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Population Analysis of Korean and Japanese Toxic *Alexandrium catenella* Using PCR Targeting the Area Downstream of the Chloroplast *PsbA* Gene

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The marine dinoflagellate genus *Alexandrium*, which produces PSP toxins, has a global distribution. As human-assisted dispersal of the species has been suggested, it is important to develop molecular tools to trace the dispersal pathway. To screen population-specific DNA sequences that differentiate Korean and Japanese *A. catenella*, we targeted the area downstream of the chloroplast *psbA* gene using PCR with population-specific DNA primers followed by RFLP (restriction fragment length polymorphism) analysis and sequencing. The RFLP patterns of the PCR products divided Korean and Japanese *A. catenella* regional isolates into three types: Korean, Japanese, and type CMC3, isolated from Korea. We sequenced the PCR products, but found no similar gene in a homology search. The molecular phylogeny inferred from the sequences separated the Korean and Japanese *A. catenella* strains, as did the RFLP patterns. However, the Japanese isolates included two slightly different sequences (types J and K), while the Korean sequence was the same as the Japanese K type. In addition, a unique sequence was found in the Korean strains CMC2 and CMC3. Population-specific PCR amplification with Japanese *A. catenella* type-specific PCR primers designed from the type J sequence yielded PCR products for Japanese strains only, showing that the unknown gene can be used for a population analysis of Korean and Japanese *A. catenella*.

Key words: *Alexandrium catenella*, RFLP (restriction fragment length polymorphism), Population-specific DNA, Population analysis

Introduction

The abundance of phytoplankton, which is responsible for primary production, acts as a measure of aquatic productivity in a water system. However, phytoplankton blooms, along with progressive eutrophication and weather variation, can cause massive, frequent red tides in marine coastal waters. Red tides, caused primarily by dinoflagellates, can cause economic loss and threaten human health. The genus *Alexandrium* contains many members that can produce paralytic shellfish poisoning (PSP) toxin, which is lethal to humans, making it the most notorious marine dinoflagellate group. Ultrastructural comparisons based on external morphology, phylogenetic relationships, and toxin analyses have been used to define and differentiate very closely related members within *Alexandrium*.

The impact of this genus, especially the toxic species, is further exacerbated by its global distribution. Several studies have demonstrated the geographic expansion of the genus *Alexandrium* (Anderson, 1989; Hallegraeff and Bolch 1991, 1992; Hallegraeff, 1993; Scholin et al., 1995), and several scenarios have been proposed to explain its dispersal. The life cycles of most members of *Alexandrium* include a dormant cyst, which is resistant to environmental stress. These tough cysts can hitchhike in the ballast water of ships or spread naturally with marine currents, resulting in the translocation of exotic organisms. Increased awareness of PSP and improved

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scientific methodology have advanced our understanding of these organisms. In addition, the enhanced growth of endemic populations caused by nutrients dumped into coastal waters has attracted attention to these organisms. These factors have all combined to accelerate the dispersal of the genus *Alexandrium*. The lack of a fossil record makes it difficult to study its evolutionary history and origin. However, whatever the reason, we cannot deny that human assistance has contributed to its spread, as demonstrated by Scholin et al. (1994, 1995). If human activity is a major contributor to the global distribution of *Alexandrium*, it must be controlled. Tracking the distribution and transport pathways might be possible if we can identify regional isolates of the genus *Alexandrium* as tracers to resolve the dispersion pathways.

Sequence analyses of the SSU and LSU rDNA have been used to resolve the phylogeny of *Alexandrium* and to resolve fine-scale differences among populations. Scholin and Anderson (1994) and Scholin et al. (1994, 1995) compared the RFLP pattern of SSU rDNA with a phylogeny inferred from the sequences of the LSU rDNA D1-D2 genes of *A. tamarense*, *A. catenella*, and *A. fundyense* collected from North America, western Europe, Japan, Australia, Thailand, and the ballast water of several cargo vessels. They showed that the 'tamarense species complex' was divided into several populations and suggested that rDNA genes containing pseudogenes are useful sequences for population analysis. Lilly et al. (2002) presented an example of the dispersal of a toxic *Alexandrium* species via human activity using thecal plate morphology, RFLP, DNA sequences, and toxin analyses of *A. catenella* in Thau Lagoon, France; they suggested that the French strain was closely related to a population found in temperate Asia.

Although rDNA sequences have been useful for grouping some populations, population-specific sequence variation in rDNA sequences is very infrequent. On the other hand, pseudogenes could potentially be used for population analysis, but their sequences are usually too diverse. Kim (2003) demonstrated this dilemma in a study of Korean and Japanese *A. catenella* and *A. tamarense* strains. There were no differences in expressed rRNA sequences, while the pseudogene types (containing the B gene of Scholin et al. 1993) were very diverse and difficult to use as population-specific markers. Therefore, it is necessary to find more variable gene regions for designing PCR primers and DNA probes to be uses as practical population markers.

This study targeted the region downstream from the 3'-end of *psbA* to develop a population-specific PCR marker for discriminating Korean and Japanese *A. catenella*, using RFLP patterns and sequence comparison.

Materials and Methods

Strains and culture condition

Nine mono-algal cultures of *A. catenella* were isolated from Korean and Japanese coastal waters for population analysis: YSC2 (Yosu, Korea), CMC2 and CMC3 (Tongyong, Korea), DPC7 and DPC8 (Busan, Korea), ACY12 (Harima Nada, Japan), TN4 and TN11 (Tanabe Bay, Japan), and OF72 (Ofunato Bay, Japan). The *A. catenella* species designation was made based on ribotype (Adachi et al., 1996; Kim, 2003). All the cultures were maintained under a 14-h light:10-h dark regime at 100 $\mu Em^{-2}S^{-1}$ with cool white bulbs at 20℃ and were grown in 2.8-L Fernbach flasks on SWII medium (Sako et al., 1990) made with filtered seawater as the base.

Total DNA extraction and purification

Cells were harvested between late exponential and early stationary growth phases by centrifugation for 5 min at 2,000 rpm. Pelleted cells were stored at -80℃. They were ground completely in liquid nitrogen using a pestle and mortar. Total DNA was extracted using a SepaGene kit (Sanko Junyaku, Tokyo, Japan) and digested using RNase A at 37℃ for 12 h. The extracted total DNA was purified by ethanol precipitation and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

PCR-amplification and RFLP (restriction fragment length polymorphism) analysis

The region downstream of the *psbA* (PS II) gene in the chloroplast genome was targeted for RFLP analysis. As shown in Table 1, four forward primers at the 3'-end of the eukaryote *psbA* gene and four reverse random primers were designed for PCR amplification. The composition of the PCR reaction mixture followed the manufacturer's recommendations (LA PCR™ kit, Takara Shuzo, Japan). The PCR amplifications were run at 96℃ for an initial 3 min, followed by 35 cycles of 94℃ for 30 sec, 60℃ for 30 sec, and 72℃ for 2 min, with a final extension at 72℃ for 7 min. The PCR products were subjected to 1.2% agarose gel electrophoresis, stained with

Table 1. Oligonucleotide primers used to analyze Korean and Japanese *Alexandrium catenella* populations

Primers	Sequence (5' to 3')
PsbAlong1F	GTCATAGCCCATCTATCTTT
Random1R	TAATTATCAGGCCCTACAGG
PsbAlong2F	CTTAGTGGCATGCAACCCTT
Random2R	GACCCTTGGTGGCTTAATTG
PsbAlong3F	ACGGGCATTATGACGCTCAT
Random3R	CTCGATGTCTTGGGCATGGA
PsbAlong4F	TGATTCAGGCATGATTCATG
Random4R	TCGTGCTTAACGCATGCTCA

ethidium bromide, and photographed under UV illumination. PCR products with clear, strong signals were excised from the agarose gel for RFLP analysis and sequence determination.

The excised PCR products for Korean *A. catenella* strains DPC7, DPC8, YSC2, and CMC3, and for Japanese strains OF72, TN11, and ACY12 were subjected to agarose gel-purification and digested with *Hae*III (Takara Shuzo, Japan) for 12 h at 37℃. The digested PCR products were subjected to 1.2% agarose gel electrophoresis, stained with ethidium bromide, and photographed, as above.

Cloning and sequencing

The PCR products from all of the *A. catenella* strains were purified as above, dissolved in TE buffer, ligated into pGEM-T Easy Vector (Promega, USA) according to the manufacturer's instructions, and transfected into competent *Escherichia coli* cells (INV αF'). Ten to 20 clones were selected for each *A. catenella* isolate, and plasmids containing the inserts were purified with a Miniprep kit (Bio-Rad). Six to 12 clones were sequenced using a *Taq* Dye Deoxy Terminator Cycle Sequencing Ready Reaction Mixture kit (Amersham Biosciences) following the DNA sequencing protocol. Sequence reactions were run on an ABI 373A automated sequencer (Applied Biosystems, USA). The nucleotide sequences obtained from each clone were analyzed using the software DNASIS-Mac (Hitachi Software Engineering, Japan).

Results

Three of the four primer sets yielded PCR products; the exception was the set PsbAlong4F and PsbAlong4R. The PCR product produced with the primer set PsbAlong1F/PsbAlong1R was an approximately 1.5-kb fragment (Fig. 1) and was subjected to RFLP analysis and sequence determination. The primer set

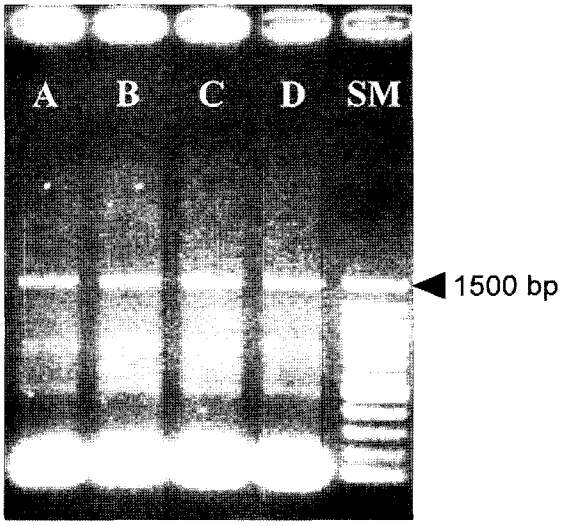

Fig. 1. The PCR product amplified with the primer pair PsbAlong1F and Random1R from *Alexandrium catenella* DPC7 (A), CMC3 (B), TN11 (C), and ACY12 (D). SM and arrowhead indicate a molecular size marker and 1.5 kb, respectively.

PsbAlong2F/PsbAlong2R produced ladder-like bands, while PsbAlong3F/PsbAlong3R resulted in a smear (data not shown). The RFLP pattern of the PCR product digested with *Hae*III divided the regional isolates into three groups: a Korean type consisting of YSC2, DPC7, and DPC8, a Japanese type comprising TN11 and ACY12, and a third type, CMC3 (Fig. 2). The Japanese strain OF72 was identical to the Korean type, although undigested PCR product remained (Fig. 2, lane 9). These different enzyme-restricted types seemed to generate from type-specific sequence variation or the gene copy numbers among *Alexandrium* strains. Unexpectedly, the partial sequences of the PCR product were similar to the chloroplast nucleomorph chromosome of *Guillardia theta* (Cryptomonadaceae), but with 70.0% homology (therefore, we considered it an unknown gene). Sequence comparisons divided the Korean and Japanese *A. catenella* strains into three types: 1) Korean strains CMC2 and CMC3 were unique; 2) the Japanese strains ACY12 and OF72 included two types, a unique Japanese type (J) and type common with the Korean type (K); and 3) the Korean strains YSC2, DPC7, and DPC8 consisting of type K only, which was identical to the Japanese type K (Fig. 3). Although ACY12 harbored two types (J and K), the latter was uncommon, being found in only 2 of 12 insert DNA clones generated from strain ACY12. Although OF72 also harbored both types, the clones sequenced were

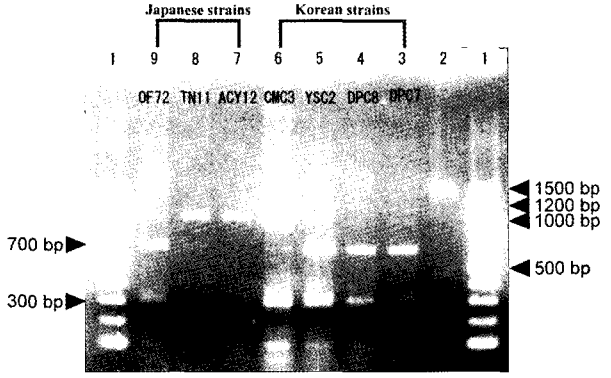

Fig. 2. The RFLP patterns generated by digesting the PCR fragment amplified from Korean and Japanese *Alexandrium catenella* regional isolates with the restriction endonuclease *Hae*III. Lanes 1 and 2 are a molecular size marker and the PCR fragment (see Fig. 1), respectively.

not sufficient to determine the proportions of the population. These sequences were subjected to a molecular phylogenetic analysis inferred from the partial sequences, as shown in Fig. 4.

In order to confirm group specificity, specific PCR primers were designed for the Japanese strain (type J in Fig. 3): JspecificF (5'-GTTTCAATCAAGTTC-ATCACG-3') and JspecificR (5'-TCGGGCTGCTCT-GAAATTGT-3') (see underlined sequences in Fig. 3). PCR amplification was carried out, with primers PsbAlong1F and Random1R as positive controls.

Fig. 5 shows that the common control primers amplified an approximately 1.5-kb PCR product in all strains (right lanes), while the Japanese *A. catenella*-specific primers amplified an approximately 1.3-kb product with some minor fragments (circled area in fig. 5) in all of the Japanese strains, except for the Korean strain CMC 3 showing smear signals.

Discussion

In order to develop a population-specific PCR marker for discriminating Korean and Japanese *A. catenella*, we PCR-amplified, cloned, and sequenced an unknown gene generated from the chloroplast DNA of Korean and Japanese *A. catenella* strains. The RFLP patterns and sequences of the gene produced three types, which divided the regional strains into Korean, Japanese, and CMC types. A prominent band

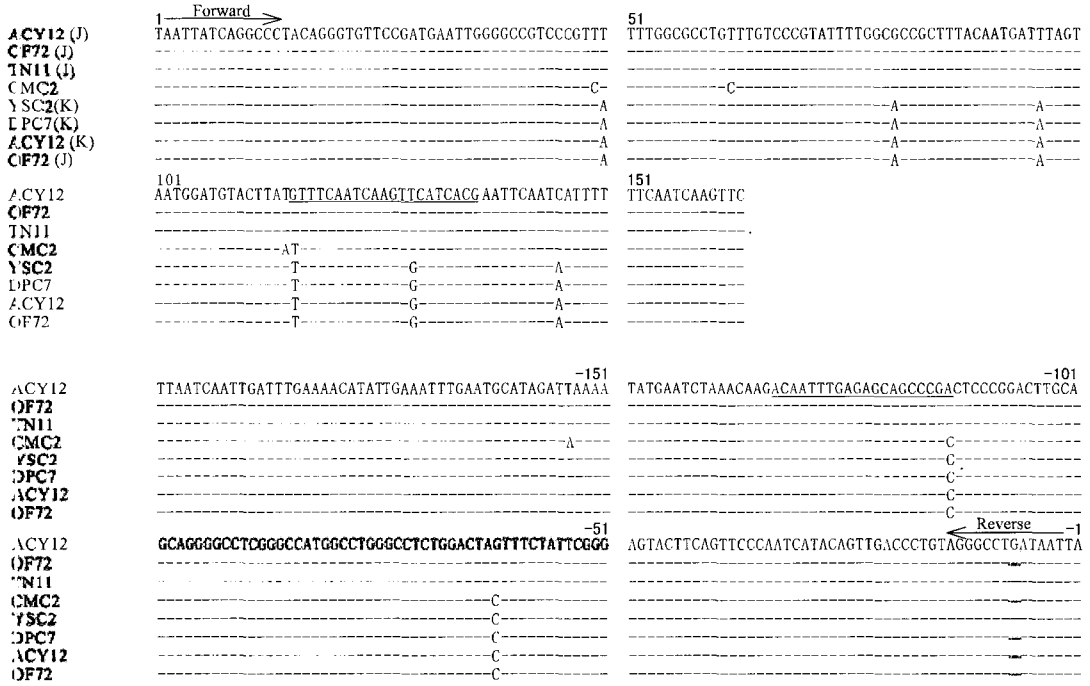

Fig. 3. Aligned partial sequences of PCR fragments amplified with primers PsbAlong1F and Random1R from Korean and Japanese *Alexandrium catenella* isolates, showing nucleotide variations between Korean and Japanese isolates. Letters in parentheses were used to differentiate each common sequence type: J, Japanese type; K, Korean type. Note that overlapped isolates (ACY12 and OF72) harbor two different sequence types. Identity with the ACY12 sequence is indicated by hyphen (-). The sequences are presented from 5' to 3'.

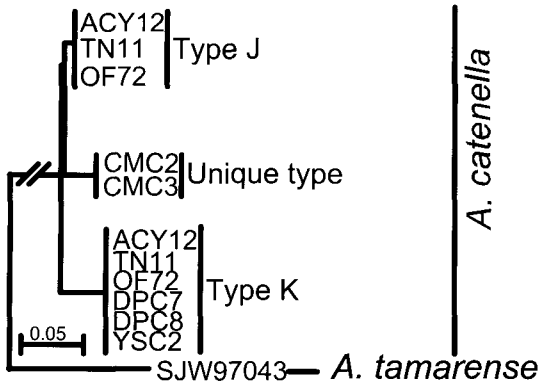

Fig. 4. Putative phylogenetic tree inferred from PCR-fragment sequences of chloroplast DNA from Korean and Japanese *Alexandrium catenella* regional isolates. The tree was constructed using the NJ method (Saitou and Nei, 1987) and Clustal X (Tomson et al., 1997). *Alexandrium tamarense* isolated from Chinhae Bay, Korea, was used as the outgroup taxon.

in the RFLP pattern grouped the Japanese OF72 with the Korean type, although the OF72 sequences included both the Korean and Japanese types. ACY12 was the Japanese type based on the RFLP pattern, but its sequences were identical to those of OF72 and included the Korean type at low frequency (6:1, Japanese: Korean type). By contrast, the sequence results showed that there was a single Korean type, except for the unique CMC2 type. The disagreement between the RFLP pattern and the sequence results might be a result of the weak signal of the RFLP pattern, *i.e.*, perhaps the Japanese strains contained bands identical to the Korean strains, but the signal was too weak to detect the latter bands because there were very few gene copies of the Korean type.

Above results imply that strains of Korean type K were transported to the Japanese coast, where they met indigenous Japanese J type strains. Although the strains differed genetically, they were compatible sexually, and genetic recombination resulted. We expected that three types will be found in the two countries: Korean unique, Japanese unique and common type, but the results of this study suggest that two types have been existed in Korea and there has been one-way transport, *i.e.*, strains of the Korean K type were transported to Japan, perhaps by the Kuroshio Current rather than in a ship's ballast waters. A more detailed population analysis using more strains from a broader region is necessary before we can draw firm conclusions.

PsbAlongF1 and PsbAlongR1 did not produce results in *A. tamarense*, but new primers designed from *A. catenella* sequence yielded PCR fragments from *A. tamarense* strains. The sequences of the *A. tamarense* strains were significantly different from those of the *A. catenella* strains, and the variability among the *A. tamarense* regional strains suggested that the sequences could be used as a population marker (data not shown).

Our results indicate that the unknown gene found in this study could be used for a population analysis of *A. tamarense* and *A. catenella*, although it is still necessary to develop a population-specific marker, such as DNA probes that can be used for fluorescent in situ hybridization (FISH) methods, and to identify the novel gene.

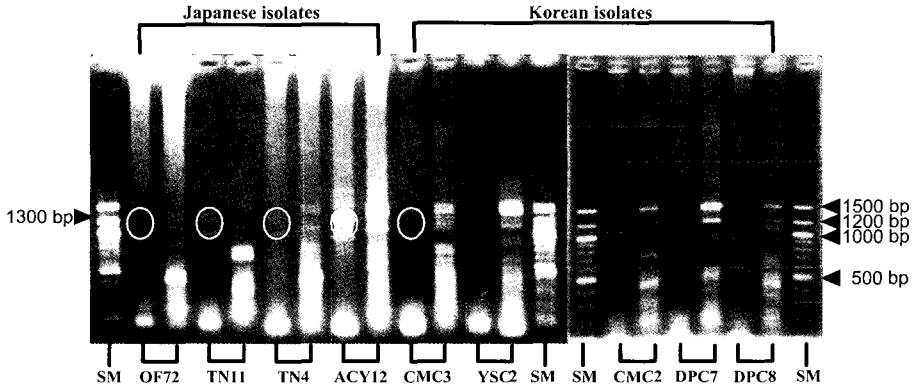

Fig. 5. PCR-amplification patterns of Korean and Japanese isolates using Japanese *A. catenella* type-specific PCR primers. PCR products with Japanese *Alexandrium catenella* type-specific and common primers were loaded in the left and right lanes, respectively. SM indicates a molecular size marker.

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