Mannitol biosynthesis in algae: more widespread and diverse than previously thought.

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Key words: Algae, primary metabolism, mannitol biosynthesis, mannitol-1-phosphate dehydrogenase, mannitol-1-phosphatase, haloacid dehalogenase, histidine phosphatase, evolution of metabolic pathways.

INTRODUCTION

Mannitol is probably the most abundant naturally occurring polyol. It is produced by a wide range of living organisms, with the noticeable exception of the Archaea and the animal kingdom. It fulfils key physiological roles, including carbon storage and protection against environmental stress (Iwamoto & Shiraiwa, 2005; Patel & Williamson, 2016). Indeed, mannitol can act as organic osmolyte, compatible solute, anti-oxidant, or thermal protectant. Among Eukaryotes, plants rely on mannose-6-phosphate reductase for mannitol production from fructose-6-phosphate (F6P) (Stoop *et al.,* 1996). Apicomplexa (Schmatz, 1989) and algae (Karsten *et al.*, 1997) have evolved a different pathway where mannitol-1-phosphate dehydrogenase (M1PDH) reduces F6P into mannitol-1-phosphate (M1P), which is then hydrolysed to mannitol by a mannitol-1-phosphatase (M1Pase) (Fig. 1a). A similar pathway occurs in fungi (Solomon *et al.*, 2007). In algae and Apicomplexa, both reactions are part of the mannitol cycle supporting production and recycling of this polyol. Genes and proteins for M1PDH have been characterized from fungi, but not for M1Pase. Conversely, both M1PDH and M1Pase genes and enzymes have been studied in the Apicomplexa *Eimeria tenella* (Schmatz, 1989; Liberator *et al.,* 1998). In algae, mannitol occurs in several lineages (Supporting Information Table S1) and can represent up to 25 % of their dry matter (Reed *et al.,* 1985). Both M1PDH and M1Pase activities have been determined in the red microalga *Dixoniella grisea* (Eggert *et al.,* 2006), the macroalga *Caloglossa leprieurii* (Karsten *et al.,* 1997), and endogenous enzymes purified from *Caloglossa* (Iwamoto *et al.,* 2001 and 2003), but the encoding genes have not been identified yet. M1PDH activity has also been measured in the green alga *Platymonas subcordiformis* (Richter and Kirst, 1987). Mannitol metabolism has been well studied in brown algae, and recombinant *Ectocarpus* M1PDH and M1Pase both characterized (Rousvoal *et al.,* 2011; Groisillier *et al.,* 2014; Bonin *et al.,* 2015). M1PDH phylogenetic analysis indicated that bacterial/fungal and apicomplexa/algal sequences formed two distinct groups among the polyol specific long chain dehydrogenase/reductase family (PSLDR) (Bonin *et al.,* 2015). Moreover, *Ectocarpus* and *Eimeria* M1Pases belong to distinct families of phosphatases: the haloacid dehalogenases (HAD-M1Pase) for the former, and the histidine phosphatases (His-M1Pase) for the latter (Liberator *et al.,* 1998; Groisillier *et al.,* 2014).

Algae represent a polyphyletic group with differing life styles (aquatic, terrestrial, extremophile, symbiotic), and their importance to aquatic ecosystems and global carbon balances makes them an important area of study (Field *et al.,* 1998). Algae arose from a complex evolutionary history involving endosymbioses and lateral gene transfers (LTGs) that have shaped their metabolic networks (Falkowski *et al.,* 2004). In this context, it was first suggested that brown algal M1PDH and M1Pase genes were acquired by LTG from actinobacteria (Michel *et al.,* 2010). Subsequently, it was

proposed that these genes were transferred from bacteria to an ancestral Ochrophyta after the separation of diatoms (Dittami *et al.,* 2011). To increase understanding of mannitol biosynthesis in algae, we had two objectives in this study: (i) to assess the occurrence of enzymes involved in mannitol biosynthesis across algal lineages, based on previously characterized algal and apicomplexa M1PDHs and M1Pases; (ii) to analyse the evolution of the mannitol biosynthetic pathway in these organisms. Mining of recently available transcriptomic and genomic resources revealed an unexpected diversity of M1PDHs and M1Pases, and we put forward hypotheses for their evolution among algae.

Materials and Methods

Algal M1PDH and M1Pases were identified using homology searches to the protein sequences of biochemically characterized M1PDH (ES0017G00030_Esil) and HAD-M1Pase (Es0100G00180_Esil) of the brown alga *Ectocarpus* (Groisillier *et al.,* 2014; Bonin *et al.,* 2015), and His-M1Pase (AF032462_Eten) of the apicomplexa *Eimeria tenella* (Liberator *et al.,* 1998). These sequences were used to interrogate the Marine Microbial Eukaryote Transcriptome Sequencing Project (MMETSP, http://marinemicroeukaryotes.org/), the 1000 plants (OneKP; https://sites.google.com/a/ualberta.ca/onekp/) transcriptome project, the Eukaryotic Pathogen Database Resources (EuPathDB; http://eupathdb.org/eupathdb/), and proteomes translated from individual algal genomes (Supporting Information Table S2). Hit sequences with E-value ≤ 1E-05 were collected for further analysis (Supporting Information Notes S1, S2, S3, S4). The molecular evolutionary genetics analysis 6 (MEGA6; Tamura *et al.,* 2013) software was used for multiple sequences alignments, performed with MUSCLE, and for phylogenetic analysis using bootstrapped Maximum Likelihood. Signal peptide and sub-cellular localization were predicted using PredAlgo (Tardif *et al.,* 2012), ASAFind (Gruber *et al.,* 2015), and PlasmoAP (Foth *et al.,* 2003). An Expanded version of the Materials and Methods section is given in Supporting Information Methods S1.

Results and discussion

Distribution of mannitol biosynthetic genes in algae

When analysing these results, it is important to consider that the abundance of particular transcripts in transcriptomes is influenced by culture conditions and life cycle stages, and that non-occurrence in the transcriptome does not necessarily equate to non-occurrence in the genome of

the corresponding organism. Similarly, it is possible that absence of sequences in genomes may result from insufficient depth of sequencing and/or the absence of prediction for genes of interest.

For algae resulting from primary endosymbiosis event (Fig. 1b), mannitol has been reported in some Chlorophyta, in a few species of Rhodophyta, but not in Charophyta and Glaucophyta. In accordance with these observations, our studies revealed no M1PDH or M1Pase genes in Charophyta or Glaucophyta species analysed. (Fig. 1b, Supporting Information Table S3). However, such genes are present in a small number of species of Chlorodendrophyceae, Mamiellophyceae, and Pyramimonadophyceae, and in one Rhodophyta species. Interestingly, the types of mannitol biosynthetic genes seen in these species are generally diverse and parallel pathways seem to exist in many of them. All Mamiellophyceae species contain a bi-functional M1PDH/HAD-M1Pase fusion protein alongside a standalone HAD-M1Pase (stHAD-M1Pase). Two distinct pathways appear to be available in species of *Tetraselmis* (Chlorodendrophyceae) and *Pyramimonas* (Pyramimonadophyceae) investigated, one based on standalone M1PDHs (stM1PDHs) and stHAD-M1Pases, the other on bi-functional proteins. A similar pattern is apparent in the Rhodophyta *Rhodella maculata*. No genomic or transcriptomic resources were available to assess the occurrence of M1PDH and M1Pase in the only Floridophyceae known to produce mannitol, *i.e.* species of the genus *Caloglossa* (Karsten and West, 1993). Mannitol biosynthetic genes are also identified in algae resulting from green-algal secondary endosymbiosis (Fig. 1b). Euglenozoa, as in Mamiellophyceae, possess a stHAD-M1Pase and a bi-modular M1PDH/HAD-M1Pase, whilst Chlorarachniophyceae contain several copies of stM1PDH and stHAD-M1Pase genes.

Among algae derived from red-algal secondary endosymbiosis (Fig. 1b), no genes of interest are observed in Cryptophyte species that were examined. In Haptophyceae, only some species of Prymnesiophyceae possess M1PDH and M1Pase genes, with two distinct profiles. While stHAD-M1Pases and standalone His-M1Pases (stHis-M1Pases) co-occur in Isochrysidales, only stHis-M1Pases have been identified in others, alongside at least one gene encoding a stM1PDH. In unicellular Ochrophyta, mannitol production has been reported in several classes, notably Bacillariophyceae (species *Thalassiosira fluviatilis*; Hellebust, 1965) and Chrysophyceae (species *Ochromonas minima*; Dittami *et al.*, 2011). However, both species were not represented in datasets analysed. Meanwhile, genes of interest are absent in all Bacillariophyceae, Chrysophyceae and Bolidophyceae inspected, and are heterogeneously distributed in other classes. Only some Dictyochophyceae species possess a bi-functional M1PDH/HAD-M1Pase. Pelagophyceae and Pinguiophyceae contain stM1PDHs and stHis-M1Pases, while Eustigmatophyceae, Raphidophyceae, Phaeophyceae, and Xanthophyceae possess stM1PDHs and stHAD-M1Pases. Among Alveolates, mannitol has been reported in *Amphidinium cartereae* (Bidwell, 1957), but no mannitol biosynthetic

genes were identified in this species. However, the corresponding genes are found in Chromerida, Apicomplexa of the *Eimeria* genus, and a minority of Dinoflagellates species examined. In these latter, a patchy distribution of M1PDH and M1Pase genes is observed, with several combinations of enzymes, including the occurrence of bi-functional proteins.

Phylogenetic analyses of algal M1PDHs and M1Pases

A phylogenetic tree of M1PDH standalone proteins and modules was obtained after alignment of the sequences (Supporting Information Fig. S1 and Notes S5), and it features several well-supported groups (Supporting Information Fig. S2). One illustrates the monophyly of green algal standalone enzymes and modules sequences, except for the module identified in *Pterosperma* (Pyramimonadophyceae). Mamiellophyceae M1PDH modules form a sub-group distinct from the one containing *Pyramimonas* and *Tetraselmis* sequences. In Chlorarachniophyceae, all stM1PDHs are derived from a common ancestor. Conversely, at least two groups of standalone sequences are observed for some lineages resulting from red algal secondary endosymbiosis, *i.e.* Haptophyceae, Raphidophyceae, Pelagophyceae, Xanthophyceae, and Phaeophyceae. However, there is no strong bootstrap support at the deeper branches for these lineages. In Dictyochophyceae, standalone and module M1PDHs are contained in separated groups. No trend for predicted localization/signal peptide can be inferred for most of these lineages (Supporting Information Table S4 and Notes S5). However, 13 out of 20 of the brown algal group 1 sequences are predicted to be plastidial or to contain a signal peptide, while only three out of 16 for group 2 sequences show such signal sequences. A third type of M1PDH is observed in Ectocarpales, probably from duplication of a group 1 sequence, and in Pelagophyceae. Alveolate M1PDHs form several distinct sub-groups. Two types of standalones are distinguished in Chromerida. The cluster of *Alexandrium* standalones is closely related to two Pinguiophyceae homologs. Standalones and modules from other Dinoflagellates are part of the same sub-group, closely related to Pelagophyceae and Haptophyceae group 1 standalones.

Analysis of HAD-M1Pases (Supporting Information Fig. S3-S4 and Notes S5) shows that modules and standalone proteins of Mamiellophyceae form two separate groups. Most Mamiellophyceae stHAD-M1Pases are predicted to be chloroplastic, in contrast to *Tetraselmis* and *Pyramimonas* sequences (Supporting Information Table S4 and Notes S5). Chlorarachniophyceae stHAD-M1Pases are comprised of two independent groups, and an additional small cluster probably corresponds to a specific duplication in *Bigelowiella* species. As for M1PDHs, no specific trend is found in the predicted subcellular localisation/presence of signal peptides in these M1Pases. Sequences from *Eutreptiella* and *Rhodella* form two distinct well-supported sub-groups, similar to

the M1PDH phylogeny. In brown algae, two distinctive groups of stHAD-M1Pases were identified. Seven of the 20 sequences of group 1 are predicted to be plastidial, while none of the 19 in group 2 are. Raphidophyceae sequences occur in two sub-groups in which both *Heterosigma akashiwo* and *Chattonella subsalsa* are represented. Dinoflagellate standalones and modules are probably monophyletic because all are contained in one moderately supported group, closely related to the cluster formed by Prymnesiophyceae sequences.

His-M1Pases were identified in all phyla derived from a red algal endosymbiont, *i.e.* Haptophyceae, Ochrophyta, Chromerida, Dinoflagellates, and Apicomplexa. Some sub-groups corresponding to these lineages are well supported (Supporting Information Fig. S5-S6 and Notes S5), such as Dictyochophyceae, Pelagophyceae and *Alexandrium*.

The phylogenetic tree built for bifunctional M1PDH/M1Pases is robust, consisting of three main groups (Fig. 2; Supporting Information Fig. S7 and Notes S5). One supports the monophyly of the green algal sequences, except for *Pterosperma*. Moreover, Mamiellophyceae, *Tetraselmis* and *Pyramimonas* sequences belong to three distinct sub-groups, apart for the sequence 0118958248_Pobo for which the location in the tree is uncertain. Most of these green algal sequences are predicted to be plastidial. A second cluster groups together *Eutreptiella*, red algal, and Dinoflagellates sequences. Finally, a third group contains the three Dictyochophyceae and the *Pterosperma* fusions.

Conclusions and hypotheses for the evolution of M1PDHs and M1Pases in algae

Previous assumptions on evolution of mannitol biosynthesis in algae (Michel *et al.,* 2010; Dittami *et al.*, 2011) were revisited by analysis of extended transcriptomic and genomic resources. M1PDH and M1Pases genes are identified in most of the phyla analysed (Fig. 3), with different combinations of standalone and fusion proteins, in good correlation with the occurrence of mannitol in algae. Algal mannitol biosynthesis was probably shaped by the occurrence of new substrate specificity within the PSLDR, HAD, and histidine phosphatase superfamilies of proteins. Since extant cyanobacteria are not able to produce mannitol, we suggest that standalone M1PDH and HAD-M1Pase genes may have been present in the non-photosynthetic eukaryotic host cell involved in primary endosymbiosis. This is also probably the case for one or several of the eukaryotic hosts involved in secondary and/or possible subsequent endosymbioses (Burki *et al.,* 2016). Little information is available about the metabolic repertoire of these heterotrophic hosts (Gould *et al.,* 2008) and their phylogenetic relationships remain unclear (Baurain *et al.,* 2010). Mannitol biosynthetic genes were then lost in Glaucophyta, Charophyta, Rhodophyta, Cryptophyta, and

Bacillariophyceae, retained as standalone proteins, or fused to create bi-functional proteins. Several specific duplication events occurred later on in several lineages. This concerns for instance M1PDHs and HAD-M1Pases in Chlorarachniophyceae, *Tetraselmis* and *Pyramimonas* species, notably for the occurrence of fusion proteins in these green algae. We also suggest that His-M1Pase may have been present in ancestors of the CASH lineages (Cryptophyta, Alveolata, Stramenopiles and Haptophyta; Baurain *et al.,* 2010), and then subsequently lost in some individual genus/species. Moreover, LTGs between eukaryotic algae may have also contributed to the uneven distribution of M1PDHs and M1Pases in these organisms. Bi-modular M1PDH/M1Pases appear independently in different lineages. Such proteins in the green alga *Pterosperma* and in the red microalga *Rhodella maculata* represent interesting cases. The former probably arose by fusion of M1PDH and His-M1Pase modules acquired possibly by LTG(s) from Pelagophycae and/or a Dictyochophyceae, both classes being closely phylogenetically related (Brown & Sorhannus, 2010). Mannitol production is limited to few Rhodophyta species, and identification of M1PDH and M1Pase in the macroalga *Caloglossa* should help to better understand evolution of this metabolic pathway in these organisms.

Identification of mannitol biosynthetic genes shows that several pathways can co-exist in some algae, such as [stM1PDH+stHAD-M1Pase] and [stM1PDH+stHis-M1Pase], or [stM1PDH+stHAD-M1Pase] and bi-functional M1PDH/HAD-M1Pase, or even all three in the Dictyochophyceae. Furthermore, some species contain several genes for the same enzymatic activity, sometimes with different predicted cellular localization. Such apparent functional redundancy may be of physiological importance, and suggests intriguing questions about evolution, subcellular localization, regulation, and ecological significance of algal mannitol synthesis pathways. This is particularly interesting in the era of “Oceans Systems Biology” (Karsenty, 2012) and the study of phytoplankton given their major contributions to carbon cycling at the planetary scale (Bork *et al.,* 2015).

Acknowledgements

We thank Dr. Erwan Corre for preliminary analysis of the MMETSP resources. This work was supported by The Leverhulme Trust Research project Grant RPG-2015-102.

Author contribution

T.T., Y.L. and S.M.M. designed the research. T.T. and Y.L. performed analyses. T.T. wrote the manuscript with input from Y.L. and S.M.M.

Figure legends

Fig. 1. Mannitol biosynthetic pathway and its distribution in algae. (a) The two steps of mannitol production from frunctose-6-phosphate catalysed by a mannitol-1-phosphate dehydrogenase (M1PDH) and a mannitol-1-phosphatase (M1Pase). (b) Occurrence of M1PDHs and M1Pases in algae. The schematic representation of algal phylogeny uses the colour code considered in the tables. A green asterisk indicates the secondary endosymbiosis of a green alga, a red asterisk the secondary endosymbiosis of a red alga. In the tables, dark colours indicate the identification of genes of interest in the organisms analysed. The name of algal classes for which mannitol production has been observed (Supplementary Information Table S1) is underlined. # indicates that mannitol has been observed in only one species of Bacillariophyceae (*Thalassiosira fluviatilis*), one species of Chrysophyceae (*Ochromonas minima*), and one genus of Floridophyceae (*Caloglossa*) which were not represented in the datasets analysed. Each combination of genes observed for a class of algae is represented by a row (see Supplementary Information Table S3 for an expanded version of these tables). A few inconsistencies were observed when comparing algae showed to produce mannitol and distribution of M1PDHs and M1Pases, and are detailed in Supporting Information Note S5.

Fig. 2. Molecular phylogenetic analysis by the maximum likelihood (ML) method of bi-functional M1PDH-M1Pases. Numbers indicate the bootstrap values in the ML analysis (100 replicates). Colour code is identical as in Fig. 1b. The origin of the sequences is indicated by a 4-5 letter abbreviation at the end of the name of the sequences, and abbreviations are defined in Supporting Information Table S3.

Fig. 3. Evolution of M1PDHs and M1Pases in algae. Primary and secondary endosymbioses are represented as described previously (Keeling, 2013). Unusual cases, such as the M1PDH/His-M1Pase fusion in the green alga *Pterosperma* sp. and the occurrence of M1PDH and M1Pase genes in the red alga *Rhodella maculata*, are not indicated for the sake of clarity; for the same reason, no gene duplication events are included in the figure. The symbol ☒ indicates the loss of mannitol biosynthetic genes. The prefix “St” was used for standalone proteins and “Fus” for bi-functional M1PDH/M1Pase fusions.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Alignment of standalone and module M1PDH sequences

Fig. S2 Molecular phylogenetic analysis by the maximum likelihood (ML) method of M1PDH standalones and modules

Fig. S3 Alignment of standalone and module HAD-M1Pase sequences

Fig. S4 Molecular phylogenetic analysis by the maximum likelihood (ML) method of HAD-M1Pase standalones and modules

Fig. S5 Alignment of standalone and module His-M1Pase sequences.

Fig. S6 Molecular phylogenetic analysis by the maximum likelihood method (ML) of His-M1Pase standalones and module

Fig. S7 Alignment of bi-functional M1PDH/M1Pases.

Table S1 List of algae known to produce mannitol

Table S2 List of transcriptomic and genomic resources considered for the analyses presented in this study

Table S3 Distribution of mannitol synthesis genes in algae and some of their related protists

Table S4 Prediction of signal peptide and of potential cellular localization

Methods S1 Expanded description of the methods

Notes S1 List of M1PDH standalones and modules used for phylogenetic and peptide signal/subcellular localization prediction analysis

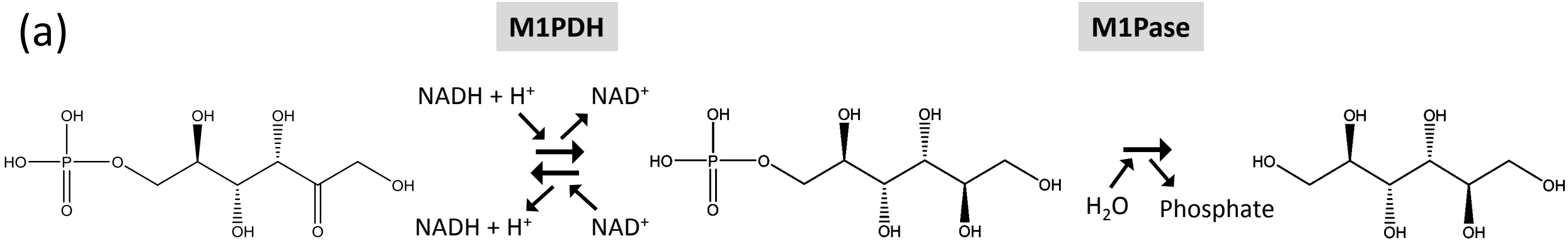
Notes S2 List of HAD-M1Pase standalones and modules used for phylogenetic and peptide signal/subcellular localization prediction analysis

Notes S3 List of His-M1Pases standalones and modules used for phylogenetic and peptide signal/subcellular localization prediction analysis

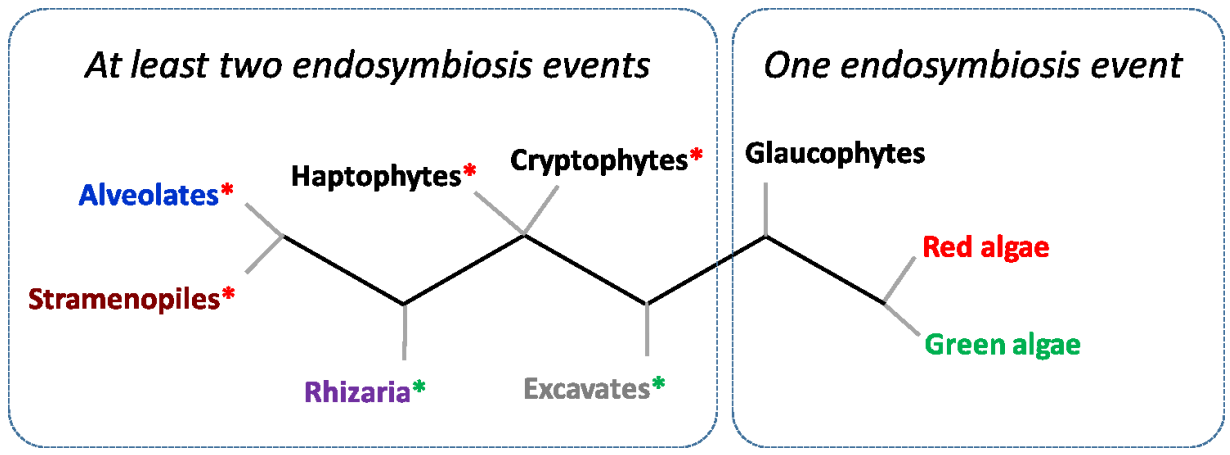
Notes S4 List of fusion M1PDH/M1Pases used for phylogenetic and peptide signal/subcellular localization prediction analysis

Notes S5 Expanded version of the Results and Discussion section

(a)



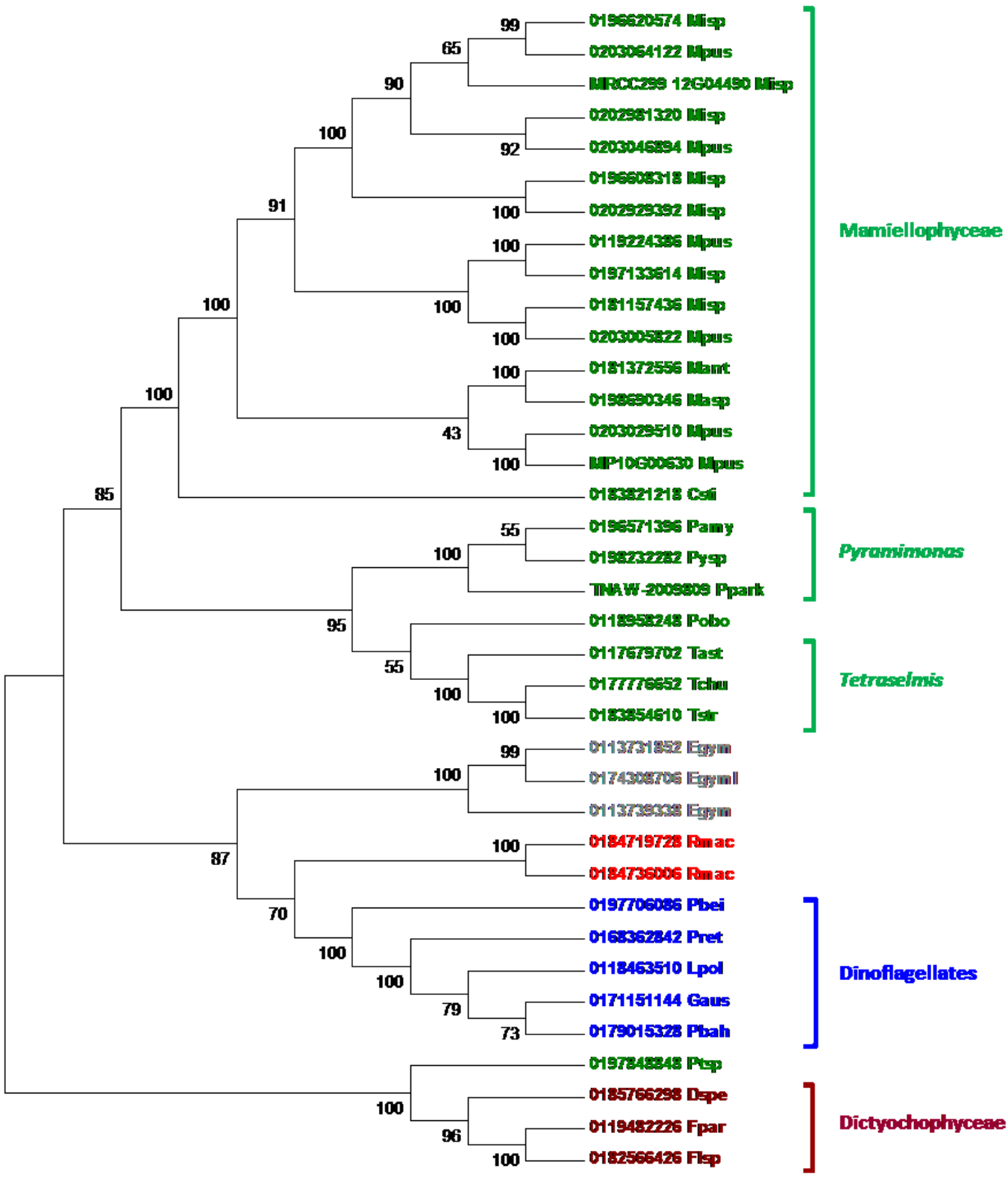
(b)



A. Standalone M1PDH
B. Standalone HAD-M1Pase
C. Standalone His-M1Pase
D. Fusion M1PDH/M1Pase

Phylum	Class	A	B	C	D
Glaucophyta	Glaucophyceae				
Charophyta	Coleochaetophyceae				
	Charophyceae				
	Klebsormidiophyceae				
	Mesostigmatophyceae				
	Zygnematophyceae				
Chlorophyta	Chlorophyceae				
	Chlorodendrophyceae	■	■		■
		■	■		
	Mamiellophyceae				
					■
			■		■
	Nephrophyceae				
	Pyramimonadophyceae				
					■
		■			■
		■	■		■
	Trebouxiophyceae				
Rhodophyta	Bangiophyceae				
	Compsopogonophyceae				
	Cyanidiophyceae				
	Florideophyceae #				
	Porphyridiophyceae				
	Rhodellophyceae	■	■		■
	Stylonematophyceae				
Euglenozoa	Euglenophyceae		■		■
Cercozoa	Chlorarachniophyceae	■	■		
			■		

Phylum	Class	A	B	C	D
Cryptophyta	Cryptophyceae				
Haptophyta	Pavlovophyceae				
	Prymnesiophyceae				
				■	
		■		■	
		■	■	■	
Ochrophyta	Bacillariophyceae #				
	Bolidophyceae				
	Chrysophyceae #				
	Dictyochophyceae				
		■	■		■
		■	■	■	■
		■		■	
		■	■	■	
		■	■		
	Eustigmatophyceae	■	■		
	Pelagophyceae	■	■	■	
		■		■	
			■		
	Phaeophyceae	■	■		
	Pinguiophyceae	■		■	
	Raphidophyceae	■	■		
	Xanthophyceae	■	■		
Chromerida	Unknown	■		■	
Dinoflagellata	Dinophyceae				
					■
		■	■	■	
			■	■	
		■			
			■		
			■		■
		■	■		■
		■		■	
				■	■


0196620574 Misp
0203064122 Mpus
MRCC299 12G04490 Misp
0202981320 Misp
0203046894 Mpus
0196608318 Misp
0202929392 Misp
0119224386 Mpus
0197133614 Misp
0181157436 Misp
0203005822 Mpus
0181372556 Mant
0198690346 Masp
0203029510 Mpus
MP10G00630 Mpus
0183821218 Csli
Mamiellophyceae
0196571396 Pamy
0198232282 Pysp
TNAW-2009809 Ppark
Pyraminonas
0118958248 Pobo
0117679702 Tast
0177776652 Tchu
0183854610 Tstr
Tetraselmis
0113731852 Egym
0174308706 Egyml
0113739338 Egym
0184719728 Rmac
0184736006 Rmac
0197706086 Pbei
0168362842 Pret
0118463510 Lpol
0171151144 Gaus
0179015328 Pbah
Dinoflagellates
0197848848 Ptsp
0185766298 Dspe
0119482226 Fpar
0182566426 Flsp
Dictyochophyceae
99
65
90
100
92
91
100
100
100
100
100
100
100
100
43
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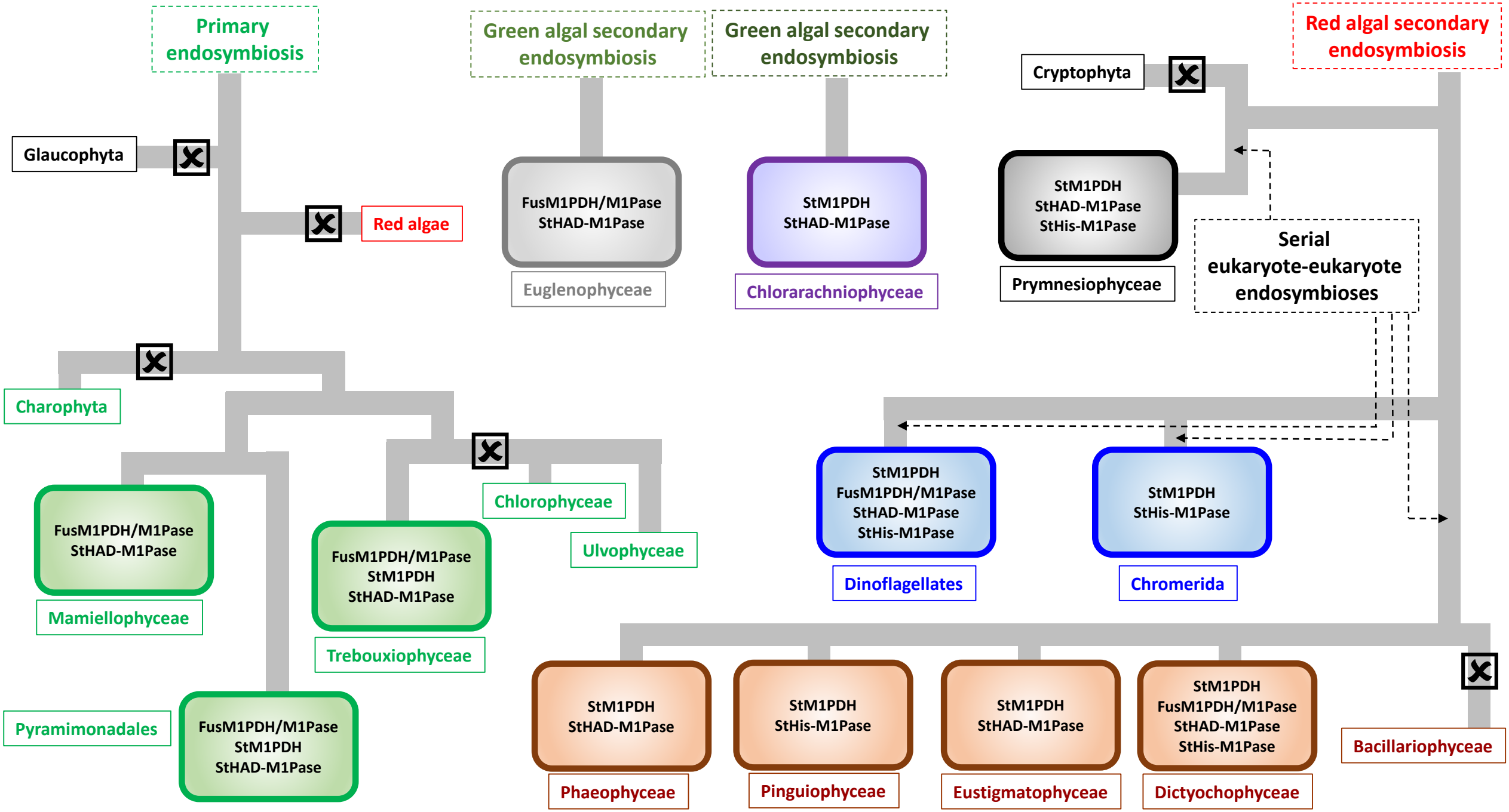
Primary endosymbiosis
Green algal secondary endosymbiosis
Green algal secondary endosymbiosis
Red algal secondary endosymbiosis
Glaucophyta
Red algae
FusM1PDH/M1Pase
StHAD-M1Pase
Euglenophyceae
StM1PDH
StHAD-M1Pase
Chlorarachniophyceae
Cryptophyta
StM1PDH
StHAD-M1Pase
StHis-M1Pase
Prymnesiophyceae
Serial eukaryote-eukaryote endosymbioses
Charophyta
Chlorophyceae
Ulvophyceae
FusM1PDH/M1Pase
StHAD-M1Pase
Mamiellophyceae
FusM1PDH/M1Pase
StM1PDH
StHAD-M1Pase
Trebouxiophyceae
StM1PDH
FusM1PDH/M1Pase
StHAD-M1Pase
StHis-M1Pase
Dinoflagellates
StM1PDH
StHis-M1Pase
Chromerida
Pyramimonadales
FusM1PDH/M1Pase
StM1PDH
StHAD-M1Pase
StM1PDH
StHAD-M1Pase
Phaeophyceae
StM1PDH
StHis-M1Pase
Pinguiophyceae
StM1PDH
StHAD-M1Pase
Eustigmatophyceae
StM1PDH
FusM1PDH/M1Pase
StHAD-M1Pase
StHis-M1Pase
Dictyochophyceae
Bacillariophyceae