aJava

Asian Journal of Animal and Veterinary Advances

Academic
Journals Inc.

www.academicjournals.com

Asian Journal of Animal and Veterinary Advances 7 (12): 1348-1355, 2012
ISSN 1683-9919 / DOI: 10.3923/ajava.2012.1348.1355
© 2012 Academic Journals Inc.

Detection of Mink (*Mustela vison*) DNA in Meat Products using Polymerase Chain Reaction PCR Assay

[1,2]Weili Sun, [1,2]Guangyu Li, [1,2]Hanlu Liu, [1,2]Wei Zhong, [1,2]Haihua Zhang, [1,2]Kun Bao, [1,2]Chao Xu, [1,3]Yahan Yang and [1,2]Zhuo Wang

[1]Institute of Special Animal and Plant Sciences, Chinese Academy of Agricultural Sciences, Jilin, 130112, People's Republic of China

[2]Jilin Provincial Key Laboratory for Molecular Biology, Special Economic Animals, Jilin, 130112, People's Republic of China

[3]Animal Production and Product Quality and Security Key Lab, Ministry of Education, Jilin Agricultural University, Changchun 130118, People's Republic of China

Corresponding Author: Guangyu Li, Institute of Special Animal and Plant Sciences, Chinese Academy of Agricultural Sciences, Changchun, 130112, Jilin, People's Republic of China Tel: +86 431 81919807 Fax: +86 431 81919807

ABSTRACT

The study was to develop a Polymerase Chain Reaction (PCR) assay for specific detection of mink meat using designed pairs of primers based on mitochondrial D-loop. Mink meat is used as fraud ingredients of false mutton or dog meat in meat markets. This study was conducted to establish Polymerase Chain Reaction (PCR) method for the sensitive and specific detection of mink (*Mustela vison*) DNA in meat products. Six pairs of primers were designed from tandem repeat region of D-loop in mitochondria after alignment of the available sequences in the GenBank database. The specific pair of primers chosen from the six designed pairs by PCR generated specific fragments of 343 bp in length for mink. The specificity of detection was conducted with DNA samples of mink, blue fox, dog, raccoon dog, swine, sheep. Then amplification of positive reaction was observed only in mink species. In this study, no adverse effects of cooking and autoclaving were found on amplification of mink DNA fragments. Then the detection limit was found to be less than 1% in mixed meat products. The PCR method described in this study proved to be very sensitive and reliable in mink DNA identification. Thus, it could be considered as a further improvement method for the detection of mink DNA in meat products processed under different manufacturing conditions.

Key words: DNA fragments, meat products, mink, polymerase chain reaction

INTRODUCTION

Mink (*Mustela vison*) is a species of carnivore, Mustelidae family and *Mustela* genus in taxonomy who originated from North America(*Mustela vision*) and Europe *Mustela lutreola*. Minks as fur bearing animals all over the world were precious descended from America *Mustela vison*. (Niethammer *et al*., 1993; Wamberga and Tausonb, 1998). Breeding and caring for fur bearing animals were conducted originally in 1950s in China. Special economic animals have becoming the special agriculture industry for its particular resources traits, economic values and growing demand.

Mink meat was mostly used as ingredients of animal feed and fewly processed food. But, in some areas, mink meat was used as substitute of other meat products because it is cheaper and easily available. Mink heart was used for pharmaceutical industry especially due to cure the Rheumatic Heart Diseases.

There are fraud phenomenons in meat and fur markets for various reasons such as public health, religious factors and abnormal competition (Arslan *et al.*, 2006; Mane *et al.*, 2006). Because mink fur products and other appendix are expensive and limited, merchants usually substitute them with other products to obtain lots of economic profits. Such as mink fur was substitution for low priced animal fur even the artificial fur. The mink hearts for drugs are substitution for chicken hearts or rabbit hearts. In recent years, the mutton adulterations have becoming growing increased substitution for mink meat, fox meat or raccoon dog meat (Sun *et al.*, 2006). At present, the quality control of meat products mostly depends upon traditional methods which mainly include macroscopical identification, microscopical examination and physical and chemical experiment. It is difficult to identify the fraud using the traditional methods especially with the development of processing technologies (Girish *et al.*, 2005). So, it is very necessary to identify mink DNA in order to inhibit the fraud phenomenon. Food safety has aroused highly attention of consumers and governments all over the world especially the meat products, animal feed.

The composition of mtDNA has no complicated intron, pseudogene or repetitive sequence which is simpler in complexity than nuclear DNA (nDNA) (Gray, 1989). Analysis of mitochondrial DNA (mtDNA) sequences has gained particular attention these years. The mtDNA is of maternal inheritance and has no recombination in all vertebrates, so that the sequence of mtDNA is more conservative (Rokas *et al.*, 2003). However, the rate of base substitution on mtDNA is higher than that of nDNA, causing a rapid evolution (Stoneking and Soodyall, 1996). On average, there are about 800-1000 mitochondria per cell and each mitochondrion contains 2-6 circular DNA molecules, making mtDNA a naturally amplified source of genetic variation (Girish *et al.*, 2004).

DNA based assays are gaining popularity in meat species identification due to their stability at high temperature and conserved structure within all individual of the species (Calvo *et al.*, 2002; Girish *et al.*, 2004). The PCR assays are employed for identification of species origin of meat using random primers (Saez *et al.*, 2004). Demmel *et al.* (2008) has reported the method of detection of lupine (*Lupinus* spp.) DNA in processed foods using real-time PCR. Many researchers (Fajardo *et al.*, 2007; Tang *et al.*, 2002a, b) identified different animals of Cervidae by using species-specific PCR method. Simultaneous detection of pathogenic vibrio species using multiplex real-time PCR has been reported by Kim *et al.* (2012). PCR-restriction fragment length polymorphism (PCR-RFLP) has been applied for the identification of deer-derived ingredients in the deer products (Kim *et al.*, 2001; Matsunaga *et al.*, 1998). The method of multiplex PCR to detect animal ingredients in feedstuffs or food products has been developed by most researchers recently (Lin and Hwang, 2008; Mane *et al.*, 2009; Dalmasso *et al.*, 2004).

This study was aimed to develop a PCR method to rapid detection the mink DNA in meat products especially in mutton and dog products, which provides a more precise detection of mink species origin for complex meat samples.

MATERIALS AND METHODS

Meat and blood samples: The study was performed during 1st November, 2011 to 20th December, 2011. Blood samples were collected in the first two weeks during this period. Whole blood

samples (1 mL) of mink, blue fox, dog, raccoon dog, swine, sheep were obtained in fur animal breeding base or animal slaughterhouse in Jilin province. The samples were collected in tube with Ethylene Diamine Tetra Acetate (EDTA) as anticoagulant. The collected blood samples were preserved at-20°C till DNA isolations.04.

In this study, the analysed meat samples were collected from local slaughterhouses which were mink, blue fox, silver fox, raccoon dog, pork, mutton and dog meat. Refrigerated packed mutton and dog meat were bought from a local store in Jilin province (China) and some meat products were bought from chaffy dish restaurant. These products were claimed from mutton or dog meat as described in their labels. Samples were kept at -20°C till further processing.

DNA extraction and purification: Genomic DNA was extracted from each blood sample according to manufacturer of Genomic DNA Extract Kit (TianGen China). Each sample of meat products was weighed 200 mg. Genomic DNA was extracted from all samples using standard procedures with minor modifications. The lysis buffer was modified with 0.5% SDS, 0.5% Triton X-100, 10 mM Tris-Cl pH 7.6, 10 mM Na_2EDTA, 8 mM $MgCl_2$and 8 mM NaCl. The quality of genomic DNA was checked by horizontal submarine agarose gel electrophoresis using 1.0% agarose. The concentration of DNA was estimated by spectrophotometry (SPECORD S600, Analytik Jena AG, Jena, Germany) and the quality and purity of DNA was evaluated by A260/A280.

Subsequently, the DNA extracts were purified with the QIAquick PCR purification kit (TianGen China) according to the manufacturer. DNA samples were diluted to 10, 5, 1, 0.5 and 0.1%, respectively in order to evaluate the test sensitivity later.

Primers design: D-loop region sequences and complete mitochondrial genome were obtained from available sequences in National Center for Biotechnology Information (NCBI) GenBank which contained 39 of Mustelidae family. Alignment of complete mitochondrial genome from *Martes zibellina* (NC011579), *Gulo gulo* (AM711901), *Nyctereutes procyonoides* (GU256221), *Vulpes vulpes* (Q374180), *Cervus nippon hortulorum* (U457433), *Canis lupus familiaris* (NC002008), *Canis lupus chanco* (NC010340) and *Oryctolagus cuniculus* (NC001913) was performed using MEGA5.0. Specific primers for mink species were then designed based upon D-loop region sequence of Martes zibellina (NC011579) using primer designing soft-ware called Primer 5.0. Then earlier 6 pairs of primer were designed to be chosen, each of which consisted of a forward primer(F) and a reverse primer (R). Designing primers was based on the principle which was base composition and annealing temperature of primers were consistent with each other as far as possible and each pair of primers had intraspecies-universality and interspecies-specificity. The designed primers were synthesised by Shanghai Sangon Biological Engineering Technology and Services Co. Ltd., Shanghai, China.

Specific primer selection and the PCR conditions: In a preliminary phase of this research, the selection of the specific primers among the 6 pairs was assessed and chosen with DNA extracted from whole blood samples in minks (Fig. 1). The reaction mixture was prepared in a 500 μL PCR tube (AXYGEN, USA) in a total volume of 25 μL containing 5 μL of forward and 5 μL of reverse primer, 12.5 μL of PCR Mix (containing buffer, dNTP, *Taq* DNA polymerase) (TaKaRa Biotechnology Co. Ltd., Dalian, China), 8.5 μL of ddH_2O, 2 μL of DNA template. The most specific primer called primer1 was as follows:

Fig. 1: PCR amplification of mink mitochondrial DNA genome extracted from blood samples with the 6 pairs of primers separately, Lane M: D2000 bp marker, Lane 1-6: Mink template with primer1-6

Table 1: PCR conditions parameters

	35 cycles				
	Pre-denaturation	Denaturation	Anneal	Extension	Preservation
Temperature (°C)	94	94	53	72	4
Time	5 min	30 sec	30 sec	90 sec	8

Forward: 5'CTTCAACCTCAACATCATCACC 3
Reverse: 5' GACATACATTGTATTCATTCTAAGCG 3'

The PCR was programmed on 2720 thermal cycler (Applied Biosystems, USA) and the PCR cycling conditions parameters are given in Table 1.

RESULTS

Amplified product detection: The specific primer was used for amplification in the species of mink, blue fox, silver fox, raccoon dog, pork, mutton and dog (Fig. 2). 0.4 g of agarose (TakaRa, China) was put in 40 mL of 1× TBE solution (Fermentas, USA) and heated to completely dissolve the agarose. Then 1 drop of (approximately 5%) ethidium bromide solution was added as gel visualising agent and mixed thoroughly. The PCR product was finally analysed using UV transilluminator and documented by gel documentation system (Alpha Imager, USA). The ready to use 100 bp ladders (Fermentas, USA) was used for present work. PCR amplified products were analyzed by electrophoresis on 1% agarose gel (TakaRa, China) contained Ethidium Bromide run in TBE buffer for 90 min at 80 V.

Sequencing and alignment: Each amplified fragment was purified by PCR Products Purification Kit (Spin-column) (TaKaRa Biotechnology Co. Ltd., Dalian, China) and cycle was sequenced

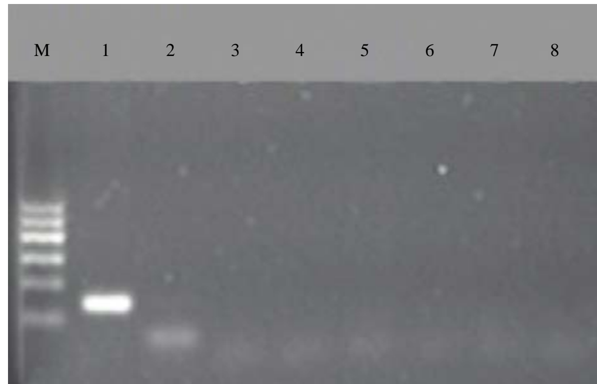

Fig. 2: PCR amplification of selected specific primers with DNA genome samples, Lane M: D2000 bp marker, Lane 1: Mink, Lane 2: Blue fox, Lane 3: Silver fox, Lane 4: Raccoon dog, Lane 5: Pork, Lane 6: Mutton, Lane 7: Dog, Lane 8: Negative control

(both strands) using PCR derived primers. The dideoxy chain termination method was performed by Shanghai Sangon Biological Engineering Technology and Services Co. Ltd., Shanghai, China. The nucleotide sequences were aligned with sequence of Martes zibellina (NC011579) downloaded from GenBank database. Similarity of amplified segment and template was 99%. The amplified segment sequence was as follows:

- AGCCAGTGACAATAACAAGCCAAGTCCCATAACTATATAAAGCCGCAATCCCCATGGCC TCCTCACTAAAAAACCCTGAATCACCCGTGTCATAAATAACTCAGTCACCTGCCCCGTTA AATTTTAACACAACTTCAACCTCAAACATCATCACCCTTCAGAATATAACAAGCAGTCAAT AGCTCAGATAATAAACCAACAATGAAAGCACCTAAAACGGCCTTATTAGAAGCCCAAAC CTCAGGATATTGCTCAGTGGCCATAGCAGTAGTATAACCAAAAACAACCAACATACCCCC CAAGTAAATCAAGAACACCATTAACCCCAGAAAAGACCCTCCAA (343 bp)

Detection limit (PCR sensitivity): The specificity of PCR assay was tested with DNA of other animal species used in this study. The DNA templates were diluted with ddH_2O containing 100%, 50,10, 5, 1, 0.5 and 0.1% mink DNA in order to test the sensitivity of PCR reaction. The non-targeted species were mink, blue fox, silver fox, raccoon dog, pork, mutton and dog meat. Finally, the detection limit was 0.5% level of adulteration of mink DNA in admixed meat products (Fig. 3). The detection limit quantity was 0.05 ng for mink.

DISCUSSION

The aim of the study was to develop and evaluate a method for detection of mink species in meat and meat products even processed under different manufacturing conditions. The variable regions of the mitochondrial gene are present in thousands of copies per cell (Greenwood and Paabo, 1999) which increases the probability of achieving a positive result due to processing conditions (Bellagamba *et al*., 2003). The species-specific PCR assay as a low-cost, precise and rapid testing method is indispensable to avoid unfair market competition and protection of consumer from fraudulent practices of meat adulteration. Some workers had suggested that mitochondrial markers were more efficient than nuclear markers for the purpose of detection and

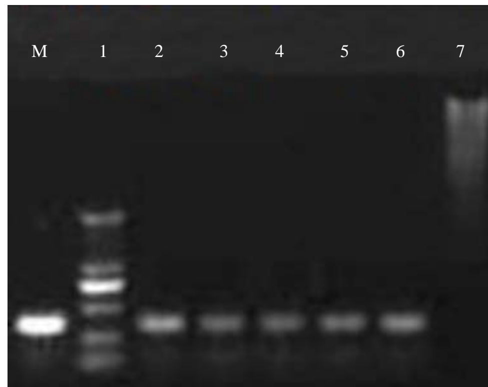

Fig. 3: PCR amplification of mink mitochondrial D-loop gene with various dilution levels up to 0.1%, Lane M: D2000 bp marker, Lane 1: 100% level, Lane 2: 50% level, Lane 3: 10% level, Lane 4: 1% level, Lane 5: 0.5% level, Lane 6: 0.1% level, Lane 7: Negative control

authentication animal species (Hopwood *et al*., 1999). The mitochondrial DNA was targeted to design species-specific primers, because mitochondrial DNA is maternally inherited so normally only one allele exists in an individual and thus no sequence ambiguities are to be expected from the presence of more than one allele (Unseld *et al*., 1995). The specific pair of primers was designed based on mitochondrial D-loop for amplification of 343 bp DNA fragments from mink DNA. Earlier, Calvo *et al*. (2002) also successfully developed swine-specific primers for detection of pork in wide range of meat and meat products in raw and cooked meats, sausages, cured meat products, hamburgers and pates.

CONCLUSION

The species-specific PCR assay was found to be precise, sensitive and rapid methods for identification of species which can be used for routine analysis of meat species, even in admixed meat and meat products under different processing conditions. Thus, it can be concluded that it was a potentially reliable technique and useful tool for detection of mink meat from other animals to protect the consumers from fraudulent practices of meat substitution.

REFERENCES

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

Bellagamba, F., F. Valfre, S. Panseri and V.M. Moretti, 2003. Polymerase chain reaction-based analysis to detect terrestrial animal protein in fish meal. J. Food Protein, 66: 682-685.

Calvo, J.H., R. Osta and P. Zaragoza, 2002. Quantitative PCR detection of pork in raw and heated ground beef and pate. J. Agric. Food Chem., 50: 5265-5267.

Dalmasso, A., E. Fontanella, P. Piatti, T. Civera, S. Rosati and M.T. Bottero, 2004. A multiplex PCR assay for the identification of animal species in feedstuffs. Mol. Cell. Probes, 18: 81-87.

Demmel, A., C. Hupfer, E.I. Hampe, U. Busch and K.H. Engel, 2008. Development of a real-time PCR for the detection of lupine DNA (*Lupinus* species) in foods. J. Agric. Food Chem., 56: 4328-4332.

Fajardo, V., I. Gonzalez, I. Lopez-Calleja, I. Martin and M. Rojas *et al.*, 2007. Identification of meats from red deer (*Cervus elaphus*), fallow deer (*Dama dama*) and roe deer (*Capreolus capreolus*) using polymerase chain reaction targeting specific sequences from the mitochondrial 12S rRNA gene. Meat Sci., 76: 234-240.

Girish, P.S., A.S.R. Anjaneyulu, K.N. Viswas, M. Anand N. Rajkumar, B.M. Shivakumar and S. Bhaskar, 2004. Sequence analysis of mitochondrial 12S rRNA gene can identify meat species. Meat Sci., 66: 551-556.

Girish, P.S., A.S.R. Anjaneyulu, K.N. Viswas, B.M. Shivakumar, M. Anand, M. Patel and B. Sharma, 2005. Meat species identification by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of mitochondrial 12S rRNA gene. Meat Sci., 70: 107-112.

Gray, W.M., 1989. Origin and evolution of mitochondrial DNA. Ann. Rev. Cell Biol., 5: 25-50.

Greenwood, A. and S. Paabo, 1999. Nuclear insertion sequences of mitochondrial DNA predominate in hair but not in blood of elephants. Mol. Ecolol., 8: 133-137.

Hopwood, A.J., K.S. Fairbrother, A.K. Lockley and R.G. Bardsley, 1999. An actin gene-related Polymerase Chain Reaction (PCR) test for identification of chicken in meat mixtures. Meat Sci., 53: 227-231.

Kim, E.J., Y.J. Jung, S.J. Kang, S.Y. Chang, K. Huh and D.H. Nam, 2001. Molecular discrimination of cervidae antlers and rangifer antlers. J. Biochem. Mol. Biol., 2: 114-117.

Kim, H.J., H.J. Lee, K.H. Lee and J.C. Cho, 2012. Simultaneous detection of Pathogenic *Vibrio* species using multiplex real-time PCR. Food Control, 23: 491-498.

Lin, W.F. and D.F. Hwang, 2008. A multiplex PCR assay for species identification of raw and cooked bonito. Food Control, 19: 879-885.

Mane, B.G., S.K. Mendiratta and A.K. Tiwari, 2009. Polymerase chain reaction assay for identification of chicken in meat and meat products. Food Chem., 116: 806-810.

Mane, B.G., V.K. Tanwar, P.S. Girish and V.P. Dixit, 2006. Identification of species origin of meat by RAPD?PCR technique. J. Vet. Public Health, 4: 87-90.

Matsunaga, T., K. Chikuni, R. Tanabe, S. Muroya and H. Nakai *et al.*, 1998. Determination of mitochondrial cytochrome b gene sequence for red deer (*Cervus elaphus*) and the differentiation of closely related deer meats. Meat Sci., 4: 379-385.

Niethammer, J., M. Stubbe and F. Krapp, 1993. Manual of the Mammals of Europe. Akademische Verlagsgesellschaft, Germany, ISBN: 9783891045282, pp: 578-580.

Rokas, A., E. Ladoukakis and E. Zouros, 2003. Animal mitochondrial DNA recombination revisited. Trends Ecol. Evol., 18: 411-417.

Saez, R., Y. Sanz and F. Toldra, 2004. PCR-based fingerprinting techniques for rapid detection of animal species in meat products. Meat Sci., 66: 659-665.

Stoneking, M. and H. Soodyall, 1996. Human evolution and the mitochondrial genome. Curr. Opin. Genet. Dev., 6: 731-736.

Sun, G., W. Wang and J. Su, 2006. Mink farming in Denmark. Chin. Wildlife, 27: 13-14.

Tang, S.Y., W. Fu, Y.J. Chen, J.Y. Wang, X. Jiang and Y.P. Zhang, 2002a. Research on the identification of Cornu Cervi Pantotrichum with molecular taxonomy. Chin. Pharm. J., 37: 258-260.

Tang, S.Y., W. Fu, Y.J. Chen, J.Y. Wang, X. Jiang and Y.P. Zhang, 2002b. Research on the identification of Penis et Testis Cervi with molecular taxonomy. J. Chinese Materia Medica, 27: 573-575.

Unseld, M., B. Beyermann, P. Brandt and R. Hiesel, 1995. Identification of the species origin of highly processed meat products by mitochondrial DNA sequences. PCR Methods Appl., 4: 241-243.

Wamberga, S. and A.H. Tausonb, 1998. Daily milk intake and body water turnover in suckling mink *Mustela vison* kits. Comp. Biochem. Physiol. A., 119: 931-939.