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A Versatile Method for DNA Sequencing of Unpurified PCR Products using an Automated DNA Sequencer and Tailed or Nested Primer Labeled with Near-infrared Dye: A Case Study on the Harmful Dinoflagellate *Alexandrium*

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DNA sequence-based typing is considered a robust tool for the discrimination of dinoflagellate species because of the availability of extensive rDNA sequences. Here, we present a rapid, cost-effective DNA-sequencing technique for various PCR products. This sequencing strategy relies on "nested" or "tailed" primer labeled with near-infrared dye, and uses a minimal volume of unpurified PCR product (ca. 5 μL) as the DNA template for sequencing reactions. Reliable and accurate base identification was obtained for several hundred PCR fragments of rRNA genes. This quick, inexpensive technique is widely applicable to sequence-based typing in clinical applications, as well as to large-scale DNA sequencing of the same genomic regions from related species for studies of molecular evolution.

Key words: *Alexandrium tamarense*, Dinoflagellate, Direct DNA sequencing, 18S rDNA

Introduction

To date, more than 30 species of the genus *Alexandrium* have been described (Balech, 1995), and some can form blooms that cause serious economic problems for aquaculture industries (Hallegraeff, 1995; Moestrup et al., 2002). To monitor the expansion and minimize the noxious effects of toxic algae, it is important to rapidly detect and correctly identify the microalgal species involved. Their identification is mainly based on morphological features, particularly the plate arrangement and apical pore complex (Han et al., 1992). These fine structures, however, are extremely difficult to discern, and it is necessary to observe them under a light microscope using high magnification because the plates are extremely small (Ki et al., 2004).

Over the last two decades, many methods based on biomolecules such as isozymes, toxins, and nucleic acids have been developed to facilitate the detection of harmful dinoflagellates. Tools that have been used recently include polymerase chain reaction (PCR) detection methods (Galac et al., 2003; Galluzzi et al., 2005), real-time PCR assays (Bowers et al., 2000; Gray et al., 2003), and sequence-based typing (Ki et al., 2004). More recently, microarray technology has been used for the simultaneous analysis of large numbers of harmful algal bloom (HAB) species (Ki and Han, 2006). Most detection methods are based on 18S, 5.8S, internal transcribed spacer (ITS), or 23-28S rDNA sequences. In fact, the number of rDNA sequences available has increased gradually, and they are easily accessible in public databases. In addition, many complete rDNA sequences, particularly 18S rDNA and ITS regions, have been determined (Ki and Han, 2005). Thus, it is not surprising that many studies have been performed on the taxonomy, phylogeny, and genetic diversity of dinoflagellates (Scholin and Anderson, 1996; Guillou et al., 2002; Murray et al., 2005; Ruiz Sebastián et al., 2005).

PCR tools are used to amplify targeted genomic DNA regions, and the products are used as template DNA in sequencing reactions. For the direct sequencing of PCR products to be useful, purification of the PCR product is required. Many commercial systems have been developed to purify PCR products by removing unincorporated components and undesired by-products to prepare the sequencing template (Leonard et al., 1998; Høgdall et al., 1999). To

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be successful, however, these systems require a considerable amount of PCR product, and small PCR products (shorter than about 150 bp in length) prepared in this way often do not provide readable sequence data (Høgdall et al., 1999), possibly because of product loss during the purification procedures.

With the rapid progress in DNA sequencing technologies in recent years, several protocols for the direct sequencing of unpurified PCR products have been developed. These include protocols using asymmetric PCR products (Gyllensten and Erlich, 1988; Wilson et al., 1990; Liu et al., 1993) and fluorescence-labeled primers (Meltzer et al., 1990; Douglas et al., 1993; Ju et al., 1995; Trower et al., 1995). The asymmetric PCR procedure is quite difficult to perform because the technique requires high optimization for each specific template-primer combination (Dowton and Austin, 1994). In addition, unreacted PCR primers and nonspecific fragments that cause rapid re-annealing of unpurified PCR products can compete with the sequencing primer in binding to the DNA template and decrease the overall nucleotide signal, preventing efficient sequencing.

Another sequencing strategy uses near-infrared dye (IRD)-labeled primers. Presumably as a result of low background fluorescence and high sensitivity, lengths in excess of 1000 bases per sample can be read at 99% accuracy with the Long READIR 4200 automated DNA sequencer (LI-COR, Lincoln, Nebraska, USA) using this method (Marziali and Akeson, 2001). However, despite the attractiveness of IRD, few studies have been conducted on the direct sequencing of unpurified PCR products using IRD-labeled primers. We sequenced the 18S ribosomal DNA genes of the toxic dinoflagellate *Alexandrium tamarense* as a model to demonstrate the potential of this strategy. Direct sequencing of unpurified PCR products using IRD was a simple and cost-saving technique because of the minimal volume of PCR product required for the sequencing reaction.

Materials and Methods

Isolation of *Alexandrium* cells

Single *A. tamarense* cells were isolated from field samples collected from Masan Bay, Korea, on 28 March 1997 using the capillary method. The isolate, HY970328M, was grown in flasks sealed with silicon stoppers for gas exchange in f/2 medium (pH 8.2; Guillard and Ryther, 1962), at 15°C under a 12:12-h light:dark cycle and photon flux density of ca. 65 μmol photons m^{-2} s^{-1}.

Cell harvest and DNA purification

Approximately 200 mL of clonal cultures reaching mid-logarithmic phase was filtered through 20-μm pore size nylon mesh and gently washed several times with fresh medium to facilitate the removal of possible bacterial contaminants. Cells concentrated on the mesh were transferred to 1.5-mL microtubes with 100 μL of 1x TE buffer (10 mM Tris-HCl [pH 8.0] and 1 mM EDTA), and the tubes were stored at -20°C until DNA extraction. Genomic DNA was isolated from the stored cells using the DNeasy Plant Mini Kit (Qiagen, Valencia, California, USA) according to the manufacturer's instructions.

PCR strategy with tailed primer

DNA templates for sequencing were prepared using PCR amplification of clean genomic DNA from *A. tamarense* (Fig. 1). A PCR primer set consisting of a forward AT18F02 (5'-AGAACGAAAGTTAAGGGATCGAAGACG-3') and a reverse AT18R01 (5'-GCTTGATCCTTCTGCAGGTTCACC-3') was designed for the conserved sequence of the 18S rDNA

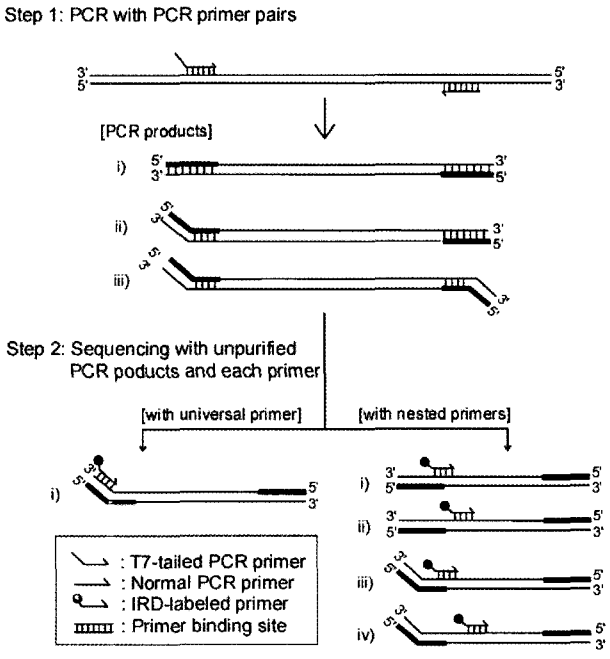

Fig. 1. Schematic representation of the DNA sequencing strategy using unpurified PCR products. In the first step, the same region of nuclear DNA is amplified using three different primer combinations (normal or tailed PCR primer). In the second step, sequencing reactions are performed using the unpurified PCR fragments and the universal or nested primer labeled with IRD. Bold lines indicate the primer parts within the PCR products; ladders represent the sites of primer binding to the templates.

of *A. tamarense*. PCR was additionally conducted usi ng a T7-tailed AT18R01 primer (5'-TACGACTCAC-TATAGGGCCTTCTGCAGGTTCACC-3'), which wa s modified by the addition of a T7 promoter sequence to the 5' end of the readily available standard AT18R0 1 primer (Fig. 1).

The PCR protocol was as follows. PCR reactions were carried out in 1x PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin), <0.1 μg of genomic DNA template, 200 μM of each of four dNTPs, 0.5 μM of each primer, and 0.2 units *Taq* polymerase (Promega, Madison, Wisconsin, USA) per 10 μL of reaction substrate. The PCR thermocycling parameters were as follows: 95°C for 5 min initially, followed by 40 cycles of denaturation at 95°C for 20 s, annealing at 52°C for 30 s, and extension at 72°C for 60 s, followed by a final extension at 72°C for 6 min, using a UNO-II Thermoblock (Biometra, Göttingen, Germany). The PCR-amplified products were checked using agarose gel electrophoresis according to standard methods (Sambrook et al., 1989).

Direct DNA sequencing

The unpurified PCR products (3-5 μL) were subjected to DNA cycle sequencing using a Thermo-Sequenase™ version 2.0 Cycle Sequencing Kit (USB, Cleveland, Ohio, USA) in the presence of 1.5 pM of SAT18R01 primer (5'-GTTACGACTTCTCCTTCC-TC-3') nested within the PCR primers or a universal T7 primer. All of the primers were labeled with IRD at the 5' end. The four base-specific reactions were subjected to 50 cycles consisting of 95°C for 20 s, 55°C for 30 s, and 72°C for 60 s in the UNO-II Thermoblock. When complete, reactions were stopped by adding 4 μL of IR2 stop/loading buffer (LI-COR), and the products were heat-denatured and analyzed on a Model 4200 Dual Dye Automated Sequencer (LI-COR) according to the manufacturer's instructions.

Results and Discussion

Our PCRs followed standard protocols and successfully amplified the target 18S rDNA regions from the genomic DNA of *A. tamarense* using the PCR primers, including a T7-tailed primer. PCR-amplified products using each primer combination (normal or tailed PCR primer) yielded fragments of around 830 bp on a gel (Fig. 2). Generally, the efficiency of amplification was not reduced with tailed primers compared to that with normal primers.

When a DNA band of the expected size was observed on a gel, the PCR products were used directly as the DNA template for DNA sequencing without further checking the DNA concentration. The concentration was >50 ng μL^{-1} because a 50-ng band of double-stranded DNA can generally be detected using ethidium bromide at concentrations as low as 0.5-1 μg mL^{-1}. When the band could not be visualized using ethidium bromide or when its signal was weak, the PCR protocol was adjusted to increase the primer concentration or the number of cycles from 30 to 35. These PCR strategy modifications efficiently improved the copy DNA yield, but also resulted in the presence of nonspecific amplified fragments. However, these unexpected by-products were negligible for DNA sequencing reactions, possibly because of the selectivity of the nested sequencing primers and low background fluorescence of the IRD (Marziali and Akeson, 2001).

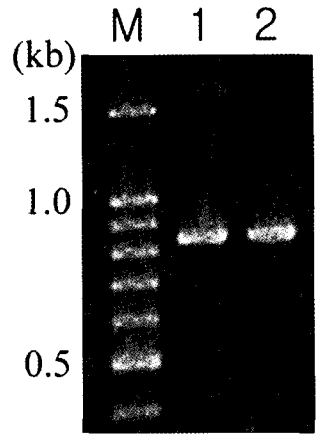

Fig. 2. PCR-amplified products using each primer set yielded fragments of around 830 bp on 1.5% agarose gel. M, molecular marker (100-bp DNA ladder); lane s 1 and 2, PCR fragments amplified from the genomi c DNA of *Alexandrium tamarense* using AT18F02 + 18R01 and AT18F02 + T7-tailed AT18R01 primer, re s- pectively.

The results of DNA sequencing using the nested primer showed improved base quality over that using the T7 primer (Fig. 3) because the nested primer reacted selectively with the target template in the sequencing reaction (see Fig. 1, step 2). These type-nested primers also worked well for DNA sequencing of unpurified PCR products of various biological samples from humans, bacteria, and plants (data not shown). Furthermore, these sequencing reactions were performed with the PCR fragments amplified using *Pfu* (Promega) and *Z-Taq* polymerase (Takara, Tokyo, Japan), and high-quality sequence ladders could be obtained using their products. The tailed-

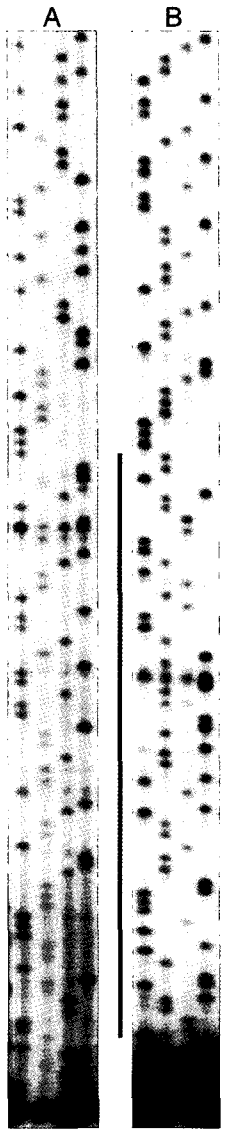

Fig. 3. Sequence data obtained by direct DNA sequencing using (A) nested IRD-labeled primer and (B) T7-tailed primer and the unpurified PCR products of 18S rRNA genes from *Alexandrium tamarense*. Sequences are shown over 100 bases from the primer and the four lanes are in order of A-T-G-C. Nucleotides from the direction of the 5' end of the PCR products could be better read by DNA sequencing using the tailed primer than using the nested primer; the line on panel B indicates these bases.

primer method, in contrast, provided lower-quality sequence data from the unpurified PCR products compared to DNA sequencing using the nested primer. This was because the primer-binding site was restricted to the universal primer part of the tailed PCR primer, and therefore, the T7 primer bound competitively to the target template and the undesired by-products in the sequencing reaction. However, when a DNA band of the expected size could be seen on a gel, the base quality obtained using the T7 primer and PCR products was reliable and accurate (Fig. 3B). The advantage of this method was not needing to synthesize a labeled primer because we used a universal primer for the sequencing reaction. Furthermore, the loss of nucleotides resulting from the binding of primer in the sequencing reaction was minimal because nucleotide elongation was initiated at the 5' end and extended from the nucleotides of the PCR primer (Fig. 3B).

We sequenced 420 PCR product templates ranging between 300 bp and 3 kb in size from 36 strains of harmful dinoflagellates using this method. The sequences that exceeded 750 bp per reaction were determined at a 98% accuracy rate. This method is very simple, rapid, and inexpensive, and very little DNA is lost during the purification process. In addition, this method is suitable for sequence-based typing (e.g., clinical diagnosis, SNP scoring, and the detection of infectious diseases in the fishery industry) and large-scale DNA sequencing of the same genomic regions from related species for molecular evolution studies. Its simplicity also makes it readily amenable to automation.

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