Evolutionary Dynamics of Chloroplast Genomes in Low Light: A Case Study of the Endolithic Green Alga Ostreobium quekettii

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Abstract

Some photosynthetic organisms live in extremely low light environments. Light limitation is associated with selective forces as well as reduced exposure to mutagens, and over evolutionary timescales it can leave a footprint on species' genomes. Here, we present the chloroplast genomes of four green algae (Bryopsidales, Ulvophyceae), including the endolithic (limestone-boring) alga Ostreobium quekettii, which is a low light specialist. We use phylogenetic models and comparative genomic tools to investigate whether the chloroplast genome of Ostreobium corresponds to our expectations of how low light would affect genome evolution. Ostreobium has the smallest and most gene-dense chloroplast genome among Ulvophyceae reported to date, matching our expectation that light limitation would impose resource constraints reflected in the chloroplast genome architecture. Rates of molecular evolution are significantly slower along the phylogenetic branch leading to Ostreobium, in agreement with the expected effects of low light and energy levels on molecular evolution. We expected the ability of Ostreobium to perform photosynthesis in very low light to be associated with positive selection in genes related to the photosynthetic machinery, but instead, we observed that these genes may be under stronger purifying selection. Besides shedding light on the genome dynamics associated with a low light lifestyle, this study helps to resolve the role of environmental factors in shaping the diversity of genome architectures observed in nature.

Key words: genome streamlining, photosynthesis, rates of evolution, boring algae, stoichiogenomics.

Introduction

Light is rapidly attenuated under water, yet some photosynthetic organisms thrive in extremely low light marine habitats (Shashar and Stambler 1992; Mock and Kroon 2002; Larkum, Douglas, et al. 2003). Specialized lifestyles may leave a footprint on organisms' genomes (Dutta and Paul 2012; Raven et al. 2013). For example, high-light and low light strains of the cyanobacterium Prochlorococcus have different genome sizes, GC contents and rates of molecular evolution, as well as other genome features that have been associated with their niche specialization (Hess et al. 2001; Rocap et al. 2003; Dufresne et al. 2005; Paul et al. 2010). Similar studies targeting the nuclear genomes of eukaryotic algae have also begun to emerge (see Raven et al. 2013 for a review). Different ecotypes of the microalga Ostreococcus, for example, show distinctive genome traits (Jancek et al. 2008), although in this case it is not clear whether low light has played a role.

The production of high-energy cofactors (ATP and NADPH) and the uptake of nitrogen are modulated by light intensity (Mactsaac and Dugdale 1972; Cochlan et al. 1991; Kirk 1994) and therefore it is logical to expect that the genome architecture of lineages living under low light conditions is influenced by resource constraints. Selection for saving resources and shortening replication times, in addition to random genetic drift, have been associated with the loss of genes, intergenic spacers and introns, a process known as genome streamlining (Giovannoni et al. 2005; Lynch 2006; Hessen et al. 2010; Wolf and Koonin 2013). Genome architecture can also be affected...
by limited supply of key elements such as nitrogen and phosphorus: different nucleotides and amino acids differ in their atomic composition, so molecules containing less atoms of the limiting nutrient may provide a selective advantage in certain niches (Acquisti et al. 2009; Elser et al. 2011; Raven et al. 2013). The Prochlorococcus strain with the smallest genome and highest content of nitrogen-poor molecules is found in surface waters, where irradiance is higher but nutrients are more depleted than in the habitat of the low light strain (Rocap et al. 2003; Dufresne et al. 2005). One could expect that when light is low enough to restrict growth rates and nitrogen uptake, organisms with small genomes and a high proportion of nitrogen-poor molecules may have better evolutionary fitness.

Sunlight may also leave footprints in a genome by directly or indirectly altering molecular rates of evolution (the molecular pacemaker). Light is a major contributor to environmental energy including solar radiation, thermal energy and chemical (metabolic) energy (Clarke and Gaston 2006). Environmental energy stimulates metabolism at many levels, and it is known that energy-rich habitats are often characterized by higher evolutionary rates (Davies et al. 2004; Clarke and Gaston 2006). Solar radiation, especially ultraviolet (UV), also plays a direct mutagenic role and may thus accelerate molecular evolution (Rothschild 1999; Willis et al. 2009). Thermal and chemical energy also depend on light: light incidence increases temperatures (e.g., in the tropics) and supports primary productivity (and consequently increases the energy available for metabolism and growth). Oxidative DNA damage generally occurs during metabolic reactions; therefore higher metabolic rates can lead to higher mutation rates (Gillooly et al. 2005). Generation times also play into it, being shorter and fixing mutations (on populations) more rapidly when the environmental energy is higher, which often happens when there is a combination of higher temperatures, metabolic rates and solar radiation (Rohde 1992; Wright and Rohde 2013). As a consequence of all these factors, it is reasonable to expect that organisms living in low-energy areas, like shaded habitats, have relatively slower rates of molecular evolution.

Challenging environments may impose particular selective regimes, which could leave a footprint of positive selection in genes undergoing adaptation. Changes in proteins that provide higher fitness in a given circumstance (e.g., low light) can be detected at the molecular level by an excess of nonsynonymous substitutions over synonymous ones (Yang 1998). Evidence of positive selection for example in the Rubisco gene (involved in carbon fixation) in mosses has been associated with its adaptation to the declining levels of atmospheric CO₂ since their origin in the Ordovician (Raven and Colmer 2016). In cases of organisms living in extremely low light, it would be reasonable to expect positive selection in genes related to the photosynthetic machinery, reflecting adaptation to low light. To our knowledge, this idea has never been tested in eukaryotic algae.

The siphonous green alga *Ostreobium* is a convenient organism to investigate photosynthesis under low light conditions (Fork and Larkum 1989; Koehne et al. 1999; Wilhelm and Jakob 2006). *Ostreobium* has an endolithic (limestone-boring) lifestyle: it bores into carbonate substrates and populates all sorts of marine limestones worldwide, including shells and coral skeletons. Only a small portion of the available light reaches *Ostreobium* in its usual habitat: ~99% of the light can be attenuated by the first millimeter of limestone (Nienow et al. 1988; Matthes et al. 2001). Other photosynthetic organisms living on the limestone substrate can further attenuate light: the living tissue of corals and their zooxanthellae, for example, absorb 95–99.9% of the available light (Halldal 1968; Schlichter et al. 1997). Even under these extreme low light conditions, *Ostreobium* carries out oxygenic photosynthesis (Kühl et al. 2008). Cyanobacteria coexisting with *Ostreobium* enhance their light interception by manufacturing far red-absorbing chlorophylls (Chl d and f; Chen and Blankenship 2011), whereas *Ostreobium* has a special chlorophyll antenna that allows it to harvest far red light (Magnusson et al. 2007). *Ostreobium* is also able to grow in quite deep waters, being abundant even at depths over 200 m where only a handful of algal species can persist (Littler et al. 1985; Dullo et al. 1995; Aponte and Ballantine 2001). Here the light is filtered strongly towards the blue end of the spectrum, with a peak at ~470–480 nm (Larkum and Barrett 1983) and a different light harvesting strategy is employed: the carotenoid siphonaxanthin transfers light energy to chlorophyll and the reaction centers (Kageyama et al. 1977). Thus the success of *Ostreobium* in terms of its cosmopolitan distribution is associated not only with its efficiency in light utilization but also its ability to employ a range of light harvesting strategies (Fork and Larkum 1989; Schlichter et al. 1997; Magnusson et al. 2007; Tribollet 2008), for which the underlying genomic basis has never been explored.

The light-driven genomic traits of *Ostreobium* can only be investigated in a comparative framework. While algal nuclear genome sequences are still scarce, chloroplast genomes are better sampled and constitute a powerful tool for molecular evolutionary studies (Lemieux et al. 2014). *Ostreobium* belongs to the Bryopsidales (Ulvophyceae), a diverse order of seaweeds for which only a handful of chloroplast genomes are available (Leilaert and Lopez-Bautista 2015). Additional chloroplast genomes of species from this order can help us investigate genomic traits correlated to low light in *Ostreobium*.

The goal of this study is to evaluate the evolutionary dynamics of the chloroplast genome of the low light alga *Ostreobium* using comparative and phylogenetic methods. Because comparative analyses in a phylogenetic context require a sufficiently large sample of genomes, we present the chloroplast genomes of four green algae, including *Ostreobium quekettii* and members from three other families in the same order, all previously uncharacterized. We used a
combination of stoichiogenomics (the study of elemental composition of macromolecules; Elser et al. 2011) and models of molecular rate variation to investigate our expectations for a lineage adapted to low light conditions. Our first expectation related to light-dependent resources limitation: if the Ostreobium lineage has evolved in low-energy and low-nutrient conditions, its chloroplast genome can be expected to be smaller, more compact (i.e., with less intergenic spacers, introns and repeats) and contain less nitrogen than the chloroplast genomes of related algae. Our second expectation was that the phylogenetic branch leading to Ostreobium has slower rates of molecular evolution (i.e., mutation rates) than other branches in the phylogeny due to fewer mutations induced by UV and slower generation times often associated with low energy niches. Lastly, we would expect genes related to its photosynthetic machinery to have experienced positive selection and enabled Ostreobium’s highly efficient light utilization.

Materials and Methods
Sequencing, Assembly and Annotation
Total genomic DNA of Ostreobium queketti, Halimeda discoidea, Derbesia sp. and Caulerpa cliftonii were extracted using a modified cetyl trimethylammonium bromide (CTAB) method described in Cremen et al. (2016) and sequenced on an Illumina platform. The collection sites and library preparation details are described in the Supplementary Materials. Sequences were submitted to European Nucleotide Archive and GenBank (accession numbers LT593849, KX808496, KX808497 and KX808498).

Sequences were assembled using CLC Genomics Workbench 7.5.1 (http://www.clcbio.com). Circularity and scaffold regions were resolved by comparing the CLC assembly with assemblies generated independently with MEGAHIT (Li et al. 2015), SOAPdenovo2 (Luo et al. 2012) and SPADES (Nurk et al. 2013). Details about the assembly settings and quality checks are reported in the Supplementary Materials. A combination of automated pipelines and manual editing was used to annotate the chloroplast genomes, which is also described in the Supplementary Materials.

Comparative Analysis
In order to compare Ostreobium with other Ulvophyceae, the chloroplast genomes of Bryopsis plumosa (NC_026795), Tydemania expedita (NC_026796), Ulva sp. (KP720616), Pseudendoclonium akinetum (AY835431) and Oltmannsiellopsis viridis (NC_008099), available in GenBank, were included in our comparative analysis. Genome features were extracted with Geneious 9.0.4 (Kearse et al. 2012). Hypothetical ORFs with <300 bp were excluded and the tilS gene was re-annotated as a pseudogene (not a CDS) in Tydemania and Bryopsis, where it has a frame shift or a stop codon in the middle of the gene. The number of repeats, including tandem and palindromic repeats, were calculated with the Geneious implementation of Phobos v.3.3.11 (Mayer 2007) and with the Emboss suite (http://www.bioinformatics.nl/emboss-explorer/); see Supplementary Materials for details.

Nitrogen (N) content quantification was based on the counts of N atoms per nucleotide or amino acid using the formula described in Acquisti et al. (2009): \( \sum (n_i \times p_i) \) where \( n_i \) is the number of N atoms in the \( i \)-th base and \( p_i \) is the proportion of each base in the chloroplast genome. For the nucleotide counts we used \( n_C = n_G = 4 \) and \( n_A = n_T = 3.5 \) (Acquisti et al. 2009). For the coding DNA sequences (exons) we used \( n_A = 5, n_T = 2, n_G = 5, \) and \( n_C = 3 \). For amino acid counts (the theoretical proteome) we used \( n = 2 \) for asparagine, glutamine, lysine and tryptophan; \( n = 3 \) for histidine; \( n = 4 \) for arginine; and \( n = 1 \) for other amino acids. Copy number and expression levels play a major role in N utilization, but neither qPCR nor transcriptome analysis could be carried out because our source materials were of different developmental stages and environmental conditions. Instead, we investigated N-content in coding sequences and amino acids on a gene by gene basis, in addition to doing so at the whole chloroplast genome level. Assuming that expression levels of genes correlate among species, the gene by gene approach should reduce the problem of differential expression between genes and make for more realistic among-species comparisons. Finally, we also evaluated whether the average length of coding sequences is smaller in Ostreobium, as gene size reduction has been observed in some endosymbionts with reduced genome sizes (Charles et al. 1999).

Phylogeny, Rates of Evolution and Selection Analysis
The coding sequences of all species were aligned at the amino acid level using a locally installed version of MAFFT v7.215 (Katoh et al. 2002), with multithreading and default parameters, and then the aligned amino acid sequences were converted back to nucleotides using RevTrans (Wernersson and Pedersen 2003). The ftsH, rpoB, rpoC1, rpoC2 and ycf1 genes could not be reliably aligned (according to a visual assessment) and were excluded along with the tilS pseudogene from downstream analyses. A maximum likelihood phylogeny was built using RAxML (Stamatakis 2006) with a GTR + Γ model, a partitioning strategy separating 1st, 2nd and 3rd codon positions, and a rapid bootstrap search of 500 replicates. Oltmannsiellopsis viridis, Pseudendoclonium akinetum and Ulva sp. were used as outgroups.

In order to test whether DNA mutation (substitution) rates were slower in the Ostreobium lineage, we studied lineage-specific rates of molecular evolution using the baseML program from the PAML v.4.7 package (Yang 2007). We chose to perform this test at the nucleotide (rather than at the amino acid) level because we expect environmental energy to affect
rates of molecular evolution at the nucleotide level (i.e., regardless whether the mutations are synonymous or nonsynonymous). We compared the fit of a model with unique rates of evolution across all branches (global clock) to a model with a different rate for the Ostreobium lineage (local clock) using the Akaike Information Criterion (AIC). Because rates of molecular evolution inherently vary among species, a model with two rates is likely to better fit the data than a single-rate model. While this is taken into consideration when calculating model fit (AIC penalizes parameter-rich models), we also verified the rates of molecular evolution under a relaxed clock model, whereby rates are free to vary on all branches of the phylogeny (see Supplementary Materials).

To evaluate whether photosynthetic genes have been under positive selection in the Ostreobium lineage, we excluded gene alignments containing less than four species and grouped (concatenated) genes into 15 gene classes (cf. Wicke et al. 2011). We analyzed this data set using the branch model implemented in PAML (Yang 1998, 2007) and the random effects branch-site model (branch-site REL) implemented in HyPhy (Kosakovsky Pond et al. 2005, 2011). The branch model was run with the codeml program, using the F3 × 4 codon model (Goldman and Yang 1994; Yang 2007). We compared the fit of a model with differential $d_0/d_1$ ratio ($\omega$) for Ostreobium and the background lineages, to a model with a universal $\omega$ for all branches (the null hypothesis) using the Akaike information criterion (AIC). This approach directly tests our hypothesis, but has a risk of returning a good fit for poor models because the null hypothesis (universal $\omega$) may be overly simple (see Kosakovsky Pond et al. 2011). Therefore we also used the branch-site REL, which allows detecting positive selection in all branches of the phylogeny and the proportion of sites under selection (Kosakovsky Pond et al. 2005, 2011). We evaluated whether positive selection had occurred in the Ostreobium lineage with the likelihood ratio test and $P$ values (with the Holm correction procedure) implemented in the branch-site REL method in HyPhy (Kosakovsky Pond et al. 2011).

Results

Four New Chloroplast Genomes of Bryopsidales

The sequence data of Ostreobium queketti, Halimeda discoidea, Derbesia sp. and Caulerpa cliftonii were assembled into complete (circular mapping) chloroplast genomes (fig. 1 and supplementary fig. S1, Supplementary Material online). The mean coverage was 235× for Ostreobium, 3,983× for Halimeda, 1,116× for Caulerpa and 469× for Derbesia (supplementary fig. S2, Supplementary Material online). Two gapped scaffold regions in Halimeda seem to have a (possibly polymorphic) number of repeats. One of these gaps was closed with an alternative assembler software (SPADES, Nurk et al. 2013) and the other was coded as stretch of Ns (see supplementary fig. S2, Supplementary Material online). The main genome features including their sizes are shown in table 1.

Gene content of Ostreobium is similar to related algae but it lacks the chloroplast envelope membrane protein gene (cemA) that is present in all other Ulvophyceae sequenced to date (supplementary table S1, Supplementary Material online). The tRNA(ille)-lysidine synthase gene (tilS) seems to be a pseudogene in Ostreobium, Halimeda and Derbesia as it contains multiple in-frame stop codons. We could not identify it at all in Caulerpa cliftonii (i.e., no tBLASTx hits with e-values < 0.001 and identity > 50%, using Bryopsis plumosa as reference), although this pseudogene has been found in another Caulerpa species (Zuccarello et al. 2009). None of our chloroplast genomes have the organelle division inhibitor factor gene (minD), supporting the notion that this gene has been lost from the chloroplasts of Bryopsidales (Leliaert and Lopez-Bautista 2015). Like Ulva, Bryopsis and Tydemania, the chloroplast genomes sequenced here do not have the quadripartite architecture often found in green algae and land plants (Lemieux et al. 2000; Pombert 2005). Despite an overall highly conserved gene content, the Ulvophyceae genomes have multiple rearrangements as indicated in the Mauve alignment (fig. 2).

Genome Economics

In order to evaluate some of our expectations regarding light-driven resource limitations on chloroplast genomes, we compared the chloroplast genome of Ostreobium with those of the eight other algae from the class Ulvophyceae in terms of size, compactness (gene-density) and nitrogen content. With 81,997 bp, Ostreobium has the smallest and most gene-dense chloroplast genome of all Ulvophyceae sequenced to date (table 1 and fig. 3). The size reduction in the Ostreobium chloroplast genome is not caused by gene loss (78 of 79 common plastid genes are present, supplementary table S1, Supplementary Material online) but by a reduction of intergenic spacers, introns and repeats (table 1 and fig. 3). Intergenic spacers compose only 11.9% of the Ostreobium chloroplast genome, compared with an average of 25.4% (std 8.4%) in other Ulvophyceae. Ostreobium also has a small number of introns, missing even the highly conserved tRNA-Leu (uua) group I intron (Simon et al. 2003) that is present in other Bryopsidales chloroplast genomes (Leliaert and Lopez-Bautista 2015; this study). Nitrogen utilization in Ostreobium did not differ substantially from other algae, either in the nucleotide composition of the complete chloroplast DNA, the coding regions, or the amino acids of predicted proteins (table 1). Likewise, the N counts on a gene by gene basis did not reveal any obvious pattern (supplementary table S2, Supplementary Material online). The average gene length in Ostreobium was found to be similar to related algae.
Rates of Evolution

To investigate whether the molecular pacemaker along the branch leading to *Ostreobium* is slower than in the remainder of the tree, we constructed a Maximum Likelihood (ML) phylogeny from the chloroplast genomes (71 genes concatenated, 47,559 bp, fig. 2) and fitted two models of molecular evolution to the same data set. We found that a model with differential rates of evolution for the branch leading to *Ostreobium* and the remaining branches of the phylogeny fits the data much better ($\Delta$AIC = 92) than a model with a homogeneous rate across the entire tree. The branch rate parameter values estimated by ML are 0.81 for the *Ostreobium* branch versus 1.00 for the remainder of the tree. In other words, the relative rate of molecular evolution along the *Ostreobium* branch is 19% slower than along the other branches of the phylogeny.

A similar result was obtained by calculating the rates of molecular evolution with a relaxed molecular clock, but some branches other than the *Ostreobium* branch also had slower rates of molecular evolution (supplementary fig. S3, Supplementary Material online). Except for *Bryopsis* and *Ostreobium*, all other Bryopsidales showed a relatively fast rate. The rate estimated for the *Ostreobium* branch corresponds to 65% of the rates averaged across all other branches of the phylogeny.

Selection on Genes Related to Photosynthesis

Our third expectation was that genes related to the photosynthetic pathway have experienced positive selection in the
lineage leading to Ostreobium. We concatenated genes encoding different subunits of the same protein to improve signal from short gene alignments. Using the branch model of Yang (1998), we tested whether the \( \omega \) ratio \((d_\text{N}/d_\text{S})\) of the branch leading to Ostreobium differs from the background \( \omega \) for other lineages in the phylogeny. If they do differ significantly, and if \( \omega > 1 \), then positive selection could be inferred (Yang 1998). However, we found no indication that genes in the branch leading to Ostreobium have been under positive selection (table 2 and supplementary table S4, Supplementary.

Table 1
Summary of the Chloroplast Genome Features of Ostreobium quekettii and Comparison with Other Ulvophyceae Chloroplast Genomes

| Species                     | Genome size (bp) | N content genome | N content coding DNA | N content proteome | GC content (%) | Introns | Repeats (50 bp+) | Tandem repeats* | Palind. seqs | Int. spacers (%) | Accession number |
|-----------------------------|------------------|------------------|----------------------|-------------------|----------------|---------|-----------------|----------------|-------------|-----------------|-----------------|
| Oltmannsiellopsis viridis   | 151,933          | 3.702            | 3.698                | 1.361             | 40.5           | 10      | 84              | 5              | 652         | 39.57           | NC_008099       |
| Pseudendoclonium akinetum   | 195,867          | 3.657            | 3.699                | 1.379             | 31.5           | 28      | 100             | 22             | 418         | 37.46           | AY835431        |
| Ulva sp.                    | 99,983           | 3.626            | 3.669                | 1.366             | 25.3           | 5       | 12              | 2              | 410         | 22.67           | KP720616        |
| Ostreobium quekettii        | 81,997           | 3.656            | 3.692                | 1.369             | 31.9           | 6       | 8               | 1              | 100         | 11.96           | LT593849        |
| Bryopsis plumosa           | 106,859          | 3.650            | 3.692                | 1.359             | 30.8           | 13      | 12              | 1              | 161         | 20.40           | NC_026795       |
| Derbesia sp.                | 115,765          | 3.644            | 3.685                | 1.374             | 29.7           | 12      | 8               | 5              | 146         | 19.09           | KX808497        |
| Caulerpa cliftonii          | 131,135          | 3.688            | 3.675                | 1.378             | 37.6           | 11      | 15              | 7              | 115         | 25.74           | KX808498        |
| Halimeda discoideaa         | 122,075          | 3.653            | 3.681                | 1.363             | 32.2           | 14      | 19              | 11             | 112         | 19.96           | KX808496        |
| Tydemania expeditionis     | 105,200          | 3.668            | 3.656                | 1.377             | 32.8           | 11      | 7               | 1              | 72          | 18.73           | NC_026796       |

**Note.**—Nitrogen (N) content in Genome and Coding DNA based on nucleotides, N content in Proteome based on amino acids counts.

*a*Only tandem repeats with 15–1,000 bp were included in the count.

*b*Excluding ORFs < 300 bp.

*c*Halimeda has one scaffold with unknown number of repeats annotated with 100 Ns.

Palind seqs, Palindromic repeats; Int. spacers, Intergenic spacers.
Material online). Instead, we observed that most of the proteins related to the photosynthetic machinery have a stronger signature of purifying selection in the Ostreobium lineage than in other branches of the phylogeny (ΔAIC > 4, table 2). We note though that these results should be interpreted with prudence given the methods’ susceptibility of returning a good fit for poor models (see Kosakovsky Pond et al. 2011). In order to verify these results in light of an alternative method, we performed a second analysis using the random effects branch-site model.

The second analysis with the branch-site REL model, which can detect selection in all branches of the tree and parts of the alignment without having to specify lineages of interest a priori (Kosakovsky Pond et al. 2011), confirmed that there are no signatures of positive selection along the Ostreobium lineage (supplementary table S5, Supplementary Material online). As in the previous analysis, the branch-site REL model suggests that several gene classes have experienced stronger purifying selection in the lineage leading to Ostreobium: many of the genes show smaller \( \omega \) values in the Ostreobium lineage (both mean \( \omega \) and \( \omega_1 \)—representing purifying selection) and a higher proportion of sites under the purifying selective regime when compared with the average values obtained for all other branches (supplementary table S5, Supplementary Material online).

**Discussion**

**An Economical Genome**

The endolthic alga Ostreobium has a remarkably small and compact chloroplast genome (figs. 1 and 3; table 1). We found that the economic nature of the Ostreobium chloroplast genome is not accomplished by a replacement of expensive nucleotides or amino acids (i.e., containing more N atoms) by more economic ones, but by an overall reduction of intergenic regions (fig. 3). Energy limitation resulting from the low light niche that this alga occupies may have contributed to an evolutionary reduction of the genome size. Due to the limited light available for photosynthesis, saving energy in any aspect of its cell biology including genome replication and transcription would result in a selective advantage. Introns significantly increase the costs of transcription (Lehninger et al. 1993; Castillo-Davis et al. 2002). Likewise, repeats and intergenic spacers consume resources, so these can be under selection towards reduction in energy-poor environments (Dufresne et al. 2005; Giovannoni et al. 2005).

Besides natural selection, neutral factors as random genetic drift and population sizes can also shape genome architecture (Lynch 2006; Lynch et al. 2006) and may have contributed to the chloroplast genome streamlining in Ostreobium. Genome reduction resulting from neutral evolution (or from a relaxation of purifying selection) is typically observed in obligate parasitic or symbiotic species, which tend to have small effective population sizes and therefore a higher influence of genetic drift (Mira et al. 2001; Wolf and Koonin 2013). Genes that are no longer essential to survival are under nearly neutral evolution, so the reduced genomes of parasitic species typically show substantial gene loss (e.g., loss of genes involved in photosynthesis) and, sometimes, an accumulation of pseudogenes (Mira et al. 2001; de Koning and Keeling 2006; McNeal et al. 2007; Wicke et al. 2013; Yan et al. 2015). Ostreobium, in contrast, has a tightly packed chloroplast genome, virtually no gene loss (except for cemA) and no sign of gene size
reduction, supporting a considerable role of adaptive processes on its genome streamlining.

Evidently, not all photosynthetic organisms living in low light environments have reduced genome sizes. *Acaryochloris marina* is a shade specialist with an 8.3 Mb genome, which is large for a cyanobacteria (Swingley et al. 2008; Larsson et al. 2011). In this case, a different mechanism can be speculated on: by producing chlorophyll *d*, *Acaryochloris* may not experience the same resource constraints on nuclear genome evolution will follow.

Photosynthetic light reactions

| Gene       | Single-α model | Two-α model | ΔAIC |
|------------|----------------|-------------|------|
| *atp*      | 0.025          | 0.028       | 0.009| 25.043 |
| *pet*      | 0.032          | 0.034       | 0.016| 3.625  |
| *psa*      | 0.019          | 0.021       | 0.007| 29.851 |
| *psb*      | 0.029          | 0.031       | 0.013| 40.538 |

Photosynthetic dark reactions

| Gene       | Single-α model | Two-α model | ΔAIC |
|------------|----------------|-------------|------|
| *chl*      | 0.022          | 0.024       | 0.012| 4.749  |
| *ccsA*     | 0.025          | 0.025       | 0.032| −1.938 |
| *rbcL*     | 0.020          | 0.023       | 0.007| 13.922 |

Translation and protein-modifying enzymes

| Gene       | Single-α model | Two-α model | ΔAIC |
|------------|----------------|-------------|------|
| *clp*      | 0.014          | 0.017       | 0.004| 2.625  |
| *intA*     | 0.039          | 0.044       | 0.020| 0.660  |
| *rpl*      | 0.039          | 0.040       | 0.029| −0.643 |
| *rps*      | 0.035          | 0.036       | 0.022| 1.179  |
| *tuAF*     | 0.023          | 0.025       | 0.011| 1.516  |

Proteins not related to photosynthesis

| Gene       | Single-α model | Two-α model | ΔAIC |
|------------|----------------|-------------|------|
| *accD*     | 0.036          | 0.041       | 0.017| 1.379  |
| *cys*      | 0.027          | 0.027       | 6.291| −2.000 |
| *rpo*      | 0.003          | 0.003       | 0.002| −1.749 |

Notes.—Two models were tested (α for all lineages and a model with different values for *Ostreobium* and all other species. The goodness of fit of the two-α over the single-α model is given by ΔAIC.
plausible explanation for why Ostreobium lineages have diversified abundantly within the endolithic niche (Marcelino and Verbruggen 2016; Sauvage et al. 2016) but are not known to have diversified out of it (i.e., given origin to nonendolithic species). Endolithic algal species are often light saturated at low light intensities but some experimental studies show that they are able to photoacclimate to light levels approaching full solar irradiance (see Tribollet 2008 for a review). There are high levels of cryptic diversity within endolithic green algae (Marcelino and Verbruggen 2016; Sauvage et al. 2016) and it is not known which species are able to cope with higher levels of light, raising the question of whether cemA has been lost in other lineages of Ostreobium and whether they acquired other mechanisms to tolerate high light.

We expected to observe a larger proportion of nitrogen-poor molecules in the Ostreobium chloroplast genome for several reasons. First, low light irradiance limits the uptake of nitrogen (Madsaac and Dugdale 1972; Cochlan et al. 1991) and it has been empirically demonstrated that Ostreobium growth is limited by nitrogen and phosphorous in naturally occurring concentrations (Carreiro-Silva et al. 2012). Second, absorption of nutrients may be difficult in endolithic environments due to limited circulation and thicker diffusive boundary layers (see Larkum, Koch, et al. 2003). However, our results indicate that the nitrogen content in the Ostreobium chloroplast genome (and predicted proteome) is similar to those of other algae in the same class (table 1 and supplementary tables S2 and S3, Supplementary Material online).

Several potential explanations can be raised. First, seaweeds in general may naturally be under nitrogen limitation (Vitousek and Howarth 1991; Harrison and Hurd 2001), resulting in all of the examined genomes having similar nitrogen content. Alternatively, genome replication and DNA repair may be less frequent in Ostreobium as a consequence of the reduced environmental energy, slow metabolism and growth, therefore a slower rate of nitrogen intake may be required and this economic aspect of Ostreobium is not reflected in its genome. Sample size could also be an issue: previous studies on N bias used nuclear genomes (Acquisti et al. 2009) and patterns may not be visible in the smaller chloroplast genome. Nitrogen limitation may also lead to overall genome reduction (Kang et al. 2015) rather than biases in nucleotide and amino acid composition. Finally, nitrogen utilization is largely dependent on the number of copies of the chloroplast genome and expression levels, which cannot be detected in our analyses. If fresh DNA extractions from algae growing in their natural conditions and belonging to the same developmental stage were available, would be interesting to perform comparative qPCR and transcriptome analyses to test whether this is the case.

In Prochlorococcus, it is the high-light strain that contains less nitrogen in its genome, although it is not substantially different from one of the low light strains (Dufresne et al. 2005). In this case, the nitrogen availability in the water column seems to play a more important role than a restricted nitrogen-uptake ability due to light limitation. Heterotrophic pathways have been observed in the genome of Prochlorococcus, especially in the low light strains, suggesting that they might use other sources of energy in addition to light (García-Fernández and Diez 2004). This potentially mitigates the effects of low irradiance on nitrogen uptake in this organism, which would explain why low light Prochlorococcus strains have more nitrogen in their genomes. A recent review (Raven et al. 2013) suggests a theoretical association between AT/GC ratios in genomes (which could culminate in nitrogen bias) and UV irradiation, but notes that this is not commonly observed in nature because multiple other factors influencing genome content may play a more significant role than light alone.

Slow Rates of Evolution

The results of two independent tests show that Ostreobium has a relatively slow rate of molecular evolution than closely related lineages. Other ulvophytes also seem to have slow rates of molecular evolution, which might be related to other species traits not analyzed here, but in Ostreobium, the most reasonable explanations relate to the effects of the low light niche that this endolithic alga occupies. Sunlight, including UV radiation, induces DNA damage, mutations and rearrangements (Ries et al. 2000; Raven et al. 2013; Kumar et al. 2014). While these changes often get repaired (see Boesch et al. 2011 for mechanisms), the frequency with which remaining mutations are passed through generations dictates the molecular pacemaker (Baer et al. 2007). Following this logic, low light lineages will likely have slower rates of molecular evolution than lineages living in high light conditions, as observed in Ostreobium and in low light strains of Prochlorococcus (Dufresne et al. 2005). In Prochlorococcus, it is likely that the loss of DNA repair genes also contributes to an increase in mutation rates in high light strains (Dufresne et al. 2005).

Sunlight also shapes evolutionary rates through environmental energy—it sustains primary productivity and ambient temperature. Energy-rich habitats are the epicenter of evolutionary change worldwide (Davies et al. 2004; Jetz and Fine 2012; Wright and Rohde 2013). This environmental energy is positively correlated to metabolic rates in many organisms (Allen et al. 2002) and the by-products of metabolic reactions (e.g., reactive oxygen and nitrogen species) are another major source of mutations (Gillooly et al. 2005; Boesch et al. 2011). It has been proposed that more solar radiation and higher temperatures increase metabolism and growth rates, shortening generation times and increasing mutation rates (Rohde 1992).
Shorter generations lead to more mutations accumulated per unit of time, so species living in high-energy habitats tend to have faster rates of molecular evolution (Bromham 2011). One could speculate that the low energy niche that Ostreobium occupies results in slow metabolic rates and generation times (although they are unknown for this alga), culminating in a slow molecular pacemaker. Longer generation times have been associated with slow rates of molecular evolution in tree ferns (Zhong et al. 2014), which are also shade plants (Page 2002).

Selection in the Ostreobium Chloroplast Genome

We did not find evidence for positive selection on genes related to photosynthesis in the lineage leading to Ostreobium (table 2 and supplementary table S5, Supplementary Material online). On the contrary, we observed some signs, though weak, of stronger purifying selection in this lineage.

Ostreobium is known to have several features that facilitate low light photosynthesis. It is able to produce red-shifted chlorophylls and uses an uncommon uphill energy transfer from these chlorophylls to photosystem II (Koehne et al. 1999; Wilhelm and Jakob 2006). The photosynthesis-related proteins that are more likely to be affected by low light (e.g., the light harvesting complex superfamily and the pigments involved in light capture) are encoded in the nucleus (Green and Parson 2003), and so innovations in these genes would not be detected in our analysis. The recently sequenced nuclear genome of the seagrass Zostera marina revealed an expanded number of light harvesting complex B genes (Olsen et al. 2016). Like Ostreobium, Zostera is adapted to a light-depleted (aquatic) niche when compared with its land plant relatives. We expect that interesting findings will result for Ostreobium with the analysis of transcriptome and nuclear genome data.

Another scenario that may have contributed to not observing selection is that the lineage leading to Ostreobium could have experienced an early burst of positive selection followed by purifying selection, and such a history may go undetected in analyses. If innovations related to low light adaptation appeared early in Ostreobium evolution and increased its fitness, it is expected that they would be immediately followed by purifying selection—especially if the loss of the cemA gene caused intolerance to high-light and confined the ancestral endolithic lineage to shaded habitats (where any mutation decreasing photosynthesis performance is likely to lead to decreased fitness). This scenario provides a plausible explanation for the stronger purifying selection on photosynthesis-related genes in the branch leading to Ostreobium when compared with other branches in the phylogeny. The available tools may not have enough power to detect faint episodes of selection, particularly if the data are saturated with synonymous substitutions or if selection occurred at deep internal branches (Kosakovsky Pond et al. 2011; Gharib and Robinson-Rechavi 2013). Although both analyses show some sign of a stronger purifying selection in the Ostreobium lineage, these results should be interpreted with caution as the phylogeny contains long branches (implying long periods of time: Ostreobium, for example, diverged 500 Ma ago), therefore substitutions may have saturated the data to a point where evolution cannot be reliably characterized by the models. Simulations mimicking the evolution of algal chloroplast genomes may help to characterize those methodological limitations. Finally, the power of these analyses will certainly increase as more genomic data of high and low light-adapted lineages become available.

Conclusion

We present the chloroplast genomes of four green algae (Bryopsidales) and investigate the genomic footprints of a low light lifestyle in the endolithic Ostreobium quekettii. This alga has the smallest and most gene-packed chloroplast genome among Ulvophyceae, which is a possible adaptation to light-related resources constraints. The molecular pacemaker is significantly slower in the phylogenetic branch leading to Ostreobium, consistent with a scenario where low energy levels reduce rates of molecular evolution. Unexpectedly, we observed some signs of higher levels of purifying selection in the photosynthesis-related genes in Ostreobium when compared with other algae. It is still unclear whether this result is allied to an early episodic positive selection followed by a strong purifying selection or to a methodological limitation, as the current methods may not have the power to detect selection in deep-branching lineages, especially if the data are saturated with substitutions. Sequencing additional chloroplast and nuclear genomes of different Ostreobium lineages and other low light adapted species will help to further clarify the genomic correlates of low light adaptations.

Supplementary Material

Supplementary figures S1–S3 and tables S1–S5 are available at Genome Biology and Evolution online (http://www.gbe.oxfordjournals.org/).

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