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Research Article

Establishment of deer heart identification method and development of the detection kit based on mitochondrial cytochrome B gene

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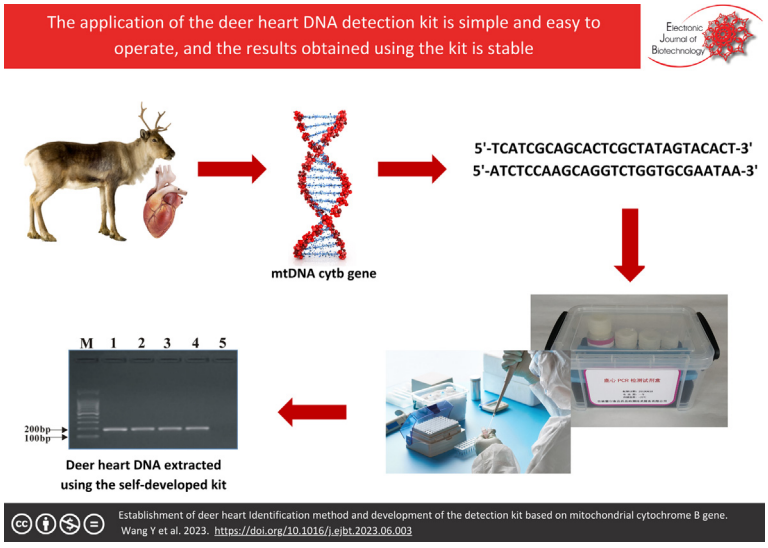
GRAPHICAL ABSTRACT



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ABSTRACT

Background: Deer heart is a valuable traditional Chinese medicinal material. At present, there is no functional component or symbol component for deer heart, and it is difficult to identify them by physical and chemical methods. In this study, we established deer heart identification method based on mtDNA cytb gene, and developed a deer heart DNA detection kit.
Results: There were specific amplification bands in reference medicinal materials and true deer hearts at 194 bp, but no amplification bands in negative control and blank control. Deer heart DNA detection kit has good specificity, reproducibility and stability, and its sensitivity can reach 0.5 mg.
Conclusions: The application of the deer heart DNA detection kit is simple and easy to operate, and the results obtained using the kit is stable, which is suitable for popularization and application.
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Specific primer
Target gene

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1. Introduction

Deer heart, a valuable traditional Chinese medicinal material, is the heart of sika deer (*Cervus nippon* Temminck) or red deer (*Cervus elaphus* Linnacus) of Cervus, Cervidae, artiodactyla, Class Mammalia [1], included in Shennong Herbal Classic. Deer heart is rich in active components such as proteins and amino acids, minerals and trace elements. It is believed in traditional Chinese medicine that deer heart has the effect of nourishing Qi, tonifying blood and calming the mind, and is mainly used for the treatment of heart diseases, or the tachycardia, and the insufficiency of heart and blood caused by shock, fatigue and long-term neuroweakness, and so on [2,3,4]. Because of its unique efficacy, accurate curative effect and high price, a large number of counterfeit products of deer heart (mostly pig heart, horse heart, cow heart, sheep heart, etc.) are sold in the market. Because the fresh deer heart is not easy to preserve, most of the deer heart sold in the market are processed, so it is difficult to distinguish them from common counterfeit products by their characters or microscopic identification [5,6,7,8]. At present, there is no functional component or symbol component for deer heart, and it is difficult to identify them by physical and chemical methods, leading to the confusion of deer heart medicinal materials in the market and seriously affecting the health and interests of consumers.

DNA fingerprinting is a method to identify species by using DNA, a genetic information vector *in vivo*, to detect the genetic characteristics of the identified objects at the molecular level, with a high species specificity [9,10,11,12]. MtDNA b (Cytochrome b, Cytb) gene is an ideal molecular genetic marker because of its simple structure, small number of genomes, moderate evolution rate and single maternal inheritance, suitable for the research and analysis of interspecies diversities and their evolution relationship, which is the focus of current research [13,14,15].

In this study, based on the polymorphism of DNA, a fingerprinting identification for deer heart was established by taking the mtDNA Cytb gene of sika deer and red deer as the target gene, selecting the specific fingerprint segments to design the specific primers and using PCR, molecular cloning and sequencing. On the basis of above works, a deer heart DNA detection kit was developed, and the identification process using this kit was simpler, rapid and accurate, indicating that the method should be more suitable for popularization. The method was aimed at checking the source of the traditional Chinese medicine to ensure the accuracy and safety of the raw drug material, with a great significance for the standardization and modernization of traditional Chinese medicines.

2. Materials and methods

2.1. Experimental materials

Deer heart samples were purchased from Jilin Longtan Mountain Deer Industry Co., Ltd., Jilin Longtan Mountain Xiangyang Deer Farm, Jiaohe City first Deer Farm and Jiaohe City third Deer Farm. Deer heart was washed with normal saline 3 times, the fat was removed, and the fat-removed deer heart was dried at 40–50°C. The reference material of deer heart was provided by Baicheng Food and Drug Inspection Institute. Pig heart, horse heart, cow heart, sheep heart, donkey heart and chicken heart were purchased from Jilin Food Market.

Deer heart DNA gene detection kit (Jilin Leibo Technology Co., Ltd); 100 bp DNA Ladder, DNA recovery kit, pGM-T vector ligation kit, DH5α competent cells, plasmid mini-preps kit, Amp, X-Gal, IPTG, and 2 × Taq PCR Master Mix (Beijing Tiangen Biochemical Technology Co., Ltd., China); Primers (Shanghai Bioengineering Co., Ltd., China).

2.2. Instruments and equipment

High-speed freezing centrifuge (D3024R, SCILOGEX, USA); handheld centrifuge (D1008E, Dalong Chuangxing Experimental Instrument Co., Ltd., China); PCR instrument (T100, BIORAD Company, USA); electrophoresis instrument and electrophoresis tank (Beijing Junyi Oriental Electroelectrophoresis equipment Co., Ltd., China); UV gel imaging analyzer (UV WHITE-2020D, BIORAD Company, USA); micronucleic acid protein analyzer (6000+, Quawell, USA); 1/1,000 electronic balance and 1/10,000 electronic balance [Ohaus Instruments (Changzhou) Co., Ltd., China); thermostat water bath (ZKW-C, Shanghai Shuli Instrument Co., Ltd., China).

2.3. Preparation of mt DNA

All samples collected in the study were fully washed with double distilled water to remove grease and dust, sterilized with 75% alcohol for 2 min, dried at 37°C in oven, and then ground into powder with a sterilized pulverator and stored in containers. 5 G of the sample were fully ground into smaller powders in a mortar, 100 mg of the sample powders were placed in a 1.5 ml centrifuge tube, 275 μl P1 digestive solution (200 μl nuclear lysis buffer, 50 μl of 0.5 mol/L ethylene diamine tetraacetic acid disodium solution, 20 μl of 20 mg/ ml proteinase K solution and 5 μl RNA enzyme solution) and 250 P2 cell nuclear lysis buffer (1 ml of 1 mol/L Tris–HCl, 2 ml of 0.5 mol/L EDTA, 0.58 g NaCl, 10 ml of 10% SDS, 1 ml of 20 mg/ml protease K) were added into the mortar, then double distilled water was added into it to a constant volume of 100 ml). The solution was evenly mixed, kept in a thermostat water bath at 55°C for 1 h, and then transferred into a DNA purification column and centrifuged at 10,000 rpm/min for 3 min. The filtrate was discarded and the residual was dissolved in 600 μl P3 eluent [26 μl of 5 mol/L potassium acetate solutio, 18 μl of l mol/L Tris-hydrochloric acid solution (pH 7.5), 3 μl of 0.5 mol/L ethylenediamine tetraacetic acid disodium solution (pH 8.0), 480 μl absolute ethyl alcohol and 273 μl sterile double-distilled water], and the solution was centrifuged at 10,000 rpm/min for 1 min, and the filtrate was discarded. The residual was repeatedly eluted with the above-mentioned eluent twice, then the residual solution was centrifuged for 2 min and the filtrate was discarded, and the DNA purification column was transferred into another centrifuge tube. 100 μl P4 sterile double-distilled water was added into the centrifuge tube, left standing at room temperature for 10–20 min, then centrifuged at 10,000 rpm/min for 2 min, and the centrifugate, that is, the test sample of DNA solution, was kept at −20°C for use.

2.4. Determination of genome DNA concentration and purity

The absorbance values of the template DNA solution extracted above at A_{260} and A_{280} were measured by an ultraviolet spectrophotometer, respectively, and the corresponding concentration and the purity were calculated.

2.5. Optimization of PCR reaction system and reaction conditions

Identification primer:

Upstream primer: 5′-TCATCGCAGCACTCGCTATAGTACACT-3′,
Downstream primer: 5′-ATCTCCAAGCAGGTCTGGTGCGAATAA-3′.

The length of the amplified fragment was 194 bp. The total volume of the PCR reaction was 25 μl, including 23 μl of the reaction system (12.5 μl of 2-Taq PCR Master Mix, 1 μl of 12.5 μmol/L Primer F, 1 μl of 12.5 μmol/L Primer R and 8.5 μl ddH_2O contained in the PCR reaction tube) and 2 μl DNA template (100 ng/μl). The reaction parameters were pre-denature at 94°C for 5 min, circular reaction 30 times (at 94°C for 30 s, 63°C for 30 s and 72°C for 30 s, respectively) and extension at 72°C for 5 min. The sterile ddH2O was taken as the blank control.

2.6. Detection by agarose gel electrophoresis

After the PCR amplification, the 2% agarose gel electrophoresis was performed on the test sample and the standard reference substance in 1 × TBE electrophoresis buffer at 80 V for 70 min. After the electrophoresis, the gel tablets were examined and photographed by a gel documentation system.

2.7. Cloning and sequencing of deer heart standard reference substance DNA

The agarose gel electrophoresis was performed on 50 μL of the PCR products, then the DNA bands were taken out under an ultraviolet transmission instrument and weighed, and AaxyPreo DNA Gel Extraction Kit 50-prep was used to recover the purified target gene. The target gene was connected with pGM-T vector at 16°C overnight to form a recombinant. The recombinant was transferred into DH5α competent cells on crushed ice, then 30 μL of the bacterial liquid were smeared on an LB solid culture medium containing Amp, X-Gal and IPTG after the proliferation for 4 h, which was incubated at 37°C overnight, and the blue-white spot screening was carried out to screen the positive recombinant. The white colonies were selected, and cultured in an LB liquid culture medium containing Amp for 12–16 h, the plasmid DNA was extracted and verified as the positive recombinant by PCR the next day, and then sent to Shanghai Bioengineering for sequencing. The samples all were sequenced by the bidirectional sequencing with the positive and negative primers, and the results of the sequencing were compared with those of the target genes of sika deer. The sequence of the amplified region DNA was identical to that of the specific fingerprint region DNA of the target gene, so the deer heart DNA positive standard reference liquid was determined.

2.8. Design of deer heart DNA detection kit

The deer heart DNA detection kit contained 20 doses and consisted of 8 parts, including the DNA extraction and PCR amplification (Table 1).

2.9. Evaluation of kit

2.9.1. Specificity

The specificity of primers was observed by the PCR amplification of genuine deer heart and its counterfeit samples.

2.9.2. Reproducibility

The same genuine deer heart and its counterfeit samples were tested by three different technicians in three different laboratories for the observation on the reproducibility of the reagents.

2.9.3. Stability

One of the kits selected randomly was used for the detection at the 3rd month, 6th month, 9th month and 12th month, respectively, for the observation on the stability of the reagents.

2.9.4. Sensitivity

The DNA extracted from 100 mg, 50 mg, 20 mg, 10 mg, 5 mg, 2 mg, 1 mg, 0.5 mg, 0.2 mg, 0.1 mg and 0.05 mg of the initial deer heart reference medicinal material samples was amplified by PCR amplification, and analyzed by agarose gel electrophoresis, respectively, for the observation on the sensitivity of the reagents. The calculation formula is as follows:

Sensitivity = (actual sample size/100) × 100%.

3. Results and analysis

3.1. Extraction of genomic DNA

The genomic DNA of deer heart was extracted using the self-developed kit. The A_{260}/A_{280} values of the extracted products were determined by UV spectrophotometry, ranging from 1.7 to 1.9, and the concentrations of DNA were 250–300 ng/μL, indicating that the genomic DNA of deer heart with a high purity could be successfully extracted by using this kit.

3.2. PCR amplification results of deer heart components

The PCR amplification on the genomic DNA of deer heart reference medicinal material and the deer heart samples was performed by using the self-developed kit. The agarose gel electrophoresis results showed that there was a specific amplification band of both the deer heart reference medicinal material and the deer heart samples at 194 bp, which was clear and bright, while there was no blank control band (Fig. 1).

3.3. Cloning and sequencing results of deer heart standard control solution DNA

The results of molecular clone blue white spot screening showed blue-white colonies on the ampicillin-positive plate (Fig. 2). The white colony was selected and cultured, then the plasmid DNA was extracted and amplified by PCR. The results of agarose gel electrophoresis showed that at an annealing temperature of 63°C, there was a specific DNA band at 194 bp (Fig. 3). The plasmid DNA of deer heart was sent to Sangon Biotech (Shanghai) for the bidirectional sequencing of the two positive and negative primers, and the sequencing results were compared with those of the target genes of silka deer and red deer, showing that the sequence of the amplified region DNA was identical to that of the mtDNACytb gene specific region DNA of silka deer and red deer, respectively, so the plasmid DNA of deer heart was determined as the standard control solution (Fig. 4) and the standard control solution was packed in the DNA detection kit.

Table 1
Composition of Deer Heart DNA detection kit.

No.	Composition	Volume	Action	Temperature	Usage
P1	a	10 ml	Cracking cells	−20°C	b
P2	a	10 ml	Separating out DNA	−20°C	b
P3	a	25 ml	Purifying DNA	−20°C	b
P4	a	5 ml	Dissolving DNA	−20°C	b
Positive control solution	a	40 μl	Positive control	−20°C	b
Negative control solution	a	40 μl	Negative control	−20°C	b
PCR reaction tube	a	23 μl		−20°C	b
Chromatographic column		20			

a: Omitted; b: Use after melting completely.

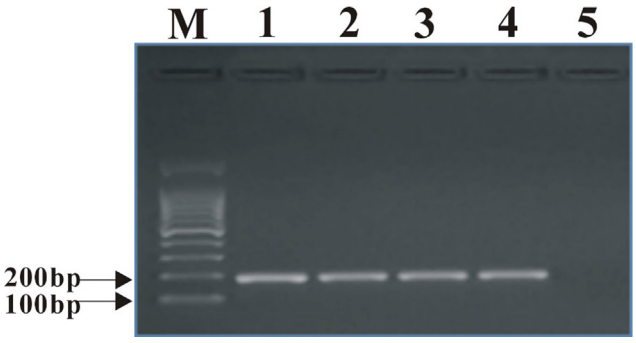

Fig. 1. Deer heart DNA extracted using the self-developed kit. M: DL 100 bp Marker; 1: Positive control; 2: Silka deer heart 1; 3: Silka deer heart 2; 4: Red deer heart; 5: Blank control.

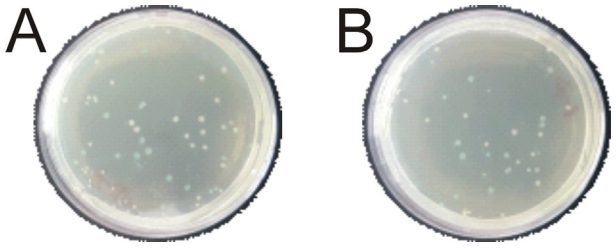

Fig. 2. Molecular cloning blue-white spot screening map of deer heart DNA. (A) Sika deer heart; (B) Red deer heart.

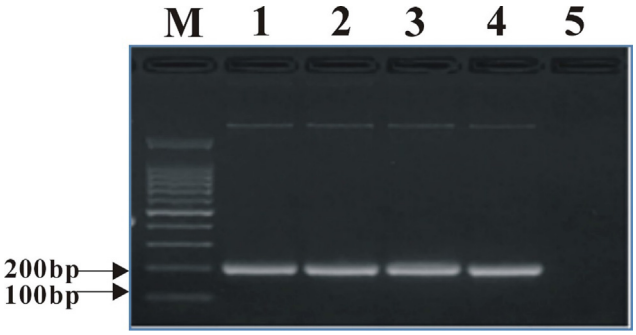

Fig. 3. PCR amplification map of deer heart plasmid DNA. M: DL 100 bp Marker; 1–2: Silka deer heart 2; 3–4: Red deer heart; 5: Blank control.

3.4. Evaluation of kit parameters

3.4.1. Specificity

The self-developed deer heart DNA detection kit was used to detect the deer heart reference medicinal material, deer heart, pig heart, horse heart, cow heart, sheep heart, donkey heart, dog heart and chicken heart samples, and the PCR amplification was carried out at 63°C. The results of agarose gel electrophoresis showed that there was a specific amplification band of both the deer heart reference medicinal material and deer heart at 194 bp, but no amplification band of pig heart, horse heart, cow heart, sheep heart, donkey heart, dog heart and chicken heart samples, and blank control (Fig. 5).

3.4.2. Reproducibility

The same samples (deer heart control medicinal material, deer heart, pig heart, horse heart, cow heart, sheep heart, donkey heart, dog heart and chicken heart) were repeatedly detected using the self-developed deer heart DNA detection kit by three different technicians in three different laboratories. The agarose gel electrophoresis results showed that after the PCR amplification at 63°C, there was a specific amplification band of both the deer heart reference medicinal material and deer heart at 194 bp, while no specific amplification band of pig heart, horse heart, cow heart, sheep heart, donkey heart, dog heart, chicken heart and the blank control at 194 bp (Fig. 6).

3.4.3. Stability

The self-developed deer heart DNA detection kit was used on the 3rd, 6th, 9th, and 12th month after the preparation of the kit for examining its stability. The results of agarose gel electrophoresis showed that after the PCR amplification at 63°C, there was a specific amplification band of both the deer heart reference medicinal material and deer heart samples at 194 bp, but no amplification band of blank control (Fig. 7).

3.4.4. Sensitivity

The self-developed deer heart DNA detection kit was used to detect the deer heart reference medicinal material and the PCR amplification was carried out at 63°C. The results of agarose gel electrophoresis showed that the brightness of the bands was dependent on the quantity of samples, that is, with the decrease of the sampling quantity, the brightness of PCR product bands decreased gradually, and the bands of PCR products were clearly visible when the sampling quantity was 0.5 mg and those were blurred when the sampling quantity was lower than 0.5 mg, so the sensitivity of the self-developed deer heart DNA detection kit was 0.5% (Fig. 8).

4. Discussion

It was considered in this study that the deer heart samples were dried at high temperature, which would lead to the serious degradation of DNA. Therefore, the procedures for the preparation of the self-developed genomic DNA extraction reagents should be simplified as much as possible and the extraction time should be shortened as soon as possible to avoid the degradation of genomic DNA by physical, chemical and biological factors [16,17,18]. The

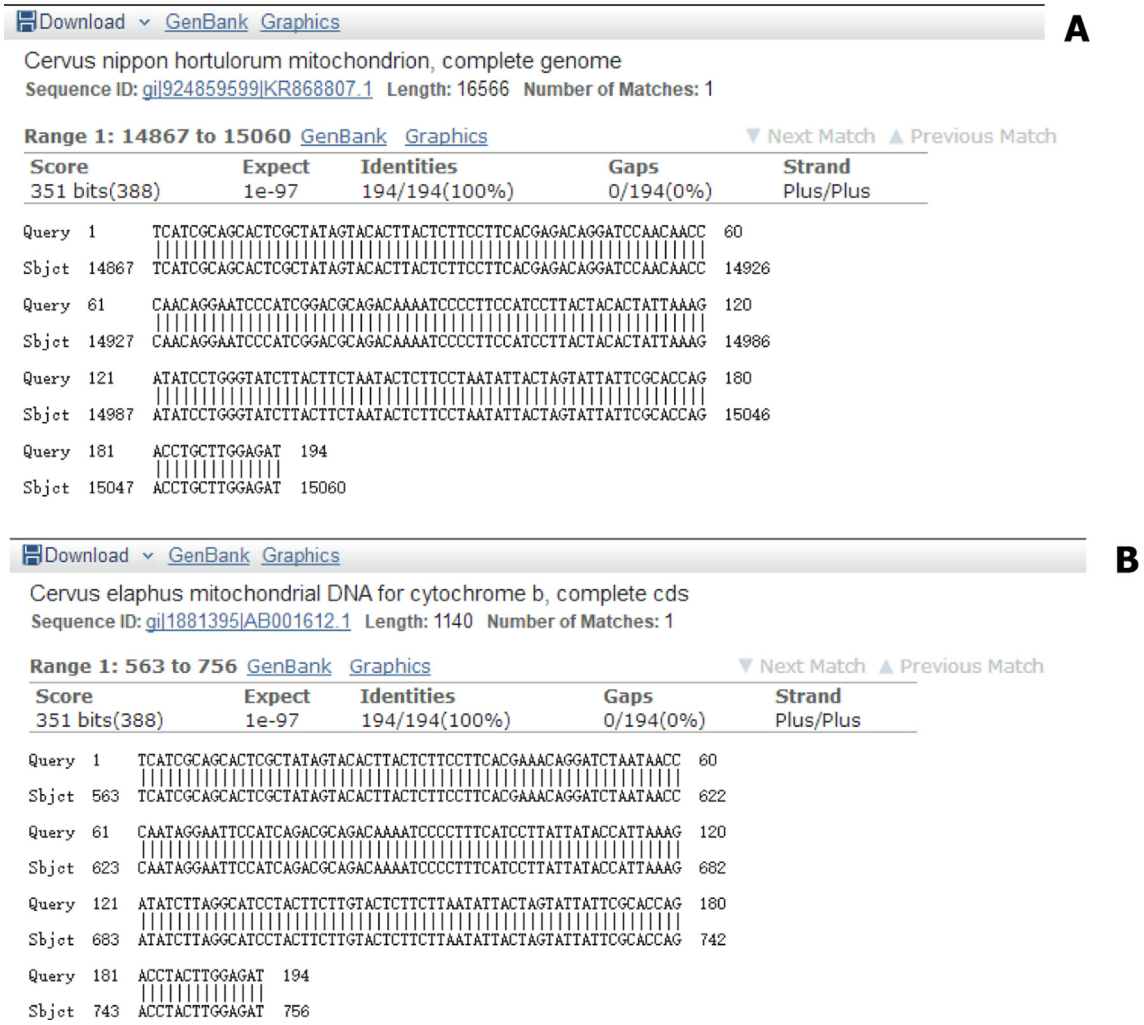

Fig. 4. Plasmid DNA sequencing and mtDNA Cytb gene alignment of deer heart. (A) Plasmid DNA sequencing and mtDNA Cytb gene alignment of sika deer heart; (B) Plasmid DNA sequencing and mtDNA Cytb gene alignment of red deer heart.

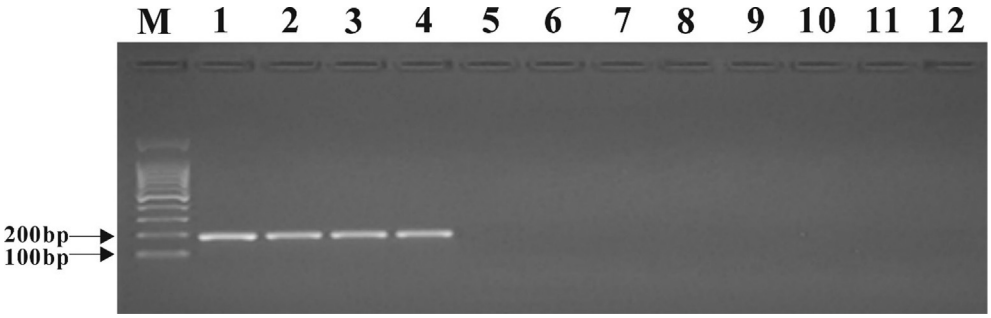

Fig. 5. PCR specific amplification map of deer heart. M: DL 100 bp Marker; 1: Positive control; 2–3: Silka deer heart; 4: Red deer heart; 5: Pig hear; 6: Horse heart; 7: Cow heart; 8: Sheep heart; 9: Donkey heart; 10: Dog heart; 11: Chicken heart; 12: Blank control.

deer heart mtDNA Cytb gene was used as the target gene, and the specific primers with a small fragment 194 bp were designed and amplified by PCR. Under the condition of annealing temperature of 63°C, the deer-derived components were successfully amplified, and the PCR amplification bands were bright and clear.

In this study, the DNA fingerprint characteristics of deer heart were studied and analyzed at the molecular level by biomolecular technology, and a specific PCR identification method for deer heart was established and a DNA fingerprint detection kit of deer heart was developed. The specificity, stability, reproducibility and sensitivity of the kit developed in this study in the identification of deer heart were detected. The results showed that using the deer heart DNA fingerprinting kit, the genomic DNA could be quickly and stably extracted from the samples and a 194 bp single DNA band could be amplified in a specific PCR system, and the repeated experiments showed that the specificity, reproducibility and stability of the kit were excellent, and the sensitivity was 0.5%.

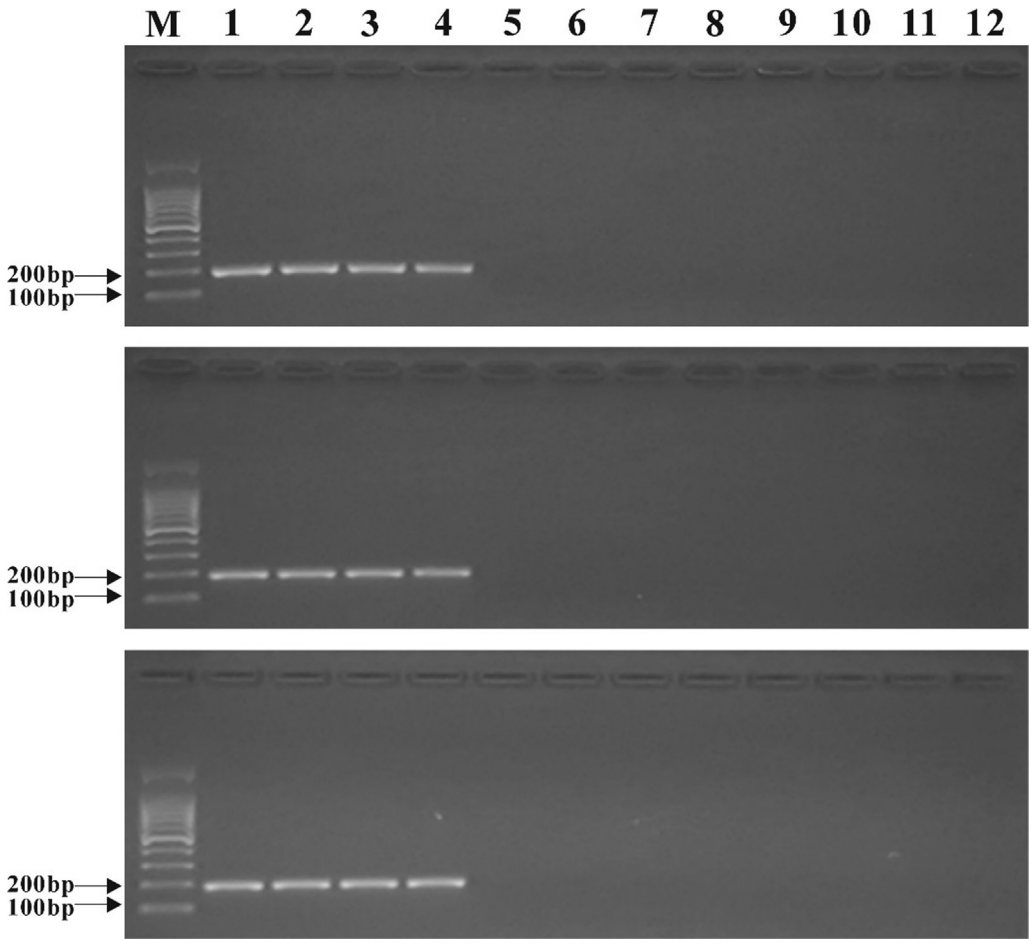

Fig. 6. PCR amplification map of deer heart DNA for the evaluation of the reproducibility. M: DL 100 bp Marker; 1: Positive control; 2–3: Silka deer heart; 4: Red deer heart; 5: Pig hear; 6: Horse heart; 7: Cow heart; 8: Sheep heart; 9: Donkey heart; 10: Dog heart; 11: Chicken heart; 12: Blank control.

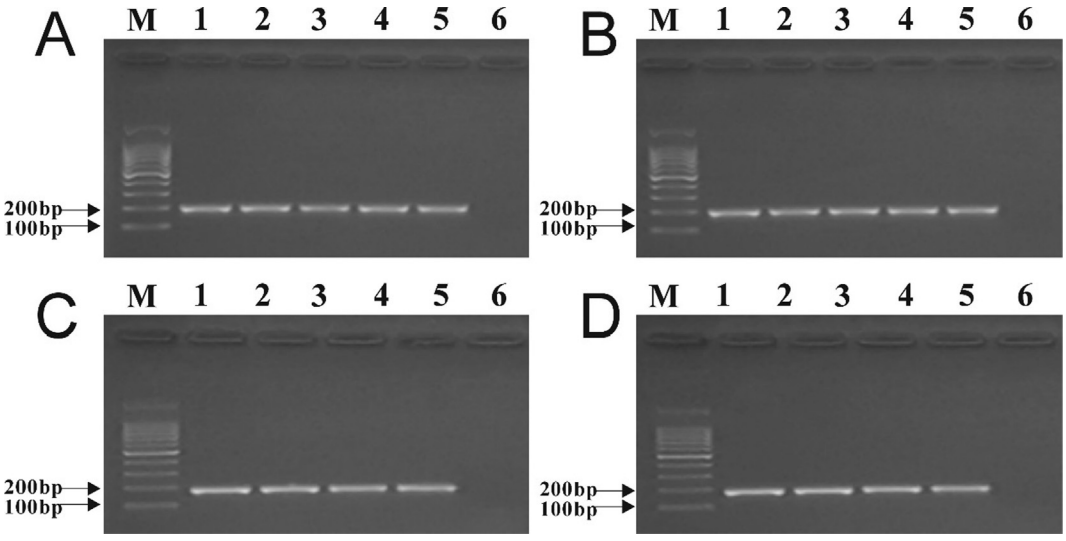

Fig. 7. PCR amplification map of deer heart DNA for examining the stability of the kit. (A) 3rd month; (B) 6th month; (C) 9th month; (D) 12th month. M: DL 100 bp Marker; 1–2: Positive control; 3–4: Silka deer heart; 5: Red deer heart; 6: Blank control.

In order to verify the specificity of the primers and the correctness of the amplified specific gene fragment sequence, the deer heart specific PCR amplification gene fragment was cloned by using a biological molecule cloning technology, a positive recombinant was screened, and the plasmid DNA of the extracted positive recombinant was used as the template for the PCR verification [19,20]. The results of agarose gel electrophoresis showed that there was only a specific amplification band at 194 bp, which

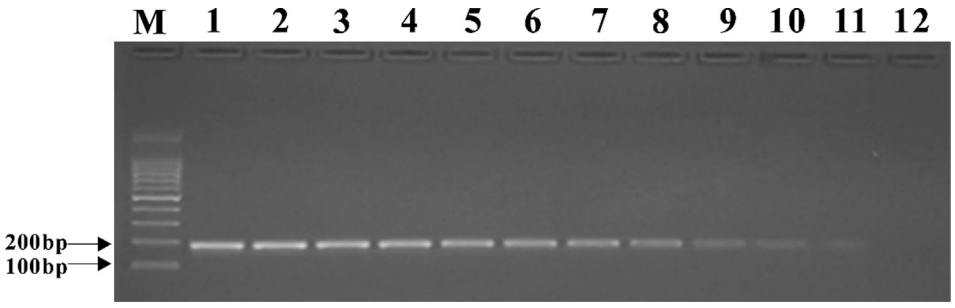

Fig. 8. Electrophoresis map for examining the sensitivity of self-developed deer heart DNA detection kit. M: DL 100 bp Marker; 1: 100 mg; 2: 50 mg; 3: 20 mg; 4: 10 mg; 5: 5 mg; 6: 2 mg; 7: 1 mg; 8: 0.5 mg; 9: 0.2 mg; 10: 0.1 mg; 11: 0.05 mg; 12: Blank control.

was identified as that of the positive recombinant, then the bidirectional sequencing was performed with positive and negative primers, and the sequencing results were compared with those of the target gene by using Basic Local Alignment Search Tool (BLAST). The analytical results showed that the sequences of the specific amplification DNA bands were identical to those of the mtDNA Cytb specific fingerprint region of sika deer and red deer, and then the clone liquid was used as the deer heart control solution packed in the kit. The most reliable and convincing method for identifying if an unknown sample belongs to a particular species is DNA sequence alignment [21,22]. Therefore, the molecular cloning and sequencing was used to verify the specificity, feasibility and reliability of the method for the identification of the deer heart DNA by using the cytb gene as the target gene in this study.

The whole process from the DNA extraction to PCR identification can be completed by using the self-developed deer heart DNA fingerprint detection kit. The reagents are complete in this kit, which can avoid the waste of reagents to save the cost, the complexity and error of reagent preparations, and the influence of reagents from different sources on the experimental results, with a good specificity, reproducibility and stability. The application of this kit can greatly simplify the operation steps since according to the standard operation flow, the identification of the true and false deer heart can be quickly and accurately completed about 2 h, and the experimental results are accurate, reliable, stable, intuitive and easy to judge, which should be easy to popularize.

5. Conclusions

In this study, we established a DNA fingerprint identification method for deer heart and develop a deer heart DNA detection kit. The application of the self-developed deer heart DNA detection kit is simple and easy to operate, and the results obtained using the kit is stable, which is suitable for popularization and application.

Ethical approval

All animal experiments were approved by the Ethical Committee of Beihua University (No.202010250018, 25 October 2020) of China. All procedures were conducted in accordance with the guidelines for the care and use of laboratory animals (China).

Author contributions

Study conception and design: J Ai.
Data collection: Y Wang.
Analysis and interpretation of results: Y Wang, L Zhang.
Draft manuscript preparation: Y Wang, L Zhang, J Ai.
Revision of the results and approval of the final version of the manuscript: Y Wang, L Zhang, J Ai.

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Conflict of interest

The authors declare that they have no competing interests.

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