Adaptive signals in algal Rubisco reveal a history of ancient atmospheric carbon dioxide

J. N. Young¹, R. E. M. Rickaby¹,*, M. V. Kapralov² and D. A. Filatov²

¹Department of Earth Sciences, Oxford University, South Parks Road, Oxford OX1 3AN, UK
²Department of Plant Sciences, Oxford University, South Parks Road, Oxford OX1 3RB, UK

Rubisco, the most abundant enzyme on the Earth and responsible for all photosynthetic carbon fixation, is often thought of as a highly conserved and sluggish enzyme. Yet, different algal Rubiscos demonstrate a range of kinetic properties hinting at a history of evolution and adaptation. Here, we show that algal Rubisco has indeed evolved adaptively during ancient and distinct geological periods. Using DNA sequences of extant marine algae of the red and Chromista lineage, we define positive selection within the large subunit of Rubisco, encoded by rbcL, to occur basal to the radiation of modern marine groups. This signal of positive selection appears to be responding to changing intracellular concentrations of carbon dioxide (CO₂) triggered by physiological adaptations to declining atmospheric CO₂. Within the ecologically important Haptophyta (including coccolithophores) and Bacillariophyta (diatoms), positive selection occurred consistently during periods of falling Phanerozoic CO₂ and suggests emergence of carbon-concentrating mechanisms. During the Proterozoic, a strong signal of positive selection after secondary endosymbiosis occurs at the origin of the Chromista lineage (approx. 1.1 Ga), with further positive selection events until 0.41 Ga, implying a significant and continuous decrease in atmospheric CO₂ encompassing the Cryogenian Snowball Earth events. We surmise that positive selection in Rubisco has been caused by declines in atmospheric CO₂ and hence acts as a proxy for ancient atmospheric CO₂.

Keywords: Rubisco; adaptive evolution; algae; palaeoclimate; carbon dioxide

1. INTRODUCTION

The living ecosystem and carbon dioxide (CO₂) and oxygen (O₂) levels in the atmosphere are inexorably linked through tight feedback mechanisms. The advent of oxygenic photosynthesis around 2.4 Ga increased atmospheric and surface ocean O₂ levels and reduced atmospheric CO₂. However, O₂ levels remained low for the next billion years despite declining CO₂ [1], partly attributed to anoxygenic photosynthesis preventing oxygenation of the deep ocean [2]. It was not until the Neoproterozoic when oxygenic photosynthesis became dominant, hypothesized to contribute to the second rise of O₂ which paved the way for evolution of complex multi-cellular life [1].

Biological innovations certainly exert an influence on the atmosphere, but atmospheric composition also drives biological adaptations. The rate-limiting step of photosynthesis, CO₂ fixation, is catalysed by the enzyme, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39). During oxygenic photosynthesis, Rubisco catalyses the two competitive reactions: CO₂ fixation for photosynthesis (carboxylation) and energy-wasting photorespiration using O₂ (oxygenation). The ability of a particular Rubisco to discriminate between non-polar, structurally similar substrates of CO₂ and O₂ is determined by the kinetic properties of Rubisco and the CO₂ and O₂ concentrations at the catalytic site of the enzyme, denoted as the specificity factor (Ω) [3]:

Ω = \frac{V_c K_c}{V_o K_o} \times \frac{[CO₂]}{[O₂]},

where \(V_c\) and \(V_o\) are maximal velocities of the carboxylase and oxygenase reactions and \(K_c\) and \(K_o\) are the Michaelis constants for CO₂ and O₂. Thermodynamic constraints dictate a trade-off between carboxylation velocity (\(V_c\)) and affinity for CO₂ that has led to suggestions that despite being conserved and sluggish, Rubisco is optimized to its physical environment [4]. The variation in Rubisco catalytic properties found within photosynthetic eukaryotes hints that it has undergone adaptations to low CO₂ [5–8], but little is known about the detailed timing of evolution of Rubisco and its relationship to the environmental change.

We aim to define the history of adaptation of Rubisco in red and Chromista algae. Our study focused on these oxygenic photosynthesizing algae because they all possess a red chloroplast containing...
the same Form 1D Rubisco [9]. Their chloroplast evolutionary history stemmed from a primary endosymbiotic event that resulted in the red algae (Rhodophyta and Cyanidiales), and subsequent secondary endosymbiosis to form the Chromalveolata [10]. Chromalveolata includes the Chromista (Haptophyta, Stramenopiles and Cryptophyta) [11] and the Alveolata. However, as there is debate on the plastid monophyly of the Chromalveolata (for review, see Keeley [12]), we focused only on the Chromista which have a well-supported single secondary endosymbiotic origin of their plastids [13]. Furthermore, many representatives of the Chromista algae, such as diatoms and Haptophyta, dominate the modern ocean and display an extensive fossil history [14,15], making it possible to date the periods of adaptive evolution in Rubisco. Form 1D Rubisco protein is made up of eight large and eight small subunits. The active sites of Rubisco are formed by large subunits which are encoded by the chloroplast gene rbcL.

To infer adaptation of Rubisco, we reconstructed the chloroplast evolutionary history of the red and Chromista algae as a template on which we identified positive selection in rbcL by applying phylogenetic analysis of maximum likelihood (PAML) [16]. PAML identifies positive selection by comparing the substitution rates of mutations that do and do not affect protein sequence (dN and dS, respectively) along a phylogeny. The former is likely to affect the survival of the organism, whereas the latter is neutral, or nearly neutral; so dS/dN > 1, dS/dN = 1 and dS/dN < 1 indicate positive, neutral and purifying selection, respectively. Bayesian tree reconstruction and dating were conducted using a number of fossil calibrations, allowing the timing of adaptive events to be constrained between branch nodes providing an evolutionary history to around 1.5 Ga.

2. MATERIAL AND METHODS

(a) DNA extraction and amplification of rbcL
DNA was extracted from Haptophyta and diatom cultures maintained at Marine Biological Association, Plymouth, UK using previously published methods [17]. About 95 per cent length of the coding region of the chloroplast rbcL gene was PCR amplified using Biomix (Bioline, MA, USA) and primers (electronic supplementary material, table S1), and the following PCR conditions: initial cycle of 95 °C for 2 min, 55 °C for 30 s, 72 °C for 30 s followed by 40 cycles of 92 °C for 30 s, 53 °C for 30 s and 72 °C for 3 min, with final elongation of 72 °C for 10 min.

DNA sequencing was performed using ABI BigDye v. 3.1 and capillary sequencers 3700 and 3730xl (Applied Biosystems Inc., CA, USA). Sequences of the rbcL gene for 17 species were uploaded to NCBI GenBank under accession numbers HQ656822–HQ656838. The new rbcL sequences were combined with a further 227 rbcL sequences from NCBI GenBank (electronic supplementary material, table S2) and aligned using CLUSTALX [18], followed by minor manual editing of the alignment and assignment of coding region using ProSeq3 [19].

(b) Phylogenetic and molecular clock analysis
The phylogeny shown in figure 1 was reconstructed using four plastid protein-coding genes used by Yoon et al. [22] with a few changes: removal or replacement of species owing to short or poor quality rbcL sequences and additional sequences of heterokonts, Haptophyta and coralline red algae. The Haptophyta tree (electronic supplementary material, figure S1) was reconstructed only using rbcL sequences. The diatom tree (electronic supplementary material, figure S2) was constructed with the 18S rRNA and rbcL genes. Bayesian tree reconstruction and dating were conducted using BEAST software [23]. The calibration constraints used for each tree are summarized in electronic supplementary material, table S3 [13–15,24–26]. Phylogeny reconstruction assumed the general time-reversible substitution model [27] with gamma-distributed rate heterogeneity. For protein-coding genes, we assumed separate rates for the three codon positions. We conducted runs with chain lengths of 5 × 10^7 to 10^8 steps assuming the Yule model of speciation process and both clock and uncorrelated lognormal-relaxed clock across the tree [28,29] with similar results. Given the standard deviation of the uncorrelated lognormal-relaxed clock (uchl.sd.stev parameter) was consistently below 1, the data appeared to fit the molecular clock model quite well, hence the latter was used for dating of individual nodes. The convergence of parameter estimates was checked using TRACER [30]. The data were saved every 10^3 steps and the first 5 × 10^3 trees were ignored as a burn-in. TreeAnnotator v. 1.5.4 was used to summarize the post-burn-in trees, and the maximum credibility tree along with 95% probability density of ages was visualized in FigTree v. 1.3.1 [31].

(c) Phylogenetic analysis of maximum likelihood
Detection of positive selection in the rbcL gene was conducted using nested maximum-likelihood models A and A1 allowing for variation in the ratio of non-synonymous to synonymous substitution rates (dN/dS) across codons and across branches implemented in PAML v. 4 [16]. The analysis was conducted assuming the phylogenies in figure 1 and electronic supplementary material, figures S1 and S2. The null model A1 allows dN/dS ratios to vary both among sites and among branches; it allows 0 < dN/dS < 1 and dN/dS = 1 for both foreground and background branches, and also two additional classes of codons with fixed dN/dS = 1 on foreground branches while restricted to 0 < dN/dS < 1 and dN/dS = 1 on background branches. The alternative A model allows 0 < dN/dS < 1 and dN/dS = 1 for both foreground and background branches, and also two additional classes of codons under positive selection with dN/dS > 1 on foreground branches while restricted to 0 < dN/dS < 1 and dN/dS = 1 on background branches. One branch at a time was labelled as a foreground branch with allowed positive selection, whereas all other branches were labelled as background branches with no positive selection allowed. Likelihood-ratio tests (LRTs) were repeated with a different branch labelled as a foreground branch until all branches had been tested [32,33]. The significance of the A/A1 LRTs was calculated assuming...
that twice the difference in the log of maximum likelihood between the two models was distributed as a chi-square distribution with one degree of freedom. The Bonferroni correction procedure implemented in SISA-Binomial [34] was used to correct the statistical significance for multiple comparisons.

These tests were applied to three datasets, red and Chromista algae, Haptophyta and diatoms, consisting of 39, 28 and 30 species, respectively (figure 1 and electronic supplementary material, figures S1 and S2).

Phylogenies shown in figure 1 and electronic supplementary material, figures S1 and S2 were also used to detect positive selection within a group using the site test M1a/M2a [35]. Four other red and Chromista algal groups were also tested using M1a/M2a LRTs consisting of 49 Rhodophyta, 45 Phaeophyceae, 28 Cryptophyta, 33 Chrysophyceae and Synurophyceae rbcL sequences (electronic supplementary material, figure S3 and table S2). Phylogenies of these groups were reconstructed using Bayesian method (MrBayes v. 3.1, GTR model with gamma-distributed rate variation across sites and a proportion of variable sites with at least $10^6$ runs) [33]. In M1a/M2a LRTs, the null model M1a (nearly neutral) that allows 0 ≤ $d_S$/dS ≤ 1 was compared with the M2a model (same as the M1a model plus an extra class under positive selection with $d_S$/dS ≥ 1). The significance of the M1a/M2a LRTs was calculated assuming that twice the difference in the log of maximum likelihood between the two models was distributed as a chi-square distribution with two degrees of freedom.

The Bayes empirical Bayes method [36] implemented in PAML was used to calculate the posterior probabilities that particular sites fall into classes with different $d_S$/dS and to identify sites with a high probability of being under positive selection (having $d_S$/dS > 1). Rubisco sites under positive selection were numbered against the spinach (Spinacia oleracea) sequence for comparison with other studies. Residue location in the tertiary structure was visualized using the structural data file of spinach Rubisco (1RBO) available on RCSB Protein Data Bank with DeepView-Swiss PdbViewer v. 3.7 [37].

3. RESULTS AND DISCUSSION

(a) Positive selection of algal Rubisco occurred during distinct geological periods

The analysis of individual branches revealed that positive selection was restricted to branches basal to the radiation of the red and Chromista algae between 1.56 and 0.41 Ga (average ages of the nodes; for the range at each node see figure 1). Positive selection in rbcL was prior to the divergence of large algal taxonomic groups, but not within the groups. To further explore this pattern, we focused on two geochemically and ecologically important groups with relatively good fossil records: the Haptophyta (including coccolithophores) and Bacillariophyta (diatoms). Once again, positive selection was restricted to a few, deep branches. The Haptophyta (electronic supplementary material, figure S1) showed a strong signal of positive selection along the branch leading to, and basal to, the radiation of the Prymnesiophyceae (which includes the coccolithophores Emiliania huxleyi, Coccolithus pelagicus and colony-forming Phaeocystis spp.) between 375 and 285 Ma. Once this group diverged, there is little evidence of positive selection apart from the branch leading to the Phaeocystaceae. In the diatoms (electronic supplementary material, figure S2), adaptation also occurred in deep branches within radial centrics (including Autacosa and Coscinodiscus spp.) at 142–97 Ma and pennates (including Fragilariapis, Phaeodactylum and Pinnularia spp.) at 73–56 Ma and in bipolar centrics (including Thalassiosira, Chaetocera and Skeletonema spp.) at 114–92 Ma.

Positive selection hints at changes of amino acids that would influence the catalytic activity of Rubisco. Indeed, positive selection does appear to explain the variation in known Rubisco specificity factors (figure 1b). However, specificity factors are only a ratio and are unable to reveal the true sensitivity of kinetic changes in Rubisco. There are few measurements of $K_C$ and $V_C$ in algae but the $K_C$ for both diatoms and Haptophyta are higher than Rhodophyta, which is higher again when compared with the Cyanidiales (figure 1b).

Using a Bayesian approach [36], we identified positively selected amino acid positions in rbcL in the basal branches where selection occurred. Only a few domains have been identified to influence activity or kinetics [9] and our sites under positive selection do not fall within these domains. However, there is some commonality to the residues under positive selection between land plants [49] and algae (figure 2), suggesting that these additional residues outside known important protein domains may also significantly affect Rubisco performance.

(b) Positive selection of Rubisco in algae contrasts to that of land plants

Our results of positive selection only in the deep branches of the algal phylogeny contrasts with the ubiquitous positive selection found throughout the land plant groups [49]. To test whether this result may be owing to differences in the analytical approaches between studies, we applied the methodology previously used for plants [49] to an expanded set of red and Chromista algal groups, including Rhodophyta, Phaeophyta, Cryptophyta, Chrysophyceae and Synurophyceae, along with Haptophyta and diatoms with phylogenies constructed using rbcL sequences (electronic supplementary material, figure S3). This analysis confirmed the absence of positive selection...
within algal taxonomic groups, with none of the expanded algal groups displaying a signal of positive selection using this test (table 1).

(c) Carbon dioxide as a driver for Rubisco adaptation

There is evidence that Rubisco adaptation in land plants was driven by a number of environmental factors in addition to atmospheric composition, including aridity and high temperatures [50]. However, in the marine realm, we propose that it is the change in atmospheric CO₂ equilibrated with surface waters which acts as the ultimate driver of positive selection in Rubisco. As Rubisco is packaged within the chloroplast, the adaptive response of Rubisco must be driven by changing intracellular conditions. Intracellular CO₂ conditions are under physiological control, and as external CO₂ becomes increasingly limited algae are known to induce carbon-concentrating mechanisms (CCMs) to boost the internal supply of CO₂ to Rubisco [5]. However, this can also lead to an elevation in intracellular O₂ of levels up to 6–6.5 times external concentrations [51], owing to reduced leakiness of both CO₂ and O₂ [52,53]. While both intracellular CO₂ and O₂ levels can change by induction of a CCM, the trigger for the employment of CCMs is driven by changes in external CO₂. CCMs are regulated by external CO₂, and on geological scales of eukaryotic evolution, changes of atmospheric CO₂ are orders of magnitude greater than changes in O₂. Additionally, the Form 1D Rubisco present in the studied algal species appears to have reduced oxygenase potential and therefore a reduced tendency to be inhibited by O₂ [6]. Finally, optimization of Rubisco efficiency is driven by the trade-off between Vcₐ and CO₂ affinity, and therefore is in tune with intracellular CO₂ concentrations [54].

In land plants, it has been established that positive selection in rbcL emerges coincident with the development of a C₄ CCM which elevates CO₂ to almost saturation at the site of Rubisco [55,56]. This relaxes pressure for Rubisco to have a high affinity for CO₂ and therefore allows an increase in Vcₐ, which results in increased photosynthetic efficiency as the plant requires less nitrogen to achieve a given CO₂ fixation capacity.
By analogy, therefore, positive selection in Rubiscos of the Haptophyta and diatoms is likely to have occurred also in response to emergence of CCMs as both Haptophyta and diatoms are thought to possess them [57]. We can infer that, like in the C₄ land plants, positive selection indicates a lowering of CO₂ affinity and an increase in $V_c$ in these algae after induction of CCMs. Indeed, modern day Haptophyta and diatoms do display lower specificities ($V$) and CO₂ affinity (i.e. higher $K_c$) than their red algal counterparts (their ancestral endosymbionts; figure 1b). So, although atmospheric CO₂ acts as the ultimate driver of Rubisco change, the mechanistic driver is the physiological innovation in boosting intracellular carbon (the CCMs) in response to declining CO₂.

The term CCM encompasses a wide variety of poorly understood mechanisms that concentrate carbon to different degrees. While the pattern of positive selection in $rbcL$ hints at a link between the presence of CCMs and positive selection within the Haptophyta and diatoms, there is insufficient knowledge to explain whether a simple relationship exists between gradational improvements in carbon concentration and positive selection in $rbcL$.

It does appear that the presence of positive selection certainly indicates elevated internal carbon. We have already outlined that positive selection in Haptophyta and diatoms suggest an increase in intracellular carbon concentrations above the Rhodophyta but many members of the Rhodophyta are also thought to possess CCMs [6], though their relative abilities to concentrate carbon are poorly documented [6]. Like the presence of positive selection that separates the Rhodophyta and Chromista (Haptophyta and diatoms), positive selection also occurs at the divergence of Rhodophyta and Cyanidiales, and again correlates with an increase in $K_c$ (figure 1b). This suggests that positive selection of $rbcL$ is linked to a gradual reduced affinity of Rubisco for CO₂, most likely driven by development of a better functioning CCM.

On the other hand, absence of positive selection does not necessarily indicate a lack of CCMs. No positive selection is detected leading to the Phaeophyta (the branch leading to *Pylaiella littoralis* in figure 1).
Rubisco adaptation correlates with declining carbon dioxide

The timing of events of positive selection in Rubisco further corroborates its relationship with atmospheric CO₂. A comparison of the occurrence of positive selection within Haptophyta and diatoms during the Phanerozoic against the most recent compilation of proxy-derived reconstructions and geochemical models of CO₂ and O₂ show that positive selection always corresponds with falling CO₂ (inset, figure 3). This correlation of Rubisco adaptation events with declining CO₂ demonstrates the potential for use of adaptation of Rubisco as an indicator of past atmospheric CO₂.

Extending the carbon dioxide record into the Proterozoic

We can apply the Phanerozoic relationship between declining CO₂ and positive selection to investigate possible CO₂ change using the deeper branches in the tree of the whole red/Chromista algal lineage (figure 3). It is challenging to constrain timing of adaptation in the Proterozoic owing to long branches and the lack of a fossil record. Nonetheless, we find positive selection on the earliest divergence of the Cyanidiales (1.56–1.14 Ga). There is a cluster of signals of positive selection in a number of branches immediately after the event of secondary endosymbiosis, the origin of the Chromista.

Table 1. PAML results for models M0 and M1a/M2a.

<table>
<thead>
<tr>
<th>group</th>
<th>tree</th>
<th>M0</th>
<th>M1a/M2a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhodophyta</td>
<td>electronic supplementary material, figure S4a</td>
<td>31 37 50 1200 21.748 2.022 37.483 0.749 0.020 0 1</td>
<td></td>
</tr>
<tr>
<td>Phaeophyceae</td>
<td>electronic supplementary material, figure S4b</td>
<td>8 29 45 1362 2.732 3.170 3.905 0.179 0.046 0.01 0.99</td>
<td></td>
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<tr>
<td>Cryptophyta</td>
<td>electronic supplementary material, figure S4c</td>
<td>4 5 33 930 8.950 0.968 14.821 0.449 0.030 0 1</td>
<td></td>
</tr>
<tr>
<td>Haptophyta</td>
<td>electronic supplementary material, figure S4d</td>
<td>10 21 32 1377 8.501 1.377 8.723 0.437 0.053 0 1</td>
<td></td>
</tr>
<tr>
<td>diatoms</td>
<td>electronic supplementary material, figure S1</td>
<td>18 23 30 900 5.617 1.380 8.178 0.444 0.054 0 1</td>
<td></td>
</tr>
<tr>
<td>red and brown algae</td>
<td>figure 1</td>
<td>28 39 40 1206 19.826 1.108 29.800 1.084 0.036 0 1</td>
<td></td>
</tr>
</tbody>
</table>

Using M0 model in PAML: nᵢ, number of families; nₒ, number of genera; nₛ, number of sequences; nₑ, number of nucleotides; BL, branch length; κ, transition/transversion rate; dₑ, synonymous substitutions; dₛ, non-synonymous substitutions; dₑ/dₛ, average dₑ/dₛ across tree (using 1 class for dₑ/dₛ). Comparing M1a (nearly neutral) with M2a (positive selection) using likelihood-ratio test (LRT), where χ² = 2 × (LRTM2a – LRTM1a) with degrees of freedom = 2 to calculate probability (p-value) for the LRT value being as high as is seen by chance.
algae, approximately 1.1 Ga. The environmental trigger for this symbiotic relationship between host and chloroplast is unknown but this relationship confirms that Rubisco evolves in response to changing intracellular conditions, plus there are certain advantages to an endosymbiotic habitat in a low CO$_2$ environment.

Figure 3. Timing of adaptation of Rubisco in response to changing atmospheric CO$_2$ and O$_2$. Timing of positive selection in figure 1 (magenta) and within Haptophyta (electronic supplementary material, figure S1, orange) and diatoms (electronic supplementary material, figure S2, green), where boxed area denotes time between age of nodes and line encompasses 95% probability density of ages. This is compared with proxy-derived Proterozoic ranges of atmospheric CO$_2$ (turquoise ovals [38–41]) and O$_2$ (grey ovals [1]). A continuous record of Phanerozoic CO$_2$ was reconstructed from proxies [43,44] binned into 10 Ma with 1 s.d. (light blue) and overlaid over modelled data (dark blue [42]). Phanerozoic O$_2$ was reconstructed from proxies showing 1 s.d. (shaded grey) and modelled data (dark grey line [45], blue line [47] and green line [46]). Dark blue blocks denote possible snowball Earths, and light blue blocks denote periods of glaciations. Inset shows Phanerozoic data on a linear scale, where coloured bars represent the average age of nodes.
The continued positive selection, on two branches subsequent to those immediately after the secondary endosymbiosis, points to sustained declining CO₂ (0.93–0.52 Ga). We present a continuous record suggesting decreasing CO₂ encompassing periods when there are currently few or no constraints. Because advances in evolutionary innovation generally occurred at times of major environmental or geochemical change, the trend of declining CO₂ during the Proterozoic suggests this billion years of time may not have been so ‘boring’. We suggest instead a backdrop of environmental change associated with the expansion of the eukaryotes during the very late Mesoproterozoic and Neoproterozoic [22]. Further, such a decrease in CO₂ likely played a role in triggering the subsequent Snowball Earth events and at least contributed to dictating Proterozoic climates [64].

4. CONCLUSION
The wide adoption of photosynthesis by life, resulting in reduced atmospheric CO₂–O₂ ratios, created increased evolutionary pressure on Rubisco which has evolved its kinetic properties in response to declining atmospheric CO₂ [8]. Determining the timing of adaptation of the CO₂–O₂ sensitive enzyme, Rubisco, presents a novel approach in understanding the biological response to changing atmosphere, and the periods of emergence of CCMs. It supports the current estimation of the periods of decreasing CO₂ beyond the Phanerozoic and provides important additional constraints to the current scarcity of proxies available during these time periods, uniquely delivering a continuous record extending 1.5 Ga. The future challenge is to further document the physiological innovations responsible for driving the Rubisco change, and to discover other genetic signatures which can open a window on past major environmental change, such as the oxygenation of the planet.

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