Lost in the Light: Plastid Genome Evolution in Nonphotosynthetic Algae

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Abstract

Photosynthesis is an awe-inspiring process. It has shaped, coloured, and diversified the biological world in innumerable ways and supplies us with the air we breathe. Photosynthetic organisms are literally our lifelines on Earth. Without them we perish. Perhaps this is why many of us are uncomfortable with and confused by the concept of a photosynthetic organism forfeiting its ability to convert sunlight into chemical energy, giving up its life-sustaining powers. Indeed, the evolutionary loss of photosynthesis, which has occurred countless times throughout evolution, remains a poorly understood and underappreciated topic, both among researchers and the general public. This is unfortunate because nonphotosynthetic plants and algae represent some of the most diverse and interesting (and even deadly) species on the planet, and they can teach us a lot about photosynthesis and biology as a whole. Here, I review the origins and evolution of nonphotosynthetic eukaryotic algae. I portray these biologically "broken light bulbs" in a contemporary framework, paying particular attention to their plastid genomes, which are much more complex and architecturally varied than one might expect. If you are anything of a rebel and prefer misfits over conformists, trouble makers over the straight-laced, and mysteries over simple plotlines, then you will not be disappointed by the eclectic assemblage of algae that have relinquished their hold on the sun.

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1. INTRODUCTION

Your absence has gone through me
Like thread through a needle.
Everything I do is stitched with its color.

W.S. Merwin

When I was an undergraduate student in Biology, I detested courses on plants and algae. Human genetics, animal behaviour, disease-causing bacteria—bring it on! Anything but botany. I still cringe at the thought of having to memorize the life cycle of a fern, and the only time I perked up in an entire semester of plant physiology was when the instructor talked about *Psilocybe* species (magic mushrooms). Who would have guessed at the time that I would go on to have a career in the plant sciences, studying the genes and genomes of eukaryotic algae? Certainly not my plant phys prof, who graciously gave me a passing grade.

I was a late bloomer. It would take another 2 years and strong persuading from my eventual PhD supervisor before I finally saw the proverbial photosynthetic light and made the scientific leap to the realm of chloroplast-containing organisms. My gateway drug into this verdant domain was not what you might expect. It wasn't some beautiful, mellifluous flower or a magnificent 200-ft. redwood. It wasn't even the bright kaleidoscopic colours of chlorophyll that first swayed me. It was something more drab and faded, and went by the name *Polytomella*.

At the first meeting with my prospective PhD supervisor, Robert Lee, he led me into the hallway outside of his cluttered office and pointed enthusiastically to a four-by-four-foot poster on the wall, which described an obscure green alga called *Polytomella*. "Have you ever heard of this critter?" asked Bob, tapping his hand against the poster. I hadn't. "That's a shame, because it is one awesome little unicell," he exclaimed. "It's free living has four flagella and a plastid, but lacks chlorophyll and can no longer derive energy from sunlight. In other words, it's a photosynthetic burnout, a green alga that isn't even green." That was my introduction to the world of non-photosynthetic algae. Being a bit of a burnout myself, I was immediately hooked and itching with curiosity.

How did achromatic algae evolve and how do they survive? Why do they lug around a plastid (the epicentre of photosynthesis) if they're non-photosynthetic? Are there different types of colourless algae, or is *Polytomella* the only one? Have certain land plants also lost photosynthetic capabilities?

Why, in Darwin's name, did I not hear anything about this in my undergraduate biology courses? And where do I sign up to start researching these organisms? Soon, I would have even more questions as I trudged through a 5-year PhD on the organelle genetics of *Polytomella* and its close relatives. I would quickly come to realize that nonphotosynthetic algae and land plants are surprisingly diverse and among the most intriguing and enigmatic species on the tree of life.

The forfeiting of photosynthesis has occurred numerous times and in disparate lineages throughout eukaryotic evolution (Blouin & Lane, 2012; Figueroa-Martinez, Nedelcu, Smith, & Reyes-Prieto, 2015; Keeling, 2013; Krause, 2008). Wherever you find photosynthesis, you will also find examples of its loss (Keeling, 2013). Nonphotosynthetic plastid-bearing species can be found in almost every kind of environment and ecosystem. They can be mind bogglingly beautiful or downright ugly, abundant or scarce, benign or deadly. Some are prolific predators, others are peaceful osmotrophs, and many are terrifying parasites with global health and economic implications (Figueroa-Martinez et al., 2015; Janouškovec et al., 2015). Most are incredibly tiny, often going unnoticed by even the keenest observers, and a few are gargantuan, by any standard of the word.

Indeed, the infamous nonphotosynthetic parasitic land plant *Rafflesia* has the largest known flower of any angiosperm, measuring, in some species, over 3 ft. in diameter and weighing over 20 lb (Meijer, 1984). But woe betide to anyone who goes looking for this floral behemoth, for if they are lucky enough to find it, they may get an unfortunate surprise:

> *Much has been made of the smell produced by* Rafflesia *flowers: an early traveler once described it as 'a penetrating odour more repulsive than any buffalo carcass in an advanced stage of decomposition' … Given their rarity and unpredictability, it is remarkable that anyone ever sees a* Rafflesia *flower in all its glory. But of course, they do. Two localities in Sabah [Borneo] offer a reasonable chance of success. … If one should bloom a sign immediately appears on the main road that a* Rafflesia *is flowering, and they charge passerby a fee to see their prized flower. Make no mistake, on a local scale this is big business, as several hundred tourists have been known to see a single flower over the course of a five- to six-day blooming period.*
>
> **Garbutt and Prudente (2006)**

Rafflesia aside, most species that have lost photosynthesis are not tourist attractions, but they are the focal point for cutting-edge research. Studies of colourless algae have improved our understanding of endosymbiosis (Janouškovec et al., 2015), cell biology (McFadden & Yeh, 2017), genome

evolution (Smith & Lee, 2014), and the diversification of life (Burki et al., 2016). They have also redefined how we view plastids (Fichera & Roos, 1997) and raised questions about what defines an alga or plant (Janouškovec et al., 2017). Some colourless lineages retain many of the features and machineries of their close photosynthetic relatives and are reliant on their plastid and plastid genome (plastome), others have completely done away with plastid DNA (ptDNA) and its associated gene expression system (Smith & Asmail, 2014), and some have gone a step further abandoning the plastid entirely (Gornik et al., 2015). If that weren't enough, there are organisms that have lost and regained plastids (Janouškovec et al., 2015).

As I tell my students whenever they get bored of my proselytizing about plastid evolution, research on colourless algae is not limited to basic science and, in fact, might hold the secrets for curing deadly diseases. For example, the malaria parasite (*Plasmodium falciparum*) and the causative agents of toxoplasmosis (*Toxoplasma gondii*) each have a nonphotosynthetic plastid called an apicoplast, and ever since it was first discovered in the mid 1990s scientists have been proclaiming its potential for therapeutic intervention (Fichera & Roos, 1997). The cyanobacterial-derived pathways within the apicoplast "are all very distant from human host metabolism and cellular processes, leaving room to design or discover specific inhibitors that would perturb the apicoplast but have no side effects" (McFadden & Yeh, 2017). Scientists are desperately trying, and have had some moderate success, in designing drugs blocking key apicoplast pathways, including those connected to the replication, transcription, and translation of ptDNA (Goodman, Pasaje, Kennedy, McFadden, & Ralph, 2016). It's not just humans who are at the mercy of parasitic nonphotosynthetic algae: the apicoplast-containing genera *Babesia*, *Eimeria*, and *Theileria* can cause serious diseases in domesticated (and undomesticated) animals, such as cattle, chickens, and other livestock (Foth & McFadden, 2003). But don't let these parasites bias you against nonphotosynthetic algae. Many, like *Polytomella*, are benign, do more good than harm, and are poised to become model research species.

Below, I explore the good and the bad sides of nonphotosynthetic algae, focusing on recent major discoveries in plastid genomics. I highlight the remarkable diversity in ptDNA architecture among colourless protists and how these data have advanced the fields of organelle genetics and plastid biology. But before we can discuss the nitty-gritty of nonphotosynthetic plastids, we first need to examine how photosynthetic plastids and their genomes came to be.

Perhaps, the most amazing thing about plastids is that they exist at all. Their labyrinthine journey from free-living bacteria to integral and inalienable components of algae and land plants involved countless winding, diverging, and colliding roads, and a lot of luck. The story of plastids has many plots, many characters, is replete with whimsy and mystery, and is still ongoing. Certain aspects of plastid evolution remain unresolved and are mired in debate, confusion, and controversy, but thankfully we now have a clear understanding of the key players and main events that first gave rise to eukaryotic phototrophy.

2. AND THEN THERE WAS LIGHT

When you think about the complexity of our natural world—plants using quantum mechanics for photosynthesis, for example—a smartphone begins to look like a pretty dumb object.

Jeff VanderMeer

Today, eukaryotic life is teeming with photosynthesis; it occurs in at least half of the currently defined supergroups (Burki, 2014). But it wasn't always like this. For the first few hundred million years of eukaryotic evolution there were no plastids. Eukaryotes owe their existence to a 1.8-billion-year-old cellular merger between two obligate heterotrophs: a bacterial endosymbiont (which resembled present-day alphaproteobacteria) and an archaeal host (which is thought to be linked to the Lokiarchaeota) (Gray, 2012; Spang et al., 2015). Early eukaryotes and the initial lineages that they gave rise to were entirely devoid of photosynthesis. Things would have remained that way until relatively recently[a] (Nowack, 2014) if it weren't for a fortuitous primary endosymbiotic event between a photosynthetic bacterium (the endosymbiont) and a unicellular nonphotosynthetic eukaryote (the host) about one and a half billion years ago (Archibald, 2015; Smith, 2017).

It makes intuitive sense why a heterotroph would want to hijack a cyanobacterium—for the sweet rewards of photosynthesis, of course—but precisely how this enslavement occurred is not so straightforward.

[a]The unicellular eukaryote *Paulinella chromatophora* (Rhizaria, Cercozoa) has a recently acquired cyanobacterial endosymbiont. Between 60 and 200 million years ago, the ancestor of this little-known amoeboid alga transitioned from a heterotrophic bacterivorous existence, sustained in part by feeding on cyanobacteria, to a phototrophic one, dependent on a cyanobacterial endosymbiont called a chromatophore (Nowack, 2014). *P. chromatophora* is the only known example of primary acquisition of a photosynthetic organelle outside of that which generated the Archaeplastida.

Undergraduate textbooks like to depict it as a single step: an image of a Pac-Man-esque eukaryote gobbling up an unsuspecting green dot. And then, voilà, a fully integrated chloroplast, with all the bells and whistles, within a modern-day plant or alga. Don't be fooled, this fast-tracked version of primary endosymbiosis is an oversimplification. Plastid organellogenesis was undoubtedly more complex, drawn out, and multifaceted than many textbooks would have us believe, occurring at a population level and an evolutionary timescale, and likely involving multiple contributing partners. Some of these complexities are described in the "shopping bag model" (Larkum, Lockhart, & Howe, 2007) of primary plastid evolution:

> *It seems unlikely that the stable [cyanobacterial] symbiont ultimately acquired by the host cell would be the first one it had ever acquired. The acquisition would almost certainly have been preceded by the uptake of other photosynthetic organisms. … [E]arly rounds of failed endosymbiosis, with some would-be endosymbionts eventually lysing and liberating DNA into the cytosol, would result in integration of endosymbiont DNA into the nuclear genome. This DNA would have persisted in the nucleus for a period of time, even if there were no longer functional symbionts in the host cytoplasm. If, finally, a symbiont [was] able to establish a balanced relationship with the host, the reservoir of sequences in the host nucleus that were derived from previous photosynthetic organisms would have provided a pool of sequences to encode proteins to be imported into the newly established plastid.*
>
> **Howe, Barbrook, Nisbet, Lockhart, and Larkum (2008)**

As provocative as the shopping bag scenario may be, it remains to be determined how many, if any, failed endosymbioses preceded the successful cyanobacterial endosymbiont—and should be stressed that early plastid evolution is an ongoing area of debate (Dagan et al., 2013; reference therein). However, there is strong evidence that the ultimate progenitor of all plastids was a fan of freshwater and hails from a newly uncovered clade called *Gloeomargarita* (Ponce-Toledo et al., 2017). Using a comprehensive phylogenomic dataset, Ponce-Toledo et al. (2017) showed that *Gloeomargarita lithophora*—a deep-branching, biofilm-forming cyanobacterium—is the closest known prokaryotic relative of plastids. What's more, the entire *Gloeomargarita* group appears to be restricted to freshwater environments, suggesting that eukaryotic photosynthesis first emerged in a terrestrial freshwater setting.

So, after a long, fortuitous start and some help from *Gloeomargarita* et al., photosynthesis became firmly established within the eukaryotic domain, eventually giving rise to the supergroup Archaeplastida (Adl et al., 2012), which is made up of red algae, green algae, land plants, and glaucophytes.

Each of these archaeplastidal lineages can trace their photosynthetic properties directly back to the *Gloeomargarita*-like endosymbiont and as such are said to have primary plastids, which contain two membranes (Keeling, 2013; Reyes-Prieto, Weber, & Bhattacharya, 2007). Not surprisingly, the first lineage to diverge within the Archaeplastida (the Glaucophyta) (Ponce-Toledo et al., 2017) is completely restricted to freshwater environments, thus, following in the footsteps of its cyanobacterial progenitor (Delwiche & Cooper, 2015). But the other archaeplastidal lineages, in addition to being found on land and in freshwater, have successfully colonized saltwater ecosystems (Keeling, 2013; Reyes-Prieto et al., 2007).

If life was simple and evolution was a straight road the story of eukaryotic photosynthesis would stop here. But as any card-carrying biologist will tell you, evolution can be a crooked and winding process, and is not opposed to taking the odd sidestep. Accordingly, plastids and photosynthesis have jumped horizontally from the Archaeplastida to other supergroups via eukaryote–eukaryote endosymbioses (Archibald, 2015; Burki, 2017; Keeling, 2013). It is a dog-eat-dog world and many heterotrophic protists make their living by devouring eukaryotic algae. Factor in a little evolutionary indigestion and some of the ideas from the shopping bag model and before you know it the photosynthetic food has become a photosynthetic endosymbiont, and then fast-forward a few more million years and it's now a bona fide photosynthetic organelle. Red algae are no stranger to this narrative, having weaved their photosynthetic powers and plastids into some pretty remote phylogenetic corners. For example, haptophyte algae (e.g. *Emiliania*), diatom algae (e.g. *Phaeodactylum*), golden algae (e.g. *Ochromonas*), and brown algae (kelp) all have red-algal-derived plastids, as do apicomplexan parasites, such as *P. falciparum*, and most dinoflagellates (e.g. *Symbiodinium*) (Archibald, 2015; Burki, 2017; Keeling, 2013). The number of eukaryote-to-eukaryote endosymbiotic events that occurred to give rise to the complex red-algal-derived plastids is hotly debated (Burki, 2017). Green algae are in on the action as well, transferring their plastids to euglenophytes (e.g. *Euglena*) and the dinoflagellate lineage *Lepidodinium* (Kamikawa, Tanifuji, Kawachi, et al., 2015) in separate secondary endosymbiotic events.

One of the major goals and outcomes of evolutionary genomics has been disentangling the convoluted history of plastids derived from one eukaryote merging with another (commonly referred to as complex plastids). As it currently stands, plastids have moved laterally from one eukaryotic lineage to another no fewer than five times (Archibald, 2015). Tracking these

movements can literally be a game of "keep your eyes on the plastid." On at least three separate occasions, a heterotrophic eukaryote has snatched a plastid (via tertiary endosymbiosis) from an alga that itself acquired its plastid secondarily from a red alga (Burki, 2017). Equally as convoluted are serial endosymbioses, whereby a secondary plastid is replaced by another plastid (Kamikawa, Tanifuji, Kawachi, et al., 2015).

In most cases, all that remains of these secondary, tertiary, or serial endosymbiotic events is the final product: an integrated, functional plastid with one or more extra membranes—a consequence of all that jumping around and the reason behind the name "complex" plastid. But sometimes the crime scene has not been entirely cleared. For cryptophytes and chlorarachniophytes, the nuclei and nuclear genomes of the engulfed primary algae—a red alga and green alga, respectively—persist in the host cell (alongside the plastid) as highly reduced organelles called nucleomorphs (Moore & Archibald, 2009).

Algae with complex plastids may seem a bit like endosymbiotic circus acts, but keep in mind that they carry out a significant proportion of the photosynthesis that occurs on Earth, and thus play an important role in reducing global atmospheric carbon dioxide levels. But, as described later, it is not always bright and sunny in the world of complex or primary plastids. Both of these kinds of plastid have discarded their photosynthetic abilities on many occasions.

3. BURNING OUT: THE EVOLUTIONARY LOSS OF PHOTOSYNTHESIS

The world breaks everyone and afterward many are strong at the broken places. But those that will not break it kills. It kills the very good and the very gentle and the very brave impartially. If you are none of these you can be sure it will kill you too but there will be no special hurry.

Ernest Hemingway—A Farewell to Arms

As counter intuitive as it may seem, a large number of algae and plants can no longer convert carbon dioxide and water into sugar and oxygen (Blouin & Lane, 2012; Figueroa-Martinez et al., 2015; Keeling, 2013; Krause, 2008). Most colourless algae are not easy to observe with the naked eye, and it is really only those who study them in the lab under a microscope that have seen one up close and personal. Nonphotosynthetic land plants, on the other hand, are hard to miss, even if they are not all as massive as *Rafflesia*; they can

even be quite beautiful and ghostly, given their lack of chlorophylls, as anyone who has gazed upon the porcelain-like petals of fringed pinesap, Indian pipe, hillside broomrape, or the flatglobe dodder can attest. Beautiful or not, why would any self-respecting and sound-minded alga or plant forsake photosynthesis, especially after all the trouble and time to acquire a plastid? The answer to this question is not as mysterious or baffling as you might expect, and has its roots in a feeding strategy called mixotrophy.

As the name implies, mixotrophic algae and plants can make use of both inorganic and organic carbon sources via photoautotrophy and chemoheterotrophy, respectively. The latter is achieved by phagocytosing entire cells (i.e. predation) or through the endocytosis or osmosis of organic compounds—or simply put: engulfing or absorbing things from the environment. Sounds like a great strategy, right? Make sugar while the sun is shining and the gettin' is good, and keep filling the coffers even if things go dark and you're stuck, for instance, under Arctic sea ice for 6 months. Being mixotrophic also means that a random mutation knocking out photosynthetic (or heterotrophic) capabilities would not necessarily be lethal, which it would be in an obligate photoautotroph.

Despite its obvious benefits, mixotrophy is a mixed blessing because it is metabolically expensive to sustain both trophic strategies, so much so that mixotrophic algae are thought to expel five times more energy and nutrients on preserving photosynthesis than on the upkeep of heterotrophy (Raven, 1997). Therefore, given the right conditions, such as when the metabolic costs of maintaining the photosynthetic machinery exceed the benefits, doing away with photoautotrophy can arguably be advantageous, even when light conditions are favourable (de Castro, Gaedke, & Boenigk, 2009). Such a view is supported by the fact that the loss of photosynthesis is not uncommon among mixotrophic species:

> *Extant colorless algal lineages have either phagotrophic or osmotrophic lifestyles, and this is generally a reflection of the heterotrophic strategy employed by their mixotrophic relatives. For example, phagotrophic colorless algae can be found among dinoflagellates, stramenopiles and cryptophytes; this lifestyle is consistent with the presence of phagotrophism in their close mixotrophic relatives. Other colorless algae, such as the chlorophyte green algae* Helicosporidium, Prototheca, Polytoma, *and* Polytomella, *are closely related to osmo-mixotrophic chlorophytes and adopted an osmotrophic strategy where the source of dissolved organic matter can be either a host (in the case of pathogenic/parasitic species) or the environment (in free-living species).*
>
> ***Figueroa-Martinez et al. (2015)***

Thus, for colourless algae, mixotrophy appears to be a prerequisite for losing photoautotrophic functions. The same theme also emerges from work on nonphotosynthetic land plants (Julou et al., 2005; Selosse, Charpin, & Not, 2017). One could debate whether photosynthetic loss is adaptive (e.g. shedding the burden of photosynthesis) or nonadaptive (e.g. random genetic drift), but there is no denying that a single mutation in the right place to the right gene is sometimes all it takes to bring down the entire photosynthetic apparatus and dramatically change phenotype and lifestyle.

Work on the model green alga *Chlamydomonas reinhardtii* has shown that point mutations to photopigment genes can shut down photosynthesis (McCarthy, Kobayashi, & Niyogi, 2004; Meinecke et al., 2010). A nonphotosynthetic mutant of *C. reinhardtii* defective for phytoene synthase—one of the first enzymes in carotenoid biosynthesis—bears a remarkable resemblance to naturally occurring colourless algae, exhibiting starch accumulation, a disorganized eyespot, and no pyrenoid (Inwood, Yoshihara, Zalpuri, Kim, & Kustu, 2008). Moreover, the lack of carotenoids leads to plastids with no stacked thylakoidal membranes, paralleling the situation in other nonphotosynthetic chlamydomonadaleans (Inwood et al., 2008). This mutant can also grow in the dark with acetate as a carbon source implying "that mutations of this type would be nearly neutral in environments where photosynthesis is not critical for carbon assimilation and offers an ecological scenario and a plausible explanation for the origin of free-living heterotrophic colourless algae" (Inwood et al., 2008).

Although colourless algae have often taken a similar route to arriving at heterotrophy, the outcome following the loss of photosynthesis can vary within and among lineages. It can result in obligate parasitism (e.g. *P. falciparum*) or an opportunistic pathogenic existence (e.g. the green alga *Prototheca wickerhamii*), a voracious predatory lifestyle (e.g. the colpodellid *Voromonas pontica*), or a harmless osmotrophic one (e.g. the green alga *Polytomella*). With respect to the Apicomplexa, the evolutionary loss of photosynthesis spawned an entire phylum of dangerous obligate animal parasites. Conversely, for green algae, nonphotosynthetic parasites, infecting everything from plants to insects to humans, have evolved multiple times independently within closely related lineages interspersed with photosynthetic taxa, and the same is true for free-living colourless green algae (Figueroa-Martinez et al., 2015). Similar trends are observed in red algae, which are estimated to have the largest number of recently photosynthetic

parasites of any major group, including nearly half of all recognized floridiophytes (Blouin & Lane, 2012). And don't get me started on the various flavours of parasitic nonphotosynthetic land plants, described in detail in Wicke (2018).

It might be easy to do away with photosynthesis, but it is not so easy to dump a plastid—all known nonphotosynthetic members of the Archaeplastida, for example, retain one (Archibald, 2015; Keeling, 2013). This is because as plastid endosymbiosis took hold, the host became dependent upon its cyanobacterial (or plastid-donating) partner for much more than photosynthesis. In plants and algae, many vital biochemical pathways unrelated to photosynthesis are outsourced entirely or partly to the plastid, such as the biosynthesis of aromatic and hydrophobic side-chain amino acids, tetrapyrroles, and terpenoids (Gould, Waller, & McFadden, 2008). Although nearly all the enzymes involved in these pathways are nuclear encoded, most nonphotosynthetic plastids still retain a genome, albeit one that is typically highly reduced with a much smaller gene content than that in photosynthetic taxa (Figueroa-Martinez et al., 2015; Graham, Lam, & Merckx, 2017; Krause, 2008). As described in the following sections, the plastomes of nonphotosynthetic species are architecturally diverse and can tell us a lot about the processes involved with and the consequences of forgoing photosynthesis.

4. GENETIC BALL AND CHAIN: PLASTOMES IN COLOURLESS ALGAE

Any half-awake materialist well knows—that which you hold holds you.

Tom Robbins

Unless you are in the field of plastid genetics, your idea of a plastome probably looks something like this: an intact, AT-rich circular molecule of approximately 150 kilobases (kb) encoding a few dozen proteins mostly involved in photosynthesis. Yes, this image fits the classic plastid genome map of *Arabidopsis* or corn or rice, but it is not representative of most ptDNAs. For both photosynthetic and colourless species, plastomes span the gamut of size, structure, and content (Green, 2011; Smith & Keeling, 2015).

The plastomes of photosynthetic algae, for instance, can be enormous, exceeding a million base pairs and 90% noncoding DNA in the red alga

Corynoplastis japonica (Muñoz-Gómez et al., 2017) and the green alga *Acetabularia acetabulum* (based on partial on ptDNA sequence; de Vries et al., 2013), or small and compact, like the 66-kb ptDNA of the dinoflagellate *Lepidodinium chlorophorum* (Kamikawa, Tanifuji, Kawachi, et al., 2015). They can be contained in long linear chromosomes with monomeric, concatenated, or branched structures (Bendich, 2004; Smith & Keeling, 2015), or fragmented into dozens of small circular molecules, as exemplified by the *Symbiodinium* ptDNA (Barbrook, Voolstra, & Howe, 2014). They can be biased in adenine and thymine or guanine and cytosine (Smith, 2012), and can contain fewer than 25 genes or as many as 250 (Janouškovec et al., 2013). And the expression of these genomes can involve nonstandard codes, the removal of dozens of introns (even introns within introns), and complicated forms of posttranscriptional processing—dinoflagellate ptDNAs are an amusement park for substitutional RNA editing (Knoop, 2011; Smith & Keeling, 2016). Thus, plastomes are much more multifarious and bizarre than most scientists might think.

The standard narrative for what happens to ptDNA after the forfeiture of photosynthesis is one of gene loss and an overall reduction in complexity. Take the 56-kb plastome of the nonphotosynthetic green alga and opportunistic animal pathogen *P. wickerhamii*. When compared to its close free-living photosynthetic relative *Auxenochlorella protothecoides*, it looks like someone came along and surgically removed nearly all of the genes connected to photosynthesis from the *P. wickerhamii* ptDNA, leaving behind 27 tRNAs, a few rRNAs, and 40 protein-coding genes (Yan et al., 2015). Nearly all of these remaining genes are involved in plastid gene expression—a complicated process involving both plastid- and nuclear-encoded machinery (Gould et al., 2008). What makes this gene loss all the more striking is that the *P. wickerhamii* and *A. protothecoides* ptDNAs are completely syntenic, photosynthetic genes notwithstanding (Yan et al., 2015).

The *P. wickerhamii* ptDNA, however, still bears the marks of its photosynthetic past, harbouring a nearly full complement of chloroplast ATP synthase subunit genes, which are typically associated with the electron transport chain of photosynthesis. These same genes have also been found in the plastomes from two other nonphotosynthetic unicellular algae (the cryptophyte *Cryptomonas paramecium* and the diatom *Nitzschia* sp.) and several parasitic plants (Donaher et al., 2009; Kamikawa, Tanifuji, Ishikawa, et al., 2015). This, alongside the absence of other photosynthesis-related genes from these genomes, has left researchers scratching their heads as to

why ATP synthase subunits are retained in some colourless plastids. A Japanese group working on *Nitzschia* has an interesting hypothesis:

> *It is possible that these ATP synthase complexes might be retained for ATP synthesis using a proton gradient generated through an as yet unknown, photosynthesis-independent mechanism. Here, we suggest an alternative function: … that following loss of photosynthesis, the ATP synthase complex in the nonphotosynthetic diatom plastids has functioned to hydrolyze ATP to maintain a proton gradient between the thylakoid lumen and stroma, required for the Tat-dependent protein translocation system. … we suggest that the Tat system also functions (or has worked) in [other] nonphotosynthetic plastids, and could again be the main reason for the retention of ATP synthase genes ….*
>
> **Kamikawa, Tanifuji, Ishikawa, et al. (2015)**

Supporting this hypothesis is the presence of a gene for Tat in the plastome of *Nitzschia* sp., but such a gene is lacking from the ptDNAs of *P. wickerhamii* and *C. paramecium* (Donaher et al., 2009; Yan et al., 2015). And by no means do the ptDNAs of all nonphotosynthetic algae contain ATP synthase genes (Figueroa-Martinez, Nedelcu, Smith, & Reyes-Prieto, 2017). In fact, at least one is a pseudogene in *C. paramecium* (Donaher et al., 2009), and they have been entirely lost from the ultracompact 37-kb ptDNA of *Helicosporidium* sp., a nonphotosynthetic pathogen and very close relative of *P. wickerhamii* (de Koning & Keeling, 2006).

Like *Helicosporidium*, the plastomes of apicomplexan parasites are paragons of compactness, ranging from about 30 to 40 kb, having as little as 5% intergenic DNA, and encoding around 30 proteins, mostly for transcribing and translating ptDNA, and none representing subunits of ATP synthase (Foth & McFadden, 2003; Janouškovec et al., 2015). For the longest time, the Apicomplexa held the record for the smallest ptDNAs ever observed. But in recent years more extreme examples of plastid genomic reduction have come from heterotrophic land plants, such as the orchid *Epipogium roseum* (19 kb) and the holoparasite *Pilostyles aethiopica* (11.4 kb) (Bellot & Renner, 2015; Schelkunov et al., 2015).

Whether you are talking about the ptDNA of colourless algae or heterotrophic plants, some common themes arise, including a small genome size, a reduced coding repertoire, a paucity of intergenic and intronic DNA, genomic rearrangements, a particularly high AT content, and elevated rates of sequence evolution (de Koning & Keeling, 2006; Figueroa-Martinez, Nedelcu, Smith, et al., 2017; Garg et al., 2014; Wicke, Müller, Quandt, Bellot, & Schneeweiss, 2016). But as biologists explore more and more ptDNAs, they are finding that these trends do not always hold. The plastome

of the free-living colourless alga *Euglena longa* is far from intron-poor, boasting 61 introns (Gockel & Hachtel, 2000)—although keep in mind that the ptDNA of its close photosynthetic relative *Euglena gracilis* has an unprecedentedly large number of introns (160) (Hallick et al., 1993). Another strange thing about *E. longa* is that its ptDNA encodes the large subunit of the enzyme RuBisCO (RBCL), and the small subunit of this enzyme (RBCS) is encoded in the nuclear genome as a precursor polyprotein comprising multiple RBCS repeats (Chan, Keller, Canaday, Weil, & Imbault, 1990). What on Earth is a nonphotosynthetic species doing with RuBisCO? A team of Czech researchers think the answer may be "absolutely nothing."

> *Both the RBCL and RBCS proteins are synthesized in* E. longa, *but their abundance is very low compared to* E. gracilis. *No RBCS monomers could be detected in* E. longa, *suggesting that processing of the precursor polyprotein is inefficient in this species. The abundance of RBCS is regulated post-transcriptionally. Indeed, blocking the cytoplasmic translation by cycloheximide has no immediate effect on the RBCS stability in photosynthetically grown* E. gracilis, *but in* E. longa, *the protein is rapidly degraded. Altogether, our results revealed signatures of evolutionary degradation (becoming defunct) of RuBisCO in* E. longa *and suggest that its biological role in this species may be rather unorthodox, if any.*
>
> **Záhonová, Füssy, Oborník, Eliáš, and Yurchenko (2016)**

The *E. longa* RuBisCO enigma exemplifies another common thread running through the field of nonphotosynthetic plastid genomics—that there are usually one or more genes kicking around in the ptDNA whose function in a nonphotosynthetic context is not easily explained. Other protein-coding genes that meet this criterion include *clpP*, *ftsH*, and *ycf1*, which have been independently conserved in the ptDNAs of diverse colourless algae (Figueroa-Martinez, Nedelcu, Smith, et al., 2017), but arguably do not have clearly defined roles in heterotrophic taxa. The *clpP* gene product (a subunit of a ClpP peptidase) is thought to be involved in protein homeostasis (Ramundo et al., 2014), that of *ftsH* is believed to be an essential protease (de Vries et al., 2013; Maul et al., 2002), and the precise function of *ycf1* is unknown (de Vries, Sousa, Bölter, Soll, & Gould, 2015; Nakai, 2015) but might be related to membrane anchorage and/or nucleic acid binding (Boudreau et al., 1997; Drescher, Ruf, Calsa, Carrer, & Bock, 2000; Ozawa et al., 2009).

The idea that nonphotosynthetic ptDNAs can harbour genes for essential pathways apart from photosynthesis is one of the main arguments for why most colourless species still sustain a plastid genome and all that entails

(Janouškovec et al., 2015). Plastid genome replication and gene expression require a complex infrastructure spanning two genetic compartments and involving hundreds of proteins. It might seem wasteful and inefficient for such an exhaustive system to persist so that only a few (or less) key metabolic genes from the ptDNA can be expressed. But if the gene or genes in question are essential and haven't successfully moved to another compartment, then the ptDNA is indispensable and the genomic bureaucracy must endure. Consequently, it was long believed that nonphotosynthetic plastids were irreversibly tied to their genomes (Barbrook, Howe, & Purton, 2006; Nair & Striepen, 2011), but now it is known that at least some species have broken free of this genetic "ball and chain."

5. ADIÓS PTDNA: THE OUTRIGHT LOSS OF A PLASTOME

Perfection is achieved, not when there is nothing more to add, but when there is nothing left to take away.

Antoine de Saint-Exupery

When I was a PhD student, my supervisor Bob (who made a cameo in the beginning of this chapter) would always march into the lab with grandiose ideas and flamboyant hypotheses. "Here's what we're going to do, Smitty," he'd say. "We're going to merge *Chlamydomonas* with *Polytomella*! What do you think—shall we call it *Chlamydomella* or *Polytomonas*?" Most of his proclamations, like *Chlamydomella*, were merely meant to produce a smile or a laugh, but sometimes he'd come up with intriguing ideas formed from years of careful observation and hours of critical thought. Shortly after I arrived in the lab, Bob became adamant that *Polytomella* (a colourless chlamydomonadalean green alga, in case you forgot) was missing a plastid genome, something the other lab members, including myself, were sceptical about. Bob's assertion was based in part on the inability to detect plastid rRNA in *Polytomella* using Northern blot or PCR experiments (Nedelcu, 2001; Nedelcu, Spencer, Denovan-Wright, & Lee, 1996). But as every scientist knows, it is much harder to prove that something doesn't exist than prove that it does exist. After a number of inconclusive experiments on the presence/absence of *Polytomella* ptDNA, next-generation sequencing technologies arrived to the rescue.

High-throughput sequencing of total cellular DNA or RNA from an alga or plant, including nonphotosynthetic ones, typically yields a large number of plastid-derived reads, which can be used to assemble complete

or nearly complete plastid genomes or transcriptomes (Shi et al., 2016; Smith, 2013). However, extensive Illumina sequencing of four different *Polytomella* species uncovered not a single identifiable ptDNA or RNA sequence (Smith & Lee, 2014). Although encouraging, this observation by itself was not enough to confidently conclude that *Polytomella* algae have no plastid genome. The real smoking gun came from an exhaustive bioinformatics search and characterization of nuclear-encoded, plastid-targeted proteins from *Polytomella*. This search uncovered a diversity of biochemical pathways occurring in the *Polytomella* plastid, such as isoprenoid biosynthesis and amino acid metabolism, but not one associated with replicating, repairing, transcribing, or translating a plastome (Asmail & Smith, 2016; Smith & Lee, 2014). So, after nearly a decade of working on the organelle genetics of *Polytomella*, Bob and I were finally able to provide sufficient data to support outright plastid genome loss in this colourless genus. On the day that the paper was accepted, we had champagne on ice ready to celebrate the first example of a plastid-bearing lineage with no ptDNA only to discover that another team had beaten us to the summit by only a few weeks. Like *Polytomella*, the nonphotosynthetic and parasitic angiosperm *Rafflesia lagascae* appears to have entirely shed its ptDNA (Molina et al., 2014).

The authors of the *Rafflesia* paper sequenced and assembled vast amounts of whole genomic DNA isolated from an *R. lagascae* floral bud and then scanned the resulting reads and contigs for plastid-derived sequences. Although they easily identified a large number of high-coverage contigs corresponding to the mitochondrial genome, they found very few with similarity to genic or intergenic sequences normally found in land plant plastomes. Moreover, not one of the plastid-like contigs contained a complete gene or an intact open reading frame, nor were they phylogenetically associated with close relatives of *Rafflesia*, but instead affiliated with species closely related to *Tetrastigma* (the plant that *R. lagascae* parasitizes). Based on these findings, Molina et al. (2014) argued that the plastid sequences recovered from the Illumina sequencing came from the nuclear (and in a few cases mitochondrial) genome and were horizontally transferred to *R. lagascae* from the plastome of *Tetrastigma*. Unfortunately, there were no accompanying data on nuclear-encoded, plastid-targeted proteins in *R. lagascae* to support the hypothesis of plastid genome loss—but see (Lee et al., 2016). Another concern with the interpretation of the data from *R. lagascae*, as pointed out by Krause (2015), is the current lack of physical evidence for the existence of a plastid compartment at all. [Note: a plastid clearly exists in

Polytomella (Moore, Cantor, Sheeler, & Kahn, 1970).] Krause goes on to suggest that something sneaky may be going on in *Rafflesia*:

> *It is feasible that the intimate association between* Rafflesia *and its host has led to parasite cells being populated with host plastids. The sequestration of host plastids could have relieved the parasite of the selective pressure to keep its own plastid genome. Thus, the phylogenetic loss of the plastid genome may be tolerable for the parasite because it can ontogenetically 'hijack' host organelles.*
>
> **Krause (2015)**

A fascinating hypothesis, and not without precedent. The appropriation of plastids by nonphotosynthetic organisms (kleptoplasty) is a well-documented phenomenon, performed by some dinoflagellates (Gast, Moran, Dennett, & Caron, 2007) and even animals, such as the sea slug *Elysia chlorotica*, which steals plastids from the heterokont alga *Vaucheria litorea* (Pelletreau et al., 2011). However, there are currently no confirmed examples of kleptoplasty being performed by any land plant, or archaeplastid for that matter.

To some, it may come as a surprise that the first convincing cases for ptDNA loss (*Polytomella* and *Rafflesia*) came from lineages whose plastids descend directly from a primary endosymbiosis of a cyanobacterium and not from those whose plastids derive from eukaryote–eukaryote endosymbioses (i.e. complex algae). However, there is mounting evidence that nonphotosynthetic plastids from certain complex algae have ditched their genomes. Genomic and/or transcriptomic analyses of the colpodellids *Alphamonas edax*, *V. pontica*, and *Colpodella angusta* (free-living heterotrophic relatives of apicomplexans), the dinoflagellates *Dinophysis acuminate*, *Noctiluca scintillans*, *Oxyrrhis marina*, as well as the perkinsid *Perkinsus marinus* (a close colourless relative of dinoflagellates) are consistent with these species harbouring a plastid but lacking ptDNA (Janouškovec et al., 2017, 2015). As scientists explore evermore remote and esoteric regions of the eukaryotic tree of life, they will likely discover many more species that have rid themselves of the burden and bureaucracy of ptDNA. I predict that not only will researchers expose many different reasons for hanging on to a plastome long after dropping photosynthesis, but they will discover a diversity of ways to discard of one.

What about scrapping the plastid completely? To the best of my knowledge, there are only two clear cases of plastid loss from the entire eukaryotic domain: the apicomplexan *Cryptosporidium parvum* (one of several species that cause cryptosporidiosis) and the basal dinoflagellate *Hematodinium* sp.

(a parasite of crustaceans) (Abrahamsen et al., 2004; Gornik et al., 2015). The fact that both of these parasites salvage metabolites from their host could have alleviated their metabolic dependence on a plastid. Outright plastid loss has never been observed in free-living heterotrophs, perhaps because they are dependent on plastid-derived metabolites that they cannot glean from their food or the environment (Janouškovec et al., 2017). But one particular free-living heterotroph has a claim to fame that no parasite has yet matched: plastid genomic inflation.

6. NONPHOTOSYNTHETIC PTDNA: NOT SO SMALL AFTER ALL

Improvement makes straight roads, but the crooked roads without improvement, are roads of genius.

William Blake

Closely related to *Polytomella* is another nonphotosynthetic lineage represented by *Polytoma uvella*, a free-living unicellular osmotroph. Despite the similar sounding names and modes of existence, the *P. uvella* and *Polytomella* lineages lost photosynthesis independently of one another, and unlike the latter, the former has a plastid genome (Figueroa-Martinez et al., 2015; Nedelcu, 2001). However, it wasn't until very recently that researchers learnt about the size and coding content of this genome (Figueroa-Martinez, Nedelcu, Smith, et al., 2017). Given the close phylogenetic proximity of *P. uvella* and *Polytomella* species, one might have expected *P. uvella* to have a very small ptDNA, but the opposite was true.

P. uvella currently has the largest plastome ever found in a nonphotosynthetic species: ~230 kb and 75% noncoding (Figueroa-Martinez, Nedelcu, Smith, et al., 2017). Even more impressive, the genome is tens of thousands of nucleotides larger than those of its closest known photosynthetic relatives, *Chlamydomonas leiostraca* (167 kb) and *C. applanata* (~203 kb), a trend not previously observed in any other close photosynthetic–nonphotosynthetic duo (Figueroa-Martinez, Nedelcu, Smith, et al., 2017). Regardless of its large size, the *P. uvella* plastome has, like other nonphotosynthetic ptDNAs, undergone significant gene loss, shedding all coding regions for photosynthetic pathways. But unlike other nonphotosynthetic ptDNAs that of *P. uvella* has highly expanded intergenic regions.

Maybe the tightening of intergenic regions in heterotrophic ptDNAs has less to do with the loss of photosynthesis and more to do with another

life-history feature common among many nonphotosynthetic lineages: parasitism. With some exceptions, the transition from a free living to a parasitic existence (particularly an obligate one) is associated with widespread genomic compaction (McCutcheon & Moran, 2012; Poulin & Randhawa, 2015). *P. uvella*, however, is free living and there is no reason to believe that it had a recent parasitic ancestor. Thus, the lack of genomic compaction in this colourless alga might partly be a consequence of it not being a parasite. One should also stress that the absence of parasitism certainly does not preclude a plastome from being compact, be it in a nonphotosynthetic or a photosynthetic species, and there are a number of nonparasitic colourless plants and algae with very little noncoding DNA in their plastomes (Donaher et al., 2009). But a parasitic lifestyle, in many cases, probably contributes to the extreme genomic compaction found in some ptDNAs (Figueroa-Martinez, Nedelcu, Reyes-Prieto, & Smith, 2017).

At first glance, the ptDNAs of *P. uvella* and *Polytomella* appear to have taken opposite paths following the loss of photosynthesis: genomic inflation vs complete genome loss. But, as noted by the authors of the *P. uvella* ptDNA sequence, such a claim might be misleading:

> *The evolutionary processes leading to these different events are not mutually exclusive and can occur in parallel. The loss of a plastid genome centers on coding DNA and involves the deletion of genes and the outsourcing of ptDNA-dependent pathways to other genetic compartments (Barbrook et al., 2006; Smith & Lee, 2014). Conversely, the expansion of a plastid genome acts on noncoding DNA, whereby error-prone DNA maintenance processes or selfish elements, for example, result in insertions in intergenic DNA. Therefore, the increase in noncoding DNA in a plastid genome does not preclude that genome from ultimately being lost. In fact, as noted above, repeat-rich noncoding DNA may even promote gene loss. In other words, there is no reason to assume that the nonphotosynthetic ancestor of* Polytomella *did not have a large, repeat-rich ptDNA or that* P. uvella *will not eventually lose its plastid genome. What is clear is that some chlamydomonadalean algae, whether they are photosynthetic or nonphotosynthetic, have a remarkable tendency toward extremes in organelle genome size.*
>
> **Figueroa-Martinez, Nedelcu, Smith, et al. (2017)**

In fact, the order to which both *Polytomella* spp. and *P. uvella* belong—the Chlamydomonadales—has a propensity for plastid genomic inflation, with at least six members known to have ptDNAs in excess of 250 kb (Featherston, Arakaki, Nozaki, Durand, & Smith, 2016).

There has been much debate about the forces driving organelle genomic expansion, with some arguing that it might be a consequence of random genetic drift, mutation rate, and/or inefficient and finicky DNA maintenance

processes (Smith & Keeling, 2015). The identification of an inflated ptDNA in a heterotrophic alga only adds a further layer of complexity to the already complicated conundrum of genome size evolution. If anything, the *P. uvella* plastome reinforces the idea that no type of chromosome is immune to genomic expansion, even those that exist in the dark.

7. CONCLUDING THOUGHTS

Well, now,
if little by little you stop loving me
I shall stop loving you little by little.
If suddenly
you forget me
do not look for me,
for I shall already have forgotten you.
If you think it long and mad,
the wind of banners
that passes through my life,
and you decide
to leave me at the shore
of the heart where I have roots,
remember
that on that day,
at that hour,
I shall lift my arms
and my roots will set off
to seek another land.

Pablo Neruda

Nonphotosynthetic algae remind us of the fallacy that evolution is progressive. No, evolution does not produce organisms perfectly suited to their environments. It leads to the survival of species with a diversity of traits—species that are "good enough" to get by, and colourless algae, despite the lack of photoautotrophy, certainly do get by. Plastid-bearing heterotrophs also reinforce the idea that evolution is not always adaptive. Through mutation and random genetic drift, a population can evolve in ways that are not necessarily catered to the environment in which it exists. Indeed, holding on to a resource heavy plastid and plastid genome long after relinquishing photosynthetic capabilities may not always be the best strategy, but it persists nevertheless. To fully appreciate the cellular and genomic architecture of nonphotosynthetic algae, we need to assess them in a range of evolutionary lights. I hope that when you think of these eclectic

organisms and their genomes, you do not just see broken light bulbs and a lack of chlorophyll, but also see them for all the dark and light shades of life that they encompass.

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