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Identification of Goose, Mule Duck, Chicken, Turkey, and Swine in Foie Gras by Species-Specific Polymerase Chain Reaction

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A specific Polymerase Chain Reaction (PCR) has been developed for the identification of goose (*Anser anser*), mule duck (*Anas platyrhynchos* × *Cairina moschata*), chicken (*Gallus gallus*), turkey (*Meleagris gallopavo*), and swine (*Sus scrofa domesticus*) in foie gras. A forward common primer was designed on a conserved DNA sequence in the mitochondrial 12S ribosomal RNA gene (rRNA), and reverse primers were designed to hybridize on species-specific DNA sequences of each species considered. The different sizes of the species-specific amplicons, separated by agarose gel electrophoresis, allowed clear identification of goose, mule duck, chicken, turkey, and swine in foie gras. Analysis of experimental mixtures demonstrated that the detection limit of the assay was ~1% for each species analyzed. This genetic marker can be very useful for the accurate identification of these species, avoiding mislabeling or fraudulent species substitution in foie gras.

KEYWORDS: Species identification; foie gras; PCR; 12S rRNA

INTRODUCTION

Within the great variety of foie gras products that can be purchased in the marketplace, first-category foie gras products are the most expensive because they can be prepared only from goose or duck foie gras (*1*). In this category "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" are included. The only first-category foie gras product in which mixing duck and goose liver is allowed is the "goose and/or duck foie gras parfait". The adulteration of meat products by the addition of low-cost meats from different species has been previously reported (*2*, *3*). Obviously this practice serves economic purposes. Nowadays, due to an increase in the number of foie gras consumers (*4*), other fraudulent practices have been introduced either to improve the texture of the product or to diminish its cost, such as the addition of fresh liver from chicken, turkey, or swine (*5*). Goose foie gras, due to its high cost, popularity, and demand, is the most susceptible to substitution using liver or meat from less valuable animal species (*1*). Moreover, the incorporation of duck in goose foie gras results in lower cost, and blending goose with duck foie gras helps to solve technical problems due to the low melting point of the goose product.

Several analytical approaches have been taken to identify animal species in fresh or processed meat products in order to protect consumers from fraud (*6*, *7*). However, adulteration of goose foie gras has been detected mainly by sensory analysis (*8*, *9*), although electrophoretic (*10*) and genetic (*11*) techniques have been also developed.

DNA techniques have become very important and are widely used nowadays. Advantages of DNA analysis are manifold: DNA is a rather stable molecule, allowing analysis of processed and heat-treated food products (*12*); it contains higher information than proteins because of the degeneracy of the genetic code, and, due to the ubiquity of DNA, all kinds of tissues can be analyzed. Early methods based on hybridization of specific probes (*13*, *14*) were complicated and time-consuming.

Polymerase Chain Reaction (PCR) easily amplifies target regions of template DNA in a rapid and sensitive manner (*15*), and it is a recommended technique for meat identification. Many sequences of mitochondrial (*16*) or genomic DNA have been analyzed from meat species (*17*), fish (*18*), and plants (*19*).

In this work, a fragment of the 12S rRNA gene has been used to develop a specific PCR for the identification of goose (*Anser anser*), mule duck (*Anas platyrhynchos* × *Cairina moschata*), chicken (*Gallus gallus*), turkey (*Meleagris gallopavo*), and swine (*Sus scrofa domesticus*) in foie gras products.

MATERIALS AND METHODS

Sample Selection. Whole duck and goose raw fat livers (foie gras), provided by Martiko (Navarra, Spain), were anatomically identified in our laboratory and used as reference samples for the development of the assay. Raw and pasteurized goose and mule duck whole foie gras samples were provided by Antonio de Miguel (Madrid, Spain). Pasteurized first-category foie gras samples were provided by Imperia

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Table 1. DNA Sequences of the Primers Used in This Work

oligo	length	sequence (5′−3′)	used as
12SREV	27	TCCGGTACACTTACCTTGTTACGACTT	reverse to sequence
12SFW	26	CCACCTAGAGGAGCCTGTTCT(AG)TAAT	common forward and common to sequence
12SG	22	CTAAATCCGCCTTCCAGAAATG	goose specific
12SD	25	CACTTACCTCATCTTTGGCATTGAC	duck specific
12SC	23	CCGTCTTAAAGTGAGCTTAGCGG	chicken specific
12ST	27	TTGAGCTCACTATTGATCTTTCAGTTT	turkey specific
12SS	23	GTTACGACTTGTCTCTTCGTGCA	swine specific

foie gras (Gerona, Spain), and commercial sterilized ones were purchased at local delicatessen markets. Seven different commercial brands of foie gras were included in the sampling. Standard commercial pasteurization treatment includes heating at 80 °C for 90 min, whereas sterilization treatments consist of heating at 102−115 °C for 60−75 min.

Raw chicken and swine livers, turkey and swine muscles, and swine fat were bought at local markets.

Samples analyzed included raw whole mule duck and goose foie gras (7 of each), pasteurized mule duck and goose foie gras (6 of each), sterilized mule duck and goose foie gras (7 of each), raw chicken liver (15 samples), raw turkey muscle (15 samples), raw swine liver (10 samples), raw swine muscle (5 samples), and raw swine fat (5 samples). Ten different chicken, turkey, and swine samples (5 g of each) were submitted to pasteurization at 65 °C for 30 min, and 5 g of the same samples was submitted to sterilization at 121 °C for 20 min and were also analyzed. Samples were transported to the laboratory under refrigeration, and they were processed immediately or stored frozen at −85 °C until used.

To prepare binary liver mixtures (swine in goose, swine in duck, chicken in goose, chicken in duck, and duck in goose), raw foie gras from goose and mule duck and raw livers from chicken and swine were used. Besides, two binary muscle−liver mixtures using turkey muscles and goose and duck foie gras and one binary fat−liver mixture using swine fat and goose foie gras were also prepared. Seven different percentages containing 0.1, 1, 5, 10, 25, 50, and 100% (w/w) for each binary mixture (liver/liver, liver/muscle, or liver/fat) were prepared in a final weight of 100 g, using a blender (Sunbeam Oster, Schaumburg, IL). Five grams of each mixture was pasteurized at 65 °C for 30 min, and 5 g was sterilized at 121 °C for 20 min.

DNA Extraction. DNA was extracted using a Wizard DNA cleanup kit (Promega, Madison, WI), as described below.

Two g of foie gras, liver, muscle, fat, or binary mixture was homogenized in 8.6 mL of extraction buffer, pH 8.0 (10 mM Tris, 150 mM NaCl, 2 mM EDTA, and 1% SDS), 1 mL of 5 M guanidine hydrochloride, and 200 μL of 20 mg/mL proteinase K (Boehringer Mannheim GmbH, Mannheim, Germany). The samples were incubated overnight at 55 °C with shaking at 60 rpm (C24KC, New Brunswick Scientific Co., Edison, NJ), and they were left to cool at room temperature. Five milliliters of chloroform (Sigma Chemical Co., St. Louis, MO) was added to the lysate before centrifugation at 8500 rpm for 10 min. The clear aqueous supernatant obtained after the centrifugation (500 μL) was used to purify DNA using the Wizard DNA cleanup system kit (Promega) with a vacuum manifold, according to the manufacturer's instructions. The DNA was eluted in 50−100 μL of sterile deionized water, and its concentration was estimated by absorbance at 260 nm.

PCR Amplification. Primers *12SFW* and *12SREV* (sequences shown in **Table 1**) were designed for the amplification of a fragment of the 12S rRNA gene, based on sequences available in the Genbank database for duck, chicken, turkey, and swine (Accession no. U59666, X52392, U83741, and AJ002189, respectively). This set of primers should produce amplicons of similar lengths in the five species analyzed in this work: 392 bp in goose DNA, 394 bp in mule duck DNA, 400 bp in turkey DNA, 402 bp in chicken DNA, and 404 bp in swine DNA.

PCR amplification reactions were performed in a total volume of 50 μL. Each reaction mixture contained 150−200 ng of template DNA, 2 mM $MgCl_2$, 5 pmol of each primer, 200 μM of each dNTP, and 2 units of *Tth* DNA polymerase (Biotools, Madrid, Spain) in a reaction buffer containing 75 mM Tris-HCl, pH 9.0, 50 mM KCl, 20 mM $(NH_4)_2SO_4$, and 0.001% BSA.

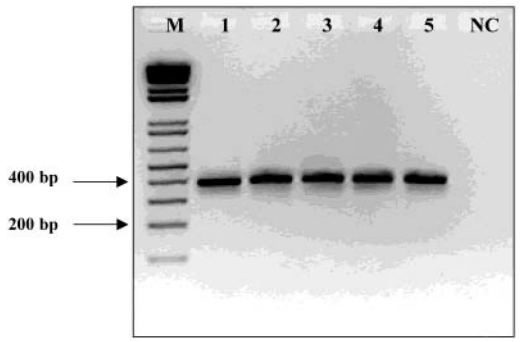

Figure 1. Electrophoretic analysis of the 12S PCR products, using primers *12SFW* and *12SREV*, obtained from goose (lane 1), mule duck (lane 2), chicken (lane 3), turkey (lane 4), and swine (lane 5). NC = negative control. M = molecular weight marker 1 kb plus DNA ladder (GibcoBRL). The picture is a reverse image of the ethidium bromide-stained gel.

PCR amplification 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 30 s at 93 °C for denaturation, 30 s at 63 °C for primer annealing, and 45 s at 72 °C for extension. The last extension step was 5 min longer.

PCR products were electrophoresed in a 1.5% D1 (Hispanlab S.A., Torrejón, Spain) agarose gel, containing 1 μg/mL ethidium bromide in Tris-acetate buffer (0.04 M Tris-acetate and 0.001 M EDTA, pH 8.0) for 45 min at 100 V. The resulting DNA fragments were visualized by UV transillumination and analyzed using Geldoc 1000 UV fluorescent gel documentation system-PC (Bio-Rad Laboratories, Hercules, CA).

Purification and Sequencing of PCR Products. PCR products obtained from goose, mule duck, chicken, turkey, and swine using oligonucleotides *12SFW* and *12SREV* were purified using the Qiaquick gel extraction kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. Purified PCR products were sequenced at the DNA Sequencing Center (Facultad de Farmacia, Universidad Complutense, Madrid, Spain). DNA sequencing was accomplished using a 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 and alignments were performed using the Wisconsing package, version 9.0 (Genetics Computer Group, Madison, WI).

Design of Species-Specific Primers and Amplification of Selected DNA Fragments. 12S rRNA gene sequences obtained from goose, mule duck, chicken, turkey, and swine PCR products were used for the design of reverse species-specific primers for each species (names and sequences are shown in **Table 1**). The forward primer *12SFW* (**Table 1**) is common for the five species analyzed in this work.

Amplification of species-specific fragments of the 12S rRNA gene was achieved in a PCR assay, using the forward primer *12SFW* and the specifically designed *12SG*, *12SD*, *12SC*, *12ST*, and *12SS* reverse primers (5 pmol of each primer). Double-stranded amplifications were carried out in a final volume of 50 μL containing 500−750 ng of template DNA. Thirty-five amplification cycles were performed with the following step-cycle profile: strand denaturation at 93 °C for 30 s, primer annealing at 63 °C for 30 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.

The detection limit of the method was determined by PCR amplification of the DNA obtained from experimental mixtures, containing 0.1−100% (w/w).

RESULTS AND DISCUSSION

Many PCR-based assays for species identification use DNA targets in the mitochondrial genome (*20*). These non-nuclear

Figure 2. DNA sequences of the 12S PCR products from goose, mule duck, chicken, turkey, and swine samples. Dots (...) indicate gaps introduced for alignment. Bold-type nucleotides indicate the position of primers *12SFW* and *12SREV* used for sequencing. Primers *12SG*, *12SD*, *12SC*, *12ST*, *12SS*, and *12SFW* are underlined.

targets possess several advantages over nuclear genes (*12*); they are generally more abundant in any given sample than single-copy nuclear genes (*21*) and, as mitochondrial DNA has a relatively high mutation rate compared with nuclear DNA, contain a greater accumulation of point mutations, which can be used to better define species differences, even in closely related species (*22*). Mitochondrial DNA tends to be inherited through the maternal germline, and the resulting lack of heterozygosity in the alleles under study simplifies analysis. Consequently, certain mitochondrial gene sequences have been reliably characterized across a variety of species (*23*). Using appropriate primer pairs, mitochondrial sequences have been amplified in many meat and fish species and the resulting differences used for species identification (*17*). The mitochondrial encoded gene for 12S rRNA was selected in this work for species identification because it has an acceptable length and an adequate grade of mutation and there are various sequences available in the databases.

Nucleotide sequences of 12S rRNA genes are available in the GenBank/EMBL databank for several of these species, such as duck (U59666), chicken (X52392), swine (AJ002189), and turkey (U83741). Comparison of the sequences available was used for the design of two primers, *12SFW* and *12SREV*, that should amplify a DNA fragment of ~400 bp in the five species (**Figure 1**). The length of the 12S rRNA gene fragments amplified from goose, mule duck, chicken, swine, and turkey was confirmed after the purification and sequencing of two individuals from each species analyzed. According to the sequences obtained (**Figure 2**) the set of primers *12SFW* and *12SREV* amplified a fragment of 392 bp in goose DNA, 394 bp in mule duck DNA, 400 bp in turkey DNA, 402 bp in chicken DNA, and 404 bp in swine DNA. These sequences were identical or very similar to those obtained from the GenBank/EMBL database.

On the basis of sequence alignment, species-specific PCR can be developed to control food authenticity, because a specific sequence can be detected very sensitively in a pool of sequences of different origins (*24*). In this work, to make the identification unequivocal for the species considered, five different reverse species-specific primers for goose (*12SG*), duck (*12SD*), chicken (*12SC*), swine (*12SS*), and turkey (*12ST*) were designed following sequence alignment and comparison (**Figure 2**). The combination of these primers, along with the forward *12SFW* oligonucleotide, would allow the amplification of specific regions of the 12S rRNA gene fragment for the five species analyzed.

The size of PCR products obtained was as expected from sequence analysis. The *12SG* primer, together with the forward *12SFW* primer, amplifies a 244 bp fragment from goose, whereas no amplification is obtained from chicken, swine, turkey, and duck DNA. The *12SD* primer, together with the forward *12SFW* primer, amplifies a 373 bp fragment from duck, whereas no amplification is obtained from chicken, swine, turkey, and goose DNA. The *12SC* reverse oligonucleotide, together with the forward *12SFW* primer, amplifies a 285 bp fragment from chicken, whereas no amplification is achieved from swine, turkey, duck, and goose DNA. The *12SS* primer, together with the forward *12SFW* primer, amplifies a 387 bp fragment from swine, whereas no amplification is obtained from chicken, turkey, duck, and goose DNA. Finally, the *12ST* primer, together with the forward *12SFW* primer, amplifies a 122 bp fragment from turkey, whereas no amplification is obtained from chicken, swine, duck, and goose DNA (**Figure 3**). All of the commercial first-category duck and goose foie gras samples

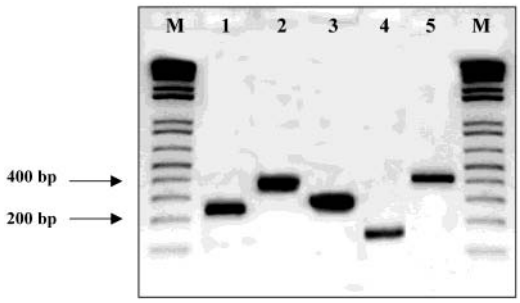

Figure 3. Electrophoretic analysis of the species-specific 12S PCR products obtained from goose (lane 1), mule duck (lane 2), chicken (lane 3), turkey (lane 4), and swine (lane 5). NC = negative control. M = molecular weight marker 1 kb plus DNA ladder (GibcoBRL). The picture is a reverse image of the ethidium bromide-stained gel.

analyzed, either raw, pasteurized, or sterilized, produced the expected duck- or goose-specific band and did not show contamination with other species (not shown).

No cross-reactivity was found when these five primer sets were tested with DNA from other meat species, such as cow, sheep, goat, horse, and rabbit (data not shown).

To determine the detection limit of the assay for the species analyzed, PCR amplification was performed on binary mixtures comprising 100, 50, 25, 10, 5, 1, and 0.1% (w/w) of the target species. For all species, it was observed that the lower the percentage of target species in the admixture, the fainter the band obtained in PCR with species-specific primer (**Figure 4**). Inhibition or unexpected PCR products were not found in the analysis of mixed species. The detection limit (lower percentage producing visible DNA amplification) of the assay was 1% or even lower for all species in the mixtures analyzed (**Figure 4**). Moreover, a detection limit of 0.1% was achieved for chicken liver in mixtures with goose or duck (**Figure 4A**,**B**) and swine liver in mixtures with goose or duck (**Figure 4E**,**F**).

The effect of thermal treatment of the samples on the technique's ability to identify species was studied through the analysis of pasteurized and sterilized experimental binary mixtures. Parts I and J of **Figure 4** show that the lower limit of chicken liver detection was not modified when the duck/chicken binary mixtures were submitted to pasteurization and sterilization treatments. Similar results were obtained for all of the species when pasteurized and sterilized binary mixtures were analyzed (not shown).

One consideration that should be mentioned is the variable amount of overall mitochondrial DNA per gram present in different kinds of tissues, such as muscle, liver, and fat. The content of mitochondria in liver cells, and thus the content of mitochondrial DNA, is ~3 times higher than in muscle cells (*25*). This could explain why the lower limit of detection for turkey muscle or swine fat in the assay is 1%, whereas detection of swine or chicken liver is possible with 0.1% swine or chicken liver in the mixtures. Besides, foie gras is obtained by submitting geese and ducks to a special diet. Force-feeding of these animals in their last weeks leads to a liver size increase due to the hypertrophy of the hepatocites, which does not happen in other cellular types (*26*). Hepatic steatosis is a reversible process (*27*) caused by an increase in the synthesis of lipids, although a hepatocites multiplication (hyperplasia) has never been demonstrated. Therefore, the rate DNA/weight of a tissue in foie gras is lower in a liver that has been submitted to force-feeding in the same species.

A genetic method for the differentiation of goose from mule duck foie gras, based on amplification of the 5S rDNA gene in

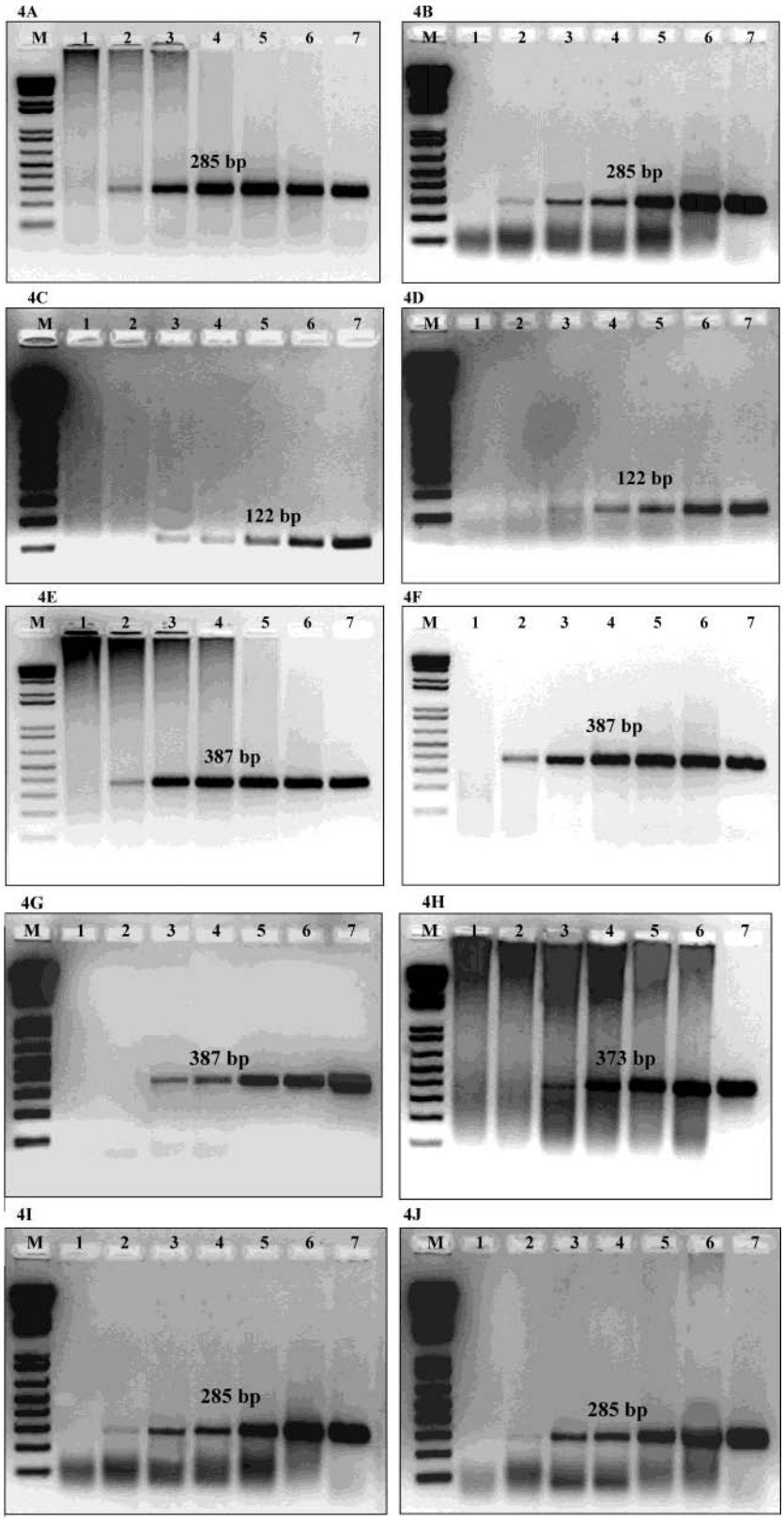

Figure 4. Electrophoretic analysis of the 12S PCR products amplified from experimental binary mixtures: (A) raw goose foie gras/chicken liver, amplified with primers *12SFW* and *12SC*; (B) raw duck foie gras/chicken liver, amplified with primers *12SFW* and *12SC*; (C) raw goose foie gras/turkey muscle, amplified with primers *12SFW* and *12ST*; (D) raw mule duck foie gras/turkey muscle, amplified with primers *12SFW* and *12ST*; (E) raw goose foie gras/swine liver, amplified with primers *12SFW* and *12SS*; (F) raw mule duck foie gras/swine liver, amplified with primers *12SFW* and *12SS*; (G) raw goose foie gras/swine fat, amplified with primers *12SFW* and *12SS*; (H) raw goose and mule duck foie gras, amplified with primers *12SFW* and *12SD*; (I) pasteurized duck foie gras/chicken liver, amplified with primers *12SFW* and *12SC*; (J) sterilized duck foie gras/chicken liver, amplified with primers *12SFW* and *12SC*. M = molecular weight marker 1 kb plus DNA ladder (GibcoBRL). Lanes 1–7 in all electrophoretic images are samples of the former species of the mixture, containing 0, 0.1, 1, 10, 25, 50, and 100% of the latter species, respectively. The pictures are reverse images of the ethidium bromide-stained gel.

a multiplex PCR assay, has been previously described (*11*). The basic unit of the 5S ADNr gene is tandemly repeated on the chromosome. Therefore, amplification of the 5S ADNr gene resulted in more than one DNA fragment, making semiquantitative detection of duck in goose/duck foie gras products difficult. Moreover, availability of more than one genetic marker is convenient as a technical support in the case of a legal requirement to demonstrate fraudulent substitution of species.

PCR is a useful method for routine species identification in foie gras, being quick and sensitive. Using the PCR method described herein, goose, mule duck, chicken, swine, and turkey are easily identified in foie gras at low levels of substitution. Besides, the method allows the semiquantitative detection of goose, mule duck, chicken, swine, and turkey in products containing different tissues (liver, muscle, and fat) or submitted to different heat treatments. Unambiguous interpretation of the results may be achieved visually without the need for computer analysis. Compared to alternative techniques such as direct sequencing of PCR products, PCR-RFLP, PCR-SSCP, RAPD, or DNA hybridization, PCR offers the advantages of being cheaper, faster, and more useful for routine analysis of large numbers of samples. The technique could be used in inspection programs to enforce labeling regulation of foie gras and related products.

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