European Journal of Phycology

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/tejp20

Galactolipids of the genus *Amphidinium* (Dinophyceae): an hypothesis that they are basal to those of other peridinin-containing dinoflagellates

Jeffrey D. Leblond, Lindsey C. Elkins, Jori E. Graeff & Kyra Sabir

To cite this article: Jeffrey D. Leblond, Lindsey C. Elkins, Jori E. Graeff & Kyra Sabir (2022): Galactolipids of the genus *Amphidinium* (Dinophyceae): an hypothesis that they are basal to those of other peridinin-containing dinoflagellates, European Journal of Phycology, DOI: 10.1080/09670262.2022.2092215

To link to this article: https://doi.org/10.1080/09670262.2022.2092215

Published online: 21 Jul 2022.

EUROPEAN JOURNAL OF PHYCOLOGY, 2022
https://doi.org/10.1080/09670262.2022.2092215

Galactolipids of the genus *Amphidinium* (Dinophyceae): an hypothesis that they are basal to those of other peridinin-containing dinoflagellates

Jeffrey D. Leblond, Lindsey C. Elkins, Jori E. Graeff and Kyra Sabir

Ecology and Evolution Group, Department of Biology, P.O. Box 60, Middle Tennessee State University, Murfreesboro, Tennessee 37132, USA

ABSTRACT
The genus *Amphidinium* is shown in many phylogenies to be basal to other peridinin-containing, photosynthetic dinoflagellates as one of the first photosynthetic genera to arise after the evolution of heterotrophic genera. As part of our continuing examination of the plastid-associated galactolipids, namely mono- and digalactosyldiacylglycerol (MGDG and DGDG, respectively), in dinoflagellates, we here examine the galactolipid composition of members of the genus *Amphidinium*. We show that this genus is characterized by an abundance of 20:5(n-3)/18:5(n-3) and 20:5(n-3)/18:4(n-3) forms of MGDG and DGDG (with *sn*-1/*sn*-2 regiochemical specificity of fatty acids), but also sometimes with generally lesser amounts of some polyunsaturated C_{18}/C_{18} forms, thus placing the examined species within a previously identified cluster of C_{20}/C_{18} MGDG- and DGDG-containing, peridinin-containing dinoflagellates. We also show that *Testudodinium testudo*, previously known as *Amphidinium testudo*, conversely falls within a previously identified C_{18}/C_{18} cluster, indicating a distinct difference in galactolipid biosynthesis capability. While it is likely that further revision of the genus may occur in the future and/or more basal peridinin-containing, photosynthetic genera may be discovered, at the current time *Amphidinium* is the currently agreed-upon most basal dinoflagellate genus for which isolates are available for biochemical characterization such as what we describe in this paper. Thus, because of the presumed basal position of the genus *Amphidinium*, we present a hypothesis that its galactolipids currently represent those that are ancestral to other genera of peridinin-containing dinoflagellates, including those within the C_{18}/C_{18} cluster.

HIGHLIGHTS
- *Amphidinium* species' galactolipids reside within the C_{20}/C_{18} peridinin dinoflagellate cluster.
- Conversely, *Testudodinium testudo* (formerly *Amphidinium testudo*) falls within the C_{18}/C_{18} cluster.
- We hypothesize *Amphidinium*'s galactolipids as basal to other peridinin dinoflagellates.

ARTICLE HISTORY Received 8 January 2022; Revised 30 April 2022; Accepted 3 June 2022

KEYWORDS *Amphidinium*; chloroplast; dinoflagellate; dinophyceae; lipid; *Testudodinium*

Introduction

The largest group of photosynthetic dinoflagellates contains those possessing the carotenoid pigment peridinin as part of a secondary plastid of red algal origin (Zapata *et al.*, 2012). This is in contrast to smaller groups of photosynthetic dinoflagellates with aberrant plastids possessing alternative carotenoids (Jeffrey *et al.*, 1975; Zapata *et al.*, 2012). As photosynthetic organisms, peridinin-containing dinoflagellates and aberrant plastid dinoflagellates possess the galactolipids mono- and digalactosyldiacylglycerol (MGDG and DGDG, respectively) as the major lipids comprising their plastid membranes (Gray *et al.*, 2009*a*; Leblond & Lasiter, 2009; Leblond *et al.*, 2019; Graeff *et al.*, 2021). These lipids are conserved across algae and plants where they fulfil the role of structural lipids within the chloroplast (Gao *et al.*, 2018; Hölzl & Dörmann, 2019; Hernández & Cejudo, 2021).

It has been established that peridinin-containing dinoflagellates can be divided into two groups (clusters) according to the particular species (forms) of MGDG and DGDG they possess (Gray *et al.*, 2009*a*; 'forms' used from here on to specify individual galactolipids so as to eliminate confusion with 'species' used with regard to algal species). Cluster 1 possesses C_{18}/C_{18} (*sn*-1/*sn*-2 regiochemistry) MGDG and DGDG as the predominant galactolipid forms, with the C_{18} fatty acids being enriched in octadecatetraenoic [18:4(n-3)] and octadecapentaenoic [18:5(n-3)] acid – the number after the colon indicates the number of unsaturations and n-3 indicates the position of the first unsaturation from the methyl end of the fatty acid – for the sake of simplicity this will be omitted from here on after its first usage for a particular fatty acid. Cluster 2 possesses C_{20}/C_{18} MGDG and DGDG as the predominant galactolipid forms,

CONTACT Jeffrey D. Leblond leblond.jeff@gmail.com

Published online 21 Jul 2022

where the C_{20} fatty acid is eicosapentaenoic [20:5 (n-3)] acid and the C_{18} fatty acids are enriched in 18:4 and 18:5.

Peridinin-containing dinoflagellates have been extensively characterized phylogenetically over the past few decades. In several of these studies using a variety of different genes within a broad sampling of dinoflagellate genera, the genus *Amphidinium* is generally placed in a position basal to other peridinin-containing dinoflagellates (Zhang *et al*., 2007; Orr *et al*., 2012; Bachvaroff *et al*., 2014; Janouškovec *et al*., 2017; Dorrell & Howe, 2015; Bolch, 2021), although as mentioned below the work of Zhang *et al.* (2007). does raise the possibility that the genus *Heterocapsa* is more basal than *Amphidinium*. However, the implication of this generally agreed upon basal position of *Amphidinium* in these phylogenies is that it likely arose as one of the first possible photosynthetic, peridinin-containing dinoflagellate genera after the appearance of heterotrophic (proto) dinoflagellates, such as *Oxyrrhis marina* Dujardin and *Amoebophrya* spp., per these phylogenies.

This implication must be considered with the following caveats which could cause our conclusions and hypothesis (see below) to change in the future. First, there are other photosynthetic dinoflagellates, such as *Spatulodinium pseudonoctilica* (Pouchet) J.Cachon & M.Cachon and dinoflagellate strains MGD and TGD, that are more basal than *Amphidinium* (Gómez *et al*., 2010; Sarai *et al*., 2020).However, while *S. pseudonoctiluca* does in some images have a reddish pigmentation per Gómez & Souissi (2007) and Gómez *et al.* (2010) that may indicate the presence of peridinin, the actual pigment content has to our knowledge not been examined to confirm that it is a peridinin-containing dinoflagellate – we are not aware of a *S. pseudonoctiluca* culture being available for further study. Strains MGD and TGD have green pigmentation (with a green algal endosymbiont; Sarai *et al*., 2020) and are thus unlikely to be peridinin-containing dinoflagellates even though they are photosynthetic. More basal peridinin-containing, photosynthetic genera may yet be discovered and, hopefully, be made available for biochemical study.

Second, there are studies based on the genes for small and large subunit ribosomal RNA where *Amphidinium* is not basal to peridinin-containing dinoflagellates (Flø Jørgensen *et al*., 2004; Horiguchi *et al*., 2012; Pinto *et al*., 2017), and one where it is debatable whether *Amphidinium* or *Heterocapsa* is more basal (in some of the phylogenetic trees produced by Zhang *et al*., 2007). However, in the phylogenies mentioned above using different genes (Zhang *et al*., 2007; Orr *et al*., 2012; Bachvaroff *et al*., 2014; Janouškovec *et al*., 2017; Dorrell & Howe, 2015; Bolch, 2021), there is general agreement that *Amphidinium* is basal to other peridinin-containing dinoflagellates. Given this, we are assuming for this work that *Amphidinium* is in such a basal position. If future studies indicate that *Heterocapsa* is indeed more basal than *Amphidinium*, then the genus *Heterocapsa* would necessitate further characterization – note that Gray *et al.* (2009*a*) examined a single isolate of *Heterocapsa niei* (A.R.Loeblich) L.C.Morrill & A.R.Loeblich and it was found to reside in the C_{18}/C_{18} cluster. More *Heterocapsa* species need to be examined to determine whether this is a uniform trait within the genus because, for example, species of the genus *Prorocentrum* examined by Gray *et al.* (2009*a*) fall into both the C_{20}/C_{18} and C_{18}/C_{18} clusters.

Third, the genus *Amphidinium* is very large with well over 100 currently listed species (Guiry & Guiry, 2021). Given this number of species, what constitutes a member of genus *Amphidinium* has frequently undergone revision as new information is collected (e.g. Flø Jørgensen *et al*., 2004; Murray *et al*., 2012; Karafas *et al*., 2017), but the named *Amphidinium* species examined in our work are all recently positioned in a clade with *Amphidinium carterae* Hulburt according to the phylogeny provided by Karafas *et al.* (2017). The relative position of these strains covered by the Karafas *et al.* (2017) phylogeny to a broader phylogeny of dinoflagellates can be inferred by the position of *A. carterae*. Note that *A. carterae* is contained within the *Amphidinium sensu stricto* group outlined by Jørgensen *et al.* (2004). In some cases, species which bear morphological resemblance to the genus *Amphidinium* sensu stricto but have a distinct genetic difference(s) have been renamed as new genera. For example, *Amphidinium testudo* Herdman has been renamed as *Testudodinium testudo* (Herdman) Horiguchi, Tamura, Katsumata & A.Yamaguchi because phylogenetic characterization of its small subunit ribosomal RNA gene (SSU rDNA), coupled with slight morphological differences, place it outside of the genus *Amphidinium* and within a clade corresponding to the new genus *Testudodinium* (Horiguchi *et al*., 2012; Pinto *et al*., 2017). According to the phylogenies presented in these studies, *T. testudo* does not occupy a basal position compared with other dinoflagellates. Nevertheless, because of its past association with the genus *Amphidinium*, we also examine the galactolipids of *T. testudo* in this work.

At the current time *Amphidinium* is the most generally agreed-upon basal dinoflagellate genus for which isolates are available for biochemical study. Thus, given the apparent basal phylogenetic placement of the genus *Amphidinium* as it relates to the large assortment of other peridinin-containing, photosynthetic dinoflagellate genera, our objective was to characterize the galactolipids of a representative selection of commercially available *Amphidinium* species with the hypothesis that its forms of MGDG and DGDG are the currently best understood 'ancestral' galactolipids of peridinin-containing dinoflagellates writ large. In other words, by examining the galactolipids of *Amphidinium* we aim to generate discussion by addressing the question of what came first, the C_{18}/C_{18} or C_{20}/C_{18} cluster as observed by Gray *et al.* (2009*a*), in terms of peridinin dinoflagellate galactolipid evolution.

Materials and methods

Culturing

Amphidinium fijiensis Karafas & C.R.Tomas ARC 114, *Amphidinium magnum* Karafas & C.R.Tomas ARC 73, *Amphidinium paucianulatum* Karafas & C.R.Tomas ARC 117, *Amphidinium theodori* Karafas & C.R.Tomas ARC 173, and *Amphidinium tomasii* Karafas ARC 388 were acquired from the Algal Resources Collection (Wilmington, North Carolina, USA). *Amphidinium* sp. CB 153240 was acquired from Carolina Biological Supply Company (Burlington, North Carolina, USA). *T. testudo* (*A. testudo*) RCC 1981 was acquired from the Roscoff Culture Collection (Roscoff, France). All species were grown autotrophically in triplicate in 2 l of f/2 medium (Guillard & Ryther, 1962; Guillard, 1975) at a salinity of 35 psu and 20°C under a 14/10 h light/dark cycle at an irradiance of ~50 μmol photons m^{-2} s^{-1} using cool white fluorescent lights. Cells were harvested via filtration onto Whatman 934-AH glass microfiber filters (GE Healthcare, Chicago, Illinois, USA) during the exponential phase of growth after approximately one month of growth at 20°C when cells were at a concentration of ~10^4 cells ml^{-1}. Filters were preserved at −80°C until lipid extraction.

Lipid processing

Total lipids were extracted according to the techniques described by Leblond & Chapman (2000), including the separation of galactolipids from other lipid classes. Briefly, the total lipid extracts were separated into five component lipid fractions on columns of activated Unisil silica (1.0 g, 100–200 mesh, activated at 120°C, Clarkson Chromatography, South Williamsport, Pennsylvania, USA). The following solvent regime was used to separate lipids according to polarity, with the fifth fraction eluting the most polar lipids (Leblond & Chapman, 2000): (1) 12 ml methylene chloride (sterol esters), (2) 15 ml 5% acetone in methylene chloride with 0.05% acetic acid (free sterols, di- and triacylglycerols, and free fatty acids), (3) 10 ml 20% acetone in methylene chloride (monoacylglycerols), (4) 45 ml acetone (chloroplast-associated galactolipids) and (5) 15 ml methanol with 0.1% acetic acid (polar lipids, including betaine lipids). All solvents were purchased from Fisher Scientific (Hampton, New Hampshire, USA) at Optima grade, the highest purity available.

Galactolipid analysis

Galactolipids were characterized as sodium adducts $[M+Na^+]$ using positive-ion electrospray ionization/mass spectrometry (ESI/MS) and electrospray ionization/mass spectrometry/mass spectrometry (ESI/MS/MS) per the original description of Gray *et al.* (2009*a*). These adducts were scanned from *m/z* 100–2000 via direct injection of a 5 μl sample volume into a methylene chloride carrier solvent at 0.1 ml min^{-1} into a Finnigan DecaXP ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Subsequent ESI/MS/MS was performed on galactolipids using a collision energy between 37.5 and 48%, and major cleaved fatty acids were identified by the differences between the masses of the original ions and their fragments. The positions of the acyl chains (*sn*-1 or *sn*-2) were determined based on Gray *et al.* (2009*a*) according to a variation of the procedure established by Guella *et al.* (2003). Additional instrument details are provided by Leblond *et al.* (2019).

Elucidation of the number and positions of unsaturations in galactolipid-associated fatty acids was accomplished by formation of fatty acid methyl esters (FAMEs) according to the derivatization procedure utilized by Leblond & Chapman (2000), followed by formation of 4,4-dimethyloxazoline (DMOX) derivatives according to the procedure of Fay & Richli (1991). Gas chromatography/mass spectrometry (GC/MS) analysis of both types of derivatives was carried out according to Leblond *et al.* (2019).

Chemotaxonomic considerations

The chemotaxonomic relationships of species of *Amphidinium* according to galactolipid composition were expressed using the Primer-e software package (Quest Research Limited, Auckland, New Zealand). This resulting clustergram using a group average method with accompanying shade plot (Fig. 2) is based on a Bray–Curtis similarity resemblance matrix of untransformed relative percentage galactolipid composition data from this study and data taken from Gray *et al.* (2009*a*).

Results

Eighteen galactolipids were observed as sodium adducts in the examined species of *Amphidinium* (Table 1). Of these were nine forms of MGDG, eight forms of DGDG and one form of trigalactosyldiacylglycerol (TGDG) – TGDG was initially described in dinoflagellates by Gray *et al.* (2009*b*).

Table 1. Relative abundance (in % of total fragment height using listed masses) of galactolipids as determined via positive-ion ESI/MS.

		A. fijiensis	*A. magnum*	*A. paucianulatum*	*A. theodori*	*A. tomasii*	*Amphidinium* sp.	*T. testudo*
Galactolipid $[M+Na]^+$	Mass[1]	ARC 114	ARC 73	ARC 117	ARC 173	ARC 388	CB 153240	RCC 1981
18:1/16:0 MGDG	779		1.0 ± 0.3					
18:5/18:5 MGDG	789	5.4 ± 0.9	17.6 ± 13.0					4.9 ± 1.3
18:5/18:4 MGDG	791	7.0 ± 2.1	16.8 ± 3.2					27.3 ± 7.1
18:4/18:4 MGDG	793	7.9 ± 1.9	9.3 ± 2.2	3.3 ± 1.3		39.9 ± 5.2	7.6 ± 1.4	9.0 ± 2.7
18:5/18:3 MGDG	793							4.7 ± 1.3
20:5/18:5 MGDG	817	0.8 ± 0.2	1.4 ± 0.2	6.1 ± 3.8	23.9 ± 8.3			
20:5/18:4 MGDG	819	16.7 ± 5.0	6.5 ± 0.6	22.6 ± 6.6	23.3 ± 4.9	7.2 ± 1.5	24.6 ± 0.9	
20:5/20:5 MGDG	845						0.1 ± 0.1	
22:6/18:4 MGDG	845						2.4 ± 2.1	
18:1/14:0 DGDG	913							6.0 ± 2.2
18:2/16:0 DGDG	939							2.1 ± 0.8
18:1/16:0 DGDG	941							3.3 ± 0.8
18:5/18:5 DGDG	951	0.7 ± 0.2	0.8 ± 0.8					
18:5/18:4 DGDG	953	5.3 ± 0.4	4.8 ± 2.0					36.3 ± 7.7
18:4/18:4 DGDG	955	4.6 ± 1.2				22.6 ± 2.6		
20:5/18:5 DGDG	979	0.8 ± 0.4	1.0 ± 0.2	1.8 ± 0.4	9.4 ± 3.7			
20:5/18:4 DGDG	981	50.9 ± 8.1	40.7 ± 5.1	66.2 ± 8.8	43.4 ± 9.6	30.3 ± 6.5	65.2 ± 2.2	
18:1/14:0 TGDG	1075							6.3 ± 0.4

[1]Mass rounded down to nearest odd number for the purpose of simplification.

To give a sense of how positive-ion ESI/MS/MS analysis was used to determine with regiochemical specificity which fatty acids are part of which galactolipids, Fig. 1 displays the positive-ion ESI/MS/MS spectra of three minor galactolipids not previously discussed in detail in our lab's manuscript series 'Mono- and Digalactosyldiacylglycerol Composition of Dinoflagellates'.

Figure 1A of data from *Amphidinium* sp. CB 153240 shows a spectrum wherein two galactolipids, 22:6(n-3)/18:4 and 20:5/20:5 MGDG, both *m/z* 845, are represented. In this spectrum, the more intense *m/z* 517 fragment represents the mass of the lipid minus 22:6 preferentially cleaved from the *sn*-1 position, while the *m/z* 569 fragment represents the mass of the lipid minus 18:4 cleaved from the *sn*-2 position. The minor *m/z* 543 fragment represents either of the 20:5 fatty acids cleaved from the *sn*-1 or *sn*-2 positions of 20:5/20:5 MGDG.

Figure 1B also of data from *Amphidinium* sp. CB 153240 shows the spectrum of 18:2/16:0 DGDG (*m/z* 939) wherein the *m/z* 659 fragment represents the mass of the lipid minus the 18:2 fatty acid preferentially cleaved from the *sn*-1 position, and the *m/z* 683 fragment represents the mass of the lipid minus the 16:0 fatty acid cleaved from the *sn*-2 position. The *m/z* 777 fragment represents the mass of the lipid, minus a single galactose residue. The 18:2 fatty acid was determined via analysis of its DMOX derivative to be 18:2(n-6; data not shown).

Figure 1C of data from *T. testudo* likewise shows the spectrum of 18:1/16:0 DGDG wherein the *m/z* 659 fragment represents the mass of the lipid minus the 18:1 fatty acid preferentially cleaved from the *sn*-1 position. The *m/z* 685 and 779 fragments represent cleavage of the 16:0 fatty acid and a galactose residue, respectively. Analysis of DMOX derivatives indicated the presence of two 18:1 fatty acids, 18:1(n-9) and 18:1(n-8; data not shown). We cannot discriminate between these two in positive-ion ESI/MS/MS analysis, thus either or both could be part of 18:1/16:0 DGDG.

Figure 2 is intended to give a visual representation of the relative percentage distribution trends observed in Table 1 and includes a clustergram to show relatedness according to galactolipid composition. Figure 2, which also includes data for two *Amphidinium* species originally published by Gray *et al.* (2009*a*) illustrates that all of the species with the exception of *T. testudo* possess C_{20} /C_{18} forms of MGDG and DGDG as the most abundant galactolipids, thus placing them clearly within Cluster 2 per Gray *et al.* (2009*a*). This does not mean that there is a lack of C_{18}/C_{18} galactolipids within these *Amphidinium* species, but rather that C_{18}/C_{18} forms are generally, but not always, comparatively minor in terms of relative percentage distribution (Table 1) – such a phenomenon was also reported in Gray *et al.* (2009*a*).

T. testudo was the only species examined in this study which produced a form of TGDG, namely 18:1/14:0 TGDG (Table 1, Fig. 2).

Discussion

The genus *Amphidinium* is a large and diverse one whose recent phylogeny indicates the formation of two clades, the Operculatum Clade based on *Amphidinium operculatum* Claparède & Lachmann and the Herdmanii Clade based on *Amphidinium herdmanii* Kofoid & Swezy, per the observations of Karafas *et al.* (2017). With the exception of *Amphidinium* sp. CB 153240, for which clade association has not been determined, the *Amphidinium* species we have examined all derive from the Operculatum Clade. Arguably the most examined

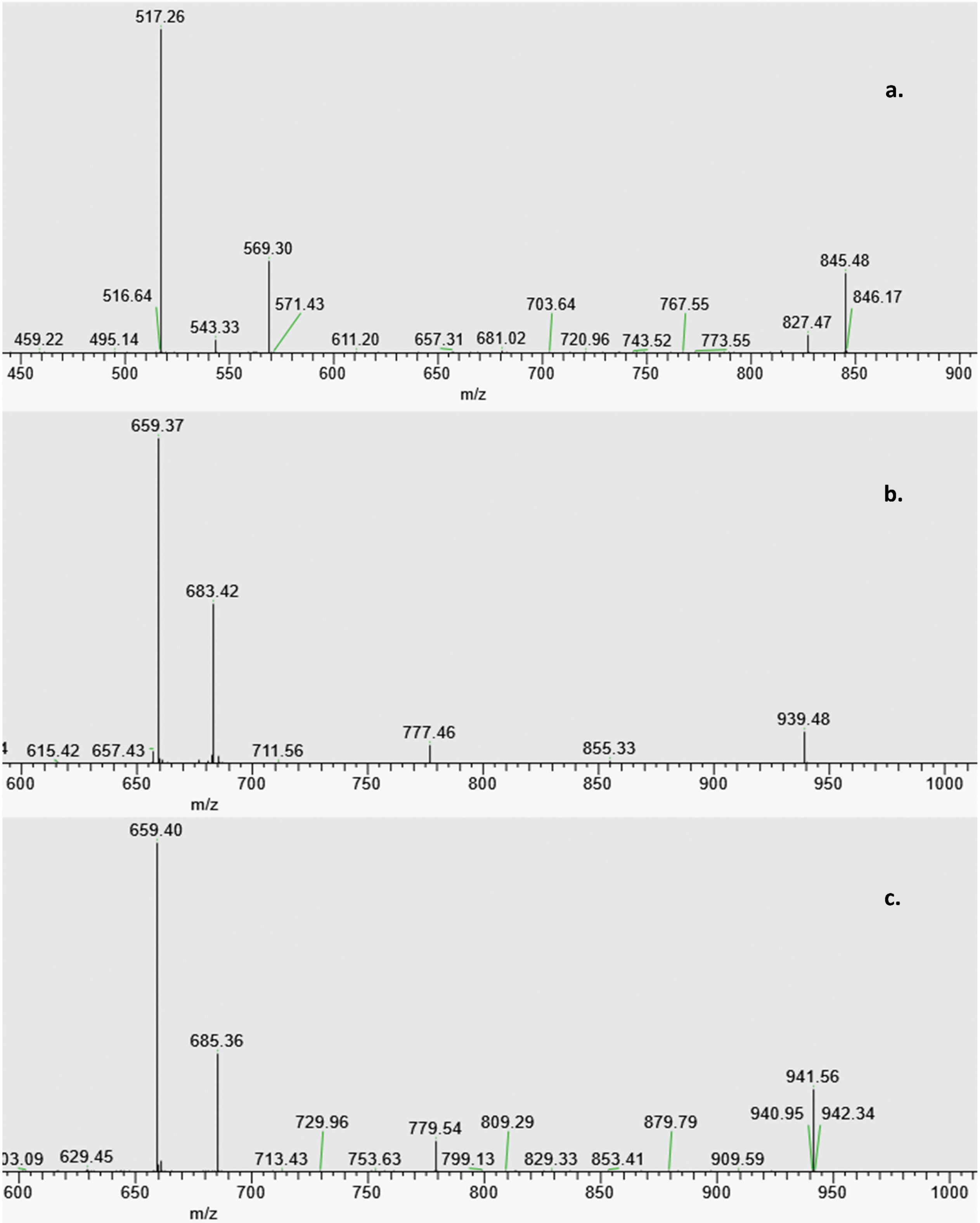

Fig. 1. Positive-ion ESI/MS/MS spectrum of sodium adducts of: (A) Mixture of 22:6/18:4 and 20:5/20:5 MGDG (both *m/z* 845) from *Amphidinium* sp. CB 153240 and (B) 18:2/16:0 DGDG (*m/z* 939) and (C) 18:1/16:0 DGDG (*m/z* 941) both from *Testudodinium testudo* RCC 1981.

species of the Operculatum Clade is *A. carterae*, which appears in many dinoflagellate phylogenies and has a basal position amongst photosynthetic dinoflagellates (cf. Bachvaroff *et al*., 2014). Thus, we interpret, with the exception of *Amphidinium* sp. and *T. testudo*, the remaining *Amphidinium* species of the Operculatum Clade examined within this study as being in a similar basal position. Future research will focus on the Herdmanii Clade outlined by Karafas *et al.* (2017).

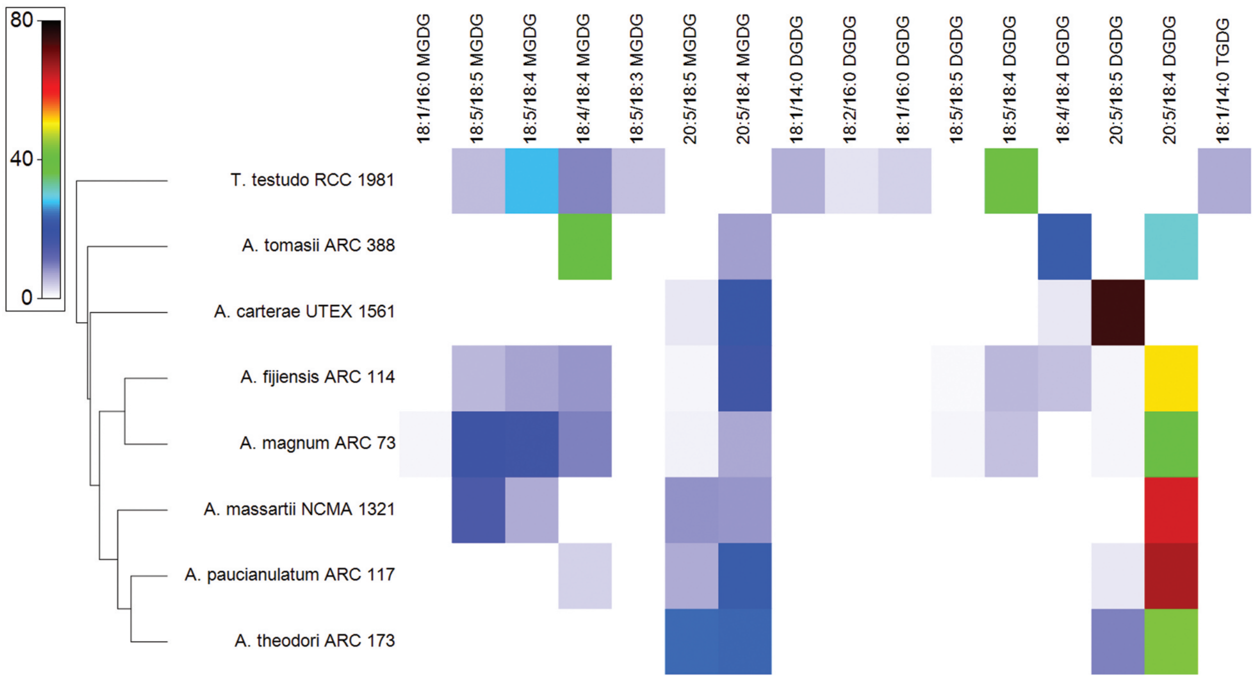

Fig. 2. Bray–Curtis similarity clustergram with corresponding shade plot of relative percentages of galactolipids in *Amphidinium* species and *Testudodinium testudo*. Data for *Amphidinium carterae* UTEX 1561 and *A. massartii* Biecheler NCMA 1821 originate from Gray *et al.* (2009*a*). All other data originate from this study. The scale indicates relative percentage values.

Regarding the data shown in Table 1 and Fig. 2, it is apparent that the members of the Operculatum Clade, as well as *Amphidinium* sp. CB 153240, generally possess 20:5/18:5 or 20:5/18:4 DGDG as the most abundant galactolipid, with lesser amounts of 20:5/18:5 and 20:5/18:4 MGDG, and polyunsaturated C_{18}/C_{18} forms of MGDG and DGDG. Thus, these members of the Operculatum Clade are located within the C_{20}/C_{18} cluster (Cluster 2) identified by Gray *et al.* (2009*a*). Regarding polyunsaturated C_{18}/C_{18} forms of MGDG and DGDG, it should be noted that some species, such as *A. tomasii*, do possess some C_{18}/C_{18} forms of MGDG and/or DGDG that are among the most abundant galactolipids. The implication of any C_{18}/C_{18} forms of MGDG and/or DGDG on our hypothesis is discussed below. Regarding the galactolipids of *T. testudo*, it is distinctly lacking in any C_{20}/C_{18} galactolipids (Table 1, Fig. 2), thus providing supporting evidence for its renaming as *T. testudo*.

With this assumption that these species of *Amphidinium* represent a group of basal photosynthetic dinoflagellates, per the phylogenies listed earlier yet recognizing the caveats we list in the Introduction, we hypothesize that their galactolipid compositions at the current time represent the most basal forms of the galactolipids of peridinin-containing dinoflagellates originally identified by Gray *et al.* (2009*a*). In other words, we hypothesize that deviation away from the galactolipid composition of these Operculatum Clade species of *Amphidinium*, such as to what is found within the C_{18}/C_{18} cluster (Cluster 1) and *T. testudo*, represents evolution from one biosynthetic capability to another. It must be noted that while C_{20}/C_{18} galactolipids are generally the most abundant in the *Amphidinium* species we examined, there are also C_{18}/C_{18} galactolipids present. Thus, these *Amphidinium* species present a mixture of C_{20}/C_{18} and C_{18}/C_{18} forms. If one considers that 18:4 and 18:5 are potential precursors to 20:5, and that members of the C_{20}/C_{18} cluster typically have one of these two polyunsaturated C_{18} fatty acids in the *sn*-2 position of the most abundant galactolipids, then it is likely that members of the C_{18}/C_{18} cluster have lost, or minimized, the ability to incorporate the 20:5 fatty acid into their galactolipids, leading to a lack of C_{20}/C_{18} galactolipids. Thus, in the biosynthetic scheme shown in Leblond *et al.* (2015), members of the C_{20}/C_{18} cluster have 20:5-containing precursor lipids migrating from the endoplasmic reticulum to the plastid, whereas this is inhibited in members of the C_{18}/C_{18} cluster. Parenthetically, note that it is much more common in published pathways, such as that by Domergue *et al.* (2002), in other groups of model algae (and plants) for 18:4 to precede 20:4(n-3) leading to 20:5, as 18:5 is not mentioned, and that it is our hypothesis that 18:5 is a potential precursor to 20:5 because it is a such common fatty acid in dinoflagellate lipids (cf. Jónasdóttir, 2019).

It is not possible to say at this time that the inherent ability to produce 20:5 is entirely absent because many of the Cluster 1 dinoflagellates produce docosahexaenoic acid (22:6) as part of their phospholipid-containing lipid fraction (Leblond & Chapman, 2000), and 20:5 is a possible precursor to 22:6 during fatty acid elongation and unsaturation within both Δ6- and Δ8-based biosynthetic pathways (Li-Beisson *et al.*, 2019). Rather, for example, perhaps there is a step(s) that prevents 20:5-containing phosphatidylcholine from accumulating in the endoplasmic reticulum (ER) and/or being transferred from the ER to the chloroplast per the model presented by Dahmen *et al.* (2013) for the Cluster 2 dinoflagellate *Lingulodinium polyedrum*, with the now corrected name of *Lingulodinium polyedra* (F.Stein) J.D.Dodge (Guiry & Guiry, 2021).

As an alternative hypothesis, it is possible that C_{18}/C_{18} cluster dinoflagellates are ancestral to C_{20}/C_{18} cluster dinoflagellates, with the ability to produce 20:5 having arisen independently amongst the C_{20}/C_{18} cluster dinoflagellates identified by Gray *et al.* (2009*a*). However, this seems less parsimonious than a single loss of function in an ancestral C_{20}/C_{18} cluster dinoflagellate leading to the dinoflagellates currently in the C_{18}/C_{18} cluster, especially given their ability to produce 22:6 in non-galactolipid, fatty acid-containing lipids and considering the additional supportive points listed below. As a second alternative hypothesis, it is also less parsimonious to hypothesize that some C_{20}/C_{18} cluster dinoflagellates independently transitioned 'back' to become C_{18}/C_{18} cluster dinoflagellates, although we cannot rule either of these alternative hypotheses out.

The chloroplast of peridinin-containing dinoflagellates is considered to have arisen from a secondary endosymbiotic event involving a red alga (see reviews by Keeling, 2004, 2010). While the red algae are an expansive group, an examination of the galactolipids of two species, *Polysiphonia* sp. and *Porphyridium* sp., has demonstrated that several 20:5-containing galactolipids are present, and that 20:5 is present singly in the *sn*-1 or doubly in the *sn*-1 and *sn*-2 positions (Carter & Leblond, 2018) – further discussion on the production of 20:5 within red algae is provided by Dodson *et al.* (2013) and Carter & Leblond (2018). Conversely, the 18:5 and 18:4 fatty acids were not found to be present within the galactolipids of these red algae, although mono- and diunsaturated C_{18} fatty acids were (Carter & Leblond, 2018). The implication in our hypothesis is that members of the Operculatum Clade inherited their ability to produce 20:5-containing galactolipids from red algae, and that the presence of 18:5 and 18:5 in the *sn*-2 position of these galactolipids was developed after inheritance of the red algal plastid. To further support our hypothesis that the Operculatum Clade possesses a set of basal galactolipids, we present the following additional information. First, we have recently examined the galactolipid composition of a newly isolated member of the Kareniaceae, *Gertia stigmatica* K. Takahashi, Benico, Wai Mun Lum & Iwataki, which differs markedly from other Kareniaceae in that it possesses a peridinin-containing plastid (lacking fucoxanthin and 19′-acyloxyfucoxanthins) of red algal origin, much like non-Kareniaceae, peridinin-containing photosynthetic dinoflagellates (Takahashi *et al.*, 2019). In this recent work we have found *G. stigmatica* to possess 20:5/18:5 MGDG and DGDG as the principal galactolipids (Leblond & Sabir, in press). This result is significant because members of the Kareniaceae have also been observed as early branching dinoflagellates (Hoppenrath & Leander, 2010; Bachvaroff *et al.*, 2014; Bolch, 2021), but direct comparison of their plastid galactolipids to those of peridinin-containing dinoflagellates has not been possible because, prior to the discovery of *G. stigmatica*, all members of the Kareniaceae had been considered to possess tertiary plastids of haptophyte origin (see for example the review by Waller & Kořený, 2017), and their galactolipids' compositions reflect the endosymbiont which has become the aberrant plastid (Leblond & Lasiter, 2009; Graeff *et al.*, 2021).

Second, examination of the heterotrophic, basal dinoflagellates *Amoebophrya* sp. and *Oxyrrhis marina* Dujardin (see example studies listed in the previous paragraph to observe their basal phylogenetic placement) revealed the absence of any of the galactolipids discussed thus far as they relate to Clusters 1 and 2 of peridinin-containing dinoflagellates (Leblond & Dahmen, 2012; Leblond *et al.*, 2013). This indicates that these galactolipids did not exist in at least two heterotrophic dinoflagellates prior to acquisition of the secondary red algal plastid.

Third, as summarized by Weatherby & Carter (2013), chromerids and peridinin-containing dinoflagellates share a common red algal plastid ancestor. The chromerid *Chromera velia* R.B.Moore *et al.* has MGDG and DGDG enriched in 20:5 but deficient in polyunsaturated C_{18} fatty acids (Botté *et al.*, 2011; Dahmen *et al.*, 2013). This lends evidence that the C_{20}/C_{18} cluster galactolipids pre-date the C_{18}/C_{18} cluster galactolipids. When considering other algal lineages, such as diatoms and haptophytes, that also trace their plastids back to red algae (cf. Keeling, 2004), it should be noted that, as discussed in Dodson *et al.* (2013) and Leblond *et al.* (2019), there are few other studies where the intact galactolipids of these algal classes have been characterized, with most studies having identified individual fatty acids derived from total lipids (i.e. without regiochemical assignment to MGDG and DGDG). Nevertheless, in the small sampling of diatoms examined they have been observed to display species-specific enrichments of polyunsaturated C_{20} and/or C_{18} fatty acids in their MGDG and DGDG (Dodson *et al.*, 2013 and references therein). Leblond & Lasiter (2009) reported MGDG and DGDG enriched in 18:5 in a single haptophyte, *Emiliania huxleyi* (Lohm.) Hay & Mohler. Thus, we are hesitant to draw firm conclusions as to the distribution of 20:5 in the galactolipids of these algal classes.

Ultimately, the origins of the galactolipid compositions of Cluster 1 and Cluster 2 peridinin-containing dinoflagellates should be elucidated via genetic characterization of relevant galactolipid biosynthesis pathways, as Riccio *et al.* (2020) have begun to accomplish for MGDG synthase as found in a wide assortment of algae, including some dinoflagellates. However, it will take phylogenetic analysis of more than a single gene to accomplish this because galactolipid biosynthesis involves several enzymes that link galactose(s) and two fatty acids to a glycerol backbone, all in the midst of an interplay between the chloroplast and ER (Benning, 2009; Sata & Awai, 2016). Such data should of course be coupled with the phenotypic data of actual galactolipid composition characterization, as we have presented here, to give the fullest picture of galactolipid origins.

Gray *et al.* (2009*a*) initially proposed a possible habitat-specific, life form-adaptive strategy to explain why a peridinin-containing dinoflagellate would be in one cluster or another, whereas a more recent study by Anesi *et al.* (2016) suggests that the galactolipid-associated fatty acid chain lengths relate more specifically to the temperature ranges encountered by a given dinoflagellate. If our hypothesis is correct, then some peridinin-containing dinoflagellates outside of the basal genus *Amphidinium* have maintained Cluster 2-type galactolipid composition, while others are more derived and have evolved to possess a Cluster 1-type.

Acknowledgement

The help of Stephanie Vandergrift in culture harvesting and lipid extraction was much appreciated. The valuable comments of two anonymous reviewers were much appreciated.

Disclosure statement

No potential conflict of interest was reported by the authors.

Author contributions

J.D. Leblond: original concept, lipid processing and analysis, drafting and editing manuscript; L.C. Elkins: culture growth, lipid processing, drafting and editing manuscript; J.E. Graeff: lipid processing, drafting and editing manuscript; K. Sabir: lipid processing.

References

Anesi, A., Obertegger, U., Hansen, G., Sukenik, A., Flaim, G. & Guella, G. (2016). Comparative analysis of membrane lipids in psychrophilic and mesophilic freshwater dinoflagellates. *Frontiers in Plant Science*, **7**: 524.

Bachvaroff, T.R., Gornik, S.G., Concepcion, G.T., Waller, R.F., Mendez, G.S., Lippmeier, J.C. & Delwiche, C.F. (2014). Dinoflagellate phylogeny revisited: Using ribosomal proteins to resolve deep branching dinoflagellate clades. *Molecular Phylogenetics and Evolution*, **70**: 314–322.

Benning, C. (2009). Mechanism of lipid transport involved in organelle biogenesis in plant cells. *Annual Review of Cell and Developmental Biology*, **25**: 71–91.

Bolch, C.J.S. (2021). Impact of molecular approaches on dinoflagellate taxonomy and systematics. In *Advances in Phytoplankton Ecology* (Clementson, L.A., Eriksen, R.S. & Willis, A., editors), 81–117, Elsevier, Amsterdam.

Botté, C.Y., Yamaryo-Botté, Y., Janouškovec, J., Rupasinghe, T., Keeling, P.J., Crellin, P., Coppel, R.L., Maréchal, E., McConville, M.J. & McFadden, G.I. (2011). Identification of plant-like galactolipids in *Chromera velia*, a photosynthetic relative of malaria parasites. *Journal of Biological Chemistry*, **286**: 29893–29903.

Carter, J.D. & Leblond, J.D. (2018). Red (hot) algae: modulation of mono- and digalactosyldiacylglycerol-associated fatty acids of *Polysiphonia* sp. and *Porphyridium* sp. in response to growth temperature. *European Journal of Phycology*, **53**: 460–470.

Dahmen, J.L., Khadka, M., Dodson, V.J. & Leblond, J.D. (2013). Mono- and digalactosyldiacylglycerol composition of dinoflagellates. VI. Biochemical and genomic comparison of galactolipid biosynthesis between *Chromera velia* (Chromerida), a photosynthetic alveolate with red algal plastid ancestry, and the dinoflagellate, *Lingulodinium polyedrum*. *European Journal of Phycology*, **48**: 268–277.

Dodson, V.J., Dahmen, J.L., Mouget, J.-L. & Leblond, J.D. (2013). Mono- and digalactosyldiacylglycerol composition of the marennine-producing diatom, *Haslea ostrearia*: comparison to a selection of pennate and centric diatoms. *Phycological Research*, **61**: 199–207.

Domergue, F., Lerchl, J., Zähringer, U. & Heinz, E. (2002). Cloning and functional characterization of *Phaeodactylum tricornutum* front-end desaturases involved in eicosapentaenoic acid biosynthesis. *European Journal of Biochemistry*, **269**: 4105–4113.

Dorrell, R.G. & Howe, C.J. (2015). Integration of plastids with their hosts: lessons learned from dinoflagellates. *Proceedings of the National Academy of Sciences USA*, **112**: 10247–10254.

Flø Jørgensen, M., Murray, S. & Daugbjerg, N. (2004). *Amphidinium* revisited. I. Redefinition of *Amphidinium* (Dinophyceae) based on cladistic and molecular phylogenetic analyses. *Journal of Phycology*, **40**: 351–365.

Fay, L. & Richli, U. (1991). Location of double bonds in polyunsaturated fatty acids by gas chromatography-mass spectrometry after 4,4-dimethyloxazoline derivatization. *Journal of Chromatography A*, **541**: 89–98.

Gao, J., Wang, H., Yuan, Q. & Feng, Y. (2018). Structure and function of the photosystem supercomplexes. *Frontiers in Plant Sciences*, **9**: 357.

Gómez, F. & Souissi, S. (2007). The distribution and life cycle of the dinoflagellate *Spatulodinium pseudonoctiluca* (Dinophyceae, Noctilucales) in the northeastern English Channel. *C. R. Biologies*, **330**: 231–236.

Gómez, F., Moreira, D. & López-García, P. (2010). Molecular phylogeny of noctilucoid Dinoflagellates (Noctilucales, Dinophyceae). *Protist*, **161**: 466–478.

Graeff, J.E., Elkins, L.C. & Leblond, J.D. (2021). Plastid-associated galactolipid composition in eyespot-containing dinoflagellates: a review. *Algae*, **36**: 73–90.

Gray, C.G., Lasiter, A.D. & Leblond, J.D. (2009a). Mono- and digalactosyldiacylglycerol composition of dinoflagellates. I. Peridinin-containing taxa. *European Journal of Phycology*, **44**: 191–197.

Gray, C.G., Lasiter, A.D. & Leblond, J.D. (2009b). Mono- and digalactosyldiacylglycerol composition of dinoflagellates. III. Four cold-adapted, peridinin-containing taxa and the presence of trigalactosyldiacylglycerol as an additional glycolipid. *European Journal of Phycology*, **44**: 439–445.

Guella, G., Frassanito, R. & Mancini, I. (2003). A new solution for an old problem: the regiochemical distribution of the acyl chains in galactolipids can be established by electrospray ionization tandem mass spectrometry. *Rapid Communications in Mass Spectrometry*, **17**: 1982–1994.

Guillard, R.R.L. (1975). Culture of phytoplankton for feeding marine invertebrates. In *Culture of Marine Invertebrate Animals* (Smith, W.L. & Chanley, M.H., editors), 26–60. Plenum Press, New York.

Guillard, R.R.L. & Ryther, J.H. (1962). Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervacea* Cleve. *Canadian Journal of Microbiology*, **8**: 229–239.

Guiry, M.D. & Guiry, G.M. (2021). *AlgaeBase*. World-wide electronic publication, National University of Ireland, Galway. https://www.algaebase.org; searched on 23 December 2021.

Hernández, M.L. & Cejudo, F.J. (2021). Chloroplast lipids metabolism and function. A redox perspective. *Frontiers in Plant Science*, **12**: 712022.

Hölzl, G. & Dörmann, P. (2019). Chloroplast lipids and their biosynthesis. *Annual Review of Plant Biology*, **70**: 51–81.

Hoppenrath, M. & Leander, B.S. (2010). Dinoflagellate phylogeny as inferred from heat shock protein 90 and ribosomal gene sequences. *PLoS ONE*, **5**: e13220.

Horiguchi, T., Tamura, M., Katsumata, K. & Yamaguchi, A. (2012). *Testudodinium* gen. nov. (Dinophyceae), a new genus of sand-dwelling dinoflagellates formerly classified in the genus *Amphidinium*. *Phycological Research*, **60**: 137–149.

Janouškovec, J., Gavelis, G.S., Burki, F., Dinh, D., Bachvaroff, T.R., Gornik, S.G., Bright, K.J., Imanian, B., Strom, S.L., Delwiche, C.F., Waller, R.F., Fensome, R.A., Leander, B.S., Rohwer, F.L. & Saldarriaga, J.F. (2017). Major transitions in dinoflagellate evolution unveiled by phylotranscriptomics. *Proceedings of the National Academy of Sciences USA*, **114**: e171–e180.

Jeffrey, S.W., Sielicki, M. & Haxo, F.T. (1975). Chloroplast pigment patterns in dinoflagellates. *Journal of Phycology*, **11**: 374–384.

Jónasdóttir, S.H. (2019). Fatty acid profiles and production in marine phytoplankton. *Marine Drugs*, **17**: 151.

Karafas, S, Teng, S.T., Leaw, C.P. & Alves-de-Souza, C. (2017). An evaluation of the genus *Amphidinium* (Dinophyceae) combining evidence from morphology, phylogenetics, and toxin production, with the introduction of six novel species. *Harmful Algae*, **68**: 128–151.

Keeling, P.J. (2004). Diversity and evolutionary history of plastids and their hosts. *American Journal of Botany*, **91**: 1481–1493.

Keeling, P.J. (2010). The endosymbiotic origin, diversification and fate of plastids. *Philosophical Transactions of the Royal Society B*, **365**: 729–748.

Leblond, J.D. & Chapman, P.J. (2000). Lipid class distribution of highly unsaturated long-chain fatty acids in marine dinoflagellates. *Journal of Phycology*, **36**: 1103–1108.

Leblond, J.D. & Dahmen, J.L. (2012). Mono- and digalactosyldiacylglycerol composition of dinoflagellates. V. The galactolipid profile of *Alexandrium tamarense* (Dinophyceae) during the course of infection by the parasitic syndinian dinoflagellate *Amoebophrya* sp. *European Journal of Phycology*, **47**: 490–497.

Leblond, J.D. & Lasiter, A.D. (2009). Mono- and digalactosyldiacylglycerol composition of dinoflagellates. II. *Lepidodinium chlorophorum, Karenia brevis*, and *Kryptoperidinium foliaceum*, three dinoflagellates with aberrant plastids. *European Journal of Phycology*, **44**: 199–205.

Leblond, J.D. & Sabir, K. (In press). Mono- and digalactosyldiacylglycerol composition of dinoflagellates. IX. Galactolipid composition of *Gertia stigmatica* (Dinophyceae), an atypical member of the Kareniaceae with a peridinin-containing, non-haptophyte-derived plastid. *Phycological Research*.

Leblond, J.D., Dodson, J. & Dahmen, J.D. (2013). Mono- and digalactosyldiacylglycerol composition of dinoflagellates. VII. Evidence against galactolipid production and plastid presence in the heterotrophic, basal dinoflagellate, *Oxyrrhis marina*. *European Journal of Phycology*, **48**: 309–317.

Leblond, J.D., Khadka, M., Duong, L. & Dahmen, J.L. (2015). Squishy lipids: temperature effects on the betaine and galactolipid profiles of a C_{18}/C_{18} peridinin-containing dinoflagellate, *Symbiodinium microadriaticum* (Dinophyceae), isolated from the mangrove jellyfish, *Cassiopea xamachana*. *Phycological Research*, **63**: 219–230.

Leblond, J.D., McDaniel, S.L., Lowrie, S.D., Khadka, M. & Dahmen, J. (2019). Mono- and digalactosyldiacylglycerol composition of dinoflagellates. VIII. Temperature effects and a perspective on the curious case of *Karenia mikimotoi* as a producer of the unusual, 'green algal' fatty acid hexadecatetraenoic acid [16:4(n-3)]. *European Journal of Phycology*, **54**: 78–90.

Li-Beisson, Y., Thelen, J.J., Fedosejevs, E. & Harwood, J.L. (2019). The lipid biochemistry of eukaryotic algae. *Progress in Lipid Research*, **74**: 31–68.

Murray, S.A., Garby, T., Hoppenrath, M. & Neilan, B.A. (2012). Genetic diversity, morphological uniformity and polyketide production in dinoflagellates (*Amphidinium*, Dinoflagellata). *PLoS ONE*, **7**: e38253.

Orr, R.J.S., Murray, S.A., Stűken, A., Rhodes, L. & Jakobsen, K.S. (2012). When naked became armored: an eight-gene phylogeny reveals monophyletic origin of theca in dinoflagellates. *PLoS ONE*, **7**: e50004.

Pinto, S.K., Terada, R. & Horiguchi, T. (2017). *Testudodinium magnum* sp. nov. (Dinophyceae), a novel marine sand-dwelling dinoflagellate from subtropical Japan. *Phycologia*, **56**: 136–146.

Riccio, G., De Luca, D. & Lauritano, C. (2020). Monogalactosyldiacylglycerol and sulfolipid synthesis in microalgae. *Marine Drugs*, **18**: 237.

Sarai, C., Tanifuji, G., Nakayama, T., Kamikawa, R., Takahashi, K., Yazaki, E., Matsuo, E., Miyashita, H., Ishida, K., Iwataki, M. & Inagaki, Y. (2020). Dinoflagellates with relic endosymbiont nuclei as models for elucidating organellogenesis. *Proceedings of the National Academy of Sciences USA*, **117**: 5364–5375.

Sata, N. & Awai, K. (2016). Diversity in biosynthetic pathways of galactolipids in the light of endosymbiotic origin of chloroplasts. *Frontiers in Plant Science*, **7**: 117.

Takahashi, K., Benico, G., Lum, W.M. & Iwataki, M. (2019). *Gertia stigmatica* gen. et sp. nov. (Kareniaceae, Dinophyceae), a new marine unarmored dinoflagellate possessing the peridinin-type chloroplast with an eyespot. *Protist*, **170**: 125680.

Waller, R.F. & Kořený, L. (2017). Plastid complexity in dinoflagellates: a picture of gains, losses, replacements and revisions. *Advances in Botanical Research*, **84**: 105–143.

Weatherby, K. & Carter, D. (2013). *Chromera velia*: the missing link in the evolution of parasitism. *Advances in Applied Microbiology*, **85**: 119–144.

Zapata, M., Fraga, S., Rodríguez, F. & Garrido, J.L. (2012). Pigment-based chloroplast types in dinoflagellates. *Marine Ecology Progress Series*, **465**: 33–52.

Zhang, H., Bhattacharya, D. & Lin, S. (2007). A three-gene dinoflagellate phylogeny suggests monophyly of Prorocentrales and a basal position for *Amphidinium* and *Heterocapsa*. *Journal of Molecular Evolution*, **65**: 463–474.