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ORIGINAL ARTICLE

Dating the cyanobacterial ancestor of the chloroplast

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Cyanobacteria have had a pivotal role in the history of life on Earth being the first organisms to perform oxygenic photosynthesis, which changed the atmospheric chemistry and allowed the evolution of aerobic Eukarya. Chloroplasts are the cellular organelles of photoautotrophic eukaryotes in which most portions of photosynthesis occur. Although the initial suggestion that cyanobacteria are the ancestors of chloroplasts was greeted with skepticism, the idea is now widely accepted. Here we attempt to resolve and date the cyanobacterial ancestry of the chloroplast using phylogenetic analysis and molecular clocks. We found that chloroplasts form a monophyletic lineage, are most closely related to subsection-I, N_2-fixing unicellular cyanobacteria (Order Chroococcales), and heterocyst-forming Order Nostocales cyanobacteria are their sister group. Nostocales and Chroococcales appeared during the Paleoproterozoic and chloroplasts appeared in the mid-Proterozoic. The capability of N_2 fixation in cyanobacteria may have appeared only once during the late Archaean and early Proterozoic eons. Furthermore, we found that oxygen-evolving cyanobacteria could have appeared in the Archaean. Our results suggest that a free-living cyanobacterium with the capacity to store starch through oxygenic CO_2 fixation, and to fix atmospheric N_2, would be a very important intracellular acquisition, which, as can be recounted today from several lines of evidence, would have become the chloroplast by endosymbiosis.
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Introduction

Evolutionary importance of cyanobacteria
Cyanobacteria form one of the most morphologically and genetically diverse group of Prokaryotes (Waterbury, 1991; Castenholz, 2001) showing cellular and colony differentiation. They are classified in five subsections and Orders, comprising unicellular and filamentous forms (Castenholz, 2001). They represent the basis of the nitrogen cycle, because the capacity to fix atmospheric N_2 is found throughout this lineage, making them essential components of past and modern ecosystems (Bergman *et al.*, 1997; Capone *et al.*, 1997; Raymond *et al.*, 2004; Tomitani *et al.*, 2006; Haselkorn, 2007). Molecular phylogenetic studies have made it clear that all photoautotrophic eukaryotes (plants and algae) share a single origin, as well as a common endosymbiotic ancestry, for cyanobacteria-derived chloroplasts (Bhattacharya and Medlin, 1995; Delwiche *et al.*, 1995; Douglas, 1998; Moreira *et al.*, 2000; Martin *et al.*, 2002; Raven and Allen, 2003; Stiller *et al.*, 2003; Hedges *et al.*, 2004; McFadden and van Dooren, 2004; Yoon *et al.*, 2004; Rodriguez-Ezpeleta *et al.*, 2005; Hackett *et al.*, 2007 among others). The work of Bhattacharya and Medlin (1995), Nelissen *et al.* (1995) and Turner *et al.* (1999) suggested the chloroplast lineage arose at the onset of diversification of the cyanobacterial lineage. Recent work by Deusche *et al.* (2008) suggested, after a careful examination of four eukaryotic and nine cyanobacterial genomes, that among cyanobacteria, *Nostoc* and *Anabaena*, within Order Nostocales, harbor more genes related to those acquired by eukaryotes. This suggests that the ancestor of the chloroplast could lie within the heterocyst-forming cyanobacteria. Heterocysts are specialized cells for N_2 fixation that lack the oxygen-generating photosystem-II (PSII). They consist of a thick isolating cell wall that is less permeable to gases, and heterocysts are connected to adjacent vegetative cells by micro-plasmodesmata, through which organic compounds (for example, sugars, amino acids) may pass. Sugar is required for respiratory reductive power, but most of the required ATP is produced through PSI, which is the only PS remaining in the heterocyst. The ATP is

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needed to fuel the activity of nitrogenase, the enzymatic complex capable to fix atmospheric N_2, which is irreversibly inhibited in the presence of oxygen (Bergman *et al.*, 1997). Tomitani *et al.* (2006) suggested, on the basis of genetic distances and fossil calibrations, an age ranging from 2450 to 2100 million years ago (MYA) for heterocystous cyanobacteria, which may predate the rise of atmospheric oxygen at about 2300 MYA. However, the work of Deusche *et al.* (2008) considered whole genomes, but cyanobacterial diversity is poorly represented in genomic studies, thus phylogenetic interpretations may be misleading at present. Recently, Deschamps *et al.* (2008) provided the first evidence of the existence of starch in bacteria within unicellular, N_2-fixing cyanobacteria, belonging to Order Chroococcales. These authors suggested that starch formation would define the genetic make-up of the ancestor of the plant kingdom related to storage polysaccharide metabolism. Unicellular N_2-fixing cyanobacteria differ phylogenetically from the heterocyst lineage, and have resolved N_2 fixation and oxygen-generating photosynthesis through temporal separation, storing polysaccharides in starch granules during the day to fuel N_2 fixation at night (Falcón *et al.*, 2004). The above suggests that the ancestor of chloroplasts had the ability to fix N_2, fix CO_2 by an oxygen-evolving type-II PS and store starch. The ancient symbiosis metabolic fluxes consisted of the export of ADP-glucose from the cyanobiont to the host, eliminating its ability to store polysaccharides, thus in habilitating its capacity to fuel N_2 fixation, demanding import of reduced nitrogen from the host to the cyanobiont (Deschamps *et al.*, 2008).

Materials and methods

To estimate the timing of phylogenetic divergence events, we used a data set including 56 cyanobacterial taxa from all subsections, and nine chloroplasts, which included members of Rhodophyta, Glaucophyta, Chlorophyta and Streptophyta. Phylogenetic relationships were estimated on the basis of nucleotide sequences of 16S *rDNA* (1255 bp), *rbcL* (1470 bp) and a concatenated set of these two loci. Bayesian phylogenetic analysis for the individual and combined loci were conducted with MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001), applying the model with best fit to each data set as identified with the Akaike Information Criterion, implemented in Modeltest (Posada and Crandall, 1998; Posada and Buckley, 2004). Each Bayesian analysis consisted of two independent Markov chain Monte Carlo runs, each formed by four differentially heated chains of 5×10^6 generations, in which a tree was sampled every 200 generations. Phylograms topologically identical to the maximum *a posteriori* (MAP) topology were recovered using PAUP* 4.0b10 (Swofford, 2002), and from these, 100 were randomly selected to conduct dating analyses.

The timing of phylogenetic divergences was estimated with penalized likelihood (Sanderson, 2002), implemented in r8s v1.71 (Sanderson, 2006). The optimal smoothing parameter for each data set was identified through a cross-validation procedure that involved pruning terminal branches. Dating analyses were conducted on the 100 phylograms topologically identical to the MAP tree. The trees were calibrated by fixing the origin of the cyanobacterial lineage at 3500 MYA, based on the age of oldest fossils represented by stromatolites (Schopf and Packer, 1987), and at 2700 MYA, the time at which oxygen-evolving cyanobacteria had likely quite originated due to reports of steranes in carbonaceous shales of northwestern Australia (Brocks *et al.*, 1999). A maximal age constraint of 460 MYA was applied to the crown group of tracheophytes, considering the oldest vascular plant fossil remains (Kenrick and Crane, 1997). Minimal age constraints of 1618 and 1253 MYA were applied to the heterocyst-forming lineage and to the origin of plastids, respectively, derived from a preliminary analysis in which the ages of these lineages were estimated without imposing minimal age constraints. Point estimates of age from each of the 100 phylograms were used to obtain mean and standard deviations of ages of nodes across the tree.

Results and discussion

Ancestry of the chloroplast

Bayesian inferences of phylogenetic relations between cyanobacteria and chloroplasts with *16S rDNA* and *rbcL* genes, plus the concatenated set, produced the following results: (1) chloroplasts constitute a monophyletic lineage and are most closely related to N_2-fixing unicellular cyanobacteria and (2) heterocyst-forming cyanobacteria are their sister group (Figure 1). Molecular clock estimates rooting the origin of cyanobacteria at 3500 and 2700 MYA gave intervals of appearance of N_2 fixation in cyanobacteria within the late Archaen and early Paleoproterozoic eons, while heterocystous and unicellular N_2-fixing cyanobacterial clades must have originated within the Paleoproterozoic (Table 1). The molecular clock estimated age for the heterocystous cyanobacterial clade coincides with the dates suggested by Tomitani *et al.* (2006) on the basis of genetic distances and fossil calibrations. Dates for the plastid lineage, considering a cyanobacterial phylogeny, occurred in the mid-Proterozoic, in agreement with previous studies (Figure 2 and Table 1).

After evolution of life, the second major event that transformed the biogeochemistry of the Earth, was oxygen-evolving photosynthesis by cyanobacteria (Dismukes *et al.*, 2001; Kopp *et al.*, 2005; Cavalier-Smith, 2006; Shi and Falkowski, 2008). The Great

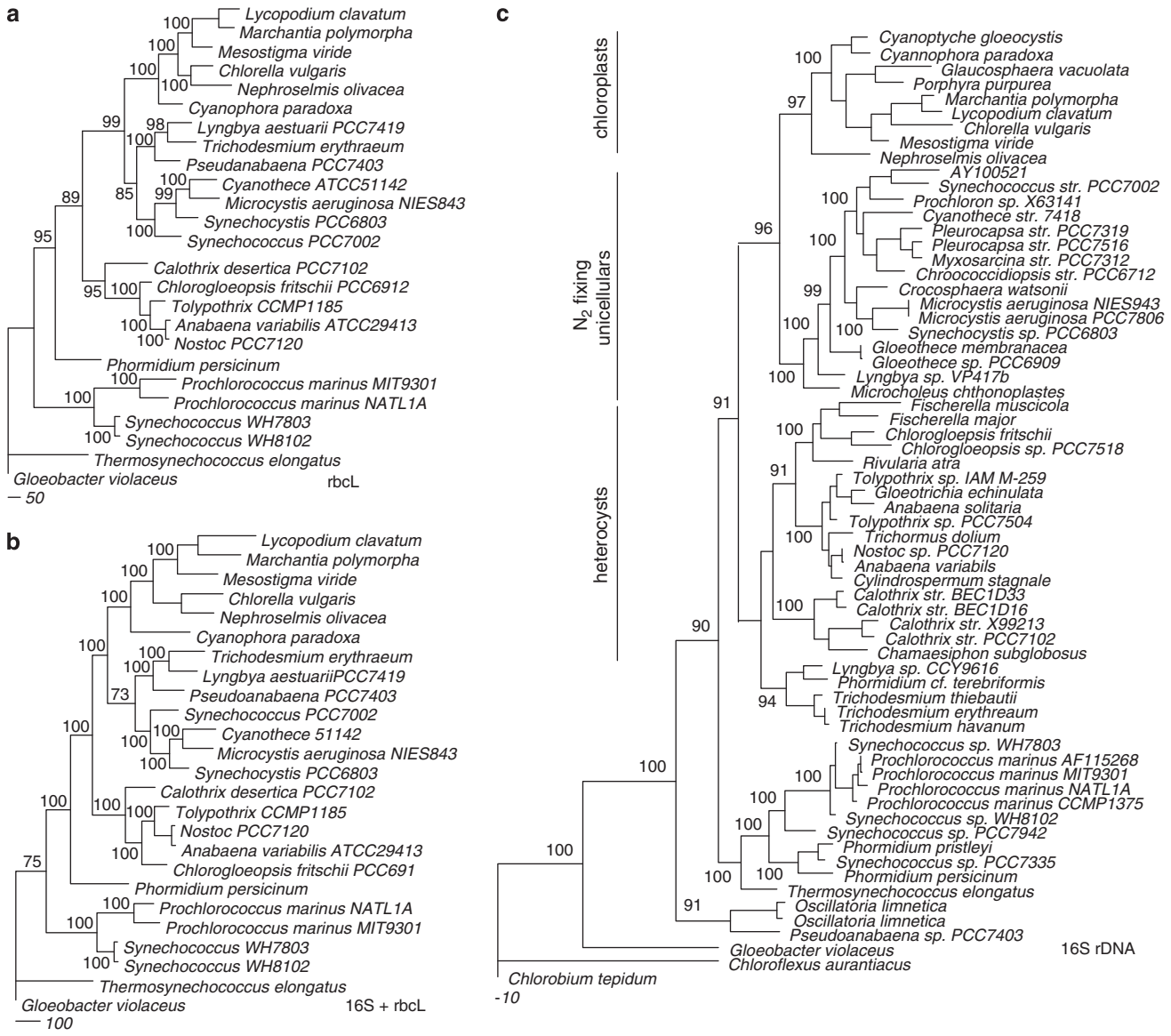

Figure 1 Phylogenetic relationships of cyanobacteria and chloroplasts rooted with (**a**) *Gloeobacter violaceus* (*rbcL* and concatenated set), (**b**) *Chlorobium tepidum* and (**c**) *Chloroflexus aurantiacus* (16S *rDNA*). Clades represent chloroplasts, unicellular cyanobacteria with the ability to degrade starch to simple sugars and use these as reductants for N_2 fixation, and heterocystous filamentous cyanobacteria. Scales represent genetic distances.

Oxidation Event (GOE), which establishes the presence of molecular oxygen in the fossil record, and thus of oxygen-producing photoautotrophs, occurred as early as 2450 MYA (Holland, 2002). However, the work of Brocks *et al*. (1999) showed that steranes were already present in the geological record by 2700 MYA, implying biologically produced molecular oxygen. Microfossils comprising six bacterium morphotypes, including cyanobacteria, have been found in Archaean rocks dating between 3200 and 3500 MYA (Schopf, 2006). Thus, current evidence suggests that the origin of oxygen-producing cyanobacteria may date from as early as, or even earlier than, 3500 MYA, and were likely extant by 2700 MYA. Nevertheless, geological features that require free environmental oxygen, for example, banded iron formations, lateritic paleosols and sulfate deposits, occur shortly before the 2300—2200 MYA global 'snowball Earth', but are not present at the ~2900 MYA Pongola glaciation (Kopp *et al*., 2005) contradicting the Archaean appearance of oxygenic photosynthesis. Further, it has been argued that the isotopic line of evidence for early >3500 MYA oxygen evolution with $\partial^{13}C$ values attributed to C-fixation, sulfate deposits (~3450 MYA) and anaerobic methanotrophy (~2700 Ma), can occur under anaerobic conditions (Hayes, 1994; Canfield *et al*., 2000; Rosing and Frei, 2004; Kopp *et al*., 2005). Eigenbrode and Freeman (2006) examined ^{13}C enrichment patterns of the Hamersley Province in Western Australia and suggested that oxygenic photosynthesis must have originated

Table 1 Dates for the chloroplast lineage and related cyanobacterial clades represented in MYA, calculated for 16S *rDNA*, *rbcL* and the concatenated set, showing mean and standard deviations from 100 identical topologies to the maximum *a posteriori* topology. Results rooting the cyanobacterial lineage at 3500 and 2700 MYA are shown

		3500 MYA (mean)	*s.d.*	*2700 MYA (mean)*	*s.d.*
16S rDNA	Appearance of	3056	95	2481	52
16SrbcL	N_2 fixation	3037	106	2614	40
rbcL		2991	80	2463	68
16S rDNA	Heterocysts/unicellulars+	2844	92	2584	41
16SrbcL	chloroplasts	2715	102	2658	27
rbcL		2550	66	2497	37
16S rDNA	Chloroplasts/	2743	111	2099	81
16SrbcL	unicellulars	2416	107	2131	46
rbcL		2685	73	2070	76
16S rDNA	Heterocysts	2092	116	1975	57
16SrbcL		2268	141	2019	53
rbcL		2274	117	2177	92
16S rDNA	Chloroplasts	1249	121	1217	73
16SrbcL		1476	98	1242	83
rbcL		1271	36	1236	74
16S rDNA	N_2-fixing unicellulars	2399	114	1850	85
16SrbcL		2300	135	1806	60
rbcL		2324	86	1841	60

sometime before 2720 MYA. This event eventually triggered the rise of aerobic ecosystems, fueling their expansion from anaerobic settings into the photic zone between 2720 and 2450 MYA. Proterozoic ocean simulations (Fennel *et al.*, 2005) suggest that rise of oxygen was delayed due to feedbacks on the N-cycle. Ammonium, in presence of oxygen, would be biologically converted to nitrate, and denitrification would have rapidly deprived the oceans of fixed inorganic nitrogen, shifting the Proterozoic ocean to a N-depleted state. In this scenario, a free-living cyanobacterium with the capacity to store starch through oxygenic CO_2 fixation, plus fix atmospheric N_2, would be a very important intracellular acquisition. As can be recounted today from several lines of evidence, this cyanobacterium would have become the chloroplast through endosymbiosis.

Our results propose the existence of oxygen-evolving cyanobacteria back to the Archaean ~2700—2500 MYA. Our results coincide with the conclusion of Eigenbrode and Freeman (2006) that the origin of oxygenic photosynthesis must have remained contrived to microbial communities, which led a transition away from purely anaerobic metabolism, fueling atmospheric oxygenation. The delay between the appearance of oxygen-evolving photosynthesis and accumulation of oxygen in Earth's atmosphere must have been of several hundred million years, as suggested by geochemical evidence (Fennel *et al.*, 2005).

Cyanobacterial N_2 fixation and climate

The molecular clock dates of appearance of N_2 fixation in cyanobacteria correspond to the late Archaean and the early Proterozoic eons at ~3000—2500 MYA, coinciding with those estimated by Shi and Falkowski (2008).

Current global rates of N_2 fixation are estimated to be much smaller than global denitrification. The balance between both processes during glacial/interglacial periods has an effect on the amount of nitrate in the ocean, influencing the rate of carbon sequestration, which is controlled by iron availability (Michaels *et al.*, 2001). A feedback system that controls carbon sequestration dynamics due to N_2 fixation/denitrification rates has been proposed, coupled with iron availability and climate on millennium time scales.

Most of the N_2 fixation (~80%) in today's oceans is attributed to *Trichodesmium* spp. These are colonial, filamentous, non-heterocystous cyanobacteria with specialized cells for N_2 fixation (Capone *et al.*, 1997; Michaels *et al.*, 2001). Molecular clock estimated the dates of appearance of *Trichodesmium* to range between 775 and 504 MYA. The 700- to 500-MYA time interval is associated to the Pan African period and it represents in the fossil record the onset of the Cambrian explosion. The increase in biodiversity within the Ediacaran and Cambrian periods is presumed to have been triggered by the split of the supercontinent Rodinia (1100—750 MYA), which preceded Pangea (Maruyama and Santosh, 2008).

Our results suggest that whereas cyanobacteria such as *Trichodesmium* could be responsible for major changes in the Earth's climate during the last ~700 MYA through their global influence on the C and N cycles, other biogeochemically relevant bacteria, such as heterocyst-forming and unicellular, N_2-fixing cyanobacteria, were possibly determinant in Earth's functioning during the last 2500 MYA, making them fundamental players in the global C and N cycles. Unicellular cyanobacteria related to the chloroplast line of descent have been acknowledged as important players in oceanic N_2 fixation (Falcón *et al.*, 2004; Montoya *et al.*, 2004). Recently, members of this clade have been reported to lack genes for the oxygen-evolving PSII and C-fixation, with implications on their evolutionary history and influence on the global C and N cycles (Zehr *et al.*, 2008).

The dates of the split between *Trichodesmium erythraeum*, now present in the Red Sea, and *Trichodesmium havanum*, from the Caribbean Sea, range between 214 and 94 MYA. The above suggests that these species of *Trichodesmium* shared a genetic pool in the Sea of Thetys and diverged with the split of Pangea. This result again places the temporality of modern biogeochemically relevant

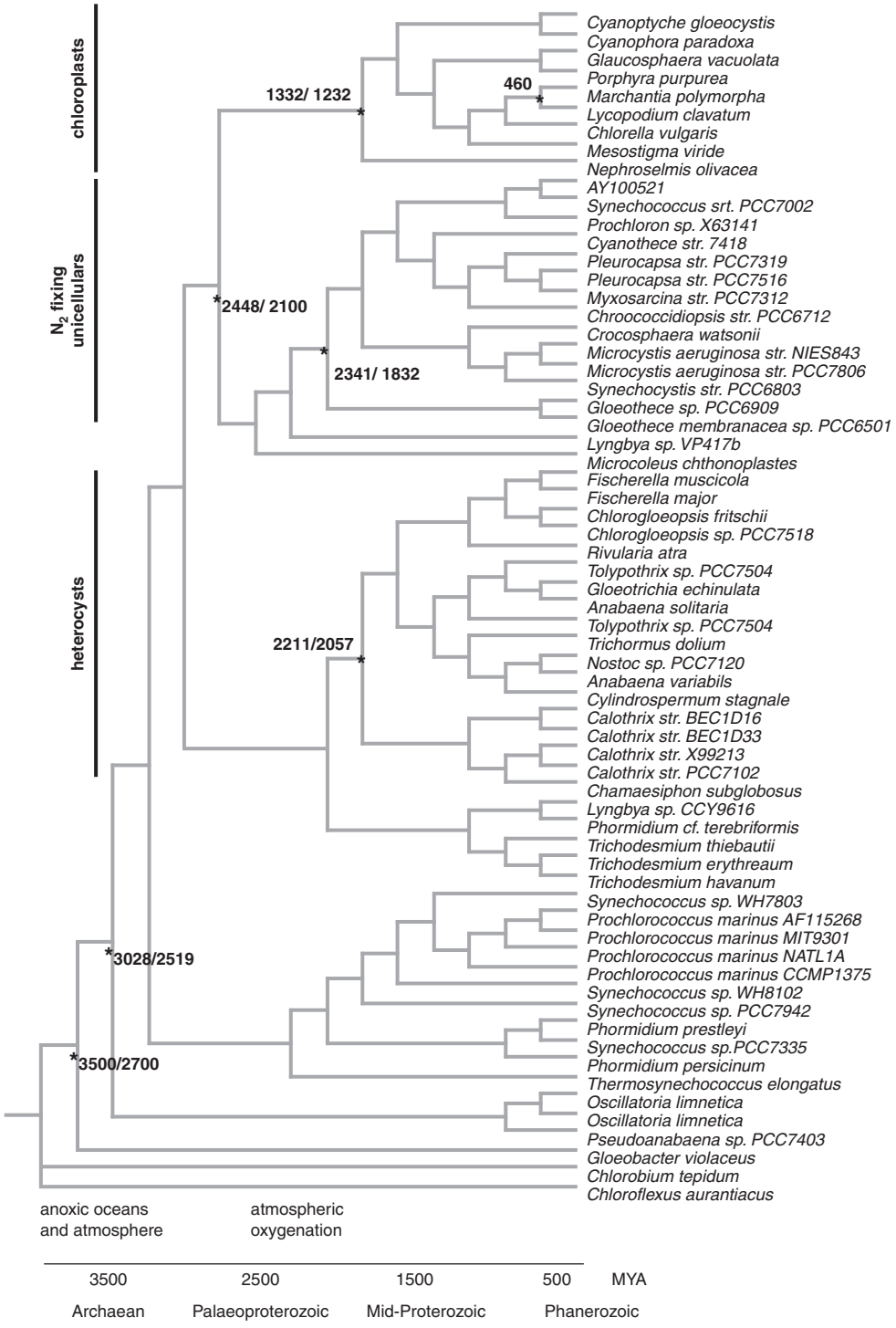

Figure 2 Divergence time estimates (MYA) among cyanobacteria and chloroplasts, including 56 cyanobacterial taxa from all subsections, and nine chloroplasts, which include a member of the oldest of the group, Cyanidiales (Rhodophyta), Glaucophyta, Chlorophyta and Streptophyta, all originating from the primary endosymbiosis event. Bayesian phylogenetic analysis showing the maximum *a posteriori* (MAP) topology, dated with penalized likelihood. Tree calibrated by imposing a fixed origin of the cyanobacterial lineage at 3500 and 2700 MYA, and a maximal age constraint of 460 MYA to the crown group of tracheophytes (indicated by asterisks).

cyanobacteria and suggests how global arrangement of emerged continents and oceanic regions had an important role in biogeography and biogeochemistry.

The closest living relatives of the plastid lineage are fundamental components of past and modern oceanic ecosystems. Their double capacity to fix N_2 through starch formation had a pivotal role in the instauration of the primary symbiosis. Our study suggests early time points of appearance of the plastid lineage and its sister clades, as well as of the cyanobacterial capacity to fix atmospheric C and N.

We conclude that small, gradual changes must have operated during the millennia after the advent of biogeochemically important cyanobacteria, throughout the history of life on Earth. The existence of different biogeochemically important metabolisms, such as oxygen-evolving photosynthesis and N_2 fixation, eventually changed the redox chemistry of the planet.

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