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ORIGINAL ARTICLE

# Harnessing Multiplex Polymerase Chain Reaction Assay for Convenient and Simultaneous Differentiation of Testudinis Carapax et Plastrum from Trionycis Carapax

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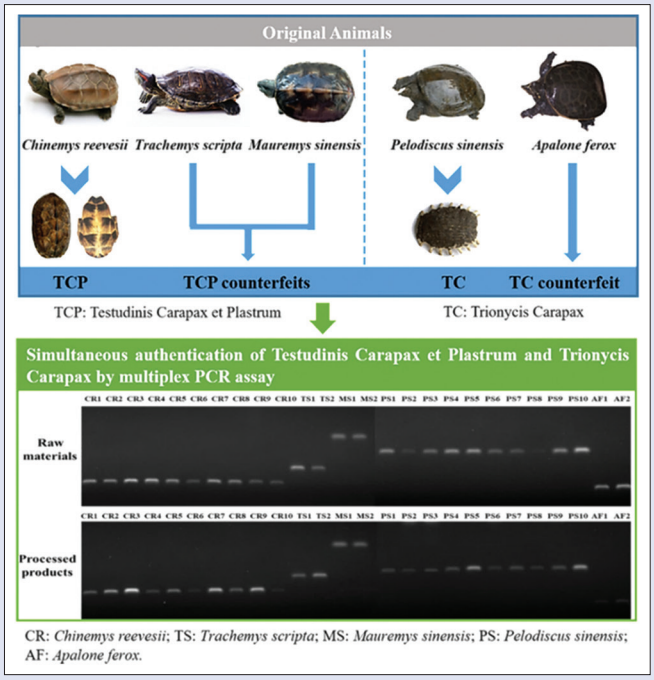
## ABSTRACT

**Background:** Testudinis Carapax et Plastrum (TCP, Guijia) and Trionycis Carapax (TC, Biejia), which made from the shell of *Chinemys reevesii* (CR) and *Pelodiscus sinensis* (PS), were two kinds of widely used animal-derived Traditional Chinese medicine (TCM). Some cheap substitutes such as the carapace and plastron of *Trachemys scripta* (TS), *Mauremys sinensis* (MS), or *Apalone ferox* (AF) shells obtained from restaurants are sometimes used, which expose the public health to a high risk and cause unfair competitions in the market. **Objective:** The objective of the study was to develop a multiplex polymerase chain reaction (PCR) approach to simultaneously differentiate five Chelonia species and identify adulteration of two natural products. **Materials and Methods:** Five novel species-specific primers were designed for CR, TS, MS, PS, and AF, followed by optimization of PCR conditions and validation for specificity and sensitivity. Then, a deliberate mixture was analyzed to verify the capability of adulteration detection. Finally, it was used to examine commercial products. **Results:** The developed method proved to be highly specific and detection limit was 1 ng for all the species tested. Particularly, it was still applicable and reliable when processed products or deliberate adulteration were examined. Two batches of commercial raw TCP products were identified to be counterfeited by TS using the newly proposed approach. **Conclusion:** The newly proposed multiplex PCR method showed sufficient merits to be readily employed as a regular means to authenticate edible and medicinal TCM products made of TCP and TC.

**Key words:** Authentication, differentiation, multiplex polymerase chain reaction, Testudinis Carapax et Plastrum, Trionycis Carapax

## SUMMARY

- A convenient and reliable multiplex polymerase chain reaction approach was developed for the first time to simultaneously differentiate five Chelonia species and identify adulteration of Testudinis Carapax et Plastrum and Trionycis Carapax. Five novel species-specific primers were designed for *Chinemys reevesii*, *Trachemys scripta*, *Mauremys sinensis*, *Pelodiscus sinensis*, and *Apalone ferox*, followed by validating their specificity and sensitivity. The developed method proved to be of high specificity and detection limit was 1 ng for all the species tested. Particularly, it was still applicable and reliable when processed products or deliberate adulteration were examined. It showed sufficient merits to be readily employed as a regular means to examine edible and medicinal Traditional Chinese medicine products made of Testudinis Carapax et Plastrum and Trionycis Carapax and were also promising in such applications for other animal-derived medicines.




**Abbreviations used:** DNA: Deoxyribonucleic acid; PCR: Polymerase chain reaction; bp: Base pair.

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## INTRODUCTION

Traditional Chinese medicine (TCM), which mainly harnesses natural medicine and its processed products in clinics, has been acting as a predominant medical practice for 1000 years in China. In the past decades, it has received more and more popularity in Asia and Western countries for its good therapeutic effect on cancer, arrhythmia, and other chronic diseases.[1-3] Moreover, it is now strongly recommended by the World Health Organization as an important step toward its long-term goal of universal health care.[4]

Testudinis Carapax et Plastrum (TCP, Guijia), the dry carapace and plastron of *Chinemys reevesii* (CR), and Trionycis Carapax (TC, Biejia), the dry shell of *Pelodiscus sinensis* (PS), are the two kinds of widely used animal-derived TCM and both of them have been documented in the earliest Materia Medica book, "Shen Nong's Classic of Materia Medica."[5,6] As reported, TCP can cure diabetic nephropathy, ischemic cerebrovascular diseases, and hypoimmunity.[7,8] In addition, TC is beneficial to cure liver fibrosis, Yin deficiency, as well as to inhibit tumor growth.[9-11] They play an important role in Chinese Patent Medicines, and they are often consumed as functional foods or health-care products for large population.

Accurate identification of original species is one of the most challenging tasks in quality assurance and certification of animal products from health and economical aspects, which is of great importance to animal-derived TCM as well.[12,13] Some cheap substitutes such as the carapace and plastron of *Trachemys scripta* (TS), *Mauremys sinensis* (MS), or *Apalone ferox* (AF) shells obtained from restaurants are sometimes used, which expose the public health to a high risk and cause unfair competitions in the market.[14] Accordingly, the development of applicable and convenient approaches is crucial for the identification of Chelonia animal carapaces. Till present, some deoxyribonucleic acid (DNA)-based methods, including real-time polymerase chain reaction (real-time PCR), nest PCR, random amplified polymorphic DNA, and restriction fragment length polymorphism, have been applied in species authentication for their high sensitivity.[15-22] Compared with them, multiplex PCR incorporating species-specific amplification was the most popular tool, which can provide a simple, specific analysis of less than two species at the same time without expensive equipment and reagents.[23-25] For example, Dai *et al.* have employed this method to identify pork, beef, chicken, and mutton in commercial deep processed meats, leatherware, and feather commodities.[26] Ali *et al.* detected five meat species (pig, dog, cat, rat, and monkey) forbidden in Islamic foods by multiplex PCR.[27] In addition, Liu *et al.* employed it to identify three Chinese medicines, namely *Panax ginseng*, *Panax quinquefolium*, and *Panax notoginseng*.[28]

In this study, a convenient and cost-effective multiplex PCR assay was developed to differentiate TCP from TC simultaneously and the specificity and sensitivity were determined. At last, commercial raw materials and processed products available have been analyzed for robustness using the newly established method.

## MATERIALS AND METHODS

### Samples

Five Chelonia species, including three kinds of Testudinidae species (CR, TS, and MS) and two kinds of Trionychidae species (PS and AF), were subjected to assay in this study. Totally, 26 batches of animal origins, including CR (CR1 to CR10), PS (PS1 to PS10), TS (TS1 to TS2), (MS1 to MS2), and AF (AF1 to AF2), were collected from different areas in the year of 2016. All of them were subjected to COI barcoding for species verification. The raw materials and processed products were then prepared using the shells of tortoises and turtles according to procedures executed in China Pharmacopoeia, as illustrated in Figure 1.[29]

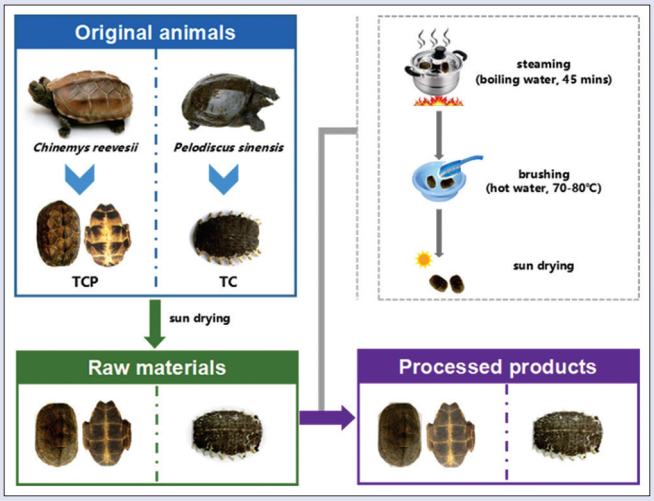


**Figure 1:** Procedures for self-made raw materials and processed products

Forty-one batches of commercial products, including one batch of TCP Reference (TCPR), raw TCP (G1 to G10), raw TC (B1 to B10), processed TCP (G11 to G20), and processed TC (B11 to B20) were collected to identify their original species by the newly developed method.

All solid samples after complete lyophilization were smashed into powder for further subsequent DNA extraction.

### Deoxyribonucleic acid extraction

SDS (Sodium dodecyl sulfate) could lysate cells and denature proteins under high temperature (55-65°C), so that nucleic acids could be released. Thus SDS-based method as described by Yang *et al.*[30,31] was used to extract DNA from each sample. The quality of DNA was examined by a BioSpec-mini spectrophotometer (Shimadzu, Japan) on the basis of absorbance at A260/A280 and they were then kept at −20°C until further experiments.

### Primer design

Species-specific primers were designed to target the mitochondrial gene of the above five Chelonia species using Oligo software (v. 7.60) (Molecular Biology Insights, Inc., Cascade, CO, USA). For that purpose, the sequences were retrieved for each target species from the GenBank database and corresponded to the following accession numbers: NC_006082.1, NC_011573.1, NC_016685.1, NC_006132.1, and NC_014054.1 for CR, TS, MS, PS, and AF, respectively. These sequences were aligned by DNAMAN (version 8.0.8.789). The designed primers were all further assessed by Oligo and DNAMAN, and then, they were synthesized by Shanghai Sangon Biotech Co., Ltd (China) and kept at −20°C prior to subsequent PCR assays.

### Polymerase chain reaction and detection

Simplex PCR was carried out before the development of the multiplex PCR method. PCR was conducted in a total volume of 25 μL, containing 2.5 μL of 10 × PCR buffer, 0.2 mM of each dNTP, 2.3 mM $MgCl_2$, 1.0 unit of Taq polymerase, 0.1–0.4 μM of each primer, 1.0 μL DNA template (50 ng), and distilled water (filled to its final volume). Amplification was conducted on a Bio-Rad T100 Thermal Cycler using the cycling program as follows: 3 min at 95°C (initial denaturation), 30 cycles of 30 s at 95°C, 30 s at 63°C, 1 min at 72°C, and 7 min at 72°C (final extension). PCR products were separated using a JY-SPFT Cell (JUNYI, Beijing) in 3% agarose gel and Tris-Borate-EDTA, (TBE) buffer which were made from tris, boric acid, EDTA and distilled water (gel size, 130 mm × 130 mm × 5 mm).

After a 70-min run, ethidium bromide was used to stain DNA and the results were then visualized by ultraviolet illumination.

## Validation of multiplex polymerase chain reaction assay

Tests of specificity, sensitivity, and robustness were performed for the validation of the developed multiplex PCR method. In detail, the specificity was carried out by amplification of DNA extracted from various batches of CR, TS, MS, PS, and AF. Then, the sensitivity was performed on DNA templates which were premixed with each target species against five concentrations from 0.01 to 100 ng/μL. At last, 41 batches of commercial products were analyzed to assess the robustness.

## Data analysis

The data of this assay were analyzed using GraphPad and SPSS. Kappa value, specificity, and sensitivity were calculated. Laboratory sensitivity and specificity were calculated using the original animal DNA from twenty "true positive" and six "true negative" samples. The diagnostic sensitivity and specificity of this assay were determined by comparing the known 26 reference samples in raw and processed forms.

# RESULTS AND DISCUSSION

## Primer specificity

The primers of five target species were designed. Selection of primers was made according to several physical parameters, including primer length, melting temperature, secondary structures, self-complementarity, and cross-reactivity inspected by DNAMAN and Oligo. Furthermore, the size of PCR amplicons must be significantly different, so they could be adequately separated by agarose gel electrophoresis. Consequently, five primer sets were selected and their information is summarized in Table 1.

Simplex PCR was performed against their target species and counterfeits for high specificity and band intensity. All the DNA sequences obtained and their homology rate (percent identity) with the available corresponding gene sequences are presented in Table 2. Then, multiplex PCR was developed after several optimizations. As shown in Figure 2, with different combinations of the samples, there showed a band of targeted PCR products without the formation of any non-specific amplified fragments.

## Evaluation of specificity and sensitivity for the multiplex polymerase chain reaction assay

Evaluation of specificity is the initial step toward the establishment of a diagnostic PCR assay. It was reported that PCR efficiency can be affected by serious mismatches in the primer binding regions, and these mismatches sometimes lead to false-negative detection. In this multiplex

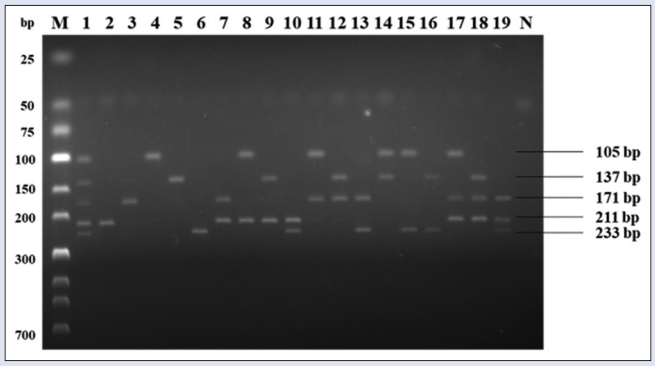


**Figure 2:** Three percent agarose gel showing the multiplex polymerase chain reaction amplified unique size DNA bands of CR (211 bp), TS (171 bp), MS (105 bp), PS (137 bp), and AF (233 bp). Lane M: DNA ladder, SN127; Lane 1: DNA mixture of CR, TS, MS, PS and AF; Lane 2-19: DNA from CR, TS, MS, PS, AF, CR + TS, CR + MS, CR + PS, CR + AF, TS + MS, TS + PS, TS + AF, MS + PS, MS + AF, PS + AF, CR + TS + MS, CR + TS + PS, CR + TS + AF, respectively; Lane N: Negative control. CR: *Chinemys reevesii*; TS: *Trachemys scripta*; MS: *Mauremys sinensis*; PS: *Pelodiscus sinensis*; AF: *Apalone ferox*; DNA: Deoxyribonucleic acid

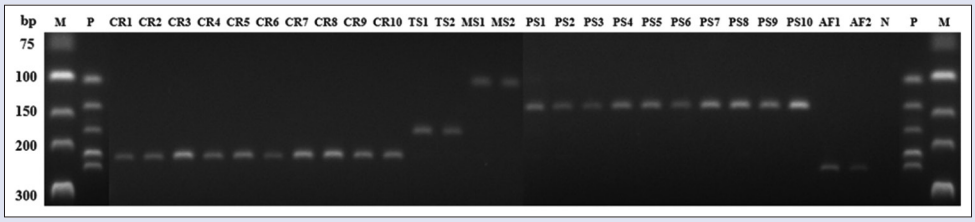


**Figure 3:** Three percent agarose gel showing the species specificity of the multiplex polymerase chain reaction assay. Lane M: DNA ladder, SN127; Lane P: Positive control; CR1~CR10: Ten batches of *Chinemys reevesii*; TS1~TS2: Two batches of *Trachemys scripta*; MS1~MS2: Two batches of *Mauremys sinensis*; PS1~PS10: Ten batches of *Pelodiscus sinensis*; AF1~AF2: Two batches of *Apalone ferox*; Lane N: Negative control; DNA: Deoxyribonucleic acid

**Table 1:** Primer sets used for polymerase chain reaction assay in this study

| Species | Primer | Sequence (5′-3′) | Target gene | Amplicon (bp) |
|---|---|---|---|---|
| CR | PC-Forward | ACTGCCATCCCTACGAGTCC | COX2 | 211 |
| | PC-Reverse | TTCTATTGGCATGACCACGCGAT | | |
| TS | PT-Forward | CATTATTCACTCTCGCAATGCC | ND5 | 171 |
| | PT-Reverse | ATGTGCCCAATAAAGTTACGTC | | |
| MS | PM-Forward | TCCTCGGGATAATCCACGAAC | ND6 | 105 |
| | PM-Reverse | CCATGGCTTTATCGTCTTGGT | | |
| PS | PP-Forward | CCTCACTCACAGCAGCCTA | ND5 | 137 |
| | PP-Reverse | GCTTCCTGCTGCTAATCGAAT | | |
| AF | PA-Forward | GGGCTTCACTATTCTTCATATGCATC | CYTB | 233 |
| | PA-Reverse | CCATTGTACTAGTGTGTTGCCAA | | |

CR: *Chinemys reevesii*; TS: *Trachemys scripta*; MS: *Mauremys sinensis*; PS: *Pelodiscus sinensis*; AF: *Apalone ferox*

**Table 2:** Deoxyribonucleic acid sequencing results of amplicons and their homology rate (percent identity) with corresponding gene sequences

| Species | Primer | Sequencing results of amplicons | GenBank Accession number | Homology rate by BLASTn (%) |
|---|---|---|---|---|
| CR | PC-F | TCATATAACAAAAATCAACAACCACACCTGACTATTAAAGCCATAGGACATCAATGATATTGAACATATGAGTACACTGATTACAAAAATCTT | AY676201.1 | 100 |
| | | GAATTTGACTCCTATATGATCCCAACCCAAAACCTTCCGAACGGACATTTCCGACTTCTAGAAGTAGACCATCGCGGTGGTCATGCCAATAGAA | | |
| | PC-R | TACTGCCATCCCTACGAGTCCTTTACCTAATAGACGAAATCAACAACCCACACCTGACTATTAAAGCCATAGGACATCAATGATATTGAA | | |
| | | CATATGAGTACACTGATTACAAAAATCTTGAATTTGACTCCTATATGATCCCAACCCAAAACCTTCCGAACGGACATTCCCGACTTCTGAAAGCCCC | | |
| TS | PT-F | TTGAGTGTCGTTTAGATTCAAAAGGCATTGCTTTATTCTGAAAACAGAAATCAAGTGTAAAAATAACATTCTTCATCTCCTTATTTCCACT | FJ392294.1 | 95.07 |
| | | ACTCCACTCAATACTATTTCCTGAACAAACCCTTTGTGACGTAACTTTATTGGGCACAT | | |
| | PT-R | TCATTATTCACTCTCGCAATGCCAATTATTATATCACTCATATCAAAAGCATTGCTTATTGTAAAAACAGAAGGAACTGTAAAAATAACA | | |
| | | TTCTTCATCTCCTTATTTCCACTACTCCACTCAATCTATTCCTAAACAAACACTG | | |
| MS | PM-F | TCAAGGAACAAACAGCGTCTCCGATAGCCCTCAACCAGCAATCAAAAGCAACCCACTACCAAGACGATAAAGCCATGG | KC333650.1 | 98.51 |
| | PM-R | TCCTCGGGATAATCCACGAACCAACTCTAACGCAACAAACAGCGTCAACAATAGCCCTCATCCAGCACGCCAATACGTTCCAAGT | | |
| PS | PP-F | AAAAAGCCGGCCTATCATTTCTTGTTCAATAGGACAACCACGATTTCAGCCCATACTTCCAATTAATGAAAATTATTACACAGCAAC | MG431983.1 | 98.10 |
| | | TAGCCCTATCATTCGATTAGCAGCAGGAAGC | | |
| | PP-R | CAAAGGCTATCCTTTGCTGTGTATATTTTCATTAATTGGAAGTATGGGCTGAAATCGTGGTTGTCCTATTTGAACAAGAATTGTGAT | | |
| | | TCGTAGGCTATAGGCTGCTGTGAGTGAGGA | | |
| AF | PA-F | TTCTTTCCTATTCGGGAACGAGGCCTATATTACGGCTCATACCTTTACAAACAAACCTGAAATACAGGTGTAATCCTACTACTATTAACC | KX882744.1 | 100 |
| | | ATAGCAACAGCATTCATAGGGTACGTCTTACCATGAGGTCAAATATCCTTCTGAGGGGCTACAGTCATCA | | |
| | | CAAACCTACTCTCAGCCATCCCTACATTGGCAACACACTAGTACAATGGA | | |
| | PA-R | TGGGCTTCACTATTCTTCATATGCATGTACCTTCGGACGAGGCATATATTACGGCTCATACCTTTACAAACAAACAT | | |
| | | GAAATACAGGTGTAATCATACTACTATTAACCATAGCAACAGCATTCATAGGGTAGGTCTTACCA | | |
| | | TGAGGTCAAATATCCTTATGAGGGGCTACAGTCATCACAAACTTATTTCAGCCTCCCCTATTTG | | |

CR: *Chinemys reevesii*; TS: *Trachemys scripta*; MS: *Mauremys sinensis*; PS: *Pelodiscus sinensis*; AF: *Apalone ferox*

PCR, the specificity was validated in 14 batches of *Testudinidae* species and 12 batches of *Trionychidae* species. As shown in Figure 3, a bright band at 211 base pair (bp) was detected for CR only, while the other two bands at 171 bp and 105 bp for TS and MS, respectively, were also successfully observed. In addition, the band at 137 bp was detected for PS and the other band at 233 bp existed for AF without any visible non-specific bands.

To examine the sensitivity of this assay, the effect of DNA concentration on PCR amplification was investigated. As shown in Figure 4, the specific bands of five species retained clear with template concentration decreasing from 100 to 1 ng/μL. However, no bands have been found after the template concentration was further decreased to 0.1 ng/μL or below. Accordingly, the detection limit of the developed method was determined as 1 ng/μL.

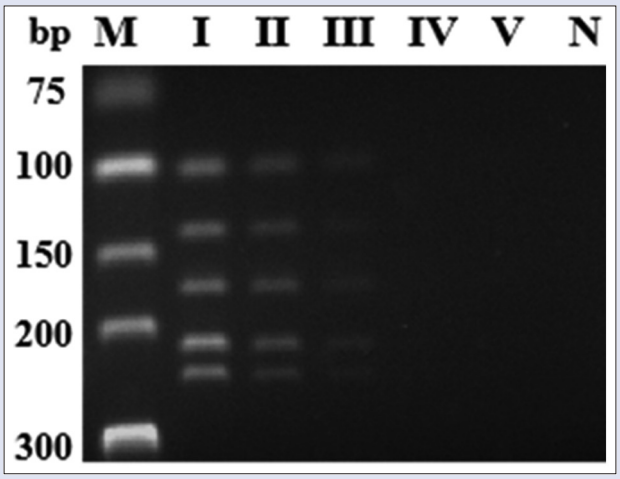


**Figure 4:** Three percent agarose gel showing the analytical sensitivity of multiplex polymerase chain reaction assay using serial dilutions of DNA as template. Lane M: DNA ladder, SN127; Lane I~V: 100 ng/μL, 10 ng/μL, 1 ng/μL, 0.1 ng/μL, 0.01 ng/μL of spiked samples; Lane N: Negative control. CR: *Chinemys reevesii*; TS: *Trachemys scripta*; MS: *Mauremys sinensis*; PS: *Pelodiscus sinensis*; AF: *Apalone ferox*; DNA: Deoxyribonucleic acid

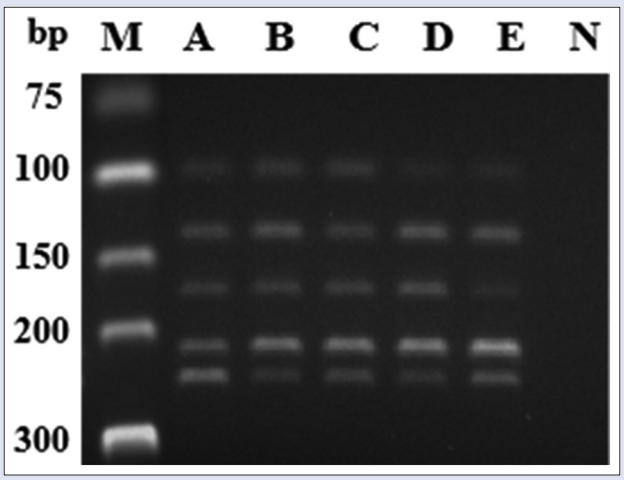


**Figure 5:** Three percent agarose gel showing analysis of reference sample mixtures by multiplex polymerase chain reaction assay. Lane M: DNA ladder, SN127; Lane A~E: Sample mixture (CR: TS: MS: PS: AF) in proportion of 2:3:4:5:6, 3:4:5:6:2, 4:5:6:2:3, 5:6:2:3:4, 6:2:3:4:5; Lane N: Negative control; DNA: Deoxyribonucleic acid

Laboratory sensitivity and specificity were calculated, and the results are summarized in Table 3. The laboratory sensitivity and specificity were all 100% for CR, TS, MS, PS, and AF, respectively.

The assay was also assessed using reference sample mixtures. The five Chelonia species (CR, TS, MS, PS, and AF) were mixed in five proportions (2:3:4:5:6, 3:4:5:6:2, 4:5:6:2:3, 5:6:2:3:4, and 6:2:3:4:5), and the weight of each sample mixture was 50 mg. DNA of those samples was extracted using the method described before, and after that, these DNA samples were amplified using the developed multiplex PCR approach. The results are shown in Figure 5; corresponding species were successfully detected in their mixture. The band intensity was increased as the higher proportion of the species added into the sample mixture. The results showed that the assay was capable of reliable detection of 10% adulterant.

## Analysis of self-made samples

The higher degree of processing results in more degradation of the original DNA in herbal medicines and this could hinder a successful DNA amplification. For this reason, the detection range of the proposed method has been determined by the analysis of 52 batches of self-made raw materials and processed products after heating. As shown in

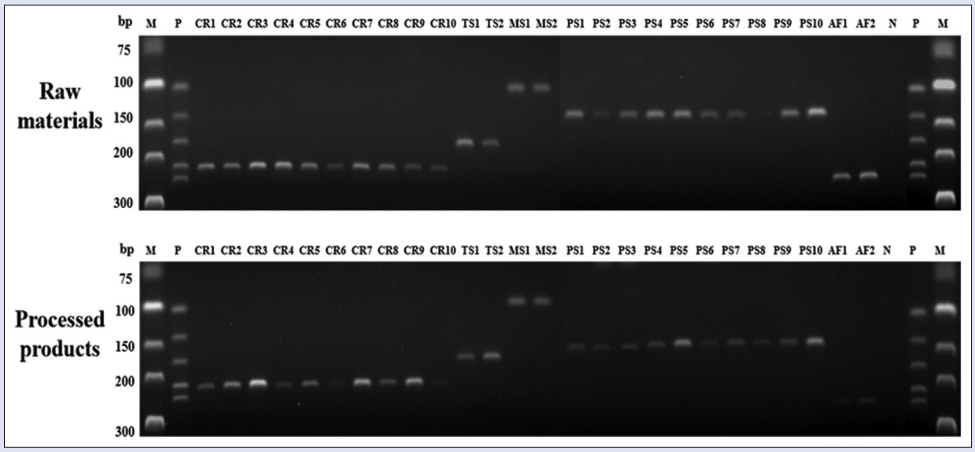


**Figure 6:** Three percent agarose gel showing analysis of self-made samples. Lane M: DNA ladder, SN127; Lane P: Positive control; Lane CR1~CR10: Ten batches of *Chinemys reevesii*; TS1~TS2: Two batches of *Trachemys scripta*; MS1~MS2: Two batches of *Mauremys sinensis*; PS1~PS10: Ten batches of *Pelodiscus sinensis*; AF1~AF2: Two batches of *Apalone ferox*; N: Negative control; DNA: Deoxyribonucleic acid

**Table 3:** Laboratory sensitivity and specificity the multiplex polymerase chain reaction test using the true positive and true negative samples

| Comparison results | | | | Sensitivity (%) | 95% CI | Specificity (%) | 95% CI |
|---|---|---|---|---|---|---|---|
| CR | Known samples | | | 100 | 69.15-100 | 100 | 79.41-100 |
| Multiplex PCR | Positive | Negative | Total | | | | |
| Positive | 10 | 0 | 10 | | | | |
| Negative | 0 | 16 | 16 | | | | |
| Total | 10 | 16 | 26 | | | | |
| TS | Known samples | | | 100 | 15.81-100 | 100 | 85.75-100 |
| Multiplex PCR | Positive | Negative | Total | | | | |
| Positive | 2 | 0 | 2 | | | | |
| Negative | 0 | 24 | 24 | | | | |
| Total | 2 | 24 | 26 | | | | |
| MS | Known samples | | | 100 | 15.81-100 | 100 | 85.75-100 |
| Multiplex PCR | Positive | Negative | Total | | | | |
| Positive | 2 | 0 | 2 | | | | |
| Negative | 0 | 24 | 24 | | | | |
| Total | 2 | 24 | 26 | | | | |
| PS | Known samples | | | 100 | 69.15-100 | 100 | 79.41-100 |
| Multiplex PCR | Positive | Negative | Total | | | | |
| Positive | 10 | 0 | 10 | | | | |
| Negative | 0 | 16 | 16 | | | | |
| Total | 10 | 16 | 26 | | | | |
| AF | Known samples | | | 100 | 15.81-100 | 100 | 85.75-100 |
| Multiplex PCR | Positive | Negative | Total | | | | |
| Positive | 2 | 0 | 2 | | | | |
| Negative | 0 | 24 | 24 | | | | |
| Total | 2 | 24 | 26 | | | | |

PCR: Polymerase chain reaction; CI: Confidence interval; CR: *Chinemys reevesii*; TS: *Trachemys scripta*; MS: *Mauremys sinensis*; PS: *Pelodiscus sinensis*; AF: *Apalone ferox*

Figure 6, all of them were successfully analyzed, indicating that the developed approach was of the strong capability to test five animal origins in processed products.

Diagnostic sensitivity and specificity of the multiplex PCR assay were figured using 26 reference samples in their raw and processed forms. The sensitivities of CR, TS, MS, PS, and AF were all 100%. The specificities of the five species were all computed as 100%. The kappa values of the five species were all 1 ± 0.0, indicating the detective results of raw materials and processed products are in "very good agreement" [Table 4].

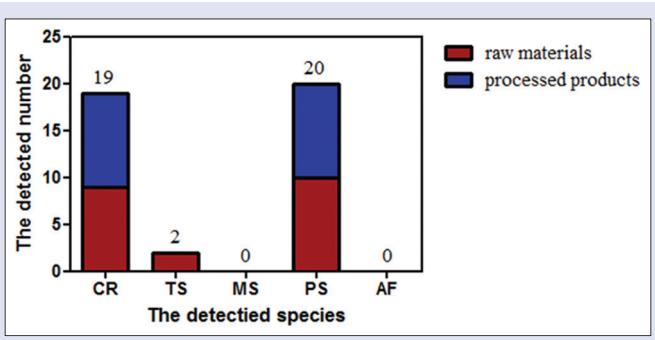


**Figure 7:** Graphical results of commercial products. CR: *Chinemys reevesii*; TS: *Trachemys scripta*; MS: *Mauremys sinensis*; PS: *Pelodiscus sinensis*; AF: *Apalone ferox*

## Assessment of commercial products

The replacement of genuine products by counterfeits or adulterants always exists on the market, in which physical identification cannot achieve high reliability without analytical supports.[32] In this session, the developed assay was carried out to assess 41 batches of commercial TCP and TC varieties to identify their animal origins simultaneously and confirm their labeling consistency. The graphical results are shown in Figure 7 [detailed information is shown in Table S1]. Overall, all DNA samples extracted from these products have been successfully amplified, and 38 out of the 41 commercial samples were authenticated as true TCP or TC products. However, two batches of commercial raw TCP (G7 and G8) were identified to be made of TS and the percentage adulteration was calculated as 18.2%.

## CONCLUSION

In the current study, the developed multiplex PCR approach is a convenient and economic method to identify CR, TS, MS, PS, and AF in TCP and TC products. It was well demonstrated for its high specificity and sensitivity, in addition to desired applicability on adulteration tests in commercial products. This newly proposed approach showed sufficient merits to be used as a regular means to examine medicinal TCM products made of CR and PS, including their raw and processed forms.

## Acknowledgements

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**Table 4:** Diagnostic sensitivity and specificity of the multiplex polymerase chain reaction test by comparing the known reference samples in raw and processed forms

| Comparison results | | | | Kappa value±SE | Sensitivity (%) | 95% CI | Specificity (%) | 95% CI |
|---|---|---|---|---|---|---|---|---|
| CR | Raw materials | | | 1±0 (very good agreement) | 100 | 69.15-100 | 100 | 79.41-100 |
| Processed products | Positive | Negative | Total | | | | | |
| Positive | 10 | 0 | 10 | | | | | |
| Negative | 0 | 16 | 16 | | | | | |
| Total | 10 | 16 | 26 | | | | | |
| TS | Raw materials | | | 1±0 (very good agreement) | 100 | 15.81-100 | 100 | 85.75-100 |
| Processed products | Positive | Negative | Total | | | | | |
| Positive | 2 | 0 | 2 | | | | | |
| Negative | 0 | 24 | 24 | | | | | |
| Total | 2 | 24 | 26 | | | | | |
| MS | Raw materials | | | 1±0 (very good agreement) | 100 | 15.81-100 | 100 | 85.75-100 |
| Processed products | Positive | Negative | Total | | | | | |
| Positive | 2 | 0 | 2 | | | | | |
| Negative | 0 | 24 | 24 | | | | | |
| Total | 2 | 24 | 26 | | | | | |
| PS | Raw materials | | | 1±0 (very good agreement) | 100 | 69.15-100 | 100 | 79.41-100 |
| Processed products | Positive | Negative | Total | | | | | |
| Positive | 10 | 0 | 10 | | | | | |
| Negative | 0 | 16 | 16 | | | | | |
| Total | 10 | 16 | 26 | | | | | |
| AF | Raw materials | | | 1±0 (very good agreement) | 100 | 15.81-100 | 100 | 85.75-100 |
| Processed products | Positive | Negative | Total | | | | | |
| Positive | 2 | 0 | 2 | | | | | |
| Negative | 0 | 24 | 24 | | | | | |
| Total | 2 | 24 | 26 | | | | | |

CI: Confidence interval; CR: *Chinemys reevesii*; TS: *Trachemys scripta*; MS: *Mauremys sinensis*; PS: *Pelodiscus sinensis*; AF: *Apalone ferox*; SE: Standard error

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## Conflicts of interest

There are no conflicts of interest.

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