Development of compound microsatellite markers in the toxic dinoflagellate *Alexandrium catenella* (Dinophyceae)

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Abstract: In this study, we identified 9 polymorphic compound microsatellite markers in the toxic dinoflagellate *Alexandrium catenella*, which was isolated from Thau lagoon (France, Mediterranean Sea), using the compound microsatellite marker technique. These new microsatellites were characterized by screening DNA templates from 43 *A. catenella* clonal strains, which were collected from a seawater sample from Inokushi Bay (Oita Prefecture, Japan). These loci provide one class of highly variable genetic marker: the number of alleles ranged from 3 to 8, and the estimate of gene diversity varied between 0.285 and 0.762. We also analyzed the same 43 DNA samples using microsatellite markers previously identified for *A. catenella*, comparing the PCR amplification success, the number of alleles and gene diversity. These three parameters were not significantly different, showing that the compound microsatellite markers have the same potential to reveal *A. catenella* genetic structure. This simple and efficient method reduces the costs for developing SSR markers and for analyzing the genetic structure of populations, therefore, suggesting the effectiveness of applying this method to other species.

Key words: *Alexandrium catenella*, microsatellite, phytoplankton, SSR markers, toxic dinoflagellate

Introduction

The genus *Alexandrium* comprises a closely related cosmopolitan toxigenic group of morphologically similar species, which is called the *Alexandrium tamarense* “species complex”, including *Alexandrium tamarense* (Lebour) Balech, *A. catenella* (Whedon & Kofoid) Balech and *A. fundyense* Balech. Within this species complex, five or six distinct ribotypes/geographic clades have been identified from phylogenetic analysis of the D1/D2 region in the LSU rDNA: western European, North American, temperate Asian, Tasmanian, tropical Asian, and Mediterranean clades (Scholin et al. 1995, John et al. 2003). These authors

also demonstrated that North American and Japanese isolates of *A. tamarense* show a high degree of genetic similarity, as do the rDNA sequences of Australian and Japanese *A. catenella*. Therefore, it was proposed that the exchange of shellfish stocks between British Columbia and Japan (F. J. R. Taylor, personal communication) and frequent shipping from Japan/Korea to Australia or other countries around the world are mechanisms whereby transfers of *A. tamarense/catenella* could occur (Scholin et al. 1995). In 1995, a bloom of *Alexandrium* sp. was first observed in Thau lagoon, French Mediterranean Sea. In 1998, another bloom leading to high levels of paralytic shellfish toxins detected in shellfish stocks, was attributed to *A. catenella* (Lilly et al. 2002). Since then, the species has been regularly recorded in the phytoplankton in this area, sometimes

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forming toxic blooms. Lilly et al. (2002) claimed that *A. catenella* was introduced from Japan into Thau lagoon via ballast water, because the sequence of the D1/D2 region in the LSU rDNA and the PCR RFLP pattern was similar to those of the Japanese temperate Asian ribotype of *A. catenella*, and until the late 1990s, the temperate Asian ribotype had not been reported outside the western Pacific. However, rDNA data are insufficient to test the dispersal theories because of insufficient polymorphism (Masseret et al. 2006). Until recently, no genetic marker was available for *A. catenella* that could unambiguously distinguish between indigenous and introduced flora. Thus, the development of highly polymorphic genetic markers was needed to test these theories.

Microsatellites (or Single Sequence Repeats, SSRs) are tandemly repeated motifs of 1–6 bases found in all prokaryotic and eukaryotic genomes investigated to date. They are present in both coding and non-coding regions and are usually characterized by a high degree of length polymorphism. Although their mechanism of evolution remains unclear, soon after their first description (Litt & Luty 1989, Tautz 1989) SSRs have been widely employed in many fields, because of their high variability, making them very powerful genetic markers for population discrimination.

We consider that an assessment of the genetic relationships among *A. catenella* populations with highly polymorphic genetic markers will provide the most promising approach to detail dispersal mechanisms. Nagai et al. (2006) have already reported 12 polymorphic microsatellite markers for *A. catenella*. However, to test the hypothesis that *A. catenella* could have been introduced from temperate Asia (e.g., Japan) into Thau lagoon through ballast water or shellfish stocks, additional microsatellite markers are needed. This is because some of the markers gave either too many null alleles, misleading bands, or excrescent ghost bands when a large number of samples were examined. In this study, we identified 9 polymorphic compound microsatellite markers in the toxic dinoflagellate *A. catenella* using a recently developed isolation technique (Lian et al. 2006, Nishitani et al. in press). We also compared the PCR amplification success (number of non-amplifying samples), the number of alleles and the gene diversity of microsatellite markers developed using different methods to evaluate the performance of the compound SSR primer method.

Materials and Methods

Total genomic DNA was extracted from three cultures isolated in Thau lagoon (France, Mediterranean Sea) in 2002 from germinated resting cysts (strains ACT1 and ACT2), and in 2003 from vegetative cells (ACT3). Cells were grown in natural seawater medium enriched according to Harrison et al. (1980). Genomic DNA was extracted using a DNeasy Plant Mini Kit (QIAGEN). The compound microsatellites were prepared according to Lian et al. (2006), as depicted in Fig. 1. The DNA was separately di-

gested with *Afa*I, *Alu*I and *Hae*III blunt-end restriction enzymes (Fig. 1-A). The DNA fragments were then ligated with a blunt adaptor, consisting of a 48-mer (5'-GTAATAC-GACTCACTATAGGGCACGCGTGGTTCGACGGCC-CGGGCTGGT-3') and a 8-mer with the 3' end capped by an amino residue (5'-ACCAGCCC-NH₂-3'), using a DNA Ligation Kit (TakaraBio, Japan) (Fig. 1-B). This blocks polymerase catalyzed extension of the shorter adaptor strand, preventing the generation of a primer binding site unless a defined, distal, gene-specific primer extends a DNA strand opposite the upper strand of the adapter (Siebert et al. 1995).

To concentrate the microsatellite regions, fragments flanked by a microsatellite at one end were amplified from the constructed libraries by compound SSR primers (AC)₆(AG)₅ or (AC)₅(TC)₇ and an adaptor primer AP2 (5'-CTATAGGGCACGCGTGGT-3') designed from the longer strand of the adaptor (Fig. 1-C). The amplified fragments were subcloned and sequenced (Fig. 1-D). For each fragment containing (AC)₆(AG)_n or (AC)₅(TC)_n compound sequences at one end, a specific primer (SP) was designed from the sequenced region flanking the microsatellite and screened (Fig. 1-E). The primer pairs of specific primer (SP) and compound SSR primer were used as a compound microsatellite marker.

To examine the suitability of the 66 primer pairs designed as compound markers, PCRs using the three DNA templates mentioned above were performed on a thermal cycler (PC-808, ASTEC) in a reaction mixture (10 µl) containing 1–10 ng of template DNA, 0.2 mM of each dNTP, 1X PCR buffer (10mM Tris-HCl, 50 mM KCl, Mg²⁺ free, pH 8.3, Applied Biosystems), 2.5 mM of Mg²⁺, 0.25 U of Ampli Taq Gold (Applied Biosystems) and 0.5 µM of each SP and a TAMRA-labelled compound primer (AC)₆(AG)₅ or (AC)₅(TC)₇. PCR cycling conditions were as follows: 9 min at 94°C, 38 cycles of 30 s at 94°C, 30 s at the primer-specific annealing temperature (Table 1), and 45 s at 72°C, and a final elongation of 5 min at 72°C. To characterize the candidate microsatellites, we screened DNA samples from 43 *A. catenella* clonal strains isolated from Inokushi Bay (Oita Prefecture, Japan), in 2006. The PCR products were denatured at 95°C for 5 min, and electrophoresed on 6% Long Ranger sequencing gels (Cambrex Bio Science) using a Gel-Scan 3000 (Corbett Robotics). The size of alleles was determined according to a size marker (GeneScan 350, Applied Biosystems) with the ONE DSCAN EX version 3.02 software (Scanalytics).

Results and Discussion

Nine of the 66 primer pairs were selected, the other 57 being rejected because of the low level of genetic variation or the occurrence of unexpected PCR bands. The characteristics of 9 compound microsatellites are described in Table 1. Each of these loci showed a single band in each individual, consistent with the haploid genome of this species. Six

Fig. 1. Schematic representation for developing compound microsatellite markers (Lian et al. 2006). The basic procedure of this method is as follows; A) digestion of genomic DNA with blunt-end restriction enzymes, B) adaptor ligation of fragmented DNAs with an unequal length adaptor, C) enrichment of compound SSR regions by PCR amplification using compound SSR primers and an adaptor primer, D) sub-cloning and sequencing of the compound SSR regions amplified by the PCR, E) design and screening of SP primers as microsatellites.

loci (FMAC06, FMAC08, FMAC09, FMAC16, FMAC49 and FMAC55) occasionally yielded no visible PCR band in some individuals, possibly due to the presence of null alleles at these loci. Linkage disequilibrium for all pairs of loci was tested with GENETPOP version 3.4 available on the web (Raymond & Rousset 1995): no significant linkage disequilibrium was found. Numbers of non-amplifying samples were 0–8 with an average plus standard deviation of 2.0 ± 2.5 . Number of alleles at the 9 loci ranged from 3 to 8 with an average plus standard deviation of 5.2 ± 1.9 , and the estimate of gene diversity (Nei 1987) varied between 0.285 and 0.762 with an average plus standard deviation of 0.543 ± 0.177 .

The same 43 DNA samples were analyzed in parallel using 11 microsatellites, out of 12 previously developed for

Alexandrium catenella by Nagai et al. (2006), for comparison with the 9 new compound microsatellite loci. We excluded one of the 12 markers previously reported (Acat44) due to a significant linkage disequilibrium with Acat06. Numbers of non-amplifying samples were 0–19 with an average plus standard deviation of 4.5 ± 6.3 . Number of alleles ranged from 1 to 8 with an average plus standard deviation of 4.7 ± 2.4 , and the estimate of gene diversity (Nei 1987) varied between 0.000 and 0.810 with an average plus standard deviation of 0.550 ± 0.265 (Table 2). The three parameters analyzed, using microsatellite markers isolated by the two different methods, were not significantly different according to the Mann-Whitney test, showing that the compound microsatellite markers have the same potential to reveal *A. catenella* genetic structure as have the loci iso-

Table 1. Primer pairs for amplification of 9 polymorphic compound microsatellite regions in the toxic dinoflagellate *Alexandrium catenella* and some characteristics of the microsatellite loci. T_a indicates annealing temperature; gene diversity was calculated after Nei (1987). The number of clonal strains screened was 43 at each locus.

Locus	Repeat motif	Primer sequence	T_a (°C)	No. of non-samples amplifying	No. of alleles	Size range (bp)	Gene diversity	Genbank accession number
FMAC06	(AC) ₆ (AG) ₈	F: TAMRA-ACACACACACACAGAGAGAG R: ACTAGCATGTTCCACAGCAGTTG	54	1	3	155–159	0.316	AB294559
FMAC08	(AC) ₆ (AG) ₁₁	F: TAMRA-ACACACACACACAGAGAGAG R: CAAGCTGCCTCCGGTTTGATAG	54	3	7	121–132	0.725	AB294560
FMAC09	(AC) ₆ (AG) ₇ CG(AG) ₂	F: TAMRA-ACACACACACACAGAGAGAG R: AAGCTCAGACGTTACCACTCG	54	8	8	159–170	0.762	AB294561
FMAC16	(AC) ₆ (AG) ₉	F: TAMRA-ACACACACACACAGAGAGAG R: CAGCCCTGTTTGTGTCCG	57	1	6	220–230	0.700	AB294562
FMAC37	(AC) ₅ (TC) ₇	F: TAMRA-ACACACACACTCTCTCTCTCTC R: CCACATTAACCTGGTAGTCGC	58	0	3	99–101	0.553	AB294563
FMAC49	(AC) ₆ (AG) ₅ (AC) ₆	F: TAMRA-ACACACACACACAGAGAGAG R: ACCCATGTGTACTGTGCCCC	54	2	7	100–112	0.559	AB294564
FMAC54	(AC) ₆ (AG) ₆	F: TAMRA-ACACACACACACAGAGAGAG R: CGAAGGGTTATATCACGGC	57	0	4	77–81	0.285	AB294565
FMAC55	(AC) ₆ (AG) ₇	F: TAMRA-ACACACACACACAGAGAGAG R: CAAACTGTTCCATGCACCA	57	3	5	100–109	0.595	AB294566
FMAC57	(AC) ₆ (AG) ₆	F: TAMRA-ACACACACACACAGAGAGAG R: GTAGTCTCTCGTGCAATGTAAGGT	57	0	4	155–165	0.388	AB294567
Average				2.0	5.2		0.543	
SD				2.5	1.9		0.177	

lated by the primer extension method (Nagai et al. 2006). The basic procedure of this method is as follows; 1) genomic DNA fragmentation to a size of 500 to 900 bp, 2) preparation of single-strand DNAs by helper phages after transformation of fragmented DNAs, 3) construction of a (CA)_n-enriched library by primer extension, 4) transformation and sequencing of the resultant double-strand DNAs, 5) design and screening of primer pairs as microsatellites (Sekino et al. 2000). By comparing the two methods used for development of microsatellite markers (Table 3), the isolation efficiency was similar in both methods and they were 13.6% and 12.6%, respectively. The numbers of non-labeled and fluorescence-labeled primers that we purchased were 66 and 2, respectively, for the compound SSR primer method (the present method), whereas they were 95×2 and 17, respectively, for the primer extension method, showing better economic performance for the present method. The time duration needed for the development of microsatellite

markers in the present method was shorter than that for the primer extension method. However, the present method may not be as effective in some species such as *Gymnodinium catenatum* Graham due to the low level of genetic variations in compound SSR regions of (AC)₆(AG)₅ or (AC)₅(TC)₇ (Nagai et al. unpublished data). Motifs of (TC)₆(AC)₅ and (CT)₆(CA)₅, (AC)₅(TC)₇ or (TC)₅(TG)₇ were useful in *Chattonella* species (Raphidophyceae) (Nishitani et al. in press) and the blue coral *Heliopora coerulea* (Pallas) (Octocorallia) (Yasuda et al. unpublished data), respectively. In conclusion, at least in *A. catenella*, the compound SSR primer method is a relatively simple, efficient and cheap method for developing SSR markers. Since the method only requires fluorescent labeling of the compound SSR primer for analyzing all related SSR markers, it also reduces the cost for analyzing the genetic structure of populations.

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Table 2. PCR amplification using the microsatellite primers for *Alexandrium catenella* previously developed by the primer extension method (Nagai et al. 2006). Gene diversity was calculated after Nei (1987). The number of clonal strains screened was 43 at each locus.

Locus	No. of non-amplifying samples	No. of alleles	Gene diversity
Acat02	14	2	0.186
Acat06	5	8	0.810
Acat10	5	5	0.749
Acat14	0	2	0.454
Acat16	1	6	0.789
Acat20	19	7	0.708
Acat22	1	6	0.753
Acat34	2	7	0.641
Acat37	0	1	0.000
Acat49	1	3	0.414
A8cat50	1	5	0.544
Average	4.5	4.7	0.550
SD	6.3	2.4	0.265

Table 3. Comparison of two different methods for the development of microsatellite markers in the toxic dinoflagellate *Alexandrium catenella*.

Method	Isolation efficiency [§]	Number of primers [‡]	Fluorescence labeled primers [†]	Duration (Weeks) [¶]
Compound SSR primer method (this study)	9/66 (13.6%)	66	2	3
Primer extension method (Nagai et al. 2006)	12/95 (12.6%)	95×2	17	4

[§], the number of microsatellite markers developed/the number of sequences determined; [‡], the number of non-labeled primers purchased; [†], the number of fluorescence-labeled primers purchased; [¶], the time duration for the development of microsatellite markers starting from DNA extraction until the end of the microsatellite primer screening.

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