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ORIGINAL ARTICLE

Identification of the Adulterated *Asini Corii Colla* with Cytochrome c Oxidase Subunit I Gene-based Polymerase Chain Reaction

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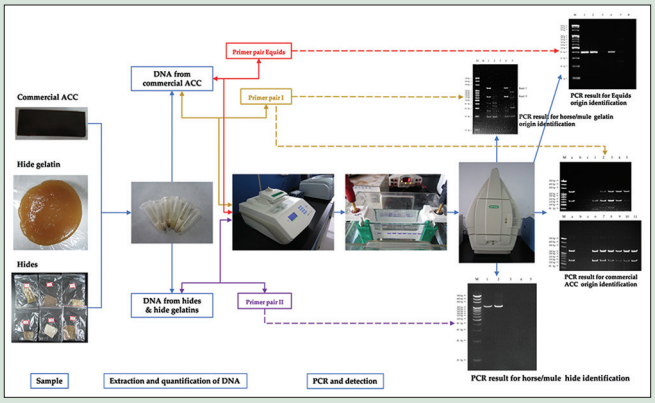
ABSTRACT

Background: *Asini Corii Colla* (ACC) (namely donkey hide gelatin, E'jiao in Chinese) was one of the most valuable tonic traditional Chinese medicines which is an infallible remedy to promote hematopoiesis. It should be produced by fresh or dried donkey hide according to Chinese Pharmacopeia (2015 edition) with a long-time decoction, while as donkey and horse (or mule) all belong to equids so their hides or their hide gelatins are share much in common, that cause the difficult in distinguishing raw materials donkey hide from horse/mule hide for manufacturer, and the challenge in the quality evaluation of ACC for regulatory authority to identify the adulterated with horse hide. **Objective:** To establish an effective quality evaluation methods for ACC focused on the qualitative-based identification of the raw material's authenticity, mainly to identify the species origin of the gelatins. **Materials and Methods:** DNA extracted from (1) Raw materials (hides of donkey, horse, mule, bovine and pig); (2) Five hide-glues (bovine, pig, donkey, horse and mule hide-glue); (3) 11 batches of ACC commercial products made by different manufactures from local drug stores. Polymerase chain reaction (PCR) method with newly designed horse-specific primers I and primer pair II. **Results:** Use the primer pair I, a 234 bp target product could be amplified sensitively from the DNA sample of horse/mule adulterated commercial ACC products, though the DNA in commercial products is severely degraded. A 219 bp product could be amplified specifically from the DNA sample of horse/mule hide, while the results were all negative for the DNA templates of donkey hide, its gelatin and ACC products without adulteration. **Conclusion:** The developed PCR method based on primer I and II provide an effective approach to identify the species origin of highly processed product ACC (primer pair I) as well as to distinguish the raw material donkey hide (primer pair II), which might enlighten a new strategy to the Quality Evaluation of ACC.

Key words: *Asini Corii Colla*, equids, polymerase chain reaction, quality evaluation, species identification

SUMMARY

- Though the quality of commercial *Asini Corii Colla* (ACC) products varies greatly and produce with nondonkey hide was one the most common adulteration, the effective method to constrain such adulteration remains to be established
- The gelatins made by donkey, horse, bovine, pig, mule shares much in common with each other, not only in contents of amino acids but also the profiles of protein in sodium dodecyl sulfate polyacrylamide gel electrophoresis, isoelectric focusing, gel filtration chromatography and two-dimensional electrophoresis
- The adulteration in ACC by using horse/mule hide, which is most difficult to detect, could be identified by Polymerase chain reaction methods with newly designed horse/mule-specific primer.



Abbreviations Used: ACC: *Asini Corii Colla*; TCMs: Traditional Chinese Medicines; SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis; IEF: Isoelectric focusing; GFC: Gel filtration chromatography; 2-DE: Two-dimensional electrophoresis; PCR: Polymerase chain reaction

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DOI: 10.4103/pr.pr_33_17

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INTRODUCTION

Asini Corii Colla (ACC) (namely donkey-hide gelatin, E'jiao in Chinese), recorded in the Chinese Pharmacopoeia (2015 Edition),[1] which has been used more than 2000 years in China as an infallible remedy, for replenishing blood or hemostasis,[2,3] suppressing tumor growth[4-6] and improving immunity,[2,7-9] the gelatin produced from fresh (or dried) skin of donkey by a long-time decocting and concentration. However, as the population of donkey decreased rapidly,[10] donkey hides became deficient and expensive. Herein, the E'jiao products in the market are usually adulterated by skins from horse, mule, hinny, pig or cattle. Horse (*Equus caballus*), donkey (*Equus asinus*) and their hybrids including mule (*E. asinus* × *E. caballus*) and hinny (*E. caballus* × *E. asinus*), which all belong to equids, share similar characters in

Cite this article as: Zuo HL, Zhao J, Wang YT, Xia ZN, Hu YJ, Yang FQ. Identification of the adulterated *Asini Corii Colla* with cytochrome c oxidase subunit I gene-based polymerase chain reaction. Phcog Res 2017;9:313-8.

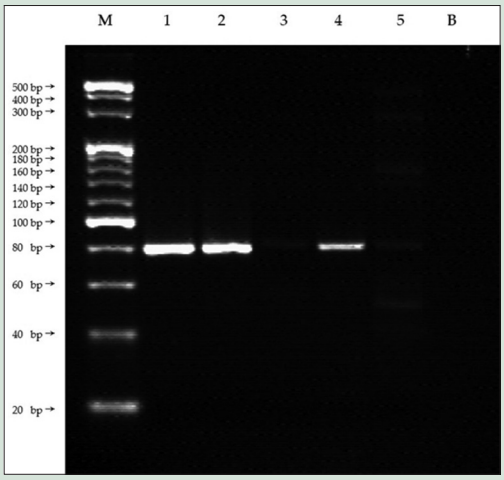

Figure 1: 8.0% polyacrylamide gel electrophoresis polymerase chain reaction products amplified with the Equids-specific primer. The PCR was conducted at anneal temperature 52 °C, 5 ng/μL DNA templates. Lane M, 20 bp DNA ladder marker; lane B, blank control (use water instead of DNA templates); lane 1, mule hide gelatin; lane 2, donkey hide gelatin; lane 3, bovine hide gelatin; lane 4, horse hide gelatin; lane 5, pig hide gelatin.

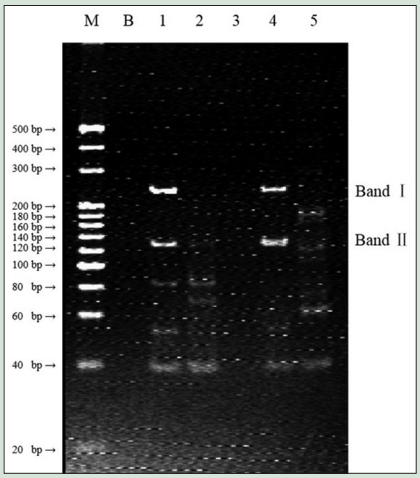

Figure 2: 8.0% Polyacrylamide gel electrophoresis of the species-specific polymerase chain reaction products amplified with primer pair I for hide gelatins. The PCR was conducted at anneal temperature 45 °C, 5 ng/μL DNA templates. Lane M, 20 bp DNA ladder marker; lane B, Blank Control (use water instead of DNA templates); lane 1, mule hide gelatin; lane 2, donkey hide gelatin; lane 3, bovine hide gelatin; lane 4, horse hide gelatin; lane 5, pig hide gelatin

appearance and nature with each other. Therefore, differentiating the skins and their produced glues from donkey, horse, and their cross hybrids is a challenging but important task for manufacturer to identify the right raw material or regulatory authority to evaluate the quality of ACC effectively.

The chromatographic methods such as high performance liquid chromatography method for the determination of amino acids[1,11] (including: L-hydroxyproline, glycine, alanine, L-proline) and nucleosides[9] could reflect the quality of the ACC in some degree, while it seems unfeasible to distinguish the source origin of gelatin considering the content of amino acid or nucleosides as criterion merely. What's more, in our previous study, the protein analysis methods including: Sodium dodecyl sulfate polyacrylamide gel electrophoresis, two-dimensional electrophoresis, isoelectric focusing and gel filtration chromatography, has also been applied for the distinguishing ACC and its adulterants, but all the results showed that they share much in common, and only a litter differences may cause by the variation of producing process,[9] but not the differences in species.

Thus, the DNA-based technology was considered to achieve the goal of species identification, as the ACC have DNA residue, though degraded severely, and the DNA-based technology has been studied in species identification of food manufacture,[12,13] such as DNA hybridization,[14,15] polymerase chain reaction (PCR),[16] the methods based on PCR (PCR product sequencing, PCR-restriction fragment length polymorphism (PCR-RFLP), species-specific primers, PCR-single-strand conformation polymorphism, random amplified polymorphic DNAs and DNA barcodes, and its application in traditional Chinese medicines[17-21] also gained much attention in recent years. The DNA-based identification method (combined analysis of nuclear and mitochondrial gene polymorphism) of the four species (hide of horse, donkey, hinny and mule) has been reported,[22] The PCR-RFLP method mainly based on the differences of restriction enzyme (Dpn II) cutting site in their protamine P1 gene (belonging to nuclear gene), cytochrome b gene (belonging to mitochondrial gene and genes hereditary character (biparental inheritance for protamine P1 gene and mitochondrial DNA [mtDNA] follows maternal heritage). However, that method cannot be used for the highly-processed ACC products, due to the DNA molecules are severely degraded into small fragments when it experienced more than dozens of hours decocting.

With molecular biology and relevant techniques developed in recent decades, PCR-based technologies, especially the "DNA barcoding" is the most popular in taxonomic classification. Hebert *et al.*[23] from University of Guelph firstly engaged in taxonomic system for the animal research has suggested that a DNA-based identification system, namely "DNA barcoding" mainly founded on the mitochondrial gene, cytochrome c oxidase subunit I (CO I) for animal classification, which has a higher genetic variation, then it was widely used in taxonomy or identification of animals,[24-29] insects[30-32] and Chinese medicines.[33]

Finding the differences of horse, donkey and their hybrids is a challenge since they are closely related species and have a high degree of sequence homology. In the present study, based on the sequence data from the NCBI database for horse and donkey, two pairs of species-specific primers were designed based on horse CO I gene for standard PCR. The developed PCR method with the species-specific primers was successfully applied to detect the components from horse or their hybrids with donkey.

MATERIALS AND METHODS

Samples

Raw materials (skins) of donkey, horse, mule, cattle and pig were collected by Gansu Tianshui Xihuang E'jiao Co., Ltd. (Gansu province, China) belong to Chongqing Taiji Industry (Group) Co., Ltd. (Chongqing, China), and they were confirmed by using the previous reported methods.[19,22] Five hide-glues, including bovine-hide glue, pig-hide glue, donkey-hide glue, horse-hide glue and mule hide-glue, were self-made according the standard operating procedure of E'jiao manufacture (provided by the E'jiao factory mentioned above). 11 E'jiao commercial products by different manufactures were purchased from local drug stores.

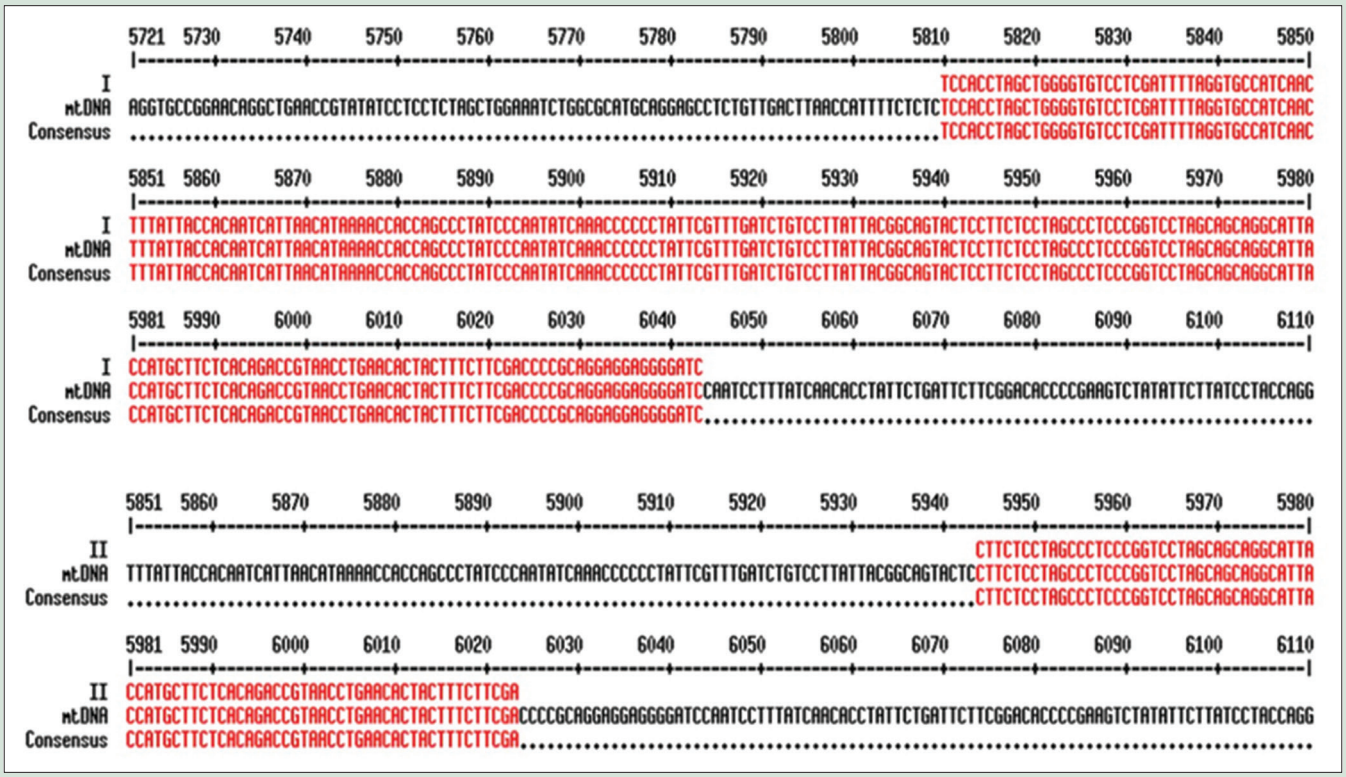

Figure 3: Alignment results of Band I and Band II to *Equus caballus* mitochondrial DNA

Chemical and biological reagents

Nucleotide Removal Kit (Cat. No. 28304, QIAquick®, QIAGEN Group, Duesseldorf, Germany). Premix Ex TaqTM Hot Start Version (Code, DRR030A, Takara Biotechnology, Dalian, China), Premix LA Taq® (Loading dye mix) (Code, D336A, Takara Biotechnology, Dalian, China), 20 bp DNA Ladder Marker (Code, D521A, Takara Biotechnology, Dalian, China), DL2,000 DNA Marker (Code, D501A, Takara Biotechnology, Dalian, China), restriction enzyme Dpn II (code R0543S, NewEast Biosciences), GelRed™ Nucleic Acid Gel Stain, 10000 × in Water (Cat. No. 41003, Biotium, Hayward, CA). And the other chemicals used for the experiments were of molecular biology grade and were purchased from Sangon (Sangon Biotech Co., Ltd., Shanghai, China). All glasswares and plasticwares and the buffers prepared in Milli-Q water were autoclave sterilized at 15 lbs for 20 min before use.

Primers design. Two pairs of primers were designed from mtDNA, cytochrome c oxidase subunit I (CO I) gene, which generally accepted that it can served as the core of a global bio-identification system for animals.[29] There are researches for the identification of horse and donkey by real-time PCR, whose primers were designed based on mtDNA.[34,35] Furthermore, the widely used DNA barcodes by taxonomic expertise in taxonomy mainly base on mtDNA CO I gene which can diagnose most closely allied species.[24] In the present study, the *E. caballus* mtDNA sequence (GenBank Accession No. NC_001640, 16660 bp), the *E. asinus* mtDNA sequence (GenBank Accession No. NC_001788, 16670 bp) and *E. caballus* COI gene sequence (GenBank Accession No. JN850774, 658 bp) were obtained from NCBI database. The sequence aligned by Florence Corpet.[23]

Due to the absent information of *E. asinus* CO I gene and the alignment of *E. caballus* shows its CO I gene located on the 5409–6066 bp of mtDNA. Thus the *E. caballus* CO I gene eventually aligned with the *E. asinus* mtDNA segment (5409–6066). Assisted with the primer designing tool Primer Premier 5.0 and specificity check function of NCBI, two pairs of primers were picked out from *E. asinus* CO I gene:

Primer pair I:

Forward primer (SEQ-1): 5'-TCCACCTAGCTGGGGTGTCC-3'

Reverse primer (SEQ-2): 5'-GATCCCCTCCTCCTGCGGGG-3'

Primer pair II:

Forward primer (GQ-1): 5'-TCTCCACCTAGCTGGGGTGTCC-3'

Reverse primer (GQ-2): 5'-GGGGTCGAAGAAAGTAGTGTTCAG-3'

In addition, another primer pair based on short interspersed nuclear element (SINE), for the identification of Equids, was designed based on previous report: [19]

Primer pair Equids:

Forward primer: 5'-CACTTGAACTACAGCTCAGC-3'

Reverse primer: 5'-GTGGTTCCGTCATAGCAG-3'

Instruments

Water-bath (Gongyi Yuhua Instrument, HH-ZK4, China), shaker (JinTan HONGKE Instrument, HY-5, China), centrifuge (Eppendorf centrifuge 5415D, Germany), ultraviolet (UV) spectrophotometer (Shimadzu, UV-2450, Japan), Thermal cycler system (Bio-Rad, S1000TM, USA), electrophoresis supply (Bio-Rad, PowerPac 300, USA) and tank (Bio-Rad, Mini-PROTEAN Tetra, USA), Biorad ChemiDoc XRS (Bio-Rad, IMAGEQUAMT400, USA).

DNA extraction

DNA extraction of hide-glues and E'jiao product: About 0.5 g powder of sample glue mixed with 3000 μL digesting buffer (10 mM Tris-HCl, 25 mM EDTA, 100 mM NaCl, 0.5% SDS, pH 8.0) in a 5 mL centrifuge tube, and pre-warm to 58°C in water bath until the solid samples were totally dissolved, centrifuge at × 12000 g for 5 min, discard the oily material that presented at the top of the tube and transferred 300 μL of

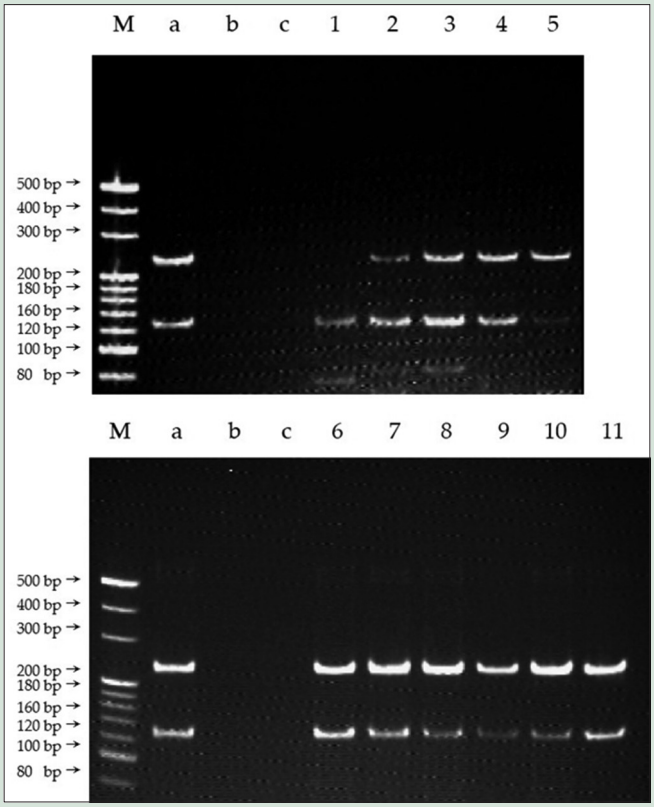

Figure 4: 8.0% Polyacrylamide gel electrophoresis of the species-specific polymerase chain reaction products amplified with primer pair I for E'jiao products. Lane M, 20 bp DNA ladder marker; lane P, Positive Control; lane B, Blank Control; lane C, E'jiao provided by Taiji Group; lane 1~lane 11, E'jiao produced by other different manufactures

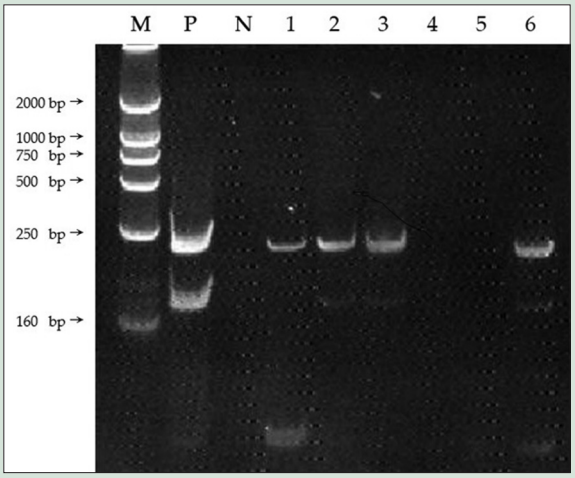

Figure 5: 8.0% Polyacrylamide gel electrophoresis of the species-specific polymerase chain reaction products amplified by primer pair I applied in hide identification. Lane M, DL2,000 DNA marker; lane P, Positive Control; lane B, Blank Control; lane 1, donkey hide DNA; lane 2, mule hide DNA; lane 3, horse hide DNA; lane 4, bovine hide DNA; lane 5, donkey hide DNA; lane 6, donkey hide DNA

the middle digested sample buffer to a new 1.5 mL tube, then add 20 μL Proteinase K, blend well and incubate at 58°C for 2 h; Next, centrifuge at × 12000 g for 3 min, discard the oily substance and precipitate (repeat 2–3 times), transferred 50 μL supernatant to a new 1.5 mL tube for the DNA extraction according to the instruction of Nucleotide Removal Kit.

DNA extraction of dry hide

One hundred mg of dry hide (vortex in 75% ethanol, to remove the exogenous DNA, then dried by air) was cut into granules and placed into a 1.5 mL tube with 400 μL digest buffer and 20 μL Proteinase K. The tube was incubated in a water bath (58°C) until the tissue was digested completely (normally more than 12 h), centrifuged for 5 min at × 12000 g to precipitate any undigested tissues, then transferred 200 μL of the supernatant into a new 1.5 mL tube (It should avoid transferring any undigested material from the bottom of the tube or any oily material that may present at the top of the tube), then extract DNA following the instruction of Nucleotide Removal Kit.

For the extracted DNA samples

Five microliter used to quantity detection and the rest were kept in a refrigerator (−20°C) until use.

DNA quantification

Five microliter DNA extraction was diluted by TE buffer to 500 μL, and detected on a UV spectrometer. The absorption at 260 nm and 280 nm was recorded for purity identification and quantitation of the extracted DNA samples. (While for DNA samples from commercial E'jiao products, the UV absorption is too weak to quantification)

Polymerase chain reaction amplification

Polymerase chain reaction solutions

A 10 μL reaction system was adopted, containing 5 μL premix ExTaqTM Hot Start version (×2 solution), 3 μL primers working solution, 2 μL DNA templates (concentration varies from different purpose of the experiments: For the inspection of the primers specificity to horse, and to confirm the anneal temperature, 10 ng/μL was employed, while for the sensitivity test, the concentration of the horse DNA templates were diluted to 10^{-6} ng/μL, for commercial products detection, the DNA extraction was used without dilution).

Amplifying program

Place the reactions in the thermal cycler and run the PCR cycling program shown in Table 1.

Detection of polymerase chain reaction products

The PCR products were separated by 8% nondenatured polyacrylamide gel, at 100 V for 50 min, and after the electrophoresis, the gels were stained in 10 mL GelRed working solution (10 μL GelRed × 10000 in water stock solution diluted in 50 mL 1 mol/L NaCl solution) for 30 min on shaker. Then observed and recorded the results by Biorad ChemiDoc XRS.

RESULTS AND DISCUSSION

The amplification results of DNA extracted from five self-made hide glue by primer pair Equids were shown in Figure 1.

The results indicated that: horse, mule and donkey are all belonged to Equids, use the PCR reaction was positive with Equids-specific primer under suitable conditions, wile for non-Equids DNA, like bovine and pig, the amplification was negative; and the reported Equids-specific primer used in PCR failed to distinguish the donkey from horse and mule.

The Figure 2 shows the amplification results of DNA extracted from five self-made hide glue by primer pair I.

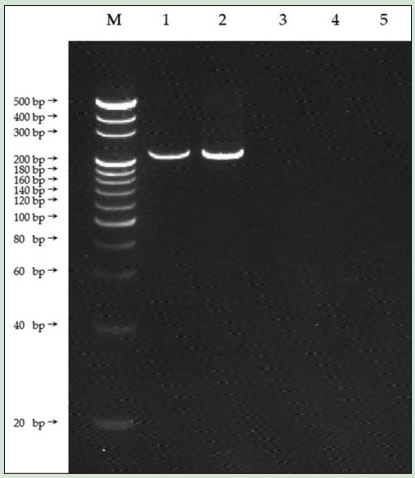

Figure 6: 8.0% Polyacrylamide gel electrophoresis of the species-specific polymerase chain reactionPCR products amplified with primer pair II for DNA from hide gelatins. The PCR was conducted at anneal temperature 55 °C, 5 ng/μL DNA templates were used. Lane M, 20 bp DNA ladder marker; lane 1, horse hide gelatin; lane 2, mule hide gelatin; lane 3, donkey hide gelatin; lane 4, pig hide gelatin; lane 5, bovine hide gelatin

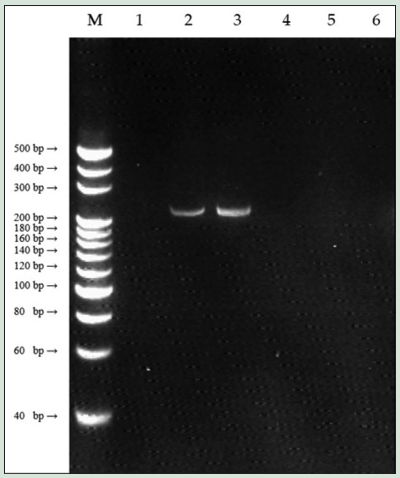

Figure 7: 8.0% Polyacrylamide gel electrophoresis of the species-specific Polymerase chain reactionPCR products amplified by primer pair II for hides' DNA. The PCR was conducted at anneal temperature 55 °C, 5 ng/μL DNA templates were used. Lane M, 20 bp DNA ladder marker; lane B, Blank Control; lane 1, mule hide DNA; lane 2, horse hide DNA; lane 3, bovine hide DNA; lane 4, donkey hide DNA; lane 5, donkey hide DNA

Table 1: Polymerase chain reaction cycling program

Segment	Number of cycle	Temperature (°C)	Duration
1	1	94	5 min
2	35	94	30 s
		45-60	30 s
		72	30 s
3	1	72	7 min
4	1	4	∞

The horse and mule show the same positive band, and pig, donkey, bovine is negative. Sequencing results of Band I (234 bp) was totally match the target sequence on *E. caballus* mtDNA CO I gene, as shown in Figure 3. The Band II was unexpected, but also specific to horse and mule DNA, and the sequencing results shows it was part of Band I.

The developed PCR method was applied to analyze the commercial E'jiao products. The results [Figure 4] showed that the collected E'jiao samples may be adulterated by horse or mule except the E'jiao provided by Taiji Group. Furthermore, the developed method was applied in the identification of different skins (hides), but false positive results often occurred [Figure 5].

To improve the specificity of the primer, it was redesigned based on primer pair I. The primer pair II was the optimized result, and successfully applied in self-made glues and hides for horse or mule origin detection, the results were shown in Figures 6 and 7.

The PCR method based on primer I can be applied in authenticity identification of highly processed E'jiao product, due to it is easily to operate and have enough sensibility to amplify the trace and highly degraded DNA. But it is not suitable for identification of skin, which genome DNA preserved relatively intact, false positive results are likely to occur. Thus, the primer II redesigned based on primer I can be used in distinguishing of horse or mule hide (skin) from donkey hide exactly.

CONCLUSION

In the present study, a PCR method with two primers (I and II) based on *E. caballus* mtDNA CO I gene were developed for authentication of the raw materials (skins) and final products of ACC. The research provides an effective approach to detect the adulteration from horse or mule and in some degree to guarantee authenticity of the source for ACC products.

ACC is a deeply manufactured product and its main constituent is protein, the current quality evaluation methods recorded in Chinese Pharmacopeia (2015 edition) have covered the limitation of the harmful ingredients (like heavy metal), or the control of the content of protein (amino acids), while the judgment only depends on traditional method maybe sometimes incorrect and use these methods to identify the adulteration seems powerless. The qualitative-based DNA relevant method is easily to control and proved to be valid, which might act as a supplementary of current quantitative methods to realize the comprehensive evaluation and control on the quality of ACC.

Acknowledgment

This work was supported by the National Natural Science Foundation of China (21275169 and 81202886). Project No. CQDXWL-2012-028 supported by the Fundamental Research Funds for the Central Universities. Project No. RC20120027 supported by the Chongqing Postdoctoral Funds.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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