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PCR-based fingerprinting techniques for rapid detection of animal species in meat products

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Abstract

A reproducible, rapid, and simple method for simultaneous identification of multiple meat species in a single step DNA-based test has been developed based on the generation of species-specific fingerprintings by two different arbitrary DNA amplification approaches (RAPD- and AP-PCR). Samples representative of various species and meat products submitted to different processing conditions were selected to verify the applicability of the techniques. RAPD-PCR fingerprintings allowed the discrimination amongst pork, beef, lamb, chicken and turkey in all cases. Samples corresponding to each species were clustered together at similarity levels $\geqslant 75\%$. The DNA profiles consisted of a discrete but reproducible number of bands, which made possible the interpretation of the results by simple visual inspection. AP-PCR also allowed identification of the five tested species in every sample although more complex patterns were generated, including some low intensity bands. In both cases, a ramp time between annealing and extension temperatures was introduced to achieve good reproducibility. Overall, the simplicity of RAPD-PCR patterns could make this technique suitable for meat authentication in routine analysis.

Keywords: RAPD-PCR; AP-PCR; Species identification; Meat products

1. Introduction

The application of quality assurance systems through the food chain requires the development of reliable and simple tools, which facilitate routine control assessments. The detection of meat species in different foods and feedstuffs deserves special attention due to the recent crisis in the meat sector (Brodmann & Moor, 2003). Consumers demand higher protection from falsely labelled meat products for a variety of economic, religious and health reasons. Thus, the prevention of fraudulent practices, nowadays, constitutes an important part of food regulatory control (Koh, Lim, Chua, Chew, & Phang, 1998). In this context, important progress on the development of robust techniques for meat species identification in commercialised products is being carried out (Calvo, Zaragoza, & Osta, 2001; Matsunaga et al., 1999; Myers, Yancy, & Farrell, 2003).

Meat proteins and DNA molecules have been used as species-specific biological markers. Several methods that rely on protein analysis have been described, including electrophoretic, immunological, chromatographic and mass-spectrometric techniques (Niederer & Bollhalder, 2001; Skarpeid, Kvaal, & Hildrum, 1998). However, the presence and characteristics of proteins are tissue-dependent and the manipulation of meat proteins during processing may alter their structure and stability. The speciation of cooked meat is especially difficult since the temperatures during heat treatment may destroy the species-specific proteins or epitopes (Calvo et al., 2001; Gouli, Mingguang, Zhijiang, Hongsheng, & Qiang, 1999). Alternatively, DNA analysis constitutes an attractive strategy for meat species identification. In comparison with proteins, DNA is stable against technological treatments and independent of the considered tissue (Martinez & Yman, 1998; Wolf, Rentsch, & Hübner, 1999). For these reasons, nucleic acid based analyses are now the preferred techniques for species identification in processed food. Initially, DNA hybridisation methods such as dot blot were applied but, currently, they have been replaced by those based on the

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amplification of genetic markers, basically, by two polymerase chain reaction (PCR) approaches. The first one is based on the definition of mono-locus-specific primers for amplification of a concrete DNA fragment and, the second one, on the use of arbitrarily chosen sequences as primers, which leads to multi-locus amplification of non-targeted DNA regions. Random amplified polymorphic DNA (RAPD) and arbitrarily primed PCR (AP-PCR) are two major methods based on the second principle (Welsh & McClelland, 1990; Williams, Kubelik, Livak, Rafalaski, & Tingey, 1990). The main differences between the two methods are the length of the primers (10-mer for RAPD-PCR and more than 18-mer for AP-PCR analysis) and the stringency of the amplification conditions.

In recent years, methods for species detection based on amplification of target DNA regions through the use of species-specific primers and universal primers have been applied (Fairbrother, Hopwood, Lockely, & Bardsley, 1998; Lockely & Bardsley, 2002; Matsunaga et al., 1999). The definition of mono-locus-specific primers is quite reliable. Nevertheless, the technique is conditioned by a priori knowledge of the nucleotide sequences flanking the loci and the use of species-specific primers may also reduce the number of species to be detected in a single PCR reaction (Lockely & Bardsley, 2002; Meyer et al., 1994). In addition, the results from the use of universal primers can be affected by the existing intraspecific polymorphisms and, normally, the identification of the amplicons requires the application of additional and more complex analytical techniques, such as sequencing, hybridisation and single-strand conformational polymorphism determination (SSCP-PCR) (Bellagamba, Valfrè, Panseri, & Moretti, 2001; Martinez and Danielsdottir, 2000; Myers et al., 2003; Wolf et al., 1999). The increased complexity of these approaches makes less realistic their application as routine quality control methods. Alternatively, amongst the non-targeted amplification methods, only RAPD-PCR analysis has been applied for meat species identification (Calvo et al., 2001; Koh et al., 1998; Martinez & Yman, 1998).

In the present work, PCR fingerprinting techniques based on the use of arbitrary primers (RAPD-PCR and AP-PCR) have been developed and compared for their ability to generate "fingerprint" patterns characteristics of five different meat species. Their potential application as reliable and simple methods for the determination of the origin of meat species is discussed.

2. Materials and methods

2.1. Samples

The samples from different animal species (pork, beef, lamb, chicken and turkey), including raw meat and meat products, were obtained from Spanish supermarkets. Pork was the declared species in samples 1–6 which included: (1) raw loin, (2) marinated loin, (3) raw sausage, (4) chorizo (dry-fermented sausage), (5) salchichón (dry-fermented sausage), and (6) cooked ham. Beef was the declared species in samples 7–9 which included: (7) raw steak, (8) undetermined raw meat, and (9) hamburger. Lamb was the declared species in samples 10 and 11 which included: (10) shoulder and (11) undetermined raw meat. Chicken was the declared species in samples 12–14, which included: (12) raw thigh, (13) raw breast and (14) cooked sausage. Turkey was the declared species in samples 15–18, which included: (15) raw wing, (16) raw breast, (17) hamburger and (18) cooked sausage.

2.2. DNA extraction

DNA extraction from meat samples was carried out with the Wizard® Genomic DNA purification Kit (Promega, Madison, WI, USA), according to the manufacturer's instructions for tissues. The concentration and quality of the DNA was estimated by measuring the absorption at 260 and 280 nm in an Ultrospec 300 UV-visible spectrophotometer (Pharmacia Biotech, Cambridge, UK) according to Sambrook, Fritsch, and Maniatis (1989).

2.3. DNA amplification by polymerase chain reaction (PCR)

Randomly Amplified Polymorphic DNA PCR (RAPD-PCR) analysis was carried out with the following 10-base primers: OPL-01 ($5'$GGC ATG ACC T$3'$), OPL-02 ($5'$TGG GCG TCA A$3'$), OPL-04 ($5'$GAC TGC ACA C$3'$) and OPL-05 ($5'$ACG CAG GCA C$3'$) (Torriani et al., 1996). Arbitrarily Primed PCR analysis (AP-PCR) was carried out with the following primers derived from the bacteriophage M13 genome and whose use has been recommended for detecting DNA polymorphisms in virtually all species: M-13 ($5'$GTT GTA AAA CGA CGG CCA GT$3'$) and DALP-232 ($5'$GTT TTC CCA GTC ACG ACG AC$3'$) (Desmarais, Lanneluc, & Lagne, 1998; Ryskov et al., 1988).

DNA was amplified in a Techne thermal cycler (Progene, Cambridge, UK). Amplifications were performed in a final volume of 50 μl containing PCR buffer (10 mM Tris–HCl, 50 mM KCl, pH 8,3), 200 μM each dNTP, 0.5 μM each primer, 1.0 U *Taq* DNA polymerase, 2.5 mM $MgCl_2$, and 100 ng of template DNA. All PCR reagents were provided from Roche (Mannheim, German) and primers from Isogen (Maarssen, The Netherlands). For RAPD-PCR the amplification programme was as follows: an initial cycle at 95 °C for 5 min; then 45 cycles of 94 °C for 1 min, 36 °C for 1 min,

and 72 °C for 2 min, using a ramp of 30 °C/min; and a final step at 72 °C for 5 min. For AP-PCR the amplification programme was as follows: an initial cycle at 95 °C for 5 min; then two cycles of amplification at low stringency of 94 °C for 5 min, 40 °C for 5 min and 72 °C for 5 min; after that, 40 cycles of amplification at high stringency of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min, using a ramp of 12 °C/min; and a final extension step at 72 °C for 5 min.

2.4. Agarose gel electrophoresis

The RAPD- and AP-PCR amplicons were separated by electrophoresis on 1% (w/v) agarose gels (15×10 cm), containing ethidium bromide (0.5 μg/ml), in 0.5×TBE (Sambrook et al., 1989). Electrophoresis took place for about 1.5 h at 100 V cm^{-1}. DNA fragments were visualized by UV transillumination and photographed under ultra violet light with a Image Master VSD (Pharmacia Biotech, Uppsala, Sweden), which allowed digitalisation of the negative photographs.

2.5. Data analysis

RAPD- and AP-PCR patterns were analysed with the software package LaneManager and 1D-Manager V2.0 (TDI, Barcelona, Spain). Calculations of similarities in the profile of bands were obtained by using the Dice coefficient and the unweighted pair group method with arithmetic average (UPGMA) clustering algorithm (Vauterin & Vauterin, 1992).

3. Results and discussion

A reproducible, rapid, and simple method for simultaneous identification of multiple meat species in a single step DNA-based test has been developed. The technique is based on the generation of species-specific fingerprintings by two different arbitrary DNA amplification procedures (RAPD- and AP-PCR) and their visualization after electrophoresis, thereby, avoiding more complex analytical steps such as DNA restriction, sequencing or hybridisation.

The processing technology (salting, drying, smoking and cooking) applied during the manufacture of meat products may affect to different extents the integrity of the extractable DNA. In general, heat treatments are those steps which mainly affect the quality of DNA causing its degradation into small size fragments whereas smoking and slating are less problematical (Dias Neto et al., 1994; Martínez & Yman, 1998). For this reason, a variety of meat products manufactured according to Spanish technologies were used as samples. Amongst those, a relatively large number of pork products were included since this is the species most frequently used in Spain. Overall, the processing conditions to which different products were submitted did not affect the yield and quality of the extracted DNA. Even in the case of cooked products high-molecular weight DNA could be extracted in sufficient amounts (3.6–6.0 μg/ml) with A_{260}:A_{280} ratios of around 1.8. These results demonstrate the applicability of DNA techniques for these kinds of samples as previously reported (Calvo et al., 2001; Dias Neto et al., 1994; Koh et al., 1998; Martínez & Yman, 1998).

3.1. Species identification by RAPD-PCR fingerprintings

The discriminating ability of the RAPD-PCR method is virtually unlimited as it is always possible to use other random primers. However, the amplification is carried out under relaxed stringency with short oligonucleotides, which are not strictly homologous to the target DNA and, therefore, the reproducibility, can be poor. In this sense, the selected primers and PCR conditions are critical to achieve high reproducibility and discrimination (Koh et al., 1998). In this study, several primers were initially tested (OPL-01, OPL-02, OPL-04 and OPL-05) and, finally, two of them (OPL-04 and OPL-05) were selected for RAPD-PCR on the basis of the number, intensity and distribution of bands able to clearly distinguish among species. The reproducibility was examined with both selected primers using DNA from two independent extractions of each sample, which were also used for two independent amplification reactions. The DNA fingerprintings were essentially the same in every case, showing excellent reproducibility (data not shown). This was achieved by the introduction of a prolonged ramp time between annealing and extension temperatures in the amplification programme (Louie et al., 1996).

Representative DNA profiles generated with the primer OPL-04 and the corresponding dendrogram are shown in Fig. 1. The profiles obtained with primer OPL-04 contained from 5 to 8 DNA bands with molecular sizes between 2.0 and 0.5 kbp. Two major clusters were defined by numerical analysis of the banding patterns at 60% similarity. One included chicken and turkey samples, which are genetically more closely related, and the other included samples from the remaining species. Five clusters representing each species could also be defined at above 80% similarity. The profiles of samples from beef were identical. The similarity amongst samples from either lamb, chicken or turkey was also high clustering at 87.5, 88.0 or 91.0%, respectively. Although pork samples constituted the most heterogeneous group their corresponding profiles still showed 82% similarity and shared, at least, three intense bands ranging from approx 1.0 to 0.6 kbp which clearly allowed their differentiation from the other

species. Although, as previously mentioned, chicken and turkey are species closely related their differentiation was also clear on the basis of the presence of an intense band of approximately 750 bp in samples from turkey that was absent in those from chicken.

The banding profile and dendrogram obtained by RAPD-PCR analysis with the primer OPL-05 are shown in Fig. 2. The profiles contained between 4 and 7 DNA bands with molecular sizes ranging from 2.0 to 0.3 kbp. Samples were also grouped into two major clusters but showing different compositions and lower similarity levels to those obtained with OPL-04. One major group included beef and pork samples (52.5% similarity), and the other included turkey, chicken and lamb samples (40.5% similarity). This analysis also allowed the definition of species-specific clusters at 75% similarity level or above. The profiles of beef samples were identical as well as those of lamb. Samples consisting of either turkey, pork or chicken produced closely related banding patterns and were clustered at 90, 79 or 75% similarity.

The similarity of the profiles of the different raw meat species using both primers, OPL-04 and OPL-05, indicated their correct commercial labelling.

Overall, RAPD-PCR fingerprintings obtained with the primers OPL-04 and OPL-05 were clear enough to allow discrimination between the common species used in Spanish meat products, even chicken and turkey. The reproducibility and simplicity of these patterns integrated by a small but reproducible number of bands makes the technique especially suitable for routine analysis due to easy interpretation of the results by visual inspection. Both aspects constitute a clear advantage in

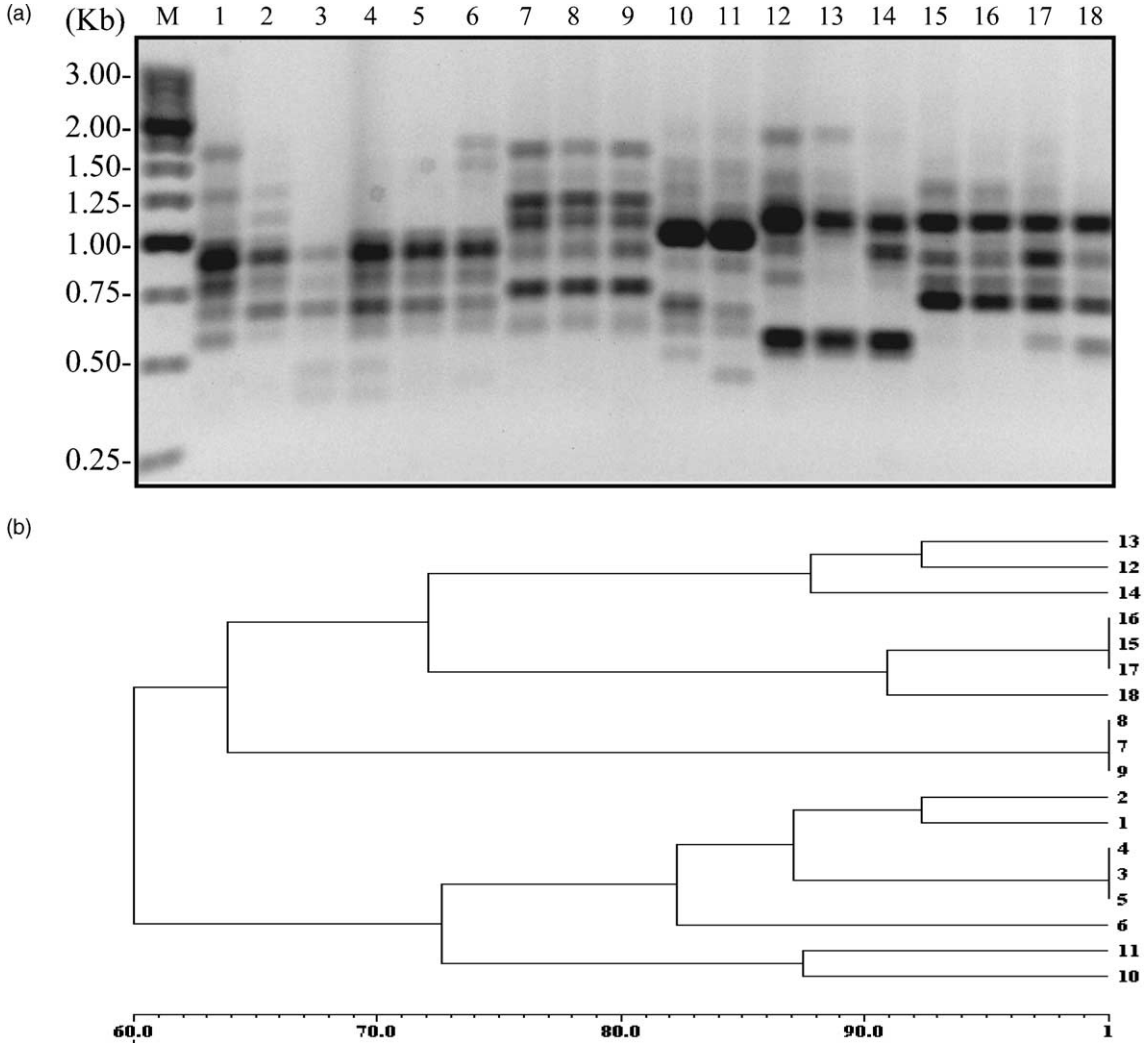

Fig. 1. Representative fingerprintings obtained by RAPD-PCR analysis using the primer OPL-04. (a) DNA banding profiles visualized on a 1% agarose gel stained with ethidium bromide. Lane M, DNA molecular weight marker XVI (Roche, Mannhein, Germany); lane 1, raw pork loin; lane 2, marinated pork loin; lane 3, raw pork sausage; lane 4, chorizo (pork dry-fermented sausage); lane 5, salchichón (pork dry-fermented sausage); lane 6, cooked pork ham; lane 7, beef steak; lane 8, undetermined part of beef; lane 9, beef hamburger; lane 10; lamb shoulder; lane 11, undetermined part of lamb; lane 12, raw thigh chicken; lane 13, raw breast chicken; lane 14, cooked chicken sausage; lane 15, raw wing turkey; lane 16, raw breast turkey; lane 17, turkey hamburger; and lane 18, cooked turkey sausage. (b) Dendrogram obtained by numerical analysis of RAPD-PCR profiles using the UPGMA method.

relation to those obtained in previous studies using other primers and amplification conditions. The major disadvantages previously detected in the application of RAPD-PCR analysis for species identification were related to the difficulties found in reproducing the DNA banding patterns and the generation of complex fingerprintings whose interpretation was not straightforward (Koh et al., 1998; Martínez & Yman, 1998).

3.2. Species identification by AP-PCR fingerprintings

AP-PCR constituted an alternative method to RAPD-PCR, which is also based on arbitrary amplification of unknown DNA sequences. However, in AP-PCR the use of longer primers and higher stringency amplification conditions seems to confer higher reproducibility and resolution (Desmarais et al., 1998). The method involves two cycles of amplification at low stringency followed by others at higher stringency (Desmarais et al., 1998; Welsh & McClelland, 1990). It has been demonstrated to be able to generate reproducible fingerprintings of complex genomes and has been used in population genetic studies and in the characterisation of strains and species of bacteria, plants and animals (Desmarais et al., 1998; Louie et al., 1996; Welsh & McClelland, 1990). Moreover, this is the first time that AP-PCR has been applied for species identification in meat and meat products. From the primers initially tested M-13 was selected for its potential application in species detection according to the distribution of the

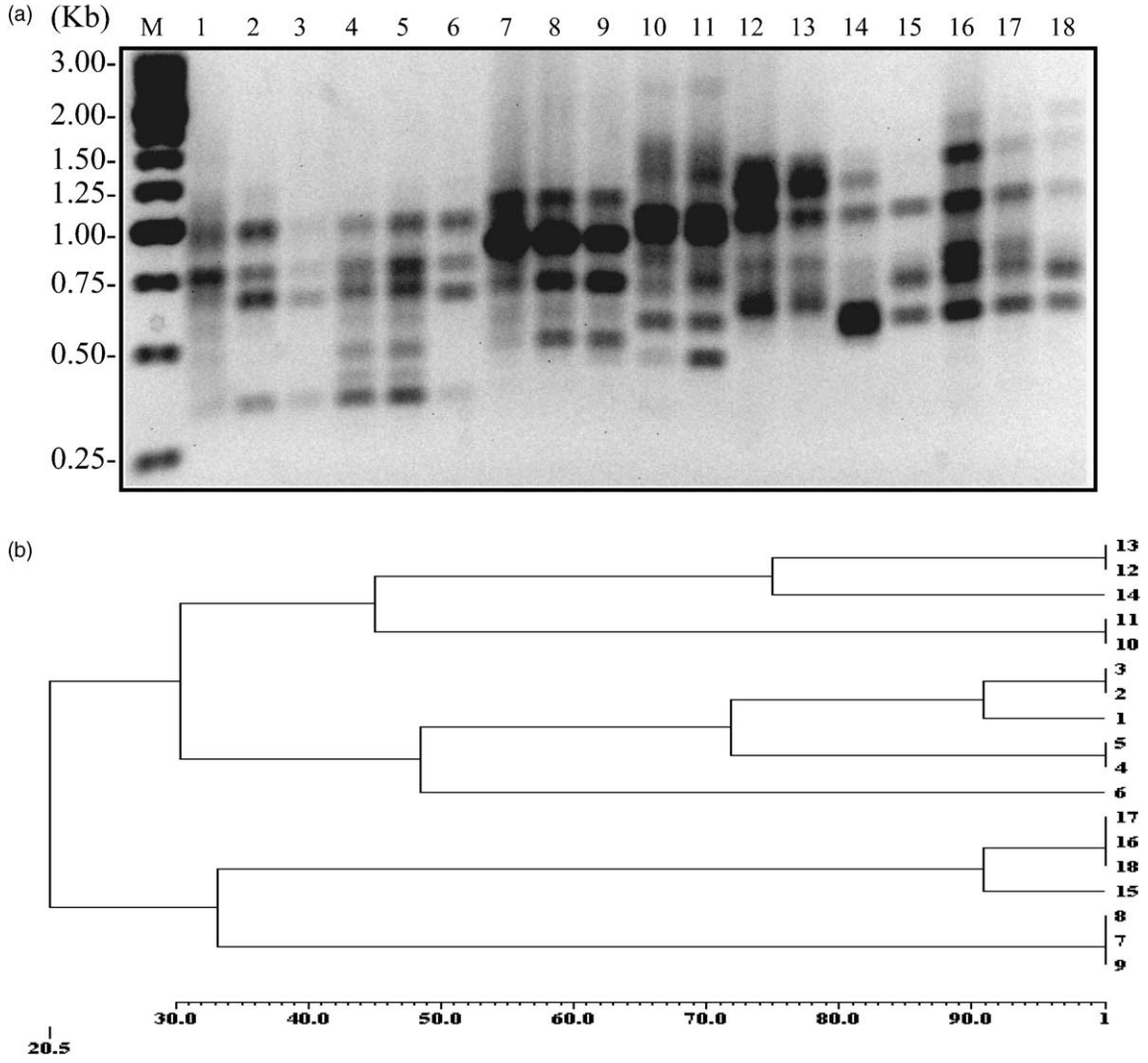

Fig. 2. Representative fingerprintings obtained by RAPD-PCR analysis using the primer OPL-05. (a) DNA banding profiles visualized on a 1% agarose gel stained with ethidium bromide. Lane M, DNA molecular weight marker XVI (Roche, Mannhein, Germany); lane 1, raw pork loin; lane 2, marinated pork loin; lane 3, raw pork sausage; lane 4, chorizo (pork dry-fermented sausage); lane 5, salchichón (pork dry-fermented sausage); lane 6, cooked pork ham; lane 7, beef steak; lane 8, undetermined part of beef; lane 9, beef hamburger; lane 10; lamb shoulder; lane 11, undetermined part of lamb; lane 12, raw thigh chicken; lane 13, raw breast chicken; lane 14, cooked chicken sausage; lane 15, raw wing turkey; lane 16, raw breast turkey; lane 17, turkey hamburger; and lane 18, cooked turkey sausage. (b) Dendrogram obtained by numerical analysis of RAPD-PCR profiles using the UPGMA method.

DNA bands. The reproducibility of the DNA profiles was verified as previously indicated for RAPD-PCR analysis. The DNA profiles and clusters obtained by their numerical analysis are shown in Fig. 3. Two major clusters were defined at 45% similarity, one included chicken and turkey samples and the other beef, pork and lamb samples as was the case for RAPD-PCR analysis with OPL-04. Samples from the five different species tested were grouped together by sharing a species-specific fingerprinting of at least 80% similarity (Fig. 3). Beef samples constituted the most homogeneous group clustering at 100% similarity. The profiles corresponding to samples made of lamb clustered at 87.5% similarity as did those corresponding to chicken. The more heterogeneous group was for pork samples, clustering at 80% similarity. The profiles of turkey samples were 79% similar. The DNA patterns from raw meat samples were similar to those of meat products, corroborating the results obtained by RAPD-PCR.

Thus, AP-PCR with the primer M-13 also allowed identification of the five tested species in different meat products. However, more complex patterns were generated including low intensity bands, which introduced some difficulty in visual interpretation.

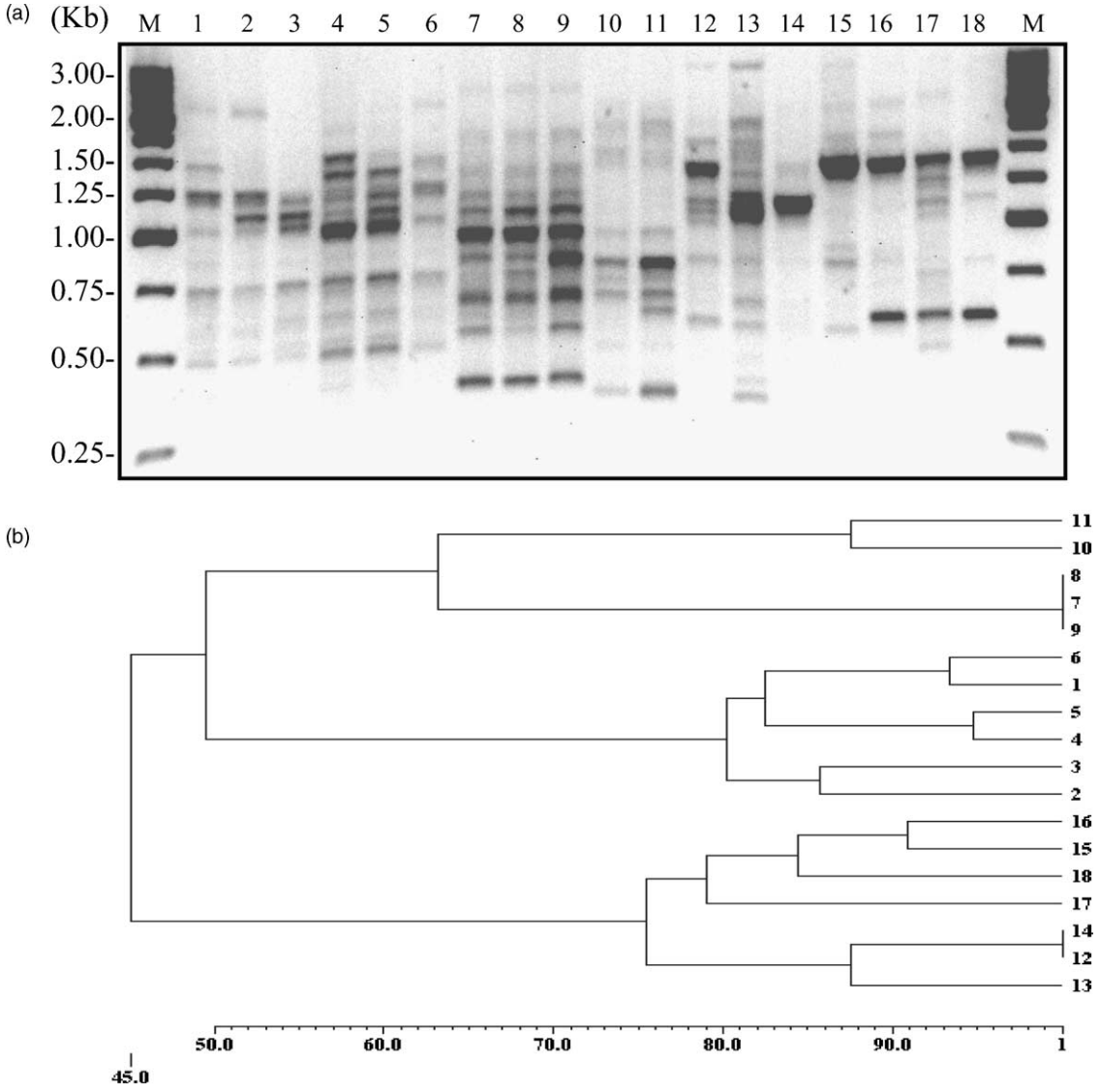

Fig. 3. Representative fingerprintings obtained by AP-PCR analysis using the primer M13. (a) DNA banding profiles visualized on a 1% agarose gel stained with ethidium bromide. Lane M, DNA molecular weight marker XVI (Roche, Mannhein, Germany); lane 1, raw pork loin; lane 2, marinated pork loin; lane 3, raw pork sausage; lane 4, chorizo (pork dry-fermented sausage); lane 5, salchichón (pork dry-fermented sausage); lane 6, cooked pork ham; lane 7, beef steak; lane 8, undetermined part of beef; lane 9, beef hamburger; lane 10; lamb shoulder; lane 11, undetermined part of lamb; lane 12, raw thigh chicken; lane 13, raw breast chicken; lane 14, cooked chicken sausage; lane 15, raw wing turkey; lane 16, raw breast turkey; lane 17, turkey hamburger; and lane 18, cooked turkey sausage. (b) Dendrogram obtained by numerical analysis of RAPD-PCR profiles using the UPGMA method.

4. Conclusions

RAPD- and AP-PCR are molecular techniques which are easier, faster and cheaper than other DNA-based techniques. The potential of the RAPD-PCR technique, using the primers OPL4 and OPL-5, for discrimination amongst the most common species (pork, beef, lamb, chicken and turkey) used in Spanish meat products produced by different processing technologies has been demonstrated. Both RAPD profiles generated by the selected primers resulted in relatively simple DNA fingerprintings from which the species of origin can be visually inferred, making this technique especially suitable for routine analysis. The application of AP-PCR using the primer M13 also allowed the detection of five species-specific fingerprintings. However, it gave a higher number of amplicons, including a significant number of faint bands, which made visual interpretation more difficult. Overall, the assayed techniques could be robust and simple methods to be considered as additional quality control tools in meat species identification.

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