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2 **Tightly constrained genome reduction and relaxation of purifying selection during**
3 **secondary plastid endosymbiosis**

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16 Abstract (250 words max)

17

18 Endosymbiosis, the establishment of a former free-living prokaryotic or eukaryotic cell as an
19 organelle inside a host cell, can dramatically alter the genomic architecture of the
20 endosymbiont. Plastids, the light harvesting organelles of photosynthetic eukaryotes, are
21 excellent models to study this phenomenon because plastid origin has occurred multiple
22 times in evolution. Here, we investigate the genomic signature of molecular processes
23 acting through secondary plastid endosymbiosis – the origination of a new plastid from a
24 free-living eukaryotic alga. We used phylogenetic comparative methods to study gene loss
25 and changes in selective regimes on plastid genomes, focusing on the green lineage that has
26 given rise to three independent lineages with secondary plastids (euglenophytes,
27 chlorarachniophytes, *Lepidodinium*). Our results show an overall increase in gene loss
28 associated with secondary endosymbiosis, but this loss is tightly constrained by retention of
29 genes essential for plastid function. The data show that secondary plastids have experienced
30 temporary relaxation of purifying selection during secondary endosymbiosis. However, this

31 process is tightly constrained as well, with selection relaxed only relative to the background
32 in primary plastids, but purifying selection remaining strong in absolute terms even during
33 the endosymbiosis events. Selection intensity rebounds to pre-endosymbiosis levels
34 following endosymbiosis events, demonstrating the changes in selection efficiency during
35 different phases of secondary plastid origin. Independent endosymbiosis events in the
36 euglenophytes, chlorarachniophytes, and *Lepidodinium* differ in their degree of relaxation of
37 selection, highlighting the different evolutionary contexts of these events. This study reveals
38 the selection-drift interplay during secondary endosymbiosis, and evolutionary parallels
39 during the process of organelle origination.

40

41 **Keywords**

42 Secondary endosymbiosis, plastids (photosynthetic organelle), selection efficiency variation,
43 drift.

44

45 Introduction

46 The endosymbiosis event leading to present-day chloroplasts is inferred to have taken place
47 ~1.5 billion years ago through the incorporation of a cyanobacterium by a heterotrophic
48 host (Yoon, et al. 2004; Price, et al. 2012; Nowack and Weber 2018). This endosymbiosis
49 event is referred to as primary endosymbiosis, with the plastids of the organisms
50 descending from this event termed primary plastids (Keeling 2010; Archibald 2015). Three
51 photosynthetic lineages emerged from this ancestor: the Chlorophyta (green algae),
52 Rhodophyta (red algae) and Glaucocystophyta. Subsequently, several red and green algae
53 have engaged in secondary endosymbiosis events, giving rise to more complex plastids.
54 Secondary endosymbiosis differs in having a eukaryotic alga (carrying a primary plastid) as
55 the photosynthetic partner being established as an organelle, and this process has spread
56 photosynthesis to many unrelated branches of the eukaryotic tree of life (Keeling 2010).
57 Despite the relevance of plastid endosymbiosis for eukaryotic evolution and algal diversity,
58 the understanding of molecular evolution during the origination of these plastids is limited.
59 Endosymbionts often experience lowered levels of natural selection (Latorre and Manzano-
60 Marín 2017; Wernegreen 2017), with the elevation of levels of stochastic genetic drift

61 leading to an accumulation of slightly deleterious mutations, resulting in genome reduction
62 and making them more susceptible to degradation (Moran 1996; Pettersson and Berg 2007;
63 Moran, et al. 2008; Bennett and Moran 2015). Plastids have retained a highly reduced
64 genome (ca. 100-200kb) characterised by accelerated rates of evolution and AT-biased
65 nucleotide composition compared to free-living cyanobacteria (Green 2011; Bennett and
66 Moran 2015). As is the case in many endosymbionts, plastid genomes have lost the majority
67 of cyanobacterial genes, some having been transferred to the nucleus. Some of the gene
68 losses are compensated by nucleus-encoded plastid-targeted genes that enable integration
69 of plastids into the host cell biology. Plastid genomes have a highly conserved set of key
70 genes encoding for core components involved in photosystem, ATP synthesis and protein
71 translation (Allen 1993, 2017) that are under strong purifying selection (Smith 2015;
72 Grisdale, et al. 2019). Several hypotheses suggest that the retention of genes in the plastid
73 genome enhances the ability of organelles to efficiently respond to fluctuating conditions
74 (Allen 1993, 2017; Johnston 2019). Strong purifying selection on the retained plastid
75 genomes distinguishes them from most other endosymbiont genomes in early stages of
76 endosymbiosis. While parallels can be expected between the evolutionary forces acting
77 during establishment of plastid endosymbiosis (e.g. (Reyes-Prieto, et al. 2010; Lhee, et al.
78 2019) and other obligate endosymbiosis events based on the similarities in their overall
79 genomic features, there has been very little work on characterising patterns of selection and
80 drift in the origination of plastid organelles.

81 Secondary endosymbiosis differs fundamentally from primary because at the start of this
82 process, the genomes of the primary plastid have already transitioned to a reduced state
83 (Green 2011), with secondary green plastids having roughly similar gene content to primary
84 green plastids (Suzuki, et al. 2016; Karnkowska, et al. 2018). Inouye and Okamoto (2005)
85 postulate that secondary endosymbiosis of plastids involves several stages, beginning with
86 permanent retention of the engulfed primary alga, followed by reduction of the
87 endosymbiont genomes (primarily the nucleus) and ultimately fixed as an organelle through
88 nuclear encoded plastid targeted genes. Recent studies have emphasized the possible role
89 of the secondary host nucleus in facilitating the integration of the incoming green plastids in
90 lineages that have hosted other plastids before (Ponce-Toledo, et al. 2018; Ponce-Toledo, et
91 al. 2019). All these previous studies related to secondary endosymbiosis are focused on the

92 endosymbiont's nuclear genome reduction, but the molecular evolution of plastid genomes
93 through the various stages of secondary endosymbiosis remains largely unexplored.

94 This study aims to characterise the molecular evolutionary processes acting on the origin of
95 secondary plastids, using secondary plastids of green algal ancestry as a model system.

96 These secondary plastids are found in three lineages, the chlorarachniophytes (a group of
97 Rhizaria), the euglenophytes (a group of excavates) and the dinoflagellate genus
98 *Lepidodinium* (Jackson *et al.* 2018). The existence of these three evolutionary events,
99 distinctly independent from each other and with clearly identifiable host and plastid donor
100 origins, makes green-type secondary plastid an excellent case study to investigate features
101 common to secondary endosymbiosis events and those unique to individual events. Here,
102 we use phylogenetic methods to examine the variation in selection on genes before, during,
103 and after endosymbiosis, and to compare how this selection varies across genes and
104 endosymbiosis events. We also quantify patterns and rates of gene loss across these events
105 of secondary endosymbiosis. Our results are interpreted in the light of evolutionary
106 processes that can contribute to variation in selection during secondary endosymbiosis.

107 **Results and Discussion**

108 *Plastid genome features*

109 Most plastid genomes, including those of secondary plastids, had small genomes (median
110 153kb), low GC proportion (0.34) and encoded an average of 80 annotated protein-coding
111 genes. Plastid genomes of chlorarachniophytes (70kb genome, 60 CDS) and *Lepidodinium*
112 (66kb, 62 CDS) are smaller with fewer CDS than those of euglenophytes plastid genomes
113 (90kb, 64 CDS) (Table S1). Codon usage bias estimated using synonymous codon usage
114 order showed that all green plastids studied had similar codon usage bias that appeared
115 proportional to nucleotide composition (Figure S1). Among the secondary plastid lineages,
116 chlorarachniophyte plastids had slightly lower GC content and higher codon usage bias than
117 euglenophytes and *Lepidodinium*. However, codon usage bias for secondary plastids was
118 within the range of that observed for primary plastids.

119 *Tightly constrained genome reduction*

120 By grouping protein-coding genes into orthogroups and estimating gene loss with Dollo
121 parsimony, it became apparent that plastid genomes experience an elevated level of
122 genome reduction during secondary endosymbiosis events, but that they retain all key
123 plastid genes encoding for core subunits related to photosynthesis, ATP and protein
124 synthesis (Figure 1 and Figure 2). Reductive genome evolution highlights the similarities in
125 molecular evolution between secondary plastid endosymbiosis and many examples of
126 bacterial endosymbiosis in insects (McCutcheon and Moran 2012). Gene loss is particularly
127 severe during primary endosymbiosis, with cyanobacteria-sized genomes (ca. 1,800-12,000
128 genes) reducing to the ca. 80-230 genes found in primary plastids (Gabr, et al. 2020). Gene
129 loss from plastids during secondary endosymbiosis was small in comparison, with our
130 estimates indicating that chlorarachniophytes lost 30 genes during secondary
131 endosymbiosis followed by euglenophytes with 24 and *Lepidodinium* with 22 gene losses
132 (Figure 1). Even though the endosymbiotic branches are among the top five branches losing
133 the most genes, the difference compared to the background is not statistically significant
134 (ANOVA and Tukey HSD tests), possibly due to the small sample size (n=3) of endosymbiotic
135 branches available for analysis.

136 When viewed as the rate of gene loss per million years of evolution, the endosymbiosis
137 branches had somewhat higher rates on average (Figure 2) but ranked lists showed that
138 chlorarachniophytes and *Lepidodinium* were not among the branches losing genes fastest.
139 So, despite most gene losses occurring on the endosymbiotic branches, the rates of loss per
140 million years for these branches are not particularly high, suggesting that gene loss is a
141 punctuated process occurring early in endosymbiosis (Moran and Mira 2001; Oakeson, et al.
142 2014). When correcting for the branch lengths of endosymbiotic branches, this punctuated
143 effect is diluted to the point of not differing from background rates. Interestingly, the three
144 independent endosymbiosis events showed similar numbers of gene losses (in the 22-30
145 range), adding to a list of similarities between secondary endosymbiosis events that also
146 includes the convergent evolution of nucleomorph architecture seen in chlorarachniophytes
147 and cryptophytes (Sarai, et al. 2020; Sibbald and Archibald 2020).

148 Our gene loss analysis showed that 17 genes were lost more than 10 times across the
149 phylogeny, including *rp32* (ribosomal protein, 30 times), *psb30* (photosystem II, 22), *tifS*
150 (tRNA Ile-Lysidine synthetase, 18), *petL* (Cytochrome b6-f complex, 16) and *ycf47* (14). Only

151 *accD* (lipid acid synthesis), *ccsA* (mediates heme attachment to c-type cytochromes) and
152 *ftsH* (cell division) were lost in all three endosymbiotic events. Some genes lost during one
153 endosymbiotic event are also absent from other secondary plastids, but were lost before
154 the endosymbiotic event. For instance, *ndh* [A:I,K](NAD(P)H oxidoreductase) was lost during
155 euglenophyte endosymbiosis but it was also lost from the green algal lineages that gave rise
156 to the chlorarachniophytes and *Lepidodinium*. Most of the genes lost during secondary
157 endosymbiosis are likely to be compensated by nuclear homologs or through an alternative
158 mechanism. For instance, the light-independent chlorophyll synthesis genes *chlB*, *chlL* and
159 *chlN* that were lost during chlorarachniophyte and euglenophyte endosymbiosis and in
160 many other primary plastids, including the ancestors of *Lepidodinium*, can be compensated
161 by the light-dependent chlorophyll production pathway (Hunsperger, et al. 2015). The *chlB*,
162 *chlL* and *chlN* genes have also been lost from some secondary plastids of cryptophyte algae
163 (Fong and Archibald 2008).

164 Homologs of *rpl12*, *rpl32*, *rps9* (small ribosomal proteins), *infA* (translational initiation
165 factor) and *ftsH* were found in the nuclear genome of the chlorarachniophyte *Bigelowiella*
166 *natans* (Curtis, et al. 2012), suggesting they may have been transferred from the plastid
167 rather than lost entirely. Similarly, homologs of *petA*, *petN*, *ycf3*, *clpP* (Clp protease
168 proteolytic subunit) and *ftsH* were recovered in the transcriptomes of the euglenophytes
169 *Euglena gracilis* and *Eutreptiella* (Hrdá, et al. 2012). Aside from the genes mentioned above,
170 all other genes lost during secondary endosymbiosis including genes with a function in
171 photosynthesis (like *psb30*, *psbM*, *psaI*) were not detected in the nuclear genomes of *B.*
172 *natans* and *E. gracilis* and may represent genuine gene losses, but some caution is
173 warranted as most of these proteins are small and may be missed in genome-wide blast
174 searches.

175 Several of the genes predicted to be lost during secondary endosymbiosis (*accD*, *infA*, *ndh*,
176 *ycf1*, *ycf3* and *ycf4*) were lost from plastid genomes in other lineages too, and compensatory
177 nuclear-encoded genes have been identified (Boudreau, et al. 1997; Millen, et al. 2001;
178 Martín and Sabater 2010; Huerlimann and Heimann 2012).

179 Losses of genes whose functions can be compensated are likely to have little impact on
180 plastid function. Loss of similar genes in parallel in different parts of the tree suggests they
181 may experience reduced selective constraints compared to key photosynthesis genes, and in

182 periods with increased drift, such genes may be more likely to be lost than genes under
183 stronger selection. Recent work shows that genes encoding central subunits of the electron
184 transport chain are more likely to be retained in the organelle (Johnston and Williams 2016). In
185 line with this, we see that across the green algal phylogeny, 48 genes including the core
186 components of photosynthesis and protein synthesis remained highly conserved (never lost
187 or lost once).

188 The role of selection in retaining genes has also been demonstrated in the chromatophore
189 genomes of *Paulinella*, a model species for the study of primary endosymbiosis (Reyes-
190 Prieto, et al. 2010; Valadez-Cano, et al. 2017; Lhee, et al. 2019). Overall, our results suggest
191 that genome reduction appears to be elevated during secondary endosymbiosis but is a
192 tightly constrained process with strong selection to retain genes with key functions. Of
193 course, the lineages with secondary endosymbionts that we study here are all
194 photosynthetic, implying that by the design of our study, we introduced a bias towards
195 endosymbiosis events that would have maintained all genes with an essential function in
196 photosynthesis. It is perfectly conceivable that other outcomes are possible in
197 endosymbiosis events that do involve loss of photosynthetic function, but we are not aware
198 of any instances where a cyanobacteria or eukaryotic alga has been retained as an
199 endosymbiont for functions other than photosynthesis, besides secondarily non-
200 photosynthetic groups such as apicomplexans.

201 *Selection dynamics through endosymbiosis*

202 For our analysis of selection dynamics through endosymbiosis, the phylogeny was divided
203 into three sets of branches representing primary plastids (P), secondary plastids (S) and
204 endosymbiosis branches (E). Selection intensity during secondary endosymbiosis was
205 quantified using a Hyphy RELAX model that contrasts the selection on the endosymbiosis
206 branches relative to all other branches. The relative selection intensity parameter (k-value)
207 of the fitted model showed that the distribution of k-values across genes is well below 1
208 (median 0.43), a clear signature of relaxation of selection in the endosymbiotic branches (E)
209 compared to all other branches (P+S) (Figure 3, and see Tables 1, S2). Of the 34 genes in the
210 analysis, 26 showed statistically supported relaxation. Two outlier genes (*psbD* and *psbE*)
211 showed slight intensification of selection ($k > 1$) for this model, but without significant
212 statistical support. The same model (denoted E × P+S) applied to a concatenated alignment

213 of all plastid genes (Table S3) returned results in line with the findings for individual genes,
214 with relative selection intensity parameter (k) value of 0.55. The $E \times P+S$ model is a
215 significantly better fit to the concatenated sequences than the null model ($p < 0.0001$ and
216 likelihood ratio = 557.65), implying a significant decrease in evolutionary selection
217 (relaxation) during endosymbiosis.

218 While the signature of relaxation is clear, this does not imply that molecular evolution is
219 neutral in endosymbiotic branches. The model categorised 82.12% of sites as being under
220 purifying selection, with ω (ratio of non-synonymous(dN) to synonymous(dS) substitutions)
221 value of 0.06 in the endosymbiotic branches indicating that most sites remain under
222 purifying selection even during endosymbiosis events. BUSTEC analyses provided additional
223 statistical support for purifying selection along endosymbiosis branches, with all genes
224 having lower AIC scores for the unconstrained model with purifying selection than for the
225 model constrained to exclude purifying selection (Table S4).

226 Selection analysis based on the $E \times P+S$ model and the BUSTEC results helps to characterise
227 the molecular evolutionary process during secondary plastid endosymbiosis. Studies of
228 insect endosymbionts suggest that relaxation of purifying selection during endosymbiosis
229 establishment in obligate endosymbionts of insects can be due to two processes: a
230 population bottleneck and decrease in functional constraints on proteins (Moran 1996;
231 Wernegreen 2004, 2015). In the case of secondary plastid endosymbiosis, it seems unlikely
232 to have much relaxation on functional constraints, in line with the observations of purifying
233 selection and tight constraints on gene loss. Also, the relaxation is observed on nearly all
234 retained genes, further shifting the balance of evidence towards population size effects on
235 plastid genomes evolution during endosymbiosis. The near-neutral theory predicts that in
236 small populations, the fate of near-neutral mutations depends on the balance between
237 selection and the stochastic effect of drift (Ohta 1972, 1992). During bottlenecks, one can
238 expect strongly deleterious mutations to continue being eliminated, while slightly
239 deleterious mutations will have higher chances of being fixed in the population by stochastic
240 drift than being eliminated by selection (Woolfit and Bromham 2003). In the chloroplast
241 genes studied here, one would expect this process to result in more non-synonymous
242 substitutions in the endosymbiotic branches, in line with the reduced selection efficiency we
243 observe.

244 Relative selection analysis using a different model comparing secondary plastids to primary
245 plastids (denoted $S \times P(E)$) suggests that relaxation of selection during endosymbiosis is
246 temporary, indicated by distribution of k -values that encompasses 1 (median 0.87) and
247 similar numbers of genes that were relaxed (13), intensified (9) or inconclusive (9) in
248 secondary branches. The analysis on concatenated sequences showed similar results
249 (median $k = 0.96$) and was not preferred over the null model, providing a clear indication
250 that following the relaxation during endosymbiosis, the purifying selection regime on plastid
251 genes returns to values similar to those before endosymbiosis.

252 Comparative studies of the genomes of endosymbionts at different stages of integration
253 have shown that genome stability increases with the age of the endosymbiont and
254 suggested this may be due to selection (Allen, et al. 2009; Martínez-Cano, et al. 2015). Our
255 findings agree with these observations, and our model system has the added advantage of
256 the endosymbiont becoming a stable organelle, fully integrated and co-diversifying with the
257 host following endosymbiosis, which was not the case in the previously studied
258 endosymbiont models. This allowed us to disentangle the molecular dynamics along the
259 endosymbiosis branch from that of a stable integrated secondary plastid, showing that the
260 purifying selection regime rebounds to near pre-endosymbiosis levels once the organelle is
261 established.

262 Our results suggest a general model for the molecular dynamics of secondary plastid
263 endosymbiosis (Figure 4). It is likely that a very small fraction of the actual population of the
264 engulfed primary alga is involved in secondary endosymbiosis, creating a drastic population
265 size bottleneck. This decrease in effective population size would then allow higher levels of
266 drift to fix slightly deleterious mutations, explaining the long branches in the phylogeny of
267 green plastids where secondary endosymbiosis events take place (Jackson et al. 2018).

268 Maintenance of the plastid genome during secondary endosymbiosis depends largely on
269 nuclear-encoded DNA replication and repair proteins (Smith and Keeling 2015). During
270 secondary endosymbiosis, nuclear-encoded proteins are often transferred from the algal
271 nucleus to the new host nucleus, with the product directed to the new plastid. This might
272 contribute to a period of reduced fidelity of plastid DNA replication during secondary
273 endosymbiosis, which might in some cases lead to failure of the secondary plastid
274 endosymbiosis. As the endosymbiont-host relationship ages, the drift acting on plastid

275 genomes could eventually decrease, with higher effective population size and level of
276 integration of plastid and host nucleus. This is reflected in the increased levels of selection
277 on secondary plastids following endosymbiosis, emphasising the important interplay
278 between drift and selection during secondary endosymbiosis and their resulting impact on
279 secondary plastid genomes.

280 *Three independent events*

281 Our analyses comparing selection regimes of the three endosymbiosis events to the
282 background individually showed distinctive scenarios. *Lepidodinium* showed the strongest
283 relaxation ($k = 0.3$) followed by chlorarachniophytes ($k = 0.45$), indicating evidence of
284 strongly relaxed selection during these two endosymbiotic events. However, euglenophytes
285 showed a k -value of 0.86, indicating a much lower level of relaxation during this
286 endosymbiosis event.

287 Tightly constrained genome reduction along with evident purifying selection across all three
288 green algal secondary endosymbioses emphasises the evolutionary parallels among these
289 independent events, but also clearly distinguishes the origin of secondary green plastids
290 from other recently established obligate endosymbionts. Differences in degrees of
291 relaxation and gene losses during these three secondary endosymbiosis events highlight the
292 different evolutionary pressures associated with them. Nutritional requirements and level of
293 mixotrophy can account for different evolutionary pressures during plastid endosymbiosis.

294 Because our analyses support increased drift, the differing relaxation intensity between the
295 events implies that there may be differences in the extent of population bottlenecks
296 underlying these events. Among the three events, euglenophytes are noticeable as they had
297 the least relaxation of selection. The endosymbiosis branch leading to the euglenophytes in
298 the phylogeny is shorter than the other two endosymbiosis branches, suggesting that plastid
299 genes have evolved through this secondary endosymbiosis with fewer substitutions (Jackson
300 et al. 2018, Figure 1). This might be due to a less intense population bottleneck with more
301 efficient integration of the plastid during euglenophyte endosymbiosis compared with the
302 other two green secondary endosymbioses. Absence of homologs of plastid origin for
303 protein import components and plastid division in euglenophytes has led to speculation that
304 integration of their plastids involved a novel/simplified process including proteins of host

305 origin (Zahonova, et al. 2018; Novák Vanclová, et al. 2020), which could have facilitated
306 more efficient integration of their plastid genomes, allowing faster recovery from
307 bottleneck. This may have enabled euglenophyte plastids to integrate with less relaxation of
308 selection.

309

310 **Material and methods**

311 *Dataset*

312 We compiled a dataset of 122 green plastid genomes spanning the primary plastids of green
313 algae (Chlorophyta, 104 genomes) and the secondary plastids of Euglenophyta (12
314 genomes), Chlorarachniophyta (5 genomes) and the dinoflagellate genus *Lepidodinium* (1
315 genome). A reference phylogeny (Figure 1) was obtained from a previous study (Jackson, et
316 al. 2018). Our dataset includes close extant relatives of ancestral green algae that were
317 involved in the secondary endosymbiosis events, making this green plastid dataset suited to
318 examining the molecular evolutionary dynamics associated with secondary endosymbiosis,
319 and investigating differences and similarities among the three independent cases of
320 secondary green plastid origination. Basic features of the plastid genomes such as number
321 of coding sequences (CDS) and genome size were recorded and GC content of CDS and
322 codon usage bias were calculated using the CodonO (Wan, et al. 2007) function from the
323 cubfits v.0.1-3 (Chen 2014) package in R v.3.5.1 (R core Team, 2018).

324 *Analysis of gene loss*

325 To investigate evolutionary patterns of gene loss, protein-coding genes were grouped into
326 orthogroups using OrthoFinder version 1.4.0 with standard parameters (Emms and Kelly
327 2015). Of the 203 orthogroups (OGs) that were present across multiple species, 116 OGs
328 corresponded to named genes with known function conserved across most plastids, while
329 the remaining OGs (mostly hypothetical genes of unknown function) were not examined
330 further. A presence/absence matrix of the 116 orthogroups corresponding to named genes
331 was constructed. Using this matrix and the reference phylogeny from Jackson et al. (2018),
332 gene gain and loss along the phylogeny was estimated using PHYLIP version 3.695
333 (Felsenstein 2005), with the Dollo parsimony method and printing the states at all nodes of

334 the tree. Gene loss and gain along each branch was extracted from the PHYLIP output using
335 OrthoMCL Tools (DOI 10.5281/zenodo.51349). The rate of gene loss and gain per million
336 years was calculated for each branch using the evolutionary time from the chronogram
337 presented by Jackson et al (2018). The estimated numbers of genes lost (and rates of gene
338 loss) were ranked from largest to smallest to see if endosymbiotic branches had greater
339 values compared to the background, and evaluated formally using ANOVA and Tukey HSD
340 tests in the stats v3.6.2 package of R core Team (2013). To investigate if the genes lost
341 during the secondary endosymbiosis may have been transferred to host nuclear genomes,
342 we performed local tBLASTn searches using orthologous genes as query against the
343 published nuclear genomes of *Bigelowiella natans* (Curtis, 2012) and *Euglena gracilis*
344 (https://www.ncbi.nlm.nih.gov/assembly/GCA_900893395.1) (e-value cut-off = 1e-05).

345 *Selection intensity analysis*

346 To study the variation in selection intensity in the protein-coding genes of secondary and
347 primary green plastids, we used the hypothesis-testing framework RELAX (Wertheim, et al.
348 2014) from the HyPhy software package version 2.3.14 (Kosakovsky Pond, et al. 2005;
349 Delpont, et al. 2010). This framework requires a predefined tree with subsets of test and
350 reference branches specified. The subset of branches that are not set as test or reference
351 remain unclassified. RELAX applies a branch-site model to estimate the strength of natural
352 selection based on the ratio of non-synonymous to synonymous substitutions (ω)
353 for three different ω categories ($\omega_1 < \omega_2 \leq 1 < \omega_3$) in the test and reference subsets. $\omega < 1$
354 represents sites under purifying selection, $\omega > 1$ represents sites under positive selection
355 and $\omega = 1$ represents sites under neutral evolution. The relative selection intensity
356 parameter (k) reflects intensification or relaxation of selection based on the relative
357 proximity of ω values to 1 (neutral evolution). If ω values of test branches are closer to 1
358 than reference branches, then selection is relaxed ($k < 1$) and in the opposite scenario,
359 selection has intensified ($k > 1$). The null model assumes identical ω values ($k = 1$) between
360 test and reference branches. The alternative model fits different sets of ω values for test
361 and reference, and thus k differs from 1, allowing a formal test of relaxed ($k < 1$) or
362 intensified ($k > 1$) selection. The likelihood ratio test (LR) performed with p-value < 0.05 by
363 comparing the null and alternate model quantifies statistical confidence for the obtained k
364 value.

365 *Models for Selection Analysis*

366 We used the HyPhy-RELAX method to study molecular evolution through the process of
367 endosymbiosis by designing different evolutionary models that allowed us to study aspects
368 of selection intensity before, during and after the endosymbiosis process. For the selection
369 analyses we included only genes that were present in all of the lineages with secondary
370 plastids (34 orthologous genes). The phylogenetic tree of algal green plastid genomes from
371 Jackson et al. (2018) was used as the predefined tree on which test and reference branches
372 were marked. In the phylogeny (Figure 1), branches leading to and connecting the species
373 containing primary plastids (i.e. the green algae) were indicated as primary branches (P),
374 and denote the state before secondary endosymbiosis. Secondary branches (S) are the
375 branches leading to and connecting the species containing secondary plastids, and denote
376 the state after secondary endosymbiosis. The endosymbiotic branches (E) indicate branches
377 connecting the backbone of green algal lineages to the lineages with secondary green
378 plastids, in other words the branches along which secondary endosymbiosis took place
379 (orange-coloured branches in Fig. 1). The *Lepidodinium* lineage includes only one plastid
380 genome so we consider this branch as the endosymbiotic branch for this case.

381 Our first model, denoted "E × P+S", has endosymbiotic (E) branches as the test set and all
382 non-endosymbiotic branches (P+S) as the reference set. This model allows us to compare
383 the selection intensity during endosymbiosis relative to before and after endosymbiosis. Our
384 second model, denoted "S × P(E)", allowed us to evaluate differences in selection intensity
385 between secondary (S) and primary (P) plastids, excluding the endosymbiont branches (E).

386 To study differences between individual endosymbiosis events, we fitted E × P+S models,
387 but specifying only a single endosymbiotic branch as test (excluding all other endosymbiotic
388 branches) and all non-endosymbiotic branches (P+S) as the reference set.

389 *Purifying selection analysis*

390 Because functional plastid genes are expected to experience purifying selection, we also
391 carried out an analysis to identify and quantify levels of purifying selection. The BUSTEC
392 method implemented in HyPhy tests for alignment-wide evidence of conservation by fitting
393 a random effects branch-site model to the entire phylogeny or a subset of tree branches
394 (Murrell, et al. 2015). The null model constrains ω values to greater than or equal to 1,

395 excluding the possibility of purifying selection. The unconstrained model allowing ω values
396 greater than and less than 1 serves as the alternate model. With endosymbiotic branches as
397 the test branches, we used BUSTEC to fit the alternative unconstrained and null constrained
398 models to these branches to quantify evidence for purifying selection during endosymbiosis.

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407 **Conflicts of Interest:**

408 The authors declare no conflicts of interests.

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537

538 **Figure Legends**

539 **Figure 1:** Evolution of green-type plastid across endosymbiosis events. The phylogeny is a
540 chronogram indicating lineages as having primary plastids (i.e green algae in wheat brown),
541 branches with secondary plastids(i.e Chlorarachniophytes, Lepidodinium, Euglenophytes in
542 pink) and branches along which endosymbiosis happens (orange). Inferred gene losses are
543 indicated along the branches.

544 **Figure 2:** Inferred rates of gene loss in branches with primary plastids (P), secondary plastids
545 (S) and branches along which endosymbiosis (E) takes place.

546 **Figure 3:** Distribution of the relative selection intensity parameter(k) values of the Hyphy
547 RELAX model for (i) Endosymbiosis (test) Vs Primary and Secondary branches (reference) [E
548 \times P+S model], (ii) Secondary(test) Vs Primary (reference), excluding endosymbiosis branches
549 [$S \times P(E)$ model]. Selection intensity is relaxed when $k < 1$ or intensified when $k > 1$. These plots
550 show that endosymbiosis branches have relaxed selection compared to the primary and
551 secondary branches and that selection on secondary branches is similar to that of primary
552 branches, indicating that the relation of selection during endosymbiosis is temporary.

553 **Figure 4:** A general model for molecular dynamics during secondary green-type plastid
554 endosymbiosis. The model illustrates the population bottleneck due to involvement of very
555 small fraction of the actual population of the engulfed primary alga in secondary

556 endosymbiosis. As the endosymbiont-host relation ages the effective population size
557 increases that counteracts the impact of stochastic drift leading establishment of secondary
558 plastids after endosymbiosis.

559 **Table 1:** The number of green algal plastid genes showing numbers of genes showing
560 relaxation and intensification for each model.

Model	Relaxation (k<1)	Significant Relaxation	Intensification (k>1)	Significant Intensification	Neither k=1)
E × P+S	32	26	2	-	-
S × P(E)	19	13	12	9	3

561 **Supplementary Materials** - <https://doi.org/10.26188/14616999>

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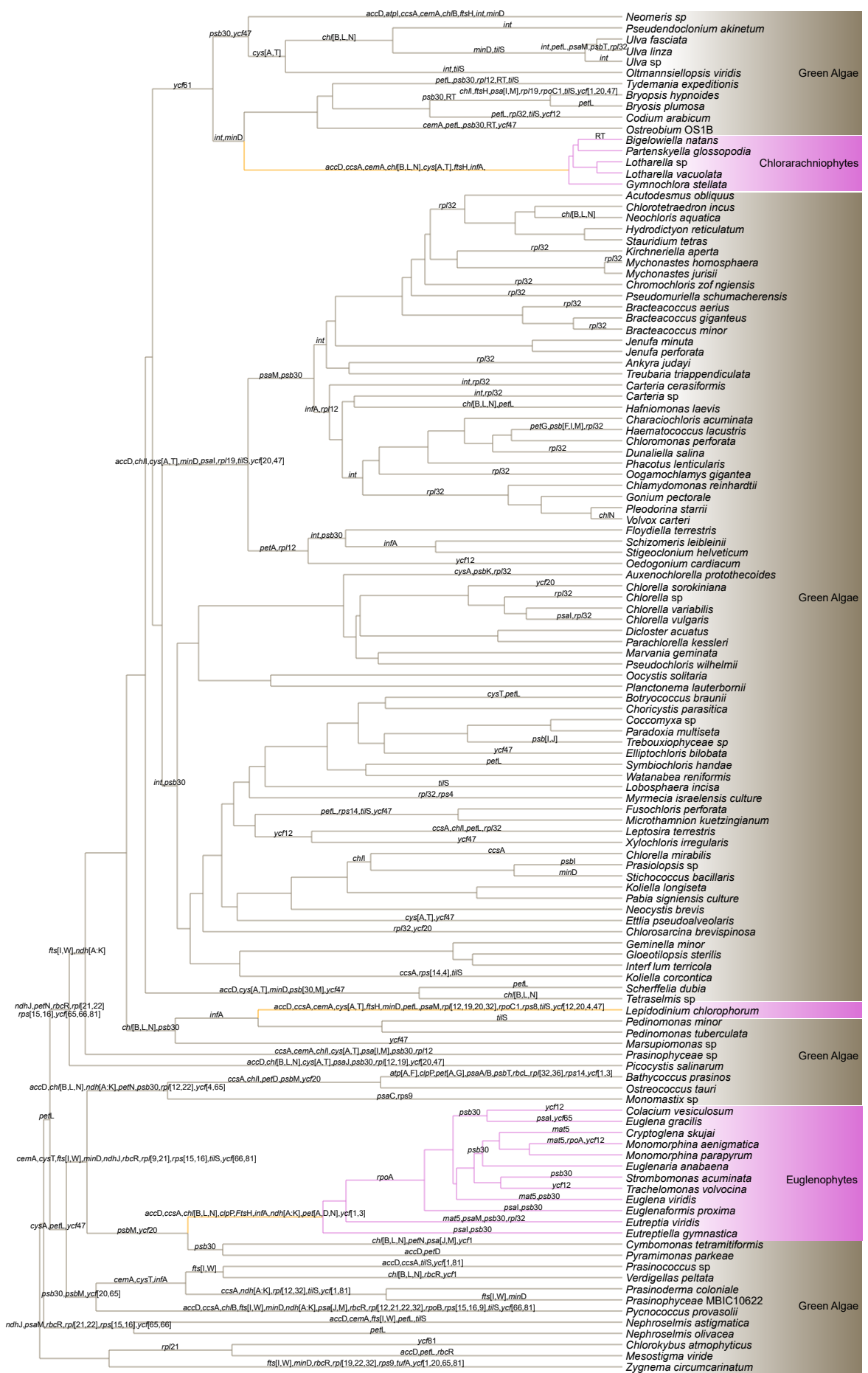


Figure 1. Evolution of green-type plastids across endosymbiosis events. The phylogeny is a chronogram indicating lineages as having primary plastids (i.e. green algae, in wheat brown), branches with secondary plastids (i.e. Chlorarachniophytes, *Lepidodinium*, Euglenophytes, in pink) and branches along which endosymbiosis happens (orange). Inferred gene losses are indicated along the branches.

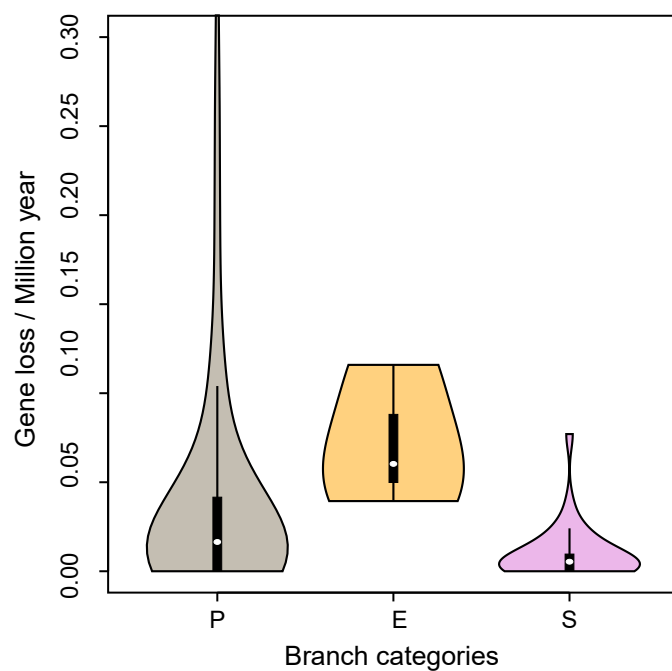


Figure 2. Inferred rates of gene loss in branches with primaryplastids(P),secondaryplastids(S) and branches along which endosymbiosis (E) takes place.

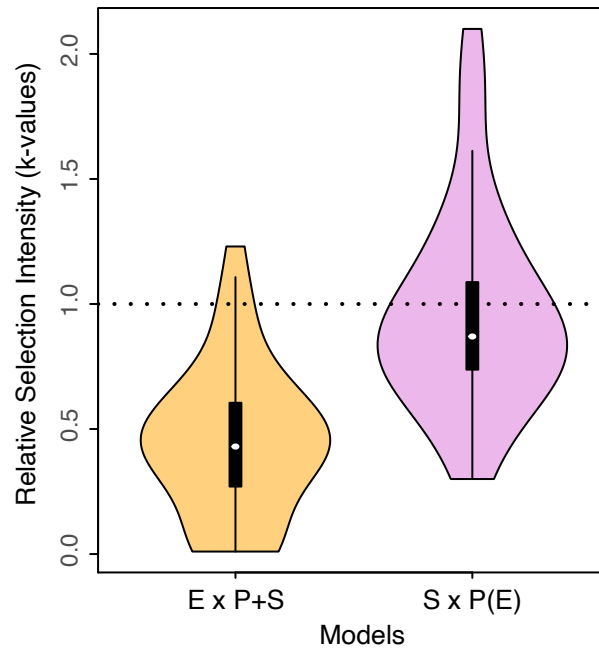


Figure 3. Distribution of the relative selection intensity parameter (k) of the HyPhy RELAX model for (i) Endosymbiosis (test) vs. Primary and Secondary branches (reference) [$E \times P+S$ model],(ii) Secondary (test) vs. Primary branches (reference), excluding endosymbiosis branches [$S \times P(E)$ model]. Selection intensity is relaxed in the test branches when $k < 1$ or intensified when $k > 1$. These plots show that endosymbiosis branches have relaxed selection compared to primary and secondary branches, and that selection on secondary branches is similar to that of primary branches, indicating that the relaxation of selection during endosymbiosis is temporary.

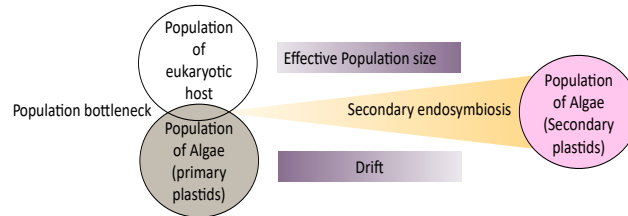


Figure 4: A general model for molecular dynamics during secondary green-type plastid endosymbiosis. The model illustrates the population bottleneck due to involvement of very small fraction of the actual population of the engulfed primary alga in secondary endosymbiosis. As the endosymbiont-host relation ages the effective population size increases that counteracts the impact of stochastic drift leading establishment of secondary plastids after endosymbiosis.