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Plastid complexity in dinoflagellates

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Plastid complexity in dinoflagellates: a picture of gains, losses, replacements and revisions

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Abstract

Dinoflagellates are exemplars of plastid complexity and evolutionary possibility. Their ordinary plastids are extraordinary, and their extraordinary plastids provide a window into the processes of plastid gain and integration. No other plastid-bearing eukaryotic group possesses so much diversity or deviance from the basic traits of this cyanobacteria-derived endosymbiont. Although dinoflagellate plastids provide a major contribution to global carbon fixation and energy cycles, they show a remarkable willingness to tinker, modify and dispense with canonical function. The archetype dinoflagellate plastid, the peridinin plastid, has lost photosynthesis many times, has the most divergent organelle genomes of any plastid, is bounded by an atypical plastid membrane number, and uses unusual protein trafficking routes. Moreover, dinoflagellates have gained new endosymbionts many times, representing multiple different stages of the processes of organelle formation. New insights into dinoflagellate plastid biology and diversity also suggests it is timely to revise notions of the origin of the peridinin plastid.

I. Introduction

Dinoflagellates represent a major plastid-bearing protist lineage that diverged from a common ancestor shared with apicomplexan parasites (Figure 1). Since this separation dinoflagellates have come to exploit a wide range of marine and aquatic niches, providing critical environmental services at a global level, as well as having significant negative impact on some habitats and communities.

As photosynthetic organisms, dinoflagellates contribute a substantial fraction of global carbon fixation that drives food webs as well as capture of some anthropogenic CO_2. Their plastids are also critical to symbiotic associations with a wide range of marine animals. These include symbioses with reef-building corals that are essential for building and maintaining diverse tropical habitats, and that in turn provide barrier protection of vast coastlines from wave erosion and inundation. The loss of the plastid-bearing symbiont leads to coral bleaching, ultimately death of the animal partners and degradation of the reef systems. Plastids of dinoflagellates also generate DMS (dimethyl sulfide) emissions that play an important role in cloud formation and drive major climatic processes, including those over terrestrial habitats (Charlson, Lovelock, Andreae, & Warren, 1987). The success of dinoflagellates in exploiting nutrient sources, however, can also result in vast blooms up to hundreds of kilometres long that can have major negative impacts. Blooms of taxa that synthesise toxins can lead to accumulation of lethal or injurious toxin doses resulting in widespread death of fish and marine mammals, as well as fatalities in humans. In other cases, the density of dinoflagellate blooms is simply enough to deplete available oxygen resulting in animal mortality through suffocation.

Not all dinoflagellates are photosynthetic, in fact only approximately half of described taxa are (Gómez, 2012). Those that are obligate heterotrophs exploit a wide range of heterotrophic strategies, including micropredation, osmotrophy and parasitism (Coats, 1999;

Gaines & Elbrächter, 1987; P. J. Hansen & Calado, 1999; Stoecker, 1999). Dinoflagellates, therefore, perform important roles in nutrient recycling and control of prey organism populations. But as parasites they can cause disease out-breaks and even collapses of fisheries stocks (Stentiford & Shields, 2005). Interestingly, most, if not all of these non-photosynthetic taxa retain relicts of a photosynthetic plastid, typically as colourless organelles but sometimes merely enzymes or biochemical pathways derived from a former resident plastid. These plastid relicts, therefore, continue to provide metabolic capacity and at least some independence from heterotrophy.

The very wide range of lifestyles that dinoflagellates have adopted have allowed wide exploitation of environmental niches. These include planktonic and sessile niches, as well as those within other organisms, be them symbiotic or parasitic partnerships (Gómez, 2012). Recent global ocean sampling of microeukaryotic abundance and diversity places dinoflagellate taxa as one of the most species rich and abundant groups, a testimony to their tremendous success and importance (de Vargas et al., 2015). Notably these data indicate that approximately three quarters of dinoflagellate taxa detected remain undescribed morphologically, begging the question of what diversity in this group remains to be explored and understood.

The pursuit of a robust understanding of the evolutionary relationships that unite and explain dinoflagellate diversity has long tormented systematists and phylogeneticists. This has been due to both convergent evolution of some traits, and high rates of molecular evolution that confounds single-gene phylogenies. Large datasets and phylogenomics, however, are now providing some clearer resolution that is concordant with evolutionary trends such as thecal development and the divergent molecular biology that exists in dinoflagellates (Figure 1) (Bachvaroff, Handy, Place, & Delwiche, 2011; Dorrell et al., 2017; Janouškovec et al., 2017). A clearer picture has also formed of the deep-branching dinoflagellate lineages and their relationships to the nearest non-dinoflagellate neighbours (Janouškovec et al., 2015). *Spathulodinium* of the Noctilucales represents the deepest branching photosynthetic dinoflagellate (Gómez, Moreira, & López-García, 2010; Janouškovec et al., 2017), and beneath that branches the chiefly parasitic Syndiniales. *Oxyrrhis* is the deepest 'true' dinoflagellate, and the nearest major group to dinoflagellates are the Perkinsozoa which share some traits characteristic of dinoflagellates but also lacks many (H. Zhang, Campbell, Sturm, Dungan, & Lin, 2011). Collectively this lineage, referred to as Dinozoa, is sister to the apicomplexan lineage that is best known for its wide range of parasites including human pathogens *Plasmodium* spp. that causes malaria. However, the apicomplexan lineage also has diverse roots including paraphyletic photosynthetic clades *Chromera* and *Vitrella* and a range of colpodellid predators (Figure 1) (Janouškovec et al., 2015).

In the context of the diversity of dinoflagellates and our current understanding of the phylogeny of this group, this chapter addresses the presence and nature of dinoflagellate plastids. Dinoflagellate plastids possess some of the most unusual character states for any plastid-containing group. Further, there have been multiple independent endosymbiotic gains of plastids within dinoflagellates, and this provides remarkable opportunities to consider the modifications to both host and symbiont following such seminal creative events that have been so important in the formation of eukaryotes. In turn, these insights provide a perspective on plastid gain and evolution that is instrumental in considering plastid origins throughout eukaryotes.

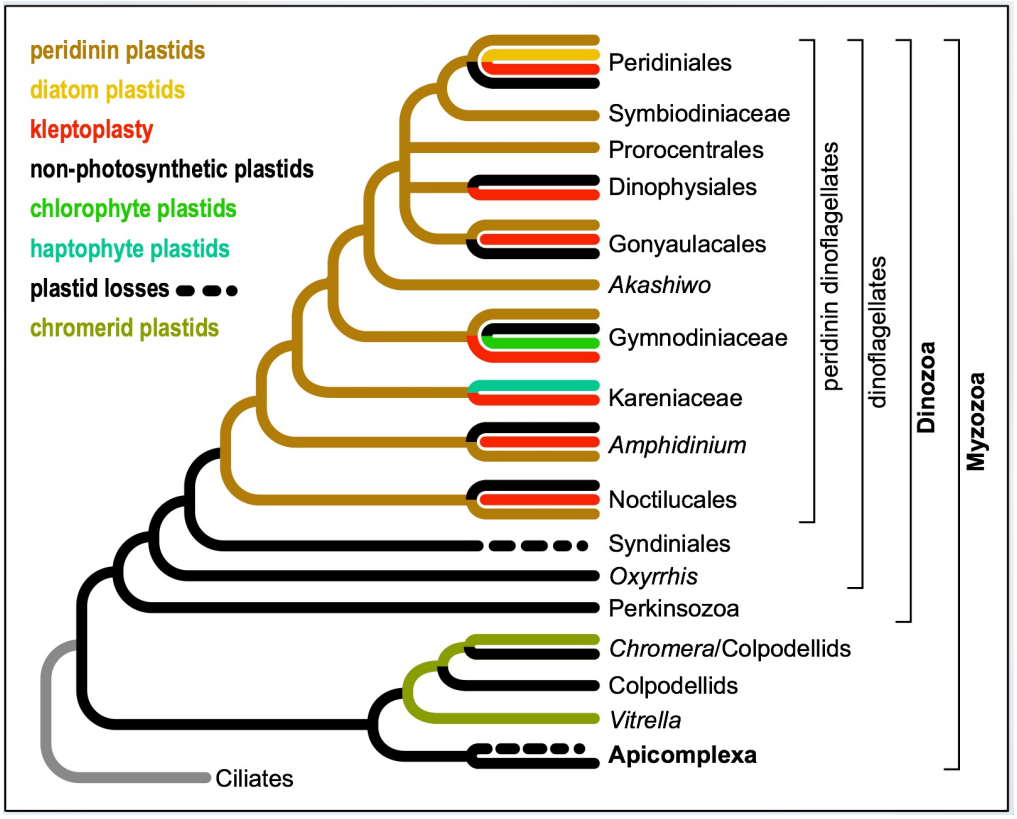

Fig 1: Schematic of dinoflagellate plastid diversity within the context of alveolate phylogeny. For each clade, the range of plastid types documented are indicated by colour. Conservative ancestral plastid state is indicated by clade branch colour where multiple lineages have a common plastid type. Phylogeny is based on Janouškovec et al. 2017.

II. Unusual features of dinoflagellates that might impact their endosymbionts

Before exploring dinoflagellate plastids in detail, and in particular the features that show variance from those characters found more widely in plastid-containing eukaryotes, it is worth considering some more general features of dinoflagellate biology that might have had impact on the establishment and development of their symbionts.

Most, if not all, photosynthetic dinoflagellates are mixotrophic (Stoecker, 1999). They gain part of their nutrition through a wide range of heterotrophic strategies that provide an alternative source of reduced carbon for energy, as well as necessary elements, minerals and/or vitamins (Gaines & Elbrächter, 1987). Growth studies for *Karlodinium veneficum* (= *Gyrodinium galatheanum*) for example, have shown that by combining feeding and photosynthesis, not only are growth rates increased, but photosynthetic efficiency is also increased, thus a synergy of these two modes of nutrition (Li, Stoecker, & Adolf, 1999). Presumably photosynthetic capacity is often limited by some key nutrients, and heterotrophy overcomes or relaxes these limitations. A further example is the coral symbiont, *Symbiodinium*, that is shown to be an effective free-living predator in order to survive in nutrient replete coral reef waters when outside of its symbiont (Jeong, Yoo, & Kang, 2012). Conversely, the copepod parasite *Dissodinium pseudolunula* retains photosynthesis which it is thought to rely upon during its dispersal stage (Stoecker, 1999). Thus, dinoflagellates show that maintaining multiple nutritional modes is a versatile strategy that can be stably maintained.

Dinoflagellates have developed some ingenious forms of food capture that enable them to hunt, track and ingest a wide range of food items. In some taxa, swimming behaviours are observed to change in response to proximity to prey, and ejectile organelles (trichocysts and nematocysts) can be discharged to immobilize and ensnare their victims (Sheng et al., 2007). Upon capture, prey can be ingested through a number of means

(Gaines & Elbrächter, 1987; P. J. Hansen & Calado, 1999; Jeong, Yoo, Kim, Seong, & Kang, 2010; Stoecker, 1999). One strategy employs a specialised appendage known as a peduncle to puncture the prey and extract cell contents through this specialised straw. Both liquid cell contents, and whole organelles are internalised into food vacuoles for digestion. This form of feeding is called myzocytosis, and is seen widely in dinoflagellates as well as lineages related to apicomplexan parasites (e.g. *Colpodella*). Indeed, the peduncle shares ultrastructural similarity to the apical complex of Apicomplexa, which in this group is the major instrument of host cell entry for parasitism (Dodge & Crawford, 1970; Norén, Moestrup, & Rehnstam-Holm, 1999; Siddall, Reece, Graves, & Burreson, 1997). This suggests that an ancestral feeding behaviour has enabled both parasitism in Apicomplexa, and ongoing heterotrophy in dinoflagellates. An alternative prey ingestion mode is phagocytosis, again seen widely in dinoflagellates. Prey cells are internalised intact and subsequently processed for digestion in food vacuoles. Interestingly, digestion might not be immediate, with factors such as temperature contributing to delay of digestion by up to a week or more in some examined cases (Li et al., 1999). Dinoflagellates have even achieved predation on cells and cell colonies many times their own cell size. A concertinaed pallium membrane can be issued to surround large prey items into which digestive enzymes are then released. Dinoflagellates with this capacity can recover digested prey nutrients without internalising such prey. Finally, osmotrophy is utilised widely by dinoflagellates, including some that specialise in saprophytic exploitation of food sources.

The ubiquity of heterotrophic behaviours in both photosynthetic and non-photosynthetic dinoflagellates likely provides options for plastid modification, including loss of function. If heterotrophy is consistently successful, loss or reduction of autotrophic capacity is possible. Indeed, many dinoflagellate groups have independently lost photosynthesis (see below, and Figure 1). Furthermore, there are also multiple cases of plastid recapture via these feeding behaviours and subsequent endosymbioses. These events have resulted in plastid replacements, and even co-existence of two evolutionarily distinct plastids in one lineage (Hehenberger, Imanian, Burki, & Keeling, 2014). Thus, there is evidence of an ongoing shifting of balance between autotrophic and non-autotrophic modes of nutrition in dinoflagellates in an equilibrium that is not so obviously seen in other plastid-containing groups. This propensity for ongoing plastid evolution is likely to be attributed, at least in part, to the persistence of mixotrophy.

A further aspect of the biology of dinoflagellates that likely impacts their plastid evolution, although in ways that might be less obvious than heterotrophy, is the long list of very bizarre dinoflagellate genomic traits (Wisecaver & Hackett, 2011). Dinoflagellate nuclear, mitochondrial, and also plastid genomes all possess unusual genomic architectures and processes of maintenance and expression. The nuclear genomes of dinoflagellates are extraordinarily large, in the order of ~1.5 to ~190 Gb (Holm-Hansen, 1969; LaJeunesse, Lambert, Andersen, Coffroth, & Galbraith, 2005). These genomes are maintained in a state of permanent condensation, and in a chromatin form that is apparently near devoid of histones (Chow, Yan, Bennett, & Wong, 2010; Gornik et al., 2012; Rizzo, 1987). When genes are expressed, most if not all mRNAs are processed through trans-splicing to receive a short 5' common leader sequence (H. Zhang et al., 2007). The mitochondrial genome is similarly unusual, although in very different ways (Nash, Nisbet, Barbrook, & Howe, 2008; Waller & Jackson, 2009). It contains very few genes, but these are fragmented and scrambled in the genomes, with at least one requiring trans-splicing to reassemble. In most dinoflagellate mitochondria, mRNAs are extensively post-transcriptionally edited, and translation evidently proceeds without conventional start or stop signals. In this environment of deviance, it is perhaps unsurprising that plastid DNAs share unusual traits in gene organisation and expression also (see below). While it is unclear what has driven the changes seen in the plastid genomes of many dinoflagellates, it is possible that processes directing divergence in the nucleus and mitochondria have affected the plastids too.

III. The peridinin plastid

The ‘peridinin plastid’ is the archetypical plastid of dinoflagellates (Figure 2). It is the one plastid type that occurs broadly in dinoflagellate orders and is, thus, presumed to be an ancestral feature of dinoflagellate radiation. It has for some time been considered to be the

product of secondary endosymbiosis of a red alga, however the simplicity of this hypothesis is discussed further in section VI below. This plastid type has several definitive features, one of the most conspicuous being its pigment composition. The dominant secondary pigment is the carotenoid peridinin that is unique to dinoflagellates. This pigment is unusual for a carotenoid in containing three closed ring structures, and this is thought to contribute to its strong blue-light absorbing properties (Rapoport et al., 2002). Furthermore, the peridinin plastid contains only a single chlorophyll c type (c_2) in addition to chlorophyll a (Prezelin, 1987). A second biochemically distinguishing feature of the peridinin plastid is its form II RuBisCO, atypical of eukaryotic types of this central photosynthetic enzyme (Morse, Salois, Markovic, & Hastings, 1995; Whitney, Shaw, & Yellowlees, 1995). Form II RuBisCO assembles as homodimers, rather than the higher order heterodimers of the large and small subunits of form I RuBisCO found through other eukaryotic plastids and their cyanobacterial forebears (Tabita, Hanson, Satagopan, Witte, & Kreel, 2008). The presence of this unusual RuBisCO suggests protein replacement via lateral gene transfer, most likely from proteobacteria where this RuBisCO form is also prevalent. The only other eukaryotes known to contain this RuBisCO form are the related 'chromerids' of the Alveolata, *Chromera* and *Vitrella* (Figure 1) (Janouškovec, Horák, Oborník, Lukeš, & Keeling, 2010).

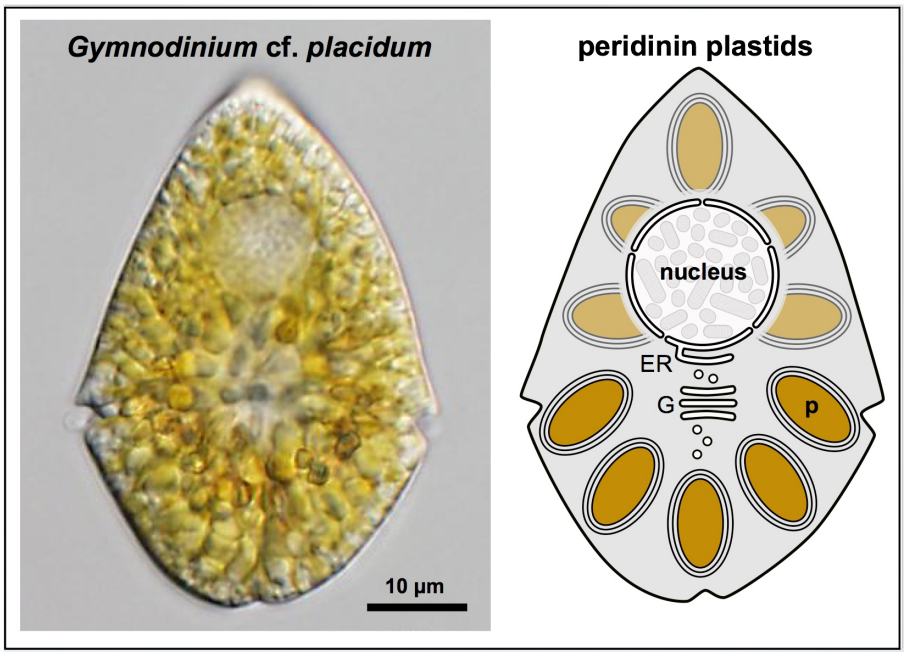

Figure 2: *Gymnodinium* cf. *placidum* containing peridinin plastids. Plastids (p) are surrounded by three membranes and nucleus-encoded proteins are trafficked to these plastids from the ER via vesicular transport through the Golgi (G). Micrograph by Gert Hansen.

Another distinctive trait of the peridinin plastid is the state of the organelle-encoded genome. Most photosynthetic plastid genomes are encoded on single molecules and contain ~90 to 250 genes (Dorrell & Howe, 2015). The peridinin plastid genome, on the other hand, has been highly reduced to less than 20 genes, and fragmented into a population of minicircles containing only one to a few genes each (Howe, Nisbet, & Barbrook, 2008b; Z. Zhang, Green, & Cavalier-Smith, 1999). These minicircles are typically 2-3 kbp in size. In each taxon they contain a common non-coding core sequence, although these core regions do not share sequence identity between taxa. The function of the core domain has not been confirmed, but it contains direct and inverted repeats and is thought to promote the poly-cistronic transcription of the circles (Moore, Ferguson, Loh, Hoegh-Guldberg, & Carter, 2003; Nelson & Green, 2005; Nisbet et al., 2008). Some minicircles are thought to lack any genes, others contain gene fragments, and some tiny 'microcircles' exist, collectively pointing to an ongoing process of recombination of these genetic elements (Barbrook, Symington, Nisbet, Larkum, & Howe, 2001; Nisbet, Koumandou, Barbrook, & Howe, 2004).

Transcribed plastid genes are unusual in receiving a polyuridine 3'-tail, although the significance of this type of post-transcriptional modification is not known (Wang & Morse, 2006). Substitutional editing of plastid transcripts has also been reported in several taxa, adding further to the complexities of these unusual genomes (Dang & Green, 2009; Mungpakdee et al., 2014; Wang & Morse, 2006; Zauner, Greilinger, Laatsch, Kowallik, & Maier, 2004).

With an organelle genome dispersed on many different molecules, these genomes are more difficult to characterise to completion than for single chromosome organelle genomes. Nevertheless, following wide sampling amongst several photosynthetic taxa, and determination of plastid genes transferred to the nucleus, a clear view of the minicircle gene content has been gained (Dorrell et al., 2017; Mungpakdee et al., 2014; Wang & Morse, 2006). This content is principally for components of the photosynthetic apparatus – photosystem II (PsbA, PsbB, PsbC, PsbD, PsbE, PsbI), photosystem I (PsaA, PsaB), cytochrome b6/f (PetB, PetD), and plastid ATP synthase (AtpA, AtpB) – and select genes for their expression (LSU and SSU rRNAs, 1-3 tRNAs and one to few ORFs). This makes the peridinin plastid's gene complement the most reduced of any known photosynthetic plastid, with the greatest portion of plastid genes relocated to the nucleus. It is possible that minicircles are more easily transferred from organelle to nucleus than components of single plastid chromosomes, making the fragmentation to minicircles a potential accelerant for transfer and contributing to this highly reduced state. Or perhaps segregation during plastid division of equal and/or complete representation of the multiple different minicircle types becomes a limiting factor. Copy number of individual minicircles has been estimated at as little as five copies during exponential growth, which would present challenges to complete sorting of multiple types of miniciricles during organelle division (Koumandou & Howe, 2007). This might have further selected for reduced gene number through relocation to the nucleus.

Genes for plastid proteins in the nucleus requires their nascent proteins to be trafficked back to these organelles and across their bounding membranes. The peridinin plastid is surround by three membranes (Figure 2), which is a further unusual state for plastids and otherwise only known for the green plastids of euglenids (Schnepf & Elbrächter, 1999). Targeted proteins possess N-terminal bipartite leader sequences consisting of a Sec61-directing signal peptide followed by a plastid-targeting transit peptide (Nassoury, Cappadocia, & Morse, 2003; Patron & Waller, 2007; Patron, Waller, Archibald, & Keeling, 2005). Unlike some other chlorophyll c-containing algae, the outermost membrane lacks continuity with the nuclear envelope or endoplasmic reticulum (ER), or obvious ribosomes bound to it. This suggests that vesicular traffic is required for delivery of proteins from the ER to the outer plastid membrane and, indeed, some nucleus-encoded plastid proteins have been detected en route to the plastid in the Golgi apparatus (Nassoury et al., 2003). Curiously, the transit peptide that follows the signal peptide has a predicted stop membrane transfer anchor in a large portion of known plastid-targeted proteins (Patron et al., 2005; Patron & Waller, 2007; Nassoury et al., 2003). This is a plastid protein feature also only known from euglenids, and is thought to maintain these proteins in a transmembrane state during their delivery to the plastid (Durnford & Gray, 2006; Durnford & Schwartzbach, 2017). The reason for such a topology is not known, nor why only a portion of plastid proteins show this apparent behaviour, but is a feature that is shared in a protein-specific manner across different dinoflagellate taxa (Patron et al., 2005). Upon fusion to the outer plastid membrane of the Golgi-derived vesicles it is thought that the transit peptide is recognised with equivalents of the translocons of the outer and inner envelope membrane of chloroplasts (TOC and TIC, respectively) to facilitate passage across the last two membranes. While TIC components Tic110 (Hehenberger et al., 2014) and Tic20 (Kořený, Lam, Waller, unpublished) are apparently present in peridinin plastids, no conserved TOC components have been identified and other typically conserved TIC components, such as Tic22, are not detected providing further evidence of an unusually derived plastid.

Plastid membrane number greater than two is widely interpreted as evidence that such plastids were gained through endosymbiosis of a eukaryote that itself contained a plastid, forming a so-called 'complex' plastid, as opposed to a primary endosymbiosis of a prokaryote forming a primary plastid (Gould, Waller, & McFadden, 2008). A complex plastid

in dinoflagellates is consistent with molecular phylogenies generally grouping peridinin plastid genes in clades with red algal and other red algal-derived complex plastids (Dorrell et al., 2017; Janouškovec et al., 2010; Waller, Patron, & Keeling, 2006a). Most complex plastids, included those in apicomplexans and chromerids, are surrounded by four membranes, and it is unclear what process might generate a three-membrane plastid. It is of note that the deepest lineage of the Dinozoa, the Perkinsozoa, have been suggested to possess plastids surrounded by four membranes (see below). If this is true, and it represents an ancestral peridinin plastid, then it suggests that the three membranes of dinoflagellate plastids arose through loss of one of an original four, well after endosymbiotic gain and integration.

Plastids derived from red algae that are surrounded by four membranes—i.e. in cryptophytes, haptophytes, heterokons and apicomplexans—share a derived version of the ER-associated degradation (ERAD) machinery, that in most eukaryotes is responsible for exporting misfolded proteins from the ER to the cytosol for proteosomal degradation (Agrawal, van Dooren, Beatty, & Striepen, 2009; Felsner et al., 2011; Stork et al., 2012). The so-called symbiont-specific ERAD-like machinery (SELMA) is an endosymbiont-derived form of this machinery present in all of these organisms' plastids. It has been repurposed for trafficking plastid proteins inward across the second outermost membrane, effectively from the Sec61 translocon on to the TOC and TIC (see Figure 3). With only three membranes surrounding the peridinin plastid rather than four, it is logical that one translocon would be missing. Peridinin-plastid-containing dinoflagellates lack any evidence for a SELMA (Kořený, Lam, Waller, unpublished), consistent with the equivalent of this second outermost membrane being missing in these plastids.

Overall, the peridinin plastid remains relatively poorly understood. This is in part due to the paucity of nuclear genomic data for dinoflagellates, with the exception of the coral symbiont *Symbiodinium* (Aranda et al., 2016; Lin et al., 2015; Shoguchi et al., 2013). Further, dinoflagellate biologists still lack reliable experimental tools such as genetic transformation. Nevertheless, the litany of unusual traits, including: form II RuBisCO; plastid minicircular chromosomes; poly-uridylated and edited gene transcripts; loss of a bounding membrane; and novel protein targeting; means that this plastid stands out as an exception amongst other complex plastids. Quite why such divergence might have occurred is unclear.

IV. Plastid reduction and loss

While dinoflagellates are often first thought of as pigmented photosynthetic algae many, perhaps even most, lack photosynthesis (de Vargas et al., 2015; Gómez, 2012). Such obligate heterotrophs are represented throughout dinoflagellate radiation and all of the major basal members of this lineage are also non-photosynthetic (Figure 1). Multiple parallel losses indicate that mixotrophy provides ongoing opportunities for lifestyle changes in dinoflagellates. Loss of photosynthesis was interpreted initially as loss of the plastid, and this was believed to have occurred multiple times independently in dinoflagellates (Saldarriaga, Taylor, Keeling, & Cavalier-Smith, 2001). However, careful scrutiny of molecular data from colourless taxa, for example *Crypthecodinium*, *Noctiluca* and *Oxyrrhis*, has consistently revealed evidence of persistent plastids, typically by way of genes for plastid-targeted proteins (Janouškovec et al., 2017; Sánchez Puerta, Lippmeier, Apt, & Delwiche, 2007; Slamovits & Keeling, 2008). Even taxa that capture kleptoplasts (*Dinophysis*) or have acquired new photosynthetic plastids ('dinotoms', see below), appear to have retained a colourless plastid (Hehenberger et al., 2014; Janouškovec et al., 2017).

The deepest branching dinozoans, Perkinsozoa, provide the earliest view of non-photosynthetic plastids in this lineage. *Perkinsus* spp. encode proteins for typical plastid functions with bipartite leader sequences consistent with targeting to complex plastids (Matsuzaki, Kuroiwa, Kuroiwa, Kita, & Nozaki, 2008; Robledo et al., 2011). *Perkinsus* plastids are one of the few dinoflagellate colourless plastids for which there are ultrastructural observations reported in the literature (Robledo et al., 2011; Teles-Grilo et al., 2007). These observations suggest either four membranes surrounding this plastid, or at least three, although direct confirmation that these structures are plastids is currently outstanding. If four, *Perkinsus* plastids could represent an ancestral state of the peridinin plastid, before one membrane was lost. We find no evidence of SELMA in *Perkinsus*

genomic data, however, suggesting either that only three membranes are present or that a novel way for proteins to translocate the extra membrane exists in *Perkinsus* spp. (Kořený, Lam, Waller, unpublished). No evidence of a plastid genome has been reported for *Perkinsus* spp., leading to the suggestion that it has been lost (Matsuzaki et al. 2008; Janouškovec et al., 2015). If the *Perkinsus* plastid had previously contained plastid minicircles primarily encoding photosynthetic genes, as for peridinin plastids, then reversion to heterotrophy would be predicted to eliminate these last vestiges of the organelle genome. This prediction extends to all other colourless dinoflagellate plastids derived from the peridinin plastid.

Throughout dinoflagellates, the biochemical functions of the colourless plastids have been inferred from the proteins predicted to be targeted to them. These indicate various synthetic roles in isoprenoid precursors, tetrapyrroles, and Fe-S clusters (Bentlage, Rogers, Bachvaroff, & Delwiche, 2016; Gornik et al., 2015; Janouškovec et al., 2017; Matsuzaki et al., 2008; Sánchez Puerta et al., 2007; Slamovits & Keeling, 2008). In eukaryotes, these pathways can all occur in either plastids or the host cytoplasm (and/or mitochondrion for tetrapyrroles), but the presence of plastids often results in loss of at least some of the host-based pathways, and thus reliance on the plastid-located ones (Waller, Gornik, Kořený, & Pain, 2016). To date, the only localisation study for any of these genes was for IspC of the isoprenoid 1-deoxy-D-xylulose 5-phosphate (DOXP) pathway performed in *Perkinsus* (Matsuzaki et al., 2008). IspC was shown to locate to a small number of puncta, suggesting multiple small plastids, although it was not verified that these represented the multi-membrane bound organelles previously seen (Teles-Grilo et al., 2007). Until more complete genomic data is available, and further proteomic studies of these plastids is undertaken, it is unknown if all dinoflagellate colourless plastids will perform the same functions. Nevertheless, these data suggest ongoing dependencies upon plastid metabolism in *Perkinsus* and dinoflagellates that are not fulfilled by heterotrophy, similar to the situation seen for the more thoroughly characterised apicoplasts of apicomplexan parasites (Ralph et al., 2004). In alveolates, an early loss of the host cell pathway for isoprenoid precursor synthesis (the mevalonate pathway) in dinoflagellates and apicomplexans has been suggested to be one factor that has driven retention of the plastid pathway (the DOXP pathway), and thus the organelle, throughout non-photosynthetic members of both groups (Janouškovec et al., 2015; 2017; Waller et al., 2016).

Despite widespread plastid retention in heterotrophic dinoflagellates, at least one taxon has apparently lost its plastid. *Hematodinium* sp. is a syndinian parasite of crustaceans with no trace of a plastid organelle (Gornik et al., 2015). Its phylogenetic position suggests that ancestrally it would have had a plastid (Figure 1) (Bachvaroff et al., 2011; Janouškovec et al., 2017), and this is corroborated by presence of one plastid-derived protein (HemD) for the tetrapyrrole pathway that now functions in the cytosol (Gornik et al., 2015). Furthermore, seven enzymes of the plastid diaminopimelate (DAP) pathway for lysine synthesis are also predicted to be present in the cytosol in *Hematodinium* sp. Although molecular phylogenies of these enzymes were unable to unambiguously identify their origin, this pathway is otherwise only known to be plastidic in eukaryotes, suggesting relocation and retention of the pathway but not the organelle (Gornik et al., 2015; Hudson, Singh, Leustek, & Gilvarg, 2006). Thus, for *Hematodinium* sp. the chance retention of some cytosol-located pathways, relocation of some plastid metabolic proteins to the cytosol, and an ability to scavenge metabolites from its host animal, have serendipitously alleviated the need for the plastid, and it has been lost. This plastid loss might be a common ancestral feature of the Syndiniales group, but appears to represent a rare evolutionarily event and is otherwise only know from the apicomplexan genus *Cryptosporidium* (Figure 1).

V. Plastid replacement

Dinoflagellates are uniquely interesting for the study of plastid endosymbiosis as there are multiple verified cases where a new photosynthetic endosymbiont has been gained and replaced or supplemented the role of the original peridinin plastid. These replacements include secondary as well as higher order (tertiary or beyond) endosymbioses, and they provide insight into the processes of plastid capture, integration and modification post gain.

A. Complex plastids derived from haptophytes

Within the Gymnodiniales, the Kareniaceae genera *Karenia*, *Karlodinium* and *Takayama* contain endosymbionts of haptophyte origin that now serve as their photosynthetic plastids (Figure 1 and 3). These dinoflagellates still maintain mixotrophy through algivorous phagocytosis (Li et al., 1999; Sheng et al., 2007), so it is plausible that this behaviour was the route to new endosymbiont capture. The foreign source of these plastids was first recognised by pigment analyses that identified a lack of peridinin but presence of signature accessory pigments of haptophytes, most notably 19'-hexanoyloxyfucoxanthin and/or 19'-butanoyloxyfucoxanthin as well as chlorophylls c_1 and c_2 (Bjørnland & Tangen, 1979; Carreto, Seguel, & Montoya, 2001). This conclusion was substantiated by occurrence of genes for plastid proteins with clear haptophyte affinities in molecular phylogenies (Burki et al., 2014; Ishida & Green, 2002; Patron, Waller, & Keeling, 2006; Takishita, Ishida, & Maruyama, 2004; Tengs et al., 2000; Yoon et al., 2005). Despite the presence of these genes, no trace of the haptophyte nucleus is retained so all such genes have been relocated a second time to the dinoflagellate nucleus (Schnepf & Elbrächter, 1999). Indeed, there is no obvious relict of the haptophyte cytosol or cytosol-contained structures, with all bounding membranes tightly appressed. The haptophyte-derived plastid, therefore, represents an intimately integrated organelle equivalent to plastids in other algae and plants.

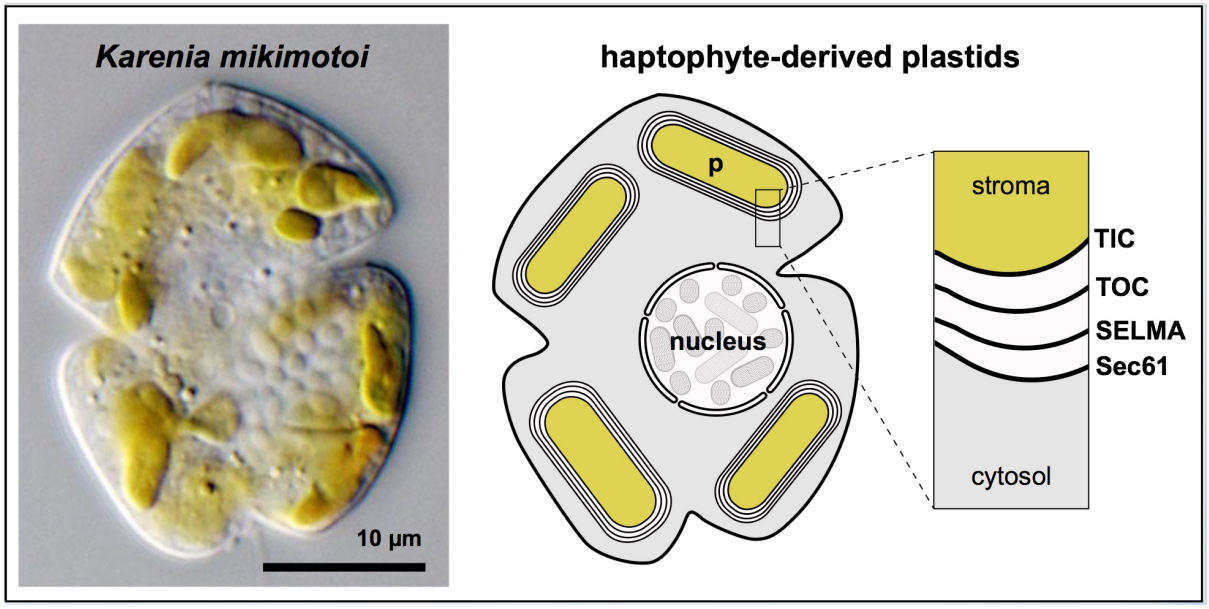

Figure 3: *Karenia mikimotoi* containing haptophyte-derived plastids (p). Protein import into these plastids is predicted to use four translocons: Sec61, SELMA, TOC and TIC. Micrograph by Gert Hansen.

While the plastid compartment in the Kareniaceae is undoubtedly derived from haptophytes, biochemically this plastid is now something of a chimera. Phylogenies of plastid-targeted proteins show that many genes for the peridinin plastid were retained and retargeted as proteins to the new symbiont, either replacing or adding to the haptophyte-derived proteins (Bentlage et al., 2016; Nosenko et al., 2006; Patron et al., 2006; Waller, Slamovits, & Keeling, 2006b). This has the further implication that during the period of haptophyte endosymbiont gain and integration, the peridinin plastid was present in some form, likely as a colourless plastid as found in many other dinoflagellates today. Presumably the new photosynthetic plastid ultimately replaced the metabolic roles of this old plastid, and it was eventually lost. However, not before peridinin plastid genes replaced some of those for the haptophyte plastid, perhaps facilitated by them being already expressed from the dinoflagellate nucleus and with targeting signals approximately appropriate for uptake into the haptophyte.

The haptophyte-derived plastid of dinoflagellates provides potentially far-reaching new insight into the establishment of protein import pathways following gain of new complex

plastids. Dinoflagellates do not typically use the SELMA machinery for protein import, but haptophytes do (Stork et al., 2012). In the case of dinoflagellates with haptophyte plastids, this machinery has been maintained from the haptophyte, and reemployed in the new context of the dinoflagellate. Proteins from all SELMA sub-complexes are present in Kareniaceae, have clear phylogenetic affinities with haptophytes, and have bipartite targeting sequences for the plastid that are otherwise lacking in the cytosolic ERAD paralogues (Kořený, Lam, Waller, unpublished). Thus, these haptophyte-derived plastids show that multiple elements of the plastid machineries are transmissible during establishment of new complex plastids (haptophyte TOC and TIC components are also present). Presence of SELMA also implies that these plastids are likely surrounded by four membranes (Figure 3), although poor ultrastructural preservation of the plastid membranes has otherwise frustrated attempts to count them directly. Curiously, while use of bipartite sorting signals of plastid-targeted proteins are maintained in this re-engineered haptophyte plastid, these proteins show subtle but distinct differences in the properties of the transit peptides from those for either peridinin plastids or free-living haptophytes. The new transit peptides for the haptophyte lack the stop-transfer membrane anchors, and are also uncharacteristically acidic in nature (Patron et al., 2006; Patron & Waller, 2007). It is possible that the period of co-existence of two different plastid types necessitated some divergence of these transit peptide properties, and these have been maintained since loss of the peridinin plastid.

The haptophyte plastid of dinoflagellates still maintains an organelle genome, and in gene content this is similar to that of other haptophytes, although with some losses compared to *Emiliania huxleyi* (Gabrielsen et al., 2011). There is evidence, however, that the sequence, architecture and expression machinery for this plastid has undergone modification since this new endosymbiosis. While Kareniaceae plastid-encoded genes branch with haptophytes in phylogenies, they show long branch lengths compared to haptophytes indicating accelerated evolution (Tengs et al., 2000; Yoon et al., 2005; Yoon, Hackett, & Bhattacharya, 2002). All genes are contained on a single organelle chromosome, but there is evidence that some additional gene copies occur on smaller extrachromosomal DNAs, potentially mimicking development of the peridinin minicircles (Espelund et al., 2012). Moreover, two processes for mature transcript production—poly-uridylation and substitutional editing—are both now found in these dinoflagellate plastids, whereas they do not occur in the plastids of haptophytes (Dorrell & Howe, 2012; Dorrell, Hinksman, & Howe, 2016; Richardson, Dorrell, & Howe, 2014). These changes all reflect unusual properties of the peridinin plastid, and suggest that the factors, presumably targeted proteins, that facilitated these changes in the peridinin plastid persist and are similarly remodelling the genetics of the new haptophyte plastid.

B. Complex plastids derived from green algae

The presence of green pigments, notably chlorophyll b, within some members of Gymnodiniaceae demonstrates a second case of plastid replacement in dinoflagellates (Figure 1 and 4) (Watanabe, Takeda, Sasa, & Inouye, 1987). *Lepidodinium chlorophorum* (= *Gymnodinium chlorophorum*) and *L. viridae* contain plastids surrounded by four membranes in total and containing plastid genomes with clear phylogenetic affinity to green algae (Kamikawa et al. 2015; Matsumoto et al., 2011; Takishita et al., 2008). An early suggestion of the presence of prasinoxanthin, a characteristic pigment of prasinophyte green algae, has not been substantiated, and phylogenetic analysis suggest that the source of this new endosymbiont is within the Pedinophyceae green algae (Matsumoto et al., 2011; Matsumoto, Kawachi, Miyashita, & Inagaki, 2012). *Lepidodinium* spp., therefore, contains green algal plastids presumably gained through secondary endosymbiosis. Uncertain monophyly of this genus might indicate that multiple similar gains have occurred, although all in closely related host taxa (Matsumoto et al., 2012).

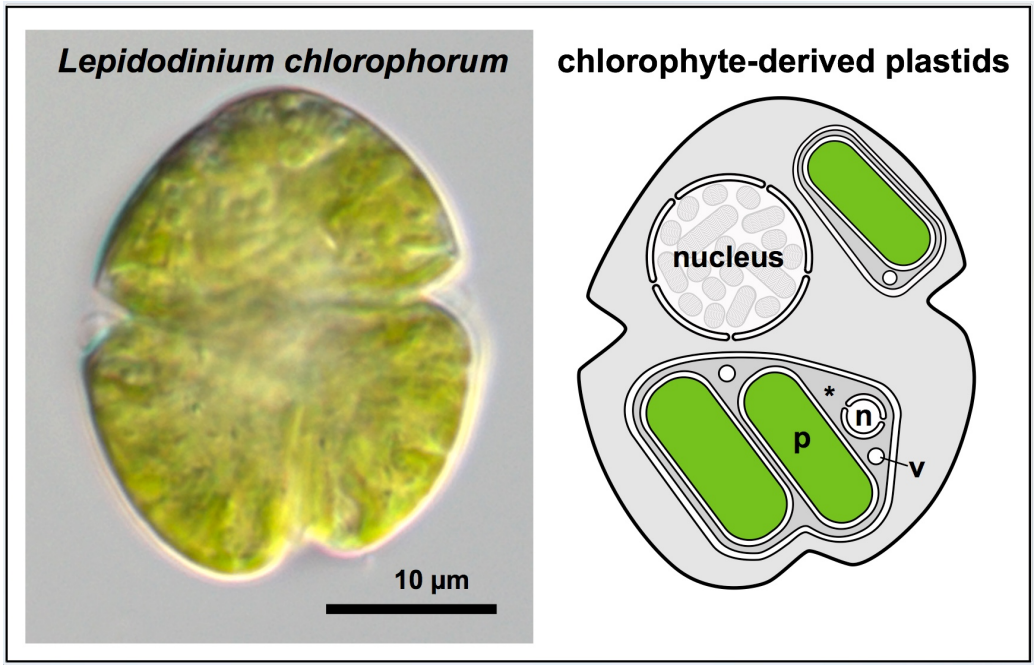

Figure 4: *Lepidodinium chlorophorum* containing chlorophyte-derived plastids. The plastids (p) are surrounded by four membranes, with a double membrane around each plastid, and a further membrane pair separating the endosymbiont from the host. Between the membrane pairs are relict structures of the endosymbiont cytoplasm including apparent ribosomes (*), vesicles (v) and a possible nucleomorph (n). Micrograph by Kazuya Takahashi.

Ultrastructural observations of *Lepidodinium* plastids show that the four bounding membranes are not all closely appressed as seen in the Kareniaceae, but rather a space occurs between the inner plastid pair containing the thylakoids, and the outer pair that separates the host from endosymbiont (Figure 4). This space contains granules consistent in size with ribosomes and membranous compartments including a double membrane-bound space with pores that is reminiscent of a small relict nucleus, known as a nucleomorph-like structure (Schnepf & Elbrächter, 1999; Watanabe et al., 1987). These observations are highly suggestive of mRNAs being translated in the symbiont cytoplasm and encoded in this small relic nucleomorph-like structure. While the presence of a relict green algal nucleus has not been formally demonstrated, *Lepidodinium* would appear to be at a state of organelle development equivalent to cryptophytes and chlorarachniophytes that possess bona fide nucleomorphs derived from endosymbiont nuclei (Moore & Archibald, 2009).

Genes from the green algal endosymbiont have been transferred to the dinoflagellate nucleus, as is evident by plastid proteins encoded on transcripts possessing the signature transcriptional element of dinoflagellate, the 5' spliced leader (Minge et al., 2010; H. Zhang et al., 2007). Several of these are of green algal origin, consistent with nucleus-to-nucleus transfer. Some, however, are peridinin plastid-derived proteins indicating that, as for the Kareniaceae, a peridinin plastid was present when the green alga was gained, and that some of its proteins have been retargeted to the new plastid. This indicates a common principle, and perhaps propensity, for dinoflagellates to support the gain of new plastids by molecular-genetic adaptations for pre-existing ones (Howe, Barbrook, Nisbet, Lockhart, & Larkum, 2008a).

The *Lepidodinium* nucleus-encoded plastid proteins all possess targeting presequences consistent with trafficking to complex plastids—that is, an ER-targeting signal peptide followed by a further extension that might act as a transit peptide. Similar to the Kareniaceae, the properties of the putative transit peptide are somewhat deviant from those of typical plastid transit peptides (Minge et al., 2010), and this might again reflect a need to be distinguished from concurrent targeting to a peridinin plastid while they co-occurred. While we currently have no insight into the translocons that operate to transport across four plastid membranes in *Lepidodinium*, it is of note that neither the dinoflagellate host nor the

pedinophyte symbiont would have possessed a SELMA-type apparatus. Therefore, it is unknown what solution has been achieved for protein transport across the third membrane. A redeployment of SELMA such as occurred in the Kareniaceae would not be possible, and a de novo solution would have likely been required in this case.

C. Complex plastid endosymbionts derived from diatoms

A further group of dinoflagellates with stable new plastid endosymbionts occur within the Peridiniales, the best studied of which are *Kryptoperidinium foliaceum* and *Durinskia baltica* (Figure 1 and 5). These dinoflagellates possess endosymbionts derived from diatoms, which is evident by both the presence of signature diatom pigments fucoxanthin as well as chlorophyll c_2 (Tamura, Shimada, & Horiguchi, 2005), and copious molecular genetic evidence including organelle genomes and extensive transcriptomes of the diatom symbiont (Burki et al., 2014; Imanian & Keeling, 2007; Imanian, Pombert, & Keeling, 2010; Imanian, Pombert, Dorrell, Burki, & Keeling, 2012). These so-called 'dinotoms' are remarkable as stable endosymbiont systems because the symbiont remains relatively intact with a high degree of autonomy. In addition to the diatom plastid, the symbiont maintains a nucleus, mitochondrion, ER and other membranous structures within its cytoplasm (Dodge, 1971; Tamura et al., 2005; Tippit & Pickett-Heaps, 1976; Tomas & Cox, 1973). The plastid and mitochondrion maintain organelle genomes that are seemingly unchanged from free-living diatoms, and the nucleus apparently encodes all genes necessary for plastid and symbiont function (Burki et al., 2014; Imanian et al., 2010; Imanian & Keeling, 2007). In fact, there is no evidence of relocation of functional genes from the symbiont to the dinoflagellate nucleus, suggesting that protein targeting back to this new symbiont has not been developed. This sets it apart from almost all other stable plastid organelles which are heavily dependent on host nucleus-encoded genes for their protein content.

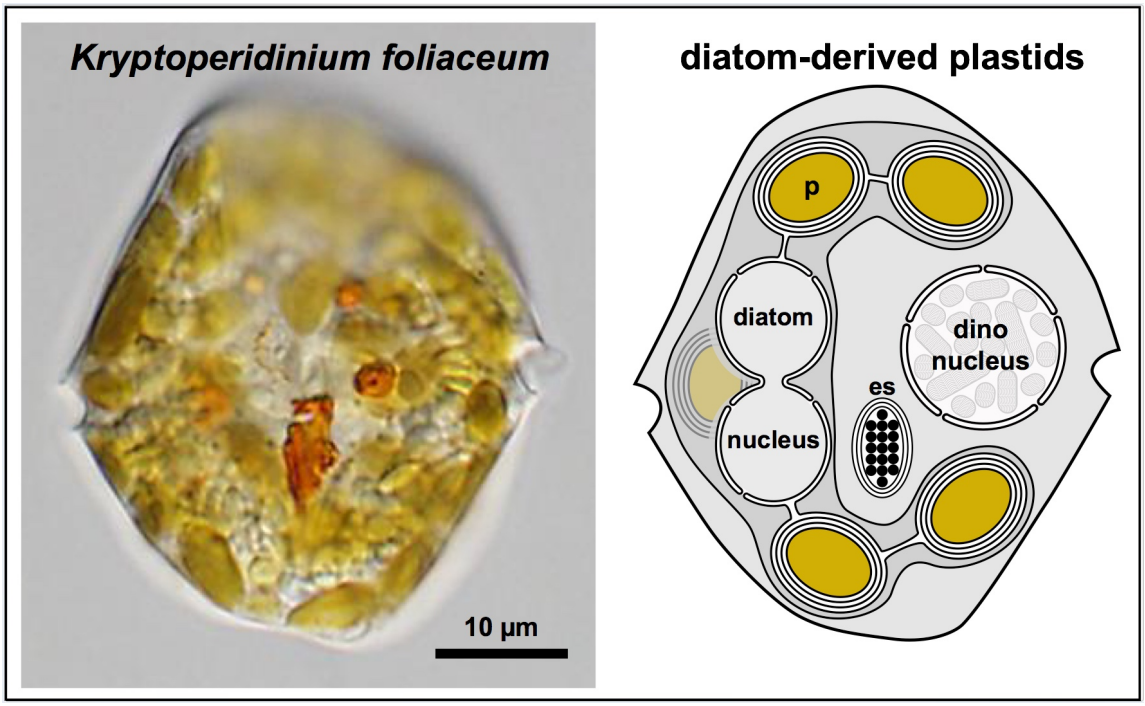

Figure 5: *Kryptoperidinium foliaceum* containing plastids (p) within a diatom endosymbiont. A single membrane separates the endosymbiont from the host, and a further four membranes surround the plastids. A three membrane-bound eye spot (es) is predicted to be a colourless peridinin plastid, implying that these 'dinotoms' contain two types of plastids. Micrograph by Gert Hansen.

The membranes surrounding this complex plastid might contribute to its apparent state of arrested development as an organelle. In addition to four membranes immediately surrounding the diatom plastid, an additional membrane then separates the diatom cytosol

and organelles from the host dinoflagellate cytosol (Dodge, 1971; Jeffrey & Vesk, 1976; Schnepf & Elbrächter, 1999; Tamura et al., 2005; Tomas & Cox, 1973). Thus, five membranes separate the host nucleus from the plastid stroma (Figure 5). Many plastid genes remain in the diatom nucleus and are targeted to the plastid via the conventional diatom pathway, including use of the diatom SELMA (Kořený, Lam, Waller, unpublished) (Burki et al., 2014). If a protein was to be targeted from the dinoflagellate cytosol, a mechanism for translocating this fifth, outermost, membrane would be required that still leaves the protein competent for translocation across the subsequent four membranes. This complexity might have created a barrier for protein targeting, and the few cases of diatom genes observed in the dinoflagellate nucleus likely represent unproductive random DNA fragment transfers (Burki et al., 2014). The outer membrane even appears to be a barrier to co-operativity between the host and symbiont mitochondria, as both appear to maintain a full suite of independent functions (Imanian & Keeling, 2007; Imanian, Carpenter, & Keeling, 2007; Imanian, Pombert, Dorrell, Burki, & Keeling, 2012).

Despite the seemingly stalled nature of this endosymbiont's development as an organelle, change is seen within this symbiont compared to free-living diatoms. The silica frustule is lost allowing an extensive lobed morphology to fill the periphery of the host cytoplasm (Dodge, 1971; Figueroa, Bravo, Fraga, Garcés, & Llaveria, 2009; Tamura et al., 2005; Tippit & Pickett-Heaps, 1976; Tomas & Cox, 1973). Further, the diatom nucleus has apparently relaxed into a permanent state of dispersed chromatin, with no evidence of the chromosome condensation or separation on a mitotic spindle as is so conspicuous in most other diatoms by microscopy (Dodge, 1971; Tippit & Pickett-Heaps, 1976). During cell division, this nucleus is pinched in half through an apparent amitotic process, although if so it is unclear how a viable segregation of genes occurs. It is possible that gene duplication has occurred on short chromosomal elements, akin to the macronucleus of ciliates, but limited details of this unusual symbiont nucleus are currently available.

Dinotoms typically have an eyespot, a conspicuous structure containing carotenoid-rich lipid globules that has long been speculated to be a relict of the peridinin plastid (Figure 5) (Dodge & Crawford, 1969; Schnepf & Elbrächter, 1999; Tamura et al., 2005). Initial evidence for this was its three bounding membranes, as seen for the peridinin plastid, and that eyespot structures are often found within the stroma of peridinin plastids. More recently, genes for plastid proteins have been found in the dinoflagellate nucleus with predicted plastid-targeting bipartite signals (Hehenberger et al., 2014). Given that the diatom plastid is apparently unable to receive proteins from the host cytosol, it is more likely that these are delivered to a plastid directly in the dinoflagellate cytosolic compartment, and the eyespot is the most likely candidate. Moreover, these genes are identified as peridinin plastid-derived, and are for metabolic pathways found in other non-photosynthetic peridinin plastids (isoprenoid precursor synthesis and tetrapyrrole synthesis) consistent with the rationale for colourless plastid retention in other dinoflagellates. The diatom symbiont nucleus also encodes genes for these pathways, and this is further evidence of redundancy and poor co-operativity between this symbiont and its host.

Dinotoms provide further insight into endosymbiosis because while the host cells form a clear monophyletic clade within the Perindiniales, the symbionts are not all derived from a single endosymbiotic event. Molecular phylogenies of plastid genes indicate that the source diatoms differ in different dinotoms—from the pennate genus *Nitzschia*, to centric genera *Chaetoceros, Cyclotella* and *Discostella* (Chesnick, Kooistra, Wellbrock, & Medlin, 1997; Horiguchi & Takano, 2006; Tamura et al., 2005; Yamada, Sym, & Horiguchi, 2017; You, Luo, Su, Gu, & Gu, 2015; Q. Zhang, Liu, & Hu, 2014). Moreover, recent detailed sampling of diatoms indicates that within dinotoms with pennate symbionts six *Nitzschia* species have been independently taken up in at least eight endosymbiotic events (Yamada et al., 2017). These data point to a process of relatively frequent endosymbiont replacements from a population of related diatoms. It is currently unclear what the longevity of each symbiont is, however in culture these cells are known to maintain the symbiont for decades. It is possible that the apparent degeneration of the diatom mitotic process might gradually undermine the viability of the symbiont, although these observations might be misinterpreted. Nevertheless, these dinoflagellates demonstrate a propensity to establish new diatom endosymbiotic relationships, and this suggests that adaptation to forming specific symbiotic relationships

likely happens before long-term establishment of symbionts. There is some evidence of a similar scenario in *Lepidodinium* spp. (above), and this might indicate a broad principle relevant to the process of endosymbiosis in general.

D. Kleptoplasts

At the furthest extreme of dinoflagellate plastid biology are taxa that exploit temporary photosynthetic plastids gained by feeding on other plastid-containing organisms. These 'stolen' plastids, or kleptoplasts, are maintained from days up to years, and during their tenure the host dinoflagellate derives metabolic benefit from the ongoing photosynthetic activity of these plastids. Some plastids can be maintained through host cell division and some are even seen within dinoflagellate cysts (Onuma & Horiguchi, 2015). However, in all cases, ongoing maintenance and division of the kleptoplast has not been achieved so the host must continue to predate on a source of these plastids to replenish its stock. Kleptoplasty is seen in a wide range of dinoflagellates. While the Dinophysiales demonstrate a particular proclivity for this behaviour (e.g., multiple *Dinophysis* spp., *Amphisolenia, Triposolenia*), kleptoplasts have been seen across several orders including: Gymnodiniales (e.g. *Nusuttodinium, Amphidinium*), Gonyaulacales (e.g. *Amylax*), Peridiniales (e.g. *Pfiesteria* and *Cryptoperidiniopsis*) and Noctilucales (e.g. *Noctiluca*) (Daugbjerg, Jensen, & Hansen, 2013; Eriksen, Hayes, & Lewitus, 2002; Gast, Moran, Dennett, & Caron, 2007; P. J. Hansen, Miranda, & Azanza, 2004; M. Kim et al., 2014; M. Kim, Nam, Shin, & Coats, 2012; Larsen, 1988; Lewitus & Glasgow, 1999; Sweeney, 1976; Takano, Yamaguchi, Inouye, Moestrup, & Horiguchi, 2014; Tarangkoon, Hansen, & Hansen, 2010; Wilcox & Wedemayer, 1985). The sources of kleptoplasts can also be diverse, with many favouring cryptophytes, but others exploiting haptophytes, pelagophytes, chlorophytes and even cyanobacteria (Daugbjerg et al., 2013; Gast et al., 2007; Sweeney, 1976; Tarangkoon et al., 2010; Wilcox & Wedemayer, 1985).

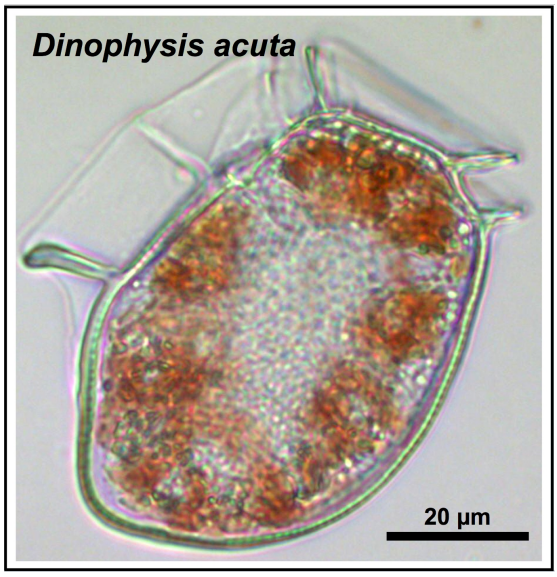

Figure 6: *Dinophysis acuta* containing pigmented kleptoplasts derived from cryptomonads. Micrograph by Susanne Busch.

Seemingly just as diverse as the source kleptoplasts is the mode of their gain, and this has consequences for the potential maintenance and longevity of these temporary organelles. Some prey organisms are engulfed whole, accumulate and persist relatively intact (P. J. Hansen et al., 2004; Li et al., 1999; Onuma & Horiguchi, 2015; Yamaguchi, Nakayama, Kai, & Inouye, 2011). Some of these symbionts undergo morphological changes within their captors and can lose surface structures such as periplasts and ejectosomes, and increase in size by up to 20-fold filling a substantial volume of the host cell (Onuma & Horiguchi, 2015; Yamaguchi et al., 2011). Other kleptoplasts are gained by processes that actively disrupt the source cell, including myzocytosis where organelles are harvested directly from the prey cytoplasm. Plastids can even loose surrounding membranes in this

process, for example cryptophyte four membrane plastids can be reduced to double membrane-bound organelles (Schnepf, Winter, & Mollenhauer, 1989). Even more remarkable, both *Dinophysis* and *Amylax* spp. acquire their kleptoplasts second-hand by preying on ciliates that themselves are utilising cryptophyte-derived kleptoplasts (M. Kim et al., 2012; 2014).

Evidence that kleptoplasts provide metabolic gain over their simple catabolic value as food has been provided for several examples, and demonstrates further that different cases of kleptoplasty represent a continuum of host benefits. For example, *Dinophysis acuminata* obtains its cryptophyte kleptoplasts indirectly from the ciliate *Mesodinium rubrum*, and with abundant prey consumption heterotrophy is the major carbon source (Riisgaard & Hansen, 2009). However, with reduced prey density, photosynthesis from the sustained kleptoplasts becomes the dominant carbon source and light-dependent growth is seen. *Cryptoperidiniopsis* grazing on algae similarly shows light-dependent growth, and in *Pfiesteria piscicida* carbon capture was seen as light-dependant (Eriksen et al., 2002; Lewitus & Glasgow, 1999). Finally, a new but undescribed Kareniaceae species from the Antarctic selectively feeds on and maintains a haptophyte (*Phaeocystis antarctica*) kleptoplast for up to 30 months (Gast et al., 2007; Sellers, Gast, & Sanders, 2014). This dinoflagellate cannot survive on this prey alone in the dark, suggesting that this dinoflagellate has become an obligate phototroph with a requirement to continuously replenish its supply of kleptoplasts.

The wide presence of kleptoplasty in dinoflagellates indicates a general capacity for establishment of extended nutritional relationships with their prey (Figure 1). Some lineages, however, show greater propensity for this behaviour, indicating that adaption has occurred to enhance the benefits derived from kleptoplasts. *Nusuttodinium*, for instance, represents a clade of both marine and freshwater species that all engage in kleptoplasty, and always with a preference for cryptophytes (Takano et al., 2014). The inheritance of this trait, even as speciation to different aquatic habitats occurred, implies stable adaptations specialised for acquiring and managing select kleptoplasts. In *Dinophysis acuta* (Figure 6), only the cryptophyte plastid is acquired, and yet these plastids display photoregulation via pigment production in response to changing light (P. J. Hansen et al., 2016). In the absence of the cryptophyte nucleus or nucleomorph, this is suggestive of some role for the host in controlling the physiology of its kleptoplast. Furthermore, in *D. acuminata* some plastid genes have been found in the dinoflagellate nucleus, implying that these might target to the cryptophyte kleptoplast to help manage or maintain it (Wisecaver & Hackett, 2010). Interpretation of these findings is confounded by the recent discovery that *D. acuminata* also retains a colourless peridinin plastid that is the likely destination for at least some of these proteins (Janouškovec et al., 2017), and protein location studies are now required to substantiate the potential for protein targeting to kleptoplasts. Finally, *Nusuttodinium aeruginosum* has achieved a limited form of coordinated kleptoplast division with host division, able to pass on a single gained cryptophyte kleptoplast through up to five divisions (and 32 offspring) (Onuma & Horiguchi, 2015). In this case, the cryptophyte nucleus is unable to divide but, nevertheless, the inheritance of this nucleus correlates with greater kleptoplast size per generation, suggesting that some nucleus-encoded plastid biogenesis processes are maintained for a time. Collectively, these examples suggest that kleptoplasty is more than just delayed prey digestion and exploitation of ongoing photosynthesis, but is a selective, deliberate behaviour with many adaptations akin to endosymbiosis.

VI. Evolution of plastids in dinoflagellates: a case to revise contemporary notions.

For much of the last one and a half decades, the ‘chromalveolate hypothesis’ has dominated discussion of the origins of complex plastids derived from red algae – i.e. those found in cryptophytes, haptophytes, heterokonts, apicomplexans and dinoflagellates (Cavalier-Smith, 1999). Central to this discussion was the assertion that minimal endosymbiotic gains of plastids was preferred for accounting for current plastid diversity, and in the hypothesised ‘chromalveolate’ clade a single ancestral plastid gain would suffice. The monophyly of this clade, however, was ultimately disproven so separate, alternative

explanations for plastid gains in these lineages have been suggested (e.g. Baurain et al., 2010; Bodył, Stiller, & Mackiewicz, 2009; Petersen et al., 2014; Puerta & Delwiche, 2008; Stiller, 2014; Stiller et al., 2014; Ševčíková et al., 2015). While the alveolates (ciliates, apicomplexans and dinoflagellates) are monophyletic, lack of any evidence of plastids in ciliates suggests more recent plastid gain(s) in the apicomplexans and dinoflagellates. This is supported by a metabolic perspective that makes an ancient plastid predating ciliates unlikely (Waller et al., 2016). The discovery of *Chromera* and *Vitrella,* that branch at the base of the apicomplexan lineage but, nonetheless, maintain photosynthetic plastids provided a welcome opportunity to compare the apicoplast with the peridinin plastid to consider if these two plastids likely shared a common endosymbiotic origin (Moore et al., 2008; Oborník et al., 2012). Select plastid features were identified as shared between the apicoplast and the 'chromerid' plastid, and the chromerid plastid with the peridinin one (see below), suggesting that a single endosymbiosis might account for these three plastid types. However, it is prudent to review all of the options for plastid gain in these lineages and, in the light of increased knowledge of the biology of dinoflagellate plastids, assess their relative support.

Below, three evolutionarily scenarios are considered that account for the presence of plastids in dinoflagellates, apicomplexans and chromerids (Myzozoa) with emphasis on considering the origin of the peridinin plastid in dinoflagellates.

A. Scenario 1: A common plastid was gained before the divergence of myzozoans.

Rationale:

A single endosymbiotic gain of a plastid in a common ancestor of all myzozoans means that the obstacles to developing stable host-symbiont partnership need to be overcome only once. In such an event, it would be predicted that shared features of these commonly derived plastid organelles would be represented in the descendent lineages, and in myzozoans some such features have been observed. All myzozoan plastids have relatively reduced organelle genomes compared to other red algal-derived plastids, with peridinin plastids most reduced in gene content, followed by apicoplasts then chromerid plastids (Janouškovec et al., 2010). Although the apicoplast and peridinin plastids share few overlapping genes due to independent organelle genome reduction, chromerids do share common plastid genes with both groups. Molecular phylogenies with either chromerid and dinoflagellate genes, or chromerid with apicomplexan genes, show a common alliance of the myzozoan sequences, although always with independent long branches amongst these taxa (Janouškovec et al., 2010; Moore et al., 2008). Perhaps more significantly, two biochemical features are common to peridinin and chromerid plastids. One is that both use the unusual form II RuBisCO in place of the form I RuBisCO seen throughout other algal groups (Janouškovec et al., 2010). The second is that both poly-uridylate at least some of their plastid mRNA transcripts (Janouškovec et al., 2010; Wang & Morse, 2006). Morphologically, a further similarity has been identified as the stacking of thylakoids with a tendency for stacks of three (Janouškovec et al., 2010; Moore et al., 2008; Oborník et al., 2012). The consistency of this character in dinoflagellates, however, is variable (Dodge, 1975).

Caveats:

Against these shared similar traits of the myzozoan plastids is a backdrop of tremendous differences also. The pigmentation of peridinin versus chromerid plastids is very different, with peridinin a novel and stable accessory pigment in dinoflagellates along with chlorophyll c, but neither are present in chromerids. The peridinin plastids also share highly modified genomes as minicircles, and transfer of many genes to the nucleus which are otherwise still present in the more conventional plastid DNAs of apicomplexans and chromerids (Janouškovec et al., 2013; Wilson & Williamson, 1997). Central to the establishment of stable organelles is the development of protein targeting, yet plastid membrane number and protein sorting methods are different also (Patron & Waller, 2007). The peridinin plastid is bounded by three membranes and lack the SELMA protein targeting machinery, but both apicomplexan and chromerid plastids have four membranes and use SELMA (Agrawal et al., 2009; Petersen et al., 2014; Stork et al., 2012). If the *Perkinsus* plastid is also surrounded by four membranes, as some images suggest (Teles-Grilo et al.,

2007), then absence of SELMA is independent of membrane loss in the dinoflagellate lineage. Finally, the metabolic division of labour between host cell and plastid after endosymbiosis typically eliminates redundant functions, for example, host versus plastid anabolic pathways for tetrapyrroles. For this pathway, apicomplexans and chromerids utilise the host 'C4' pathway that starts with glycine in the mitochondrion, while dinoflagellates with peridinin plastids maintain the plastid-based 'C5' pathway that starts with glutamyl-tRNA (Janouškovec et al., 2017; Kořený, Sobotka, Janouškovec, Keeling, & Oborník, 2011; Ralph et al., 2004). Basal dinoflagellate lineages Perkinsozoa, *Oxyrrhis* and Syndiniales, however, all exclusively maintain the C4 host pathway (Janouškovec et al., 2017; Waller et al., 2016). In the scenario of a common plastid, maintenance of metabolic redundancy is required for some time during the radiation of dinoflagellates before differential loss of this redundant feature. Thus, if a common myzozoan plastid occurred as hypothesised in Scenario 1 many independent character gains and losses, as well as maintenance of redundancy, are required to account for extant myzozoan plastid diversity.

B. Scenario 2: Plastids were gained independently after divergence from the common myzozoan ancestor

Rationale:

An alternative explanation for the many divergent characters that separate the peridinin plastid from those of apicomplexans and chromerids is that these plastids were gained independently. Thus, their differences might by the products of independent processes of integration with their hosts.

The processes necessary to establish a new endosymbiotic organelle are likely to promote a period of accelerated organelle evolution—the "evolutionary upheavals associated with endosymbiosis" (Stiller, 2014). In particular, the functional relocation of genes from symbiont to host nucleus is likely to drive this change. Organelle DNA insertion into host nuclei is thought to be relatively random (Timmis, Ayliffe, Huang, & Martin, 2004), but from such transfers some symbiont genes will come to be expressed, translated and ultimately their proteins delivered back to the organelle where they are required. When these imported proteins achieve sufficient function in the organelle, the resident organelle gene is redundant and can be lost through mutational erosion. During this sequence of events, many processes are likely to promote change in both the nucleus and organelle copies of these genes and, thus, the pathways they define. Regulation of the transferred gene will initially occur in the context of a new host environment determined by foreign transcriptional, translational and import control processes acting on the organelle protein. Even during a process of selection for restored function, this varied regulation is likely to have biochemical impact on the organelle. Furthermore, initial selection for function of the transferred gene will be relaxed due to the second gene copy still in the organelle, so accumulation of some mutations is likely prior to fixation of the functional copy. For gene transfers from the symbiont nucleus to the host nucleus, which are the majority of transfers in complex plastid formation, mis-splicing of introns in the foreign nucleus might contribute to further evolution of transferred genes. Finally, during the window of time when two genes provide function, both a host nucleus and organelle copy, partial or full redundancy will relax selection on the organelle-encoded gene also, allowing it to independently evolve. Chance fixation of the organelle gene, that might be subsequently retransferred, will again have promoted evolution of the organelle. It is likely that successful transfer of genes to the host will often requires multiple attempts, and during all of these there is potential for the biochemistry of the organelle to be progressively, incrementally changed. Thus, the drivers for gene relocation, irrespective of what these are, promote rounds of gene duplication and divergence, and in effect an increased rate of evolution genes and the processes they define.

Such a period of accelerated evolution during organelle establishment might explain some of the novelty found in the peridinin plastid. For instance, it is easy to envisage how the biochemical pathway for pigment synthesis such as the carotenoids could be impacted by changed regulation, stoichiometries and primary sequence of the synthetic enzymes, allowing potential evolutionary exploration of novel molecular structures such as the peridinin carotenoid. The development of the recombined minicircular organelle DNAs might

similarly be the product of transient perturbation of the genome replication and maintenance processes.

Caveats:

Two independent gains of plastid organelles in lineages without pre-existing plastids is necessarily twice as complex as a single gain and integration. While complexity does not eliminate possibility, in these simple terms it could be argued as less likely. Scenario 2 also requires the two unusual plastid traits, use of form II RuBisCO and poly-uridylation of transcripts, to have been acquired independently in chromerids and dinoflagellates. Given that these traits are otherwise apparently only found in these groups, complete independence of their gains seems unlikely also.

C. Scenario 3: A common plastid was present in the myzozoan common ancestor, but the dinoflagellate peridinin plastid represents a replacement by a new endosymbiont

Rationale:

Plastid replacement in dinoflagellates has happened many times (Figure 1). We know from the Kareniaceae, *Lepidodinium* spp. and dinotoms that independent gains of stable plastids have occurred, and in dinotoms a similar endosymbiont type has been gained multiple times within this closely related clade. Moreover, the broad occurrence of kleptoplasty in dinoflagellate orders, including cases of adaptation for symbiont preference and sustained maintenance of the plastid, further indicates the propensity for dinoflagellates to experiment with symbiosis. In this context, an early plastid replacement giving rise to the peridinin plastid is easy to imagine. Nay, one must almost find reason to argue that it has not occurred.

An independent gain of the peridinin plastid would provide opportunity for the accelerated evolution, postulated above during genetic integration of the endosymbiont, to lead to the many novel derived characters seen today in dinoflagellates. Furthermore, the difference in plastid protein targeting signals, membrane number and import machinery (lack of SELMA) could be explained by these basic organelle biogenesis processes evolving anew independently. If a former myzozoan plastid was present and replaced after concurrent occupancy, this might further drive the new import processes to be distinct from those seen in apicomplexans and chromerids, as is also observed in plastid replacements in the Kareniaceae and *Lepidodinium* (Minge et al., 2010; Patron et al., 2006).

If a plastid replacement did occur an obvious question is when. If it occurred after the Syndiniales diverged then this new peridinin plastid might have restored photosynthesis in dinoflagellates. Independent loss of autotrophy in all multiple basal lineages would not be required to explain its conspicuous absence in early dinozoans (Figure 1). Similarly, the maintenance of redundancy in C4 and C5 pathways for tetrapyrrole synthesis would not be required for this span of dinoflagellate evolution. Instead the myzozoan C4 pathway would simply have been lost in favour of the plastid C5 pathway upon new plastid gain. This hypothesised late replacement implies that the relict plastids in Perkinsozoa and *Oxyrrhis* do not represent derivatives of the peridinin plastid. This implication is difficult to assess in these highly-reduced organelles for which there is currently relatively little direct data. However, if *Perkinsus* and *Oxyrrhis* plastids are derived from the peridinin plastid, then an earlier replacement event, before their divergence, must be considered.

Caveats:

The use of form II RuBisCO and mRNA poly-uridylation in both peridinin and chromerid plastids would seemingly require independent gain of these unusual traits in the scenario of different endosymbiotic events. However, the process of plastid replacement is known to utilise proteins and processes from pre-existing resident plastids. In the Kareniaceae several peridinin plastid proteins have been maintained and retargeted to the haptophyte plastid, and both substitutional RNA editing and poly-uridylation have been gained (Dorrell & Howe, 2012; Patron et al., 2006). Therefore, transfer of poly-uridylation following plastid replacement has biological precedent, and the adoption of the pre-existing alternative RuBisCO protein is also plausible. So, neither of these features present a serious obstacle to Scenario 3. If the peridinin plastid's form II RuBisCO was inherited from an

earlier plastid, then it might seem unlikely that a long non-photosynthetic period would have preceded the new plastid gain. This might favour an early plastid replacement event, rather than one after the Syndiniales. It is of note, however, that there is precedent also for maintenance of RuBisCO in non-photosynthetic algae such as *Euglena longa* (Záhonová, Füssy, Oborník, Eliáš, & Yurchenko, 2016). An alternative explanation is that a chromerid-type cell, already in possession of these two traits, was itself the source of a new plastid in dinoflagellates that ultimately resulted in the peridinin plastid.

Each of the three scenarios presented here have both strengths and weaknesses, and there is no unambiguous consensus to which best accounts for the evolution of the peridinin plastid. The discussion of plastid origins, however, has been dominated by schemes that seek to minimise the number of endosymbiotic events. For dinoflagellates in particular, the empirical evidence indicates that this is poorly justified, and equal consideration should be given to possible plastid replacement and/or independent origin.

VII. Conclusion

Dinoflagellate biology provides countless subjects of fascination. Be they: highly complex cell structures for sight, defence and prey capture (Gavelis et al., 2015; 2017); novel nuclear and chromatin conformations; symbioses with animals; or toxic bloom formation: this group of organisms show a penchant for the diverse and unusual, although not at the expense of their broad global impact and importance. As such they are potent subjects for studying organism evolution and function. The biology of their plastids, in particular, offers many insights into endosymbiosis and organellogenesis. Dinoflagellates are seen to develop taxon-selective feeding behaviours, and exploitation of temporary plastids over a wide range of time-scales. These behaviours have led to repeated cases of stable endosymbioses within closely related dinoflagellate and prey taxa, as is particularly evident in dinotoms. Further, we see new cases of genetically integrated organelles in the Kareniaceae and *Lepidodinium* that show both recycling of complex protein import machineries as well as development of novel mechanisms for organelle biogenesis. In these plastid replacement events the impact of former plastids on the new ones can be seen with adoption of genes and genetic processes from the old to the new, in doing so forming an amalgam of plastid biochemistries and histories. These somewhat more recent and, indeed, ongoing cases of endosymbiosis in dinoflagellates bring into sharper focus the processes of organelle formation that otherwise lose resolution with antiquity. Many of the principles displayed in plastid formation in dinoflagellates, therefore, might just as likely have contributed to the formation of plastids throughout eukaryotic diversity, be they primary or complex.

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