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Construction of genomic marker sets based on the chloroplast genome of a green alga, *Ulva pertusa* (syn. *Ulva australis*), leads to simple detection of *Ulva* species

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In closed sea areas such as Tokyo Bay, a phenomenon known as a green tide often occurs, in which large amounts of *Ulva* seaweed grow abnormally and form mats along the coast. This is currently a serious environmental problem. Green tides are generated by the explosive growth of multiple types of *Ulva* algae. However, many *Ulva* species show similar characteristics to each other and are indistinguishable by appearance, making it difficult to identify the *Ulva* algae in green tides. In this study, we determined the entire nucleotide sequence of the chloroplast genome of *Ulva pertusa* (syn. *Ulva australis*) and identified two large inversions of gene order, suggesting the occurrence of genome inversions. We also detected structural polymorphisms among *Ulva* chloroplast genomes. *Ulva pertusa* was classified in a different clade from that containing *U. lactuca* and *U. ohnoi*, suggesting that *U. pertusa* is evolutionarily divergent from these species. Based on this knowledge, we constructed a genetic diagnosis system for *Ulva* algae. Using this approach, we established a simple method that can determine the species of *Ulva* algae by PCR using specific molecular markers, through which representative *Ulva* species such as *U. lactuca*, *U. ohnoi* and *U. pertusa* were easily distinguished.

Key words: chloroplast genome, complete nucleotide sequence, genotyping marker, polymorphic DNA, species identification

INTRODUCTION

The genus *Ulva* (Ulvophyceae, Chlorophyta), consisting of common green macroalgae that often form algal beds, is found in intertidal zones in bays. Many kinds of *Ulva* species are important primary producers that support the ecosystem in bay areas (Zertuche-González et al., 2009). Although most algal species in the genus *Ulva* exhibit a simple multicellular organization, their morphological and cytological properties are highly divergent. *Ulva* presents flat lettuce-like blades with two cell layers, while *Enteromorpha* produces monostromatic tubular thalli. Therefore, these algae were formerly classified as different genera because of differences in their morphological characteristics. However, they are currently considered to belong to the same genus based on their genetic proximity, such as the similarity of their nuclear-encoded internal transcribed spacer (ITS) sequences (Tan et al., 1999; Hayden et al., 2003; Shimada et al., 2003; Kraft et al., 2010; Guidone et al., 2013).

Ulva shows phenotypic plasticity, which includes the typical morphology formed with the aid of specific bacteria (Lovlie, 1969; Coates et al., 2015; Wichard et al., 2015). Therefore, *Ulva* is an interesting taxon for the investigation of evolution from unicellular organisms to multicellular organisms and of embryology leading to morphological diversity. However, it is also difficult to determine the species of these algae based on their morphological characteristics because their morphological plasticity varies due to environmental factors and symbiotic bacteria.

Recently, the nucleotide sequences of the ITSs, the *rbcL* gene and the *cox* genes have been analyzed in *Ulva* species. These genetic analyses have revealed that some of these algae previously considered to be different species are the same species: *U. armoricana* and *U. scandinavica* have been renamed as *U. rigida* (Malta et al., 1999; Kawai et al., 2007), *U. fasciata* and *U. lactuca* are also identical

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(Hughey et al., 2019), and *U. pertusa*, a common *Ulva* species in Japan, is the same as *U. australis* in Australia (Couceiro et al., 2011; Hanyuda and Kawai, 2018).

Although green alga species show similar morphological characteristics, there is a great divergence in their genome structures. Nucleotide diversity is generally high in the chloroplast genomes of green alga species compared with that in their nuclear genes (Leliaert et al., 2012). This situation indicates that the identities of individual species can be efficiently determined based on the diversity of their chloroplast genome sequences. Therefore, a large number of chloroplast genomes have been analyzed. These nucleotide sequences exhibit considerably different sizes, as exemplified by *U. linza* (86,726 bp), *U. lactuca* (96,005 bp), *U. ohnoi* (103,313 bp) and *U. mutabilis* (119,866 bp) (accession numbers KX058323, KT882614, AP018696 and MK069584, respectively).

In this study, we report the entire nucleotide sequence of the *U. pertusa* chloroplast genome and its structural differences from other previously reported chloroplast genomes. Based on these data, we constructed simple molecular markers that allowed the discrimination of *Ulva* species living in Tokyo Bay such as *U. pertusa*, *U. lactuca* and *U. ohnoi*, whose characteristics are highly similar.

MATERIALS AND METHODS

Algae and culture conditions Unialgal cultures of *U. pertusa* (=*U. australis*) and other green alga species were obtained from the stock center of the Kobe University Macro-Algal Culture Collection (KU–MACC); these cultures included *U. pertusa* (=*U. australis*) (KU-1658), *U. ohnoi* (KU-1529, KU-1525), *U. lactuca* (KU-1539, KU-1540), *U. fenestrata* (KU-1603), *U. intestinalis* (KU-1534), *U. compressa* (KU-1634) and *U. flexuosa* (KU-1526, KU-1532). They were cultured at 18 °C under a 16–8 h light–dark cycle in sea water (filtered, UV-sterilized and autoclaved) containing 0.5% KW21 salt (Daiichi Seimo, Kumamoto, Japan) in a culture flask with gentle shaking.

Preparation of DNA from algal samples, PCR, and DNA sequence analysis Chloroplast DNA was prepared using a Chloroplast Isolation Kit (Sigma-Aldrich, St. Louis, MO, USA) and purified with the QuickExtract DNA extraction kit (Lucigen, Middleton, WI, USA). Genomic DNA was also prepared using the REDExtract-N-Amp TM Plant PRC Kit (Merck, Darmstadt, Germany). PCR was performed using KOD Fx Neo DNA polymerase (Toyobo, Osaka, Japan). The reaction cycles were set with an initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 98 °C for 10 s, annealing at 50–60 °C for 30 s, extension at 68 °C for 30 s kb^{-1}, and a final extension at 68 °C for 7 min. Primers for PCR amplification of fragments of the *U. pertusa* chloroplast genome were designed based on the chloroplast genome of *U. lactuca* (acc. no. KT882614). Primers used for PCR amplification are listed in Supplementary Table S1. The PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). Sequencing reactions were performed using a Sanger sequencing platform, the ABI 3730 XL automated sequencer (Applied Biosystems, Foster City, CA, USA). The nucleotide sequence of the chloroplast genome was determined using the PCR-amplified fragments via the direct sequencing method based on Sanger sequencing.

Annotation and mapping of chloroplast genes The primary approach for the identification of putative genes was reciprocal BLAST analysis. tRNA genes were submitted to tRNAscan-SE 2.0 (http://lowelab.ucsc.edu/tRNAscan-SE/) using the default model (Lowe and Chan, 2016). Thereafter, the chloroplast genes were annotated by GeSeq (https://chlorobox.mpimp-golm.mpg.de/geseq.html) (Tillich et al., 2017). The circular genome map was drawn using OGDRAW v1.3.1 (https://chlorobox.mpimp-golm.mpg.de/OGDraw.html) (Greiner et al., 2019). The resulting annotated sequence has been deposited at the DDBJ under accession number LC507117.

Phylogenetic tree analysis Sequence datasets for *Ulva* species were obtained from the GenBank database. They were aligned using MAFFT (Katoh et al., 2005), and then edited using trimAI (Capella-Gutiérrez et al., 2009). A maximum likelihood (ML) phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA) X10.1 (Kumar et al., 2018). The ML tree was constructed with 1,000 bootstrap replicates obtained based on the General Time Reversible model (Felsenstein, 1985; Nei and Kumar, 2000).

RESULTS

Structural characteristics of the *U. pertusa* chloroplast genome The entire nucleotide sequence of the *U. pertusa* chloroplast genome was determined. This genome is a circular DNA molecule consisting of 102,899 bp (Fig. 1), which is similar to the reported sizes for *U. ohnoi* (103,313 bp), *U. flexuosa* (89,414 bp), *U. lactuca* (96,005 bp) and other *Ulva* species (Cai et al., 2017; Wang et al., 2017; Suzuki et al., 2018).

The *U. pertusa* chloroplast genome was predicted to contain 104 genes, including 74 predicted protein-coding genes, 3 ribosomal RNA genes and 27 tRNA genes, similar to other chloroplast genomes (Table 1). No inverted repeat (IR) sequences were found in the *U. pertusa* chloroplast genome. The numbers of introns were different between genes in the chloroplast genomes (Supplementary Table S2). Some genes lacked introns; no introns

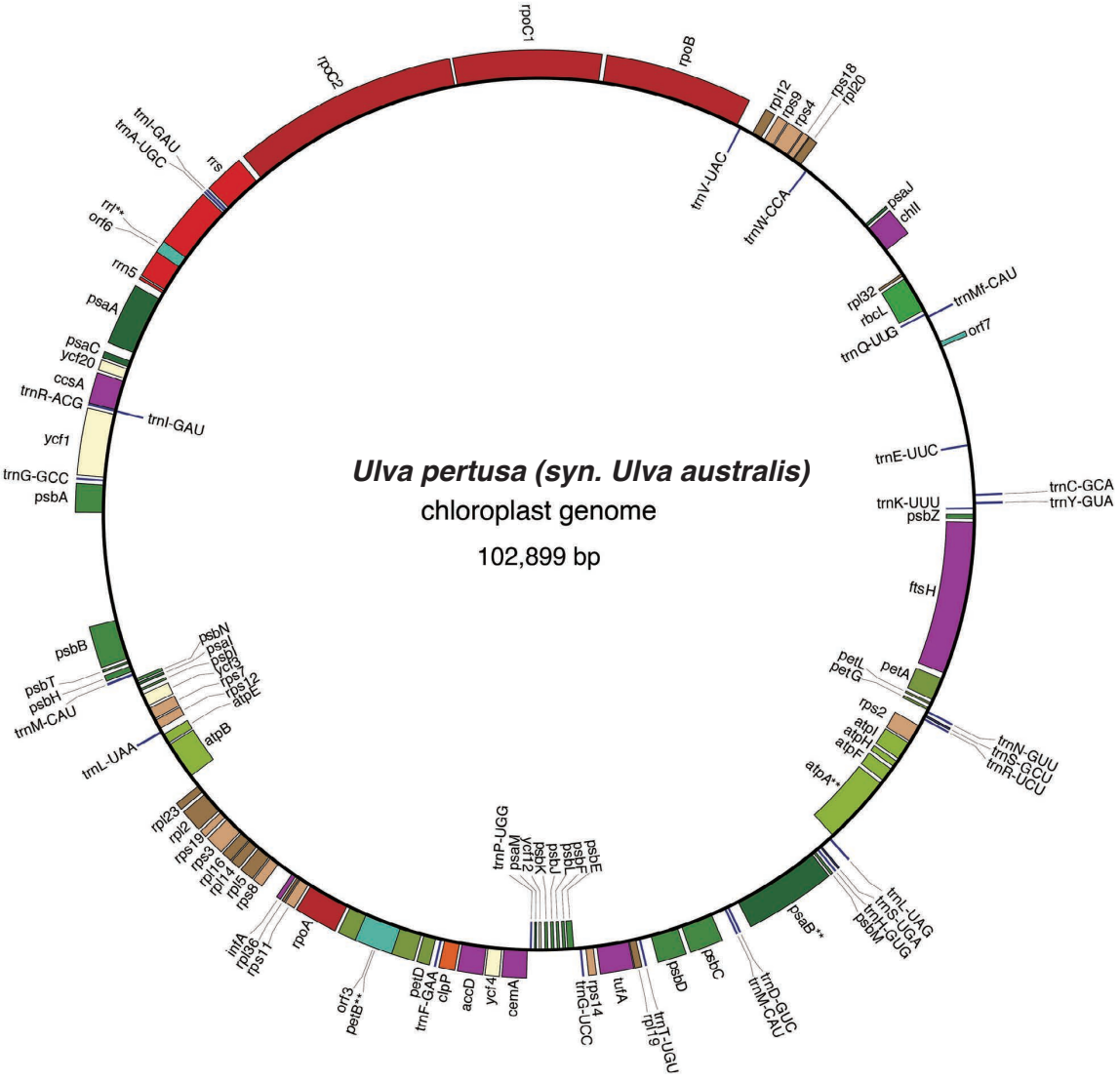


Fig. 1. Map of the chloroplast genome of *U. pertusa*. Genes shown inside the circle are transcribed clockwise, and those drawn outside the circle are transcribed counterclockwise. Functionally annotated genes are shown as colored boxes. The list of genes in the chloroplast genome is provided in Supplementary Table S2.

Table 1. Number of genes in the chloroplast genomes of *Ulva* species

	Genome size (bp)	rRNA	tRNA	Transcription and translation	Photosynthesis	Others	*Ycf*	ORF
U. pertusa	102,899	3	27	25	27	14	5	3
U. mutabilis	119,866	3	27	25	27	14	5	1
U. ohnoi	103,313	3	28	25	27	14	5	7
U. lactuca	96,005	3	28	25	27	14	5	6
U. linza	86,726	3	28	25	27	14	5	0
U. flexuosa	89,414	3	28	25	27	14	5	0
U. prolifera	93,066	3	28	25	27	14	5	1

Genes in the chloroplast genomes are listed. These genes were classified into rRNA genes (shown by "rRNA"), tRNA genes ("tRNA"), genes for transcription and translation ("Transcription and translation"), genes for photosynthesis ("Photosynthesis"), genes involved in other functions ("Others"), *Ycf* genes ("*Ycf*") and other ORFs ("ORF").

were found in the *psbD* or *atpB* genes, which include introns in *U. ohnoi* and *U. lactuca*.

The *U. pertusa* chloroplast genome contained a set of tRNA genes, each of which corresponded to an individual residue among 20 amino acids (Table 2). Different numbers of tRNA genes were predicted to exist in the chloroplast genomes of *U. pertusa* and *U. mutabilis* in comparison with other *Ulva* species. In the *U. pertusa* and *U. mutabilis* chloroplast genomes, no *trnF(AAA)* was predicted to exist. Only *trnF(GAA)* was found in these genomes for tRNA-Phe, whereas both *trnF(AAA)* and *trnF(GAA)* occur in many *Ulva* species, such as *U. ohnoi* and *U. lactuca* (Table 2). A nucleotide sequence that showed partial similarity to *trnF(AAA)* was found in the region between *psbN* and *trnM(CAU)* of the *U. pertusa* chloroplast genome, where the *trnF(AAA)* gene is located in the *U. lactuca* and other chloroplast genomes (Fig. 2).

Table 2. tRNA genes in *Ulva* species chloroplast genomes

	U. pertusa *U. mutabilis*	*U. ohnoi* *U. lactuca* *U. linza* *U. flexuosa* *U. prolifera*
trnA(UGC)	1	1
trnC(GCA)	1	1
trnD(GUC)	1	1
trnE(UUC)	1	1
trnF(AAA)	0	1
trnF(GAA)	1	1
trnG(GCC)	1	1
trnG(UCC)	1	1
trnH(GUG)	1	1
trnI(GAU)	2	2
trnK(UUU)	1	1
trnL(UAA)	1	1
trnL(UAG)	1	1
trnM(CAU)	2	2
trnfM(CAU)	1	1
trnN(GUU)	1	1
trnP(UGG)	1	1
trnQ(UUG)	1	1
trnR(ACG)	1	1
trnR(UCU)	1	1
trnS(GCU)	1	1
trnS(UGA)	1	1
trnT(UGU)	1	1
trnV(UAC)	1	1
trnW(CCA)	1	1
trnY(GUA)	1	1

The numbers of copies of each tRNA gene are listed.

Comparison with other chloroplast genomes *Ulva pertusa* exhibits a phenotype of lettuce-like morphology similar to the morphology of *U. ohnoi* and *U. lactuca*. However, evolutionary studies on these algae have suggested that *U. pertusa* is closely related to *U. flexuosa* and *U. linza*, and this species has been classified into a clade separate from that containing *U. ohnoi* and *U. lactuca* (Kraft et al., 2010; Ichihara et al., 2015; Matsumoto and Shimada, 2015). Using the complete chloroplast genome sequences, we generated a phylogenetic tree of *Ulva* species, including *U. pertusa*. Our results also indicated that *U. pertusa* is evolutionarily distant from *U. ohnoi* and *U. lactuca* and relatively close to *U. mutabilis*, *U. flexuosa*, *U. prolifera* and *U. linza* (Fig. 3).

The structure of the *U. pertusa* chloroplast genome showed a major difference from those of the representative chloroplast genomes. The order of genes was inverted in the 25-kb region lying between the *psbB* and *rpl19* genes of the *U. pertusa* chloroplast genome (Fig. 4). In addition, the 3-kb region containing *psbD* and *psbC* was inverted in *U. pertusa* and *U. mutabilis* (Fig. 4). These characteristics suggest that rearrangement events have occurred in the *U. pertusa* and *U. mutabilis* chloroplast genomes.

Construction of genotyping markers allowing the detection of polymorphic DNAs in chloroplast genomes The nucleotide sequences of individual genes were highly conserved, but large differences were found at many points in the chloroplast genomes, such as in introns and intergenic regions. Using the unique nucleotide sequences in the *U. pertusa* chloroplast genomes, we attempted to design appropriate genotyping markers and applied them to establish a simple method for the identification of individual *Ulva* species.

We designed four genotyping markers that corresponded to sequences located near the *psbD*, *atpA* and *atpB* genes and in the region between the *rbcL* and *chl1* genes (Table 3). Using these markers, we set genotyping markers that could detect genetic polymorphisms among the *Ulva* species. The PCR-amplified fragments detected apparent polymorphisms due to different fragment sizes depending on the individual *Ulva* species (Fig. 5). These results suggest that these markers can be used for the differentiation of *U. pertusa* from other *Ulva* species.

Next, we set out to distinguish individual *Ulva* species using these markers. Polymorphic DNA fragments specific to each *Ulva* species, namely *U. ohnoi*, *U. fenestrata*, *U. lactuca*, *U. flexuosa*, *U. compressa*, *U. intestinalis* and *U. pertusa*, were detected. In this analysis, markers A to D generated a unique set of sizes of the amplified fragments for each of these species (Fig. 6A–D). The same

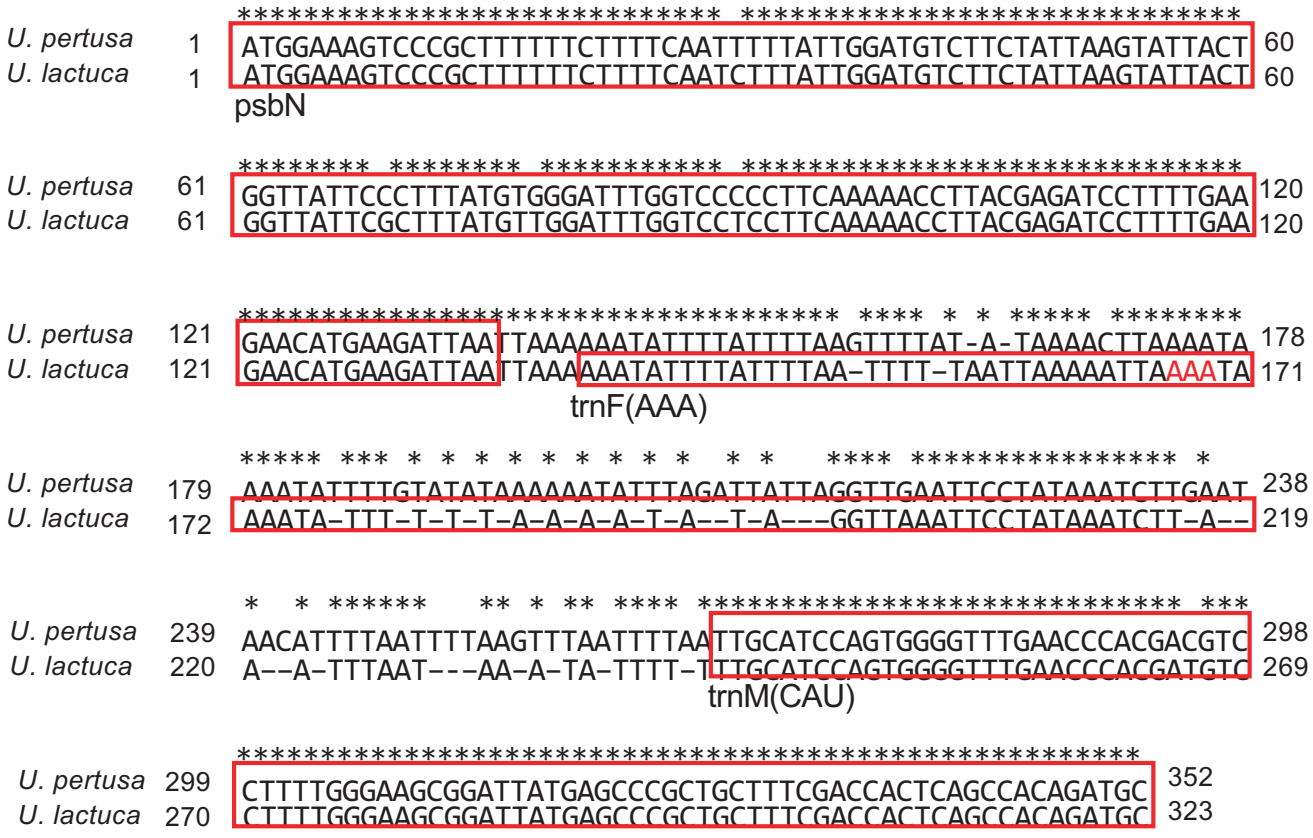


Fig. 2. Comparison of the nucleotide sequences in the region around the *trnF(AAA)* gene. Nucleotide sequences of the *U. pertusa* and of *U. lactuca* chloroplast genomes are shown. The *psbN* and *trnM(CAU)* regions of both species and the *U. lactuca* trnF(AAA) region are boxed. Identical nucleotides between the species are indicated by asterisks. The region corresponding to the anticodon of *trnF(AAA)* of *U. lactuca* is indicated by red letters.

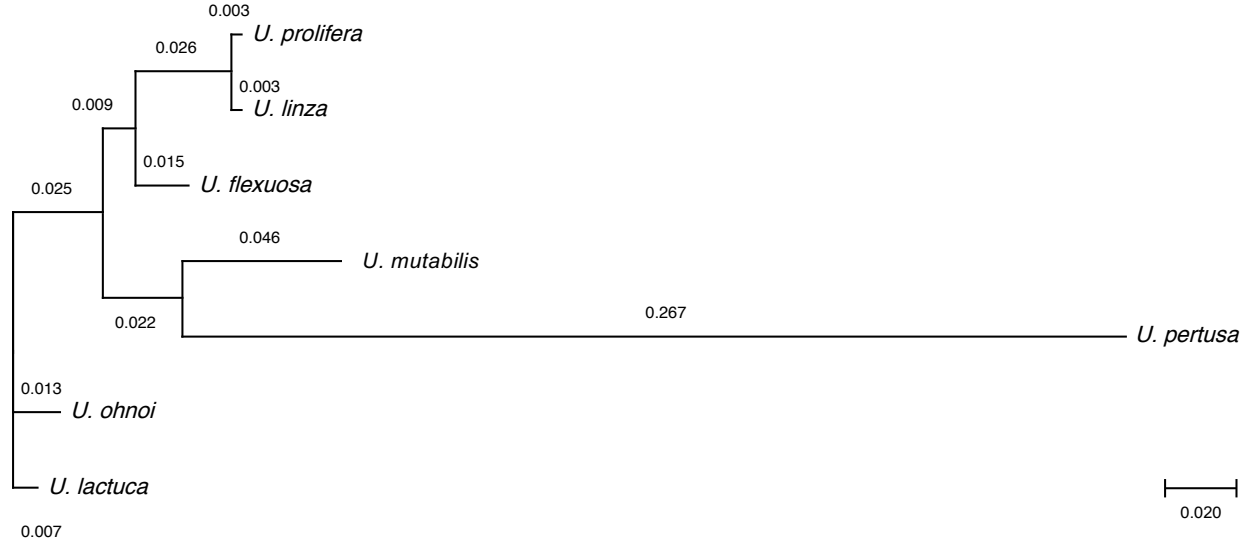


Fig. 3. Phylogenetic tree of *Ulva* species. Evolutionary analysis of the complete chloroplast genome sequences was performed using the maximum likelihood method (Nei and Kumar, 2000).

analysis conducted on *U. ohnoi*, *U. lactuca* and *U. flexuosa* showed the same results, indicating the reproducibility of our genetic diagnosis. The nucleotide sequence of the *U. mutabilis* chloroplast genome has been reported (acc. no. MK069584). Based on these sequence data, we estimated what the sizes of the amplified fragments of the *U.*

mutabilis chloroplast genome would be when they were amplified using the primers A to D, and predicted them to be 1.5 kb, 1.4 kb, 1.8 kb and 6.5 kb, respectively. Due to the differences in the amplified fragment patterns specific to each individual species, the species were clearly distinguished from each other (Fig. 6E).

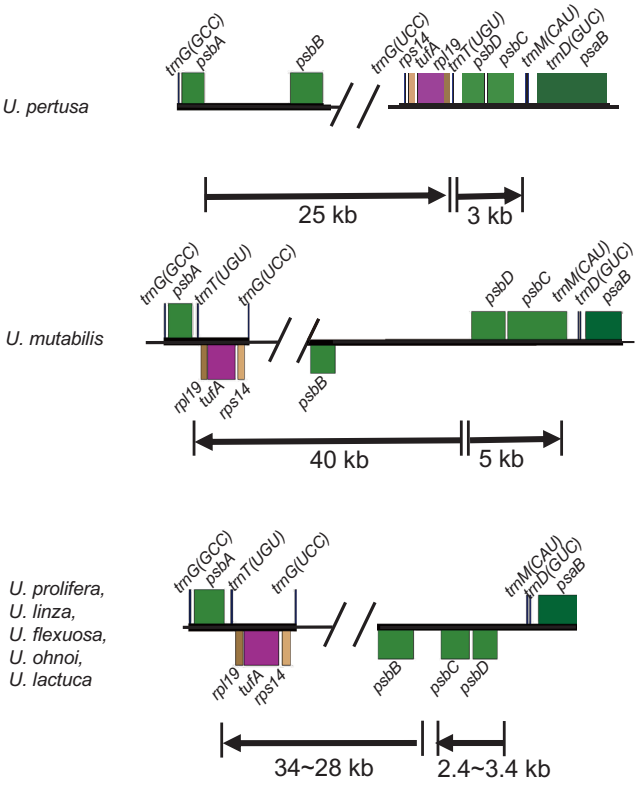


Fig. 4. Schematic representation of the order of genes in the chloroplast genomes of *Ulva* species. Regions containing predicted sequence inversions are shown. A gene above the line is transcribed in the clockwise direction, as in Fig. 1, and a gene below the line is transcribed in the counterclockwise direction. Arrows indicate the orientation of the gene order in the chloroplast genomes. Detailed maps of the genes are shown in Supplementary Fig. S1.

DISCUSSION

We determined the entire nucleotide sequence of the *U. pertusa* chloroplast genome. The structural characteristics of this genome were similar to those of other algal chloroplast genomes (Fig. 1). The size of the chloroplast genome of *U. pertusa* (102,899 bp) was similar to those of other *Ulva* species, such as *U. ohnoi* (103,313 bp), *U. flexuosa* (89,414 bp) and *U. lactuca* (96,005 bp). In higher plant chloroplast genomes, IR sequences have been reported (Shinozaki et al., 1986; Hiratsuka et al., 1989). However, no IR sequences were found in the *U. pertusa* chloroplast genome, although they are found in

Table 3. Primer list of the genotyping markers

Primer set		Sequences
A	Forward	5'-GGTGCTGTACTAATGGGTGAAG
	Reverse	5'-GACCAATTGCAACATAAACAC
B	Forward	5'-TACCTTCTGTTAAAGCTTTTTCCATATC
	Reverse	5'-CAGGCGTGTAATAAGTTAAACG
C	Forward	5'-CGACGTAATGGATGACTGGT
	Reverse	5'-CATAAAGAAGTGAAGCCAACG
D	Forward	5'-TTAATGCGAGGAATGGAAGC
	Reverse	5'-CCGGTTGTATAAACCGTGG

PCR primers used for genotyping markers are listed. Markers A to D are the sets of primers (forward and reverse). The sites of the A, C and D primers are located upstream and downstream of the *atpA*, *psbD* and *atpB* genes. The marker B primers correspond to sequences in the 5' regions of the *rbsL* and *chl1* genes.

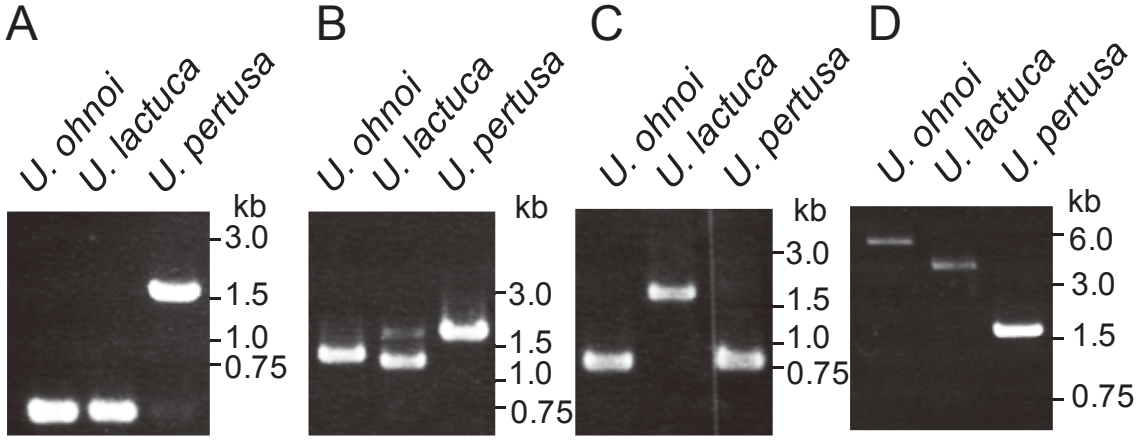
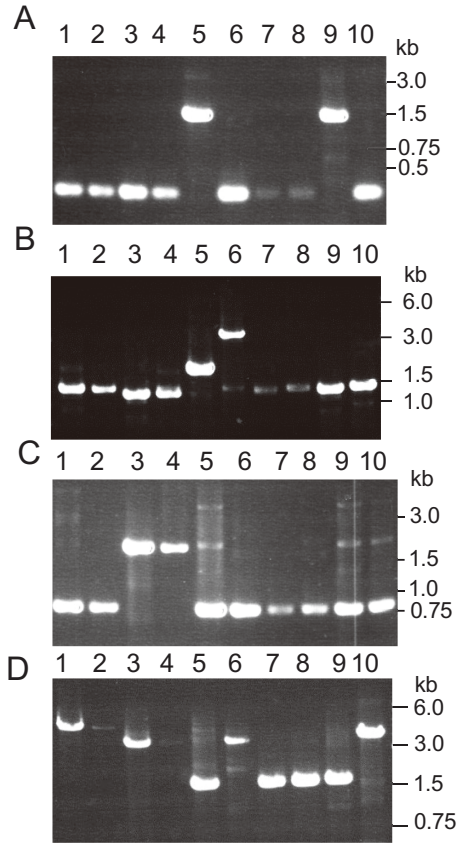


Fig. 5. Evaluation of the genotyping markers. PCR-amplified fragments obtained using primers for the specific genotyping markers are shown. Panels A to D display fragments amplified from the genomic DNA of *U. ohnoi*, *U. lactuca* and *U. pertusa* using specific primers A to D, respectively, which are listed in Table 3. Molecular sizes are shown on the right of each panel.



E

	A	B	C	D
U. pertusa	1.5	2.0	0.8	1.5
U. ohnoi	0.4	1.5	0.8	6.0
U. lactuca	0.4	1.3	1.6	4.0
U. fenestrata	0.4	3.0	0.8	4.0
U. flexuosa	0.4	1.5	0.8	1.5
U. intestinalis	1.5	1.5	0.8	1.5
U. compressa	0.4	1.5	0.8	4.0
U. mutabilis	1.5	1.4	1.8	6.5
	amplified fragments			

Fig. 6. Detection of polymorphic DNA fragments obtained using genotyping markers A to D. Panels A to D display PCR-amplified fragments obtained using specific primers A to D, respectively. Numbers in the panels indicate the DNA samples prepared from green algae: 1: *U. ohnoi* (KU-1525), 2: *U. ohnoi* (KU-1529), 3: *U. lactuca* (KU-1539), 4: *U. lactuca* (KU-1540), 5: *U. pertusa* (KU-1658), 6: *U. fenestrata* (KU-1603), 7: *U. flexuosa* (KU-1526), 8: *U. flexuosa* (KU-1532), 9: *U. intestinalis* (KU-1534), 10: *U. compressa* (KU-1634). These are independently isolated and cultured lines maintained at the Kobe University Research Center for Inland Seas. Molecular sizes are shown on the right. Panel E summarizes the results of amplification using the genotyping markers. The sizes of the amplified fragments (kb) obtained when the genetic diagnosis is performed on each *Ulva* species are shown. The row for *U. mutabilis* exhibits the predicted sizes of fragments that would be amplified using the genotyping markers A to D. They were estimated using the nucleotide sequence data of the *U. mutabilis* chloroplast genome (acc. no. MK069584).

those of other *Ulva* species (Cai et al., 2017).

Protein-coding genes and ribosomal RNA genes in the chloroplast genome were highly conserved. It has been shown that higher plants possess 30–31 tRNA genes in their chloroplast genomes (e.g., Shinozaki et al., 1986; Hiratsuka et al., 1989). However, the *Ulva* chloroplast genomes exhibit fewer tRNA genes, although they include sequences corresponding to all 20 amino acid residues. A lack of any *trnF(AAA)* gene was predicted in the *U. pertusa* and *U. mutabilis* chloroplast genomes, which are classified in the same clade, although *trnF(AAA)* is highly conserved in other *Ulva* species (Table 2). Instead, there was a region showing similarity to *trnF(AAA)* in the position corresponding to *trnF(AAA)* in the other *Ulva* chloroplast genomes (Fig. 2). In this region, there was an "AAA" sequence that could correspond to the anticodon sequence of *trnF(AAA)*. However, this trinucleotide lay in an A-rich region, but a secondary structure of the tRNA was predicted in *U. pertusa*. Therefore, this sequence may be a pseudogene, indicating that the gene has been disrupted.

Sea lettuce and green laver had been considered to be different genera because of their morphological divergence, but recently they have been classified in the same genus based on the similarity of their DNA sequences (Hayden et al., 2003). Phylogenetic trees have been constructed using the *rbcL* gene and ITS sequences (Hayden et al., 2003; Kraft et al., 2010). Our phylogenetic tree analysis of *Ulva* species indicated that *U. pertusa* and *U. mutabilis* are closely related. This also suggested that they were more similar to *U. flexuosa*, *U. linza* and *U. prolifera*, which had been classified in the genus *Enteromorpha*, than to *U. ohnoi* and *U. lactuca* (Fig. 3). This result represents a DNA-based classification of *Ulva* species that differs from the previous classification based on morphological characteristics.

Large genome inversions were found in the approximately 25-kb region between the *psbB* and *rpl19* genes, and in the 3-kb region containing the *psbD* and *psbC* genes of the *U. pertusa* chloroplast genome (Fig. 4). The inversion of the 25-kb region was only found in the *U. pertusa* chloroplast genome. The inversion of the 3-kb region was detected in the chloroplast genomes of *U. pertusa* and *U. mutabilis* (Fig. 4). These findings suggest that two genome inversion events occurred sequentially; the 3-kb region was inverted in the ancestral chloroplast genome before *U. pertusa* and *U. mutabilis* diverged, after which the 25-kb region underwent inversion in the *U. pertusa* genome.

Ulva algae often form green tides in closed seas such as Tokyo Bay (Yabe et al., 2009). Various organisms, including alien species of seaweed, have been introduced into broad sea areas through transport in media such as ballast water. These species subsequently often show abnormal increases and become a major problem along

coasts due to the formation of green tides (Verlaque et al., 2002; Aguilar-Rosas et al., 2008).

Under such circumstances, it is desirable to monitor the growth of the species before the formation of a green tide. In Tokyo Bay, three major *Ulva* species are commonly present: *U. pertusa*, *U. ohnoi* and *U. lactuca*. However, these algae show very similar morphologies that can be altered in a plastic manner by environmental conditions, and it is very difficult to distinguish them from each other (Bryhni, 1974; Spoerner et al., 2012). In this study, we developed novel genotyping markers based on differences in the nucleotide sequences of the chloroplast genomes of these species. Since the PCR-amplified fragments obtained using these markers showed polymorphisms, we suggest that these markers can lead to the establishment of a simple method for identifying individual *Ulva* species (Figs. 5 and 6). We anticipate that our genotyping marker system will be applied in various scenarios. This system enables precise determination of species that show similar morphologies. Even if morphorogical changes occur due to the growing environment, the algae can be identified. This makes it easy to establish which *Ulva* species are growing in Tokyo Bay, and also helps to quickly identify alien species that have entered the bay.

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