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Development of real-time PCR assay for genetic identification of the mottled skate, *Beringraja pulchra*

In Kwan Hwang [a,*], Hae Young Lee [a], Min-Hee Kim [b], Hyun-Su Jo [c,1], Dong-Ho Choi [a], Pil-Won Kang [a], Yang-Han Lee [b], Nam-Soo Cho [b], Ki-Won Park [b], Ho Zoon Chae [d]

[a] DNA Analysis Division, Seoul Institute, National Forensic Service, Seoul, South Korea
[b] Forensic DNA Division, National Forensic Service, Wonju, South Korea
[c] Fisheries Researchs and Environment Division, West Sea Fisheries Research Institute, Incheon, South Korea
[d] School of Biological Sciences and Technology, Chonnam National University, Gwangju, South Korea

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ABSTRACT

The mottled skate, *Beringraja pulchra* is one of the commercially important fishes in the market today. However, *B. pulchra* identification methods have not been well developed. The current study reports a novel real-time PCR method based on TaqMan technology developed for the genetic identification of *B. pulchra*. The mitochondrial cytochrome oxidase subunit 1 (COI) nucleotide sequences of 29 *B. pulchra*, 157 skates and rays reported in GenBank DNA database were comparatively analyzed and the COI sequences specific to *B. pulchra* was identified. Based on this information, a system of specific primers and Minor Groove Binding (MGB) TaqMan probe were designed. The assay successfully discriminated in 29 specimens of *B. pulchra* and 27 commercial samples with unknown species identity. For *B. pulchra* DNA, an average Threshold Cycle (Ct) value of 19.1 ± 0.1 was obtained. Among 27 commercial samples, two samples showed average Ct values 19.1 ± 0.0 and 26.7 ± 0.1, respectively and were confirmed to be *B. pulchra* based on sequencing. The other samples tested showed undetectable or extremely weak signals for the target fragment, which was also consistent with the sequencing results. These results reveal that the method developed is a rapid and efficient tool to identify *B. pulchra* and might prevent fraud or mislabeling during the distribution of *B. pulchra* products.

1. Introduction

The mottled skate, *Beringraja pulchra*, is a species of big skate, family Rajidae, in the Rajiforms order. The Rajiforms consist of 245 valid species [1], which spread across three families and 30 genera [1–4]. *B. pulchra* was initially classified as *Raja pulchra* until a new genus *Beringraja* was established by Ishihara [4]. It has been found in the Sea of Okhotsk, the Yellow Seas along South Korea, and even as far as the East China Sea [4–7]. It was extremely common about 20 years ago in the Yellow Sea, especially around the Daecheong-do and Heuksan-do Islands of South Korea. However, with severe population decline, it is currently listed as a vulnerable species by the International Union for Conservation of Nature [8]. The 11 skate species belonging to five genera have been identified in Korea to date and *B. pulchra*, which is one of the most expensive fishes sold in South Korea, reaching US$ 10–80 per kilogram, is the most economically valuable species, consumed in raw or fermented forms in South Korea [8]. However, with the increase of its value, food crime like substitution or ambiguous labeling of *B. pulchra* products, which makes morphological identification impossible, has been relatively increasing and the police are currently focusing on to tracking down such cases in South Korea.

However, suitable identification methods for *B. pulchra* have not yet been developed. Currently, protein-based methods such as high-performance liquid chromatography, electrophoretic techniques and enzyme-linked immunosorbent assays [9] are sensitive for processed materials and can differentiate between closely related species but they are often time-consuming and tend to show be unsatisfactory results due to protein denaturation [10,11].

In the present work, RT-PCR analysis was used and based on the use of a Minor Groove Binding (MGB) TaqMan probe, designed to bind at the diagnostic sites of a target DNA and fluoresce during

* Corresponding author at: DNA Analysis Division, Seoul Institute, National Forensic Service, 139 Jiyang-ro, Yangcheon-gu, Seoul 158-707, South Korea. Tel.: +82 2 2600 4861; fax: +82 2 2600 4866.
E-mail address: inkai@korea.kr (I.K. Hwang).
[1] Present address: Department. of Marine Science & Production, Kunsan National University, Gunsan, South Korea.

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amplification. In addition, the mitochondrial cytochrome oxidase subunit 1 (COI) gene was applied to unknown commercial and *B. pulchra* samples as a genetic marker for species identification. Mitochondrial (mt) genes have a high copy number allowing a greater yield of mtDNA to be recovered from trace samples compared to nuclear DNA [12]. Also, mtDNA genes typically lack recombination promoting the loss or fixation of mtDNA haplotypes, reducing within species diversity and thus enabling species identification [13,14].

In this study, we describe the development of a TaqMan real-time PCR technique, based on amplification of COI gene fragment, for specific identification of *B. pulchra*. Applicability of the *B. pulchra* specific system was assessed through the analysis of COI sequences of 29 *B. pulchra*, 27 unknown commercial samples and 157 skates and rays reported in GenBank DNA database. The reported real-time PCR assay provides a useful tool for authenticating and differentiating *B. pulchra* from its relatives and probable substitutes in the market.

2. Materials and methods

2.1. Collection of samples and DNA extraction

Tail tissue samples were isolated from 29 wild *B. pulchra* specimens captured near coasts of South Korea. Unknown commercial samples composed mainly of fermented fish were obtained in supermarkets of Seoul and Jeolla province in Korea. They were preserved in 100% ethanol and then transported to the laboratory for DNA extraction. About 0.2 g of tissue was selected from each sample for DNA extraction. Total DNA was isolated from each sample using QIAamp DNA microkit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted total DNA was quantified using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Barrington, IL, USA) and stored at −20 °C.

2.2. PCR amplification and DNA sequencing

The DNA fragment of all samples on COI gene were amplified by using the three primers described in Table 1. PCR amplifications were conducted in 25 μl solutions containing 10 ng template DNA, 2.5 μl Gold ST*R 10× buffer (Promega, Madison, WI, USA), 2.5 U AmpliTaq Gold® DNA polymerase (Applied Biosystems, Foster City, CA, USA) and 1.0 μM of each primer. Thermal cycling was performed on a GeneAmp® PCR System 9700 (Applied Biosystems) under the following conditions: 95 °C for 11 min; 30 cycles of 94 °C for 1 min, annealing time at each 58, 54, and 52 °C for three sets of primers for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 7 min. The amplified PCR Products with QX Size 50–800 base pair (bp) and 15 bp/1000 bp alignment Marker (Qiagen) were analyzed by QIAxcel Advanced system (Qiagen) which utilizes QX cartridges comprising an array of 12 capillaries prefilled with gel polymers.

Table 1
Description of the primers used in PCR amplification of *B. pulchra* and unknown commercial samples.

PCR round	Primer name	Sequences (5□–3□)	Position[a]	Expected size (bp)
First	BPCOI-F1	GTGCCTGAGCAGGTATGGT	21–39	600
	BPCOI-R1	CCGGGTCAAAGAAAGTTGTG	601–620	
Second	BPCOI-F2	ATAATGTCATTGTTACAGCCCA	114–135	507
	BPCOI-R1	CCGGGTCAAAGAAAGTTGTG	601–620	
Third	BPCOI-F1	GTGCCTGAGCAGGTATGGT	21–39	439
	BPCOI-R2	ATAATTGTGGTGATGAAGTTAAT	437–459	

[a] Position is defined according to the sequence of the *Beringraja pulchra* (GenBank accession number EU327184).

Sequencing reactions were performed with BigDye Terminatior v3.1 Cycle Sequencing Kit (Applied Biosystems), following the manufacturer's instruction and sequencing was carried out by capillary electrophoresis using a 3500xL Genetic Analyzer (Applied Biosystems). Raw data have been analyzed using the Sequence Analysis software v.5.4 (Applied Biosystems). The sequences have been analyzed with Molecular Evolutionary Genetics Analysis (Mega) Version 5.1 software [15] and the results were authenticated with BLAST on GenBank (Basic Local Alignment Search Tool, National Centre for Biotechnology Information, Bethesda, Maryland; www.ncbi.nlm.nih.gov/).

2.3. Design of primers and probe

The identification of *B. pulchra* species provided by National Fisheries Research and Development Institute (NFRDI) and unknown commercial samples confiscated in supermarket was performed by using primers based on the mitochondrial COI gene of *B. pulchra* (GenBank accession number EU327184.1) as given in Table 1.

The mitochondrial sequences such as 12S rRNA, cytochrome b, and COI from our database and GenBank were analyzed to find a suitable region for primer and probe design for real-time PCR. Sequences of mitochondrial COI region were selected and aligned to design a specific MGB Taq-Man Primer-probe set for *B. pulchra*. Primer Express software (Applied Biosystems) was used for selection the sequence of the primers and probe set.

The sequences of the primers-probe set designed in this study are the following:

BPCOIRT-F (forward): 5?-GGGCCGGAACAGGTTGA-3?
BPCOIRT-R (reverse): 5?-CCCAGCGTGGGCTATATTTC-3?
BPCORT-P (probe): 5?-CTGTGTACCCCCCCTT-3?

The probe was labeled with the fluorochrome FAM in the 5? end and the Minor Groove Binding (MGB) in the 3? end.

2.4. Real-time PCR amplification

Real-time PCR reactions were performed in a total volume of 25 μl in MicroAmp fast optical 96-well reaction plate (Applied Biosystems), covered with MicroAmp optical adhesive film (Applied Biosystems). The experiments were conducted to detect emitted FAM fluorescence from a probe for COI and VIC fluorescence from a probe for Internal Positive Control (IPC), a synthetic sequence not found in nature (Applied Biosystems). Each reaction contained 10 ng of DNA, 12.5 μl of TaqMan Fast Universal PCR Master Mix no UNG Amperase (2×) and a final concentration of 900 nM for each primer and 225 nM for the probe. TaqMan reactions were run on 7500 PCR System (Applied Biosystems) with the following thermal cycling protocol: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Fluorescence data from the plates were analyzed using the 7500 system SDS software v1.3.0. A negative control was included and positive control was the IPC for amplification and inhibition control of the sample. Each Ct value was obtained by the average of three replicates. The average Ct value calculated with *B. pulchra* samples was compared with the average Threshold Cycle (Ct) value obtained with unknown commercial samples.

3. Results and discussion

3.1. Identification of B. pulchra and unknown commercial samples by FINS

Until now, there has been limited studies on rays and skates species with regard to their mitochondrial region sequences such