Subcellular compartments interplay for carbon and nitrogen allocation in *Chromera velia* and *Vitrella brassicaformis*

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ABSTRACT

Endosymbioses necessitate functional cooperation of cellular compartments to avoid pathway redundancy and streamline the control of biological processes. To gain insight into the metabolic compartmentation in chromerids, phototrophic relatives to apicomplexan parasites, we prepared a reference set of proteins probably localized to mitochondria, cytosol and the plastid, taking advantage of available genomic and transcriptomic data. Training of prediction algorithms with the reference set now allows a genome-wide analysis of protein localization in *C. velia* and *V. brassicaformis*. We confirm that the chromerid plastids house enzymatic pathways needed for their maintenance and photosynthetic activity, but for carbon and nitrogen allocation, metabolite exchange is necessary with the cytosol and mitochondria. This indeed suggests that the regulatory mechanisms operate in the cytosol to control carbon metabolism based on the availability of both light and nutrients. We discuss that this arrangement is largely shared with apicomplexans and dinoflagellates, possibly

stemming from a common ancestral metabolic architecture, and supports the mixotrophy of the chromerid algae.

KEYWORDS: chromerid, protein localization, prediction algorithm, endosymbiosis, plastid integration, mixotrophy

34 INTRODUCTION

35 Endosymbiotic organelles play crucial roles in cellular biochemistry.
36 Mitochondria, alpha-proteobacterial endosymbionts of eukaryotes (Oborník 2019;
37 Gruber 2019), represent an energetic hub, where balancing of catabolic and anabolic
38 processes takes place tightly regulated with the speed of respiration (e.g. Searcy 2003;
39 Pagliarini and Rutter 2013; Gray 2015). In plastids, domesticated cyanobacteria (Gruber
40 2019; Oborník 2019), inorganic carbon is fixed into sugars and several essential
41 compounds are synthesized, such as fatty acids, isoprenoid units, tetrapyrroles, and
42 amino acids (e.g. Tetlow et al. 2017). These semiautonomous endosymbiont-derived
43 organelles contain genetic information and their own translation apparatuses, but by far
44 do not encode all the proteins required for their function. Due to endosymbiotic gene
45 transfer, most essential genes were transferred from their genomes to the nuclear
46 genome and the organelles are greatly dependent on the import of proteins synthesized
47 in the cytosol (e.g. Mallo et al. 2018). Sorting of proteins to subcellular locations
48 specifically is therefore crucial for the correct function of both the proteins and the
49 organelles, and thus targeting signals and protein translocation are key to our
50 understanding of organellar biology (Kunze & Berger 2015).

51 Proteins destined to plastids and mitochondria typically encode an N-terminal
52 motif, i.e. a targeting presequence. The targeting presequence of mitochondrial proteins
53 is termed the mitochondrial transit peptide (mTP) and has the physicochemical
54 properties of an amphiphilic helix. Similarly, the chloroplast transit peptides of primary
55 algae (rhodophytes, chlorophytes and glaucophytes) and plants are amphiphilic helices,
56 though they are typically enriched in hydroxylated amino acids and less positively
57 charged than mTPs (Kunze & Berger 2015; Garg & Gould 2016). In comparison, complex
58 algae (those that maintain eukaryotic endosymbionts) including the chromerids, target

59 proteins to the plastid via the endomembrane (secretory) pathway, using chloroplast
60 transit peptides directly preceded by an ER signal peptide, which are referred to as
61 bipartite targeting sequences (BTS) (reviewed in Patron & Waller 2007).

62 Reconstruction of ancestral traits (Joy et al. 2016) allows us to unveil changes in
63 lifestyle and genome organization in an evolutionary perspective and to compare
64 functionalities among the organisms of interest. The discovery and genome
65 characterization of chromerids *Chromera velia* and *Vitrella brassicaformis*, the closest
66 known photosynthetic relatives of apicomplexan parasites, have provided an excellent
67 framework to study the transition from free-living phototrophs to obligate parasites
68 (Moore et al. 2008; Oborník et al. 2009; Janouškovec et al. 2010; Burki et al. 2012;
69 Janouškovec et al. 2015; Woo et al. 2015; Füssy & Oborník 2017b). Much knowledge has
70 accumulated about the function of the apicomplexan remnant plastid, the apicoplast
71 (reviewed in Boucher et al. 2018), which given their shared origin structurally and
72 molecularly resembles the photosynthetic plastid of chromerids (Moore et al. 2008;
73 Janouškovec et al. 2010; Woo et al. 2015). Nevertheless, the protein composition of the
74 chromerid plastid is largely unknown, except for a recent work that focused on *C. velia*
75 photosystems (Sobotka et al. 2017), and therefore a pre-transition model of the
76 apicoplast could not be studied in detail. The mitochondrial genome of apicomplexans is
77 massively reduced in gene content and found to contain only three protein-coding
78 genes, *cox1*, *cox3* and *cyb*, with the majority of mitochondrial proteins requiring import
79 from cytosol (Nash et al. 2008; Janouškovec et al. 2013; Flegontov et al. 2015).
80 Strikingly, the *C. velia* mitochondrion was found to contain only two of these genes, *cox1*
81 and *cox3* (Flegontov et al. 2015). It has been hypothesized that the reduction of the
82 apicomplexan (and chromerid) mitochondrial genome could be linked with the change
83 in lifestyle strategy, particularly a change to facultative anaerobiosis (Dorrell et al.

84 2013), which is consistent with the observed reduction of the respiratory chain in all
85 myzozoans (dinoflagellates, chromerids and apicomplexans) (Flegontov et al. 2015;
86 Oborník & Lukeš 2015).

87 The relatively small nuclear genome size (up to 193 Mb) and largely complete
88 sequence data of chromerids (Woo et al. 2015) make them ideal for large-scale targeting
89 signal recognition and, by extension, organellar proteome prediction. Up to now, plastid
90 proteomes have been determined in only a handful of organisms, mainly plants and
91 green algae (van Wijk & Baginsky 2011; Terashima et al. 2011; Dorrell et al. 2017), but
92 also a handful of complex algae and protist parasites (Hopkins et al. 2012; Boucher et al.
93 2018). Similarly, mitochondrial proteomic data are rather scarce and focused on model
94 organisms, such as humans (Calvo & Mootha 2010; Palmfeldt & Bross 2017), yeast
95 (Gonczarowska-Jorge et al. 2017), plants (Huang et al. 2013), and protists (Smith et al.
96 2007; Atteia et al. 2009; Panigrahi et al. 2009; Danne et al. 2013; Gawryluk et al. 2014).

97 The aim of the work is to define and characterize the subcellular proteomes of
98 chromerids by bioinformatic tools with an emphasis on plastid- and mitochondrion-
99 destined proteins. For the analysis, we compiled sets of compartment-specific proteins
100 of *C. velia* and *V. brassicaformis* and optimized the performance of the ASAFind (Gruber
101 et al. 2015) prediction tool with the reference amino acid frequency matrices. Our
102 analyses bring first implications on carbon and nitrogen allocation among the plastid,
103 cytosol and mitochondria in chromerids, suggesting interplay of these compartments is
104 in place for efficient carbon metabolism under changing light and nutrient conditions.
105 This work also confirms biochemical peculiarities ancestrally shared with
106 apicomplexans and dinoflagellates, such as the lack of the canonical mitochondrial
107 pyruvate decarboxylase and cytosolic amino acid synthesis.

108

109 DATA SOURCES AND METHODS

110 The sequence data of the chromerid algae *Chromera velia* CCMP2878 and *Vitrella*
111 *brassicaformis* CCMP3155 were retrieved from CryptoDB (www.cryptodb.org, version
112 34). Additional transcriptomic data were retrieved from NCBI GenBank (Dorrell et al.
113 2014; Woehle et al. 2011) and MMETSP sequence databases (MMETSP0290 and
114 MMETSP1451, (Keeling et al. 2014; Cohen et al. 2016). The sequence data were
115 annotated using the information available at KEGG servers (Kanehisa et al. 2017) and
116 using the InterProScan annotation tool of Geneious (Jones et al. 2014; Kearse et al.
117 2012).

118 Plastid-targeted reference sequences were identified based on several lines of
119 evidence; a) the protein had a clear role in the plastid metabolism (in synthesis of
120 pigments and cofactors, or being a subunit of the photosynthetic machinery, etc.), with
121 an emphasis on filling the gaps between well-defined enzymatic steps; b) reassuring
122 sequence completeness, an N-terminal extension (40-80 amino acids) that could
123 possibly encode a bipartite targeting sequence preceded the mature protein (as
124 determined by InterProScan), though essentially no targeting sequence prediction was
125 employed to avoid circular reasoning (including predictor-positive proteins and using
126 them to evaluate this predictor); c) there was a phylogenetic relationship to another
127 plastid-targeted protein among chromerids or Apicomplexa (in case of ribosomal
128 proteins; Gupta et al. 2014). Mitochondrial references were compiled similarly, only the
129 N-terminal extension was found shorter. Cytosolic references lacked an extension and
130 secretory proteins had an identifiable role in the endomembrane system or at the
131 cytoplasmic membrane. Metabolic gaps were filled by targeted BLAST searches in the
132 genomic (CryptoDB) as well as transcriptomic data (CryptoDB, GenBank, MMETSP)
133 using known-function apicomplexan sequences and KEGG orthologs as queries.

134 To define the best tool for the subcellular localization of proteins, the sets of
135 reference sequences of *Chromera* and *Vitrella* were analyzed by prediction algorithms.
136 The tools tested were selected to be suitable for large-scale analyses and included:
137 TargetP (Emanuelsson et al. 2000), SignalP (v. 4.1) (Petersen et al. 2011), ASAFind
138 (Gruber et al. 2015), HECTAR v1.3 (Gschloessl et al. 2008), MultiLoc2 (Blum et al. 2009),
139 PrediSi (Hiller et al. 2004) and PredSL (Petsalaki et al. 2006). All the prediction
140 algorithms except HECTAR were run locally with default parameters; SignalP was run
141 with sensitive cutoff values (-u 0.3 -U 0.3). The sensitivity (proportion of recognized true
142 positives) and precision (proportion of positive results, also termed the positive
143 predictive value) of the prediction algorithms were compared, setting a certain
144 threshold specified for each of the predictors. Sensitivity was computed as:

145 (Eq.1) *sensitivity = (true positives) / (true positives + false negatives)*

146 and positive predictive value was computed as:

147 (Eq.2) *precision = (true positives) / (true positives + false positives).*

148 An optimal threshold would cover maximum positive proteins while including a low
149 number of false positives (proteins falsely predicted to the organelle in question).

150 Bit score-weighted matrices of amino acid positions surrounding the signal
151 cleavage sites were calculated separately for *C. velia* and *V. brassicaformis*, as described
152 by Crooks et al. (2004). Only cleavage sites which were consensually (by majority
153 agreement) predicted by PrediSi, PredSL, SignalP and ASAFind were considered. The
154 transit peptide sequence logos and frequency plots of plastid-targeted proteins from *C.*
155 *velia* and *V. brassicaformis* were created with WebLogo (Crooks et al. 2004;
156 http://weblogo.berkeley.edu/; version 2.8.2).

157 Where applicable, closest hits for proteins were found using BLAST against nr or
158 RefSeq databases, and using DIAMOND (Buchfink et al. 2014) against an in-house made

159 database consisting from sequences collected from NCBI, MMETSP (Keeling et al. 2014;
160 Cohen et al. 2016) and Ensembl Genomes (release 37; Kersey et al. 2016). Sequences
161 were aligned using MAFFT v.7 (Katoh & Standley 2013) and automatically trimmed by
162 trimAL (Capella-Gutiérrez et al. 2009). Maximum likelihood trees were inferred from the
163 trimmed alignments using the best-fitting substitution model as determined by the IQ-
164 TREE –m TEST option limited to LG matrix by -mset (Nguyen et al. 2015). Branch
165 supports were determined by rapid bootstrapping followed by 1,000 ultra-fast
166 bootstrap replicates (-bb 1000).
167
168
169 RESULTS
170 **Sequence completeness and reference compilation**
171 During the annotation and alignment of reference proteins, we noticed that some
172 contigs retrieved from CryptoDB (Woo et al. 2015) are apparently truncated at their 5'-
173 ends. The sequence data appear gene-rich but are still highly fragmented, with 5,966
174 and 1,064 genomic scaffolds assembled for *C. velia* and *V. brassicaformis*, respectively,
175 although the number of chromosomes in *C. velia* is estimated to be much smaller (Vazač
176 et al. 2017). Since it is essential to identify a protein's complete N-terminus to predict its
177 localization to the plastid or to mitochondria (see Introduction), we pursued an
178 independent assessment of N-termini completeness. We searched for (almost) identical
179 transcripts in chromerid transcriptomes available in GenBank and MMETSP and used
180 these contigs to extend those from CryptoDB towards their 5'-end, where possible.
181 While these fused contigs are indeed chimeras of orthologs from different strains (the
182 transcripts were not completely identical), we assume they code for *bona fide* N-termini
183 in all these sequenced strains of *C. velia* and *V. brassicaformis*. These chimeric contigs are

184 for clear distinction marked in the reference sequence list (Supplementary Table S1).
185 Other, apparently truncated contigs were omitted from the reference sets.

186 We performed a systematic search of housekeeping and metabolically active
187 proteins to obtain the reference sets from *C. velia* and *V. brassicaformis* data. To find
188 plastid references, we searched for pathways intimately linked with their biogenesis and
189 photosynthesis, including the biosynthesis of fatty acids (type II FAS) and lipids, iron-
190 sulfur clusters, terpenoids and terpenoid derivatives (photosynthetic pigments,
191 vitamins), tetrapyrrole cofactors, protein translocator subunits and components of the
192 photosynthetic electron transport chain (recently characterized by Sobotka et al. 2017)
193 (Fig. 1), and the enzymes of carbon fixation (Fig. 2). Among mitochondrial pathways, we
194 looked for the enzymes of the tricarboxylic acid (TCA) cycle and the components of the
195 respiratory chain (Flegontov et al. 2015) (Fig. 2). From pathways localized in the
196 cytosol, we identified the enzymes of glycolysis, storage amylopectin biosynthesis and
197 breakdown (Coppin et al. 2005), and the enzymes of the pentose phosphate cycle that
198 we distinguished from the enzymes of the plastid carbon fixation by their N-terminal
199 extensions (Fig. 2). Using phylogenetic analyses (not shown), we also identified
200 ribosomal proteins from all three translationally active compartments (Gupta et al.
201 2014). We also included biosynthesis pathways of several amino acids and other
202 compounds, where enzymatic steps showed consistent localization (Fig. 2). Organellar
203 genome-encoded proteins were dropped from the reference sets (Supplementary
204 Table 1). In a scarcity of experimental data, we believe this is the most reliable approach
205 to compile reference sequences.

206 [insert Fig. 1]

207 Though most of the searched pathways are near-complete, we failed to identify
208 representatives of some enzymatic steps in the available transcriptomic and genomic

209 data. These include the acyl-[acyl carrier protein (ACP)] ligase (in both chromerids) and
210 two lipid phosphatases of the glycero(phospho)lipid biosynthesis (phosphatidate
211 phosphatase in *C. velia*, phosphatidylglycerophosphatase in *V. brassicaformis*), several
212 enzymes of the carotenoid (neoxanthin synthase), terpenoid-quinone (tocopherol
213 cyclase), and amino acid biosynthesis pathways (undescribed steps of Lys and Pro
214 synthesis), and one enzyme of the pentose phosphate cycle (cytosolic ribose 5-
215 phosphate isomerase; Fig. 1, Fig. 2). Some of these steps are possibly catalyzed by
216 distantly related enzymes that were not recognized by our searches, but other absences
217 may be of biological relevance. Ribose 5-phosphate isomerase is necessary to recycle
218 ribulose 5-phosphate, but a cytosolic isoform was not recovered, and maybe
219 alternatively spliced transcripts encode for a truncated, non-plastid-targeted protein.
220 Similarly, the final steps of fucoxanthin biosynthesis are missing. Although there are
221 biochemical data that *Chromera* and *Vitrella*, respectively, accumulate (iso)fucoxanthin
222 and vaucheriaxanthin – both derivates of neoxanthin – as accessory photosynthetic
223 pigments (Moore et al. 2008; Oborník et al. 2012), neoxanthin synthase was not found in
224 our data, while the last enzymes of the respective pathways are unknown (Mikami &
225 Hosokawa 2013). Plant neoxanthin synthase is a neofunctionalized lycopene cyclase
226 (Bouvier et al. 2000), opening the possibility that a promiscuous activity of the latter
227 enzyme is responsible for neoxanthin synthesis in chromerids. In comparison, the
228 absence of acyl-ACP synthase might be compensated if fatty acids are not released from
229 the bond with the ACP but rather directly used for lipid synthesis (Bisanz et al. 2006).
230 Alternatively, intermediate glycerolipids (diacylglycerol esterified with fatty acids of
231 various length and saturation) could be imported from other cell compartments (Jouhet
232 et al. 2007).
233 [insert Fig. 2]

234

235 **Prediction performance**

236 ASAFind and HECTAR (Gruber et al. 2015; Gschloessl et al. 2008) were designed
237 to predict the BTS of plastid proteins in complex algae, particularly in heterokonts.
238 ASAFind identifies plastid proteins based on the output of SignalP (Petersen et al. 2011)
239 and a sliding-window scan for the highly conserved Phe residue around the predicted
240 cleavage site. HECTAR uses a combination of predictors in three decision modules and
241 aims to classify proteins based on presence of four types of targeting modules: signal
242 peptides, type II signal anchors, chloroplast transit peptides, and mitochondrial transit
243 peptides. To predict the mTP, several predictors are available. These include TargetP,
244 MultiLoc2, and HECTAR (Emanuelsson et al. 2000; Blum et al. 2009; Gschloessl et al.
245 2008).

246 Both ASAFind and HECTAR were specifically trained on stramenopile sequences,
247 though ASAFind offers the possibility to use an alternative bit score matrix derived from
248 a training set. TargetP and MultiLoc2 were trained using plant and animal sequences.
249 Having reference dataset at hand, we could test the performance of these algorithms on
250 chromerid datasets, which has been unknown. Our results are summarized in Table 1
251 (full results in Supplementary Table S2). Mitochondrial predictors offered only
252 moderate sensitivity and precision, with *V. brassicaformis* sequences being better
253 resolved. We defined two thresholds for each predictor, one having higher sensitivity
254 (around 75 %), the other more selective (with around 85 % precision). HECTAR offers
255 higher precision with the 75%-level sensitivity compared to other mitochondrial
256 predictors (Supplementary Fig. S3). With plastid sequences, ASAFind and HECTAR
257 performed comparably, having both high sensitivity and precision. However, it must be
258 noted that the threshold for HECTAR is very low and most of our plastid controls were

259 marked as "secretory proteins" by this predictor. Our datasets might be over-fit by
260 lacking enough endomembrane system proteins as negative controls, therefore
261 precision is expected to drop with broader reference sets. We also used our plastid
262 references to derive bit score-weighted matrices of amino acids flanking the cleavage
263 site independently for *C. velia* and *V. brassicaformis* (as in Gruber et al. 2015). Using this
264 matrix, we could further improve the performance of ASAFind (Table 1). This suggests
265 that indeed chromerid plastid-targeting presequences differ from those of
266 stramenopiles, but possibly also from each other.

267 [insert Table 1]

268 To visualize the amino acid enrichment around the signal cleavage site, we
269 created logo-plots of amino acid frequencies in this motif in chromerids. We found that
270 both chromerids have a conserved Phe residue just after the cleavage site, though in *C.*
271 *velia* the Phe motif is more frequently found (Fig. 3).

272 [insert Fig. 3]

273

274 **Plastid housekeeping**

275 Most of the enzymes we expected to localize in chromerid plastids are directly or
276 indirectly related to photosynthesis. These include the photosystems core subunits, the
277 proteins of light-harvesting antennae and the photosynthetic electron transport chain,
278 but also insertase proteins TAT, SEC and SRP/Alb3 that embed these factors in the
279 thylakoid membrane (Fig. 2, discussed in further detail by Sobotka et al. 2017).
280 Furthermore, we detected the many enzymatic steps that produce photosystems
281 cofactors and accessory pigments (Fig. 1). Tetrapyrrole synthesis is one of the prime
282 biochemical pathways of plastids, since heme is a vital component to the electron
283 transport chain and retrograde plastid signaling, while chlorophyll is a fundamental

284 cofactor in light harvesting complexes (de Souza et al., 2017). In *C. velia*, the tetrapyrrole
285 pathway starts curiously with the delta-aminolevulinic acid (ALA) synthesis from
286 glycine and succinyl-CoA in the mitochondrion, but the rest of the pathway is predicted
287 to reside in the plastid (Kořený et al. 2011). This feature is shared by *V. brassicaformis*
288 and indeed supported by our results (Fig. 1). Heme is also required for the function of
289 cytosolic and mitochondrial hemoproteins (e.g., respiratory chain components), so it is
290 exported from the chromerid plastid, as there is apparently no tetrapyrrole synthesis
291 activity in other compartments besides ALA synthesis.

292 Chlorophyll *a* (Chl *a*) is the only chlorophyll species employed by chromerids
293 (Moore et al. 2008). During the last step of Chl *a* synthesis, a terpenoid (phytyl) moiety is
294 attached to the Mg^{2+}-coordinated tetrapyrrole, chlorophyllide *a*. Phytyl diphosphate is
295 synthesized from methyl-D-erythritol 4-phosphate (MEP) and the pathway also resides
296 in the chromerid plastid. The alternative cytosolic (mevalonate) pathway for terpenoid
297 biosynthesis is missing, but terpenoid diphosphates are apparently exported from the
298 plastid for further elongation by cytosolic and mitochondrial polyprenyl diphosphate
299 synthases to produce, among others, ubiquinone for the mitochondrial respiratory chain
300 (Supplementary Table S1, Imlay & Odom 2014).

301 Terpenoid derivatives include carotenoids and terpenoid quinones. Carotenoids
302 violaxanthin and (iso)fucoxanthin are accessory photosynthetic pigments involved in
303 light-harvesting, and the de-epoxidation of violaxanthin was suggested to be
304 fundamental for photoprotection in *C. velia* (Kotabová et al. 2011). Plastoquinol is an
305 electron transport chain electron mediator, while tocopherol has a protective role in
306 oxidative stress (Müller & Kappes 2007). The plastid localization of these pathways
307 therefore conditions the photosynthetic abilities of chromerids.

308 Fatty acid and lipid synthesis are important for the generation of plastid
309 membranes and the modulation of their physicochemical properties. Chromerid plastids
310 house the type II fatty acid synthesis pathway that is fed with acetyl-CoA by the plastid
311 pyruvate dehydrogenase complex (Foth et al. 2005). The produced acyl-ACP molecules
312 may then be directly esterified with glycerol 3-phosphate to become intermediates of
313 glycerolipid synthesis. Galactolipids of the mono- and digalactosyl-diacylglycerolipid
314 groups (MGDG and DGDG) are major lipids of plastids in *C. velia* (Botté et al. 2011),
315 probably required for proper embedding of photosystems into the thylakoid
316 membranes but possibly also involved in plastid protein translocation (Schleiff et al.
317 2003). Their biosynthesis is limited to plastids in both *C. velia* and *V. brassicaformis*, as
318 the respective protein contain plastid-targeting presequences (Fig. 1).

319 Flavin cofactors are critical for multiple enzymes of the TCA cycle, fatty acid
320 oxidation, photosynthesis, respiratory chain, and *de novo* pyrimidine biosynthesis. In
321 chromerids, riboflavin is synthesized in the plastid from a Calvin cycle intermediate,
322 ribulose 5-phosphate, and flavin nucleotides are then supposedly exported from the
323 plastid (Fig. 2).

324 Iron-sulfur clusters are components of redox proteins and in plastids, they
325 function as the prosthetic group of the cytochrome b_6f and the ferredoxin redox relay
326 that signals the plastid redox state to downstream enzymes. Plastid iron-sulfur clusters
327 are typically synthesized by the SUF system, using ferrous ions and cysteine-derived
328 sulfur as substrates. Both Cys synthesis and SUF system are found in chromerid plastids
329 (Fig. 1), suggesting that plastid client proteins have regular access to Fe-S clusters.

330 Unexpectedly, only few enzymes of amino acid biosynthesis localize to plastids in
331 *C. velia* and *V. brassicaformis*. We could reliably predict that only three enzymes of Cys
332 synthesis reside in the plastid, but this amino acid is directly metabolized by the SUF

333 system to assemble Fe-S clusters. Strikingly, plant chloroplasts synthesize several amino
334 acids (Lys, Arg, Ala, Trp, Tyr, Phe; Van Dingenen et al. 2016) and participate on nitrogen
335 assimilation with the glutamine synthase / glutamine oxoglutarate aminotransferase
336 (GS/GOGAT) cycle. Apparently, chromerid plastids are much less involved in nitrogen
337 metabolism than previously studied systems (see below).

338 In summary, chromerid plastids are well-equipped for the biogenesis and
339 maintenance of the photosynthetic machinery. In addition, these organelles prime fatty
340 acid and terpenoid biosynthesis by the formation of short-chain intermediates (short-
341 chain saturated fatty acids and monoterpenoid diphosphates), that are however
342 exported for further processing. Heme is another crucial compound produced in this
343 compartment. All these processes seem closely coupled to photosynthesis and,
344 importantly, to carbon fixation by the Calvin cycle, which will be overviewed in the next
345 section.

346

347 **Compartment interplay**

348 Calvin cycle is the very center of the second phase of photosynthesis; it utilizes
349 ATP and NADPH produced by the light-dependent reactions to fix carbon dioxide into
350 carbohydrates. We identified in chromerids all the enzymes of the cycle, discriminating
351 them from the cytosolic carbohydrate metabolism variants by their N-terminal
352 extensions having BTS characteristics. For three molecules of CO_2, the Calvin cycle
353 produces one triose phosphate molecule (dihydroxyacetone phosphate – DHAP, or 3-
354 phosphoglycerate) that can be metabolized by other pathways (see above), exported to
355 the cytosol or reintroduced to the cycle to produce sugar phosphates with more carbons.

356 Triose phosphates enter the cytosol via the triose phosphate transporters, and
357 those identified in *Plasmodium falciparum* prefer DHAP and phospho*enol*pyruvate (PEP)

358 as substrates, while also accepting 3-phosphoglycerate (Lim et al. 2010). Upon the entry
359 of triose phosphates to the cytosol, they can take two major paths, depending on the
360 metabolic state. Gluconeogenetic pathway leads to the accumulation of storage
361 saccharides (amylopectin), while glycolysis yields pyruvate, a hub compound for both
362 anabolic and catabolic reactions. Generally, favorable and illuminated growth of algae
363 promote storage and anabolic pathways, while dark growth and starvation promote
364 spending of sugar phosphates in the TCA cycle and respiration.

365 We found enzymes involved in all processes of polysaccharide accumulation and
366 degradation (Coppin et al. 2005). Some of the storage sugar enzymes encode signal
367 peptides or transmembrane domains, suggestive of their extracellular or membrane-
368 associated activity. This is consistent with chromerids forming a thick cellulose cell-wall
369 (Moore et al. 2008; Oborník et al. 2012; Füssy & Oborník 2017a).

370 The Calvin cycle is not only a source of triose phosphates for cytosolic
371 glycolysis/gluconeogenesis, but also of pentose phosphates that are the starting
372 substrates for nucleotide (ribose 5-phosphate), amino acid (ribose 5-phosphate and
373 erythrose 4-phosphate), and vitamin synthesis (ribulose 5-phosphate). In this respect it
374 complements the cytosolic pentose phosphate cycle that also provides these sugar
375 phosphates. Based on our predictions, the plastid ribulose 5-phosphate pool serves as
376 the substrate for flavin nucleotide synthesis, while cytosolic ribulose 5-phosphate is
377 used for pyridoxin synthesis. Notably, we could not find any cytosolic 5-phosphoribosyl-
378 1-pyrophosphate (PRPP) synthases that would provide PRPP for the synthesis of His,
379 Trp and nucleotides in this compartment. Two pairs of orthologs are found in
380 chromerids. Each of these sequences has a presequence, though only two orthologs have
381 BTS (the other pair have mTP-like N-termini and were discarded from predictions due
382 to uncertain localization; Supplementary Table S1). Comparison with PRPP synthase

383 sequences from apicomplexans revealed that some of those too have predicted
384 presequences (including both *Toxoplasma gondii* ME49 paralogs), but proteins having
385 these presequences do not cluster together (Supplementary Table S1). The localization
386 of PRPP synthases is therefore questionable and might be achieved by alternative
387 splicing.

388 Other anabolic reactions also stem from the cytosolic pool of sugar phosphates.
389 Biosynthesis of most amino acids in chromerids depends on glycolysis and pentose
390 phosphate cycle intermediates. Cys, Met and Ser derive from 3-phosphoglycerate, while
391 Phe, Trp and Tyr are synthesized from erythrose 4-phosphate via chorismate, and His is
392 a derivative of PRPP (but see above). Ala, Arg, Asp, Asn, Lys, Pro and Thr derive from 2-
393 oxoglutarate (2-OG) transaminated to form Glu by the GS/GOGAT cycle (Fig. 2). Notably,
394 the cytosolic 2-OG pool for GS/GOGAT cycle exists by the action of cytosolic copies of
395 TCA cycle enzymes (Fig. 2, Supplementary Table S1). Chromerid plastids do not
396 principally participate in the amino acid biosynthesis, with a few exceptions. A part of
397 the Arg pathway (ornithine synthesis) is predicted to localize in the plastid, which might
398 be a rate-limiting, regulatory measure on ornithine production via dependence on
399 stromal ATP levels. This is not unexpected, as in plants Arg synthesis is regulated at the
400 level of N-acetylglutamine phosphorylation (Ferrario-Méry et al. 2006). Notably,
401 chromerids utilize argJ (Supplementary Table S1), which is a glutamate transacetylating
402 enzyme that allows to recycle N-acetylglutamate after the production of ornithine. This
403 reaction does not produce free acetate and thus does not require additional ATP for
404 acetyl-CoA recycling. In *Plasmodium*, *Cryptosporidium* and *Eimeria*, Arg (via ornithine) is
405 used for polyamine synthesis (Cook et al. 2007) but this pathway shows cytosolic
406 localization congruently in apicomplexans and chromerids (Supplementary Table S1;
407 Shanmugasundram et al. 2013). Gly may be produced by glycine

408 hydroxymethyltransferase and threonine aldolase in mitochondria, cytosol, and plastids
409 (Supplementary Table S1), reflecting its involvement in multiple pathways as a reaction
410 intermediate (for plastid formylmethionine and mitochondrial ALA synthesis, for
411 instance). There is some incongruence about the localization of Ile, Leu and Val
412 synthesis among chromerids, as *V. brassicaformis* appears to localize Val and Ile
413 biosynthesis to both mitochondria and plastid while Leu biosynthesis is cytosolic. In
414 comparison, all these pathway steps are cytosolic in *C. velia*. Chromerid plastids thus
415 appear to have minor roles in amino acid biosynthesis.

416 Both catabolic and anabolic reactions were found to take place in the chromerid
417 mitochondria, though they are missing some common components. Both apicomplexans
418 and chromerids ancestrally lack the respiratory complex I (and *C. velia* also lacks
419 complex III) and utilize alternative NADH dehydrogenases that pass electrons to
420 ubiquinone but do not contribute to the proton gradient (Fig. 2, Flegontov et al. 2015).
421 Like Apicomplexa, chromerids also lack a canonical mitochondrial pyruvate
422 dehydrogenase and instead take advantage of the promiscuous activity of the branched-
423 chain amino acid dehydrogenase (BKCDH) (Foth et al. 2005; Danne et al. 2013;
424 Oppenheim et al. 2014). The NAD^+-dependent isocitrate dehydrogenase is missing and
425 replaced by an $NADP^+$-dependent isozyme, which could be linked to the loss of the
426 canonical NADH dehydrogenase (respiratory complex I). $NADP^+$-dependent isocitrate
427 dehydrogenase might in turn support the activity of NADPH-dependent enzymes (Danne
428 et al. 2013). This is notable because in mammals both NAD^+ and $NADP^+$ isocitrate
429 dehydrogenases are operational, the latter typically in reverse (reductive) direction
430 (Sazanov & Jackson 1994; Yoo et al. 2008). In contrast with apicomplexans, both
431 fumarate hydratase types are present in chromerids (apicomplexans express only type I;
432 Bulusu et al. 2011), while an ortholog of the conserved apicomplexan malate:quinone

433 oxidoreductase is missing (Danne et al. 2013). Chromerids localize to mitochondria
434 several enzymes of amino acid decomposition (Supplementary Table S1), suggesting
435 that at least some catabolic pathways feed into the mitochondrial metabolism. A set of
436 lactate dehydrogenases in chromerids allow fermentation of pyruvate to lactate under
437 temporary dark anaerobic conditions (Flegontov et al. 2015; Oborník & Lukeš 2015) and
438 participate in methylglyoxal detoxication (Cordeiro et al. 2012). Furthermore, the
439 mitochondrial metabolism is equipped with malic enzyme (decarboxylating malate
440 dehydrogenase), which allows the regeneration of pyruvate for anabolic reactions when
441 the cycle is fed by fatty acid beta-oxidation. Therefore, mitochondria are well-integrated
442 in the chromerid carbon metabolism in both catabolic and anabolic directions.

443 Carbon metabolism also affects the synthesis rates of nitrogen pathways. The
444 GS/GOGAT cycle is responsible for nitrogen (ammonia) assimilation into amino acids in
445 most phototrophs. In chromerids, GOGAT is an NADH-dependent cytosolic enzyme, and
446 the restriction of amino acid pathways to the cytosol suggests that they are decoupled
447 from the redox state of the plastid and rather reflect the redox state of the cytosol
448 (Fig. 2). This also suggests that the plastids of chromerids are not as deeply involved in
449 primary metabolism as the plastids of primary algae and plants by lacking the ability to
450 synthesize amino acids and polysaccharides. Instead, plastid activity is crucial in lower
451 carbohydrate and fatty acid metabolism and appears to be sensed indirectly, through
452 the supply of triose phosphates. Under favorable conditions, the cytosol is fed with
453 photosynthetic sugar phosphates, and reducing agents and ATP are produced by
454 glycolysis. This promotes anabolic reactions that allow the accumulation of
455 polysaccharides and production of amino acids and lipids. In the dark or under nutrient
456 scarcity, the shortage of energy must be compensated by the reactions of the TCA cycle
457 and respiration in mitochondria. Unfortunately, there are no published large-scale

458 quantitative data suitable for tracing in more detail the metabolic flows through the
459 described pathways.
460
461
462 DISCUSSION
463 The physicochemical character of mitochondrial and plastid targeting
464 presequences remains quite similar across long evolutionary distances among
465 eukaryotes. Still, the predicting power of localization algorithms decreases with
466 divergence between the reference and the analyzed sets. Prediction algorithms perform
467 best if trained using lineage-specific datasets, usually based on available experimental
468 data (e.g. (Emanuelsson et al. 2007; Kaundal et al. 2013; Gruber et al. 2015).
469 Consistently, plastid-localization signals show specific variability among algal clades
470 (Patron & Waller 2007). Although many tools have been implemented to determine
471 mitochondrion- and plastid-localized proteins in *C. velia* and *V. brassicaformis* (Kořený et
472 al. 2011; Woehle et al. 2011; Petersen et al. 2014; Flegontov et al. 2015; Woo et al. 2015;
473 Sobotka et al. 2017), none of them have been tested on reference proteins in terms of
474 predictive power. To find a suitable tool to predict protein localization in chromerids,
475 we prepared a manually curated inventory of references that included proteins from
476 plastid, mitochondrion, and several other compartments, as negative controls. To date,
477 two works (Flegontov et al. 2015; Sobotka et al. 2017) have investigated the metabolism
478 of chromerids on organellar level, and only the latter work supports the localization of
479 analyzed (plastid) proteins with proteomic data. Our dataset included sequences of
480 typical plastid-targeted proteins as well as proteins with unambiguous localization to
481 mitochondria and other compartments, conserved in other eukaryotic lineages. To
482 ensure that our sequences are largely complete at their N-termini, we used protein

483 models generated by two independent sequencing initiatives, EuPathDB (deposited at
484 CryptoDB; (Woo et al. 2015) and MMETSP (Keeling et al. 2014).

485 We analyzed the performance of several algorithms based on their sensitivity
486 (percentage of true positive sequences passing a threshold) and precision (percentage
487 of false positives in the set passing the same threshold). For plastid proteomes, ASAFind
488 using a species-specific bit score matrix was found to be the most efficient for each
489 chromerid species. The sensitivity of ASAFind with *Phaeodactylum tricornutum*
490 sequences was comparable to our results (80%; Gruber et al. 2015). With mitochondrial
491 references, we could not observe predictive differences for *V. brassicaformis* sequences.
492 With *C. velia* datasets, HECTAR was more precise at higher sensitivity levels, and
493 MultiLoc2 could not reach an 85% precision (Table 1). TargetP performed similarly to
494 HECTAR (TargetP is indeed part of HECTAR's mitochondrial module) and is widely used
495 for finding mitochondria-targeted genes with sensitivity around 60-75% (depending on
496 the model and reliability class) in various organisms (Emanuelsson et al. 2007),
497 including the chromerids (Kořený et al. 2011; Flegontov et al. 2015; Woo et al. 2015).
498 This accuracy is also relatively lower because a portion of mitochondrial proteins use
499 alternative routes or signals for translocation to this organelle (Sun & Habermann
500 2017). Several of our plastid and mitochondrial reference sequences were recovered as
501 false negatives not passing the probability threshold. Indeed, 136 of the 1,141 reference
502 sequences had alternative open reading frames or were unannotated and had to be
503 manually adjusted using homology annotation, alignments and phylogenetic analyses
504 (Supplementary Table S1). For instance, we could not obtain consistent localizations for
505 the enzymes of the MEP pathway that is thought to be exclusively plastid-localized
506 (Fig. 1). With untreated data, the false negative discovery rate would be much higher,
507 leading to orphan enzymes predicted to unexpected compartments. This points out the

508 problems with automated analyses – there is an essential need for highly complete
509 sequence data, which worsens large-scale predictions in the chromerids.

510 Although targeting presequences are generally not generally not conserved on
511 sequence level, the conservation of amino acids flanking the BTS cleavage sites was
512 found to be crucial for proper plastid targeting (Gruber et al. 2007). Proteins targeted to
513 the rhodophyte-derived complex plastids generally expose an invariant Phe at their N-
514 terminus after signal peptide cleavage (Gould et al. 2006; Patron & Waller 2007). Based
515 on our frequency matrices, in *Vitrella* plastid-targeted proteins the Phe motif is not
516 strictly conserved, while most plastid-targeted proteins of *Chromera* do possess this Phe
517 (Fig. 3). This suggests there is some versatility of the translocation machineries in
518 chromerids. Our observations are similar to the results of Woehle et al. (2011), although
519 the frequency of Phe in their *C. velia* dataset appears higher. AT richness above 57 %
520 was shown to correlate with a shift in amino acid composition of transit peptides
521 towards AT-rich codons (Ralph et al. 2004) but the difference in the GC percentage of
522 the plastid reference transcripts in chromerids appears unlikely to be the cause for a
523 diminished Phe (53.0 % and 59.6 % GC in *C. velia* and *V. brassicaformis*, respectively).
524 Lastly, we cannot exclude the possibility that mis-identified cleavage sites or mis-
525 assembled transcripts in our data affected the amino acid frequencies. Nevertheless, the
526 Phe motif is less strongly retained in *Toxoplasma* and apparently absent in *Plasmodium*
527 apicoplast-targeted proteins (Patron & Waller 2007), consistent with our results. In
528 addition, not all rhodophyte-derived lineages retain a high percentage of plastid-
529 targeted proteins with the Phe; despite Phe occurs predominantly in cryptophytes
530 (*Guillardia theta*) and heterokonts (*Thalassiosira pseudonana* and *Phaeodactylum*
531 *tricornutum*), haptophytes apparently do not rely on Phe in their transit peptide
532 presequences (Kilian & Kroth 2005; Patron & Waller 2007; Gruber et al. 2015). Phe

533 motif is also absent from the transit peptides of chlorophyte-derived algae (Patron &
534 Waller 2007).

535 The pathways that localize into plastids are typically associated with
536 photosynthesis. We identified enzymes responsible for the synthesis of tetrapyrroles,
537 terpenoids, carotenoids, lipids, iron-sulfur clusters and carbohydrates, hence
538 compounds required for the proper assembly and function of the photosynthetic
539 machinery. Our findings are consistent with previous biochemical analyses, showing
540 that chromerids have a limited set of photosynthetic pigments (Moore et al. 2008;
541 Kotabová et al. 2011) and that they exhibit unique structural changes to the
542 photosystems (Sobotka et al. 2017). We show that terpenoid and lipid pathways are
543 primed with substrates produced by the plastid carbohydrate metabolism (DHAP,
544 pyruvate, and PEP) and linked with the rate of carbon fixation by substrate availability
545 (Fig. 1). This is not unprecedented, as plant chloroplasts show a similar arrangement in
546 photosynthetically active and inactive plastids (e.g. Neuhaus and Emes 2000).

547 Nevertheless, plastid products might be essential beyond photosynthesis, which
548 is best illustrated by comparison of algae with relatives that lost photosynthetic abilities
549 (e.g., Hadariová et al. 2018). Indeed, the biology of non-photosynthetic plastids has been
550 best studied in apicomplexans to find suitable weak-spots of these infamous parasites.
551 The apicoplast produces fatty acids and terpenoids and participates in heme synthesis
552 to sustain parasite growth in hosts where salvage of these compounds is not possible
553 (reviewed by van Dooren and Striepen 2013). This dependence on a remnant plastid can
554 be regarded as an evolutionary constraint that cannot be easily overcome once parallel
555 pathways in the cytosol or mitochondria are lost (though losses of plastids occasionally
556 happen, see Füssy & Oborník 2018; Oborník 2018). Consistent with this view,
557 apicomplexans lack apicoplast-independent pathways for terpenoid (via mevalonate),

558 fatty acid (using type I fatty acid synthase, or FASI) and tetrapyrrole synthesis. Some
559 apicomplexans appear to possess FASI-like enzymes, though functional analyses suggest
560 that *Cryptosporidum* FASI is not involved in *de novo* fatty acid synthesis and rather
561 accepts long-chain fatty acyl thioesters as substrates for elongation (Zhu et al. 2004).
562 The importance of *Toxoplasma* FASI remains unclear, while other apicomplexan FASI-
563 like enzymes might be involved in polyketide synthesis (Kohli et al. 2016).

564 The photosynthetic relatives to apicomplexans, chromerids are also likely to lack
565 cytosolic pathways for fatty acid, terpenoid and tetrapyrrole synthesis and thus rely
566 entirely on the plastid synthesis. In addition, chromerid plastids synthesize FAD
567 cofactors and a nitrogen metabolism intermediate, ornithine (see below). Conversely,
568 chromerid plastids do not host any fatty acid elongases or desaturases, therefore short-
569 chain fatty acids need to be exported, processed and reimported for incorporation into
570 plastid lipids (Botté et al. 2011). This is analogous to the fatty acid synthesis architecture
571 in apicomplexans (Mazumdar & Striepen 2007). Similarly, chromerid tetrapyrrole
572 biosynthesis relies on the import of the starting substrate, ALA, from the cytosol
573 (Kořený et al. 2011). Therefore, chromerid plastids pathways are interdependent with
574 the cytosol, pointing out possible feedback regulatory mechanisms to limit their
575 biosynthetic activity.

576 In the dark, the reduced triose phosphate supply from the chromerid plastid must
577 be counterbalanced by catabolic reactions of the mitochondrial TCA cycle and
578 respiration. The chromerid TCA cycle and respiratory chain represent modifications to
579 the canonical mitochondrial pathways (see Results). The list of chromerid mitochondrial
580 enzymes is largely shared with parasitic apicomplexans (Flegontov et al. 2015; Jacot et
581 al. 2016). As yet, it is unclear what impact this arrangement has on the metabolism of
582 photoautotrophic organisms, but it stands out that through the TCA cycle chromerid

583 mitochondria are metabolically versatile and integrate catabolic pathways with enzymes
584 priming anabolic reactions and enzymes typically associated with anaerobiosis
585 (Flegontov et al. 2015). Intracellular stages of *Toxoplasma gondii* actively catabolize host
586 glucose via the oxidative TCA cycle to produce energy efficiently (MacRae et al. 2012). In
587 comparison, for asexual stages of *Plasmodium*, purine salvage from oxaloacetate is vital,
588 while the importance of the TCA cycle is diminished (Bulusu et al. 2011; Ke et al. 2015).
589 Similarly, cytosolic ATP citrate lyase contributes to acetyl-CoA production in *T. gondii*
590 (Tymoshenko et al. 2015). As such, chromerid mitochondria are likely to have major
591 influence on metabolic fluxes in the cell.

592 We observed that amino acid biosynthesis pathways consistently showed
593 cytosolic localizations, with minor exceptions. This is probably not due to mis-
594 annotation as we could not extend the sequences towards an alternative N-terminus in
595 most cases. Chromerid plastids thus appear to host only parts of Cys and Arg synthesis,
596 suggesting that algae do not necessarily synthesize amino acids in plastids as plants do
597 (Van Dingenen et al. 2016). While plastid Cys synthesis is required by the Fe-S cluster
598 assembly system SUF, the plastid production of ornithine for Arg biosynthesis might
599 have a regulatory role in chromerids. Indeed, nitrogen metabolism is energy-demanding
600 and subject to intense cross-talk with carbon metabolism, and plastids play a crucial role
601 in balancing the fluxes (Stitt 2002; Németh et al. 2018). As yet, the primarily cytosolic
602 localization of nitrogen metabolism in chromerids appears extraordinary among
603 phototrophs (Allen et al. 2011; Bromke 2013; de la Torre et al. 2013; Dorrell et al. 2017).
604 Of studied apicomplexans, *Toxoplasma* has the broadest capacity to produce amino acids
605 *de novo* or secondarily from specific precursors, being auxotrophic only for Arg, His and
606 Trp (Chaudhary et al. 2014; Tymoshenko et al. 2015). Although the middle steps of Pro
607 and Lys synthesis are currently unknown (Shanmugasundram et al. 2013), none of these

608 amino acid synthesis pathways appears to be placed exclusively in the apicoplast, in line
609 with our results. Arg scavenging from outer sources might have therefore triggered the
610 loss of the ornithine cycle in the ancestor of apicomplexans. Reliance on amino acid
611 import from the host resulted in even greater reduction of amino acid synthesis
612 capabilities. Thus, *Plasmodium* synthesizes six amino acids (Gly, Glu, Gln, Pro, Asp, Asn),
613 while *Cryptosporidium,* which entirely lacks an apicoplast (Keithly et al. 2000;
614 Chaudhary et al. 2014), synthesizes only Gly, Glu and Pro. It is tempting to speculate that
615 the major role of the cytosol in amino acid synthesis facilitated the plastid loss in a
616 distantly related non-photosynthetic dinoflagellate, *Hematodinium* (Gornik et al. 2015).

617 In apicomplexans, indeed, the main source of energy is glycolysis of host-drawn
618 glucose, but the parasites appear to use the same carbohydrate subpathways as
619 chromerids. These include the compartment exchange of triose phosphates via
620 phosphate transporters (Lim et al. 2010), parallel cytosolic and plastid glycolysis to
621 pyruvate (Fleige et al. 2007), cytosolic accumulation of storage polysaccharides (Coppin
622 et al. 2005) and a notable relocation of the canonical pyruvate dehydrogenase in the
623 apicoplast to feed the fatty acid synthesis (Foth et al. 2005; Fleige et al. 2007). There are
624 also obvious commonalities in the arrangement of mitochondrial metabolism, including
625 the modifications of the canonical TCA cycle, the respiratory chain, and the anabolic
626 reactions of mitochondria. As a previous comparative analysis of mitochondrial
627 metabolism of apicomplexans and dinoflagellates showed, this arrangement is largely
628 shared among all myzozoans, and likely represents an ancestral state employed by the
629 dinoflagellate-apicomplexan progenitor (Danne et al. 2013).

630 The overall architecture of biosynthetic pathways in chromerids suggests that
631 their cytosol represents the compartment which integrates the cellular energy status.
632 Through metabolite flow it can directly regulate anabolic and catabolic reactions based

633 on photosynthate supply from the plastid and nutrient availability. Hypothetically, such
634 a central role for the cytosol could have been employed by a free-living unicellular
635 eukaryovore/algivore that ingests its prey into a food vacuole and gradually digests it,
636 much like the one we picture was the ancestor of Myzozoa (Tikhonenkov et al. 2014).
637 Indeed, also dinoflagellates employ a remarkable spectrum of trophic strategies
638 (reviewed in Waller & Kořený 2017). Despite this diversity, there are commonalities in
639 the arrangement of mitochondrial and plastid pathways among myzozoans (Danne et al.
640 2013; Gornik et al. 2015; Waller et al. 2016), pointing out that the cytosolic pyruvate
641 hub may be persisting through plastid endosymbiotic events.

642 The apparently ancestral potential to exploit external resources and the richness
643 of apicomplexan cell-surface transporters raises the question whether chromerids also
644 take considerable advantage of extracellular nutrients. Both chromerids were isolated as
645 coral-associated organisms (Moore et al. 2008; Oborník et al. 2012) and intriguing data
646 have been presented on mixotrophy of *C. velia* and its association with corals (Cumbo et
647 al. 2013; Foster et al. 2014; Mohamed et al. 2018). This question therefore needs to be
648 addressed in more detail, as it might present implications on the early evolution of
649 Apicomplexa. However, experimental data are currently unavailable to present an in-
650 depth metabolomic model for chromerids and we can merely compare our results with
651 those from other alveolates. Here we present a benchmark set of biochemical pathways
652 that can be investigated by quantitative or phylogenetic approaches for a deeper
653 understanding of one of the "alveolate ways" of trophic transition from photosynthetic
654 algae to obligatory parasites.

655

656

657 CONCLUSIONS

658 Chromerids occupy a pivotal position in the tree of alveolates and hold the key to
659 our understanding of transition to parasitism; they are free-living phototrophic algae
660 with relatively canonical chromosomes and branch sister to the apicomplexans. To
661 sketch a few more pathways on the metabolic map of chromerids, we prepared
662 reference datasets of plastid, cytosolic and mitochondrial enzymes. We have manually
663 curated these protein sequences so that most of them are complete, which is crucial for
664 correct predictions of their subcellular localization. We unveiled that chromerid plastids
665 vividly exchange compounds with the cytosol and mitochondria in order to produce
666 terpenoids, lipids and tetrapyrroles. In contrast, chromerid plastids appear to have only
667 a minor role in amino acid synthesis, as most of these pathways reside in the cytosol.
668 Uniquely, chromerids were found to use a plastid ornithine cycle combined with a
669 cytosolic Arg cycle for synthesis and decomposition of this amino acid. We outlined a
670 major hub represented by lower glycolysis and gluconeogenesis enzymes that appears
671 to regulate carbon and nitrogen metabolite flow depending on the photoactivity of the
672 plastid. When compared to apicomplexan metabolic pathways, our model confirms a
673 conserved architecture of carbon and nitrogen metabolism in these groups. Further
674 analyses are though needed to gain insight into the regulation of these pathways in
675 response to various cues. Using the suggested prediction tools, it is now also possible to
676 introduce more enzymatic steps to the picture.
677
678
679 ABBREVIATIONS: 2-OG – 2-oxoglutarate, ACP – acyl-carrier protein, ALA – delta-
680 aminolevulinic acid, BTS – bipartite (plastid) targeting signal, Chl *a* – chlorophyll *a*,
681 DGDG/MGDG – di-/monogalactosyl-diacylglycerolipid, DHAP – dihydroxyacetone
682 phosphate, Ery-4P – erythrose 4-phosphate, FAD – flavin adenine dinucleotide,

683 GS/GOGAT – glutamine synthase / glutamine oxoglutarate aminotransferase, IMS –
684 inter-membrane space, MEP – methyl-D-erythritol 4-phosphate, mTP – mitochondrial
685 transit peptide, OAA – oxaloacetate, orn – ornithine, PEP – phospho*enol*pyruvate, PQ –
686 plastoquinol, PRPP – 5-phosphoribosyl-1-pyrophosphate, pyr – pyruvate, Ru-5P –
687 ribulose 5-phosphate, TCA – tricarboxylic acid, THF - tetrahydrofolate, UQ - ubiquinone.
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699 MO and ZF conceived the study. ZF and TF performed all the bioinformatic
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711
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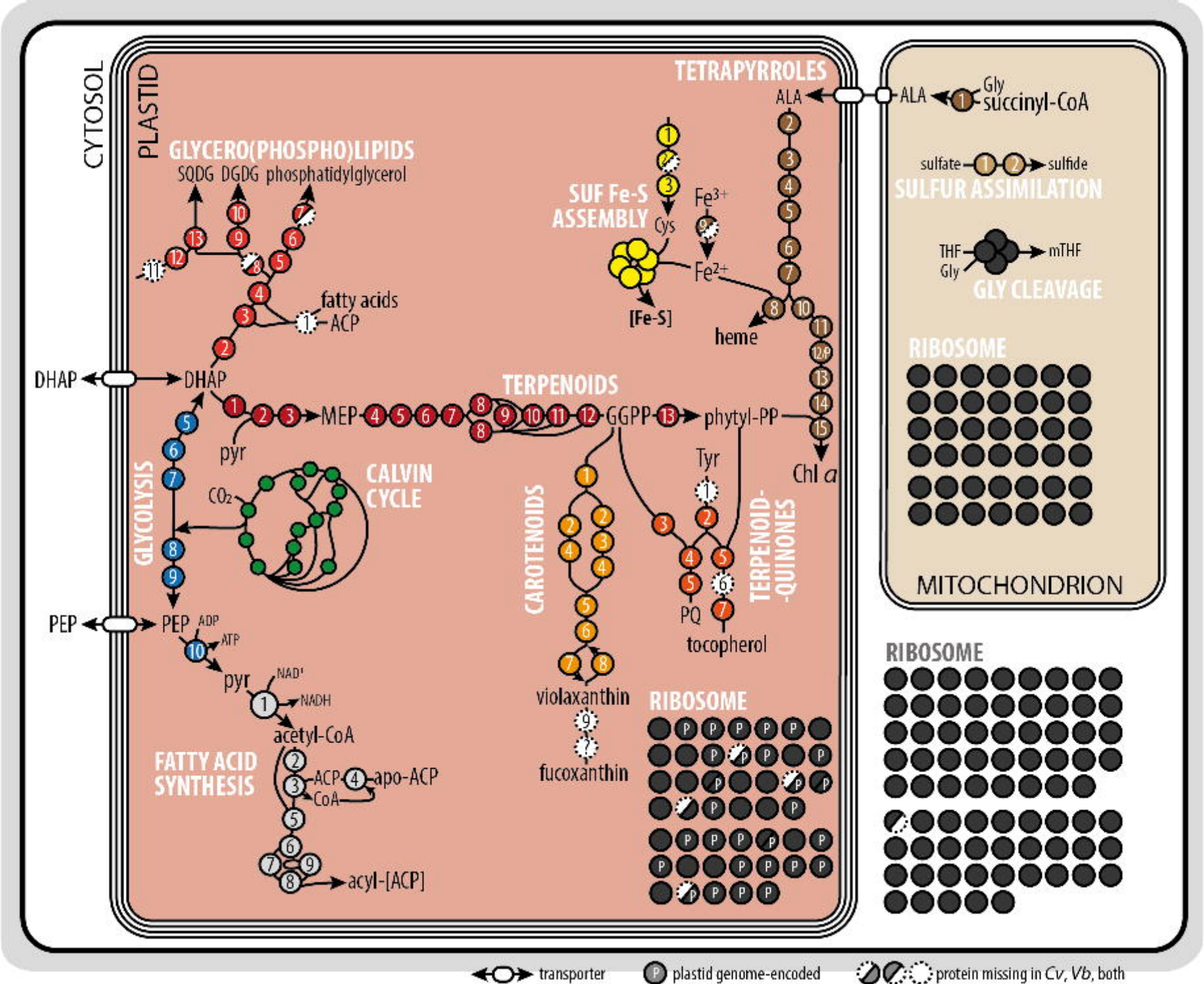
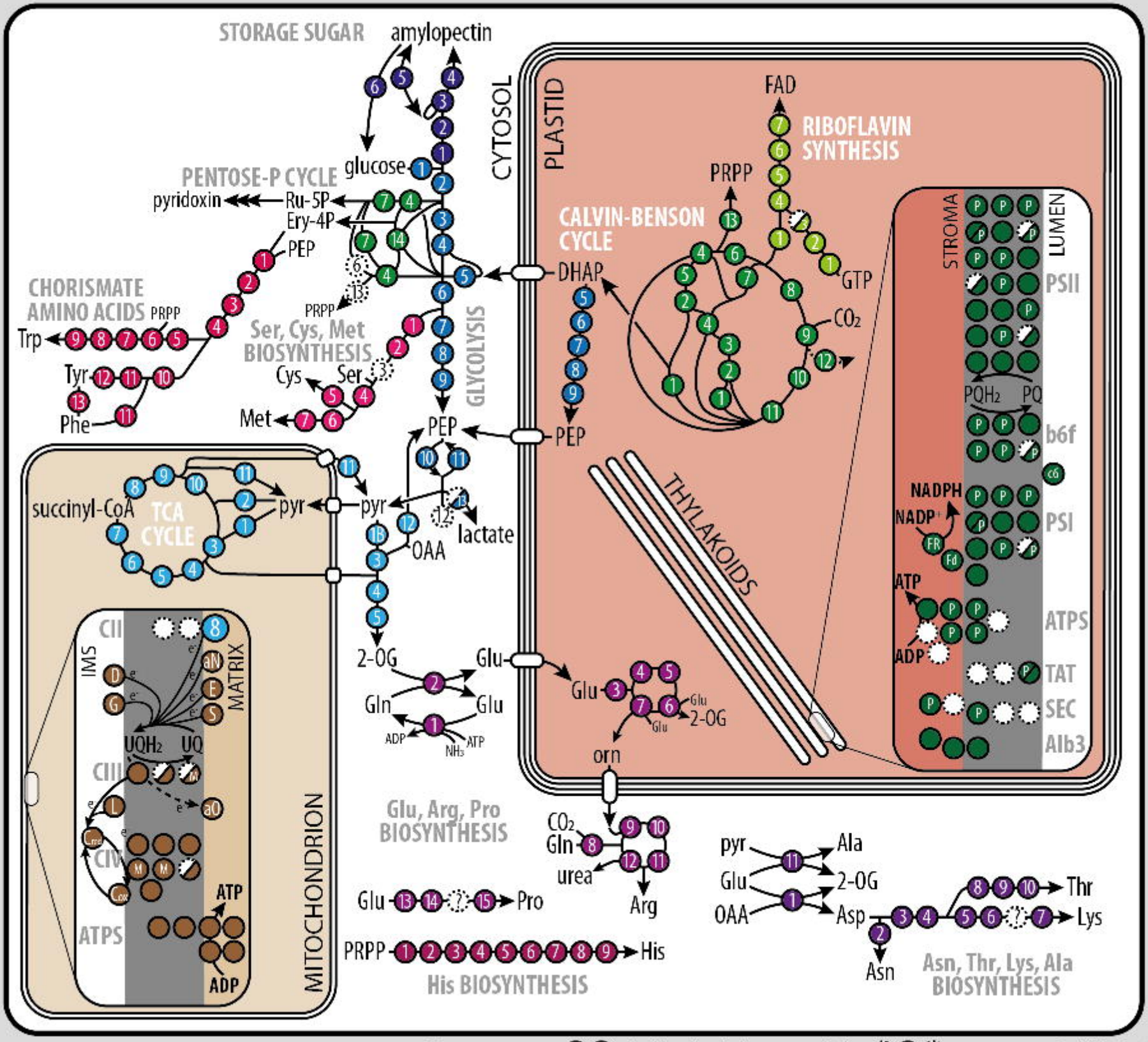
1047
1048
1049 FIGURES:
1050 **Figure 1: Overview of reference pathways**, focused on the inter-connected reactions
1051 of terpenoid, lipid and tetrapyrrole biosynthesis, mitochondrial sulfur assimilation and
1052 glycine cleavage, and ribosomal proteins. Pathways are color-coded and
1053 enzymes/proteins are numbered according to the Supplementary Table S1.
1054 Abbreviations: ACP – acyl-carrier protein, ALA – delta-aminolevulinic acid, Chl *a* –
1055 chlorophyll *a*, DGDG – digalactosyldiacylglycerolipids, DHAP – dihydroxyacetone
1056 phosphate, GGPP – geranylgeranyl pyrophosphate, IMS – inter-membrane space, MEP –

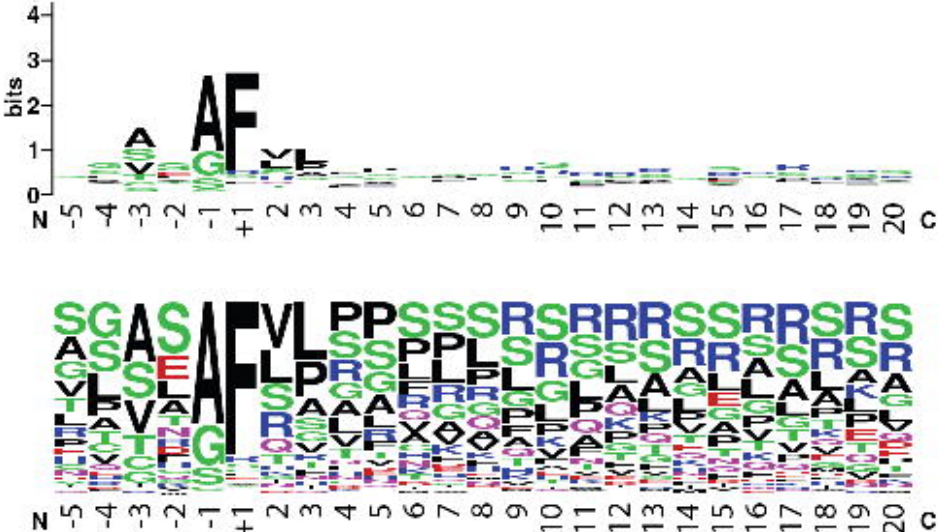
1057 methyl-D-erythritol 4-phosphate, PEP – phospho*enol*pyruvate, pyr – pyruvate, SQDG –
1058 sulfoquinovosyldiacylglycerolipids, THF – tetrahydrofolate.
1059
1060 **Figure 2: Overview of reference pathways, continued**, focused on the inter-
1061 connected reactions of carbohydrate and amino acid biosynthesis, mitochondrial
1062 respiration and plastid photosynthesis. Pathways are color-coded and
1063 enzymes/proteins are numbered or coded according to the Supplementary Table S1. 2-
1064 OG – 2-oxoglutarate, DHAP – dihydroxyacetone phosphate, Ery-4P – erythrose 4-
1065 phosphate, FAD – flavin adenine dinucleotide, IMS – inter-membrane space, OAA –
1066 oxaloacetate, orn – ornithine, PEP – phospho*enol*pyruvate, PQ – plastoquinol, PRPP – 5-
1067 phosphoribosyl-1-pyrophosphate, pyr – pyruvate, Ru-5P – ribulose 5-phosphate, TCA –
1068 tricarboxylic acid, THF – tetrahydrofolate, UQ – ubiquinone.
1069
1070 **Figure 3: Sequence motifs surrounding the signal cleavage site differ in**
1071 **chromerids.** While *C. velia* retains a highly conserved Phe residue that follows after the
1072 cleavage site (marked by +1), in *V. brassicaformis* Phe seems less conserved. Logoplot
1073 created by WebLogo (Crooks et al. 2004).
1074
1075 **Table 1: Performance of various localization predictors as employed in this study.**
1076 ASAFind and HECTAR-plastid were used for plastid reference assessment. Note that
1077 ASAFind has a cumulative score, with our data reaching up to values of 6. ASAFind+
1078 denotes ASAFind with species-specific bit score matrices designed with *C. velia* and *V.*
1079 *brassicaformis* plastid references. TargetP, HECTAR-mitochondrion and MultiLoc2 were
1080 used for mitochondrial predictions and two thresholds are presented for these

1081 predictors, one aimed at higher sensitivity (s – 75%), the other aimed at high precision
1082 (p – 85%).
1083
1084 SUPPLEMENTARY MATERIAL:
1085 **Supplementary Table S1: Reference protein lists grouped by metabolic function;**
1086 **inferred phylogenies of argJ, polyprenyl-PP synthases, and PRPP synthase; set of**
1087 **updated reference protein sequences.** Trees in Newick format, accessions are marked
1088 on leaves. The maximum-likelihood trees were inferred by the IQ-TREE software (see
1089 main text Methods). Sequence updates are based on homology searches within
1090 alternative transcriptomic datasets (elongated sequences) and comparison of
1091 orthologous sequences in *C. velia* and *V. brassicaformis* (merged contigs).
1092
1093 **Supplementary Table S2: Prediction scores for reference proteins and the species-**
1094 **specific ASAFind scoring matrices of *C. velia* and *V. brassicaformis*.**
1095
1096 **Supplementary Figure S3: Targeting peptide score distributions for reference**
1097 **sequences.** Reference colors: green – plastid, blue – mitochondrial, grey – other
1098 (cytosolic or non-plastid endomembrane route).
1099

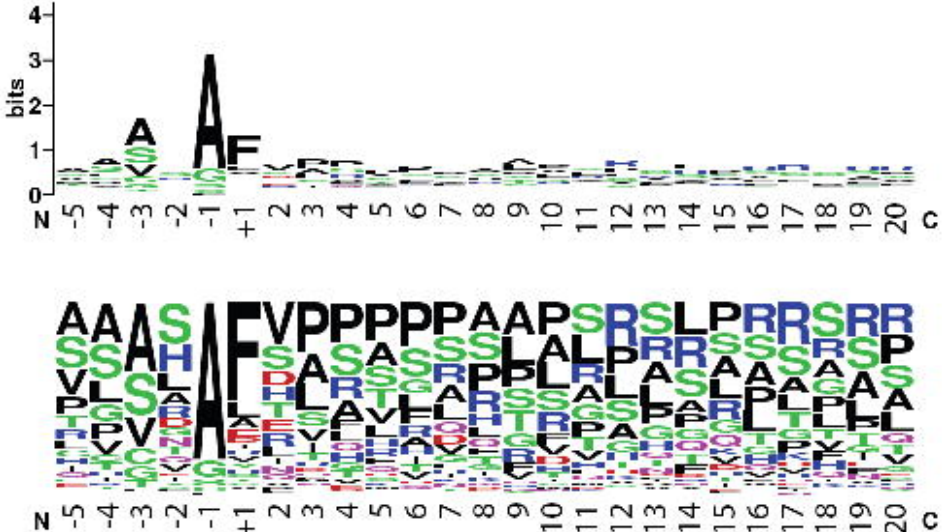

CYTOSOL
PLASTID
TETRAPYRROLES
ALA
ALA
Gly
succinyl-CoA
GLYCERO(PHOSPHO)LIPIDS
SQDG DGDG phosphatidylglycerol
SUF Fe-S ASSEMBLY
Cys
Fe3+
Fe2+
[Fe-S]
heme
sulfate
sulfide
SULFUR ASSIMILATION
THF
Gly
mTHF
GLY CLEAVAGE
fatty acids
ACP
RIBOSOME
DHAP
DHAP
TERPENOIDS
MEP
GGPP
phytyl-PP
pyr
Chl a
CALVIN CYCLE
CO2
GLYCOLYSIS
CAROTENOIDS
Tyr
TERPENOID-QUINONES
MITOCHONDRION
PEP
PEP
ADP
ATP
PQ
tocopherol
pyr
NAD+
NADH
violaxanthin
RIBOSOME
acetyl-CoA
FATTY ACID SYNTHESIS
ACP
CoA
apo-ACP
fucoxanthin
acyl-[ACP]
RIBOSOME
transporter
plastid genome-encoded
protein missing in Cv, Vb, both


STORAGE SUGAR
amylopectin
glucose
CYTOSOL
PLASTID
PENTOSE-P CYCLE
pyridoxin
Ru-5P
Ery-4P
PEP
CHORISMATE
AMINO ACIDS
PRPP
Trp
Tyr
Phe
Ser, Cys, Met
BIOSYNTHESIS
Cys
Ser
Met
GLYCOLYSIS
PEP
pyr
lactate
OAA
succinyl-CoA
TCA
CYCLE
2-OG
Glu
Gln
ADP
ATP
NH3
Glu, Arg, Pro
BIOSYNTHESIS
CO2
urea
Arg
Pro
His
His BIOSYNTHESIS
pyr
Ala
2-OG
OAA
Asp
Asn
Thr
Lys
Asn, Thr, Lys, Ala
BIOSYNTHESIS
FAD
RIBOFLAVIN
SYNTHESIS
PRPP
CALVIN-BENSON
CYCLE
DHAP
GTP
CO2
THYLAKOIDS
orn
STROMA
LUMEN
PSII
PQH2
PQ
b6f
NADPH
NADP+
PSI
ATP
ADP
ATPS
TAT
SEC
Alb3
CII
IMS
MATRIX
UQH2
UQ
CIII
CIV
ATPS
MITOCHONDRION
transporter
plastid/mitochondrial genome-encoded
protein missing in Cv, Vb, both

Chromera velia



Vitrella brassicaformis



	ASAFind	ASAFind+	HECTAR	TargetP - mTP		HECTAR - mTP		MultiLoc2 - mTP	
C. velia				(s)	(p)	(s)	(p)	(s)	(p)
THRESHOLD:	1.4	1.4	0.05	0.35	0.8	0.14	0.41	0.25	0.95
SENSITIVITY:	0.774	0.830	0.769	0.757	0.586	0.748	0.586	0.748	0.361
PRECISION:	0.921	0.921	0.872	0.375	0.844	0.529	0.855	0.485	0.736
V. brassicaformis									
THRESHOLD:	1	1	0.16	0.56	0.81	0.18	0.37	0.64	0.89
SENSITIVITY:	0.759	0.897	0.744	0.757	0.586	0.748	0.559	0.755	0.582
PRECISION:	0.886	0.916	0.967	0.712	0.855	0.664	0.861	0.697	0.842