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# Authentication of Equine DNA from Highly Processed Donkey-Hide Glue (*Colla Corii Asini*) Using SINE Element

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## ABSTRACT

In order to detect the DNA from highly processed medicinal donkey-hide glue *Colla Corii Asini* from adulterants blended or totally substituted with horse, cattle, and pig tissues, various species-specific PCR assays based on highly repetitive SINE elements were established. For horse and donkey tissues, a two-step PCR assay was adopted, including an initial detection of equine DNA based on the equine SINE ERE-1 and a further detection of horse DNA based on the horse specific satellite. The assay can detect the equine DNA and the horse DNA from binary solid glue mixtures spiked with 0.1% *Colla Corii Asini* and 1% horse-hide glue respectively. In addition, for bovine and porcine detection, the primer pairs were designed upon 1.711B bovine repeat and PRE-1 respectively. The developed methods can detect bovine and porcine DNA from binary solid glue mixtures spiked with 0.1% cattle-hide glue and 0.1% pig-hide glue respectively. The result shows that our developed PCR assays were useful, convenient and sensitive for *Colla Corii Asini* identification.

Key words: donkey-hide glue, *Colla Corii Asini*, highly processed material, molecular identification, SINE

## INTRODUCTION

Donkey (*Equus asinus*) is one of the most valuable domestic animals for the economy in many developing countries[(1)]. Donkey meat is a healthy food with high protein but low fat and cholesterol contents[(2)] while donkey milk composition is similar to human milk and more suitable for infants compared with those of other mammals[(3)]. A traditional Chinese medicine or health-care food *Colla Corii Asini*, also named as donkey-hide glue[(4)], has been derived from donkey skin. *Colla Corii Asini* is effective in promoting hematopoiesis, arresting bleeding, and also in treatment of gynecologic diseases and some chronic diseases[(5)].

As recorded in Chinese Pharmacopoeia[(4)], *Colla Corii Asini* is made of dry skin of *Equus asinus* by decoction and concentration. However, as donkey population decreased seriously in most countries[(6)], donkey skin became deficient. Skin or even bone from other animals such as cattle, pig and horse was sometimes processed and disguised as the *Colla Corii Asini* product or are blended into *Colla Corii Asini* production. This kind of adulterations or substitutions may harm people's health seriously[(7-9)], thus establishment of a strategy to distinguish *Colla Corii Asini* from the frauds becomes important.

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Because traditional morphological inspections of shape, color or texture were subjective and unreliable for accurate authentication of *Colla Corii Asini*, several spectroscopy-based methods, including two dimensional correlation infrared spectroscopy[(7)], near-infrared spectroscopy[(8)] and X-ray fluorescence analysis[(9)], were developed. However, these methods were inconvenient due to sample processing variability.

As the development of biotechnology, DNA-based molecular biomarkers have been widely used for food identification with respect to religious, ethical, economic and health purposes[(10)]. For DNA-based molecular authentication of *Colla Corii Asini*, the key step is to detect its donkey origin to meet the standard of Chinese Pharmacopoeia. Up till now, various approaches have been developed for donkey DNA detection, such as random amplified polymorphic DNA (RAPD) technology[(11)] and species-specific polymerase chain reaction (PCR) aimed at a target sequence from mitochondrion DNA elements[(12-14)]. The former method can make a rapid detection without any subsequent sequencing or restriction enzyme analysis, but it has trouble dealing with severely degraded DNA samples. In contrast, the latter strategy can be applied successfully to processed food products like cooked sausages[(13)]. Using the primers derived from mitochondrion DNA, PCR could genetically detect donkey DNA at a substitution level of 0.1% from meat mixture heated

at 100°C for 30 min or at a substitution level of 0.5% from meat mixture heated at 120°C for 30 min[15]. However, *Colla Corii Asini* is derived from donkey skin through decoction for about 10 h at above 120°C at 0.1 MPa, pH 5.0 - 6.5 followed by evaporation at 120 - 130°C for 15 - 20 h, most of the DNA molecules are severely degraded into much smaller fragments than those of commonly processed meat products[16,17]. Thus the above mentioned approaches are not suitable for such highly degraded DNA samples.

In recent years, many studies focused on short interspersed nuclear element (SINE) as a powerful tool for phylogenetic research[18]. SINE is a class of repetitive molecules with length ranging from 70 - 500 bp. It has more than $10^6$ total copies per haploid genome[19], while the widely used mitochondrion DNA (including cytochrome b) and ribosomal DNA only have about 2,500 and 5,000 copies, respectively[20]. This makes SINE an ideal molecular marker for forensic DNA detection[21]. In recent years, it has been applied to species-specific detection of meat samples successfully[22,23].

The SINE ERE-1 family has been found in most equine genomes with an estimated copy number of 20,000 to 80,000[23]. ERE-1 is Perissodactyla species-specific[24] and distributes in white rhino, Malayan tapir and equine such as horse or donkey genome. However, it can be used for equine DNA detection in meat[23] by the fact that most Perissodactyla species are rare except the domestic horse or donkey in our everyday life in the meat industry.

In order to detect the donkey DNA from highly processed traditional Chinese medicine *Colla Corii Asini*, we developed a two-step PCR detection method. First, primers derived from the equine SINE ERE-1 were designed and optimized for equine DNA detection, aimed to detect the donkey origin. Because the result might be misled by the presence of horse DNA, as ERE-1 distributes in both donkey and horse, a second PCR based on horse specific satellite DNA localized on the centromere of equine submetacentric chromosome 1[25] was subsequently conducted to detect the horse DNA specifically. After that, the *Colla Corii Asini* derived from donkey could easily be identified. In addition, the porcine SINE PRE-1 and bovine SINE derived satellite 1.711B bovine repeat were also used to detect DNA from pig and cattle specifically. Of these elements, the former contains about 100,000 copies per genome[26] while the latter covers about 7.1% of the bovine genome[27]. The method was convenient, reliable and efficient to detect the severely degraded DNA extracted from the glue samples.

## MATERIALS AND METHODS

### I. *Samples*

*Colla Corii Asini* derived from donkey skin (*Equus asinus*), *Colla Carapacis et Plastri Testudinis* derived from tortoise shell (*Chinemys reevesii*) and *Colla Cornus Cervi* derived from deer horn (*Cervus elaphus*) were produced in Shandong Donge E-jiao Co. of Shandong Province. Cattle-hide glue derived from cattle skin (*Bos taurus*), pig-hide glue derived from pig skin (*Sus scrofa*), and horse-hide glue derived from horse skin (*Equus caballus*) were prepared in the laboratory of Shandong Donge E-jiao Co. following the same procedure as that for *Colla Corii Asini* production.

Meat samples from donkey (*Equus asinus*), horse (*Equus caballus*), cattle (*Bos taurus*), pig (S*us scrofa*), tortoise (*Chinemys reevesii*), and deer (*Cervus elaphus*) used as positive control were purchased from local supermarkets.

### II. *DNA Extraction, Quantification and Sequencing*

#### (I) *DNA Extraction and Quantification*

The method for extracting trace amounts of severely degraded DNA fragments from various solid glues[16] was described as follows.

For each DNA preparation, 1 g solid glue was ground into powder and transferred into a 60 mL centrifugal tubes with 1 mL DNA extraction buffer (10 mM Tris-HCl, 25 mM EDTA, 100 mM NaCl, 0.5% SDS, pH 8.0) and 20 μL proteinase K (20 mg/mL). After incubating the tube for 1-2 h at 56°C with occasional shaking, 10 mL PN buffer (Qiagen, Duesseldorf, Germany) was added and mixed completely. Then, a QIAquick spin column (Qiagen) was loaded with about 600 μL sample and centrifuged for 1 min at 10,000 g. After discarding the flow-through appropriately, the step was repeated 12-14 times until all volume was centrifuged through the same QIAquick spin column. After that, the column was washed 3 times with 500 mL PE buffer (Qiagen) and dried by evaporation for 5 min at room temperature. Then, the DNA sample was eluted with 100 μL pre-heated TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) by centrifuging the column for 2 min at 12,000 g and stored at -20°C until use.

Genomic DNA was extracted from meat samples using the TIANamp Genomic DNA kit (Tiangen Biotech, Beijing, China). Before DNA preparation, 1 g meat sample was washed several times with sterile water and cut into small pieces. After homogenizing the tissue, about 0.1 g homogenate was transferred to 1.5 mL centrifuge tube and washed again with sterile water. The tube was then centrifuged at 12,000 g for 1 min. The pellets derived from meat homogenate were resuspended in 200 μL nuclei lyses buffer GA (Tiangen Biotech). Afterwards, the kit instructions were followed for the extraction of DNA from tissue samples.

DNA extracts from either solid glues or meat samples were quantified by UV absorption spectrophotometry at 260 nm on a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

#### (II) *Sequencing*

After the PCR products were fractionated by electrophoresis, fragments of less than 100 bp from solid glues DNA extracts or equine genomes were purified using TIANgel Midi Purification Kit (Tiangen Biotech) and subsequently ligated into pMD™ 19-T Simple Vector (Takara, Otsu,

Japan). The vector was then sequenced on a 3730 DNA sequencer (Applied Biosystems).

(III) *Preparation of Glue Mixtures*

To determine the detection limits of the equine-, horse-, pig- and bovine-specific PCR assays, various glue mixtures were prepared. For equine DNA detection, various contents (0.1%, 1%, 10%) of *Colla Corii Asini* were thoroughly mixed and blended with pig-hide glue before grinding into powders. For horse DNA detection, horse-hide glue / C*olla Corii Asini* mixtures were prepared by thoroughly mixing different contents (0.1%, 1%, 10%) of horse-hide glue and *Colla Corii Asini* before grinding into powders. For porcine or bovine DNA detection, different contents (0.1%, 1%, 10%) of pig-hide glue or cattle-hide glue were mixed with *Colla Corri Asini*. Afterwards, 1 g of each glue mixture was weighed for DNA extraction.

III. *Primer Design*

The oligonucleotide primer pair (Equine-up: 5'-CGGACATGGCACTGCTCAT-3' and Equine-down: 5'-TATATTCTTCGTTGTGGGTCCTTCT-3') for species-specific detection of equine DNA was designed from equine SINE ERE-1 sequence (GenBank Accession No. D26565). The primer pair (Horse-up: 5'-CACTTGAACTACAGCTCA GC-3' and Horse-down: 5'-GTGGTTCCGTCATAGCAG-3') for species-specific detection of horse DNA was designed from horse specific satellite DNA localized on the centromere of equine submetacentric chromosome 1 element (GenBank Accession No. AJ937277). The primer pair (Pig-up: 5'-CGAATCCGACTAGGAACCA-3' and Pig-down: 5'-ACCACATCTCACGGCTACG-3') for species-specific detection of pig DNA was designed from porcine SINE PRE-1 sequence (GenBank Accession No. Y00104). The primer pair (Bovine up: 5'-GTTTGCAGGAAGAAAGCC-3' and Bovine down: 5'-TGCATAGAGGATAATGGG-3') for species specific detection of bovine DNA was designed from bovine SINE derived satellite 1.711B bovine repeat (GenBank Accession No. V00116). All the primers were synthesized by Invitrogen (Shanghai, China).

IV. *PCR Detection*

During amplification of the DNA sample by PCR, more cycles were needed to obtain a positive result because DNA extracted from solid glue was highly degraded to short fragments of less than 100 bp[16].

For equine DNA detection, the primer pair equine-up/down was used in a 25 μL reaction volume containing 12.5 μL Premix Taq (Ex Taq Version) (Takara Biotechnology, Dalian, China) and 0.4 μM reverse and forward primer. The templates were 5 μL and 1 μL DNA extracted from glue samples (diluted to a final concentration of 10 ng/μL) and meat samples (diluted to a final concentration of 1 ng/μL), respectively. PCR amplification was conducted with an initial denaturation of 6 min at 94°C, followed by 40 amplification cycles of 30 s at 94°C, 30 s at 60°C, 30 s at 72°C for solid glue extracted DNA while 30 amplification cycles for genomic DNA, and a terminal elongation of 7 min at 72°C.

For horse DNA detection, using the primer pair Horse-up/down, PCR annealing temperature is 50°C, and the number of thermal cycle was 30 for both solid glue and genomic DNA extracts. The rest PCR condition was the same as that for equine DNA detection.

For pig DNA detection, using the primer pair Pig-up/down, PCR conditions changes with annealing temperature switched to 54°C, and the thermal cycles to 30 for both solid glue and genomic DNA extracts, while the rest condition remains.

For bovine DNA detection, using the primer pair Bovine-up/down, PCR conditions were the same as for equine DNA detection, except that the annealing temperature was 50°C and the thermal cycles were 40 and 30 for solid glue and genomic DNA extracts, respectively.

All the PCR experiments were conducted in a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA). After amplification, 10 μL PCR products were fractionated by electrophoresis on a 2 - 3% TAE agarose gel, stained with GelRed$^{TM}$ Nuleic Acid Gel Stain (Biotium, Hayward, CA) and visualized under UV light.

## RESULTS

I. *Specificity of Species Specific PCR Detection*

The reported mitochondrion DNA based method[12-14] was tried but no PCR product was detected from DNA extracts of *Colla Corii Asini* (data not shown), just as the failure of 12S rDNA based tortoise DNA detection from tortoise shell glue[28]. In the present study, several primer pairs were designed based on equine SINE ERE-1 element for the establishment of equine DNA detection method. After a brief comparison and optimization (data not shown), the base pair Equine-up/down was selected for good specificity and sensitivity in equine DNA detection. The specificity was confirmed by PCR amplification of equal amount of donkey, horse, bovine, porcine, tortoise, and cervine genomic DNA. A PCR fragment corresponding in size to the predicted 81 bp was clearly amplified and observed from only donkey and horse genomic DNAs while no cross-amplification existed in other DNA samples and the negative control (Figure 1A). The PCR product was sequenced for further analysis, only leading to a consensus with our predicted sequence of ERE-1 element (Figure 3A). The results repeated well. The method also showed good specificity to various solid glue samples (Figure 2A), where the predicted PCR product could only be generated from DNA extracts derived from *Colla Corii Asini* and horse-hide glue, not from pig-hide glue or cattle-hide glue.

Since the equine SINE ERE-1 based PCR method could not distinguish species between donkey and horse, the second detection aimed for horse material contamination had

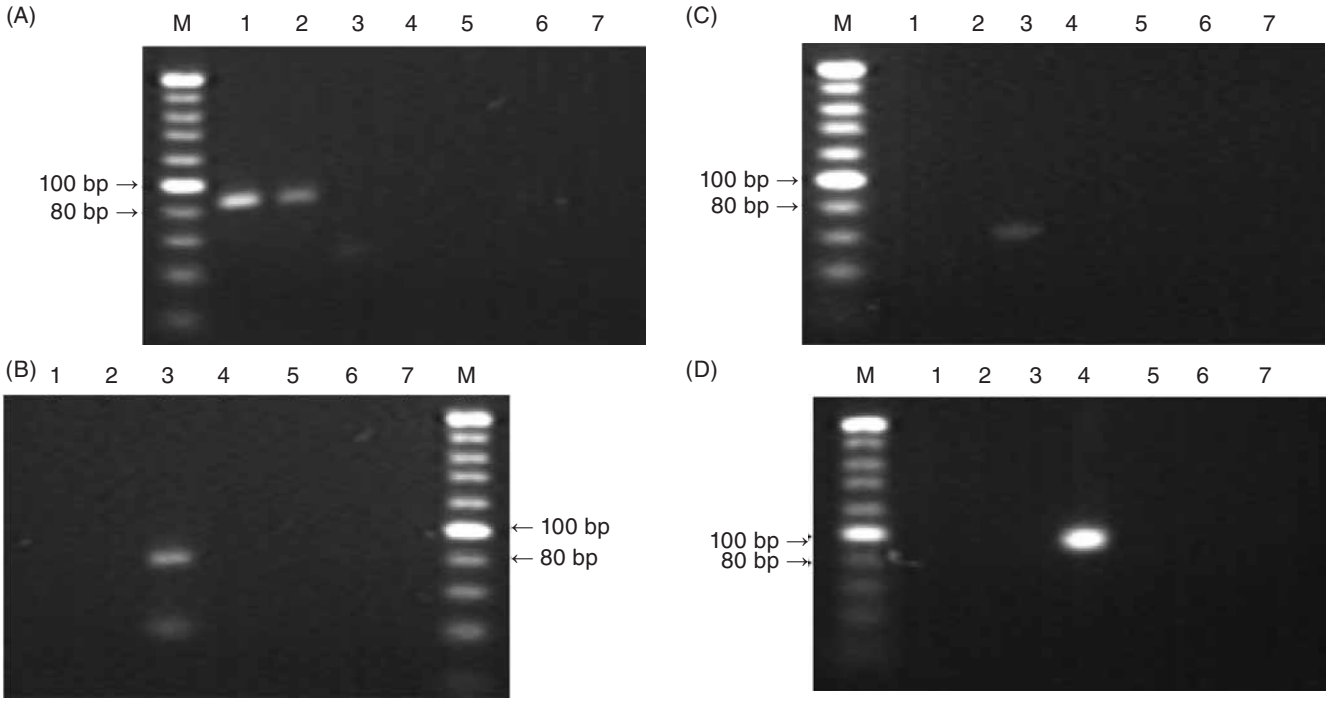


**Figure 1.** Agarose electrophoresis of the species specific PCR products obtained from genomic DNA of 6 species. (A) ERE-1 based equine DNA detection. Lane 1, donkey; lane 2, horse; lane 3, bovine; lane 4, porcine; lane 5, tortoise; lane 6, cervine; lane 7, blank; M, 20 bp ladder DNA marker. (B) Horse specific satellite based horse DNA detection. Lane 1, blank; lane 2, donkey; lane 3, horse; lane 4, cattle; lane 5, porcine; lane 6, tortoise; lane 7, cervine; M, 20 bp ladder DNA marker. (C) 1.711B bovine repeat based bovine DNA detection. Lane 1, donkey; lane 2, horse; lane 3, bovine; lane 4, porcine; lane 5, tortoise; lane 6, cervine; lane 7, blank; M, 20 bp ladder DNA marker. (D) PRE-1 based porcine DNA detection. Lane 1, donkey; lane 2, horse; lane 3, bovine; lane 4, porcine; lane 5, tortoise; lane 6, cervine; lane 7, blank; M, 20 bp ladder DNA marker.

to be developed. Unlike the mitochondrion DNA based horse detection strategy for common use, we adopted a class of specific satellite DNA localized on the centromeric region of equine chromosome 1 for primer design. As another kind of highly repetitive element, satellite DNA would also be more suitable for trace DNA detection. Also after screening several primer pairs on the base of their specificity and sensitivity (data not shown), Horse-up/down was selected for further study. The primer pair could generate a 78 bp fragment from horse tissue. The specificity for horse detection was also confirmed by PCR amplification of donkey, horse, bovine, porcine, tortoise, and cervine genomic DNA. The predicted PCR product was generated only from horse genomic DNA whereas no cross-amplification was observed from the DNA of other species (Figure 1B). Consequently, the PCR product was also sequenced and aligned with predicted sequence with good identity (Figure 3B). The results repeated well. When applied to various solid glue samples derived from different origins, the method was also specific for horse DNA (Figure 2B), where DNA extracts of other solid glues but horse-hide glue could generate no PCR products at all.

For PCR detection of bovine DNA, bovine SINE derived satellite 1.711B bovine repeat was used for primer design. After screening through different PCR conditions, the primer pair Bovine up/down was selected. The predicted 64 bp PCR products could only be amplified from bovine genome (Figure 1C) and cattle-hide glue DNA extracts (Figure 2C). An alignment of these PCR products and predicted sequence of 1.711B bovine repeat also shows good identity (Figure 3C).

Since the pig tissue might be blended or substituted for *Colla Corii Asini* production, porcine DNA detection was also essential and was carried out using PRE-1 element. Following the same optimization procedure, primer pair Pig-up/down was selected because of its good performance in specificity and sensitivity. The predicted 88 bp PCR product could only be generated from porcine genome (Figure 1D) and pig-hide glue (Figure 2D) DNA extracts. Furthermore, an alignment of the PCR products and the predicted sequence of PRE-1 also shows good identity (Figure 3D).

II. *Sensitivity of Species Specific PCR Detection*

After the equine SINE based PCR assay was established and applied to various solid glues successfully, detection limit of the assay for highly processed solid glue DNA extracts was determined by equine DNA detection from binary mixtures of *Colla Corii Asini* and pig-hide glue at levels of 0.1%, 1%, 10% by the developed method. Using primer pair Equine-up/down, the desired 81 bp amplicon was generated from the glue mixtures containing only 0.1% *Colla Corii Asini* (Figure 4A) while no amplicon was amplified from pig-hide glue and negative control, indicating that the method was very sensitive in practical use.

DNA extracts of binary mixtures of 0.1%, 1%, 10%

horse-hide glue and *Colla Corii Asini* were also used for detection limit determination for the horse specific assay. Only DNA extracts from glue mixtures with more than 1% horse-hide glue could generate the desired 78 bp PCR products while no amplicon was obtained from DNA extracts of glue mixtures with *Colla Corii Asini*, 0.1% horse-hide glue as well as the negative control (Figure 4B). The result meant that the horse satellite based PCR identification method was

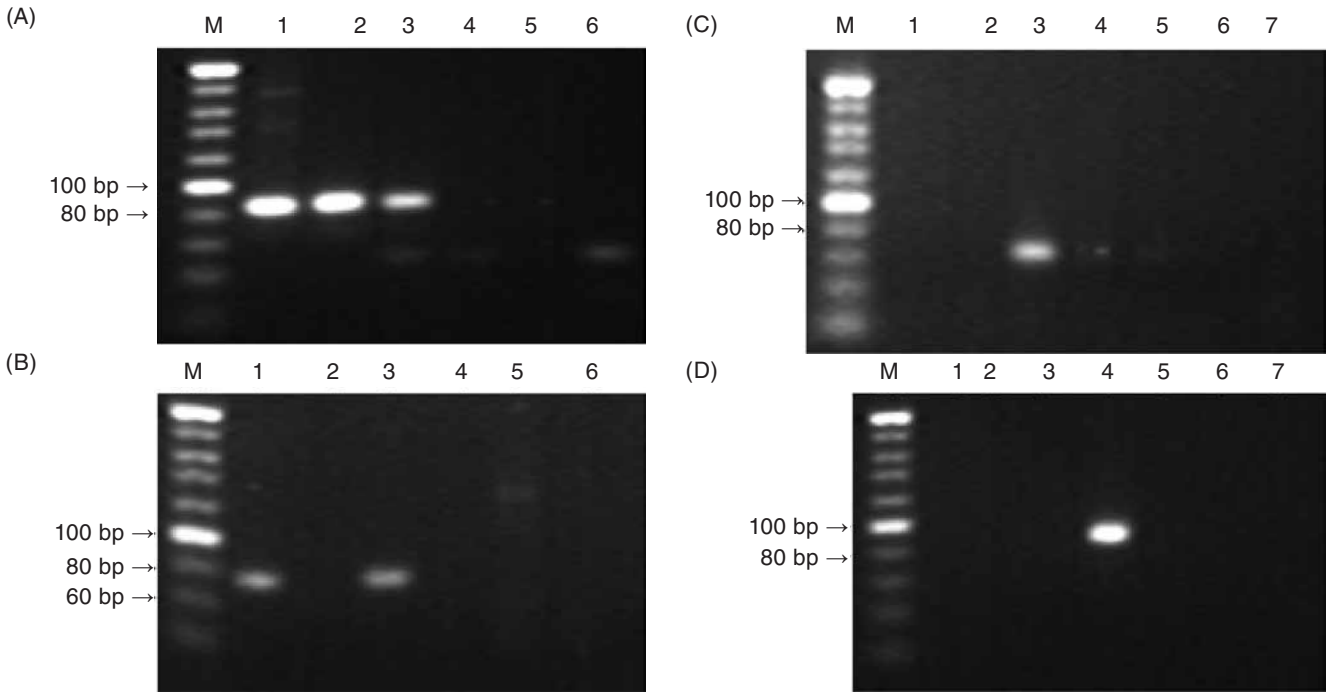


**Figure 2.** Agarose electrophoresis of the species specific PCR products amplified from DNA extracts of various solid glues. (A) ERE-1 based equine DNA detection. Lane 1, positive control (donkey genomic DNA); lane 2, *Colla Corii Asini*; lane 3, horse-hide glue; lane 4, cattle-hide glue; lane 5, pig-hide glue; lane 6, blank; (B) Horse specific satellite based horse DNA detection. Lane 1, positive control (horse genome); lane 2, *Colla Corii Asini*; lane 3, horse-hide glue; lane 4, cattle-hide glue; lane 5, pig-hide glue; lane 6, blank; (C) 1.711B bovine repeat based bovine DNA detection. Lane 1, *Colla Corii Asini*; lane 2, horse-hide glue; lane 3, cattle-hide glue; lane 4, pig-hide glue; lane 5, *Colla Carapacis et Plastri Testudinis*; lane 6, *Colla Cornus Cervi*; lane 7, blank; (D) PRE-1 based porcine DNA detection. Lane 1, *Colla Corii Asini*; lane 2, horse-hide glue; lane 3, cattle-hide glue; lane 4, pig-hide glue; lane 5, *Colla Carapacis et Plastri Testudinis*; lane 6, *Colla Cornus Cervi*; lane 7, blank; M, 20 bp ladder DNA marker.

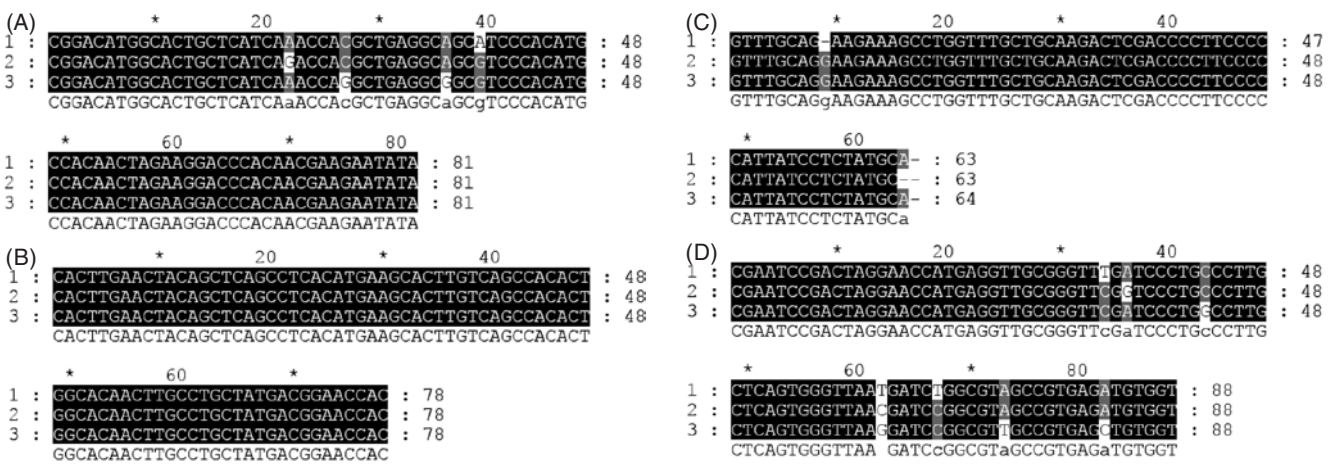


**Figure 3.** Alignment of PCR products and the predicted sequence for species specific DNA detections. (A) ERE-1 based equine DNA detection. Lane 1, *Colla Corii Asini*; lane 2, donkey genome; lane 3, the predicted sequence of equine SINE ERE-1 element; (B) Horse specific satellite based horse DNA detection. Lane 1, horse genome; lane 2, horse-hide glue; lane 3, the predicted sequence of horse specific satellite DNA element; (C) 1.711B bovine repeat based bovine DNA detection. Lane 1, bovine genome; lane 2, cattle-hide glue; lane 3, the predicted sequence of 1.711B bovine repeat; (D) PRE-1 based porcine DNA detection. Lane 1, pig genome; lane 2, pig-hide glue; lane 3, the predicted sequence of PRE-1 element.

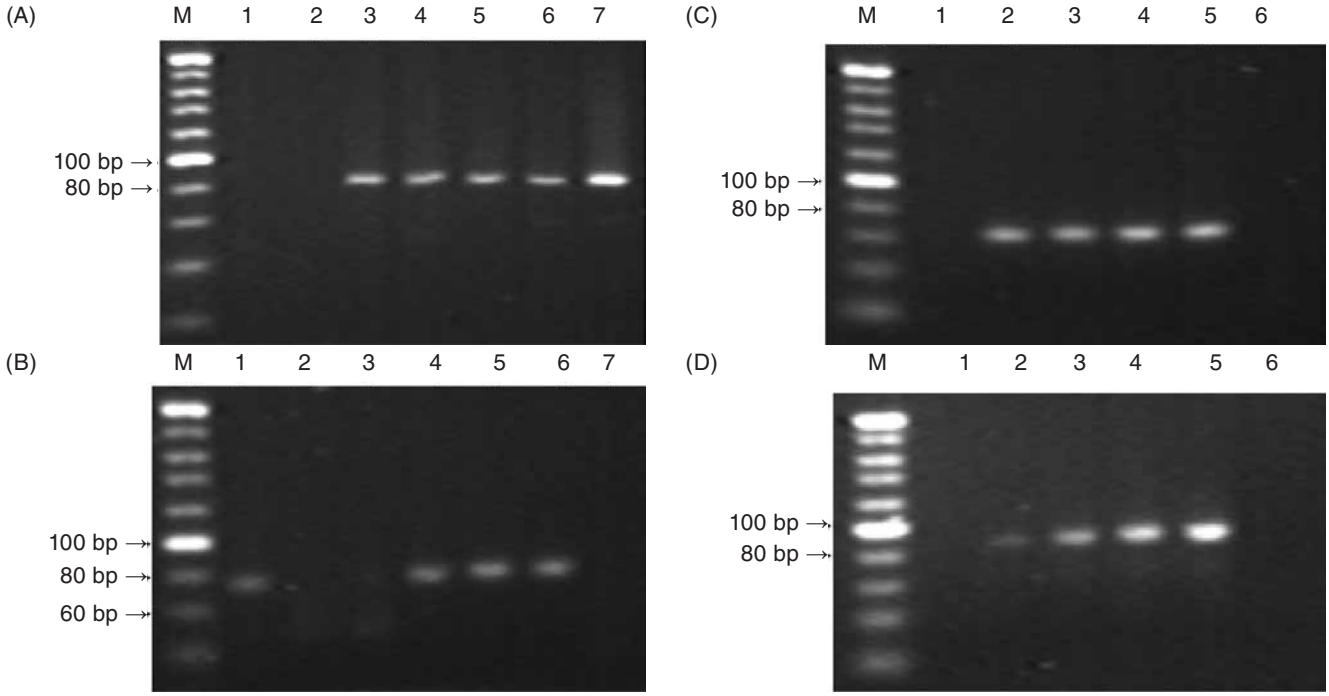


**Figure 4.** Species specific PCR detection of DNA extracts from binary mixtures of solid glues. (A) ERE-1 based equine DNA detection with pig-hide glue spiked with different amount of *Colla Corii Asini*. Lane 1, blank; lane 2, pig-hide glue; lane 3, 0.1% *Colla Corii Asini*; lane 4, 1% *Colla Corii Asini*; lane 5, 10% *Colla Corii Asini*; lane 6, *Colla Corii Asini*; lane 7, positive control (donkey genome); (B) Horse specific satellite based horse DNA detection with *Colla Corii Asini* spiked with different amount of horse-hide glue. Lane 1, positive control (horse genome); lane 2, *Colla Corii Asini*; lane 3, 0.1% horse-hide glue; lane 4, 1% horse-hide glue; lane 5, 10% horse-hide glue; lane 6, horse-hide glue; lane 7, blank; (C) 1.711B bovine repeat based bovine DNA detection with *Colla Corii Asini* spiked with different amount of cattle-hide glue. Lane 1, *Colla Corii Asini*; lane 2, 0.1% cattle-hide glue; lane 3, 1% cattle-hide glue; lane 4, 10% cattle-hide glue; lane 5, cattle-hide glue; lane 6 blank; (D) PRE-1 based porcine DNA detection with *Colla Corii Asini* spiked with different amount of pig-hide glue. Lane 1, *Colla Corii Asini*; lane 2, 0.1% pig-hide glue; lane 3, 1% pig-hide glue; lane 4, 10% pig-hide glue; lane 5, pig-hide glue; lane 6, blank; M, 20 bp ladder DNA marker.

also very sensitive and could detect the horse origin from highly processed solid glues containing only 1% horse tissue.

Sensitivity of the bovine specific PCR assay was also determined using binary solid glue mixtures containing 0.1%, 1%, 10% cattle-hide glue and *Colla Corii Asini*. The predicted 64 bp products could be generated from 0.1% cattle-hide glue sample (Figure 4C), indicating that our developed bovine specific PCR assay could even detect bovine DNA from solid glues containing only 0.1% cattle tissue.

In order to determine the detection limit of porcine specific PCR assay, binary mixtures of 0.1%, 1%, 10% pig-hide glue and *Colla Corii Asini* were used. The predicted 88 bp PCR products could be amplified from DNA extracts of glue mixtures containing only 0.1% pig-hide glue (Figure 4D), which represents the good sensitivity of the porcine specific PCR assay.

## DISCUSSION

*Colla Corii Asini* is one of the most valuable traditional Chinese medicine. In this study, different species-specific identification strategies were established to authenticate *Colla Corii Asini* from the products labeled fraudulently, especially from those blended with or totally substituted with horse, cattle and pig tissues.

Donkey and horse are very close species. To distinguish them from each other, almost all the reported methods were focused on mitochondrion DNA. The approach was also reported effective and sensitive while applied to some processed samples[12-14]. Our previous study showed that little residual DNA could be extracted from the glue samples and only fragment less than 100 bp could be amplified[16,17]. The large amounts of degraded collagen peptides might also affect the PCR efficiency[29], and thus the commonly used mitochondrion DNA based technology is not suitable for the genetic identification of highly processed medicinal glue.

SINE is a kind of highly repetitive element located in eukaryotic genome with the copy number of 20,000 - 80,000[23], which is much higher than those of mitochondrion DNA and ribosomal DNA (2,500 - 5,000)[20]. The length of SINE ranges from 70 to 500 bp. Besides, the insertion of SINE could be species specific and is a powerful tool for tracking lineages. Because of all these features, SINE is an ideal and preferable molecular marker for forensic DNA research[21]. By SINE-based species-specific detection method, bovine, porcine, and chicken DNAs were detected at 0.005% (0.5 pg), 0.0005% (0.05 pg), and 0.05% (5 pg), respectively, for meat samples[22], which is much more sensitive than mitochondrion or ribosomal DNA-based methods. So for highly

processed solid glue samples, the SINE family was selected for probe design in this study.

Since few researches were focused on donkey genome, the SINE element distributed specifically in donkey genome has not been reported yet. In this study, the equine SINE ERE-1 was selected for the first round detection of equine DNA that covered both donkey and horse. Although ERE-1 is also found in other Perissodactyla species[24], such as white rhino, Malayan tapir and zebra, they are unlikely be blended into *Colla Corii Asini* production because of scarcity of origin. However, horse has to be selected out. For this purpose, a second PCR assay based on horse-specific satellite DNA localized on the centromere of equine submetacentric chromosome 1[25] was developed. In this two-step PCR assay, the first round PCR was so sensitive that it could detect as little as 0.1% equine tissue from highly processed solid glues which made it accurately to determine whether equine tissue was added in solid glue preparation, while the second round PCR could detect horse DNA from solid glues mixtures containing only 1% horse-hide glue which was also sensitive enough for the detection of horse tissue. The tiny difference in sensitivity of these two assays might be contributed by different copy number of the satellite DNA and the SINE element. Furthermore, in order to assure if other animal tissues were blended into *Colla Corii Asini* production, the bovine DNA detection assay based on SINE derived satellite 1.711B bovine repeat and porcine DNA detection assay based on PRE-1 were also developed with the detection limit as little as 0.1%.

In conclusion, the PCR-based molecular identifications for equine, horse, cattle and pig DNA from highly processed solid glues were established in this study. The method was suitable for analysis of severely degraded DNA fragments (less than 100 bp) and thus could be used to prevent unethical practices that may occur not only in highly processed solid glue but in the highly processed meat products. Additionally, the method allows the detection without subsequent RFLP or sequence analysis. It was also economic and does not require expensive instruments. To our knowledge, it is the first time that the horse species-specific satellite DNA localized on the centromere of equine submetacentric chromosome 1[25] is used for horse tissue detection effectively.

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