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Identification of Turtle Shell and Its Preparations by PCR-DNA Sequencing Method

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ABSTRACT

A polymerase chain reaction (PCR) with DNA sequencing method has developed to identify the species of turtle shell specimens, skinned and bleached turtle shells, and its preparations. Two pairs of universal primer of mitochondrial gene, 12S rRNA (L:1373, H:1478) and cytochrome b (L:14181, H:15149), were applied to perform PCR and test for the optimal PCR conditions. The PCR products of about 165-bp and 376-bp in size were obtained from all 12 authentic turtle shells by primers of the 12S rRNA gene and the cytochrome b gene, respectively. Following DNA sequencing analysis of both bands without primers and GeneBank database search, DNA sequences of about 108-bp of 12S rRNA fragments were well differentiated among the 12 authentic specimens. However, DNA sequences of 307-bp of cytochrome b fragments were not completely distinguished for those authentic specimens. The PCR method with the primers of mitochondrial 12S-rRNA can also be successfully applied to the skinned and bleached turtle shell, and its powder preparation to identify their corresponding species. Thirty samples of skinned and bleached turtle shells were identified and categorized as three different species of *Siebenrockiella crassicollis*, *Indotestudo elongata*, and *Cuora amboinensis*, respectively. Furthermore, one extract and three powders of turtle shell preparations were identified as *Siebenrockiella crassicollis*, and one of turtle extract preparations was shown the cross-amplification with the other animal. Turtle jelly preparation, however, did unidentifiable. The results also indicated that the endangered species of turtle listed in CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) Appendix I and the prohibition of wild fauna announced by the Committee on Chinese Medicine and Pharmacy, Department of Health, Taiwan were not used as raw materials in the Chinese pharmaceutical manufacturers.

Key words: turtle shell, PCR, DNA sequence, 12S rRNA

INTRODUCTION

Endangered animals, such as rhinoceros, tiger, moschus, antelope, bear, pangolin, and turtle etc., have been used as traditional Chinese medicines (TCM) in oriental countries. The plastron is the main useable part of turtle shell, which consists of protein (about 32%), collagen (fibrous protein about 7%), and calcium carbonate (about 50%)[(1)]. The function of turtle shell is the antipyretic and analgesic effect documented in Chinese Materia Medica[(2)], including remedy for calcium insufficiency and source of protein uptake. According to 1992-1998 Directorate General of Customs import reports[(3)], the average amount of turtle shell imported from mainland China and Southeast Asian countries to Taiwan exceeds 120 metric tons per year for TCM used. More than 20 species of turtle were imported. Although not on the list of endangered species of CITES, *Cuora amboinensis*, *Malayemys subtrijuga* and *Siebenrockiella crassicollis*, constituted 75% of imported shells. However, endangered species may be found in the remaining 25% of imported turtle shells.

Using endangered animal as the raw material for TCM is criticized, condemned, and even prosecuted by CITIES. This is one of most important animal protection issues for regulatory authorities in oriental countries. The species of animal and plant covered by CITES are listed in three appendices, according to the degree of protection needed. Appendix I includes species threatened with extinction. Since these species are the most endangered, CITES generally prohibits international trade of these species. Appendix II includes species not necessarily now threatened with extinction. Impart of these species may be authorized with an export permit or re-export certificate; import permit is not necessary. Appendix III contains species that are protected in at least one country. Almost all species of turtle are listed in all three appendices of CITES. Furthermore, 26 species of Asian turtles were newly adopted into Appendix II on the 2002 conference of CITIES[(4)]. The Committee on Chinese Medicine and Pharmacy (CCMP), Department of Health (DOH) had also announced 14 species of conservative animal including turtle of *Chinemys reevesii* banned for prescription as raw material in TCM in June, 2000.

In general, turtle shell is identified by its shape, color, stripe and size[(5)], but turtle shell as the raw material for TCM used is skinned, bleached and cracked. Since loss of appearance and lack of marked constituents, origin of turtle shell is extremely difficult to identify by chemical analysis and morphological examination even with a product certificate provided by the wholesaler. Developing a reliable analytical method to identify the turtle shell and its ingredient in the preparations is important and valuable for

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the post-marketing surveillance and regulatory management. Differential thermal analysis[6] and the circular dichroism analysis[7] had been used for identification of commercial turtle shell, donkey-hide glue, antler glue, tortoise shell glue and their forgeries, respectively, but these methods were not specific and suitable for the analysis of turtle species. Wu *et al.*, had used the mitochondrial 12S rRNA gene to identify the tissues (blood, muscle) and shell of turtle in the market by PCR-DNA sequencing method for the Chinese three-keeled pond turtle *Chinemys reevesii* documented in the China Pharmacopoeia[8]. The purpose of this study was to develop a molecular biotechnology method by modifying Wu's method to identify whether the turtle shells were from the endangered species listed in CITIES or the conserved animal announced by CCMP.

MATERIALS AND METHODS

I. *Chemicals and Reagents*

Sodium dodecyl sulfate (SDS), Tris base, disodium ethylenediaminetetraactate•2H_2O (EDTA), chloroform/isoamylalcohol (24:1, v/v) and phenol/chloroform/isoamylalcohol (25:24:1, v/v/v) were purchased from Merck Inc. (Darmstadt, Germany), and TAE (Tris-Acetic acid-EDTA) buffer (50×) was bought from BDH (England). QIAquick PCR purification kit was obtained from Qiagen (Germany). Tag polymerase and dNTP were purchased from New England BioLabs (USA).

II. *Specimens and Samples*

Twelve authentic specimens of turtle shell without skinning preservation in our laboratory were chosen for references. Based on the textbook of CITES Identification Guide-Turtles and Tortoises[9], the specimens were identified by their appearance and morphology as *Cuora amboinensis* (Ca), *Chinemys reeresii* (Cr), *Heosemys grandis* (Hg), *Indotestudo elongata* (Ie), *Kachuga tecta* (Kt), *Manouria impressa* (Mi), *Morenia ocellata* (Mo), *Malayemys subtrijuga* (Ms), *Melanochelys trijuga* (Mt), *Platysternon megacephalum* (Pm), *Siebenrockiella crassicollis* (Sc) and *Sacalia quadriocellata* (Sq), respectively. The turtles of Kt and Mo are listed in CITES Appendix I, the turtles of Ca, Ie, Mi, Pm, Hg, and Sc are listed in CITES Appendix II, and the turtle of Cr is listed in the Taiwan Wild Animal Protection Act. The remaining three turtles are non-conservative. Thirty skinned and bleached turtle shell samples and three pieces of rusa (water deer, *Cervus unicolor* Korr, Cu) horn references were provided by Chinese pharmaceutical manufacturers. Powder, extract and jelly of turtle shell preparations were purchased from Chinese drug stores.

III. *Primers*

The published sequences for mammal and reptile mitochondria RNA (mtRNA) were used for the primers[10]. The sequences of the two primers were as follows: letters L and H refer to the light and heavy strands; the number refers to the position of the 3' base of primer in the template human mtRNA sequence: 12S rRNA, L1373 (CGCTCGA GAGAAATGGGCTACATTTTCT) and H1478 (TGACTG CAGAGGGTGACGGGCGGTGTGT)[8,11]; Cytochrome b, L14841 (AAAAAGCTTCCATCCAACATCTCAGCATGA TGAAA), H15149 (AAACTGCAGCCCCTCAGAATGATA TTTGTCCTCA)[12-14], respectively. The four primers were provided by Perkin Elmer Company (USA).

IV. *DNA Extraction*[12]

About 100~120 mg of sample was placed in a 2.0-mL micro-centrifuge tube and mixed with 20 volume of 0.5 M EDTA (pH 8.0) solution. The mixture solution was incubated in a water bath at 56°C for 36~48 hr and then centrifuged at 3,000 rpm. The supernatant of EDTA solution was decanted and discarded. DNA was extracted from decalcium sample residue by digestion in 1 mL of buffer solution (consisting of 100 mM Tris-HCl, pH 8.0, 100 mM EDTA, 10% SDS, 1 μg proteinase K/mL) at 56°C for 40 min. The extracted DNA was further purified by extracting the above clear solution with 1 mL of phenol/chloroform/isoamylalcohol (25:24:1, v/v/v) mixture for three times, then extracting with 1 mL of chloroform and isoamyl alcohol (24:1, v/v) mixture. The aqueous solution was transferred into another 2.0-mL micro-centrifuge tube. Total DNA was precipitated by adding 3/5 volume of isopropanol and 1/30 volume of 3 M sodium acetate. The resulting solution was centrifuged at 13,000 rpm for 3 min. The precipitated DNA so obtained was washed twice with 70% ethanol, and then the residue dissolved in TE buffer or sterile distilled water. The amount of DNA is determined by the nucleotide analyzer (Pharmacia Biotech Gene Quanut II, Hong Kong). When the absorption at $OD_{260/280}$ is greater than 1.4, the quality of DNA is enough for PCR amplification.

V. *Polymerase Chain Reaction and rDNA Amplification*[13]

Purified DNA was dissolved in sterile distilled water and stored at -20°C before PCR amplification. DNA amplification was performed in 50 μL of solution containing about 1~30 ng DNA with 5 μL of 10× Taq buffer, 1.0 μL of 50 mM primers, 8 μL of 10 mM dNTP, and 2.5 Unit Taq polymerase. A negative-control tube (no template) was incubated with each run. The above mixtures were incubated in a PCR reactor (GeneAmp PCR system 9600, Perkin Elmer Co., USA) under following program: an initial DNA denaturation step at 94°C for 2 min followed by 30 cycles consisting a denaturation step at 94°C for 40 sec, an annealing primers step at 52°C for 1 min, and an extension step at 72°C for 1 min. The last extension step was kept at 72°C for another 5 min. The PCR products were purified with PCR purification kit according kit manual. About 10 μL of purified PCR products and 100-bp DNA ladder

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maker were added 2 μL of 6× gel loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol), and mixed well. The mixture solution was loaded onto a 2% agarose gel containing ethidium bromide (0.5 mg/mL, in 1 × TAE buffer). After electrophoresis under 100 volts for 15 min, the gel was photographed using UV illumination at 365 nm.

VI. *Sequencing and Phylogenetic Analysis*

DNA sequencing was analyzed by Mission Biotech (Taiwan) using the automatic sequence analyzer (Perkin Elmer Applied Biosystems, ABI PRISM 337). Sequence data were compared with the Genbank database and uploaded to "The Biology WorkBench" website of San Diego Supercomputer Center (http://workbench.sdsc.edu). The aligned DNA sequences were analyzed phylogenetically using CLUSTAL W. for multiple sequence alignment, whereas the rooted dendrogram was generated by the program of Phylip's Drawgram.

RESULTS AND DISCUSSION

Before extraction and purification of the mitochondria DNA, the calcium of turtle shell was removed with EDTA. However, the calcium content in turtle shell preparations was found less than in turtle shell, and DNA extraction can be directly carried out. There are three methods to extract DNA from turtle shell. The first method is using the commercial kit to extract DNA. The extracted DNA is of low quantity but high purity. The second method, a traditional method, uses the phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v) mixture to extract DNA. The third method directly extracts DNA from the sample by the traditional method. The obtained DNA from the third method is notably less than from the first extraction method, but the amount and purity of DNA are enough to perform PCR. The second method was used in this study for cost consideration. The quantity of DNA obtained is more than the first method and the purity is acceptable.

In general, the 12S rRNA and cytochrome b genes of mitochondria are usually used as template for identification of relative species in mammalian and reptiles[10]. The strategy of this study is to use the authentic specimens without skinned to assess which gene is suitable for identification of their species by PCR-DNA sequencing method.

Two pairs of universal primer L1373 and H1478 for 12S rRNA and primer L14181 and H15149 for cytochrome b were applied for PCR amplification. The PCR products of 12S rRNA and cytochrome b genes of 12 turtle references were about 165-bp and 376-bp, respectively (Figures 1 and 2). Therefore, the PCR products of these two genes could not be directly distinguished among the 12 turtle references. Sequencing of the PCR products without primers obtained about 108-bp for 12S rRNA and about 307-bp for cychrome b. Sequence data of the turtles were compared with Genebank for database search and assigned accession numbers. The accession numbers and locations of 12 turtle references at 12S rRNA gene in Genebank database were AF043394 and 285-392 bp (Ca); AF043387 and 282-390 bp (Cr); AF043400 and 282-388 bp (Hg); AF175338 and 289-394 bp (Ie); - and - (Kt); AF043411 and 283-390 bp (Mi); AF043409 and 288-395 bp (Mo); AF043398 and 284-931 bp (Ms); AF043405 and 285-392 bp (Mt); AF043412 and 281-387 bp (Pm); AF043406 and 285-392 bp (Sc); AF043392 and 285-392 bp (Sq); respectively. Comparison sequences between turtle references and results of Genebank database search, the similarities were more than 98%. Besides turtle of Kt, sequences of turtle references matched sequences of searched turtle in Genebank.

The PCR products of cytochrome b gene of 12 turtle references were directly sequenced for evaluation due to lack of sequence data of cytochrome b gene in Genebank. After sequences of Pair-Wise alignment analysis of PCR products of 12S rRNA and cytochrome b genes, the rooted dendrogram were generated as shown in Figure 3. Sequences of 12S rRNA fragment could be properly distinguished among the 12 turtle references, with sequence difference of each pair of about 7~23%.

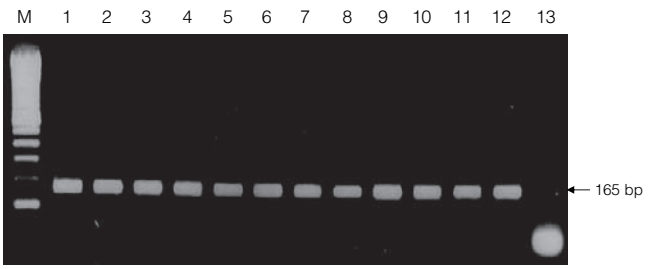

Figure 1. Agarose gel electrophoresis of PCR amplifications of gene fragment of 12S rRNA with primer L1373/H1478 of authentic turtle specimens. Lane 1-12: PCR products of 12 turtle shells, numbers: 1: Ie, 2: Sc, 3: Kt, 4: Mo, 5: Ca, 6: Ms, 7: Sq, 8: Mi, 9: Mt, 10: Cr, 11: Pm and 12: Hg, respectively; lane 13: extract control; lane M: DNA 100-bp ladder size marker.

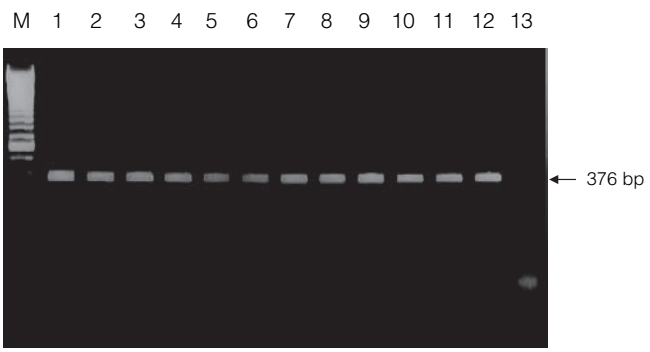

Figure 2. Agarose gel electrophoresis of PCR amplifications of gene fragment of Cytochrome b with primer L14841/H15149 of authentic turtle specimens. Lane 1-12: PCR products of 12 turtle shells, numbers: 1: Ie, 2: Sc, 3: Kt, 4: Mo, 5: Ca, 6: Ms, 7: Sq, 8: Mi, 9: Mt, 10: Cr, 11: Pm and 12: Hg, respectively; lane 13: extract control; lane M: DNA 100-bp ladder size marker.

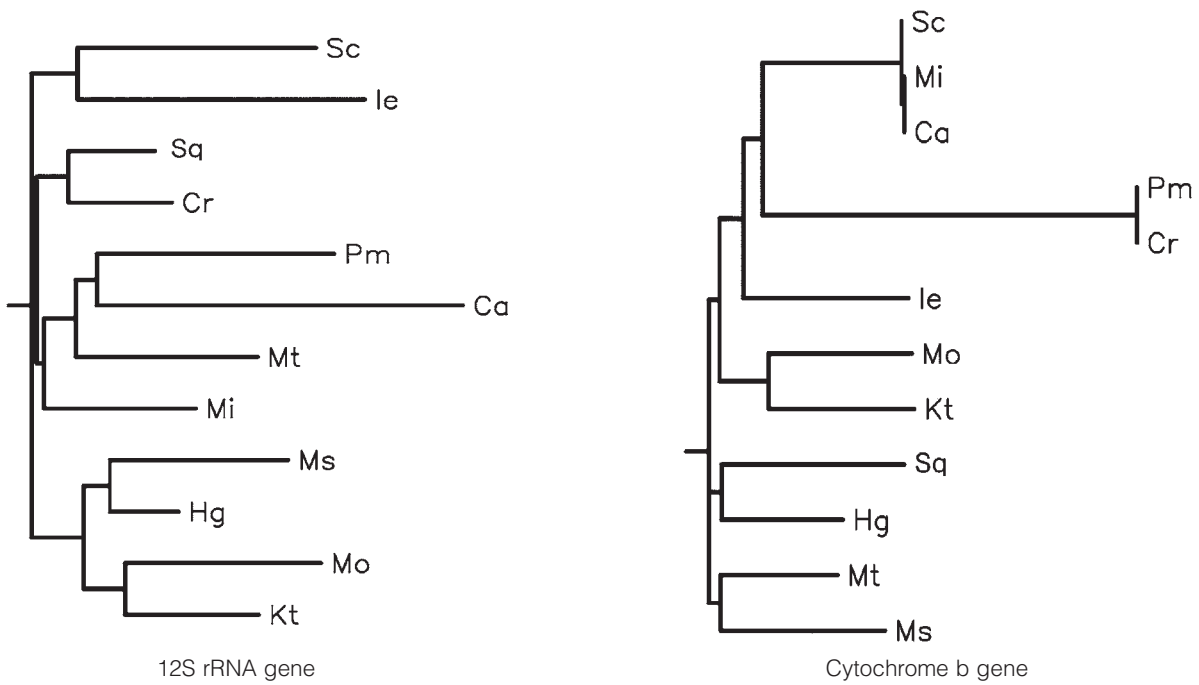

Figure 3. Phylogeny rooted dendrogram for 12 species of authentic turtle specimens constructed from DNA sequences of about 108-bp of 12S rRNA gene fragment and about 307-bp of cytochrome b gene fragment.

However, sequences of cytochrome b gene could not be properly identified since sequences of turtle of Cr and Pm were identical, and the turtle of Ca, Mi and Sc also had the same sequences in this fragment. Eventually, the identified results of 12 turtle references indicated that 12S rRNA gene and the applied primers could dependably identify the turtle references by PCR-DNA sequencing method, and could also support the appearance examination of turtle references.

The universal primers of 12S rRNA and PCR-DNA sequencing method were then applied to 30 skinned and bleached samples obtained from domestic Chinese pharmaceutical manufacturers to identify their species. Sequence data and results of Genebank searches are shown in Figure 4. Species of turtle samples were identified and categorized as three different species. They were 18 samples of Sp-1 for Sc, 9 samples of Sp-2 for Ie, and three samples of Sp-3 for Ca, respectively. These three species of turtle are all listed in CITES Appendix II. The investigation demonstrated that the main raw material of turtle used in Chinese pharmaceutical manufacturers was the Sc. No endangered species (CITES Appendix I) of turtles provided as the raw material for registration was found in this study.

Furthermore, this study focused on how to identify the finished products claimed to contain turtle shell ingredient. Five powder samples were chosen for this study. One sample was the turtle shell powder directly ground from the shell, two samples were turtle shell extracts and the other two samples were the Kuei-Lu-Erh-Hsien-Chiao (KLEHC) preparation consisting of turtle shell, deer horn, ginseng and Lycii at a ratio of about 5:10:1:1. After PCR amplification, three powder samples and two KLEHC preparations respectively obtained a 165-bp band of PCR product. Sequencing of the PCR products, three powder samples having the same sequences as Sp-1 were identified as Sc, but the two KLEHC samples were identified as one Sc and one rusa, respectively.

The finished product of KLEHC is approved by the CCMP, DOH. It must contain turtle shell ingredient in the formula or it is illegal and violates the GMP criteria. The content of turtle shell in the KLEHC is about 30% and the turtle shell to deer horn ratio is 1:2. The amounts of two other botanical materials are minor and their DNAs could not be proliferated by the primers used in this method. Basically, the DNA fragments of 12S rRNA gene of turtle and deer shall be simultaneously amplified by PCR in the experiment. In order to ensure the sample result of deer, the sample along with three rusa references were re-tested by this method. The PCR products of rusa also obtained a band of 165-bp in the same size as of the turtle. The sequences of the PCR products of three rusa references and one finished product of KLEHC are shown in Figure 4. The four 108-bp DNA sequences divided into two groups were almost identical but had only one nucleotide different at the 17th position. Sequence data between rusa and authentic turtles were significantly different, and the similarity of sequence data was about 54~65%. The re-test results demonstrated that PCR product of the KLEHC was the rusa not the turtle. After reviewing the primers and the 12S rRNA gene of deer, we found the sequences of primer L1373 at 5' position from 9th to 28th were the same as the sequence number from 704th to 723th in Genebank search number. When the constituent of turtle to deer ratio in the product is less

```
       1                                                                                     50
Sc     A C A C T A G A A A   T A A A C T C A C G   A A A A G G A A C T   A T G A A A C T A G   T C C T A G A A G T
Sp-1   ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦   ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦   ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦   ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦   ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦
Ie     ◦ A ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦   ◦ – ◦ T T ◦ T ◦ ◦ ◦   G ◦ ◦ ◦ – ◦ ◦ ◦ ◦ ◦   ◦ ◦ ◦ ◦ ◦ ◦ ◦ A ◦ ◦   ◦ ◦ T ◦ ◦ T ◦ ◦ ◦ ◦
Sp-2   ◦ A ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦   ◦ – ◦ T T ◦ T ◦ ◦ ◦   G ◦ ◦ ◦ – ◦ ◦ ◦ ◦ ◦   ◦ ◦ ◦ ◦ ◦ ◦ ◦ A ◦ ◦   ◦ ◦ T ◦ ◦ T ◦ ◦ ◦ ◦
Ca     ◦ T ◦ T ◦ ◦ ◦ ◦ ◦ ◦   ◦ T T ◦ T ◦ ◦ ◦ ◦ ◦   G ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦   ◦ ◦ ◦ ◦ ◦ ◦ T A ◦ ◦   ◦ ◦ ◦ C ◦ C ◦ ◦ ◦ ◦
Sp-3   ◦ T ◦ T ◦ ◦ ◦ ◦ ◦ ◦   ◦ T T ◦ T ◦ ◦ ◦ ◦ ◦   G ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦   ◦ ◦ ◦ ◦ ◦ ◦ T A ◦ ◦   ◦ ◦ ◦ C ◦ C ◦ ◦ ◦ ◦
Cu     ◦ A T ◦ ◦ ◦ A G ◦ ◦   ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦   G ◦ ◦ ◦ ◦ T T ◦ T ◦   ◦ ◦ ◦ ◦ ◦ ◦ T ◦ ◦ A   ◦ A A C C A ◦ ◦ ◦ G
Cu-1   ◦ A T ◦ ◦ ◦ A G ◦ ◦   ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦   G ◦ ◦ ◦ ◦ T T ◦ T ◦   ◦ ◦ ◦ ◦ ◦ ◦ T ◦ ◦ A   ◦ A A C C A ◦ ◦ ◦ G
KLEHC  ◦ A T ◦ ◦ ◦ A G ◦ ◦   ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦   G ◦ ◦ ◦ ◦ T T ◦ T ◦   ◦ ◦ ◦ ◦ ◦ ◦ T ◦ ◦ A   ◦ A A C C A ◦ ◦ ◦ G
Cu-2   ◦ A T ◦ ◦ ◦ A G ◦ ◦   ◦ ◦ ◦ ◦ ◦ ◦ A ◦ ◦ ◦   G ◦ ◦ ◦ ◦ T T ◦ T ◦   ◦ ◦ ◦ ◦ ◦ ◦ T ◦ ◦ A   ◦ A A C C A ◦ ◦ ◦ G

       51                                                                                    100
Sc     A G G A T T T A G C   A G T A A G T C G G   G A T C A – G A G T   G C C C A A C T T A   A G T T G G T C C T
Sp-1   ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦   ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦   ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦   ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦   ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦
Ie     ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦   ◦ ◦ ◦ ◦ ◦ A ◦ A A ◦   ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ A ◦   ◦ ◦ ◦ ◦ T ◦ T ◦ ◦ ◦   ◦ ◦ C C ◦ ◦ C ◦ ◦ ◦
Sp-2   ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦   ◦ ◦ ◦ ◦ ◦ A ◦ A A ◦   ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ A ◦   ◦ ◦ ◦ ◦ T ◦ T ◦ ◦ ◦   ◦ ◦ C C ◦ ◦ C ◦ ◦ ◦
Ca     ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦   ◦ ◦ ◦ ◦ ◦ ◦ C ◦ ◦ ◦   ◦ ◦ ◦ ◦ ◦ ◦ A ◦ A C   ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦   ◦ ◦ ◦ C ◦ ◦ ◦ ◦ ◦ ◦
Sp-3   ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦   ◦ ◦ ◦ ◦ ◦ ◦ C ◦ ◦ ◦   ◦ ◦ ◦ ◦ ◦ ◦ A ◦ A C   ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦   ◦ ◦ ◦ C ◦ ◦ ◦ ◦ ◦ ◦
Cu     ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦   ◦ ◦ ◦ ◦ ◦ A C T A A   ◦ ◦ A T ◦ G A G T G   C T T A G T T G A ◦   T T A G ◦ C C A T G
Cu-1   ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦   ◦ ◦ ◦ ◦ ◦ A C T A A   ◦ ◦ A T ◦ G A G T G   C T T A G T T G A ◦   T T A G ◦ C C A T G
KLEHC  ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦   ◦ ◦ ◦ ◦ ◦ A C T A A   ◦ ◦ A T ◦ G A G T G   C T T A G T T G A ◦   T T A G ◦ C C A T G
Cu-2   ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦   ◦ ◦ ◦ ◦ ◦ A C T A A   ◦ ◦ A T ◦ G A G T G   C T T A G T T G A ◦   T T A G ◦ C C A T G

       101             109
Sc     G A G G C G C G C
Sp-1   ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦ ◦
Ie     A ◦ ◦ ◦ ◦ A ◦ ◦ ◦
Sp-2   A ◦ ◦ ◦ ◦ A ◦ ◦ ◦
Ca     ◦ ◦ ◦ ◦ T ◦ ◦ ◦ ◦
Sp-3   ◦ ◦ ◦ ◦ T ◦ ◦ ◦ ◦
Cu     ◦ ◦ ◦ C A C G C
Cu-1   ◦ ◦ ◦ C A C G C
KLEHC  ◦ ◦ ◦ C A C G C
Cu-2   ◦ ◦ ◦ C A C G C
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Figure 4. DNA sequences of about 108-bp of 12S rRNA gene fragment of 30 skinned and bleached turtle shell samples, deer horn references and turtle shell product. Sc represents turtle of *Siebenrockiella crassicollis* in Genebank; Sp-1 represents 18 samples of turtle shell. Ie represents turtle of *Indotestudo elongata* in Genebank; Sp-2 represents 9 samples of turtle shell, Ca represents turtle of *Cuora amboinensis* in Genebank; and Sp-3 represents 3 samples of turtle shell. Cu represents deer of *Cervus unicolor* in Genebank; Cu-1 represents one of deer horn reference. Cu-2 represents two of deer horn references. KLEHC is the turtle preparation. Dots represent sequence identity with the Sc, and dashes indicate deletions.

than 1/2 (original ratio) at a certain critical value, more DNA was extracted from deer, and oppositely, less DNA were extracted from turtle. The DNA templates obtained from deer and turtle may perform amplification competition in the PCR process. The cross-amplification phenomena had occurred as the universal primers of 12S rRNA were applied. This might be the reason why the claimed turtle product was not found in the turtle but in the deer.

Finally, using this method to identify the turtle jelly preparation (Kuei-Ban-Chiao, KBC), a glue-like residue produced by prolonged boiling of turtle shells and concentrated with evaporation was attempted in this study. Many bases without discriminated as denote of N were appeared in the sequences of PCR product. If the jelly preparation is processed at the high temperature for a long time, the DNAs may be destroyed to form many small fragments. The PCR amplification could not obtain appropriate DNA fragments as the template by the universal primers. Thus, this method could not determine the presence of turtle shell constituent in finished products of both KLEHC and KBC. As for the long-term boiled sample or sample containing other animal DNA, the PCR-DNA sequencing method needs further modification.

CONCLUSIONS

The PCR-DNA sequencing method can be successfully applied for identification of references of turtle specimen, raw material of skinned and bleached turtle shell and directly

powdered turtle shell preparation. The above method was not suitable for identification of turtle shell products of the KLEHC preparation and jelly preparation (KBC) in this study. The universal primers of 12S-rRNA need further modification to solve this problem in the future.

Before this study, no precise analytical methods and reports for identification of endangered species of turtle shell used in Taiwan were available. The results revealed that the endangered species of turtle listed in CITES and the conservation turtles banned by the CCMP, DOH were not used as the raw material by Taiwan's Chinese pharmaceutical manufacturers in this investigation.

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