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1 **Chimeric origins of ochrophytes and haptophytes revealed through an ancient plastid**
2 **proteome**

3
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19
20 **Abstract**

21
22
23 **Plastids are supported by a wide range of proteins encoded within the nucleus and**
24 **imported from the cytoplasm. These plastid-targeted proteins may originate from the**
25 **endosymbiont, the host, or other sources entirely. Here, we identify and characterise 770**
26 **plastid-targeted proteins that are conserved across the ochrophytes, a major group of**
27 **algae including diatoms, pelagophytes and kelps, that possess plastids derived from red**
28 **algae. We show that the ancestral ochrophyte plastid proteome was an evolutionary**
29 **chimera, with 25% of its phylogenetically tractable proteins deriving from green algae. We**
30 **additionally show that functional mixing of host and plastid proteomes, such as through**
31 **dual targeting, is an ancestral feature of plastid evolution. Finally, we detect a clear**
32 **phylogenetic signal from one ochrophyte subgroup, the lineage containing pelagophytes**
33 **and dictyochophytes, in plastid-targeted proteins from another major algal lineage, the**
34 **haptophytes. This may represent a possible serial endosymbiosis event deep in eukaryotic**
35 **evolutionary history.**

36
37 **Introduction**

38
39 Since their origin, the eukaryotes have diversified into an extraordinary array of organisms,
40 with different genome contents, physiological properties, and ecological adaptations¹⁻³.
41 Perhaps the most profound change that has occurred within individual eukaryotic cells is the
42 acquisition of plastids via endosymbiosis, which has happened at least eleven times across
43 the tree of life¹. All but one characterized group of photosynthetic eukaryotes possess
44 plastids resulting from a single ancient endosymbiosis of a beta-cyanobacterium by an
45 ancestor of the archaeplastid lineage (consisting of green algae and plants, red algae, and
46 glaucophytes)¹.

47
48 Photosynthesis has subsequently spread outside of the archaeplastids through secondary,
49 tertiary, or more complex endosymbiosis events. By far the most ecologically successful of
50 these lineages are those that possess plastids derived from secondary or more complex
51 endosymbioses of a red alga^{1,4,5}. These are the "CASH lineages", consisting of

52 photosynthetic members of the cryptomonads, alveolates (such as dinoflagellates),
53 stramenopiles (also referred to as heterokonts) and haptophytes^{1,4} (see Table 1 and Fig. 1-
54 figure supplement 1 for definitions). The most prominent of these are the photosynthetic
55 members of the stramenopiles, termed the ochrophytes^{2,6,7}. The ochrophytes include the
56 diatoms, which are major primary producers in the ocean^{8,9}, multicellular kelps, which serve
57 as spawning grounds for marine animals¹⁰, and the pelagophytes, small free-living algae
58 frequently associated with harmful blooms¹¹ (Fig. 1, panel A; Fig. 1- figure supplement 1).
59 The stramenopiles also contain many aplastidic and non-photosynthetic lineages (e.g.,
60 oomycetes), which diverge at the base of the ochrophytes and play important roles as
61 pathogens and in microbial food webs^{6,12} (Fig. 1- figure supplement 1).
62

63 Following their acquisition, plastids have undergone a number of evolutionary changes that
64 bound them more intricately with the biology of the host. These include the transfer of
65 plastid-derived genes to the host nucleus^{3,13,14} and the targeting of proteins encoded within
66 the nucleus to the plastid^{15,16}. Previous studies have shown that many plastid-targeted
67 proteins are not derived from the endosymbiont genome¹⁷. Proteins encoded by genes
68 acquired from other sources, such as laterally acquired genes^{18,19} or previous endosymbiotic
69 organelles historically possessed by the host^{20,21}, or proteins that have been repurposed
70 from endogenous host organelles^{22,23} have important roles in supporting the biology of
71 plastid lineages. Other gene transfer events, e.g. from food sources²⁴, bacterial symbionts²⁵,
72 viruses²⁶, or diazotrophic non-plastid cyanobacterial endosymbionts^{27,28} have also played
73 major roles in the evolution of photosynthetic eukaryotes, and it remains to be determined
74 which of these have contributed to the diverse range of plastid proteins observed today. It
75 nonetheless remains largely unknown which proteins had the most fundamental roles in
76 establishing current plastid lineages³, i.e., which plastid proteins represent the ancestral
77 components of plastid-targeted proteomes.
78

79 Ochrophytes represent an excellent system in which to reconstruct the origins of plastid
80 proteomes. Firstly, plastid-targeting sequences in different ochrophytes are relatively well
81 conserved, enabling *in silico* prediction of plastid-targeted proteins from a wide range of
82 different species^{29,30}, in contrast to plastid-targeting sequences within archaeplastid
83 lineages, which are extremely variable^{31,32}. Secondly, compared to other CASH lineages
84 (haptophytes, cryptomonads, and dinoflagellates), ochrophytes represent an extremely well
85 characterised system for experimental and bioinformatic investigation, with (to date) eleven
86 complete genomes, and transcriptome libraries available for over 150 species through
87 MMETSP^{33,34}. Reliable transformation and other manipulation strategies are also available
88 for multiple species, such as the model diatom *Phaeodactylum tricorutum*³⁵⁻³⁷.
89

90 Thirdly, the origin of the ochrophyte plastid is an evolutionarily valuable topic to
91 understand. It is currently not known when the ochrophyte plastid was acquired: whether it
92 originated recently, predates the radiation of aplastidic stramenopile relatives^{5,6,12}, or was
93 acquired prior to the divergence of stramenopiles from their closest relatives, the
94 alveolates³⁸. Verifying a late origin for the ochrophyte plastid would thus enable insights into
95 the cellular changes that accompany the transition from a solely heterotrophic to a
96 phototrophic lifestyle^{6,12}, which is currently not possible for archaeplastids^{39,40}, and difficult
97 for haptophytes and cryptomonads, in which these relatives respectively remain unknown
98 or understudied at a genomic level^{39,41}. It has additionally been proposed, based on the
99 presence of large numbers of genes of putative green algal origin in diatom genomes^{42,43},
100 that the ancestor of ochrophytes once possessed a green algal endosymbiont, which was
101 subsequently replaced via the serial endosymbiosis of a red algal-derived plastid^{1,44}. This
102 hypothesis remains controversial⁴⁵⁻⁴⁷, in particular due to issues associated with the

103 distinction of genes of red and green algal origins in ochrophyte genomes⁴⁸⁻⁵⁰. A final
104 evolutionary suggestion regarding ochrophytes is that they have acted as endosymbiotic
105 donors into other CASH lineages. One recent study proposed that haptophytes possess
106 plastids acquired via the endosymbiosis of an ochrophyte⁵, although the exact identity of
107 this endosymbiotic acquisition remain unresolved. Characterising the ancestral ochrophyte
108 plastid proteome might therefore help answer major questions about the ways in which
109 plastids become established in the host cell, and provide valuable insights into the origins
110 and diversification of other ecologically important algal lineages.

111
112 In this study, we present an experimentally verified *in silico* reconstruction of the proteins
113 targeted to the plastid of the last common ochrophyte ancestor. We show that this ancestral
114 plastid proteome was an evolutionary mosaic, containing 770 proteins from a range of
115 different sources. Our dataset indicates that the ochrophyte plastid was acquired late in
116 stramenopile evolution, following the divergence of extant aplastidic relatives, that plastid-
117 targeted proteins of green algal origin played a significant role in its origin, and that there
118 has been bidirectional integration of the biology of the ochrophyte host and plastid
119 proteomes, such as the ancient recruitment of proteins from both host and endosymbiont
120 to dually support the biology of the plastid and mitochondria. Finally, we show evidence for
121 an ancient endosymbiosis of a specific ochrophyte lineage, an ancestor of the pelagophytes
122 and dictyochophytes, by a common ancestor of the haptophytes, which we propose- based
123 on discrepancies between the origins of the haptophyte plastid proteome and genome-
124 reveals a possible serial endosymbiosis event early in haptophyte evolution, preceding the
125 origins of the current haptophyte plastid. Our work resolves several long-standing questions
126 of ochrophyte evolution, and provides new insights into the origins and diversification of
127 CASH lineages as a whole.

128

129 **Results**

130

131 **1. *In silico* reconstruction of an ancestral plastid proteome**

132

133 We developed an *in silico* pipeline for identifying putatively ancestral plastid-targeted
134 proteins across the ochrophytes (Fig. 1). We screened a large composite library, comprising
135 eleven different ochrophyte genomes, together with transcriptome data from a further 158
136 ochrophyte species (Table S1- sheet 1¹⁴⁵) using the ochrophyte plastid targeting predictors
137 ASAFind (Table S2- sheet 1¹⁴⁵)²⁹ and HECTAR (Table S3- sheet 1¹⁴⁵)³⁰. Sequences with
138 predicted plastid localisation were binned into eleven taxonomic sub-categories within three
139 major groups (chrysisita, hypogyrista, and diatoms) based on recent multigene phylogenies¹²
140 (Fig. 1, panel A; Fig. 1- figure supplement 1), then assembled by sequence similarity into
141 homologous plastid-targeted protein groups (HPPGs, Materials and Methods).

142

143 We next tested the level of conservation best able to identify truly ancestral HPPGs. We
144 selected three patterns of conservation that identified the largest number of HPPGs from a
145 positive control dataset of proteins with previously identified plastid-associated functions,
146 and minimised the number identified from a negative control dataset of HPPGs generated
147 using seed sequences from three other published CASH lineage genomes, for which no
148 plastid-targeted orthologues were detected in any ochrophyte genome sequence (Materials
149 and Methods; Table S2- sheet 2, sections 1-2; Table S3- sheet 2, sections 1-2¹⁴⁵). The
150 selected conservation patterns were: the presence of the protein in a majority of chrysisitan
151 sub-categories and a majority of either diatom or hypogyristean sub-categories; or presence
152 in at least one chrysisitan sub-category and a majority of both diatoms and hypogyristea (Fig.
153 1, panel B). We extracted HPPGs matching the conservation patterns defined above and

154 verified their monophyly within ochrophytes via alignment and single-gene trees (Fig. 1,
155 panel C; Table S4- sheet 1¹⁴⁵). From this, we identified 770 proteins that were probably
156 targeted to the ancestral ochrophyte plastid (Fig. 1, panel D; Table S4- sheet 2¹⁴⁵). This
157 dataset is significantly enriched in proteins from within the positive control dataset and
158 contains significantly fewer proteins from the negative control dataset than would be
159 expected through random assortment (chi-squared test, $P < 1 \times 10^{-10}$; Fig. 1), confirming its
160 specificity towards probable ancestral plastid-targeted proteins.

161

162 **2. Experimental verification of ancestral ochrophyte HPPGs**

163

164 We wished to verify that the ancestral ochrophyte plastid-targeted proteins inferred from
165 the *in silico* pipeline are genuinely plastid-targeted. 106 of our inferred ancestral HPPGs
166 include a *P. tricornutum* protein with prior experimental plastid localization, or unambiguous
167 plastid function (Fig. 1, panel D), but the remainder do not. We selected ten proteins for
168 experimental localisation (Fig. 2, panel A; Table S5¹⁴⁵). These were chosen on the basis of
169 having only non-plastid annotations on the first 50 BLAST hits against the NCBI nr database
170 excluding ochrophytes, thus arguing against their predicted plastid localization beyond these
171 organisms. In each case, all of the ochrophyte protein sequences within the alignment had a
172 well conserved central domain, and a highly variable N-terminal domain of between 30 and
173 50 amino acids containing an ASAFAP motif, consistent with a conserved plastid targeting
174 sequence²⁹ (Fig. 2- figure supplement 1).

175 The selected proteins included five aminoacyl-tRNA synthetases that yielded BLAST top hits
176 only against enzymes with cytoplasmic annotations, or of probable prokaryotic origin (Fig. 2-
177 figure supplement 2). Also included were a GroES-type chaperonin of inferred mitochondrial
178 origin, an Hsp90-type chaperonin of inferred endoplasmic reticulum origin and a
179 pyrophosphate-dependent phosphofructokinase, which is related to cytosolic enzymes from
180 other lineages (Fig. 2- figure supplement 3), and is distinct from the ATP-dependent
181 phosphofructokinases used by primary plastid lineages⁵¹. The Mpv17 membrane protein is
182 most closely related to enzymes with peroxisomal functions and localisation^{52,53}, but lacks
183 any identifiable peroxisomal targeting sequence (PSL, KRR, or a PTS1 motif)⁵⁴ in its C-
184 terminus. Novel protein 1 lacks any conserved domains, and yielded no BLAST matches
185 outside of the ochrophytes below an expect value of 1×10^{-05} (except for one dinoflagellate
186 sequence), and hence might constitute an entirely novel plastid-targeted protein (Fig. 2-
187 figure supplement 4; Table S5¹⁴⁵).

188 We generated C-terminal GFP-fusion constructs for each of these proteins using *P.*
189 *tricornutum* genes and transformed wild-type *P. tricornutum* (Fig. 2, panel B; Fig. 2- figure
190 supplement 5; Table S5¹⁴⁵). In each case, we identified GFP fluorescence associated with the
191 plastid. In one case (the peroxisomal membrane protein; Fig. 2, panel B), the GFP
192 accumulated in a ring around the plastid equator, consistent with a periplastid compartment
193 (PPC) localisation^{88,55}. In other cases (such as the five aminoacyl-tRNA synthetases, Fig. 2-
194 figure supplement 5), the GFP signal localised both within and external to the plastid,
195 consistent with a multipartite localisation within the cell. However, in all cases the proteins
196 tested were at least partially targeted to the plastid.

197 We additionally generated heterologous GFP fusion constructs for five of the proteins using
198 sequences from the "dinotom" *Glennodinium foliaceum*, a dinoflagellate alga that harbours
199 permanent endosymbionts of diatom origin^{20,56}, and the eustigmatophyte *Nannochloropsis*
200 *gaditana*, which as a member of the "PESC clade" is distantly related to *P. tricornutum* on
201 the ochrophyte tree¹². We expressed these constructs in *P. tricornutum* (Fig. 2, panel B; Fig.
202 2- figure supplement 6), and, in each case, detected plastid-localized GFP fluorescence

203 similar to the patterns observed with the *P. tricornutum* gene constructs. Overall, our data
204 therefore supports that the ancestral HPPG dataset consists of genuinely conserved plastid-
205 targeted proteins, rather than misidentified proteins of non-plastid function.

206

207 **3. Evolutionary origins of the ochrophyte plastid**

208

209 *The ochrophyte plastid is an evolutionary mosaic*

210

211 We wished to identify the evolutionary affinity of each ancestral HPPG in our dataset. In
212 particular, we assessed whether proteins that are of unconventional origin, such as the
213 products of genes endogenous to the host, or genes that have been acquired from other
214 sources such as prokaryotes and green algae, have significantly contributed to the origins of
215 the ochrophyte plastid^{1, 44}.

216

217 We accordingly determined the closest relative of each ancestral HPPG (Materials and
218 Methods). Due to ongoing controversies regarding the evolutionary composition of
219 ochrophyte genomes^{46, 47}, we utilised a combined phylogenetic and BLAST top hit approach
220 to robustly infer the most probable origin of each HPPG (Materials and Methods; Table S4-
221 sheet 2¹⁴⁵). For both the BLAST and phylogenetic analyses, stringent criteria were applied to
222 avoid misidentification due to topological ambiguity, or contamination within individual
223 sequence datasets^{57, 58} (Materials and Methods). We took the union of these two analyses to
224 produce a dataset of 263 HPPGs for which both phylogenetic and BLAST top hit analyses
225 indicated the same clear evolutionary origin. These origins were grouped into six
226 evolutionary categories, red algae, green algae, aplastidic stramenopiles, other eukaryotes,
227 prokaryotes, and viruses (Fig. 3, panel A).

228

229 Of the 263 HPPGs that were resolved from the combined analysis, 149 (57%) were of red
230 algal, i.e. endosymbiont origin (Fig. 3, panel A; Table S4- sheet 3¹⁴⁵). This is analogous to
231 results from studies of archaeplastid plastid proteomes, in which approximately half of the
232 plastid-targeted proteins are of endosymbiont origin^{18, 32}. The remaining 114 HPPGs resolved
233 with other sister-groups, consistent with a mosaic origin of the ochrophyte plastid
234 proteome. The most significant of these lineages was green algae (67 HPPGs, 25%), followed
235 by aplastidic stramenopiles (26 HPPGs, 10%), and prokaryotes (21 HPPGs, 8%) (Fig. 3, panel
236 A). None of the HPPGs were clearly assigned to other eukaryotes or to viruses, consistent
237 with previous assertions that these lineages have contributed very little to ochrophyte
238 evolution⁵⁹ (Fig. 3, panel A).

239

240 *Late origin of ochrophyte plastids*

241

242 We wished to determine whether the ochrophyte plastid was acquired by a common
243 ancestor of all stramenopiles or later in ochrophyte evolution. We reasoned that if the
244 ochrophyte plastid was acquired early, i.e., before the divergence of aplastidic relatives,
245 endosymbiotic gene transfer from the red algal symbiont to the host nucleus would have
246 commenced prior to the radiation of the stramenopiles⁶⁰. Based on the primary evolutionary
247 affinities of each ancestral HPPG (Fig. 3, panel A), we would expect at least half of the
248 aplastidic stramenopile-derived proteins to show a deeper red algal origin. We accordingly
249 profiled the deeper evolutionary affinity of each ancestral HPPG of aplastidic stramenopile
250 origin by a combined phylogenetic and BLAST top hit analysis, as before.

251

252 First, we noted that the majority (20/26) of the ochrophyte HPPGs with aplastidic
253 stramenopile origins specifically resolved as a sister-group to oomycetes, as opposed to the

254 deeper-branching labyrinthulomycetes or slopalinids (Fig. 3, panel B; Table S4- sheet 3¹⁴⁵).
255 Because oomycetes are the sister-group of ochrophytes^{6,12}, this suggests that our dataset
256 retains useful phylogenetic signal.

257

258 Next, from the 26 ancestral HPPGs of aplastidic stramenopile origin, we identified a clear
259 sister-group to the stramenopile clade for 16 HPPGs using BLAST, and for 18 HPPGs using
260 single-gene trees (Fig. 3, panel B). However, only one BLAST top hit and four trees showed a
261 deeper red algal affinity (Fig. 3, panel B). These proportions are significantly smaller than the
262 proportions of ochrophyte proteins of red origin in the entire ancestral HPPG dataset
263 (expected frequencies: 9.54 BLAST top hits, 10.7 sister-groups; chi-squared-test, $P \leq 0.01$; Fig.
264 3, panels A, B). In five cases we identified the same deeper affinity through combined BLAST
265 top hit and tree sister-group analysis, but none of these were of red algal origin (Fig. 3, panel
266 B). We conclude that plastid-targeted proteins in ochrophytes that are related to aplastidic
267 stramenopile proteins are predominantly not of red origin. This is consistent with a late
268 origin for the ochrophyte plastid, following the divergence of the ochrophytes and
269 oomycetes.

270

271 *A significant green algal contribution to ochrophyte plastid evolution*

272

273 Previous reports of green genes in ochrophyte genomes have been controversial due to a
274 paucity of red algal sequence data^{44, 47, 59}. We were able to avail in our pipeline of sequence
275 information from five complete red algal genomes^{48, 49, 61-63} and twelve red algal
276 transcriptomes^{34, 64}, allowing us to more clearly infer the reliability of the green signal in
277 ochrophytes. We tested whether the inferred green algal origin could be due to a protein
278 family's absence from red algal lineages (Fig. 4, panel A). For the majority of our green
279 HPPGs (40/67), an orthologue was identified in at least four of the five major red algal sub-
280 categories considered (cyanidiales, bangophytes and florideophytes, compsoogonophytes
281 and stylonematophytes, porphyridiophytes, and rhodellophytes; Fig. 4, panel B; Fig. 4- figure
282 supplement 1; Table S4- sheet 4¹⁴⁵). We therefore conclude that these green genes were not
283 misidentified as the result of undersampling within red sequence libraries, or secondary
284 gene loss events in the red algae^{45, 50}.

285

286 We then considered whether the green genes in our dataset originate from a specific source
287 within the green algae. Phylogenetic analyses of the HPPGs of verified green origin exhibited
288 a strong bias toward chlorophyte origins. Ochrophytes branched as sister-groups to
289 individual or multiple chlorophyte lineages in 51 of the 67 trees (Fig. 4, panel C; Fig. 4- figure
290 supplement 2). Similarly, we noted a strong predominance of chlorophyte lineages amongst
291 BLAST top hits (56/67) despite the fact that these lineages only correspond to approximately
292 25% of the green sequences present in our libraries (Fig. 4- figure supplement 3; Table S4-
293 sheet 3¹⁴⁵). In contrast, only 16 of the single-gene trees recovered a sister-group relationship
294 between ochrophytes and all green lineages (chlorophytes and streptophytes), none
295 recovered a specific sister-group relationship between ochrophytes and streptophytes (Fig.
296 4, panel C), and only 11 of the BLAST top hits were to streptophyte sequences (Fig. 4- figure
297 supplement 2; Table S4- sheet 3¹⁴⁵). This bias is inconsistent with the green ancestral HPPGs
298 being of misidentified red origin, or originating at a deeper position within the green algae,
299 in which case they should show a more stochastic distribution of evolutionary affinities
300 across all green lineages⁴⁶.

301

302 Next, we tested whether our data supported a single origin for the green genes within the
303 chlorophytes, or whether the HPPGs of green origin arose through gene transfer events
304 from multiple chlorophyte lineages. We identified all amino acids that were uniquely shared

305 between ochrophytes and chlorophytes in the 31 green HPPGs for which we found no
306 evidence of gene duplication or subsequent lateral gene transfer into green algae,
307 ochrophytes, or other major photosynthetic eukaryotes (Table S6- sheets 1, 2¹⁴⁵; Materials
308 and Methods). We then inferred the most probable origin in the green algal tree for each
309 uniquely shared residue as well as the earliest possible origin, taking into account gapped
310 and missing positions (Fig. 4, panel D; Fig. 4- figure supplement 4; Table S7- sheets 1, 3¹⁴⁵). In
311 both analyses the majority of the uniquely shared residues were inferred to have originated
312 in a common ancestor of all chlorophytes, or of all chlorophyte lineages excluding the basal
313 *Prasinoderma/ Nephroselmis* sub-category (189/289 positions in observed analysis; 100/147
314 positions in the earliest possible analysis; Fig. 4, panel D; Fig. 4- figure supplement 4; Table
315 S7- sheets 1, 3¹⁴⁵). All other nodes within the green tree, including all specific green sub-
316 categories, shared much smaller numbers of residues with ochrophytes (Fig. 4, panel D; Fig.
317 4- figure supplement 4; Table S7- sheets 1, 3¹⁴⁵). Thus, our data is congruent with the
318 majority of the ochrophyte green genes originating from deep within the chlorophyte
319 lineage.

320
321 Finally, we considered whether the green genes that function in ochrophyte plastids were
322 more likely to have been acquired through endosymbiosis, or through lateral gene transfers,
323 for example from a food organism^{65,66} or other intracellular symbiont³. We reasoned that if
324 the green genes in ochrophytes were predominantly of endosymbiotic origin, they should
325 encode more plastid-targeted proteins than genes of alternative origin, in the same manner
326 as genes of cyanobacterial origin retained in archaeplastid genomes are biased towards
327 encoding proteins with plastid functions²⁰. We accordingly constructed a secondary dataset,
328 consisting of 7140 non-redundant gene families that are broadly distributed across the
329 ochrophytes, and tested the targeting preferences of proteins from each HPPG (Fig. 4, panel
330 E; Fig. 4- figure supplement 5; Table S8- sheet 1¹⁴⁵). 871 gene families resolved with the
331 green algae per BLAST top hit analysis (Fig. 4- figure supplement 6; Table S8- sheet 2¹⁴⁵).
332 Using both ASAFind²⁹ and HECTAR³⁰, gene families of predicted green algal origin were
333 significantly more likely to encode proteins with plastid-targeting predictions than the
334 dataset as a whole (chi-squared, $P < 1E^{-03}$; Fig. 4, panel E; Fig. 4- figure supplement 5; Table
335 S8- sheet 3¹⁴⁵). We also observed a similar, though stronger, bias towards plastid-targeted
336 proteins among the proteins of red algal origin (chi-squared, $P < 1E^{-40}$; Fig. 4, panel E; Fig. 4-
337 figure supplement 5; Table S8- sheet 3¹⁴⁵). Collectively, our data support the presence of
338 genes of chlorophyte origin in the last common ochrophyte ancestor, the majority of which
339 have predicted plastid localisations, consistent with an acquisition through a plastid
340 endosymbiosis event.

341 342 **4. Functional consequences of mosaic origins for the ochrophyte plastid**

343 *Metabolic completeness of the ochrophyte plastid*

344
345
346 We identified effectively complete core plastid metabolism pathways within the ancestral
347 HPPG dataset (Fig. 5, panel A; Fig. 5- figure supplement 1; Table S9- sheet 1¹⁴⁵). The majority
348 of the remaining proteins remain plastid-encoded in some ochrophyte lineages, or are
349 dispensible for the metabolic pathway (Fig. 5- figure supplements 1, 2)⁶⁷⁻⁶⁹. In four cases
350 (isopropylmalate synthase, sedoheptulose bisphosphatase, 3-dehydroquinase synthase, and
351 shikimate kinase) lateral gene transfer and replacement events have occurred into individual
352 ochrophyte lineages since their radiation, preventing identification of a single HPPG within
353 the ancestral dataset (Fig. 5, panel A; Fig. 5- figure supplements 2-6). Taking these
354 exceptions into account, we conclude that the ancestral ochrophyte plastid proteome
355 contained the fundamental components of core plastid metabolism.

356
357 *Mosaic origins of ochrophyte plastid metabolism*

358
359 Given the mosaic evolutionary origins of ancestral ochrophyte plastid-targeted proteins, we
360 wondered whether certain evolutionary affinities might correlate with specific metabolic
361 functions. It has previously been speculated, for example, that genes acquired by diatoms
362 from green algae might have a specific role in tolerating variable light regimes^{42, 70, 71} or
363 eliminating toxic substances from diatom plastids⁷². We noted that many of the pathways in
364 the ochrophyte plastid utilise a mixture of genes of red, green, host and prokaryotic origin
365 (Fig. 5- figure supplement 1), which would suggest a converse scenario: that the mosaic
366 origins of the ochrophyte plastid have led to the functional mixing of enzymes with disparate
367 evolutionary origins.

368
369 Consistent with this latter idea, we found very little evidence that individual categories of
370 HPPG (i.e., red algal, green algal, prokaryotic or host origin) are associated with particular
371 KOG annotations, as inferred by chi-squared testing ($P < 0.05$) against a null hypothesis that
372 all KOG families and classes are homogenously distributed across the ancestral HPPG
373 dataset, independent of evolutionary origin (Fig. 5, panel B; Fig. 5 – figure supplement 7;
374 Table S9- sheet 2¹⁴⁵). The notable exceptions are prokaryotic HPPGs being elevated in
375 information storage and processing proteins, particularly those involved in translation, while
376 HPPGs of host origin were enriched in proteins involved in cellular processes and signalling
377 relative to the ancestral HPPG set as a whole (Fig. 5, panel B; Fig. 5 – figure supplement 7;
378 Table S9- sheet 2¹⁴⁵). In contrast, several KOG categories were more highly represented in
379 the ancestral HPPG set than in HPPGs as a whole (Fig. 5, panel B; Fig. 5 – figure supplement
380 7; Table S9- sheet 2¹⁴⁵).

381
382 A related question is whether proteins that catalyse adjacent steps of a biochemical
383 pathway tend to have shared or different evolutionary affinities. Multiple sets of non-native
384 proteins might be preferentially utilised by ochrophyte plastids, over homologous proteins
385 of endosymbiont origin, due to performing concerted steps in individual metabolic pathways
386 or cellular processes^{1, 42, 73}. In this instance, pairs of proteins that interact with one another
387 would be more likely to come from the same evolutionary origin than would be expected by
388 random association. Alternatively, early ochrophyte plastids might have had no preference
389 for utilising interacting proteins of the same evolutionary origin, in which case proteins
390 involved in specific metabolic pathways might frequently have different evolutionary origins
391 to adjacent enzymes in the same pathway. Of the 313 pairs of such biochemical neighbours
392 identified in the ancestral HPPGs, only 44 shared the same evolutionary origin, which is no
393 different than that which would be expected by chance (expected number 41.05; chi-
394 squared, $P=0.541$; Fig. 5, panel C; Table S9- sheet 3¹⁴⁵). Thus, interactions between proteins
395 of different evolutionary origin were forged early in the evolution of the ochrophyte plastid.

396
397 Finally, we sought correlations between expression dynamics and evolutionary affinity,
398 taking advantage of microarray data from *P. tricornutum* and *T. pseudonana*⁷⁴ (Table S10-
399 sheets 1-4¹⁴⁵). We found no evidence that ancestral HPPG genes of any evolutionary origin
400 had more similar expression dynamics to each other than to those of other evolutionary
401 origins (ANOVA, $P \leq 0.05$; Fig. 5, panel D; Fig. 5- figure supplements 8, 9; Table S10- sheet
402 5¹⁴⁵). For example, in both species, genes of green origin show a weaker average positive
403 coregulation with one another than they do to genes from the same species of red or of
404 prokaryotic origin (Fig. 5, panel D). Thus, the chimeric origins of the ochrophyte plastid has
405 enabled extraordinary functional mixing of proteins from early in its evolution, with each of

406 the different donors contributing proteins with a broad range of biochemical functions and
407 transcriptional patterns in response to changing physiological conditions.

408

409 *Ancient origins of chimeric plastid-targeted proteins*

410

411 We considered whether the mixing of proteins from different evolutionary sources might
412 have more substantially changed the biology of the ochrophyte plastid. It has been reported
413 by Méheust et al.⁷⁵ that proteins of chimeric evolutionary origin, generated by the fusion of
414 domains from different evolutionary sources, form a significant component of plastid
415 proteomes. Thus, the chimeric origins of the ochrophyte plastid might have enabled the
416 creation of syncretic proteins not found in the endosymbiont or host ancestors. We
417 identified orthologues of seven chimeric proteins identified in this study within our dataset,
418 underlining their importance for the establishment of the ochrophyte plastid (Fig. 6, panel
419 A)⁷⁵.

420

421 Next, we assessed whether the mosaic composition of the ochrophyte plastid proteome had
422 also enabled the establishment of novel chimeric fusion proteins, unique to ochrophyte
423 plastids. Using the taxonomic subdivisions erected for this study, we identified further
424 chimerism events in members of 42 ancestral HPPGs (Fig. 6, panel B; Table S9- sheet 1,
425 sections 4, 5; Table S11¹⁴⁵). These include three HPPGs (e.g. NADH-ubiquinone
426 dehydrogenase) in which chimeric proteins have formed through the fusion of modules of
427 prokaryotic origin to others of eukaryotic origin, and seven HPPGs (e.g. translation factor EF-
428 3b, and an N6-adenine DNA methyltransferase) in which fusion events have occurred
429 between modules of red origin and modules of green origin (Fig. 6, panel B). To our
430 knowledge, neither of these types of fusion event have previously been reported for plastid-
431 targeted proteins⁷⁵. The chimeric proteins contain domains from a wide range of
432 evolutionary origins: 20 (47.6%) contain a domain of inferred green origin and 18 (43.8%)
433 contain a domain of host origin.

434

435 Amongst the chimeric proteins identified, we found two that probably fused in the
436 ochrophyte ancestor (Fig. 6, panels A, B). In one case, a bifunctional protein containing an N-
437 terminal 3,4-dihydroxy-2-butanone 4-phosphate (DHBP) synthase and C-terminal GTP
438 cyclohydrolase II protein, which performs two consecutive steps of riboflavin biosynthesis⁷⁶,
439 has formed through the fusion of a cyclohydrolase domain of probable host origin to a
440 synthase domain of probable red algal or actinobacterial origin (Fig. 6- figure supplements
441 1,2). While bifunctional DHBP synthase/ GTP cyclohydrolase proteins are known in bacteria,
442 red algae and plants (Fig. 6- figure supplement 1)^{48, 76}, in these taxa the DHBP synthase
443 domain is located at the protein C-terminus; thus, an analogous but topographically distinct
444 fusion protein has evolved in ochrophytes. In a second, previously reported case⁷⁵, a C-
445 terminal plastid-targeted Tic20 subunit of red algal origin has become fused to an N-terminal
446 EF-hand motif, for which no clear evolutionary outgroup (to an e value of below 1×10^{05})
447 could be found (Fig. 6- figure supplement 3). Thus, the fusion of proteins of different
448 evolutionary origins has generated new functions in the ochrophyte plastid proteome.

449

450 *Ancestral and bidirectional origins of dual targeting in ochrophytes*

451

452 Finally, we considered whether the acquisition of the ochrophyte plastid might have also
453 fundamentally altered the biology of the host cell, by contributing proteins to host processes
454 and structures outside the plastid. As an exemplar system, we considered dual targeting of
455 proteins to plastids and mitochondria, which is known to occur extensively in plants^{77, 78}, and
456 has recently been documented in diatoms⁷⁹ and in other complex plastid lineages^{79, 80}.

457 Previous studies have speculated that dual targeting may arise early in plastid evolution, for
458 example through the retargeting of proteins from the host mitochondria to the plastid, or
459 equally via the adaptation of proteins of plastid origin to the mitochondria^{18,77}.

460
461 We indeed identified proteins that appeared to be dual targeted to the plastid and a
462 secondary organelle (Fig. 2- figure supplements 5, 6), which we verified to be the
463 mitochondria using Mitotracker orange (Fig. 7 panel A). In at least two cases (histidyl- and
464 prolyl-tRNA synthetase) this dual targeting is a conserved feature, as we identified the same
465 fluorescence patterns both in *P. tricornutum* and using heterologous expression constructs
466 from *G. foliaceum* and *N. gaditana* (Fig. 7, panel A; Fig.7- figure supplement 1). To determine
467 whether dual targeted proteins were ancestrally present in the ochrophyte plastid, we
468 developed an *in silico* pipeline, based on experimental data, to identify probable dual
469 targeted proteins from within the HPPG dataset (Fig. 7- figure supplement 2; Table S12-
470 sheet 1¹⁴⁵). In total, we identified 1103 HPPGs that included at least one member that was
471 probably dual targeted to plastids and mitochondria (Table S12- sheet 1¹⁴⁵). 34 of these
472 HPPGs passed the conservation thresholds previously inferred to signify an ancestral origin
473 (Table S12- sheet 1¹⁴⁵). Thus, dual targeting is an ancestral feature of the ochrophyte plastid.
474

475 We then considered the origins of the ancestrally dual targeted ochrophyte proteins. 15 of
476 the 34 putative ancestrally dual targeted HPPGs were orthologous to HPPGs of clear
477 evolutionary origin; of these, the majority (11/15; 73%) were of red algal, i.e., probable
478 endosymbiont origin (Fig. 7, panel B; Table S12- sheet 2¹⁴⁵). To determine how these dual
479 targeted HPPGs have altered the biology of the host, we searched for gene families
480 corresponding to aminoacyl-tRNA synthetases within the 7140 non-redundant gene families
481 previously identified to be shared across the ochrophytes (Table S8- sheet 1¹⁴⁵). To enable
482 function of the translational machinery, each genome within the ochrophyte cell (i.e.,
483 nucleus, mitochondrion, and plastid) requires aminoacyl-tRNA synthetase activity for each
484 amino acid⁷⁹; thus, if any class of aminoacyl-tRNA synthetase is represented by fewer than
485 three genes, then individual tRNA synthetases must support the biology of multiple
486 organelles through dual targeting. We identified seven classes of tRNA synthetase for which
487 there were only two gene families in the ochrophyte ancestor, one corresponding to a
488 cytosolic enzyme, and the other to an enzyme that was probably dual targeted to both the
489 mitochondria and plastid. These include five cases in which the dual targeted tRNA
490 synthetase was of apparent red algal, i.e., endosymbiont origin (Fig. 7, panel C). Thus, the
491 acquisition of the ochrophyte plastid also altered the biology of the mitochondria, with dual
492 targeted proteins of endosymbiont origin functionally replacing endogenous mitochondrial-
493 targeted homologues.
494

495 **5. Complex evolutionary origins of CASH lineage plastids**

496
497 *A pelagophyte/ dictyochophyte origin of the haptophyte plastid proteome*
498

499 We considered whether our dataset provides evidence for any of the other CASH lineage
500 plastids (cryptomonads, haptophytes, or photosynthetic alveolates) originating within the
501 ochrophytes^{1,5,7}, or evidence for gene transfer from ochrophytes into lineages with complex
502 plastids of green algal origin (chlorarachniophytes and euglenids)^{81,82}. In a majority
503 (243/437) of trees in which they could be assigned a clear origin, plastid-targeted proteins
504 from haptophytes resolved at a position within the ochrophyte clade (Materials and
505 Methods; Fig. 8, panel A; Table S4- sheet 5¹⁴⁵). All other groups (except for dinotoms, which
506 have well-defined plastids of diatom origin^{20,56}) generally branched externally rather than
507 within the ochrophyte clade (Fig. 8, panel A). Indeed, the proportion of haptophyte proteins

508 that resolved within the ochrophytes was found to be significantly greater than any of the
509 other groups except for dinotoms (chi-squared, $P < 1 \times 10^{-05}$; Table S4- sheet 5¹⁴⁵).

510
511 We noted that the plastid-targeted haptophyte proteins of ochrophyte origin were biased
512 towards specific origins, with over half of the proteins that grouped with a specific
513 ochrophyte lineage (100/178) resolving with members of the hypogyristera (i.e.,
514 pelagophytes, dictyochophytes, and bolidophytes; Fig. 8- figure supplement 1; Table S4-
515 sheet 5¹⁴⁵). No such bias could be observed in any other CASH lineage, in which invariably a
516 significantly smaller proportion of proteins were found to resolve with hypogyristeran
517 lineages (chi-squared $P < 0.01$; Fig. 8- figure supplement 1; Table S4- sheet 5¹⁴⁵). We
518 additionally explored whether there might be unique synapomorphies shared between one
519 ochrophyte lineage and the haptophytes. We found 53 ASAFind-generated HPPGs that
520 contained a majority ($\geq 2/3$) of the haptophyte sub-categories and contained at least one
521 member of the hypogyristera, but contained no other ochrophyte orthologues (Fig. 8, panel
522 B; Table S2- sheet 2, section 3¹⁴⁵). This was significantly more than would be expected (28.3,
523 chi-squared $P = 0.00013$) through a random assortment of all HPPGs that were uniquely
524 shared between haptophytes and one ochrophyte lineage, corrected for the relative size of
525 each dataset (Materials and Methods). We similarly found a significantly larger number of
526 HPPGs to be uniquely shared between a majority of both the haptophytes and a majority
527 ($\geq 2/3$) of the hypogyristeran sub-categories (15, expected number 8.0, $P = 0.034$; Fig. 8, panel
528 B) or shared between a majority of hypogyristera and at least one haptophyte sub-category
529 (28, expected number 12.9, $P = 0.00073$; Table S2- sheet 2, section 3¹⁴⁵; Fig. 8, panel B). Thus,
530 our data supports a specific gene transfer event between the hypogyristera and the
531 haptophytes.

532
533 We investigated whether there is a more specific origin for the ochrophyte sequences in
534 haptophyte plastids. First, we tabulated the individual ochrophyte sub-categories identified
535 in the first sister group to haptophyte sequences, of which the greatest number (94)
536 resolved specifically with pelagophyte and dictyochophyte sequences, rather than with
537 bolidophytes, non-hypogyristeran lineages, or more ancestral nodes (Fig. 8, panel C; Fig. 8-
538 figure supplement 2). Next, we extracted all of the haptophyte plastid-targeted sequences
539 assembled into each ancestral ochrophyte HPPG, performed BLAST top hit analysis (Table
540 S13- sheets 1-3¹⁴⁵), and identified sequences for which the best hit was from the same
541 ochrophyte lineage (diatoms, hypogyristera, or chrysisita) as the tree sister group (Table S13-
542 sheet 4¹⁴⁵). We performed separate analyses for query sequences from each of the three
543 haptophyte sub-categories considered in our analysis (pavlovophytes, prymnesiales, or
544 isochrysidales). In each case, at least 50% of the sequences that produced an evolutionarily
545 consistent series of top hits resolved either with the pelagophytes or dictyochophytes (Fig.
546 8- figure supplement 3; Table S13- sheet 4¹⁴⁵). Thus, these proteins originated within an
547 ancestor of the pelagophyte/ dictyochophyte lineage.

548
549 We next tested the probable direction of the gene transfer events. We reasoned that if the
550 genes identified within our study had been transferred from an ancestor of pelagophytes
551 and dictyochophytes into the haptophytes, then we should also see a strong secondary
552 signal linking the haptophytes to earlier ancestors of the pelagophyte/ dictyochophyte clade,
553 for example the common ancestor of hypogyristera and diatoms. We inspected the
554 secondary BLAST top hits associated with genes shared between haptophytes and
555 hypogyristera (Fig. 8- figure supplement 4; Table S13- sheet 5¹⁴⁵), and the next deepest sister-
556 groups to haptophyte proteins that are of probable pelagophyte or dictyochophyte origin in
557 each single-gene tree (Fig. 8- figure supplement 4; Table S4- sheet 2, section 6¹⁴⁵). The
558 majority of haptophyte proteins of hypogyristeran origin in single-gene trees (65/100) clearly

559 resolved within a broader HPPG containing multiple ochrophyte lineages, and this bias was
560 corroborated by the specific sister groups associated with each protein as inferred by heat
561 map analysis (Fig. 8- figure supplement 4, panel A). Moreover, the majority of haptophyte
562 proteins with hypogyrustean BLAST top hits, and hypogyrustean proteins with haptophyte
563 BLAST top hits (48/ 86 sequences total) had next best BLAST hits against diatoms (Fig. 8-
564 figure supplement 4, panel B). We additionally tabulated the earliest and latest possible
565 origin points of amino acid residues that were uniquely shared between haptophytes and
566 some but not all ochrophyte lineages, from a dataset of 37 HPPGs for which there was a
567 clear evolutionary affinity between haptophytes and ochrophytes and strict subsequent
568 vertical inheritance (Fig. 8, panel D; Fig. 8- figure supplement 5; Table S6- sheets 3, 4¹⁴⁵). A
569 greater number of the uniquely shared residues were found to be conserved between the
570 haptophytes and the common ancestor of hypogyrustea and diatoms, than were specifically
571 only shared with pelagophyte and dictyochophyte sequences, both per the latest possible
572 origin (139 residues shared with hypogyrustea and diatoms; 99 residues with pelagophytes
573 and dictyochophytes; Fig. 8, panel D; Table S7- sheets 2, 3¹⁴⁵) and per the earliest possible
574 origin (46 residues shared with hypogyrustea and diatoms; 41 residues with pelagophytes
575 and dictyochophytes; Fig. 8- figure supplement 5; Table S7- sheets 2, 3¹⁴⁵). This specifically
576 supports a transfer of plastid-targeted proteins from an ancestor of the pelagophyte/
577 dictyochophyte clade into the haptophytes, rather than the other way around.

578
579 Finally, we tested whether these proteins were likely to have been acquired through an
580 endosymbiotic event. We reasoned that the genes acquired by haptophytes through
581 endosymbiotic events should encode a greater proportion of plastid-targeted proteins than
582 would be observed with genes of alternative origin. We accordingly constructed a dataset of
583 12,728 non-redundant gene families that were broadly distributed across the haptophytes
584 (Table S14- sheet 1¹⁴⁵), of which 772 were of probable hypogyrustean origin (Fig. 8- figure
585 supplement 6; Table S14- sheet 2¹⁴⁵). A significantly larger proportion of the ancestral
586 haptophyte gene families of hypogyrustean origin were predicted by ASAFind to be targeted
587 to the plastid than would be expected by random distribution of the data (observed number
588 43, expected number 22.8, chi-squared $P= 2.2 \times 10^{-05}$; Fig. 8, panel E; Table S14- sheet 3¹⁴⁵),
589 consistent with an endosymbiotic origin. Thus, our data support an endosymbiotic uptake of
590 an ancestor of the pelagophytes and dictyochophytes by an ancestor of the haptophytes.

591 *Phylogenetic discrepancies between the haptophyte plastid proteome and genome*

592
593 The transfer of plastid-targeted proteins from the pelagophyte/dictyochophyte clade into
594 the haptophytes is surprising, as previous studies have indicated that the haptophyte plastid
595 genome originates either as a sister-group to the entire ochrophyte lineage⁵ or to the
596 cryptomonads^{83,84}. To verify this discrepancy we constructed two plastid trees, one using 54
597 conserved proteins that are encoded in all sequenced red lineage and glaucophyte plastids
598 (Fig. 9, panel A; Table S15- sheet 1¹⁴⁵), and one using a smaller subset of 10 plastid-encoded
599 proteins that were detected in many of the transcriptome libraries used in this study (Fig. 9,
600 panel B; Table S15- sheet 1¹⁴⁵).

601
602
603 A specific sister-group relationship between the cryptomonads and haptophytes was
604 recovered, with moderate to strong bootstrap support, in both the gene-rich tree (Fig. 9,
605 panel A) and the taxon-rich tree (Fig. 9, panel B). Both trees also strongly supported the
606 monophyly of ochrophyte plastid genomes (Fig. 9). Alternative topology tests rejected any
607 possibility that the haptophyte plastid originated within the ochrophytes (Fig. 9- figure
608 supplement 1; $P \leq 0.05$). Similarly, trees calculated from alignments in which fast-evolving
609 sites and clades had been serially removed, and in which the alignment had been recoded to

610 minimise amino acid composition biases (Fig. 9- figure supplement 2; Table S15- sheet 2;
611 Table S16¹⁴⁵) either recovered a sister-group relationship between haptophytes and
612 cryptomonads, or placed haptophytes as the sister group to all ochrophytes. We additionally
613 generated and inspected single-gene tree topologies for each of the constituent genes used
614 to generate each concatenated multigene alignment, and could not find any that confidently
615 resolved a sister-group relationship between haptophytes and the pelagophyte/
616 dictyochophyte clade (Fig. 9- figure supplement 3; Table S15- sheet 3¹⁴⁵). Finally, we found
617 only three residues in the alignment that were uniquely shared among all four haptophytes
618 and the sole representative of pelagophytes and dictyochophytes (*Aureococcus*) in the gene-
619 rich dataset, and no residues that were shared between a majority of the haptophytes and
620 at least one pelagophyte or dictyochophyte sequence in the taxon-rich dataset (Fig. 8, panel
621 C; Table S17- sheet 4¹⁴⁵). In contrast, we found large numbers of residues that were shared
622 uniquely by haptophytes and other lineages (Fig. 9, panel C; Table S17- sheet 4¹⁴⁵). This
623 strong support for a relationship between haptophytes and cryptomonads is inconsistent
624 with phylogenetic artifacts such as coevolution between specific protein complexes^{58, 85} or
625 gene duplication and differential loss of paralogues⁸⁶, in which case there should still be a
626 detectable underlying signal linking it to the pelagophytes and dictyochophytes. We
627 conclude that while many plastid-targeted haptophyte proteins originate from an ancestor
628 of the pelagophytes and dictyochophytes, the haptophyte plastid genome does not.

629 Discussion

630
631
632 In this study, we have reconstructed an experimentally verified dataset of 770 plastid-
633 targeted proteins that were present in the last common ancestor of all ochrophytes (Figs. 1,
634 2). Our dataset accordingly provides windows into the evolutionary origins of the
635 ochrophyte plastid lineage. These include evidence for a green algal contribution to
636 ochrophyte plastid evolution and a late acquisition of the ochrophyte plastid following
637 divergence of the ochrophyte lineage from oomycetes (Figs. 3, 4). This latter finding is
638 particularly interesting as molecular divergence estimates place the ochrophytes as
639 diverging from the oomycetes no more than 90 million years prior to the radiation of
640 ochrophyte lineages^{87, 88}. Assuming that these estimates are reliable, our dataset represents
641 some of the earliest proteins to support the ochrophyte plastid following its endosymbiotic
642 uptake. We also provide evidence for widespread mixing of proteins of different
643 evolutionary origin in the ancestral ochrophyte plastid (Fig. 5), including evidence for the
644 formation of new fusion proteins through the recombination of domains of different
645 evolutionary origins (Fig. 6), and a bidirectional mixing of proteins derived from the
646 endosymbiont with proteins from host organelles via dual targeting (Fig. 7). A schematic
647 outline of these results is shown in Fig. 10.

648
649 Many questions nonetheless remain to be answered. It remains to be determined whether
650 the *in silico* prediction facilitated by programmes such as ASAFind and HECTAR are sufficient
651 to enable the identification of all ochrophyte plastid proteins^{29, 30}. This is particularly
652 pertinent in the context of dual targeted proteins, insofar as the dataset of 34 potentially
653 ancestrally dual targeted proteins identified in this study may not include proteins that are
654 dual targeted to the plastid and other cellular organelles, such as the ER⁸⁹, cytoplasm⁹⁰, or
655 nucleus⁹¹. We note also that, based on the fluorescence patterns observed with the
656 exemplar proteins within this study (Figs. 2, 7), ASAFind and HECTAR may identify proteins
657 targeted to the periplastid compartment, as well as to the plastid stroma. While these
658 periplastid and multipartite proteins probably form an important part of plastid physiology,
659 it will be interesting to dissect the specific signals associated with the targeting of proteins to
660 individual sub-compartments within CASH lineage plastids^{55, 92}.

661

662 Another major question concerns the origins of plastid-targeted proteins of green algal
663 origin in ochrophytes. Overall, our data supports the targeting of a significant complement
664 of proteins of chlorophyte origin to the ochrophyte plastid (Fig. 4). It remains to be
665 determined, however, what the exact chlorophyte donor was, and how these genes may
666 have been acquired. It is possible that the green genes were transferred into the ochrophyte
667 lineage via lateral gene transfer, either from a range of different green algal sources or
668 repeatedly from one lineage (for example, a semi-permanent intracellular symbiont³),
669 although neither scenario would explain the bias in green algal genes in ochrophyte
670 genomes towards encoding proteins of plastid function (Fig. 4, panel D). An alternative
671 possibility might be a cryptic green algal endosymbiosis in the evolutionary history of the
672 host, as has been previously suggested^{1,44} (Fig. 10), or a more convoluted pattern of
673 acquisition. We note, for example, that the green genes identified in our study are not only
674 plastid-targeted across the ochrophytes, but are apparently shared with haptophytes and
675 cryptomonads (Fig. 10- figure supplement 1), which would be equally consistent with them
676 having been present in a common ancestor of the CASH lineage plastid, and relocated to
677 each host nuclear lineage following endosymbiosis (Fig. 10). Thus, pinpointing the exact
678 nature and timing of the green gene transfer into ochrophytes rests not only on more
679 extensive sequencing of deep-branching chlorophyte lineages, but also on characterising the
680 genome composition of the closest aplastidic relatives of extant ochrophytes (e.g.,
681 *Develorapax*, *Pirsonia*⁶), and the closest red algal relative of CASH lineage plastids, which
682 remains unknown^{1,4}.

683

684 We also provide evidence for a chimeric origin of the haptophyte plastid (Figs. 8, 9). A
685 schematic outline of these results is shown in Fig. 10- figure supplement 2. We have shown
686 that a significant number of plastid-targeted proteins found in haptophytes originate from
687 an ancestor of the pelagophytes and dictyochophytes (Fig. 8). This relationship is supported
688 by multiple lines of evidence- i.e., uniquely shared proteins, single-gene tree topologies,
689 BLAST top hit analysis, and analysis of synapomorphies in multigene alignments (Fig. 8 and
690 supplements). Alongside the bias of haptophyte genes of hypogyristean origin encoding
691 proteins of plastid function (Fig. 8- panel E), these observations argue against these genes
692 having been acquired through multiple independent lateral gene transfer events, and
693 instead support an endosymbiosis event. We note that other studies have shown strong
694 evidence for gene transfers between haptophytes and individual members of the
695 hypogyristea: for example, Stiller *et al.* have demonstrated a strong enrichment in BLAST top
696 hits against haptophytes, from the genome of the pelagophyte *Aureococcus*
697 *anophageferrens*, compared to other ochrophyte genomes⁵. We additionally note that an
698 ancestral gene transfer from a pelagophyte/ dictyochophyte ancestor into the haptophytes
699 is a chronologically realistic scenario: molecular clock estimates place the pelagophytes and
700 dictyochophytes diverging between 300 and 700 million years before present^{87,93}, which
701 broadly overlaps with the molecular dates estimated for the radiation of the haptophytes in
702 the same studies^{87,93}, and precedes the first haptophyte microfossils, identified ca. 220
703 million years before the present⁹⁴.

704 Finally, we verify that the evolutionary links between haptophyte and the pelagophyte/
705 dictyochophyte clade in terms of plastid-targeted proteins are not supported by phylogenies
706 of the haptophyte plastid genome (Fig. 9). Other multigene phylogenies of red lineage
707 plastid genomes have similarly demonstrated that the haptophyte plastid genome instead
708 resolves as a sister-lineage either to cryptomonads or to all ochrophytes^{5, 38, 83, 84}.
709 Furthermore, the structure and content of haptophyte and hypogyristean plastid genomes
710 are dissimilar: for example, haptophyte plastids possess an *rpl36* gene that has been laterally
711 acquired from a bacterial donor and is shared with cryptomonad plastids but absent from

712 ochrophytes⁹⁵, and ochrophyte plastids no longer retain genes encoding the plastid division
713 machinery proteins *minD* and *minE*, which remain plastid-encoded in haptophytes and
714 cryptomonads⁹⁶. Similarly, extant haptophyte plastids have comparatively large plastid
715 genomes and possess a conventional quadripartite structure⁹⁷, whereas extant pelagophyte
716 plastids have a reduced coding content compared to other photosynthetic ochrophytes,
717 cryptomonads and haptophytes, and have secondarily lost the plastid inverted repeat^{98,99},
718 although it is not yet known whether dictyochophyte plastids share this reduced structure.

719 The discrepancy between the pelagophyte/ dictyochophyte origin of the haptophyte plastid
720 proteome and the clear non-ochrophyte origin of its plastid genome might be explained by
721 several different evolutionary scenarios. One possibility would be a serial endosymbiosis
722 event deep in haptophyte evolutionary history, in which an ancient plastid derived from a
723 pelagophyte/ dictyochophyte ancestor was acquired by the haptophyte common ancestor,
724 then replaced subsequently by a plastid of non-ochrophyte origin (Fig. 10- Figure
725 supplement 2). Verifying this scenario, or its alternatives (such as lateral gene transfer from
726 pelagophyte or dictyochophyte algae into the algal ancestors of the haptophyte plastid)
727 rests on identifying the exact origin of the current haptophyte plastid genome, and in
728 particular demonstrating that the haptophyte plastid genome originates from within (rather
729 than forms a sister-group to) a major lineage of eukaryotic algae other than ochrophytes
730 (Fig. 10- Figure supplement 2). For this, sequence data from early-diverging members of the
731 cryptomonads and haptophytes will be particularly important^{41,100,101}. It also remains to be
732 determined whether other CASH lineage plastids, such as the peridinin-type plastids found
733 in most photosynthetic alveolates, originate within the ochrophytes^{7,20}. Similar plastid
734 proteome reconstructions, using bespoke datasets for these species, will be particularly
735 useful in unravelling their disparate evolutionary origins.

736 Overall, our dataset provides valuable and deep insights into the chimeric origins and
737 complex fates of a major group of eukaryotic algae. Further studies using more sensitive
738 pipelines, or using analogous datasets from other major CASH lineages, may elucidate the
739 evolutionary and physiological diversification of plastids in the open ocean.

741 **Materials and Methods**

742 **Identification of ancestral plastid-targeted ochrophyte proteins**

743 Ancestral plastid-targeted proteins in ochrophytes were identified via a composite pathway,
744 consisting of *in silico* prediction, identification of conserved proteins using BLAST, alignment,
745 and single-gene tree building. First, the complete protein libraries annotated from eleven
746 ochrophyte genomes (the diatoms *Phaeodactylum tricornutum*⁵⁹, *Thalassiosira*
747 *pseudonana*⁹, *Thalassiosira oceanica*¹⁰², *Fistulifera solaris*¹⁰³, *Fragilariopsis cylindrus*, *Synedra*
748 *acus*¹⁰⁴, and *Pseudonitzschia multiseriis*; the pelagophyte *Aureococcus anophagefferens*¹¹;
749 the eustigmatophytes *Nannochloropsis gaditana* and *Nannochloropsis salina*^{37,105}; and the
750 kelp *Ectocarpus siliculosus*¹⁰; Table S1- sheet 1¹⁴⁵), were screened using the ochrophyte
751 plastid-targeting predictors ASAFind²⁹ (used in conjunction with SignalP version 3.0¹⁰⁶; Table
752 S2¹⁴⁵) and HECTAR³⁰ (integrated into a Galaxy¹⁰⁷ instance available at <http://webtools.sb-roscoff.fr>; Table S3¹⁴⁵). All proteins that were deemed to possess plastid-targeting sequences
753 (regardless of the confidence score applied by ASAFind²⁹) were retained for further
754 inspection.
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758

759 Possible conserved plastid-targeted sequences (i.e. homologous plastid-targeted protein
760 groups, or HPPGs) were next identified using a customised BLAST protocol. First, a library of
761 non-redundant proteins was generated to serve as seed sequences for further searches.

762 Each plastid-targeted protein identified from ochrophyte genome sequences was searched
763 by BLASTp against a modified Uniref¹⁰⁸ library, and the expect values for all top hits were
764 extracted, to yield a floating BLAST threshold below which orthologous proteins were
765 identified. All sequences from lineages with a history of secondary endosymbiosis were first
766 removed from the Uniref library in order to avoid the confounding effects of gene transfer
767 from current and former symbionts^{5, 7, 81, 82}. The removed lineages included cryptomonads,
768 centrohelids, telonemids, haptophytes, alveolates, rhizaria, euglenids, and plastid-bearing
769 stramenopiles. All of the ochrophyte genome-derived plastid-targeted proteins were
770 searched against one another by BLAST, and proteins that matched one another with an
771 expect score lower than the first outgroup hit (or were retrieved as a stronger match than
772 the outgroup hit if the expected values of both were zero), and thus likely correspond to
773 different proteins within the same monophyletic plastid protein cluster, were merged. Only
774 one protein was retained as the seed sequence for subsequent growth of each cluster: this
775 was defined first via organism (in order of preference: *P. tricornutum*, *T. pseudonana*, *P.*
776 *multiseriis*, *F. cylindrus*, *S. acus*, *A. anophagefferens*, *E. siliculosus*, *N. gaditana*, *N. salina*, *T.*
777 *oceanica*, *F. solaris*) and, where more than one protein was available for a given organism,
778 the protein with the lowest BLAST expect value against the corresponding uniref top hit.
779

780 Next, plastid-targeted protein sequences were sought from all available ochrophyte
781 sequence data. A search database was built from all eleven completed ochrophyte genomes,
782 147 ochrophyte sequence libraries from the Marine Microeukaryote Transcriptome
783 Sequence Project³⁴, eleven further ochrophyte transcriptome sequencing projects^{64, 109, 110}
784 and uniref. Cross-contamination was removed from MMETSP transcriptomes as previously
785 described⁵⁷. Briefly, this procedure compares the nucleotide sequences of contigs assembled
786 from each MMETSP library by pairwise BLAST, and defines a separate cross-contamination
787 threshold for each pair of MMETSP libraries based on their distribution of BLAST percent
788 identities. These distributions should each contain a peak centered on the average
789 nucleotide percent identity of transcripts between the two species. In addition, in the
790 presence of cross-contamination, there should be a second peak at 100% identity. The
791 procedure defines the cross-contamination threshold as the minimum between these two
792 peaks; above the threshold, contigs (and the proteins predicted from them) are considered
793 to be potentially cross-contaminated. In total, 2.5% of the MMETSP contigs were discarded
794 through this method. A summary of the number of contigs discarded is provided in Table S1-
795 sheet 2, section 1¹⁴⁵.
796

797 Each decontaminated sequence was trimmed at the N-terminus to the first methionine
798 present, and binned into one of eleven different evolutionary categories, based on recent
799 multigene phylogenetic trees for ochrophytes and diatoms^{12, 111-113} (fig. 1, panel A; Table S1-
800 sheet 1¹⁴⁵). These consisted of: three chrysostran lineages (the "PX clade" of phaeophytes,
801 xanthophytes and related lineages; raphidophytes; and the "PESC clade" of pinguiophytes,
802 eustigmatophytes, synchromophytes, and synurophytes/chrysophytes), three hypogyrystean
803 lineages (pelagophytes; dictyochophytes; and bolidophytes), and five diatom lineages (the
804 basally divergent genus *Corethron*; radial centric lineages such as Coscinodiscophytes and
805 Rhizosoleniaceae; the polar centric Thalassiosirales and Skeletonemataceae, which appear
806 to be relatively distantly related to pennate diatoms^{111,113}; polar centric lineages such as
807 Odontellids and Chaetocerotales that appear to be more closely related to pennate
808 diatoms^{111,113}; and finally all pennate lineages). These binned sequences were then searched
809 for plastid-targeted proteins by ASAFind and HECTAR as before.
810

811 The seed sequences for the resulting non-redundant HPPGs were searched against the
812 enlarged plastid sequence library using BLASTp. Proteins that matched against seed

813 sequences with a lower expect value than the outgroup best hit (or were retrieved as a
814 stronger match than the outgroup hit if the expected values of both were zero), were added
815 to each HPPG. Next, three custom thresholds were defined that were particularly successful
816 in distinguishing probable proteins of true plastid localisation from false positives (fig. 1,
817 panel B). For this, conservation patterns were selected that maximised the relative
818 enrichment in proteins with unambiguous plastid functions (i.e., were annotated to function
819 in photosynthesis, to constitute integral parts of the plastid thylakoid or inner membranes,
820 or corresponded to the expression products of genes that are plastid-encoded in red algae
821 but have been apparently relocated to the ochrophyte nucleus⁹⁷ or that corresponded to
822 proteins previously verified experimentally to localise to ochrophyte plastids^{29, 30, 114, 115}), and
823 thus should contain relatively fewer examples of mispredicted proteins within the dataset.
824 At the same time, conservation patterns were selected that minimised the number of HPPGs
825 identified as conserved from a negative control dataset (consisting of HPPGs assembled
826 using seed sequences from the published genome sequences of the cryptomonad *Guillardia*
827 *theta*¹⁷ or the haptophytes *Emiliania huxleyi*¹¹⁶ and *Chrysochromulina tobin*¹¹⁷, and for which
828 no plastid-targeted orthologues were detected in any of the ochrophyte genome sequences
829 used in this study). The thresholds corresponded to: orthologues in a majority ($\geq 2/3$) of
830 chrysistan and a majority ($\geq 3/5$) of diatom lineages; a majority of chrysistan and a majority
831 ($\geq 2/3$) of hypogyristean lineages; and at least one chrysistan, and a majority of both
832 hypogyristean and diatom lineages (fig. 1).

833
834 All of the HPPGs that passed at least one threshold were extracted, and homology for each
835 HPPG was confirmed individually (Table S4- sheet 1¹⁴⁵). First, each HPPG was aligned using
836 20 iterations of MUSCLE v8¹¹⁸, followed by the in-built alignment programme integrated into
837 GeneIOUS v 4.76¹¹⁹, under the default criteria. Each HPPG alignment was manually
838 inspected, and proteins that failed to align with the genomic sequences, clearly terminated
839 within the conserved region of the protein, or were truncated at the N-terminus by a length
840 of greater than 50 amino acids (i.e. the approximate length of an ochrophyte plastid-
841 targeting sequence^{29, 114}) were removed, following which HPPGs that no longer passed the
842 taxonomic criteria defined for conservation were eliminated (Table S4- sheet 1¹⁴⁵). Next,
843 each HPPG was enriched with the sequences for the top 50 hits obtained when the seed
844 sequence was searched against the modified uniref library as detailed above, alongside the
845 single best hit for composite transcriptome and genome libraries constructed for 36
846 eukaryotic sub-categories (Table S1- sheet 1¹⁴⁵), and realigned against this reference. The
847 transcriptome components of the reference sequence libraries were cleaned of residual
848 contamination as defined above, and 23 individual MMETSP libraries were additionally
849 excluded due to evidence of further contamination (Table S1- sheet 2¹⁴⁵). Sequences that
850 failed to align were removed, and HPPGs that failed to meet the criteria for conservation
851 following alignment were eliminated (Table S4- sheet 1¹⁴⁵).

852
853 Finally, each HPPG was trimmed at the N- and C-termini to (respectively) the first residue
854 and last residue visually identified to be conserved in > 70% of the sequences in the
855 alignment, corresponding to the probable conserved domain of the protein. Each HPPG was
856 then trimmed with trimAl using the -gt 0.5 option¹²⁰. 100 trees were calculated for each
857 trimmed alignment using RAxML, with the JTT substitution model + gamma correction¹²¹.
858 The consensus tree from the 100 bootstrap replicates was manually inspected for the
859 presence of a clade of ochrophyte proteins, containing sufficient sequences to pass the
860 criteria for conservation defined above, that was either monophyletic, or paraphyletic to the
861 inclusion of only one of five different non-ochrophyte groups (prokaryotes, red algae, green
862 algae, aplastidic stramenopiles, and all other eukaryotes excluding CASH lineages, rhizaria

863 and euglenids; Table S4- sheet 1¹⁴⁵). HPPGs that passed this final stage of analysis were
864 deemed to correspond to ancestrally plastid-targeted proteins (Table S4- sheet 2¹⁴⁵).

865

866 All identified plastid-targeted proteins, HPPGs, full aligned HPPGs, and single-gene trees
867 have been made publically accessible through the University of Cambridge dSpace server
868 (<https://www.repository.cam.ac.uk/handle/1810/261421>¹⁴⁵).

869

870 **Generation of fluorescence expression constructs for *Phaeodactylum tricornerutum***

871

872 *Phaeodactylum tricornerutum* 1.86 (CCMP2561), *Nannochloropsis gaditana* CCMP526, and
873 *Glenodinium foliaceum* PCC499 were maintained in liquid cultures of f/2 medium
874 supplemented with vitamins, and 100 µg/ ml each of ampicillin, streptomycin, kanamycin
875 and neomycin, in a constant 19°C environment in a 12h: 12h cycle of 150 µE m⁻² s⁻¹ light:
876 dark. *P. tricornerutum* was maintained on an orbital shaker at 100 rpm, while *N. gaditana* and
877 *G. foliaceum* were maintained as stationary cultures. Large volume cultures of *P.*
878 *tricornerutum* (e.g. cultures grown for transformation by bombardment) were grown in
879 artificial seawater, supplemented with vitamins but without antibiotics.

880

881 Total cellular RNA was extracted from c. 30 ml volumes of late log phase culture from each
882 species using a modified Trizol phase extraction and DNase treatment protocol as described
883 elsewhere²¹. Each RNA sample was tested for integrity by gel electrophoresis and quantified
884 by a nanodrop spectrophotometer, and confirmed to be free of residual DNA contamination
885 by direct PCR using universal eukaryotic 18S rDNA primers¹²². Approximately 200 ng purified
886 RNA from each species was used as the template for cDNA synthesis, using a Maxima First
887 Strand cDNA Synthesis Kit (Thermo), following the manufacturer's instructions.

888

889 Nucleotide sequences encoding plastid-targeted proteins of unusual provenance were
890 identified using the complete genome sequences of *Phaeodactylum tricornerutum* and
891 *Nannochloropsis gaditana*^{37, 59}, and the *Glenodinium foliaceum* CCAP1116/3 transcriptome
892 library assembled as part of MMETSP^{34, 123} (Table S5¹⁴⁵). Two primers were designed for each
893 sequence: a PCR forward primer corresponding to the 5' end of the ORF, and a
894 translationally in-frame PCR reverse primer positioned a minimum of 45 bp into conserved
895 domain of the protein sequence (Table S5¹⁴⁵). These primers were respectively fused to 5'
896 fragments complementing the 3' end of the *P. tricornerutum* FcpA promoter, and the 5' end of
897 the GFP CDS. For one gene (the novel plastid protein), PCR reverse primers were designed
898 complementary to the 3' end of the CDS of each gene due to the lack of a verifiable CDD; a
899 full-length PCR reverse primer was additionally designed against the histidyl-tRNA
900 synthetase sequence from *Nannochloropsis gaditana* due to failure to obtain functional
901 expression from N-terminal constructs (data not shown).

902

903 High-fidelity PCR products were amplified with each primer pair from the corresponding
904 cDNA product using Pfu DNA polymerase (Thermo), per the manufacturer's instructions. In
905 two cases (*Nannochloropsis gaditana* peroxisomal membrane protein, and the novel plastid
906 protein) inserts were amplified from synthetic, codon-optimised constructs, designed to
907 maximise expression levels in *Phaeodactylum tricornerutum* (Eurofins). Each product was
908 separated by DNA gel electrophoresis, cut, purified using a PCR gel extraction column kit
909 (Macherey-Nagel), quantified using a nanodrop spectrophotometer, and verified by Sanger
910 sequencing (GATC Biotech). The purified products were then used for Gibson ligation
911 reactions¹²⁴ (NEB), following the manufacturer's instructions, using linearised and DpnI-
912 treated vector sequence generated from the pPhat-eGFP vector³⁵, and transformed into
913 chemically competent Top10 *E. coli* cells, prior to selection on LB-1% agar plates containing

914 100 µg/ ml ampicillin. Individual colonies were picked, verified to contain the insert
915 sequence by PCR, and grown as overnight liquid cultures on LB medium supplemented with
916 100 µg/ ml ampicillin, prior to purification of the plasmids by alkaline lysis and isopropanol
917 precipitation¹²⁵. Purified plasmids were integrated into *P. tricornutum* cells via biolistic
918 transformation, using the Biolistic PDS-1000/He Particle Delivery System (BioRad),
919 essentially as previously described^{35, 126}.

920

921 Colonies obtained from each transformation were transferred to liquid f/2 supplemented
922 with vitamins and 100 µg/ ml zeocin, and were left to recover under the same growth
923 conditions as used for liquid cultures of untransformed cells. Expression of GFP was
924 visualised using a TCS SP8 confocal microscope (Leica), an excitation wavelength of 488 nm
925 and emission wavelength interval of c. 510-540 nm. Chlorophyll fluorescence (using an
926 emission interval of 650-700 nm) and bright field images were simultaneously visualised for
927 each cell. Wild-type cells that did not express GFP were used to identify the maximum
928 exposure length possible without false detection of chlorophyll in the GFP channel (Fig. 2-
929 figure supplement 7).

930

931 Possible mitochondrial localisations of dual targeted proteins were identified by staining
932 cells with approximately 100 mM Mitotracker orange, dissolved in filtered seawater, for 25
933 minutes under standard culture conditions⁵⁵. Cells were rinsed and resuspended in fresh
934 filtered seawater prior to visualisation, using the same conditions as stated above for GFP,
935 and a 548 nm excitation laser and 575-585 nm absorbance window for the Mitotracker
936 signal. To ensure that there was no possible crosstalk between the two signals, negative
937 controls consisting of an unstained GFP-expressing wild-type line, and stained wild-type
938 cells, were used respectively to determine the maximum exposure length possible without
939 (respectively) false detection of GFP in the Mitotracker channel, and false detection of
940 Mitotracker in the GFP channel (Fig. 7- figure supplement 1).

941

942 **Reconstruction of evolutionary origins of ancestral plastid-targeted proteins**

943

944 The most probable evolutionary origins of individual plastid-targeted proteins were
945 identified via the combined products of BLAST top hit analysis and phylogenetic sister-group
946 inference. First, a composite reference sequence library was generated by appending the
947 uniref outgroup library previously used for BLAST-based assembly of ancestral HPPGs, with
948 twenty-two combined eukaryotic transcriptome and genomic libraries of taxa with no
949 suspected history of serial endosymbiosis, which was previously used to enrich each single-
950 gene tree (Table S1- sheet 1¹⁴⁵). Each sequence within the library was then assigned a
951 taxonomic affinity consisting of one of six lineages (green algae, red algae, aplastidic
952 stramenopiles, all other eukaryotes, prokaryotes, and viruses) and one of 48 sub-categories,
953 (Table S1- sheet 1, section 1¹⁴⁵). Next, each seed protein sequence within each ancestral
954 HPPG was searched by BLASTp against the composite library, with a threshold e-value of $1 \times$
955 10^{-05} . Sequences were annotated by the lineage and sub-category of the first hit obtained,
956 and by the number of consecutive top hits obtained within the same lineage (Table S4- sheet
957 2, section 2¹⁴⁵). To minimise misidentification due to any residual contamination in individual
958 sequence libraries, only sequences for which the first three or more BLAST hits resolved
959 within the same lineage were deemed to be unambiguously related to that lineage.

960

961 Sister-group relationships were additionally inferred for each ancestral HPPG from the
962 previously generated single-gene trees (Table S4- sheet 2, section 3¹⁴⁵). To ensure that only
963 true sister-group relationships were recorded, and to avoid potential misidentifications of
964 individual sister-group relationships due to species-specific gene transfer or contaminants

965 that had not previously been excluded by screening individual species libraries, only trees in
966 which ochrophytes were monophyletic, (i.e., not paraphyletic with regard to any one of the
967 five outgroups), for which a single sister-group could be identified (using the most
968 phylogenetically complex node as the outgroup), and for which the sister-group contained at
969 least two monophyletic or paraphyletic sequences, from different sub-categories of the
970 same lineage, were used for subsequent analysis.

971

972 **Reconstruction of evolutionary relationships between ochrophytes and other CASH** 973 **lineage plastids**

974

975 To identify the probable relationships between ochrophytes and other CASH lineage
976 plastids, each ancestral HPPG tree was enriched with sequences from six different groups of
977 organisms with histories of serial endosymbiosis (cryptomonads, haptophytes, dinotoms,
978 other alveolates, euglenids, and chlorarachniophytes), subdivided into thirteen sub-
979 categories (Table S1¹⁴⁵). For the cryptomonad, haptophyte and dinotom sequences, as
980 plastid-targeted proteins from these lineages may be identified using targeting predictors
981 trained on diatoms such as HECTAR⁶ and ASAFind^{29,30}, each of the HPPGs initially generated
982 was enriched with plastid-targeted sequences from each cryptomonad, haptophyte and
983 dinotom sub-category identified by *in silico* prediction with these programmes (Table S2-
984 sheet 1; Table S3- sheet 1¹⁴⁵).

985

986 The position of each group of organisms within the tree was then annotated as falling into
987 one of eight different categories, four of which were internal to the ochrophytes (diatoms;
988 hypogyristea; chrysisita; or an ambiguous internal position) and four of which were external
989 to the ochrophytes (as an immediate sister-group to all ochrophytes prior to the first
990 outgroup lineage previously identified; within the red algae; within the green algae; and at
991 any other position external to the ochrophytes; Table S4- sheet 2, sections 5-6¹⁴⁵). To
992 minimise the incorporation of contaminant and non-plastid sequences, tree positions were
993 only recorded if the branch containing sequences from that particular lineage included at
994 least two of the sub-categories considered (for alveolates, cryptomonads, and haptophytes),
995 contained at least one predicted plastid-targeted sequence (for dinotoms, cryptomonads
996 and haptophytes), and for which only one category could be applied (i.e., the tree only
997 contained one evolutionarily distinct group for each lineage, which could be unambiguously
998 allocated one category over all others). Each tree annotation was repeated three times
999 independently, and only tree annotations that were recorded consistently in each case were
1000 retained for further analysis.

1001

1002 To identify proteins that were uniquely shared between haptophytes and other lineages,
1003 every HPPG initially generated was screened for the inclusion of only two of five different
1004 lineages (diatoms including dinotoms, hypogyristea, chrysisita, haptophytes, and
1005 cryptomonads; Table S2- sheet 2, section 3; Table S3- sheet 2, section 3¹⁴⁵). The frequencies
1006 of these proteins were then compared to the numbers expected in a random distribution of
1007 all uniquely shared HPPGs across the entire dataset: for example, if half of all uniquely
1008 shared HPPGs were shared with diatoms and one other lineage, and half were shared with
1009 haptophytes and one other lineage, then one-quarter of all uniquely shared HPPGs should
1010 be shared between haptophytes and diatoms.

1011

1012 The specific evolutionary relationships associated with haptophyte plastid-targeted proteins
1013 incorporated into ancestral HPPGs were investigated using a modified BLAST top hit
1014 technique. Firstly, all of the plastid-targeted proteins assembled into each ancestral HPPG
1015 were extracted and separated into each separate sub-category (Table S13- sheet 1¹⁴⁵). Each

1016 sub-category list was then reduced to only leave one, randomly selected sequence per HPPG
1017 (Table S13- sheet 2¹⁴⁵). Finally, each sequence retained in the reduced list was searched by
1018 BLAST against a composite library, consisting of the library previously used for outgroup top
1019 hit analysis, enriched with all of the plastid-targeted proteins identified for ochrophytes,
1020 haptophytes and cryptomonads , except for those that corresponded to the same particular
1021 lineage as the query sequence (Table S13- sheets 1,3¹⁴⁵). For example, in the case of
1022 haptophytes, plastid-targeted sequences that had been separated into three individual
1023 categories (pavlovophytes, prymnesiales, and isochrysidales¹²⁷) were searched against a
1024 composite library consisting of all outgroup sequences, and plastid-targeted sequences from
1025 diatoms, hypogyristera, chrysisita, and cryptomonads, but excluding haptophytes. BLAST top
1026 hit analysis was then performed as described above (Table S13- sheets 1, 3¹⁴⁵). Finally, to
1027 enable the identification of genes with consistent results from multiple analyses, the lineage
1028 of the BLAST top hit was compared to the lineage of the haptophyte sister-group in the
1029 single-gene tree analysis (Table S4- sheet 2, section 5; Table S13- sheet 4¹⁴⁵).

1030

1031 **Identification of uniquely shared residues in multigene HPPG datasets**

1032

1033 To identify residues that are uniquely shared between ochrophytes and other lineages,
1034 multigene datasets were constructed of a) ancestral HPPGs of green algal origin, and b)
1035 ancestral HPPGs for which haptophytes show origins within the ochrophytes. To minimise
1036 the incorporation of sequences of misidentified origin, in each case only the HPPGs for
1037 which the proposed evolutionary origin were identified both by BLAST top hit and single-
1038 gene tree analysis were included. To avoid introducing artifacts due to lineage-specific gene
1039 transfers, paralogy events, or other phylogenetic incongruencies that could otherwise bias
1040 the eventual results^{86, 128}, the single-gene tree generated for each HPPG was manually
1041 inspected to exclude any that contain multiple clades (defined as monophyletic groups
1042 containing more than one sequence from a particular lineage, separated from one another
1043 by at least two sequences from outside that particular lineage) for each of the major
1044 lineages of interest within the tree:

1045

1046

- 1047 • For the green gene dataset, HPPG trees containing more than one clade of
1048 ochrophyte, cryptomonad, haptophyte, red algal, or green algal sequences were
1049 excluded. To account for the possibility that CASH lineage sequences might
1050 originate from within the green algae, the green algae were allowed to be
1051 paraphyletic with regard to the cryptomonad, haptophyte and ochrophyte
1052 sequences, but were not allowed to incorporate sequences from other lineages.
1053 Similarly, to account for the possibility that subsequent gene transfers may have
1054 occurred from ochrophytes into other CASH lineages, the ochrophytes were
1055 allowed to be paraphyletic with regard to cryptomonad and haptophyte sequences,
1056 but not to any other lineages.
- 1057 • For the haptophyte gene dataset, HPPG trees containing more than one clade of
1058 ochrophyte, haptophyte, diatom, hypogyristeran, or chrysisitan sequences were
1059 excluded. To account for the possibility that haptophytes arose within the
1060 ochrophytes, the ochrophyte, diatom, hypogyristeran and chrysisitan sequences
1061 were allowed to incorporate sequences from haptophytes. Similarly, due to the
1062 paraphyly of hypogyristera with regard to diatoms, the hypogyristeran sequences
1063 were allowed to incorporate sequences from diatoms, but not from other lineages.
- 1064 • In all cases, sequences from chlorarachniophytes, euglenids, and alveolates were
1065 not incorporated into any of the clade assessments, due to uncertainty over the
1066 gene transfer events that have occurred in each lineage^{7, 81, 82}.

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This left datasets consisting of 32 HPPGs for which the ochrophytes were of clear green algal origin, and 37 HPPGs in which the haptophytes were of clear ochrophyte origin, with no conflicting phylogenetic signal. The rationale for inclusion and exclusion of each HPPG in each analysis is presented in Table S6, sheets 1 and 3¹⁴⁵.

Next, to eliminate individual sequences remaining within each HPPG that might have arisen through species-specific gene transfer or contamination events, each trimmed sequence within each approved alignment was inspected using a composite BLAST approach. First, each sequence was searched against a composite library containing all uniref, jgi and MMETSP sequences from every lineage within the tree of life, and the top ten hits were tabulated for each sequence. In each case, only sequences for which at least the first three hits were of the same lineage as that of the query were retained. For the haptophyte multigene alignment, the ochrophytes were separately analysed as each of the three component lineages (chryista, hypogyristea, and diatoms), which is to say that a query obtained from a member of the hypogyristea would only be retained if the first three BLAST top hits originated from other hypogyristean sequences, rather than other ochrophytes.

Next, each of the component sequences within each cleaned alignment were searched against all other component sequences within the same alignment using BLASTp, and the top ten hits within the alignment were ranked. In each case, sequences were only approved for incorporation into the multigene dataset if the first non-self hit was to a different sub-category within the same lineage, e.g. if a query sequence from a red alga yielded a top hit against a red algal sequence from a different red sub-category. To allow for possible cases of paraphyly and/or absence of sequences within each alignment, the following modifications were applied:

- Green algal sequences within the confirmed green origin alignments were allowed to yield top hits against ochrophytes, cryptomonads, and haptophytes, but were required to yield a best hit against another green alga with an expect value lower than the top hit against red algal or glaucophyte sequences.
- Glaucophyte sequences were deemed to be of correct origin if they yielded a top hit against cyanobacteria, red algae, or green algae, due to the incorporation (in general) of only one glaucophyte sequence in each alignment.
- Ochrophyte sequences were deemed to be of correct origin if they yielded a top hit against any other ochrophyte sub-category (regardless of whether this was of diatom, hypogyristean or chryistan origin). Ochrophyte sequences were additionally allowed to yield top hits against cryptomonads (in the green gene alignments), and haptophytes (in both green and haptophyte gene alignments), but were required to yield a best hit against another ochrophyte with an expect value lower than the best hit against green algal, red algal or glaucophyte sequences.
- Sequences for which no top hits were found for a different sub-category within the same lineage, but for which at least one top hit were found within the same sub-category within the lineage, and for which the first ten BLAST hits did not directly indicate a contamination event, were deemed to be of correct origin.

Tabulated outputs for each BLAST analysis are provided in Table S6, sheets 2 and 4. Finally, each dataset was reduced to leave only one randomly selected sequence for each given sub-category within each HPPG alignment.

1117 The number of residues that were uniquely shared between ochrophytes and green algae in
1118 the green gene dataset, and haptophytes and ochrophytes in the haptophyte dataset, were
1119 then tabulated (Table S7¹⁴⁵). Briefly, residues were inferred to be uniquely shared between
1120 ochrophytes and green algae if they were present in at least 2/3 of the ungapped
1121 ochrophyte sequences, one or more green algal sequence, and if none of the red algal or
1122 glaucophyte sequences shared the residue in question, but at least one of these sequences
1123 had a non-matching (i.e. non-gapped) residue at that position (Table S7- sheet 1, section
1124 2¹⁴⁵). Similarly, residues were inferred to be uniquely shared between ochrophytes and
1125 haptophytes if they were present in at least 2/3 of the ungapped haptophyte sequences,
1126 one or more ochrophyte sequence, and if none of the green algal, red algal, glaucophyte or
1127 cyanobacterial sequences shared the residue in question, but at least one of these
1128 sequences had a non-matching (i.e., non-gapped) residue at that position (Table S7- sheet 2,
1129 section 2¹⁴⁵). The origin point of each uniquely shared residue was then inferred by
1130 comparison to reference topologies respectively of green algae¹²⁹ and of ochrophytes (per
1131 Fig. 1). Residues were assumed to have originated in a common ancestor of a particular
1132 clade if that clade contained more lineages with matching than non-matching or gapped
1133 residues (Table S7- sheets 1-2, section 5¹⁴⁵). A second analysis was additionally performed in
1134 which all gapped residues were deemed to be matching, to identify the earliest possible
1135 origin point for each uniquely shared residue, taking into account secondary loss^{45, 50} and
1136 absence of sequences from each alignment^{46,47}.

1138 **Analysis of targeting preferences of ancestral ochrophyte and haptophyte genes.**

1139
1140 Two libraries of non-redundant gene families that were broadly conserved across
1141 ochrophytes or haptophytes, and thus might represent gene products of the ancestral
1142 genomes of these lineages, were generated using a similar BLAST-based assembly pipeline
1143 as used to construct HPPGs (Table S8; Table S14¹⁴⁵). Ochrophyte gene families were deemed
1144 to be conserved if orthologues were detected in one of three different patterns of
1145 ochrophyte sub-categories previously defined to correspond to ancestral plastid-targeted
1146 proteins (Fig. 1, panel B; Table S8- sheet 1, section 3¹⁴⁵). Haptophyte gene families, built
1147 through a similar pipeline using seed sequences from the *Chrysochromulina tobin* and
1148 *Emiliana huxleyi* genomes^{116,117}, were deemed to be ancestral if orthologues were identified
1149 in at least two of the three haptophyte sub-categories considered (pavlovophytes,
1150 prymnesiales, and isochrysidales; Table S14- sheet 1, section 3¹⁴⁵).

1151
1152 The most probable evolutionary origin of each gene family was inferred by BLAST top hit
1153 analysis of the seed sequence (Table S8- sheets 1, 2; Table S14- sheets 1, 2¹⁴⁵). Ochrophyte
1154 sequences were searched against the composite uniref + MMETSP library used to previously
1155 identify the most likely outgroup to each ancestral plastid-targeted protein (Table S8- sheet
1156 1, section 6¹⁴⁵), while haptophyte sequences were searched against the enriched library that
1157 also contained all ochrophyte and cryptomonad sequences, to enable the distinction of
1158 proteins of probable CASH lineage plastid origin from proteins that had evolved through
1159 independent gene transfer events between haptophytes and non-CASH lineage organisms
1160 (Table S14- sheet 1, section 6¹⁴⁵). Targeting preferences for each protein encoded within
1161 each gene family were identified using SignalP v 3.0 and ASAFind v 2.0^{29,106}, and with
1162 HECTAR³⁰, as previously discussed (Table S8- sheet 3; Table S14- sheet 3¹⁴⁵). Targeting
1163 preferences that were identified in a plurality of sequences and in $\geq 2/3$ of the sequences
1164 within each ochrophyte gene family were recorded (Table S8- sheet 2, sections 4-5¹⁴⁵). As
1165 only three haptophyte sequences were assembled for each ancestral haptophyte gene
1166 family, only targeting predictions that were identified in $\geq 2/3$ of the sequences within the
1167 HPPG were inferred to be genuine (Table S14- sheet 2, sections 4-5¹⁴⁵).

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Functional and physiological annotation of ancestral plastid-targeted proteins

Core plastid metabolism pathways were identified using recent reviews of ochrophyte metabolism, or reviews of homologous plant plastid metabolic pathways where ochrophyte-specific reviews have not yet been published^{51, 97, 115, 130-136}. The probable function and KOG classification of each HPPG were annotated using the pre-existing annotations associated with seed protein sequence (if these existed), or if not the annotated function of the top uniref hit previously identified by BLAST searches of the seed sequence (Table S9¹⁴⁵). Expression dynamics for each ancestral HPPG within the genomes of the model diatoms *Phaeodactylum tricornutum* and *Thalassiosira pseudonana* were inferred using microarray data integrated into the DiatomPortal server⁷⁴ (Table S10- sheets 1,2¹⁴⁵). Correlation coefficients were calculated between each pair of *P. tricornutum* and *T. pseudonana* genes that were incorporated into an ancestral HPPG, across all microarray libraries within the dataset (Table S10- sheets 3,4¹⁴⁵), with average values being calculated from all pairwise correlations for different evolutionary categories of protein (Table S10- sheet 5¹⁴⁵).

Possible chimeric proteins, resulting from the fusion of proteins of different evolutionary origins, were identified in the dataset using a modified version of a previously published protocol⁷⁵ (Table S9- sheet 1, sections 4,5; Table S11¹⁴⁵). Each protein within each HPPG was searched using BLASTp against the composite outgroup MMETSP-enriched library, using the same taxonomic classification used for the identification of the evolutionary origin of each seed protein within the dataset, and all hits with an expect value of 1×10^{-05} . Component sequences were then grouped into component families according to the following rule: if two component sequences overlapped by more than 70% of their lengths on the protein composite, they belonged to the same component family. Overlapping and/ or nested component families were additionally merged if one family was included by more than 70% of its length into the other one. Component families were then assigned a broad evolutionary origin corresponding to their taxonomic composition. If the three best component sequences, according to their BLAST bitscore against the composite gene, matched with the same lineage (e.g., green algae, red algae, aplastidic stramenopiles, or other eukaryotes), the component was considered to have originated from that lineage.

Possible dual targeted proteins were identified within the dataset by screening all possible plastid-targeted proteins with Mitofates, using a cut-off targeting threshold of 0.35¹³⁷, which was inferred to be more effective in identifying experimentally verified ochrophyte mitochondria-targeted proteins (Fig. 7- figure supplement 2)²⁹ than other threshold values or targeting prediction programmes such as TargetP¹³⁸ or Mitoprot¹³⁹. The default Mitofates positive cutoff value was modified from 0.38 to 0.35 in order to maximise the capture of experimentally localised mitochondrial proteins, without admitting proteins with unambiguous plastid localisation (Fig. 7- figure supplement 2). As dual targeting to plastids and mitochondria may be achieved either by distinct protein isoforms resulting from ambiguous targeting peptides or alternative internal translation initiation sites that allow production of mitochondrial targeting sequences^{77, 80}, each protein was screened with Mitofates using both the full-length N-termini, and N-termini predicted to result from the next downstream methionine within 30 residues. Possible conserved dual targeted proteins were then identified via the same BLAST-based assembly pipeline and stringency thresholds used to identify probable ancestral HPPGs (Table S12- sheet 1¹⁴⁵). All putative dual targeted proteins have been made publically accessible through the University of Cambridge dSpace server (<https://www.repository.cam.ac.uk/handle/1810/261421>)¹⁴⁵.

1219 **Construction and inspection of concatenated and exemplar phylogenetic trees**

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1221 For the plastid genome phylogenetic analysis, single-gene alignments were constructed by
1222 BLAST searches of published red lineage and glaucophyte plastid genomes (for the gene rich
1223 analysis) or of these genomes plus all MMETSP libraries for the same lineages (for the taxon
1224 rich analysis), using the *Phaeodactylum tricornutum* protein sequence as query and a
1225 threshold e-value of 1×10^{-05} , followed by alignment using GeneIOUS v 4.76¹¹⁹, as before.
1226 The gene rich analysis included protein sequences from 54 genes that were identified in 22
1227 different non-green lineage plastid genomes while the taxon-rich analysis included 10
1228 different plastid genes that were identified in all 22 plastid genomes and at least 30 different
1229 MMETSP libraries³⁴ (Table S15- sheet 1¹⁴⁵). For the taxon-rich analysis, only species that
1230 were represented in $\geq 6/12$ of the single-gene alignments were included in the concatenated
1231 alignment. Each concatenated alignment was trimmed using trimal¹²⁰ using the -gt 0.8
1232 option.

1233

1234 Single-gene alignments for four plastid-targeted proteins predicted to be of polyphyletic
1235 origin in ochrophytes (3-dehydroquinate synthase, isopropylmalate dehydratase,
1236 sedoheptulose biphosphatase, and shikimate kinase) were generated using a similar BLAST-
1237 based assembly and alignment pipeline as used to verify ancestral plastid-targeted proteins.
1238 In this case, all non-redundant (as inferred by BLAST top hit evalue) plastid-targeted
1239 sequences for each protein identified from ochrophyte genomes were used as independent
1240 queries for the identification of plastid-targeted orthologues, 50 uniref top hits, and top hits
1241 from the combined MMETSP and genomic libraries from 36 eukaryotic sub-categories, as
1242 before. HPPGs were independently generated, aligned and trimmed for each seed sequence;
1243 all HPPGs generated for each protein were then merged, realigned and retrimmed using
1244 trimAl to generate a single-gene alignment. Single-gene alignments for each of the
1245 constituent genes in each concatenated plastid genome tree were generated by splitting the
1246 alignment into its component genes. All alignments have been made publically accessible
1247 through the University of Cambridge dSpace server
1248 (<https://www.repository.cam.ac.uk/handle/1810/261421>)¹⁴⁵.

1249

1250 Trees were inferred for each concatenated and exemplar single-gene alignment (Table S15-
1251 sheet 2¹⁴⁵) using the MrBayes and RAxML programmes in-built into the CIPRES web-
1252 server^{121, 140, 141}. Bayesian trees were inferred using three substitution models (GTR, Jones,
1253 and WAG), a minimum of 600000 generations, and an initial burn-in discard value of 0.5.
1254 Trees were only utilised if the final convergence statistic between the two chains run was \leq
1255 0.1, and tree calculation was automatically stopped if the convergence statistic fell below
1256 0.01. RAxML trees were inferred using three substitution models (GTR, JTT, and WAG) with
1257 automatic bootstopping, as previously described⁵⁸. The best tree topology for each RAxML
1258 tree was inferred, and bootstrapping was performed using a burnin value of 0.03.
1259 Alternative tree topologies were tested for the RAxML + JTT tree inferred from each
1260 concatenated alignment using CONSEL¹⁴², under the default conditions. Tree outputs have
1261 been made publically accessible through the University of Cambridge dSpace server
1262 (<https://www.repository.cam.ac.uk/handle/1810/261421>)¹⁴⁵.

1263

1264 Modified alignments were generated for both of the plastid concatenated multigene
1265 datasets from which individual clades of organisms (diatoms, hypogyristera, chrysissta,
1266 haptophytes, cryptomonads, red algae, and different combinations of green algae) had been
1267 removed (Table S15- sheet 2¹⁴⁵). Fast-site removal was performed using TIGER¹⁴³. Site rate
1268 evolution characteristics were calculated for each alignment using the -b 100 option, and
1269 modified alignments were constructed from which the rate categories corresponding to the

1270 fastest evolving 40-50% of sites were serially removed (Table S15- sheet 2¹⁴⁵). Amino acid
1271 composition for each plastid alignment were calculated, and two modified alignments were
1272 generated from which glycines (which in all alignments occur at significantly lower
1273 frequencies in ochrophytes than in haptophytes or cryptomonads; chi-squared, $P \leq 0.05$;
1274 Table S16- sheet 3¹⁴⁵), and from which seven amino acids (alanine, aspartate, glycine,
1275 histidine, leucine, asparagine, threonine and valine) which were found in at least one
1276 alignment to occur at significantly different frequencies in ochrophytes compared to
1277 haptophytes or to cryptomonads ($P \leq 0.05$; Table S16- sheet 3¹⁴⁵) had been removed. Trees
1278 were inferred for each modified alignment using RAxML with the JTT substitution, and
1279 MrBayes with the Jones substitution, and bootstrap calculation as previously described.
1280 Modified alignments and tree outputs have been made publically accessible through the
1281 University of Cambridge dSpace server
1282 (<https://www.repository.cam.ac.uk/handle/1810/261421>)¹⁴⁵.

1283
1284 Uniquely shared residues were manually tabulated for both of the plastid genome multigene
1285 alignments (Table S17¹⁴⁵). For the gene-rich plastid multigene alignment, residues that were
1286 present in all haptophyte sequences and only found in a maximum of one other lineage (red
1287 algae, glaucophytes, cryptomonads, diatoms, hypopyristea, or chryista) were tabulated
1288 (Table S17- sheet 1¹⁴⁵). For the taxon-rich alignment, to take into account gaps and missing
1289 characters, residues were tabulated if they were found in a majority of haptophyte
1290 sequences, and one other lineage, as before (Table S17- sheet 2¹⁴⁵). The total number of
1291 residues shared, and uniquely shared, with each non-haptophyte species and lineage are
1292 respectively tabulated in Table S17, sheets 3 and 4¹⁴⁵.

1293

1294 **Data deposition**

1295

1296 All supporting datasets for this study, including supplementary tables predicted plastid-
1297 targeted and dual targeted protein libraries, single gene and multigene alignments, and tree
1298 outputs, have been made publically and freely accessible through the University of
1299 Cambridge dSpace server (<https://www.repository.cam.ac.uk/handle/1810/261421>)¹⁴⁵.

1300

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1302

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1318

1319 **Competing interests.**

1320

1321 The authors declare no competing financial or non-financial interests in this project.

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1692

1693 **Table 1- Glossary Box**

1694
1695 A schematic figure of eukaryotic taxonomy, showing the evolutionary origins of nuclear and
1696 plastid lineages, adapted from previous reviews³, is shown in Fig. 1- figure supplement 1.
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Complex plastids	Plastids acquired through the endosymbiosis of a eukaryotic alga. These include secondary plastids of ultimate red algal origin (such as those found in ochrophytes, haptophytes and cryptomonads), secondary plastids derived from green algae (such as those found in euglenids or chlorarachniophytes), or tertiary plastids such as those found in dinotoms and certain other dinoflagellates (resulting from the endosymbioses of eukaryotic algae that themselves contain plastids of secondary endosymbiotic origin).
CASH lineages	The four major lineages of algae with plastids of secondary or higher red origin, that is to say <u>C</u> ryptomonads, <u>A</u> lveolates (dinoflagellates, and apicomplexans), <u>S</u> tramenopiles, and <u>H</u> aptophytes.
Stramenopiles	A diverse and ecologically major component of the eukaryotic tree, containing both photosynthetic members (the ochrophytes), which possess complex plastids of red algal origin, and aplastidic and non-photosynthetic members (e.g. oomycetes, labyrinthulomycetes, and the human pathogen <i>Blastocystis</i>), which form the earliest-diverging branches. It is debated when within stramenopile evolution the extant ochrophyte plastid was acquired.
Ochrophytes	Photosynthetic and plastid-bearing members of the stramenopiles, including many ecologically important lineages (diatoms, kelps, pelagophytes) and potential model lineages for biofuels research (<i>Nannochloropsis</i>). Ochrophytes form the most significant component of eukaryotic marine phytoplankton ^{1,2} .
Haptophytes	Single-celled, photosynthetic eukaryotes, possessing complex plastids of

	ultimate red origin. Some haptophytes (the coccolithophorids) are renowned for their ability to form large blooms (visible from space), and to form intricate calcareous shells ^{1,94} , which if deposited on the ocean floor go on to form a major component of limestone and other sedimentary rocks.
HPPG	"Homologous plastid protein group". Proteins identified in this study to possess plastid-targeting sequences that are homologous to one another, as defined by BLAST-based HPPG assembly and single gene phylogenetic analysis.

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Figure Legends

Fig. 1. Procedure for identification of conserved plastid-targeted proteins in ochrophytes.

Panel A shows a schematic unrooted ochrophyte tree, with the three major ochrophyte lineages (chrysisista, hypogyristea, and diatoms) denoted by different coloured labels. "PX" refers to the combined clade of phaeophytes, xanthophytes and related taxa, and "PESC" to pinguiphytes, eustigmatophytes, synchromophytes, chrysophytes and relatives. A global overview of the eukaryotic tree of life, including the position of ochrophytes relative to other lineages is shown in figure supplement 1. **Panel B** shows the number of inferred positive control HPPGs (i.e., HPPGs encoding proteins with experimentally confirmed plastid localisation, or unambiguously plastid function) and negative control HPPGs (i.e., HPPGs encoding proteins with no obvious orthologues in ochrophyte genomes, but found in haptophyte and cryptomonad genomes) detected as plastid-targeted in different numbers of ochrophyte lineages using ASAFind (**i**) and HECTAR (**ii**). The blue bars show the number of positive controls identified to pass a specific conservation threshold, plotted against the left hand vertical axis of the graph, while the red bars show the number of negative controls that pass the same conservation threshold, plotted against the right hand vertical axis of the graph. The number of different sub-categories included in each conservation threshold is shown in a heatmap below the two graphs, with the specific distribution for each bar in the graph shown in the aligned cells directly beneath it. Each shaded cell corresponds to an identified orthologue in one sub-category of a particular ochrophyte lineage: orange cells indicate presence of chrysisistan sub-categories; light brown cells the presence of hypogyristean sub-categories; and dark brown cells the presence of diatom sub-categories. In each graph, black arrows label the conservation thresholds inferred to give the strongest separation (as inferred by chi-squared P-value) between positive and negative control sequences. The table (**iii**) tabulates the three conservation patterns identified as appropriate for distinguishing probable ancestral HPPGs from false positives. **Panel C** shows the complete HPPG assembly, alignment and phylogenetic pathway used to identify conserved-targeted proteins. **Panel D** tabulates the number of HPPGs built using ASAFind and HECTAR predictions, and the number of non-redundant HPPGs identified in the final dataset. The final total represents the pooled total of non-redundant HPPGs identified with both ASAFind and HECTAR.

Fig. 2. Verification of unusual ancestral plastid-targeted proteins. Panel A lists the ten proteins selected for experimental characterisation and their most probable previous localisation prior to their establishment in the ochrophyte plastid, based on the first 50 nr BLAST hits. Exemplar alignments and single-gene tree topologies for some of these proteins are shown in figure supplements 1-4. **Panel B** shows the localisation of GFP constructs for copies of two proteins with an unambiguous plastid localisation (a pyrophosphate-dependent PFK, which localises to the pyrenoid, and a novel plastid protein, with

1740 cosmopolitan distribution across the plastid) and one protein with a periplastid localisation
1741 (a predicted peroxisomal membrane protein) from the diatom *Phaeodactylum tricornutum*,
1742 the diatom endosymbiont of the dinoflagellate *Glenodinium foliaceum* and the
1743 eustigmatophyte *Nannochloropsis gaditana*, expressed in *P. tricornutum*. All scale bars = 10
1744 μm . Expression constructs for seven additional *P. tricornutum* proteins and three additional
1745 *N. gaditana* proteins with multipartite plastid localisations are shown in figure supplements
1746 5 and 6, and control images (wild-type cells, and cells expressing untargeted eGFP) are
1747 shown in figure supplement 7.

1748
1749 **Fig. 3. Evolutionary origins of the ochrophyte plastid proteome.** Panel A displays the origins
1750 inferred by BLAST top hit, phylogenetic analysis, and combined analysis for all ancestral
1751 HPPGs. Panel B shows (i) a schematic diagram of stramenopile taxonomy, with the
1752 evolutionary relationships between labyrinthulomycetes, oomycetes, slopalinids and
1753 ochrophytes proposed by recent multigene studies¹², and the probable closest stramenopile
1754 relative (as inferred by BLAST top hit analysis) of the 26 ancestral HPPGs verified by
1755 combined analysis to be of aplastidic stramenopile origin, and (ii) the next nearest relative,
1756 as inferred through BLAST top hit, phylogenetic and combined analysis, of the 26 aplastidic
1757 stramenopile HPPGs verified by combined analysis. The evolutionary categories in this graph
1758 are shaded as per in panel A.

1759
1760 **Fig. 4. Verification and origins of the green signal in ochrophyte plastids.** Panel A shows a
1761 schematic tree of the 11 archaeplastid sub-categories with which each green HPPG
1762 alignment was enriched prior to phylogenetic analysis. The topology of the red and green
1763 algae are shown according to previously published phylogenies^{129, 144}. Green sub-categories
1764 are in green text; red algal sub-categories in red text; and other sub-categories are in blue
1765 text. Five ancestral positions within the green algal tree inspected in subsequent analyses
1766 are labelled with coloured boxes. Panel B shows the number of HPPGs of verified red (red
1767 bars) or green origin (green bars) for which orthologues were identified in different numbers
1768 green sub-categories (plotted on the x-axis) and red sub-categories (plotted on the z-axis).
1769 An equivalent graph showing only HPPGs for which a glaucophyte orthologue was detected
1770 is shown in figure supplement 1. Panel C compares the number of trees in which HPPGs of
1771 verified green origin resolve as a sister group to all green lineages (including chlorophytes
1772 and streptophytes); to multiple chlorophyte sub-categories but to the exclusion of
1773 streptophytes; and to individual chlorophyte sub-categories only. A detailed heatmap of the
1774 evolutionary distribution of the green sub-categories detected in each sister-group is shown
1775 in figure supplement 2, and the distribution of BLAST top hits within each sub-category is
1776 shown in figure supplement 3. Panel D lists the number of residues inferred from a dataset
1777 of 32 ochrophyte HPPGs of verified green origin, which have been subsequently entirely
1778 vertically inherited in all major photosynthetic eukaryotic lineages, to be uniquely shared
1779 between ochrophytes and some but not all green lineages, hence might represent specific
1780 synapomorphic residues. Residues are categorized by inferred origin point within the tree
1781 topology shown in panel A, i.e., each of the five ancestral nodes labelled. A final category
1782 shows all of the residues inferred to be specifically shared with one green sub-category, and
1783 not with any other. The distribution of residues based on the earliest possible origin point
1784 (taking into account gapped and missing residues in each HPPG alignment) is shown in figure
1785 supplement 4. Panel E shows the number of the 7140 conserved gene families inferred to
1786 have been present in the last common ochrophyte ancestor that are predicted by ASAFind
1787 to encode proteins targeted to the plastid, subdivided by probable evolutionary origin, and
1788 the number expected to be present in each category assuming a random distribution of
1789 plastid-targeted proteins across the entire dataset, independent of evolutionary origin.
1790 Evolutionary categories of proteins found to be significantly more likely (chi-squared test,

1791 P=0.05) to encode plastid-targeted proteins than would be expected are labelled with black
1792 arrows. An equivalent distribution of plastid-targeted proteins inferred using HECTAR is
1793 shown in figure supplement 5.
1794

1795 **Fig. 5. Functional mixing of the ancestral ochrophyte HPPGs.** **Panel A** tabulates nineteen
1796 different fundamental plastid metabolism pathways and biological processes recovered in
1797 the ancestral HPPG dataset. Detailed information concerning the origin and identity of each
1798 component of each pathway is provided in figure supplement 1, and an overview and
1799 phylogenetic trees of each of the non-vertically inherited enzymes identified are provided in
1800 figure supplements 2-6. **Panel B** compares the distribution of individual KOG families in the
1801 complete HPPG library, the ancestral HPPG dataset, and HPPGs of verified prokaryotic origin.
1802 KOG families pertaining to metabolism are shown in shades of green, families pertaining to
1803 information storage are shown in shades of red, and families pertaining to cellular processes
1804 are shown in shades of blue. Families with unknown KOG classification or general function
1805 predictions only are not shown. KOG classes that are enriched in the ancestral HPPG dataset
1806 compared to relative proportions of each KOG class found in the full HPPG dataset, or in
1807 individual ancestral HPPGs of prokaryotic origin compared to the ancestral HPPG dataset (as
1808 inferred by chi-squared test, $P < 0.05$), are labelled with black horizontal arrows. No such
1809 enrichments were observed in any evolutionary category of ancestral HPPGs other than
1810 prokaryotes, hence analogous distributions of HPPGs of red algal, green algal and host origin
1811 are not shown. Overviews of the broader KOG classes that are enriched either in the
1812 ancestral HPPG dataset, or in specific evolutionary categories of ancestral HPPG, are shown
1813 in figure supplement 7. **Panel C** tabulates the number of ancestral HPPGs performing
1814 consecutive metabolic functions, or that are likely to have direct regulatory interactions,
1815 alongside the number of these protein pairs in which both members are of verified
1816 evolutionary origin; the number observed where both members possess the same
1817 evolutionary origin; the expected number of protein pairs where both members possess the
1818 same evolutionary origin; and the chi-squared probability of similarity between the observed
1819 and expected values. **Panel D** shows heatmaps for the pairwise correlation coefficients of
1820 expression for genes encoding different evolutionary categories, as verified using combined
1821 BLAST top hit and single-gene tree analysis, of ancestral HPPGs in the model diatoms
1822 *Phaeodactylum tricornutum* (i) and *Thalassiosira pseudonana* (ii). A scale bar showing the
1823 relationship between shading and correlation coefficient is shown to the right of the
1824 heatmaps. Boxplots comparing the individual expression profiles of different categories of
1825 ancestral HPPG, and the associated ANOVA P values calculated, are shown in figure
1826 supplements 8 (for *P. tricornutum*) and 9 (for *T. pseudonana*).
1827

1828 **Fig. 6. Origins of chimeric proteins in the ochrophyte plastid.** **Panel A** tabulates eight
1829 ancestral HPPGs containing domains of cyanobacterial and non-cyanobacterial origin, as
1830 previously identified by Méheust et al⁷⁵ that were inherited by the ochrophyte plastid, and
1831 two chimeric ancestral HPPGs which are probably of specific ochrophyte origin. **Panel B**
1832 shows a complete tabulated list of all ancestral HPPGs (listed by identifier, with the
1833 predicted function given in brackets) in which at least one chimerism event between
1834 domains of red algal, green algal, aplastidic stramenopile, other eukaryotic, and prokaryotic
1835 origin was detected. In each case, the inferred evolutionary origins of the N-terminal (NTD)
1836 and C-terminal (CTD) components of the chimeric members of the HPPG are given,
1837 according to the colour key within the figure, followed by its distribution across all
1838 ochrophyte lineages. The two chimeric HPPGs inferred to have arisen in the ochrophyte
1839 ancestor are shown in bold text and labelled with horizontal arrows. Exemplar alignments
1840 and phylogenies of the two chimeric proteins inferred to have originated in the ochrophyte
1841 ancestor are shown in figure supplements 1-3.

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Fig. 7. Ancient and bidirectional connections between the ochrophyte plastid and mitochondria. Panel A shows Mitotracker-Orange stained *P. tricornutum* lines expressing GFP fusion constructs for the N-terminal regions of histidyl- and prolyl-tRNA synthetase sequences from *P. tricornutum* and the eustigmatophyte *Nannochloropsis gaditana*. Targeting constructs for an additional four dual targeted proteins in *P. tricornutum* and one dual targeted protein in *G. foliaceum*, alongside Mitotracker-negative and wild type control images, are shown in figure supplement 1. Panel B profiles the predicted evolutionary origins of the 34 ancestral dual targeted HPPGs, as inferred by BLAST top hit and single-gene tree analysis. Data supporting the thresholds used to identify probable dual targeted HPPGs *in silico* are supplied in figure supplement 2. Panel C shows seven classes of tRNA synthetase for which only two copies were inferred in the genome of the last common ochrophyte ancestor. Evolutionary origins are inferred from combined BLAST top hit and single-gene tree analysis for dual targeted proteins, and from BLAST top hit analysis alone for cytoplasmic proteins. In five cases the dual targeted isoform is inferred to be of ultimate red algal origin, indicating that a protein derived from the endosymbiont has functionally replaced the endogenous host mitochondria-targeted copy.

Fig. 8. Footprints of an ancient endosymbiosis in the haptophyte plastid proteome. Panel A indicates the number of ancestral ochrophyte HPPGs that included sequences from other algal lineages in single-gene tree analyses, and whether those algal lineages branched within or external to ochrophytes. An overview of the specific origins of proteins of ochrophyte origin in each lineage is shown in figure supplement 1. Panel B compares the number of ASAFind-derived HPPGs that are uniquely shared between hypogyristera (i) or haptophytes (ii) and one other CASH lineage. Values are given for proteins found in a majority of sub-categories in hypogyristera/ haptophytes and at least one sub-category from only one other lineage (light bars), and proteins found in a majority of sub-categories in hypogyristera/ haptophytes and a majority of sub-categories from only one other lineage (dark bars). Values that are significantly greater than would be expected through random distribution are labelled with black arrows. Panel C shows a schematic ochrophyte tree, with six different ancestral nodes within this tree labelled with coloured boxes, and the most probable origin point for each of the 243 haptophyte plastid-targeted proteins of probable ochrophyte origin within this tree, as inferred by inspection of the nearest ochrophyte sister-group in single-gene trees. A detailed heatmap of the ochrophyte sub-categories contained in each lineage is shown in figure supplement 2, and BLAST top hit analyses corresponding to each plastid-targeted protein are shown in figure supplement 3. Panel D shows the number of residues that are uniquely shared between haptophytes and each node of the ochrophyte tree for 37 genes in which there has been a clear transfer from ochrophytes to haptophytes, and entirely vertical subsequent inheritance. A similar graph, showing the earliest possible inferred origin of each uniquely shared residue, is shown in figure supplement 4. Panel E shows the number of the 12728 conserved gene families inferred to have been present in the last common haptophyte ancestor that are predicted by ASAFind to encode proteins targeted to the plastid, subdivided by probable evolutionary origin, and the number expected to be present in each category assuming a random distribution of plastid-targeted proteins across the entire dataset, independent of evolutionary origin. Evolutionary categories of proteins found to be significantly more likely (chi-squared test, P=0.05) to encode plastid-targeted proteins than would be expected by random distribution are labelled with black arrows. The evolutionary origins of the ancestral gene families are shown in figure supplement 5.

1892 **Fig. 9. Non-ochrophyte origins of the haptophyte plastid genome. Panels A and B,**
1893 respectively, show gene-rich and taxon-rich phylogenies of plastid-encoded proteins from
1894 red algae and plastids of red algal origin with the glaucophyte *Cyanophora paradoxa* as
1895 outgroup. **Panel A:** Combined Bayesian and Maximum Likelihood analysis (MrBayes +
1896 RAxML, GTR, JTT, WAG) of a 22 taxa x 12103 aa alignment of 54 proteins encoded by all
1897 published red and red-derived plastid genomes. **Panel B:** analysis of a 75 taxa x 3737 aa
1898 alignment of 10 conserved plastid-encoded proteins detectable in a broad range of red
1899 lineage MMETSP libraries. Nodes resolve with robust support (posterior probabilities of 1 for
1900 all Bayesian trees and > 80% bootstrap support for all ML trees) are shown with filled circles;
1901 individual support values for each analysis are shown for the remaining nodes are shown as
1902 detailed in the box below panel B. Alternative topology tests, the results of fast-site and
1903 clade deduction analysis for each tree, and heatmap comparisons of sister-group
1904 relationships identified for single-gene trees of each constituent gene within each
1905 concatenated alignment are shown in figure supplements 1-3. **Panel C** shows the number of
1906 residues in each alignment that are uniquely shared between haptophytes and only one
1907 other lineage. For the gene-rich alignment (i), which is gap-free, residues are included that
1908 are found in all four haptophyte sequences and at least one sequence from the lineage
1909 under consideration. For the taxon-rich alignment (ii), to account for the presence of gapped
1910 positions, residues are included that are found in at least 11 of the 22 haptophyte sequences
1911 and at least one sequence from the lineage under consideration.

1912
1913 **Fig. 10. Schematic diagram of events giving rise to the ancestral ochrophyte plastid**
1914 **proteome.** Each cell diagram depicts a different stage in the ochrophyte plastid
1915 endosymbiosis; each protein depicted represents on or more proteins inferred in this study
1916 to have been nucleus-encoded and plastid-targeted in the last common ancestor of all
1917 ochrophytes. An ancient ochrophyte ancestor, which had already diverged from oomycetes
1918 and other aplastidic stramenopile relatives, and which may have possessed a green algal
1919 plastid (A), acquired a red lineage plastid via secondary or higher endosymbiosis (B). Both
1920 the host and the endosymbiont are likely to have been evolutionary chimeras, possessing
1921 proteins encoded by genes acquired from endosymbiotic and/or lateral gene transfer
1922 events. Both host and symbiont are additionally likely to have possessed chimeric proteins,
1923 generated through the fusion of genes of different evolutionary origins, and a large number
1924 of mitochondrial-, ER- and (in the case of the red endosymbiont) potentially dual targeted
1925 proteins. Following genetic integration of the red endosymbiont with its stramenopile host,
1926 the first ochrophytes (C) thus possessed a wide range of proteins of plastid function
1927 acquired from different sources, with no apparent functional bias in the types of proteins
1928 that were retained from different sources. Chimeric proteins and dual targeted proteins,
1929 either acquired directly from the endosymbiont, or generated de novo, were also
1930 widespread features of this ancestral plastid proteome. Detailed information regarding the
1931 relationship between ultimate the evolutionary origins of each HPPG, and its presence or
1932 absence in other CASH lineages, is provided in figure supplement 1. A schematic diagram of
1933 possible models through which the haptophyte plastid may have originated is shown in
1934 figure supplement 2.

1935
1936 **Supporting figure and dataset legends.**

1937
1938 **Fig. 1- figure supplement 1. Overview of eukaryotic diversity.** This figure, adapted from a
1939 previous review³, profiles the diversity of different eukaryotic nuclear lineages. Each grey
1940 ellipse corresponds to one major clade, or “supergroup” of eukaryotes. A brown ellipse
1941 within the stramenopile clade delineates the ochrophyte lineages. Dashed lines denote
1942 uncertain taxonomic relationship. For each taxon, a type species (defined either by the

1943 presence of a complete genome, extensive transcriptome library, or of particular anthropic
1944 significance) is given in brackets. Taxa that lack plastids are labelled in grey, and taxa with
1945 plastids are shaded according to the evolutionary origin of that plastid lineage.

1946
1947 **Fig. 2- figure supplement 1- Exemplar ochrophyte plastid protein alignments.** This figure
1948 shows untrimmed GeneIOUS alignments for two ancestral HPPGs of unusual provenance. In
1949 each case the full length of the protein (labelled i) and N-terminal region only (ii) are shown,
1950 demonstrating the broad conservation of the N-terminus position. Sequences for which
1951 exemplar targeting constructs (*Phaeodactylum tricornutum*, *Nannochloropsis gaditana*,
1952 *Glenodinium foliaceum*) are shown at the top of each alignment.

1953
1954 **Fig. 2- figure supplement 2. Tree of ochrophyte glycyl-tRNA synthetase sequences.** This
1955 tree shows the consensus unrooted Bayesian topology for a 95 taxa x 487 aa alignment of
1956 glycyl tRNA synthetase sequences. The font colour of each sequence corresponds to the
1957 taxonomic origin (see legend below for details) and are labelled with the taxonomic
1958 identifiers previously defined in Table S1. Sequences labelled with chl_ possess apparent
1959 plastid targeting sequences recognisable by CASH lineage plastids. The ancestral ochrophyte
1960 plastidic isoform, of apparent chlamydiobacterial origin, is labelled with a blue ellipse. Black
1961 circles at each node denote posterior probabilities of 1.0 in Bayesian inferences with three
1962 different substitution matrices (GTR, Jones, and WAG), and grey circles indicate posterior
1963 probabilities of 0.8 with at least two of these matrices. Support values for all remaining
1964 nodes, using both Bayesian and RAxML analysis, is provided in the form MrBayes posterior
1965 probabilities: GTR/Jones/WAG RAxML best tree likelihoods: GTR/ JTT/ WAG

1966
1967 **Fig. 2- figure supplement 3. Tree of ochrophyte pyrophosphate dependent phosphofructo-**
1968 **1- kinase sequences.** This tree shows the consensus Bayesian topology inferred for a 94 taxa
1969 x 449 aa alignment of pyrophosphate-dependent PFK, with taxa and support values shown
1970 as per Fig. 2, figure supplement 2. The ancestral ochrophyte plastid isoform, of probable
1971 aplastidic stramenopile origin, is labelled with a cyan ellipse.

1972
1973 **Fig. 2- figure supplement 4. Tree of a novel ochrophyte plastid-targeted protein.** This tree
1974 shows the consensus Bayesian topology inferred for a 16 taxa x 103 aa alignment of a
1975 plastid-targeted protein seemingly restricted to ochrophytes and one dinoflagellate lineage.
1976 Taxa are labelled and support values are shown as per fig. 2- figure supplement 2.

1977
1978 **Fig. 2- figure supplement 5. Multipartite *Phaeodactylum* plastid-targeted proteins.** This
1979 figure shows the localisation of GFP overexpression constructs for copies of seven proteins
1980 from the diatom *Phaeodactylum tricornutum* that are of non-plastid origin, but show
1981 multipartite localization to the plastid and one other organelle (the mitochondria, or in the
1982 case of the “ER heat shock protein” to the endoplasmic reticulum).

1983
1984 **Fig. 2- figure supplement 6. Heterologous expression constructs of multipartite plastid-**
1985 **targeted proteins.** This figure shows the localisation of GFP overexpression constructs for
1986 copies of two proteins from the dinotom *Glenodinium foliaceum* (**Panel A**), and three
1987 proteins from the eustigmatophyte *Nannochloropsis gaditana* (**Panel B**) that are of non-
1988 plastid origin, but show multipartite localisation to the plastid and one other organelle, per
1989 Fig. 2, figure supplement 5.

1990
1991 **Fig. 2- figure supplement 7. Exemplar control images for confocal microscopy.** This figure
1992 shows fluorescence patterns for wild-type *Phaeodactylum tricornutum* cells (i), and
1993 transformant *Phaeodactylum* cells expressing GFP that has not been fused to any N-terminal

1994 targeting sequence (ii), both visualised under the same conditions used for all other
1995 transformant cultures.

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Fig. 4- figure supplement 1. Sampling richness associated with ancestral HPPGs of green algal origin. This figure shows the number of sub-different archaeplastid orthologues for ancestral HPPGs verified by combined BLAST top hit and single-gene tree analysis to be of either green algal origin (green bars) or red algal origin (red bars), for which glaucophyte orthologues could also be identified.

Fig.4- figure supplement 2. Heatmaps of nearest sister-groups of ancestral HPPGs of verified green origin. This figure shows the specific topologies of single gene trees for HPPGs verified to be of green origin by combined BLAST and phylogenetic analysis. **Panel A** shows a reference topology of evolutionary relationships between green lineages, defined as per Leliaert et al. 2011. Six ancestral nodes that might correspond to the origin point of ochrophyte HPPGs are labelled with coloured boxes. **Panel B** shows the presence and absence of each green subcategory in the immediate sister-group to the ochrophyte HPPG in each single tree of HPPGs of verified origin. HPPGs are grouped by the inferred origin point within the green algae, with the number of HPPGs identified for each origin point given with round brackets.

Fig. 4- figure supplement 3. Specific origins of green HPPGs as inferred from BLAST top hit analyses. These charts show (i) the number of BLAST top hits against each of the individual green sub-categories from HPPGs for which a green origin was identified both from BLAST top hit and single-gene tree analysis, and (ii) the total number of non-redundant sequences from each green sub-category included in the BLAST library.

Fig. 4- figure supplement 4. Earliest evolutionary origins of shared plastid residues. This figure shows the number of residues in the concatenated alignment of HPPGs of verified green origin, which have been subsequently vertically inherited in all major photosynthetic eukaryotes that are present in green algae and ochrophytes, and are not found in red algae and glaucophytes. Residues are divided by inferred origin point, and are shown as per fig. 4, panel D. The values here are calculated as the earliest possible origin point for each uniquely shared residue, in which all gapped and missing positions within the alignment are treated as potential identities. 100 of the 147 residues inferred to have originated within green algae in this analysis originated either within a common ancestor of all chlorophytes, or in a common ancestor of all chlorophytes excluding the basally divergent lineages *Prasinoderma*, *Prasinococcus* and *Nephroselmis*.

Fig. 4- figure supplement 5. Origins and HECTAR based targeting tests of proteins encoded by conserved ochrophyte gene clusters. **Panel A** shows the most probably evolutionary origin, identified using BLAST top hit analysis, for 7140 conserved gene clusters inferred to have been present in the last common ochrophyte ancestor. **Panel B** shows the number of these gene families that are predicted by HECTAR to encode proteins targeted to the plastid, subdivided by probable evolutionary origin, and the number expected to be present in each category assuming a random distribution of plastid-targeted proteins across the entire dataset, independent of evolutionary origin. Categories inferred to be significantly enriched above the expected values are labelled with black arrows.

Fig. 5- figure supplement 1. Reconstructed metabolism pathways and core biological processes in the ancestral ochrophyte plastid. This figure tabulates each of the ancestral ochrophyte HPPGs corresponding to 350 central plastid metabolism and other biological

2045 processes. The "origin" column shows the probable evolutionary source for each HPPG as
2046 defined by combined BLAST tophit and single-gene tree analysis. The origin of each ancestral
2047 HPPG is either assigned a "high confidence" value (in which the same origin was robustly
2048 supported both by single-gene tree and by BLAST tophit analysis) or a "low confidence"
2049 value (in the absence of robust and consistent support through both techniques;
2050 corresponding to the tree sister-group if one could be clearly assigned, or the BLAST tophit
2051 identity if not). A dash indicates the corresponding protein was not identified in the
2052 ancestral HPPG dataset due to either being plastid-encoded or alternative reasons; detailed
2053 explanations for the enzymes that are neither plastid-encoded nor detected in the ancestral
2054 HPPG dataset are provided in figure supplement 2.

2055

2056 **Fig. 5- figure supplement 2. Core plastid metabolism proteins not identified within the**
2057 **ancestral HPPG dataset.**

2058

2059 **Fig. 5 - figure supplement 3. Tree of ochrophyte sedoheptulose- 7-bisphosphatase**
2060 **sequences.** This figure shows the consensus Bayesian topology inferred for a 218 taxa x 303
2061 aa alignment of sedoheptulose-7-bisphosphatase sequences, shown as per fig. 2, figure
2062 supplement 2. Two different ochrophyte plastid isoforms- one restricted to chrysoista, and of
2063 probable red algal origin, and one found in hypogyrystea and diatoms, of probable green
2064 algal origin- are shown respectively by red and green ellipses.

2065

2066 **Fig. 5- figure supplement 4. Tree of ochrophyte 3-dehydroquinase synthase sequences.**
2067 This figure shows the consensus Bayesian topology inferred for a 324 taxa x 387 aa
2068 alignment of 3-dehydroquinase synthase, shown as per fig. 2, figure supplement 2. Three
2069 ochrophyte plastid isoforms are shown with coloured ellipses: a probable bacterial isoform
2070 restricted to pelagophytes and dictyochophytes (blue ellipse), and two isoforms of
2071 ambiguous red/ green origin found respectively in raphidophytes and eustigmatophytes, and
2072 in diatoms (green ellipses with red borders).

2073

2074 **Fig. 5 - figure supplement 5. Tree of ochrophyte isopropylmalate dehydrogenase**
2075 **sequences.** This tree shows the consensus Bayesian phylogeny inferred for a 202 taxa x 592
2076 aa alignment of isopropyl malate dehydrogenase sequences, shown as per fig. 2- figure
2077 supplement 2. Two ochrophyte plastid isoforms are shown with coloured ellipses: an
2078 isoform of green algal origin restricted to diatoms and hypogyrystea (green ellipse), and a red
2079 algal isoform found in diatoms, pelagophytes and xanthophytes (red ellipse).

2080

2081 **Fig. 5- figure supplement 6. Tree of ochrophyte shikimate kinase sequences.** This figure
2082 shows the consensus Bayesian topology inferred for a 127 taxa x 262 aa alignment of
2083 shikimate kinase sequences. The WAG Bayesian topology was excluded from the consensus
2084 due to non-convergence between the two chains, hence the tree is produced from the
2085 consensus of GTR and Jones substitution matrices only, but is otherwise presented
2086 identically to fig. 2, figure supplement 2. Two distinct ochrophyte plastid isoforms are shown
2087 with coloured ellipses: a green algal isoform conserved across diatoms, dictyochophytes and
2088 raphidophytes (red ellipse), and a pelagophyte isoform of uncertain origin (grey ellipse).

2089

2090 **Fig. 5- figure supplement 7. KOG classes associated with different categories of HPPGs.**
2091 These pie charts profile the distribution of different KOG classes across (i) all HPPGs except
2092 for those with general function predictions only, or without any clear KOG function, (ii) the
2093 same, but restricted to ancestral HPPGs and (iii) the same, for ancestral HPPGs of
2094 unambiguous red, green, prokaryotic and aplastidic stramenopile origin as identified by
2095 combined BLAST top hit and single-gene tree analysis. KOG classes that occur at elevated

2096 frequency in the ancestral HPPG dataset compared to the complete HPPG dataset, and one
2097 KOG class enriched in the prokaryotic HPPG dataset compared to the ancestral HPPG dataset
2098 (chi-squared test, $P < 0.05$) are labelled with horizontal arrows.
2099

2100 **Fig. 5- figure supplement 8. Coregulation of genes incorporated into HPPGs of different**
2101 **origin in the model diatom *Phaeodactylum tricornutum*.** Panel A shows boxplots of the
2102 correlation coefficients between the expression profiles of genes encoding members of
2103 ancestral HPPGs of red algal origin (i), green algal origin (ii), prokaryotic origin (iii) or host
2104 origin (iv), compared to genes encoding members of other HPPGs. Each HPPG is separated
2105 by evolutionary origin on the x-axis of each graph: for example, the box labelled “green
2106 algae” on the “red algae” graph shows the correlation coefficients between genes encoding
2107 members of ancestral HPPGs of red origin, and ancestral HPPGs of green origin. Panel B
2108 shows the P value statistics of mean separation calculated when comparing genes encoding
2109 members of ancestral HPPGs of the same origin (shown by row) to members of ancestral
2110 HPPGs of different origin (shown by column). For example, the intersect between the “red”
2111 row and “green” column shows the difference in mean correlation coefficient between pairs
2112 of genes that both encode members of ancestral HPPGs of red origin, and gene pairs of
2113 which one encodes an ancestral HPPG member of red origin, and the other an ancestral
2114 HPPG member of green origin. None of the P values calculated are significant, i.e. there are
2115 no categories of ancestral HPPG in which the internal correlation coefficients of gene
2116 expression are any different to those observed across the dataset as a whole.
2117

2118 **Fig. 5- figure supplement 9. Coregulation of genes incorporated into HPPGs of different**
2119 **origin in the model diatom *Thalassiosira pseudonana*.** Boxplots (Panel A) and P value
2120 statistics (Panel B) are shown as per Fig. 5- figure supplement 8. Only two of the correlation
2121 value ANOVA tests (comparison of red-red and red-host correlations, and prokaryotic-
2122 prokaryotic and prokaryotic-host correlations, shaded in green) reveal a significantly higher
2123 correlation coefficient between pairs of genes encoding members of HPPG of the same
2124 evolutionary origin than pairs of genes encoding members of HPPGs with different
2125 evolutionary origins. These differences most probably reflect the extremely weak correlation
2126 coefficients associated with genes encoding HPPGs of host origin to all other genes
2127 considered (compare “Host” category on boxplots i, ii and iii to all other categories);
2128 however, detailed comparison of the correlation values between genes encoding ancestral
2129 HPPGs of host origin and genes encoding ancestral HPPGs of different evolutionary origin
2130 (Panel A, boxplot iv; Panel B, bottom row) reveals no specific difference in the pairwise
2131 correlation values observed between genes encoding ancestral HPPGs of host origin, and
2132 genes encoding ancestral HPPGs of all other origins within the dataset.
2133

2134 **Fig. 6- figure supplement 1. Alignments of an ochrophyte-specific riboflavin biosynthesis**
2135 **fusion protein.** Panel A shows alignments of the full length (i) and cyclohydrolase domain
2136 only (ii) of a plastid-targeted GTP cyclohydrolase II/ 3,4-dihydroxy-2-butanone 4-phosphate
2137 synthase protein conserved across the ochrophytes. Coloured bars adjacent to each
2138 sequence correspond to the phylogenetic identity of the sequence. The cyclohydrolase
2139 domain of the ochrophyte protein is positioned in the N-terminal region, and the synthase
2140 domain in the C-terminal region. Three uniquely shared residues at the N-terminus of the
2141 cyclohydrolase domain confirm that it has been inherited from the aplastidic stramenopile
2142 ancestor of the ochrophytes.
2143

2144 **Fig. 6- figure supplement 2. Origins of ochrophyte plastid 3,4-dihydroxy-2-butanone 4-**
2145 **phosphate synthase.** This figure shows the consensus Bayesian topology inferred for a 22
2146 taxa x 206 aa alignment of 3,4-dihydroxy-2-butanone 4-phosphate synthase domains from

2147 different lineages, inferred using Jones and WAG matrices, and shown as per fig. 2, figure
2148 supplement 2. The ochrophyte plastid isoforms branch with red algal and actinobacterial
2149 sequences.

2150

2151 **Fig. 6- figure supplement 3. An ochrophyte-specific Tic20 fusion protein.** This figure shows
2152 alignments of the full length (i) and conserved region only (ii) of plastid Tic20 sequences,
2153 displayed as per figure supplement 1.

2154

2155 **Fig. 7- figure supplement 1. Experimental verification of additional ochrophyte dual-**
2156 **targeted proteins. Panel A** shows Mitotracker-orange stained *Phaeodactylum tricornerutum*
2157 lines expressing four additional dual-targeted proteins (glycyl-, leucyl-, and methionyl-tRNA
2158 synthetases, and a predicted mitochondrial GroES-type chaperone) from *Phaeodactylum*
2159 *tricornerutum*, and a dual-targeted histidyl-tRNA synthetase from *Glenodinium foliaceum*.

2160 **Panel B** shows control images that confirm an absence of crosstalk between GFP and
2161 Mitotracker: wild-type *Phaeodactylum* cells stained with Mitotracker, and cells expressing
2162 the *Glenodinium* histidyl-tRNA synthetase–GFP fusion construct and visualised with the
2163 Mitotracker laser and channel in the absence of Mitotracker stain.

2164

2165 **Fig. 7- figure supplement 2. Comparison of different in silico targeting prediction**
2166 **programmes for the identification of dual-targeted ochrophyte proteins. Panel A** shows
2167 Mitofates scores for ochrophyte proteins verified experimentally to be dual targeted in this
2168 and a previous study⁹. **Panel B** shows Mitofates scores for all ochrophyte proteins for which
2169 a subcellular localisation has been identified in previous studies. The red lines in each graph
2170 show the Mitofates default cutoff (0.385) and the green lines indicate our chosen cutoff
2171 (0.35). **Panel C** compares different in silico targeting prediction algorithms with respect to
2172 predicted mitochondrial localization by experimentally validated localization. Mitofates
2173 strikes the best balance between high true positives and low false positives.

2174

2175 **Fig. 8- figure supplement 1. Origin of proteins of ochrophyte origin in different CASH**
2176 **lineages.** This figure profiles the evolutionary origins of proteins inferred by single-gene
2177 phylogenetic analysis to have been transferred from the ochrophytes into other lineages
2178 that have acquired plastids through secondary or more complex endosymbioses. Proteins
2179 are divided into the three major ochrophyte lineages (i.e. diatoms, chrysiata, and
2180 hypopyristea); all remaining proteins (inferred to have been acquired from an ancestor of
2181 multiple ochrophyte lineages, or of ambiguous but clearly ochrophyte origin) are grouped as
2182 a final category. The haptophyte proteins that could be attributed to a specific ochrophyte
2183 lineage are particularly skewed (100/178 proteins) to origins within the hypopyristea.

2184

2185 **Fig.8- figure supplement 2. Heatmaps of nearest sister-groups to haptophytes in ancestral**
2186 **ochrophyte HPPG trees.** This figure shows the specific ochrophyte lineages implicated in the
2187 origin of haptophyte plastid-targeted proteins, as inferred from the nearest ochrophyte
2188 sister-groups to haptophytes in trees of 242 haptophyte proteins of probable ochrophyte
2189 origin from combined BLAST top hit and single-gene tree analysis. At the top a schematic
2190 tree diagram of the ochrophytes is shown as per fig. 1, with six major nodes in ochrophyte
2191 evolution labelled with coloured boxes. The heatmap below shows the specific distribution
2192 of sister-groups in each tree, shown as per figure 4- figure supplement 2.

2193

2194 **Fig. 8- figure supplement 3. Internal evolutionary affinities of haptophyte plastid-targeted**
2195 **proteins incorporated into ancestral ochrophyte HPPGs.** This figure profiles the
2196 evolutionary origins of haptophyte plastid-targeted proteins incorporated into ancestral
2197 ochrophyte HPPGs by BLAST top hit analysis. Separate values are provided for query

2198 sequences from each of the three haptophyte sub-categories (pavlovophytes,
2199 prymnesiophytes, and isochrysidales) considered within the analysis. Only sequences for
2200 which a consistent origin could be identified by both BLAST top hit and single-gene tree
2201 analysis are included. For each haptophyte lineage > 50% of the sequences verified by
2202 combined analysis to be of a specific ochrophyte origin have either pelagophyte or
2203 dictyochophyte top hits.

2204

2205 **Fig. 8- figure supplement 4. Evidence for gene transfer from pelagophytes and**
2206 **dictyochophytes into haptophytes.** Panel A shows the next deepest sister groups identified
2207 for haptophyte proteins of hypogyrustean origin in single-gene trees. The pie chart (i)
2208 compares the number of single-gene trees in which the combined clade of haptophyte and
2209 hypogyrustean proteins resolves within a larger clade comprising the ochrophyte HPPG,
2210 compared to the number that resolves in external positions, either with other lineages or as
2211 a sister-group to all other sequences within the HPPG clade. Sequences for which no clear
2212 next deepest sister group affinity could be identified are listed as “not determined”. The
2213 heatmap (ii) shows the specific sister-group sequences associated with 65 HPPGs in which
2214 the haptophyte sequences specifically resolve with the pelagophyte/ dictyochophyte clade
2215 and for which a clear internal or external position for the haptophyte/ hypogyrustean group
2216 relative to the remaining ochrophyte HPPG clade could be identified. Both analyses indicate
2217 a clear bias for haptophyte sequences branching within a deeper ochrophyte clade, not just
2218 restricted to the immediate sister-groups. Panel B tabulates the BLAST next best hits for
2219 haptophyte sequences for which a phylogenetically consistent (>3 consecutive top hits) top
2220 hit to hypogyrustea could be identified, and pelagophyte/ dictyochophyte sequences for
2221 which a phylogenetically consistent top hit to haptophytes could be identified. In each case
2222 either the largest number of sequences, or (in the case of pavlovophytes) the joint largest
2223 number of sequences for which a phylogenetically consistent next best hit could be
2224 identified resolved with diatoms, indicating that these sequences were probably present in
2225 the common ancestor of diatoms and hypogyrustea, and subsequently transferred to the
2226 haptophytes.

2227

2228 **Fig. 8- figure supplement 5. Earliest possible origin points of uniquely conserved sites in**
2229 **haptophyte plastid-targeted proteins.** This figure shows the total number of residues that
2230 are uniquely shared between a 37 proteins that have clearly been transferred between the
2231 ochrophytes and haptophytes, and are of subsequently entirely vertical origin, assuming the
2232 earliest possible origin point for each residue (i.e. in which gapped or missing positions were
2233 interpreted as identities). 87/ 128 of the uniquely shared residues inferred to originate
2234 within the ochrophytes were congruent to gene transfers between the haptophytes and
2235 pelagophyte and dictyochophyte clade; of these, slightly more than half (46) are inferred to
2236 have originated in a common ancestor of all hypogyrustea and diatoms, consistent with the
2237 gene transfer having occurred from an ancestor of the pelagophytes and dictyochophytes
2238 into the haptophytes, rather than the converse.

2239

2240 **Fig. 8- figure supplement 6. Evolutionary origin of ancestral haptophyte genes.** This figure
2241 shows the most likely evolutionary origin assigned by BLAST top hit analysis to the 12728
2242 conserved gene families inferred to have been present in the last common haptophyte
2243 ancestor.

2244

2245 **Fig. 9- figure supplement 1. Alternative topology tests of plastid genome trees.** Tests were
2246 performed with the RAxML + JTT trees inferred for the gene-rich (panel A) and taxon-rich
2247 (panel B) plastid-encoded protein alignments. In each case, a schematic diagram of the tree
2248 topology obtained is given (i). The black box corresponds to the branch position of

2249 haptophytes in the consensus tree; alternative branching positions for the haptophyte
2250 sequences are labelled with numbered boxes. The table below (ii) lists the probabilities for
2251 each alternative position under eight different tests performed with CONSEL. Alternative
2252 positions that are not rejected by a topology test are shaded. All possible trees in which the
2253 haptophyte sequences branch within the ochrophytes are clearly rejected under all
2254 conditions, confirming that its plastid genome is of non-ochrophyte origin. The legend at the
2255 bottom of panel B gives full names for each test performed.

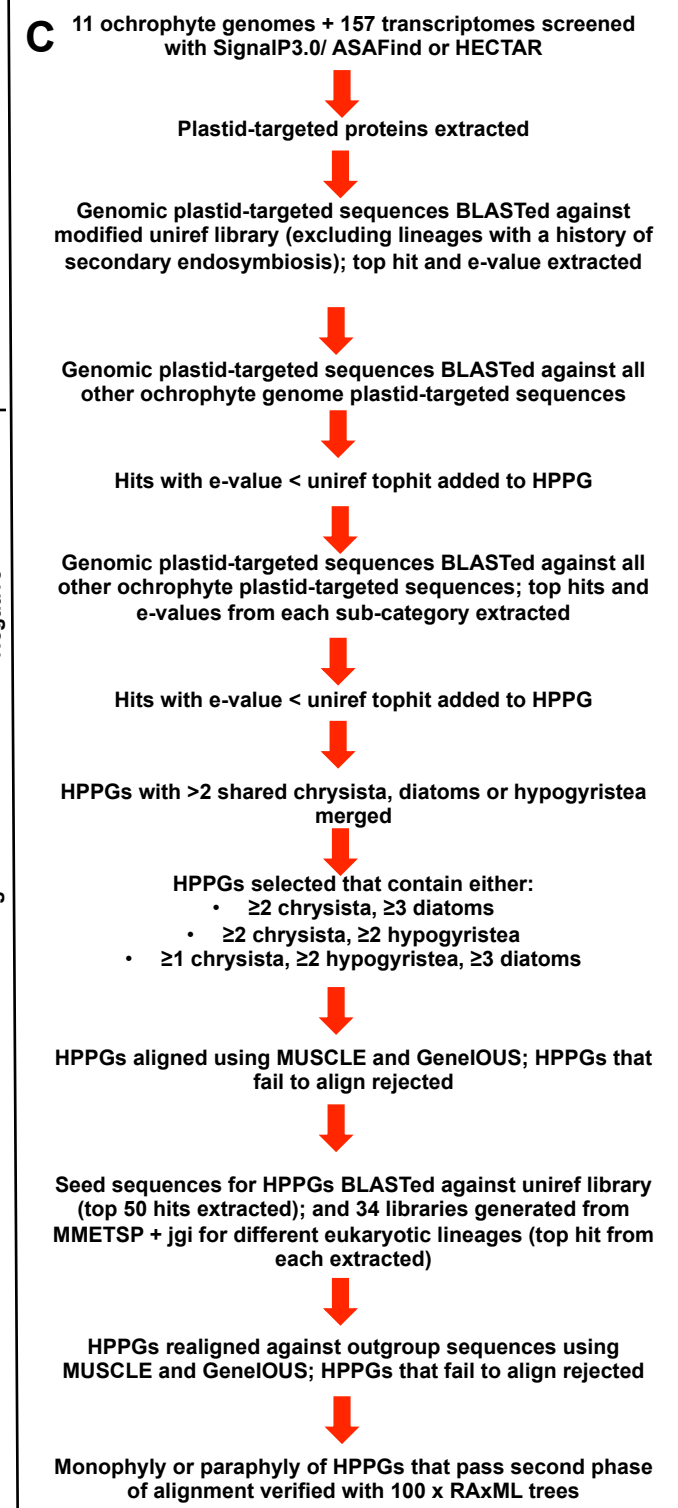
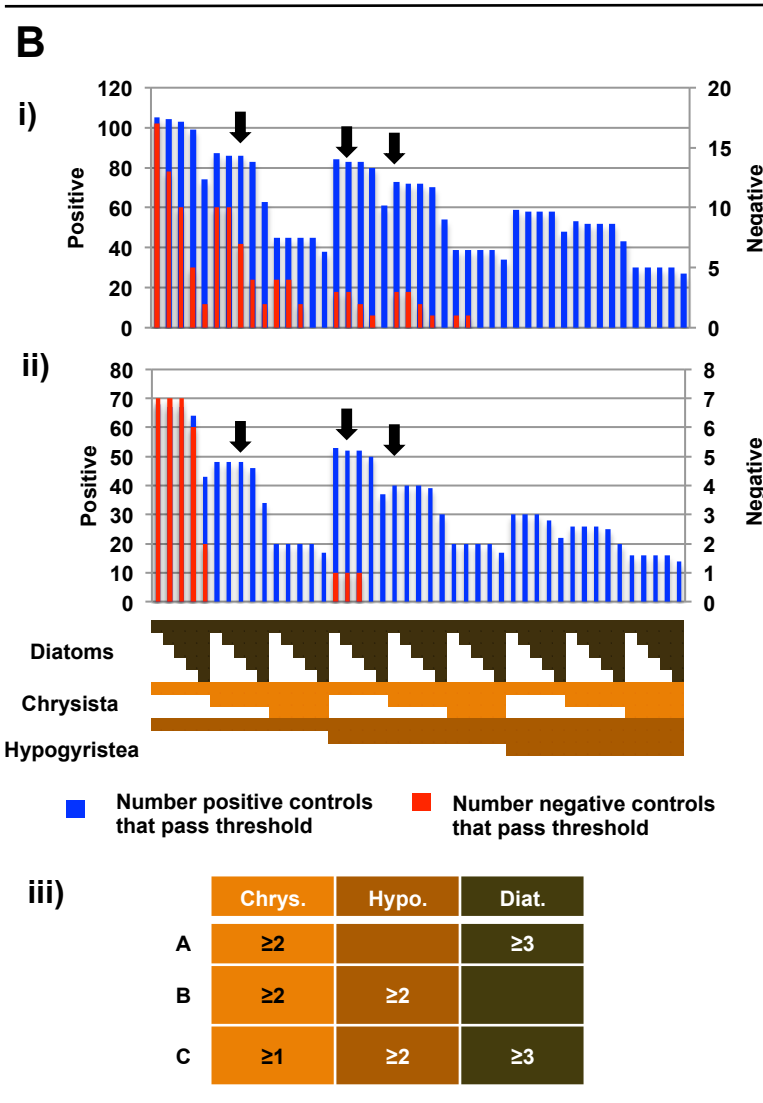
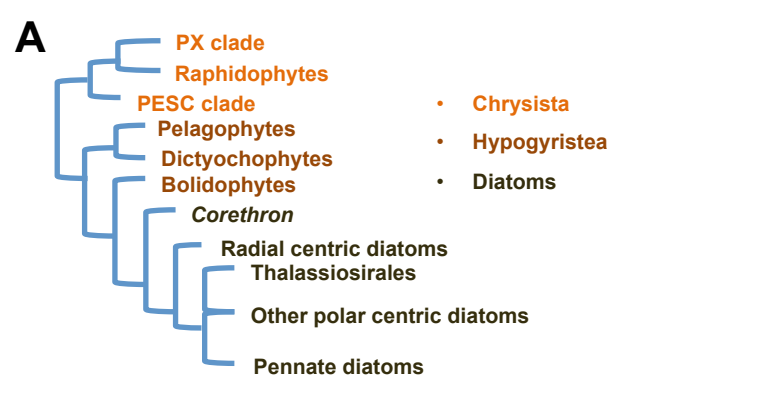
2256
2257 **Fig. 9- figure supplement 2. Fast site removal and clade deduction analysis of plastid**
2258 **genome trees. Panel A** shows the support values obtained for Bayesian + Jones trees
2259 inferred from modified versions of the taxon-rich plastid multigene alignment from which
2260 the 13 fastest evolving site categories had been removed for four different branching
2261 relationships pertaining to the placements of haptophyte and hypogyrstean sequences. The
2262 % of residues from the original alignment retained in each modified alignment are shown
2263 with grey bars. **Panel B** tabulates the support obtained for two different evolutionary
2264 relationships (haptophytes as a sister group to all cryptomonads, and as a sister group to all
2265 ochrophytes) in gene-rich (i) and taxon-rich (ii) alignments modified to remove all amino
2266 acids that occur at different frequencies in haptophytes to ochrophyte lineages, and
2267 modified to remove individual or pairs of CASH lineages. “x” indicates that the topology in
2268 question was not obtained.

2269
2270 **Fig. 9- figure supplement 3. Single-gene tree topologies associated with individual plastid-**
2271 **encoded genes.** These heatmaps show the first sister-groups identified to haptophytes, and
2272 members of the pelagophyte/ dictyochophyte clade, in single-gene trees of component
2273 genes included in concatenated trees of plastid-encoded proteins using both the gene-rich
2274 (i) and taxon-rich (ii) alignments. Topologies are given for trees inferred with MrBayes using
2275 the Jones substitution matrix, and RAxML trees inferred using JTT, under the same
2276 conditions as the multigene trees. The identity of the first sister-group is shaded according
2277 to the legend given below. Only three single-gene trees (labelled with black arrows) support
2278 any sister-group relationship between haptophytes and the pelagophyte/ dictyochophyte
2279 clade; however, in each case (explained beneath the legend) this topology is not robustly
2280 supported, either due to polyphyly of one of the constituent lineages, or conflicting
2281 topologies identified via alternative methods.

2282
2283 **Fig. 10- figure supplement 1. Complex origins of different ancestral ochrophyte HPPGs**
2284 **Panel A** shows the evolutionary positions of lineages with histories of secondary
2285 endosymbiosis in trees of ancestral ochrophyte HPPGs verified by combined BLAST top hit
2286 and single-gene tree analysis to be either of red algal (i) or green algal origin (ii). In both
2287 cases, in more than half of the constituent trees, haptophyte and cryptomonad sequences
2288 resolve as closer relatives to the ochrophytes than the red or green algal evolutionary
2289 outgroup, either due to resolving in the ochrophyte HPPG or forming a specific sister-group
2290 to the ochrophyte lineages. **Panel B** plots the distribution of cryptomonads (i) and
2291 haptophytes (ii) in trees for different categories of ancestral ochrophyte HPPG of verified
2292 evolutionary origin. HPPGs of green algal origin more frequently show internal or sister
2293 positions for the cryptomonad sequences than all other categories of HPPG, and in more
2294 than 50% of cases resolve internal or sister positions for the haptophyte sequences. This
2295 might be consistent with a green algal contribution in the endosymbiotic ancestor of
2296 cryptomonad, haptophyte and ochrophyte plastids.

2297
2298 **Fig. 10 –figure supplement 2. Different scenarios for the origins of haptophyte plastids.**
2299 This schematic tree diagram shows different possibilities for the origins of the haptophyte

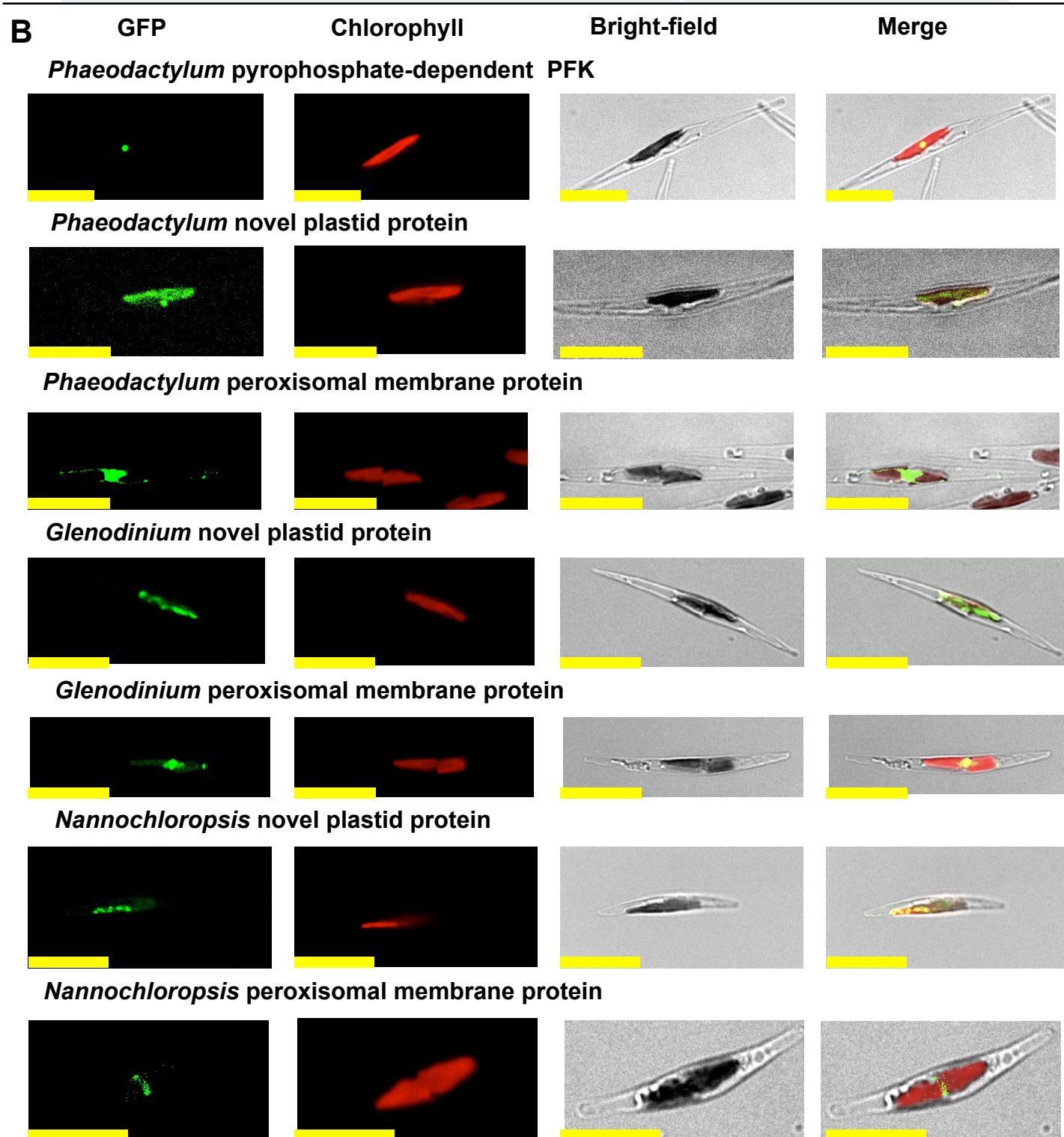
2300 plastid as predicted from the data within this study. No inference is made here regarding the
2301 ultimate origin of the ochrophyte plastid, although it is noted that the ochrophyte,
2302 cryptomonad and haptophyte plastids are likely to be closely related to one another within
2303 the red plastid lineages. First, a common ancestor of the pelagophytes and dictyochophytes
2304 was taken up by a common ancestor of the haptophytes (point **1**), yielding a permanent
2305 plastid that contributed genes for a large number of plastid-targeted proteins in extant
2306 haptophytes. This plastid was subsequently replaced via serial endosymbiosis (point **2**)
2307 yielding the current haptophyte plastid and plastid genome. This serial endosymbiosis event
2308 either involved a close relative of extant cryptomonads (**2A**) or a currently unidentified
2309 species that forms a sister-group in plastid gene trees to all extant ochrophytes, but is
2310 evolutionarily distinct from the pelagophytes (**2B**). It is possible that the haptophyte plastid
2311 may have been acquired through the secondary endosymbiosis of a different lineage of red
2312 algae to the ochrophyte, either via a cryptomonad intermediate (**2C**) or directly (**2D**).
2313
2314

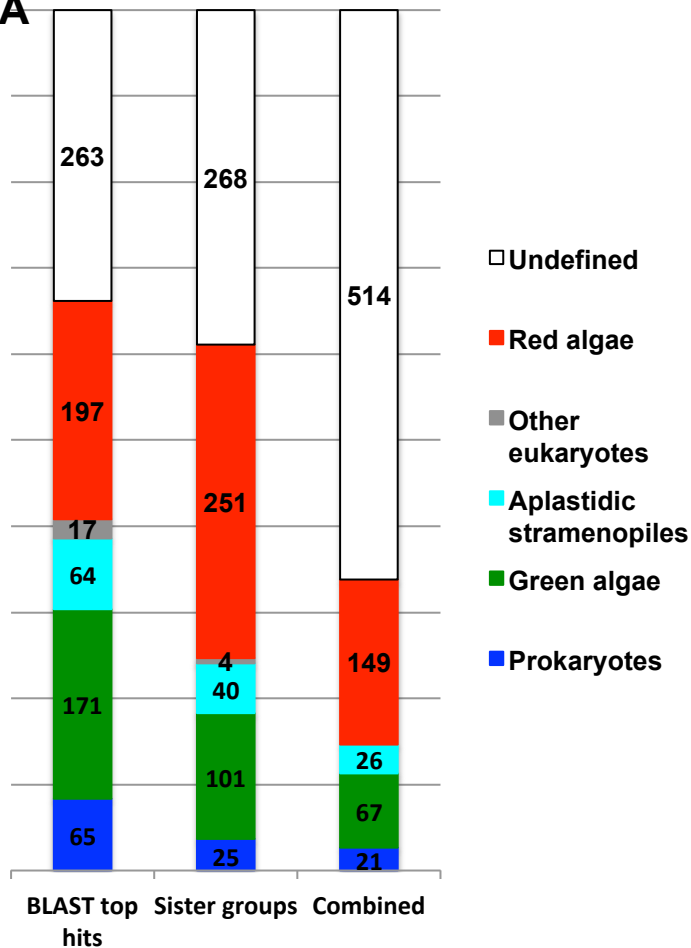
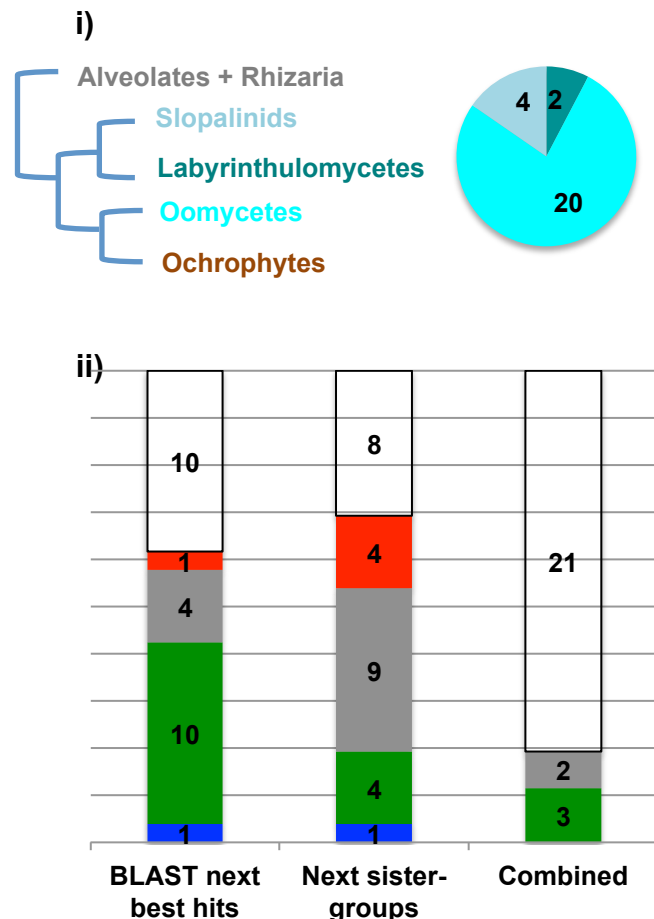


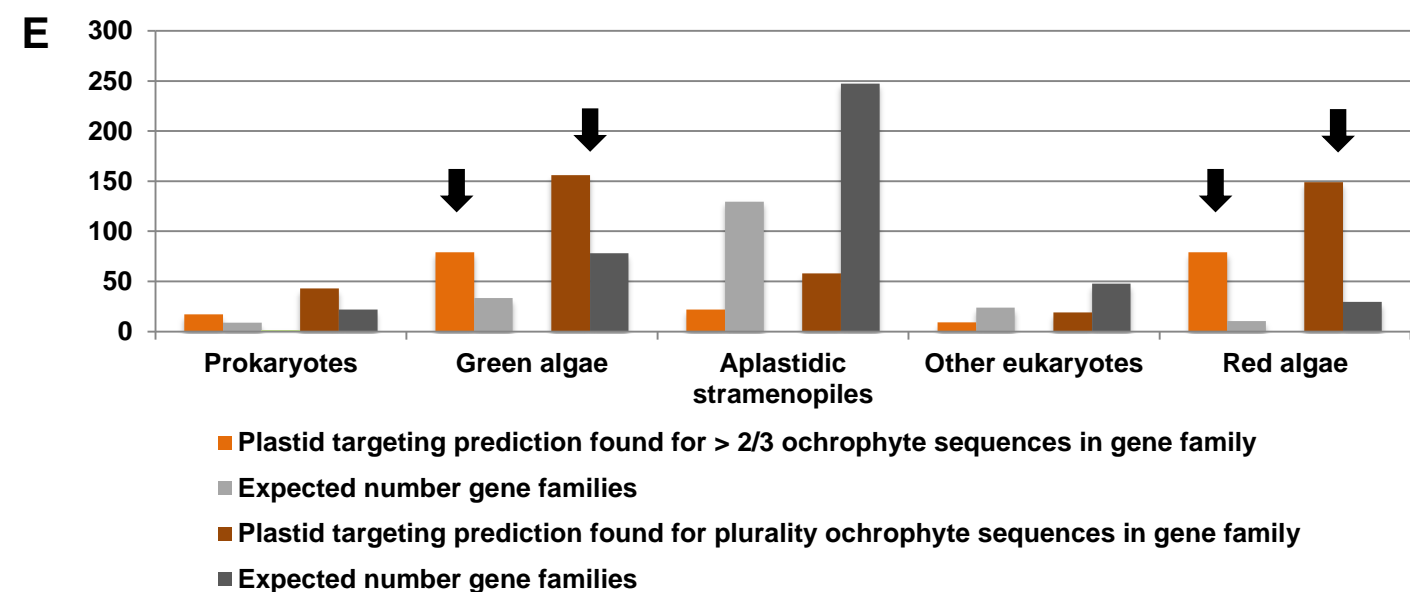
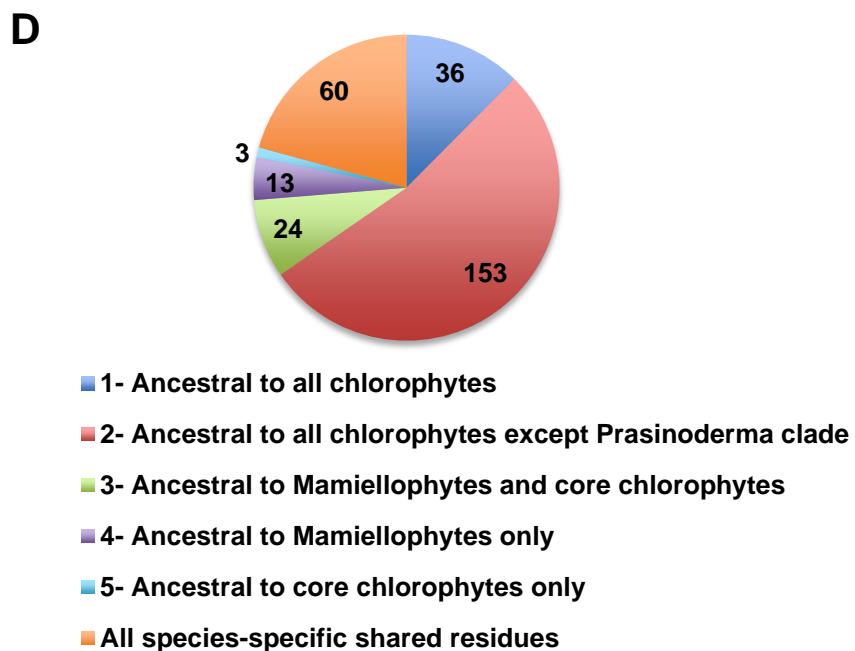
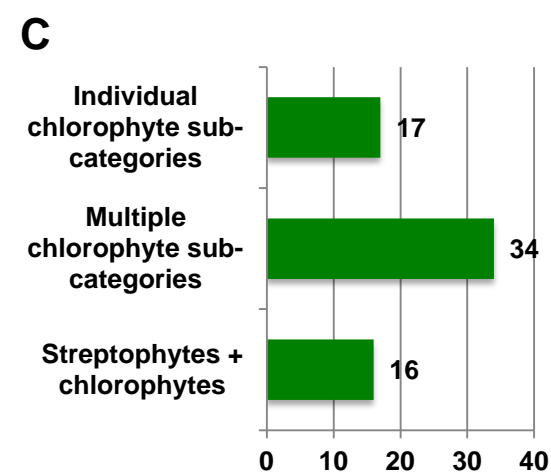
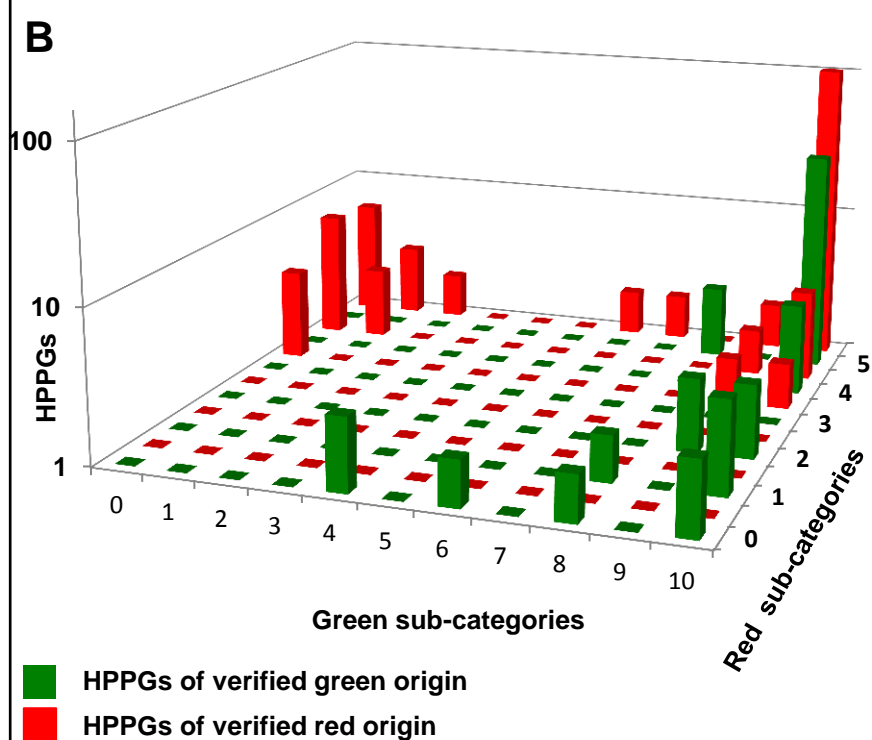
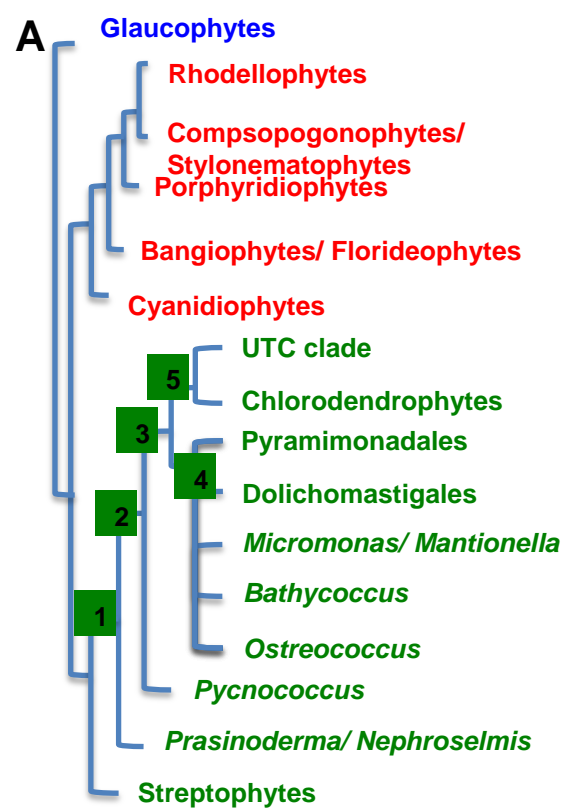
D

HPPGs	Total	+ve	-ve	Total	+ve	-ve
ASAFind			HECTAR			
Total	7238	181	1970	2858	155	493
Passed HPPG assembly	924	104	7	291	65	3
Ancestral	731	102	2	278	60	2
Total ancestral homologous plastid-targeted protein groups (HPPGs)= 770						
Total positive controls= 106						
Total negative controls= 4						

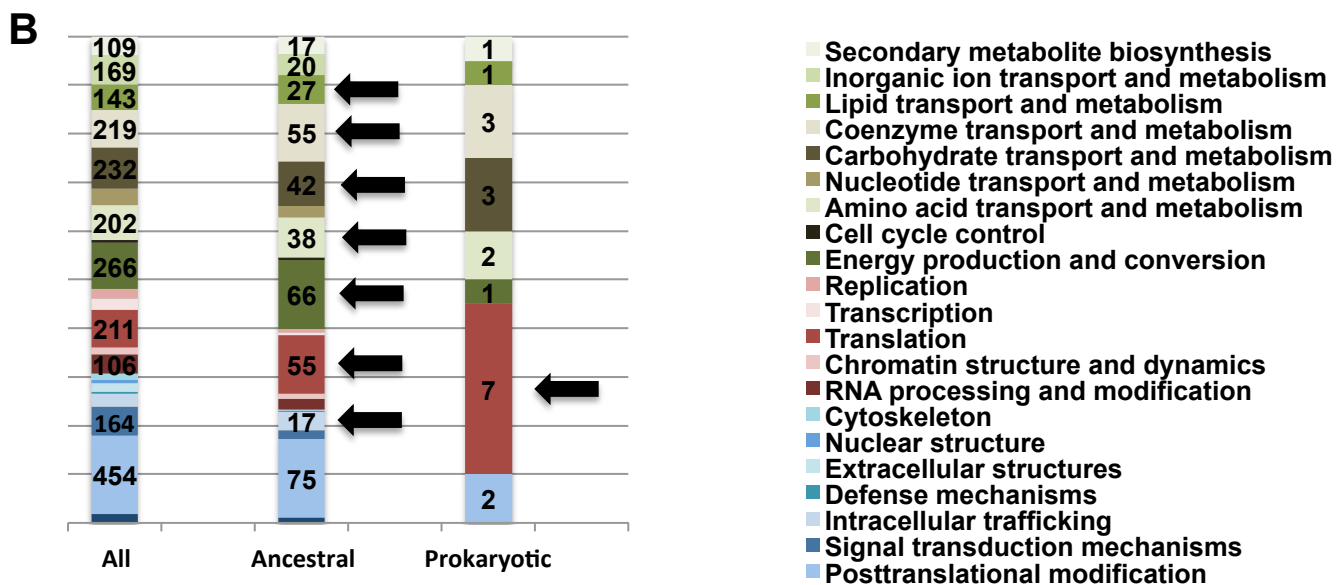
A	Protein	Probable origin
	ER Heat Shock Protein	Host ER
	Glycyl tRNA synthetase	Bacterial
	Histidyl tRNA synthetase	Host cytoplasm
	Methionyl tRNA synthetase	Bacterial
	Leucyl tRNA synthetase	Host cytoplasm
	Mitochondrial GroES chaperonin	Host mitochondria
	Pyrophosphate-dependent phosphofructokinase	Symbiont cytoplasm
	Peroxisomal membrane protein MPV17	Symbiont peroxisome
	Prolyl tRNA-synthetase	Symbiont cytoplasm
	Novel protein 1	Unknown



A**B**

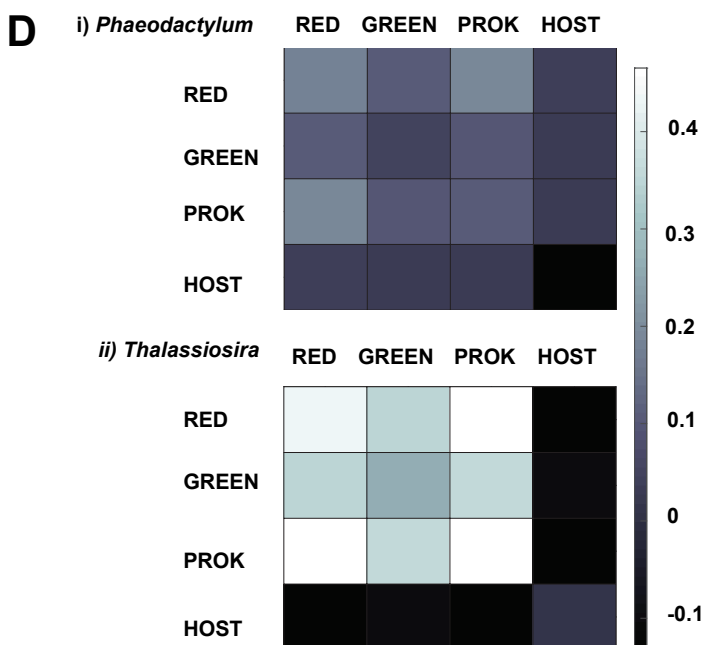


Biological process	Identified	Plastid-encoded	Dispensible	Non-vertical
Light-harvesting proteins	14	-	-	-
Photosynthesis	28	45	-	-
Central carbon metabolism	27	2	-	1
Lipid synthesis	16	-	-	-
Tetrapyrrole synthesis	24	1	1	-
Carotenoid synthesis	18	-	1	-
Fe-S cluster synthesis	8	2	-	-
Riboflavin synthesis	2	-	-	-
Glu/Gln/Asp/Lys synthesis	16	-	-	-
Phe/Trp/Tyr synthesis	13	-	-	2
Ile/Leu/Val synthesis	6	1	-	1
Ser/Cys synthesis	8	-	1	-
tRNA synthesis	22	-	-	-
Nucleotide synthesis	4	-	-	-
Ribosomal proteins	8	45	1	-
Translation initiation	7	2	-	-
Protein import complexes	8	4	-	-
Division	2	0	-	-
Clp protease complex	8	1	-	-
Total	239	103	4	4



C

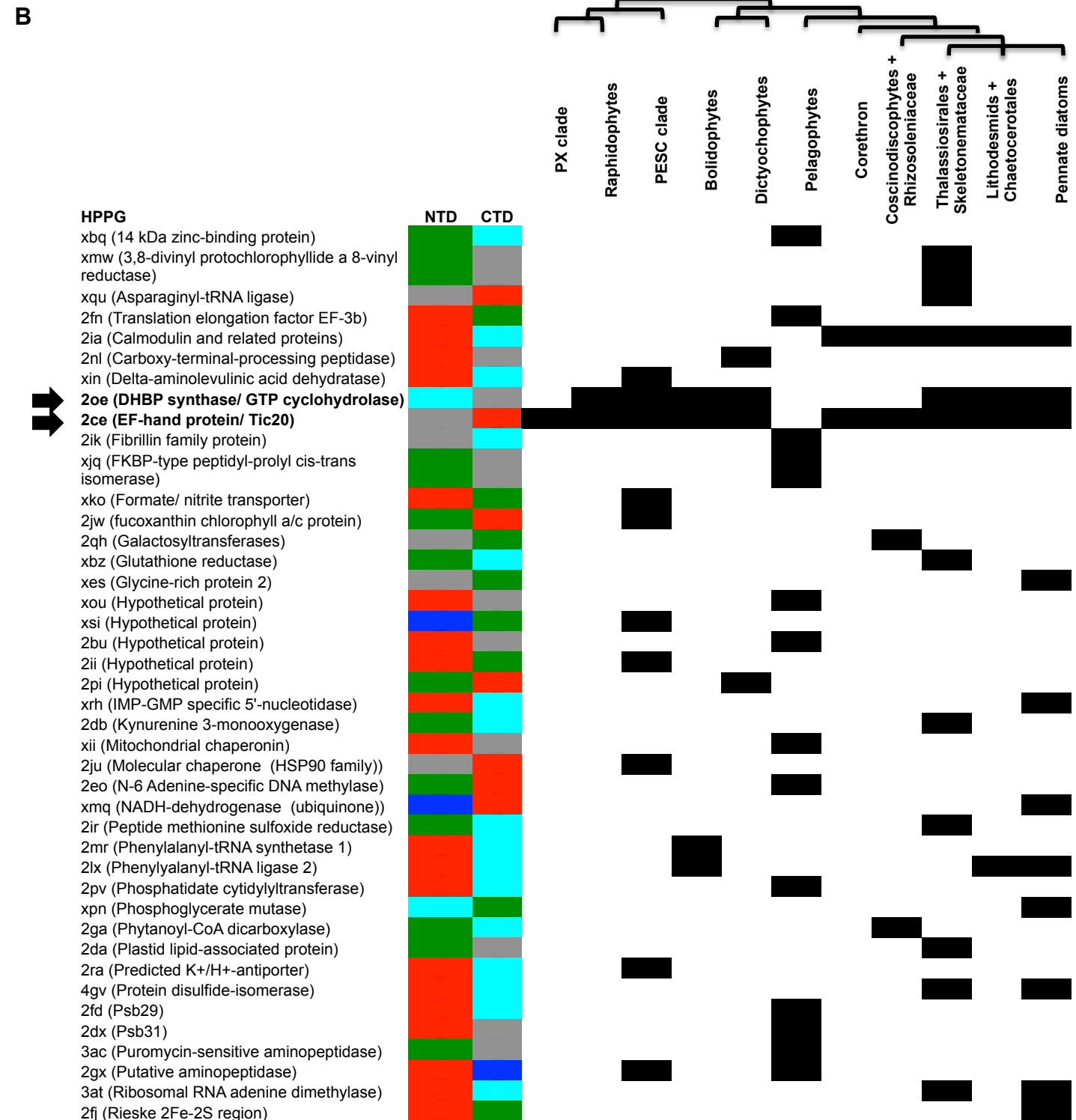
Number protein pairs	313
Number protein pairs between HPPGs of clear evolutionary origin	95
Observed number protein pairs between HPPGs of same origin	44
Expected number protein pairs between HPPGs of same origin	41.05
Chi-squared P	0.541

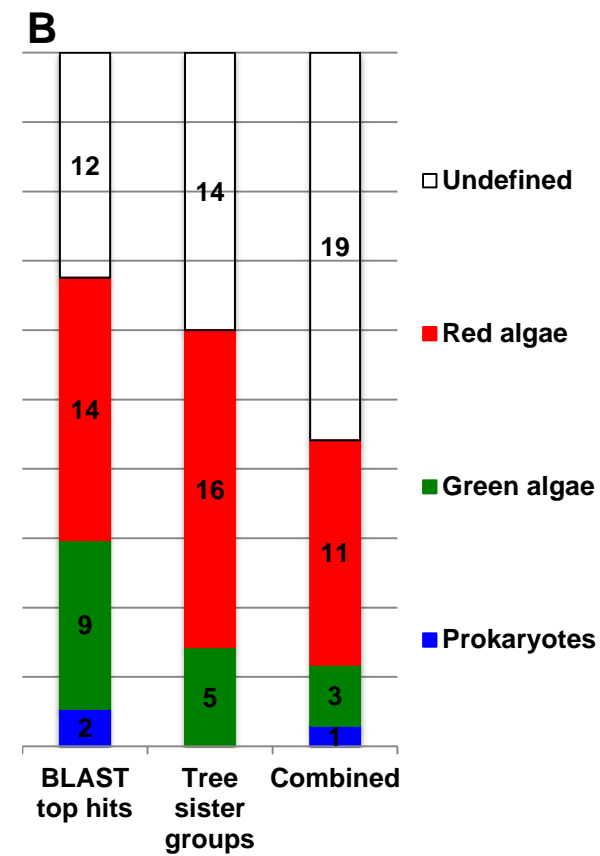
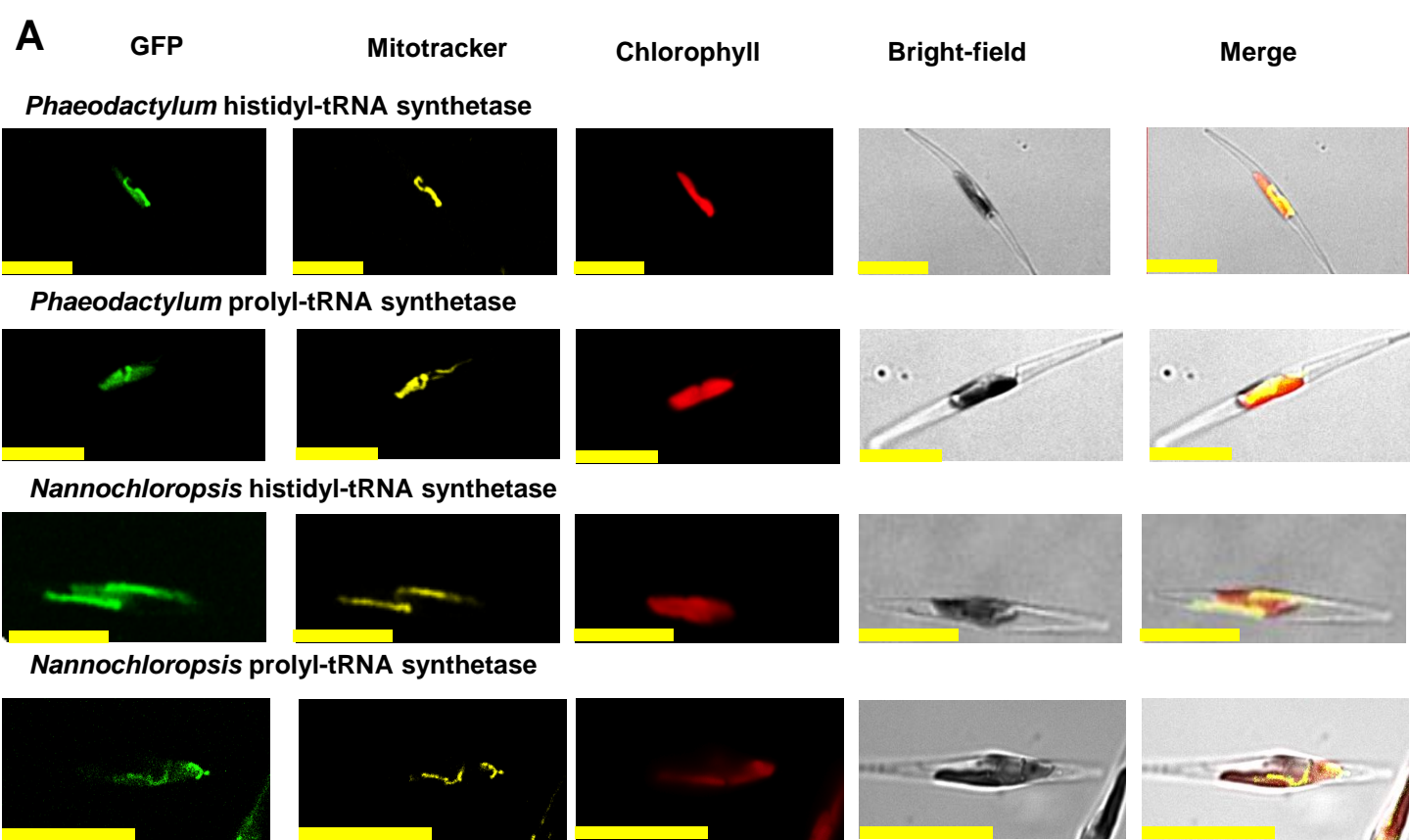


A

i) Chimeras inherited by the ochrophyte ancestor	Origin in ochrophytes	NTD	CTD
PpiC-type peptidyl-prolyl cis-trans isomerase	Ambiguous	Firmicutes/ Proteobacteria	Cyanobacteria
Hypothetical protein	Red	Firmicutes	Cyanobacteria
Rieske 2Fe-2S region	Green	Cyanobacteria	Proteobacteria
Probable heme-binding protein	Red	Cyanobacteria	Proteobacteria
Acyl-CoA:diacylglycerol acyltransferase (DGAT)	Host	Cyanobacteria	Actinobacteria
Phenylalanyl-tRNA synthetase	Red	Cyanobacteria	Proteobacteria

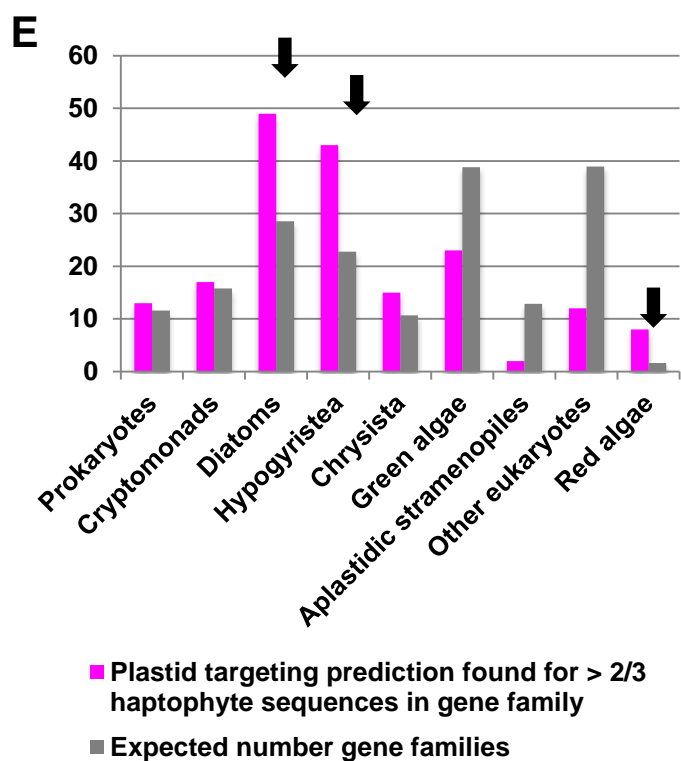
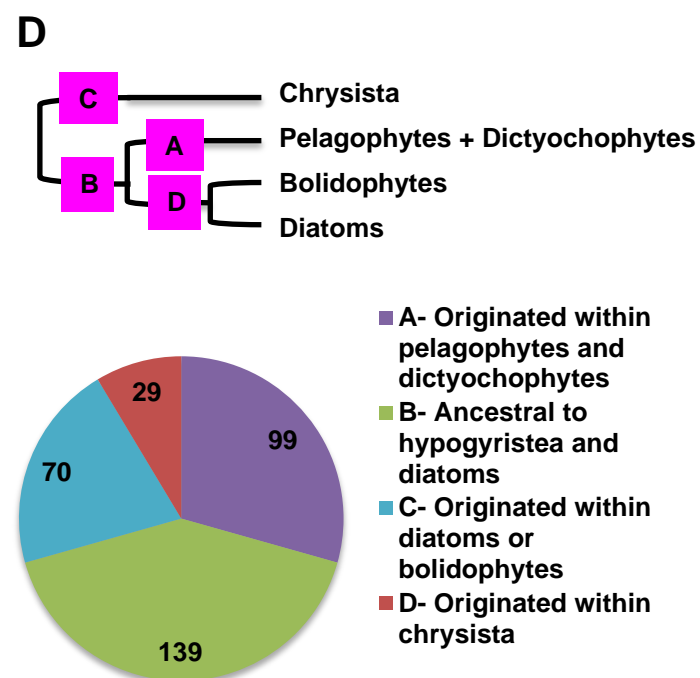
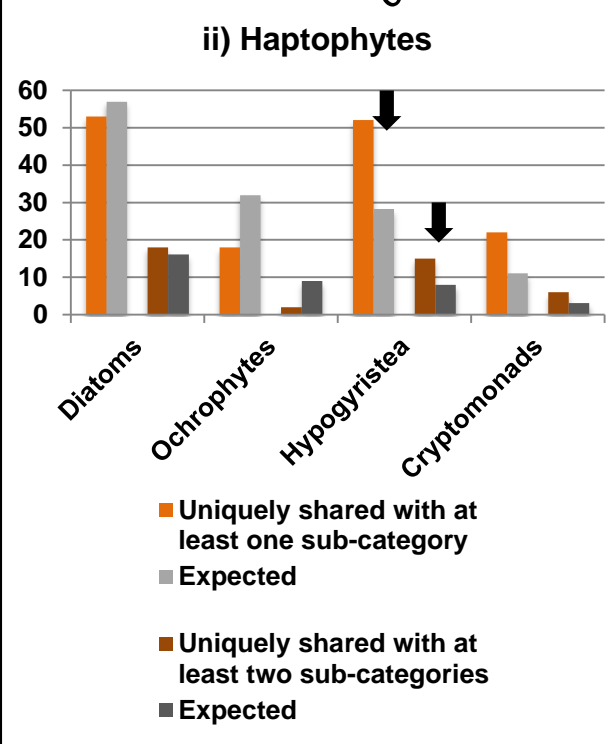
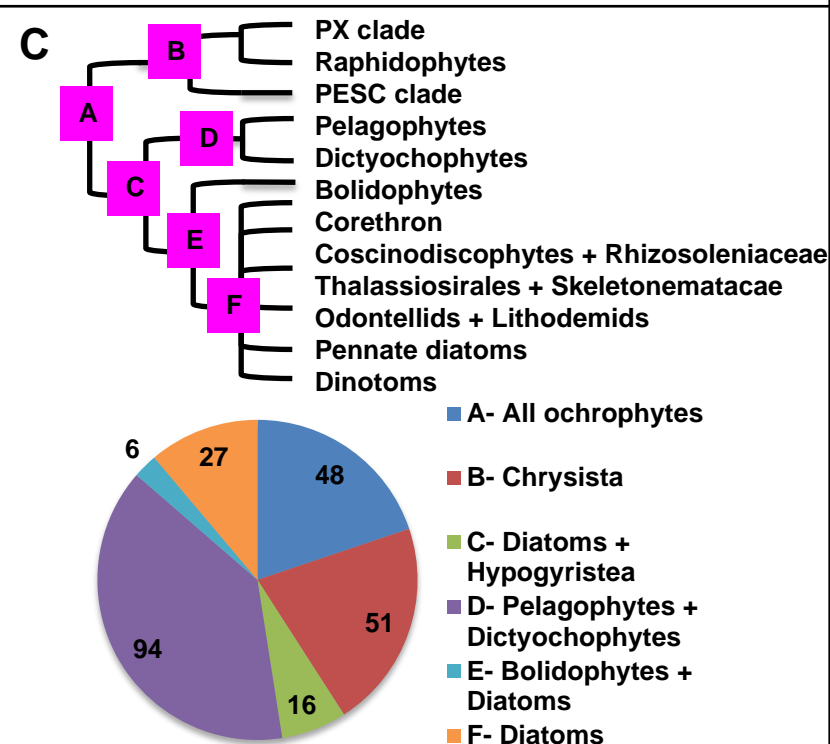
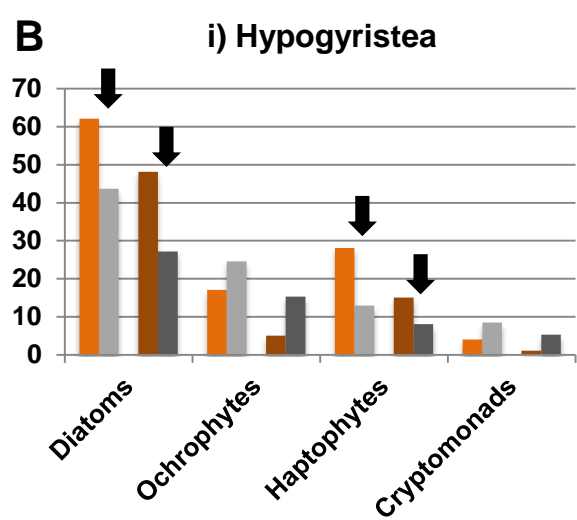
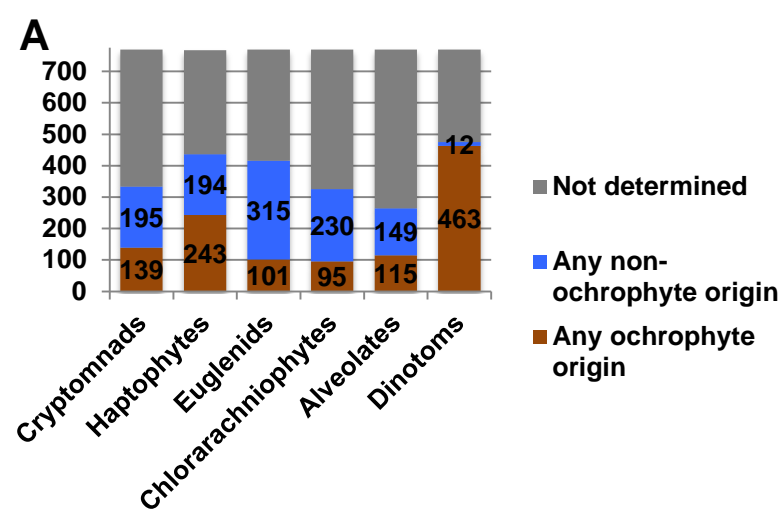
ii) Chimeras endogenous to ochrophytes	NTD	CTD
Calmodulin and related proteins/ Tic20	Unknown	Red algae
DHBP synthase/ GTP cyclohydrolase	Aplastidic stramenopiles	Actinobacteria/ Red algae

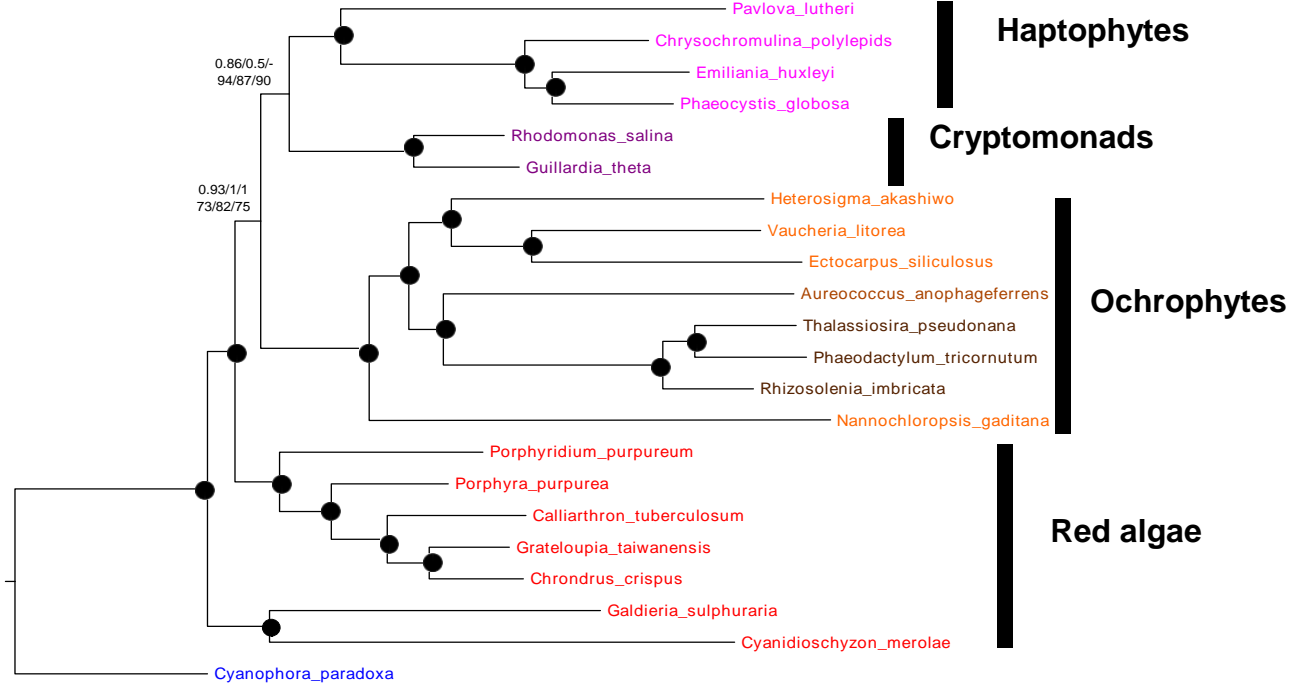
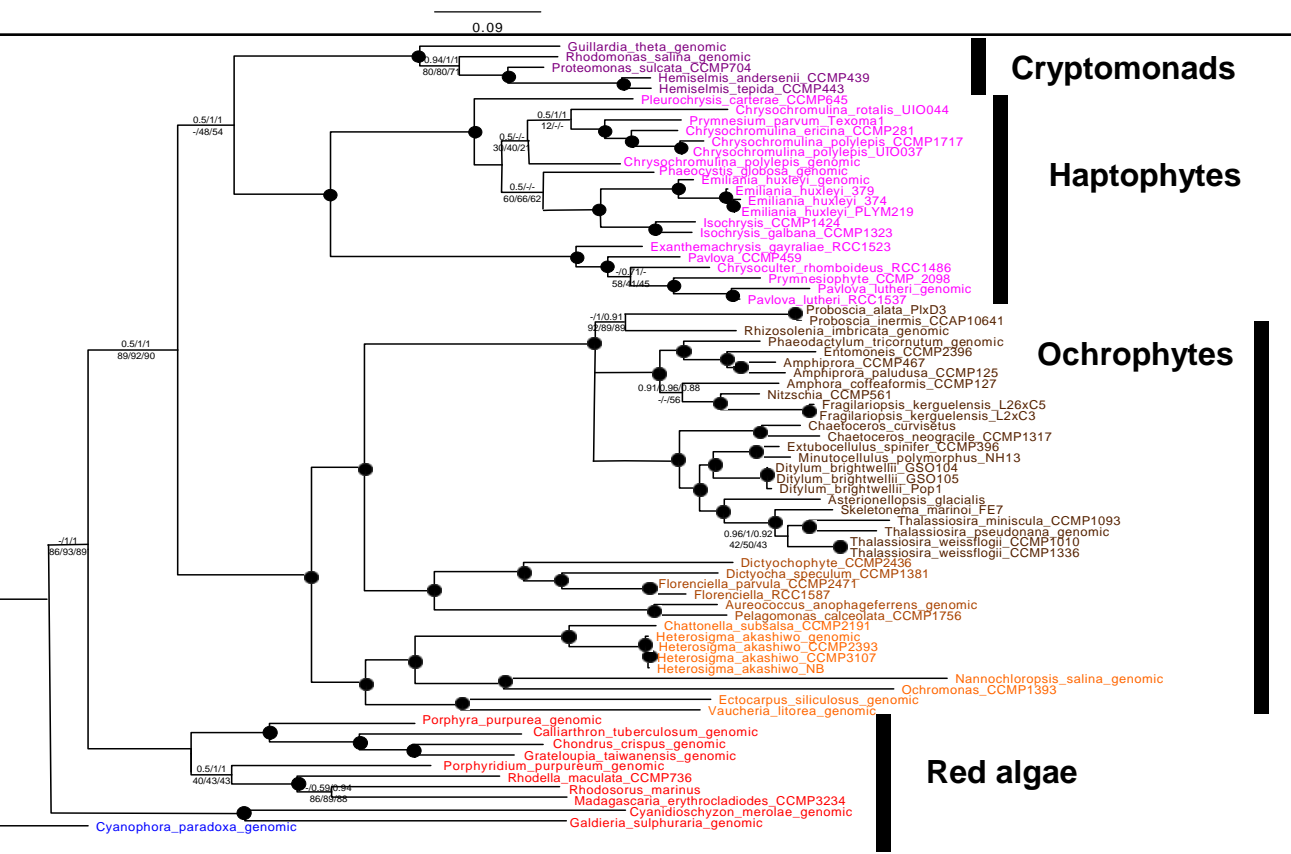




C

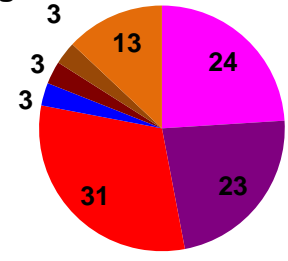
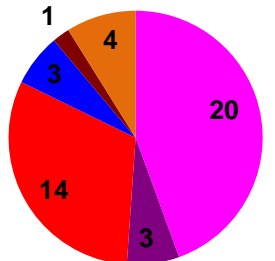
tRNA synthetase	Cytoplasmic isoform	Dual-targeted isoform
Ser	Aplastidic stram	Prokaryotic
Ala	Aplastidic stram	Aplastidic stram
Trp, Arg, Asn, Asp, Val	Aplastidic stram	Red algal



A**B****Key to support values**

● Support value 1.0 in all MrBayes consensus trees; > 80% all ML best trees

Other nodes
MrBayes: GTR/ Jones/ WAG
RAxML: GTR/ JTT/ WAG

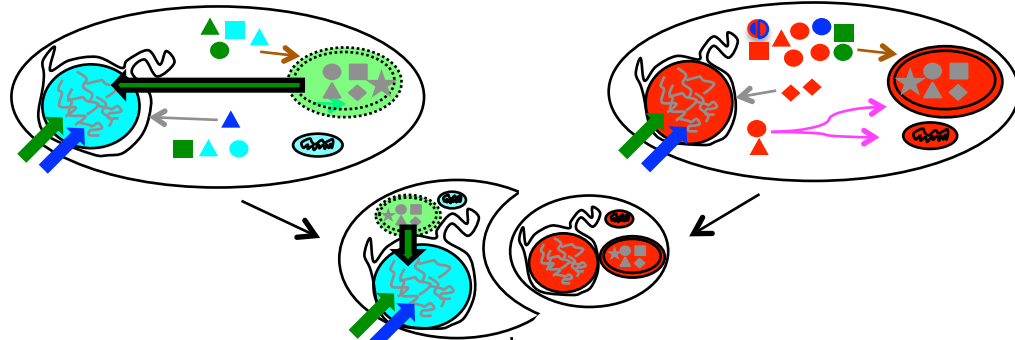
C**i) gene-rich dataset****ii) taxon-rich dataset**

■ Exclusive to haptophytes
■ Cryptomonads
■ Red algae
■ Glaucophytes
■ Diatoms
■ Hypogyristera

Stramenopile host

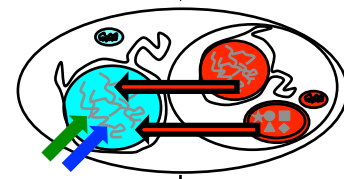
Red lineage symbiont

A



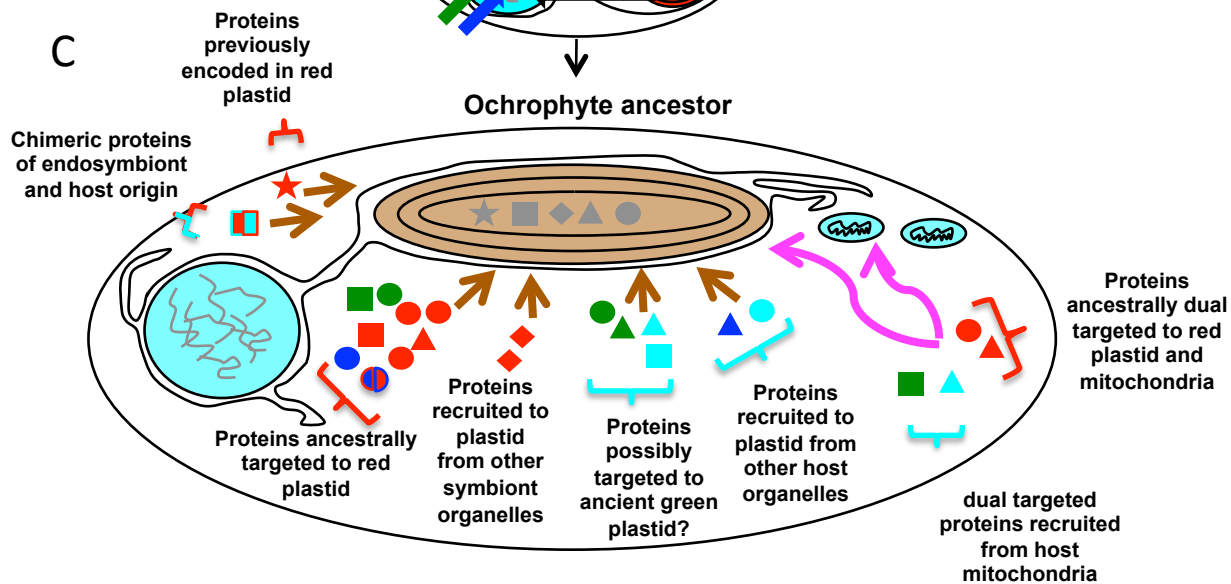
B



















Endosymbiotic intermediates



C

Ochrophyte ancestor



-  Nucleus
-  Mitochondrion
-  Red lineage plastid
-  Putative green plastid
-  LGT from green algae
-  LGT from prokaryotes
-  Uniquely plastid-targeted proteins
-  Uniquely mitochondria-targeted proteins
-  Uniquely other (e.g. ER)-targeted proteins
-  Dual mitochondria and plastid-targeted proteins
-  Different proteins that are nucleus-encoded and plastid-targeted in ochrophytes
-  Chimeric/ fusion plastid proteins
-  Proteins of acquired from endosymbiont (regardless of origin)
-  Proteins acquired from host (regardless of origin)
-  Proteins endogenous to red algae
-  Proteins endogenous to stramenopile host
-  Proteins acquired from green algae
-  Proteins acquired from prokaryotes

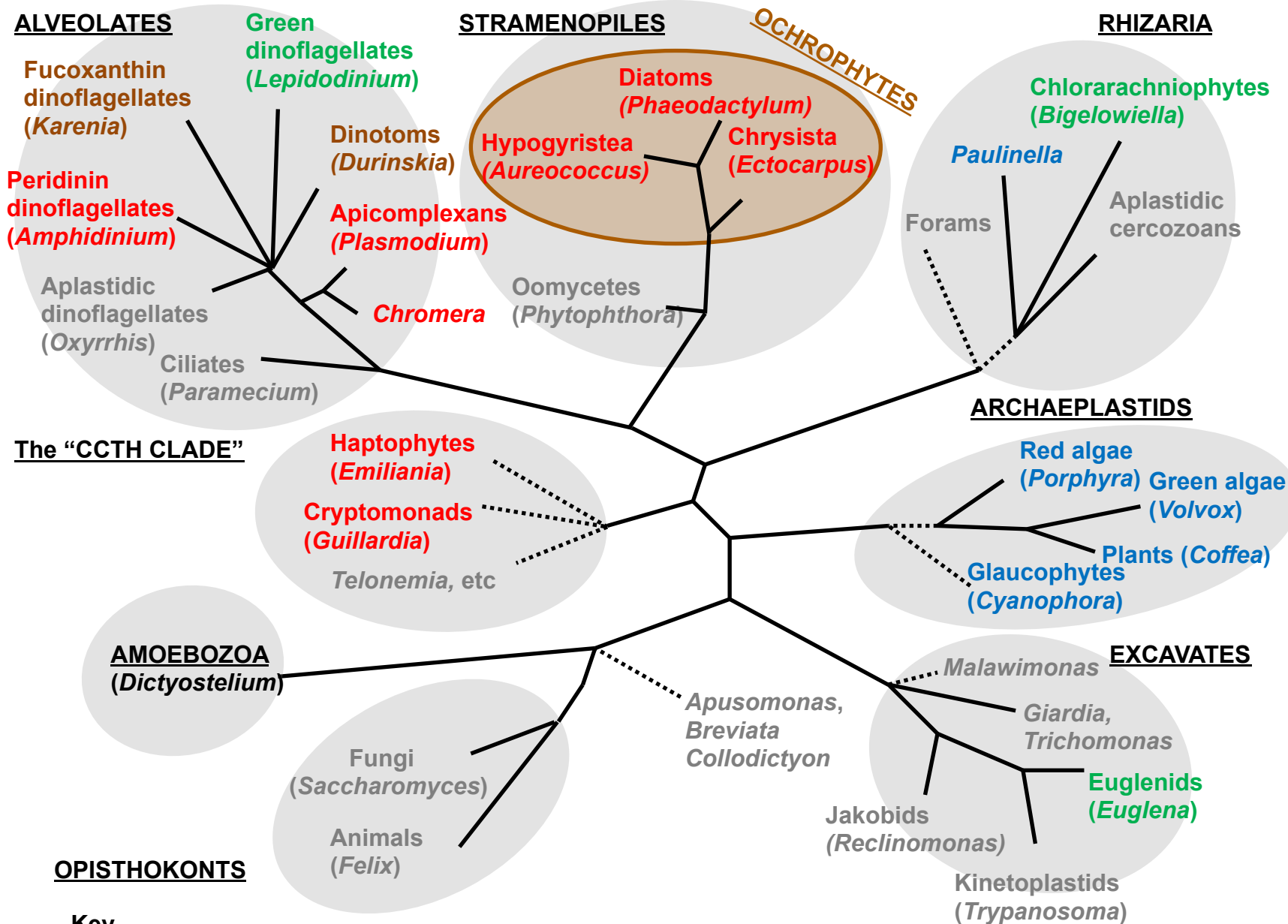


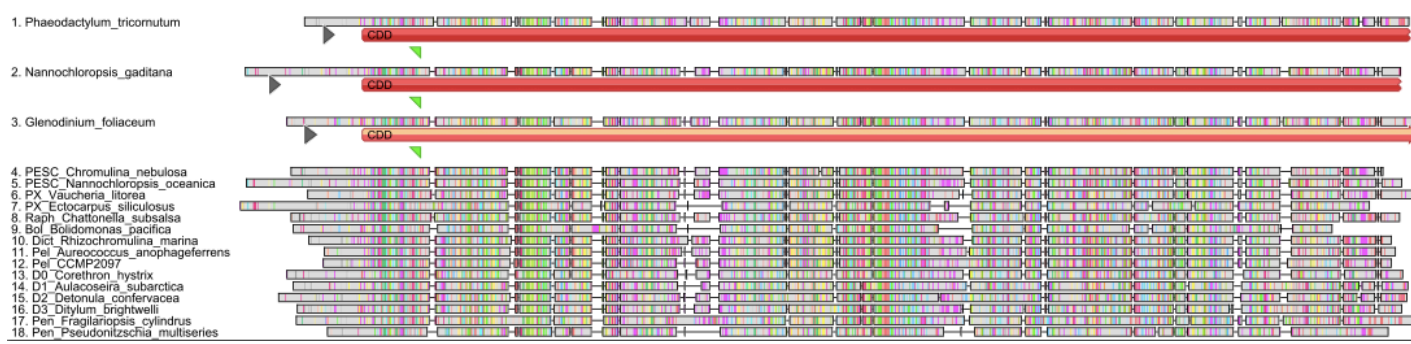
Fig. 1- figure supplement 1. Overview of eukaryotic diversity. This figure, adapted from a previous review³, profiles the diversity of different eukaryotic nuclear lineages. Each grey ellipse corresponds to one major clade, or “supergroup” of eukaryotes. A brown ellipse within the stramenopile clade delineates the ochrophyte lineages. Dashed lines denote uncertain taxonomic relationship. For each taxon, a type species (defined either by the presence of a complete genome, extensive transcriptome library, or of particular anthropic significance) is given in brackets. Taxa that lack plastids are labelled in grey, and taxa with plastids are shaded according to the evolutionary origin of that plastid lineage.

- Key**
- Primary plastid
 - Complex plastid of red origin
 - Complex plastid of green origin
 - Plastid unambiguously agreed to have originated from another CASH lineage

Fig. 2- figure supplement 1- Exemplar ochrophyte plastid protein alignments. This figure shows untrimmed GeneIOUS alignments for two ancestral HPPGs of unusual provenance. In each case the full length of the protein (labelled i) and N-terminal region only (ii) are shown, demonstrating the broad conservation of the N-terminus position. Sequences for which exemplar targeting constructs (*Phaeodactylum tricornutum*, *Nannochloropsis gaditana*, *Glenodinium foliaceum*) are shown at the top of each alignment.

A i) full length

ER heat-shock protein

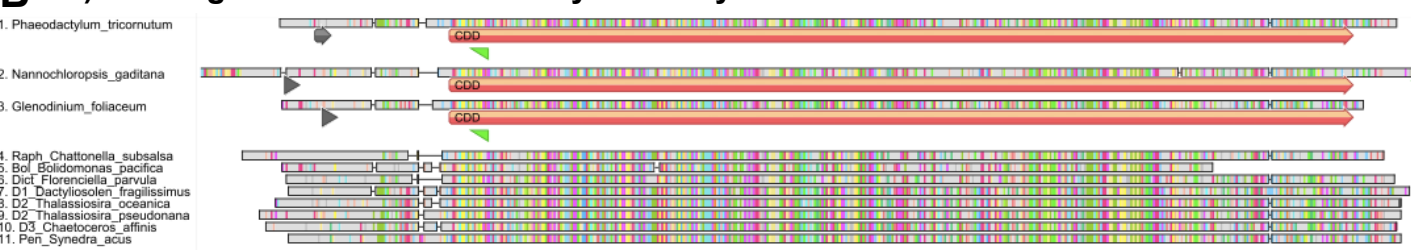


ii) NTD



B i) full length

Histidyl tRNA-synthetase



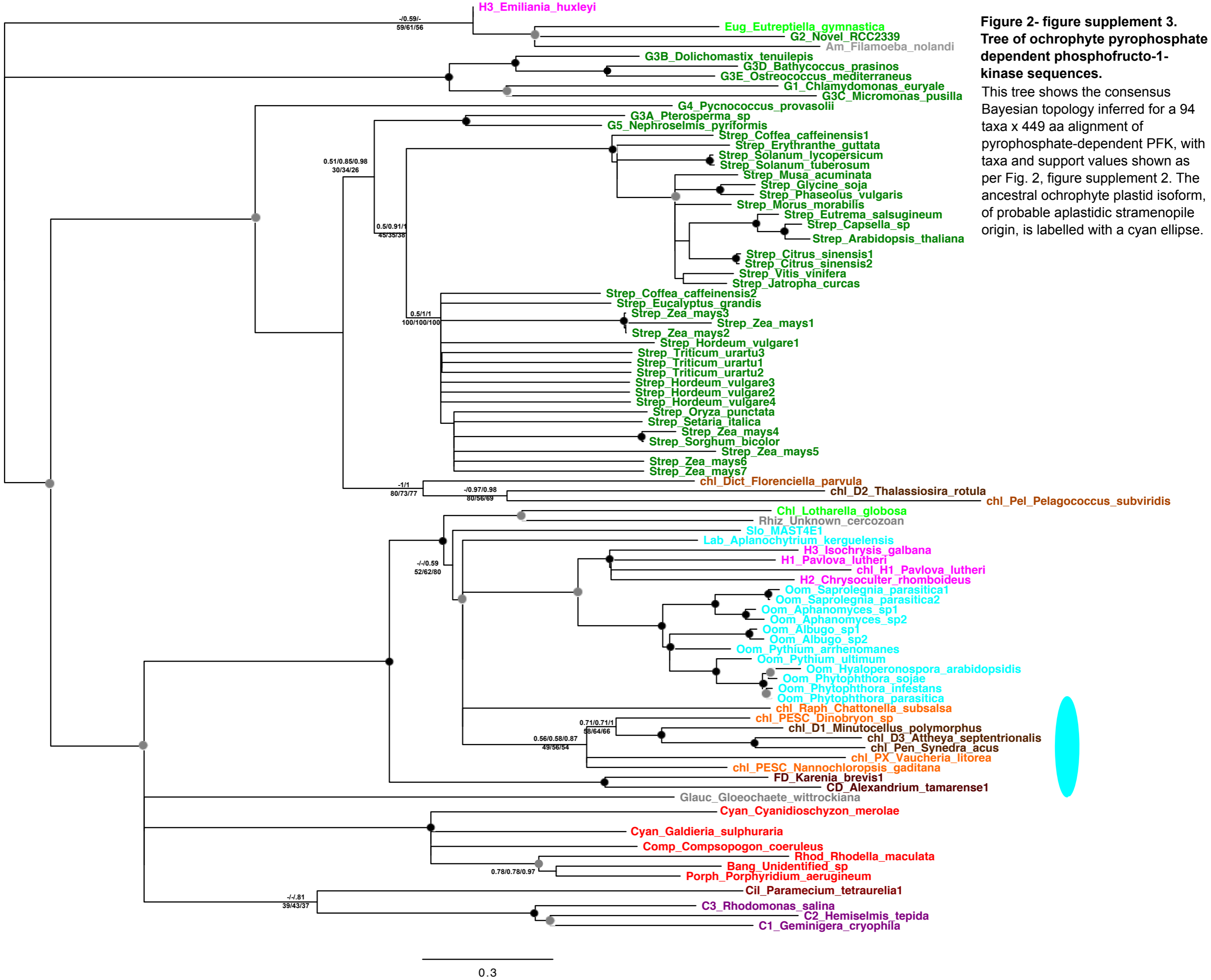
ii) NTD



Key

- ASAFAF motif
- Conserved domain
- Position of PCR reverse primer





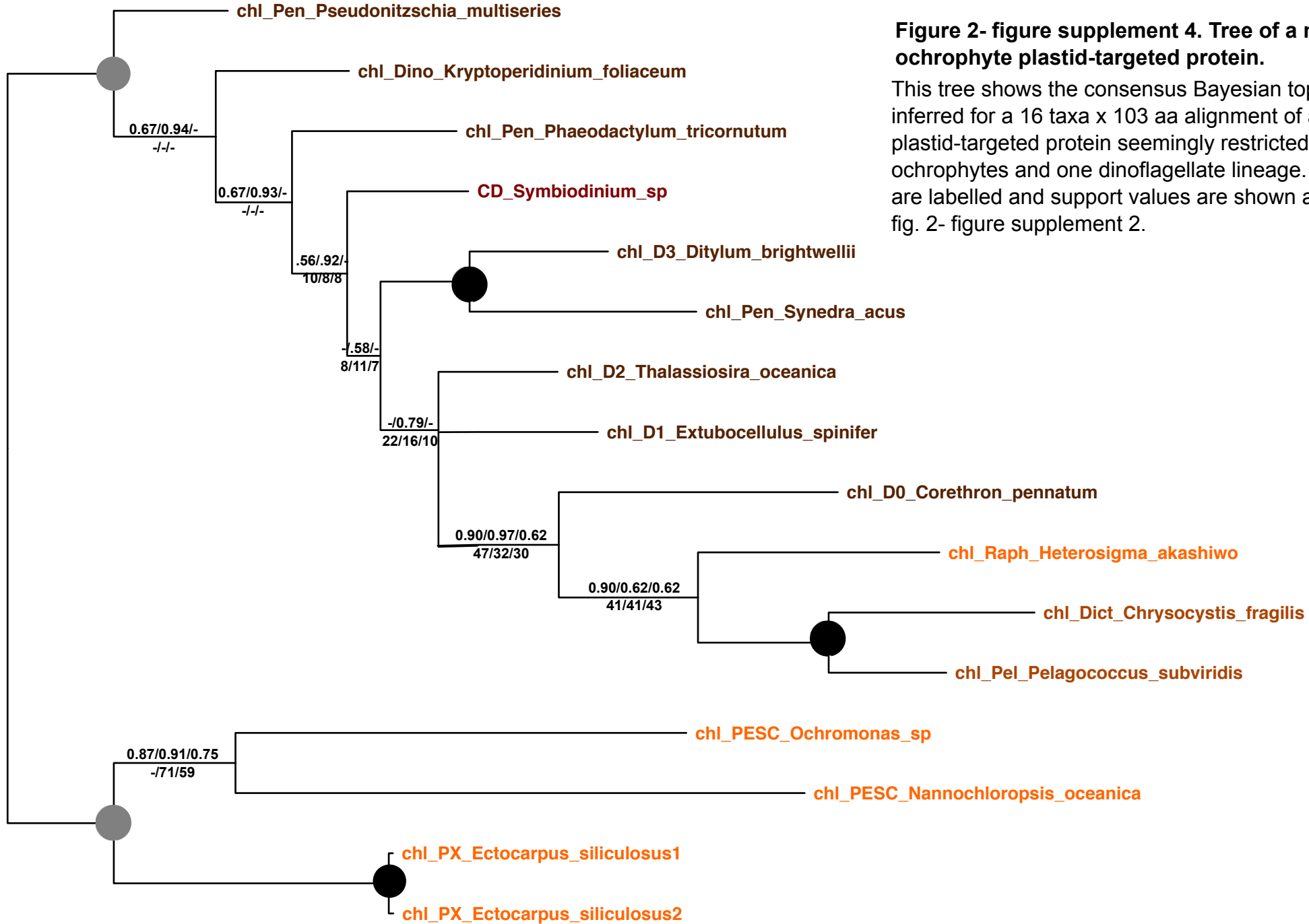


Figure 2- figure supplement 4. Tree of a novel ochrophyte plastid-targeted protein.
 This tree shows the consensus Bayesian topology inferred for a 16 taxa x 103 aa alignment of a plastid-targeted protein seemingly restricted to ochrophytes and one dinoflagellate lineage. Taxa are labelled and support values are shown as per fig. 2- figure supplement 2.

0.2

Fig. 2- figure supplement 5. Multipartite *Phaeodactylum* plastid-targeted proteins. This figure shows the localisation of GFP overexpression constructs for copies of seven proteins from the diatom *Phaeodactylum tricornutum* that are of non-plastid origin, but show multipartite localisation to the plastid and one other organelle (the mitochondria, or in the case of the “ER heat shock protein” to the endoplasmic reticulum).

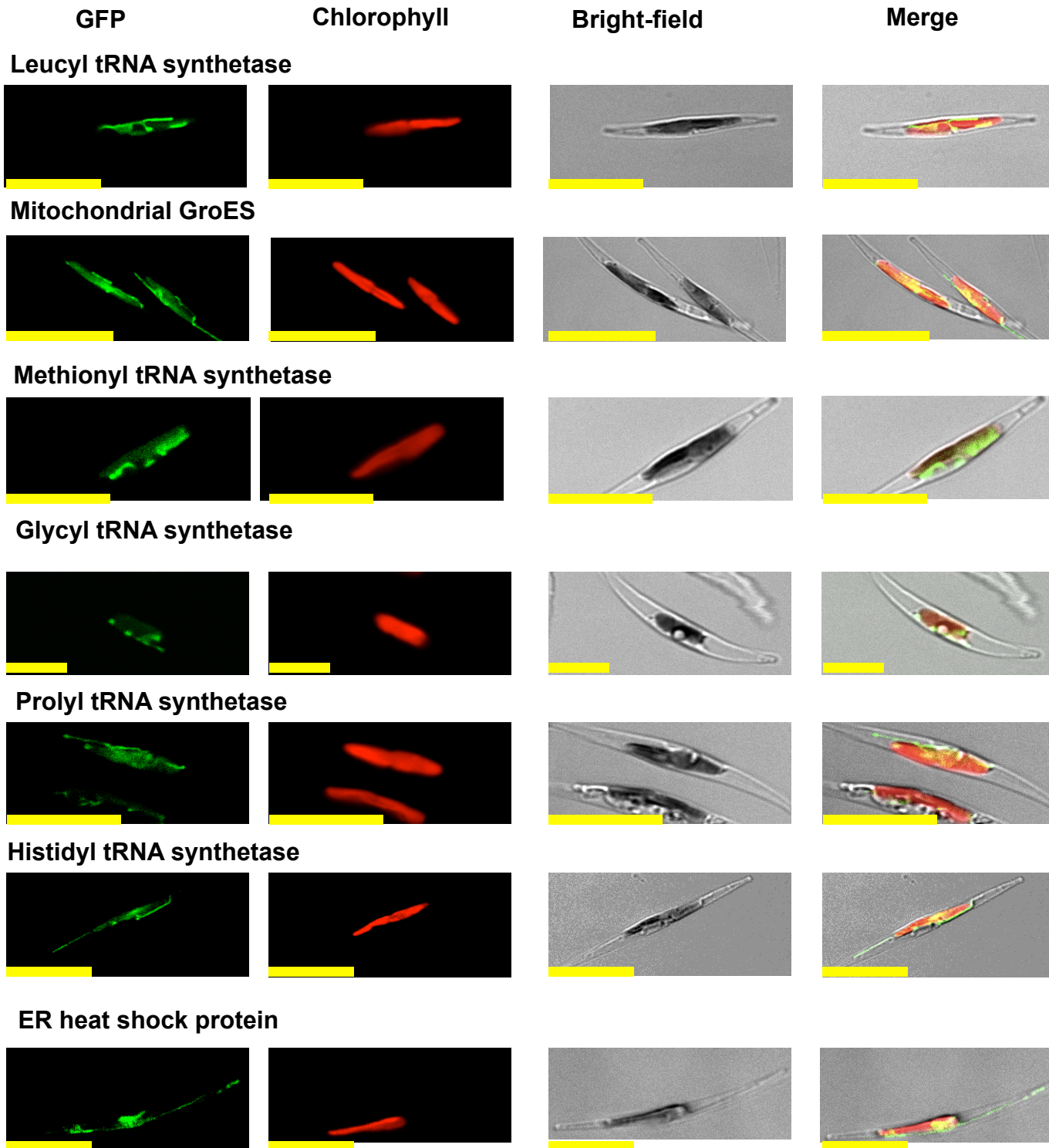
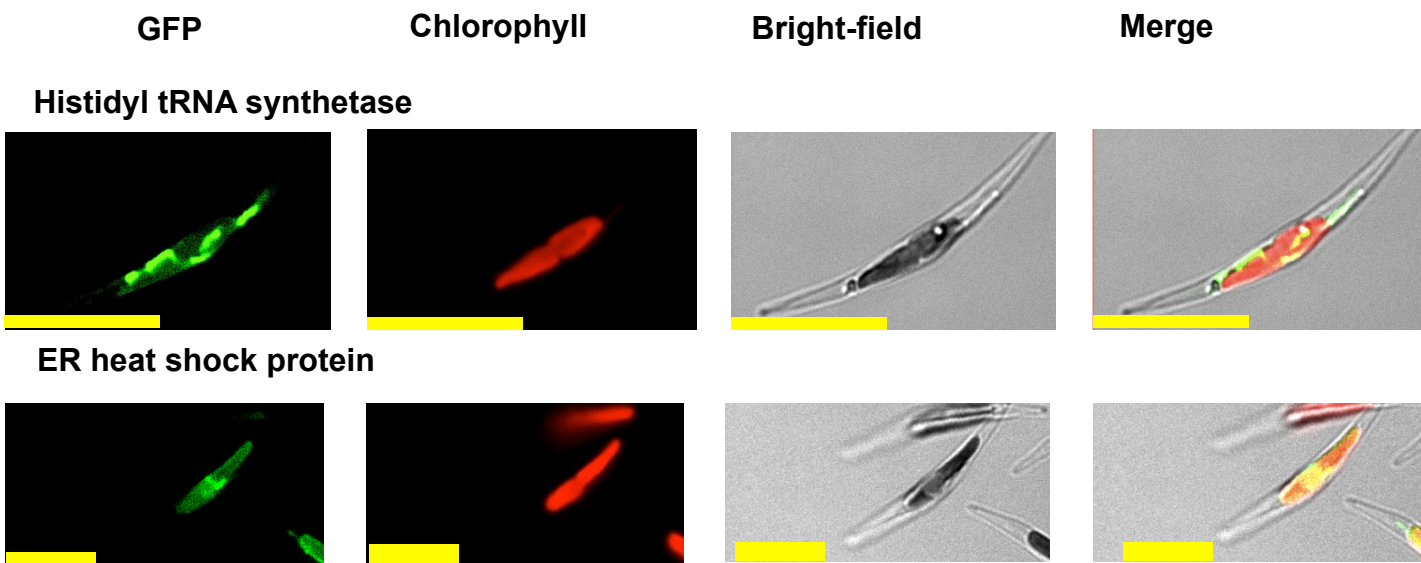


Fig. 2- figure supplement 6. Heterologous expression constructs of multipartite plastid-targeted proteins. This figure shows the localisation of GFP overexpression constructs for copies of two proteins from the dinotom *Glenodinium foliaceum* (**Panel A**), and three proteins from the eustigmatophyte *Nannochloropsis gaditana* (**Panel B**) that are of non-plastid origin, but show multipartite localisation to the plastid and one other organelle, per Fig. 2, figure supplement 5.

A *Glenodinium foliaceum*



B *Nannochloropsis gaditana*

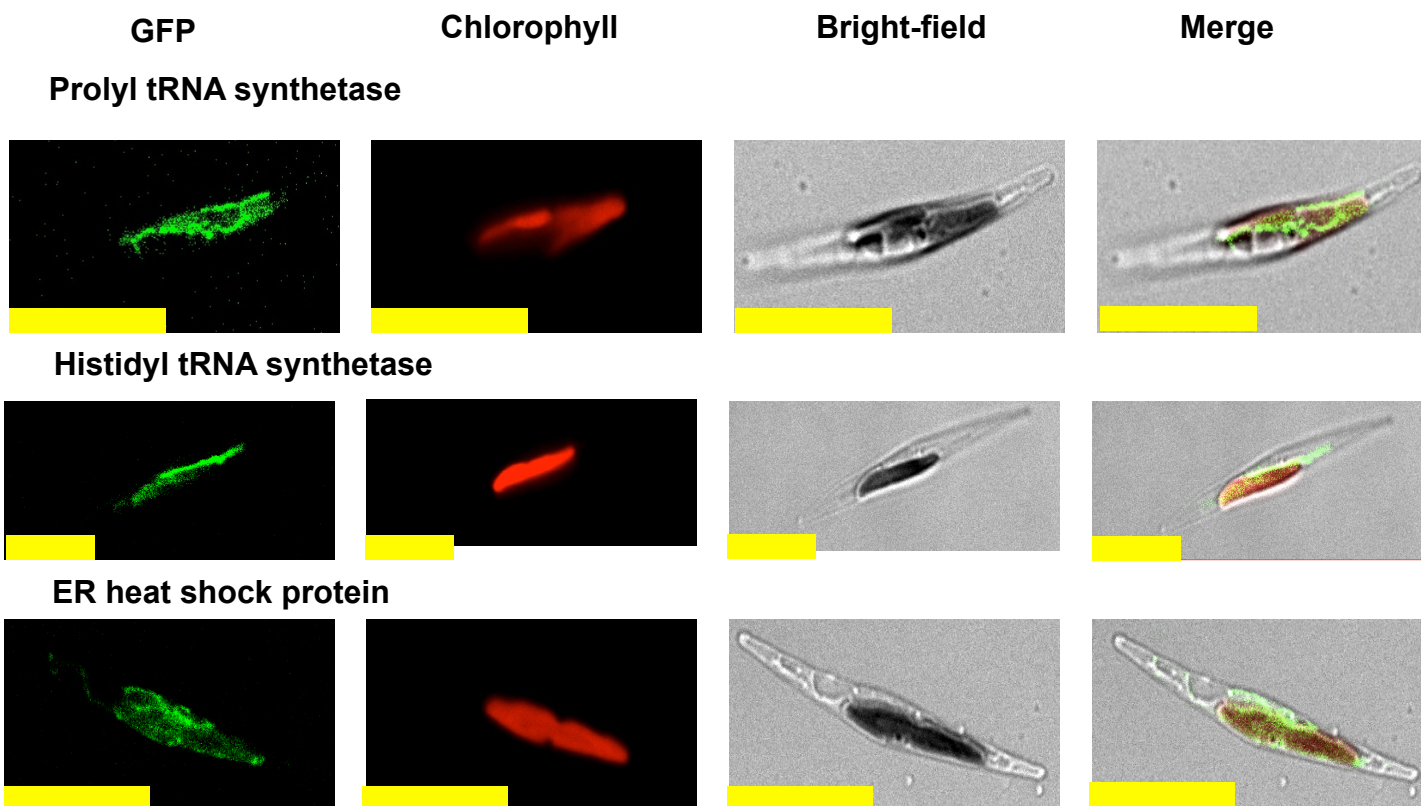


Fig. 2- figure supplement 7. Exemplar control images for confocal microscopy. This figure shows fluorescence patterns for wild-type *Phaeodactylum tricornutum* cells (i), and transformant *Phaeodactylum* cells expressing GFP that has not been fused to any N-terminal targeting sequence (ii), both visualised under the same conditions used for all other transformant cultures.

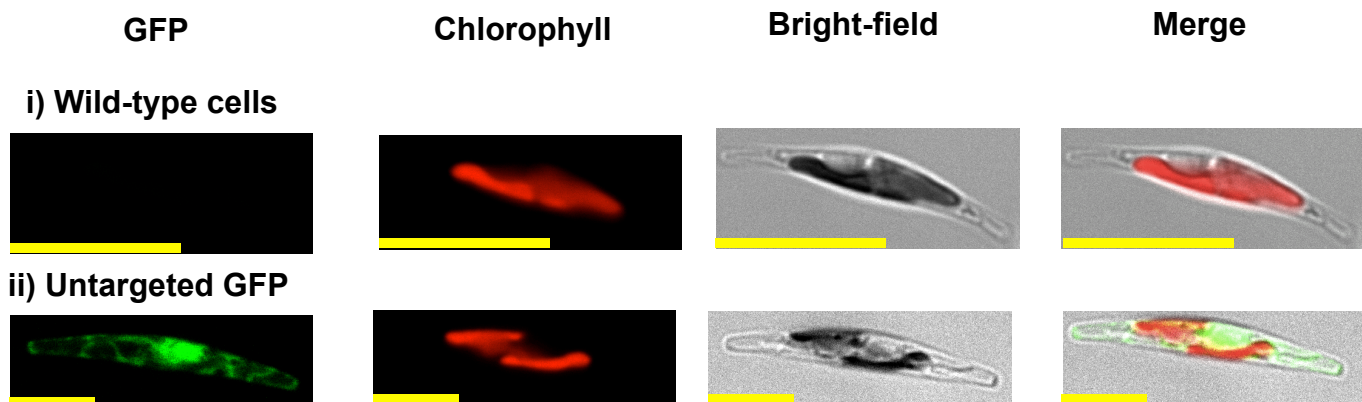


Fig. 4- figure supplement 1. Sampling richness associated with ancestral HPPGs of green algal origin. This figure shows the number of sub-different archaeplastid orthologues for ancestral HPPGs verified by combined BLAST top hit and single-gene tree analysis to be of either green algal origin (green bars) or red algal origin (red bars), for which glaucophyte orthologues could also be identified.

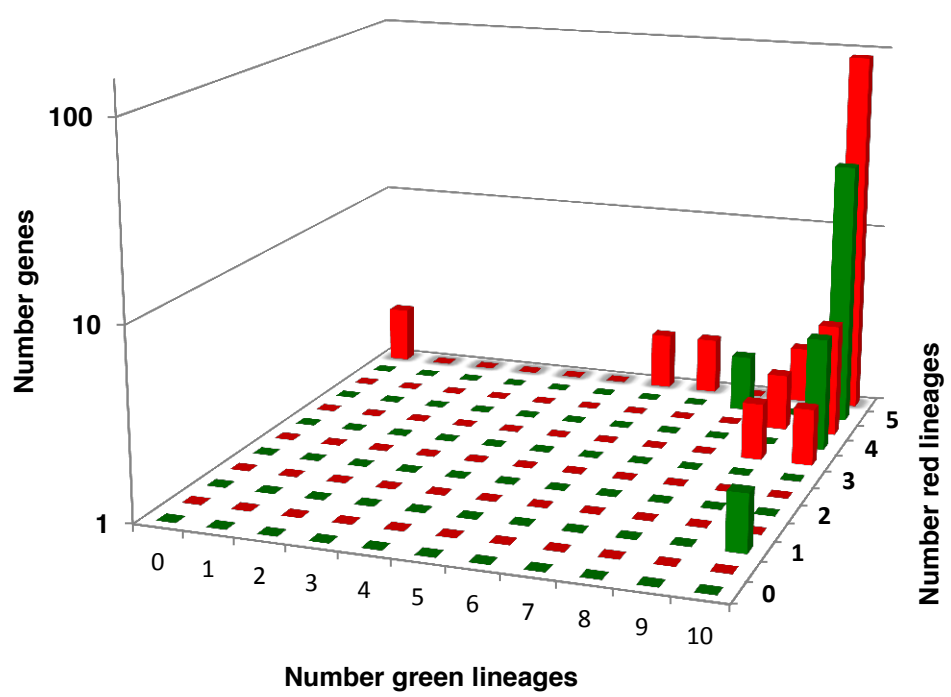


Fig.4- figure supplement 2. Heatmaps of nearest sister-groups of ancestral HPPGs of verified green origin. This figure shows the specific topologies of single gene trees for HPPGs verified to be of green origin by combined BLAST and phylogenetic analysis. **Panel A** shows a reference topology of evolutionary relationships between green lineages, defined as per Leliaert et al. 2011. Six ancestral nodes that might correspond to the origin point of ochrophyte HPPGs are labelled with coloured boxes. **Panel B** shows the presence and absence of each green sub-category in the immediate sister-group to the ochrophyte HPPG in each single tree of HPPGs of verified origin. HPPGs are grouped by the inferred origin point within the green algae, with the number of HPPGs identified for each origin point given with round brackets.

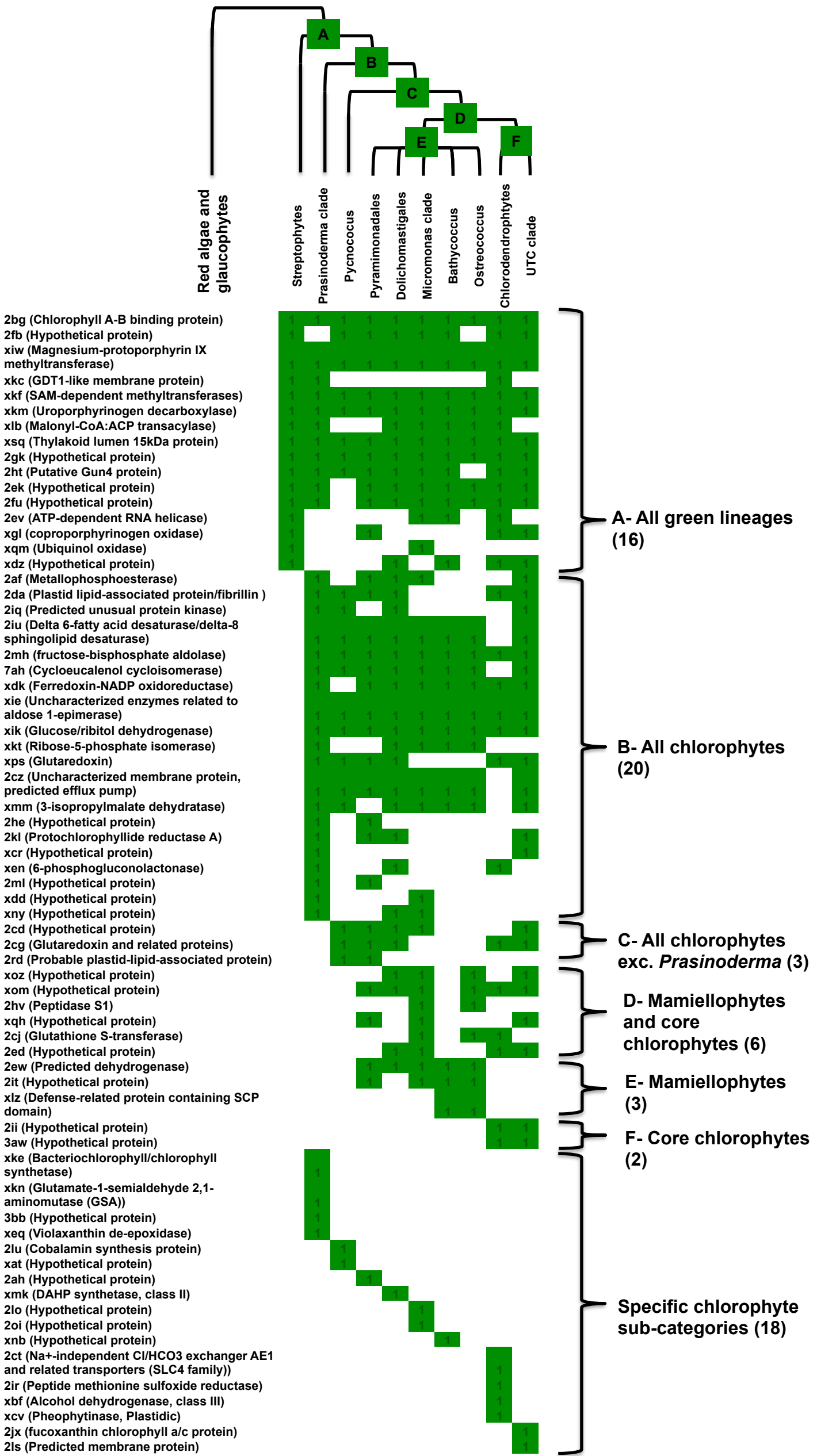
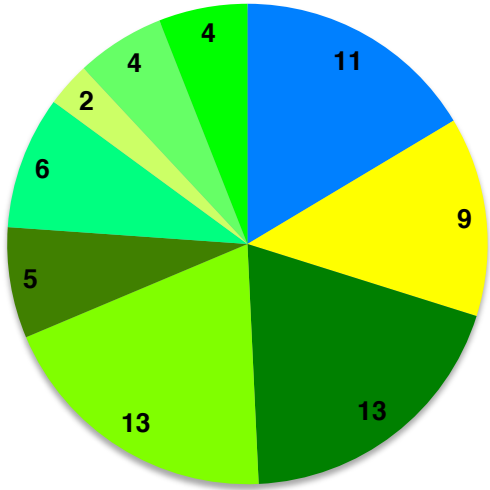


Fig. 4- figure supplement 3. Specific origins of green HPPGs as inferred from BLAST top hit analyses. These charts show (i) the number of BLAST top hits against each of the individual green sub-categories from HPPGs for which a green origin was identified both from BLAST top hit and single-gene tree analysis, and (ii) the total number of non-redundant sequences from each green sub-category included in the BLAST library.

i) Number top hits



- Streptophytes
- UTC clade
- Chlorodendrophytes
- Pyramimonadales
- Dolichomastigales
- Micromonas + Mantoniella
- Bathycoccus
- Ostreococcus
- Pycnococcus
- Prasinoderma + Nephroselmis

ii) Dataset size

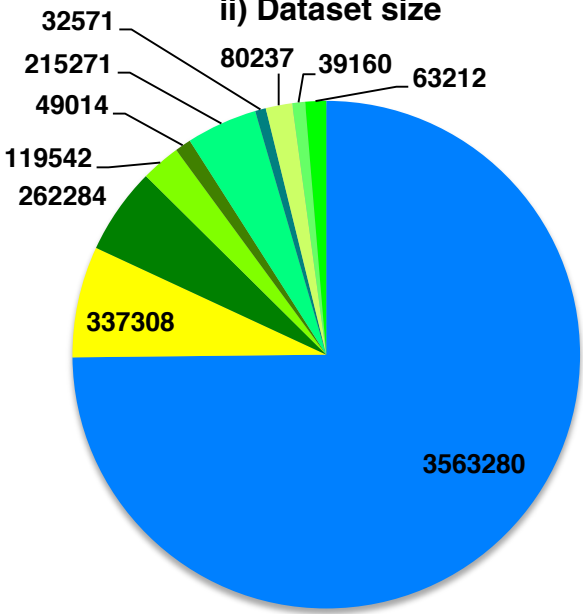


Fig. 4- figure supplement 4. Earliest evolutionary origins of shared plastid residues. This figure shows the number of residues in the concatenated alignment of HPPGs of verified green origin, which have been subsequently vertically inherited in all major photosynthetic eukaryotes that are present in green algae and ochrophytes, and are not found in red algae and glaucophytes. Residues are divided by inferred origin point, and are shown as per fig. 4, panel D. The values here are calculated as the earliest possible origin point for each uniquely shared residue, in which all gapped and missing positions within the alignment are treated as potential identities. 100 of the 147 residues inferred to have originated within green algae in this analysis originated either within a common ancestor of all chlorophytes, or in a common ancestor of all chlorophytes excluding the basally divergent lineages *Prasinoderma*, *Prasinococcus* and *Nephroselmis*.

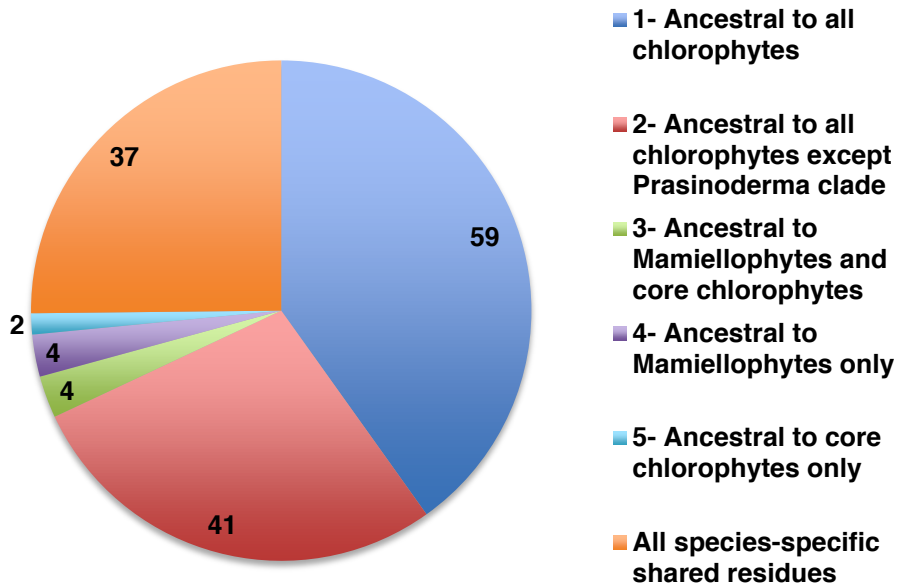
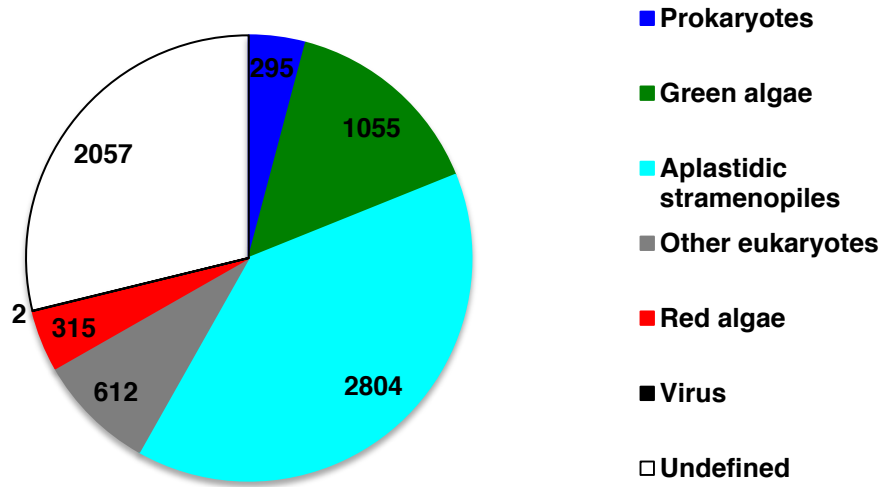


Fig. 4- figure supplement 5. Origins and HECTAR based targeting tests of proteins encoded by conserved ochrophyte gene clusters. Panel A shows the most probably evolutionary origin, identified using BLAST top hit analysis, for 7140 conserved gene clusters inferred to have been present in the last common ochrophyte ancestor. **Panel B** shows the number of these gene families that are predicted by HECTAR to encode proteins targeted to the plastid, subdivided by probable evolutionary origin, and the number expected to be present in each category assuming a random distribution of plastid-targeted proteins across the entire dataset, independent of evolutionary origin. Categories inferred to be significantly enriched above the expected values are labelled with black arrows.

A



B

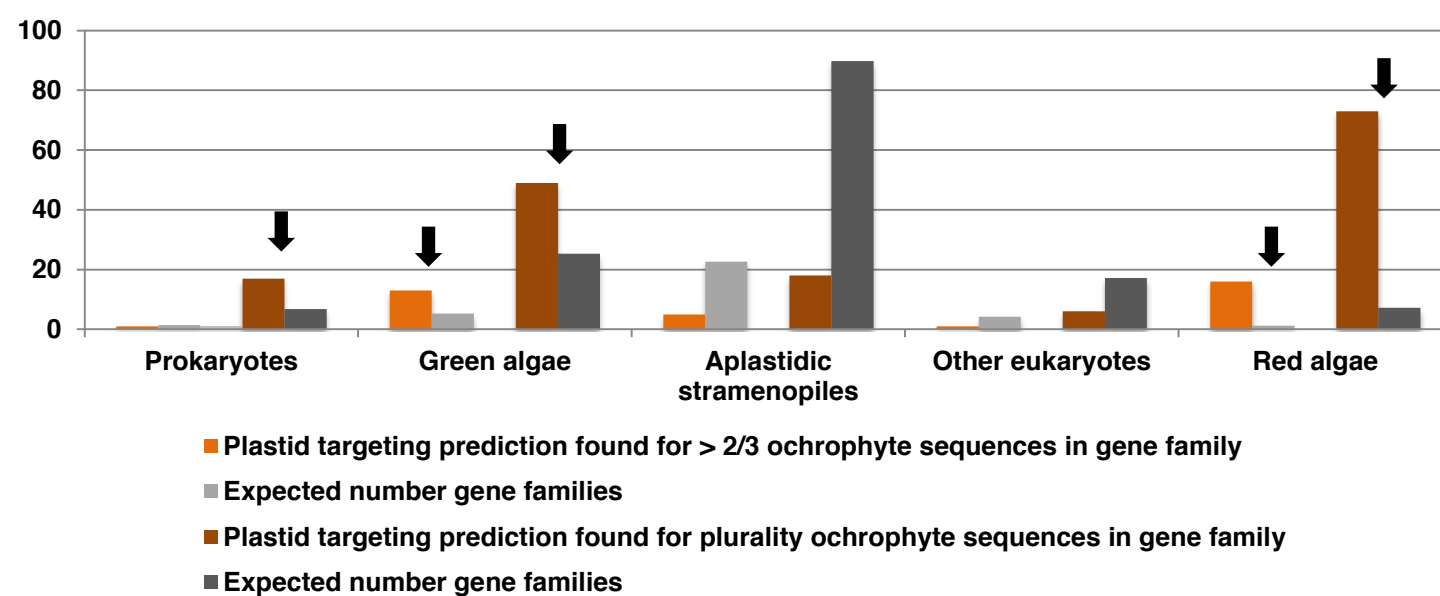
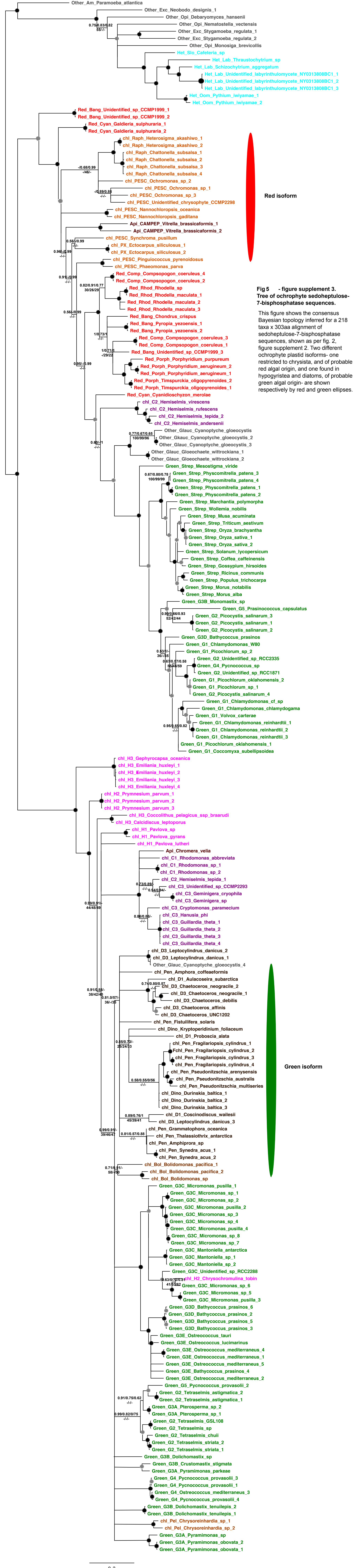


Fig. 5- figure supplement 2. Core plastid metabolism proteins not identified within the ancestral HPPG dataset.

Enzyme	Pathway	Distribution	Probable explanation	References
Sedoheptulose-bis-phosphatase	CBB cycle	Multiple isoforms	Functionally conserved, but with different LGT events in different ochrophyte lineages	Fig. supplement 3
Transaldolase	CBB cycle	Hypogyristera and diatoms	Functionally complemented by sedoheptulose-bis-phosphatase/ fructose-bisphosphate aldolase	Kroth et al., 2008
Isopropylmalate dehydrogenase	Leucine biosynthesis	Multiple isoforms	Functionally conserved, but with different LGT events in different ochrophyte lineages	Fig. supplement 4
3-dehydroquinase	Shikimate biosynthesis	Multiple isoforms	Functionally conserved, but with different LGT events in different ochrophyte lineages	Fig. supplement 5
Shikimate kinase	Shikimate biosynthesis	Multiple isoforms	Functionally conserved, but with different LGT events in different ochrophyte lineages	Fig. supplement 6
APS kinase	Fe-S cluster biosynthesis	Not found	Functionally dispensible; may be complemented by PAPS reductase	Gutierrez-Marcos et al. 1996
Magnesium protoporphyrin IX methylmonoester cyclase	Chlorophyll biosynthesis	Not found	Not known to be essential for chlorophyll metabolism outside of green lineage	Tanaka and Tanaka 2007
Isopentenyl diphosphate isomerase	Carotenoid biosynthesis	Not found	Dispensible for isoprenoid metabolism	Ershov et al. 2000; Rohdich et al. 2002
rps15	Ribosomal small subunit	Not found	Not known outside of green lineage	Green 2011



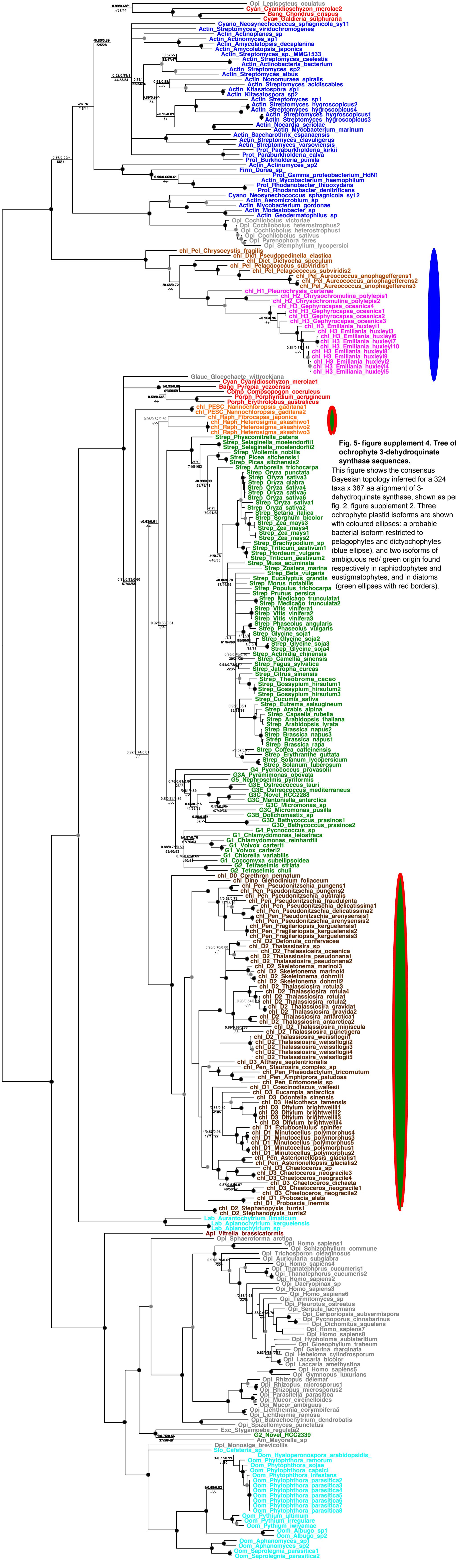


Fig. 5- figure supplement 4. Tree of ochrophyte 3-dehydroquinase sequences.

This figure shows the consensus Bayesian topology inferred for a 324 taxa x 387 aa alignment of 3-dehydroquinase synthase, shown as per fig. 2, figure supplement 2. Three ochrophyte plastid isoforms are shown with coloured ellipses: a probable bacterial isoform restricted to pelagophytes and dictyochophytes (blue ellipse), and two isoforms of ambiguous red/ green origin found respectively in raphidophytes and eustigmatophytes, and in diatoms (green ellipses with red borders).

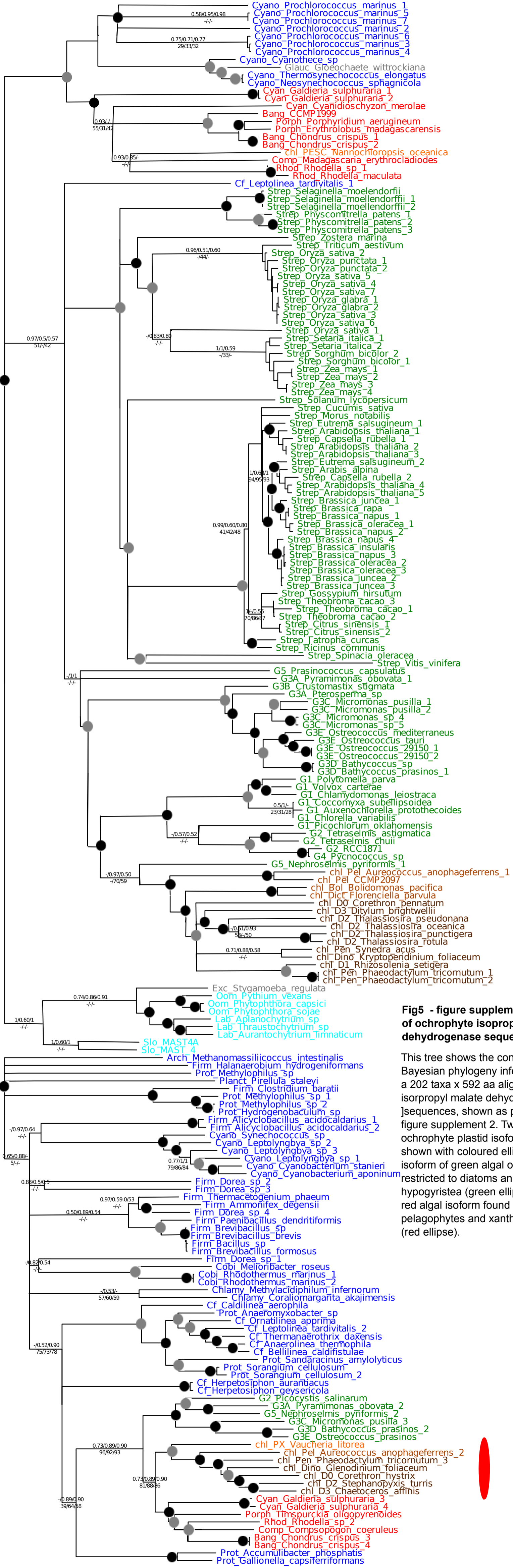


Fig5 - figure supplement 5. Tree of ochrophyte isoropropylmalate dehydrogenase sequences.

This tree shows the consensus Bayesian phylogeny inferred for a 202 taxa x 592 aa alignment of isoropropyl malate dehydrogenase sequences, shown as per fig. 2-figure supplement 2. Two ochrophyte plastid isoforms are shown with coloured ellipses: an isoform of green algal origin restricted to diatoms and hypogrystea (green ellipse), and a red algal isoform found in diatoms, pelagophytes and xanthophytes (red ellipse).

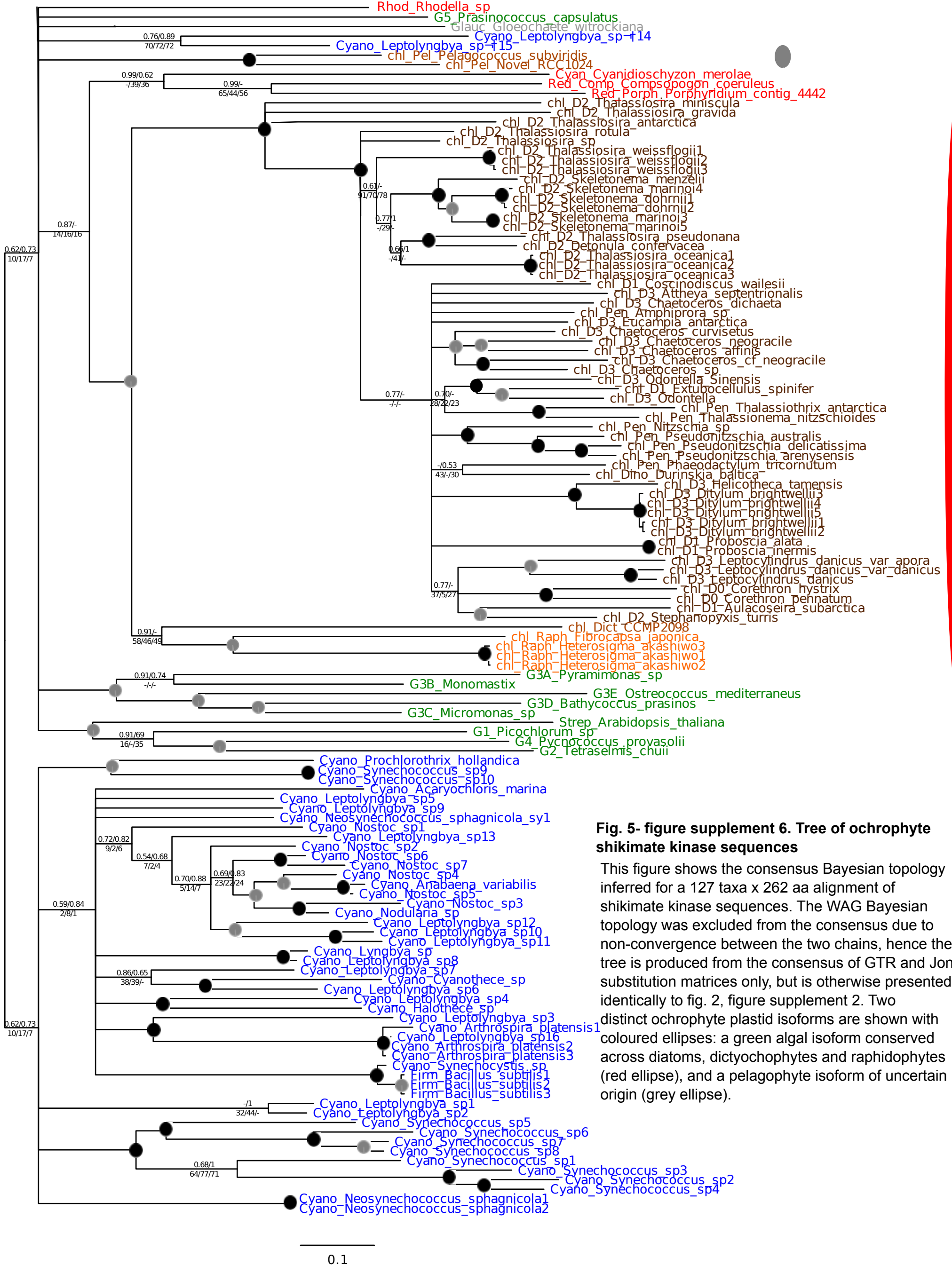


Fig. 5- figure supplement 6. Tree of ochrophyte shikimate kinase sequences

This figure shows the consensus Bayesian topology inferred for a 127 taxa x 262 aa alignment of shikimate kinase sequences. The WAG Bayesian topology was excluded from the consensus due to non-convergence between the two chains, hence the tree is produced from the consensus of GTR and Jones substitution matrices only, but is otherwise presented identically to fig. 2, figure supplement 2. Two distinct ochrophyte plastid isoforms are shown with coloured ellipses: a green algal isoform conserved across diatoms, dictyochophytes and raphidophytes (red ellipse), and a pelagophyte isoform of uncertain origin (grey ellipse).

Fig. 5- figure supplement 7. KOG classes associated with different categories of HPPGs.

These pie charts profile the distribution of different KOG classes across (i) all HPPGs except for those with general function predictions only, or without any clear KOG function, (ii) the same, but restricted to ancestral HPPGs and (iii) the same, for ancestral HPPGs of unambiguous red, green, prokaryotic and aplastidic stramenopile origin as identified by combined BLAST tophit and single-gene tree analysis. KOG classes that occur at elevated frequency in the ancestral HPPG dataset compared to the complete HPPG dataset, and one KOG class enriched in the prokaryotic HPPG dataset compared to the ancestral HPPG dataset (chi-squared test, $P < 0.05$) are labelled with horizontal arrows.

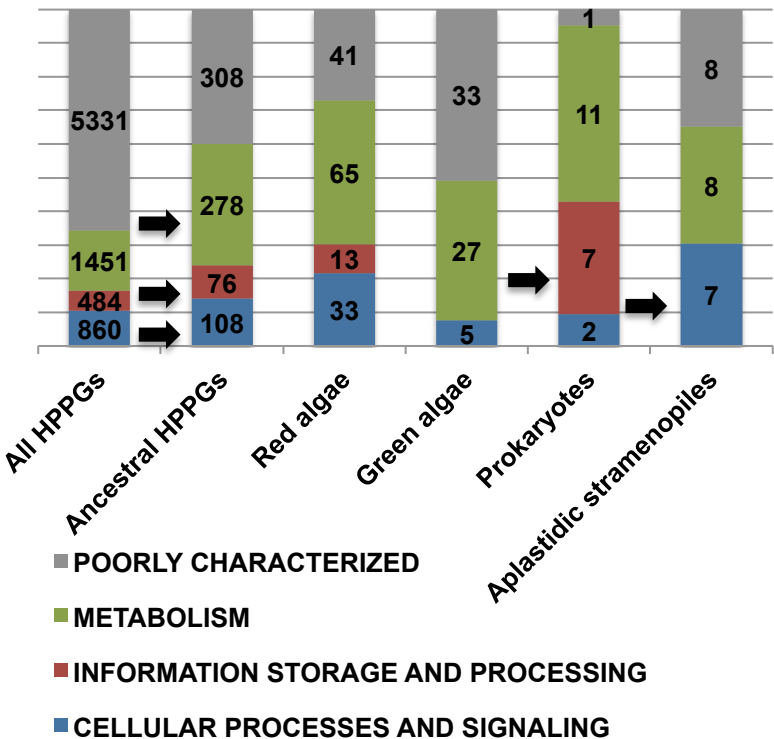
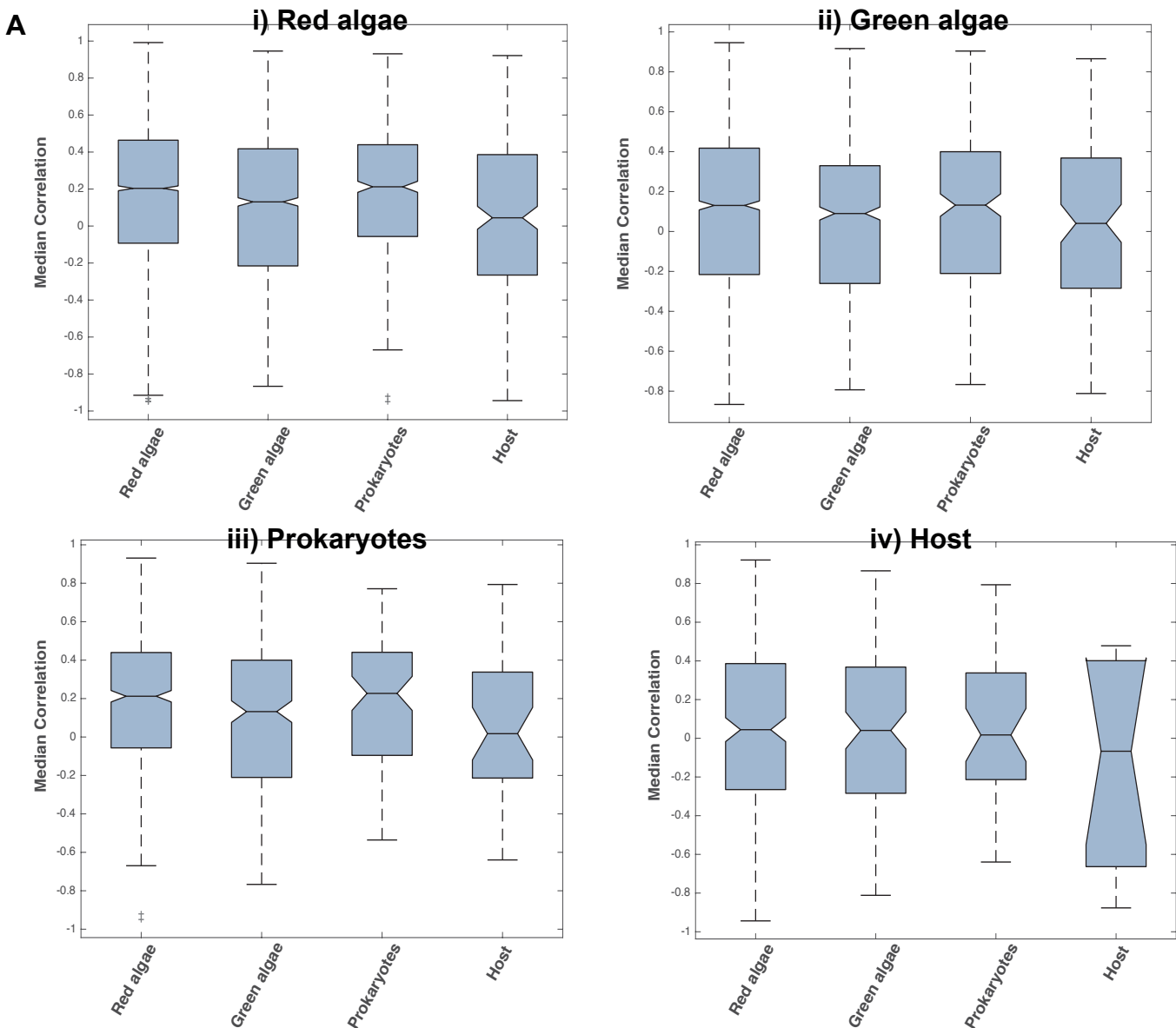


Fig. 5- figure supplement 8. Coregulation of genes incorporated into HPPGs of different origin in the model diatom *Phaeodactylum tricornutum*. Panel A shows boxplots of the correlation coefficients between the expression profiles of genes encoding members of ancestral HPPGs of red algal origin (i), green algal origin (ii), prokaryotic origin (iii) or host origin (iv), compared to genes encoding members of other HPPGs. Each HPPG is separated by evolutionary origin on the x-axis of each graph: for example, the box labelled “green algae” on the “red algae” graph shows the correlation coefficients between genes encoding members of ancestral HPPGs of red origin, and ancestral HPPGs of green origin. Panel B shows the P value statistics of mean separation calculated when comparing genes encoding members of ancestral HPPGs of the same origin (shown by row) to members of ancestral HPPGs of different origin (shown by column). For example, the intersect between the “red” row and “green” column shows the difference in mean correlation coefficient between pairs of genes that both encode members of ancestral HPPGs of red origin, and gene pairs of which one encodes an ancestral HPPG member of red origin, and the other an ancestral HPPG member of green origin. None of the P values calculated are significant, i.e. there are no categories of ancestral HPPG in which the internal correlation coefficients of gene expression are any different to those observed across the dataset as a whole.

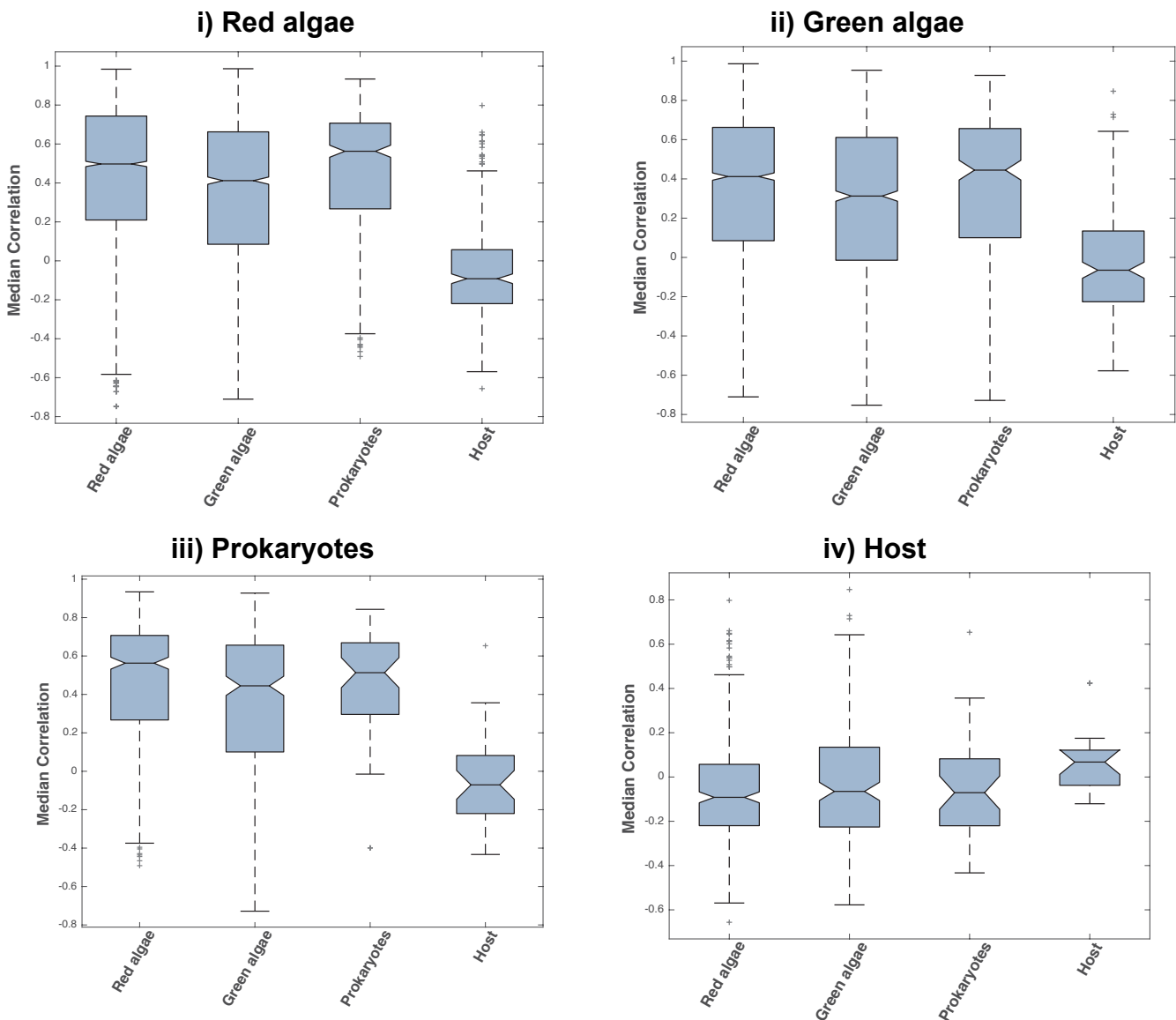


B

	Red	Green	Prokaryotic	Host
Red		0.393	-0.945	0.491
Green	-0.555		-0.780	0.905
Prokaryotic	-0.358	-0.432		0.564
Host	-0.925	0.598	-0.475	

Fig. 5- figure supplement 9. Coregulation of genes incorporated into HPPGs of different origin in the model diatom *Thalassiosira pseudonana*. Boxplots (**Panel A**) and P value statistics (**Panel B**) are shown as per Fig. 5- figure supplement 8. Only two of the correlation value ANOVA tests (comparison of red-red and red-host correlations, and prokaryotic-prokaryotic and prokaryotic-host correlations, shaded in green) reveal a significantly higher correlation coefficient between pairs of genes encoding members of HPPG of the same evolutionary origin than pairs of genes encoding members of HPPGs with different evolutionary origins. These differences most probably reflect the extremely weak correlation coefficients associated with genes encoding HPPGs of host origin to all other genes considered (compare “Host” category on boxplots **i**, **ii** and **iii** to all other categories); however, detailed comparison of the correlation values between genes encoding ancestral HPPGs of host origin and genes encoding ancestral HPPGs of different evolutionary origin (**Panel A**, boxplot **iv**; **Panel B**, bottom row) reveals no specific difference in the pairwise correlation values observed between genes encoding ancestral HPPGs of host origin, and genes encoding ancestral HPPGs of all other origins within the dataset.

A



B

	Red	Green	Prokaryotic	Host
Red		0.296	-0.833	0.005
Green	-0.376		-0.564	0.093
Prokaryotic	0.279	0.473		0.019
Host	-0.951	0.478	0.323	

Fig. 6- figure supplement 1. Alignments of an ochrophyte-specific riboflavin biosynthesis fusion protein. Panel A shows alignments of the full length (i) and cyclohydrolase domain only (ii) of a plastid-targeted GTP cyclohydrolase II/ 3,4-dihydroxy-2-butanone 4-phosphate synthase protein conserved across the ochrophytes. Coloured bars adjacent to each sequence correspond to the phylogenetic identity of the sequence. The cyclohydrolase domain of the ochrophyte protein is positioned in the N-terminal region, and the synthase domain in the C-terminal region. Three uniquely shared residues at the N-terminus of the cyclohydrolase domain confirm that it has been inherited from the aplastidic stramenopile ancestor of the ochrophytes.

A) i) Full sequence length



ii) Cyclohydrolase domain only



KEY

- █ Ochrophyte
- █ Aplastidic stramenopile
- █ Green alga
- █ Prokaryote
- █ Red alga

- ➔ GTP cyclohydrolase
- ➔ 3,4-dihydroxy-2-butanone 4-phosphate synthase
- ➔ Residue unique to stramenopiles
- ➔ Residue unique to stramenopiles and green algae

Fig. 6- figure supplement 2. Origins of ochrophyte plastid 3,4-dihydroxy-2-butanone 4-phosphate synthase. This figure shows the consensus Bayesian topology inferred for a 22 taxa x 206 aa alignment of 3,4-dihydroxy-2-butanone 4-phosphate synthase domains from different lineages, inferred using Jones and WAG matrices, and shown as per fig. 2, figure supplement 2. The ochrophyte plastid isoforms branch with red algal and actinobacterial sequences.

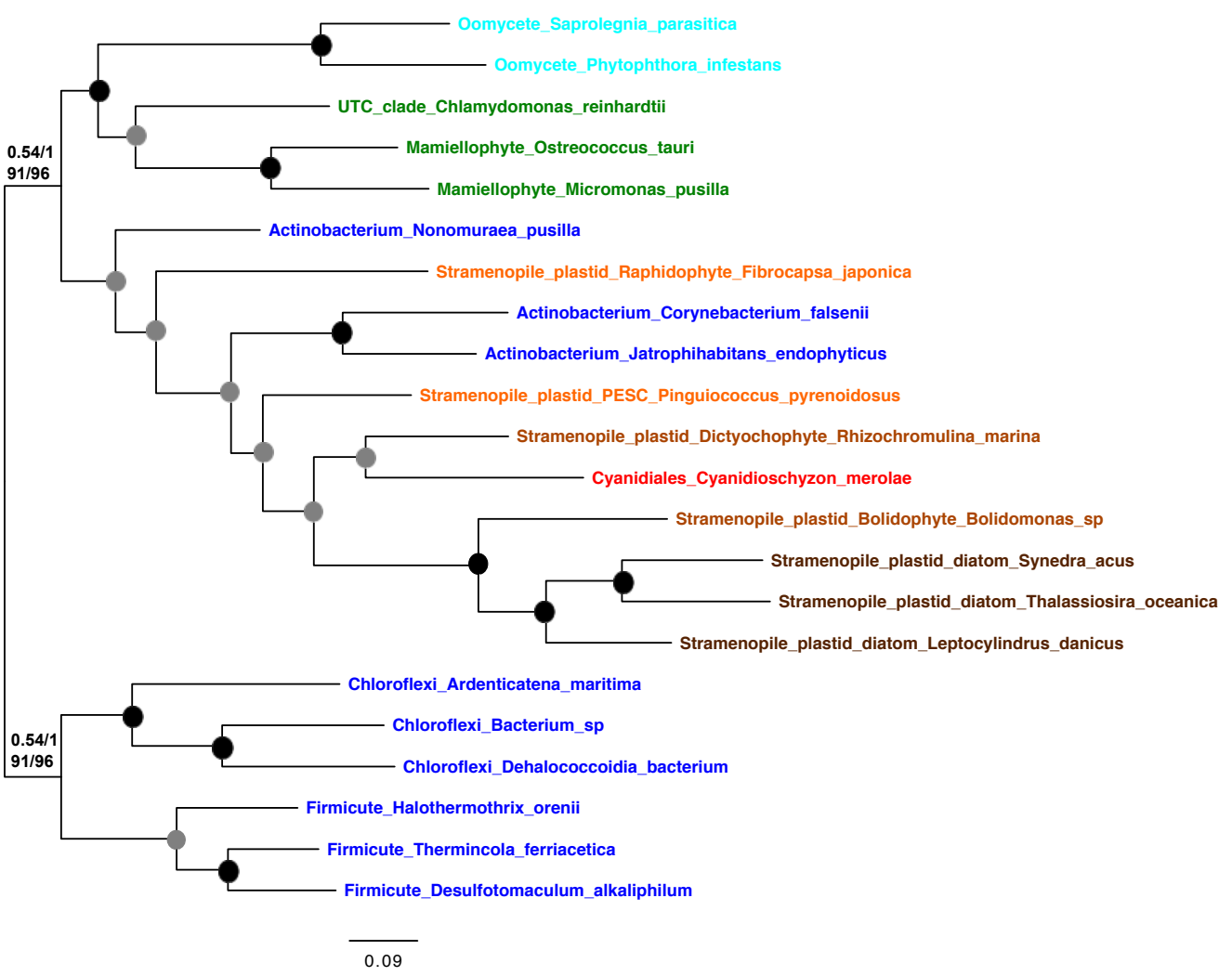


Fig. 6- figure supplement 3. An ochrophyte-specific Tic20 fusion protein. This figure shows alignments of the full length (i) and conserved region only (ii) of plastid Tic20 sequences, displayed as per figure supplement 9.

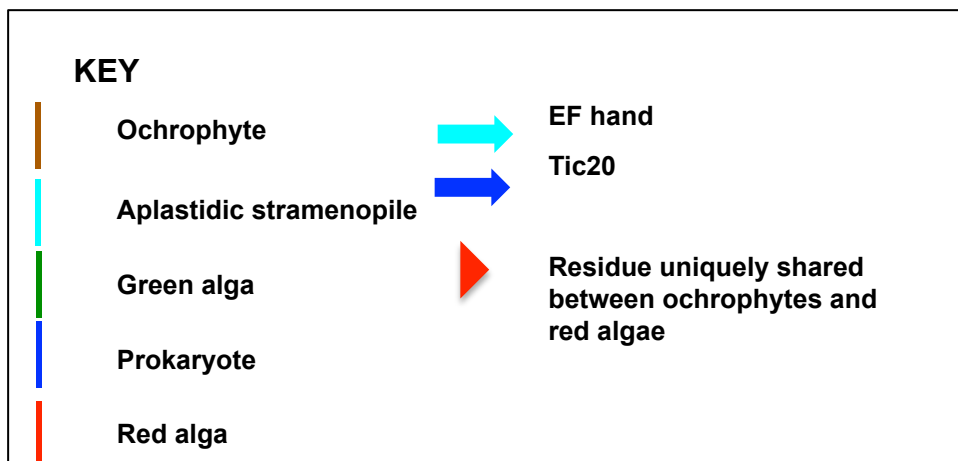
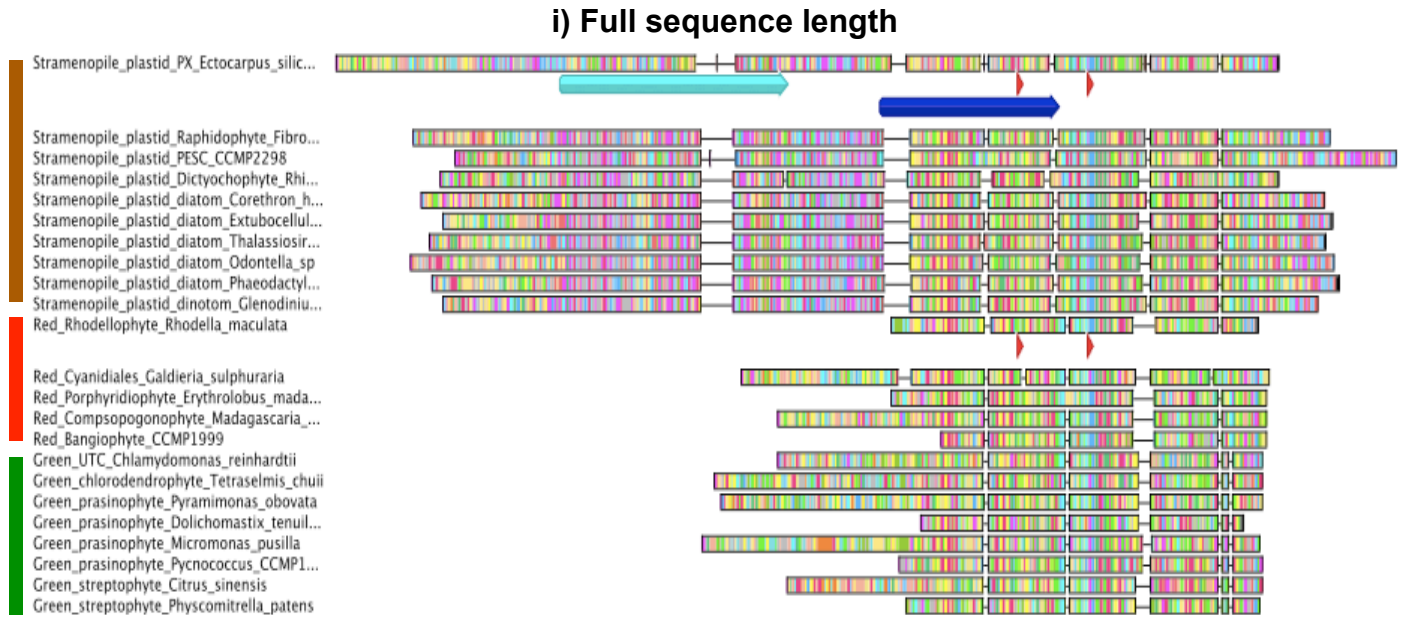


Fig. 7- figure supplement 1. Experimental verification of additional ochrophyte dual-targeted proteins. Panel A shows Mitotracker-orange stained *Phaeodactylum tricornutum* lines expressing four additional dual-targeted proteins (glycyl-, leucyl-, and methionyl-tRNA synthetases, and a predicted mitochondrial GroES-type chaperone) from *Phaeodactylum tricornutum*, and a dual-targeted histidyl-tRNA synthetase from *Glenodinium foliaceum*. **Panel B** shows control images that confirm an absence of crosstalk between GFP and mitotracker: wild-type *Phaeodactylum* cells stained with mitotracker, and cells expressing the *Glenodinium* histidyl-tRNA synthetase–GFP fusion construct and visualised with the mitotracker laser and channel in the absence of mitotracker stain.

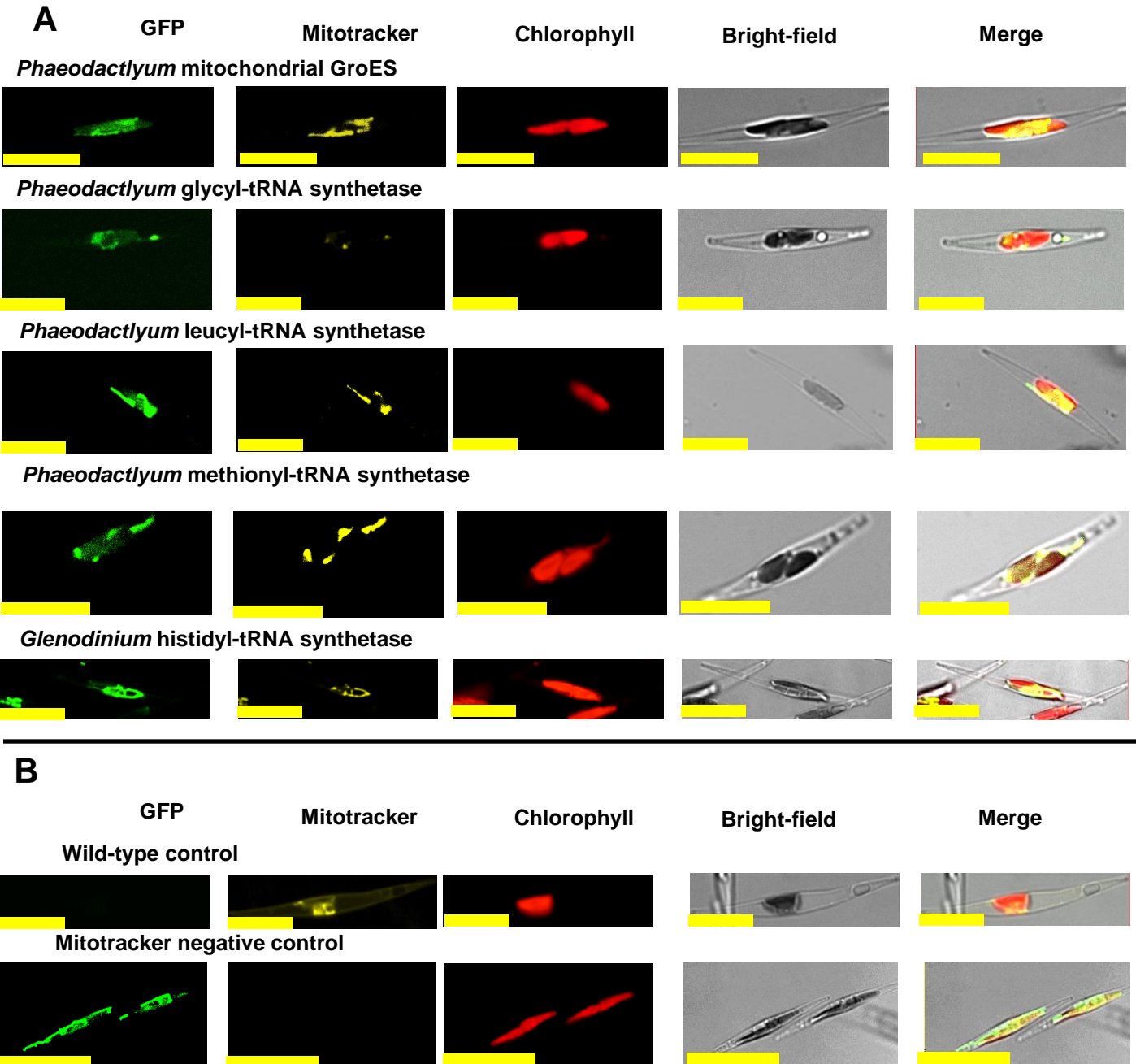
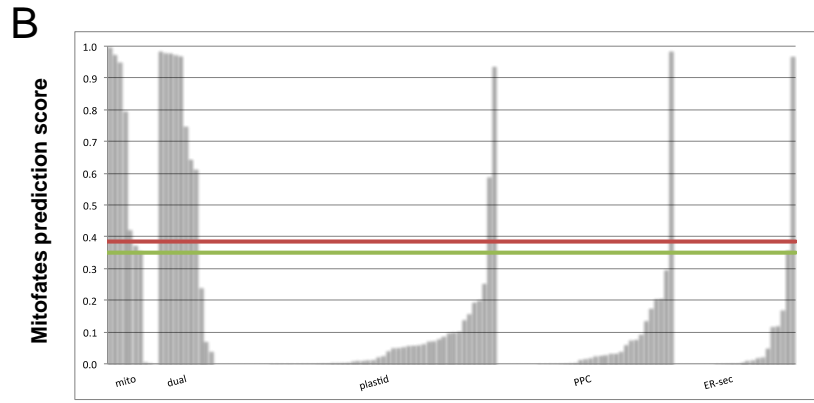
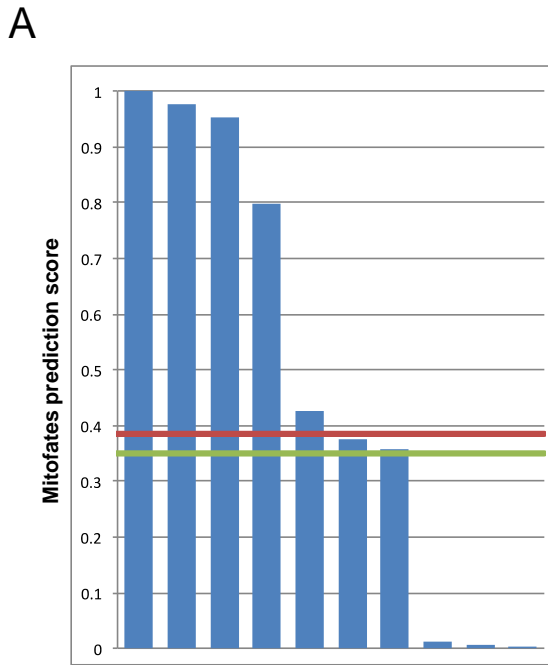


Fig. 7- figure supplement 2. Comparison of different in silico targeting prediction programmes for the identification of dual-targeted ochrophyte proteins. Panel A shows Mitofates scores for ochrophyte proteins verified experimentally to be dual targeted in this and a previous study⁹. **Panel B** shows Mitofates scores for all ochrophyte proteins for which a subcellular localisation has been identified in previous studies. The red lines in each graph show the Mitofates default cutoff (0.385) and the green lines indicate our chosen cutoff (0.35). **Panel C** compares different in silico targeting prediction algorithms with respect to predicted mitochondrial localization by experimentally validated localization. Mitofates strikes the best balance between high true positives and low false positives.



C

validated localization	% predicted mitochondrially targeted			
	mitofates >0.35	mitoprot >0.9	targetP >0.9	targetP >0.7
mitochondrion	70	70	40	60
mitochondrion and plastid	67	83	42	83
plastid	4	11	0	2
PPC	3	9	6	9
endomembrane or secreted	8	17	4	4

Fig. 8- figure supplement 1. Origin of proteins of ochrophyte origin in different CASH lineages. This figure profiles the evolutionary origins of proteins inferred by single-gene phylogenetic analysis to have been transferred from the ochrophytes into other lineages that have acquired plastids through secondary or more complex endosymbioses. Proteins are divided into the three major ochrophyte lineages (i.e. diatoms, chrysisista, and hypogyristea); all remaining proteins (inferred to have been acquired from an ancestor of multiple ochrophyte lineages, or of ambiguous but clearly ochrophyte origin) are grouped as a final category. The haptophyte proteins that could be attributed to a specific ochrophyte lineage are particularly skewed (100/178 proteins) to origins within the hypogyristea.

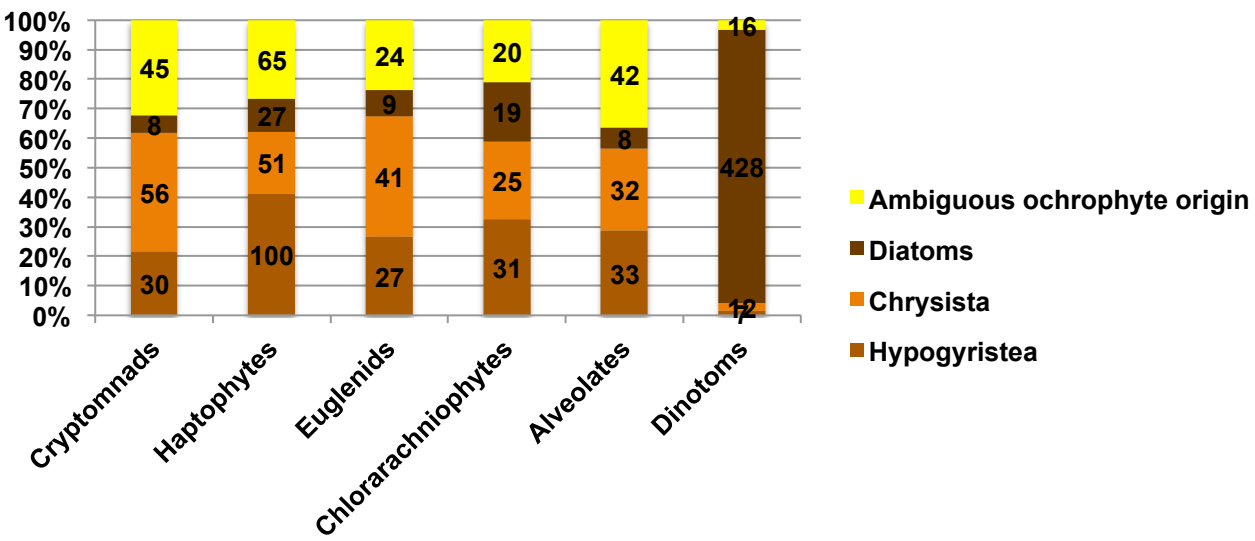


Fig.8- figure supplement 2. Heatmaps of nearest sister-groups to haptophytes in ancestral ochrophyte HPPG trees. This figure shows the specific ochrophyte lineages implicated in the origin of haptophyte plastid-targeted proteins, as inferred from the nearest ochrophyte sister-groups to haptophytes in trees of 242 haptophyte proteins of probable ochrophyte origin from combined BLAST top hit and single-gene tree analysis. At the top a schematic tree diagram of the ochrophytes is shown as per fig. 1, with six major nodes in ochrophyte evolution labelled with coloured boxes. The heatmap below shows the specific distribution of sister-groups in each tree, shown as per figure 4- figure supplement 2.

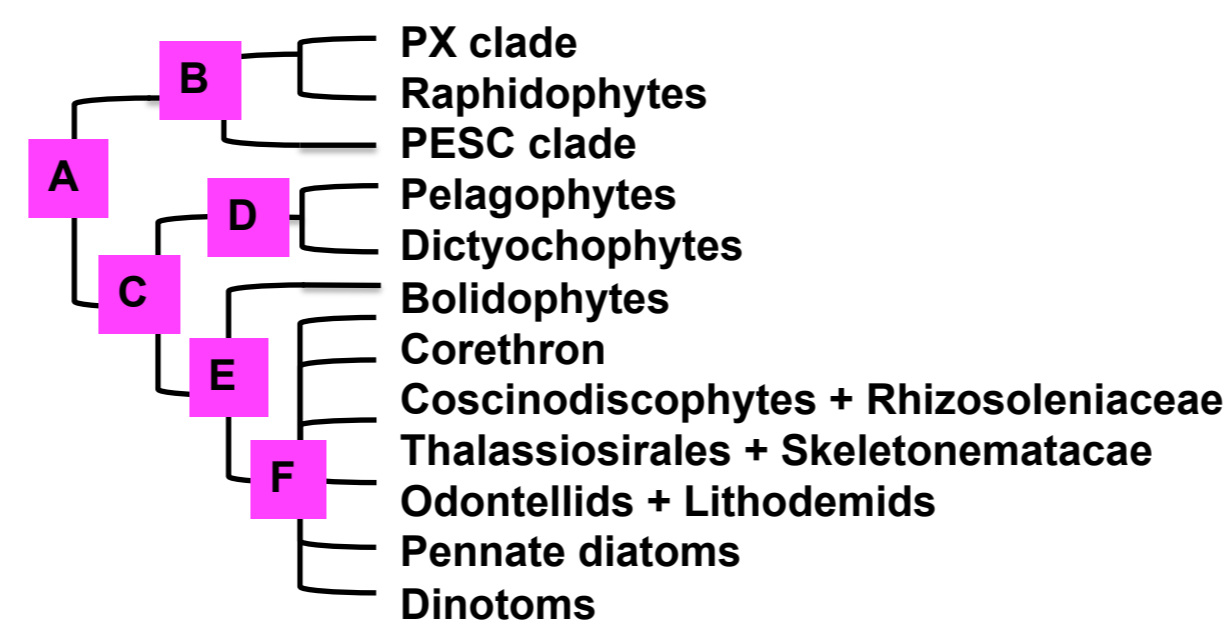


Fig. 8- figure supplement 3. Internal evolutionary affinities of haptophyte plastid-targeted proteins incorporated into ancestral ochrophyte HPPGs. This figure profiles the evolutionary origins of haptophyte plastid-targeted proteins incorporated into ancestral ochrophyte HPPGs by BLAST top hit analysis. Separate values are provided for query sequences from each of the three haptophyte sub-categories (pavlovophytes, prymnesiophytes, isochrysidales) considered within the analysis. Only sequences for which a consistent origin could be identified by both BLAST top hit and single-gene tree analysis are included. For each haptophyte lineage > 50% of the sequences verified by combined analysis to be of a specific ochrophyte origin have either pelagophyte or dictyochophyte top hits.

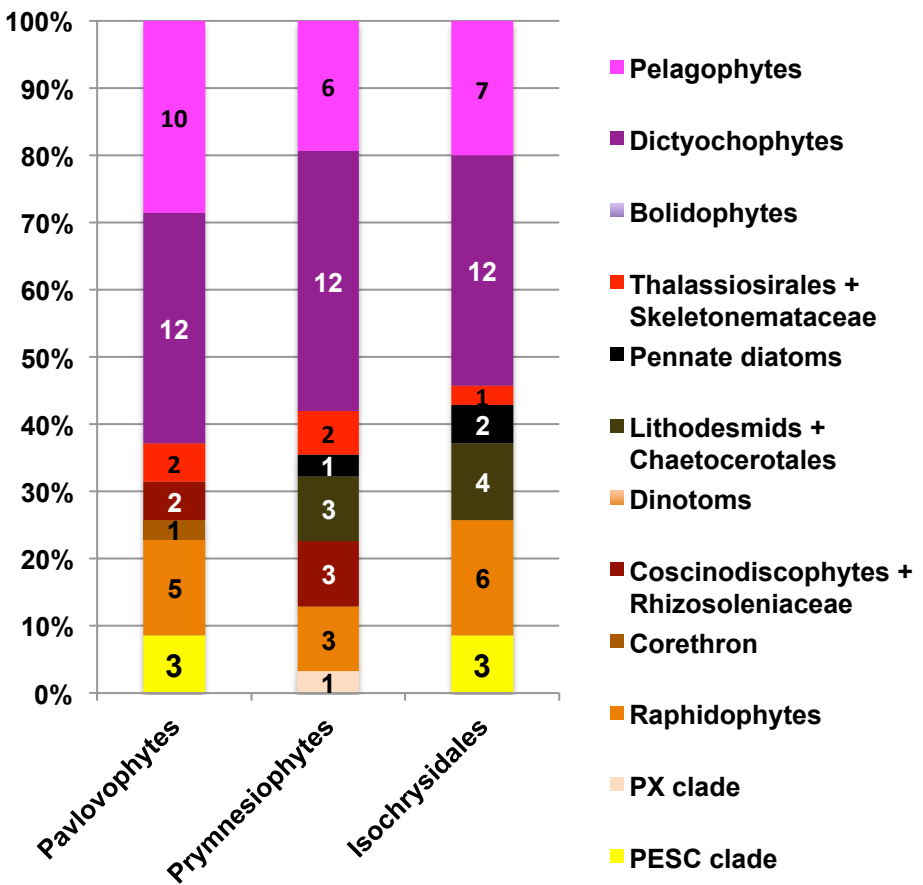


Fig. 8- figure supplement 4. Evidence for gene transfer from pelagophytes and dictyochyophytes into haptophytes. Panel A shows the next deepest sister groups identified for haptophyte proteins of hypogyrustean origin in single-gene trees. The pie chart (i) compares the number of single-gene trees in which the combined clade of haptophyte and hypogyrustean proteins resolves within a larger clade comprising the ochrophyte HPPG, compared to the number that resolves in external positions, either with other lineages or as a sister-group to all other sequences within the HPPG clade. Sequences for which no clear next deepest sister group affinity could be identified are listed as “not determined”. The heatmap (ii) shows the specific sister-group sequences associated with 65 HPPGs in which the haptophyte sequences specifically resolve with the pelagophyte/ dictyochyophyte clade and for which a clear internal or external position for the haptophyte/ hypogyrustean group relative to the remaining ochrophyte HPPG clade could be identified. Both analyses indicate a clear bias for haptophyte sequences branching within a deeper ochrophyte clade, not just restricted to the immediate sister-groups. **Panel B** tabulates the BLAST next best hits for haptophyte sequences for which a phylogenetically consistent (>3 consecutive top hits) top hit to hypogyrustea could be identified, and pelagophyte/ dictyochyophyte sequences for which a phylogenetically consistent top hit to haptophytes could be identified. In each case either the largest number of sequences, or (in the case of pavlovophytes) the joint largest number of sequences for which a phylogenetically consistent next best hit could be identified resolved with diatoms, indicating that these sequences were probably present in a common ancestor of diatoms and hypogyrustea, and subsequently transferred to haptophytes.

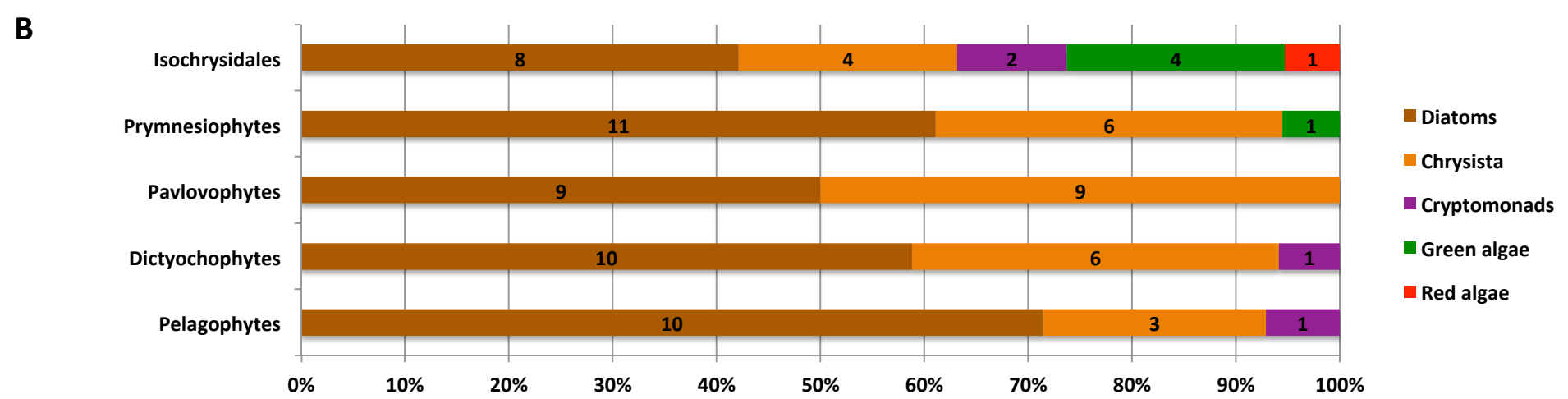


Fig. 8- figure supplement 5. Earliest possible origin points of uniquely conserved sites in haptophyte plastid-targeted proteins. This figure shows the total number of residues that are uniquely shared between a 37 proteins that have clearly been transferred between the ochrophytes and haptophytes, and are of subsequently entirely vertical origin, assuming the earliest possible origin point for each residue (i.e. in which gapped or missing positions were interpreted as identities). 87/ 128 of the uniquely shared residues inferred to originate within the ochrophytes were congruent to gene transfers between the haptophytes and pelagophyte and dictyochophyte clade; of these, slightly more than half (46) are inferred to have originated in a common ancestor of all hypogyristera and diatoms, consistent with the gene transfer having occurred from an ancestor of the pelagophytes and dictyochophytes into the haptophytes, rather than the converse.

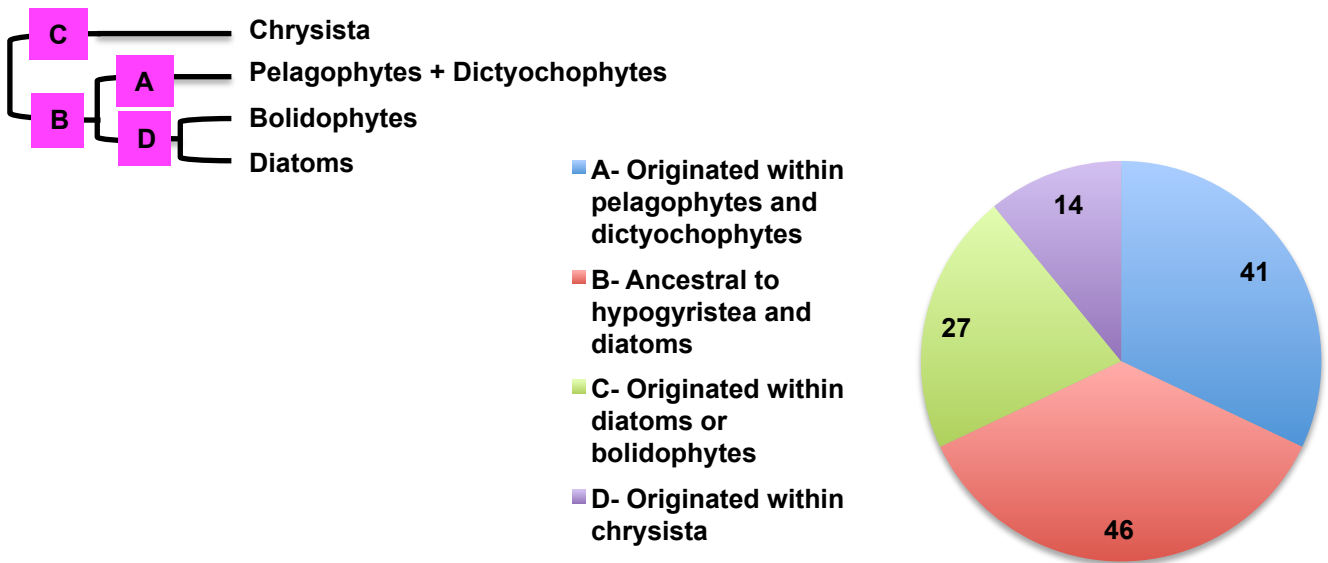


Fig. 8- figure supplement 6. Evolutionary origin of ancestral haptophyte genes. This figure shows the most likely evolutionary origin assigned by BLAST top hit analysis to the 12728 conserved gene families inferred to have been present in the last common haptophyte ancestor.

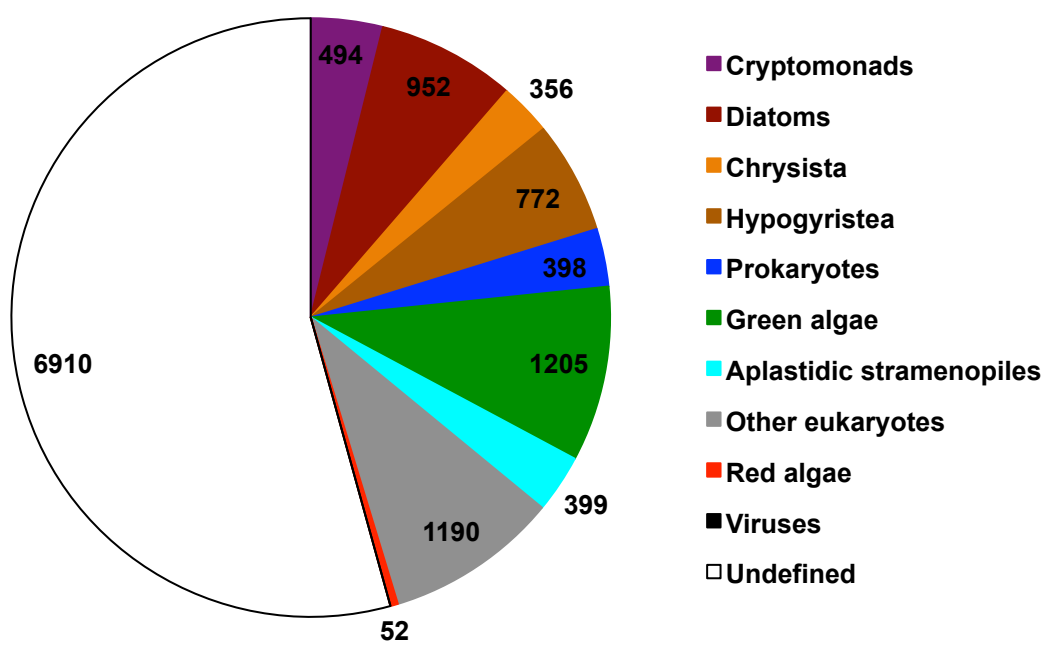
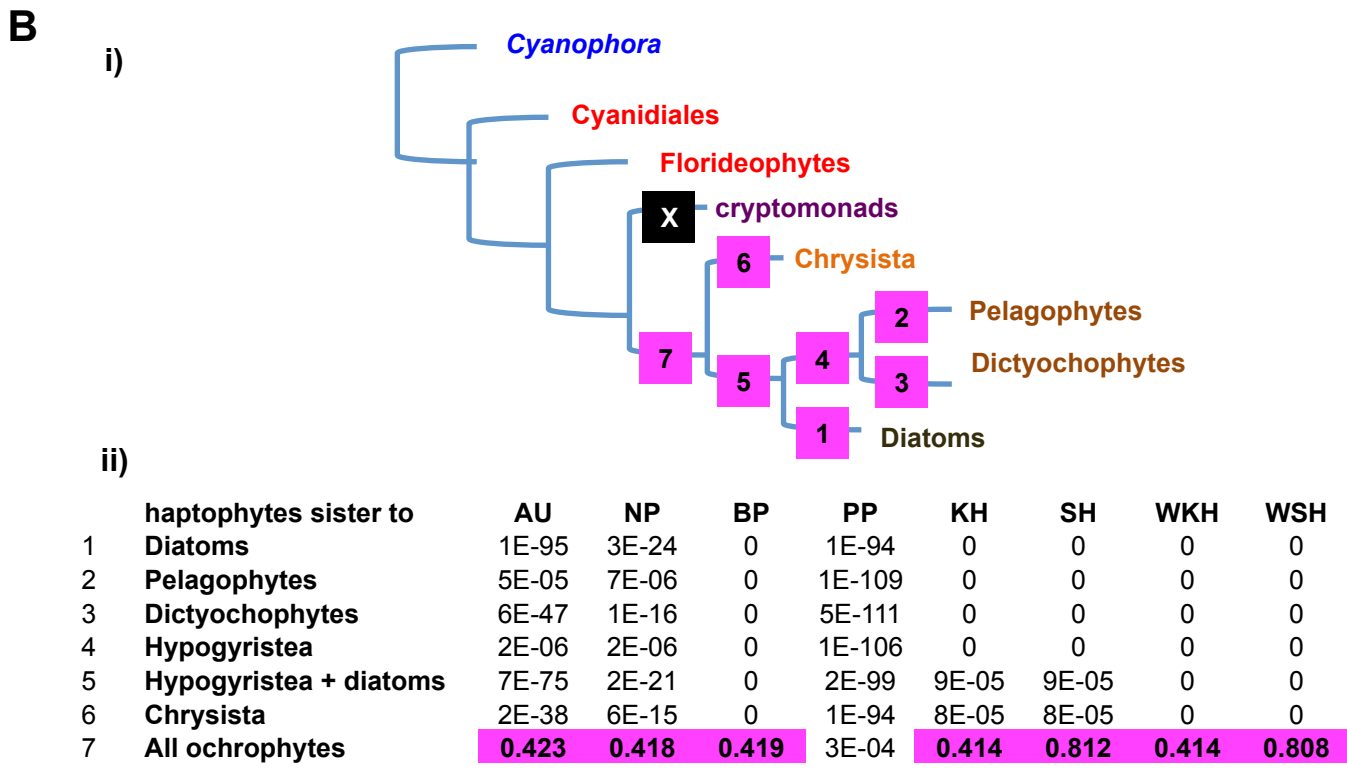
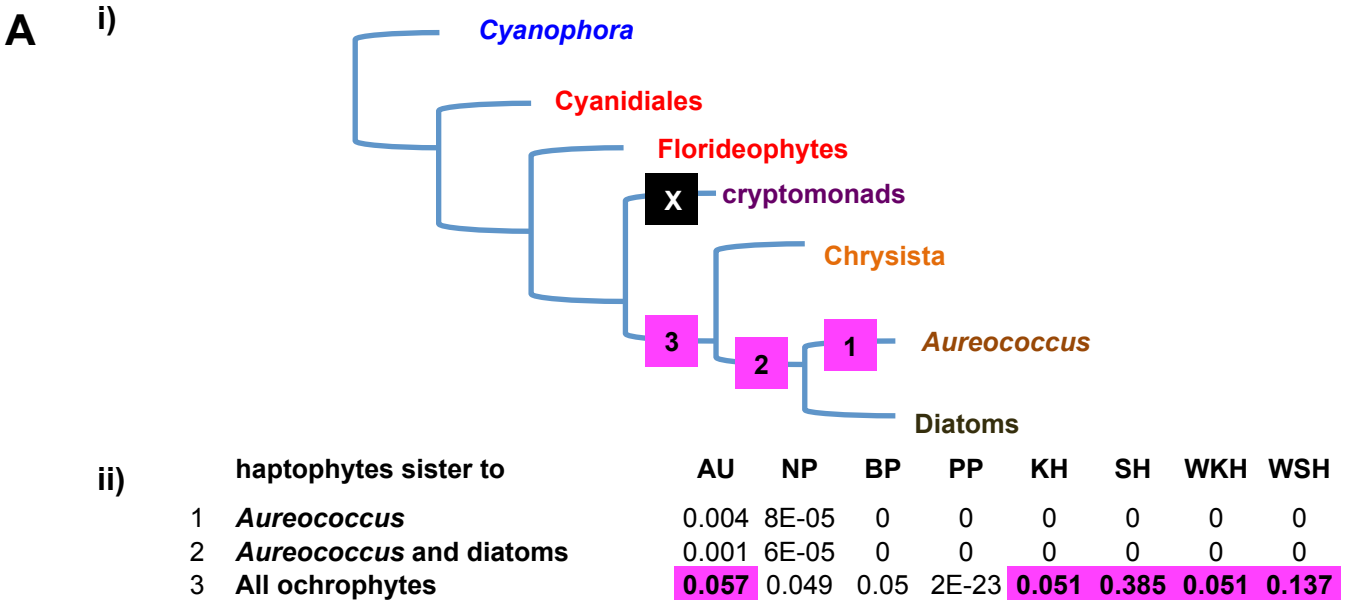
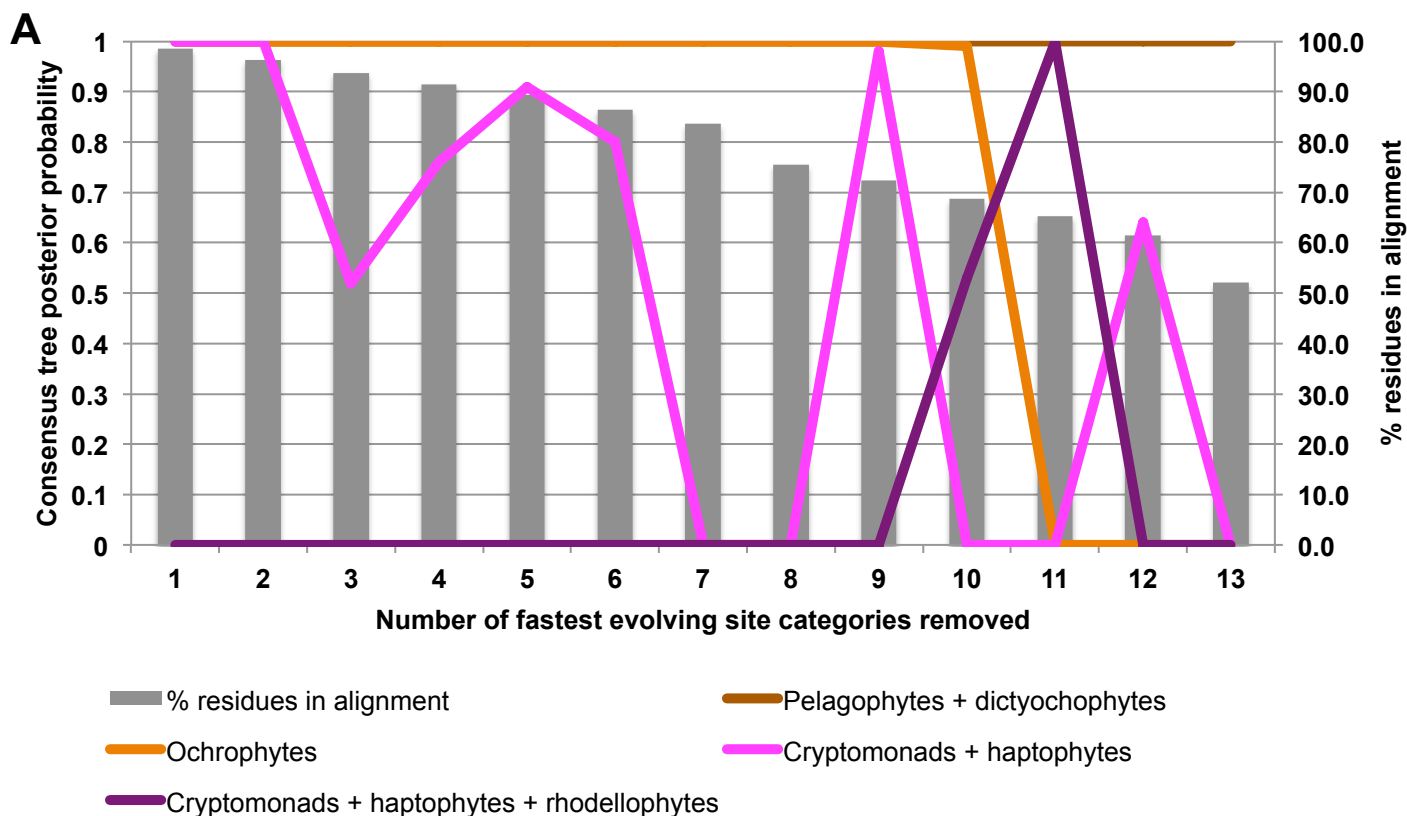


Fig. 9- figure supplement 1. Alternative topology tests of plastid genome trees. Tests were performed with the RAxML + JTT trees inferred for the gene-rich (**panel A**) and taxon-rich (**panel B**) plastid-encoded protein alignments. In each case, a schematic diagram of the tree topology obtained is given (i). The black box corresponds to the branch position of haptophytes in the consensus tree; alternative branching positions for the haptophyte sequences are labelled with numbered boxes. The table below (ii) lists the probabilities for each alternative position under eight different tests performed with CONSEL. Alternative positions that are not rejected by a topology test are shaded. All possible trees in which the haptophyte sequences branch within the ochrophytes are clearly rejected under all conditions, confirming that its plastid genome is of non-ochrophyte origin. The legend at the bottom of panel B gives full names for each test performed.



- AU - approximately unbiased test
- NP & BP - bootstrap probabilities for the selection
- PP - bayesian posterior probability (using BIC)
- KH - Kishino-Hasegawa test
- SH - Shimodaira-Hasegawa test
- WKH & WSH - weighted versions of the above two tests

Fig. 9- figure supplement 2. Fast site removal and clade deduction analysis of plastid genome trees. Panel A shows the support values obtained for Bayesian + Jones trees inferred from modified versions of the taxon-rich plastid multigene alignment from which the 13 fastest-evolving site categories had been removed for four different branching relationships pertaining to the placements of haptophyte and hypogyrystean sequences. The % of residues from the original alignment retained in each modified alignment are shown with grey bars. **Panel B** tabulates the support obtained for two different evolutionary relationships (haptophytes as a sister group to all cryptomonads, and as a sister group to all ochrophytes) in gene-rich (i) and taxon-rich (ii) alignments modified to remove all amino acids that occur at different frequencies in haptophytes to ochrophyte lineages, and modified to remove individual or pairs of CASH lineages. “x” indicates that the topology in question was not obtained.



B

Topology	Tree	No glycines	No variant aa	No diatoms	No chrysisista	No cryptomonads	No diatoms + chrysisista	No diatoms + cryptomonads	No chrysisista + cryptomonads
i) Gene-rich alignment									
cryptomonads + haptophytes	MrBayes	1	1	1	1	x	x	x	x
cryptomonads + haptophytes	RAxML	95	97	98	62	x	30	x	x
haptophytes + ochrophytes	MrBayes	x	x	x	x	1	x	1	1
haptophytes + ochrophytes	RAxML	x	x	x	x	100	x	100	100
ii) Taxon-rich alignment									
cryptomonads + haptophytes	MrBayes	1	0.84	1	1	x	x	x	x
cryptomonads + haptophytes	RAxML	35	x	x	x	x	x	x	x
haptophytes + ochrophytes	MrBayes	x	x	x	x	1	1	1	1
haptophytes + ochrophytes	RAxML	x	x	43	73	100	69	100	100

i) Gene-rich dataset

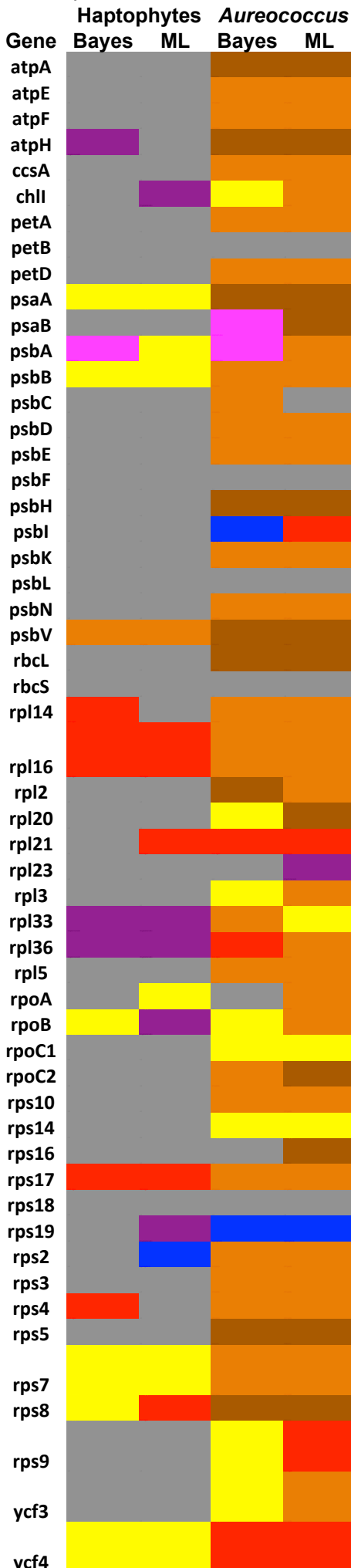
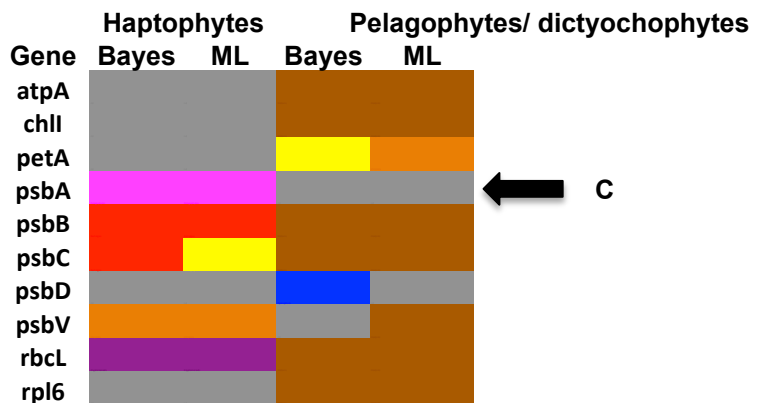


Fig. 9- figure supplement 3. Single-gene tree topologies associated with individual plastid-encoded genes. These heatmaps show the first sister-groups identified to haptophytes, and members of the pelagophyte/dictyochophyte clade, in single-gene trees of component genes included in concatenated trees of plastid-encoded proteins using both the gene-rich (i) and taxon-rich (ii) alignments. Topologies are given for trees inferred with MrBayes using the Jones substitution matrix, and RAxML trees inferred using JTT, under the same conditions as the multigene trees. The identity of the first sister-group is shaded according to the legend given below. Only three single-gene trees (labelled with black arrows) support any sister-group relationship between haptophytes and the pelagophyte/dictyochophyte clade; however, in each case (explained beneath the legend) this topology is not robustly supported, either due to polyphyly of one of the constituent lineages, or conflicting topologies identified via alternative methods.

ii) Taxon-rich dataset



Key

- Diatoms
- Chrysisista
- Multiple ochrophyte lineages
- Cryptomonads
- Red algae
- Glaucophytes
- Ambiguous topology
- Sister-group relationship between haptophytes and pelagophytes/ dictyochophytes

Detailed topological explanations of labelled trees

- A** *Aureococcus* resolves with *Pavlova*, but not other haptophytes
- B** *Aureococcus* and haptophytes resolve as sister-groups under Bayes only
- C** Haptophytes resolve with pelagophytes, but not dictyochophytes

Fig. 10- figure supplement 1. Complex origins of different ancestral ochrophyte HPPGs Panel A shows the evolutionary positions of lineages with histories of secondary endosymbiosis in trees of ancestral ochrophyte HPPGs verified by combined BLAST top hit and single-gene tree analysis to be either of red algal (i) or green algal origin (ii). In both cases, in more than half of the constituent trees, haptophyte and cryptomonad sequences resolve as closer relatives to the ochrophytes than the red or green algal evolutionary outgroup, either due to resolving in the ochrophyte HPPG or forming a specific sister-group to the ochrophyte lineages. **Panel B** plots the distribution of cryptomonads (i) and haptophytes (ii) in trees for different categories of ancestral ochrophyte HPPG of verified evolutionary origin. HPPGs of green algal origin more frequently show internal or sister positions for the cryptomonad sequences than all other categories of HPPG, and in more than 50% of cases resolve internal or sister positions for the haptophyte sequences. This might be consistent with a green algal contribution in the endosymbiotic ancestor of cryptomonad, haptophyte and ochrophyte plastids.

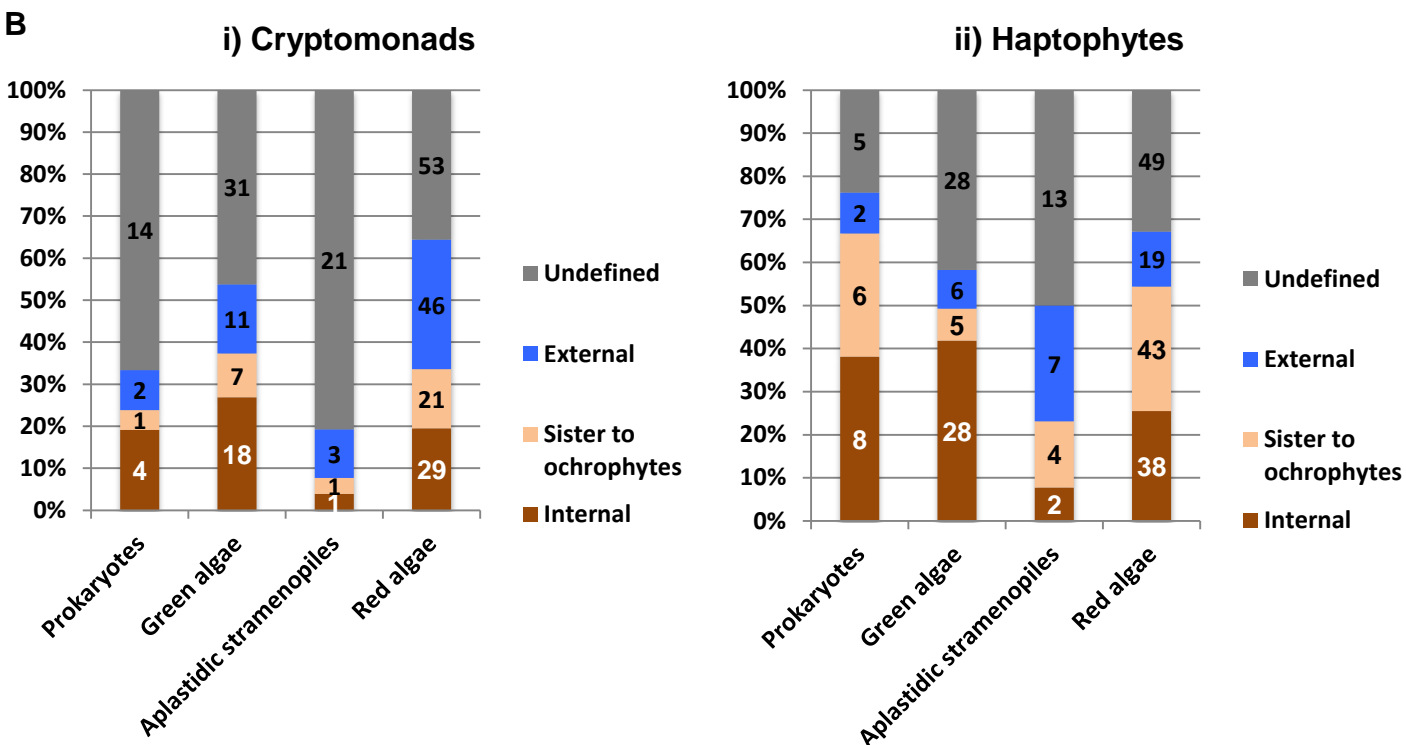
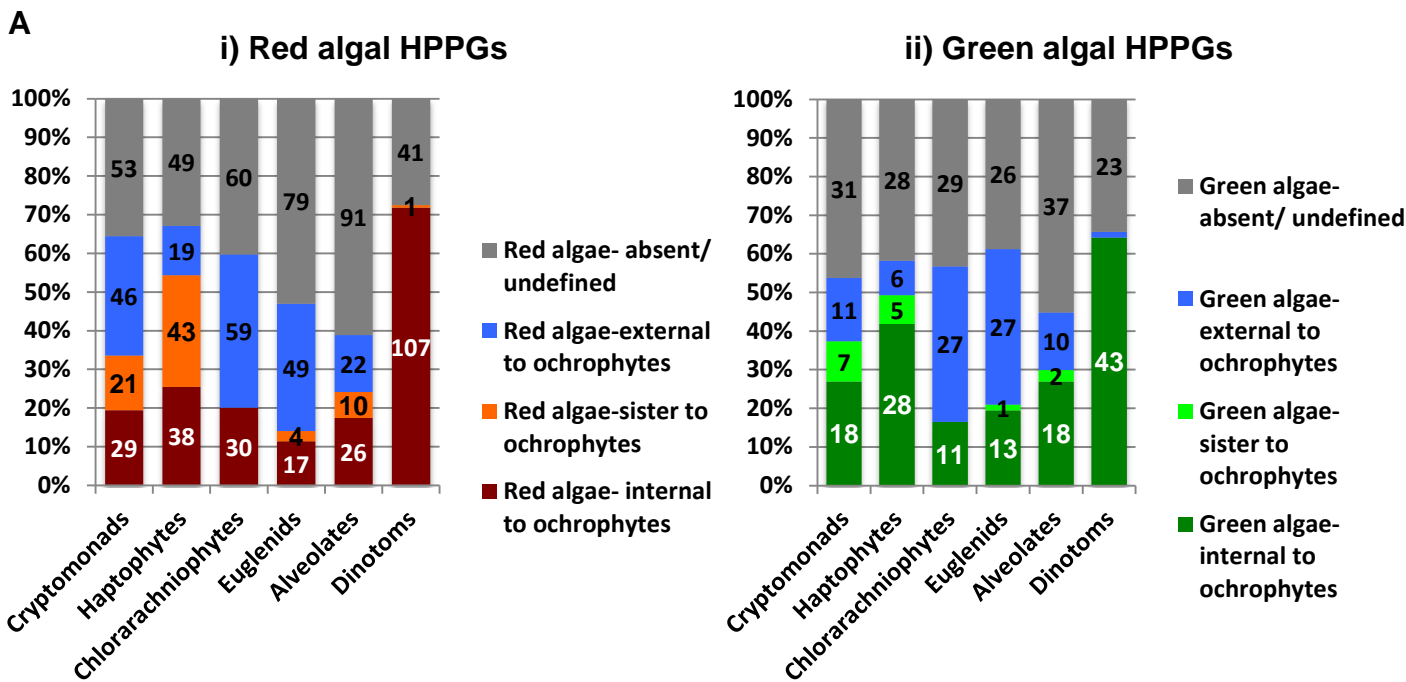


Fig. 10 –figure supplement 2. Different scenarios for the origins of haptophyte plastids. This schematic tree diagram shows different possibilities for the origins of the haptophyte plastid as predicted from the data within this study. No inference is made here regarding the ultimate origin of the ochrophyte plastid, although it is noted that the ochrophyte, cryptomonad and haptophyte plastids are likely to be closely related to one another within the red plastid lineages. First, a common ancestor of the pelagophytes and dictyochophytes was taken up by a common ancestor of the haptophytes (point 1), yielding a permanent plastid that contributed genes for a large number of plastid-targeted proteins in extant haptophytes. This plastid was subsequently replaced via serial endosymbiosis (point 2) yielding the current haptophyte plastid and plastid genome. This serial endosymbiosis event either involved a close relative of extant cryptomonads (**2A**) or a currently unidentified species that forms a sister-group in plastid gene trees to all extant ochrophytes, but is evolutionarily distinct from the pelagophytes (**2B**). It is possible that the haptophyte plastid may have been acquired through the secondary endosymbiosis of a different lineage of red algae to the ochrophyte, either via a cryptomonad intermediate (**2C**) or directly (**2D**).

