# Lipids of *Pedinomonas minor*, *Pedinomonas* sp., *Pyramimonas parkeae, Pyramimonas obovata*: A Quest to Reconcile These Tertiary and Secondary Plastid Ancestors of the Dinoflagellate *Lepidodinium chlorophorum* and Euglenid *Euglena gracilis*.

by

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science in Biology

Middle Tennessee State University

August 2021

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## ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Jeffrey D. Leblond for his expert advice, encouragement, and support throughout all stages of this thesis; and my invaluable committee members Dr. Mary B. Farone and Dr. Rebecca Seipelt-Thiemann for their guidance, and contributions that helped improve the quality of this work. My deepest appreciation goes to Middle Tennessee State University Department of Biology; I would not have been able to accomplish my dream of going to graduate school if not for the graduate assistantship awarded to me. I would also like to thank Dr. Cynthia Chappell, for her generous summer stipend support that ensured I had a roof over my head, and food in my belly during the summer without an assistantship. Finally, I am thankful, and grateful to my family and friends for their continuous support and prayers.

## ABSTRACT

The goal of this study was to investigate the plastid origins of the only green-pigmented dinoflagellate genus, represented by *Lepidodinium chlorophorum*, and the evolutionary model euglenid *Euglena gracilis*. Intact forms of mono- and digalactosyldiacylglycerol (MGDG and DGDG, respectively) present in the thylakoid membrane of the green algae *Pedinomonas minor* and *Pedinomonas* sp., putative plastid ancestors of *L. chlorophorum*, and *Pyramimonas parkeae* and *Pyramimonas obovata*, putative plastid ancestors of *E. gracilis*, were elucidated via positive-ion mode electrospray ionization/mass spectrometry (ESI/MS) and ESI/MS/MS. I hypothesized that *P. minor* and *Pedinomonas* sp. would bear forms of MGDG and DGDG similar to forms of MGDG and DGDG present in *L. chlorophorum*, and *P. parkeae* and *P. obovata* would share homology with forms present in *E. gracilis*. All four species produced abundant forms of $C_{18}/C_{16}$ (*sn*-1/*sn*-2 regiochemistry) MGDG and scarce forms of $C_{18}/C_{16}$ DGDG. Specifically, *Pedinomonas* sp. and *P. minor* possessed 18:3/16:4 MGDG as the dominant galactolipid, which relates to a prior observation that *L. chlorophorum* produces the 16:4 fatty acid at the *sn*-2 position of both MGDG and DGDG. *P. parkeae* and *P. obovata* also possessed 18:3/16:4 MGDG and DGDG, but unlike the *Pedinomonas* pair, the *Pyramimonas* and Euglena pair was almost an exact match to each other. I also examined sterols of *Pedinomonas* and *Pyramimonas*. *Pedinomonas* did not produce any sterol similar to *L. chlorophorum* but *Pyramimonas* produced poriferasterol, a sterol found in *E. gracilis.*

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# CHAPTER I: INTRODUCTION

Plastids are membrane-bound photosynthetic organelles found in plants and algae. The origin of the plastid is considered to have evolved from a primary endosymbiotic event involving cyanobacteria (internalized by a primitive eukaryotic host). Without involving the quintessential process of phagocytosis by digesting cyanobacteria in the food vacuole, the host cell retained the free-living cyanobacterium leading to photosynthetic ability by the host cell (Keeling, 2010).

Dinoflagellates are unicellular organisms found in both marine, and freshwater habitats. Some species are photosynthetic autotrophs while others can be parasitic and/or heterotrophic (Dagenais-Bellefeuille & Morse, 2013). Most photosynthetic dinoflagellates possess secondary plastids (Kamikawa et al., 2015). A secondary endosymbiosis event implies that an organism formed because of primary endosymbiosis was further engulfed by another host, hence the plastids acquired from secondary endosymbiosis are referred to as secondary plastids (Cavalier-Smith, 2003). Most photosynthetic dinoflagellates possess plastids that contain the photosynthetic pigment peridinin (Dorrell & Howe, 2015), a light-harvesting apocarotenoid associated with chlorophyll indicating that the plastid originated from a red alga. A small fraction gained plastids that do not contain peridinin, but rather contain pigments associated with other green algal groups, haptophytes (a different clade of algae), and diatoms (Ishida & Green,

2002; Minge et al., 2010). *Lepidodinium chlorophorum* is a member of the only dinoflagellate genus that harbors a green tertiary plastid (Shalchian-Tabrizi et al., 2010).

Organisms called euglenoids are single-celled flagellates that are mostly photosynthetic and autotrophic, but they can be heterotrophic (Yamaguchi et al., 2012). They are both free-living and/or parasitic (Adl et al., 2012). This study however focuses on *Euglena gracilis*, a photosynthetic flagellate (Yamaguchi et al., 2012) which can be used to produce biofuels, a non-polluting energy source which is also renewable (Grimm et al., 2015). As seen in the case of *L. chlorophorum*, *E. gracilis* harbors a secondary green plastid derived from a green alga (Jackson et al., 2018). Some efforts to unravel the origin of these elusive plastids found in *L. chlorophorum* and *E. gracilis* have been made (Shalchian-Tabrizi et al., 2006; Leblond & Lasiter, 2009; Kamikawa et al., 2015; Jackson et al., 2018), but much more remains to be learned. Background literature to provide further insight on plastid complexity is described below.

*Plastid Complexity*

Plants possess chloroplasts surrounded by two membranes (Heldt & Sauer, 1971). The presence of these membranes represents the membranes of cyanobacteria implying that at one time, symbiogenesis occured between a cyanobacterium and a host, with the cyanobacterium retaining its photosynthetic ability, regardless of the transfer of genes between the endosymbiont and the host nucleus (Shi et al., 2016). In direct contrast to this, dinoflagellates have tertiary plastids that are enveloped by four membranes (Kamikawa et al., 2015), implying that the eukaryotic host that underwent endosymbiosis

was further engulfed by a dinoflagellate. A distinguishing feature of secondary and tertiary plastids derived from green algae is that the outer bilipid-membrane layer appears to be slightly separated from the inner bilipid-membrane layer indicating that there is a boundary between the green algal host and its symbiont (Walker & Kořený, 2017). Also, the presence of four plastid membranes housed in green dinoflagellates brings into question as to how the symbiont-host relationship was established. Plastid-targeted proteins serve as a pathway to study the transfer of genes between the endosymbiont and host nucleus. Sequencing of plastid-targeted genes existing in dinoflagellate plastid of green-algal origin strikingly revealed that not all these genes were of green-algal origin. These genes shared homology with genes from streptophytes (land plants and closely related green algae), red algae, and other types of dinoflagellates (Shalchian-Tabrizi et al., 2010), substantiating plastid complexity in dinoflagellates.

### *Origin of Plastids Derived from Green Algae*

The ability to photosynthesize makes dinoflagellates and euglenophytes an important part of global carbon fixation, and the photosynthetic capacity of *L. chlorophorum* is due to the occurrence of a tertiary plastid derived from green algae (Turmel et al., 2009). Multiple studies have focused on determining the origin of plastid donors of endosymbiosis events. Shalchian-Tabrizi et al. (2006) utilized combined heat shock protein 90 and ribosomal RNA sequence to determine the evolutionary origin of tertiary plastids in *L. chlorophorum*. Results from this study indicated that the plastids originated from a prasinophyte, which implies that its original secondary plastid was

replaced by a prasinophte endosymbiont. Almost a decade later, Kamikawa et al. (2015) completely sequenced the plastid genome of *L. chlorophorum* which showed this dinoflagellate plastid originated from a pedinophyte. It should be noted, however, that pedinophytes and prasinophytes are sister clades under the phylum Chlorophyta (Lemieux et al., 2014). However, only a single species of *Pedinomonas* was used in the study so conclusions could not be confidently drawn if the plastid found in *L. chlorophorum* was inherited from a pedinophyte or rather from an algal group closely related to a pedinophyte. To unravel the origin of the secondary plastid present in *Euglena,* Turmel et al. (2009) compared gene orders between *Pyramimonas* (a green algae) and *Euglena* reporting a striking similarity between these genomes, but evolutionary analyses suffer from the use of only marine species of *Pyramimonas,* because members of the *Euglenophyceae* are found in both marine, brackish, and freshwater environments (Satpati & Pal, 2017). The next paragraph provides a closer look into the phylogeny of *Pedinomonas* and *Pyramimonas*.

### *Pedinomonas and Pyramimonas*

*Pedinomonas* and *Pyramimonas* are sister clades within the phylumChlorophyta (Moestrup, 1991; Daugbjerg et al., 2019). *Pedinomonas* species are unicellular flagellates with a single emergent flagellum (Daugbjerg & Moestrup, 1993). Originally classified to be closely related to viridiplants, *Pedinomonas* has been the subject of phylogenetic complexity (Turmel et al., 2009; Marin, 2012). Turmel et al. (2009) placed *Pedinomonas* within the Chlorellales (Trebouxiophyceae), but molecular phylogenetic analysis of

plastid-encoded rRNA identified several novel pedinophyte lineages (Marin, 2012). As a result, *Pedinomonas* is currently classified as a distinct genus. *Pyramimonas* is also unicellular, but its cells are usually inversely pyramidal (Daugbjerg & Moestrup, 1993). Some *Pyramimonas* species possess four flagella while some have been found to bear up to sixteen flagella and are characterized by large scales manufactured in the Golgi apparatus (Daugbjerg & Moestrup, 1992; Daugbjerg & Moestrup, 1993).

### *Lipids as Biomarkers*

Mono- and digalactosyldiacylglycerol (MGDG and DGDG) are two major glycolipids found in plastids of dinoflagellates and other algae (Al-Fadhli et al., 2006; Anesi et al., 2016). MGDG and DGDG present in thylakoids play important roles in photosynthesis (Kobayashi, 2016). Leblond & Chapman (2000) devised a strategy to separate MGDG and DGDG into distinct fractions from total lipids present in dinoflagellates and other algae. Because the structure of these lipids includes two acyl groups attached to a glycerol backbone which is then linked to a galactose sugar(s), the use of electrospray ionization/mass spectrometry/mass spectrometry (ESI/MS/MS) has been established as the ideal technique to study intact form of these glycolipids (Gray et al., 2009). In addition to elucidating the forms of fatty acids contained in these glycolipids, ESI/MS/MS also provides additional information as to the regiochemical distribution of the acyl groups on the glycerol backbone (Guella et al., 2003). To elucidate these findings, in-depth analysis was carried out by comparing intact forms of glycolipids found in *L. chlorophurum* with glycolipids found in a prasinophyte (Leblond & Lasiter, 2009). Findings from this study

indicated that the MGDG and DGDG composition of *L. chlorophorum* and a model prasinophyte *Tetraselmis* sp. were similar in some cases but not all (see discussion).

Sterols are a class of lipids present in eukaryotes. Aside from being membrane reinforcers in eukaryotes, sterols play a major role in cell regulation and homeostasis (Dufourc, 2008). Dinoflagellates and *Euglena* are known to produce a wide variety of sterols (Taipale *et al.,* 2016; Volkman et al., 1999). Some of the sterols already identified in *Euglena* include: ergosterol (24-methylcholesta-5,7,22E-trien-3β-ol), and chondrillasterol ((22E)-5α-poriferasta-7, 22-dien-3β-ol) (Taipale *et al.,* 2016). In dinoflagellates, dinosterol (4α,23,24-trimethyl-5α-cholest-22E-en-3β-ol) (Kaku & Hiraga, 2003), is a biomarker that is distinctive of dinoflagellates from green algae. Green algae by far, contain the most diverse kinds of sterols, and sometimes these sterols can be used to denote taxonomical relationships (Patterson, 1971).

*Objectives and Hypothesis*

In this study, ESI/MS/MS was used to compare intact molecular forms of MGDG and DGDG present in *Pedinomonas minor* and *Pedinomonas* sp., putative ancestors to *L. chlorophrum*, and *Pyramimonas parkeae* and *Pyramimonas obovata*, putative ancestors to *E. gracilis,* to investigate their evolutionary history, and to show that, the MGDG and DGDG composition of these algae shows similarity to that of their potential plastid derivatives. Additionally, sterols present in *Pedinomonas* and *Pyramimonas* were also compared to sterols of *L. chlorophorum* and *E. gracilis*, which had been published previously, to check for any form of homology. I hypothesized that the algal lipids, would

show some form of homology, but not identity to their supposed plastid descendants. By doing so, I aimed to highlight variation in lipid composition within each studied algal taxon, provide support to the notion of complexity in secondary and tertiary plastids derived from green algae, and describe how this complexity affects pinpointing the exact ancestral species.

# CHAPTER II: MATERIALS AND METHODS

## *Culturing and Lipid Processing*

*Pedinomonas minor* and *Pedinomonas* sp. strains (UTEX 1350, and UTEX 1027) were acquired from the University of Texas (UTEX) Culture Collection of Algae, while *Pyramimonas parkeae* and *Pyramimonas obovata* (CCMP 725 and CCMP 722) were acquired from the Provasoli-Guillard National Center for Marine Algae and Microbiota (previously known as Center for the Culture of Marine Phytoplankton CCMP). Each strain was grown in quadruplicate at 20$^{o}$C and 30$^{o}$C using 2 L of modified Woods Hole MBL medium (Nichols, 1973) for *Pedinomonas*. The *Pyramimonas* strains were grown in quadruplicate at 20$^{o}$C using L1 medium (Guillard, 1975). These cultures were harvested according to procedures described by Leblond & Chapman (2000). Lipid extraction and fractionation were also carried out in concordance with techniques elucidated by Leblond & Chapman (2000).

## *Analysis of intact forms of MGDG and DGDG using ESI/MS/MS*

Glycolipids were dissolved in a solution of methanol/chloroform/sodium acetate such that the ratio was 60:133:0.6 according to Welti et al. (2002) to produce positively charged sodium adducts [M+Na$^{+}$]. These adducts were subjected to a positive-ion ESI/MS scan from *m/z* 100–2000 via direct injection (5 µl sample volume into a methylene chloride carrier solvent at 0.5 ml/min) into a Finnigan DecaXP ion trap mass spectrometer

(Waltham, MA, USA) in conformity with Gray et al. (2009). Further ESI/MS/MS analysis was performed according to procedures established by Gray et al. (2009).

*Sterol Analysis*

Sterol esters and free sterols from fractions 1 and 2, respectively, were saponified and derivatized to form trimethylsilyl (TMS)-ether derivatives according to Leblond & Chapman (2002). The derivatives were analyzed via gas chromatography/mass spectrometry (GC/MS) with a Thermo TSQ Quantum Gas Chromatograph/Mass Spectrophotometer (Austin, TX) and a Restek Rxi-5Sil MS column (30 m x 0.25 µm film thickness, Restek Corp., Bellefonte, PA, USA) in positive-ion electron impact (EI) mode using the following conditions: 1 µl injected via splitless injection with injector set at 280°C, transfer line set at 275°C, helium carrier at 28 cm/s, 70 eV with a scanning range 50–600 amu, and a cycle time of 1.1 s. The GC temperature was 50°C for 1 min, 50–170°C at 15°C/min, and 170–300°C at 10°C/min with a hold of 11 min. The transfer line was set at 275°C. Relative retention times (RRT) to cholesterol were calculated using retention times (RT) according to the methodology of Jones et al. (1994). Comparison was made to the TMS-ether derivative of an authentic standards of cholesterol from Alfa Aesar (Haverhill, MA, USA) , fucosterol (5-cholesten-24(28)-ethylidene-3β-ol), from Steraloids Inc. (Newport, RI, USA), β-sitosterol (5-cholesten-24β-ethyl-3β-ol) with campesterol (24-methylcholesta-5-en-3β-ol), from TCI America (Portland, OR, USA), and ergosterol (24-methylcholesta-5,7,22E-trien-3β-ol), from Sigma-Aldrich (St. Louis, MO, USA).

# CHAPTER III: RESULTS

*Galactolipids*

*Pedinomonas* sp. and *P. minor* both produced abundant forms of MGDG and scarce forms of DGDG, however, these forms were similar, but not identical (Table 1). Both *Pedinomonas* sp. and *P. minor* produced abundant 18:3/16:4 MGDG (*m/z* 767, see Fig. 1), and scarce 18:1/16:0 DGDG (*m/z* 941). Notably, *P. minor,* however, produced 18:1/16:4 MGDG (*m/z* 771), and 20:4/16:4 MGDG (*m/z* 793, see Fig. 2); these forms of MGDG were not produced by *Pedinomonas* sp. In fact, *Pedinomonas* sp. did not produce eicosatetraenoic acid ($C_{20:4}$) at the *sn*-1 or *sn*-2 position of either MGDG or DGDG. My study revealed the production of trace quantities of polyunsaturated fatty acid (PUFA) eicosatetraenoic acid by *P. minor* (see Table 1). Eicosatetraenoic acid in green-pigmented microalgae has been reported in trace concentrations (Lopez et al 2019). Similarly, *Pedinomonas* sp. produced forms of MGDG and DGDG not present in *P. minor*. These forms included 18:3/18:3 MGDG (*m/z* 797), 18:2/16:0 DGDG (*m/z* 939), 18:4/18:4 DGDG (*m/z* 955), and 18:3/18:3 DGDG (*m/z* 959). Production of octadecatrienoic acid ($C_{18:3}$) and octadecatetraenoic acid ($C_{18:4)}$ at the *sn*-2 position by *Pedinomonas* sp. once more emphasizes the dissimilarity between forms of MGDG and DGDG produced by *P. minor* and *Pedinomonas* sp.

Table 1: Relative abundance of MGDG and DGDG. Average relative percentages of lipids in *Pedinomonas* sp. and *Pedinomonas minor* grown in triplicate at 20°C and 30°C.

| | | *Pedinomonas* sp. | | | *Pedinomonas minor* | | |
|---|---|---|---|---|---|---|---|
| Mass | Lipid | 20°C | 30°C | *p*-value | 20°C | 30°C | *p*-value |
| 743 | 16:1/16:4 MGDG | 0.43 (0.21) | 0.61 (0.19) | 0.4 | 0.58 (0.11) | 0.74 (0.13) | **0.02** |
| 767 | 18:3/16:4 MGDG | 79.25 (8.12) | 68.86 (3.88) | 0.1 | 72.31 (6.65) | 52.29 (0.82) | **0.04** |
| 771 | 18:1/16:4 MGDG | | | | 7.86 (1.76) | 19.89 (1.40) | **0.02** |
| 793 | 20:4/16:4 MGDG | | | | 1.77 (0.05) | 1.31 (0.54) | 0.3 |
| 797 | 18:3/18:3 MGDG | 0.53 (0.16) | 0.84 (0.19) | 0.1 | | | |
| 909 | 18:3/16:0 DGDG | 1.36 (0.69) | 0.95 (0.14) | 0.5 | 0.59 (0.10) | 1.08 (0.32) | 0.1 |
| 929 | 18:3/16:4 DGDG | 3.59 (0.14) | 4.18 (0.50) | 0.2 | 3.68 (2.58) | 2.02 (0.39) | 0.3 |
| 931 | 18:3/16:3 DGDG | 4.99 (1.01) | 7.19 (1.24) | 0.1 | 4.02 (1.26) | 2.38 (0.15) | 0.1 |
| 933 | 18:3/16:2 DGDG | 2.90 (1.70) | 5.11 (0.37) | 0.1 | 3.13 (0.54) | 3.41 (0.18) | 0.4 |
| 935 | 18:2/16:2 DGDG | 1.80 (1.54) | 4.01 (1.06) | 0.2 | 2.99 (1.02) | 6.73 (0.58) | 0.06 |
| 937 | 18:1/16:2 DGDG | | | | 1.96 (0.83) | 5.96 (0.83) | **0.01** |
| 937 | 18:3/16:0 DGDG | 2.30 (1.58) | 3.74 (0.94) | 0.1 | | | |
| 939 | 18:2/16:0 DGDG | 1.18 (0.83) | 1.90 (0.45) | 0.1 | | | |
| 941 | 18:1/16:0 DGDG | 0.83 (0.65) | 1.07 (0.20) | 0.4 | 1.10 (0.66) | 4.19 (0.55) | 0.05 |
| 955 | 18:4/18:4 DGDG | 0.32 (0.05) | 0.42 (0.21) | 0.6 | | | |
| 959 | 18:3/18:3 DGDG | 0.53 (0.30) | 1.10 (0.34) | 0.1 | | | |

Standard deviations are listed in parentheses. p-values with statistical significance are in bold. MGDG (monogalactosyldiacylglycerol); DGDG (digalactosyldiacylglycerol).

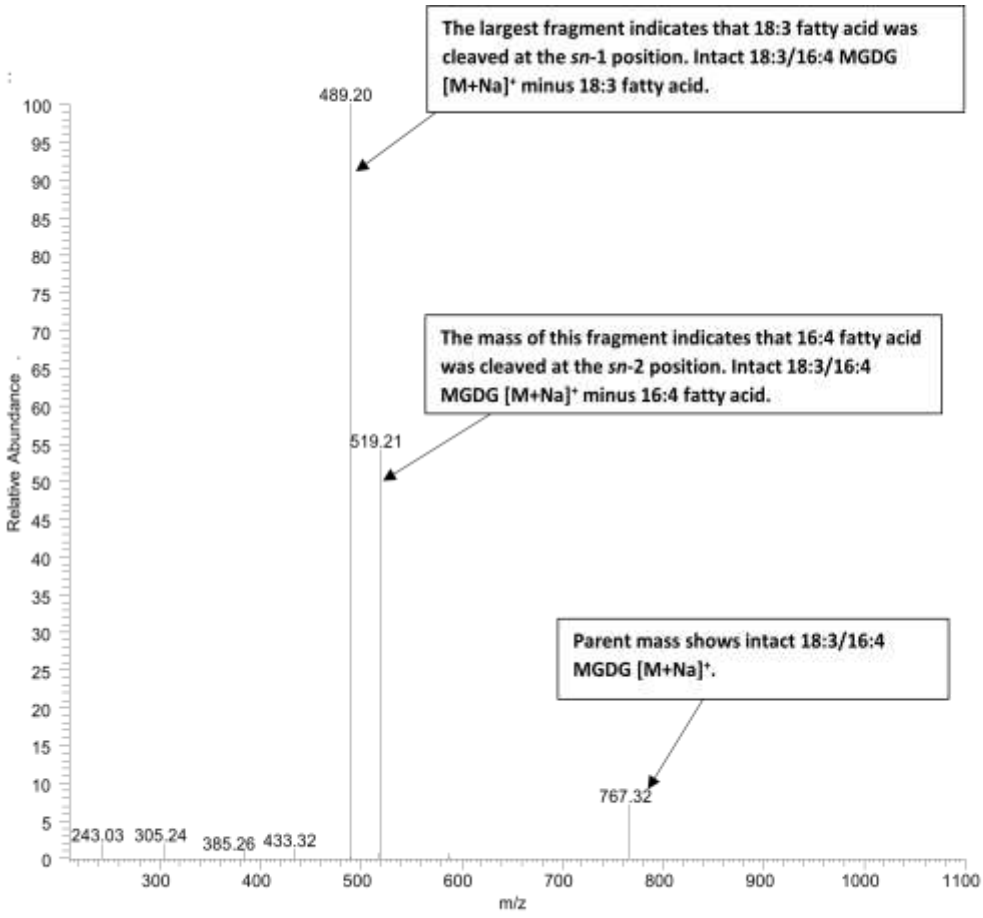


Figure 1: Positive-ion ESI/MS/MS spectrum of 18:3/16:4 MGDG from *Pedinomonas sp.*

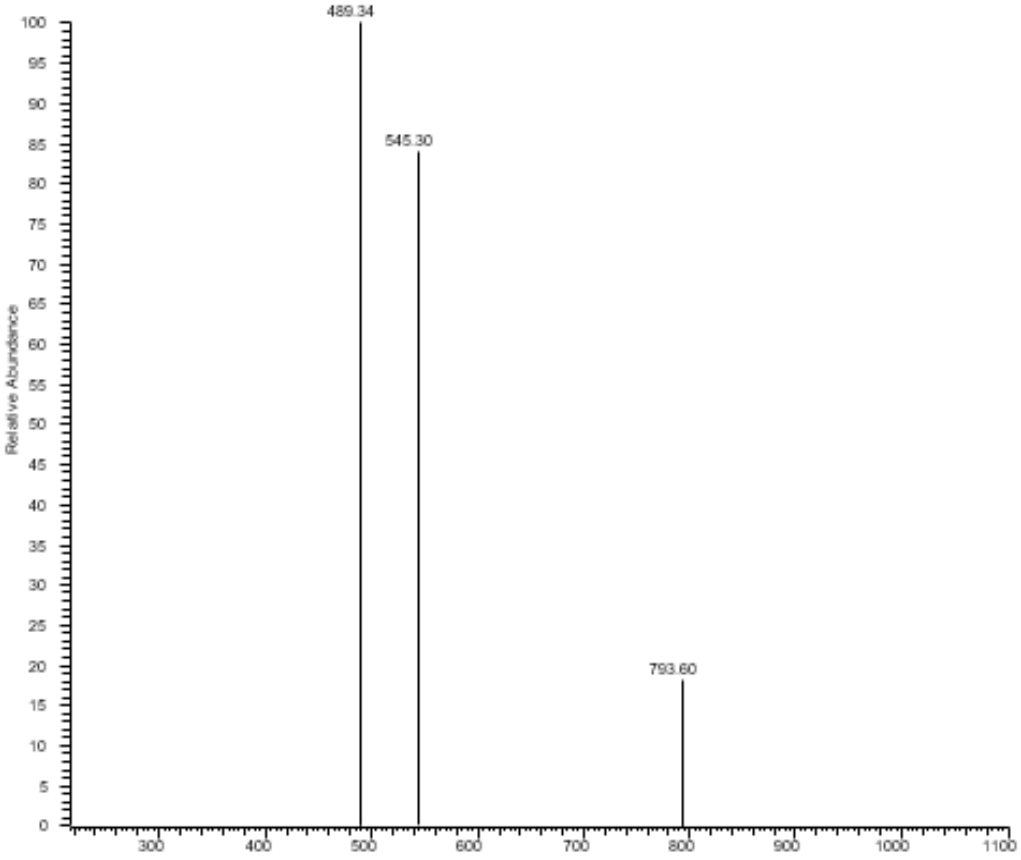


Figure 2: Positive-ion ESI/MS/MS spectrum of 20:4/16:4 MGDG from *Pedinomonas minor.*

Another observation was the production of 18:3/16:0, and 18:1/16:2 DGDG (*m/z* 937) by *Pedinomonas* sp. and *P. minor*, respectively. In *Pedinomonas* sp., the positive-ion ESI/MS/MS spectra of parent *m/z* 937 revealed *m/z* 659 as the main cleavage and *m/z* 681 as the minor cleavage. The main cleavage product was the sodium adduct of the lipid minus 18:3 at the *sn*-1 position, while the minor cleavage indicated 16:0 to be in the *sn*-2 position. In the case of m/z 937 in *P. minor*, the major and minor cleavage was *m/z* 655

and *m/z* 685 respectively, revealing 18:1 at the *sn*-1 position and 16:2 at the *sn*-2 position (Figs. 3 & 4).

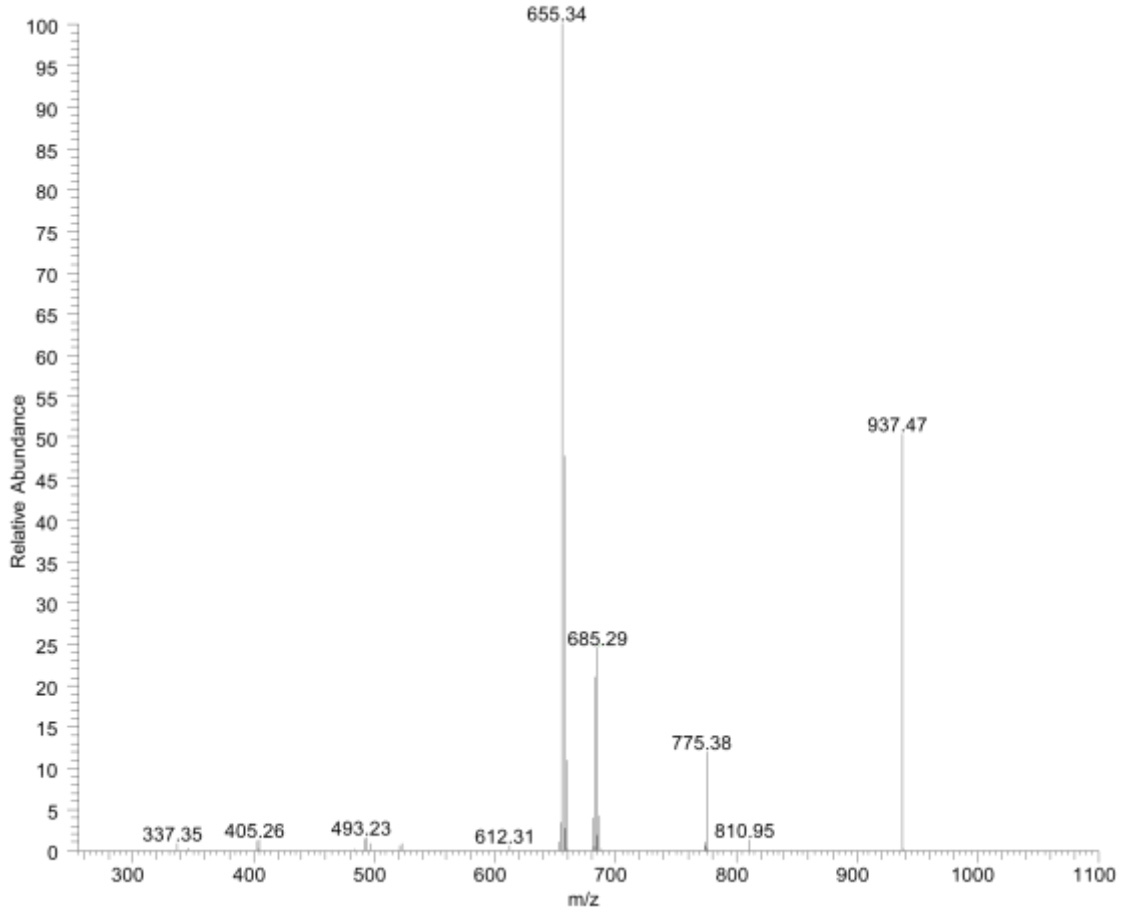


Figure 3: Positive-ion ESI/MS/MS spectrum of 18:1/16:2 DGDG from *Pedinomonas minor*.

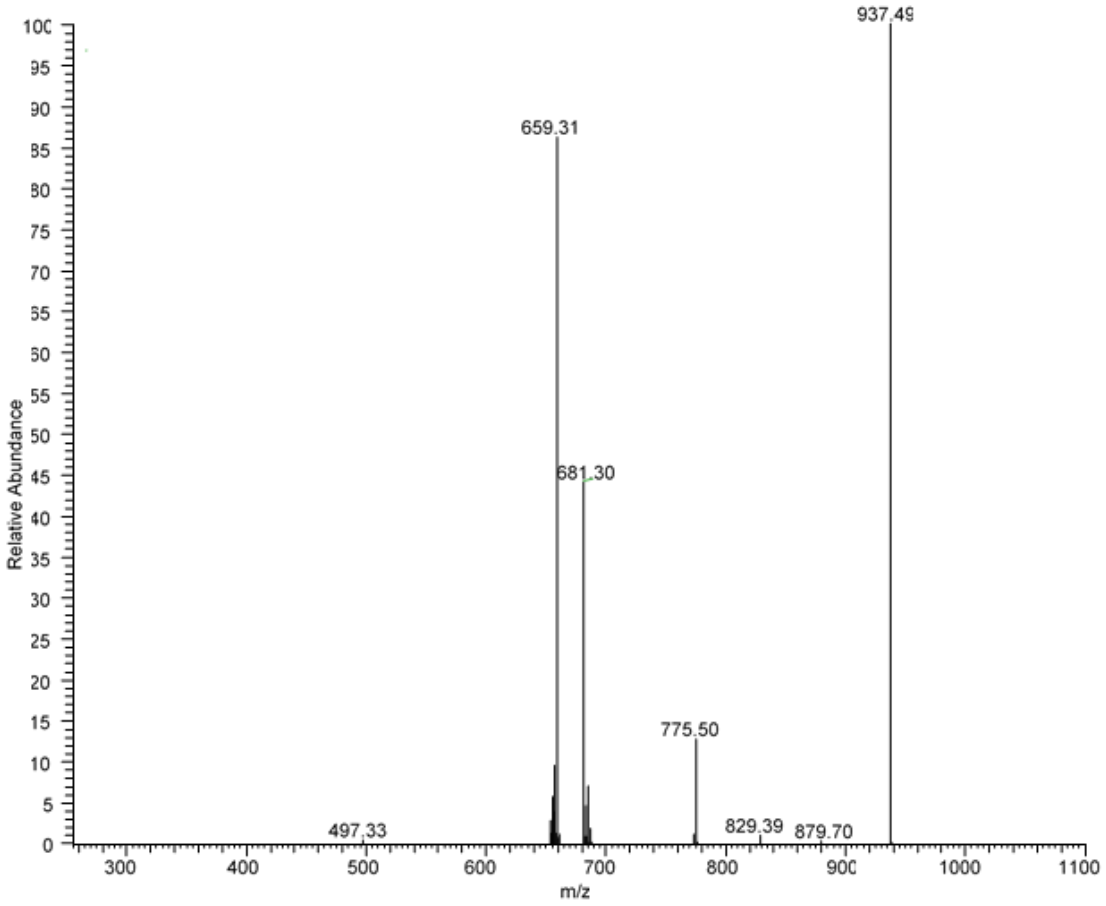


Figure 4: Positive-ion ESI/MS/MS spectrum of 18:3/16:0 DGDG from *Pedinomonas sp*.

*Pyramimonas* produced predominant $C_{18}/C_{16}$ MGDG and minor forms of $C_{18}/C_{16}$ DGDG (Table 2). Predominant forms of MGDG included 18:3/16:4 MGDG (*m/z* 767), 18:3/16:3 MGDG (*m/z* 769), and 18:5/16:4 MGDG (*m/z* 763). Scarce forms of DGDG included 18:3/16:2 DGDG (*m/z* 933), and 18:3/18:3 DGDG (*m/z* 933). Although both *P. parkeae* and *P. obovata* produced identical forms of MGDG and DGDG, the concentration of each form differs. Over 70% of total MGDG and DGDG produced by *P. parkeae* was

18:3/16:4 MGDG while *P. obovata* only produced 50% relative abundance of that same lipid. The most abundant form of DGDG observed in *Pyramimonas* was the 18:3/16:2 DGDG in *P. obovata* with a 10.7% relative abundance, as opposed to the trace concentration of that same form of DGDG present in *P. parkeae*. *P. parkeae* produced a total of 89.96% relative abundance of MGDG while *P. obovata* produced 76.92% relative abundance of MGDG. However, *P. obovata* produced 20.34% relative abundance of DGDG while *P. parkeae* produced 10.04% relative abundance of DGDG.

Table 2: Relative abundance of MGDG and DGDG. Average relative percentages of lipids in *Pyramimonas parkeae*. and *Pyramimonas obovata* grown in triplicate at 20°C.

| | | *Pyramimonas parkeae* | | *Pyramimonas obovata* | |
|---|---|---|---|---|---|
| Mass | Lipid | 20°C | SD | 20°C | SD |
| 743 | 16:1/16:4 MGDG | 3.40 | 0.22 | 1.70 | 0.57 |
| 763 | 18:5/16:4 MGDG | 1.32 | 1.05 | 11.92 | 2.71 |
| 767 | 18:3/16:4 MGDG | 72.58 | 2.48 | 50.89 | 35.82 |
| 769 | 18:3/16:3 MGDG | 11.23 | 1.06 | 9.81 | 0.36 |
| 793 | 18:4/18:4 MGDG | 1.43 | 1.44 | 2.60 | 0.17 |
| 929 | 18:3/16:4 DGDG | 4.58 | 0.88 | 3.99 | 2.54 |
| 933 | 18:3/16:2 DGDG | 0.97 | 0.09 | 10.72 | 3.67 |
| 937 | 18:3/16:0 DGDG | 2.33 | 0.73 | 4.09 | 0.64 |
| 959 | 18:3/18:3 DGDG | 2.16 | 1.20 | 1.54 | 0.39 |

SD = standard deviation; MGDG = monogalactosyldiacylglycerol;

DGDG = digalactosyldiacylglycerol. *Pyramimonas* did not grow at 30°C.

This study also examined the effect of growth temperatures at 20°C and 30°C, to observe temperature modulation of the fatty acid, at either the *sn*-1 or *sn*-2 position of both MGDG and DGDG because fatty acids are desaturated at lower temperature but are saturated at increasing temperature. The purpose of temperature comparisons in this study was to observe how individual species deal with different temperatures as its growth condition changes. Temperature modulation in terms of degree of saturation/unsaturation was not observed, however in terms of concentration, the relative percentages at which each form of MGDG and DGDG was produced did differ (Table 1; Figs. 5 & 6). No temperature comparison was made for *Pyramimonas* because the cultures did not grow at 30°C. T-test statistical analysis was used to compare the difference between the means of cultures grown in triplicates at 20°C and 30°C. Level of significance was set at $p \leq 0.05$ with 95% confidence level. Out of the four types of MGDG produced by *P. minor*, differences in three were found to be statistically significant. *P. minor* favored the production of 18:3/16:4 MGDG at 72.31% relative abundance at 20°C compared to a 52.29% relative abundance of that same lipid at $30^0$C, 16:1/16:4 MGDG at 0.58% and 0.74% relative abundance, and 18;1/16:4 MGDG at 7.86% and 19.89% relative abundance. Out of the seven forms of DGDG observed in *P. minor*, only 18:1/16:2 DGDG with relative percentages of 1.96% at 20°C and 5.96% at 30°C, was statistically significant. Statistical analysis revealed that the relative percentages of MGDG and DGDG produced by *Pedinomonas* sp. was not significant.

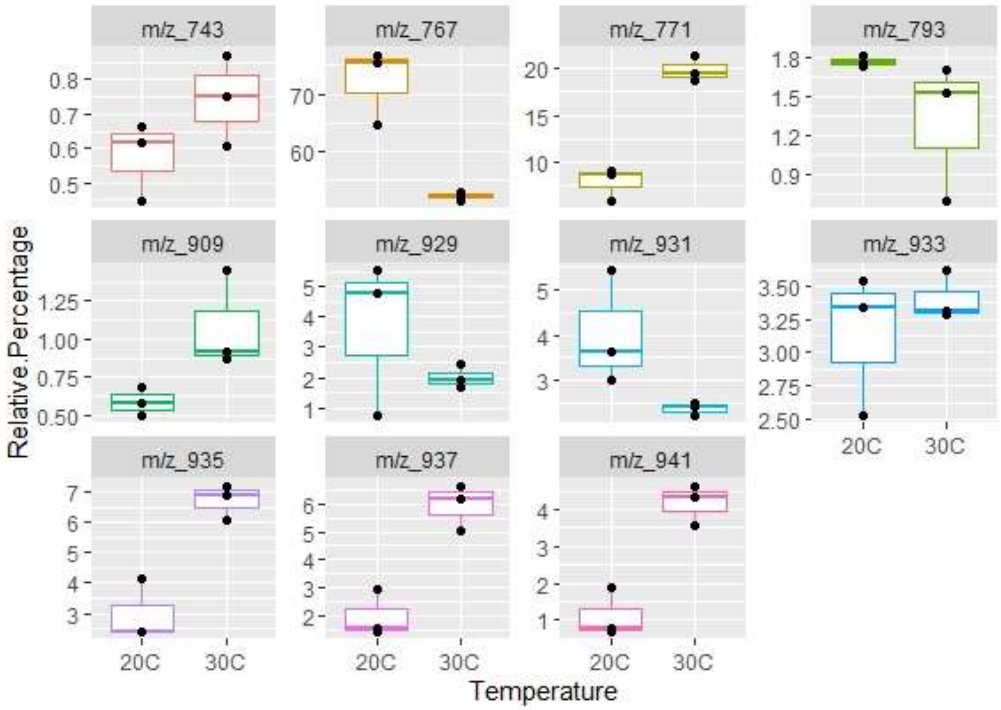


Figure 5: Boxplot showing difference in the forms of MGDG and DGDG produced by *Pedinomonas minor.* grown at 20°C and 30°C. The box displays the summary between the first, and third quartiles. The horizontal line that divides the box into two parts shows the median. The vertical line that goes through the box represents the range of each data point and is denoted by black dots.

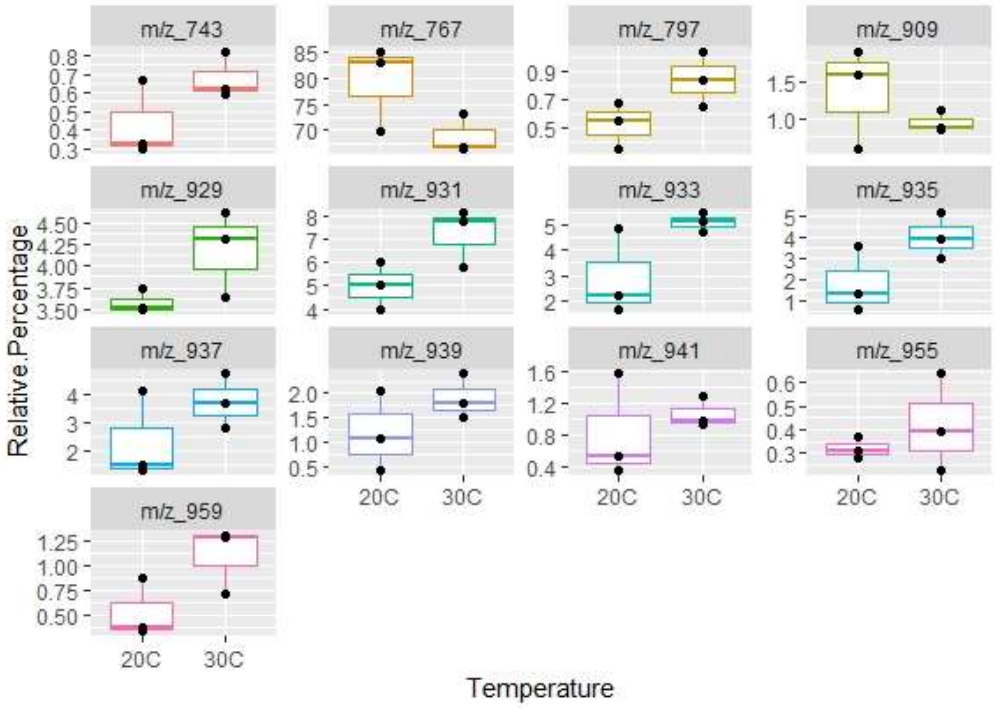


Figure 6: Boxplot showing difference in the forms of MGDG and DGDG produced by *Pedinomonas* sp. grown at 20°C and 30°C. The boxplot displays the summary between the first, and third quartiles. The horizontal line that divides the box into two parts shows the median. The vertical line that goes through the box represents the range of each data point and is denoted by black dots.

*Sterols*

Not only is galactolipid composition a good indicator of species relatedness, but sterols can also be used. To investigate this, sterol composition of the four species were undertaken. Further inquiry into the sterol composition of *Pedinomonas* and *Pyramimonas* revealed that *Pedinomonas* favored the production of 24-melthyl sterols (Table 3) while *Pyraminonas* favored production of mostly 24-ethyl sterols (Table 4).

Table 3: Relative percentage distribution of individual sterols within free sterol fraction of *Pedinomonas* sp. and *Pedinomonas minor*.

| Suggested Structure | MW | RT | RRT | *Pedinomonas* sp. | *P. minor* |
|---|---|---|---|---|---|
| 24-methylcholesta-5,7,22E,24(28)-tetraen-3β-ol | 466 | 38.03 | 1.08 | 7.61 | 4.00 |
| 24-methylcholesta-5,7,22E-trien-3β-ol (ergosterol) | 468 | 38.56 | 1.04 | 53.12 | 16.64 |
| 24-methyl-5α-cholesta-7,22-dien-3β-ol (stellasterol) | 470 | 38.77 | 1.09 | 2.79 | 4.13 |
| $C_{29:2}$ sterol | 484 | 39.14 | 1.19 | 4.00 | 9.56 |
| $C_{28:2}$ sterol | 474 | 39.33 | 1.24 | 2.26 | 11.66 |
| 24-methyl-5α-cholest-7en-3β-ol (fungisterol) | 470 | 39.68 | 1.62 | 28.04 | 50.10 |
| $C_{28:0}$ sterol | 482 | 39.91 | 1.32 | 2.19 | 3.91 |

MW= Molecular weight of sterols as TMS derivatives.
RRT= Relative retention time to the TMS derivative of cholesterol.

Table 4: Relative percentage distribution of individual sterols within free sterol fraction of *Pyramimonas parkeae* and *Pyramimonas obovata*.

| Suggested Structure | MW | Retention Time (Min) | RRT | *P. parkeae* | *P. obovata* |
|---|---|---|---|---|---|
| $C_{28:2}$ sterol | 470 | 39.48 | 1.28 | 10.84 | 4.25 |
| 24-ethylcholest-5,24(28)E-dien-3β-ol (fucosterol) | 484 | 40.19 | 1.45 | | 51.38 |
| $C_{29:2}$ sterol | 484 | 40.48 | 1.52 | 18.04 | 1.74 |
| 24-ethylcholest-5,24(28)Z-dien-3β-ol (isofucosterol) | 484 | 41.10 | 1.68 | 53.50 | 42.64 |
| 24S-ethylcholest-5,22E-dien-3β-ol (poriferasterol) | 484 | 39.66 | 1.32 | 17.63 | |

MW= Molecular weight of sterols as TMS derivatives.
RRT= Relative retention time to the TMS derivative of cholesterol.

In *Pedinomonas* we found seven sterols were identified with suggested structures of 24-methylcholesta-5,7,22E,24(28)-tetraen-3β-ol (*m/z* 466, $C_{28:1}$, RT =38.03, RRT =1.08), 24-methylcholesta-5,7,22E-trien-3β-ol (ergosterol) (*m/z* 468, $C_{28:3}$, RT =38.56, RRT =1.04),

24-methyl-5α-cholesta-7,22-dien-3β-ol (stellasterol) (*m/z* 470, $C_{28:2}$, RT =38.77, RRT =1.09), $C_{29:2}$ sterol (*m/z* 484, RT =39.14, RRT =1.19), $C_{28:2}$ sterol (*m/z* 474, RT =39.33, RRT =1.24), 24-methyl-5α-cholest-7en-3β-ol (fungisterol) (*m/z* 470, $C_{28:2}$, RT =39.68, RRT =1.62), and a $C_{28:0}$ sterol (*m/z* 482, RT =39.91, RRT =1.32).

The most abundant sterol observed in *Pedinomonas* sp. was ergosterol (Fig. 8) with relative abundance of 53.12%. The mass spectrum of ergosterol in *Pedinomonas* sp. produced the same fragments (m/z 468, 378, 363, 337, 253, 131) as the mass spectrum of authentic ergosterol standard (Fig. 7). These fragments are synonymous with the TMS-derivatives of ergosterol observed by Jones et al. (1994).

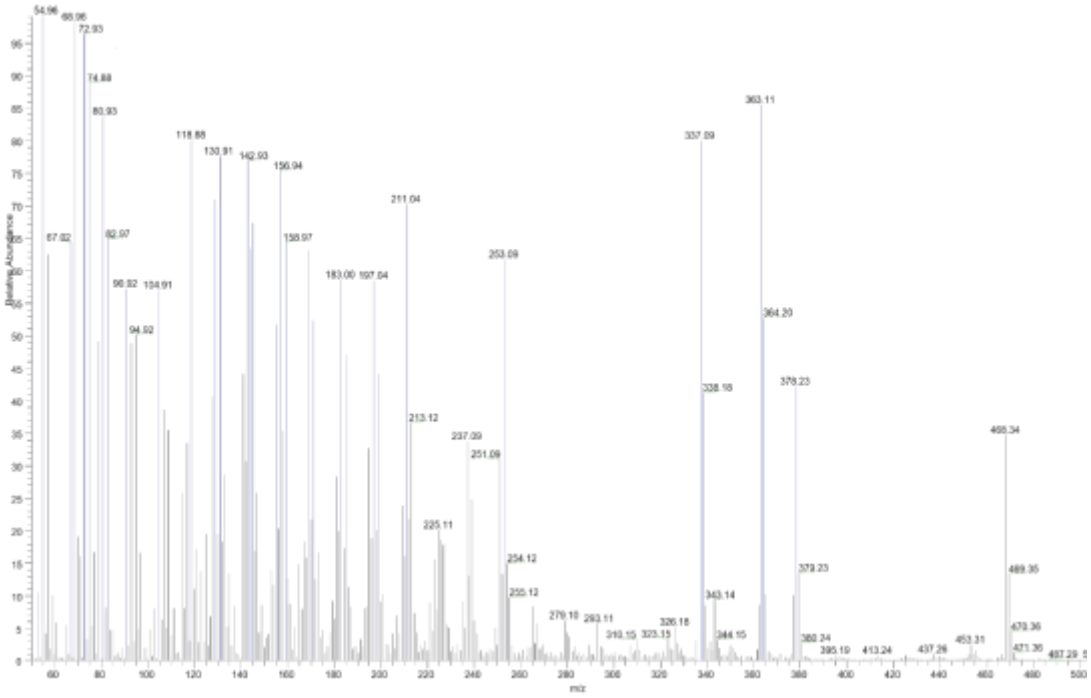

Figure 7: Mass spectrum of authentic ergosterol standard as its TMS derivative.

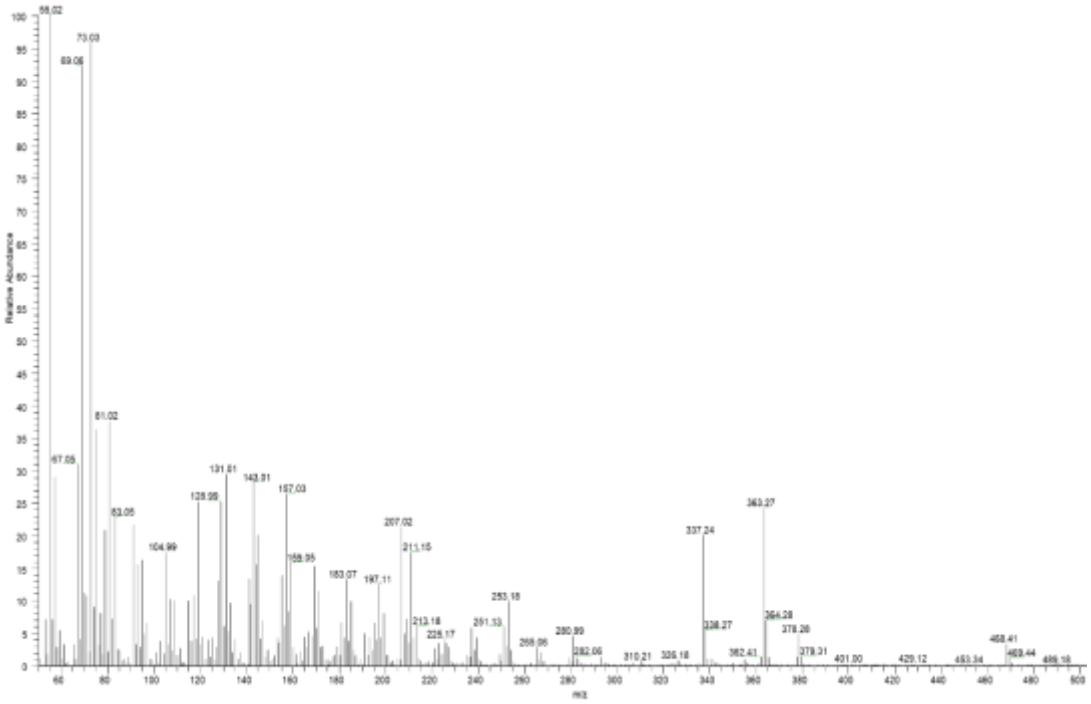

Figure 8: Mass spectrum of ergosterol as its TMS derivative in *Pedinomonas* sp.

Five types of sterols were observed in *Pyramimonas*. Out of the five, three were identical sterols of both *P. parkeae* and *P. obovata*, but two were not. The three identical sterols observed in both cultures were 24-ethylcholest-5,24(28)Z-dien-3β-ol (isofucosterol) (*m/z* 484, $C_{29:2}$, RT =41.10, RRT =1.68), $C_{28:2}$ sterol (*m/z* 470, RT =39.48, RRT =1.28), $C_{29:2}$ sterol (*m/z* 484, RT =40.48, RRT =1.52). Fucosterol (*m/z* 484, $C_{29:2}$, RT =40.19, RRT =1.45) was observed in *P. obovata* with a relative percentage of 51.38%, but not in *P. parkeae*. Conversely, poriferasterol (*m/*z 484, $C_{29:2}$, RT =39.66, RRT =1.32) was observed in *P. parkeae* but was not found in *P. obovata*. Fucosterol was the most abundant sterol observed in *P. obovata*. The mass spectrum of fucosterol sterol standard (Fig. 9) displayed

the same fragments as the mass spectrum of fucosterol identified in *P. obovata* (Fig. 9 & 10), with RRT of 1.43 and 1.45, respectively. These fragments (m/z 484, 371, 355, 296, 281) corroborate the TMS-derivatives of fucosterol observed by Jones et al. (1994).

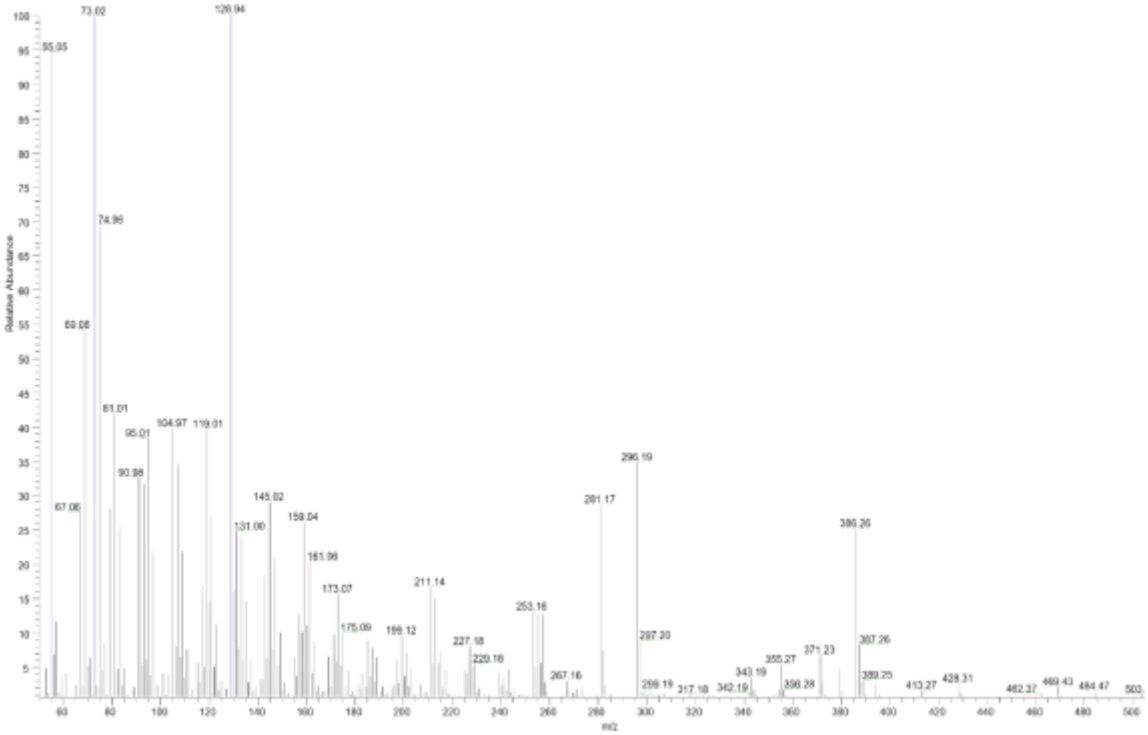


Figure 9: Mass spectrum of authentic fucosterol standard as its TMS derivative.

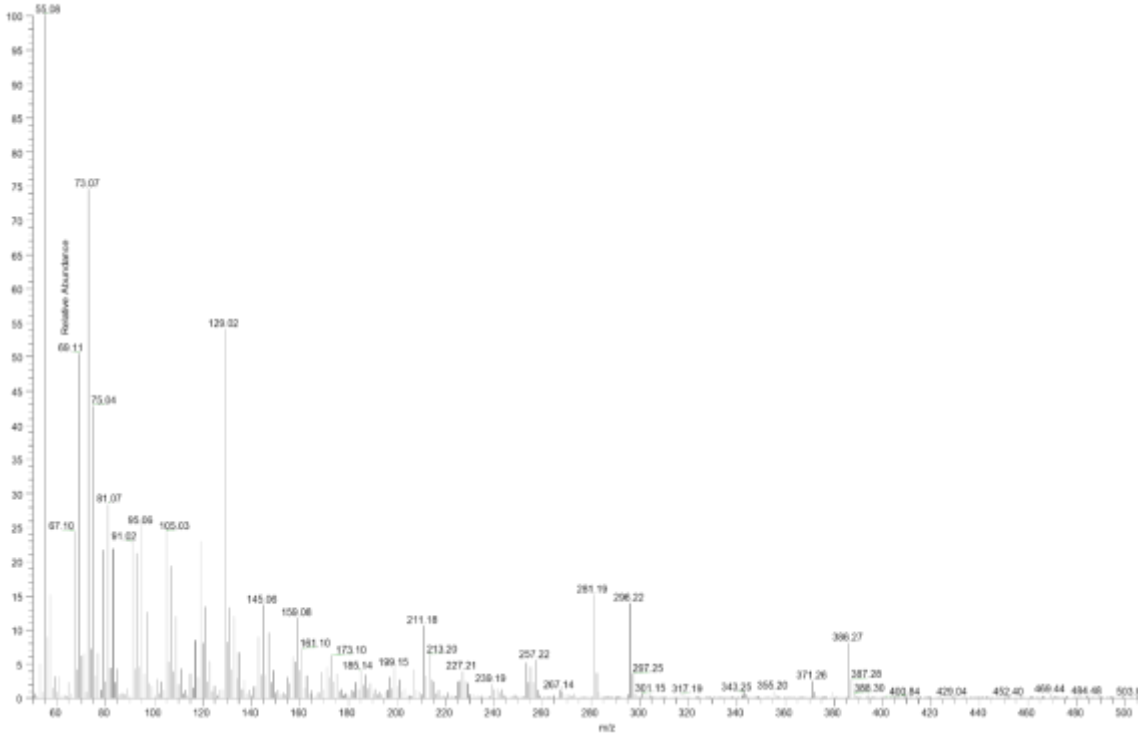


Figure 10: Mass spectrum of fucosterol as its TMS derivative in *Pyramimonas obovata.*

# CHAPTER IV: DISCUSSION

The quest to unravel the origin of secondary and tertiary plastids in *L. chlorophorum* and *E. gracilis* has been the subject of many publications (Shalchian-Tabrizi et al., 2006; Leblond & Lasiter, 2009; Kamikawa et al., 2015; Jackson et al., 2018). It was initially suggested that the tertiary plastids in *L. chlorophorum* originated from a prasinophyte (Shalchian-Tabrizi et al., 2006), but phylogenetic analysis of the *L. chlorophurum* genome indicated the possibility of the tertiary plastid to be of pedinophyte origin (Kamikawa et al., 2015). While genomic comparisons of plastid genes does serve as one way of studying the evolutionary relationships between algae, analysis of the stoichiometry of glycolipids produced provides additional evolutionary insight (Gray et al., 2009; Leblond & Lasiter, 2009). The plastid origin of *L. chlorophorum* was investigated by examining MGDG and DGDG, and sterols, of two pedinophytes presumed to be plastid ancestors of *L. chlorophorum*. The analysis revealed that these pedinophytes possessed 18:3/16:4 MGDG and 18:3/16:4 DGDG, whereas previous examination of the MGDG and DGDG in *L. chlorophurum* showed the production of 18:5/16:4 MGDG, 18:5/18:5 MGDG, and 20:5/16:4 DGDG (Leblond & Lasiter, 2009).

Surprisingly, 18:5 or 20:5 fatty acidswere not found at either the *sn*-1 or *sn*-2 position of *Pedinomonas*. The only similarity to *L. chlorophorum* in this case was the production of hexadecatetraenoic acid ($C_{16:4}$) at the sn-2 position of both MGDG and DGDG. However, a wide range of green algae are equally notable producers of the 16:4

fatty acid (Khotimchenko et al., 2002); it is present in the green macro alga *Ulva lactuca* (Harwood & Jones, 1989), a member of the *Chlamydomonadales Dunaliella primolecta* (Lang et al., 2011), and *P. parkeae* and *P. obovata*, the two prasinophytes utilized in this study produced it. Interestingly, production of the 16:4 fatty acid in a dinoflagellate is rare, but the dinoflagellate *Karenia mikimotoi* produces it at the *sn*-2 position (Leblond et al., 2019). It should be noted however that the genus *Karenia* has a haptophyte-derived tertiary plastid (Takashita et al., 2004). Production of the 16:4 fatty acid at the *sn*-2 position of MGDG and DGDG in both *L. chlorophorum* and *Pedinomonas* is not enough grounds to assign the exact origin of the tertiary plastid in *L. chlorophorum* to a pedinophyte because tertiary plastids in dinoflagellates are seemingly complex. Waller & Kořený (2017) provide an extensive explanation on how the transition from mixotrophy to phototrophy may have affected plastids in dinoflagellates during the endosymbiotic process. Taking this into consideration, we suggest that this dinoflagellate must have acquired the genes necessary to produce the 16:4 fatty acid at the *sn*-2 position from green algae; while the alga symbiont lost the genes required to produce the $C_{18}$ fatty acid at the *sn*-1 position. In addition, a closer look into the phylogeny of the *Pedinophyceae* reveals a deep, yet complex, relationship with other members of the Chlorophyta like Trebouxiophyceae, and Ulvophyceae (Marin, 2012; Lemieux et al., 2014).

On the comparison of glycolipids present in *E. gracilis* with its presumed plastid ancestor *Pyramimonas*, *Pyramimonas* possessed forms of $C_{18}/C_{16}$ MGDG and DGDG that are identical to forms of MGDG and DGDG in *E. gracilis* (18:3/16:4 MGDGD, 18:3/16:4

DGDG) already elucidated by Leblond et al. (2015). This striking resemblance indicates that the secondary plastid present in *E. gracilis* evolved from a secondary endosymbiotic event involving a member of the order *Pyramimonadales.* As a model organism of early eukaryote evolution (Henze et al., 1995), the plastid genome and lipid composition of *E. gracilis* has been extensively studied (Geimer et al., 200; Leblond et al., 2015; Schwarzhans et al., 2015). The secondary plastid of *E. gracilis* is composed of three membranes, the two innermost membranes are generally assumed to have originated form the primary plastid while the outermost membrane originated from a eukaryote (Tomečková et al., 2020). However, one proteomic study suggested that the middle membrane may have originated from the eukaryotic host endomembrane system (Novak et al., 2020), but not until recently was it discovered that the lipid composition of the middle plastid membrane bears an interesting similarity in lipid composition to that of the primary plastid envelope (Tomečková et al., 2020). In fact, this resemblance bears a more striking form of homogeneity than other eukaryotic membranes (Tomečková et al., 2020), implying that much more remains to be learned through study of the lipid composition of organisms bearing secondary and tertiary plastids.

Also, production of abundant forms of 18:3/16:4 MDGD, and scarce forms of 18:3/16:4 DGDG, by algae utilized in this study substantiates previous discoveries that “algal” plastids have MGDG as their main lipid, with smaller amounts of DGDG (Harwood & Jones, 1989). Also, *Pedinomonas* belongs to the Chlorophyceae*,* while *Pyramimonas* belongs to the Prasinophyceae; observation of large concentrations of predominantly $C_{18}$

and $C_{16}$ fatty acids by both *Pedinomonas* and *Pyramimonas* is valid because, members of these classes are notable producers of large quantities of $C_{18}/C_{16}$ fatty acids (Lang et al., 2011, Widianingsih et al., 2013). Historically, fatty acids are incorporated into glycerolipids via the prokaryotic and eukaryotic pathways. Via the prokaryotic pathway, $C_{16}$ fatty acid is esterified to the *sn*-2 position of glycerol inside the plastid, while in the eukaryotic pathway, $C_{18}$ fatty acids are incorporated at the *sn*-2 position of glycerol in the endoplasmic reticulum (see Ohlrogge & Browse (1995) for an extensive discussion on glycerolipid biosynthesis). Production of 16:3 and 18:3 fatty acids at the *sn*-2 position of MGDG in *Pedinomonas* and *Pyramomonas* indicates the use of both the prokaryotic and eukaryotic pathways for lipid biosynthesis.

In contrast, analysis of *Pedinomonas* sterols did not reveal any similarity to past study on the sterols of *L. chlorophorum* (Leblond & Lasiter, 2012). Sterols produced by *L. chlorophorum* described in that past study were prominent 4α-methyl sterols such as dinosterol, and dinostanol (Leblond & Lasiter, 2012), but this study revealed that *Pedinomonas* possessed 24-methyl sterols (Table 3). This interesting dissimilarity between sterols in *L. chlorophorum* and *Pedinomonas* supports the notion of plastid complexity in dinoflagellates (Waller & Kořený, 2017). It therefore implies that during the endosymbiotic event, loss of the genes necessary for sterol production by the once-free living alga was requisite to allow for formation of the symbiont. This study also compared sterols produced by *Pyramimonas* to that already identified in *E. gracilis.* Sterols that have been reported in *Euglena* included cholesterol, poriferasterol, chondrillasterol, and

corbisterol, with the most common being ergosterol (Brandt et al., 1970, Taipale et al., 2016). Production of ergosterol was not observed by either *P. parkeae* or *P. obovata* but other members of the *Pyramimonadales* like *Pyramimonas grossii* has been found to produce ergosterol (Patterson et al., 1992). The predominant sterol common to both *P. parkeae* and *P. obovata* was isofucosterol with a 53.50% and 42.64% relative abundance, respectively. Similarly, isofucosterol has also been observed in large quantities in the marine *Pyramimonas gelidicola* (Volkman, 1986). The wide variety of sterols produced by species of *Pyramimonas* draws heavily on the taxonomical variation within members of this genus.

Out of the five sterols produced by *P. parkeae and P. obovata,* (Table 4) poriferasterol sterol was the only sterol common to *E. gracilis*. *E. gracilis* is photosynthetic/green when grown in light, but a mutant form is white/bleached in the absence of light (Brandt et al., 1970). In the study where poriferasterol was identified in *Euglena* (Brandt et al., 1970), only the white/bleached mutant produced it. Furthermore, based on extended taxon-sampling, it has been suggested that the endosymbiotic event resulting in the possession of secondary plastids in *E. gracilis* likely took place in a marine habitat. Interestingly, of the two species of *Pyramimonas* utilized in this study, only *P. parkeae* – the strictly marine strain- was observed to possess poriferasterol at a 17.63% relative abundance. It should be noted however that *Pyramimonas chordata*, another marine species of *Pyramimonas*, has poriferasterol as the main sterol (Ponomarenko et al., 2004). As an essential feature of eukaryotic membranes, sterols are highly conserved

through evolution; in light of this, it therefore implies that, there is a possibility that *E. gracilis* acquired the ability to produce poriferasterol from its marine alga symbiont.

# CHAPTER V: CONCLUSION

Plastid lipids serve as an additional tool for comparative and evolutionary analysis. Dissimilarity between MGDG and DGDG composition of two *Pedinomonas* cultures and the slight resemblance to MGDG and DGDG already identified in *L. chlorophorum* hints that more work needs to be done using a wide number of members of the order Pedinomonadaceae, or algae closely related to the Pedinomonadaceae, to see if any will bear striking similarity to the forms of MGDG and DGDG present in *L. chlorophorum*. In terms of temperature modulation, we have shown that the concentration of MGDG and DGDG, produced at 20$^{o}$C, and 30$^{o}$C differs in *P. minor* but not in *Pedinomonas* sp. Regarding sterol composition, we have shown that; *Pedinomonas* did not produce sterols that are similar to already identified sterols in *L. chlorophorum*. MGDG and DGDG in *Pyramimonas* was almost an exact match to that of *E. gracilis*, but in the case of *Pyramimonas* sterols, only the strictly marine species produced poriferasterol, a sterol common to *E. gracilis*.

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