JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

# PCR-RFLP Analysis of Nuclear Nontranscribed Spacer for Mackerel Species Identification

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*Scomber* mackerel have been marketed in fresh and frozen forms and as processed seafood worldwide, and three species of Japanese mackerel *S. japonicus*, Pacific mackerel *S. australasicus*, and Atlantic mackerel *S. scombrus* have constituted a significant part of absolute Scombrid consumption in Japan. The present study was undertaken to develop a rapid and reliable method not only for differentiation of *Scomber* mackerel from related Scombrid fish by PCR amplification using *Scomber* genus-specific primers but also for identification of three *Scomber* mackerel species by PCR-RFLP analysis. Alignment of nucleotide sequences of the nuclear 5S ribosomal RNA gene (5S rDNA) among Scombrid fish allowed the selection of oligonucleotide primers specific for the *Scomber* genus. These primers enabled amplification of the nontranscribed spacer (NTS) of the 5S rDNA from *S. japonicus*, *S. australasicus*, and *S. scombrus*, whereas no amplification was demonstrated from other Scombrid fish. RFLP analysis of the PCR products with *Sca*I endonuclease generated unique restriction patterns for each *Scomber* species. This simple, robust, and reproducible PCR-RFLP technique using *Scomber* genus-specific primers can serve as a routine food inspection program to enforce labeling regulations of marketed Scombrid fish.

**KEYWORDS: Genus-specific primers; *Scomber*; PCR; RFLP; species identification; 5S ribosomal DNA (5S rDNA); nontranscribed spacer (NTS)**

## INTRODUCTION

The family Scombridae comprises 15 genera and 54 species (*1*). Scombrid fish are generally migratory pelagic fish forming large schools near the marine surface (*2*). Because they are globally distributed from tropical to subtropical oceans, Scombrid fish have so far been one of the most economically important fishery resources worldwide (*3*). *Scomber* mackerel is the representative genus of the Scombridae and is currently classified into three species. Japanese mackerel *Scomber japonicus* is distributed over the Pacific and Indian Oceans but is apparently absent from Indonesia and Australia. Pacific mackerel *Scomber australasicus* inhabits the West Pacific Ocean including Indonesia and Australia. Atlantic mackerel *Scomber scombrus* dominantly migrates in the East Atlantic Ocean. In addition, *S. japonicus* appears along the east coast of the North and South Americas in the West Atlantic Ocean, and some fish taxonomists claim that the Atlantic population might be recognized as a separate species with *Scomber colias* (*4*).

Scomber mackerel have been extensively traded in both fresh and frozen forms and as processed seafood such as smoked and canned products (*5*). In Japan, because *S. japonicus* and *S. australasicus* are domestically caught by fishery and *S. scombrus* is imported mainly from northern Europe, all three species have constituted a significant part of absolute Scombrid consumption (*6*). Due to unique morphological features in the skin, *Scomber* mackerel can be discriminated not only from other marine fish but also among these three species, which allows us to discover fraudulent or unintentional mislabeling of commercially unprocessed *Scomber* mackerel following the Japanese Labeling Regulation (Japanese Agricultural Standard). However, once the skin is removed, processed products containing *Scomber* mackerel are generally unidentifiable, and even a trace of *Scomber* mackerel sometimes causes serious food allergenic episodes (*7*). The present study was undertaken to develop a rapid and reliable method not only for the differentiation of *Scomber* mackerel from related Scombrid fish by PCR amplification using *Scomber* genus-specific primers but also for the identification of three *Scomber* mackerel species by PCR-RFLP analysis.

## MATERIALS AND METHODS

**Fish Samples.** Specimens of five closely related Scombrid mackerel, Japanese mackerel *S. japonicus*, Pacific mackerel *S. australasicus*, and Atlantic mackerel *S. scombrus* belonging to the genus *Scomber*, frigate mackerel *Auxis thazard*, and Spanish mackerel *Scomberomorus niphoniuss*, were purchased from local fish suppliers in Yokohama, Japan. All specimens were morphologically identified by fish taxonomists at the National Institute of Fisheries Science, Yokohama, Japan, and then they were cut into fillets and immediately stored in a laboratory freezer at −20 °C until DNA preparation.

**DNA Preparation.** Total genomic DNA was prepared from small scraps (∼20 mg) of frozen fillets according to the modified urea−SDS−Proteinase K method. Samples were immersed in TESU6 buffer (10 mM Tris-HCl, pH 8.0, 20 mM EDTA, pH 8.0, 2% SDS, 6 M urea) containing 25 μg of Proteinase K (Sigma), mixed by vortex, and incubated at 55 °C with shaking for 15 min. A 0.1 volume of 5 M

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10.1021/jf0484881 CCC: $30.25 © 2005 American Chemical Society
Published on Web 01/14/2005

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                 5S21F                                                                                    80
S. japonicus     tacgcccgatctcgtccgat ctcggaagctaagcagggtc gggcctggttagtacttgga tgggagaccgcctgggaata
S. australasicus .................... .................... .................... ....................
S. scombrus      .................... .................... .................... ....................
A. thazard       .................... .................... .................... ....................
S. niphoniuss    .................... ...................A .................... ....................

                                                            Saba-18F                                 160
S. japonicus     ccaggtgctgtaagcttTTT CCTCTCAGCTGTCACCAAAG GAGGGCGCTGTTGCTCCATC AC----CGCACACAGGGCTT
S. australasicus .................... .................... .................... ..----..............
S. scombrus      .................... .................G.. .................... ..----.............G.
A. thazard       .................... .T...GGTG....GT.G.TT ..........C.TG.....T .TTTCT.A....G.C....G
S. niphoniuss    ...................G .A.T.-GCG.CCA.A..GCA C..A..A....T.A..C..G TA---AAAAAGAAAAATACC

                                                                                                     240
S. japonicus     TGAGGA----ACAAAAGCTG CAATCATTCCA-GCTGTGTC TGAATGCACGCCAGAGA--- -GGTGGCACTGAGACGCCTC
S. australasicus ......----.......... ...........-........ .................--- -...................
S. scombrus      ....A.----....-..... ...........-........ ........G.G......--- -.......A...........
A. thazard       ..C..GCAAC...GGT..AC T...G.G.AAGT...CA.A. AAC.GC...C.A.A...TAA AAA.AAT.A.A.A.AAAAAA
S. niphoniuss    A.C.TGTCAT...C.T.GCT TTG.G.CAT..-------.T .AC.CCGG.C.TTC.TCT-- -TT.CATG.A.CAG.AGTGT

                                                                                                     320
S. japonicus     ACTTTCCAAGTAGTTTTTAG GGTTCCAGTTCTCACTGTTA CAGCCTGTAAAAAGCCTGCA ----------ATCATCCCGC
S. australasicus .................... .................... ..................T. ----------.AA.G..T..
S. scombrus      .................G.. ......T...G......... ........T........... ----------.T..T.TTAT
A. thazard       .AAAAAA..ATGAAGGAA.A ATAAAA.AGA.CTC..AC.. G..TAAA..GG.GC..CAT. TGTTGTGATA.AG.C...CA
S. niphoniuss    GTCA.GTACT.G.C...AT. ACA..ATT.A.A.CG.CC.T TGAT..T.TC.TGC.G.AGT -------GTA.CT.GGATTA

                                                                                                     400
S. japonicus     ATGTAC---ACAAAT-CACA AATGGCCTGAGAAACAAGGT TGCAGTCCTTCCAGCTGTGT GTTTCTCCGCAGAAAAAAAA
S. australasicus .ATC.TCCTG..TG.A.... ...A................ ....A............... ........A........---
S. scombrus      .A.C-----G..TG.A.... ...A.............--- -------------------- --------------------
A. thazard       .A..GAAAGTTTC.GC..T. G...AG.CTTA..TAG.CT. .AG.TGT.AAT.TATGC.C. ..GA..TT.A.T.T.G.T.T
S. niphoniuss    .....AAATT.C...CTCTC GTGAT...C.AT.TTT.C.G A.A.CCGTG.T...GAT.CC C.......T-----------

                              ScaI ↓                                                                 480
S. japonicus     CAACTATTCCT-ATCAAAGT ACTGTTAAAGGATAGCATGT GTTTGATGGCTGAGACA-CC ATTATCCGCCACTC--AGCT
S. australasicus -..........-..GC...C .A.................. .................-.. ..............--....
S. scombrus      ---.A......-..G..... .A.................. .................-.. ..............--....
A. thazard       A.G..G..TTGG.AGTG.CA GA..GA.CGT..GT.G...A C.......TAC.TC...ATT .AGGATGGA..GGAGG....
S. niphoniuss    ---..C....C----CG..- GT.C.CCC..T.CGTA...A A.CA..G.AT.TTA..--.. .CG.C..T..C.CG-----.

                             Saba-20 R                                                5S21R          560
S. japonicus     GGTGTGAGCAACTGTCAGTG GTGTCACAGCATGTTTAAAT GGGCTTTCTGCGGCCTGTTT CGTCgcttacggccatacca
S. australasicus .................... .................... ...........A.T...... .A..................
S. scombrus      ....A...G........... .................... ...........T.G...... .C..................
A. thazard       A.GCA...GGTTCAATCA.. AA..G.GGAG.A........ A....G...A.AC....AAC TT..................
S. niphoniuss    AA.TAC.TGT.AA.CTGA.. AC----TGAC.CG.AT.TAA ..CT.A.G..TGTT.AC... G.CA................

                     565
S. japonicus     gcctg (534 bp)
S. australasicus ..... (534 bp)
S. scombrus      ..... (486 bp)
A. thazard       ..... (565 bp)
S. niphoniuss    ..... (514 bp)
```

**Figure 1.** Aligned nucleotide sequences of the 5S rDNA comprising 5S rRNA (lower case letters) and NTS (capital letters) from *Scomber japonicus*, *Scomber australasicus*, *Scomber scombrus*, *Auxis thazard*, and *Scomberomorus niphoniuss*. Underlined bold type nucleotides in 5S rRNA and NTS of *S. japonicus* sequence indicate the positions of fish 5S rDNA universal primers and *Scomber* genus-specific primers, respectively. The *Sca*I restriction site for *S. japonicus* is shaded.

NaCl was added and mixed by inversion. An equal volume of phenol/chloroform/isoamyl alcohol was added and mixed by inversion. Following centrifugation at 10000*g* for 5 min, the upper aqueous phase was collected, and a 0.6 volume of isopropyl alcohol was added, mixed by vortex, and spun down. The DNA pellet was washed with ethanol, dried in a Speed-vac, and resuspended in 10T0.1E (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, pH 8.0).

**PCR Amplification.** PCR amplification of the 5S rDNA was performed in 10 $\mu$L of PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.01% gelatin, 0.005% Brij35, 10 mM DTT) containing 200 $\mu$M each dNTP, 0.25 unit of *Taq* DNA polymerase (Sigma), 0.5 $\mu$M each primer, and 10 ng of template DNA. The primers used were 5S21F (5′-TACGC CCGAT CTCGT CCGAT C-3′) and 5S21R (5′-CAGGC TGGTA TGGCC GTAAG C-3′), corresponding to 5S1 and 5S2, respectively, for PCR amplification of the 5S rDNA in flatfish (*8*). Shuttle PCR was carried out in a Techgene thermal cycler (Techne) programmed as 2 min at 96 °C, 30 cycles of 10 s at 96 °C and 20 s at 68 °C, and finally 5 min at 68 °C. A 5 $\mu$L portion of amplicon was migrated at 15 V/cm for 40 min on a 2.0% agarose gel and visualized under UV illumination in an EDAS290 Gel Documentation System (Invitrogen).

**DNA Sequencing.** PCR products were purified by chloroform extraction and poly(ethylene glycol) (PEG) precipitation. The DNA pellet was washed with ethanol, dried in a Speed-vac, and resuspended in 5 $\mu$L of ligation mixture containing 1 unit T4 DNA ligase (Invitrogen), ligation buffer (Promega), 5% PEG8000 (Sigma), and 25 ng of *Xcm*I-digested plasmid T-vector (*9*). After TA-cloning at room temperature for 30 min, transformation was performed using DH5α Ca-competent cells followed by blue-white selection (*10*). Colony direct PCR was performed on randomly chosen white colonies using M13-

M4 (5′-GTTTT CCCAG TCACG AC-3′) and M13-RV (5′-CAGGA AACAG CTATG AC-3′). PCR products comprising the expected size of insert were cleaned with an Exo/SAP kit (Amersham Pharmacia), and DNA sequencing of both strands was accomplished by a CEQ DTCS kit (Beckman Coulter) and M13(−20) (5′-GTTGT AAAAC GACGG CCAGT-3′) or T7 (5′-TAATA CGACT CACTA TAGGG-3′) in a CEQ2000XL DNA Analysis System (Beckman Coulter).

**PCR-RFLP Analysis.** On the basis of the alignment of forward and reverse nucleotide sequences of the 5S rDNA obtained from five Scombrid mackerel, *Scomber* genus-specific primers were designed as Saba-18F (5′-GGGCG CTGTT GCTCC ATC-3′) and Saba-20R (5′-ATGCT GTGAC ACCAC TGACA-3). The respective primers corresponded to nucleotides 28−45 and 241−260 of the NTS region of *J. mackerel*. *Scomber* genus-specific PCR amplification of the 5S rDNA NTS region was performed as above, except that Saba-18F and Saba-20R were substituted for 5S21F and 5S21R. Without further purification, a 5 $\mu$L portion of amplicon was incubated with 10 units of *Sca*I restriction enzyme at 37 °C for 2 h, and then electrophoretic analysis was performed as above.

## RESULTS AND DISCUSSION

In higher eukaryotes, tandem arrays of rRNA genes are organized in two distinct multigene families (*11*). One class is represented by the 45S rDNA that codes for the 18S, 5.8S, and 28S rDNA and internal transcribed spacers (ITS) and a second class by the 5S rDNA that codes for the 5S rDNA (*12*). 5S rDNA repeats consist of 120 bp long coding sequences separated from each other by the NTS. These noncoding rDNA spacer segments including NTS and ITS evolve much more rapidly than the coding rDNA, which is highly conserved even among nonrelated taxa (*13*).

Among nuclear markers available for species identification of commercial fish and seafood products, noncoding rDNA spacer segments can be good candidates. The NTS region was reported to be useful for the discrimination of related fish species, which belong to different genera but are morphologically indistinguishable when being processed (*8*, *14*). It remains uncertain whether the NTS region allows us to discriminate related fish species belonging to even the same genus. In the present study, a simple and reliable PCR-RFLP analysis using the NTS region was developed for accurate discrimination of closely related mackerel species belonging to the *Scomber* genus.

By means of fish universal primers, 5S21F and 5S21R based on conserved sequences of the 5S rDNA, apparently single PCR products at a size from 490 to 560 bp were successfully amplified from five Scombrid mackerel (**Figure 1**), all of which are popularly marketed in both fresh and frozen forms and as processed seafood in Japan (*6*). Nucleotide sequences of these PCR products showed that each of them corresponded to a single repeat of the 5S rDNA. The size of PCR products amplified using 5S21F and 5S21R varied among fish species, but those ranging ∼500−650 bp were frequently observed, for example, Greenland halibut *Reinhardtius hippoglossoides* (*8*), Nile perch *Lates niloticus*, grouper *Epinephelus guaza*, and wreck fish *Polyprion americanus* (*14*), *Brycon insignis* (*15*), rainbow trout *Salmo gairdneri*, brown trout *Salmo trutta*, and Atlantic salmon *Salmo salar* (*16*), tilapia *Oreochromis niloticus* (*17*), and several marine fish species (F. Aranishi, unpublished data).

To differentiate *Scomber* mackerel from other Scombrid species by a simple PCR technique, new primers were designed as Saba-18F and Saba-20R based on conserved sequences of the NTS region among three *Scomber* mackerel (**Figure 1**). PCR amplification using these *Scomber* genus-specific primers enabled us to obtain single PCR products from three *Scomber* mackerel, whereas no amplification was verified in *Auxis thazard* and *Scomberomorus niphoniuss* (**Figure 2**), showing accurate discrimination of *Scomber* mackerel from other Scombrid species. In addition, the PCR products apparently varied between *S. scombrus* and two other *Scomber* mackerel. The exact lengths of the PCR products were determined from nucleotide sequence analysis to be 416 bp for *S. japonicus*, 416 bp for *S. australasicus*, and 366 bp for *S. scombrus* (**Figure 1**). It is noteworthy that the lengths of a single repeat of the 5S rDNA are the same between *S. japonicus* and *S. australasicus*.

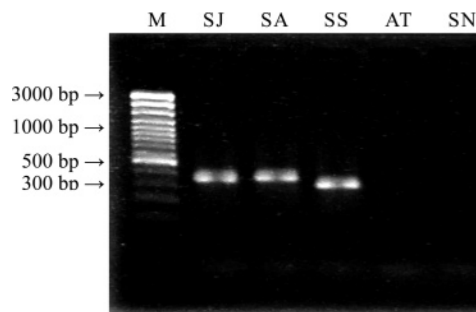


**Figure 2.** PCR amplification of the 5S rDNA NTS using Saba-18F and Saba-21R primers from *Scomber japonicus* (SJ), *Scomber australasicus* (SA), *Scomber scombrus* (SS), *Auxis thazard* (AT), and *Scomberomorus niphoniuss* (SN): lane M, 100 bp molecular size marker.

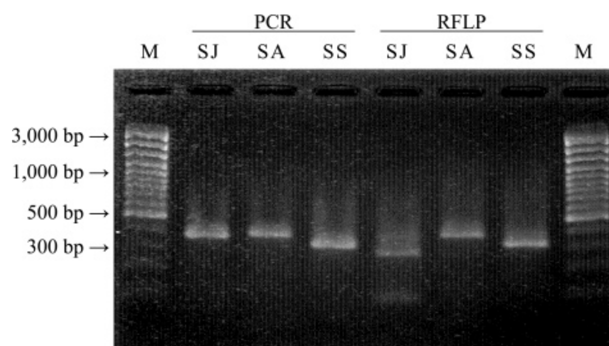


**Figure 3.** PCR-RFLP analysis of the 5S rDNA NTS amplified using Saba-18F and Saba-21R primers from *Scomber japonicus* (SJ), *Scomber australasicus* (SA), and *Scomber scombrus* (SS). PCR and RFLP indicate the PCR products and those after *Sca*I digestion, respectively: lane M, 100 bp molecular size marker.

In searching for appropriate restriction enzyme identifying PCR products amplified using *Scomber* genus-specific primers from *S. japonicus* and *S. australasicus*, computer sequence analysis was carried out using the program NEBcutter (*18*). Although the sequence homology between them was extremely high at 94.7%, a unique recognition site of *Sca*I restriction enzyme was found only in *S. japonicus* (**Figure 1**). As expected, *Sca*I restriction enzyme digestion yielded two fragments at sizes of 89 and 327 bp from the PCR product of *S. japonicus*, whereas a single PCR product at a size of 426 bp remained undigested in *S. australasicus* (**Figure 3**). For 5−10 individual specimens per each *Scomber* mackerel, nucleotide sequencing was performed in both strands, and no intraspecies polymorphism was verified at the *Sca*I recognition site. Hence, species-specific PCR-RFLP electrophoretic profiles made it possible to identify *S. scombrus*, *S. japonicus*, and *S. australasicus*, indicating that the NTS region is a useful nuclear marker in the discrimination of related fish species belonging even to the same genus.

This simple, robust, reliable, and inexpensive protocol involving PCR amplification of a selected NTS region using *Scomber* genus-specific primers followed by RFLP analysis with *Sca*I restriction enzyme can be routinely performed not only in differentiating *Scomber* mackerel from related Scombrid fish

but also in identifying three economically important *Scomber* mackerel in food inspection laboratories. We also report the modified method for megabase DNA preparation using TESU6 buffer containing 6 M urea. A high concentration of >4 M of urea has the potential ability to degenerate proteins constituting soft and hard tissues, but no effect on nucleotide structures (*19*). TESU6 buffer is available both for preservation of samples at ambient temperature with no DNA degradation by latent DNase and for genomic DNA extraction from tissues with no requirement of mechanical homogenization.

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**Received for review September 10, 2004. Revised manuscript received November 17, 2004. Accepted November 17, 2004. This work was supported in part by grants from the Agriculture, Forestry and Fisheries Research Council, Ministry of Agriculture, Forestry and Fisheries of Japan.**

JF0484881