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Identification of pork (*susscrofa*), horse (*Equuscaballus*) and donkey (*Equusasinus*) by PCR-RFLP-DHPLC analysis on the mitochondrial *COI* gene

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Abstract: *Donkey flesh is a popular food in China. To ensure the quality of donkey products plays a vital role in protecting the interests of consumers. Pork and horse are potential in donkey flesh food because of their easier availability at cheaper prices. In present study, PCR-RFLP-DHPLC technology was established for distinguishing the pork, horseflesh and donkey flesh in donkey flesh food. The assay combined the species-specific primers to selectively amplify a short fragment of mitochondrial cytochrome c oxidase subunit I (COI) gene from a heterogeneous background of genomic DNAs followed by RFLP-DHPLC analysis. The assay was sensitive enough to detect 5ng PCR products and finally 7 of marked donkey products were contaminated in 60 samples. Besides pork and horse, no other animal derived ingredients were blended in donkey in these samples.This research brings a promising way to rapid detect any contamination in a specific flesh food.*
Key words: *PCR-RFLP-DHPLC, donkey, COI gene*

I. Introduction

Donkey meat has moderate tenderness, good juiciness, moderate IMF (intramuscular fat) and rich perfume. There are many popular food made from donkey flesh in China, such as a pie with donkey meat and donkey-hide glue. Pork and horse are potential forgery for donkey flesh food because of their easier availability at cheaper prices. Since meat adulteration and mislabeling are illegal and raise many health and economic issue, the detection of adulteration and identification of adulterants in meat products is crucial for the enforcement of labeling legislation and prevention of unfair competition. This is also important for the implementation of national standard as well as protection of the consumer preference [1]. A number of methods have been published on the identification of pork, horse and donkey [2-6]. Presently, real-time PCR methods are routinely used for the species authentication as they can amplify a specific target sequences from a few copies to easily detectable quantities even in a very complex pool of genomic sequences and fluorescently labeled probes used in this technique allow signal generation to be measured in real-time, thus eliminating the need for electrophoresis [7-9]. The major drawbacks of real-time PCR include the soaring cost of the specifically designed instrument and reagents and also the difficulties in appropriate probe design.

PCR-RFLP (PCR-restriction fragment length polymorphism) is one of the most widely used methods for species identification [10, 11]. This method involves the amplification of a preselected DNA fragment with universal primers, followed by digestion with restriction endonucleases, which recognize specific short sequences (four to six nucleotides) of the amplified fragment and cut the DNA at those sites.Denaturing high-pressure liquid chromatography (DHPLC) can be used to separate DNA fragments according to length automatically, and the resolution is 1/100 nucleotide [12, 13]. DHPLC can make up for the deficiency of PCR-RFLP which are in low degree of automation and easy to cause cross-contamination of samples. But there is no such report on combining thePCR-RFLP and DHPLCto a solution for testing meat. In this manuscript, PCR-RFLP-DHPLC was established as a novel high efficient method to identify the contamination in the products marked with donkey flesh. The advantage of this technology lies in a wide range of detection and not having to design the specific probe for different species.

II. Materials and Methods

Primer design

The region to be amplified was searched on the cytochrome b (*cyt*b) gene of mitochondrial DNA. The sequence alignment was carried out with DNA man. It allowed a fragment to be identified that could be amplified in all the mammal species using two universal primers, named *COI*-F (primer *forward*) and *COI*-R(primer *reverse*), whose sequence were reported in Table 1. These primers were designed to amplify a fragment of *cyt*b in a length of 446bp.

Table 1 Sequences of universal primers in different species

COI-F: CGNATAAAYAAYATRAGCTTYTGA
COI-R: TANACTTCDGGRTGNCCRAARAATCA
R:A/G; Y:C/T; D:G/A/T; N:A/T/G/C

PCR amplification

The amplification reaction was set up using Q5 Hot Start High-Fidelity PCR kit (New England BioLabs) according to the instruction. Negative template control of PCR reaction (absence of template DNA) was included to ensure the purity of PCR reaction mixture from contaminating DNA. The products of PCR were analyzed by DHPLC.

RFLP

The sequences of 446bp fragments of the tested species from GenBank database were aligned using the NEBcutter V2.0 (http://n2c.neb.com/NEBcutter2/) to detect the presence of one or more restriction sites that could character each species. On the basis of such analysis some restriction enzymes were selected for carrying out the RFLP analysis: *Hpa* II and *Hind* III. The endonucleases with the position of restriction sites and the length of expected fragments in each of the tested species are reported in table 2. The restriction digestion was carried out in a total volume of 20 uL reaction mixture containing 7uL unpurified PCR product, 2uLof 1×enzyme buffer, and 2-5 units of enzyme. The final volume was made up to 20uL with autoclaved sterile dH_2O. Restriction reactions were then incubated for 1h at 37℃ followed by a 20min water bath at 80℃ to inactivate enzyme. The restriction fragments were separated by DHPLC.

Table2 Positions of restriction sites of selected endonucleases on the amplified 446bp fragment of *cytb* gene and restriction fragment length

species	Enzymes		
	*Hpa*II	*Hind* III	Number of fragments (bp)
pork (*susscrofa*)	/	74	2 (74, 372)
horse (*Equuscaballus*)	15	80, 235, 313	5 (15, 65, 78, 133,155)
Donkey (*Equusasinus*)	15	/	2 (15, 431)

DHPLC

The products of enzyme-digested were analyzed by DHPLC with a universal linear model at 50℃. The condition was shown in Table3. The samples were analyzed according to three samples following a blank sample order.

Table 3： DHPLC conditions of fragment separation（universal model）

Gradient Name	Time(min)	%A	%B
Loading	0.0	65	35
Start Gradient	1.0	60	40
Stop Gradient	17.0	28	72
Start Clean	17.1	0	0
Stop Clean	18.1	0	0
Start Equilibrate	18.2	65	35
Stop Equilibrate	20.2	65	35

Establish digestion fingerprint maps of positive COI fragments

The COI sequences of horseflesh (equus caballus|KT757764.1), donkeyflesh (equus asinus|x97337.1) and pork (sus scrofa|KF569218.1) were obtained from NCBI database. The sequences between the prime (forward: 5`-CAA CCA CAA AGA CAT TGG CA-3`, reverse: 5`-GGT GTC CGA ARA AYC ARA A-3`) of 3 species were synthesized by Sangon Biotech company and cloned in PUC57 vectors. These vectors were transformed into DH5α and stored at -20℃respectively. Specific fragments (446bp) of *cytb* were selectively amplified with *COI*-F and *COI*-R from the vectors in 20uL Q5 reaction mixture according to the instruction of Q5 Hot Start High-Fidelity PCR kit. The products of PCR were digested by *Hpa* II and *Hind* III. The products of digestion were analyzed by DHPLC with a universal model. The results of the analysis were determined by sequencing.

Sample selection and DNA extraction

60 samples of donkey meat were obtained at random from restaurant and supermarkets in Beijing, China. Total genomic DNA was extracted from the samples using Universal Genomic DNA Extraction Kit (TaKaRa) according to the instruction.

Sequencing

The PCR products were cloned in pMD20-T vector (TaKaRa). 100 clones per sample were collected to sequence in Sangon Biotech Company.

III. Results and Discussion

Method exploration

The universal primers (*COI*-F and *COI*-R) used in this study can amplify 445-449bp fragment of the mammals and birds *COI* genes（Fig.1）. All their sizes were consistent.

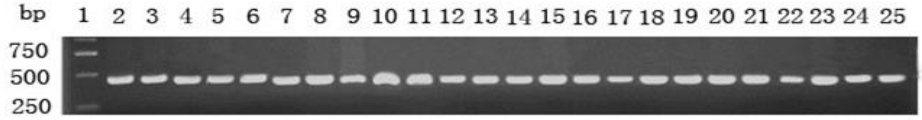

Fig.1 446bp PCR products which were amplified from mammals and birds *COI* genes. These fragments were separated by 2% agarose gel electrophoresis.

(1) Marker; (2) *ovis aries;* (3) *capra hircus;* (4) *equus caballus;* (5) *equus asinus;* (6) *bos Taurus;* (7) *sus scrofa;* (8) *bos mutus;* (9) *bubalus bulalis;* (10) *oryctolagus cuniculus;* (11) *felis catus;* (12) *rattus norvegicus;* (13) *vulpes;* (14) *canis familiaris;* (15) *nyctereutes procyonoides;* (16) *cervus elaphus;* (17) *camelus bactrianus ferus;* (18) *gallus;* (19) *anas;* (20) *coturnix japonica;* (21) *anser cygnoides;* (22) *columba livia;* (23) *meleagris gallopavo;* (24) *cervus Nippon;* (25) *capreolus pygargus.*

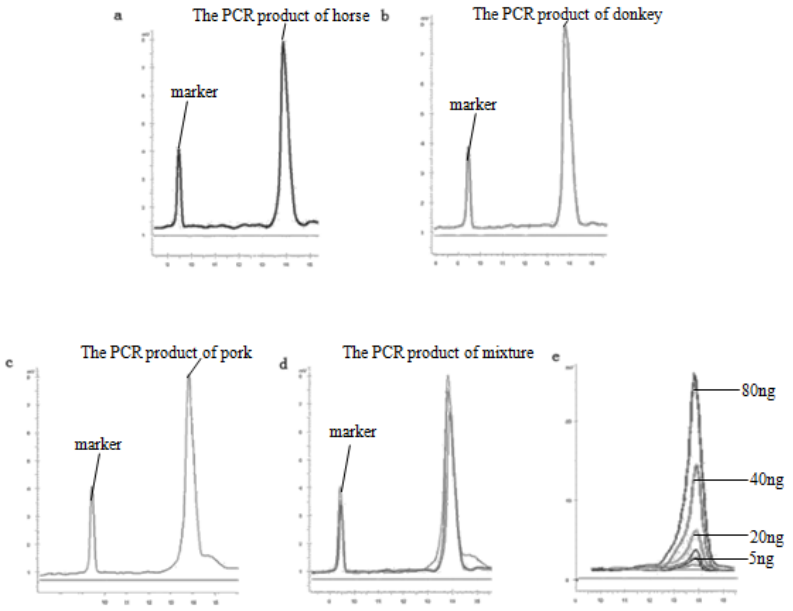

Fig.2 The DHPLC maps of PCR products which were amplified using the universal primer and the sensitivity of DHPLC

(a) the DHPLC map of PCR products of horse ; (b) the DHPLC map of PCR products of donkey;
(c) the DHPLC map of PCR products of pork; (d) the mixture of picture a-c; (e) the sensitivity of DHPLC.

The plasmids containing the *COI* gene of pork, horseflesh and donkey flesh were extracted using a plasmid purification kit (TaKaRa). The *COI* genes in plasmids were amplified as template by PCR using the universal primers and the PCR products were analyzed by DHPLC. 100bp fragments were added into samples

as markers in order to ensure the reliability and stability of the test. The lengths of the fragments amplified from horse (*equus caballus*|KT757764.1), donkey (*equus asinus*|x97337.1) and pork (*sus scrofa*|KF569218.1) were in the same size, 446bp (Fig.2a, 2b, 2c). If the fingerprintswere compared together in one map, the same chain lengths were detected among them (Fig.2d). As the results shown, the detection sensitivity of DHPLC is 5ng (Fig.2e).

The positive PCR fragments were digested by *Hind* III and *Hpa* II and the products were analyzed by DHPLC. The digestion fingerprint maps were shown in Fig.3. The abortion peaks of 15bp fragments in horse and donkey were not shown in the fingerprint maps because the fragment is too short. But the phenomenon does not affect the identification results of donkey and horse.

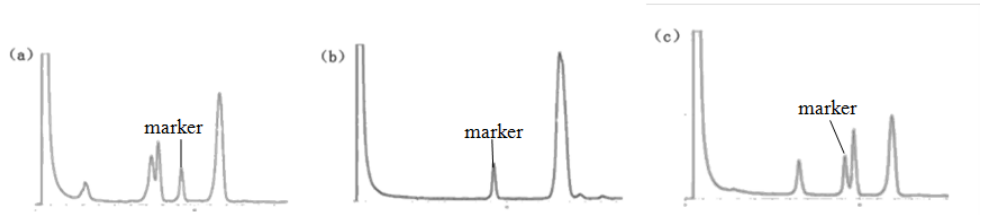

Fig.3 The digestion fingerprint maps of positive *COI* fragments

(a) The digestion fingerprint maps of horse *COI* fragment
(b) The digestion fingerprint maps of donkey *COI* fragment
(c) The digestion fingerprint maps of pork *COI* fragment

In order to appraise the identification effect of positive COI digestion fingerprint maps, 10 samples of pork, donkey flesh and horseflesh were collected. The genomic DNAs of samples were extracted using Universal Genomic DNA Extraction Kit (TaKaRa) according to the instruction. The PCR products were amplified using the universal primers and digested by *Hind* III and *Hpa* II. The digestion products were detected by DHPLC. The digestion fingerprint maps of pork and donkey flesh are consistent with the positive maps. While there are two different digesting maps presented in horse samples like Fig.4. One pattern is the same to the positive maps (Fig.4a) and the other is not at all (Fig.4b). The PCR product of Fig.4b was sequenced in order to further determine the identification results. Compared with the PCR products sequence of Fig.4a, the 314 nucleotide site of Fig.4b *COI* sequence had a mutation in G-C, which makes the fragment lose an *Hpa* II enzyme digestion site. There are 6 samples which digestion fingerprint maps of *COI* gene present the same pattern as Fig.4b. The results mean that this type of horseflesh is not a minority in China market. In order to verify the testing capacities using these fingerprint maps, 60 samples of donkey products were purchased from markets. The digestion products were analyzed using DHPLC and all the detection results were further conformed by sequencing. There are three samples of donkey products mixed with pork and one sample is the horseflesh mixed with pork. Only pork was detected in two samples and only horseflesh was detected in one sample. The single donkey ingredients were detected in the remaining 53 samples.There was no other ingredients of animal origin were detected in these samples. Some test results were shown in Figure 5. Only pork, donkey flesh and horseflesh were detected in sample A, sample B and simple C respectively, as shown in Fig.5A, Fig.5B and Fig.5C. The sample D is a mixture of pork and donkey (Fig.5D) and the sample E is a mixture of pork and horse (Fig.5E).

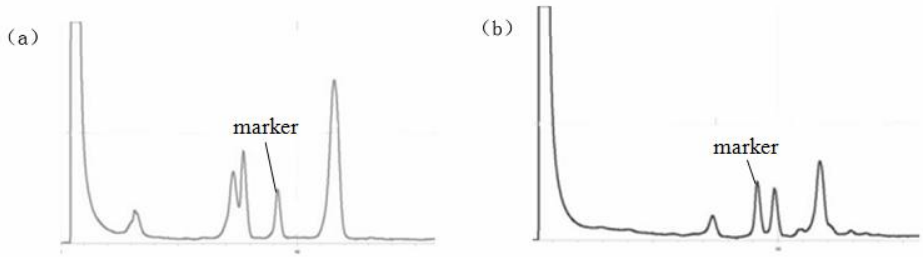

Fig.4 Two different digestion fingerprint maps of horse samples

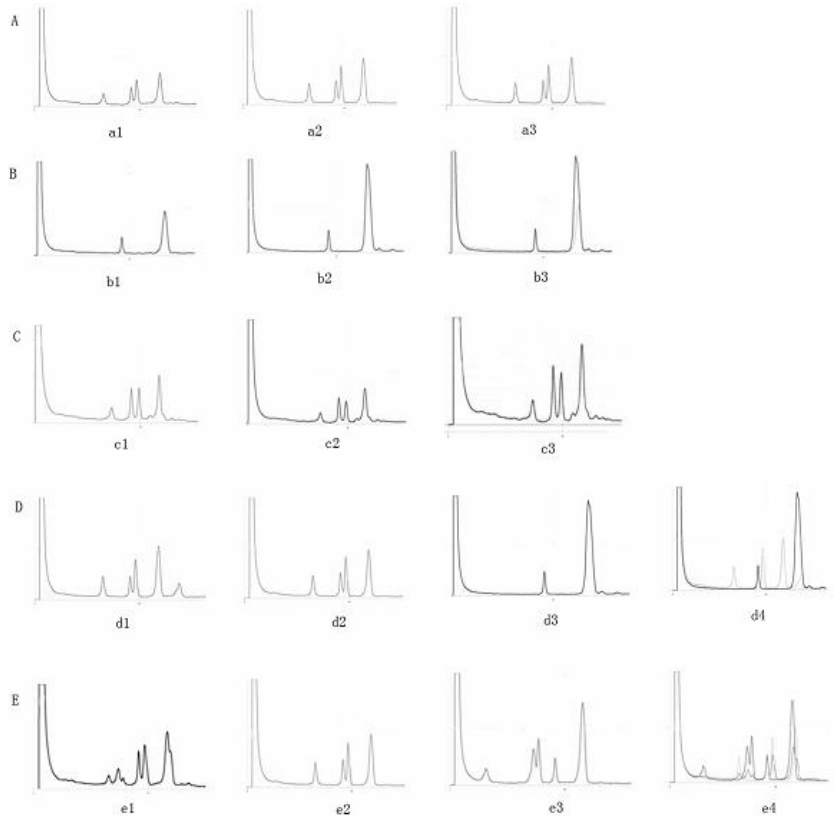

Fig.5 Species identification in donkey samples from the market by fingerprint maps of DHPLC

a1: fingerprint map of sample A; a2: fingerprint map of pork; a3: mixture of map a1 and a2
b1: fingerprint map of sample B; b2: fingerprint map of donkey; b3: mixture of map b1 and b2
c1: fingerprint map of sample C; c2: fingerprint map of horse; c3: mixture of map c1 and c2
d1: fingerprint map of sample D; d2: fingerprint map of pork; d3: fingerprint map of donkey;
d4: mixture of map d1, d2 and d3
e1: fingerprint map of sample E; e2: fingerprint map of pork; e3: fingerprint map of horse;
e4: mixture of map e1, e2 and e3

IV. Conclusion

In this manuscript, pork, horseflesh and donkey flesh can be distinguished using PCR-RFLP-DHPLC. Detection sensitivity can reach 5ng PCR products. Now it is well known that real time PCR is a kind of popular technology which is popularly used to detect the specific meat species in products. Compared with real time PCR, the main advantage of PCR-RFLP-DHPLC is low cost, as well as the identification of the unlimited species range. When the abnormal elution peaks appeared in the maps which were not matched with the specified fingerprint maps, contamination can be found in donkey flesh food. The practical application in field shows that this technology has high purging efficiency in detection of unknown flesh resource.

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Reference

[1]. Wang, Q., et al., *Identification of 12 animal species meat by T-RFLP on the 12S rRNA gene.* Meat Sci, 2010. **85**(2): p. 265-9.
[2]. Chisholm, J., et al., *The detection of horse and donkey using real-time PCR.* Meat Sci, 2005. **70**(4): p. 727-32.
[3]. von Bargen, C., J. Brockmeyer, and H.U. Humpf, *Meat authentication: a new HPLC-MS/MS based method for the fast and sensitive detection of horse and pork in highly processed food.* J Agric Food Chem, 2014. **62**(39): p. 9428-35.
[4]. Zhao, C.J., et al., *Differentiating among horse (Equus caballus), donkey (Equus asinus) and their hybrids with combined analysis of nuclear and mitochondrial gene polymorphism.* J Anim Breed Genet, 2005. **122**(4): p. 285-8.
[5]. Soares, S., et al., *Quantitative detection of poultry meat adulteration with pork by a duplex PCR assay.* Meat Sci, 2010. **85**(3): p.

531-6.
[6]. Doosti, A., P. Ghasemi Dehkordi, and E. Rahimi, *Molecular assay to fraud identification of meat products.* J Food Sci Technol, 2014. **51**(1): p. 148-52.
[7]. Tanabe, S., et al., *A real-time quantitative PCR detection method for pork, chicken, beef, mutton, and horseflesh in foods.* Biosci Biotechnol Biochem, 2007. **71**(12): p. 3131-5.
[8]. Murugaiah, C., et al., *Meat species identification and Halal authentication analysis using mitochondrial DNA.* Meat Sci, 2009. **83**(1): p. 57-61.
[9]. Ali, M.E., et al., *Nanoparticle sensor for label free detection of swine DNA in mixed biological samples.* Nanotechnology, 2011. **22**(19): p. 195503.
[10]. Haider, N., I. Nabulsi, and B. Al-Safadi, *Identification of meat species by PCR-RFLP of the mitochondrial COI gene.* Meat Sci, 2012. **90**(2): p. 490-3.
[11]. Hellberg, R.S. and M.T. Morrissey, *Advances in DNA-based techniques for the detection of seafood species substitution on the commercial market.* J Lab Autom, 2011. **16**(4): p. 308-21.
[12]. Kodama, C.S., et al., *Use of PCR-DHPLC with fluorescence detection for the characterization of the bacterial diversity during cassava (Manihot esculenta Crantz) fermentation.* Genet Mol Res, 2014. **13**(1): p. 1304-13.
[13]. Mounier, J., et al., *Use of denaturing high-performance liquid chromatography (DHPLC) to characterize the bacterial and fungal airway microbiota of cystic fibrosis patients.* J Microbiol, 2014. **52**(4): p. 307-14.