Identification of Sole *(Solea solea)* and Greenland Halibut *(Reinhardtius hippoglossoides)* by PCR Amplification of the 5S rDNA Gene

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Polymerase chain reaction (PCR) amplification of the nuclear 5S rDNA gene, has been used for the identification of sole *(Solea solea)* and Greenland halibut *(Reinhardtius hippoglossoides)*. Two species-specific primers were designed to amplify specific fragments of the 5S rDNA gene in each species. The remarkably different size of the amplicons obtained gives, by simple agarose gel electrophoresis, two distinguishable band patterns for both flatfish species. This genetic marker can be very useful for the accurate identification of *S. solea* and Greenland halibut, to enforce labeling regulations.

Keywords: *Species identification; Solea solea; Reinhardtius hippoglossoides; 5S rDNA; nuclear marker*

INTRODUCTION

There has been a tremendous growth in seafood consumption due to changes in consumer attitudes toward health and nutrition. Some of the world's fish catch is sold unprocessed, but the market of processed fishery products is steadily increasing. Whenever the distinguishing features are removed at the processing stage, species are no longer recognizable. As a result of that, for closely related species such as *Solea solea* and Greenland halibut, fraudulent or unintentional mislabeling can pass undetected (Domínguez et al., 1997).

Numerous analytical methods have been developed for fish species identification (Mackie, 1996), mainly including electrophoretic techniques (LeBlanc et al., 1994; Cancalon, 1995; Gallardo et al., 1995; Huang et al., 1995a; Chen et al., 1996), high performance liquid chromatography (Osman et al., 1987; Amstrong and Leach, 1992), and immunoassays (Verrez-Bagnis and Escriche-Roberto, 1993; Taylor et al.,1994; Huang et al., 1995b; Carrera et al., 1996, 1997). Despite their individual merits, these methods rely upon the analysis of proteins. Unfortunately, proteins lose their biological activity soon after the fish has died, their presence and characteristics depend on the cell type being examined and many of them are heat-labile. For species identification, it would be preferable to analyze DNA rather than proteins. DNA carries an organism's genetic information that is the same in all cell types, and also, it is a very stable and long-lived biological molecule (Bartlett and Davidson, 1992; Ferguson et al., 1995).

Most of the genetic approaches to determine species identity are based on the use of conserved mitochondrial or nuclear DNA primers for PCR amplification, followed by sequencing (Bartlett and Davidson, 1991) or restriction fragment length polymorphism studies (PCR-RFLPs) (Meyer et al., 1995; Borgo et al., 1996; Ram et al., 1996; Carrera et al., 1998; Céspedes et al., 1998a,b; Quinteiro et al., 1998) of the amplicons obtained. In this work we have focused on the 5S ribosomal DNA gene (5S rDNA) because it presents a noteworthy structure, which would allow direct species identification by PCR amplification, without the need of later sequencing, or digestion with restriction enzymes (Pendás et al., 1994).

We report in this article a method for the identification of *S. solea* and Greenland halibut *(R. hippoglossoides)*, on the basis of PCR amplification of species-specific fragments in the 5S rDNA gene. This method can be applied to the detection of fraudulent or unintentional mislabeling of these species, in the market of processed products.

MATERIALS AND METHODS

Sample Selection and DNA Extraction. *S. solea* specimens were obtained from MercaMadrid (Madrid, Spain) and other local markets. Frozen samples of Greenland halibut *(R. hippoglossoides)* were obtained directly from manufacturers in Vigo (Spain) and Valencia (Spain). Every specimen was morphologically identified following the keys of Bauchot and Pras (1993). Fifteen individuals of each species were analyzed. Genomic DNA was extracted from fish muscle samples, according to a previously described procedure (Céspedes et al., 1998a).

PCR Amplification of a Unit of the 5S rDNA Gene. The set of primers used for PCR amplification of the 5S rDNA gene in *S. solea* and Greenland halibut, were designated as follows: (5S1) 5′-TACGCCCGATCTCGTCCGATC-3′ (forward primer) and (5S2) 5′-CAGGCTGGTATGGCCGTAAGC-3′ (reverse primer), consisting of nucleotides 1−21 and 24−45, respectively, of the coding region in the 5S rDNA gene. These oligonucleotides correspond to primers A (5S1) and B (5S2), designed by Pendás et al. (1994) for the amplification of one unit of any tandemly arranged 5S rDNA in salmon, brown trout, and rainbow trout.

Double-stranded amplifications were carried out in a final volume of 50 μL, containing 10 mM Tris-HCl, pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 0.2 mM each of dATP,

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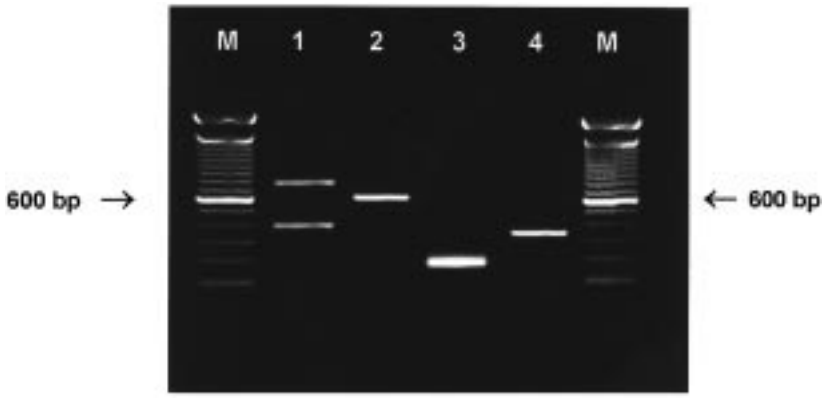

Figure 1. Electrophoretic analysis of the 5S rDNA gene PCR products, obtained from (lanes 1 and 3) sole *(S. solea)* and (lanes 2 and 4) Greenland halibut *(R. hipoglossoides).* Oligonucleotides 5S1 and 5S2 were used for PCR amplification on samples 1 and 2. Primers 5S1, 5SF. and 5SL were used for PCR amplification on samples 3 and 4. M indicates 100 bp ladder for molecular weight marker.

dTTP, dGTP, and dCTP, 10 pmol of each primer, 10 ng of template DNA, and 2 U of DynaZyme II DNA polymerase (Finnzymes Oy, Espoo, Finland).

The DNA was amplified in a Progene Thermal Cycler (Techne Ltd., Cambridge, U.K.). Thirty-five cycles were performed, with the following step-cycle profile: strand denaturation at 93 °C for 45 s, primer annealing at 65 °C for 45 s, and primer extension at 72 °C for 45 s. The last extension step was 5 min longer. An initial denaturation at 93 °C for 2 min was carried out to improve the final result.

Electrophoresis of a 10-μL portion of the amplification product was performed for 1 h at 100 V in a 1.5% D1 (Hispanlab S. A., Alcobendas, Spain) agarose gel, containing ethidium bromide (1 μg/mL) in Tris-acetate buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0). DNA fragments were visualized by UV transillumination and analyzed using Geldoc 1000 UV Fluorescent Gel Documentation System-PC (Bio-Rad Laboratories, Hercules, CA).

Cleanup and Sequencing of PCR Products. Electrophoresis of 100 μL of PCR products from each species was performed for 45 min at 100 V in a 1.5% LM-2 (Hispanlab) agarose gel, containing ethidium bromide (1 μg/mL) in Tris-acetate buffer. Each DNA fragment was excised from the agarose gel using a sterile scalpel. The gel slice was purified with the Qiaquick gel extraction kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. The DNA was eluted in 25 μL of sterile distilled water. The concentration of the purified PCR product was estimated by agarose gel electrophoresis using a standard (Mass Ruler, Bio-Rad) as reference marker. A Geldoc 1000 System-PC (Bio-Rad) was used for that purpose.

Purified PCR products from *S. solea* and Greenland halibut were sequenced at the Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas (Madrid, Spain). DNA sequencing was accomplished using dRhodamine terminator cycle sequencing ready reaction kit (Perkin-Elmer/Applied Biosystems Division, Foster City, CA) and both 5S1 and 5S2 primers, in an ABI Prism model 377 DNA sequencer (Perkin-Elmer). Direct and reverse sequences of each PCR fragment were obtained for two samples of each *S. solea* and Greenland halibut species.

Design of Species-Specific Primers for *S. solea* and Greenland Halibut 5S rDNA. The 5S rDNA gene sequences of *S. solea*, obtained by PCR amplification with oligonucleotides 5S1 and 5S2, were used for the design of *S. solea* specific primer, 5SL, 5′-CAAGGCTGAAACTAGAAGGA-3′, corresponding to nucleotides 166−187 of the 5S rDNA gene (on the NTS fragment). This 5SL primer together with the 5S1 oligonucleotide amplifies a 187 bp fragment in *S. solea*, whereas no amplification is possible in Greenland halibut. Likewise, the 5S rDNA gene sequences from Greenland halibut *(R. hippoglossoides)* were used for the design of oligonucleotide, 5SF, 5′-TTCTGATGGTGAGTAAACTCCCT-3′, which is specific for this species. This latter primer, corresponding to nucleotides 346−368 of the Greenland halibut 5S rDNA gene (on the NTS fragment) amplifies, together with 5S1 oligonucleotide, a 368 bp fragment in Greenland halibut, while no amplification is possible from *S. solea* DNA.

Sequences analysis of the 5S rDNA gene and primer design were performed with the aid of the Wisconsin Package, version 9.0 (Genetics Computer Group, Madison, WI) and the Amplify software (Engels, 1992).

PCR Amplification of Specific Fragments of the 5S rDNA Gene. The set of primers used for PCR amplification of DNA from *S. solea* and Greenland halibut samples were the forward primer 5S1 and the specifically designed 5SL and 5SF reverse primers. Although these primers were tested all together (5S1, 5SL, and 5SF) and separately in two different sets (5S1 and 5SF and 5S1 and 5SL), the amplification conditions were identical in all cases. PCR was performed in a total volume of 50 μL. Each reaction mixture contained 10 ng of template DNA, 10 pm of each primer, 200 μM of each dNTP, and 2 U of DynaZyme II DNA polymerase (Finnzymes Oy, Espoo, Finland) in a reaction buffer containing 10 mM Tris-HCl, pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, and 0.15% Triton X-100.

PCR was carried out in a Progene Thermal Cycler (Techne Ltd., Cambridge, U.K.) programmed to perform a denaturation step of 93 °C for 2 min, followed by 35 cycles consisting of 45 s at 93 °C, 45 s at 65 °C, and 45 s at 72 °C. The last extension step was 5 min longer.

Electrophoresis of a 10-μL portion of the amplification products was performed for 1 h at 100 V in a 1.5% D1 (Hispanlab S. A., Alcobendas, Spain) agarose gel, containing ethidium bromide (1 μg/mL) in Tris-acetate buffer. DNA fragments were visualized by UV transillumination and analyzed using the Geldoc System.

RESULTS AND DISCUSSION

Among nuclear markers, the 5S rDNA gene has a special interest in species identification because of its noteworthy structure that makes it a species-specific gene in higher eukaryotes (Pendás et al., 1994; Belkhiri et al., 1997). The 5S rDNA gene comprises a 120 bp highly conserved coding sequence (5S rRNA) and a nontranscribed spacer (NTS), the length of which may vary from species to species. This basic unit (5S rRNA + NTS) is tandemly repeated a variable number of times on the chromosome, depending on the species.

The oligonucleotides 5S1 and 5S2, based on the conserved region of this gene in *Oncorhynchus mykiss*, had already been used to amplify a whole unit of the 5S rDNA gene (coding sequence + NTS) from salmon and trout templates (Pendás et al., 1995). These primers, allowed us to amplify two PCR products from *S.*

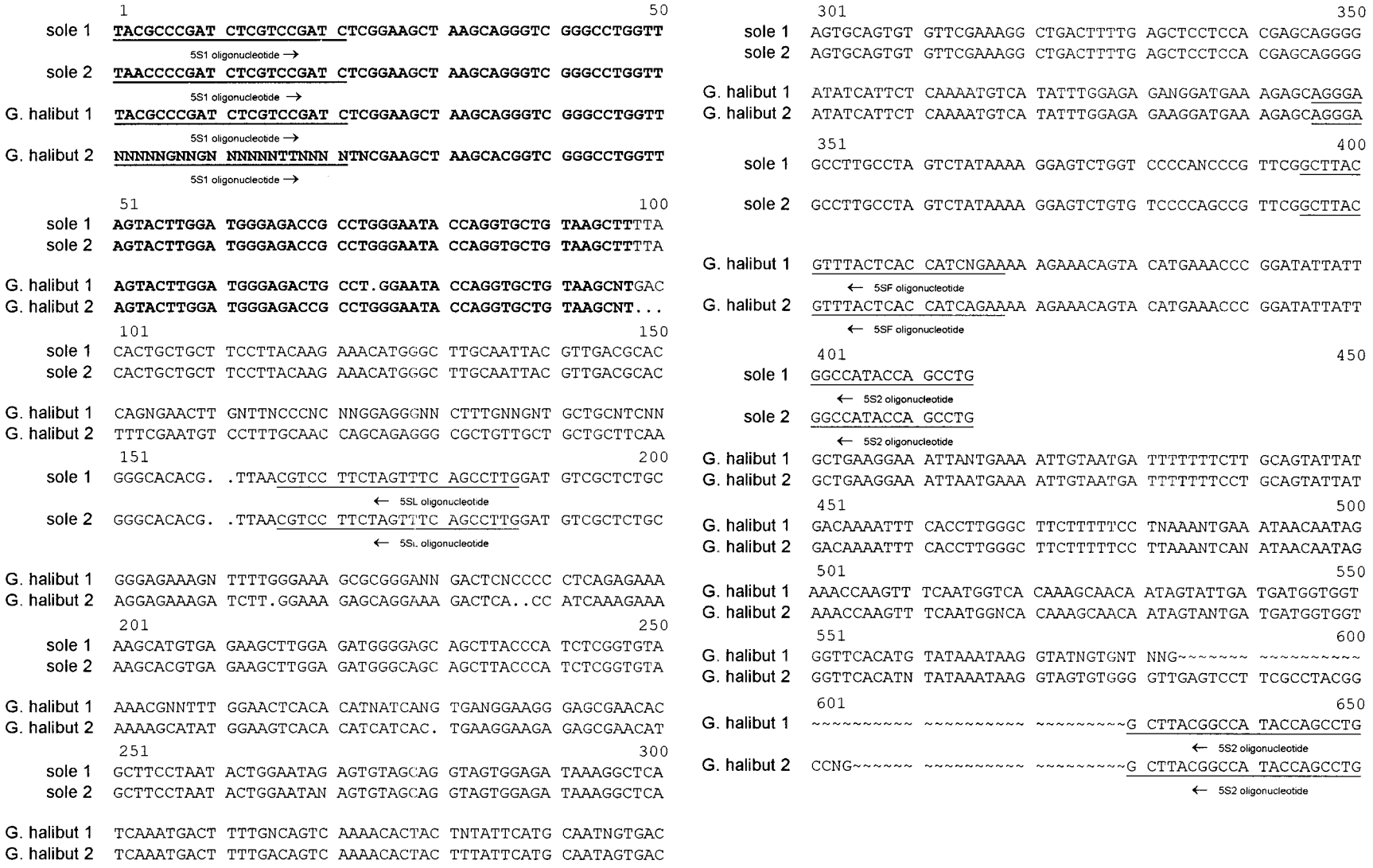

Figure 2. DNA sequences of the 5S rDNA gene from two sole *(S. solea)* and two Greenland halibut *(R. hipoglossoides)* samples. The corresponding coding sequence of the 5S rDNA gene is indicated in boldface type. Primers 5S1, 5S2, 5SL, and 5SF are underlined.

solea DNA (415 and 830 bp long) and one single PCR product of about 650 bp from Greenland halibut DNA (Figure 1, lanes 1 and 2). The size of amplification product was estimated to be the same in 15 individuals of each species.

We purified and sequenced each amplification product of *S. solea* and the amplification product of Greenland halibut (Figure 2). The results showed that the 415 bp PCR product of *S. solea* corresponded to one unit of the 5S rDNA gene and that the 830 bp fragment was composed of two tandemly arranged units of this gene. Similarly, the 650 bp PCR fragment from Greenland halibut DNA was confirmed to be one unit of the 5S rDNA gene. Accordingly, the variable NTS was determined to be about 295 bp in *S. solea* and 530 bp in Greenland halibut.

Although the length of the NTS appears to be species specific in fish species, as it has been shown for salmon and trout (Pendás et al., 1994) as well as for *S. solea* and Greenland halibut within this study, small differences in size could not be detected by simple agarose gel electrophoresis. As a result, an unknown species could be wrongly identified as *S. solea* or Greenland halibut if its NTS fragment were similar in length. For this reason and to make the identification specific for both flatfish studied, we decided to design a specific primer for *S. solea* (5SL) and another for Greenland halibut (5SF) which would allow, in combination with the 5S1 oligonucleotide, the amplification of specific regions of the 5S rDNA gene for both species.

We used the 295 bp NTS of *S. solea* to design the 5SL primer. This reverse oligonucleotide, together with the direct 5S1 primer, amplifies a 187 bp fragment from *S. solea* DNA, while no amplification is achieved from Greenland halibut DNA. Similarly, the 530 bp NTS of Greenland halibut, was used to design the 5SF oligonucleotide. This reverse primer, together with the direct 5S1 oligonucleotide, amplifies a 368 bp fragment from Greenland halibut DNA, whereas no amplification is obtained from *S. solea* DNA.

To confirm the effectiveness of the designed 5SL and 5SF primers, we tested them separately. When only primers 5S1 and 5SL were included in the PCR mix, no amplification was observed in Greenland halibut, whereas the expected 187 bp product appeared in all *S. solea* samples studied. Likewise, when only 5S1 and 5SF primers were included in the PCR mix, no amplification was observed in *S. solea*, while DNA from Greenland halibut yielded the expected 368 bp fragment. Since our purpose was to make the identification as simple and straightforward as possible, the PCR amplifications were carried out using the three primers 5S1, 5SL, and 5SF in the same reaction. The results showed that no disturbance occurred among the three oligonucleotides during the amplification reaction and that only the primers specific of each DNA sample annealed on their target sequences. As a result, the products of the amplification were identical to those observed when the primers were tested separately (Figure 1, lanes 3 and 4).

The PCR amplification of selected NTS fragments by species-specific oligonucleotides 5SL and 5SF, together with 5S1 primer, is a powerful technique for the identification of Greenland halibut and *S. solea*, because of its simplicity, specificity, and sensitivity. With this method the identification relies not only on the different size of the amplicons obtained, but also on the presence of the target sequences specific of each flatfish studied. This simple PCR amplification provides a powerful method to detect mislabeling or willful as well as fraudulent substitution of these species, particularly in the market of frozen fillets of flatfish.

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