COMPARATIVE GENOMIC ANALYSIS ILLUSTRATES EVOLUTIONARY DYNAMICS OF MULTI-SUBUNIT TETHERING COMPLEXES ACROSS GREEN ALGAL DIVERSITY

YASINEE PHANPRASERT

MASTER OF SCIENCE
IN
BIOLOGICAL SCIENCE

SCHOOL OF SCIENCE
MAE FAH LUANG UNIVERSITY
2022

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ACKNOWLEDGEMENTS

I would like to thank the following people: my supervisor, Asst. Prof. Dr. Eleni Gentekaki who provided research skills and knowledge; my co-supervisor, Prof. Dr. Joel B. Dacks who provided knowledge and research skills and his lab members at University of Alberta, Canada; Dr. Shweta Pipaliya; Kiran More; Dr. Kristína Záhonová; and Dr. Kacper Maciszewski who support me technical advice, thesis revise and suggestions. I'm grateful for my examiner committee, Assoc. Prof. Dr. Eunsoo Kim for sharing and advising the useful data, Asst. Prof. Dr. Siam Popluechai, head of Gut Microbiome research group as well as his lab member, and Dr. Nakarin Pamornchainavakul for basic programming skills. I also appreciated helps from Suthee Wipasa who supported me and relieved my stresses for the past two years of study. Finally, my biggest thanks to my family and my friends who were not mentioned here for all support in the culmination of two years of distance learning.

Yasinee Phanprasert

Thesis Title	Comparative Genomic Analysis Illustrates Evolutionary Dynamics of Multi-subunit Tethering Complexes Across Green Algal Diversity
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ABSTRACT

Green algae are the prevalent group of photosynthetic eukaryotes. Although a vast majority of them carry out oxygenic photosynthesis, certain species can also at times transition into mixotrophs, free living heterotrophs, or even parasites. The physical characteristics of green algae are highly diverse – they vary greatly in size, shape, color, and habitat. Nonetheless, all of them share the immense cellular complexity, a key constituent of which is the complex web of interacting membrane-bound organelles, collectively known as the endomembrane system. It is strictly controlled by an array of proteins, such as tethering factors. Among them are the multi-subunit tethering complexes (MTCs), which promote the initial interaction between a vesicle and its destination organelle. The aims of this study are to compare the multi-subunit tethering complexes in various green algae and study their evolutionary dynamic across the diversity of Chlorophyta. Our results reveal that while green algae carry a generally conserved and unduplicated complement of MTCs, some intriguing variation exists. Notably, we identified incomplete sets of TRAPPII, exocyst, and HOPS/CORVET components in all Mamiellophyceae, and what is more, not a single subunit of Dsl1 has been found in *Cymbomonas*

tetramitiformis. As absence of Dsl1 have been correlated with having unusual peroxisomes, we searched for peroxisome biogenesis machinery, finding very few components in *Cymbomonas*, suggestive of peroxisomal lack. Overall, we demonstrate conservation of MTCs across green algae with some notable taxon-specific losses possibly indicative of unusual endomembrane systems.

Keywords: Multi-subunit Tethering Complexes, Exocyst, TRAPPII, Dsl1, Peroxisome, membrane-trafficking, Prototheca, Cymbomonas

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ABBREVIATION AND SYMBOL

COG	Conserved Oligomeric Golgi
CORVET	Class C Core Vacuolar/Endosomal Tethering
GARP	Golgi Associated Retrograde Protein
HOPS	Homotypic Fusion and Vacuolar Protein Sorting
MTC	Multi-subunit Tethering Complexes
MTS	Membrane Trafficking System
TRAPP	Transport Protein Particle

CHAPTER 1

INTRODUCTION

In general, green algae are valuable and play a key ecological role in the environment. They have been recognized as the basal species in food webs, habitats for others aquatic animals, and even as indicators for assessing environmental status (Wehr & Sheath, 2015). Various green algae are beneficial to human welfare and economy – they can be used as commercial foods, supplementary foods, pharmaceutical products, and ingredients of cosmetic products (Aditya et al., 2016; Hermawan et al., 2018; Kusumaningrum, 2008; Silva et al., 2020). The emergence of photosynthetic eukaryotes has been explained by an endosymbiotic scenario, in which a heterotrophic eukaryotic cell enveloped a cyanobacterium, which eventually evolved into a primary plastid (Cavalier-Smith & Lee, 1985; Fang et al., 2017; Oborník, 2019). This event started the distinct evolutionary path of the Archaeplastida – the group which subsequently split into three lineages: *Viridiplantae* (green algae and plants), *Rhodophyta* (red algae) and *Glaucophyta*. The "green" lineage *Viridiplantae* is subdivided into two phyla: *Streptophyta* and *Chlorophyta*, the former of which encompasses the land plants and their closest relatives, charophyte algae, while *Chlorophyta* comprise the rest of the green algae.

The physical characteristics of green algae are highly distinct and diverse. They vary widely in size, shape, color, and habitat. Green algae can be motile or non-motile, and their habitat ranges from freshwater to saltwater, or even land. Cell organization can be unicellular or multicellular, while some taxa are filamentous, forming cell networks (Leliaert et al., 2012; Tang et al., 2020). Among green algae, Mamiellophyceae (order Mamiellales) comprise the most dominant phytoplankton in the marine environment (Tragin & Vaulot, 2019). This order consists of only three genera,

including the smallest known photosynthetic green alga (0.8 μm cell in diameter), *Ostreococcus,* the first described picoplanktonic species, *Micromonas*, and the distinctly pyramid-shaped planktonic microalga *Botryococcus* (Piganeau et al., 2011).

Green algae are generally autotrophic owing to their oxygenic photosynthetic activity. Nonetheless, some of them might lead mixotrophic or heterotrophic lifestyles. Mixotrophy is an intermediate nutritional strategy, merging autotrophy and heterotrophy in the same organism to maximize the efficiency of intake of organic carbon or other nutrients (Selosse et al., 2017). This strategy is common among diverse groups of protists combining phototrophy and phagotrophy (Flynn et al., 2019). Recent studies have revealed that the protist plankton, exhibiting photo- and phago-mixotrophy, constitute common and important members of the global microbial community (Leles et al., 2017, 2019).

A perfect example of a mixotrophic green alga is the genus *Cymbomonas.* These algae generally utilize photosynthesis, but they also have the ability to engulf bacterial cells via phagocytosis (Gagat & Mackiewicz, 2017). In others, however, heterotrophy is the only means of energy acquisition, as previous studies have shown that multiple representatives of green algae have lost their photosynthetic ability independently; for example, within *Chlorellales*, the loss has occurred at least two times (Figueroa-Martinez et al., 2015). The relationship between the loss of photosynthesis and parasitism still remains poorly understood; nonetheless, studies suggest that loss of photosynthesis occurs early on during the shift to parasitism (Poulin & Randhawa, 2015). Some of the extant members of *Ulvophyceae* and *Trebouxiophyceae* are dangerous pathogens. For instance, the Ulvophyte, *Cephaleuros* causes red rust on crops (Pitaloka et al., 2015; Sunpapao et al., 2016), while in Trebouxiophyceae, a clade comprising parasitic green algae with diverse host preferences, *Helicosporidium* infects the guts of arthropods (Tartar, 2013), while its close phylogenetic relative, *Prototheca,* has the ability to infect mammalian hosts (Lass-Flörl & Mayr, 2007). *Prototheca* has been isolated from the natural environment, including slime flux of trees, grass, fresh and salt water, and wastewater, as well as tissues of numerous animals – e.g. cattle, deer, dogs, cats and humans – in which it causes protothecosis, a rare skin infection (Lass-Flörl & Mayr, 2007; Shave et al., 2021; Sykes, 2014). It is worth noting that in other parasitic protists, studies have shown that membrane trafficking system-mediated

vesicle transport of various virulence factors can contribute to their capabilities for host invasion (Dacks et al., 2005; Rai & Johnson, 2019).

Cellular complexity is one of the main characteristics of eukaryotes and it is crucial for the transport of proteins and other molecules through the endomembrane system (Gurkan et al., 2013). There are many organelles involved in membrane trafficking machinery, such as the endoplasmic reticulum (ER), Golgi complex, endosomes, and the specific proteins that mediate membrane trafficking, such as Soluble NFS attachment receptor (SNAREs), tethering complexes, and small GTPase (Cai et al., 2007). These proteins are the coordinators for membrane fusion and vesicle transport (Angers & Merz, 2011). Membrane fusion is a multi-step process, including budding/scission, tethering, docking, and fusion. During the membrane fusion, the small GTPase in its functional form (with GTP loaded) activates tethering factors (Stenmark, 2009). Then, SNAREs from both membranes will lead the two layers of donor and acceptor organelles to fuse together (Barlow & Dacks, 2018; Carr & Rizo, 2010). Before the fusion occurs, however, correct identification of the target membrane requires specific recognition factors to reach their destination. When this process occurs uncontrollably, the proteins can form non-fusogenic complexes early in the secretory pathway (Dubuke & Munson, 2016).

To prevent premature localized fusion and ensure correct cargo delivery, target membrane binding must be tightly controlled by additional accessory proteins, such as tethering factors (Bröcker et al., 2010; Dubuke & Munson, 2016). Multi-subunit tethering complexes (MTCs) promote the initial interaction between the vesicle and the target organelle by binding with Rab-GTPase as well as SNAREs and leads to membrane fusion (Bröcker et al., 2010; Dubuke & Munson, 2016). MTCs can be classified into three groups based on structural similarities, the first of which are the complexes associated with tethering containing helical rods (CATCHR), consisting of Conserved Oligomeric Golgi (COG), Dsl1, Golgi associated retrograde protein (GARP), and exocyst. COG controls the retrograde transportation of vesicle tethering at the Golgi apparatus. The COG complex is a hetero-octameric protein that consists of eight subunits named COG1 through COG8 (Bröcker et al., 2010). The smallest MTC is Dsl1, which consists of three subunits including Dsl1, tip20 and sec20 localized at endoplasmic reticulum. (Dubuke & Munson, 2016). GARP regulates retrograde

transportation from endosomes to the *trans*-Golgi network (TGN), consisting of four core subunits, which are Vps51 to Vps54 (Bröcker et al., 2010; Dubuke & Munson, 2016). Exocyst is an octameric protein complex, which recognizes the fusion of secretory vesicle to plasma membrane. Similarly to COG, this complex comprises eight subunits (Ahmed et al., 2018; Heider et al., 2016; Mei & Guo, 2018). The second group, the homotypic fusion and vacuolar protein sorting (HOPS) complex and class C core vacuolar/endosomal tethering (CORVET), are specifically involved in endosomal transportation (Balderhaar & Ungermann, 2013). The last group of MTCs constitutes the transport protein particle (TRAPP) complex. There are two forms of this complex including TRAPPI and TRAPPII. TRAPPI functions in tethering the coated vesicles during transport from ER to Golgi, while TRAPPII involves tethering of coated vesicle *intra*-Golgi trafficking and early endosome to late Golgi (Bröcker et al., 2010; Dubuke & Munson, 2016). Generally, MTCs are universally represented in eukaryotes, as they are the conserved components of membrane trafficking machinery, however, reduction of MTCs has occurred in many lineages as well (Santana-Molina et al., 2021).

Particularly, MTCs have been studied in many lineages across eukaryotic diversity. For instance, Apicomplexa have drastically reduced MTC components, with Dsl1 and exocyst complexes mostly absent (Klinger et al., 2013). Moreover, the complete set of Dsl1 complex is missing across ciliate diversity, as well as COG, exocyst, and TRAPPII, which were subsequently reduced across ciliate genomes (Richardson & Dacks, 2022). All eight COG subunits were present in *Chromera* and *Vitrella* as well as the entire complexes of HOPS and CORVET, however, none of the exocyst complements were identified (Woo et al., 2015). To deepen our understanding of how such ancestrally autotrophic organisms could evolve sophisticated adaptations for heterotrophic or mixotrophic lifestyles, it is important to consider the evolutionary processes underlying the remodeling of cellular trafficking machinery, starting with the multi-subunit tethering complexes that are involved in the initial process of membrane fusion. This study aims to investigate the MTC components across the diversity of the green algae via comparative genomic analysis.

CHAPTER 2

LITERATURE REVIEW

2.1 The Diversity of Green Algae

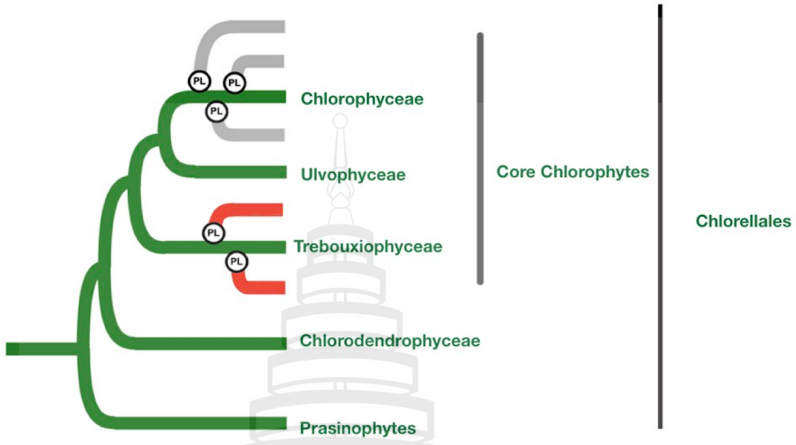
Green algae are valuable and have important ecological roles in the environmental. They have been recognized as the basal species in food webs (Wehr & Sheath, 2015), habitats of other aquatic animals, and even as indicators of environmental status. In addition, green algae are economically important to humans, as they can be used as commercial foods, dietary supplements, potential pharmaceutical products, and ingredients of cosmetic products (Ben-Amotz, 1999; Kusumaningrum, 2008; Silva et al., 2020). For instance, *Dunaliella salina*, a unicellular green alga that plays a key role in hypersaline environments (Oren, 2005, pp. 1905–2005), is one of the most popular commercial dietary supplements, because of its high beta-carotene content (Ben-Amotz, 1999). Astaxanthin, an antioxidant in carotenoid pigment extracted from Trebouxiophyceae, has been also used as a powerful supplement food (Aditya et al., 2016). With regard to industrial application, the *Cladophorales*, which have unique physical appearance and special cell wall characteristics, have been used as a filter membrane, reinforcement fiber, etc (Mihranyan, 2011). Green algae belong to Viridiplantae one of the three groups of *Archaeplastida* along with Glaucophyta and Rhodophyta. Viridiplantae, is further subdivided into Streptophyta and *Chlorophyta*. Streptophyta is comprised of land plants and charophyte algae, while Chlorophyta comprise the rest of the green algae (Fang et al., 2017). Typically, green algae are considered to be primary producers – they have the ability to produce energy and biomass via photosynthesis. In the past, chlorophytes were classified into four main clades, based on morphological characteristics: Chlorophyceae, Trebouxiophyceae, Ulvophyceae, and Prasinophyceae (Leliaert, 2019; Lewis & McCourt, 2004).

The physical characteristics of green algae are highly distinct and diverse. They vary widely in size, shape, color, and habitat. Green algae can be motile or non-motile, and their habitat ranges from freshwater to saltwater, or even land. Cell organization can be unicellular or multicellular, while some taxa are filamentous, forming cell networks (Leliaert, 2019; Lewis & McCourt, 2004; Tang et al., 2020). Among green algae, Mamiellophyceae (order Mamiellales) are one of the most dominant in the environment. This order consists of only three genera, including the smallest known photosynthetic green alga (0.8 μm cell diameter), *Ostreococcus,* the first described picoplanktonic species, *Micromonas*, and the distinctly pyramid-shaped planktonic microalga *Botryococcus* (McKie-Krisberg & Sanders, 2014; Worden et al., 2009).

Green algae are generally considered as autotrophic due to their oxygenic photosynthetic activity; on the other hand, some of them might lead mixotrophic or heterotrophic lifestyles (Oborník, 2019). Mixotrophy is an intermediate nutritional strategy, merging autotrophy and heterotrophy in the same organism to maximize the efficiency of the intake of organic carbon or other nutrients (Flynn et al., 2019; Selosse et al., 2017). This strategy is common among diverse groups of protists combining phototrophy and phagotrophy. Although generally photoautotrophic, mixotrophic species have been described and may well be more prevalent than previously suspected. Indeed, recent studies have revealed that protist plankton, exhibiting photo- and phago-mixotrophy, constitutes common and important members of the global microbial community (Flynn et al., 2019; Oborník et al., 2021; Selosse et al., 2017).

The most prominent example of a mixotrophic green alga is the genus *Cymbomonas.* These algae generally utilize photosynthesis, but still retain the ability to engulf bacterial cells via phagocytosis (Maruyama & Kim, 2013). In others, however, heterotrophy is the only means of energy acquisition, as previous studies have shown that multiple representatives of green algae have lost their photosynthetic ability independently; for example, within *Chlorellales*, the loss has occurred at least two times (Figueroa-Martinez et al., 2015) (Figure 2.1). For example, loss of photosynthesis in *Prototheca* and *Helicosporidium* may have occurred during the transition from mixotrophy to parasitism. Mixotrophic organisms spend five times more energy to maintain photosynthetic abilities but produce in a smaller magnitude. This means that photosynthesis has a relatively smaller yield of energy. This might be a selective

pressure related to how the organisms have lost photosynthesis (Figueroa-Martinez et al., 2015).



Source Figueroa-Martinez et al. (2015)

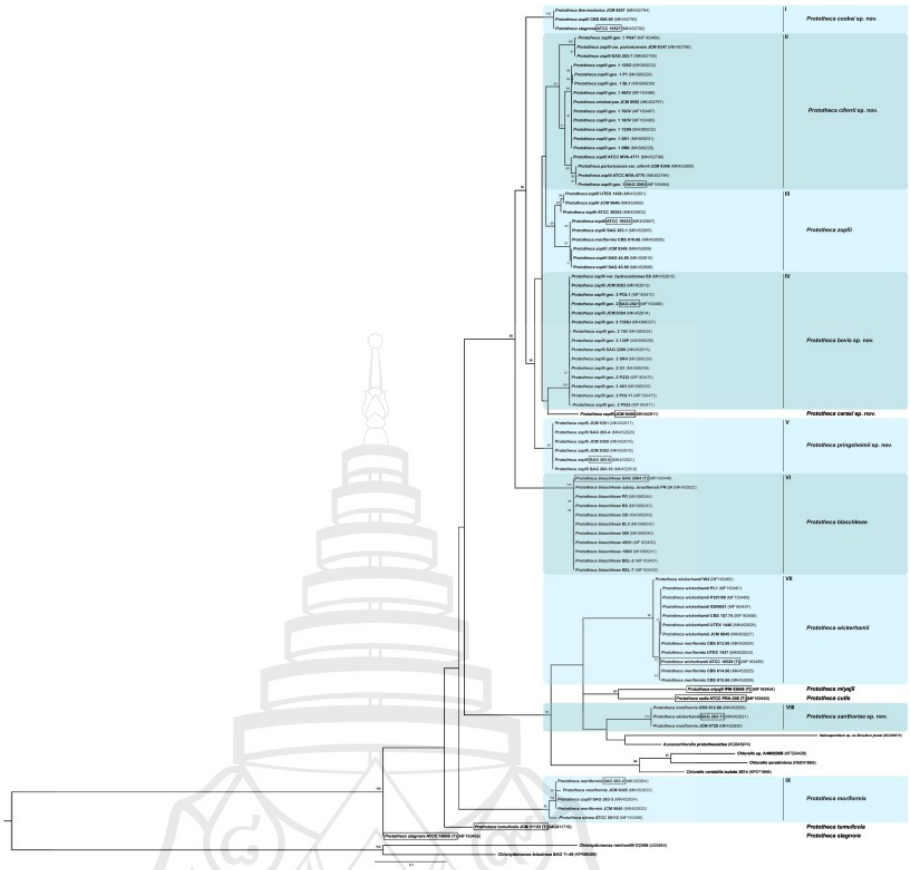
Figure 2.1 Independent losses of photosynthesis in chlorophytes and parasitism of Trebouxiophyceae, *Prototheca* and *Helicosporidium* (red color), PL abbreviated from photosynthesis loss

The relationship between the loss of photosynthesis and parasitism remains poorly understood. However, some green algae, members of *Ulvophyceae* and *Trebouxiophyceae*, have been considered as pathogens. For example, the ulvophyte *Cephaleuros* causes leaf spot or rest rust disease on commercial crops (Sunpapao et al., 2016). Another example of this is a clade in Trebouxiophyceae comprising of parasitic non-photosynthetic members; *Helicosporidium*, an obligate parasite and *Prototheca,* an opportunistic pathogen. *Helicosporidium* infects the guts of arthropods (Tartar, 2013), while its close phylogenetic relative, *Prototheca* has the ability to infect mammalian hosts (Lass-Flörl & Mayr, 2007). A common feature of these green algal pathogens is the loss of photosynthetic ability. Nonetheless, studies suggest that loss of photosynthesis occurs early in the shift to parasitism

2.2 Prototheca; A Parasitic Green Alga

Prototheca spp. are spherical unicellular (3-30 μm in diameter), achlorophyllic, heterotrophic algae (Lass-Flörl & Mayr, 2007). *Prototheca* belongs to kingdom Viridiplantae, phylum Chlorophyta, class Trebouxiophyceae, order Chlorellales, family Chlorellaceae. Prototheca species can be cultured on agar and their colonies are white to cream color, smooth, moist, yeast-like and rapidly growing. *Prototheca* used to be considered a fungus based on its yeast-like colonies. However, it was later re-classified as a green alga due to its spore production characteristics and cell wall components (Lass-Flörl & Mayr, 2007; Sykes, 2014). *Prototheca* reproduces asexually; its life cycle begins with the process of endosporulation, which results in a sporangium containing multiple sporangiospores. Sporangia release the spores into the environment and the cycle repeats (El-Ani, 1967; Rapuntean et al., 2009).

Prototheca is divided into nine phylogenetically distinct clades based on the *cytb* gene: *P. cookei, P. ciferrii, P. zopfii, P. bovis, P. pringsheimii, P. blaschkeae. P.wickerhamii, P. xanthoriae,* and *P. moriformis* (Jagielski et al., 2019).



Source Jagielski et al. (2019)

Figure 2.2 Maximum likelihood phylogenetic analysis of 86 species of *Prototheca* based on the *cytb* gene

Prototheca has been isolated from living organisms and the environment, including slime flux of tree, grass, fresh and salt water, and wastewater. In animals, the organism has been isolated from cattle, deer, dogs and cats. In cattle, *Prototheca* has been linked to mastitis (Ranjan et al., 2006). Five species of *Prototheca* are known to cause infection: *P. blaschkeae*, *P. cutis*, *P. wickerhamii*, *P. ciferrii* (previously known as *P. zopfii* genotype 1) and *P. bovis* (previously known as *P. zopfii* genotype 2) (Jagielski et al., 2019; Lass-Flörl & Mayr, 2007; Sykes, 2014).

In humans, *Prototheca* species cause protothecosis, a rare infection. The major species causing human protothecosis is *P. wickerhamii*, whereas, in animals it is *P.*

bovis (Sykes, 2014). This infection is caused by direct contact with potential contaminated sources such as sewage. Protothecosis develops in immunosuppressed patients and those with underlying diseases (Lass-Flörl & Mayr, 2007). *Prototheca bovis* may also be transferred to humans through contaminated milk. Previous studies have suggested that *P. bovis* has the capability to invade and survive within host epithelial cells in cell line cultures and induce the innate immune response of host cells, resulting in inflammation and apoptosis (Leonel Gonçalves et al., 2015; Shahid et al., 2017). However, the mechanism of how *Prototheca* invades and survives in the host cell remains unclear. In Thailand, it is very interesting to note that *Prototheca bovis* has been found in feces samples from asymptomatic individuals (Jinatham et al., 2021). Therefore, further studies should be focused on the pathogenesis of *Prototheca* species, particularly its membrane trafficking system. A biological system that is commonly adapted in many parasitic lineages is MTS, which is also one of the critical pathogenic mechanisms of parasites utilized to respond to host immune responses.

2.3 Parasitism

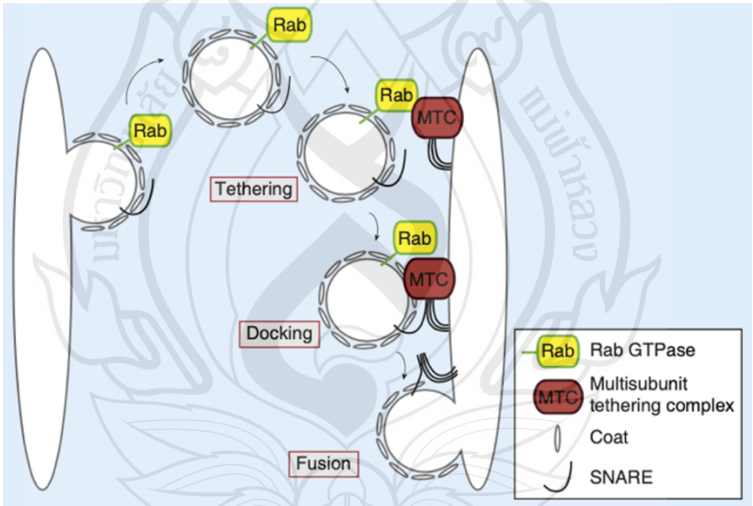
Parasitism is the association between two organisms, whereby the parasite consumes nutrients from its host. The word ‘parasite’ comes from the Greek; *parasitos*, which means dinner guest; however, parasites are the organisms that benefit from the host, while negatively impacting them (Lafferty, 2008). There are many independent transitions to parasitism across eukaryotic lineages, and many biological characteristics that it evolves from the non-pathogenic one (Poulin & Randhawa, 2015). Parasites often display loss or reduction of complex morphological structures and organelles. For example, the mitochondria of parasites from various eukaryotic lineages that have adapted to anaerobic environments have been reduced (Poulin & Randhawa, 2015). Some of these changes reflect adaptations towards an anaerobic lifestyle, multi-host life cycle, asexual multiplication in the intermediate host, and facultative truncation of the life cycle (Rohde, 2013). Many parasites also increase their reproductive capacity, thus producing a greater number of offspring than free-living forms (Figueroa-Martinez et al., 2015).

At the genome level, parasites display gains and losses. Some parasites have increased cellular complexity for functional gain. For example, many parasites have duplications of genes related to evading the host immune response or invading the host (Sun et al., 2010). Nonetheless, nuclear and organellar genome reductions are widespread in various parasitic species across eukaryotes possibly due to selective constraints (Poulin & Randhawa, 2015). Typically, most parasites show loss/simplification/reduction of several metabolic pathways accompanied by loss of some function. Parasites reduce many of their biological systems and biogenesis of new organelles.

Moreover, some parasites have reduced the number of genes associated with membrane trafficking machinery, for example, *Giardia*, apicomplexans, and kinetoplastids. The gut parasite *Giardia*, causative agent of giardiasis, displays reduction of its endomembrane system, whereby only the ER and peripheral vacuoles are remaining (Faso & Hehl, 2011; Klinger et al., 2016). Apicomplexans, the causative agents of malaria and toxoplasmosis, have a unique invasion organelle, the apical complex, a divergent endo-lysosome and its compartments and reduced MTC components (Baum et al., 2008; Klinger et al., 2013). Kinetoplastids, causes of diseases in diverse host species, have modified their endocytic pathway that is adapted for evasion of host immune system, and expanded their transport genes reflecting a reorientation of membrane function (Jackson et al., 2016; Manna et al., 2014). *Entamoeba*, an intestinal parasite that lacks stacked Golgi complexes, damages host cells by secretion of virulence factors through phagocytosis. This parasite has an expanded set of paralogous genes of MTS components (Ralston, 2015; Watanabe et al., 2020). In contrast, some parasites have expanded their membrane trafficking complements, such as *Entamoeba* (Klinger et al., 2016). In general, a group of mostly parasitic protists have evolved from a free-living ancestor into a parasitic organism under different selective pressures and the influence of the environment. A large body of research has focused on relatively well-known parasites, such as Apicomplexans, amoeba or fornicates. However, not much information is available for other lineages including green algae.

2.4 Membrane Trafficking System

Cellular complexity is one of the main characteristics of eukaryotes. There are many organelles involved in membrane trafficking machinery such as the endoplasmic reticulum (ER), Golgi complex, and endosomes, and the specific proteins that mediate membrane trafficking, like, Soluble NFS attachment receptor (SNAREs), tethering complexes and small GTPase. These proteins are the coordinators for membrane fusion and process of vesicle transportation (Gurkan et al., 2013). There are many steps involved in membrane fusion including budding/scission, tethering, docking, and fusion. For the membrane fusion, small-GTPase in functional from (with GTP-loaded) activates tethering factors. Then, SNARE from both membranes will lead the two layers of donor and acceptor organelles to fuse together (Figure 2.3) (Barlow & Dacks, 2018; Bröcker et al., 2010; Cai et al., 2007)

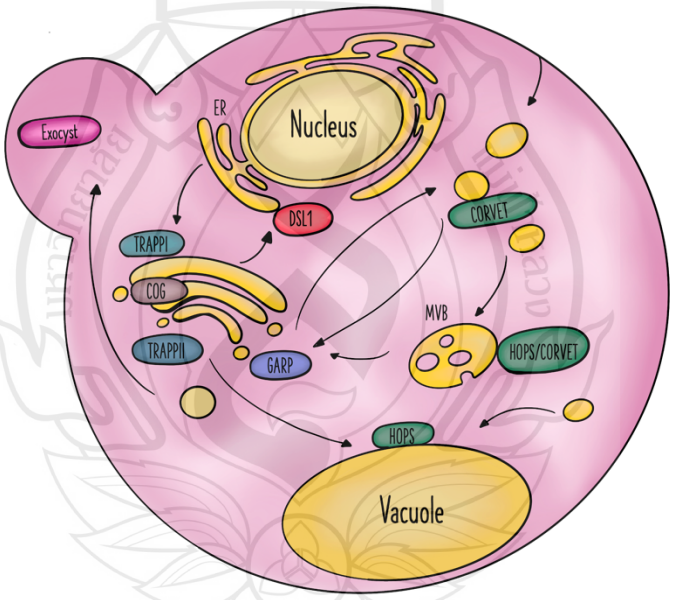


Source Bröcker et al. (2010)

Figure 2.3 Role of MTCs in membrane trafficking system

The recognition of membrane requires specific recognition factors to reach their destination. When this process is not controlled, the proteins can form non-fusogenic

complexes early in the secretory pathway. To prevent premature localized fusion and ensure correct cargo delivery, this process must be tightly controlled by other inhibitory and subsequent proteins such as tethering factors (Dubuke & Munson, 2016). Multi-subunit tethering complexes (MTCs) promote the initial interaction between a vesicle and target organelles by binding with Rab-GTPase as well as SNAREs and subsequently leads to membrane fusion (Figure 2.3). MTCs can be classified into three groups based on structural similarities including complexes associated with tethering containing helical rods (CATCHR), which consist of COG, Dsl1, GARP, and the exocyst. The second group, HOPS and CORVET are involved in endosomal transport. The last group of MTCs are the TRAPP complexes. The protein sizes of these tethering factors are between 50 and 140 kDa per unit, and the complexes form multiple subunits (Bröcker et al., 2010; Marcotte & Munson, 2016).



Note ER for (endoplasmic reticulum), MVB for (multivesicular bodies). Organelles are indicated in yellow color.

Figure 2.4 General trafficking pathway of MTCs. Overview of multi-subunit tethering complexes (MTCs) pathways across intracellular trafficking system. MTCs are indicated in different colors

Table 2.1 Properties of mutisubunit tethering complexes

Tethering complexes	Localization	Function between	Subunits
Dsl1	ER	Golgi to ER	Dsl1
			Sec20
			Tip20
COG	Golgi	endosome to Golgi via retrograde vesicular trafficking	COG1
			COG2
			COG3
			COG4
			COG5
			COG6
			COG7
			COG8
GARP	TGN	endosome to TGN	Vps51
			Vps52
			Vps53
			Vps54
Exocyst	Plasma membrane	Vesicle to plasma membrane	Sec3
			Sec5
			Sec6
			Sec8
			Sec10
			Sec15
			Exo70
			Exo84
CORVET	Endosome	TGN to early endosomes	Vps8
			Vps3
HOPS	vacuole	MVB to vacuole, and vacuole to vacuole	Vps11
			Vps16

Table 2.1 (continued)

Tethering complexes	Localization	Function between	Subunits
HOPS	vacuole	MVB to vacuole, and vacuole to vacuole	Vps16
			Vps18
			Vps33
			Vps39
			Vps41
TRAPPI	Golgi	ER to Golgi	Trs20
			Trs23
			Trs31
			Trs33
			Trs85
			Bet3
			Bet5
TRAPPII	Golgi	Endosome to Golgi	Trs65
			Trs120
			trs130

Source Bröcker et al. (2010)

2.4.1 HOPS/CORVET

The homotypic fusion and vacuolar protein sorting (HOPS) complex and class C core vacuolar/endosomal tethering (CORVET) perform as the coordinators of early and late endosome and lysosome fusion (Figure. 2.4) (Bröcker et al., 2010). CORVET acts in the early endosomal pathway, while HOPS is involved in the membrane fusion of late endosome and lysosome (Balderhaar & Ungermann, 2013). Both are hexameric complexes, share four subunits, interact with Rab-GTPase, activate and proof-read SNAREs (Solinger & Spang, 2013). Defects in CORVET and HOPS are associated with neurodegeneration and pigment disorders (van der Beek et al., 2019).

2.4.2 TRAPPs

The Transport protein particle (TRAPP) complex is also known as the trafficking protein particle. There are two forms of this complex including TRAPPI and TRAPPII. TRAPPI functions in tethering coated vesicles during ER to Golgi transportation (Bröcker et al., 2010). TRAPPII functions in tethering coated vesicle *intra*-Golgi trafficking and early endosome to late Golgi. This complex can act as multimeric guanine exchange factors (GEFs) that activate Rab-Ypt1 in yeast (Dubuke & Munson, 2016; Marcotte & Munson, 2016).

2.4.3 COG

Conserved oligomeric Golgi (COG) complex, a multi-subunit tethering complex of the CATCHR, controls the retrograde transportation of vesicle tethering at the Golgi apparatus (Figure 2.4) (Bröcker et al., 2010). The COG complex is a hetero-octameric protein that consists of eight subunits named COG1 through COG8. The complex is distributed into two functional distinct subunit-loops which are loop A (COG1 to COG4) and loop B (COG5 to COG8). COG interacts with both vesicle and target membranes (Dubuke & Munson, 2016; Marcotte & Munson, 2016; Smith & Lupashin, 2008). In humans, defect in this complex leads to neurodegenerative disease (Climer et al., 2015). This complex is the most closely related to the exocyst complex (Blackburn et al., 2019).

2.4.4 Exocyst

Exocyst is an octameric protein complex of the CATCHR family. It recognizes secretory vesicle fusion to plasma membrane (Figure 2.4). Similarle to COG, this complex comprises eight subunits (Dubuke & Munson, 2016). Exocyst regulates many biological processes, such as trafficking and fusion of Golgi-derived vesicles to the plasma membrane, cell migration, autophagy, cytokinesis, and endocytosis (Ahmed et al., 2018). Exocyst associates with small GTPase resulting in docking of the vesicle to plasma membrane and, finally, upon fusion mediated by SNARE, the biological material is released to the extracellular space (Heider et al., 2016; Marcotte & Munson, 2016). Mutation of this complex in some species results in lethality.

2.4.5 GARP

The Golgi associated retrograde protein (GARP) complex regulates retrograde transportation from endosomes to the *trans*-Golgi network (TGN) (Bröcker et al., 2010). For both early and late endosomes, GARP is another small tethering unit consisting of four core subunits, which are Vps51 to Vps54 (Bröcker et al., 2010; Dubuke & Munson, 2016; Marcotte & Munson, 2016).

2.4.6 Dsl1

The smallest MTC is DSl1, which is the simplest tethering complex of the CATCHR family. It consists of three subunits including Dsl1, tip20 and sec20, localizing at the endoplasmic reticulum (Figure. 2.4). DSl1 is involved in retrograde transportation from Golgi to ER that recognizes Golgi-derived COPI vesicles leading into the fusion machinery (Meiringer et al., 2011). DSl1 is also involved in vesicular transport and required for protein transportation between Golgi and ER (Bröcker et al., 2010). This complex has two functions: tethering COPI vesicle and increasing the efficiency of the fusion machinery through acceleration of the assembly of SNARE complexes (Meiringer et al., 2011). It is also involved in peroxisome biogenesis at the ER in yeast. The Dsl1 complex has also been shown to correlate with degenerate peroxisome organelles in various microbial eukaryotes (Jansen et al., 2021). Independent Dsl1 reduction could indicate degenerate peroxisome biology.

2.5 Peroxisomes

Peroxisomes are single-bound organelles that are present in most eukaryotic lineages (De Duve & Baudhuin, 1966; Gabaldón, 2010). They are defined by a group of conserved proteins called peroxins, which are required for peroxisome biogenesis. Peroxins are encoded by Pex genes, and are also called Pex proteins (Distel et al., 1996). All Pex proteins are encoded by the nuclear genome and translated by ribosomes in the cytoplasm (Gabaldón, 2010). In general, peroxisomes contain a variety of metabolic functions, such as fatty acid oxidation, beta-oxidation, and others. One of the major features that most peroxisomes share is that they possess oxidases, which reduce molecular oxygen to hydrogen peroxide, and catalases for hydrogen peroxide

detoxification (De Duve & Baudhuin, 1966). Other types of peroxisomes have also been described. For example, some plant peroxisomes are known as glyoxysomes (Hayashi et al., 2000), peroxisomes in trypanosomatid species are named as glycosomes, where certain glycolytic reactions take place (Michels et al., 2006), and Woronin bodies, a particular type of peroxisome in filamentous fungi, which maintain cellular integrity (Würtz et al., 2009). Peroxisomes most likely originated from the endoplasmic reticulum (ER) with highly enzymatic contents. (De Duve & Baudhuin, 1966).

Notably, peroxisomes are absent in many eukaryotes such as *Giardia* and *Trichomonas*. Peroxisomes are generally missing in anaerobic eukaryotes (Le et al., 2020). The relationship between anaerobic protists and unusual peroxisomes have been observed in many lineages across eukaryotes such as *Blastocystis*, *Entamoeba histolytica*, *Mastigamoeba balamuthiit*, and *Pelomyxa schiedti* (Gentekaki et al., 2017; Le et al., 2020; Verner et al., 2021; Záhonová et al., 2022). The transition of eukaryotes from aerobiosis into anaerobiosis is reflected by the reduction of mitochondrial metabolism. The loss of mitochondria with reduction of peroxisomes is not unexpected due to the function of these organelles being tightly coupled (Gawryluk & Stairs, 2021; Müller et al., 2012). Although the bulk of evidence points towards a correlation between anaerobiosis in protists and having both nonconventional peroxisomes and mixotrophic lifestyle, the relationship between mixotrophy and unusual peroxisomes still requires more studies.

2.6 Objectives

The aims of this study are to investigate the evolutionary dynamics (gains or losses) of the group of protein complexes that involves in the beginning process of membrane fusion called the multi-subunit tethering complexes across green algae diversity and study the evolutionary history of MTCs in the total of 1000 proteins across 25 selected species of Chlorophyta, Streptophyta, and Prasinodermophyta.

CHAPTER 3

METHODOLOGY

In this study, the methodology was divided into three parts. In the first part, data management, the data was prepared for the next step of the analysis. In the second part, homology searches were performed for identification of orthologous and paralogous genes. Lastly, phylogenetic analysis was performed to classify unambiguous protein complexes and identification of paralogous genes

3.1 Data Collection

In this study, there are three types of data including genomic sequences, transcriptomic assemblies, and protein sequences. The available predicted proteins, transcriptomic assemblies, and genomic sequence data available on public databases were downloaded for database construction and carried out using a Linux based server. Transcriptome data of *Prototheca zopfii*, *Prototheca wickerhamii,* and *Polytomella parva* were translated into predicted proteins by using GeneMarkS-T (Tang et al., 2015) for the next step. Then, the predicted proteins were then used for database construction. Whole genome sequences of the species lacking predicted proteins and transcriptomic assemblies were downloaded and used to construct a database. The annotated protein sequences of multi-subunit tethering complexes (MTCs) of *Homo sapiens*, *Saccharomyces cerevisiae*, and *Arabidopsis thaliana* were downloaded to use as queries for homology identification. Twenty-five species were utilized to analyze the results (Figure 3.1) including four Streptophyta, one Prasinodermophyta, and 20 Chlorophyta (Trebouxiophyceae, Chlorophyceae, Ulvophyceae, Mamiellophyceae, and Pylamimonadales) in order to compare the evolutionary dynamic (gains or losses) of MTC components within the group. In the case of five different species were used

including parasitic Prototheca; *P. zopfii*, *P. wickerhamii*, *P. cutis*, *P. ciferrii* and a non-parasitic *P. stagnorum* were added to investigate the evolutionary transition into an opportunistic pathogen. *Helicosporidium* was added to study the evolutionary transition into an obligate parasite. In addition, *Polytoma parva*, a free-living green alga lacking photosynthetic activity was used to observe the differences among non-photosynthetic alga and photosynthetic algae as well. The data of *Chlamydomonas reinhardtii*, *Dunaliella salina*, *Chromochloris zofingiensis*, *Micromonas pusilla*, *Coccomyxa subelipsoidea*, and *Botrycoccus braunii* are available at the US Department of Energy Joint Genome Institute available on https://phytozome-next.jgi.doe.gov (Goodstein et al., 2012). *Prasinoderma coloniale*, was obtained from the China National GeneBank website (https://ftp.cngb.org).

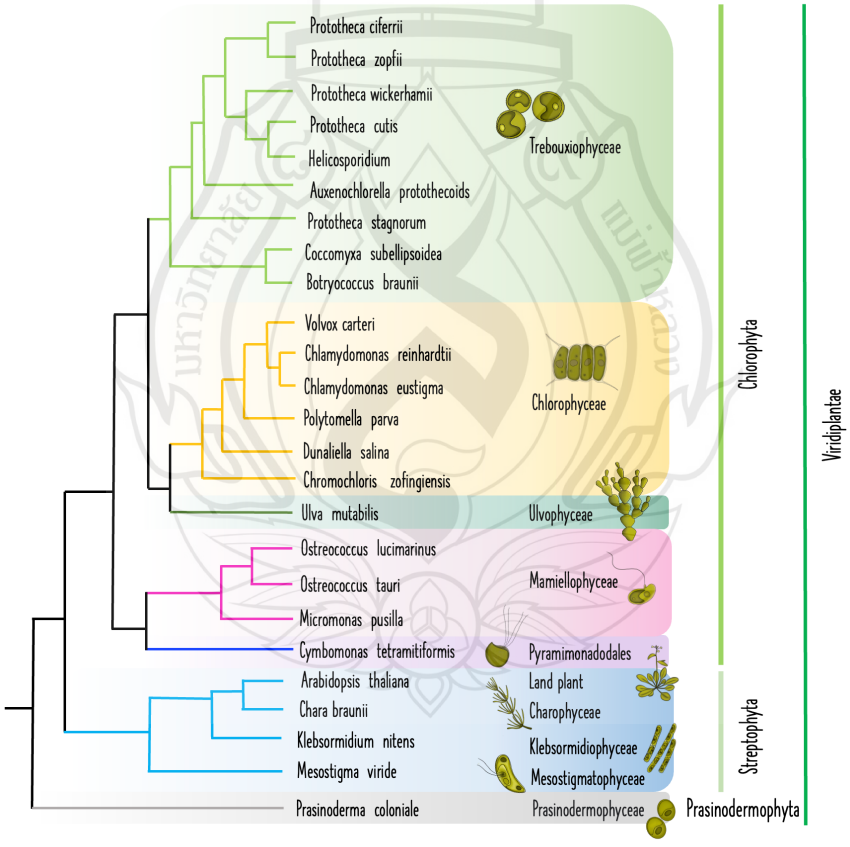


Figure 3.1 Taxon selection of 25 species including four species of Streptophyta, one species of Prasinodermophyta, and 20 species of Chlorophyta

3.2 Homology Searching

Homology searching was carried out using the BLAST algorithm (Altschul et al., 1990), the identified MTCs sequences of *Arabidopsis thaliana*, *Saccharomyces cerevisiae*, and *Homo sapiens* were used as a queries in preliminary searches. In the case of protein databases, protein-protein blast version 22.2.29+ was used for homology searching.

In the case of divergent sequences, homology searching was performed with the high sensitivity method HMMER version 3.1.1. Specifically, a multiple sequence alignment (MSA) of human, yeast, thale cress and other identified homologous proteins from the previous step were aligned with MUSCLE version 3.8.31. HMM profiles were constructed with HMMER version 3.1.1 from the MSA alignments (Johnson et al., 2010). All the hits from the preliminary search step that had e-values less than or equal to 0.05 were used as query sequences to search against the *Arabidopsis* predicted proteins database. The positive hits of *Arabidopsis* MTCs were then used as queries to search against the *Homo sapiens* predicted proteins database.

If the homologous genes could not be identified or proteins and transcriptomic assemblies were not available, the whole genome sequences were searched by using Protein Query-translated Subject Blast v. 2.2.29+ (tBLASTn). The MTC sequences of *Arabidopsis thaliana*, *Saccharomyces cerevisiae*, *Homo sapiens*, and close relative sequences were used as queries. The protein domains of positive sequences were confirmed by using Interproscan (https://www.ebi.ac.uk/interpro/search/) (Mitchell et al., 2019) and pfam (https://pfam.xfam.org) (Mistry et al., 2021).

In the case of peroxisome biogenesis machinery, a list of sequences was used to build a custom database (Supplementary table). BLASTp and tBLASTn were performed against the custom database. The positive hits with e-value less than or equal to 0.05 were used as queries to search against the *Arabidopsis thaliana* predicted proteins database. In the case of *Arabidopsis thaliana* PEX proteins, were search into homo sapiens predicted proteins. The domain of the PEX proteins was confirmed by interproscan.

3.3 Phylogenetic Analysis

In the case of some protein complexes, to classify the unambiguous deeply paralogous protein complexes from each other, phylogenetic analysis was carried out. The homology search results of the same complexes were aligned and trimmed by using MUSCLE v.3.8.115 and trimAL (version 1.4), respectively (Capella-Gutiérrez et al., 2009; Edgar, 2004). Maximum likelihood analysis with non-parametric bootstrap 1000 replicates was conducted using IQ-TREE V.2 (Minh et al., 2020). Mr. Bayes v3.2.7a was used to observe the best tree topology and associated posterior probability values with one million generations (Ronquist et al., 2012), with 25% of burnin value. Trees were visualized and manually curated using Fig Tree v.1.4.4. All the homology search results were reported as Coulson plots in order to investigate the evolutionary patterns of MTCs complexes and PEX proteins. Pie charts display comparative genomic data, each pie is used to describe a complex of each taxon, and it is divided into sectors of protein in a complex. Filled pie sectors indicate identified homologue, while unfilled sections indicate that a homologue was not identified.

CHAPTER 4

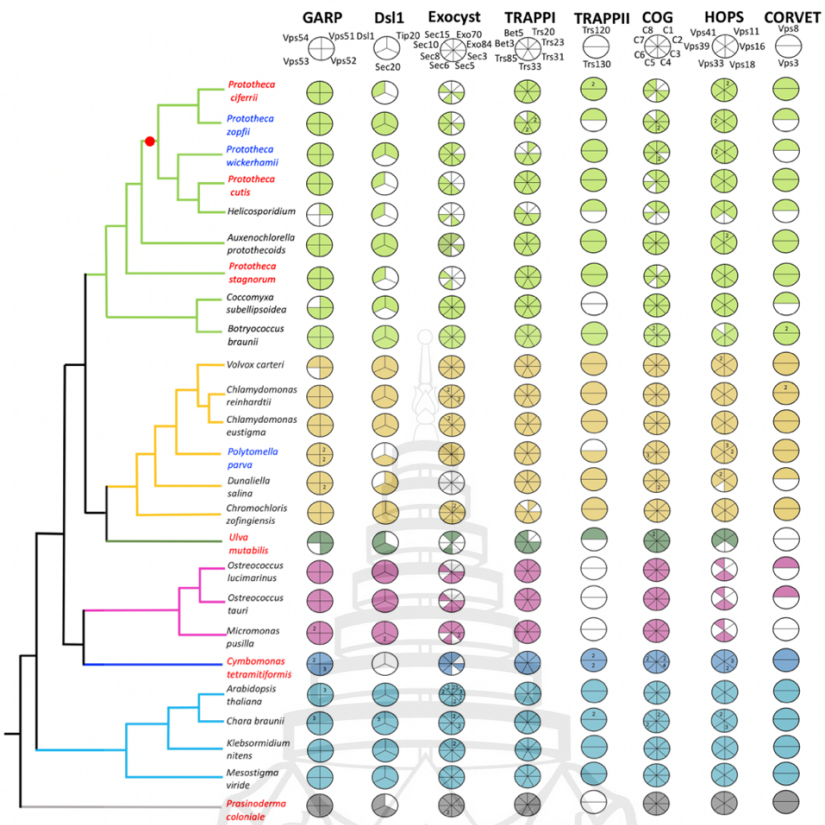
RESEARCH RESULTS

4.1 Trends of MTCs Dynamic Across Chlorophyta

In order to understand the diversity and the extent of conservation of multi-subunit tethering complexes across Chlorophyta, we sampled a taxonomically broad range of green algal genomes and performed a comparative analysis including 25 species covering Chlorophyta, Streptophyta, and Prasinodermophyta. As MTC composition is generally conserved within eukaryotes, we expected to find a similar complement of these proteins in the investigated organisms, and indeed we identified a near complete set of tethering factors across the studied lineages, with few cases of gene duplication (Figure 4.1). Comparative genomics revealed all eight systems to possess a general pattern of conservation – GARP, Dsl1, exocyst, TRAPPI, TRAPPII, COG, HOPS, and CORVET were present in 94.6%, 76%, 74.5%, 93.7%, 72%, 93.5%, 92%, and 78% of the datasets, respectively. We have identified all of the MTC components in *Chlamydomonas reinhardtii* (in line with previous studies; (Woo et al., 2015)), as well as *Arabidopsis thaliana* (Vukašinović & Žárský, 2016) as a positive control of our analysis.

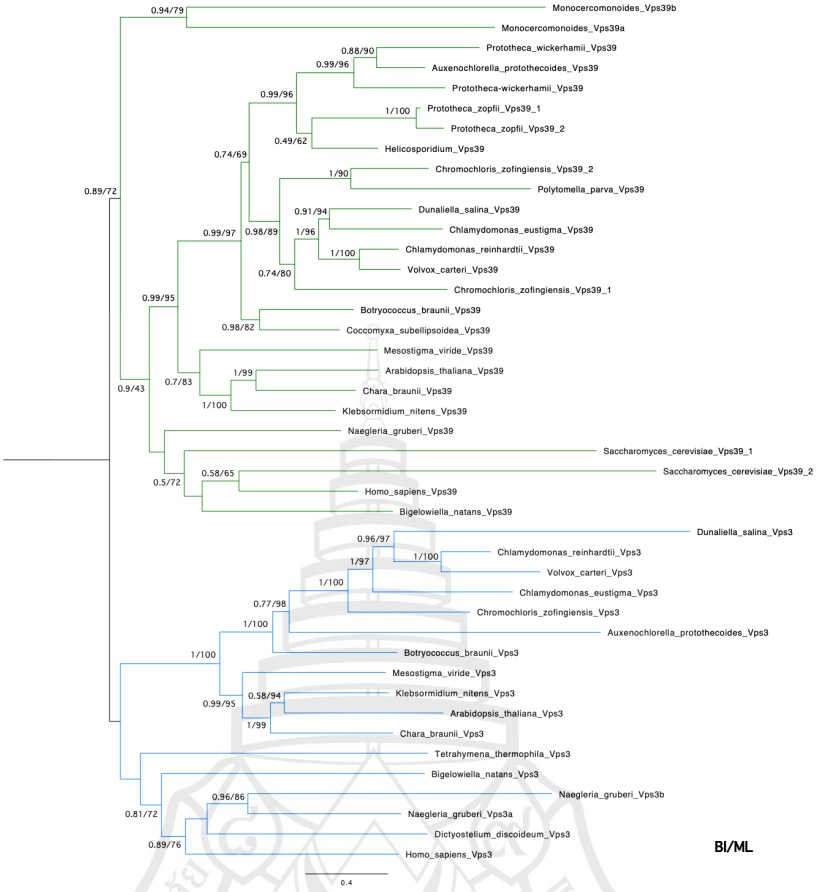
However, we identified certain notable cases of subunit absence: for instance, we recovered less than half (45%) of the investigated components of MTCs in the *Helicosporidium* genome, an obligate parasitic green alga. In comparison, its sister taxon, *P. cutis*, retained 80% of MTC proteins. Notably, we identified none of the Dsl1 complex components in *Cymbomonas tetramitiformis*. This complex correlates with peroxisome biogenesis. For *Prototheca* spp., an opportunistic parasitic green alga, there was only a slight reduction of the MTC complement, when compared with their sister

taxon, the obligate parasite *Helicosporidium*. In the Mamiellophyceae, the picoplanktonic green algae, many MTC subunits were lost, including parts of exocyst, HOPS, CORVET, and all TRAPPII complexes. Moreover, phylogenetic analysis demonstrated that Vps3/39 and Vps8/41 are robustly separated into two groups of sequences around the central nodes (Figure 4.2 and 4.3)



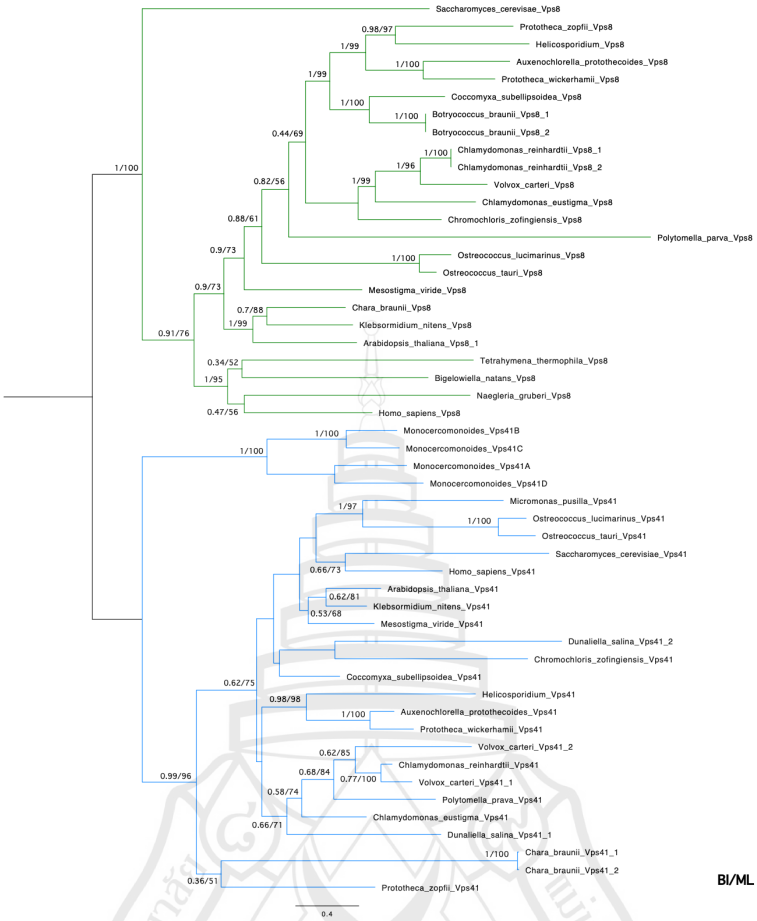
Note The analysis illustrates the distribution of MTC complexes in representatives of the main green algal lineages. Coulson plot shows the presence of the investigated proteins in filled circles, along with the numbers of paralogues, while unfilled indicate that no homologue was identified. Colors on the plot were used to visually distinguish lineages. Trebouxiophyceae is labeled in green color, Chlorophyceae in yellow, Ulvophyceae in dark green, Mamiellophyceae in pink, Pyramimonadales in dark blue, Streptophyta in blue, and Prasinodermophyta in grey. The phylogenetic relationships of the taxa in question are shown on the left side of the figure. Taxa with predicted proteomes derived from genomic data are shown in black text, those with genomic data (but no predicted proteins) in red text, while those derived from transcriptomic data are in blue text. Red dot indicates the parasitic clade.

Figure 4.1 Comparative genomic survey of MTC components across the diversity of green algae



Note This figure reveals the separation of Vps3 and Vps39 proteins from green algal diversity. Branch colors are for visual proposes

Figure 4.2 Phylogenetic analysis separating Vps3 and Vps39 which are paralogous



Note This figure reveals the separation of Vps8 and Vps41 proteins from green algal diversity. Branch colors are for visual proposes

Figure 4.3 Phylogenetic analysis separating Vps8 and Vps41 which are paralogous

4.2 Mamiellophyceae Have Distinctly Reduced MTCs

The Mamiellophyceae is one of the largest lineages of prasinophytes, which is considered the earliest-divergent group within the Chlorophyta. This group is further divided into three orders: Mamiellales, Dolichomastigales and Monomastigales (Not et al., 2012), the first of which includes common tiny green algae, e.g., *Micromonas*, *Ostreococcus*, and *Bathycoccus* (Grimsley et al., 2012). We found that TRAPPII complexes are completely lost in *Micromonas pusilla*, *Ostreococcus tauri*, and *Ostreococcus lucimarinus*, while HOPS, CORVET, and exocyst have been partially

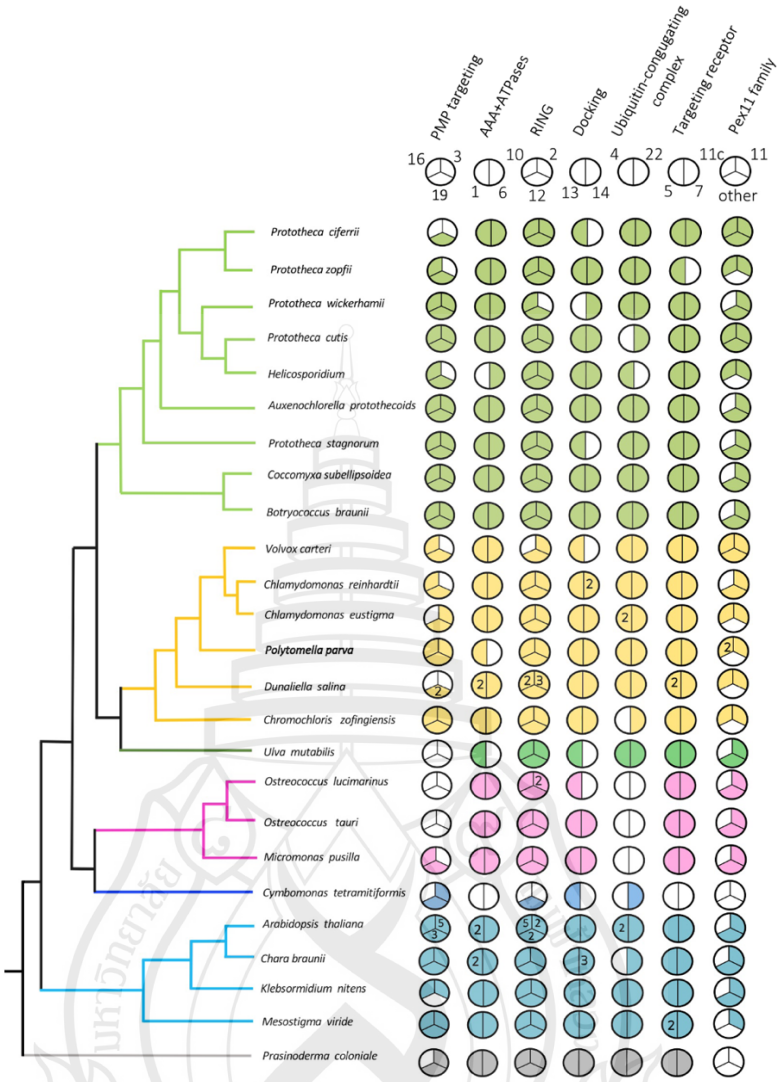
reduced in this group, with the last one having the most peculiar distribution. Exocyst is an octameric protein complex of the CATCHR family, whose role is the recognition of a secretory vesicle directed to the plasma membrane. Similar to COG, this complex comprises eight subunits: Exo70, Exo84, Sec3, Sec5, Sec6, Sec8, Sec10 and Sec15. Exocyst regulates many biological processes such as trafficking and fusion of Golgi-derived vesicles to the plasma membrane, as well as cell migration, autophagy, cytokinesis, and endocytosis (Ahmed et al., 2018). Exocyst binds with the small GTPase, resulting in docking of the vesicle to the plasma membrane, which then leads to SNARE-mediated vesicle fusion, during which the biological material is released into the extracellular space (Mei & Guo, 2018). Notably, we found that *Dunaliella salina* lost all eight exocyst subunits, while *Prototheca* spp. and *Helicosporidium* lost the Exo84 protein, which indicates partial reduction of their exocyst complexes.

4.3 Peroxisome Biogenesis Machinery Across Green Algae

Prototheca species were also observed to possess reduced Dsl1 complex – the simplest and smallest tethering complex of the CATCHR family. This system, consisting of only three subunits (Dsl1, Tip20 and Sec20), localizes at the endoplasmic reticulum membrane, where its function is tethering and assisting in membrane fusion of incoming Golgi-derived vesicles (Meiringer et al., 2011, p. 1). Moreover, it is involved in peroxisome biogenesis at the ER, which has been deduced from the observation that the lack of peroxisomes correlates with absent or reduced Dsl1 (Grimsley et al., 2012; Not et al., 2012).

Furthermore, we found all of the Dsl1 components to be absent in *Cymbomonas tetramitiformis*. This finding is especially puzzling, considering that our comparative genomic analysis of MTCs illustrates that there is no known significant degeneration of peroxisome biogenesis machinery in any other investigated green algae. We therefore hypothesized that the *Prototheca* species and *C. tetramitiformis* underwent unique, independent Dsl1 reduction that most likely impairs their peroxisome biogenesis. To verify this assumption, we performed a comparative genomic analysis of peroxisome biogenesis-related proteins (PEX family) across the chlorophyte

diversity. The Coulson plot of Pex proteins in green algae illustrates that although the peroxisome biogenesis machinery remains largely intact in all chlorophytes including *Prototheca* spp., it has been drastically reduced in *Cymbomonas tetramitiformis,* which we identified to possess only few Pex proteins in their genome (Figure 4.4).



Note The analysis shows the distribution of PEX proteins across green lineages nearly complete in all complexes. Coulson plot identified the presence and absence of those proteins in filled circles with the numbers of paralogues. No association between the color and the results. The phylogenetic relationship of each taxon is shown to the side of the figure.

Figure 4.4 Comparative genomic survey of peroxisome biogenesis machinery across the diversity of green algae

Finally, our results demonstrated that the Pex proteins appear to be well-conserved across green algae. Their components were present and functional across

most of green algal diversity, except *Cymbomonas tetramitiformis,* a phago-mixotrophic marine alga, which lost all of its Dsl1 components and the majority of its peroxisome biogenesis machinery. The relationship between anaerobic protists and unusual peroxisomes has been observed in many lineages across eukaryotes such as *Blastocystis*, *Entamoeba histolytica*, *Mastigamoeba balamuthii*, and *Pelomyxa schiedti*. It is normal that peroxisomes are missing in some anaerobic eukaryotes. The transition of eukaryotes into anaerobiosis is reflected by the reduction of mitochondrial metabolism. The loss of mitochondria with reduction of peroxisomes is not unexpected due to the function of these organelles are tightly coupled. Although the bulk of evidence points towards a correlation between anaerobiosis in protists and having both nonconventional peroxisomes and mixotrophic lifestyle, the relationship between mixotrophy and unusual peroxisomes still requires more studies. It would be very interesting to study the co-evolution between peroxisomes and Dsl1 complexes in order to understand the evolutionary relationship between mixotrophic lifestyles across eukaryotes.

CHAPTER 5

DISCUSSION AND CONCLUSION

5.1 DISCUSSION

In this study, we observed the evolutionary dynamics (gains or losses) of the multi-subunit tethering complexes (MTCs), protein complexes involved in the beginning process of membrane fusion, across 25 species of green algae including Chlorophyta, Streptophyta, and Prasinodermophyta. Particularly, MTCs have been studied in many lineages across eukaryotic diversity. For instance, apicomplexans have drastically reduced MTC components, with Dsl1 and exocyst complexes mostly absent (Klinger et al., 2013). Moreover, the complete set of Dsl1 complex is missing across ciliate diversity, as well as COG, exocyst, and TRAPPII were subsequently reduced across ciliate genomes (Richardson & Dacks, 2022). In contrast, all eight COG subunits were present in *Chromera* and *Vitrella*, basal group of Apicomplexans, as well as the entire complexes of HOPS and CORVET, however, no exocyst complements were identified (Woo et al., n.d.).

A limitation of this study is that not all representatives of free-living and parasitic green algal lineages were included in the analysis due to unavailability of the data in order to understand the evolutionary path among green algal diversity. In our analyses we employed extra caution to eliminate falsely positive results by performing forward and reciprocal searches with E-value of 0.05 and confirming the hits with protein domain identification. When the candidate proteins could not identified, tBLASTn was performed by using the identified protein sequence of close relative species to search into genomic data The majority of the investigated protein complexes were not divergent and most subunits were detected by initial BLAST searches. However, the possibility that some subunits could not be identified due to low levels of

transcriptome expression cannot be excluded.

Mamiellophyceae, more specifically reduction of all TRAPPII with incomplete set of exocyst, and HOPS/CORVET. So, these proteins share similar functions in the endomembrane system that involved in endocytic/late secretory system. (Bröcker et al., 2010; Cai et al., 2007; Dubuke & Munson, 2016). The size of mamiellophyte genomes is dramatically reduced possibly due togenomic streamlining (Derelle et al., 2006; Palenik et al., 2007; Worden et al., 2009). Thus, it is very likely that these complexes have undergone simultaneous reduction. Since these algae have the ability to live in oligotrophic environments, they might have selected to maintain the autotrophic lifestyle instead of having a complex endomembrane system (Cardol et al., 2008; Derelle et al., 2006; Farrant et al., 2016; Kulk et al., 2013). Moreover, *Helicosporidium*, the unique obligatorily parasitic green alga, underwent even more pronounced MTC reduction, as only 45% of its components were recovered when compare with its sister taxa, *Prototheca cutis*, which retains 80% of the MTC components.

It is worth noting that there have been over a hundred independent transitions to parasitism across eukaryotes, all of which likely affected many physiological characteristics of the organisms involved. Parasites often display loss or reduction of morphologically and functionally complex structures and organelles. For example, the mitochondria of parasites from various eukaryotic lineages that have adapted to anaerobic environments have been reduced (Poulin & Randhawa, 2015). Some of these changes reflect adaptations towards an anaerobic lifestyle, multi-host life cycle, asexual reproduction in the intermediate host, and facultative streamlining of the life cycle in general (*Encyclopedia of Biodiversity - 2nd Edition*, n.d.). Many parasites also increased their reproductive capacity, thus producing greater numbers of offspring than their free-living relatives (Figueroa-Martinez et al., 2015).

Nonetheless, some parasites, such as *Giardia*, apicomplexans and kinetoplastid, have reduced their complement of genes associated with the membrane trafficking machinery. The gut parasite *Giardia*, causative agent of giardiasis, displays reduction of its endomembrane system, of which only the ER and peripheral vacuoles remain (Faso & Hehl, 2011). Apicomplexans, a group of specialized parasites causing, among others, malaria and toxoplasmosis, possess a unique invasion organelle called the apical complex, while diverging from their relatives by having lost endo-lysosomal

compartments (Baum et al., 2008) and certain MTC components (Klinger et al., 2013). Kinetoplastids, which cause a variety of diseases in diverse host species (e.g. African sleeping sickness in humans), have modified their endocytic pathways in order to evade the host immune system, and their expanded transport genes reflect reassignment of membrane functions (Jackson et al., 2016). Additionally, *Entamoeba*, an intestinal parasite that lacks stacked Golgi body, can damage host cells by secretion of virulent factors using novel systems, which evolved by paralogous expansion of MTC complexes involved in phagocytosis (Ralston, 2015; Watanabe et al., 2020).

A variety of parasitic protists have evolved from free-living ancestors into specialized parasites by responding in diverse ways to different selective pressure and environmental conditions. Still, the bulk of research on these organisms has focused on large groups of diverse and widespread parasites, such as the Apicomplexa, amoebae or fornicates. On the other hand, the available information on evolution of parasitism in other lineages, especially ones where parasites are rare, such as green algae, remains very scarce. *Prototheca*, an opportunistically parasitic green alga, showed no significant distinction from the free-living chlorophytes in its evolutionary dynamics of MTC proteins due to the transition into a opportunistic parasitic organism.

We did not find any divergence in the MTC conservation pattern that would correlate with the loss of photosynthesis in *Prototheca* and *Helicosporidium.* This permanent shift in trophic mode has most likely occurred during the transition from mixotrophy to parasitism, as the maintenance of dormant photosynthetic capabilities vastly increases the costs of energy production, compared to the alternative of obtaining it through heterotrophy (Figueroa-Martinez et al., 2015). These costs might be a powerful enough selective pressure factor to promote the eventual loss of photosynthesis in mixotrophs, which, if applicable, most likely occurs early during the transition to parasitic organisms. Although we observed that both *Prototheca* species and *Helicosporidium* possess a reduced exocyst complex, with patchy distribution of all eight of its subunits, the entire system was also lost in the photosynthetic chlorophyte *D. salina*.

This finding is particularly surprising, considering the previously mentioned hypothesis that the loss of exocyst paved the way for the acquisition of the apical complex in the apicomplexans. However, complete exocyst losses have also been

reported in other, predominantly free-living eukaryotic lineages as well, including ciliates and haptophytes. It is noteworthy that ciliates are the protists with most unusual endocytosis and exocytosis pathways, and they display yet another convergence with *Prototheca,* which is the reduction of the Dsl1 complex across all of their diversity (Richardson & Dacks, 2022). As the absence of Dsl1 components has been correlated with peroxisome biogenesis machinery, resulting in degenerated or unusual peroxisomes (Gentekaki et al., 2017; Karnkowska et al., 2019; Klinger et al., 2013), it might be worthwhile to study the peroxisomes of *Prototheca* in further detail.

Another notable finding was the complete loss of exocyst components in *Dunaliella salina*, a halophile green unicellular micro-alga found in hypersaline environments. The effect that loss of exocyst might produce in this alga is unclear. Complete exocyst losses have also been reported in other protistan lineages, including the Apicomplexa and the predominantly free-living ciliates. *Dunaliella salina* lives in relatively extremophilic environments and so perhaps this is an adaptation to this ecological niche (Oren, 2005). The loss of exocyst might be related to adaptation of plasma membrane to survice in extreme environments.

Finally, our results demonstrated that the PEX proteins are well-conserved across green algae. Their components were present and functional across most of green algal diversity, except for *Cymbomonas tetramitiformis,* a phago-mixotrophic marine alga (Gagat & Mackiewicz, 2017), which lost all of its Dsl1 components and the majority of its peroxisome biogenesis machinery. The relationship between anaerobic protists and unusual peroxisomes has been observed in many lineages across eukaryotes, such as *Blastocystis*, *Entamoeba histolytica*, *Mastigamoeba balamuthiit*, and *Pelomyxa schiedti* (Gentekaki et al., 2017; Le et al., 2020; Verner et al., 2021; Záhonová et al., 2022). It is generally accepted that peroxisomes are missing in many anaerobic eukaryotic lineages. The transition of eukaryotes into anaerobiosis is reflected by the reduction of mitochondrial metabolism. The loss of mitochondria with reduction of peroxisomes is not unexpected due to the function of these organelles being tightly coupled (Gawryluk & Stairs, 2021; Le et al., 2020; Müller et al., 2012). Although the bulk of evidence points towards the correlation between anaerobiosis in protists and having both nonconventional peroxisomes and mixotrophic lifestyle, the relationship between mixotrophy and unusual peroxisomes still requires more studies.

It would be very interesting to study the co-evolution between peroxisomes and Dsl1 complexes in order to understand the evolutionary relationship between mixotrophic lifestyles across eukaryotes.

5.2 Conclusion

To determine the evolutionary dynamics (gains or losses) of the MTCs across green algae, we performed a comparative genomic analysis to investigate the presence and absence of various MTCs with their respective organisms' physiological traits and phylogenetic position. Our results showed MTC conservation in Chlorophyta, with very few lineage-specific losses that might be related with their other distinguishing traits, e.g., the drastic reduction of peroxisome biogenesis machinery in a mixotrophic species *Cymbomonas tetramitiformis*. Finally, the sequencing and annotation of genomes from underinvestigated taxa in the studied clade will be of great importance for further works aiming to broaden our understanding of the evolution of membrane trafficking in green algae.

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