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Tortoise DNA Detection from Highly Processed Tortoise Shell Using SINE Element

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ABSTRACT

Colla carapax et plastrum testudinis is one of the most valuable traditional Chinese medicine referring to highly processed tortoise shell glue which contains trace amount of severely degraded DNA. In this work, a simple and fast methodology was first developed for the identification of highly processed medicine from substitutions which were labeled fraudulently as tortoise shell glue but derived from tissues of other animals such as horse, cattle or pig. The method is based on polymerase chain reaction (PCR) amplification of tortoise-specific Pol III/SINE element and allows the detection of tortoise DNA in glue mixtures, which contain only 0.1% *colla carapax et plastrum testudinis*. A comparison of five various polymerases for the assay was also conducted, based on their performances in PCR. Finally, the method was applied for the detection of tortoise DNA from six commercial tortoise shell glue products; one of them was tested negative, indicating none or less than 0.1% tortoise shell in there.

Key words: tortoise shell glue, *colla carapax et plastrum testudinis*, molecular identification, short interspersed nuclear element, traditional Chinese medicine

INTRODUCTION

Tortoise shell is a valuable traditional Chinese medicine (TCM) that has been widely used as tonic food for a long time especially in some East Asian countries[(1)]. In recent years, some studies focused on its pharmaceutical effect have revealed that the powder and extracts of tortoise shell are useful in promoting hepatic function and relieving stress[(2)]. They also exert anticancer[(3)] and immunomodulation activities[(4)]. From tortoise shell, two major nutraceuticals were derived: one is Kwei Ling Ko (Tortoise shell-Rhizome Jelly) which is well known for its anti-inflammatory properties[(5)], the other is *colla carapax et plastrum testudinis* (glue of tortoise shell) which can nourish blood and arrest bleeding[(6)].

As recorded in the Chinese Pharmacopoeia, *colla carapax et plastrum testudinis* is the solid glue prepared from tortoise shell by decoction and concentration[(6)]. However, as the demand for turtles grows rapidly during these years[(1)], in some batches of the TCM, there exist substitutions derived from skins or even bones from other animals such as horse, cattle or pig which were fraudulently labeled as tortoise shell glues. These substitutions own little pharmaceutical effect but may harm people's health seriously. Due to the fact that morphological characters are not reliable enough to distinguish solid glues of different origin, it is essential to establish an effective, reliable and applicable method to identify tortoise shell glue in order to ascertain people's health.

As the development of biotechnology, DNA based molecular markers have been widely used for TCM identification because it is more effective, specific, reliable, convenient and less affected by environmental and processing conditions[(7)]. For example, Japanese green tea can be identified by STS-RFLP analysis[(8)] while ginseng by direct amplification of minisatellite region[(9)]. However, up to now, such method was mainly applied to crude and raw TCM products with relatively intact DNA, but few were developed for highly processed TCM materials like solid glue. DNAs extracted from the latter were usually severely degraded in short length and trace amount, thus rendering the efficiency of PCR[(7)]. For example, from 1g *colla corii asini* (ass-hide glue), only as few as 3-5 μg DNA fragments could be extracted and

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only amplicon of less than 100 bp could be amplified, i.e., the average length of DNA fragments derived from *colla corii asini* was less than 100 bp[16].

Up till now, various methods have been developed for the identification of tortoise shell, including ATR-FTIR spectroscopy[10], FT-Raman spectroscopy[11] and molecular identification based on PCR amplification of cytochrome b sequence[1] and 12S rRNA gene[12]. However, all these reported methods are suitable for raw or lightly processed tortoise shell but less effective for highly processed tortoise shell-derived products like *colla carapax et plastrum testudinis*, which is processed through an initial decoction for about 10 hr, at nearly 120°C, pH 5.0-6.5, followed by further evaporation at 120-130°C for 15-20 hr. After this drastic processing, *colla carapax et plastrum testudinis* may contain only trace amount of severely degraded DNA fragments but large amount of degraded collagen peptides (more than 80%), just like that in *colla corii asini*. For example, the spectroscopy based method was less applicable to samples processed in different degrees of manufacture[10,11]. The widely used cytochrome b based PCR identification method was not proven effective on processed tortoise shell material[1] while the 12S rRNA gene based method couldn't identify the tortoise jelly preparations[12]. Therefore, new strategies are demanded for the detection of tortoise DNA from highly processed tortoise shell.

In recent years, short interspersed nuclear element (SINE) was increasingly reported to be used as a specific sequence for species identification[13]. It is a class of repetitive sequences with length ranging from 70 to 500 bp and can have > 10^6 total copies per haploid genome, more than those of the widely used mitochondrial DNA (including cytochrome b and 12S rRNA), chloroplast DNA of about 2500 copies and ribosomal DNA of about 5000 copies[14]. Besides, SINE can be used to establish genealogies at sub-species level[15]. Because of all these features, SINE was an ideal genetic marker for DNA detection of highly processed product.

In our previous work, the DNA of highly processed solid glue was extracted successfully which could be subject to amplifications by PCR[16], albeit short in length and trace in amount. Here, in order to detect the tortoise DNA from highly processed tortoise shell to ascertain whether the product is labeled fraudulently, i.e. a substitution, we firstly developed a SINE-based PCR identification method. The method is effective, reliable and convenient in practical use.

MATERIALS AND METHODS

I. *Samples*

In this study, the solid glues were purchased from the following sources: *colla carapax et plastrum testudinis* made from tortoise shell, *colla corii asini* (ass-hide glue) made from donkey skin (*Equus asinus*) and cattle-hide glue made from cattle skin (*Bos taurus*) were produced by Shandong Donge E-jiao Co., Ltd. (Shandong Province, China), pig-hide glue was prepared from the skin of *Sus scrofa* in the laboratory of Shandong Donge E-jiao Co., Ltd, while 6 kinds of different *colla carapax et plastrum testudinis* products were collected in January, 2008, from a pharmacy store. Three of them were produced in Henan Province, China while the other 3 samples were produced in Shandong Province, China.

Meat samples from tortoise (*Chinemys reevesii*), cattle (*Bos taurus*), pig (*Sus scrofa*), horse (*Equus caballus*) and donkey (*Equus asinus*) used as positive controls were purchased from local supermarket.

II. *DNA Extraction and Quantification*

DNAs were extracted from various solid glues according to the previously established method[16], as follows.

For each DNA preparation, in order to avoid the false-positive result derived from the external contamination, each side of the glue surface was irradiated by UV for 2-3 h. Then about 1g of glue was ground into powder and transferred into a 60-mL centrifugal tube where 1 mL of DNA extraction buffer (10 mM Tris-HCl, 25 mM EDTA, 100 mM NaCl, 0.5% SDS, pH 8.0) and 20 μL of proteinase K (20 mg/mL) were added. After incubating for 1-2 h at 56°C with occasional shaking, the sample was added in 10 mL of PN buffer (Qiagen, Duesseldorf, Germany) and mixed completely. About 600 μL of samples was then added into the QIAquick spin column (Qiagen, Duesseldorf, Germany) and centrifuged for 1 min at 10,000 ×g. After discarding the flow-through appropriately, the step was repeated several times (about 12-14 times) until all volume was centrifuged through the same QIAquick spin column. The column was washed 3 times with PE buffer (Qiagen, Duesseldorf, Germany) (500 μL each time), and dried by evaporation for 5 min at room temperature. Later on, DNA was eluted with 100 μL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) by centrifuging for 2 min at 12,000 ×g and stored at -20°C until use.

Genomic DNAs were extracted from meat samples using the TIANamp Genomic DNA kit [Tiangen Biotech (Beijing) Co., Ltd., Beijing, China]. For each DNA preparation, 1 g of meat was washed several times with sterile water and cut into small pieces. The tissue was homogenized and about 0.1 g homogenate was transferred to a 1.5-mL microcentrifuge tube, washed again with sterile water and centrifuged at 10,000 rpm for 1 min. The pellets derived from meat homogenate were suspended in 200 μL of nuclei lyses buffer GA [Tiangen Biotech (Beijing) Co., Ltd., Beijing, China], and used for DNA extraction by following the kit instruction for tissue samples.

DNA extracts were measured quantitatively by UV absorbance at 260 nm on a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

III. *PCR Reaction*

The oligonucleotide primer pair (Tor-up: 5'-GAGCATTGGCCTGCTAAACC-3' and Tor-down: 5'-TTTTGCCCCAGATCCCTAAA-3') for species-specific detection of tortoise DNA was designed and optimized from the 5' region of tortoise Pol III/SINE[17] based on the fact that different tortoise Pol III/SINE elements of cryptodiran turtles including CryI and CryII share the same 5' regions (position 1 to 95)[18]. The primer pair was synthesized by Invitrogen.

For tortoise DNA detection, the primer pair Tor-up/down was used in a 25 μL reaction volume containing 12.5 μL Premix Ex-taq™ Hot Start Version (TaKaRa Dalian, Dalian, China), 0.4 μM reverse and forward primer, 5 μL and 1μL of template DNA from glue (10 ng/μL) and meat samples (1 ng/μL), respectively. PCR amplification was performed as follows: first denature: 4 min at 94°C; and the following amplification cycles: 40 cycles for glue sample and 30 cycles for meat sample of 30 s at 94°C, 30 s at 50°C, 30 s at 72°C; and a terminal elongation of 7 min at 72°C.

PCR amplification was conducted in a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA). PCR products (10 μL) were electrophoresed in a 2-3% TAE agarose gel and further visualized under UV using GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, CA).

IV. *Sequencing*

The PCR product was sequenced and aligned with the reference from tortoise Pol III/SINE to confirm the analysis. After electrophoresis, the 72 bp fragments derived from both the DNA extracts of *colla carapax et plastrum testudinis* and tortoise genome were purified from agarose gel using TIANgel Midi Purification Kit [Tiangen Biotech (Beijing) Co., Ltd., Beijing, China], and consequently ligated into pMD™ 19-T Simple Vector (Takara, Otsu, Japan). The plasmid was then sequenced on a 3730 DNA sequencer (Applied Biosystems, Foster City, CA).

V. *Preparation of Gelatin Mixtures*

Colla carapax et plastrum testudinis / *colla corii asini* mixtures were prepared by thoroughly mixing various percentages (0.1%, 1%, 10%) of *colla carapax et plastrum testudinis* and *colla corii asini* before grinding into powders. One gram of each gelatin mixture was weighed for DNA extraction and extracted DNA was diluted to 10 ng/μL. for the assessment of minimum detection limit of tortoise species-specific SINE-based PCR assay for solid glue.

VI. *Polymerase Efficiency Evaluation*

To investigate the efficiency of five commercial DNA polymerases, including 2x Taq PCR MasterMix [Tiangen Biotech (Beijing) Co., Ltd., Beijing, China] , Premix Ex-taq™ Hot Start Version (Takara, Otsu, Japan), Pyrobest™ DNA polymerase (Takara, Otsu, Japan), PrimeSTAR HS DNA Polymerase (Takara, Otsu, Japan) and Ampli Taq Gold™ (Applied Biosystems, Foster City, CA) used for PCR detection of DNA extracts from *colla carapax et plastrum testudinis*, each PCR amplification was conducted in a 25 μL of reaction mixture containing recommended amount of DNA polymerase as recorded in the instructions, 0.4 μM reverse and forward primer (Tor-up/do) and 5 μL of DNA extracts (diluted to a final concentration of 10 ng/μL). The PCR reaction with an initial denaturation at 94°C for 4 min, 40 cycles of 30 s at 94°C, 30 s at 50°C, 30 s at 72°C; and a final extension of 7 min at 72°C were carried out in triplicate for each polymerase.

RESULTS

I. *Specificity of the Tortoise SINE Based PCR*

In this study, several primer pairs were initially designed based on the 5' region of tortoise Pol III/SINE (position 1 to 95)[18]. PCR conditions were individually optimized for each primer pair. The specificity and sensitivity in tortoise DNA detection were compared and evaluated for various genomic DNAs (data not shown). Tor-up and Tor-down pair was thus selected due to their good performance. A PCR product with expected size of 72 bp was clearly observed on electrophoregram from tortoise genome whereas none was amplified from horse, donkey, cattle and pig genomes under the same experimental conditions (Figure 1). The PCR product was cloned and sequenced for further analysis. Alignment of the sequence and the reference tortoise Pol III/SINE showed a good match (Figure. 2).

II. *Application of the Method to Solid Glues*

Based on the fact that the highly processed *colla corii asini* contains only trace and short DNA fragments[16], *colla carapax et plastrum testudinis* derived from tortoise shell by the same processing procedure, may also contain trace amount of highly degraded DNA fragments. Therefore, the primer pairs were designed to generate amplicon with size limited to 80 bp. Since the specificity and sensitivity were verified, the methodology was then applied to detect the tortoise DNA from various solid glues. Just as predicted, only DNA extracts derived from *colla carapax et plastrum testudinis* could generate amplicon in accordance with that from positive control, while no PCR products could be obtained from DNA extracts of other solid glues at all, including *colla corii asini* (ass-hide glue), cattle-hide glue and pig-hide glue (Figure 3). The PCR products were sequenced and aligned with the reference Pol III/SINE, showing good

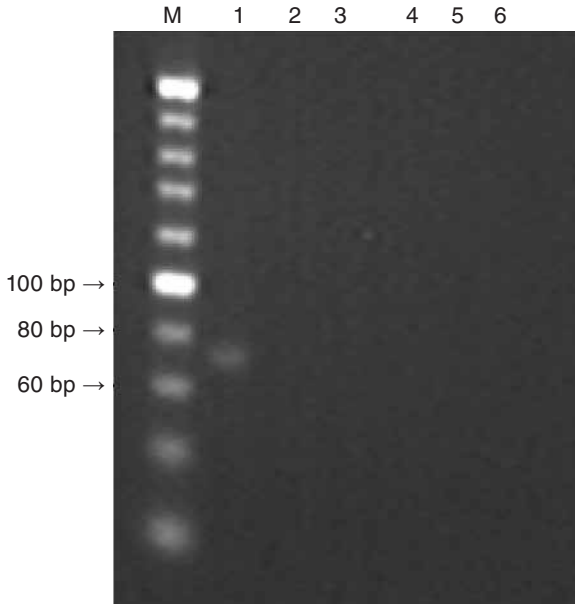

Figure 1. Agarose electrophoresis of the tortoise Pol III/SINE based PCR products amplified from genomic DNA of 5 species. Lane 1: tortoise; lane 2: donkey; lane 3: horse; lane 4: cattle; lane 5: pig; lane 6: blank; M : 20 bp DNA ladder.

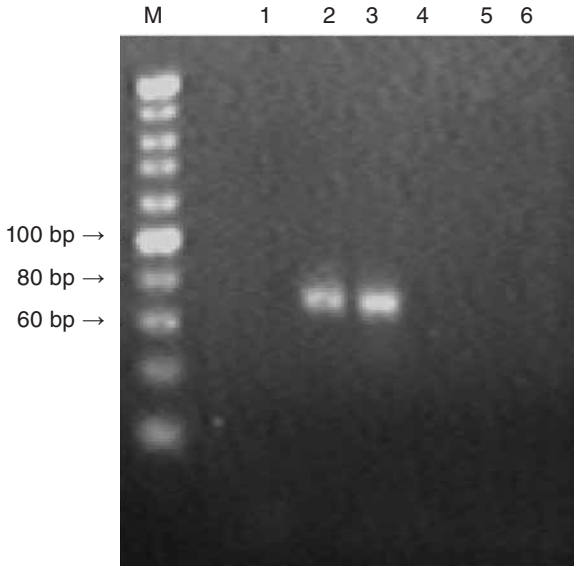

Figure 3. Agarose electrophoresis of the tortoise Pol III/SINE based PCR products amplified from DNA extracts of 4 TCM solid glues. Lane 1: blank; lane 2: positive control with tortoise genome DNA; lane 3: *colla carapax et plastrum testudinis*; lane 4: *colla corii asini*; lane 5: cattle-hide glue; Lane 6: pig-hide glue; M represents 20 bp DNA ladder.

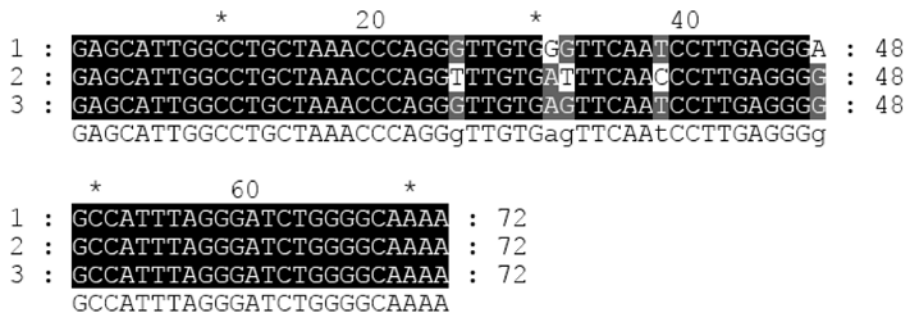

Figure 2. DNA sequences of about 72 bp tortoise Pol III/SINE based PCR products amplified from *colla carapax et plastrum testudinis* (line 1) and tortoise genome (line 2). Line 3 is the predicted sequence of 5' region of tortoise Pol III/SINE element (position 23 to 94)[18]. The consensus sequence of each subgroup is indicated in the bottom line. Small letters indicate those present predominantly at a certain position in the consensus sequence. The * mark represents a 10 nucleotides interval.

identities (Figure 2), indicating that the method was suitable for tortoise DNA detection in highly processed tortoise shell materials.

III. *Polymerase Efficiency Evaluation*

To select a suitable and inexpensive DNA polymerase used for further molecular authentication of *colla carapax et plastrum testudinis*, 5 commercially purchased DNA polymerases were compared with their PCR performance. Each of the polymerase was tested for PCR detection of DNA extracts derived from *colla carapax et plastrum testudinis* in triplicates. The result showed that all polymerases performed well in tortoise shell DNA detection with the intensity of bands obtained nearly the same (Figure 4). The commonly used taq was effective enough for tortoise DNA detection in highly processed tortoise shell.

IV. *Evaluation of Effective Detection Limit*

DNA extracted from binary mixtures of *colla carapax et plastrum testudinis* and *colla corii asini* at levels of 0.1%, 1%, 10% were used to evaluate the effective detection limit of tortoise species-specific SINE-based PCR identification in practical application. Using the Tor-up/down primer pair, the desired 72 bp fragment could be amplified from the templates of binary mixtures blended with 0.1%, 1%, 10% and 100% *colla carapax et*

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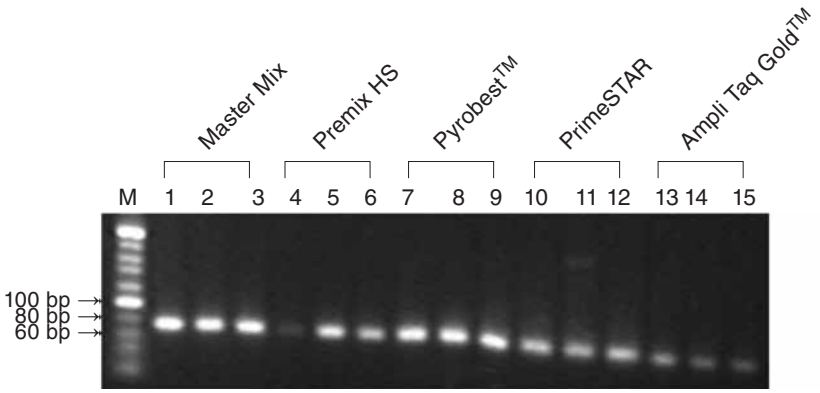

Figure 4. Tortoise Pol III/SINE based PCR detection of DNA extracts from *colla carapax et plastrum testudinis* using 5 different PCR polymerases. Lanes 1-3: 2x Taq PCR MasterMix; Lanes 4-6: Premix Ex-taq™ Hot Start Version; lanes 7-9: Pyrobest™ DNA polymerase: lanes 10-12, PrimeSTAR HS DNA Polymerase; lanes 13-15: Ampli Taq Gold™; M represents 20 bp DNA ladder.

plastrum testudinis (Figure 5). In contrast, no amplicon could be obtained from blank and the *colla corii asini* DNA extracts, indicating that our developed tortoise species specific SINE based PCR identification method can detect the tortoise DNA from gelatin mixtures containing only 0.1% tortoise shell glue, which is sensitive enough to meet the practical use.

V. *DNA Extraction from 6 Commercially Purchased Colla Carapax et Plastrum Testudinis*

The method for tortoise DNA detection from highly processed tortoise shell material was established to detect the tortoise DNA from gelatin containing only 0.1% tortoise shell glue and then employed to identify *colla carapax et plastrum testudinis* products purchased from drug stores. Six samples were collected for PCR based detection. Tortoise DNA was detected in 5 of the 6 samples although the strength of PCR products varies which may reflect to various degree of degradation of DNA extracts. The failure in obtaining the expected amplicon in 1 of the 6 samples indicated no tortoise shell was used in its production process or its tortoise origin was less than 0.1% (Figure 6, lane 5). This result implied that this sample was labeled fraudulently.

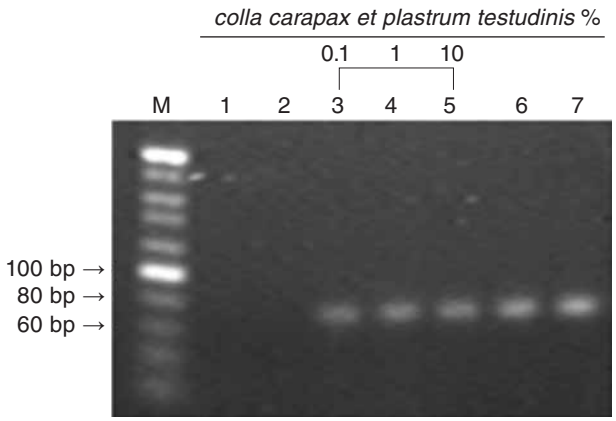

Figure 5. Tortoise Pol III/SINE based PCR detection of DNA extracts from binary mixtures of *colla carapax et plastrum testudinis* and *colla corii asini*. Lane 1: blank; lane 2: *colla corii asini*; lane 3: 0.1% *colla carapax et plastrum testudinis*; lane 4: 1% *colla carapax et plastrum testudinis*; lane 5: 10% *colla carapax et plastrum testudinis*; lane 6: *colla carapax et plastrum testudinis*; lane 7: positive control with tortoise genome DNA; M: 20 bp DNA ladder.

DISCUSSION

DNA based identification of TCM depends heavily on both DNA extraction and PCR amplification procedures[19]. Just like other solid glues[16], tortoise shell glue is a kind of highly processed product. PCR detection of the severely degraded DNA fragments is very difficult and posts a big obstacle for its identification. Since the issue concerning the extraction of DNA fragments from shell glue samples has been resolved successfully, as described in our previous work[16], we thus focused on the detection of DNA of tortoise origin using specific

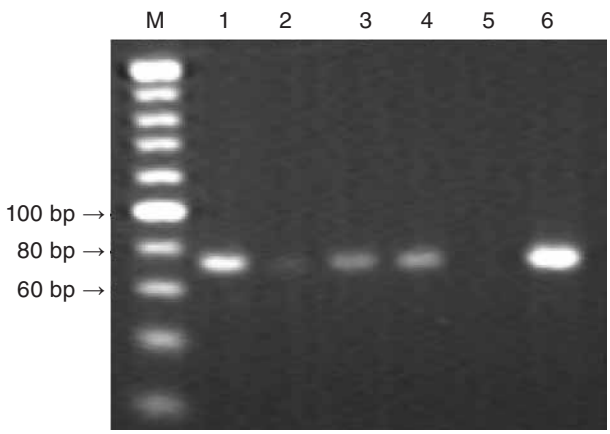

Figure 6. Tortoise Pol III/SINE based PCR detection of DNA extracts from 6 commercially purchased *colla carapax et plastrum testudinis* samples. Lane 1-6 represents 6 different *colla carapax et plastrum testudinis*; M: 20 bp DNA ladder.

DNA marker. The reported 12S rRNA gene method is not applicable to the identification of turtle jelly[12]. So that we developed a molecular identification method using Pol III/SINE and applied it to tortoise glue *colla carapax et plastrum testudinis* successfully. The method was reliable, effective and convenient. So far as we know, the SINE element has never been used for TCM identification[7][19].SINE is a class of mobile repetitive nucleotides widely spread in the eukaryotic genomes with size ranging from 70 to 500 bp. The absence or existence of SINE insertion at certain loci of the genome can be used for tracking linkages[20]. In addition, much more copies of SINE exist in the genome than mitochondrial DNA, chloroplast DNA or rRNA genes[14], which is beneficial for PCR detection of highly degraded DNA in a sample. Thus, SINE has many advantages as a molecular marker for species specific DNA detection. First, the method would be more sensitive with a lower detection limit[21], which means it is the most likely choice to detect trace amount of highly degraded DNA from forensic samples[22] or DNA extracts from highly processed material. Second, the method could be very convenient to conduct by single step PCR without subsequent restriction fragment length polymorphism (RFLP) analysis. Finally, the length of the amplicon can be designed easily from less than 100 to several hundreds bp based on different processed target templates. In this study, for the detection of trace amount of short DNA fragments, the PCR target was limited to less than 80 bp. Although SINE has never been used for TCM identification before, its potential in this field could be predicted especially for highly processed TCM such as tortoise shell glue.

Tortoise Pol III/SINE element was found uniquely in hidden-necked tortoise genomes[23]; it contains 2 subgroups, including CryI and CryII. In different turtle species, CryI has 4 different forms, namely as CryIA, CryIB, CryIC and CryID, while CryII has 5, including CryIIA, CryIIB, CryIIC, CryIID and CryIIE[18]. All these nine Pol III/SINE sequences vary in their 3' regions but their 5' regions are homologous[18], so our primers were designed within their homologous 5' regions (position 1 to 95) to assure that the PCR amplicon exists in DNA extracts out of all the tortoise species.

Although the assay can not discriminate among different tortoise species, it is useful to distinguish tortoise DNA from other animals such as donkey, horse, pig or cattle. So, it could be used to identify tortoise shell glue from substitutions derived with tissue of donkey, horse, pig or cattle more rapidly. If no Pol III/SINE could be amplified from the tortoise shell glue *colla carapax et plastrum testudinis*, this product was labeled fraudulently. Furthermore, the method can detect tortoise DNA from glue mixtures containing only 0.1% tortoise shell glue, indicating that it also owns good sensitivity and can accurately identify whether the glue products labeled as *colla carapax et plastrum testudinis* contain any tortoise tissues at all.

Finally, the assay was applied successfully to 6 commercially *colla carapax et plastrum testudinis* products with one sample tested negative. It is most likely prepared with none or less than 0.1% tortoise shells, thus being regarded as a substitution. However, it is possible that this sample was made from tortoise but the extracted DNA was severely degraded and became undetectable through PCR. As a result, in order to evaluate the quality of the DNA extracts, for this sample, PCR detection of other animal tissues like pig, cattle and horse should be conducted to ascertain its original constitutes.

When applying the method in practical use, more concerns should be addressed on the removal of contamination during PCR processes, because little contamination in PCR reaction may lead to false positive results thus lower the efficiency. Therefore, strict manipulations and clean environment are essential to make this assay reliable. Based on the fact that more than one kind of solid glue are usually processed in a factory and all these glues could be cross-contaminated with each other during processes, so the surface irradiation of the product is essential because the contaminated DNA on the surface can be eliminated.

On the other hand, in this study, PCR of 30 cycles could amplify no band (data not shown), however, the number of cycles is still limited to no more than 40, because more cycles might increase the risk of false positive results derived from inadequate handling[24]. In fact, the number of PCR cycles could also be monitored and optimized according to the samples from different manufacturing processes.

In conclusion, SINE-based molecular identification method for tortoise DNA detection from highly processed tortoise shell glue was established successfully which shows a new way for TCM identification at DNA level, especially for those highly processed materials. As we know, this is the first report on tortoise shell glue identification by SINE-based molecular method. The method might also evolve into one kind of basic technology for standardization, modernization and sustainable development of TCM.

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