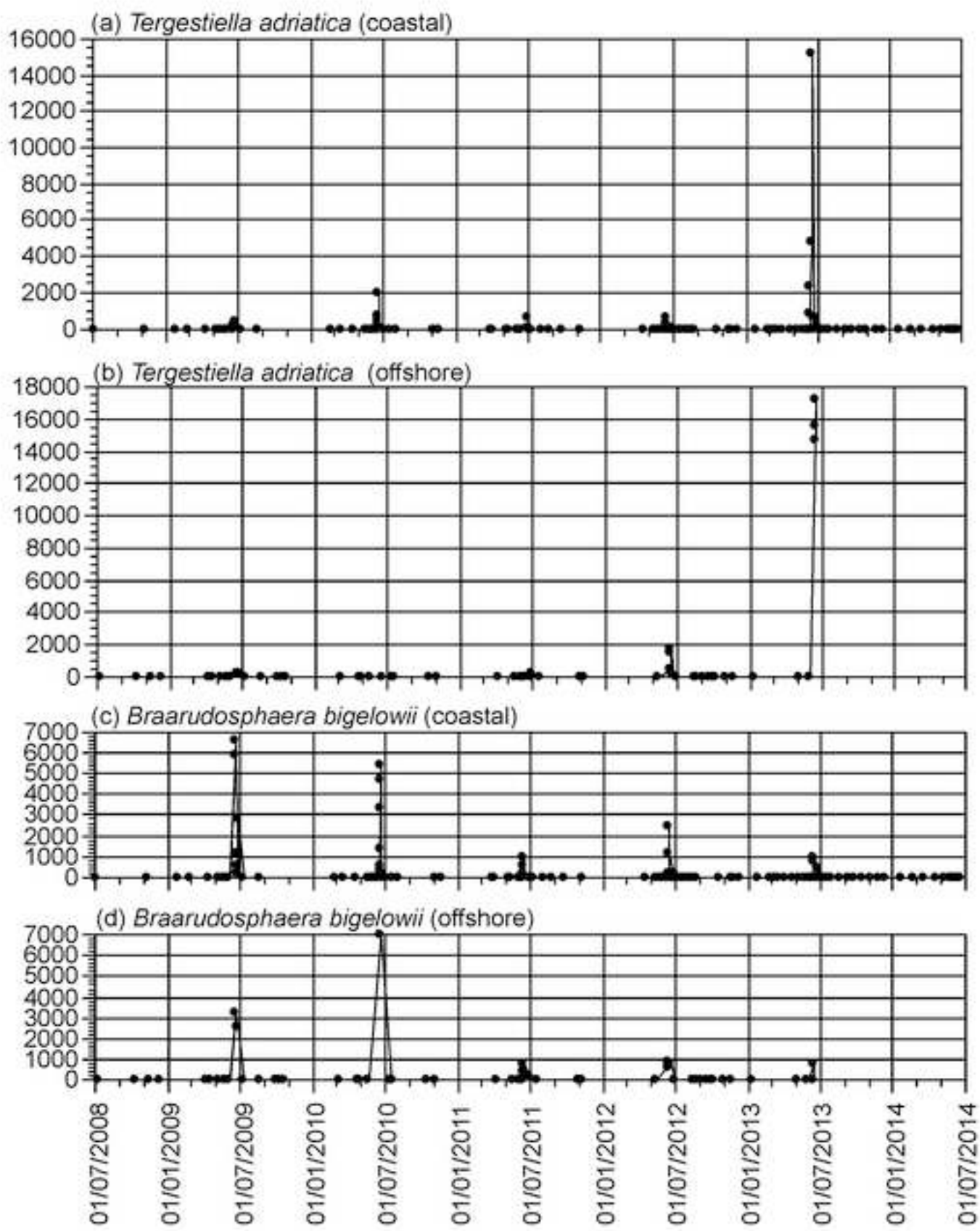


Fig. 6, Hagino et al.



# Re-discovery of a “living fossil” coccolithophore from the coastal waters of Japan and Croatia

K. Hagino et al. 2015 *Marine Micropaleontology*

## Supplementary Materials

### 1. Methods

#### 1-1. Sampling interval in Tomari

The sampling interval in Tomari was adjusted according to the season and purpose of studies in the area (Appendix 1). 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 culture studies of other coccolithophore species, and less than once a month during the coccolithophore-scarce periods, from mid-July to mid-September and from mid-December to early March. From April 2012 to June 2014, sea-surface water samples were collected at least once a month throughout the year, in order to characterize the seasonal succession of coccolithophores.

### 2. Results and Discussion

#### 2-1. Comparison of *Tergestiella* and *Cyclagelosphaera*

Our *Tergestiella adriatica* specimens closely resemble the well-known extinct Mesozoic genus *Cyclagelosphaera*, a member of the family Watznaueriaceae. The Watznaueriaceae form robust placolith coccoliths that frequently dominated Mesozoic nannofossil assemblages from the Early Jurassic to the end-Cretaceous (Lees et al., 2006). The structure of Watznaueriaceae coccoliths was described in detail by Young and Bown (1991), based on *Watznaueria fossacineta*. *Cyclagelosphaera* coccoliths differ from those of *Watznaueria* in being circular with a closed central area, but otherwise 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 (Figs 3a and 3j). 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). 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 coccoliths grow during 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 are connected to the peg-like elements on the proximal surface forming a cycle of wedge-shaped tube-core elements, and these elements have 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 (arrow on Figs 3c). 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, (arrow on 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). Furthermore, 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 (compare Figs 2e-f with Figs 2k-l). 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 that form coccoliths similar in shape to those of *T. adriatica* but these have distinctly different structures, for example, in the Calcidiscaceae and Coccolithaceae the distal shield is formed from V-units, whilst in the Noelaerhabdaceae the V-units are vestigial. The morphological similarity of *Cyclagelosphaera* and *Tergestiella* is therefore significant and strongly suggests that *T. adriatica* is a direct descendant of *Cyclagelosphaera*.

#### 2.2. Taxonomic notes

Two fossil species of *Cyclagelosphaera* are commonly recorded in sediments of late Maastrichtian and early Paleocene age (Bown, 1998): *Cyclagelosphaera margerelii* and *Cyclagelosphaera reinhardtii*. Of these *C. margerelii* has a weakly developed cone in the central area (Fig. 3k), whereas *C. reinhardtii* has a prominent cone (Fig. 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, 3g, and 3j). *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 and flat cycle with irregular sutures between the central cone and distal shield was reported as *Cyclagelosphaera* sp. cf. *C. reinhardtii* from post K/Pg deposits from the Netherlands (Mai et al., 1997, Pl. 9, fig. 3). Our specimen resembles it in the general morphology of the median tube core cycle, but differs from it in having a cycle of pores between the flat cycle and distal shield elements. 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.

### 2-3. Re-examination of filter samples used in previous studies

We re-examined nine samples, which were known to contain *B. bigelowii* from previous studies conducted in other parts of the seas of Japan (Fig. 1a, Appendix 3), using cross-polarized LM. The large coccoliths of *T. adriatica* are very distinctive in cross-polarized light and so it was possible to rapidly scan large areas of filter for all these samples. The re-examined samples did not contain any fragments of *T. adriatica* (Appendix 3).

Subsequent to this rediscovery of *Tergestiella*, we participated in a research cruise, D366, around the NW European continental shelf in June/July 2011 (Fig. 1a) (Achterberg and Richier, 2011). Samples were taken from 74 CTD station depth profiles and 200 additional surface water underway samples. During LM counting of these samples 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.

### 2-4. New Jersey Margin Fossil Material

We re-evaluated the data presented in Mountain et al. (2010) in order to consider whether the *Cyclagelosphaera?* sp. specimens were part of the biocoenosis or a result of reworking (Appendix 4). Oligocene sediments at Site M0027 contain only sporadic reworked Eocene specimens, primarily in the upper Oligocene (Zone NP24-25). Rare specimens of *Cyclagelosphaera?* sp. occur in Oligocene sediments assigned to Zones NP22-25, although in some cases these specimens co-occur with the reworked Eocene taxa (e.g., *Neochiastozygus*). Lower and middle Miocene sediments at all three sites (M0027-29) contain some calcareous nannofossil species typical of mid-Eocene to Oligocene assemblages (e.g., *Reticulofenestra umbilicus*, *R. samodurovii*, *R. reticulata*, *R. lockeri*, *Transversopontis* spp.), as well as *Cruciplacolithus* spp. and *Cyclagelosphaera?* sp.

Mountain et al. (2010) reported the *Cruciplacolithus* spp. occurrences as reworked specimens, but there is a possibility that these were in situ and not reworked, since the genus *Cruciplacolithus* is extant (Bown, 2010; McIntyre and Bé, 1967). *Cyclagelosphaera?* sp. occurs nearly consistently in lower to middle Miocene (NN4-NN5) sediments at all three sites, together with only a few specimens of presumed reworked taxa in the lowermost part of the NN4 section. In the upper NN4 to NN5 section, *Cyclagelosphaera?* sp. and *Cruciplacolithus* spp. occur together without any other recorded Paleogene taxa. Additionally, we observed coccospheres of *Cyclagelosphaera?* sp. in multiple samples from the middle Miocene of Site M0029 (Fig. 3j). These results strongly suggest that these Oligocene to Miocene *Cyclagelosphaera* specimens from the New Jersey Shelf were part of a nearshore biocoenosis and not reworked.

Katz et al. (2013) estimated paleo-water depths for Sites M0027-29 using benthic foraminifer faunal assemblages interpreted within a sequence stratigraphic framework. At

Site M0027, *Cyclagelosphaera* occurs sporadically in rare numbers within the lowermost Oligocene sediments (NP22/23) and upper Oligocene (NP24/25 boundary) (Mountain et al., 2010). Water depths at those times were estimated to be 100-120 m to around 100 m, respectively. Lower Miocene sediments (mid-NN2) also contain sporadic occurrences of *Cyclagelosphaera* in rare to few numbers, with paleo-water depths estimated at 50-80 m. *Cyclagelosphaera* is consistently present in rare to few numbers throughout the upper lower Miocene and middle Miocene section (NN4 to NN6/7). Paleo-water depths during that time were generally less than 50 m, with several short-lived highstands when water depths increased to 75-100 m.

At Site M0028, no paleo-water depth estimate is available for a single occurrence of *Cyclagelosphaera* in the lower Miocene mid-NN2 section. *Cyclagelosphaera* is consistently present in rare to few numbers throughout the upper lower to middle Miocene interval (NN4-5). Paleo-water depths are estimated at 50-100 m for the upper lower Miocene interval, decreasing to 10-25 m by the middle Miocene.

At site M0029, *Cyclagelosphaera* is absent from lower Miocene sediments, but consistently present in rare to few numbers in upper lower to middle Miocene sediments (NN4 to NN6/7). Paleo-water depths during the late early Miocene and early middle Miocene were 75-100 m, shallowing to 25-80 m during the middle Miocene.

These paleodepth estimates indicate that the cored sediment was deposited in shallow, shelfal waters, supporting the nearshore affinity of *Cyclagelosphaera/Tergestiella*. In fact, the observed specimens of *Cruciplacolithus* that were reported as reworked in (Mountain et al., 2010) could also be part of the in situ assemblage. Extant *Cruciplacolithus neohelis* shows affinity to nearshore waters such as a tidal pools and lagoons (e.g., (Kawachi and Inouye, 1994; Konno and Jordan, 2006).

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Appendix 2. Sampling date, location, sampling depth, temperature, salinity, occurrence data and estimated cell density of coccolithophore taxa per liter for sampling locality in offshore Rovinj, Croatia. VR indicates very rare.

Date	station no.	Latitude	Longitude	Sampling depth (m)	temperature	salinity	<i>Acartoica</i> spp.	<i>Calcipapirus rigidus</i>	<i>Emiliania huxleyi</i>	<i>Ophiaster</i> spp.	<i>Rhabdosphaera clavigera</i>	<i>Syracosphaera</i> spp.	<i>Tergestiaella adriatica</i>	Miscellaneous	Total cell density
18-Aug-09	RV001	45°4'40"N	13°36'29"E	0	26.6	36.1	0	2838	0	0	3040	380	0	5676	11934
18-Aug-09	RV001	45°4'40"N	13°36'29"E	5	26.2	36.4	0	5676	0	0	8514	0	0	5676	19866
18-Aug-09	RV001	45°4'40"N	13°36'29"E	10	23.5	37.0	0	0	14190	0	8514	11360	0	5676	39740
18-Aug-09	RV001	45°4'40"N	13°36'29"E	20	16.7	37.7	0	0	5676	2838	1419	0	0	2838	12771
18-Aug-09	RV001	45°4'40"N	13°36'29"E	27	16.4	37.8	0	0	1419	1419	0	0	0	2838	2838
28-Aug-09	RV001	45°4'40"N	13°36'29"E	0	27.5	35.0	0	5676	5676	0	0	0	0	1419	12771
28-Aug-09	RV001	45°4'40"N	13°36'29"E	5	27.5	35.1	0	5676	5676	0	2838	1419	VR	0	15609
28-Aug-09	RV001	45°4'40"N	13°36'29"E	10	26.1	36.1	0	190	0	0	0	0	0	0	190
28-Aug-09	RV001	45°4'40"N	13°36'29"E	20	17.1	37.8	1419	0	7100	0	0	0	0	0	8519
28-Aug-09	RV001	45°4'40"N	13°36'29"E	27	16.5	37.8	0	0	2838	0	0	0	0	2838	5676
19-Aug-10	RV001	45°4'40"N	13°36'29"E	0	24.9	34.5	0	0	2838	190	0	0	0	0	3028
19-Aug-10	RV001	45°4'40"N	13°36'29"E	5	24.9	34.5	0	0	0	0	0	0	VR	2838	2838
19-Aug-10	RV001	45°4'40"N	13°36'29"E	10	22.7	36.3	0	0	0	0	1419	1419	0	2838	5676
19-Aug-10	RV001	45°4'40"N	13°36'29"E	20	15.3	37.4	0	0	27690	0	0	0	0	1419	29109
19-Aug-10	RV001	45°4'40"N	13°36'29"E	27	14.9	37.4	0	0	24140	0	0	0	0	1419	25559

Appendix 3 Sampling date, location, sampling depth, temperature, occurrence data and estimated cell density of coccolithophore taxa per liter for the samples known to contain *B. bigelowii* from previous studies.

Date	Cruise	Station no.	Latitude	Longitude	Sampling depth	Temperature	<i>Emiliania huxleyi</i>	<i>Acanthoica quattrosipina</i>	<i>Coccolithus pelagicus</i>	<i>Gephyrocapsa oceanica</i>	<i>Gephyrocapsa ericsonii</i>	<i>Syracosphaera</i> sp.	<i>Helicosphaera wallichii</i>	<i>Braarudosphaera bigelowii</i>	Miscellaneous	Total cell density	Reference
23/06/1990	KT90-9	1	41.14N	142.00E	0	15.5	4181	0	25	2604	0	275	0	376	50	7,510	Hagino et al. (2005)
23/06/1990	KT90-9	2	41.12N	142.44E	0	15.0	320	0	0	350	0	9	0	16	5	701	Hagino et al. (2005)
24/06/1990	KT90-9	3	41.11N	142.85E	0	14.5	1911	0	134	2129	0	0	0	855	0	5,029	Hagino et al. (2005)
28/06/1990	KT90-9	33	39.33N	142.90E	0	15.3	7176	0	53	394	0	53	0	210	0	7,886	Hagino et al. (2005)
28/06/1990	KT90-9	34	38.55N	142.48E	0	17.8	1127	0	0	140	135	55	10	10	25	1,502	Hagino et al. (2005)
02/07/2006	-	Funahama	43.18N	141.04E	0	19.5	VR	0	0	VR	0	0	0	VR	0	VR	Hagino et al. (2009)
17/06/2007	-	Shukutsu	43.23N	141.02E	0	17.0	VR	0	0	388	0	0	0	194	0	583	Hagino et al. (2009)
24/06/2007	-	Shukutsu	43.23N	141.02E	0	17.5	0	0	0	0	188	0	0	0	0	188	Hagino et al. (2009)
02/07/2007	-	Shukutsu	43.23N	141.02E	0	18.0	162	0	0	162	0	0	0	974	0	1,298	Hagino et al. (2009)
19/06/2011	D366	CTD25	45.49N	-7.19E	5m	15.1	2000	0	0	0	0	0	0	250	0	2250	Young et al. (2014)
29/06/2011	D366	CTD55	57.00N	5.01E	16m	13.8	43537	2700	0	0	0	0	0	8100	0	54337	Young et al. (2014)
02/07/2011	D366	CTD65	56.49N	3.62E	26m	10.2	234012	0	0	0	0	0	0	2700	0	236712	Young et al. (2014)
27/06/2011	D366	U198	55.64N	4.35E	5m	14.5	25699	0	0	0	0	0	0	4535	0	30234	Young et al. (2014)
01/07/2011	D366	U248	56.88N	6.56E	5m	13.8	24490	0	0	0	0	2721	0	2721	0	29932	Young et al. (2014)
01/07/2011	D366	U249	56.87N	6.47E	5m	13.8	38095	0	0	0	0	2721	0	8163	0	48979	Young et al. (2014)
01/07/2011	D366	U253	56.90N	5.68E	5m	13.4	57142	0	0	0	0	0	0	2721	0	59863	Young et al. (2014)
01/07/2011	D366	U254	56.80N	5.36E	5m	13.8	81632	0	0	0	0	0	0	8163	0	89795	Young et al. (2014)
01/07/2011	D366	U255	56.71N	5.00E	5m	13.8	40816	0	0	0	0	0	0	10884	0	51700	Young et al. (2014)
02/07/2011	D366	U262	56.64N	3.69E	5m	13.6	244896	0	0	0	0	0	0	21769	0	266665	Young et al. (2014)
05/07/2011	D366	U299	59.86N	-6.41E	5m	12.0	336732	0	4131	0	0	77520	0	1377	1377	419760	Young et al. (2014)

