Retention, erosion, and loss of the carotenoid biosynthetic pathway in the nonphotosynthetic green algal genus *Polytomella*

**Introduction**

The evolutionary loss of carotenoid biosynthesis is often tied to the loss of photosynthesis, which is not surprising. In plants and algae, carotenoids are primarily associated with photosynthetic processes, from light absorption to photosystem assembly to protection from photodamage (Lohr, 2009; Cazzonelli, 2011; Santabarbara *et al.*, 2013), and they are also key constituents of algal eyespots – specialized, typically plastid-located optical devices that detect light and direct phototaxis (Kreimer, 2009; Ladygin & Semenova, 2014). In fact, a nonphotosynthetic mutant of the model green alga *Chlamydomonas reinhardtii* lacking phytoene synthase – one of the first enzymes in carotenoid biosynthesis (Fig. 1a) – bears a remarkable resemblance to naturally occurring colourless algae, exhibiting starch accumulation, a disorganized eyespot, and no pyrenoid (Inwood *et al.*, 2008). This observation has led some to suggest that mutations to the carotenoid pathway could be responsible for the evolution of nonphotosynthetic algae (Inwood *et al.*, 2008), many of which are missing the genes for carotenoid production (Borza *et al.*, 2005; Pombert *et al.*, 2014; Figueroa-Martinez *et al.*, 2015), with some notable exceptions (Tonhosolo *et al.*, 2009).

One algal system that could prove particularly useful for investigating the relationship between carotenoid biosynthesis and a heterotrophic existence is *Polytomella*: a monophyletic genus of nonphotosynthetic, free-living unicells closely related to *Chlamydomonas reinhardtii* (Pringsheim, 1955; Smith *et al.*, 2013), and not to be confused with the polyphyletic genus *Polytomella* (MacDonald & Lee, 2015). Entire plastid-located optical devices that detect light and direct phototaxis (Kreimer, 2009; Ladygin & Semenova, 2014). Entire plastid-located optical devices that detect light and direct phototaxis (Kreimer, 2009; Ladygin & Semenova, 2014). Entire plastid-located optical devices that detect light and direct phototaxis (Kreimer, 2009; Ladygin & Semenova, 2014).

For *Polytomella magna*, we recovered complete transcript and gene sequences for all of the enzymes in the carotenoid pathway up to and including lycopene β-cyclase (LCYB), which synthesizes β-carotene through the cyclization of lycopene (Fig. 1a,c). This finding is consistent with *P. magna* having an eyespot – an organelle that is known to be rich in carotenoids (Ladygin & Semenova, 2014). Entire *P. magna* transcripts and genes were also collected for carotene β-ketolase (BKT) and carotene β-hydroxylase (CHYB), which are involved in canthaxanthin and zeaxanthin synthesis, respectively. The nucleotide sequences for the remaining carotenoid enzymes, however, were not found in either the transcriptome or genome of *P. magna*, implying that its carotenoid pathway ends shortly downstream of LCYB (Fig. 1c).

In contrast to *Polytomella magna*, the entire carotenoid pathway appears to be missing from both *P. parva* and *P. piriformis*, which are the two most derived and closely
related of the four *Polytomella* spp. explored here (Fig. 1b). Neither transcripts nor genes for any of the carotenoid enzymes could be identified from *P. parva* or *P. piriformis* (Fig. 1c), which is in line with their lack of both pigmentation and eyespot structures (Pringsheim, 1955). Pseudogenes showing resemblance to carotenoid enzymes were also unde-
tectable in these two taxa.

The most unexpected observations came from *Polytomella capuana*: the genetic architecture of its carotenoid pathway is intermediate to those of *P. magna* and *P. parva*/*P. piriformis*. Like with *P. magna*, we uncovered *P. capuana* transcript and gene sequences for each of the carotenoid enzymes leading to and including LCYB and CHYB (Fig. 1c; Table S1). Unlike *P. magna*, however, the nucleotide and putative amino acid sequences of the *P. capuana* transcripts and genes were abnormal, containing large insertions and showing very poor sequence identity relative to the carotenoid transcripts/genes of *P. magna*, *Chlamydomonas reinhardtii*, and *Volvox carteri*, and in three cases they had premature stop codons (Fig. 1c,d). Moreover, the enzyme BKT, which was present in the transcriptome of *P. magna*, was missing from that of *P. capuana*, but a likely BKT pseudogene was discovered in the *P. capuana* genome (Table S1). Together, these data suggest that the *P. capuana* carotenoid pathway is in a state of decay.
**Polytomella capuana** genes for carotenoid biosynthesis: another one bites the dust

Close inspection of the *Polytomella capuana* genomic and transcriptomic sequence data indicate that the genes encoding carotenoid enzymes are under relaxed selective constraints and are accumulating deleterious mutations (Fig. 1d; Table S1). Indeed, three of the seven recovered transcripts contained premature stop codons (Fig. 1c) and six contained one or more large (>50 amino acid) insertions; these same features were also observed in the corresponding *P. capuana* genomic sequences. One of the most extreme examples of genetic degeneration within the *P. capuana* carotenoid pathway is the transcript representing LCYB: not only does it have an internal stop codon, but its putative coding sequence – again, because of a series of large insertions – is potentially >1000 nt longer than the LCYB transcripts from *P. magna*, *Chlamydomonas reinhardtii*, and *Volvox carteri* (Fig. 1d; Table S1). Likewise, the *P. capuana* CHYB transcript contained so many insertions that it was not possible to accurately align it to those from other chlamydomonadalean algae (Table S1). Signs of relaxed selection were also observed in the CHYB and BKT genes of *P. magna*, both of which catalyse reactions at the very end of its carotenoid pathway (Fig. 1c,d). Similar to the carotenoid genes from *P. capuana*, the *P. magna* BKT coding sequence had three large (>150 nt) insertions and was >600 nt longer than the BKT transcripts of *C. reinhardtii* and *V. carteri* (Fig. 1d; Table S1).

The aberrant carotenoid gene sequences in *Polytomella capuana* and *P. magna*, and the complete loss of these genes from *P. parva* and *P. piriformis* signify that the carotenoid pathway in *Polytomella* is at various stages of degradation and loss, which is presumably linked to the presence or absence of an eyespot among its members. Furthermore, this degradation follows a phylogenetic pattern whereby the pathway is present in the deepest branching lineage (*P. magna*), lost in the most derived one (*P. parva/P. piriformis*), and is in an intermediate stage of loss in *P. capuana*, which branches between the former two lineages (Fig. 1b). This pattern could make *Polytomella* an attractive group for studying the evolution, function, and loss of carotenoid biosynthesis in algae.

**Polytomella: a model system for studying the retention and loss of carotenoids**

Research into carotenoids has major implication for medicine, industry, and evolution (Cazzonelli, 2011; Shumskaya & Wurtzel, 2013). Carotenoids are crucial for human health, providing precursors for vitamin A biosynthesis, but they need to be acquired through diet, which has led to the genetic engineering of β-carotene-rich crops, such as ‘golden rice’ (Ye et al., 2000). Carotenoids are also manufactured on an industrial scale for use in nutritional supplements, medicines, and cosmetics (Cazzonelli, 2011). Among the best-studied organisms for synthesizing, harvesting, and genetically modifying carotenoids are chlamydomonadalean algae (e.g. Cordero et al., 2011), including *Haematococcus pluvialis* and *Dunaliella salina* (Fassett & Coombes, 2011), which are close relatives of *Polytomella* (Figueroa-Martínez et al., 2015). Consequently, *Polytomella* is well situated within the tree of green algae for comparative studies on carotenoid biosynthesis with model photosynthetic species. The next obvious step is a detailed biochemical characterization of the pigments within *Polytomella* taxa. Whether or not *Polytomella* spp. could be suitable for harvesting carotenoids largely depends on the amount and types of carotenoids they can produce, and if they are confined to an eyespot or exist in additional storage compartments.

Although there has yet to be a transformation system developed for *Polytomella* spp., it is conceivable that they could serve as a biological factory for carotenoid production. *Polytomella* spp. are fast growing (c. 6 h doubling time) and can be cultivated at room temperature in a simple, well-defined medium. They also constitute a potentially cost-effective industrial system since, in contrast to photosynthetic algae, their growing conditions do not require a photobioreactor, and the absence of a cell wall makes cell disruption easy.
More broadly, *Polytomella* spp. could yield insights into why the carotenoid pathway (as well as an eyespot) is maintained in some heterotrophic algae and lost in others. Although the presence of a carotenoid biosynthetic pathway is considered quite rare among well-studied nonphotosynthetic species, new data from diverse lineages are showing that it is not as rare as once thought. For instance, the nonphotosynthetic apicomplexan parasite *Plasmodium falciparum* has a red-algal-derived plastid (but no eyespot) and can synthesize carotenoids, which appear to have an important metabolic role (Tonhosolo et al., 2009); and the same might be true for other apicomplexan parasites, such as *Toxoplasma gondii* (Nagamune et al., 2008). *Polytomella*, however, appears to be the first example of a nonphotosynthetic genus in which some members have retained the carotenoid pathway whereas others have lost it. If anything, these data support the idea that in nature the evolutionary loss of photosynthesis (at least with respect to *Polytomella* spp.) can lead to the loss of carotenoid biosynthesis, rather than the other way around, which some have hypothesized (Inwood et al., 2008); that said, the data do not exclude the alternative scenario from happening in other groups. It will be interesting to see if other aspects of *Polytomella* nuclear genomic architecture follow a similar pattern to those observed here for the carotenoid pathway. An earlier study on *Polytomella* mitochondrial genomes showed that palindromic genes were lost or retained to various degrees in the four known lineages highlighted here (Smith et al., 2013). If we have learned anything about *Polytomella* genomic architecture over the past decade, it is that anything can go.

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**Author contributions**

S.R.A. and D.R.S. designed the research, performed the experiments, analysed the data, and wrote the manuscript.

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**References**


**Supporting Information**

Additional supporting information may be found in the online version of this article.

**Methods S1** Mining carotenoid genes from *Polytomella* transcriptomes and genomes.
Table S1 Carotenoid pathway transcript sequences and their sources for *Chlamydomonas reinhardtii*, *Volvox carteri*, and *Polytomella* spp.

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