Differential DNA Rearrangements of Plastid Genes, psbA and psbD, in Two Species of the Dinoflagellate Alexandrium

Satoko Iida1, Atsushi Kobiyama2, Takehiko Ogata2 and Akio Murakami1,*

1Kobe University Research Center for Inland Seas, 2746 Iwaya, Awaji, 656-2401 Japan
2School of Marine Biosciences, Kitasato University, 160-4 Okiraiazautou, Sanriku, Ofunato, 022-0101 Japan
*Corresponding author: E-mail, akiomura@kobe-u.ac.jp; Fax, +81-799-72-2907
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Plantomes of the peridinin-containing dinoflagellates are composed of a limited number of genes, which are carried individually on small circular molecules, termed ‘minicircles’. Although the prevalent plastid chromosome of most algae and plants has only a single copy of each gene, our previous study showed that low copy numbers of multiple variants of the gene psbA co-exist with the ‘ordinary’ gene encoding the D1 protein in minicircles of Alexandrium tamarense. Although none of the psbA variants encoded the entire protein, they persisted in culture. In this study, we compared the distribution and structure of psbA and psbD variants in two species of Alexandrium to characterize DNA rearrangement within these genes. In addition to four previously reported psbA variants, three psbD variants were found in A. tamarense minicircles. The ordinary psbA and psbD genes also co-existed with variants in another species, A. catenella. The sequences of the ordinary genes were virtually identical in the two species. All the variants comprised insertion or deletion mutations, with no base substitutions being identified. Duplicated parts of the coding sequences were contained in most of the insertions. Short direct repeats (4–14 bp) and/or adenine+thymine-rich motifs were present in all mutation regions, although the position and/or the sequence of each DNA rearrangement was unique to each variant. The results indicated that replication-based repeat-mediated recombination was responsible for generation of the variants.

Introduction

Plastids carrying their own genetic information are crucial to the function of plants (Pyke 2009). In land plants, plastomes (plastid genomes) typically consist of a 120–160 kbp circular chromosome, with a quadripartite structure comprising two inverted repeats (20–30 kbp) and small and large single-copy regions (Sugiura 1992, Maier and Schmitz-Linneweber 2004, Bock 2007). Approximately 100 protein-coding genes, in addition to rRNA and tRNA genes, are contained and shared among these chromosomes. In various algal lineages, plastid chromosomes are less homogeneous in size, organization and gene content, and unusual plastome structures are found in some dinoflagellates (Simpson and Stern 2002, Maier and Schmitz-Linneweber 2004, Kim and Archibald 2008).

The peridinin-containing dinoflagellates are an algal group of Alveolata with plastids that originated from secondary endosymbiosis involving a red algal lineage (Simpson and Stern 2002, Maier and Schmitz-Linneweber 2004, Kim and Archibald 2008). Unlike other algae and plants, the plastomes of the dinoflagellates do not consist of a single chromosomal unit (Zhang et al. 1999). To date, only a limited number of genes have been found on independent small circular molecules, termed ‘minicircles’, which have been identified in several dinoflagellate species (reviewed in Green 2004, Howe et al. 2008). Most of the genes that are usually carried in the plastid in other algae and plants are nuclear genes in the dinoflagellates (Hackett and Bhattacharya 2006). Each minicircle molecule (2–10 kbp) carries one or a limited number of genes, in addition to non-coding regions. These non-coding regions have little similarity to known genes of any species. As the non-coding regions contain inverted repeats and/or other secondary structures and are moderately conserved across minicircles of a given species carrying different genes, they are proposed to be related to the replication and/or segregation of the minicircles (Zhang et al. 1999, Zhang et al. 2001, Zhang et al. 2002). In addition to ‘ordinary’ minicircles carrying full-length genes, there...
are unusual forms of minicircles apparently derived from ordinary minicircles by DNA recombination (reviewed in Howe et al. 2008).

*psbA* is a plastid gene encoding the D1 core subunit of the PSII reaction center. Sequences of *psbA* are well conserved in different evolutionary lineages, probably because of functional constraints on the D1 protein (Douglas 1994). Only one form of *psbA* has been reported for all algae and plants, except for a species of the dinoflagellates, *Alexandrium tamarense*. Low copy numbers of four minicircles carrying truncated *psbA* genes (designated as ‘*psbA* variants’) co-exist with the ordinary (standard or full-length) *psbA*-carrying minicircle in *A. tamarense* (Iida et al. 2009). Curiously, the sizes and sequences of the minicircles carrying these *psbA* variants are closely related to the ordinary (full-length) *psbA*-carrying minicircles, including the coding and the non-coding regions. Although the *psbA* variants comprise the minority of the copies of the *psbA* molecules, they persisted in the minicircle genome even after culture for several hundred generations (Iida et al. 2009).

DNA recombination has been implicated in both the replication and repair of plastid genomes in plants and green algae (Day and Madesis 2007, Maréchal and Brisson 2010). The presence of various rearranged minicircles is one of the peculiar features of minicircle genomes (Howe et al. 2008) and could be associated with their replication and repair. In this study, we initially examined the occurrence of variants of the *psbD* gene in *A. tamarense* and of the *psbA* and the *psbD* genes in *A. catenella*. Identification of the variants with structures closely related to the ordinary genes enabled the characterization of DNA rearrangements involved in their generation. We show that the gene variants, in both *psbA* and *psbD* and in different *Alexandrium* species, were possibly generated by illegitimate recombination at short direct repeats. Replication-mediated processes could also be involved in their generation.

### Results

The **psbD** variants in *Alexandrium tamarense* minicircles

To determine the presence of *psbD* variants, gene cloning was carried out using the *A. tamarense* strain examined in our previous studies (strain OFAT0105-8; Iida et al. 2008, Iida et al. 2009). Three *psbD* variants, in addition to the ordinary gene, were found (the ordinary *psbD* gene, AB531477; AT*psbD*-V1, AB531478; AT*psbD*-V2, AB531479; and AT*psbD*-V3, AB531480) (Fig. 1). As with the variants of *psbA*, substitution mutations

![Multiple variants of the ordinary plastid genes, psbA (left) and psbD (right), from the dinoflagellate *Alexandrium tamarense* (strain OFAT0105-8). In addition to the ordinary *psbD* gene, three *psbD* variants with one to three deletions were found in the minicircle genome of *A. tamarense* (AT*psbD*-V1, AT*psbD*-V2 and AT*psbD*-V3). Apart from the deletions, the variants were identical to the ordinary *psbD* sequence. For comparison, previously reported ordinary *psbA* and its four variants (AT*psbA*-V1, AT*psbA*-V2, AT*psbA*-V3 and AT*psbA*-V4, Iida et al. 2009) are also indicated. As in the *psbD* variants, the *psbA* variants retained sequences identical to the ordinary *psbA* at regions other than the DNA rearrangements (insertion or deletion). All insertions included a partial *psbA* sequence identical to the sequence immediately upstream or downstream of the respective insertion (indicated in the same color as the *psbA* coding region), resulting in generation of direct repeats in each variant. Primer sites targeting both the ordinary gene and the variants are also shown. See Fig. 4A, B, and Supplementary Figs. S1, S2 for sequences of the DNA rearrangement sites.](https://academic.oup.com/pcp/article-abstract/51/11/1869/1852040)
were not found in psbD. One to three deletions of varying lengths (11–68 bp) were present in each variant. The psbD variant sequences were identical to the ordinary psbD at regions other than these deletions. The results indicated that the occurrence of variants of the ordinary plastid gene was not a phenomenon unique to psbA.

Co-amplification of the ordinary plastid gene and the variants

The multiple variants of both psbA and psbD did not contain substitution mutations and retained partial or complete psbA and psbD coding sequences. They were examined by PCR amplification using primers targeting both the ordinary gene and the variants. The results of electrophoresis (Fig. 2) were consistent with those of the cloning experiments of the psbA (Iida et al. 2009) and the psbD variants (described above) from A. tamarense. The amplification of A. tamarense included products with sizes expected for the variants and the ordinary gene (Fig. 2A, B). The absence of products larger than the ordinary psbD suggested that insertion variants were absent, or were present with frequencies too low to detect by PCR. Multiple amplification products were also detected from the minicircles of the other three Alexandrium species (A. catenella, A. insuetum and A. fraterculus) in addition to those expected for the ordinary genes (Fig. 2A, B), suggesting that psbA and psbD variants were also present in the other species but with different structures. We further analyzed A. catenella, which is the species most closely related to A. tamarense (Adachi et al. 1996, John et al. 2003, Janson and Hayes 2006, Lilly et al. 2007), and compared the structures of variants in the two species.

The psbA and the psbD variants in Alexandrium catenella minicircles

Gene cloning was carried out using minicircle DNA from A. catenella (strain OFAC9982-101). Almost complete sequences of ordinary psbA (910 bp, AB531481) and ordinary psbD (1,011 bp, AB531483) were determined in A. catenella (Fig. 3). The psbA and psbD sequences were identical in A. catenella and A. tamarense, except for a single silent substitution in psbA.

However, the structures of the variants in A. catenella were different from those of A. tamarense. In addition to the ordinary psbA, one psbA variant was identified in A. catenella (ACpsbA-V1, AB531482) (Fig. 3), corresponding to an amplification product smaller than the ordinary psbA (Fig. 2A). An 80 bp deletion was present in ACpsbA-V1. Apart from the 80 bp deletion, no additional mutations were found. ACpsbA-V1 was likely to be specific to A. catenella, as it was not detected in A. tamarense (Iida et al. 2009; Figs. 1, 2A). Although it was not cloned and sequenced, an amplification product larger than the ordinary psbA (>1.0 kb, Fig. 2A) suggested that another specific psbA variant with an insertion could also be present in A. catenella.

In A. catenella, four psbD variants were identified (ACpsbD-V1, AB531484; ACpsbD-V2, AB531485; ACpsbD-V3, AB531486; and ACpsbD-V4, AB531487) (Fig. 3). The variants had 141–351 bp insertions. A small 2 bp deletion was also found in ACpsbD-V1, in addition to the insertion. psbD variants with larger deletions, such as those found in A. tamarense, were not detected, although they are expected to be easily amplified and cloned if present. All four insertions in A. catenella psbD were located at homologous positions. Common sequence motifs were shared among these insertions (Supplementary Fig. S3). The motifs also occurred repeatedly in each variant. For example, two 48 bp sequence motifs and 45 bp of the same motif constituted a 141 bp insertion of ACpsbD-V1, and a 81 bp sequence comprising four sequence motifs appeared three times in an insertion of ACpsbD-V4. None of the insertions gave significant BLAST hits for known introns, transposons or related elements. Some of the insertions included short partial psbD sequences (30 bp in ACpsbD-V2 and ACpsbD-V3, and 27 and 42 bp in ACpsbD-V4), each of which was identical to the

![Fig. 2 Multiple products from amplification targeting psbA (A) and psbD (B) in four species of the dinoflagellate genus Alexandrium. The positions of the primers are indicated in Fig. 1. The products of the ordinary genes are present in all species. Due to the presence of the psbA variants (ATpsbA-V1, 673 bp; ATpsbA-V2, 597 bp; ATpsbA-V3, 667 bp), the electrophoresis pattern in A. tamarense psbA is a smear around 0.6–0.7 kb with an additional faint product around 0.1 kb (ATpsbA-V4, 143 bp). Amplification products expected for the variants are also present in A. tamarense using the primers for psbD (ATpsbD-V1, 864 bp; ATpsbD-V2, 912 bp; ATpsbD-V3, 883 bp). The ordinary gene products and the variants were also contained in the amplification of the other three Alexandrium species. M, 100 bp DNA ladder.]

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sequence of the immediate upstream region (Fig. 3). Other than these mutations, all other sequenced samples were identical to the ordinary psbD gene. No base substitution was found among the psbD variants or the ordinary gene.

**DNA rearrangements of the psbA and the psbD genes were possibly mediated by short direct repeats**

The DNA rearrangement sites of *A. tamarense* were bordered by short direct repeats (4–14 bp), regardless of modes of mutation (insertions or deletions) and genes (psbA or psbD) (Table 1, Fig. 4A, B, Supplementary Figs. S1, S2). Patterns of ten direct repeats were homologous and two were complementary. The distance between repeats was 5–376 bp. In addition to the direct repeats, adenine-thymine-rich (A+T-rich) motifs were present in either one or both boundaries of the rearrangement sites and in relatively long rearranged (inserted or deleted) sequences (Supplementary Table S1).

Deletion junctions of *A. catenella* psbA were bordered by 8 bp homologous direct repeats (Table 1, Fig. 4C, Supplementary Fig. S2). A+T-rich motifs were abundant in the deleted sequence (Supplementary Table S1).

Bordering the insertion site of *A. catenella* psbD, there were 9 bp homologous direct repeats (Table 1, Fig. 4D). The four psbD variants were probably derived from a single common prototype as their rearrangements had occurred at homologous positions and their inserted sequences were locally identical. Each psbD variant was modeled to have been generated by single- or multiple-step DNA rearrangements of ACpsbD-pV, one possible structure of a prototype of the four psbD variants (Table 1, Fig. 4D, Supplementary Fig. S3). Short direct repeats were always present at the junctions of each rearrangement site (Supplementary Fig. S3).

**Species-specific occurrence of the psbA variants**

We have shown that the plastid gene variants distinguish *A. catenella* strain OFAC9982-101 from *A. tamarense* strain OFAT0105-8. To clarify whether such differences were maintained within each species, the distribution of the psbA variants was assessed in both strains (Fig. 5). The amplification products differed between species but were similar among strains of the same species (Fig. 5A). Of the four psbA variants originally discovered in *A. tamarense* strain OFAT0105-8, all except ATpsbA-V3 were detected in all of the three additional strains of *A. tamarense* examined in this study (Fig. 5B). Similarly, the psbA variant ACpsbA-V1 originally found in *A. catenella* strain OFAC9982-101 was detected in all of the other three strains (Fig. 5C). These data suggest that the plastid gene variants with distinct DNA rearrangements are maintained in the population of each species for at least several years.

**Discussion**

The existence of gene variants in both psbA and psbD suggests that their generation and persistence is a characteristic feature of the minicircle genomes of the dinoflagellate *Alexandrium*.
species (Figs. 1–5). The direct repeat sequences bordering the rearrangement sites of psbA and psbD genes examined in this study (Table 1, Fig. 4, Supplementary Figs. S1–S3) are too short to serve as a substrate for homologous recombination (Persky and Lovett 2008). The distances between the short direct repeats subjected to DNA rearrangement were within several hundreds base pairs and perhaps were related to the dimensions of a replication fork. In our previous study, we proposed that intramolecular interaction within a distance of <1kb during DNA recombination could have given rise to ATpsbA-V1 and ATpsbA-V4, i.e. a 112 bp segment in the upstream sequence of the same minicircle was inserted into ATpsbA-V1 and a 392 bp segment of the same minicircle into ATpsbA-V4 (Iida et al. 2009). The proximity of the interacting sequences also supports the idea that these recombinations occurred in the context of replication forks (Bzymek and Lovett 2001).

The role of specific sequences in promoting illegitimate recombination is largely unexplored (Bzymek and Lovett 2001). However, we found A+T-rich motifs in the majority of both rearrangement junctions and rearranged (either inserted or deleted) sequences (Fig. 4, Supplementary Table S1). Although the actual role of A+T-rich motifs cannot be identified, our earlier sequence analyses have implicated A+T-rich motifs at the junction of a 138 bp insertion in ATpsbA-V1 (TATTATT in Fig. 4A, Supplementary Table S1) as the illegitimate recombination site that could have given rise to a precursor of ATpsbA-V1 (Fig. 1 and Fig. S6 in Iida et al. 2009). A stretch of adenines and thymines could have a greater tendency to melt and favor the DNA recombination. In summary, the characteristics of the DNA rearrangements suggest that replication-based repeat-mediated recombination might be the underlying mechanism for the generation of plastid gene variants.

In the dinoflagellate species, many 'aberrant' minicircles appear to have been generated by recombination of formerly normal gene-carrying minicircles (Howe et al. 2008). Aberrant minicircles reported in Heterocapsa triquetra contained truncated coding sequences of two or three plastid genes that have experienced differential DNA rearrangements (insertion, deletion and duplications) (Zhang et al. 2001). Similar to those of A. tamarense, the rearrangement sites of Heterocapsa were bordered by short direct repeats, and A+T-rich motifs were present in some rearrangement junctions and/or rearranged sequences (Supplementary Table S2). This implies the presence of specific DNA rearrangement mechanisms common to the dinoflagellates.

DNA repair mechanisms and selection for functional and intact genomes are suggested to exist in the plastid of plants and green algae (Day and Madesis 2007). Several families of nuclear-encoded proteins are involved in the suppression of recombination between repeated DNA sequences in plants (reviewed in Maréchal and Brisson 2010). It is possible that the rearranged molecules of the dinoflagellate minicircles

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### Table 1

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The four psbD variants of A. catenella were probably derived from a single common prototype (ACpsbD-pV).

\* Only direct repeats that have associated with generation of the ACpsbD-pV are described. Refer to Fig. 4 for the positions of the mutations and Supplementary Fig. S3 for the repeats at the borders of the DNA rearrangements which have led to generation of four psbD variants from ACpsbD-pV.

\* One possible structure of a single common prototype of the four psbD variants of A. catenella.
Fig. 4 Examples of short direct repeats bordering DNA rearrangement sites in plastid genes of *Alexandrium tamarense* (A, B) and *A. catenella* (C, D). DNA rearrangements are proposed based on sequence alignment between the ordinary gene (psbA and psbD) and the variants (ATpsbA-V1, ATpsbD-V3 and ACpsbA-V1, ACpsbD-V3) or a hypothetical common prototype of four psbD variants in *A. catenella* (ACpsbD-pV). In addition to short direct repeats, A+T-rich motifs were found in one or both ends of the rearrangement junctions and in all rearranged (either inserted or deleted) sequences (cf. Supplementary Table S1). The A+T-rich motif of the rearranged sequence is only indicated in red when it is longer than 4 bp.

**A** 138-bp insertion in ATpsbA-V1

| psbA | GGCCTTGGGA TTTTCTAGC TTTTTGGGCA CCAATTAATG CAGGCAACGC TGTATTATTT |
| ATpsbA-V1 | GGCCTTGGGA TTTTCTAGC TTTTTGGGCA CCAATTAATG CAGGCAACGC TGTATTATTT |

| psbA | CTTGGTATGA GTGTTACA |
| ATpsbA-V1 | CTTGGTATGA GTGTTACA |

**B** 11-bp and 35-bp deletions in ATpsbD-V3

| psbD | TTTTGGTTCA TCTTTGTTAA CACATGGTTA AGCTACCTCT ATTTAAGTT GTTGAAATTT TTTAATGCT GCTGATCAA |
| ATpsbD-V3 | TTTTGGTTCA TCTTTGTTAA CACATGGTTA AGCTACCTCT ATTTAAGTT GTTGAAATTT TTTAATGCT GCTGATCAA |

| psbD | CACCAGCAA CAGTATGG |
| ATpsbD-V3 | CACCAGCAA CAGTATGG |

**C** 80-bp deletion in ACpsbA-V1

| psbA | TTTCTATCCT GAAAGGAGAT CACATGGTTA TTTAAGCGTG TTTATCTATG TGTGGGACFA TCAATTATTA GTGCTTGCAT |
| ACpsbA-V1 | TTTCTATCCT GAAAGGAGAT CACATGGTTA TTTAAGCGTG TTTATCTATG TGTGGGACFA TCAATTATTA GTGCTTGCAT |

| psbA | TTTCCCTTCT TGTATGGTAC GAGATGGGCA |
| ACpsbA-V1 | TTTCCCTTCT TGTATGGTAC GAGATGGGCA |

**D** Hypothetical insertion (239-bp) in a common prototype of four psbD variants in *A. catenella* (ACpsbD-pV)

| psbD | CTCTTTTGGTAA TTTAGACCTTA TGGTTACCTT CAGTGTGTTA AGTTAAAATT TTTTTTTTTTTT |
| ACpsbD-pV | CTCTTTTGGTAA TTTAGACCTTA TGGTTACCTT CAGTGTGTTA AGTTAAAATT TTTTTTTTTTTT |

| psbD | TTTTGGTATGA GTGTTACA TTTAAGCGTG TTTATCTATG TGTGGGACFA TCAATTATTA GTGCTTGCAT |
| ACpsbD-pV | TTTTGGTATGA GTGTTACA TTTAAGCGTG TTTATCTATG TGTGGGACFA TCAATTATTA GTGCTTGCAT |

| psbD | TTTTGGGATCA ACTATGACTA GACCAAAGTT TTTATCTATG TGTGGGACFA TCAATTATTA GTGCTTGCAT |
| ACpsbD-pV | TTTTGGGATCA ACTATGACTA GACCAAAGTT TTTATCTATG TGTGGGACFA TCAATTATTA GTGCTTGCAT |

| psbD | TTTTGGGATCA ACTATGACTA GACCAAAGTT TTTATCTATG TGTGGGACFA TCAATTATTA GTGCTTGCAT |
| ACpsbD-pV | TTTTGGGATCA ACTATGACTA GACCAAAGTT TTTATCTATG TGTGGGACFA TCAATTATTA GTGCTTGCAT |

| NNNNN | or NNNNN : Short homologous or complementary direct repeats flanking DNA rearrangement |
| NNNNN | : Adenine+thymine-rich motif at the rearrangement junction or at the rearranged sequence |
| NNNNN | : Duplication of partial coding sequences at immediate upstream region (*****)

Fig. 4 Examples of short direct repeats bordering DNA rearrangement sites in plastid genes of *Alexandrium tamarense* (A, B) and *A. catenella* (C, D). DNA rearrangements are proposed based on sequence alignment between the ordinary gene (psbA and psbD) and the variants (ATpsbA-V1, ATpsbD-V3 and ACpsbA-V1) or a hypothetical common prototype of four psbD variants in *A. catenella* (ACpsbD-pV). In addition to short direct repeats, A+T-rich motifs were found in one or both ends of the rearrangement junctions and in all rearranged (either inserted or deleted) sequences (cf. Supplementary Table S1). The A+T-rich motif of the rearranged sequence is only indicated in red when it is longer than 4 bp. Note, a duplicated partial coding sequence is present in an insertion in ATpsbA-V1 and in ACpsbD-pV. Further single or multiple DNA rearrangements of ACpsbD-pV at short direct repeats might have generated four psbD variants of *A. catenella* (Supplementary Fig. S3).
constitute a by-product of recombination processes which are required for DNA repair. In the dinoflagellates, either a specific plastome–nuclear interaction associated with their secondary endosymbiotic origin (e.g. Hackett et al. 2004) or selective pressure against photosynthesis (e.g. Iida et al. 2008) might have rendered the recombination machinery more error prone.

The minicircle genome of the peridinin-containing dinoflagellates includes a variety of rearranged molecules of plastid genes. Some of them were probably maintained as a consequence of strong selection for the replicative advantage of small size (Zhang et al. 2001). In A. tamarense, the sizes and sequences of the minicircles carrying the psbA variants are closely related to the ordinary psbA-carrying minicircles, and many resources are consumed in their replication, segregation, transcription and RNA editing (Iida et al. 2009). As the psbA variants do not show apparent replicative advantage, a low copy number of the variants could be eliminated from the population in the evolutionary time scale although they were tolerated by the plastid for several years. The absence of the ATPsbA-V3 variant in three strains of A. tamarense could indicate operation of such moderate purifying selection (Fig. 5).

However, it is equally possible that some of the variants are preserved because they provide an important source of genetic diversity in a cell. PSII is susceptible to high light damage, and synthesis of the psbA and psbD gene products (the D1 and D2 core proteins of PSII) is regulated in transcriptional and/or translational stages in plants and algae (Liere and Börner 2007, Peled-Zehavi and Danon 2007). Curiously, sequences corresponding to the whole coding region of the ordinary genes were differentially retained in three psbA variants of A. tamarense (Fig. 1) and three psbD variants of A. catenella (Fig. 3). Insertions in each of these variants contained direct repeat sequences generated by duplications of a partial coding sequence. If excision of sequences between the direct repeats occurs, an extra copy of the ordinary gene is generated. Certain direct repeat sequences are thought to mediate rapid amplification of sequences between the repeats and lead to gene copy number variation (Hastings 2007, Maréchal and Brison 2010). Although both A. tamarense and A. catenella have been regarded as similar ‘toxic bloom-forming species’ (Janson and Hayes 2006) and their distributions overlap geographically (Ogata et al. 1982), analyses of an expressed sequence tag library demonstrated that some protein-coding information is not homogeneous between the two species (Uribe et al. 2008). Indeed, although both species tend to bloom in the same coastal area, this occurs at different times and depths (Ogata et al. 1982). Some of the psbA and psbD variants could be related to gene amplification events as part of a regulatory mechanism for PSII. It will be interesting to examine whether the apparent genomic instability of minicircles is related to as yet unidentified beneficial characteristics.

Fig. 5 Distribution of the psbA variants in strains of Alexandrium tamarense and A. catenella. (A) Co-amplification of multiple products by the psbA primer set as in Fig. 2A. (B) Detection of the psbA variant in A. tamarense by gene-specific PCR. (C) Detection of the psbA variant in A. catenella by gene-specific PCR. The size of the product for the variant in A. catenella is 445 bp (ACpsbA-V1) and those in A. tamarense are indicated in the legend to Fig. 2. Strains of A. tamarense: (a) OFAT0105-8, (b) OFAT0105-4, (c) OFAT0305-5, (d) OFAT0305-18; strains of A. catenella: (e) OFAC9982-101, (f) OFAC9982-102, (g) OF0707AC-4, (h) OF0707AC-7.

Materials and Methods

Strains and cultures

Four species of the dinoflagellate genus Alexandrium were used: A. tamarense, A. catenella, A. insuetum and A. fraterculus. All strains were isolated and cultured in the same way as for A. tamarense strain OFAT0105-8 (Kobiyma et al. 2006, Iida et al. 2009).

Isolation of minicircular DNA

The DNA used in the present study was minicircle DNA isolated using a QIAprep Miniprep Kit (Qiagen) according to the manufacturer's instructions for plasmid isolation.

Gene cloning from Alexandrium tamarense

psbD, a plastid gene encoding the D2 protein and carried on minicircles, was examined. Minicircular DNA of A. tamarense strain OFAT0105-8, examined by Iida et al. (2009), was used.
as a template for the following PCRs. Standard PCR was carried out using primers (118, 5′-TCTGTTACTTTATCTGAT GACTGG-3′; 1096, 5′-CTTGGACACCACTCACAAGCG-3′) designed for conserved regions of algal and plant psbD genes. Based on the sequences of the initial PCR products, additional primers targeting Alexandrium psbD were designed. An inward-directed PCR product was obtained by 25 cycles at 94°C for 15s, 58°C for 30s and 68°C for 60s using primers C3s (5′-TTCTACTCAATTTCTGGCAACG-3′) and C3as (5′-GAGAA TTACCCTTTGGCAATAC-3′). The PCR product was ligated into the pTA2 vector (Toyobo) after purification by a GeneClean Kit (Q-BIOgene). An outward-directed PCR product was obtained by 25 cycles at 94°C for 15s, 58°C for 30s and 68°C for 5 min using primers m1as (TATCCCAGCTACACCCTCAG) and m1s (CAAGATGGAGGTTCACTTCCAC) and was ligated into the pTA2 vector (Toyobo) after purification by Wizard SV Gel and a PCR Clean-Up System (Promega). High-fidelity KOD DNA polymerase (Toyobo) was used for both PCRs. Templates for sequence analyses were prepared using a BigDye Terminator v3.1 kit (Applied Biosystems). Sequencing was carried out using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). All sequence alignments were conducted with MEGA version 3.1 (Kumar et al. 2004), and optimized manually.

**Gene cloning from Alexandrium catenella**

Minicircle DNA of A. catenella strain OFAC9982-101 was used as a template for the following PCRs with two primer sets targeting Alexandrium psbA (primers l and m in lida et al. 2009) and Alexandrium psbD (C3s and C3as described above). The other cloning procedures were the same as in the inward-directed PCR for A. tamarense DNA.

**Detection of multiple variants in the other species or strains**

The presence of multiple psbA and psbD variants in different species of *Alexandrium* was determined by the detection of multiple products of amplification with primers targeting psbA (153, 5′-GACACGCTTATTCTGGCAACAC-3′; IPas2, 5′-ATACATCCCAGGACTTCTG-3′) or psbD (118 and 1096 described above). A PCR product was obtained by 25–35 cycles at 94°C for 15s, 58°C for 30s and 72°C for 60s using Taq polymerase (Toyobo).

Gene-specific PCRs were performed to examine the distribution of psbA variants in strains of *A. catenella* and *A. tamarense* using gene-specific primers targeting the boundaries of DNA rearrangements (insertions or deletions) in each psbA variant. Two rounds of amplification were performed. Reaction conditions for the first round of PCR were the same as those for multiple products but with different primers (128A, 5′-GTGG GCTGGCAATTATGTTATA-3′; and IPas1, 5′-CAAGACAAAA TAACCATGACAGC-3′). The amplified products were purified using a GeneClean Kit (Q-BIOgene) and used as templates in the second round of PCR. The second-round PCR products were obtained by 25 cycles at 94°C for 10s, 60°C for 20s and 72°C for 20s. In addition to the psbA primer (IPas2 described above), a primer specific to ACpsbA-V1 was used in *A. catenella* (S1s1, 5′-TTCTATCTGAATGGGAGTTG-3′). Gene-specific primers for the psbA variants of *A. tamarense* were the same as those reported in the previous study (a, e, n, o and p in lida et al. 2009).

**Supplementary data**

Supplementary data are available at PCP online.

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**References**


