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Multiple Intraspecific Variations of Mitochondrial Genomes in the Green-Tide Forming Alga, *Ulva compressa* Linnaeus (Ulvophyceae, Chlorophyta)

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To gain further insights into intraspecific evolution of *Ulva* mitochondrial genomes, the mitogenomes of three morphotypes of the green-tide forming alga, *Ulva compressa* Linnaeus from China and United States, were sequenced and compared with the available data from Ulvophyceae. The *U. compressa* mitogenomes displayed substantial genome size variation at intraspecific level ranging from 61,700 to > 66,587 bp, due to different acquisitions of foreign DNA fragments, gain or loss of both group I and II introns, and non-coding intergenic spacer regions. The *U. compressa* mitogenomes harbored variable gene content ranging from 69 genes (including *orf*s) in *Uco1* to 76 in *Uco5*, and contained different intron content from 4 introns in *Uco3* and *Uco4* to 7 in *Uco1*. A total of 63 genes and only two group IB introns (intron *cox1*-1107 and *cox1*-1125) were shared by these five mitogenomes. The *U. compressa* mitogenomes accumulated many more inverted repeat (IR) elements, ranging from 45 in *Uco1* to 88 in *Uco2*, than that of the other *Ulva* species (3–34). A locally collinear block of eight genes (*rps11-rps19-rps4-rpl16-trnR-trnQ-trnE-trnS*) with the size of 3,631 bp has been inverted only in *Uco1* indicating that the rearrangement event happened after its divergence from *Uco2–5* and might be related to a specific IR element. The majority of the common genes (˜76%) displayed high identity (>98%) among these five mitogenomes, while some low values were observed in six genes mainly due to duplication and insertion/deletion mutations of small DNA fragments. Our study presented the first case of multiple intraspecific variations in ulvophycean mitogenomes, and indicated that the mitogenome will be a valuable tool for understanding the native or non-indigenous nature of the cosmopolitan *Ulva* species.

Keywords: green algae, mitochondrial genome, intraspecific evolution, genome rearrangement, Ulvophyceae, intron

INTRODUCTION

The class Ulvophyceae is one of the five classes of green algae in the core Chlorophyta (Burger and Nedelcu, 2012; Leliaert et al., 2012; Fuèíková et al., 2014), and contains ten orders and more than 1,900 species thus far (Guiry and Guiry, 2020). Species in Ulvophyceae have developed four main cytomorphological types including non-motile uninucleate unicells, multicellular filaments or blades consisting of uninucleate cells, multicellular thalli composed of multinucleate cells, and coenocytic thalli (Mine et al., 2008; Cocquyt et al., 2010; Leliaert et al., 2012). Phylogenetics is required to develop a comprehensive inventory of the Ulvophyceae to understand the evolutionary history and biodiversity over time. Mitochondrial genomes (mitogenomes) have been useful in phylogenetics, phylogeography and population genetic studies at various taxonomic levels due to the high mutation rates, abundance in cells, and genome-level content (Gray et al., 2001; Hanyuda et al., 2016).

Despite the rich biodiversity and various morphological types in Ulvophyceae, the mitochondrial genomes have been sequenced for only 16 ulvophycean taxa including nine in the order Ulvales (all *Ulva* spp.), three in Ulotrichales, three in Bryopsidales, and one in Oltmannsiellopsidales (**Table 1**). The reported ulvophycean mitogenomes exhibit large variation in genome size, architechtures, gene density, and intron content (Liu et al., 2017). Species in Bryopsidales harbor the inflated mitogenomes ranged from 197,427 bp in *Caulerpa ashmeadii* to 241,739 bp in *Ostreobium quekettii*, due to expansion of non-coding DNA and proliferation of introns (Sauvage et al., 2019; Repetti et al., 2020). Species in Ulotrichales tend to contain large mitogenomes, such as the 105,236-bp *Gloeotilopsis planctonica* and 95,880-bp *Pseudendoclonium akinetum* mitogenomes, which also display characteristics of the 'expanded-derived' patterns of evolution (Pombert et al., 2004; Turmel et al., 2016). The reported *Ulva* mitogenomes ranged from 61,614 bp in *Ulva lactuca* to 73,493 bp in *Ulva* sp. UNA00071828, which were smaller than that of Bryopsidales and Ulotrichales (Melton et al., 2015; Liu et al., 2017; Suzuki et al., 2018). The 56,761-bp mitogenome of *Oltmannsiellopsis viridis* (Oltmannsiellopsidales) is the smallest one reported in Ulvophyceae so far (Pombert et al., 2006).

The reported mitogenomes of nine *Ulva* species were conserved in genome arrangement, gene order and content of core genes, while they displayed intraspecific variation in genome size, due to intron gain or loss, insertion of foreign DNA fragments, and changes of repeat regions (Liu et al., 2017; Liu M. et al., 2020). Two mitogenomes of *Ulva pertusa*, which is currently regarded as a taxonomic synonym of *Ulva australis*, exhibited intraspecific variation of intron content, due to the insertion of two group II introns in the larger one, and the changes of tandem repeats (Liu et al., 2017). Two mitogenomes of the notorious bloom-forming alga, *Ulva prolifera* (Wang et al., 2019), exhibited a difference in genome size, which was mainly caused by an insertion of 1878 bp foreign DNA sequence harboring *trnL(uag)* and two *orfs* (*orf253* and *orf173*) into the *rnl-trnY(gua)* intergenic spacer region (Liu and Pang, 2016). A similar insertion sequence was present in the *Ulva linza* mitogenome, and contained *orf457* representing a fusion of *orf253* and *orf173* (Zhou et al., 2016a,b). Three mitogenomes of *U. lactuca* contain the same number of introns, although the algal thalli were sampled at three greatly distant places (Melton and Lopez-Bautista, 2016; Hughey et al., 2019), while differences in genome size among these three mitogenomes were mainly caused by duplication mutations and insertion/deletion mutations of short DNA sequences (Liu M. et al., 2020).

Ulva compressa Linnaeus has a worldwide geographic distribution in marine and estuarine environments (Guiry and Guiry, 2020). This alga is known for its rapid, proliferous growth in eutrophic conditions, even forming green tides (Blomster et al., 2002; Liu et al., 2013). *U. compressa* usually displays great morphological plasticity due to temperature, salinity, irradiance, wave exposure, and nutrient content (Steinhagen et al., 2019). Previously, two mitochondrial genomes of *U. compressa*, one complete mitogenome (GenBank accession number KX595276) from Southern Yellow Sea, China (Cai et al., 2018a), and another nearly complete mitogenome (MK069586) from China, had been sequenced. To gain further insights into the evolution of *Ulva* mitogenomes, in this study, three morphotypes of *U. compressa* samples were collected from China and the United States, and their complete mitogenomes were sequenced to compare with the available mitogenomes in Ulvophyceae.

MATERIALS AND METHODS

Sample Collection and DNA Extraction

The algal thalli of three morphotypes of *Ulva compressa* Linnaeus were sampled from Swansboro, NC, United States (**Figure 1A**); Huiquan Bay, Qingdao, Shandong, China (**Figure 1B**); and Subei Shoal, Jiangsu, China (**Figure 1C**), respectively (**Supplementary Table S1**). The sample from the United States (*Uco1*) was preserved in silica gel and as a herbarium voucher, which was submitted to University of Alabama Herbarium (UNA00072686). The dried thallus was used to extract DNA with a Qiagen Plant DNA Extraction Kit (QIAGEN, Valencia, CA, United States). Two algal samples from China (*Uco2* and *Uco3*) were kept in coolers (5–8°C) and transported to the laboratory in IOCAS within 48 h after collection. Fresh tissue from one individual algal thallus was used for DNA extraction using a Plant Genomic DNA Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. These three *U. compressa* samples showed different morphological features, which have been noted in this species (**Supplementary Table S1**). Two common DNA markers, the internal transcribed spacer DNA (ITS) region including the 5.8S rDNA gene and plastid-encoded large subunit of the ribulose 1,5-bisphosphate carboxylase gene (*rbc*L) (Hayden and Waaland, 2002), were used for species identification according to the protocol as previously described (Liu et al., 2010). The identification results confirmed that all of these three samples were *U. compressa* (**Supplementary Figures S1, S2**).

Sequencing and Assembly

The concentration and quality of isolated DNA were measured with a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher

Scientific, Waltham, MA, United States). For the sample from the USA, paired-end reads (150 bp) were sequenced at Cold Spring Harbor Laboratory on an Illumina MiSeq platform. For two samples from China, the purified DNA was fragmented into 350 bp and used to construct short-insert libraries. The short fragments were sequenced using an Illumina Hiseq 4000 sequencing platform. The mitogenomes of *U. compressa* were constructed using a combination of *de novo* and reference-guided assemblies. Genome assembly for the sample from the USA was done with both A5 (Tritt et al., 2012) and Geneious R7 (Kearse et al., 2012). For samples from China, the filtered reads were assembled into contigs using SOAPdenovo2.04 (Luo et al., 2012). Incomplete genomes were closed by iteratively mapping the trimmed reads on to the contigs with Geneious 7.1 software[1] (**Supplementary Table S2**).

Genome Annotation

Protein-coding genes (PCGs) were annotated by Open Reading Frame (ORF) Finder[2], DOGMA(Wyman et al., 2004) and ORF finder in Geneious. The ORFs with more than 100 codons were identified in the *U. compressa* mitogenomes. Ribosomal RNA genes (rRNAs) were identified by a BLAST of the non-redundant databases at the National Center for Biotechnology Information (Altschul et al., 1997) and by comparing newly sequenced *U. compressa* mitogenomes with rRNA genes from the other ulvophyceaen mitogenomes. Transfer RNA genes (tRNAs) were searched for by reconstructing their cloverleaf structures using the tRNA scan-SE 1.21 software with default parameters (Schattner et al., 2005).

Intron Analysis

Introns were identified by aligning the homologous genes from 17 *Ulva* mitogenomes. We detected that some introns were not correctly annotated in the reported *Ulva* mitogenomes from GenBank, thus re-annotation of these introns were performed in this study. Intron insertion-sites were determined manually based on the alignments of nucleotide (nt) sequences for the homologous mitochondrial genes and/or amino acid (aa) sequences for PCGs in the mitogenome of *U. compressa* (KY626327) (*Uco3*). Intron name was defined as host gene plus insertion site. The class and core structure of all these introns were determined using the software RNAweasel (Lang et al., 2007) and Mfold (Zuker, 2003). The core domains of intronic *orf*s were determined by significant Pfam-A matches (Punta et al., 2012). In group IIA and IIB introns, the conserved reverse transcriptase (RT) domains with relatively complete structure were searched from the aa sequences of their intronic *orf*s, and 7 group IIB introns were omitted because their RT domains were degenerated or lost. Finally, the aa sequences of RT domains from 27 group IIA and IIB introns were subjected to concatenated alignments using ClustalX 1.83 with the default settings (Thompson et al., 1997). Maximum Likelihood (ML) phylogenetic tree was constructed based on the Jones et al. w/freq.

[1] Biomatters, http://www.geneious.com.

[2] http://www.ncbi.nlm.nih.gov/gorf/orFigurecgi

TABLE 1 | The known 24 mitochondrial genomes from 16 ulvophycean taxa in the class Ulvophyceae.

Order	Species	Accession number	Abbreviation	Size (bp)	References
Ulvales	*Ulva compressa*	MH013469	*Uco1*	61,700	This study
	Ulva compressa	MH093740	*Uco2*	62,791	This study
	Ulva compressa	KY626327	*Uco3*	62,477	This study
	Ulva compressa	KX595276	*Uco4*	62,311	Cai et al., 2018a
	Ulva compressa	MK069586	*Uco5*	>66,587	GenBank
	Ulva lactuca	KU182748	*Ula1*	62,021	Liu M. et al., 2020
	Ulva lactuca	KT364296	*Ula2*	61,614	Melton and Lopez-Bautista, 2016
	Ulva lactuca	MH763013	*Ula3*	>61,125	Hughey et al., 2019
	Ulva australis	KX530816	*Uau1*	69,333	Liu et al., 2017
	Ulva australis	KX530817	*Uau2*	64,602	Liu et al., 2017
	Ulva prolifera	KT428794	*Upr1*	63,845	Liu and Pang, 2016
	Ulva prolifera	KU161104	*Upr2*	61,962	Zhou et al., 2016a
	Ulva linza	KU189740	*Uli*	70,858	Zhou et al., 2016b
	Ulva flexuosa	KX455878	*Ufl*	71,545	Cai et al., 2018b
	Ulva sp.	KP720617	*Usp*	73,493	Melton et al., 2015
	Ulva ohnoi	AP018695	*Uoh*	65,326	Suzuki et al., 2018
	Ulva expansa	MH730971	*Uex*	64,143	Hughey et al., 2018
Ulotrichales	*Tupiella akineta*	AY359242	*Tak*	95,880	Pombert et al., 2004
	Gloeotilopsis planctonica	KX306823	*Gpl*	105,236	Turmel et al., 2016
	Rhexinema sarcinoideum	KX306822	*Rsa*	85,108	Turmel et al., 2016
Bryopsidales	*Caulerpa lentillifera*	KX761577	*Cle*	209,034	Zheng et al., 2018
	Caulerpa ashmeadii	MH745227	*Cas*	197,427	Sauvage et al., 2019
	Ostreobium quekettii	MN514984	*Oqu*	241,739	Repetti et al., 2020
Oltmannsiellopsidales	*Oltmannsiellopsis viridis*	DQ365900	*Ovi*	56,761	Pombert et al., 2006

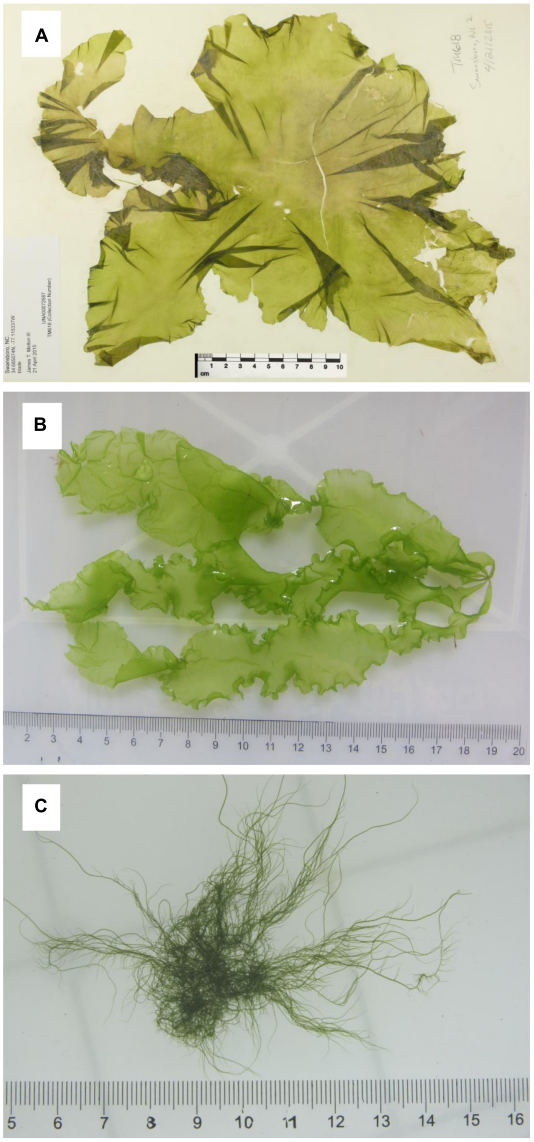

FIGURE 1 | Three morphotypes of *Ulva compressa* collected from China and USA: **(A)** *Uco1* with foliated, distromatic blade, **(B)** *Uco2* with ribbon, distromatic blade and monostromatic, tubular margins, and **(C)** *Uco3* with filamentous, tubular blade, and dense branches.

model (Jones et al., 1992) with 1000 bootstrap replicates using the software MEGA 7.0 (Kumar et al., 2016). Initial trees for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using a JTT model. All positions containing gaps and missing data were eliminated. There were a total of 123 positions in the final dataset.

Comparative Genomic and Phylogenomic Analyses

Base composition of the 17 *Ulva* mitogenomes was determined using MEGA 7.0 (Kumar et al., 2016). Tandem repeats (TRs) were found with Tandem Repeats Finder using the default settings (Benson, 1999). Inverted repeats (IRs) were identified with Inverted Repeats Finder using the default settings and the additional constraint that repeats had to be > 75% similar[3]. A total of 61 genes including 29 core PCGs, 2 rRNAs, 27 tRNAs and 3 conserved free-standing *orf*s (cfORFs) were shared among the 17 *Ulva* mitogenomes. Differences and identity values of these gene sequences were calculated by use of the BioEdit v7.1.9 software (Hall, 1999). The nt sequences of 17 mitogenomes were subjected to concatenated alignments using ClustalX 1.83 with the default settings (Thompson et al., 1997). The evolutionary history was inferred by using the Maximum Likelihood (ML) method based on the Tamura-Nei model (Tamura and Nei, 1993). Initial trees for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. There were a total of 142,736 positions in the final dataset. Phylogenetic analyses were conducted with 1,000 bootstrap replicates using MEGA 7.0 (Kumar et al., 2016).

RESULTS AND DISCUSSION

Comparison of Genome Features in *U. compressa* Mitogenomes

The three newly sequenced mitogenomes of *U. compressa* ranged from 61,700 bp in *Uco1* to 62,791 bp in *Uco3*, which were similar to that of *Uco4* in size, but were shorter than that of *Uco5* (>66,587 bp) (**Table 1**). The variation of *U. compressa* mitogenome size was mainly caused by differences in the content of genome-specific free-standing *orf*s (sfORFs), both group I and II introns, and the non-coding intergenic spacer regions (**Figure 2**). The content of non-coding spacer in *U. compressa* mitogenomes was 22.29–26.93% (**Figure 2**), which was within the known range for *Ulva* mitogenomes (20.51–32.94%), but was lower than the mitogenomes of Bryopsidales (34.8–46.0%) (Zheng et al., 2018; Repetti et al., 2020), Ulotrichales (43.3–49.0%) (Pombert et al., 2004; Turmel et al., 2016), and Oltmannsiellopsidales (34.1%) (Pombert et al., 2006), displaying the most compact architectures among the Ulvophyceae so far.

The overall A + T content in the United States sample (*Uco1*) was 61.96%, which was the lowest value recorded in *Ulva* mitogenomes sequenced so far (Liu et al., 2017). For each *U. compressa* mitogenome, the non-coding intergenic spacer regions was mostly richer in A + T content, while the A + T content in introns was much lower than that of the mitogenome. The non-coding intergenic regions tends to become richer in A + T content, as was observed in the vast majority of the mitogenomes (e.g., Joardar et al., 2012; Liu and Pang, 2015). The A + T contents in core protein-coding genes (PCGs), rRNA genes (rRNAs), tRNA genes (tRNAs) and conserved free-standing *orf*s (cfORFs) maintained relatively stable values among these five *U. compressa* mitogenomes, while the intron A + T content

[3]http://tandem.bu.edu/cgi-bin/irdb/irdb.exe

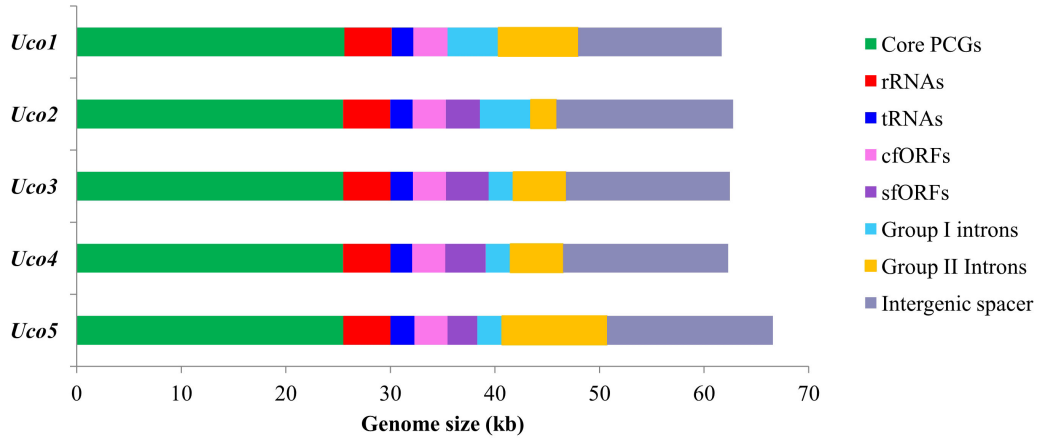

FIGURE 2 | Contributions of 29 core PCGs, rRNA genes (rRNAs), tRNA genes (tRNAs), conserved free-standing *orf*s (cfORFs), genome-specific free-standing *orf*s (sfORFs), group I, and II introns, and non-coding intergenic spacer regions in five *U. compressa* mitogenomes. Horizontal bars represent the size of mitogenomes.

TABLE 2 | Comparison of genome features in the five mitochondrial genomes of *Ulva compressa* (*Uco1 – 5*).

Genome features	***Uco1***	***Uco2***	***Uco3***	***Uco4***	***Uco5***
Collection sites	**Swansboro, North Carolina, Uited States**	**Qingdao, Shandong, China**	**Subei Shoal, Jiangsu, China**	**Southern Yellow Sea, Jiangsu, China**	**China**
Overall A + T content (%)	61.96	63.52	63.03	63.08	62.27
PCG A + T content (%)	62.94	63.08	63.00	62.98	62.99
rRNA A + T content (%)	61.00	61.08	61.05	61.01	61.05
tRNA A + T content (%)	57.48	57.46	57.36	57.25	58.04
cfORF A + T content (%)*	63.57	64.14	64.11	64.05	64.11
sfORF A + T content (%)**	–	64.61	66.40	66.87	66.88
Intron A + T content (%)	59.06	61.45	56.90	56.87	55.63
Spacer A + T content (%)	63.39	66.18	66.24	66.39	66.15
Spacer content (%)	22.29	26.93	25.11	25.36	23.83
Intron content (%)	20.24	11.66	11.82	11.85	18.65
Introns	7	5	4	4	6
Group I introns	4	4	2	2	2
Group II introns	3	1	2	2	4
Genes	69	73	73	72	76
Core PCGs	29	29	29	29	29
Core tRNA genes	28	28	28	27	28
Specific tRNA genes	0	1	1	1	3
rRNA genes	2	2	2	2	2
Conserved free-standing *orfs*	3	3	3	3	3
Specific free-standing *orfs*	0	5	6	6	5
Intronic *orfs*	7	5	4	4	6

**The cfORF A + T content (%) represented the A + T content of three conserved free-standing orfs. **The sfORF A + T content (%) represented the A + T content of the genome-specific free-standing orfs.*

showed variations ranging from 55.63% in *Uco5* to 61.45% in *Uco2* (**Table 2**).

Variation of Gene Content in *U. compressa* Mitogenomes

The five mitogenomes of *U. compressa* harbored variable gene content ranging from 69 genes (including *orf*s) in *Uco1* to 76 in *Uco5* (**Table 2**). The differences were mainly due to the acquisition of foreign DNA fragments, which carried different numbers of genome-specific tRNAs and *orf*s, and gain or loss of group I and II introns which usually harbored one intronic *orf*. The five mitogenomes of *U. compressa* shared a total of 63 genes including 29 core PCGs, two rRNAs, 27 tRNAs, three cfORFs (**Figure 3**) and two intronic *orf*s situated in two group IB introns (i.e. intron *cox1*-1107 and *cox1*-1125) (**Table 3**). The 29 PCGs including five ATPase subunits (*atp1*, *4*, *6*, *8* and

9), apocytochrome b (*cob*), three cytochrome oxidase subunits (*cox1-3*), eight NADH dehydrogenase complex subunits (*nad1-7*, and *4L*), and 12 ribosomal proteins (*rpl5*, *14*, and *16*; *rps2-4*, *10-14*, and *19*) were conserved in the *Ulva* mitogenomes. The three cfORFs including *orf541*/*511*, *orf316* and *orf227* were present in all sequenced *Ulva* mitogenomes, but their function is unknown based on the BLASTP searches in public databases of NCBI.

The *trnI(uau)* between *cox3* and *rpl14* was absent in *Uco4*, but was present in the other four *U. compressa* mitogenomes (**Figure 3**), suggesting that this tRNA gene was subject to a recent loss or transfer in *Uco4*. Previously, we found that its counterpart was missing from two *U. australis* mitogenomes (Liu et al., 2017), but present in the other sequenced *Ulva* mitogenomes. This evidence indicates that *trnI(uau)* experienced at least two independent losses in the *Ulva* lineage. Interestingly, this tRNA gene displayed a conserved structure in DHU and TΨC stem-loops, but exhibited variable nucleotide sequences in the anticodon stem-loop. Instead of *trnI* with the anticodon TAT in *U. compressa*, it was predicted as *trnK* with TTT in *U. australis*, *U. flexuosa* and *U. ohnoi*, *trnP* with TGG in *U. prolifera* and *U. linza*, *trnV* with TAC in *U. expansa*, and *trnX* with TTTT in *Ulva* sp. (**Figure 4**). Although unusual, such a variation or modification of tRNA genes has been observed before in various organisms. For example, the tRNA gene located between *nad4* and *nad5* in brown algae displayed a variable anticodon among different species, which might play a new role instead of old function (Liu and Pang, 2015).

Among the five *U. compressa* mitogenomes, a few genome-specific tRNAs and free-standing *orf*s (more than 100 codons) were observed in three intergenic regions, e.g., *cox1-trnM1*, *trnA1-trnR1,* and *cox3-trnI2*. The *orf114* with unknown function was located in the intergenic region of *cox1-trnM1* only in *Uco2*, while a DNA fragment carrying two pseudo tRNA genes (*trnX1* and *trnX2*) and *orf121* was present in the intergenic region of *cox3-trnI2* only in *Uco5* (**Figure 3**). Considering these two DNA fragments were absent in other *Ulva* mitogenome, it makes sense that two independent integration events of foreign DNA fragments happened in *Uco2* and *Uco5*, respectively. Mitochondrial genomes have been observed to be able to accept foreign DNA fragments, which usually carry some specific tRNA genes and/or *orf*s, from diverse sources including chloroplast and nuclear sequences, bacteria, plasmid, virus, and jumping DNA/RNA-protein complex (e.g., Seif et al., 2005; Wang et al., 2007; Darracq et al., 2011; Apitz et al., 2013; Gandini and Sanchez-Puerta, 2017). However, the source of these foreign fragments observed in mitogenomes of *U. compressa* is still unknown due to little information on the possible function of these *orf*s. The insertion of large foreign DNA sequences could increase the size of mitochondrial genomes. The mitogenomes of *Silene vulgaris* (angiosperms) ranged in size from 361 to 429 kb and the unprecedented amounts of intraspecific diversity were due to frequent acquisition and rapid turnover of foreign sequences (Sloan et al., 2012).

It is worth noting that four samples from China (*Uco2–5*) shared a specific DNA fragment, which harbored 4–6 *orfs* and one tRNA gene (*trnA2*), in the intergenic region of *trnA1-trnR1*. Of these sfORFs, three have sequence similarity to the DNA-directed RNA polymerase (*rpo*), while the others had little sequence similarity to any PCGs in the GenBank database (**Figure 3**). Based on the comparative sequence analysis, it was shown that *Uco3* has a more complete DNA fragment in this region, which contains a collinear block of seven genes (*orf211-orf121-orf413-trnA2-orf203-orf182-orf229*) compared with *Uco2*, *Uco4* and *Uco5*. Subsequent insertion/deletion events have occurred in *Uco2* and *4*, which caused frameshift mutations and even gene loss in this region. However, this DNA fragment was absent in the United States sample *Uco1* as well as the mitogenomes of the other *Ulva* species, indicating that the acquisition of this foreign fragment occurred in the common progenitor of *Uco2-5* after its divergence from *Uco1*, as was consistent with the result of phylogenomic analysis (see a section below).

Variable Intron Content and Intronic *orf*s in *U. compressa* Mitogenomes

The *U. compressa* mitogenomes contained different intron content ranging from 4 introns in *Uco3* and *Uco4* to 7 in *Uco1* (**Table 3**). These introns totally occupied 11.66% of the mitogenome in *Uco2* to 20.24% in *Uco1* (**Table 2**), suggesting that variation in intron content greatly caused changes in mitogenome size at the intraspecific level in *U. compressa*. Considering the previous similar findings in *U. australis* (Liu et al., 2017) and *U. linza-prolifera* (LP clade) (Liu and Pang, 2016; Zhou et al., 2016a,b), intraspecific variation of intron content probably occurs widely in *Ulva* species. Nine distinct insertion sites in four genes (*cox1*, *cox2*, *nad3* and *rnl*) were detected among these five *U. compressa* mitogenomes. Comparative analysis of intron insertion-sites in *U. compressa* mitogenomes with that in other *Ulva* species indicated that *cox1* tended to contain more introns, followed by *rnl* and *rns*, which was similar to that observed in other green algae, plants, Ochrophyta and fungi (e.g., Joardar et al., 2012; Sloan et al., 2012; Liu F. et al., 2020). However, group I introns prevailed in *cox1* and *rnl* in *Ulva* species, as was significantly different from that observed in Ochrophyta (e.g., diatom) which harbored almost all group II introns (Liu F. et al., 2020).

Each intron in these five *U. compressa* mitogenomes contained one intron-encoded protein (*orf* with over 100 codons), which was believed to participate in intron propagation and/or intron splicing (maturases) (Dai et al., 2003; Haugen et al., 2005). All of the group I introns in *U. compressa* mitogenomes belonged to group IB and harbored an intronic *orf* which encoded a putative LAGLIDADG homing endonuclease (LHE), while all of the *orf*s in group IIA and IIB introns encoded putative reverse transcriptase/maturases (RTMs) in *U. compressa*. We found that these RTMs encoded by group IIA and IIB introns in *Ulva* species fall into two different lineages, group IIA and group IIB (**Figure 5**), which were observed to coevolve with the group II intron RNA structure (ribozyme components) (Fontaine et al., 1997; Toor et al., 2001; Zimmerly et al., 2001). However, a few of group II introns (i.e., intron *cox1*-874, *rnl*-2080, *rnl*-2698, *rns*-420 and *rns*-670) which harbored the homing endonucleases assigned to LAGLIDADG family (Liu et al., 2017), and a group ID intron

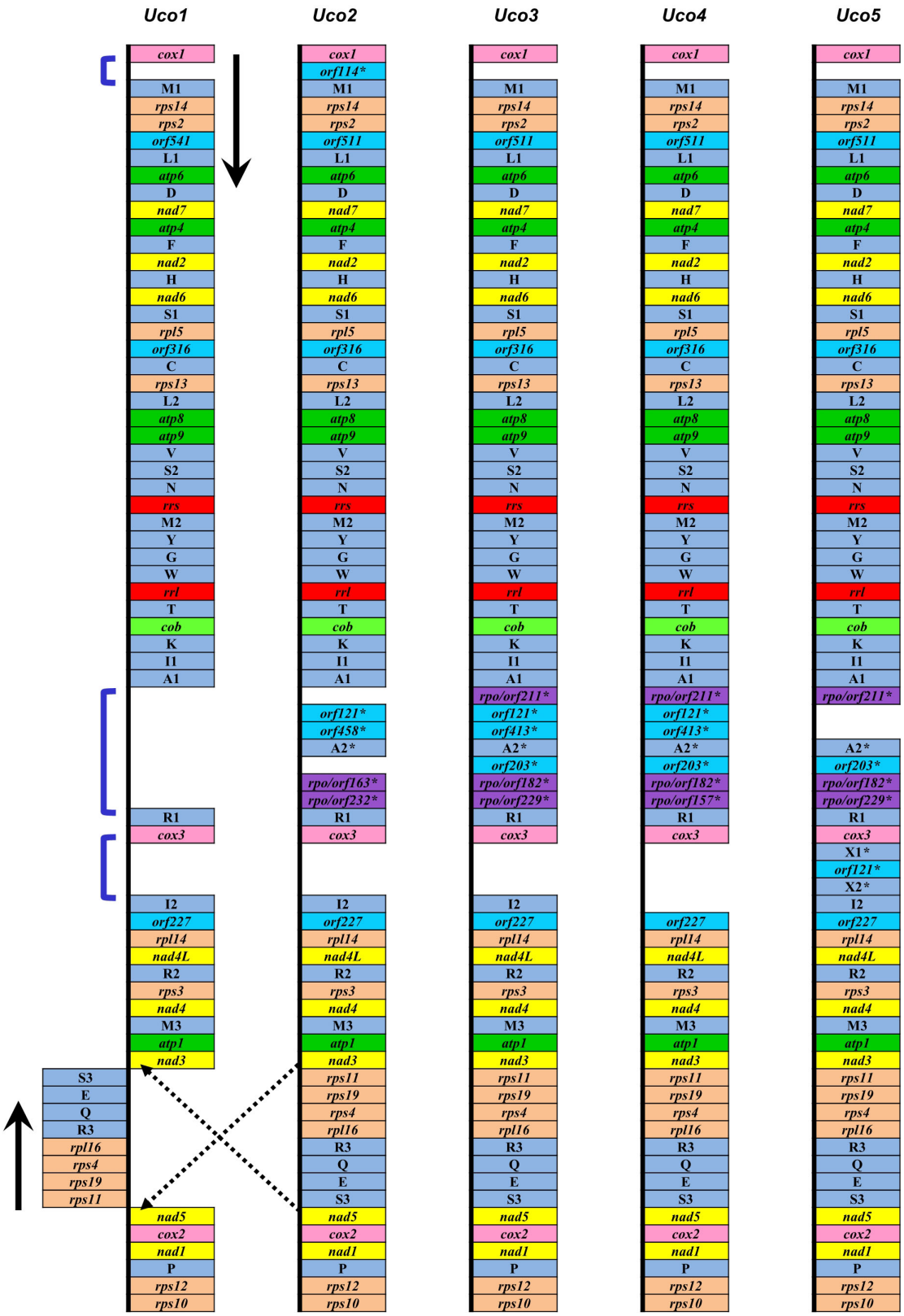

FIGURE 3 | Comparison of genome organization and gene order of five *U. compressa* mitogenomes. The dark blue brackets showed three intergenic regions with the genome-specific tRNAs and *orf*s, which were indicated by an asterisk (*).

TABLE 3 | Comparison of insertion site, size and group of introns in the 17 *Ulva* mitogenomes.

Introns*	*Uco1*	*Uco2*	*Uco3*	*Uco4*	*Uco5*	*Ula1*	*Ula2*	*Ula3*	*Uau1*	*Uau2*	*Upr1*	*Upr2*	*Uli*	*Ufl*	*Usp*	*Uoh*	*Uex*
atp1-540									1,157	1,157					1,345		1,303
atp1-990																2,589	
cox1-199					2,501								2,525				
cox1-281						1,401	1,401	1,401			1,408	1,408	1,406				
cox1-386	1,446	1,370				1,249	1,248	1,249	1,270	1,269	1,277	1,276	1,277	1,293	1,287	1,269	
cox1-643	2,508																
cox1-709											1,434	1,433	1,434	1,594	1,590		
cox1-734														1,125			
cox1-760									2,604								
cox1-874											1,509	1,509	1,509	1,591			1,157
cox1-1107	1,142	1,195	1,188	1,188	1,212	1,109	1,109	1,109	1,129	1,129			1,156	1,128	1,162	1,127	1,157
cox1-1125	1,147	1,148	1,156	1,156	1,156						1,117	1,117	1,118	1,139	1,240	1,134	1,170
cox2-424	2,561		2,581	2,581	2,581				2,463							2,539	
cox2-751		2,459	2,460	2,460	2,460				2,380	2,380							
nad3-215															2,727		
nad3-216	2,542				2,507	2,481	2,481	2,481			2,503	2,503	2,516			2,498	
nad5-800											1,227	1,227	1,232		1,309		1,180
rnl-1963									2,364	2,364							1,681
rnl-2080														1,550			1,546
rnl-2698													1,393	1,380			
rnl-2747	1,139	1,149									1,202	1,202	1,250	1,198			566
rns-420													1,402	1,368	1,319		619
rns-670											1,098	1,098	1,481	1,004	1,534		623
rns-780									2,415	2,415					2,449	1,028	

**Intron insertion-sites were determined by comparing homologous genes relative to the mitogenome of U. compressa (KY626327) (Uco3). Intron name was defined as host gene plus insertion site. Different colored boxes denoted different introns: group IB (complete), blue; group ID, green; group IIA, red; group IIB, orange; and group II (LHE), pink.*

Species	Accession number	tRNA	Acceptor stem		DHU				Anticodon				TΨC			Acceptor stem
					stem	loop	stem		stem	loop	stem		stem	loop	stem	
Uco1	MH013469	*trnI*	AGAAAA	TG	ATGT	AATGGC-A	ACAT	G	CCCGC	TTTATGA	GCTGG	ATAT	GTGGG	TTCAAAT	CCTAC	TTTTCT
Uco2	MH093740	*trnI*	AGAAAA	TG	ATGT	AATGGC-A	ACAT	G	CCCGC	TTTATGA	GCTGG	ATAT	GTGGG	TTCAAAT	CCTAC	TTTTCT
Uco3	KY626327	*trnI*	AGAAAA	TG	ATGT	AATGGC-A	ACAT	G	CCCGC	TTTATGA	GCTGG	ATAT	GTGGG	TTCAAAT	CCTAC	TTTTCT
Uco5	MK069586	*trnI*	AGAAAA	TG	ATGT	AATGGC-A	ACAT	G	CCCGC	TTTATGA	GCTGG	ATAT	GTGGG	TTCAAAT	CCTAC	TTTTCT
Ula1	KU182748	*trnK*	AGAAAA	TG	ATGT	AATGGCTA	ACAT	GC	CCG	CTTTTTAGC	TGG	ATAT	GTGGG	TTCAAGT	CCTAC	TTTTCT
Ula2	KT364296	*trnK*	AGAAAA	TG	ATGT	AATGGCTA	ACAT	GC	CCG	CTTTTTAGC	TGG	ATAT	GTGGG	TTCAAGT	CCTAC	TTTTCT
Ula3	MH763013	*trnK*	AGAAAA	TG	ATGT	AATGGCTA	ACAT	GC	CCG	CTTTTTAGC	TGG	ATAT	GTGGG	TTCAAGT	CCTAC	TTTTCT
Upr1	KT428794	*trnP*	AGAAAA	TA	ATGT	AATAGGTA	ACAT	G	CCAGC	TTTGGGT	GCTGG	ATAT	GTGGG	TTCAAGT	CCTAC	TTTTCT
Upr2	KU161104	*trnP*	AGAAAA	TA	ATGT	AATAGGTA	ACAT	G	CCAGC	TTTGGGT	GCTGG	ATAT	GTGGG	TTCAAGT	CCTAC	TTTTCT
Uli	KU189740	*trnP*	AGAAAA	TA	ATGT	AATAGGTA	ACAT	G	CCAGC	TTTGGGT	GCTGG	ATAT	GTGGG	TTCAAGT	CCTAC	TTTTCT
Ufl	KX455878	*trnK*	AGAAAA	TG	ATGT	AATGGCTA	ACAT	G	CCCG	CTTTTAG	CTGG	ATAT	GTGGG	TTCAAGT	CCTAC	TTTTCT
Usp	KP720617	*trnX*	AGAAAA	TG	ATGT	AATGGCTA	ACAT	G	CCTGC	TTTTTA	GCTGG	ATAT	GTAGG	TTCAAGT	CCTAC	TTTTCT
Uoh	AP018695	*trnK*	AGAAAA	TG	ATGT	AATGGCTA	ACAT	GC	CCG	CTTTTTAGC	TGG	ATAT	GTGGG	TTCAAGT	CCTAC	TTTTCT
Uex	MH730971	*trnV*	AGAAAA	TG	ATGT	AATGGCTA	ACAT	G	CCCGC	TTTACAA	GCTGG	ATAT	GTGGG	TTCAAAT	CCTAC	TTTTCT

FIGURE 4 | The aligned sequences of tRNA genes located between *cox3* and *rpl14* in the 14 *Ulva* mitogenomes.

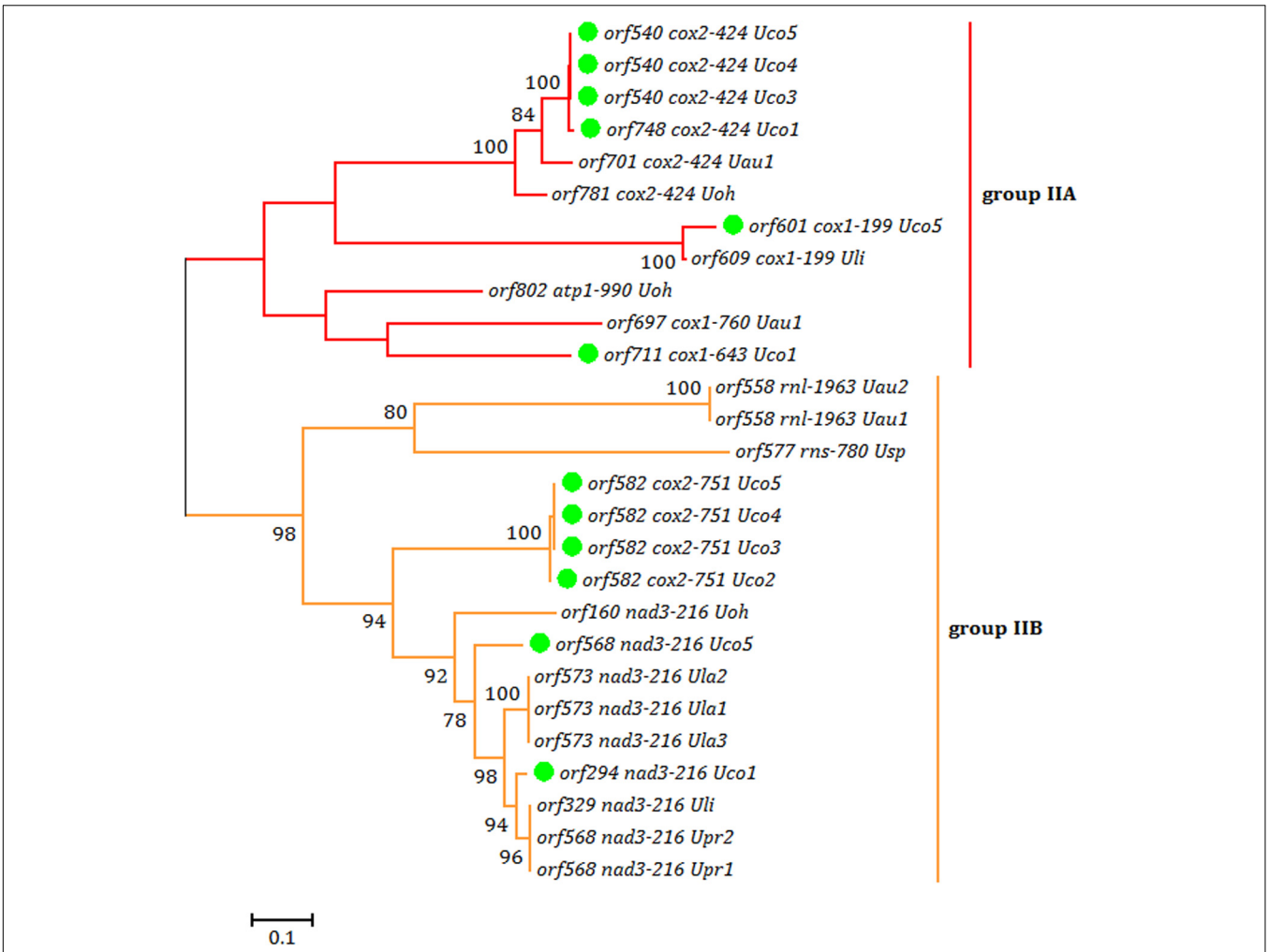

FIGURE 5 | Phylogenetic tree based on Maximum Likelihood (ML) analysis of amino acid (aa) sequences of 27 reverse transcriptase (RT) domains from intronic *orf*s in group IIA and IIB introns. The ML analysis was conducted with 1,000 bootstrap replicates using MEGA 7.0. Branch lengths were proportional to the amount of sequence change, which were indicated by the scale bar below the trees.

(i.e., intron *cox1*-709) which contained a putative LHE, were absent in these five mitogenomes of *U. compressa* (**Table 3**).

It is notable that none of the introns was shared by all of the sequenced *Ulva* mitogenomes, suggesting that both group I and II introns were mobile genetic elements transferred across species (Dai et al., 2003; Haugen et al., 2005) and had the ability to invade specific target sites within some genes (Lang et al., 2007). Only two group IB introns in *cox1* (i.e. intron *cox1*-1107 and *cox1*-1125) were shared by these five *U. compressa* mitogenomes (**Table 3**). These two group IB introns were common in most of *Ulva* species as well as species in Ulotrichales (Turmel et al., 2016). It seems more reasonable that these two group IB introns were inherited vertically from a common ancestor in the Ulvales/Ulotrichales lineage, and had experienced at least one or two independent losses in the *Ulva* lineage. Two group II introns in *cox2* (i.e. intron *cox2*-424 and *cox2*-751) were present in four out of five *U. compressa* mitogenomes, but they belong to group IIA and IIB introns, respectively. Although these two group II introns were also present in one or two other *Ulva* species, they were more likely to be from multiple horizontal transfers to these specific insertion sites in the *Ulva* lineage (Turmel et al., 2016; Liu et al., 2017). Remarkably, intron *cox1*-643 (IIA) which contained a complete RTM protein was specific to *Uco1*, and absent in *Uco2-5* and other *Ulva* species, indicating this group IIA intron should be the result of a recent independent invasion event.

Both group I and II introns from the same insertion site were homologous among the *Ulva* mitogenomes, indicating that cognate introns should have descended from a common progenitor (Ikuta et al., 2008). For the cognate introns of both group I and II, the RNA secondary structures of ribozyme components were highly conserved, while the intronic *orf* (LHE or RTM) might have been degenerated and even completely lost in some introns (**Table 3**). We observed that a total of 20 introns (˜16.5%) in the 17 mitogenomes displayed degenerate *orf*s or no informative *orf* due to deletion/insertion and

frameshift mutations. The *U. expansa* mitogenome harbored more degenerate introns than the other *Ulva* mitogenomes, of which some (e.g., intron *rnl*-2747, *rns*-420, and *rns*-670) completely lost the intronic *orf*s (i.e., LHEs). These degenerate introns have probably lost mobility competence, but they must retain splicing competence because they are located in conserved genes (Robart and Zimmerly, 2005).

Repeat Sequences and Intraspecific Genome Rearrangement

Numerous repeat sequences including tandem repeats (TRs) and inverted repeats (IRs) were distributed in the five mitogenomes of *U. compressa*. Most of these repeat sequences were restricted to the intergenic or intronic non-coding regions. The AT-rich TR, which was previously identified in the *nad6-trnS(gcu)* intergenic region among *Ulva* mitogenomes (Liu and Pang, 2016; Liu et al., 2017), showed differences in periodic sequence and copy number not only at interspecific level, but also at intraspecific level. This TR exhibited four types of periodic sequence × copy number in *U. compressa*, e.g. 16 bp × 5 in *Uco1*, 14 bp × 16.7 in *Uco2*, 4 bp × 71.8 in *Uco3* and *4*, and 44 bp × 4.4 in *Uco5*. Comparative analysis indicated that *U. compressa* mitogenomes contained many more IRs, ranging from 45 in *Uco1* to 88 in *Uco2*, than that of the other *Ulva* species (3 - 34).

All of the genes annotated in *Uco2-5* were coded on the same strand and shared a high level of conservation in gene synteny, as was the same as that in the other *Ulva* mitogenomes (Melton et al., 2015; Liu et al., 2017), but was different from the mitogenomes of Ulotrichales, Bryopsidales and Oltmannsiellopsidales which had their genes distributed on the two strands (Pombert et al., 2006; Turmel et al., 2016; Repetti et al., 2020). However, a locally collinear block of eight genes (*rps11-rps19-rps4-rpl16-trnR-trnQ-trnE-trnS*) with the size of 3,631 bp has been inverted only in *Uco1* (**Figure 6**), indicating that this rearrangement event happened after its divergence from *Uco2-5*.

Interestingly, a GC-rich (61.26%) IR element was located in the boundary of this inverted gene block in *Uco1*. The copies of this specific IR (left vs right: 150 vs 152 bp) differed by 1.97% mismatches and 1.32% indels, and its loop region was 3.7 kb in size carrying the eight genes (**Figure 6**). The absence of this IR from mitogenomes of *Uco2-5* as well as the other *Ulva* mitogenomes not only eliminated the possibility that the IR was lost from the mitogenomes of *Uco2-5*, but also indicated that the IR appeared in *Uco1* after its divergence from *Uco2-5*. IRs are common elements widespread in most of organelle genomes as well as the genomes of eukaryotes and prokaryotes. These elements could mediate genome rearrangement through excision of the repeat associated regions (Bi and Liu, 1996). IR sequences capable of forming stem-loop structures are the substrates for intragenomic recombination (Lin et al., 2001; Alverson et al., 2011). The mitogenomes of animals, fungi, plants and green algae are thought to undergo genome rearrangements mediated by IRs or stem-and-loop structures. The rearrangement event observed in *Uco1* was probably related to the appearance of this specific IR element.

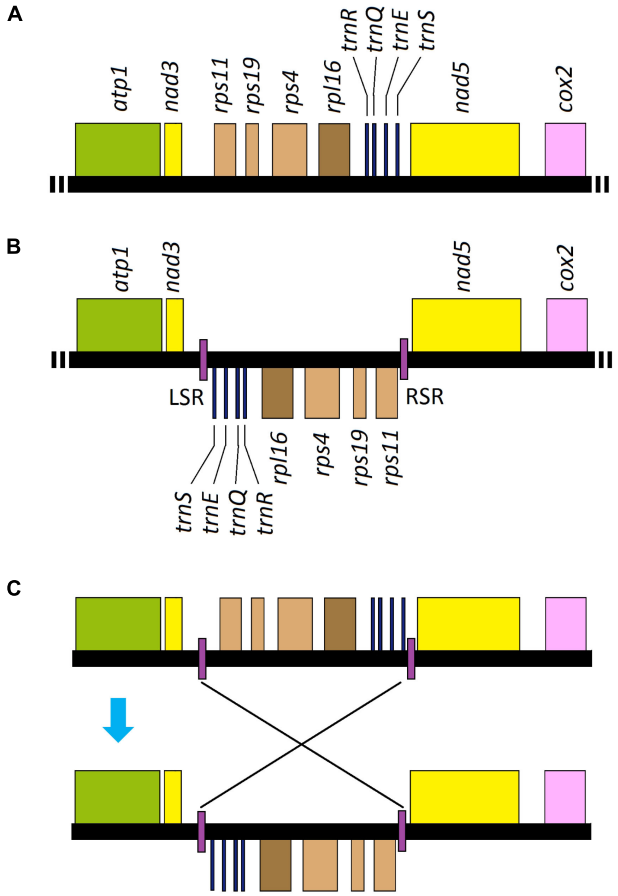

FIGURE 6 | Intraspecific genome rearrangement of *U. compressa* mitogenome: **(A)** gene order from *nad3* to *nad5* in mitogenomes of *Uco2-5* as well as the other *Ulva* species; **(B)** gene order from *nad3* to *nad5* in mitogenome of *Uco1* with the specific inverted repeat (IR), and its left stem region (LSR) and right stem region (RSR) were located in two boundaries of the inverted eight gene block, respectively, and **(C)** hypothesis of the rearrangement event related to the specific IR element in *Uco1*.

Intraspecific Gene Mutations and Phylogenomic Analysis

The majority of the common genes (76%) including 24 core PCGs, 2 rRNAs, 26tRNAs and 2 cfORFs displayed high identity values (> 98%) among these five mitogenomes of *U. compressa* (**Supplementary Table S3**), while some low values were observed in six genes (*nad3*, *rps2*, *rps3*, *rps4*, *rps14* and *orf541*/*511*) mainly due to duplication mutations and insertion/deletion mutations of small DNA fragments at the intraspecific level. A 15-bp deletion mutation occurred at position 217 of *nad3* in *Uco1*, not in *Uco2-5*. Interestingly, an identical sequence with a 27-bp hairpin structure was inserted three times into *rps2* of *Uco1* (position 756, 1662 and 1920), and twice into *rps3* of *Uco1* (position 375 and 406), and was not detected in the other four mitogenomes of *U. compressa*. The *rps4* in *Uco4* was shorter due to a C-insertion mutation at position 447 which caused the early appearance of stop codon. A duplication mutation of GCCCAAAGGCAAGGG happened

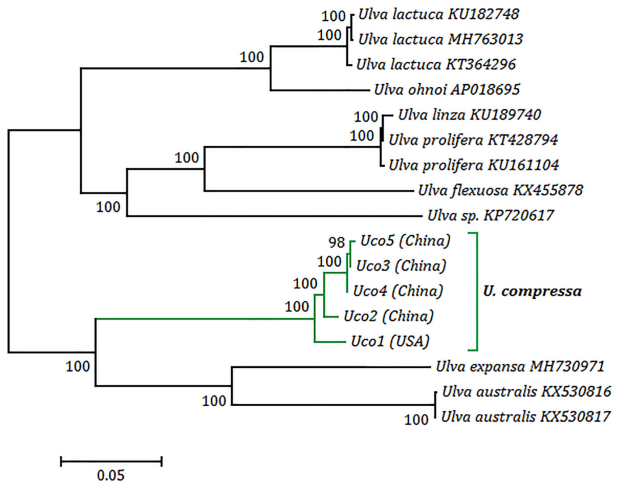

FIGURE 7 | Phylogenomic tree based on Maximum Likelihood (ML) analysis of nucleotide sequences of 17 *Ulva* mitogenomes. The ML analysis was conducted with 1,000 bootstrap replicates using MEGA 7.0. Branch lengths were proportional to the amount of sequence change, which were indicated by the scale bar below the trees.

TABLE 4 | Differences (bp, upper-right) and identity values (%, bottom-left) of the aligned nucleotide sequences of 61 genes included 29 core PCGs, 2 rRNAs, 27 tRNAs and 3 cfORFs in the five mitogenomes of *U. compressa*.

	Uco1	*Uco2*	*Uco3*	*Uco4*	*Uco5*
Uco1	–	468*	411	620	430
Uco2	98.6**	–	225	434	242
Uco3	98.8	99.3	–	209	19
Uco4	98.2	98.7	99.4	–	228
Uco5	98.7	99.3	99.9	99.3	-

**The number of alignment positions where two sequences differ. **The percentage of overlapping alignment positions where two sequences agree.*

at position 452 of *rps14* in *Uco1* and *3-5*, but not in *Uco2*. Two insertion sequences with 51-bp and 39-bp hairpin structures were observed at position 587 and 677 of *orf541* only in *Uco1*, which caused it to be longer than its counterparts (*orf511*) in the other four mitogenomes of *U. compressa*.

Phylogenomic analysis revealed that five samples of *U. compressa* formed a highly supported *U. compressa* clade (100% bootstrap) (**Figure 7**). Two samples from China (*Uco3* and *Uco5*) closely grouped together with *Uco4* from Southern Yellow Sea China, forming a subclade representing the filamentous, highly branched morphotype. This morphotype is common in Subei Shoal, Jiangsu, China and has partly contributed to the annual large-scale green tides in the Yellow Sea (Liu et al., 2013; Cai et al., 2018a). These three samples clustered more closely with *Uco2* from Qingdao, Shandong, China than *Uco1* from Swansboro, North Carolina, United States. The former is a ribbon, blade-like morphotype which was common in Qingdao coasts, China, but not found in the Subei Shoal, while the latter is a foliated, free-floating morphotype from the USA. The phylogenetic relationships among these five mitogenomes were consistent with the observed genome variations including integration of foreign DNA fragments, genome rearrangement, and gene mutations.

Although these samples of *U. compressa* exhibited diverse morphotypes, we found that they shared highly identical sequences of mitochondrial genes and other barcoding markers (ITS and *rbc*L). Based on a comparative analysis of the aligned nucleotide sequences of 61 mitochondrial genes included 29 core PCGs, 2 rRNAs, 27 tRNAs and 3 cfORFs, the sequence identity values ranged from 98.2% (different in 620 bp) in a pair of *Uco1*/*4* to 99.9% in *Uco3*/*5* (different in 19 bp) among these five *U. compressa* samples (**Table 4**). It is worth noting that based on comparative mitogenome information among these five samples of *U. compressa*, new DNA markers could be designed to study the population diversity and phylogeography of the green-tide forming alga (Steinhagen et al., 2019). Considering the marked intraspecific variations in genome size, intron content, genome architecture and gene mutations in *U. compressa*, the mitogenome will be a valuable tool to help us understand the native or non-indigenous nature of the cosmopolitan *Ulva* species.

CONCLUSION

Although the sequenced mitogenomes from nine *Ulva* species showed some conserved features in core gene content and gene order, we still know little on the evolution of *Ulva* mitochondrial genomes at the intraspecific level. In this study, the mitogenomes of *U. compressa* displayed marked multiple intraspecific variations in genome size, gene content, intron content and genome architecture. This was mainly due to insertion of foreign DNA fragments, frequent gain or loss of both group I and II introns, enrichment of repeat sequences, and rearrangement by inversion of a syntenic block with eight collinear genes. Our study not only presented the first case of multiple intraspecific variations in ulvophycean mitogenomes, but also indicated that the mitogenome will be a valuable tool for understanding the native or non-indigenous nature of the cosmopolitan *Ulva* species. These results will enhance our understanding of the evolution of mitochondrial genomes in green algae.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

FL and JM designed the study. FL, JM, and JL-B performed the experiments. FL, JM, and NC performed the analysis. FL wrote

the manuscript. All authors have read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2020.00714/full#supplementary-material

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