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Presence of vitamin B₁₂ metabolism in the last common ancestor of land plants

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Summary

Vitamin B₁₂, or cobalamin, (hereinafter B₁₂) is an essential organic cofactor for methionine synthase (METH) and is only synthesised by a subset of bacteria. Plants and fungi have an alternative methionine synthase (METE) that does not need B₁₂, and are typically considered not to utilise it. Some algae facultatively utilise B₁₂ because they encode both METE and METH, while other algae are dependent on B₁₂ as they encode METH only. We performed phylogenomic analyses of METE, METH and eleven further proteins involved in B₁₂ metabolism across >1,600 plant and algal genomes and transcriptomes (e.g., from OneKp), demonstrating the presence of B₁₂-associated metabolism deep into the streptophytes. METH, and five further accessory proteins (MTRR, CblB, CblC, CblD and CblJ) were detected in the hornworts (Anthocerotophyta), and two (CblB and CblJ) were identified in liverworts (Marchantiophyta) in the bryophytes, suggesting a retention of B₁₂-metabolism in the last common land plant ancestor. Our data further show more limited distributions for other B₁₂-related proteins (MCM, RNR-II), and B₁₂-dependency in several algal orders. Finally, considering the collection sites of algae that have lost B₁₂ metabolism, we propose freshwater-to-land transitions and symbiotic associations to have been constraining factors for B₁₂ availability in early plant evolution.

Introduction

Since their endosymbiotic origin over a billion years ago, photosynthetic eukaryotes have diversified into a wide range of different lineages with diverse and complex metabolic functions (reviewed in (1)). The majority of photosynthetic eukaryote taxonomic diversity relates to marine and freshwater algae, including unicellular, colonial and multicellular (e.g., giant kelp)(2) forms. Plants only form one very small component, in the subgroup Streptophyta of the broader lineage Viridiplantae (otherwise including Chlorophyta, or green algae) (3). Plants however comprise surprising morphological and functional diversity, with two major groups, Tracheophyta (or vascular plants), in which the sporophyte (diploid) form constitutes the majority of the life-cycle, and Bryophyta (including mosses, liverworts and hornworts) in which the gametophyte (haploid) form is predominant (3). Amongst these different plant orders, hornworts (or Anthocerotophyta) stand apart through the retention of many features reminiscent of their closest algal relatives. These features include: algal-style carbon concentrating mechanisms (CCM), using a biophysical CCM and pyrenoids (4, 5); an absence of genomic features such as expanded transcriptome factor repertoires and whole-genome duplications associated with higher plants (6); and the pervasive adoption of symbioses with cyanobacteria, particularly in the context of nitrogen fixation (7). As hornworts are projected to have diverged from other plant orders over 400 million years ago, understanding what features link their biology both to higher plants, and to extant algal relatives in the Streptophyta, may provide us with clues about the deep evolution and terrestrial origin of the land plant lineage (1, 6).

One aspect of metabolism that differs between plants and microalgae is the use of cobalamin, or vitamin B₁₂, as an enzyme cofactor. B-vitamins are organic micronutrients taken up by organisms from the environment that are essential for central metabolic processes (8). Plants, fungi and microorganisms also require these compounds for their metabolism, but are able to synthesise them *de novo*. The exception is for vitamin B₁₂, a cobalt-containing corrinoid molecule that is not synthesised by any eukaryote (9). In humans, B₁₂ is essential for two enzymatic activities: B₁₂-dependent methionine synthase (METH) in the C1 cycle (10) and methylmalonyl-coA mutase (MCM), responsible for the metabolism of odd-chain fatty acids and branched-chain amino acids via the propionate shunt (11). B₁₂ deficiency and its associated pathologies (including pernicious anaemia and methylmalonic acidemia) may be chronic in subsistence economies, with

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3
4 particularly adverse impacts on child development and during pregnancy (12). Addressing how to mitigate B₁₂ deficiency
5 is therefore a key challenge to sustainably feeding a growing planetary population (13, 14).

6
7 Historically, B₁₂ in the human diet has been obtained from different sources. These include from ruminant animals and
8 their derivatives (i.e., dairy products) (15), direct consumption from soil (e.g., via geophagia (16), from fortified food
9 (nutritional yeast) and from edible seaweeds across the algal tree of life (17). Indeed, many microalgae also encode
10 METH and/or MCM and many are B₁₂-dependent, with one study for example finding 171/326 algal species were unable
11 to grow in the absence of supplemented B₁₂ (18). Some microalgal lineages may encode further B₁₂-dependent enzymes
12 (e.g. form II ribonucleotide reductase (RNR_{II}), first documented in the green microalga *Euglena*) (19), which is not found
13 in humans.

14
15 B₁₂ biosynthesis involves over twenty enzymatic steps from the tetrapyrrole intermediate uroporphyrinogen III, and has
16 only been described in a subset of bacteria and archaea (20). Algae may acquire synthesised B₁₂ by scavenging from the
17 environment (21), phagotrophic consumption of B₁₂-containing organisms (22) or via symbiotic exchanges with B₁₂-
18 producing commensals (18). Additionally, there are a number of B₁₂ variants with different axial ligands and different
19 bio-availabilities. Intrinsic factor, the B₁₂-binding protein found in the human ileum, has a much higher affinity for
20 cobalamin, which has 5,6-dimethylbenzimidazole (DMB) as the lower axial ligand, than for pseudocobalamin, which has
21 adenine as the lower axial ligand (23). Eukaryotic microalgae have a similar preference for cobalamin over
22 pseudocobalamin, although a B₁₂ remodelling pathway has been documented in some species (24), which enables them to
23 convert pseudocobalamin to cobalamin if supplied with DMB.

24
25 Little is known of algal B₁₂ uptake or cellular transport, but the pathways are well documented in humans (**Fig. 1**).
26 Following its endocytic internalisation, cobalamin is released into the cytosol via a chaperone CblC and the lysosomal
27 transporters CblF/ CblJ. This involves the reductive removal of any preceding upper-axial ligands associated with the
28 cob(III)-alamin and conversion into cob(I)- and cob(II)alamins complexed with CblC. Cob(I,II)-alamins complexed with
29 CblC can then be transferred to the conjugate protein CblD, which subsequently directs the synthesis of different
30 cobalamin-dependent enzymes (25). METH requires methylcobalamin, produced in the cytosol from CblD-associated B₁₂
31 by methionine synthase reductase (MTRR) (26), whereas MCM requires adenosylcobalamin, produced in the
32 mitochondria via a probable B₁₂ transporter protein CblA (27) and an adenosyltransferase CblB (19). A few additional
33 proteins (e.g., CblX, epi-CblC) have been shown to have epistatic effects on cobalamin uptake in humans, but are of
34 unknown function (25). Finally, RNR-II is cytosolic and utilises adenosylcobalamin.

35
36 B₁₂ may be scarce in the environment, including in soil (28) and large regions of the ocean (29), and uptake and
37 processing of the vitamin is energetically costly (30). In contrast to the widespread utilisation of B₁₂ by aquatic algae,
38 land plants have widely lost their metabolic dependence on B₁₂ (31). Plants are able to nonetheless synthesise methionine
39 and complete the methylation cycle in C1 metabolism, despite the loss of METH, because of a B₁₂-independent
40 methionine synthase (METE) that arose separately, which can replace METH, albeit with a lower catalytic activity (32).
41 METE is also present in many METH-containing eukaryotic algae, which renders them facultative B₁₂ users (31). The
42 most parsimonious explanation is an ancestral presence of both METE and METH in eukaryotes with obligately B₁₂-
43 dependent species having lost METE, and plants, fungi, and other species that do not have B₁₂-associated metabolism
44 having lost METH.

45
46 In the last five years, the dramatic expansion in plant and algal sequence resources, such as through the OneKp
47 project (3), a global initiative to sequence 1,293 publically available transcriptomes from across the plants, and close
48 (particularly freshwater) algal relatives in the Viridiplantae (33), has provided unprecedented insights into early plant
49 evolution. This dataset has provided particular insight into the phylogenetic relationships of plants, including the probable
50 monophyly of bryophytes (mosses, liverworts, and hornworts) within the plants (3). Data from within OneKp further
51 support the stepwise accumulation of evolutionary innovations associated with the colonisation of land in the closest
52 streptophyte relatives of land plants (34), and the importance of gene losses as well as gene family expansions for the
53 post-terrestrial diversification of both bryophytes and vascular plants (35). These expanded genomic resources prompted
54 us to revise our current understanding of the distribution of B₁₂-dependent metabolism across photosynthetic eukaryotes,
55 considering in which taxonomic and ecological contexts B₁₂-dependent metabolism occurs.

56 Results

57 *Distribution of encoded METH/ METE proteins indicate B₁₂ presence in hornworts*

58
59 We identified homologues of the thirteen B₁₂-associated proteins using available information from from algal
60 (*Chlamydomonas reinhardtii*, *Euglena gracilis*, *Phaeodactylum tricoratum*) and human genome sequences (**Dataset S1**,
sheet 1). These were used to search a composite genome and transcriptome library of photosynthetic eukaryotes (**Dataset**
S1, sheet 2). The library contained: 53 algal and plant genomes, including those of the hornworts *Anthoceros agrestis* and
A. punctigera (6); decontaminated versions of the 1,000 plant transcriptomes (OneKp ; 1,293 libraries) (3, 33) and the

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2
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4 Marine Microbial Eukaryote Transcriptome Sequencing Project (MMETSP; 305 libraries) (36); and 32 further eukaryotic
5 transcriptomes, as described previously (37). Certain libraries were represented in more than one library type (e.g.,
6 genome plus MMETSP; genome plus OneKp) with 1,666 unique strains in the final dataset. Homologues were retrieved
7 by reciprocal BLAST best-hit (RbH), and further evaluated by single-gene RAxML trees, and PFAM domain analysis,
8 and were categorised taxonomically following recently published multigene phylogenies of plant and algal diversity (3,
9 37). Full outputs are provided in **Dataset S1**, sheets 3-4.

10
11 The evolutionary distributions of each protein are shown schematically in **Fig. 2**. The patterns underline the
12 widespread occurrence of the B₁₂-dependent form of methionine synthase, METH, across eukaryotic algae, found in all
13 major marine groups, and in streptophyte relatives of land plants (3). METH, alongside METE, was further detected in
14 multiple distantly related hornwort genera, suggesting widespread conservation of B₁₂-dependent methionine synthases
15 across the class Anthocerotophyta (**Dataset S1**, sheet 5) (3, 5). These included OneKp homologues from *Megaceros* (*M.*
16 *tosanus*, OneKp transcript- UCRN2004435; *M. vincentianus*- TCBC2004163), *Nothoceros* (*N. aenigmaticus*
17 DXOU2038410), *Paraphymatoceros* (*Paraphymato. hallii*: FAJB2057847) and *Phaeoceros* (*Phaeo. carolinianus*,
18 RXRQ2022853, RXRQ2022854), as well as probable METH in *Anthoceros agrestis* Bonn (geneID:
19 Sc2ySwM_228_5027); *A. agrestis* Oxford (utg0000031_252) and *A. punctigera* (utg0000981_165) genomes. METH was
20 not found elsewhere within the land plant lineage, except for one potential METH homologue identified by RbH in the
21 liverwort *Blasia sp.* that resolved during preliminary phylogenetic analyses with bacterial sequences and showed (>99%)
22 high sequence similarity with a *Mucilaginibacter* METH (38). Previous studies have documented bacterial contamination
23 in the *Blasia* transcriptome, (33), and we consider that this is unlikely to be a liverwort METH.

24
25 The METH phylogeny robustly placed the hornwort sequences with other streptophyte sequences (RAxML
26 bootstrap support : 100%), and as a sister-group to the charophytes / coleochaetales (**Fig. S1** ; **Dataset S1**). This group
27 was positioned more deeply within Viridiplantae, as a sister to the chlorophytes (RAxML bootstrap support: 100%; **Fig.**
28 **S1**). Each of the *Megaceros*, *Nothoceros*, *Phaeoceros* and *Paraphymatoceros* sequences possessed all five PFAMs
29 associated with METH (homocysteine S-methyltransferase, PF02574 ; pterin-binding, PF00809 ; B₁₂-binding, PF02607
30 and PF02310 ; and the activation domain, PF02965) with similar e-values to functionally characterised equivalents from
31 algae (**Fig. S2**) (39). The three identified *Anthoceros* homologues (*A. agrestis* Bonn : 228_5027_1_ *A. agrestis* Oxford
32 utg0000031_252_1; *A. punctigera*, _utg0000981_165_1) possessed all PFAMs apart from the first B₁₂-binding (PF02607)
33 domain. However, this PFAM is detected in alternative gene models in each genome (*A. agrestis* Bonn: 362_443; *A.*
34 *agrestis* Oxford: utg0000031_664; *A. punctigera*, utg0001451_284). We note that at the very least the *A. agrestis* Oxford
35 N- and C-terminal models are encoded on the same chromosomal element (utg0000031), suggesting that *Anthoceros* also
36 possesses an active METH. The hornwort sequences all had conserved active sites (e.g., the substrate-binding pocket of
37 the pterin-binding site) associated with METH activity (**Fig. S2**) (39).

38 We also considered the distribution of the methionine synthase reductase (MTRR) necessary for METH activity.
39 Well-conserved homologues were detected across hornwort genomes and transcriptomes, but were absent from other
40 plant groups (**Fig. 2**). The hornwort MTRR sequences grouped with other streptophyte sequences within the Viridiplantae
41 (**Fig. S1**), and had well-conserved PFAM domains (flavodoxin 1- PF00258 , FAD-binding- PF00667 , oxidoreductase-
42 PF00175 ; **Fig. S2**).

43 *Widespread occurrence of two distinct METE isoforms in plants*

44
45 All plant libraries studied possessed *METE* sequences, including hornworts, indicating that both genes were
46 present in the last common plant ancestor (**Fig. 2**). The hornwort *METE* resolved with other plant homologues (**Fig. S1**)
47 and possessed well-conserved N-terminal (PF08267) and C-terminal catalytic (PF01717) PFAM domains (**Fig. S2**),
48 indicating a vertical origin and possible functionality.

49
50 The plant *METE* enzymes resolved phylogenetically in two discrete families: a conventional isoform with 1,768
51 recovered examples (labelled “Clade I”); and a less frequently observed isoform (“Clade II”) with 125 observed
52 examples (**Fig. S1** ; **Dataset S1**). Both isoforms contained both PFAM domains, i.e. are likely to function as methionine
53 synthases (**Fig. S2** ; **Dataset S1**). In certain cases, Clade I and II were found in the same organism (e.g., the hornworts
54 *Megaceros vincentianus* and *Phaeoceros carolinianus*). Both Clade I and Clade II isoforms were detected in vascular
55 plant, moss, liverwort, hornwort and streptophyte sequences (**Fig. S1**), suggesting their presence in the last plant common
56 ancestor. All three characterised *Arabidopsis* methionine synthases (At5g17920; At3g03780; At5g20980) resolved within
57 Clade I, but the Clade II enzymes all showed greater proximity by BLASTp analysis to these than to *Arabidopsis*
58 adenosyl-methionine synthases (At1g02500; At4g01850; At3g17390; At2g36880) (**Dataset S1**, sheet 6) (40). Both Clade
59 I and Clade II isoforms were distantly positioned to chlorophyte *METE* sequences, which formed a sister-group to red
60 algae, cryptomonads and chromerids (**Fig. S1**). The most parsimonious explanation for this distribution would be the
ancestral replacement of the streptophyte *METE*, still retained in chlorophytes, with the Clade I and Clade II isoforms,
preceding the loss of METH within specific plant groups.

Distribution of METE sequences confirms widespread B₁₂ auxotrophy in algae

METE was absent from many of the algal libraries searched (**Fig. 2**). Transcriptome libraries may under-report the presence of METE, as it may be transcriptionally repressed in B₁₂-supplemented cultures (32), and therefore we limit our consideration to lineages with at least one sequenced genome. Consistent with previous studies (41), the METH and METE distributions project B₁₂ auxotrophy in haptophytes (including three genomes included: *Emiliania huxleyi*, *Chrysochromulina tobinii*, and *Pavlova* sp. CCMP2436). Only one potential haptophyte METE homologue (from *Pavlova lutheri* UTEX LB1293) was found by RbH, but was excluded as a probable green algal contaminant (**Dataset S1**, Sheet 21). We note of a recently discovered and alternative METE-related proteins in the haptophyte *Phaeocystis*, discussed below (42). No occurrences of METE were found in the prasinophyte class Mamiellophyceae (including *Micromonas* and *Ostreococcus* sp. genomes) or the ochrophyte class Pelagophyceae (including two genomes: *Aureococcus anophagefferens*, and Pelagophyceae sp. CCMP2097) (**Fig. 2**). The absence of METE from these three groups is particularly interesting given their environmental abundance (43), underlining the importance of B₁₂ acquisition for marine photosynthesis.

Further instances of obligate B₁₂ requirements were found at more phylogenetically localised scales, including most immediate relatives of the model green algal species *C. reinhardtii* (44) and the glaucophytes, considered as the sister-group to the Viridiplantae; and Palmophyllophyceae, either a sister-group to all other chlorophytes or to all other Viridiplantae (45, 46). Finally, dinoflagellates (including three genomes from *Symbiodinium* sp.) were found to possess only partial METE sequences, which lacked the conventional N-terminal PFAM domain (MetH_synt_1; PF08267), following previous studies (39) (**Dataset S1**). We did, however, identify complete METE sequences containing both PFAM domains in the chromerids *Chromera velia* and *Vitrella brassicaformis*, which are the closest photosynthetic relatives of dinoflagellates (47). The dinoflagellate METE-like sequences were distantly related to other algae and instead resolved with Archaea (**Fig. S1**), which likewise lacked the N-terminal PFAM domain. This may suggest a horizontal acquisition of the dinoflagellate METE-like gene accompanied by the loss of the complete METE isoform.

Finally, the METE sequences of the red algae *Porphyridium* and *Cyanidioschyzon* and the chrysophyte *Poteroispumella* resolved with bacterial homologues (**Dataset S1**; **Fig. S1**). In each case, these acquisitions were shared between multiple related strains, and are likely to be bacterial horizontal gene transfers as opposed to contaminants.

MCM and RNR II enzymes are restricted to individual algal lineages

Next, we considered the distributions of B₁₂-dependent MCM and RNR II across our dataset (**Fig. 2**). Neither enzyme was detected in streptophytes (including hornworts), and were absent from chlorophytes, except for sporadic occurrences that are likely consistent with horizontal acquisitions (discussed below). MCM homologues were primarily detected in algae with secondary red chloroplasts (e.g., diatoms, cryptomonads, haptophytes, ochrophytes), typically possessing well-conserved PFAM domains, and predictable mitochondrial-targeting sequences (**Dataset S1**; **Fig. S3**). MCM is implicated in the catabolism of branched-chain amino acids in diatoms under nitrate exhaustion, but lacks an apparent RNAi mutant phenotype (48), so its significance for B₁₂ dependence in eukaryotic algae remains to be determined. We additionally detected MCM in a small number of glaucophytes, red algae (*Galdieria sulphuraria*) and green algae (*Tetraselmis*, *Chlorella*) (**Fig. 2**). The MCM phylogeny retrieves a sister-group relationship between green algae and haptophytes (**Fig. S1**), implying it was probably acquired by horizontal gene transfer.

The B₁₂-dependent type II ribonucleotide reductase (RNR II), first detected in the secondary green chloroplast-containing alga *Euglena gracilis* (49) was also found in the distantly related haptophytes and chlorarachniophytes, reflecting possible ancestral horizontal gene transfers between all three lineages (50). Intriguingly it is also present in the Chlamydomonadales (although not *C. reinhardtii*), where it has most likely been acquired by an independent horizontal gene transfer from bacteria (**Figs. 2, 3**).

Distribution of uptake proteins suggests deeper retention of B₁₂-dependent metabolism within the bryophytes

We further considered the distribution of six proteins associated with the uptake and intracellular transport of B₁₂ (CblA, B, C, D, F, and J) and two epistatic regulators in humans (CblX, epi-CblC) (25). We could identify widespread presence of CblC, D and J proteins in hornwort libraries (**Fig. 2**). The hornwort sequences resolved within clades of other Viridiplantae, and contained well-conserved PFAM domains (**Fig. S2**; **Fig. S4**), suggesting vertical origin and functionality. That said, very few carried credible signal peptides that might suggest participation in an endosomal B₁₂ uptake pathway (**Fig. S3**). The only protein for which we could identify consistent endomembrane targeting predictions was CblF (**Fig. S3**), but this was almost exclusively detected amongst dinoflagellates (**Figs. 2, 4**). We tentatively propose that the B₁₂ intracellular trafficking pathways may not be identical across different branches of the eukaryotic tree of life.

Surprisingly, homologues of CblB, responsible for the synthesis of adenosyl-cobalamin (19), were found in hornworts (**Figs. 2, S1**). This was despite the absence of MCM, which uses adenosyl-cobalamin, alongside the CblB

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3
4 partner enzyme CblA (**Fig. 2**) (27). We could further detect homologues of CblB and indeed CblJ in liverworts, which
5 grouped monophyletically with homologues from hornworts (**Fig. S4**) and possess well-conserved PFAM domains (**Fig.**
6 **S2**). It remains to be determined, e.g. via the functional characterisation of putative adenosyl-cobalamin dependent
7 enzymes, if some form of adenosyl-cobalamin dependent metabolism is retained in hornworts and liverworts.

8
9 We also searched for homologues of the newly characterised CBA1 protein that is required for cellular B₁₂
10 uptake in the microalgae *C. reinhardtii* and *P. tricornutum* (51), using similar methodology. While the *Chlamydomonas*
11 and *Phaechoactylum* CBA1 proteins do not possess the same PFAM domains, they show moderate reciprocal similarity to
12 one another (BLASTp- 27.8% pairwise identity; e-value 1x 10⁻¹⁴), and thus were used to construct a consensus hmm for
13 validation of individual sequences. Consistent with the findings of the initial study documenting its role (51), potential
14 CBA1 homologues were found across the tree of life, including in hornworts (**Fig. 2; S4**), grouping within a
15 monophyletic clade of streptophyte sequences that suggest vertical inheritance. Many of the CBA1 homologues
16 possessed signal peptides and could thus have potential extracellular or plasma membrane localisations (**Fig. S3**). That
17 said, plausible CBA1 homologues were found using the same approach in vascular plants, although not in mosses or
18 liverworts. Thus, we tentatively propose that hornworts could uptake extracellular B₁₂ using CBA1-type proteins,
19 although the role of CBA1 homologues in hornworts and indeed in other plant species awaits experimental validation,
20 e.g. via complementation assays with *Chlamydomonas* CBA1 mutants (51).

21 *Rare occurrences of B₁₂-independent algae are biased towards terrestrial and symbiotic species*

22
23 Finally, considering B₁₂ independence as the presence of METE but absence of METH/ MTRR, RNR II or
24 MCM activities, we identify eleven algal species that may, like vascular plants, have secondarily lost B₁₂-dependent
25 metabolism completely (**Table 1**). We further identified four species that possess METE and either MCM or RNR II, but
26 lack METH/MTRR, suggesting a loss of B₁₂-related methionine synthesis (**Table 1**).

27
28 These B₁₂-independent species mainly originate from freshwater (e.g., *Leptosira obovata*, *Porphyridium*
29 *cruentum*, *Poterospumella* JBC07) or terrestrial habitats (*Coccomyxa pringsheimii*, *Cyanidioschyzon merolae*, *Galdieria*
30 *sulphuraria*). Freshwater habitats may be limited by the abundance of B₁₂-producing bacteria in the water column (as
31 opposed to sediments) (52), and in certain cases by cobalt scarcity (53). Similarly, *Geminigera cryophila*, which is the
32 only cryptomonad within our dataset to lack METH and encode METE, was collected from the Antarctic, which is
33 characterised by vitamin B₁₂-limitation (**Fig. 1**) (29). Lastly, many of the B₁₂-independent algal species are symbionts of
34 other organisms (*Leptosira obovata* and Trentepohliales, from lichens (54); *Vitrella brassicaformis*, from coral (47); and
35 *Brandtodinium nutriculum*, a foraminiferan symbiont (55)), and may be subject to B₁₂ limitation, or even potentially
36 receive methionine and as folate from their hosts.

37 **Discussion**

38
39 We have queried the distribution of thirteen B₁₂-associated proteins across more than 1,600 plant and algal
40 genomes and transcriptomes, in particular profiting from sequence data from the OneKp transcriptome project (3) (**Fig. 1,**
41 **Dataset S1**, sheet 2). Our data suggest the hitherto undocumented retention of vertically inherited B₁₂-associated
42 methionine synthesis pathways (METH, MTRR) and B₁₂ uptake-associated proteins (CblB, C D, J) in hornworts, and the
43 retention of potential CblB and CblJ homologues in liverworts (**Fig. 2**). Formally, the active use of B₁₂ by hornworts
44 awaits functional characterisation, e.g. by bioassay of B₁₂ content (56), from experimental inference of B₁₂ use such as
45 B₁₂-dependent suppression of hornwort METE (32), or via uptake of fluorescently-labelled B₁₂ analogues (57) by
46 hornwort cells.

47
48 Overall, our data indicate the presence of B₁₂-dependent metabolism in the last common plant ancestor following
49 the transition to land, with subsequent losses in mosses, vascular plants, and (dependent on the functions of the detected
50 CblB and CblJ homologues), liverworts (**Fig. 3**). It remains to be determined when this loss occurred during plant
51 evolution. Some studies have proposed hornworts as the earliest-diverging land plant group (58, 59), which would imply
52 a single METH loss in the common liverworts, mosses and vascular plant ancestor. Other studies, including the
53 *Anthoceros* genome and OneKp transcriptome projects (3, 6, 35), strongly recover bryophyte monophyly, indicating
54 independent losses of B₁₂-dependent metabolism in vascular plants and mosses (**Fig. 3**). In either case, the presence of B₁₂-
55 -dependent methionine synthesis in hornworts sits alongside other phenomena (e.g., biophysical CCMs in hornworts (5),
56 and auxin transporters in charophytes (34)) that blur the physiological boundaries between algae and plants.

57
58 It remains to be determined why B₁₂-dependent metabolism was lost in early plant lineages. Many hornwort
59 species are characterised by the presence of cyanobacterial symbionts, which might produce pseudocobalamin, although
60 this is not bioavailable to microalgal species (23, 60). It is possible that hornworts can utilise pseudocobalamin, and thus
the retention of METH may be linked to their microbiome. Equally, the repeated losses of METH and its associated
enzymes in other freshwater, terrestrial and symbiotic algae may suggest specific ecological niches in which the loss of

B₁₂-associated metabolism is more likely to have occurred (**Table 1**). Comparative studies of the cell biology of these species may elucidate reasons for why B₁₂ metabolism was lost in most land plant lineages.

The data presented in our current study are based on B₁₂-associated proteins with known structures and functions from model species, and relate only to the diversity of these proteins across plants and algae. Beyond these known pathways and functions, yet undetected diversity may exist in B₁₂ metabolism across the tree of life. A recent study identified a novel fusion gene project that appears to confer B₁₂-independent methionine synthesis in haptophytes, projected in our data and elsewhere to have universally lost METE (41, 42) (**Fig. 2**). Deeper inspection of plant and algal genomes, and meta-genome assembled genomes e.g. from the *Tara* Oceans expedition, for novel genes showing structural similarity to the full diversity of archaeal, bacterial and eukaryotic B₁₂-independent synthases, may provide clues about the diversity of strategies used by plants and algae for tolerating environmental B₁₂ scarcity (61, 62).

Ultimately, determining why most plants, excluding hornworts, have apparently lost B₁₂-associated metabolism is particularly important due to the prevalence of vitamin B₁₂ insufficiency/deficiency in populations consuming plant-based diets (12). Understanding the significance of B₁₂ utilisation across algae may facilitate the reintroduction of B₁₂ uptake into crop species, or algal cultivation for dietary consumption (57), sustainably feeding the human planetary population (14).

Materials and Methods

Homologue detection : Potential homologues of thirteen Vitamin B₁₂-associated enzymes (listed in **Dataset S1**, sheet 1) (31) were searched across a composite library of 1,663 non-redundant plant and algal genomes and transcriptomes (**Dataset S1**, sheets 2-4) (3, 36, 37) by BLASTp with threshold e-value 10⁻⁰⁵. Potential matching sequences were extracted and searched by BLASTp against the complete *Arabidopsis thaliana* genome (63), which uniquely encodes METE, supplemented with the query sequences defined above. Sequences that retrieved a best-scoring match to a query sequence were retained for downstream phylogenetic analysis (**Dataset S1**, sheets 5-17). For METE which is retained in plants, sequences that retrieved one of the three *Arabidopsis* methionine synthases were likewise retained for downstream analysis (40).

Phylogeny : Inferred homologues were aligned against the query sequence, and best-scoring homologues obtained from parallel BLASTp searches of 51 combined genome and transcriptome libraries corresponding to different prokaryotic and non-photosynthetic eukaryotic taxonomic groups from across the tree of life (37) by MAFFT v 7.487 using the -gt auto setting (64). The resulting alignments were imported into GeneIOUS v 10.0.9 and initially screened using the in-built NJ tree function with 100 replicates and random starting seeds; and highly divergent branches (defined visually as branches with > 1.0 calculated substitutions/ site) were iteratively removed (65). Curated alignments were trimmed with trimal v 1.4 using the -gt 0.5 setting (66), then inspected with RAXML v 8.0 using the PROTGAMMAJTT substitution matrix and 350 bootstrap replicates (67). The best-scoring tree was inspected for branches of contaminant origin (e.g. sequences from one algal library only that resolved with bacterial homologues). Homologues that passed the initial RAXML curation were passed through a second iteration of mafft alignment, manual curation, and RaxML phylogeny, prior to enumeration of homologue presence/ absence (**Dataset S1**, sheets 18-21).

Functional, targeting and biogeographical annotation : PFAM domains were searched in each homologue retrieved by the initial RbH search by HMMER v 3.3.2 against the Pfam v 35.0 library (68). Sequences that retrieved PFAM domains associated with each query protein with threshold e-value 10⁻⁰⁵ were recorded (**Dataset S1**, sheet 22). For CbA1, where no PFAM is associated with the *Chlamydomonas reinhardtii* sequence, a specific hmm was generated from the characterised *Phaeodactylum* and *Chlamydomonas* proteins and used to screen homologues (**Dataset S1**, sheet 23). Localisations for each protein were inferred following previous studies (37), considering the consensus predictions of WolfPSort, SignalP, ASAFind, TargetP and HECTAR (**Dataset S1**, sheet 23). Collection sites were recorded for each algal species using the corresponding culture collection records considering strain synonyms, and where appropriate direct information from the literature or collector (69-71).

Data deposition : An interactive map of all algal collection sites identified within the dataset, shadeable either via phylogenetic affiliation or inferred Vitamin B₁₂ metabolic status, is available via <https://tinyurl.com/3esfpkmv>.

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Tables

Table 1

Species	Lineage	Strain	Ecological context	Collection latitude	Collection longitude	Notes
Land Plants	Mosses, Vascular plants, liverworts?					
<i>Blastophysa cf. rhizopus</i>	Chlorophytes	M3368	Marine ; Epiphyte	28.4	14.4	Based on PFAM only
<i>Botryococcus braunii</i>	Chlorophytes	UTEX 2441	Freshwater	-13.5	-72.1	Based on PFAM only
<i>Brantodinium nutriculum</i>	Dinoflagellates	RCC3387	Marine; foraminiferan symbiont	43.7	7.3	
<i>Cephaleuros virescens</i>	Chlorophytes	SAG 28.93	Leaf parasite	-29.9	31.0	Based on PFAM only; Trentepohliales
<i>Coccomyxa pringsheimii</i>	Chlorophytes	SAG 216.7	Freshwater; lichen symbiont	N/A	N/A	
<i>Cyanidioschyzon merolae</i>	Red algae	10D	Hot spring	40.9	14.3	
<i>Galdieria sulphuraria</i>	Red algae	074W	Fumarole	-7.6	11.2	Encodes MCM, CblA
<i>Geminigera cryophila</i>	Cryptomonads	CCMP2564	Marine ; Antarctic	-77.8	-163	
<i>Ignatius tetrasporus</i>	Chlorophytes	UTEX2012	Freshwater	N/A	N/A	May encode RNR II
<i>Leptosira obovate</i>	Chlorophytes	SAG 445.1	Freshwater	47.5	7.9	
<i>Porphyridium cruentum</i>	Red algae	UTEX 161	Freshwater	47.6	7.6	Encodes CblB
<i>Poteriospumella JBC07</i>	Chrysophytes	JBC07	Freshwater	31.5	120.2	
<i>Pseudoscofieldia marina</i>	Chlorophytes	SCCAP K/0017	Estuarine	59.4	10.6	Encodes CblB
<i>Trentepohlia annulata</i>	Chlorophytes	SAG 20.94	Freshwater	49.7	16.0	Based on PFAM only; Trentepohliales
<i>Vitrella brassicaformis</i>	Alveolates	CCMP3155	Marine, coral	-23.5	152	

Figure and table captions

Fig. 1. B₁₂ uptake and utilisation pathways in eukaryotes. This figure shows a schematic cell, with potential B₁₂-associated proteins detected in previous studies across the eukaryotes, per (25) and (31). NB that many of these proteins have been established from human cellular models, and further functional diversity in B₁₂ metabolism may exist in non-model species.

Fig. 2. Bar plots of the occurrence of B₁₂-associated metabolism across photosynthetic eukaryotes. These graphs show the number of species from fourteen different algal phylogenetic or functional groups inferred to possess METH, METE, or both, by RbH, PFAM domain and single-gene phylogenetic analysis ; and the number of species from these groups for which at least one B₁₂-associated enzyme was detected inferred to possess homologues of MTRR, MCM, RNR II, CBLA, CBLB, CBLC, CBLD, CBLF, CBLJ, CBLX, epi-CBLC or CBA1 via the same methodology. The total number of species assessable in each case is given to the right-hand side of each plot. Complete tabulated occurrences per lineage and species are provided in **Dataset S1**, sheets 3-4; and individual homologue lists per gene in **Dataset S1**, sheets 5-18.

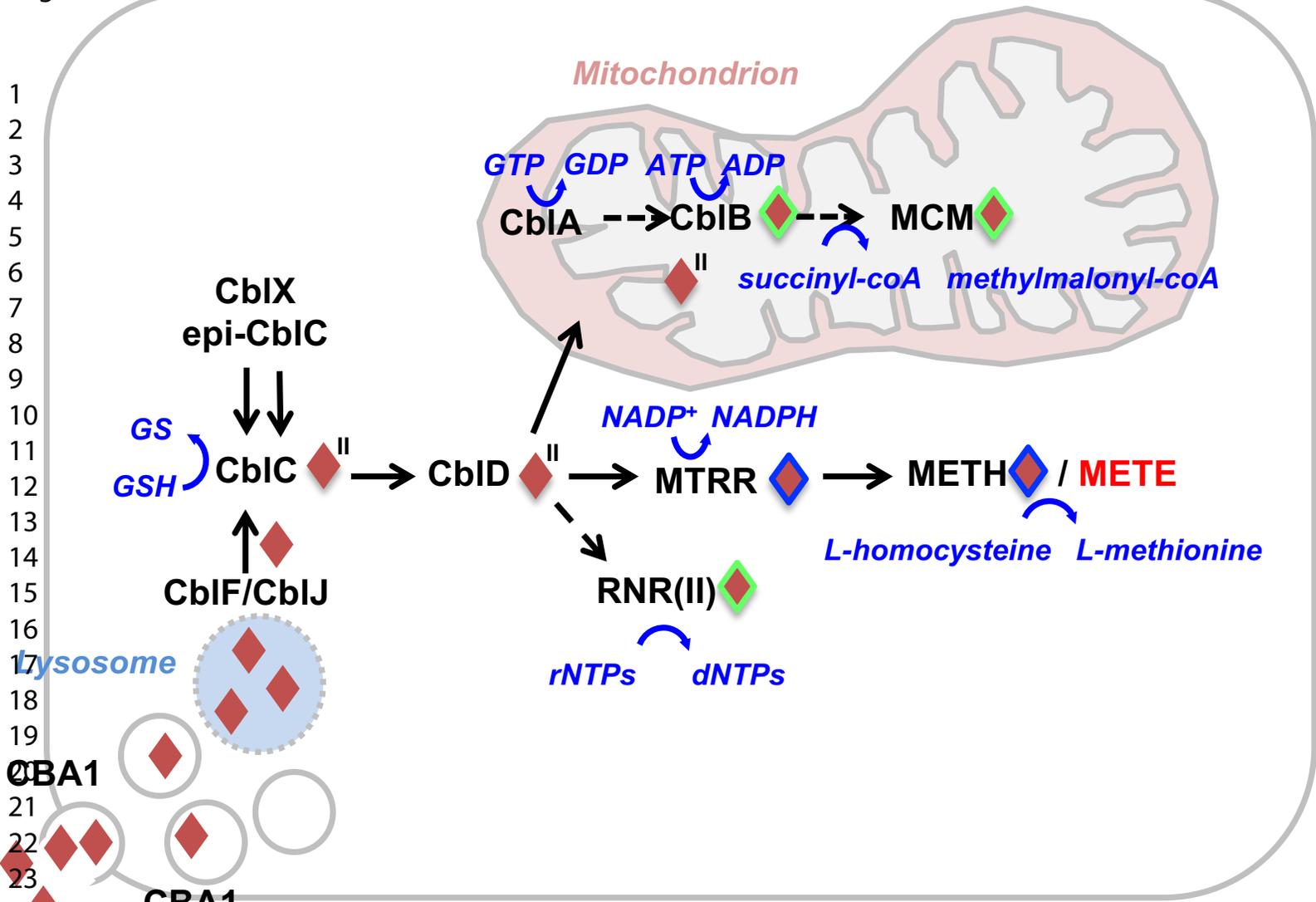
Fig. 3. Vertical inheritance of hornwort B₁₂ metabolism. This figure shows a concatenated multigene tree topology for all OneKp transcriptomes, taken from (3). Taxa names are coloured by origin: dark green- vascular plants and mosses; light green- liverworts; cyan- hornworts; dark yellow- other streptophytes; orange- chlorophytes; brown- other algae. Branches are shaded by B₁₂ status: red- B₁₂-dependent metabolism only; purple- B₁₂-dependent and -independent metabolism; light blue: B₁₂-independent metabolism only; grey- unknown. Vascular plants are collapsed to a single branch. Liverworts are marked as « unknown » due to the uncertain function of their encoded CblB and CblJ proteins. Major algal lineages are labelled, alongside species of interest. A nexus format topology is provided in **Dataset S1**, sheet 22.

Table 1. Species inferred to have lost Vitamin B₁₂-associated metabolism, based on the retention of METE and absence of METH and MTRR sequences. A map of collection sites of all algae included in the dataset is provided at <https://tinyurl.com/3esfpkmv> .

Figures

Figure 1

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Key

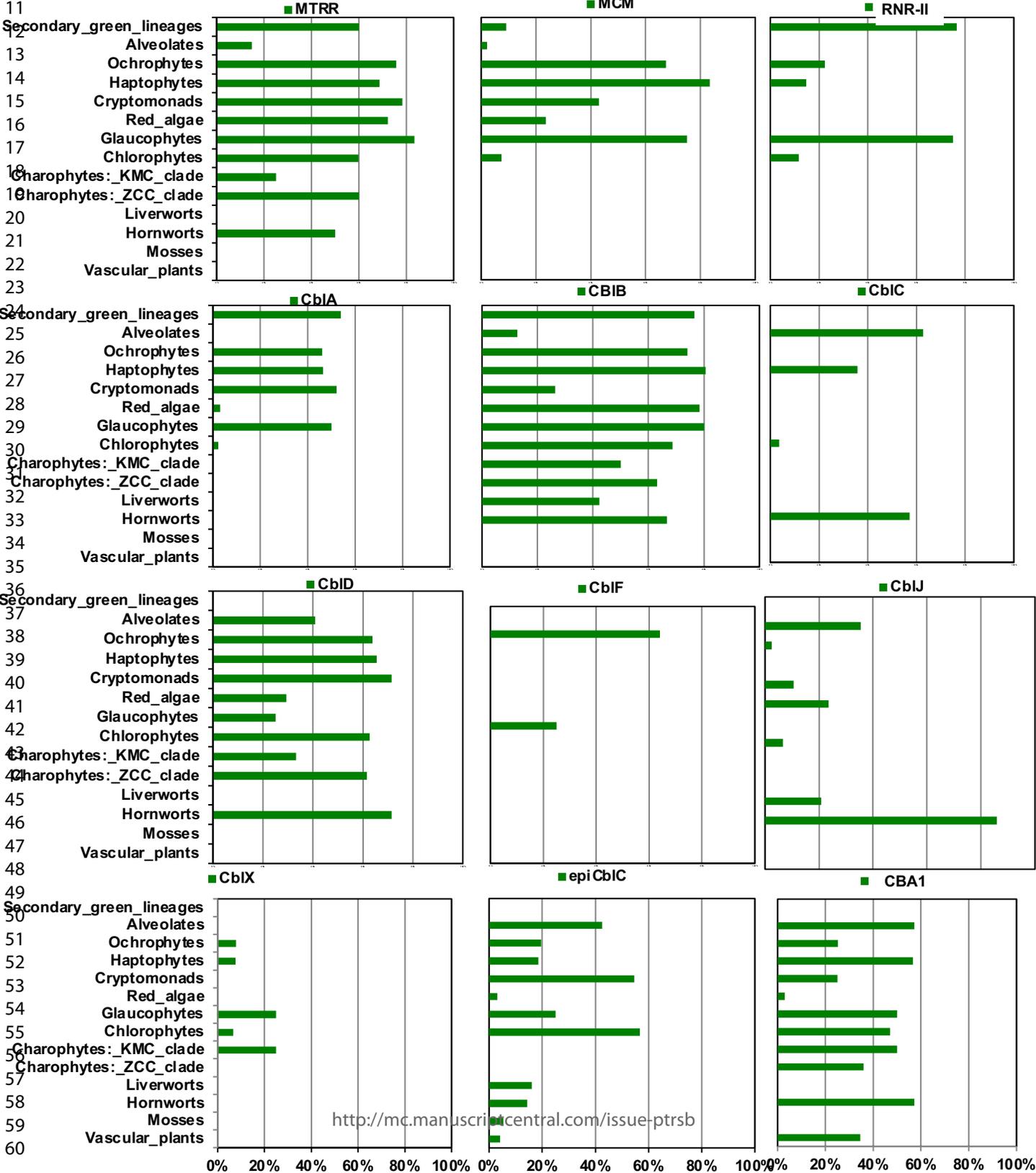
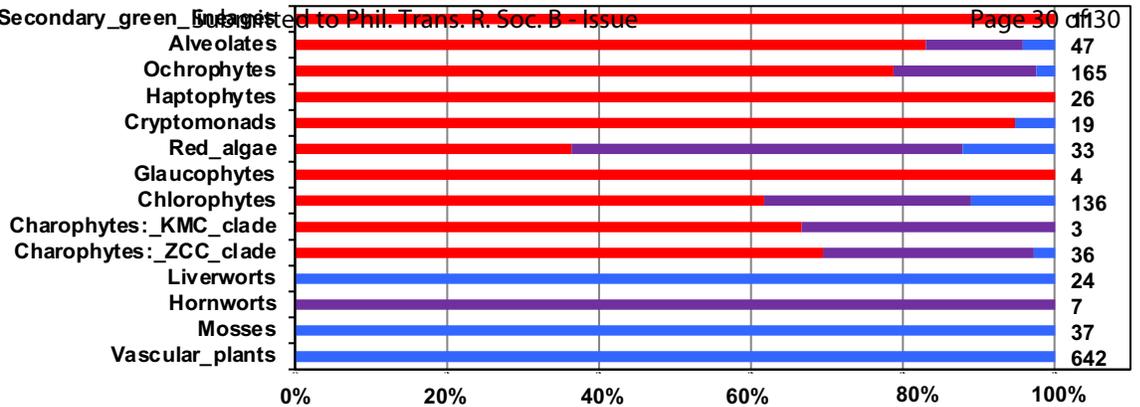
External source

- ◆ Cob(III)- alamin
- ◆^{II} Cob(II)-alamin
- ◆ Adenosyl-cobalamin
- ◆ Methyl-cobalamin
- ◆ B₁₂-associated protein
- ◆ B₁₂-independent isoform
- ◆ Metabolite

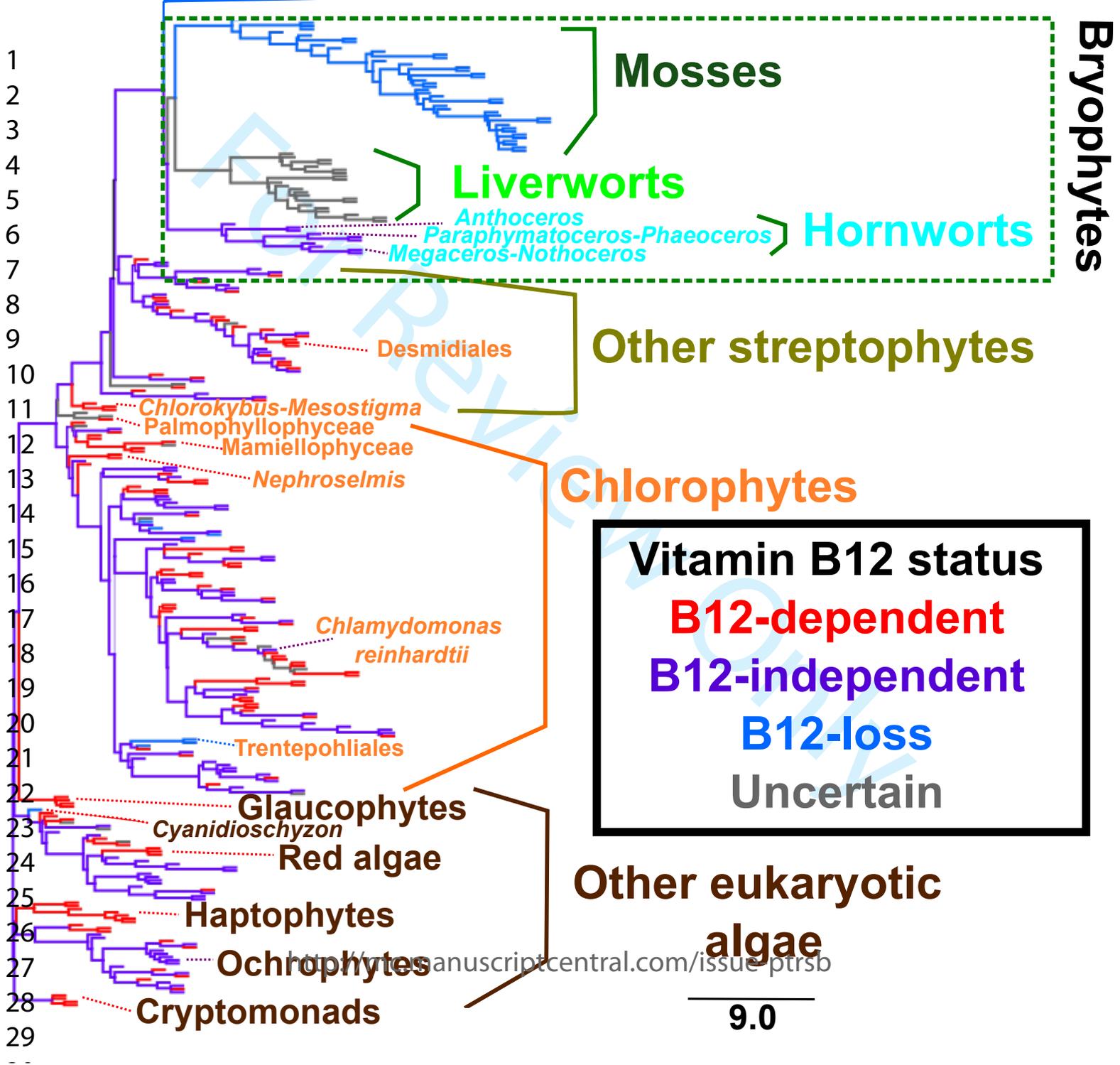
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Key

- 1 ■ Species with MetE homologue only
- 2 ■ Species with Meth homologue only
- 3
- 4 ■ Species with MetE and MetH homologue
- 5
- 6 ■ Species with MetE/ Meth and protein homologue
- 7
- 8 ■ Species with MetE/Meth but no protein homologue
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Vascular Plants



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