



Complete plastid genome of *Kappaphycus alvarezii*: insights of large-scale rearrangements among Florideophyceae plastid genomes

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Abstract

Kappaphycus alvarezii is a large commercial tropical marine red alga. In this study we used next-generation sequencing to determine the complete plastid genome of *K. alvarezii*. The plastid genome mapped a 178,205-bp circular DNA molecule with a GC content of 29.6%, without any inverted repeat region, and encoded a standard set of 202 protein-coding genes, including 4 open reading frames, 30 transfer RNA genes, 3 ribosomal RNA genes, and 1 transfer-messenger RNA gene. Only one group II intron interrupting the *trnMe* gene was detected. Comparative analysis of the seven plastid genomes from three orders including Gigartinales, Gracilariales, and Peyssonneliales revealed that the genomes were more conserved except five remarkable gene rearrangements. However, an approximately 12.5-kb gene fragment from the *psaM* to *ycf21* gene in *K. alvarezii* plastid genome was completely reversed compared to that in published plastid genomes from the Florideophyceae. To our knowledge, the gene rearrangement was identified for the first time in the plastid genomes of red algal species. In addition, phylogenetic analysis of 49 Florideophyceae species based on 144 shared protein-coding genes suggested that different species from the same class clustered together according to their sources: three species of Gigartinales formed one group as the subbranch of Florideophyceae, but *K. alvarezii* was the most closely related to *Riquetophycus* sp. from the Peyssonneliales.

Keywords *Kappaphycus alvarezii* · Rhodophyta · Plastid genome · Gene rearrangements · Phylogenetic analysis

Introduction

Kappaphycus alvarezii (Solieriaceae, Rhodophyta) is a large tropical red seaweed which is an important raw material to extract carrageenan; it is mainly cultivated in Southeast

Asian countries such as Indonesia and the Philippines (Bindu and Levine 2011; Hurtado et al. 2015). Because of its important economic value in the food and carrageenan industry and the relatively simple aquaculture (seeding breeding mainly via vegetative reproduction), large-scale

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cultivation of *K. alvarezii* has expanded to tropical and subtropical countries, including Malaysia, Fiji, Vietnam, Tanzania, and South Africa (Hurtado et al. 2014; Msuya et al. 2014). In the 1980s, *K. alvarezii* was first introduced to China for culture (Wu et al. 1988). At present, large-scale farming of *K. alvarezii* is performed in the sea areas of Hainan Province of Southern China and *K. alvarezii* has become an important seaweed resource in China.

In recent years, studies focusing on *K. alvarezii* have gradually increased. *K. alvarezii* is a good seaweed resource that can be used as a raw material to produce renewable biofuel. Previous studies showed that extracting bioethanol from the acid hydrolysate of the carrageenophyte *K. alvarezii* was possible (Khambhaty et al. 2012; Meinita et al. 2012, 2019). However, several species of *Kappaphycus* are considered to be invasive algae that can grow rapidly throughout the reef habitats and kill corals (Conklin and Smith 2005; Chandrasekaran et al. 2008). Most studies have generally focused on the maximum utilization of *Kappaphycus* with regard to its culture, including growth rate experiments and protoplast culture (Hayashi et al. 2007; Zhang et al. 2014). However, little information is available on the molecular biology of *K. alvarezii*. Existing studies involved only in markers for barcoding, species delimitation, and molecular phylogenetic analyses based on single gene sequence, such as plastid *rbcl*, mitochondrial *cox1*, nuclear ITS, and 28S rRNA (Conklin et al. 2009; Liu et al. 2012; Sun et al. 2014). At present, plastid genomes from Solieriaceae have been rarely explored. Further, the complete plastid genomes of only *Chondrus crispus* (NC_020795) and *Mastocarpus papillatus* (NC_031167) from Gigartinales order are available (Janoukovec et al. 2013; Sissini et al. 2016). Conducting detailed molecular systematic researches on red algae of Solieriaceae family and phylogenetic analysis at the whole plastid genome level might provide more comprehensive and accurate data to clarify the evolution of plastids.

With the development of high-throughput sequencing technologies, whole genome sequencing projects have been established for numerous species. Organelle genomes of mitochondrion and plastid are small in size and have relatively compact structure; thus, obtaining the whole genomes in a short time is usually possible. In this study, we obtained the red algae plastid genome pool by sequencing the large marine red alga *K. alvarezii*. The data represented the first completely characterized plastid genome from the species belonging to Solieriaceae. By conducting comparative analysis of plastid genomes of six red algal species from Florideophyceae, we explored the changes in the characteristics of plastid genome structure during the evolution of these species. Further, we provided new molecular data that might form a basis for phylogenetic studies on tropical red algae as well as comparative genomics of Solieriaceae species.

Materials and methods

Sampling and DNA extraction

Kappaphycus alvarezii was collected from Lingshui County, Hainan Province of China. Algal material was deposited in the Culture Collection of Seaweed at the Ocean University of China in Qingdao (sample number: 2012040002). Fresh alga was cultivated at 24 °C in sterilized seawater with nutrients (4 mg L⁻¹ NaNO₃, 0.4 mg L⁻¹ KH₂PO₄) under fluorescent light (~40.5 μmol photons m⁻² s⁻¹; 12 h light/dark cycles). The alga was thoroughly cleaned with sterilized seawater before extracting DNA. Total DNA was extracted from fresh alga according to a modified CTAB method (Doyle and Doyle 1990). The quality and quantity of extracted DNA were determined using a NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific Inc.).

Genome sequencing and assembly

DNA library sequencing and construction were performed by the Beijing Genomics Institute (BGI) in Shenzhen, China. We used approximately 5 μg of purified DNA for the construction of short-insert libraries following manufacturer's instructions (Illumina Inc., USA). The sequenced libraries included short pair-end libraries with insert lengths of 250, 300, and 500 bp. The constructed DNA library was extracted using the Illumina HiSeq 2000 system.

The raw reads were assembled into contigs by using SOAPdenovo software (Luo et al. 2012) with default assembly parameters (SOAPde-novo all -s assembly.conf -o mysample -k 49 -p 8&). The proportion of plastid-related contigs was determined using the plastid genome of *Chondrus crispus* (NC_020795) as reference sequence by using Basic Local Alignment Search Tool (BLAST) software (Altschul et al. 1997). Subsequently, all plastid-related contigs were aligned and ordered into a circular structure by using CodonCode Aligner software (CodonCode Corporation, USA). Gaps between contigs were filled using polymerase chain reaction (PCR) and Sanger sequencing by using the primers listed in Table S1, until the complete circular sequence was obtained. For junction regions between rearranged segments, additional primers (Table S1) were designed to ensure the accuracy of the sequence by conducting PCR and Sanger sequencing.

Annotation and comparative analysis

The protein-coding, ribosomal RNA (rRNA), intron and transfer-messenger RNA (tmRNA) genes of the assembled plastid genome were determined by sequence alignment with the corresponding plastid genome of *C. crispus* by using Geneious R 10 (Biomatters Ltd., New Zealand; available from

<http://www.geneious.com/>). To detect the open reading frames (ORFs), we used NCBI ORF-finder, and alignments were conducted using BLASTX searches at NCBI. Transfer RNA (tRNA) genes were predicted using tRNAscan-SE 1.21 software (Lowe and Eddy 1997). The plastid genome map of *K. alvarezii* was obtained using Organellar Genome OGDRAW software (Lohse et al. 2007). The plastid genomes of seven species from Florideophyceae class were compared using the multiple genome alignment software MAUVE (Darling et al. 2004).

Phylogenetic analysis

Phylogenetic analysis was conducted using 144 conserved plastid protein-coding genes from 50 red algal plastid genomes publicly available at the NCBI GenBank (including our one new plastid genome). Every gene was found in all taxa (50 species); some protein-encoding genes previously reported as missing were reannotated in this study (Table S2). Each protein sequence was aligned individually by using MEGA 5.0 software (Tamura et al. 2011), and then the entire concatenated alignment was generated and edited manually using BioEdit software (Hall 1999). The Gblocks server (http://molevol.cmima.csic.es/castresana/Gblocks_server.html; Castresana 2000) was used to generate concatenated alignments and remove poorly aligned regions, resulting in the alignment reducing from 36,690 to 29,175 position. To reconstruct the phylogenetic tree, the best-fit model for maximum likelihood (ML) was selected using ProtTest 3.4.2 (Darriba et al. 2011). ML search and ML bootstrap analysis were performed using RAxML (Stamatakis 2006) with 1000 replications under the CpREV + G + I + F model. The Bayesian tree (BI) was conducted using MrBayes v. 3.1.2 (Huelsenbeck and Ronquist 2001). Bayesian analysis was performed using two separate sequence analyses for four Markov chains (by using default heating values), which were run for 2,000,000 generations until the average standard deviation of split frequencies was below 0.01 (Ronquist and Huelsenbeck 2003). In addition, the first 25% of trees were discarded as burn-in. The remaining trees were used to build a 50% majority rule consensus tree, accompanied with posterior probability values. FigTree v1.3.1 software was used to display and edit the phylogenetic tree (Rambaut 2009).

Results

Structure and features of the plastid genome

The complete plastid genome of *K. alvarezii*, sequenced using next-generation sequencing (NGS) method, was assembled as a circular molecule of 178,205 bp in length with an overall A + T content of 70.44%; the nucleotide composition was 34.92% A,

14.83% C, 14.74% G, and 35.52% T (GenBank accession number; KU892652). No inverted repeat (IR) region was identified in *K. alvarezii*. IR was described in most of the early-diverged classes (Bangioophyceae), whereas most Florideophyceae species found merely single or partially inactivated (pseudogenes) duplicated rDNAs. The plastid genome of *K. alvarezii* encoded 202 protein-coding genes, 30 tRNA genes, 3 rRNA genes, and 1 tmRNA gene; only one group II intron (with intronic *ORF436*) interrupting the *trnMe* gene was detected (Fig. 1; Table 1). The similarity of intronic *ORF436* between *K. alvarezii* and *C. crispus* was 49.89% based on amino acid sequence alignment using MEGA 5.0 software (Fig. S1). And the published plastid genomes of red algae suggested that the intronic ORFs of all Florideophyceae species were a homologous group derived from prokaryotes (Lee et al. 2016). The coding region was 145,809 bp, accounting for 81.82% of the total genome. The tRNA sequences ranged from 71 to 89 bp, and most of the tRNA genes had the standard cloverleaf secondary structures (Fig. S2). All genes were encoded on both the heavy and light strands.

Kappaphycus alvarezii plastid genome had three types of start codons (Table S3). ATG was used as the start codon for nearly all plastid genes (94%), whereas TTG was used as the start codon for the *ycf20*, *ycf27*, and *trxA* genes. In addition, many plastid genes (including *psbC*, *rps8*, *rpl24*, *rpl3*, *petN*, *rbcS*, *infC*, *chlI*, *ycf63*, and *ycf64*) used GTG as the start codon. Three typical types of stop codons were noted for *K. alvarezii* plastid genes: TAA, TAG, and TGA (Table S3). Of these, the predominant codon was TAA with the highest proportion of 70.3%; TGA and TAG also accounted for a considerable proportion of 10.4% and 19.3%, respectively. In addition, the plastid genome of *K. alvarezii* was compact; ten pairs of gene overlaps with overlap length of 1–26 bp (*carA-ycf53*, *ccs1-trpG*, *ycf60-rps6*, *psbD-psbC*, *ORF146-groEL*, *trnH-ycf29*, *rpl24-rpl14*, *rpl23-rpl4*, *atpF-atpD*, and *psaL-trnT*) were found.

Comparative genome analysis and gene rearrangement

Kappaphycus alvarezii as well as *C. crispus* and *Mastocarpus papillatus* belong to the same order of Gigartinales; the overall gene content and structure of *K. alvarezii* plastid genome were nearly the same as those of the plastid genomes of *C. crispus* and *M. papillatus* (Table 1). However, global plastid genome comparison indicated some remarkable differences among different Gigartinales species as follows: the *dfr* gene was present in *K. alvarezii* and *M. papillatus* between the *ycf63* and *psbE* genes, but not in *C. crispus*. Further, interestingly, the *pbsA* gene was absent between the *bas1* and *rpl35* genes in *K. alvarezii*, as well as in *M. papillatus*, but not in *C. crispus*; this gene was commonly found in other red algal plastid genomes of Florideophyceae and Bangioophyceae species. However, a fragment of similar length was still found at the

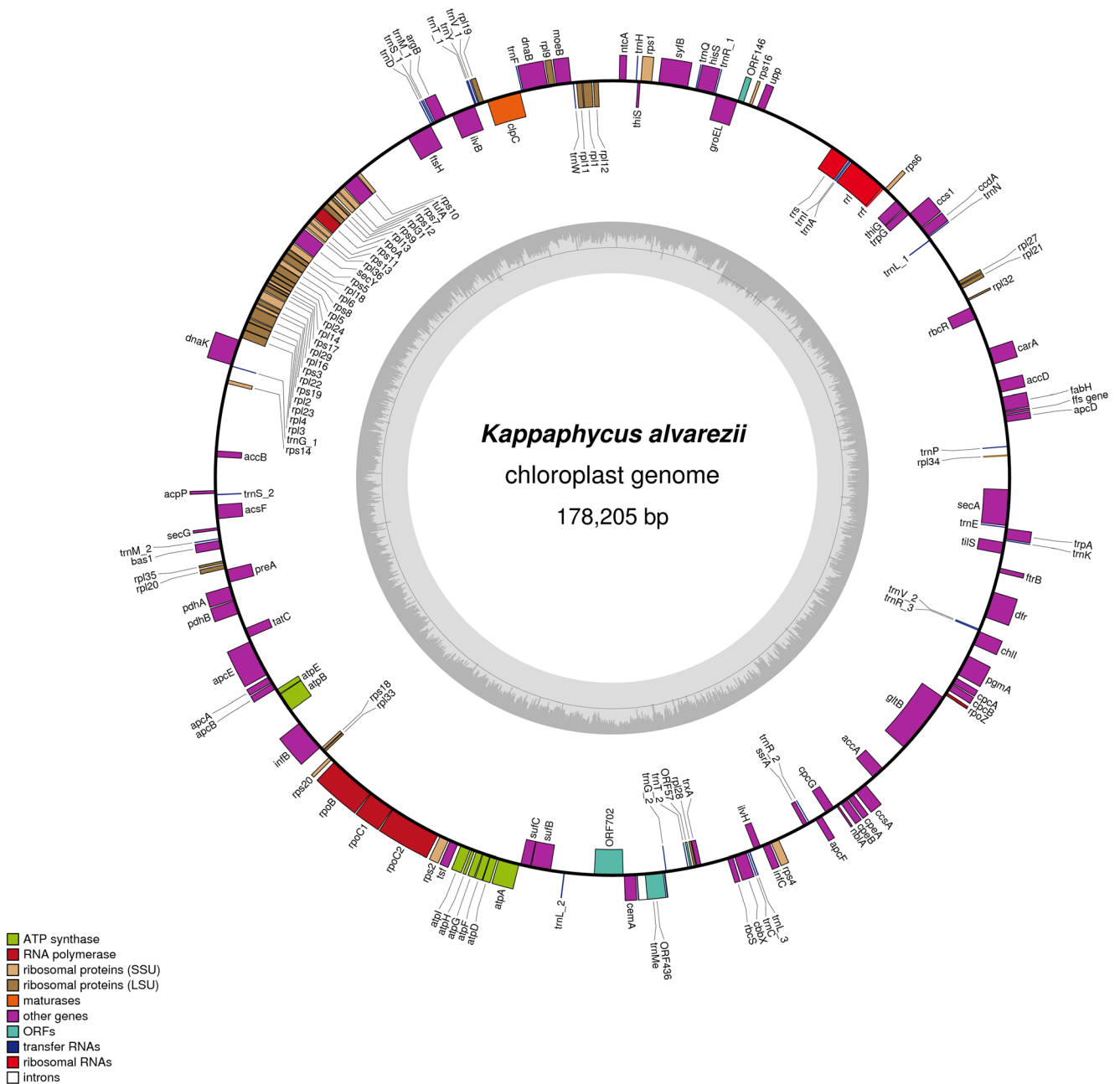


Fig. 1 The physical map and organization of *Kappaphycus alvarezii* plastid genome. Color indicates different gene classifications. Genes inside the circles are transcribed clockwise and those outside are transcribed counterclockwise

original location of the *pbsA* gene, which had a low homology of 62.5% with the *pbsA* gene from *C. crispus* (Fig. 2). Several stop codons were produced ahead of time in the fragment, probably because of many base mutations at different sites, or perhaps because a frameshift by one base mutation caused multiple stop codons downstream of the shifting point. Obtaining an effective length of the ORF was not possible in this study, although we attempted to shift the reading frame or change the transcriptional direction. Therefore, we speculated that the *pbsA* gene was absent in the plastid genome of *K. alvarezii*. In addition, notably, a gene cluster containing

16 genes of about 12.5 kb in length from the *psaM* to *ycf21* genes in the *K. alvarezii* plastid genome was completely reversed compared to that from *C. crispus* and *M. papillatus*. The junction regions related to the fragment were verified by PCR and Sanger sequencing by using primers listed in Table S1. The gene fragment having the same gene content and order was located at the same position in the plastid genome, but in a different transcriptional direction (Fig. 3).

Comparison of three plastid genomes from Gigartinales, one known plastid genome of *Riquetophycus* sp. from Peyssonneliales and three previously sequenced species from

Table 1 General features of the plastid genomes of red algal species

Order	Gigartinales			Gracilariales			Peyssonneliales
	Species	<i>Chondrus crispus</i>	<i>Mastocarpus papillatus</i>	<i>Kappaphycus alvarezii</i> *	<i>Gracilaria salicornia</i>	<i>Gracilaria tenuistipitata</i> var. <i>liui</i>	
Genome size (bp)	180,086	184,382	178,205	179,757	183,883	183,013	180,384
GC content (%)	28.7	29.1	29.6	28.8	29.2	27.4	28.8
Coding content (%)	81.14	78.96	81.82	80.88	80.40	79.60	85.29
Spacer content (%)	14.56	17.38	13.69	15.15	15.43	14.86	13.90
Total number of genes	240	235	238	235	238	241	237
Protein-coding genes	204	202	202	203	203	204	202
tRNA genes	30	30	30	29	29	32	30
rRNA genes	3	3	3	3	3	3	4
tmRNA	1	0	1	0	1	1	0
Intron	1	1	1	1	1	1	1
GenBank Accession	NC_020795	NC_031167	KU892652	NC_023785	AY673996	KP330491	KX284710

The *Mastocarpus papillatus trnM* gene and group II introns are not annotated in GenBank. One group II intron (139,732 > 141,733) interrupting the *trnM* gene is detected (as unannotated 139,696 > 139,733 and 141,734 > 141,783). Asterisk indicates newly sequenced in this study

Gracilariales, including *Gracilaria salicornia*, *Gracilaria tenuistipitata* var. *liui* and *Gracilariopsis lemaneiformis*, revealed significant synteny. The overall sequences of the red algal plastid genomes were found to be fairly conserved, with similar gene content and genome organization. However, global plastid genome comparison by using MAUVE alignment indicated a large number of rearrangements among different species (Fig. 3). Five reversed fragments were detected between seven Florideophyceae species, corresponding to the regions marked with A, B, C, D, and E in Fig. 3, with fragment lengths of about 18.5 kb (*ycf46-trnN*), 4.6 kb (*ccdA-rps6*), 10.5 kb (*psb28-rpl9*), 4.4 kb (*dnaB-clpC*), and 12.5 kb (*ycf21-psaM*), respectively. These reversed fragments showed the following characteristics: (1) long conserved gene clusters with almost the same gene content and order; (2) same location in the plastid genome structure; and (3) reversal of fragment, indicating that the transcriptional orientation of the entire fragment had changed.

Phylogenetic analysis

Based on the previously published plastid genomes of Florideophyceae, including 49 species, we used a common dataset of 144 protein-coding genes to conduct phylogenetic analysis; *C. merolae* was used as an outgroup. The ML and BI phylogenetic trees with very high posterior probabilities are shown in Fig. 4. The results revealed that the Florideophyceae taxa were clearly separated into 20 orders. Although the phylogenetic relationship obtained in this study was similar with the one revealed by Lee et al. (2016), it reconducted the phylogenetic relationship in the order Gigartinales. Three species from Gigartinales and one from Peyssonneliales formed a clade. Within this clade, *C. crispus* showed a closer relationship with *M. papillatus*; however, *K. alvarezii* was included in a strongly supported clade with *Riquetophycus* sp., which belonged to Peyssonneliales.

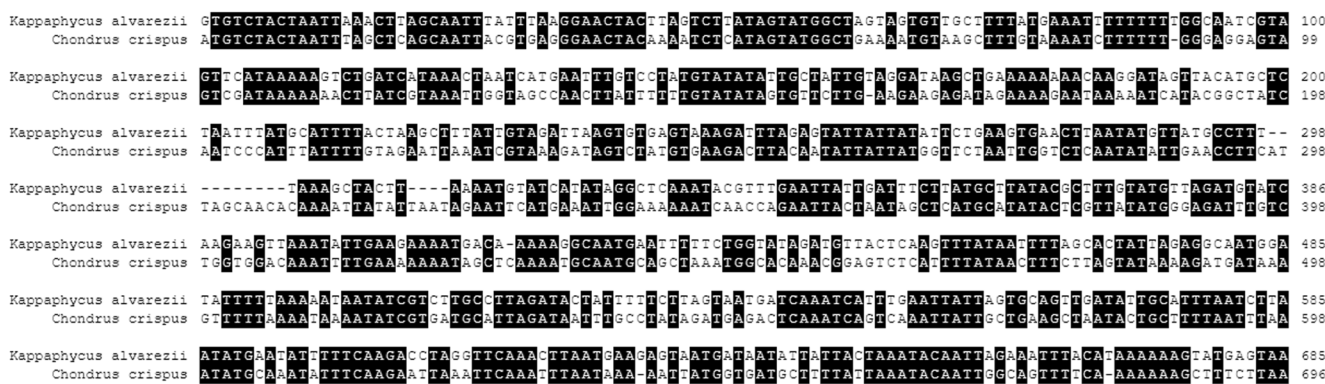


Fig. 2 Sequence alignment of the pseudogene *pbsA* from *Kappaphycus alvarezii* with *Chondrus crispus* that contained entire *pbsA* gene. The homology bases are shown by a black box

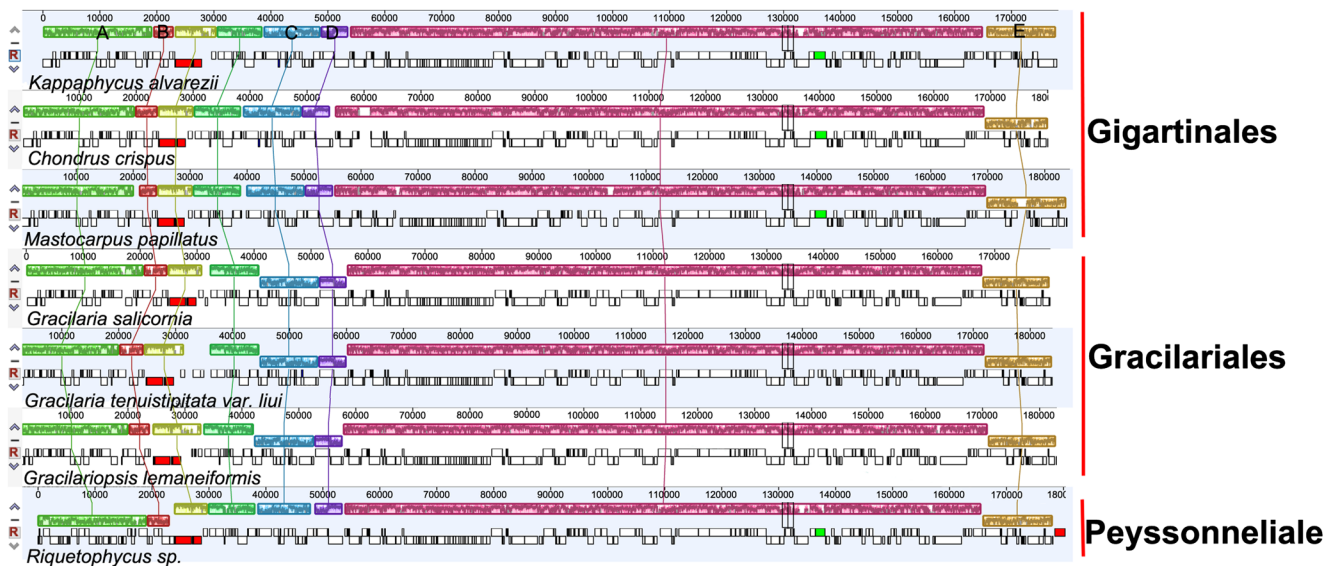


Fig. 3 Plastid genome comparison among seven red algal species from Florideophyceae by using MAUVE. Color box below the line has an inverse orientation relative to *Kappaphycus alvarezii*. Letters A, B, C,

D and E represent three inverted fragments among seven species, respectively, in which the fragment E in *K. alvarezii* is unique compared with that in the other six species

Discussion

The plastid genomes of Florideophyceae species were conserved in gene content and genome structure except

Hildenbrandiales (Lee et al. 2016). The plastid genome of *K. alvarezii* was fairly conserved, with similar gene content and number. However, the plastid genome size differed among the Florideophyceae species: the average length was

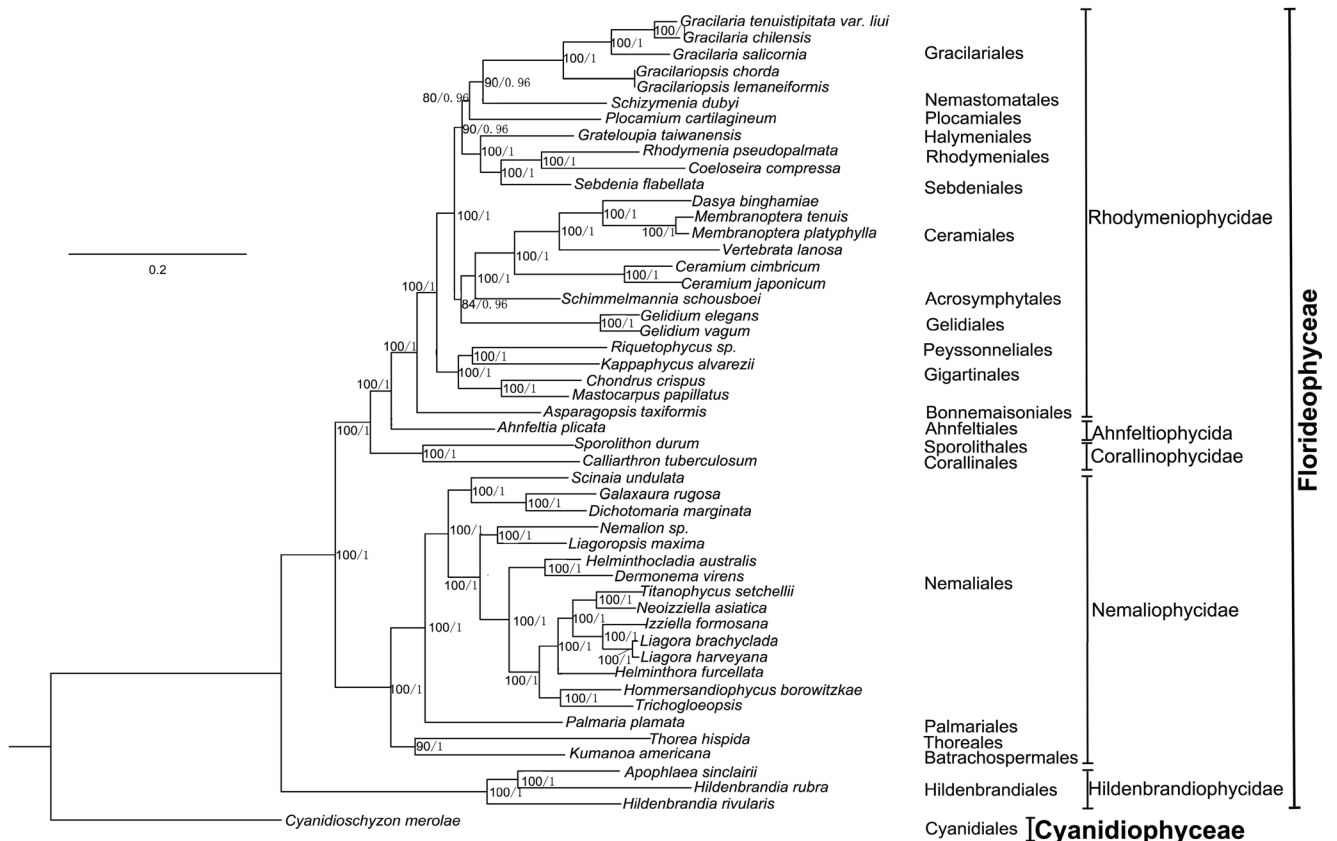


Fig. 4 ML and BI phylogenetic trees generated using 144 concatenated plastid protein-coding genes from 49 Florideophyceae species. *Cyanidioschyzon merolae* was set as an outgroup; the support values

for each node are shown from maximum likelihood bootstrap and Bayesian posterior probabilities. Asterisk indicates newly sequenced *Kappaphycus alvarezii* in this study

181 kb, and average AT content was 70.71%. The plastid genome size of *K. alvarezii* was the smallest among those of Gigartinales species. The three members of Gigartinales consisted of one group II intron, interrupting *trnMe* gene (Table 1). Within Florideophyceae species, it was found to contain one intron or two introns encoding maturase ORF (RT/*mat*) (Janoukovec et al. 2013). For example, *Calliarthron tuberculosum* additionally contained a second group II intron in *chlB*, except one group II intron in *trnMe*. It was likely that Florideophyceae species should have two introns in *chlB* and *trnMe*, while *chlB* intron or *trnMe* intron had been missed in some Florideophyceae plastid genomes during the time of Florideophyceae divergence; only a handful of species had been able to retained both two.

In the plastid genomes of red algae, in addition to ATG, TTG and GTG also served as initiation codons. TTG was a rare alternative initiation codon in organisms known to use a non-standard genetic code. In bacteria, in addition to the standard start codon ATG, the triplets GTG, ATT, and TTG serve as start codons (Golderer et al. 1995). A previous study showed that TTG was the start codon for the *tatC* gene from the mitochondrial genome of *Kappaphycus striatus* (Tablizo and Lluisma 2014). In addition to TTG, GTG was also occasionally used as the start codon in *K. alvarezii*. Analysis of brown algal plastid genomes revealed that several genes used GTG as the start codon (Corguille et al. 2009; Wang et al. 2013). Additionally, as a red algae plastid descendant, a few brown algal species also used ATT as a start codon (Zhang et al. 2015a, b). However, TTG as a start codon was noted only in the plastid genomes of red algal species.

One of the most remarkable characteristics in *K. alvarezii* was the absence of the *pbsA* gene. Analysis of the published plastid genomes of Gigartinales species showed that the *pbsA* gene was absent in *K. alvarezii* and *M. papillatus*. In algae, the *pbsA* gene was homologous with the heme oxygenase (*HO*) gene for phycobilin synthesis (Reith and Munholland 1995). *HO* is a key enzyme in the synthesis of the chromophoric part of the photosynthetic antennae in cyanobacteria, red algae, and Cryptophyceae. Richaud and Zabulon (1997) cloned and sequenced the *pbsA* gene from the chloroplast genome of red alga *Rhodella violacea* and found that it was split into three distant exons. No large insertion was noted at the assumed region of the *pbsA* gene; therefore, it probably did not contain any intron. The loss of gene function should have been caused by base mutation. In the present study, the *pbsA* gene was encoded in most red algal plastid genomes, and the phylogenetic data suggested *pbsA* gene was derived from cyanobacteria, while the loss of *pbsA* gene in red algal plastids was rare and a stochastic process, probably because the gene played an unnecessary role. Furthermore, it has been found that some red algal species presented three heme oxygenase genes; two nuclear-encoded heme oxygenase types, *HMOX1* and *HMOX2*; and *pbsA* gene in plastid genome (Costa et al. 2016; Cho et al. 2018).

MAUVE analysis from seven species showed that the gene content and order of the three plastid genomes of *G. salicornia*, *G. tenuistipitata* var. *liui*, and *G. lemaneiformis* from the order Gracilariales were almost completely consistent. Nonetheless, *K. alvarezii*, *M. papillatus*, and *C. crispus* from Gigartinales order and *Riquetophycus* sp. from the Peyssonneliales order showed marked differences compared with those in the three species of order Gracilariales. In *Riquetophycus* sp., there are two long and conserved gene clusters, in which the gene order was almost exactly the same (areas A and B in Fig. 3) and completely reversed compared with other species. Similar to *Riquetophycus* sp., two gene clusters (areas C and D in Fig. 3) from the three Gracilariales species were reversed compared with those in the three Gigartinales species and one Peyssonneliales species. Interestingly, whereas in *K. alvarezii*, another gene cluster (area E in Fig. 3) was reversed compared to that in the other six species, and this region was unique based on comparison with the Florideophyceae plastid genomes published in the NCBI dataset. Therefore, the rearranged fragments of *K. alvarezii* from Solieriaceae were identified for the first time in the plastid genomes of red algae. Thus, the results showed that red algal plastid genomes included four rearrangements between those three orders, with the feature of a large conserved gene cluster reversed at the same location. Only in this study, for Florideophyceae, including seven species from three different orders, a total of five long reversed fragments were detected; furthermore, such rearrangements not only occurred between species from different orders but were also noted at the family level. Usually, gene rearrangements such as reordering of genetic elements occur via repeated inversion. However, no similar structures such as inverted sequences were found at the junction area between these rearranged fragments and adjacent to the relevant region, or any common feature was not determined between these fragments. The detailed mechanism of these rearrangements will warrant further analysis.

The phylogenetic analysis did not well supported the previous observation of the evolutionary process of the class Florideophyceae, either we added plastid genome of newly sequenced specie, *K. alvarezii*; the result reconstructed the phylogenetic relationship in Gigartinales. Our phylogenetic analysis suggested that the Florideophyceae species, except Hildenbrandiophycidae (*Hildenbrandia rivularis*, *Hildenbrandia rubra*, and *Apophlaea sinclairii*), formed a tight group, in which the Florideophyceae taxa were clearly separated into different groups corresponding to their subclass (Nemaliophycidae, Corallinophycidae, Ahnfeltiophycidae and Rhodymeniophycidae). However, three species from the order Gigartinales and *Riquetophycus* sp. from Peyssonneliales clustered as a subbranch in a large branch of the subclass Rhodymeniophycidae, consistent with the findings for *M. papillatus* described in the phylogenetic analysis of Florideophyceae mitogenomes (Sissini et al. 2016). Addition of the newly sequenced specie *K. alvarezii* revealed

that *K. alvarezii* had a closer relationship with *Riquetophycus* sp., whereas *C. crispus* and *M. papillatus* formed another cluster within the Gigartinales order.

In conclusion, to our knowledge, the complete plastid genome of *K. alvarezii* from Gigartinales was determined for the first time in this study. Comparative analysis indicated that a new gene rearrangement was observed in the *K. alvarezii* plastid genome, with the complete reversion of an approximately 12.5 kb gene fragment from the *psaM* to *ycf21* genes, unlike that in other published Florideophyceae plastid genomes. Moreover, inclusion of a new species helped in the reconstruction of the phylogenetic relationship of Florideophyceae species, in which *K. alvarezii* was found to have a closer relationship with *Riquetophycus* sp. from the Peyssonneliales. Additionally, the plastid genome of *K. alvarezii* could be of significance for studies on evolutionary relationship among red algal species.

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