Identification of Goose (*Anser anser*) and Mule Duck (*Anas platyrhynchos x Cairina moschata*) Foie Gras by Multiplex Polymerase Chain Reaction 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 goose and mule duck foie gras. Two species-specific reverse primers were designed and used in a multiplex reaction, together with a forward universal primer, to amplify specific fragments of the 5S rDNA in each species. The different sizes of the species-specific amplicons, separated by agarose gel electrophoresis, allowed clear identification of goose and mule duck foie gras samples. This genetic marker can be useful for detecting fraudulent substitution of the duck liver for the more expensive goose liver.

Keywords: *Species identification; Anser anser; Anas platyrhynchos x Cairina moschata; 5S rDNA; foie gras*

INTRODUCTION

Foie gras is a traditional luxury food prepared from the liver of specially reared geese or ducks. The history of foie gras began thousands of years ago in ancient Egypt. However, during the last two centuries it has been linked to the French gastronomy. Contemporary consumption of products derived from goose and mule duck (such as foie gras) is beginning to increase worldwide (*1*). A great variety of these products is available and the French legislation (*2*) is used as a reference in all the European countries that lack specific regulations for this kind of products.

First-category foie gras are exclusively made of specially force-fed duck or goose liver and can be sold, in order of decreasing selling prices, as "goose (or duck) whole foie gras", "goose (or duck) foie gras", "goose (or duck) lump of foie gras" and "goose (and/or duck) foie gras parfait". Mixing of duck and goose liver is allowed only in the latter product, whereas the former products have to be entirely pure. These products are commonly pasteurized or sterilized. Fraudulent labeling practices exist because goose foie gras is the most appreciated by consumers and also the most expensive. The development of analytical methods for species identification is, therefore, necessary to detect and avoid fraudulent or unintentional mislabeling of these products.

Morphological atributes and sensory differences are commonly used for species identification, although these criteria may lack reliability when products are processed, heated, and mixed with spices and other ingredients (*3*, *4*). Protein-based analytical methods for differentiation of goose and mule duck foie gras are scarce, and are limited to polyacrylamide disc-gel electrophoresis (*5*) and immunological methods (*6*, *7*). However, their reliability might be compromised in heat-treated products, because severe heat treatments denaturate the soluble proteins and may destroy or alter the species-specific epitopes recognized by the antibodies (*8*).

In recent years, nucleic-acid-based analytical methods have become more popular for the differentiation and identification of meat products (*9*−*12*). 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 (*13*, *14*). 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 (*15*) or restriction fragment length polymorphism studies (PCR−RFLP) of the amplicons obtained (*16*−*20*).

We report in this article a method for the identification of goose (*Anser anser*) and mule duck (*Anas platyrhynchos x Cairina moschata*), on the basis of multiplex PCR amplification of species-specific fragments in the nuclear 5S rDNA gene. This method can be applied to the detection of fraudulent or unintentional mislabeling of these species in the market of foie gras products.

MATERIALS AND METHODS

Sample Selection and DNA Extraction. Raw and pasteurized goose and mule duck whole foie gras were provided by Antonio de Miguel (Madrid, Spain). Other pasteurized first category foie gras samples were provided by IMPERIA foie gras (Girona, Spain), and sterilized samples were purchased at local delicatessen markets. Seven different commercial brands were included in the sampling. Standard pasteurization treatment includes heating at 80 °C for 90 min, whereas sterilization treatment consists of heating at 102−115 °C for 60−75 min.

Genomic DNA was extracted from foie gras samples essentially according to the method described by DeSalle et al. (*21*). Briefly, 100 mg of foie gras was homogenized in 500 μL of TSM buffer (0.2 M Tris, 0.1 M EDTA, 1% SDS), and 6 μL of 20 mg/mL proteinase K (Boehringer Mannheim GmbH, Man-

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nheim, Germany) was added. The samples were incubated for 3 h at 55 °C with shaking at 60 rpm. After incubation, tubes were placed on ice for 30 min. They were then centrifuged at 9000*g* for 10 min. The aqueous phase was transferred to a clean tube, leaving behind the upper fat layer and the pellet. DNA was extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol in a 25:24:1 ratio, and extracted once with an equal volume of chloroform. Then the DNA was precipitated twice with ethanol at −20 °C for 8 h. The pellets were allowed to dry at 20 °C, resuspended in 50 to 100 μL of sterile distilled water, and the concentration of DNA was estimated by absorbance at 260 nm.

DNA was extracted from pasteurized and sterilized first category foie gras samples of 15 different individuals of each fowl species analyzed.

PCR Amplification of the 5S rDNA Gene. The set of primers used for PCR amplification of the 5S rDNA gene in goose and mule duck was designated as follows: forward primer 5S1 (5′-TACGCCCGATCTCGTCCGATC-3′) and reverse primer 5S2 (5′-CAGGCTGGTATGGCCGTAAGC-3′), consisting of nucleotides 24−45 and 1−21, respectively, of the coding region in the 5S rDNA gene. These oligonucleotides correspond to primers B (5S1) and A (5S2) designed by Pendás et al. (*22*) for the amplification of one unit of any tandemly arranged 5S rDNA in rainbow trout. Double-stranded amplifications were carried out in a final volume of 50 μL, containing 10 mM Tris-HCl, pH 8.8, 2 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 0.2 mM each of dATP, dTTP, dGTP, dCTP, 10 pmol of each primer, 500 ng of template DNA and 2U of *Tth* DNA polymerase (Biotools, Madrid, Spain).

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 94 °C for 45 s, primer annealing at 63 °C for 45 s, and primer extension at 72 °C for 45s. The last extension step was 5 min longer. An initial denaturation at 94 °C for 3 min was carried out to improve the final result. The PCR products (10 μL) were mixed with 2 μL of gel loading solution (Sigma Chemical Co., St. Louis, MO) and electrophoresed in a 1.5% D1 (Hispanlab S. A., Torrejón, 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) for 40 min at 100V. DNA fragments were visualized by UV transillumination and analyzed using Geldoc 1000 UV fluorescent gel documentation system−PC (Bio-Rad Laboratories, Hercules, CA).

Purification, Cloning, and Sequencing of PCR Products. The PCR products (120 μL) were loaded on a 1.5% LM-2 (Hispanlab) agarose gel, containing ethidium bromide (1 μg/mL) in Tris-acetate buffer and electrophoresed at 100V for 40 min. Each DNA fragment was excised from the agarose gel under UV light 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 15 μL of sterile distilled water. The concentration of the 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.

The purified PCR products were ligated into the plasmid pGEM-T easy, using a pGEM-T easy vector system II (Promega, Madison, Wisconsin), and subsequently transformed into *E. coli* JM109 high-efficiency cells and plated on LB-ampicillin plates with IPTG and X-Gal. Recombinant clones were then selected as white colonies and subcultured for subsequent PCR amplification using the set of vector primers M13F (5′-dGTTTTCCCAGTCACGAC-3′) and M13R (5′-dCAGGAAACAGCTATGAC-3′). The PCR program for amplification consisted of a denaturation step of 92 °C for 5 min, followed by 30 cycles of 2 min at 92 °C for denaturation, 2 min at 60 °C for annealing, and 2 min at 72 °C for extension. Two individual clones of each species were sequenced using the vector primers and also the internal primers 5S1 and 5S2.

Sequences were determined at the Centro de Investigaciones Biológicas (Consejo Superior de Investigaciones Científicas, Madrid, Spain). DNA sequencing was accomplished using

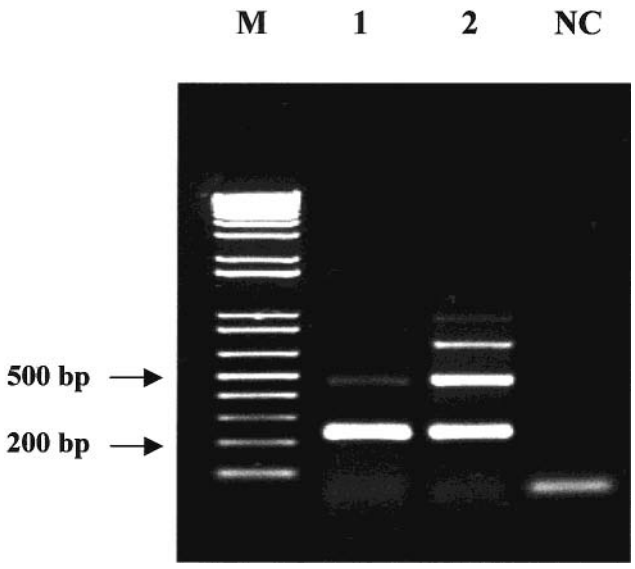

Figure 1. Electrophoretic analysis of the 5S rDNA PCR products obtained from mule duck (lane 1) and goose (lane 2). M, 100 bp ladder molecular weight marker (GibcoBRL); NC, negative control.

dRhodamine terminator cycle sequencing ready reaction kit (Perkin-Elmer/Applied Biosystems Division, Foster City, CA), in an ABI Prism model 377 DNA sequencer (Perkin-Elmer).

Sequence analysis, alignments, and restriction maps were performed using the Wisconsing package, version 9.0 (Genetics Computer Group, Madison, WI).

Design of Species-Specific Primers for Mule Duck and Goose. The 5S rDNA gene sequences obtained from mule duck were used for the design of a reverse primer specific for this species, 5SD: 5′-CCGCAAAAGCCCCCC-3′, corresponding to nucleotides 114−128 of the 5S rDNA (on the NTS fragment). Likewise, the 5S rDNA gene sequences from goose were used to design the reverse primer 5SG: 5′-TCTTCCCCACACCTGCACC-3′, which is specific for this species, and corresponds to nucleotides 175−193 of the goose 5S rDNA (on the NTS fragment). The forward primer 5S3, 5′-CGGAAGCTAAGCAGGGTCG-3′, was designed in the conserved coding region (nucleotides 23−41) of the 5S rDNA in order to be used in a multiplex PCR assay for both species.

PCR Amplification of Specific Fragments of the 5S rDNA Gene. Amplification of species-specific fragments of the 5S rDNA gene was achieved in a multiplex PCR format, using the forward primer 5S3 and the specifically designed 5SD and 5SG reverse primers (5 pmol of each primer). Double-stranded amplifications were carried out in a final volume of 50 μL containing 20 ng template DNA. Thirty-five amplification cycles were performed with the following step-cycle profile: strand denaturation at 94 °C for 45 s, primer annealing at 66 °C for 45 s, and primer extension at 72 °C for 45s. The last extension step was 5 min longer. An initial denaturation at 94 °C for 3 min was carried out to improve the final result.

RESULTS AND DISCUSSION

We have focused on the 5S ribosomal RNA gene as a suitable candidate for the genetic identification of related species because of its structure, which makes it a species-specific gene in higher eukaryotes (*22, 23*).

The 5S rDNA gene comprises a 120 bp highly conserved coding sequence (5S rRNA) and a nontranscribed spacer (NTS), which length and sequence 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. Because these repetitive DNA sequences show both high intraspecific homogeneity and high interspecific variability, they

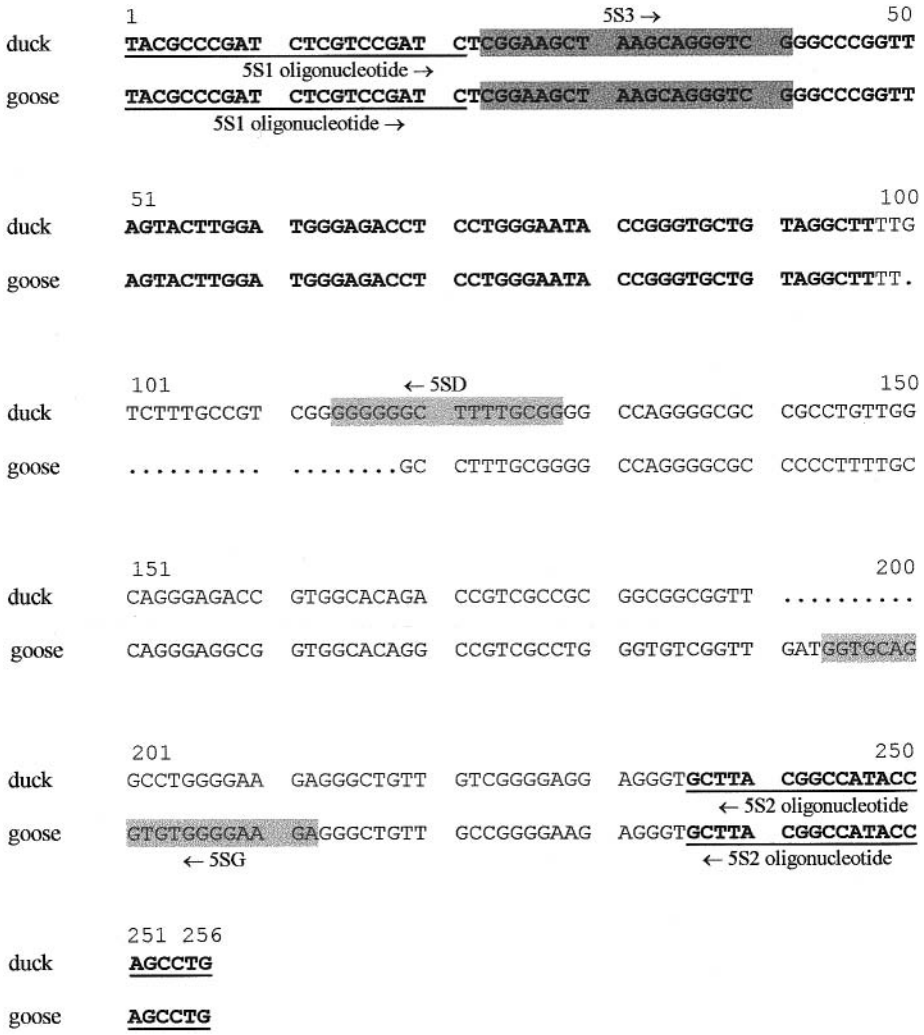

Figure 2. DNA sequences of the 5S rDNA gene from goose and mule duck samples. The coding sequence of the 5S rDNA gene is indicated in boldface type. Primers 5S1 and 5S2 are underlined. The forward primer 5S3 and the reverse species-specific primers 5SD and 5SG are shown with shadowing.

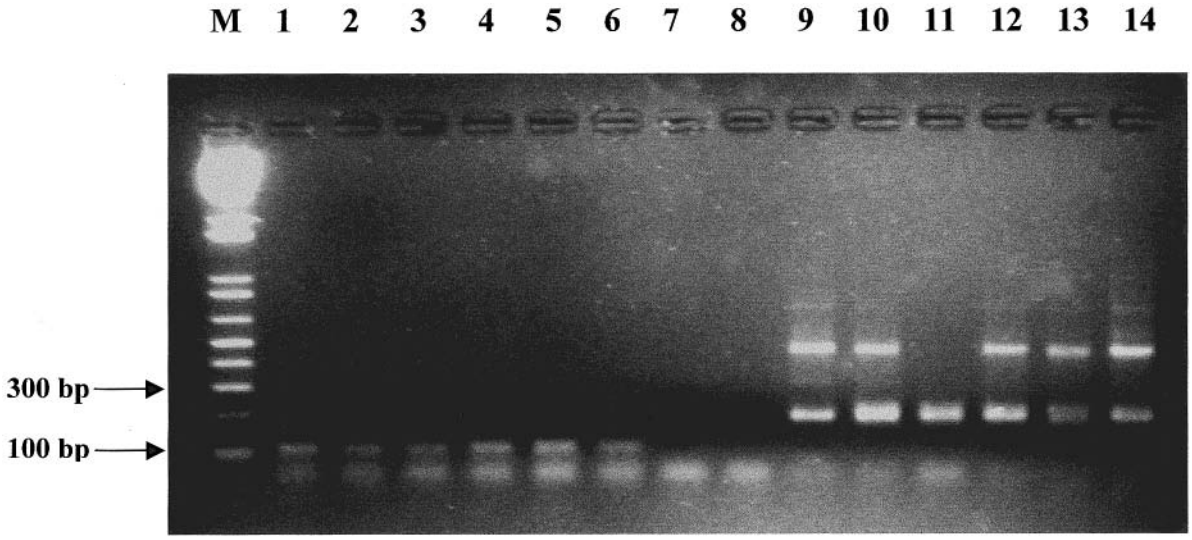

Figure 3. Electrophoretic analysis of the 5S rDNA multiplex PCR obtained from duck (lanes 1−6), chicken (lane 7), pork (lane 8), and goose (lanes 9−14) DNAs, using oligonucleotides 5S3, 5SD, and 5SG. Heat treatments of the samples are raw (lanes 1, 2, 7, 8, 9, and 10), pasteurized (lanes 3 and 4), and sterilized (lanes 5, 6, and 11−14). M, 100 bp ladder molecular weight marker (GibcoBRL).

have been considered as informative genetic markers for the identification of a species (*24*, *25*). They are also used for the determination of the phylogenetic relationship among closely related species in a wide variety of organisms including various vertebrates (*26*, *27*). The oligonucleotides 5S1 and 5S2, on the basis of the

conserved region of this gene in *Oncorhynchus mykiss*, have already been used to amplify a whole unit of the 5S rDNA gene (coding sequence + NTS) from salmon and trout templates (*28*, *29*). Using these primers, two DNA bands of approximately 250 and 500 bp were amplified from mule duck DNA, and four DNA bands of approximately 250, 500, 750, and 1000 bp were amplified from goose DNA (Figure 1). The sizes of amplification products were estimated to be the same in 15 individuals of each species. The smallest band of the PCR products (250 bp long) from goose and mule duck was purified and sequenced in two individuals from each species. According to the sequences obtained (Figure 2) the set of primers 5S1 and 5S2 amplified a fragment of 246 bp from mule duck and 237 bp from goose DNA, corresponding to one unit of the 5S rDNA gene. Accordingly, the variable NTS was determined to be 128 bp long in mule duck and 119 bp long in goose. These small differences in length of the NTS fragments for both species could not be clearly detected by simple agarose gel electrophoresis. Moreover, an unknown sample belonging to a different species could be wrongly identified as goose or mule duck if its NTS fragment were similar in length. To make the identification unequivocal for the species of interest, two different primers sequence-specific for duck (5SD) and goose (5SG) were designed. The combination of these primers, along with the 5S3 oligonucleotide, would allow the amplification of specific regions of the 5S rDNA gene for the two species analyzed.

For the design of the primers, the 5S rDNA sequences from duck and goose were aligned and compared. Figure 2 shows that goose and mule duck 5S rRNA coding region are identical in length and sequence, whereas the NTS region has 85% similarity. Therefore, we selected the NTS region to design the 5SD and 5SG species-specific primers. Alignment of the NTS sequences created a 10 bp gap in the mule duck and a 19 bp gap in the goose sequence that were particularly useful for this purpose. The 5SD reverse oligonucleotide, together with the forward 5S3 primer, amplifies a 106 bp fragment from duck, whereas no amplification is achieved from goose DNA. The 5SG primer, together with the forward 5S3 primer, amplifies a 171 bp fragment from goose, whereas no amplification is obtained from duck DNA.

To make the identification as simple as possible, a multiplex PCR amplification was carried out by using the three primers (5S3, 5SD, and 5SG) in the same reaction. The results showed (Figure 3) that no disturbance occurred among the three oligonucleotides during the amplification reaction and that only the primers specific to each DNA sample annealed on their target sequences. The same results were obtained in the analysis of products submitted to pasteurization and sterilization treatments.

Even though optimization of parameters that influence the reaction might be more complex than those in common PCR, multiplex PCR is becoming a rapid and convenient screening assay in both the clinical and the research laboratories, and has been successfully applied in many areas of DNA testing, including analyses of polymorphisms, quantitative assays, and reverse transcription PCR (*30*).

The results obtained in this work suggest that multiplex PCR amplification of selected 5S rDNA fragments using species-specific oligonucleotides 5SD and 5SG, together with 5S3 primer, is a powerful technique for the identification of mule duck and goose. With this method, the identification relies not only on the different sizes of the amplicons obtained, but also on the presence of the target sequences specific to each species studied. Because it is rapid, reliable, and easy to perform, the method described here offers a promising alternative for detection of fraudulent species substitution in the market of foie gras and other processed products containing goose or mule duck tissues.

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