

Research Article

Species Identification of Bovine Bone Marrow from Nonbovine Products Using Multiplex PCR Technology

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Bovine bone marrow is traditionally regarded as a highly nutritious food that has been widely used as a medicinal and health food for several decades in China. A large number of adulterated and counterfeit bone marrows from pigs and donkeys have been used in place of bovine bone marrow in commercial products, which are almost identical morphologically between species. Therefore, we explored the feasibility of multiplex PCR technology to differentiate bovine bone marrows from different domestic animals. Three pairs of specific primers for bovine, pig, and donkey were designed according to the conserved sequence in mitochondrial cytochrome b. A modified method was used to extract the genomic DNA from common domestic animals' bone marrows. The optimal reaction conditions for triple PCR were optimized. A three-fold PCR detection assay was successfully established to identify three species of bovine, pig, and donkey. Three primers have good specificity and high sensitivity. Additionally, the assay sensitivity test confirmed that the extracted DNA concentration was the lowest in bovine bone marrow at 10^6 pg/ μ L. The assay also showed 100% specificity. Rapid authentication of bovine bone marrow and differentiation from nonbovine products can be achieved using an improved SDS alkali denaturation method and species-specific PCR assay. Both species-specific PCR methods described in this study can be potentially applied for the quality evaluation of functional food and drug resources.

1. Introduction

The bovine bone marrow (BBM) consists of bone marrow from the two bovine species, *Bos taurus domesticus* Gmelin and *Bubalus bubalis* Linnaeus. BBM contains protein, fat, stearic acid, linoleic acid, and other nutrients. BBM is a healthy food that nourishes the lungs, detoxifies the kidneys, and replenishes bone marrow. Many bone-strengthening powders and calcium supplement products use BBM as a natural source of supplemental calcium [1, 2]. In addition, BBM is noted as a major component of Chinese patent medicine in the List of traditional Chinese medicine (TCM) Resources in Jilin Province, which has not been recorded in the Chinese Pharmacopoeia [3]. Due to its popularity, the quantity of BBM required for food, medicinal, and health products is increasing. Authentication of the natural components in the BBM is critical for effective quality control. A

large number of adulterated and counterfeit bone marrows from pigs and donkeys have been used in place of BBMs in commercial products, which are almost identical morphologically to the species [4, 5]. These products are not readily distinguishable from those containing BBM. Differentiation of BBM from the bone marrow of other species is performed using a range of analytical techniques, such as microscopy and thin-layer and high-performance liquid chromatography. Identification of physical traits in BBM mainly refers to microscopic morphological features such as appearance, size, and color, as well as epidermal features and odor [6, 7]. These methods are simple but subjective, and as the medicinal form is usually incomplete, the features are difficult to identify. Thin-layer and high-performance liquid chromatography are used to measure distinct BBM components. However, these methods have limited specificity, as the chemical structures of similar substances are sometimes

difficult to differentiate from those of BBM-derived materials [8].

In recent years, molecular identification methods have been developed to identify fraudulent or adulterated meat or TCM based on the specific genetic targets of animal-derived components in food or medicinal products [9]. DNA fingerprint patterns, including random amplified polymorphic DNA and PCR-amplified fragments length polymorphism, have been applied in the animal fields of biochemistry, genetics, and molecular biology to separate or purify genomic DNA, exhibiting characteristic banding patterns owing to electrophoretic mobility classification and species identification [10].

Our research team focuses on translational medicine with DNA fingerprinting technology for the identification of TCM and meat products based on the mitochondrial cytochrome b gene (Cyt b). We have developed a series of species-specific PCR kits to identify *Penis et testis cervi*, *Zaocys dhumnades*, and mink hearts [11–13]. However, the identification of BBM using DNA fingerprints has not been reported. Therefore, this study aimed to evaluate a species-specific PCR assay to differentiate BBM from those of pig and donkey species.

2. Materials and Methods

2.1. Collection of Samples. Two batches of fresh BBM samples originating from *Bos taurus domesticus* Gmelin (voucher specimens were 2016BH001 and 2016BH002) were collected from the standard specialized *Bos taurus domesticus* Gmelin breeds (Jilin Nong-an and Yong-ji *Bos taurus domesticus* Company, China). Two batches of samples originating from *Bubalus bubalis* Linnaeus (voucher specimens were 2016BH003) were also collected from standard specialized *Bubalus bubalis* Linnaeus breeds (Yunnan Qu-jing *Bubalus bubalis* Company, China). These samples were used as standard references. Three batches of counterfeit samples, including pig, sheep, and donkey bone marrow samples, were purchased from randomly selected supermarkets located in the region (Jilin Yong-ji, China). All samples were identified by the Chinese Food and Drug Supervision and Management, Jilin (Figure 1). Additionally, 37 batches of commercially available instant-frozen bovine bone marrow samples, labeled “BBM,” were purchased from 37 randomly selected supermarkets located in four cities of Jilin Province, China. In the meanwhile, three batches of bone marrows from horse and deer species were purchased, and artificially made bone marrow was used as a counterfeit in the study. All animal samples collected and test protocols were approved by the Medicine Institutional Animal Care and Use Committee, Beihua University (number: 2018-1-22). All specimens and DNA extracts were stored at -80°C at the Herbaria of BBM, Changchun Food and Drug Administration, and Innovation Center for DNA Fingerprint Detection Technology at TCM, Jilin Province, China.

2.2. Extraction and Detection of Genomic DNA from All Samples. One Gram of each fresh or frozen tissue sample was washed three times with a cold saline solution,



FIGURE 1: Morphological characteristics of bone marrow from four species. 1: Bone marrow from *Bos taurus domesticus* Gmelin; 2: bone marrow from *Bubalus bubalis* Linnaeus; 3: bone marrow from a species of pig; 4: bone marrow from a species of donkey; 5: bone marrow from a species of sheep.

completely cleaned of all connective tissue and fat, and cut into pieces. Total DNA was extracted using a modified SDS denaturation method as previously described [11]. The extracted DNA was electrophoresed in a 0.8% agarose gel (containing Gel Red) at 3 V/cm for 2 h and then recorded on a UV gel analyzer. The purity and concentrations of the extracted DNA (A260/A280) were determined using a trace nucleic acid analyzer (Q6000, Quawell, USA). The genomic DNA was dried at room temperature, dissolved in TE buffer, and preserved at -20°C until required for use.

2.3. Primer Designs. The Cyt b gene sequences for *Bubalus bubalis* (LOC102394407), *Bos taurus* (KT260196), *Sus scrofa* (AF163100), and *Equus asinus* (JF7188884.1) were downloaded from GenBank. Three specific primers of Cyt b were designed by the NCBI Primer-BLAST online primer design software and synthesized by Sangon Biotech (Shanghai, China) Co. Ltd (Table 1).

2.4. Establishment and Optimization of Multiplex PCR System. The total multiplex PCR system was 40 μL , including 2 \times Taq PCR Master Mix 20 μL (Takara, Shiga, Japan), template DNA (100 ng/ μL) of each species was 0.5 μL , upstream and downstream primers (10 ng/ μL) were 0.15–1 μL , and all were supplemented with sterile double-distilled water. PCR was performed on a conventional PCR machine (T100 Thermal Cycler, Biorad, CA), and the reaction conditions are as follows: predenaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 30 s, extension at 72°C for 10 min, preservation at 4°C . PCR-amplified products were subjected to electrophoresis on a 2% agarose gel containing 0.5 $\mu\text{L}/\text{mL}$ GelRed (Biotium, USA) for 50 min at 70 V and then analyzed under a UV light.

2.5. Evaluation of Assay's Specificity and Sensitivity. A double-blind method was utilized to estimate the assay's specificity and sensitivity. To validate the assay's specificity,

TABLE 1: Oligonucleotide sequence primers were used in the study.

Species	Sequences (5'→3')	Expected length (bp)
<i>Bubalus bubalis</i> (LOC102394407) and <i>Bos taurus</i> (KT260196)	F: CATCAAACATCTCATCTTGATG R: GTGTAAGACCCGTAATATAAG	254
<i>Equus asinus</i> (JF718884.1)	F: TGTGGGACGAGGACTA R: TGTGGGAATGGAGC	447
<i>Sus scrofa</i> (AF163100)	F: GTAATCTTGCTTTTC R: AGGATTAGTATTATAAATAAGGC	595

the simplex-PCR with bovine primers and the multiplex PCR were conducted; respectively, 2 batches of authentic BBM and three batches of bone marrows from sheeps, horses, pigs, deer, and donkeys were randomly numbered. For the sensitivity test, approximately 1-2 g of each authentic BBM DNA extract was diluted with ddH₂O to 100 ng/ μ L and then diluted to 0.01 pg/ μ L. All sample DNA extracts, PCR systems, and reaction conditions were performed as described above.

2.6. Evaluation of Commercially Available Samples. Thirty-seven batches of commercially available samples from different places of origin were selected to evaluate the assay's specificity. All PCR amplification was undertaken using 0.5 μ L BBM or sample DNA extract as the template.

3. Results

3.1. Analysis of Genomic DNA Extracted from All Samples. Agarose gel electrophoresis showed a bright band with an approximate size of 20 kb, demonstrating that target genomic DNA was successfully extracted by the modified method (Figure 2(a)). The A260/A280 ratios of the samples were in the range of 1.75–1.85, indicating high DNA purity with no protein contamination.

3.2. Construction of Multiplex PCR Approaches. The sample template loading, annealing temperature, and cycle number of amplicons were optimized. Primer concentrations were adjusted according to the band brightness of each primer pair in the gradient combination system to amplify uniform, clear, bright, and specific target bands. When the annealing temperature was 58°C, the bands were clear, and there were nonspecific bands or primer dimers (Figure 2(b)).

3.3. Evaluation of Assay's Specificity and Sensitivity. Regarding the assay's specificity, only bone marrows from bovine, pig, and donkey species showed the expected length amplification products with the multiplex PCR and specific primers. Whereas, the results of the bovine primers' simplex-PCR demonstrated that the assay could distinguish all authentic BBM from nonbovine products included in the study. The negative control and the other species showed negative results, demonstrating that the assay specificity was 100% (Figures 3(a) and 3(b)).

DNA extracts of BBM samples were diluted to 100 ng/ μ L and then diluted to 100 ng/ μ L, 10 ng/ μ L, 1 ng/ μ L, 100 pg/ μ L, 10 pg/ μ L, 1 pg/ μ L, and 0.1 pg/ μ L in a series of 10-fold

gradients. The results showed that the band became shallower with decreasing concentration. The results showed that the detection limit of DNA in BBM was 10 pg/ μ L (Figure 3(c)).

3.4. Characteristics Fingerprinting of Commercially Available Samples with Two Methods. All 37 batches of commercially available samples showed electrophoresis patterns with multiplex PCR. About 17 samples were found to be authentic BBM. Others were distinctly dissimilar to those of BBM, which were identified as counterfeits from pig or donkey species (Table 2).

4. Discussion

The *Cyt b* gene is an appropriate gene by which to analyze genetic diversity and evolutionary relationships between intraspecific and related species [14–16]. It is a valuable tool for phylogenetic classification and for evaluating the genetic diversity of organisms [17, 18]. Therefore, the authentication of specific components of healthy foods and of TCM based on the molecular characterization of *Cyt b* is more accurate and reliable than other methods [19, 20]. The application of molecular genetic markers to identify BBM has the advantage of being species-specific with high accuracy and reproducibility. This approach avoids the inherent limitations of macroscopic identification in TCM by authenticating at the molecular level, thereby facilitating the standardization and internationalization of TCM compounds [20–22].

Optimization of an SDS alkaline denaturation method to extract sufficient amounts of high-purity DNA was the key here to the success of subsequent genetic analyses. This optimization included the use of SDS to dissolve nuclear membranes and alkaline denaturation to remove proteins. DNase enzymes digest nuclear DNA attached to the outer membrane of mitochondria, and differential centrifugation removes cell debris. The method is easy and rapid and requires minimal laboratory equipment and reagents to directly isolate genomic DNA from intact cells without the isolation and purification of mitochondria.

In this study, a multiplex PCR method was developed for the detection of three species of products. A multiplex PCR technology can simultaneously amplify and analyze multiple target genes, save operating time and cost, and provide increasingly accurate information for food safety detection [23–25]. It has the advantages of high efficiency, systematicness, economy, and simplicity. The design of specific

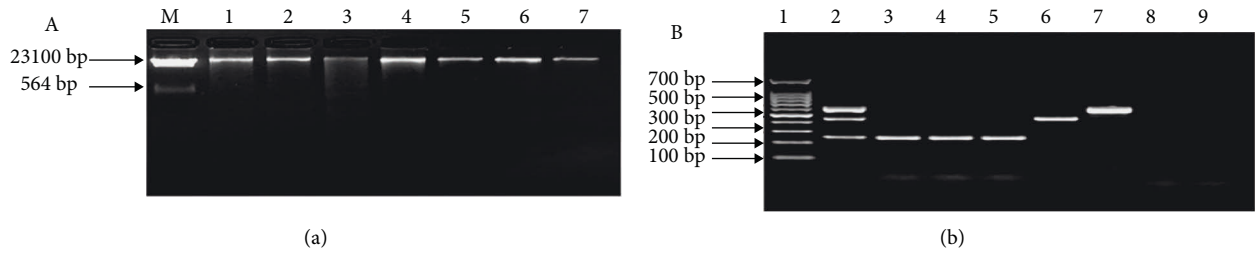


FIGURE 2: Agarose gel electrophoresis of genomic DNA extracts and species-specific fragments by multiplex PCR. (a) Agarose gel electrophoresis of genomic DNA extracted from different animals' bone marrows using an SDS alkaline lysis method. M: Marker; 1~3: BBM listed as 2016BH001, 2016BH002, and 2016BH003; 4: donkey bone marrow; 5: pig bone marrow; 6: sheep bone marrow; 7: a mixture of DNA extracted from horse and deer bone marrow (1:1). (b) Agarose gel electrophoresis of species-specific fragments by multiplex PCR. 1: Marker; 2: a mixture of DNA extracted from three species (1:1:1); 3~5: BBM listed as 2016BH001, 2016BH002, and 2016BH003; 6: donkey bone marrow; 7: pig bone marrow; 8: sheep bone marrow; 9: a mixture of DNA extracted from horse and deer bone marrow (1:1).

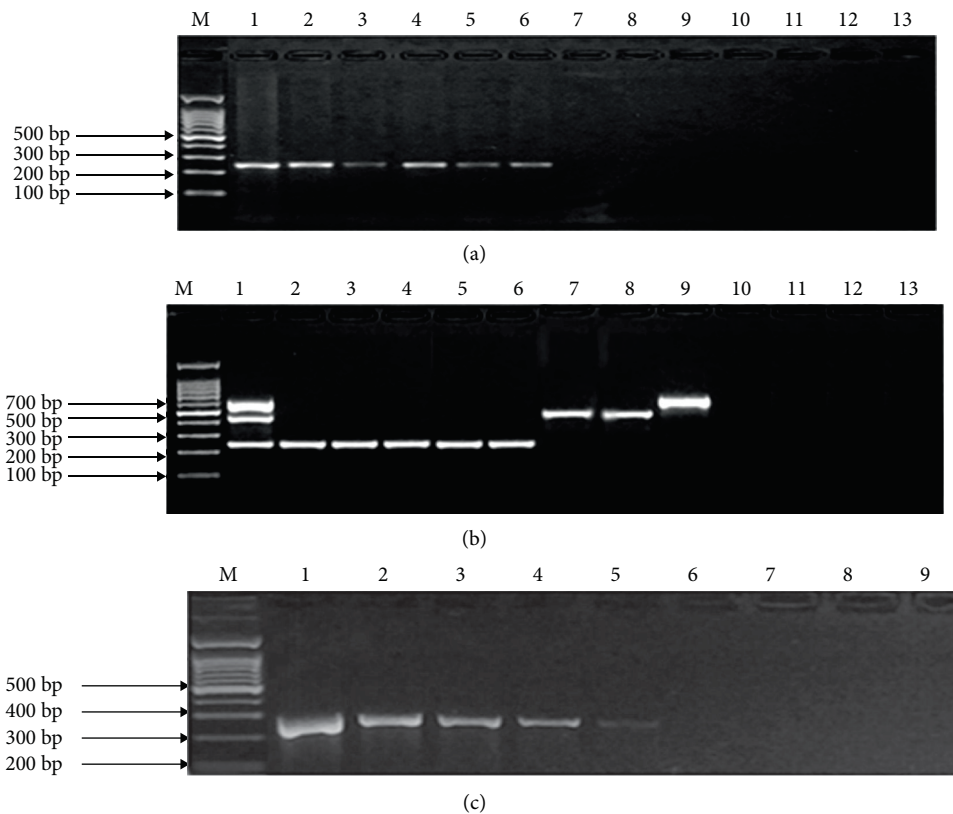


FIGURE 3: Specificity and sensitivity tests of the PCR assay using gel electrophoresis. (a) Specificity tests of the simplex-PCR assay using gel electrophoresis. M: Marker; 1: a mixture of DNA extracted from three species; 2~4: bovine bone marrow listed as 2016BH001, 2016BH002, and 2016BH003; 5~6: commercially available BBM; 7~8: donkey bone marrow; 9: pig bone marrow; 10: sheep bone marrow; 11: deer bone marrow; 12: horse bone marrow; 13: negative control. (b) Specificity tests of the multiplex PCR assay using gel electrophoresis. M: Marker; 1: a mixture of DNA extracted from three species; 2~4: bovine bone marrow listed as 2016BH001, 2016BH002, and 2016BH003; 5~6: commercially available BBM; 7~8: donkey bone marrow; 9: pig bone marrow; 10: sheep bone marrow; 11: deer bone marrow; 12: horse bone marrow; 13: negative control. (c) Sensitivity tests of the PCR assay using gel electrophoresis. M: Marker; 1: DNA extracts of BBM samples diluted to 100 ng/μL; 2: 10 ng/μL; 3: 1 ng/μL; 4: 100 pg/μL; 5: 10 pg/μL; 6: 1 pg/μL; 7: 0.1 pg/μL; 8: 0.01 pg/μL; and 9: negative control.

primers is another key to the identification of BBM using genetic analysis. We selected the *Cyt b* gene of each species to design specific primers, compared and analyzed the gene sequence homology of domestic animals' species with Clustalx software, and used NCBI Primer-Blast online primer design software to design species-specific primers for

bovine, pig, and donkey. We validated the assay's applicability for vouchers and real-world samples. The multiplex PCR could accurately identify all voucher bone marrow from bovine, pig, and donkey species and did not react with nonbovine species as well as the simplex-PCR with bovine primers. In addition, the limit of detection was 10 pg/μL

TABLE 2: Evaluation of commercially available samples collected from different cities in China using a multiplex PCR.

Serial No.	Collection place	Batches (0.5 kg)	Multiplex PCR
JLB-1	Jilin city	5	BBM
JLB-2	Jilin city	5	BBM
JLB-3	Jilin city	5	BBM
JLB-4	Jilin city	5	Pig
JLB-5	Jilin city	5	BBM
JLB-6	Jilin city	10	BBM
GZLB-1	Gongzhuling city	5	Pig
GZLB-2	Gongzhuling city	5	Pig
GZLB-3	Gongzhuling city	5	BBM
GZLB-4	Gongzhuling city	3	BBM
GZLB-5	Gongzhuling city	5	Pig
GZLB-6	Gongzhuling city	5	Pig
SPB-1	Siping city	3	Pig
SPB-2	Siping city	5	Pig
SPB-3	Siping city	5	Pig
SPB-4	Siping city	5	Pig
SPB-5	Siping city	10	Pig
SPB-6	Siping city	5	Pig
SPB-7	Siping city	5	BBM
SPB-8	Siping city	5	BBM
SPB-9	Siping city	5	BBM
SPB-10	Siping city	5	BBM
SPB-11	Siping city	5	BBM
SPB-12	Siping city	5	Pig
SPB-13	Siping city	5	Pig
CCB-1	Changchun city	5	Pig
CCB-2	Changchun city	6	Pig
CCB-3	Changchun city	5	BBM
CCB-4	Changchun city	5	BBM
CCB-5	Changchun city	5	BBM
CCB-6	Changchun city	5	BBM
CCB-7	Changchun city	5	BBM
CCB-8	Changchun city	5	Pig
CCB-9	Changchun city	5	Donkey
CCB-10	Changchun city	5	Donkey
CCB-11	Changchun city	10	Donkey
CCB-12	Changchun city	5	Donkey

comparable to our previously published PCR assays [11–13]. Our findings demonstrate that a rather trivial Cyt *b* gene segment contains genetic information that can be used in the development of sensitive and specific molecular techniques to study inter and intraspecies evolution. Similar to the findings reported by others [26–28].

Morphologically, the similar features with BBM come from those species of pig, donkey, horse, sheep in addition to deer. However, the bone marrows from horse and deer species are difficult to get as an alternative to the BBM. The shape of the bone marrow from sheep species is smaller than that of cattle's; it is easy to be identify with the naked eye. The most common counterfeits are the bone marrows from pig and donkey species, both of which lack medicinal or health functions. In fact, the problematic identification of BBM is often misidentified by the molecular method owing to improper labeling of BBM contents mixed with other domestic animals. With real-world samples labeled BBM, the assay performed successfully and was able to detect authentic BBM. Taken together, a species-specific, PCR-based

method to identify BBM is an essential research tool for improving the accuracy of detecting TCM compounds in medicinal herbs [29–32]. PCR-based methods are simple, highly specific, sensitive, and accurate, thereby complementing existing Chinese herbal medicine detection methods [33–35]. Next, we will adopt SDS-PAGE analysis of whole-cell proteins on gel fingerprints among three species, as previously described [36].

5. Conclusion

The study demonstrated that rapid authentication of BBM and differentiation from nonbovine products can be achieved using an improved SDS alkali denaturation method and species-specific PCR. The assay reported here is highly sensitive and suitable for the authentication of BBM DNA in fresh and frozen BBM materials. The species-specific PCR as described in this study can be potentially applied for the quality evaluation of functional food and drug resources.

Data Availability

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethical Approval

All animal samples collected complied with the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines, and test protocols were approved by the Medicine Institutional Animal Care and Use Committee, Beihua University (number: 2018-1-22).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Liyuan Sun and Mingcheng Li have contributed equally to this work. Yize Guan conceived and designed the experiments and wrote a draft manuscript. Liyuan Sun and Mingcheng Li analyzed and interpreted the results of the experiments and revised the manuscript. Yize Guan, Nan Li, and Tancheng Li performed the experiments. All authors read and approved the final manuscript.

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