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Short Communication

Nucleotide substitution analyses of the glaucophyte *Cyanophora* suggest an ancestrally lower mutation rate in plastid vs mitochondrial DNA for the Archaeplastida

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ABSTRACT

A lot is known about the evolution and architecture of plastid, mitochondrial, and nuclear genomes, but surprisingly little is known about their relative rates of mutation. Most available relative-rate data come from seed plants, which, with few exceptions, have a mitochondrial mutation rate that is lower than those of the plastid and nucleus. But new findings from diverse plastid-bearing lineages have shown that for some eukaryotes the mitochondrial mutation rate is an order of magnitude greater than those of the plastid and nucleus. Here, we explore for the first time relative rates of mutation within the Glaucophyta—one of three main lineages that make up the Archaeplastida (or Plantae *sensu lato*). Nucleotide substitution analyses from distinct isolates of the unicellular glaucophyte *Cyanophora paradoxa* reveal 4–5-fold lower rates of mutation in the plastid and nucleus than the mitochondrion, which is similar to the mutational pattern observed in red algae and haptophytes, but opposite to that of seed plants. These data, together with data from previous reports, suggest that for much of the known photosynthetic eukaryotic diversity, plastid DNA mutations occur less frequently than those in mitochondrial DNA.

1. Introduction

Some of the first insights into the relative mutation rates among plastid, mitochondrial, and nuclear DNA (ptDNA, mtDNA, and nucDNA) came from flowering plants (Wolfe et al., 1987). By comparing synonymous-site divergence levels within ptDNA, mtDNA, and nucDNA, Wolfe et al. (1987) showed that the mitochondrial genome mutation rate is about 3 and 10 times lower than that of the plastid and nucleus, respectively. More recent analyses of seed plants have supported these findings (Drouin et al., 2008), with some notable exceptions (Mower et al., 2007; Sloan et al., 2008, 2012). But we still know very little about the relative mutation rates within plastid-containing protists, which together represent most of the known photosynthetic eukaryotic diversity (Falkowski et al., 2004). This is largely because comparing substitution rates among plastid, mitochondrial, and nuclear genomes requires large amounts of nucleotide sequence data from three different genetic compartments for at least two distinct populations or "species". Moreover, the two species must be closely enough related that the synonymous-site divergence, which is used as an entrée into the mutation rate (Kimura, 1983), has not reached saturation. These are not trivial requirements when considering that most protist groups are poorly sampled and poorly studied (del Campo et al., 2014).

The data that are available from plastid-bearing protists present a very different picture of organelle and nuclear DNA mutational patterns than those observed in seed plants. In the red algal genus *Porphyra*, for instance, the mitochondrial mutation rate is estimated to be 3–5 times that of the plastid and nucleus (Smith et al., 2012). Similarly, in the haptophyte genus *Phaeocystis*, which has a plastid that derives from the secondary endosymbiosis of a red alga (Keeling, 2010), the mtDNA mutation rate is predicted to be 10 and 3 times those of ptDNA and nucDNA, respectively (Smith et al., 2014). An almost identical mitochondrial vs plastid mutational ratio has been observed in other lineages with secondary, red-algal plastids, including the stramenopile alga *Heterosigma akashiwo* and the nonphotosynthetic apicomplexan parasite *Babesia bovis* (Smith and Keeling, 2012). In the model green algal lineages *Chlamydomonas* and *Mesostigma* similar relative rates of mutation are predicted for all three genetic compartments, with slightly higher rates of mutation estimated for the mitochondrion vs plastid (Popescu and Lee, 2007; Hua et al., 2012).

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Relative-rate statistics are lacking for the Glaucophyta, which is a phylum of unicellular freshwater algae. Glaucophytes, red algae, and green plants have plastids that descend directly from the primary endosymbiosis of a cyanobacterium, and together they form the monophyletic supergroup Archaeplastida (Adl et al., 2012). The plastids of glaucophytes (called cyanelles) contain a unique peptidoglycan layer (between the inner and the outer cyanelle membranes) not found in the plastids of other eukaryotes (Pfanzagl et al., 1996; Löffelhardt et al., 1997). Unraveling cyanelle evolution is key to understanding the origin of plastids within the Archaeplastida and whether they derive from a single or multiple primary endosymbiotic events; most data strongly support the former (Price et al., 2012).

The best-studied glaucophyte is the globally distributed, freshwater flagellate *Cyanophora paradoxa*. All three of its genomes have been sequenced, including the 135.6 kb ptDNA, which has ~180 densely packed genes (Stirewalt et al., 1995); the 51.6 kb mtDNA, which has ~70 genes; and the ~70 Mb nuclear genome, containing ~28,000 genes (Price et al., 2012). By taking advantage of these sequence data and the availability of distinct *C. paradoxa* geographical isolates from culture collections, we explore, for the first time, relative rates of mutation within the Glaucophyta.

2. Materials and methods

C. paradoxa NIES-763 (obtained from the Microbial Culture Collection at the National Institute for Environmental Studies, Japan) was grown in DY-V medium (Andersen et al., 1997) at 18 °C (14/10 h light/dark cycle) and harvested at stationary phase. A ~300 μL cellular pellet was submerged in 12 mL of lysis buffer (10 mM Tris–HCl pH 7.6, 10 mM EDTA, 10 mM NaCl), treated with Proteinase K and SDS (final concentrations 30 μg/mL and 0.5%, respectively) for 20 min at 50 °C, vortexed for 1 min with 1.5 mL of 0.5 mm glass beads, then incubated for 1 h at 50 °C. Nucleic acids were extracted once with an equal volume of phenol, followed by phenol/chloroform (1:1), then chloroform, and precipitated using standard ethanol/sodium acetate methods. RNA was removed by the addition of RNase A (25 μg/mL final concentration). Total DNA was sequenced using Illumina technology (HiSeq 2000) at the Roy J. Carver Center for Genomics, University of Iowa, giving ~76.5×10^6 paired-end reads (length = 100 nt; average insert size = 450 nt).

The *C. paradoxa* NIES-763 organelle DNA sequences were assembled *de novo* with Ray v2.2.0 (Boisvert et al., 2010) using *k*-mers of 21, 27, 31, and 37. The resulting contigs were scanned for mitochondrial and plastid sequences using BLAST and the mitochondrial and plastid genome sequences of *C. paradoxa* CCMP 329/UTEX LB 555 (Stirewalt et al., 1995; Price et al., 2012) as queries. Hits to organelle DNA were assembled into larger contigs using read-mapping approaches with Geneious v6.1.2 (Biomatters Ltd., Auckland, NZ). The average Illumina read coverage for the organelle contigs was ~1300 reads/site. Nuclear genes were assembled using the same approach. The *C. paradoxa* NIES-763 mtDNA and ptDNA sequences are deposited in GenBank (accession numbers KM198929–KM198930) and the nucDNA data are in Supplementary Tables S1 and S2. Organelle and nuclear gene data for CCMP 329 (or its synonymous strain UTEX LB 555) came from GenBank (accessions CPU30821 & HQ849544) and the *C. paradoxa* Genome Portal: http://cyanophora.rutgers.edu/cyanophora/home.php.

Organelle and nuclear genes were aligned with MUSCLE (Edgar, 2004), implemented through Geneious, using default settings. Synonymous and nonsynonymous substitutions were measured with the CODEML program of PAML v4.3 (Yang, 2007), employing the maximum-likelihood method and the F3x4 codon model. Substitutions in non-protein-coding regions were estimated with BASEML of PAML using the HKY85 model.

3. Results and discussion

3.1. Nucleotide substitution rates in Cyanophora paradoxa

Using the maximum-likelihood method (Yang, 2007), we measured nucleotide substitution rates (i.e., pairwise divergence) between two distinct geographical isolates of *C. paradoxa*: strain NIES-763 (isolated from freshwater in Mitsukaido, Japan, in 1987) and strain CCMP 329 (isolated from freshwater in England, UK, in 1943). The latter strain was previously used to generate the *C. paradoxa* draft nuclear genome sequence and complete mtDNA sequence (Price et al., 2012), and it is synonymous with strain UTEX LB 555, which was used to sequence the first cyanelle genome (Stirewalt et al., 1995). Our dataset included complete mtDNA and ptDNA gene sets as well as 43 functionally diverse nuclear genes (Supplementary Table S1).

The nucleotide divergence between the two *C. paradoxa* strains, for all categories of nucleotide site, was lowest for the ptDNA and highest for the mtDNA (Table 1). Within protein-coding regions, the average number of pairwise substitutions per synonymous site (d_S) for the mitochondrial genome (5.29 ± 3.2) was ~5 times that of the plastid (1.01 ± 1.2) and ~4 times that of the nucleus (1.21 ± 0.8). Concatenated protein-coding datasets revealed even greater differences in d_S between the mtDNA (~5.0) vs the ptDNA (~0.7) and nucDNA (~0.9) (Table 1).

For the mitochondrial genome, d_S differed by more than an order of magnitude (1.5–98.6) among the individual protein-coding genes, with *rps*4, *rps*10, and *nad*6 showing particularly high levels of synonymous substitution (Supplementary Table 1). Synonymous substitution rates for the various plastid- and nuclear-encoded genes were generally much lower than those observed in the mitochondrion, ranging from 0 to 8.1 (ptDNA) and 0.2 to 4.4 (nucDNA) (Supplementary Table S1).

The relative rates of substitution at non-synonymous codon positions (d_N) paralleled those observed at synonymous sites. When looking at averages among protein-coding loci, d_N for the mtDNA (0.14 ± 0.11) was respectively 5 and 3.5 times that of the ptDNA (0.027 ± 0.04) and nucDNA (0.04 ± 0.03) (Table 1; Supplementary Table S1). The d_N/d_S ratio, which is often used to gauge the intensity and directionality of natural selection, was similar for the organelle and nuclear genomes, with averages of 0.02 (mtDNA), 0.04 (ptDNA), and 0.06 (nucDNA). This is consistent with strong purifying selection acting on the protein-coding genes in the different genomes (Table 1). The nucleotide substitution rates at rRNA-coding sites were low in all three genetic compartments (<0.1), but were still ~10 times greater for the mtDNA than the ptDNA and nucDNA; a similar trend was also observed for tRNA-coding regions (Table 1).

Together, the data on pairwise divergence between CCMP 329 and NIES-763 suggest that the rate of mutation in the mitochondrial genome of *C. paradoxa* is respectively 5 and 4 times that of the plastid and nucleus. However, the levels of synonymous substitution for many of the mtDNA-encoded genes, unlike those in the ptDNA and nucDNA, were saturated (Supplementary Table S1). Thus, we may have underestimated d_S in the mitochondrial compartment, and the relative mtDNA mutation rate may be even greater than that predicted here.

3.2. Relative rates in the Archaeplastida and beyond

Data on the relative mutation rates among mitochondrial, plastid, and nuclear genomes are slowly accumulating for diverse species. When including the present study, relative-rate statistics now exist for all major archaeplastidal lineages: red algae, green algae, land plants, and glaucophytes (Table 2; Fig. 1). Although

Table 1
Plastid, mitochondrial, and nuclear DNA (ptDNA, mtDNA, and nucDNA) substitution rates between two geographically distinct isolates of *Cyanophora paradoxa*.

	Substitutions per site			Substitution rate ratios (pt: mt: nuc)
	ptDNA	mtDNA[a]	nucDNA	
Synonymous sites				
Average (SD)	1.007 (1.22)	5.288 (3.17)	1.214 (0.83)	1: 5.3: 1.2
Concatenation	0.667	4.940	0.868	1: 7.4: 1.3
Nonsynonymous sites				
Average (SD)	0.027 (0.04)	0.135 (0.11)	0.040 (0.03)	1: 5: 1.5
Concatenation	0.024	0.111	0.041	1: 4.6: 1.7
d_N/d_S (SD)	0.041 (0.08)	0.022 (0.02)	0.060 (0.03)	–
rRNAs[b]	0.007	0.093	0.008	1: 13.3: 1.1
tRNAs[c]	0.010	0.076	–	1: 7.6: –

SD: standard deviation; d_N/d_S: ratio of nonsynonymous to synonymous substitutions per site, based on averages of individual loci not concatenated datasets. The substitution rate statistics for the individual loci within the organelle and nuclear compartments, including those that were derived from concatenated datasets, are shown in Supplementary Table S1.

[a] Average synonymous site substitution rate for mtDNA does not include the following loci, which had extremely high d_S values: *atp*8, *nad*6, *rpl*5, *rps*4, *rps*7, and *rps*10. When these loci are included in the analysis, the average d_S is ~15.

[b] For mtDNA and ptDNA includes the concatenation of all rRNA-coding regions. For nucDNA includes 18*S* and 26*S* rRNA-coding regions.

[c] Based on concatenation of all tRNA-coding regions. Analysis not performed for nucDNA.

Table 2
Synonymous substitution rates in plastid, mitochondrial, and nuclear genomes of various archaeplastidal lineages and the haptophyte genus *Phaeocystis*.

	Substitutions per synonymous site			Substitution rate ratios (pt: mt: nuc)
	ptDNA	mtDNA	nucDNA	
ARCHAEPLASTIDA				
Glaucophytes				
Cyanophora	1.01 (1.22)	5.29 (3.17)	1.21 (0.83)	1: 5.3: 1.2
Green algae[a]				
Chlamydomonas	0.30 (0.11)	0.29 (0.05)	0.37 (0.29)	1: 1.0: 1.2
Mesostigma	0.11 (0.06)	0.17 (0.11)	0.27 (0.18)	1: 1.5: 2.5
Seed plants[b]				
Angiosperms	0.39 (0.01)	0.13 (0.01)	2.11 (0.09)	1: 0.3: 5.4
Gymnosperms	0.61 (0.03)	0.28 (0.02)	1.23 (0.09)	1: 0.5: 2.0
Red algae[c]				
Porphyra	0.47 (0.22)	1.76 (0.58)	0.43 (0.18)	1: 3.7: 0.9
HAPTOPHYTA[d]				
Phaeocystis	0.25 (0.16)	2.41 (0.97)	0.85 (0.54)	1: 9.6: 3.4

Synonymous-site substitution rates are based on averages among loci, not concatenations; standard deviation in brackets.

[a] Data from Popescu and Lee (2007), Hua et al. (2012).

[b] Data from Drouin et al. (2008).

[c] Data from Smith et al. (2012).

[d] Data from Smith et al. (2014).

these data are restricted to a small number of taxa, a general trend is emerging: within archaeplastidal species, the mtDNA mutation rate typically exceeds or is similar to that of the ptDNA (Table 2; Fig. 1). The obvious exception to this is seed plants, where the mitochondrial mutation rate is estimated to be approximately a third of that of the plastid (Fig. 1). Recent studies, however, have uncovered some seed plant lineages, including certain *Silene* species, with exceptionally high mtDNA mutation rates (Mower et al., 2007; Sloan et al., 2008, 2012). Seed plants aside, when looking across the Archaeplastida there is a tendency toward lower rates of mutation in ptDNA vs mtDNA, with the rates for nucDNA being more varied (Table 2; Fig. 1). In some cases, these predicted mutation rate differences are quite pronounced. For example, the data presented here on *Cyanophora* and earlier results on red algae reveal a 4–10-fold higher mutation rate in mtDNA as compared to ptDNA and nucDNA (Table 2; Fig. 1).

A higher mutation rate in mtDNA vs ptDNA is consistently observed in plastid-bearing taxa outside the Archaeplastida as well. Studies of diverse photosynthetic eukaryotes with red-algal-derived plastids, including haptophyte and stramenopile algae, have uncovered ~5–20 times greater mutation rates in mtDNA relative to ptDNA (Smith and Keeling, 2012), and, in some cases, nucDNA (Table 2; Fig. 1). A similar trend has also been exposed in non-photosynthetic apicomplexan parasites with red-algal plastids (Smith and Keeling, 2012).

Given all of this, it is hard to ignore that there is a general tendency toward an mtDNA/ptDNA mutation-rate ratio of >1 in a diversity of species across the eukaryotic tree. We propose that this mutational pattern represents the norm rather than the exception for plastid-bearing protists, and that it existed in the photosynthetic ancestor(s) of the Archaeplastida, later shifting toward an mtDNA/ptDNA mutational-rate ratio of <1 in the ancestor of seed plants (Fig. 1). It is important to stress that relative rates of mutation within a species or lineage do not necessarily reflect absolute rates of mutation between different species or lineages. So although an mtDNA/ptDNA mutation-rate ratio of >1 may be common in various eukaryotes, the absolute rates within mitochondrial, plastid, and nuclear genomes likely differ substantially among lineages.

The levels of synonymous-site nucleotide divergence between *C. paradoxa* CCMP 329 and NIES-763, particularly for the mtDNA, are high compared to those observed within other photosynthetic

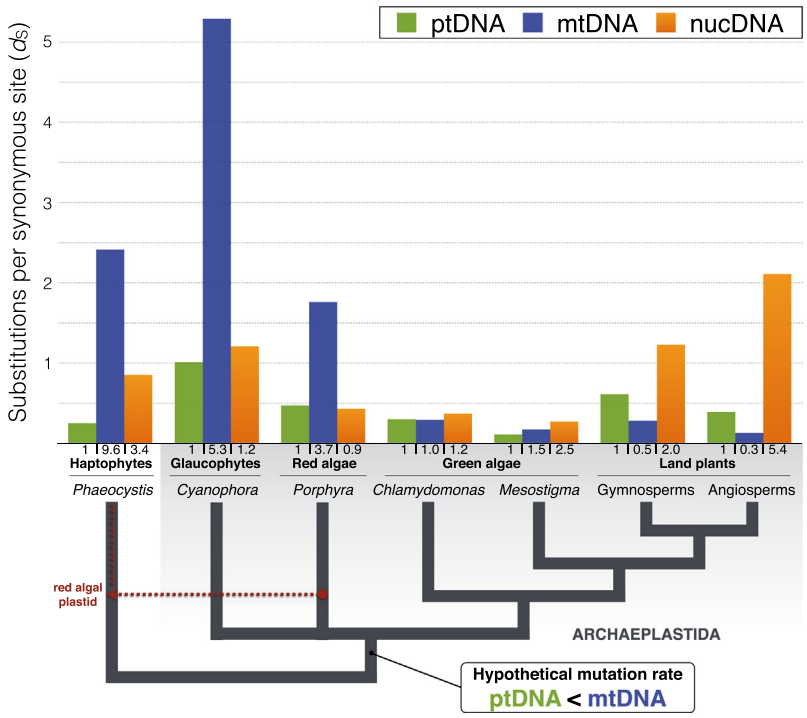

Fig. 1. Synonymous substitution rates in the organelle and nuclear genomes from archaeplastidal lineages and the haptophyte genus *Phaeocystis*. Plastid DNA (ptDNA) is green, mitochondrial DNA (mtDNA) is blue, and nuclear DNA (nucDNA) is orange. Synonymous-site substitution rates are based on averages among loci, not the concatenated datasets (see Table 2 for a complete list of substitution rates and its sources). Substitution rate ratios are shown beneath the *x*-axis. The Archaeplastida (i.e., Plantae *sensu lato*) comprises glaucophytes, red algae, green algae, and land plants, all of which have primary plastids. The haptophyte genus *Phaeocystis* has a secondary, red-algal-derived plastid. Based on the date shown, it is hypothesized that in the plastid-containing ancestor of the Archaeplastida the plastid genome mutation rate was lower than that of the mitochondrial genome.

lineages (Fig. 1). This may suggest that the absolute mutation rates within *C. paradoxa* are high, but this is speculative, as we do not know how long ago CCMP 329 and NIES-763 shared a common ancestor. The number of generations separating these two strains may be much larger than that separating strains (or species) employed in other substitution rate studies, such as *Mesostigma viride* SAG 50-1 vs NIES-296 (Table 2; Fig. 1). Nonetheless, the elevated nucleotide divergence between CCMP 329 and NIES-763, at the very least, implies that these isolates represent distinct populations or "species" of *Cyanophora* (Chong et al., 2014).

3.3. Updating our view of organelle genome mutation rates

Next-generation sequencing technologies and improvements in microbial culturing techniques have made it quick and easy to examine the genomes of previously unexplored plastid-bearing lineages. A single run of total DNA (or RNA) from a eukaryotic alga on a high-throughput sequencing platform typically produces enough data to assemble complete mitochondrial and plastid genomes (Smith, 2012, 2013). As a consequence, scientists are now generating, at an unprecedented rate, organelle genome sequences from a wide array of algal groups and are using these data to address a broad range of questions, from understanding the evolution of the malaria parasite (Taylor et al., 2013) to the development of biofuels (Hannon et al., 2010) to tracing the history of ancient Arctic vegetation (Willerslev et al., 2014). Thus, it is crucial that we understand the underlying mutational processes acting on these genomes.

Studies of seed plants have provided a strong foundation for our knowledge of plastid and mitochondrial genetics. But the patterns of organelle genome evolution in seed plants, as we are finding out, do not necessarily reflect those in plastid-bearing protists. If the relative rates of mutation between mitochondrial and plastid genomes for *C. paradoxa* and other plastid-containing protists examined thus far are representative of those in a diversity of microbial eukaryotes then it could have far-reaching implications on how organelle DNA is used for genetic analyses. If it turns out that within most protists the plastid mutation rate is lower than that of the mitochondrion, it would mean that ptDNA is a more suitable genetic marker for broad-scale comparative analyses, such as those attempting to resolve relationships between distantly related species or groups. Conversely, mitochondrial genomes, with their proclivity toward higher rates of mutation, may end up being better "fine-tune" genetic markers, ones that could be used for addressing population-level problems. Whatever the ultimate outcome, there is no denying that the data that are available point toward lower rates of mutation in ptDNA vs mtDNA for a variety algal species.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2014.07.001.

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