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The Mitochondrial Genome of *Grateloupia taiwanensis* (Halymeniaceae, Rhodophyta) and Comparative Mitochondrial Genomics of Red Algae

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Abstract. Although red algae are economically highly valuable for their gelatinous cell wall compounds as well as being integral parts of marine benthic habitats, very little genome data are currently available. We present mitochondrial genome sequence data from the red alga *Grateloupia taiwanensis* S.-M. Lin & H.-Y. Liang. Comprising 28,906 nucleotide positions, the mitochondrial genome contig contains 25 protein-coding genes and 24 transfer RNA genes. It is highly similar to other red algal genomes in gene content as well as overall structure. An intron in the *cox*1 gene was found to be shared by *G. taiwanensis* and *Grateloupia angusta* (Okamura) S. Kawaguchi & H. W. Wang. We also used whole-genome alignments to compare *G. taiwanensis* to different groups of red algae, and these results are consistent with the currently accepted phylogeny of Rhodophyta.

Introduction

Red algae (division Rhodophyta) are a monophyletic group of mostly multicellular photosynthetic eukaryotes. They occur primarily in tropical to temperate marine habitats, though freshwater and extremophilic species are known. Currently around 7000 species of red algae have been named, and the total number of red algal species on Earth has been estimated at around 14,000 (Guiry, 2012). Economically, red algae are the source for agar and carrageenan, industrially valuable hydrocolloids (Bixler and Porse, 2011); ecologically, red algae serve as primary producers as well as providing microhabitats and substrates for other organisms.

On the tree of life, the red algae occupy a distinctive position, serving as a link between primary and secondary endosymbiosis. They bear plastids of primary endosymbiotic origin, having arisen after an ancient phagotrophic eukaryote engulfed a cyanobacterium. Consequently they are grouped with Viridiplantae (green algae and land plants) and Glaucophyta (glaucophytes, a small unicellular group) in the supergroup Plantae, all of which are characterized by primary plastids. In addition, they are the source of the secondary plastids found in the numerous "brown" algal lineages, such as heterokonts, cryptophytes, and dinoflagellates, although it is unclear exactly how the secondary plastid of red algal origin was inherited among these groups (Keeling, 2013). Red algae are a link between these primary and secondary endosymbiotic lineages, which has allowed for many gene transfer events between these groups (Qiu *et al.,* 2013). Thus, genomic methods have been used to test various hypotheses regarding the inheritance of the "red" secondary plastid (*e.g.,* Baurain *et al.,* 2010; Burki *et al.,* 2012), necessitating a large amount of sequence data for red algae as well as for other groups.

Red algal genomic research is progressing rapidly with increasing computational capacity and falling sequencing costs. The first fully sequenced red algal genome, *Cyanidioschyzon merolae* P. De Luca, R. Taddei, & L. Varano (Matsuzaki *et al.,* 2004), was shown to be highly compact, with unusually low numbers of introns, transfer RNA genes, and ribosomal RNA genes compared to other eukaryotes.

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Abbreviations: bp, base pair; DCJ, double-cut-and-join; LCB, locally collinear block; ORF, open reading frame.

These findings reflect the specialized ecological niche of *C. merolae,* a unicellular extremophile found in very hot and acidic environments. Compact genomes were also found in *Porphyridium purpureum* (Bory de Saint-Vincent) K. M. Drew & R. Ross (Bhattacharya *et al.,* 2013), *Pyropia yezoensis* (Ueda) M. S. Hwang & H. G. Choi (Nakamura *et al.,* 2013), and *Chondrus crispus* Stackhouse (Collén *et al.,* 2013), suggesting that the common ancestor of red algae lived in an extreme environment, which led to a reduction of the genome that was subsequently inherited by its non-extremophilic descendants.

In addition, many red algal organellar genomes have been published in recent years. These genomes are functional remnants of the original bacterial endosymbionts, retaining only a few genes related to cellular respiration or photosynthesis in a single circular chromosome. Genes that were not retained were either transferred to the nucleus *via* endosymbiotic gene transfer or simply lost (Timmis *et al.,* 2004). The remaining mitochondrial and plastid genes are useful for studying organellar function as well as for phylogenetic analysis. Since next-generation sequencing technologies have become more accessible, many red algal genomics researchers have used these techniques to generate sequence data for organellar genomes (*e.g.,* Hwang *et al.,* 2013; Campbell *et al.,* 2014; S. Y. Kim *et al.,* 2014). However, relative to the total number of red algae, few mitochondrial and plastid genomes have been sequenced, with many large taxonomic groups unrepresented.

At present, the phylogeny of Rhodophyta is unresolved at deep nodes. Molecular systematics studies of red algae, using single or several phylogenetic markers, have not produced an unambiguous topology, and the branching pattern is unclear at the class and ordinal levels, despite numerous studies dealing with this issue (Freshwater *et al.,* 1994; Ragan *et al.,* 1994; Oliveira and Bhattacharya, 2000; Saunders and Hommersand, 2004; Yoon *et al.,* 2006; Le Gall and Saunders, 2007). The paucity of sequence data available for red algae is a major limiting factor for resolving the phylogeny, both in taxon sampling and character (*i.e.,* marker) sampling. Next-generation sequencing technologies can generate very large amounts of sequence data in a short time, making them highly valuable in improving phylogenetic resolution for Rhodophyta.

The current study presents mitochondrial genome sequence data from *Grateloupia taiwanensis* S.-M. Lin & H.-Y. Liang, a mesophilic, multicellular benthic red alga that grows on rocks in the shallow subtidal zone and has a disjunct distribution. It was first described in the Pacific (Lin *et al*., 2008), but it was later identified as a non-native species in the Gulf of Mexico, closely related to the aggressive invasive species *Grateloupia turuturu* Yamada (DePriest and López-Bautista, 2012). More generally, the genus *Grateloupia* is used as food and in pharmaceutical research, making *Grateloupia* an interesting target for future study. The plastid genome of *G. taiwanensis* was published by DePriest *et al.* (2013), who demonstrated patterns of gene retention and genome structure among red algal plastids. Similar methods are used in the current study to examine the mitochondrial genome of *G. taiwanensis*, synthesizing data from a selection of the 17 mitochondrial genomes previously available for red algae.

Materials and Methods

Sample collection and DNA extraction

The *Grateloupia taiwanensis* specimen used in this study was collected in April 2011 from a jetty in Orange Beach, Alabama (30°16′27.6″N 87°33′34.0″W). A large portion of the thallus (approximately 60 cm^2) was removed and preserved in silica gel for DNA extraction, and the remainder was vouchered on a herbarium sheet and deposited in The University of Alabama Herbarium. DNA was extracted from the silica-preserved sample using the QIAGEN DNEasy Plant mini kit (QIAGEN, Valencia, CA). To maximize DNA yield, the large

Table 1

Characteristics of red algal mitochondrial genomes used in this study

	Grateloupia taiwanensis	*Grateloupia angusta*	*Chondrus crispus*	*Gelidium vagum*	*Gracilaria salicornia*	*Rhodymenia pseudopalmata*	*Sporolithon durum*	*Pyropia haitanensis*	*Cyanidioschyzon merolae*
Size (bp)	28,906	27,943	25,836	24,901	25,272	26,166	26,202	37,023	32,211
G+C (%)	31.4	30.2	28.9	30.4	28.4	29.5	28.4	30.7	27.2
Protein-coding genes	26	26	26	23	25	24	24	23	34
tRNAs	24	19	23	18	20	21	19	24	25
GenBank Accession	KM999231	KC875853	NC_001677	KC875754	KF824534	KC875752	KF186230	JQ736808	NC_000887
Reference	This study	S. Y. Kim *et al.* (2014)	Leblanc *et al.* (1995)	Yang *et al.* (2014)	Campbell *et al.* (2014)	K. M. Kim *et al.* (2014)	K. M. Kim *et al.* (2013)	Mao *et al.* (2012)	Ohta *et al.* (1998)

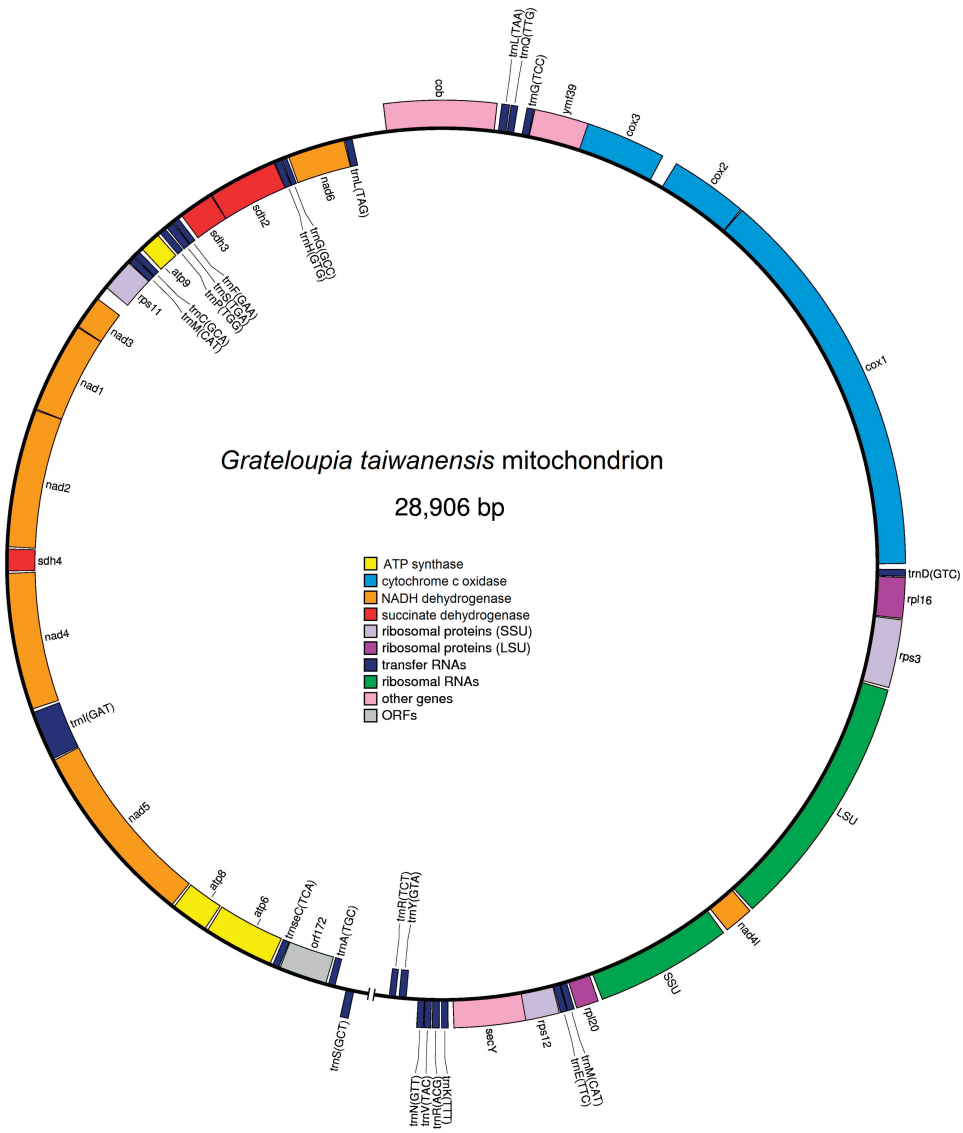

Figure 1. The mitochondrial genome of *Grateloupia taiwanensis,* drawn by OGDRAW software (Bock *et al.,* 2007). The gap between trnS(GCT) and trnR(TCT) indicates the ends of the linear contig.

sample was separated into eight pieces, and DNA was extracted from each piece individually. DNA samples were then pooled for genome sequencing.

Whole-genome sequencing

Whole genomic DNA from *G. taiwanensis* was sequenced in the laboratory of Debashish Bhattacharya (Rutgers University, New Jersey). The procedure for whole-genome sequencing of this sample was described in DePriest *et al.* (2013): The sequencing library was prepared using the Nextera DNA Sample Prep kit (Illumina, San Diego, CA) per the manufacturer's protocol and sequenced on one-half lane of an Illumina Genome Analyzer IIX using the TruSeq SBS kit v5 (Illumina) in a 150 × 150-bp paired-end run. The data were adapter- and quality-trimmed (error threshold = 0.05, *n* ambiguities = 2) using CLC Genomics Workbench (CLC Bio, Aarhus, Denmark) prior to *de-novo* assembly with the same software (automatic bubble size, minimum contig length = 100 bp). The raw reads were then mapped to the assembly contigs (similarity = 90%, length fraction = 75%), and regions with no evidence of short-read data were removed.

Identification of mitochondrial genome

The assembly contained about 62.7 Mbp separated into 130,269 contigs. The N50 statistic, defined as the sequence length for which 50% of the total base pairs in the assembly are contained in contigs at least as long, was 4703 bp.

Despite the somewhat poor assembly results, one contig of length 28,906 was identified as the mitochondrial genome, on the basis of several criteria. First, when the assembly was queried for known red algal mitochondrial genes using BLAST (Altschul *et al.,* 1997), the resulting significant hits ($e \leq 10^{-20}$) were found in this contig. Second, the size of the contig (28,906 bp) is similar to the lengths of other red algal mitochondrial genomes (Table 1). Third, multicellular red algae are known to have large numbers of mitochondria per cell (Garbary and Pei, 2006) and, therefore, many copies of the mitochondrial genome per cell, causing relative enrichment of the mitochondrial genome in the extracted DNA. Thus it is reasonable that the mitochondrial genome resolved as a single contig after assembly.

Annotation of genes

The goal of annotation is to indicate the start and stop positions of genes, including protein-coding genes, tRNAs, and rRNAs, in the mitochondrial genome. These data will be compared between taxa in the analysis. The mitochondrial genome contig was then isolated from the assembly, imported to Geneious R7 (Geneious, 2014), and set to represent a circular topology for visualization and genome comparison purposes only. To detect open reading frames (ORFs), the Geneious ORF Finder was used. For the genetic code, the Mold/Protozoan Mitochondrial option was selected; this codon translation scheme has been shown in red algae of the classes Florideophyceae and Bangiophyceae (Boyen *et al.,* 1994; Leblanc *et al.,* 1995; Burger *et al.,* 1999), but not Cyanidiophyceae (Ohta *et al.,* 1998). Only one codon is different between the Standard genetic code and the Mold/Protozoan Mitochondrial code: in the Standard code, the codon UGA is a "stop" codon, but in the Mold/Protozoan Mitochondrial code, UGA codes for the amino acid tryptophan.

To determine which ORFs likely represented actual mitochondrial genes, the *G. taiwanensis* mitochondrial genome was aligned with that of *Grateloupia angusta* (Okamura) S. Kawaguchi & H. W. Wang (S. Y. Kim *et al.,* 2014), using the Mauve genome alignment ver. 2.3.1 (Darling *et al.,* 2004) plugin in Geneious with the progressiveMauve algorithm (Darling *et al.,* 2010) and default settings. Protein-coding gene annotations in *G. angusta* that corresponded to an ORF in *G. taiwanensis* were annotated on the *G. taiwanensis* mitochondrial genome. These annotations were confirmed with a BLAST search against the UniProtKB sequence database (UniProt, 2014), with $e \leq 10^{-10}$ as the cutoff for a positive identification.

To find tRNA sequences, the mitochondrial genome sequence was submitted to the tRNAscan-SE ver. 1.21 server (Lowe and Eddy, 1997; Schattner *et al.,* 2005). The tRNA annotations were then annotated on the genome. To locate ribosomal RNA sequences, rRNA sequences were extracted

Table 2

Comparison of named protein-coding genes in red algal mitochondria; presence indicated by a dot; absence by a dash

	atp6	atp8	atp9	ccmA (yejW)	ccmF (yejR)	cob (cytB)	cox1	cox2	cox3	nad1	nad2	nad3	nad4	nad4l	nad5	nad6	rpl16	rpl20	rps3	rps4	rps8	rps11	rps12	rps14	sdh2 (sdhB)	sdh3 (sdhC)	sdh4 (sdhD)	secY	yejU	yejV	ymf16	ymf39
Grateloupia taiwanensis	•	•	•	–	–	•	•	•	•	•	•	•	•	•	•	•	•	•	•	–	–	•	•	–	•	•	•	•	–	–	•
Grateloupia angusta	•	•	•	–	–	•	•	•	•	•	•	•	•	•	•	•	•	•	•	–	–	•	•	–	•	•	•	•	–	–	•
Chondrus crispus	•	•	•	–	–	•	•	•	•	•	•	•	•	•	•	•	•	–	•	–	–	•	–	–	•	•	–	–	–	•	–
Gelidium vagum	•	•	•	–	–	•	•	•	•	•	•	•	•	•	•	•	–	•	•	–	–	•	–	–	•	•	•	•	–	–	–
Gracilaria salicornia	•	•	•	–	–	•	•	•	•	•	•	•	•	•	•	•	•	•	•	–	–	•	–	–	•	•	•	•	–	–	–
Rhodymenia pseudopalmata	•	•	•	–	–	•	•	•	•	•	•	•	•	•	•	•	–	–	•	–	–	•	–	–	•	•	•	•	–	–	–
Sporolithon durum	•	•	•	–	–	•	•	•	•	•	•	•	•	•	•	•	•	–	•	–	–	•	–	–	•	•	•	•	–	–	–
Pyropia haitanensis	•	•	•	–	–	•	•	•	•	•	•	•	•	•	•	•	•	–	•	–	–	•	–	–	•	•	–	•	–	–	•
Cyanidioschyzon merolae	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	–	•	•	–	•

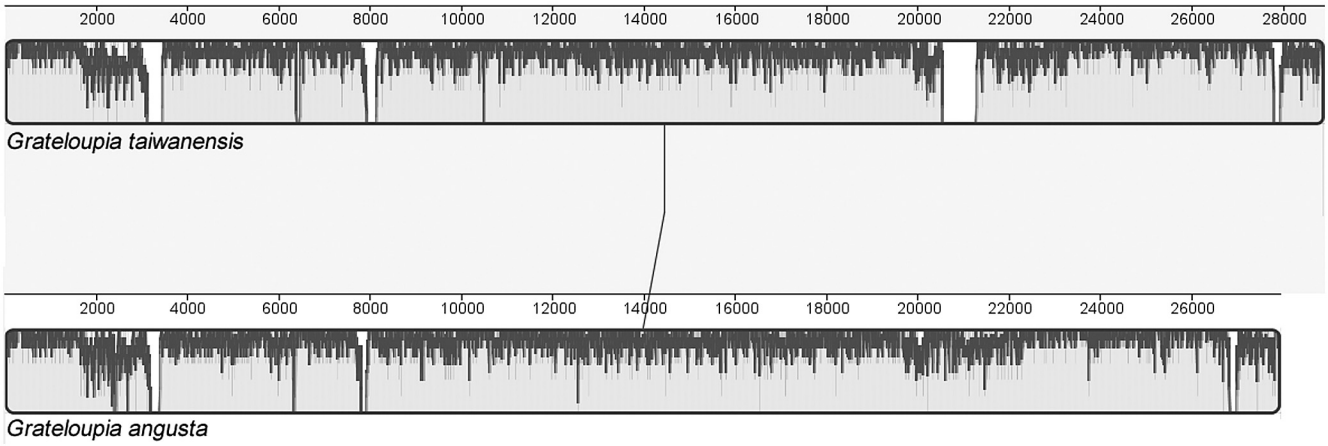

Figure 2. Mauve genome alignment of *Grateloupia taiwanensis* and *Grateloupia angusta.*

from the *G. angusta* mitochondrial genome and queried against the *G. taiwanensis* genome using BLAST; after finding the location of each gene, it was annotated.

Comparison with other mitochondrial genomes

Selected red algal mitochondrial genomes for comparison of gene content were downloaded from NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/). Lists of gene annotations in Geneious were copied and compared manually. Names of apparently unmatched genes were checked by searching UniProt for the gene name and using the preferred name only, in order to avoid using multiple names for each species. Uncharacterized ORFs were not included.

Mauve genome alignment

Mauve genome alignments are useful because they show conserved regions and rearrangements in the genome between taxa. For this analysis, several Mauve alignments were performed using default settings, with several selected combinations of red algae, based on their taxonomic relationships: (1) *G. taiwanensis* and *G. angusta;* (2) *G. taiwanensis* and various Rhodymeniophycidae—*Chondrus crispus*, *Gelidium vagum* Okamura, *Gracilaria salicornia* (C. Agardh) E. Y. Dawson, and *Rhodymenia pseudopalmata* (J. V. Lamouroux) P. C. Silva; (3) *G. taiwanensis* and *Sporolithon durum* (Foslie) R. A. Townsend & Woelkerling, subclass Corallinophycidae*;* (4) *G. taiwanensis* and *Pyropia haitanensis* (T. J. Chang & B. F. Zheng) N. Kikuchi & M. Miyata*,* class Bangiophyceae; and (5) *G. taiwanensis* and *Cyanidioschyzon merolae,* class Cyanidiophyceae. To aid in visualization, the *cox*1 gene, a commonly used phylogenetic and barcoding marker, was designated as position 1 of each circular genome, as Mauve requires sequences to be linearized. Images of Mauve alignments were exported from Geneious in PNG format and edited for publication.

Results

General characteristics and gene content

The mitochondrial genome contig is 28,906-bp long and has a GC content of 31.4%. It includes 25 protein-coding genes, 24 tRNAs, and 2 rRNA subunits (Fig. 1). The *Grateloupia taiwanensis* mitochondrial genome is most similar to that of *G. angusta* and *Chondrus crispus* in these aspects (Table 1). Two introns were found in genes in the *G. taiwanensis* genome: one in *cox*1 and one in *trn*I(GAT). A set of 20 named protein-coding genes (that is, excluding uncharacterized open reading frames [ORFs]) is shared across all species in the analysis (Table 2). When considering only named genes, *G. taiwanensis*, *G. angusta,* and *Gracilaria salicornia* are identical in gene content, containing the 20 "core" genes in addition to *rpl*20, *rps*11, *sec*Y, and *ymf*39. With minor differences, species of the subphylum Eurhodophytina (containing Florideophyceae and Bangiophyceae) are mostly alike in mitochondrial gene content. The mitochondrial genome of *Cyanidioschyzon merolae,* however, contains 30 genes, including 6 that are not present in any of the Eurhodophytina.

Genomic structure and rearrangements

The genomes of *G. taiwanensis* and *G. angusta* are highly similar with no rearrangements (Fig. 2); full bars represent higher similarity between the two genomes, and lower or absence of bars represents lower or absent similiarity between the *G. taiwanensis* and *G. angusta* mitocondrial genomes. Sequence similarity is consistent throughout, but a region of reduced similarity can be observed roughly between positions 1800 and 3000 in the alignment (all base pair positions given in this section refer to positions in the alignment, rather than positions in either genome). This region corresponds to the intron found in the *cox*1 gene.

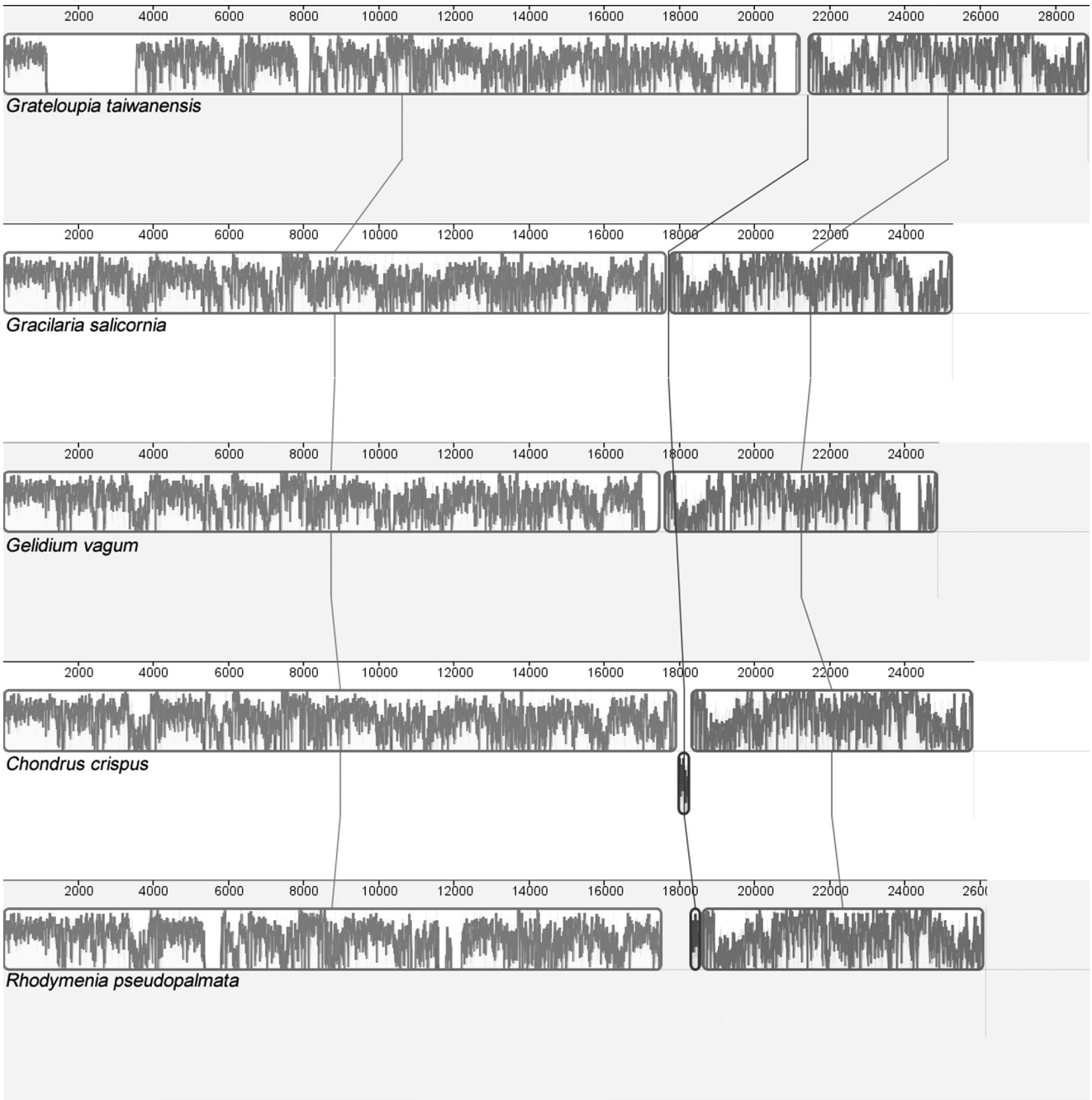

Figure 3. Mauve genome alignment of *Grateloupia taiwanensis* and Rhodymeniophycidae species.

From positions 21,108 to 21,859, *G. taiwanensis* was found to have a region including three additional tRNA genes—*trn*Y(GTA), *trn*R(TCT), and *trn*S(GCT); this region absent in *G. angusta* also includes the gap created when the contig was circularized for representational purposes.

When compared to other members of subclass Rhodymeniophycidae (Fig. 3), *G. taiwanensis* again appears highly similar. The group II intron shared by *G. taiwanensis* and *G. angusta* in *cox*1 is now represented by a large gap, as this intron is not present in *Chondrus crispus, Gelidium vagum, Gracilaria salicornia,* or *Rhodymenia pseudopalmata.* The three tRNA genes found in *G. taiwanensis* but missing from *G. angusta* are also missing from these four Rhodymeniophycidae species. A small region from bases 24,454 to 24,491 in *Gelidium vagum* (18,420 to 18,454 reverse in *Gracilaria salicornia*) may be an alignment artifact and is unlikely to represent homology, as this region

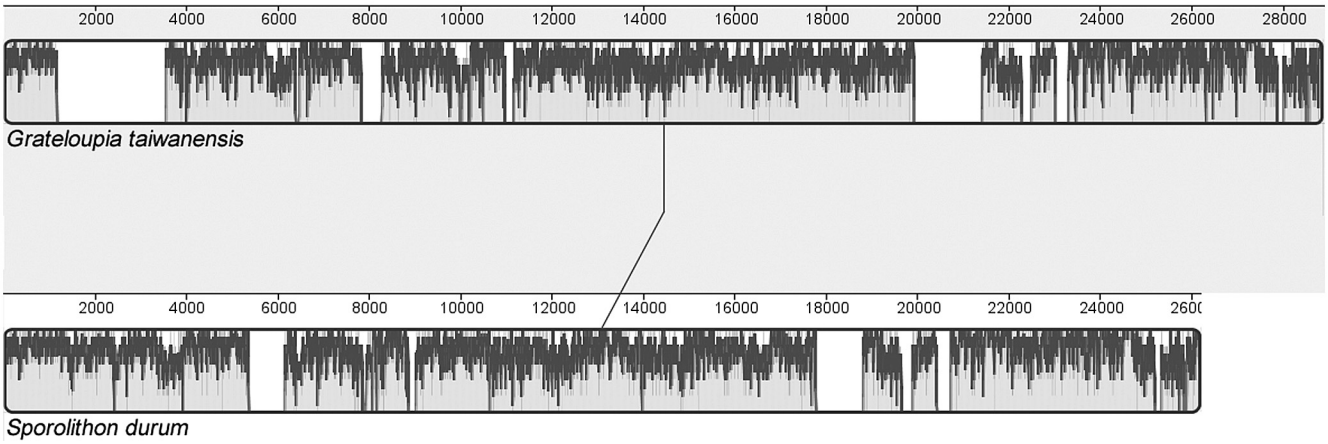

Figure 4. Mauve genome alignment of *Grateloupia taiwanensis* and *Sporolithon durum.*

occurs inside different genes between the two species. Excluding this tiny block, the Mauve alignment resulted in three locally collinear blocks (LCBs) shared across all five species, but no evidence of rearrangements was found.

Sporolithon durum, belonging to a different subclass (Corallinophycidae), is still highly similar to *G. taiwanensis* (Fig. 4). The group II *cox*1 intron and the three additional tRNA genes of *G. taiwanensis* are absent in *S. durum* as well. At position 21,163 in *G. taiwanensis,* a gap corresponding to the *G. taiwanensis orf*172 is evident; this gene is not present in *S. durum,* which has a different annotation, *orf-Sdur*34, immediately after this gap, at its position 21,621. These two ORFs do not appear to be homologous.

Several rearrangements are evident between *G. taiwanensis* and *Pyropia haitanensis* (class Bangiophyceae) (Fig. 5). The alignment recognized four LCBs, with three large-scale rearrangements, indicated by the double-cut-and-join (DCJ) distance value of 3. The *cox*1 gene contains many introns in *P. haitanensis,* indicated by gaps in the alignment inside this gene, which is located in the LCB at position 1. Two intronic ORFs are found within the *P. haitanensis cox*1 gene, but it is unclear whether either of these ORFs correspond to the one found in *G. taiwanensis.* A small LCB located at position 32,540 in *P. haitanensis* (21,111 in *G. taiwanensis*) contains two of the three tRNA genes—*trnR*(TCT) and *trnY*(GTA)—previously found in *G. taiwanensis* but none of the other Florideophyceae species. A region of genes from positions ~30,500 to the end of the *P. haitanensis* genome, excluding the aforementioned small LCB, is located outside any LCB. These genes are present in *G. taiwanensis,* but they are contained in the larger LCBs. This may indicate genome rearrangements of a small scale—that is, of single genes.

Between *G. taiwanensis* and *Cyanidioschyzon merolae*

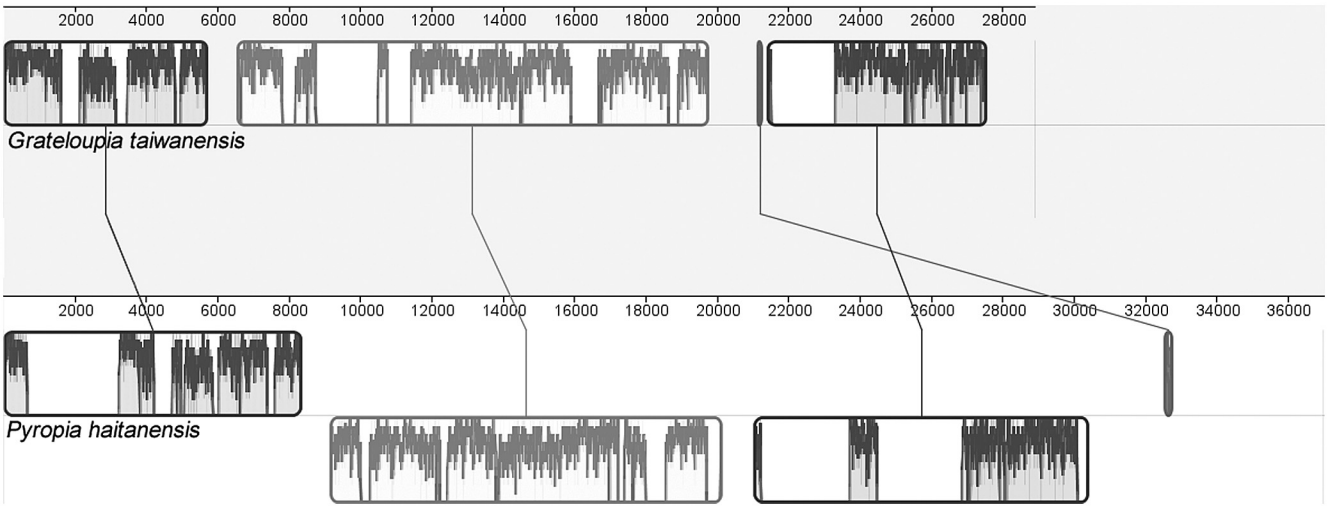

Figure 5. Mauve genome alignment of *Grateloupia taiwanensis* and *Pyropia haitanensis.*

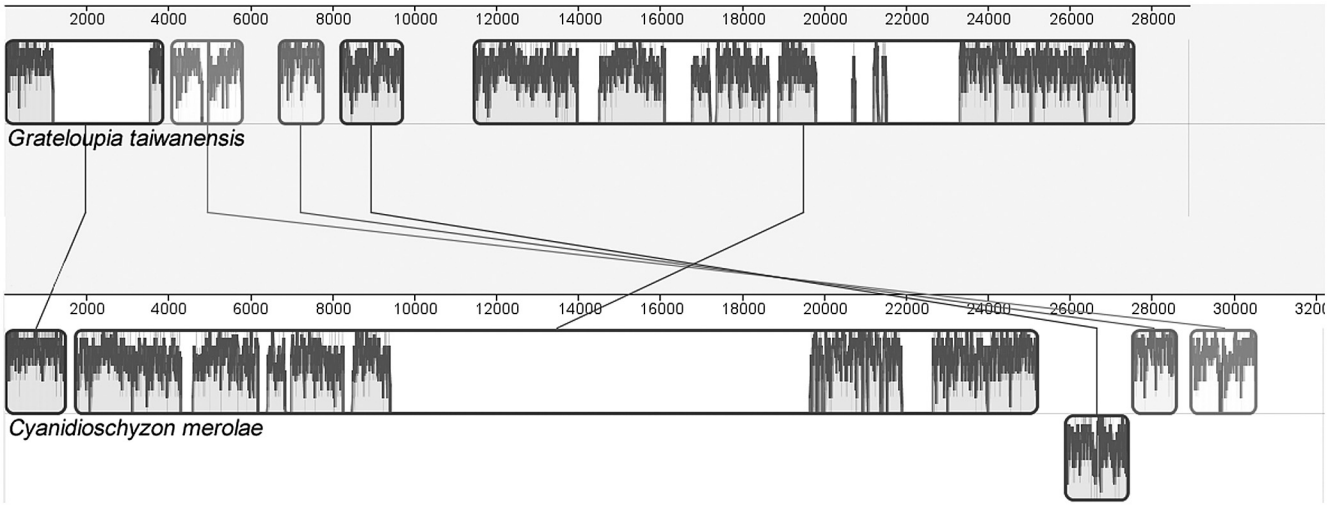

Figure 6. Mauve genome alignment of *Grateloupia taiwanensis* and *Cyanidioschyzon merolae.*

(class Cyanidiophyceae), the same number of rearrangements are apparent (Fig. 6), again with a DCJ distance of 3, but with five LCBs. The *cox*1 intron of *G. taiwanensis* is not present in *C. merolae.* A large gap region between positions ~9,500 and ~20,000 in *C. merolae* includes several genes that are not present in *G. taiwanensis.*

Discussion

The mitochondrial genome of *Grateloupia taiwanensis* is typical of red algae, especially Florideophyceae, in overall structure and characteristics. Red algal mitochondria seem to vary less in their genomes than in their plastids; however, these genomes are very different in size, with plastid genomes about five times larger than mitochondrial genomes. Larger genomes with more genes would hypothetically have more possibilities for gene losses and genome rearrangements, but a definitive conclusion on this topic cannot be drawn from these results.

On the other hand, the mitochondrial genome of *Cyanidioschyzon merolae* is very different from that of the other taxa in our analysis, being largest in size (32,211 bp) and containing the most protein-coding genes (34). It may seem counter-intuitive that an extremophilic organism with a highly reduced nuclear genome retains a relatively large mitochondrial genome. But this supports the hypothesis that the common ancestor of Rhodophyta was itself an extremophile, occurring in acidic hot springs as do present-day Cyanidiophyceae. *Cyanidioschyzon merolae* may possess many characteristics of this ancestor, including several mitochondrial genes that are not present in other groups of red algae, as over time these genes have been either lost or transferred out of the mitochondrion. However, of the seven classes of red algae, only three (Cyanidiophyceae, Bangiophyceae, and Florideophyceae) currently have mitochondrial genomes available, and the unrepresented classes are all phylogenetically placed between Cyanidiophyceae and the others. Additional sequencing is necessary so that patterns of gene retention may be further investigated.

Besides several additional tRNA genes found in *Grateloupia taiwanensis,* the mitochondrial genome of *G. taiwanensis* is highly similar to that of *Grateloupia angusta,* which would be expected of species belonging to the same genus. It should be noted, though, that *Grateloupia* is a genus undergoing extensive taxonomic revision. Gargiulo *et al.* (2013) split *Grateloupia s.l.* into several genera, on the basis of both morphological and sequence data. This includes several resurrected taxa and some new genera, which the authors intend to describe in a forthcoming paper. *Grateloupia taiwanensis* was not included in their analysis, but considering previous phylogenetic analyses (Lin *et al.,* 2008; DePriest and López-Bautista, 2012), *G. taiwanensis* appears to belong to a clade that Gargiulo *et al.* (2013) suggest should become a new genus based on *Grateloupia subpectinata* Holmes. *Grateloupia angusta* does not belong to this clade, instead belonging to a clade corresponding to the genus *Pachymeniopsis* Y. Yamada ex S. Kawabata (Gargiulo *et al.,* 2013). Therefore, although we refer to two species of *Grateloupia* in the current study, it is most likely that neither one actually belongs to *Grateloupia s.s.* In this case, the unique *cox*1 intron shared by these two species would have a wider taxonomic distribution than simply one genus, possibly present in the entire family or order.

We have demonstrated a simple set of methods for investigating a new organellar genome, from sequencing and annotation to large-scale comparisons among species. With the increasing popularity and efficiency of next-generation sequencing, many red algal organellar genomes have recently been quickly published in short papers simply to

make the data available. However, we have shown that a more in-depth characterization of a new organellar genome can produce scientifically interesting results, such as the differences in gene content between *Grateloupia taiwanensis* and *Grateloupia angusta,* with simple methods based on next-generation sequencing technology and publicly available software. Future genome sequencing efforts in red algae should focus on unsampled taxonomic groups so that the full potential of red algal organellar genomes can be revealed and allow for a better understanding of deeper phylogenomic relationships among red algal groups.

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