RESEARCH NOTE

PCR-based detection of allergenic mackerel ingredients in seafood

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Introduction

Fish is one of the most common causes of food allergy, especially in coastal countries such as Japan. An investigation conducted by the Japanese Government in 1997 reported that fish was ranked second among various causes of food allergy for adults and that approximately 20% of food allergic adults in Japan were sensitive to fish. Several fish species and seafood products are known to cause allergy, and were classified into four groups consisting of (i) salmon, sardine and mackerel, (ii) cod and tuna, (iii) octopus and squid, and (iv) crab and shrimp, by a cluster analysis of immunoglobulin reactivities of children (Tanaka *et al*. 2000). *Scomber* mackerel belonging to the family Scombridae are one of the most commercially important seafoods in Japan, where the annual consumption of *Scomber* mackerel is much larger than that of salmon, sardine, cod and tuna, all of which are also allergenic fish.

Three species – Japanese mackerel (*Scomber japonicus*), Pacific mackerel (*Scomber australasicus*) and Atlantic mackerel (*Scomber scombrus*) – constitute a significant part of the absolute mackerel consumption, and are considered to be most frequently involved in incidents of fish allergy in Japan. Since *Scomber* mackerel possess unique characteristics in the skin, they are easily differentiated from other whole fish, but processed foods containing *Scomber* mackerel with the skin removed are difficult to identify. In addition, foods containing even a trace of *Scomber* mackerel sometimes cause serious allergenic episodes (Sackesen and Adalioglu 2003).

In this study, we report a rapid and reliable method to detect and differentiate *Scomber* mackerel fish from other Perciformes marine fish by a simple PCR technique using new *Scomber* genus specific primers. Nucleotide sequences of the nuclear 5S ribosomal RNA gene (5S rDNA) were compared among 15 marine fish species belonging to 13 genera in 6 families of Perciformes. Alignment of their sequences allowed the selection of a pair of oligonucleotide primers specific for *Scomber* mackerel. These primers enabled PCR amplification of the flanking nontranscribed spacer region of the 5S rDNA from *S. japonicus, S. australasicus* and *S. scombrus,* but no amplification from other fish including closely related Scombridae fish. This simple and robust method can serve in routine detection and authentication of allergenic *Scomber* mackerel ingredients in seafood in food inspection laboratories.

Materials and methods

Fish samples

Specimens of 15 marine fish species comprising 13 genera in 6 families of Perciformes (table 1) were purchased from local fish suppliers in Japan. All specimens were morphologically identified by fish taxonomists and then immediately stored in laboratory freezers at -20°C or -40°C until DNA preparation.

DNA preparation

High quality megabase genomic DNA was prepared from frozen small muscle (~20 mg) by the modified urea–SDS–proteinase K method (Aranishi and Okimoto 2004). Briefly, samples were immersed in 100 μL of TESU6 buffer (10 mM Tris-HCl, pH 8.0, 20 mM EDTA, pH 8.0, 2% SDS, 6 M urea) containing 25 μg proteinase K (Sigma) and incubated at 55°C for 15 min. Ten μl of 5 M NaCl was added and mixed by inversion. An equal volume of phenol–chloroform–isoamyl alcohol (25:24:1) was added and mixed.

Following centrifugation at 10,000 g for 5 min, aqueous phase was collected and then subjected to Ethanol precipitation. The DNA pellet was washed twice with 70% Ethanol dried, and resuspended in 100 μL of 10T0.1E (10 mM

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Tris-HCl pH 8.0, 0.1 mM EDTA pH 8.0).

PCR amplification

PCR amplification of the 5S rDNA was performed in 10 μl of PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.01% gelatin) containing 200 μM each dNTP, 0.25 unit *Taq* polymerase (Sigma), 0.5 μM of each primer, and 10 ng of template DNA in Techgene thermal cycler (Techne) programmed as 2 min at 94°C, 30 cycles of 10 sec at 94°C, 20 sec at 55–64°C, depending on fish species, 40 sec at 72°C, and finally 5 min at 72°C. The primers used were 5S21F (5'-TACGCCCGATCTCGTCCGATC-3') and 5S21R (5'-CAGGCTGGTATGGCC GTAAGC-3') for PCR amplification of the 5S rDNA in flatfish (Cespedes *et al.* 1999). A 5 μL portion of amplicons was run at 15 V/cm on a 2.0% agarose gel and visualized under UV illumination using an EDAS290 Gel Documentation System (Invitrogen).

DNA sequencing

PCR products were purified by NaOAc-Ethanol precipitation followed by rinse with 70% Ethanol and subsequent drying. The DNA pellet was resuspended in 2 μl of Blunting-phosphorylation-ligation buffer (55 mM Tris-NaOAc, pH 7.9, 110 mM KOAc, 25 mM $Mg(OAc)_2$, 2.5 mM ATP, 25 mM DTT, 2.5 mM hexaminecobalt trichloride, 100 μM each dNTP). The mixture was incubated with 1 unit T4 polynucleotide kinase (Invitrogen) and 0.2 unit T4 DNA polymerase (Invitrogen) at 37°C for 30 min, and the enzymes were inactivated at 75°C for 10 min. Ligation was carried out into *Sma*I-digested pUC19 with 1 unit T4 DNA ligase (Invitrogen) followed by transformation into EC100 Ca^{2+}- competent cells (Epicentre) (Pope and Kent 1996). Colony direct PCR was performed on randomly chosen white colonies on LB-ampicillin/IPTG/X-gal agar. DNA sequencing of colony direct PCR products comprising the expected size of insert was accomplished with a CEQ DTCS kit using a CEQ2000XL DNA Analysis System (Beckman Coulter).

Results and discussion

Among nuclear markers, the 5S rDNA is of special interest in taxonomic identification because of its unique structure making it a species specific gene in higher eukaryotes, including teleost fish (Pendas *et al.* 1995; Sajdak *et al.* 1998). The 5S rDNA consists of a 120 bp conserved coding region (5S rRNA) and an intergenic spacer (NTS), and the length and sequence of the NTS may vary among species. The oligonucleotides 5S1 and 5S2, based on the conserved 5S rRNA, had been used to amplify a whole unit of the 5S rDNA from some fish species (Pendas *et al.* 1995; Cespedes

Table 1. List of Perciformes marine fish species used to obtain reference nucleotide sequences of 5S rDNA.

Family	Species	Common name	Lane*
Scombridae	*Scomber japonicus*	Japanese mackerel	1
	Scomber australasicus	Pacific mackerel	2
	Scomber scombrus	Atlantic mackerel	3
	Auxis thazard	Frigate mackerel	4
	Scomberomorus niphonius	Spanish mackerel	5
Carangidae	*Trachurus japonicucs*	Jack mackerel	6
	Decapterus maruadsi	Round mackerel	7
	Seriola quinqueradiata	Yellowtail amberjack	8
Sparidae	*Acanthopagrus schlegelii*	Black seabream	9
	Sparus sarba	Silver seabream	10
	Pagrus major	Red seabream	11
	Dentex tumifrons	Yellow seabream	12
Centrolophidae	*Hyperoglyphe japonica*	Japanese butterfish	13
Sciaenidae	*Pennahia argentata*	White croaker	14
Haemulidae	*Parapristipoma trilineatum*	Chicken grunt	15

* Numbers indicate the lane numbers on agarose gel electrophoresis shown in Figure 1.

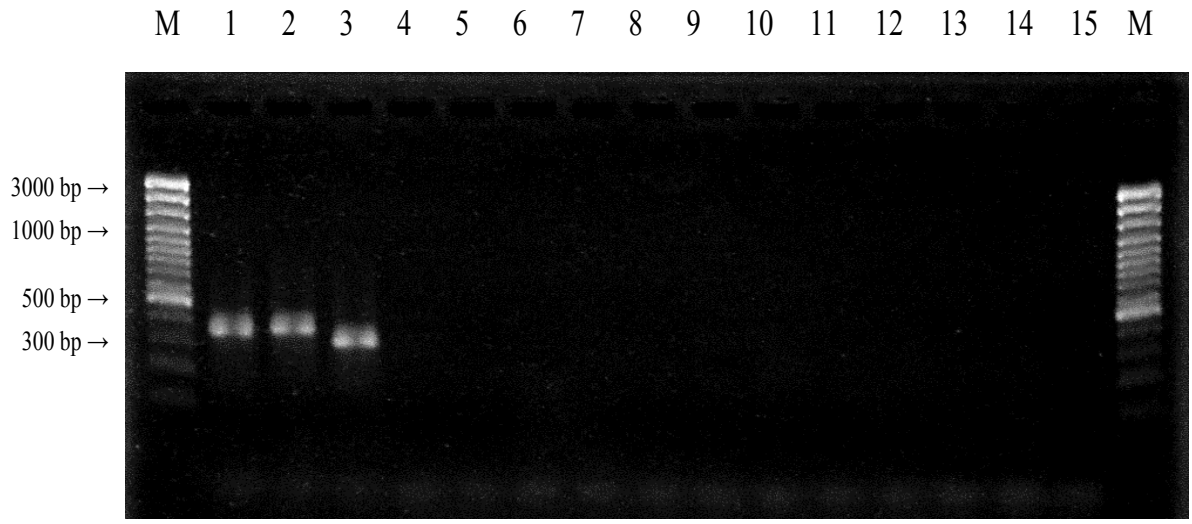

Figure 1. PCR amplification of the 5SrDNA NTS with Saba-18F and Saba-21R primers from 15 Perciformes marine fish species belonging to 13 genera, namely *Scomber* (lanes 1-3), *Auxis* (lane 4), *Scomberomorus* (lane 5), *Trachurus* (lane 6), *Decapterus* (lane 7), *Seriola* (lane 8), *Acanthopagrus* (lane 9), *Sparus* (lane 10), *Pagrus* (lane 11), *Dentex* (lane 12), *Hyperoglyphe* (lane 13),

et al. 1999; Asensio *et al.* 2001). In this study, these primers allowed amplification of various sizes of PCR products from 15 marine fish species, and their nucleotide sequences were determined. When the sequences of the NTS were compared between three *Scomber* species and 12 other fish, a pair of novel oligonucleotides was designed as PCR primers for the conserved sequences of the NTS among the *Scomber* mackerel genus.

Shuttle PCR for the 5S rDNA NTS with the new oligonucleotide primers, Saba-18F (5'-GGGCGCTGTTGCTCCATC-3') and Saba-20R (5'-ATGCTGTGACACCACTGACA-3'), were conducted under the conditions at 96°C for 2 min, 30 cycles of 10 sec at 94°C and 20 sec at 68°C, and finally at 68°C for 3 min. Figure 1 shows single PCR products amplified only from *S. japonicus* (lane 1), *S. australasicus* (lane 2) and *S. scombrus* (lane 3), and their sizes were 359 bp, 359 bp and 311 bp, respectively. Meanwhile no product was detected in other 12 species. It is noteworthy that *Auxis thazard* (lane 4) and *Scomberomorus niphonius* (lane 5) were negative, although they are closely related to *Scomber* mackerel in the family Scombridae.

Thus, the results indicate that a pair of Saba-18F and Saba-21R oligonucleotides is *Scomber* specific, and it is the first report of genus specific primers in Perciformes marine fish. This procedure is a straightforward and sophisticated method for genus identification of *Scomber* mackerel with no requirement of special reagents and equipments. It thus can be routinely performed to detect and differentiate allergenic *Scomber* mackerel ingredients in seafood in food inspection laboratories.

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