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# Molecular Clocks and Inferring Evolutionary Milestones and Biogeography in the Microalgae

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## ABSTRACT

Molecular clocks are proving to be very useful tools for unravelling the evolution of protistan taxa relative to geological change. Molecular clocks have been used to reconstruct the biogeographic history of non-fossilized microalgae from calibration of trees/clocks based on taxa with a fossil record. They have also been used to extrapolate back to the origin of microalgal lineages, to document the appearance of new morphotypes in the fossil record that should be recognised as new species, to estimate the timing of major evolutionary events, viz., endosymbioses and to infer possible explanations for selective survival during global change extinction events.

**Keywords:** Molecular clocks, Phylogeny, Historical biogeography

## INTRODUCTION

Molecular clocks are proving to be very useful tools for unravelling the evolution of protistan taxa. Molecular clocks have been used to reconstruct biogeographic histories, divergence times of many protists ranging from their origins to the divergence of cryptic species. Microalgae, such as diatoms, dinoflagellates and coccolithophores, have mineralised walls that preserve well. These microalgal groups have better preserved fossil records than their metaphyton and metazoan counterparts and molecular clocks made using calibrations from microalgae are better calibrated. Many microalgal groups suffered greatly at mass extinctions only to radiate after the event, whereas others pass through the events relatively unscathed. Mass extinctions are important to macroevolution not only because they involve a sharp increase in extinction intensity over “background” levels, but also because they bring a change in extinction selectivity and these quantita-

tive and qualitative shifts set the stage for evolutionary recoveries [1].

Before constructing a molecular clock, calibrated studies must be performed to determine the reliability/test intrinsic error margins of clocks and to provide baseline estimates of molecular evolution rates. Calibration of the molecular tree is possible in two ways: First, a calibration point can be established where a clade in the tree has a derived character that is unique to that clade and that can be traced in the fossil record. In this case one can infer that (a) the character must have evolved on the branch of the tree leading to the base of the clade and (b) that the character must have evolved prior to its first occurrence in the fossil record. One can refer to these as character-based constraints. Alternatively, a calibration point can be established where sister taxa in the tree have good fossil records, then the first occurrences (FOs) of the taxa can be used as a minimum age constraint for the node in the tree at which the two taxa diverge. If both taxa have sufficient de-

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rived characters to be certain they are monophyletic, then the FO of the older taxon can be used for the constraint, and a range extension for the younger taxon inferred. One can refer to these as divergence-based constraints.

In constructing a molecular clock, one must take account of the rates of evolution that will likely vary across the tree of interest. There are basically two methods that can be used to correct for this variation. One can average the rate of evolution across the tree, e.g. in a linearised tree [2], after eliminating all taxa from the tree that vary in their rate of evolution outside a poisson distribution. This is a strict molecular clock. Branch lengths are regressed against fossil dates and displayed in the model provided by Ref 3. Nodes without dates can be inferred from the regression line. Alternatively, one can allow the rate of evolution to vary across the tree [4]. This is a relaxed molecular clock and is a preferred method because it helps to remove one of the biases of constructing a molecular clock.

In this paper I will review the use of molecular clocks in the microalgae to estimate their evolutionary milestones and to infer the biogeography of selected genera.

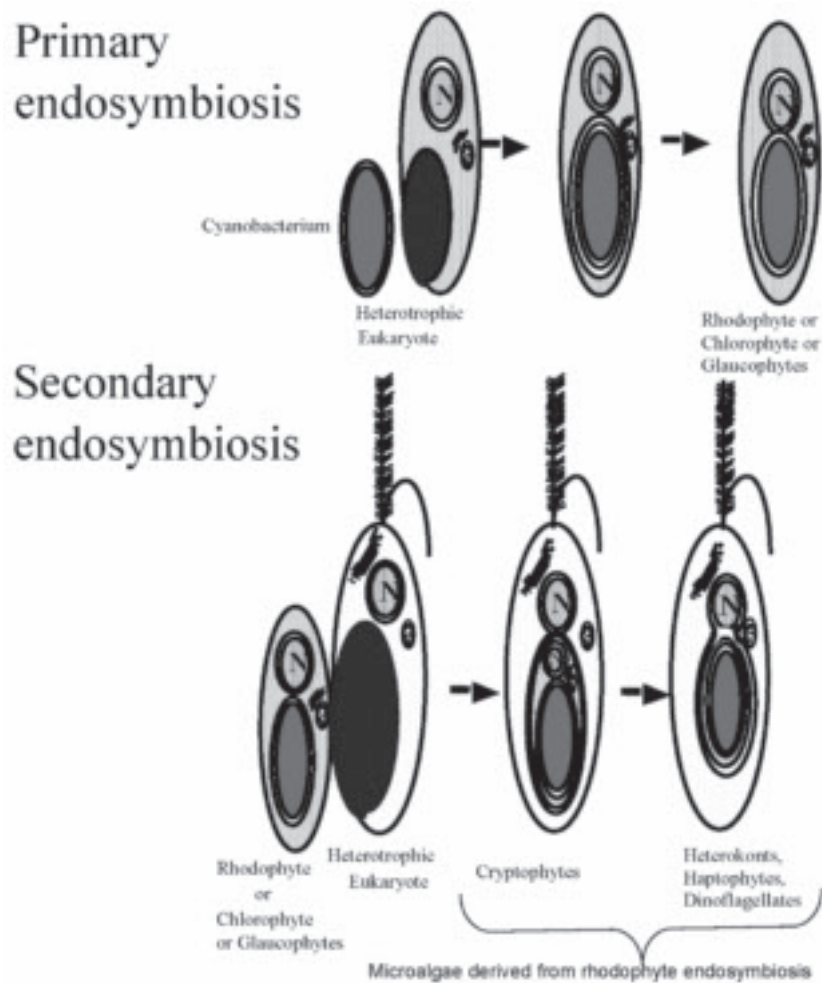
### The Origin of the Plastids

Because photosynthesis has played such a fundamental role in shaping the biosphere, the origins of the plastids have remained one of the most intriguing and well researched topics in biology [see reviews in 5, 6, 7]. The origin of the initial photosynthetic eukaryote likely involved the phagotrophic uptake of a cyanobacterium by a heterotrophic host cell. This endosymbiosis resulted in formation of three primary algal lineages: the green algae, the red algae and the glaucophytes. Each of these algal groups contains plastids that are surrounded by two membranes, which constitute the two membranes of the original cell membrane of the cyanobacterium (Fig. 1).

Although many early single gene phylogenetic analyses suggested that this may have happened multiple times, the analysis of multiple gene phylogenies of the host plant and the plastid have indicated that the red, green and glaucophytes host and plastid lineages are monophyletic [8] and there is a single endosymbiotic event giving rise to these algae. Following this endosymbiotic event, a secondary event took place, which one of the primary endosymbiotic algae was engulfed by a second heterotrophic host. If the engulfed alga was green algae, then the resultant new eukaryote cell was a

euglenoid or chlorarachniophyte alga. If the engulfed alga was a red alga, then the resultant new eukaryotic cell was a cryptomonad, haptophyte, heterokont or dinoflagellate alga. Each of these new eukaryote cells has 3–4 membranes around the plastid, each one representing a different membrane in the serial endosymbiosis (Fig. 1).

The number and timing of these events has been controversial. Early single gene phylogenies showed that there were multiple events and the timing of these events was placed at a recent date [9]. Multiple gene phylogenies of the plastid clearly showed that the plastid were monophyletic [6] and molecular clocks using calibration dates from the outgroup taxa and not any ingroup taxa estimated the origin of the red algae plastid to be soon after the primary endosymbiotic event. Host gene phylogenies have proven very difficult to recover that supported a monophyletic origin of the hosts [10]. Even now that both primary and the red lineage secondary endosymbioses appear to have happened only once, it is clear that an early timing of this event does not match the fossil record of the phytoplankton who are the modern components of the red algal secondary endosymbiotic event [11]. Clearly, the host lineages did not take immediate advantage of their newly acquired organelle and photosynthetic function. All of the early divergences in the heterokont tree are heterotrophic and they appear to have lost the plastid from their secondary endosymbiosis. There is a final divergence in this lineage of all of the autotrophic golden brown and brown algae (Fig. 2A). In the haptophytes, there is a long branch from the origin before the divergence of the two classes and after that there is another long branch in each of the classes until the classes diverge into orders and families (Fig. 3A). The same appears to be true for the dinoflagellates, whose basal lineages are heterotrophic or parasitic and there is a later divergence of the photosynthetic lineage with many multiple losses of their plastids and serial replacement of the original plastid with other algal groups [12] (Fig. 4). Using a molecular clock we are able to estimate the divergence of the autotrophic heterokonts and the dinoflagellates and the divergences of the modern diversity in the haptophyte lineage. Each of these major groups of secondary endosymbiotic algae appear to have radiated after 250 Ma. In other words, the plastid did not really confer any adaptive advantage before this time because all earlier lineages do not retain their plastids and are heterotrophic in the case of the pigmented heterokonts of which the diatoms are a member and the dinoflagellates or are all



**Fig. 1** Scenario showing the serial endosymbiosis leading to the algae in the primary endosymbiosis (top row) and the red algae in the secondary endosymbiosis (bottom row). Drawn by Wiebe Kooistra.

extinct in the case of the haptophytes. At 250 Ma, the world suffered a major mass extinction with approximately 98% of all life in the ocean going extinct. It is at this time that the red algal plastid containing lineages began to proliferate and diversify. It would seem that the red alga plastid had an adaptive advantage at this time when the ocean chemistry changed [13].

#### **Origin of the genus *Alexandrium* and historical biogeography of the *Alexandrium tamarense* species complex [14]**

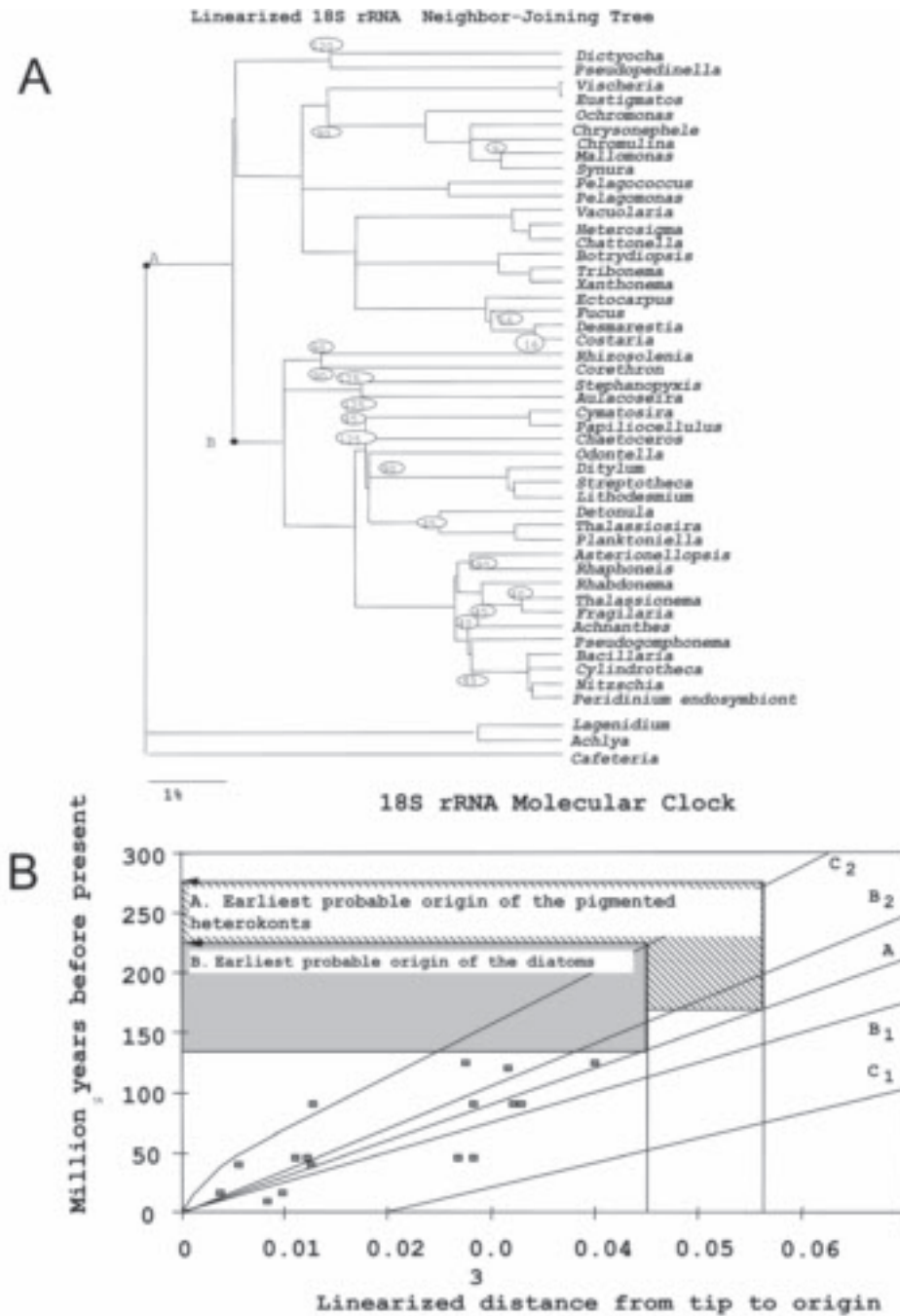
Within the genus *Alexandrium*, *A. tamarense*, *A. fundyense* and *A. catenella* comprise a closely related cosmopolitan toxigenic grouping of morphology-based species (“morphospecies”), the “*Alexandrium tamarense*” species complex, that play a prominent role in HABs. Individual morphospecies are identi-

fied by differences in cell shape and in the geometry of the apical pore complex (APC), by the presence or absence of a ventral pore on the apical plate (1'), and by the tendency to form chains or not. Phylogenetic studies of the *Alexandrium tamarense* species complex, based on 18S rDNA, the D1/D2 region of 28S rDNA and ITS sequences [see review in ref 14], have yielded results that contrast with the conventional morpho species. Strains within the *A. tamarense* species complex are distributed geographically. Indeed, several of the geographic ribotypes contain specimens of each of the three morphospecies of the *A. tamarense* species complex. Thus, at least for molecular phylogenetic purposes, the three morphospecies are generally referred to collectively as the *A. tamarense* species complex. Within the *A. tamarense* species complex, six different ribotypes/geographic clades have been previously identified: western Eu-

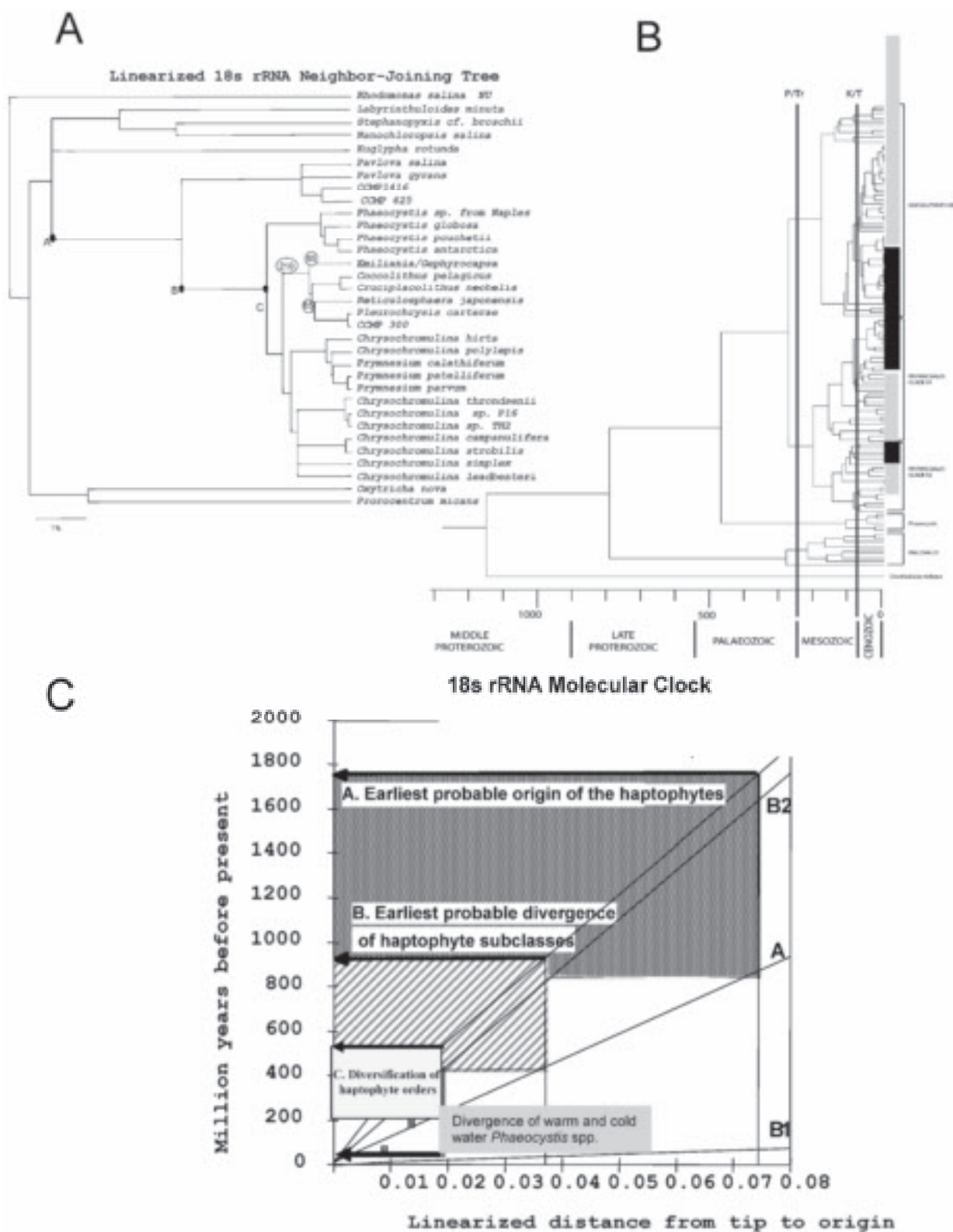
ropean (WE), North American (NA), Mediterranean (ME) temperate Asian (TA), Tasmanian (TASM), and tropical Asian (TROP) clades. The NA, TA, and TROP clades consist only of toxic strains, whereas the WE, ME and TASM clades are exclusively non-toxic.

**Calibration of the molecular clock**

Linearised branch lengths were regressed against the three fossil dates from the dinoflagellates to calculate a molecular clock according to the method described by ref. 3. We used the dates: 190 Ma for the Peridinales; 180 Ma for the Gonyaulacaceae; and 145 Ma for the Ceratiaceae. The molecular



**Fig. 2** Linearised tree for the 18S rDNA gene for the heterokonts (A) and a molecular clock for the group showing the average and earliest possible age of origin for the pigmented heterokonts and the origin of the diatoms (B). Redrawn from [9].

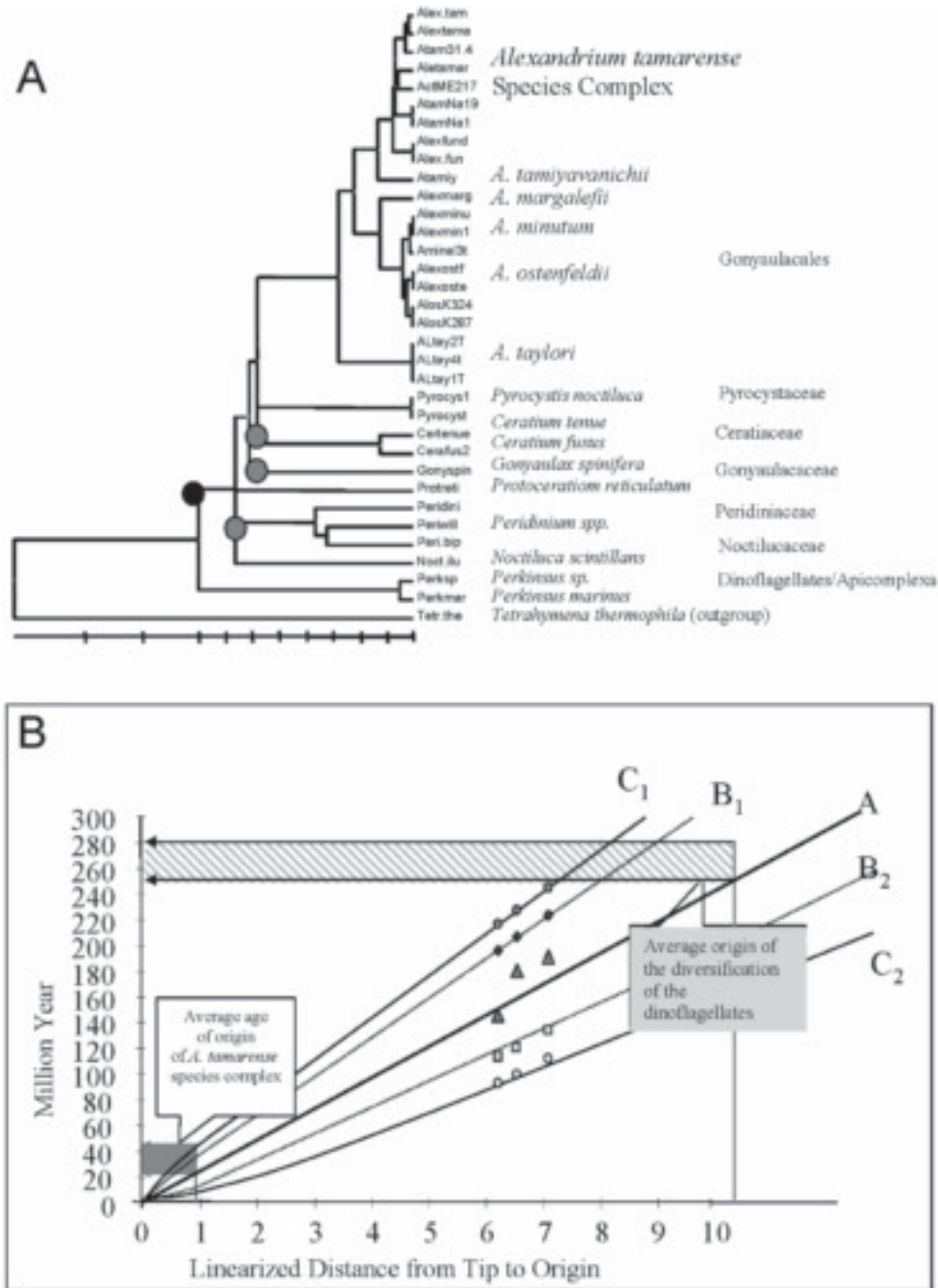


**Fig. 3** Linearised tree for the 18S rDNA gene for the haptophytes (A), a similar tree (B) using a relaxed molecular clock made with the r8s program [4] and a molecular clock (C) showing the average and earliest possible age of origin for the haptophytes, the divergence of its classes, the divergence of its orders and the divergence of the warm and cold water species of the genus *Phaeocystis*. Redrawn from [9].

clock thus constructed was then used to extrapolate dates for the nodes for the origin of the genus *Alexandrium* and the *Alexandrium tamarensis* species complex. The average time of origin for the genus *Alexandrium* (77 Ma) and the *Alexandrium tamarensis* species complex (23 Ma) was calculated from

the average branch lengths of each group, respectively.

Our molecular clock provides a hypothetical model to investigate the biogeographic distribution of the *A. tamarensis* ribotypes, because the relationships among the geographic clades exhibit vicariant events



**Fig. 4** Linearised tree for the 18S rDNA gene for the dinoflagellates (A) and a molecular clock (B), for the group showing the average and earliest possible age of diversification of the group and for the divergence of the *Alexandrium tamarensis* species complex. Redrawn from [9].

rather than dispersal events [see review in 13]. We estimate that the average age of the genus *Alexandrium* is 77 Ma (Late Cretaceous), and no earlier than 119 Ma (mid Cretaceous); these dates do not conflict with the 105 Ma date for the closest dinoflagellates with similar tabulation and fossilizable cysts. At 120 Ma, climate and water temperature were much warmer than today. Shallow seas covered much of the continental areas, with sea levels up to 200 m higher than today. These continental areas were arranged such that there was a global circum-equatorial current within the Tethys Ocean. Between 65 Ma and 55 Ma, two catastrophic events affected global biodiversity: the end Cretaceous mass extinction event (65 Ma); and the Late Paleocene thermal maximum (55 Ma), with a deep-sea temperature increase of 5–6°C that killed benthic foraminifera and apparently caused planktonic microalgae, including dinoflagellates to proliferate. In the early Paleogene (40–60 Ma), the ocean basins were significantly re-arranged as Tethys Sea closed, new oceans opened, resulting in lowered sea level and a cooler seasonal global climate. Permanent polar ice sheets formed and both the length of global coastlines and the area of continental shelves increased. Coastal regions became more heterogeneous in topological, hydrodynamic and climatic conditions, thus promoting regional differences.

Under these mid Cenozoic conditions, *Alexandrium* likely diverged into several taxa (Fig. 5A). The *A. tamarensis* species complex diverged probably around the early Neogene (23 Ma), but no earlier than the late Paleogene (45 Ma). A global distribution of planktonic species was possible through the eastern Indian Ocean, Tethys and the Pacific Ocean, with counter currents for anti-clockwise distributions. At 36 Ma, the Tasmania-Antarctica and Drake passages opened, forming the Antarctic Circumpolar Current (ACC) and intensifying conditions favorable for the build up of increasing Antarctic ice sheets and ocean fertility. When the Tethys Ocean closed, populations became isolated in various ocean basins. This regionalizing effect was enhanced when, from about 3–13 Ma, the Isthmus of Panama was uplifted, cutting of the tropical Pacific-Atlantic connection and reorganizing Northern Hemisphere ocean circulation. As a result, surface waters cooled through North Atlantic deep water formation, which could have increased precipitation of the Northern Hemisphere and promoted glaciation after 2.5–3 Ma. These geological events likely lead to allopatric speciation of global planktonic populations.

Given mid Cenozoic paleoclimatic and geological

changes, we propose the following scenario to explain the modern distribution of the strains within the *Alexandrium tamarensis* species complex [see ref 13]. Our scenario starts with a globally distributed ancestral population, which diverges first into eastern and western Pacific populations as a response to a relatively short but deep glacial maximum around 23 Ma. The eastern Pacific population was connected to Atlantic populations through the Central American Seaway and its counter currents, whereas the western Pacific population was connected to eastern Atlantic populations through Tethys (Fig. 5A). The heterogeneous climatic and oceanic conditions between 40–65 Ma likely promoted genetic differentiation within the *A. tamarensis* species complex. When the Tethys Ocean closed, the western Pacific population diverged into TA (pale stars in Fig. 5) and WE clades (dark stars in Fig. 5). As the Isthmus of Panama uplifted, ancestral populations in the subtropical Atlantic (starbursts in Fig. 5) were separated from those in the eastern Pacific (NA clade: light pinnacles in Fig 5). The closing of Tethys, the formation of the Mediterranean Sea, and the uplift of the Panama Isthmus created significant changes in circulation and paleoclimate. Around 5 Ma, the Mediterranean Sea dried up and was subsequently refilled by tropical and sub-tropical Atlantic water with sub-tropical Atlantic *A. tamarensis* populations. Eventually, indigenous sub-tropical Atlantic populations became extinct because of unfavorable environmental conditions, leaving relict populations, the ME clade (starbursts in Fig. 5), in the Mediterranean. Relict populations of the ancient sister group of the *A. tamarensis* species complex can be found in tropical waters (dark pinnacles in Fig. 5).

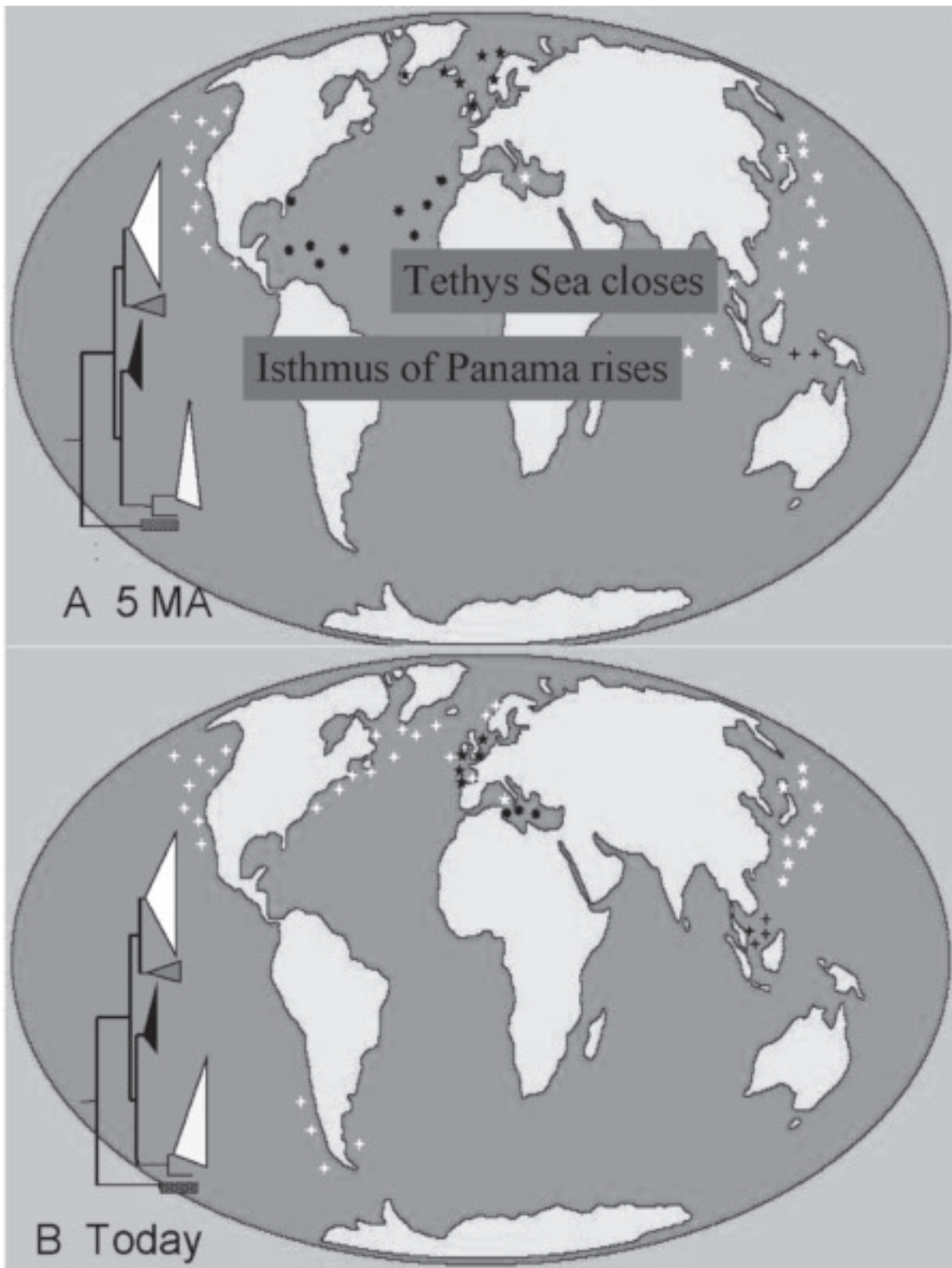
### Origins of the Haptophyte and the historical biogeography of selected species

A molecular clock has been constructed from our 18S rDNA data set and calibrated with fossil dates from the haptophyte coccolithophorid species (Fig. 3). This clock has been done with two methods: the strict clock using a linearised tree (Fig. 3A) and the relaxed clock using the R8S program (Fig. 3B).

### Origin of the Haptophyta

The age of divergence of Haptophyta from other eukaryotes predicted by this clock is ca. 1,200 Ma [9], which is broadly congruent with that from other studies [15, 16], although because this is based on extrapolation from much younger calibration points, a substantial error margin (at least + / – 30%) should be assigned to this estimate (Fig. 3A). The very





**Fig. 5** Biogeographic representation of the distribution of geographic ribotypes in the *Alexandrium tamarense* species complex at 5 MA (A) and today (B). Temperate Asian ribotype = pale stars, Western European ribotype = dark stars, North American ribotype = pale pinnacles, Mediterranean ribotype = dark starbursts, Topical Asian = dark pinnacles. Redrawn from [14].

long branch leading to the next divergence indicates that we have likely lost many of the early divergences in this group through extinction, or that they have not yet been sampled. The next divergence of the Class Pavlovaceae from the Class Prymnesiophyceae is still very deep-pre-Cambrian or Early Palaeozoic, (ca. 800 Ma). Endosymbiosis of chloroplasts is presumed to have taken place at the origin of the group and thus would have an ancient origin before the class divergence. The order Phaeocystales diverges from all other Prymnesiophyceae at ca 490 Ma and then the Prymnesiales diverge from the Coccolithales plus Isochrysidales at ca. 260 Ma. Order level cladogenesis in the haptophytes thus appears to be a Late Palaeozoic/Early Mesozoic event and may be correlated with P/Tr boundary. Molecular diversification within the orders is earlier within the Prymnesiales than it is within the Coccolithales plus Isochrysidales where most of these latter divergences occur fairly late in our phylogenetic tree. The divergences within the Coccolithales plus Isochrysidales occur predominantly after the K/T boundary, as predicted by the fossil record. Mesozoic coccolithophores have been intensively studied and at the K/T boundary an abrupt extinction is documented in the fossil record with ca. 90% of end-Cretaceous species disappearing. Subsequently, there is a major radiation in the Early Cenozoic with new clades rapidly diversifying and forming the origins of the modern coccolithophore biota.

#### Historical biogeography of the genus *Phaeocystis*

*Phaeocystis* Lagerheim is a cosmopolitan bloom forming alga that is often recognized both as a nuisance alga and an ecologically important member of the phytoplankton [see review in Ref. 17]. *Phaeocystis* has a polymorphic life cycle with both colonial and flagellated cells. The colonial stage, with cells very loosely interconnected and enclosed in a thin skin is most easily recognized, although some new species may form mucilaginous colonies or do not seem to make a colonial stage. Because it is a gelatinous microalga, it has no fossil record and we have inferred the biogeographic history of its species using the molecular clock of the haptophytes calibrated with the coccolithophorid algae.

*Phaeocystis* is one of the first divergences in the Prymnesiaceae in the 18S rDNA tree. Unicellular species are the first to diverge following by the divergence of the larger colonial species, which fall into two groups. The warm water *Phaeocystis* species diverged from the cold water species at approximately 30 Ma (Fig. 3C), which coincides with the

time that the Drake Passage opened and the ACC system was formed. This would have effectively isolated ancestral populations in the Antarctic sufficiently to allow them to speciate from their warm water ancestors. The separation of the polar species *Ph. pouchetii* from *Ph. antarctica* is approximately 15 Ma, which coincides with a major warming event in the world's oceans at this time. Before this time populations must have been able to cross the equator from the south to the north because water temperatures were cool enough to allow survival, but this warming event separated the two polar populations to allow them to diverge into the two species we have today at the poles. Similar results have been found for foraminifera. Isolates from the ACC gradually seed the continental gyres of the Antarctic Ocean to establish a cosmopolitan population of *Ph. antarctica* around the Antarctic.

#### Selective Extinction of the Haptophyta at the K/T boundary

We used a molecular clock to help interpret the extinction of the coccolithophores at the K/T boundary [18]. It is commonly suggested that diatoms and dinoflagellates show reduced extinction rates at the K/T boundary as compared to the coccolithophores and silicoflagellates because they produce resting stages that would facilitate survival from an environmental disturbance. One would expect that the non-calcified haptophytes would have the same rate of extinction as the calcified ones, because neither group commonly produces resting stages. However, from our rRNA tree and the placement of the K/T boundary across the cladogram there is no evidence of bottlenecks in the non-calcified taxa at this time. There are many clades with deep divergences in the non-calcified taxa. Our tree has 25 lineages in the non-calcifying taxa crossing the K/T boundary representing 50 OTUs, whereas in the calcifying lineage there are 11 lineages representing 65 OTUs.

The divergences extend from before the K/T boundary to the present day. This suggests that there must be an alternative mechanism by which these non calcifying without resting stages survived the K/T boundary. The resting stage hypothesis is generally not applicable for these algae. One alternative hypothesis is that there was a selective extinction of oceanic species followed by a recolonization from the coastal realm. Among the calcifying species, the molecular tree supports the well-documented palaeontological interpretation of the K/T boundary as being a period of extinction followed by a major diversification because divergences occur in all clades

following this time point, but most particularly in the calcifying algae. Among the calcifying algae, the divergence of one major neritic group, the Pleurochrysidaceae occurs at the K/T boundary. Also at this time, there was a selection against calcifying algae, which enhanced their extinction.

Another possible explanation lies in the mode of nutrition in the two haptophyte lineages. The non-calcifying haptophytes are known for their ability to switch between autotrophic and heterotrophic nutrition. Thus, when nutrients are abundant, they photosynthesize. However, when the reverse is true, they engulf prey and survive heterotrophically. At the K/T boundary, it is likely that light quality was poor and impaired photosynthetic ability. Those taxa with either the ability to form resting stages, such as the diatoms and the dinoflagellates, or those taxa with the ability to switch mode of nutrition could have an adaptive advantage over those who have neither of these traits. Coccolithophores are not commonly known to form resting stages and it appears that they are predominantly obligate autotrophs. Microalgal EST libraries produced under stress conditions have shown what appears to be a universal response to stress, i. e., under stress conditions, photosynthesis genes are down regulated. Thus, at the K/T boundary, the stress induced by reduced light quantity and quality, could have shut down photosynthesis. Cells that could switch nutrition or form resting stages could have a better chance of survival. Either of these reasons could be offered as an explanation as to why the coccolithophores suffered a greater extinction at this time.

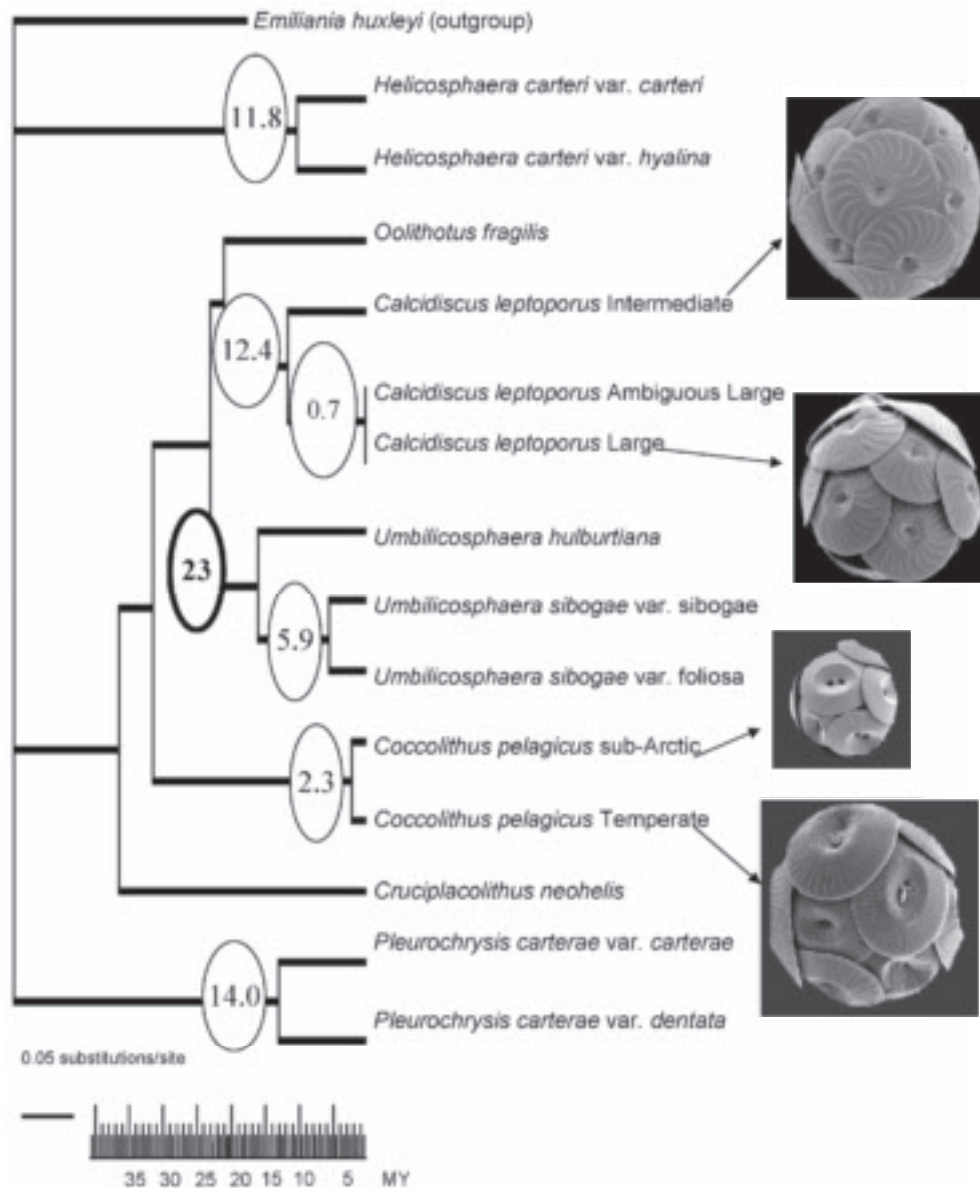
#### **Confirmation of cryptic coccolithophorid species and their divergence times**

We examined morphotype variants of five recognized species of coccolithophores [19, Fig. 6). We confirmed that the morphological fine-scale variation observed within each of them correlates well with distinct genotypes, obtained from three different genes of two cellular organelles, the nucleus and the chloroplast. This strongly supports previous views of reproductive isolation among recognized species of coccolithophores. Cryptic or pseudo-cryptic species indicate optimal phenotypes subject to strong stabilizing selection. That is, despite the lack of gene flow between closely related taxa (Fig. 6), they remain very similar, with just minute morphological differences separating them. Strong stabilizing selection may be acting on their phenotypes, which implies that the forms of their coccoliths are functionally relevant to their survival.

In our tree we constructed a molecular clock using one calibration point: the divergence of *Umbilicosphaera* from *Calcidiscus* at 23 Ma to estimate the divergence times of the cryptic species pairs. For each pair, our clock corroborated the first appearance of the morphotypes in the fossil record. The recognition of extant pseudo-cryptic species with a fossil record may also have more practical consequences. Biostratigraphers have often used subtler morphological criteria than biologists in discrimination of species, and our results justify this approach. Taxonomic subdivisions finer than previously assumed can thus provide useful biostratigraphic markers. To achieve this goal it is necessary to find morphological characters that can accurately discriminate “cryptic” species. Once these characters are found, then the cryptic species become “pseudo-cryptic” species, i.e., species that are morphologically recognized as such only after other methods have unveiled their existence. Our data suggest that the conventional morphological differences separating the varieties of *Umbilicosphaera sibogae*, *Pleurochrysis dentata*, and *H. carteri* can be used at the species level. *Calcidiscus leptoporus*, subspecies *quadriperforatus* and *C. leptoporus*\* have a zone of obscured sutures around the central area, which is absent in *C. leptoporus* subspecies *leptoporus* and *C. leptoporus* SMALL. The relatively recent distinction between *C. leptoporus* subspecies *quadriperforatus* and *C. leptoporus*\* is only qualitative at present, and we have not been able to establish characters that permit a clear morphological separation, although they are clearly molecularly distinct. The subspecies of *C. pelagicus* are distinguished by the coccolith size, although some overlap exists.

#### **Origin of the Bacillariophyta**

Diatoms are eukaryotic, unicellular or colonial microorganisms. Their hallmark is a unique type of silica cell wall, the frustule, which consists of two intricately shaped and ornamented compound structures called the epitheca and the hypotheca, which provides them with a good fossil record. A molecular clock constructed from four genes has placed the average age of the diatoms c. 135 Ma ago, with their earliest possible age being 240 Ma ago [20] (Fig. 2B). This agrees well with the first fossil record of the diatoms at 180 Ma. The diatoms are composed of two major clades, the radial centrics, which now belong to the subdivision Coscinodiscophytina, and the bipolar centrics and the pennate diatoms, which now belong to the Bacillariophytina [see ref. 21]. These new subdivisions are defined on



**Fig. 6** Linearised tree for the *tufA* gene for the coccolithophorid algae using one calibration point at 23 MA. All other dates on the nodes of the trees are inferred from the molecular clock. Examples of cryptic species pairs are placed on the nodes. Redrawn from [19].

the presence of scales in the sexual cycle and a GERM unit arrangement for the Golgi bodies in the former and the presence of scales and bands in the sexual cycle and a perinuclear arrangement of the Golgi bodies in the latter. Coscinodiscophytina contains one class Coscinodiscaceae, the radial centric diatoms, and the Bacillariophytina contains two classes: the Mediophyceae or bipolar centric diatoms and the Bacillariophyceae or pennate diatoms. The primary feature separating is the structure of the specialised zygote or auxospore in the diatoms

and how that zygote and forms its initial cell following cell enlargement in the diatom sexual cycle.

## CONCLUSIONS

Although molecular clocks can be controversial, they have been particularly useful in reconstructing major evolutionary events, estimating divergence times in the microalgae [16, 22], reconstructing historical biogeography [14, 18] and evaluating survival mechanisms of major extinction events [19].

Perhaps the microalgae are particularly well suited for this because of their well documented fossil record, their relatively stable rates of evolution and the extensive data set now available from several molecular markers.

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