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Short Communication

Discrimination between Atlantic and Pacific Subspecies of Northern Bluefin Tuna (*Thunnus thynnus*) by Magnetic-Capture Hybridization Using Bacterial Magnetic Particles

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Abstract: The previously developed magnetic-capture hybridization technique employing bacterial magnetic particles was applied to discriminate between Atlantic and Pacific subspecies of the northern bluefin tuna (*Thunnus thynnus*) using specific DNA sequences. Nucleotide sequences of a 925-bp fragment (*ATCO*) flanking the mitochondrial ATPase and cytochrome oxidase subunit III genes in these two subspecies were compared. Two regions having single-nucleotide and three-nucleotide differences between the subspecies were adopted to design DNA probes (NR1, 21-mer; NR2, 29-mer), and two internal primer sets were designed to amplify DNA fragments containing these regions. The DNA probes were immobilized on bacterial magnetic particles via streptavidin-biotin conjugation and subjected to magnetic-capture hybridization with the digoxigenin-labeled fragments amplified using the internal primers. The luminescence intensities of DNA on bacterial magnetic particles obtained by hybridization between the probes and the complementary fragments were higher than those obtained by hybridization with noncomplementary fragments. These data suggest that this system employing DNA on bacterial magnetic particles may be useful for discrimination of these two subspecies by recognizing a single-nucleotide difference.

Key words: tuna species identification, mtDNA, northern bluefin tuna, bacterial magnetic particles, magnetic-capture hybridization.

INTRODUCTION

Exact identification of the species and origin of marine products is necessary, particularly for foreign trade. The northern bluefin tuna (*Thunnus thynnus*) is most highly rated as a "sashimi" product for the Japanese market, which may consequently have accelerated illegal fishing and trading irrespective of the decreasing stocks. Currently, the fishing season and amount of catch and trading in the Atlantic subspecies (*T. thynnus thynnus*) are severely regulated. However, discrimination of this species from the Pacific subspecies (*T. thynnus orientalis*) is nearly impossible when the few diagnostic external and internal morphological characteristics are removed, or when they are filleted. Therefore, genetic species identification should be useful.

Protein electrophoresis has been performed for tuna species identification (Sharp and Pirages, 1978; Chow and

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Kishino, 1995), but has failed to discriminate Atlantic and Pacific northern bluefin tuna. Chow and Inoue (1993) and Chow and Kishino (1995) performed analyses based on polymerase chain reaction (PCR) of nucleotide sequence and analyses of restriction fragment length polymorphism on a DNA fragment (*ATCO*) flanking the mitochondrial ATPase and cytochrome oxidase subunit III genes of all tuna species. They reported distinct differences in the restriction profiles and nucleotide sequences between Atlantic and Pacific northern bluefin tunas. However, the nucleotide sequences reported were partial and the analytical procedure was time-consuming, which is undesirable in the field.

We have analyzed the entire nucleotide sequences of the *ATCO* fragments of these two subspecies to find DNA sequences to be used in the design of specific DNA probes. Here, we introduce a system to discriminate between Atlantic and Pacific northern bluefin tuna by magnetic-capture hybridization using bacterial magnetic particles (BMPs), which have previously been used for fluoroimmunoassay (Nakamura et al., 1991, 1993; Nakamura and Matsunaga, 1993), messenger RNA recovery (Sode et al., 1993), as DNA carriers for ballistic transformation (Takeyama et al., 1995), and chemiluminescence enzyme immunoassay (Matsunaga et al., 1996).

Materials and Methods

DNA Sequence Analysis of *ATCO* Region Flanking Mitochondrial ATPase and Cytochrome Oxidase III Genes

Samples of Atlantic northern bluefin tuna (*T. thynnus thynnus*) and the Pacific northern bluefin tuna (*T. thynnus orientalis*) were derived from the laboratory collection of the National Research Institute of Far Seas Fisheries, Shimizu, Japan. Standard phenol-chloroform extraction was used to extract crude DNA from frozen or ethanol-preserved muscle tissue, and DNA samples were dissolved in TE buffer prior to PCR amplification. A PCR primer set and amplification conditions to amplify a flanking region between mitochondrial ATPase and cytochrome oxidase III genes (*ATCO* region) are from Chow and Inoue (1993) and Chow and Kishino (1995). The primer nucleotide sequences are 5′-CTTCGACCAATTTATGAGCCC-3′ (L8562) and 5′-GCCATATCGTAGCCCTTTTTG-3′ (H9432). For nucleotide sequence analysis, amplified PCR fragments were electrophoresed on 1% agarose gel, excised, and cloned into pUC18, which was subsequently used to transform *Escherichia coli* DH5αMCR. Clones containing the target fragment were screened and sequenced using an automatic DNA sequencing machine (DSQ-1000L, Shimadzu).

Immobilization of Biotinylated Oligonucleotide on Bacterial Magnetic Particles

Bacterial magnetic particles 50 to 100 nm in size were extracted from *Magnetospirillum magneticum* AMB-1 according to the method of Matsunaga et al. (1996). Heterobifunctional reagent, Sulfo-LC-SPDP with one *N*-hydroxysuccinimide ester and one pyridyl disulfide, was used for covalent linkage of streptavidin to the BMPs' surface (Matsunaga et al., 1999). Sulfo-SMCC (1 mg) was added to 500 μl of streptavidin solution (200 μg/ml) and incubated for 30 minutes at 30°C with continuous shaking. Streptavidin was preactivated using Sulfo-SMCC that is reactive toward sulfhydryl groups. The sample was then purified on a NAP-5 column (Pharmacia, Uppsala, Sweden) and eluted with 1 ml of phosphate-buffered saline (PBS: 20 mM phosphate buffer, pH 7.2, 150 mM NaCl, 1 mM EDTA, 0.02% sodium azide) according to the manufacturer's instructions. Sulfo-LC-SPDP (2.0 mM) was added to 1 ml of BMP suspension (1 mg/ml). The suspension was treated by sonication and incubated for 30 minutes at room temperature with pulsed sonication (1-minute pulses at 5-minute intervals).

After the incubation, modified BMPs were separated magnetically from the reaction mixture using a neodium-boron magnet and washed three times with 1.0 ml of acetate buffer (100 mM sodium acetate buffer, 100 mM NaCl, pH 4.5). The modified BMPs were dispersed in 1 ml of 0.16 M dithiothreitol (DTT) in acetate buffer and incubated for 30 minutes at room temperature with pulsed sonication for the exchange reaction of thiol group. The surface was subjected to the immobilization of maleimide-activated streptavidin. After washing with 1 ml of PBS, the BMPs having a thiol group were incubated with Sulfo-SMCC-modified streptavidin (maleimide-activated) in aqueous solution for 20 hours at 4°C. Streptavidin-BMP conjugates were washed with PBS three times to remove excess unconjugated streptavidins.

Biotinylated tuna subspecies-specific oligonucleotide probe (130 pmol) was coupled to 50 μg of streptatidinylated BMPs by incubation in 100 μl of PBS for 30 minutes at room temperature. The probe-immobilized BMPs (DNA-BMPs) were washed again with PBS and stored at 4°C.

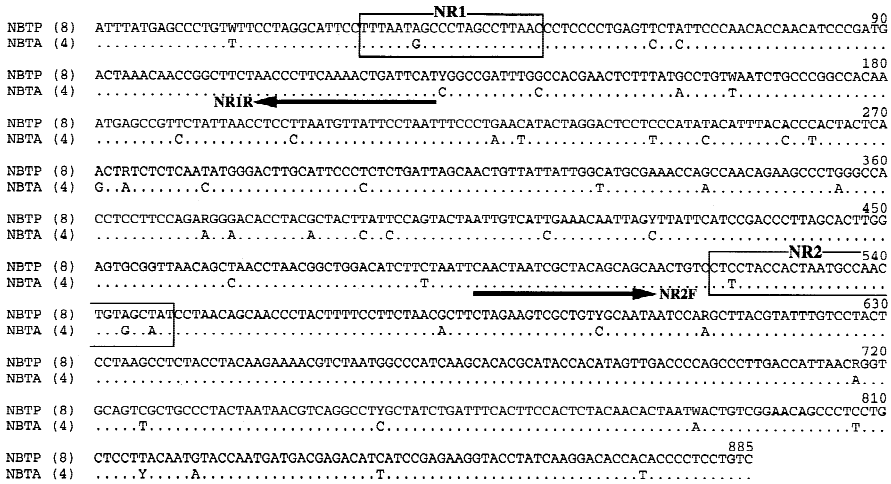

Figure 1. Nucleotide sequence alignment of 885-bp fragments (*ATCO*) between the mitochondrial DNA ATPase and cytochrome oxidase subunit III genes of eight individuals of Pacific northern bluefin tuna (NBTP) and four of Atlantic subspecies (NBTA). R indicates A or G; W, A or T; and Y, C or T. Position and priming directions of two internal primers (NR1R and NR2F) are indicted by arrows underlining the sequence, and positions of NR1 and NR2 probes are boxed. Nucleotide sequences of an individual in each subspecies are available in GenBank under accession numbers AF115272 and AF115276.

Tuna Identification Using Subspecies-Specific Oligonucleotide Probe Immobilized on Bacterial Magnetic Particles

Two internal primers were designed to amplify two internal sequences in the *ATCO* region. PCR amplifications were performed with 25 μM of each deoxynucleoside triphosphate with addition of 10% digoxigenin (DIG)–labeled dUTP as dTTP.

DIG-labeled PCR product (100 ng) was denatured and subjected to hybridization reaction with DNA-BMPs (50 μg). Hybridization was performed at 65°C for 10 minutes in 100 μl of hybridization buffer (50 mM Tris buffer, pH 8.3, 250 mM KCl, 15 mM $MgCl_2$, 0.04 U poly(dI-dC)–poly(dI-dC). The hybridized DNA-BMPs were collected using a neodium-boron magnet and washed three times with PBS (100 μl) containing 1% bovine serum albumin (BSA) and 0.05% Tween 20 to remove unbound DIG-labeled PCR products. The collected DNA-BMPs were then incubated at 37°C for 30 minutes in 100 μl of PBS containing an anti-DIG Fab′ fragment labeled with alkaline phosphatase (AP) (1.0 U of anti-DIG-AP; Boehringer Mannheim, Mannheim, Germany). They, they were magnetically collected again and washed twice with PBS buffer containing 1% BSA and 0.05% Tween 20 to remove unreacted anti-DIG-AP. The luminescence intensity of the hybrid BMPs resuspended in 50 μl of PBS buffer was measured after the addition of substrate (CDP-star with SAPPHIRE II, Roche Diagnostics, Basel, Switzerland) for AP.

Results and Discussion

Nucleotide Sequence Analysis

The amplified fragments were 927 bp in length, and no length differences were observed between individuals. The aligned nucleotide sequences of 885 bp excluding priming sites of eight individuals of the Pacific (NBTP) and four of the Atlantic (NBTA) subspecies are shown in Figure 1. Two regions (NR1 and NR2, boxed in Figure 1) were adopted for designing specific DNA probes. NR1 and NR2 were 21-mer and 29-mer, respectively, and contained single-nucleotide and three-nucleotide differences between the subspecies. Positions and priming directions of two internal primers (NR1R and NR2F) are underlined with arrows in Figure 1. NR1R and NR2F were designed to amplify two fragments containing the NR1 and NR2 regions by combining with L8562 and H9432 primers, respectively.

Tuna Identification by Magnetic-Capture Hybridization Using DNA-BMPs

As the surface of BMPs is negatively charged (Matsunaga et al., 1996), nonspecific binding of target DNA on the BMP surface was not significantly observed (data not shown). However, nonspecific binding of anti-DIG-AP on the surface of BMPs was observed when washing buffer without BSA was used. The addition of BSA to the washing buffer significantly decreased nonspecific binding of anti-DIG-AP

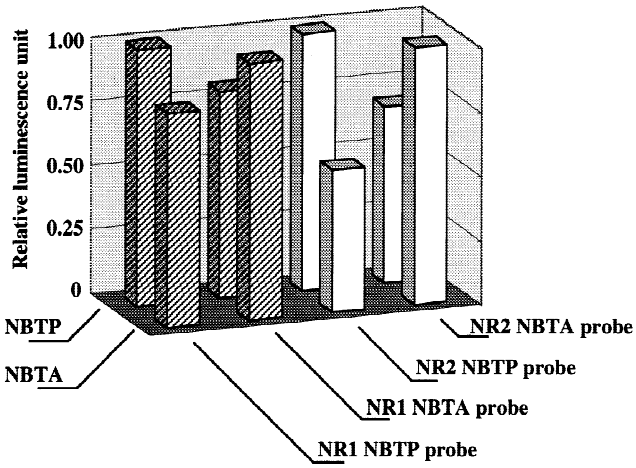

Figure 2. Luminescence intensities obtained by hybridization between subspecies-specific probes (NR1/NBTP, NR1/NBTA, NR2/NBTP, and NR2/NBTA) immobilized on BMP and DIG-labeled target DNA fragments from NBTP and NBTA genome. Luminescence intensities obtained by hybridization with noncomplementary sequences are presented as relative units to those obtained with complementary sequences.

because the BMP surface was masked by BSA. From these data, 1.0% BSA was determined to be optimal for inclusion in washing buffer.

PCR attempts using two primer sets (L8562/NR1R and NR2F/H9432) amplified 150-bp and 406-bp fragments, respectively. These DIG-labeled PCR products were subjected to hybridization reaction with DNA-BMPs. Figure 2 shows the results of experiments evaluating hybridization between subspecies-specific probes (NR1/NBTP, NR1/NBTA, NR2/NBTP, and NR2/NBTA) immobilized on BMPs and target PCR products amplified from NBTP and NBTA genome. The luminescence intensities from hybridization between the probes and the noncomplementary fragments were presented as relative values to those obtained between the probes and the complementary fragments. When amplified fragment containing the NR1 region of NBTP was hybridized with NR1/NBTA probe, the relative luminescence intensities (± SD) were reduced to 0.81 ± 0.16. When amplified fragment containing NR1 region of NBTA was hybridized with NR1/NBTP, the relative luminescence intensities were reduced to 0.84 ± 0.05. Likewise, in hybridization between the NR2 fragment of NBTP and NR2/NBTA probe, the luminescence intensities were reduced to 0.69 ± 0.05, and those between NR2 fragment of NBTA and NR2/NBTP were reduced to 0.56 ± 0.05.

The luminescence intensities between the probes and the noncomplementary fragments were apparently lower than those between the probes and the complementary fragments, even when there was only a single-nucleotide difference. The number of nucleotides that differ between the probes and the target fragment as well as those nucleotide length may result in the luminescence intensity different. These data suggest that this system employing DNA-BMPs may be useful for discriminating these two subspecies by recognizing single-nucleotide differences. Further investigation on hybridization conditions, length of target sequence, and the positional effects of nucleotide substitution are necessary to optimize this detection system.

Magnetic-capture hybridization using BMPs is useful for further development of an automated system of high-throughput analysis, which may make species identification more rapid and less costly. Nucleotide sequence analysis for the other tuna species is under way, by which better DNA probes may be designed to discriminate all tuna species using this system.

Acknowledgments

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