African Journal of Biotechnology Vol. 11(89), pp. 15590-15595, 6 November, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.1753
ISSN 1684–5315 ©2012 Academic Journal

Full Length Research Paper

Species specific polymerase chain reaction (PCR) assay for identification of pig (*Sus domesticus*) meat

Arun Kumar, Rajiv Ranjan Kumar*, Brahma Deo Sharma, Sanjod Kumar Mendiratta, Deepak Sharma and Palanisamy Gokulakrishnan

Division of Livestock Products Technology, Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India.

Accepted 4 October, 2012

A highly specific single step polymerase chain reaction (PCR) is described for the detection of pig (*Sus domesticus*) meat. A PCR assay was successfully optimized for amplification of 629 and 322-bp DNA fragment extracted from pig meat using designed species-specific primer pairs based on mitochondrial D-loop and 12S ribosomal ribonucleic acid (rRNA) gene, respectively. The optimized PCR assay was subsequently validated for its specificity with deoxyribonucleic acid (DNA) extracted from cattle, buffalo, sheep, goat and pig. PCR amplification of target DNA with pig-specific primers was repeated 15 times, with consistent results observed. The specificity of pig-specific PCR provides a valuable tool for identification of pig meat and to avoid its fraudulent substitution and adulteration.

Key words: Pig meat, adulteration, polymerase chain reaction (PCR), mitochondrial D-Loop, 12S ribosomal ribonucleic acid (rRNA) gene.

INTRODUCTION

The adulteration/substitution of meat has always been a concern for various reasons such as public health, religious factors, wholesomeness and unhealthy competition in meat market (Arslan et al., 2006). Consumer should be protected from these malicious practices of meat adulterations by quick, precise and specific identification of meat animal species. Several analytical methodologies have been employed for meat authentication based on anatomical, histological, microscopic, organoleptic, chemical (Matsunaga et al., 1999a; Ilhak and Arslan, 2007), electrophoretic (Renon et al., 2003; Vallejo et al., 2005), chromatographic (Ashoor et al., 1988; Toorop et al., 1997) or immunological (Chen and Hsieh, 2000; Hajmeer et al., 2003; Giovannacci et al., 2004) principles. However, by virtue of their inherent limitations (Calvo et al., 2001), most of these techniques have been replaced by the recent DNA-based molecular techniques.

The reasons why DNA is the method of choice for the purpose of species identification include (i) its stability, allowing species identification even in heated and processed products; (ii) the conserved nature of DNA structure in all tissues of an individual (Saini et al., 2007); and (iii) higher discriminating power of closely related species due to informative nucleotide algorithms of adenine (A), thymine (T), guanine (G) and cytosine (C) (Wolf et al., 1999). The information available in the DNA molecule is enormous as compared to proteins. Hence, DNA based techniques are the most preferred for the purpose of meat speciation.

Different DNA-based techniques used for animal species identification include DNA hybridization (Baur et al., 1987; Janssen et al., 1998), polymerase chain reaction (PCR) and its variants (Matsunaga et al., 1999a, b), polymerase chain reaction- restriction fragment length

*Corresponding author: E-mail: dr_rajivranjan@yahoo.com. Tel: 09897299102.

Abbreviations: PCR, Polymerase chain reaction; **PCR-RFLP,** polymerase chain reaction-restriction fragment length polymorphism; **PCR-SSCP,** polymerase chain reaction single-strand conformation polymorphism.

polymorphism (PCR-RFLP) (Girish et al., 2005), random amplification of polymorphic DNA- polymerase chain reaction (RAPD-PCR) (Sebastio et al., 2001; Mane et al., 2006; 2008), polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) (Weder et al., 2001) and PCR-sequencing (Bartlett and Davidson, 1992). In the early developments of animal species identification, species-specific probes were hybridized to DNA extracted from meat samples (Lenstra et al., 2001).

The species-specific probes (satellite repetitive DNA) enabled improved specificity and paved way for the detection of animal species (Lenstra and Buntjer, 1999). A probe generated by PCR amplification of species-specific satellite complementary DNA (cDNA) has also been used in hybridization. Based on the conserved sequence contained in the actin multigene family, the genomic DNA was digested with BamHI, electrophoresed and hybridized to actin cDNA probe to differentiate beef, pork, lamb, horse, chicken and fish species (Janssen et al., 1998). However, the major disadvantages of DNA hybridization include time and labor, cross-reactivity of closely related species. Further, in view of the complicated procedure and consequent to the unprecedented developments in molecular biology, the PCR has almost replaced the DNA hybridization for the purpose of species identification. On the other hand, other DNA based methods are costly, time consuming and require post-PCR analysis either in the form of restriction digestion (PCR-RFLP) or in-silico analysis (RAPD-PCR and PCR sequencing) for precise identification.

However, species-specific PCR assay was found to be rapid and cost effective for identification of meat species due to specific detection of target sequence without the need of further sequencing or digestion of the PCR products with restriction enzymes (Rodriguez et al., 2004) and successfully used for identification of various species of meat (Ilhak and Arslan, 2007; Martin et al., 2007; Frezza et al., 2008; Mane et al., 2007). These PCR assays targets genomic as well as mitochondrial DNA for the purpose of meat species identification. However, in the present study the mitochondrial DNA was used for meat species identification because of the maternal inheritance of mitochondria, where normally only one allele exists in an individual and thus no sequence ambiguities are expected from the presence of more than one allele (Unseld et al., 1995).

The variable regions of the mitochondrial gene are present in thousands of copies per cell (Greenwood and Paboo, 1999), which increases the probability of achieving a positive result even in severe DNA fragmentation due to intense processing conditions (Bellagamba et al., 2001). Mitochondrial DNA evolves much faster than nuclear DNA (Brown et al., 1982) and presents more sequence diversity, thus facilitating the identification of closely related species (Pfeiffer et al., 2004). Here, we describe a species specific PCR assay which holds a unique advantage over other DNA based methods in terms of specificity, sensitivity, robustness and rapidity. Keeping these considerations in view, the present work was designed to develop highly specific PCR using newly developed primers for identification of pig meat in raw meat samples.

MATERIALS AND METHODS

Sample selection and DNA extraction

The fresh meat samples from five different species of food animals that is, pig (*Sus domesticus*), cattle (*Bos indicus* and *Bos taurus*), buffalo (*Bubalus bubalis*), sheep (*Ovis aries*), and goat (*Capra hircus*) were collected from the local slaughterhouses and experimental abattoir of the Institute. The collected samples, one from each species were transported to the laboratory under refrigeration, and were stored frozen at -20°C prior to analysis.

DNA was extracted from the samples using the DNeasy® blood and tissue kit (QIAGEN, Germany) according to the manufacturer's instructions. Subsequently, the quality of DNA was assessed by agarose gel electrophoresis using 0.8% agarose gel (AMRESCO, USA) stained with ethidium bromide. The purity and concentration of DNA was estimated spectrophotometrically using Nanodrop® ND-1000 spectrophotometer (Thermo Scientific) at 260 and 280 nm. The DNA sample showing the $OD_{260:280\ nm}$ value of 1.70 to 1.90 was considered as good quality.

Design of PCR primers

Pig mitochondrial D-loop and 12S rRNA gene sequences were downloaded from the GenBank database and aligned using the "Megalign" program (Lasergene software; DNAStar, Inc., Madison, Wisconsin, USA). The primer sites with nucleotide sequence variation between species and homology within a species (free from SNPs) were located. The oligonucleotide primers were designed against such unique species-specific sites so as to yield appropriately sized PCR products using the "Primer-Select" program (Lasergene software; DNAStar, Inc.).

Selected primers were screened for primer specificity (species specificity) and cross reactivity using local alignment tool "BLAST" (http://www.ncbi.nlm.nih.gov/blast). Finally, selected primers were custom synthesized (Eurofins Genomics India Pvt Ltd. Bangalore, India) and used for PCR amplification. Information on the four primers (MDL-F, MDL-R, 12S rRNA-F and 12S rRNA-R) used in this study is shown in Table 1.

PCR amplification

Polymerase chain reaction (PCR) was performed in 25 µl of reaction mixture containing 50 ng of genomic DNA, 200 µm of each dNTP, 1.5 mM $MgCl_2$, 5 pmoles of each primer, 1 unit GoTaq® Flexi DNA Polymerase (Promega, Madison, WI, USA), 1x PCR-colored buffer (Promega, Madison, WI, USA), and nuclease-free water to make a final volume. Amplification was performed on a PTC-200 DNA Engine® thermal cycler, (Bio-Rad, USA) using 0.2 ml reaction tubes. The PCR program consisted of 5 min denaturation at 94°C, followed by 34 cycles of denaturation (94°C, 45 s), annealing (63°C, 45 s for mt D-loop primers and 70°C, 45 s for 12S rRNA primers), and primer extension (72°C, 45 s). The final cycle was followed by extension at 72°C for 10 min and indefinite hold time at 4°C.

Table 1. The details of the pig specific primers used in the present investigation.

Primer name	Gene	Primer length (bp)	Sequence (5'- 3')	Primer combination	Amplicon size (bp)
MDL-F	mt D-loop	20	GCAACCCGCTTGGCAGGGAT	MDL-F and MDL-R	629
MDL-R	mt D-loop	20	TTTGGGGTTTGGCAAGGCGT		
12S rRNA-F	12S rRNA	21	TGGCGGTGCTTCACATCCACC	12S rRNA-F and 12S rRNA-R	322
12S rRNA-R	12S rRNA	20	GCGGTGTGTGCGTGCTTCAT		

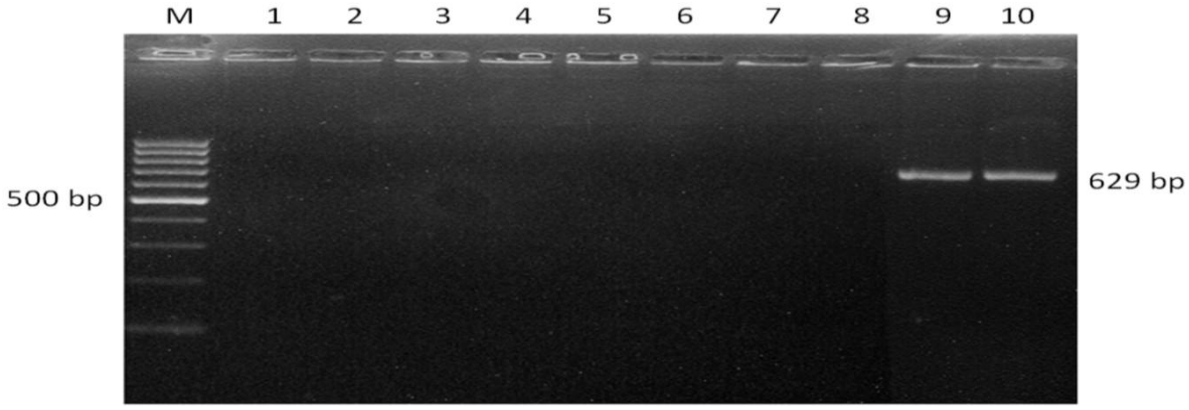

Figure 1. PCR amplification of mitochondrial d-loop region in pig. Lane M, 100 bp DNA ladder; lanes 1 and 2, cattle; lanes 3 and 4, buffalo; lanes 5 and 6, sheep; lane 7 and 8, goat; lanes 9 and 10, pig (629 bp amplicon).

Gel electrophoresis

The submarine horizontal agarose gel electrophoresis was used for analysis of PCR products. Two percent agarose was used for preparation of gel. For that 0.4 g of agarose (AMRESCO, USA) was put in 20 ml of 1x TBE solution (Fermentas, USA) and heated to completely dissolve the agarose. Then 1 µl (5%) ethidium bromide solution was added as gel visualizing agent and mixed thoroughly. The electrophoresis was done for 90 min at 80 V. The PCR product was finally analyzed using UV transilluminator and documented by gel documentation system (Alpha Imager, USA). The ready to use 100 bp ladders (O'Gene Ruler, Fermentas, USA) was used for the present work.

RESULTS AND DISCUSSION

A fragment of 629-bp from pig mitochondrial d-loop gene and 322-bp from pig 12S rRNA gene was amplified. Different primer concentrations (5 to 15 picomoles) and annealing temperatures (55 to 70°C) were employed for the standardization of PCR. An optimum primer concentration of 5 picomoles and annealing temperature of 63°C (for mt D-loop primers) and 70°C (for 12S rRNA primers) were found suitable for amplification. In a similar study, Matsunaga et al. (1999a) reported cytochrome-b gene based species-specific primers for the identification of pig meat (398 bp amplicon) along with poultry, cattle, sheep, goat and horse meats.

Earlier, restriction digestion based PCR assays targeting different mitochondrial genes, namely cytochrome-b gene (Partis et al., 2000; Sun and Lin, 2003; Maede, 2006), 12S rRNA gene (Girish et al., 2005) and d-loop gene (Malisa et al., 2006), have been employed for identification of pig meat. However, the pig specific assay developed in the present study, being a single step method, offers advantages over the previously reported techniques with respect to its simplicity, specificity and accuracy. The occurrence of 629 and 322-bp PCR product on gel electrophoresis confirms the presence of pig DNA and does not require further analysis for confirmation. Similar findings were reported by Nagappa (2008), who targeted mitochondrial d-loop gene for differentiation of meats from six meat animal species including pig.

The possibility of cross amplification was precluded by analyzing pig specific primers with DNA extracted from cattle, buffalo, sheep and goat meats. The 629 and 322 bp amplicon was evident in pig DNA only and no amplification was observed in the DNA of other species including a negative control (Figures 1 and 3). Specificity of the PCR assay was confirmed 15 times by testing pig-specific primers with DNA isolated from different pig meat samples, where invariably 629 and 322-bp amplicon was

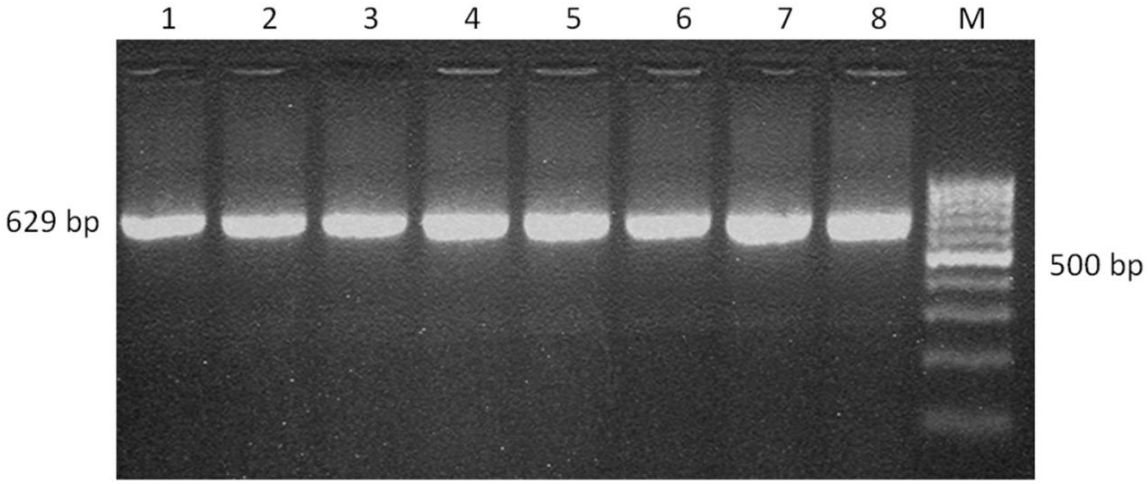

Figure 2. PCR amplification of mitochondrial d-loop region in different pig meat samples for validation of PCR. Lanes 1 to 8, different pig meat samples (629 bp amplicon); lane M, 100 bp DNA ladder.

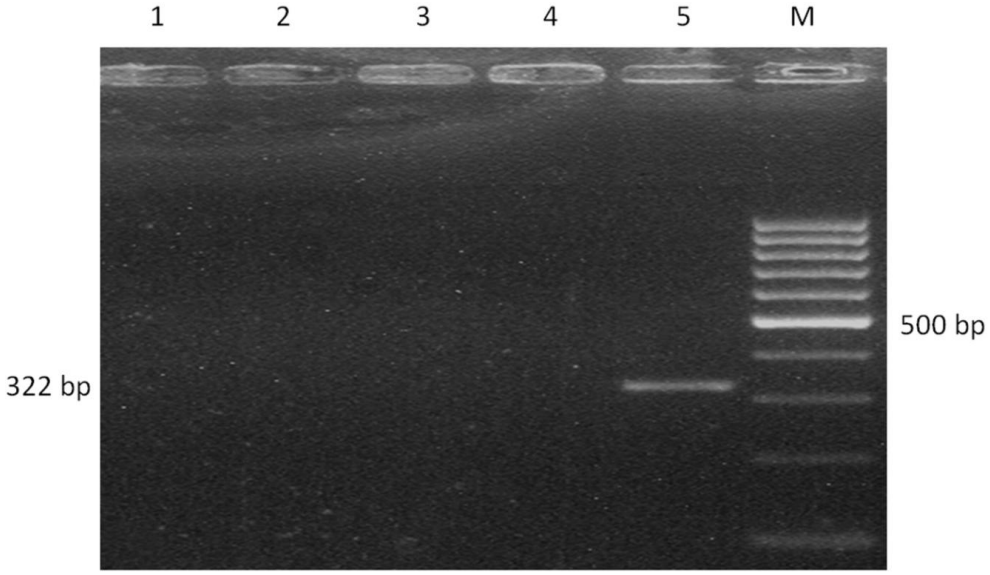

Figure 3. PCR amplification of 12S rRNA gene in pig. Lane 1, cattle; lane 2, buffalo; lane 3, sheep; lane 4, goat; lane 5, pig (322 bp amplicon); lane M, 100 bp DNA ladder.

obtained (Figures 2 and 4).

Conclusions

In conclusion, the pig-specific PCR assay developed in the present work presents an easy and reliable single step PCR assay for identification of pig meat. The suitability of this PCR assay lies in simplicity in result interpretation. The present work would help in addressing social, religious, economic, forensic and public health issues related to pig species identification and, further, it could also be used for routine analysis of suspected meat samples.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Indian Veterinary Research Institute (IVRI) and Central Avian Research Institute (CARI), Izatnagar, India for providing necessary facilities to accomplish this research. The help rendered

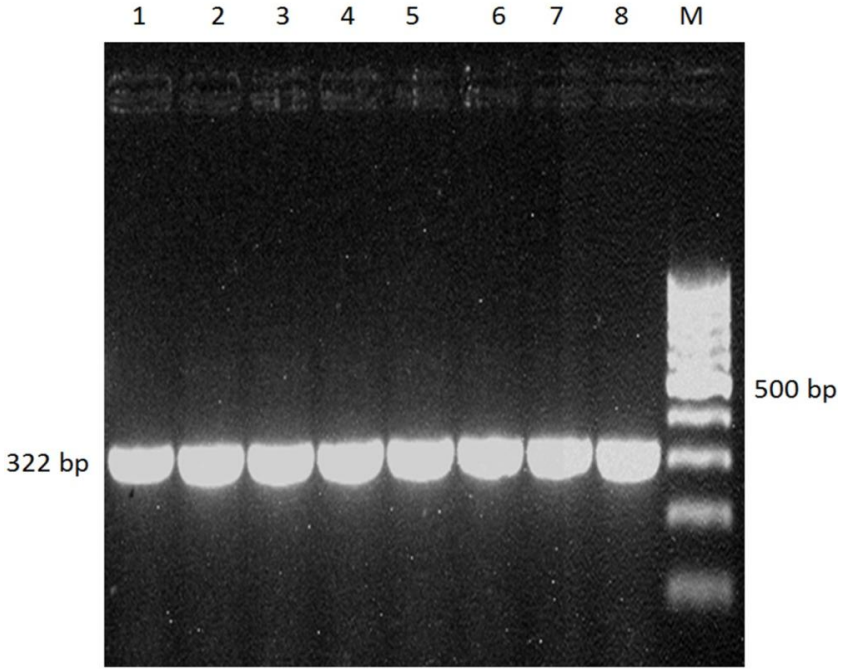

Figure 4. PCR amplification of 12S rRNA gene in different pig meat samples for validation of PCR. Lanes 1 to 8, different pig meat samples (322 bp amplicon); lane M, 100 bp DNA ladder.

by Drs. Girraj Goyal and Rahul Arya is highly acknowledged.

REFERENCES

Arslan A, Irfan-Ilhak O, Calicioglu M (2006). Effect of method of cooking on identification of heat processed beef using polymerase chain reaction (PCR) technique. Meat Sci. 72:326-330.

Ashoor SH, Monte WC, Stiles PG (1988). Liquid chromatographic identification of meats. J. AOAC Int. 71:397-403.

Bartlett SE, Davidson WS (1992). FINS (forensically informative nucleotide sequencing): a procedure for identifying the animal origin of biological specimens. Biotechnology 12:408-411.

Baur C, Teifel-Greiding J, Leibhardt E (1987). Spezifizierung hitzedenaturierter Fluschproben durch DNA-analyse. Arch. Lebensmittelhyg. 38:172-184.

Bellagamba F, Moretti VM, Comincini S, Valfre F (2001). Identification of species in animal feedstuffs by polymerase chain reaction–restriction fragment length polymorphism analysis of mitochondrial DNA. J. Agric. Food Chem. 49:3775-3781.

Brown WM, Prager EM, Wang A, Wilson AC (1982). Mitochondrial DNA sequences of primates: tempo and mode of evolution. J. Mol. Evol. 18:225-239.

Calvo JH, Zaragoza P, Osta R (2001). Technical note: a quick and more sensitive method to identify pork in processed and unprocessed food by PCR amplification of a new specific DNA fragment. J. Anim. Sci. 79:2108-2112.

Chen FC, Hsieh YH (2000). Detection of pork in heat-processed meat products by monoclonal antibody-based ELISA. J. AOAC Int. 83:79-85.

Frezza D, Giambra V, Chegdani F, Fontana C, Maccabiani G, Losio N (2008). Standard and light-cycler PCR methods for animal DNA species detection in animal feedstuffs. Innov. Food Sci. Emerg. Technol. 9:18-23.

Giovannacci I, Guizard C, Carlier M, Duval V, Martin JL, Demeulemester C (2004). Species identification of meat products by ELISA. Int. J. Food Sci. Technol. 39:863-867.

Girish PS, Anjaneyulu ASR, Viswas KN, Shivakumar BM, Anand M, Patel M, Sharma B (2005). Meat species identification by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of mitochondrial 12S rRNA gene. Meat Sci. 70:107-112.

Greenwood A, Paboo S (1999). Nuclear insertion sequences of mitochondrial DNA predominate in hair but not in blood of elephants. Mol. Ecol. 8(1):133-137.

Hajmeer M, Cliver DO, Provost R (2003). Spinal cord tissue detection in comminuted beef: comparison of two immunological methods. Meat Sci. 65:757-763.

Ilhak OI, Arslan A (2007). Identification of meat species by polymerase chain reaction (PCR) technique. Turk. J. Vet. Anim. Sci. 31:159-163.

Janssen FW, Hagele GH, Buntjer JB, Lenstra JA (1998). Species identification in meat using PCR-generated satellite probes. J. Ind. Microbiol. Biotechnol. 21:115-120.

Lenstra JA, Buntjer JB (1999). On the origin of meat. Food Chem. 64: 1.

Lenstra JA, Buntjer JB, Janssen FW (2001). On the origin of meat-DNA techniques for species identification in meat products. Vet. Sci. Tomorrow pp. 1-15.

Maede D (2006). A strategy for molecular species detection in meat and meat products by PCR-RFLP and DNA sequencing using mitochondrial and chromosomal genetic sequences. Eur. Food Res. Technol. 224:209-217.

Malisa AL, Gwakisa P, Balthazary S, Wasser SK, Mutayoba BM (2006). The potential of mitochondrial DNA markers and polymerase chain reaction-restriction fragment length polymorphism for domestic and wild species identification. Afr. J. Biotechnol. 5(18):1588-1593.

Mane BG, Mendiratta SK, Tiwari AK, Bhilegaokar KN, Ravindra PV, Raut AA (2007). Precise and rapid identification of ovine-caprine origin of cooked meat by specific PCR assay. J. Vet. Pub. Health

5(2):107-111.
Mane BG, Tanwar VK, Girish PS, Sharma D, Sonawane AA (2008). Differentiation of Meat Species By Means of PCR Technique. Int. J. Food Saf. Nutr. Pub. Health 1(1):51-57.
Mane, BG, Tanwar VK, Girish PS, Dixit VP (2006). Identification of Species Origin of Meat by RAPD-PCR Technique. J. Vet. Pub. Health 4(2):87-90.
Martin I, Garcia T, Fajardo V, Lopez-Calleja I, Rojas M, Hernandez YE, Gonzalez I, Martin R (2007). Mitochondrial markers for the detection of four duck species and the specific identification of Muscovy duck in meat mixtures using the polymerase chain reaction. Meat Sci. 76(4):721-729.
Matsunaga T, Chikuni K, Tanabe R, Muroya S, Shibata K, Yamada J, Shinmura Y (1999a). A quick and simple method for the identification of meat species and meat products by PCR assay. Meat Sci. 51:143-148.
Matsunaga T, Shibata K, Yamada J, Shinmura Y (1999b). Effects of processing condition on species identification of meat products. J. Jpn. Soc. Food Sci. Technol. 46:187-194.
Nagappa K (2008). Molecular identification of animal species using polymerase chain reaction based techniques. Ph.D thesis, G.B. Pant Univ. Agric. Technol. Pantnagar, India. pp. 263, 145.
Partis L, Croan D, Guo Z, Clark R, Coldham T, Murby J (2000). Evaluation of a DNA fingerprinting method for determining the species origin of meats. Meat Sci. 54:369-376.
Pfeiffer I, Burger J, Brenig B (2004). Diagnostic polymorphisms in the mitochondrial cytochrome b gene allow discrimination between cattle, sheep, goat, roe buck and deer by PCR-RFLP. BMC Genet. 5:30.
Renon P, Bernardi C, Scocca S, Cantoni C, Gridavilla G (2003). IEF (Isoelectric focusing) and gas chromatography to identify wild ruminant species. Ind. Aliment. 42:496-500.
Rodriguez MA, Garcia T, Gonzalez I, Asensio L, Hernandez PE, Martin R (2004). PCR identification of beef, sheep, goat and pork in raw and heat-treated meat mixtures. J. Food Prot. 67(1):172-177.
Saini M, Das DK, Dhara A, Swarup D, Yadav MP, Gupta PK (2007). Characterization of peacocks (*Pava cristatus*) mitochondrial 12S rRNA sequence and its use in differentiation from closely related poultry species. Br. Poult. Sci. 48(2):162-166.
Sebastio P, Zanelli P, Neri TM (2001). Identification of anchovy (*Engraulis encrasicholus L*.) and gilt sardine (*Sardinella aurita*) by polymerase chain reaction, sequence of their mitochondrial cytochrome b gene, and restriction analysis of polymerase chain reaction products in semipreserves. J. Agric. Food Chem. 49:1194-1199.
Sun YL, Lin CS (2003). Establishment and application of a fluorescent polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method for identifying porcine, caprine, and bovine meats. J. Agric. Food Chem. 51(7):1771-1776.
Toorop RM, Murch SJ, Ball RO (1997). Methodology and development of prediction equations for the determination of pork substitution in veal. Food Res. Int. 30:629-636.
Unseld M, Beyermann B, Brandt P, Hiesel R (1995). Identification of the species origin of highly processed meat products by mitochondrial DNA sequences. PCR Meth. Appl. 4:241-243.
Vallejo B, Gonzalez AF, Mazorra MA, Rodriguez R (2005). Capillary electrophoresis for the analysis of meat authenticity. J. Sep. Sci. 28:826-836.
Weder JK, Rehbein H, Kaiser J (2001). On the specificity of tuna-directed primers in PCR-SSCP analysis of fish and meat. Eur. Food Res. Technol. 213:139-144.
Wolf C, Rentsch J, Hubner P (1999). PCR-RFLP analysis of mitochondrial DNA: a reliable method for species identification. J. Agric. Food Chem. 47:1350-1355.