

Development and Application of qPCR Assay for Species-specific Identification of Two Animal-derived Tonics: Vinegar-quenched *Testudinis Carapax et Plastrum* and Vinegar-quenched *Trionycis Carapax*

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Abstract

Background: Vinegar-quenched *Testudinis Carapax et Plastrum* (TCP) and vinegar-quenched *Trionycis Carapax* (TC) are made from the shells of *Chinemys reevesii* and *Trionyx sinensis*. However, some cheap substitutes from non-medicinal species such as *Trachemys scripta*, *Mauremys sinensis*, *Chelydra serpentina*, and *Apalone ferox* are often seen on the market, which exposes public health to high risk. Conventional methods are not applicable for the identification of these two products since their intrinsic constituents are largely destroyed after being highly processed.

Objectives: A new quantitative polymerase chain reaction (qPCR) method was developed to detect DNA components of Vinegar-quenched TCP and vinegar-quenched TC based on simple sequence repeat differential genes of animal-derived mitochondria.

Materials and Methods: Six species-specific primer reactions were designed and further validated for specificity, sensitivity, and repeatability. Finally, the developed method was used to assess the authenticity of their commercial products.

Results: The assay exhibited good specificity, and the limit of detection was above 1×10^2 copies/ μ L. There was good linearity in the concentration, ranging from 1×10^2 to 1×10^7 copies/ μ L. In addition, the method was validated through repeatability assessment (coefficient variation $<1\%$). While qPCR was applied for the analysis of 36 batches of commercial products of these two tonics, it could effectively identify the authenticity of the products, and consequently, seven batches were fake or adulterated.

Conclusion: This newly proposed method is promising for the quality evaluation of highly processed animal-derived Chinese medicines, which will assist in ensuring safety and efficacy in clinical practice and protect fair trade in the market.

Keywords

Vinegar-quenched *Trionycis Carapax*, Vinegar-quenched, *Testudinis Carapax et Plastrum*, real time polymerase chain reaction, species-specific primers, natural medicines

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Introduction

Trionycis Carapax (TC) is the carapace of *Trionyx sinensis* Wiegmann and *Testudinis Carapax et Plastrum* (TCP) is the carapace and plastron of *Chinemys reevesii* (Gray) (Chinese Pharmacopoeia Committee, 2020), both of which were consumed as important animal-derived medicinal materials in Traditional Chinese Medicine (TCM). They are usually subjected to a vinegar-quenched process, and these highly processed products are more widely administered in clinical practice to nourish Yin and subdue Yang. Vinegar-quenched

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TCP is often used to improve osteoporosis (Chen *et al.*, 2016), alleviate gastric ulcers (Zhao *et al.*, 2020), and enhance immunity, and vinegar-quenched TC is mainly used to treat liver cancer (Li *et al.*, 2018), liver fibrosis (Sun *et al.*, 2018), strengthen immunity (Chen *et al.*, 2021), as well as inhibit angiotensin I converting enzyme (Liao *et al.*, 2018). They play an important role in Chinese Patent Medicines, like some well-known and well-developed herbal medicines (Lu *et al.*, 2023). However, due to the limitation of *Chelonia* resources, some fake products made from non-medicinal species, including *Trachemys scripta*, *Mauremys sinensis*, *Chelydra serpentina*, and *Apalone ferox*, were often seen on the market, which exposed public health to a high risk and caused unfair economic competition. Vinegar-quenched TC and vinegar-quenched TCP are obtained by sand-scalding their clean products until the surface is light yellow, followed by immediate quenching in vinegar (Figure 1). After this deep processing, the complete shape of the carapace is broken, and the external characteristics of the prepared pieces are mostly lost, which increases the difficulty of identification.

Conventional identification based on morphology is not suitable for the authenticity of highly processed products and their powder. Rather than proteins and small molecules, specific DNA fragments are present in most biological samples even after being processed and can be readily extracted from even a very small portion of tested products. Therefore, they are an ideal and powerful tool for the identification of original material existing in final products

(Han *et al.*, 2018; Kim & Kang, 2023; Liu *et al.*, 2022; Lo & Shaw, 2018). In recent decades, polymerase chain reaction (PCR)-based methods to verify the origin of species have been considered the most preferred technology due to the favorable specificity and stability of DNA fragments (Geng, 2015). Previous studies (Yang *et al.*, 2018) showed that only the authenticity of medicinal materials and raw products of TCP or TC could be identified by simple PCR; however, none of the species was detected in 10 out of 12 batches of commercial Vinegar-quenched TCP products. Authentication of the highly processed animal-derived Chinese medicines cannot be accomplished yet due to improper primers as well as insufficient limitation of detection as desired, which often leads to false negative results. Accordingly, the development of applicable and convenient approaches is crucial for the identification of highly processed *Chelonia* animal carapaces.

SYBR Green-based qPCR is widely used because of its convenience in designing the assay and its relatively low setup and running costs. SYBR Green dye intercalates into double-stranded DNA to monitor the amplification of the target gene specifically initiated by specific primers (Kang & Tanaka, 2018; Rio-Lavín *et al.*, 2021). For instance, specific primer pairs and probes were designed based on the mitochondrial cytochrome b gene, followed by the establishment of a qPCR detection method to detect fresh donkey meat and different processed donkey meat products (Kim *et al.*, 2020). In addition, a convenient qPCR method was well developed to identify 10 shrimp varieties using five gene-specific primers

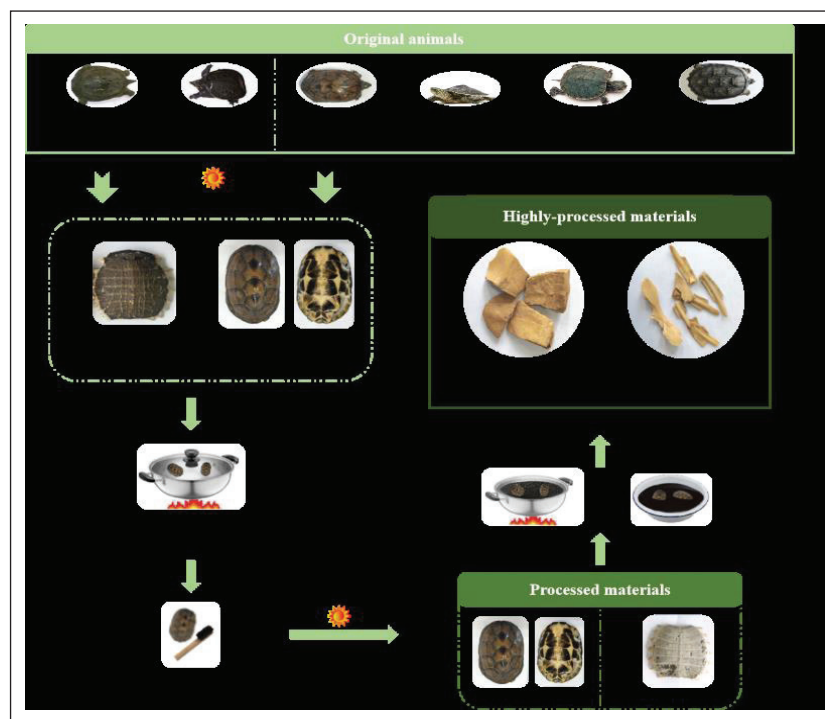


Figure 1. Procedures for Vinegar-quenched TC and Vinegar-quenched TCP.

Abbreviations: TC, *Trionycis Carapax*; TCP, *Testudinis Carapax et Plastrum*.

and distinguish mislabeled seafood or seafood adulterated with other varieties (Mondal & Mandal, 2020).

In this study, an SYBR Green-based qPCR assay was proposed for the identification of Vinegar-quenched TC and Vinegar-quenched TCP, which targeted the two ends of the simple sequence repeats (SSR) of six *Chelonia* species, and the specificity, sensitivity, and repeatability were determined. At last, the commercial products available have been quantitatively detected using the newly established method.

Materials and Methods

Materials

In this study, a total of 20 batches of six animal origins from *Chelonia*, including *C. reevesii*, *M. sinensis*, *T. scripta*, *C. serpentina*, *T. sinensis*, and *A. ferox*, were collected in recent years (Table S1), and all the species were verified by COI barcoding before any subsequent experiments. The protocols are described in the Chinese Pharmacopoeia (Ch.P., 2020 edition) (Figure 1). Ethical approval (No. 2017-03-03) was obtained from the Animal Ethics Committee of Jiangsu University prior to the experiments. The medicinal materials were purchased from aquaculture markets and breeding bases in different regions, but not wild animals from the environment.

In addition, 36 batches of commercial vinegar-quenched TC and vinegar-quenched TCP were purchased from TCM hospitals (Table S2).

Reagents

Proteinase K from *Tritirachium album* (Lot#020M8627) was purchased from Sigma-Aldrich (USA). DNA extraction phenol reagent (Lot#1027Z011) was purchased from Solarbio (China). BeyoFast™ SYBR Green Qpcr Mix (2×, High ROX) (Lot#030521220105) was purchased from Beyotime (China). PEASY®-Basic Seamless Cloning and Assembly Kit (Lot#P61026) was purchased from TransGen Biotech (China). Other reagents were all of analytical grade and supplied by Sinopharm Chemical Reagent Co. Ltd.

DNA Extraction

All samples were cleaned with 70% ethanol (EtOH) and pulverized after the removal of EtOH by air drying, then ground into a fine powder. Sodium dodecyl sulfate (SDS)-based protocols, as described by Yang *et al.* (2018), were used to extract DNA from each sample. In detail, 50 mg of the homogenized sample was mixed with 995 µL of extraction buffer (100 mM NaCl, 10 mM Tris-HCl, 25 mM ethylenediaminetetraacetic acid [EDTA], 0.5% SDS) and 5 µL proteinase K (20 mg/mL), and the mixture was incubated at 56°C for 3 h. After centrifugation at 12,000 rpm for 10 min,

900 µL of the supernatant was transferred to a new clear tube. An equal volume of phenol chloroform isoamyl alcohol solution and chloroform isoamyl alcohol solution were sequentially mixed with the supernatant for further purification. Then, the supernatant was precipitated by 850 µL of isopropanol and 85 µL of 5 M potassium acetate. The supernatant was removed after centrifugation, and the resulting DNA pellet was washed with 500 µL of 70% EtOH and finally reconstituted in 25 µL of Tris-EDTA (TE) buffer for subsequent experiments. SDS acts as an anionic surfactant to lyse cells at high temperatures (50°C–60°C) and denature proteins. Meanwhile, protease K was added to further degrade proteins and release nucleic acids from the sample matrix. The concentration and purity of the resulting DNA were determined by a NanoDrop1000 analyzer (Thermo Fisher Scientific, USA). The purified DNA was stored at –20°C for subsequent experiments.

Primers Design

Complete mitochondrial genome sequences of six species, including *C. reevesii*, *T. scripta*, *M. sinensis*, *C. serpentina*, *T. sinensis*, and *A. ferox* (Accession No.: NC_006082.1, NC_011573.1, NC_016685.1, NC_011198.1, NC_006132.1, NC_014054.1) were individually imported into SSRHunter1.3 software to retrieve their respective SSR sequences, and primers were designed by Oligo 7 software using the SSR sequences (*ca.* 300 bp) as templates for each species. Then, primer sets were further evaluated by Oligo and online NCBI Primer-BLAST (Figure S1), and the resulting primer candidates (Table 1) were synthesized by Sangon Biotech (Shanghai) Co. Ltd.

Real-time PCR

The DNA of vinegar-quenched TC and vinegar-quenched TCP was largely degraded after high processing including sand scalding and vinegar tempering, so the amount of template DNA in the samples has been much reduced. Accordingly, a more sensitive qPCR was adopted for the identification of six *Chelonia* species. In brief, the reaction was conducted in a total volume of 20 µL, containing 10 µL of SYBR Green Qpcr Mix, 2 µL of forward and reverse primers, 2 µL of DNA template (10 ng/µL), and 6 µL of deionized distilled water. At the cycling stage, initial denaturation was performed at 95°C for 2 min, followed by amplification for 35 cycles (95°C, 15 s; 64°C, 15 s). Then, the reaction system was kept at 95°C for 15 s prior to incubation at 60°C for 1 min. At the end of PCR, the temperature was increased to 95°C at a slope of +0.3°C/s, and maintained for 15 s. The annealing temperature and cycle number were optimized to establish the optimal amplification conditions (Table 2), and the specificity and sensitivity of the primer were investigated for method validation.

Table 1. DNA Sequences of Primers Used in this Study.

Species	Code	Sequence (5'-3')	Amplicon size
<i>Trionyx sinensis</i>	PS-F	TCTGTGGTCTCAGGTCCCAT	95 bp
	PS-R	GCACCTTTTAGTCTATGCGCGAA	
<i>Apalone ferox</i>	AF-F	ATTCATCGCCCTATCACTACACC	130 bp
	AF-R	CGTGATGATGCTACTAATAATGACACC	
<i>Chinemys reevesii</i>	CR-F	ACAGCCCAATCTATAAACACATTCC	81 bp
	CR-R	GTTTAGTCCTCCCCATCCTCC	
<i>Trachemys scripta</i>	TS-F	CACCATCCGCCGTAGCTTC	99 bp
	TS-R	TTTATGTGGTGGGGTTTGTGTT	
<i>Mauremys sinensis</i>	MS-F	ACCAACCGCGTTAGCTC	62 bp
	MS-R	AAGATTTGAACCATTGTAGGCTT	
<i>Chelydra serpentina</i>	CS-F	ATTAGCCCGAATGGTCACTTGC	74 bp
	CS-R	ACTAGCGTTTATGTCAGTTTGGTCA	

Abbreviations: PS-F, *Trionyx sinensis* forward primer; PS-R, *Trionyx sinensis* reverse primer; AF-F, *Apalone ferox* forward primer; AF-R, *Apalone ferox* reverse primer; CR-F, *Chinemys reevesii* forward primer; TS-F, *Trachemys scripta* forward primer; TS-R, *Trachemys scripta* reverse primer; MS-F, *Mauremys sinensis* forward primer; MS-R, *Mauremys sinensis* reverse primer; CS-F, *Chelydra serpentina* forward primer; CS-R, *Chelydra serpentina* reverse primer.

Table 2. Optimized PCR Conditions for the Six Species Analyzed in this Study.

Amplification Program	Primers					
	CR	TS	MS	CS	PS	AF
Initial denaturation				95°C (2 min)		
Denaturation				95°C (15 s)		
Annealing			63°C (15 s)		64°C (15 s)	
Cycle number	30	31	31	33	28	30

Abbreviations: PCR, Polymerase chain reaction.

Construction of Recombinant Standard Plasmid

The DNA amplicons of *C. reevesii* or *T. sinensis* were subjected to PCR by the primer sets and then inserted into the amplified pGEX-4T-1 (Fenghui Biotechnology, China) plasmid by the pGEX primer set (Table 3) using the pEASY-Basic Seamless Cloning and Assembly Kit. Primer pGEX, PSC, and CRC were used to amplify the pGEX-4T-1 plasmid and the amplicons of *C. reevesii* and *T. sinensis*, respectively. The resulting recombinant vector was transformed into *Escherichia coli* Top10 competent cells (Sangon Biotech, China), and the correct cloning was confirmed by Sanger sequencing (Sangon Biotech, China). DNA was quantified by the NanoDrop1000 analyzer (Thermo Fisher Scientific, USA). Moreover, the standard plasmid copy number (N) was calculated by the following formula:

$$N(\text{copies}/\mu\text{L}) = 6.02 \times 10^{14} \times C(\text{ng}/\mu\text{L}) / [B(\text{bp}) \times 660].$$

C is the DNA concentration, and B is the sum of base numbers in the plasmid and in the insert.

Calibration Curve

The standard plasmid was diluted 10-fold serially and amplified under optimized qPCR conditions at a concentration ranging from 1×10^2 to 1×10^7 copies/ μL . To construct a calibration curve with abscissa as the logarithm of plasmid copy numbers and ordinate as the C_t value.

Specificity Assay

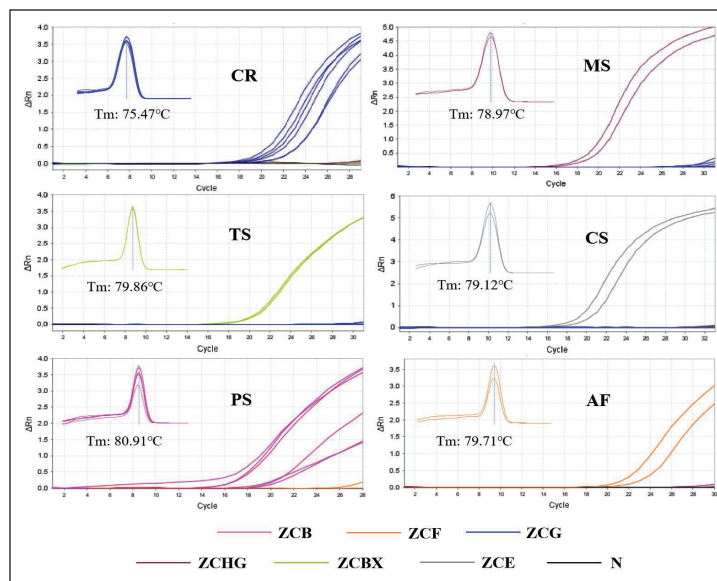
The DNA (10 ng/ μL) purified from self-made vinegar-quenched TC or vinegar-quenched TCP and their counterfeits was used as a template, and a qPCR assay was then carried out to verify the specificity under the optimized conditions. The DNA amplicons were sequenced by Sangon Biotech (Shanghai) Co. Ltd. to verify the accuracy of the specific amplification.

Sensitivity Assay

The DNA was extracted from six standard plasmids, and dissolved into TE buffer. Then, 10-fold dilution was performed six times, which resulted in the DNA solution

Table 3. Primers Used for Cloning the Amplicons to Construct the Calibration Curve.

Primer	Sequence (5'–3')
pGEX-F	CGCATCGTGACTGACTGACGATCT
pGEX-R	CGGGGATCCACGCGGAACC
PSC-F	GGTTCGCGTGATCCCCGGTCTGTGGTCTCAGGTCCCCAT
PSC-R	GATCGTCAGTCAGTCACGATGCGCACCTTTTAGTCTATGCGCGAA
CRC-F	GGTTCGCGTGATCCCCGGACAGCCCAATCTATAAACACATTCC
CRC-R	TCGTCAGTCAGTCACGATGCGTTTAGTCCTCCCCATCCTCC

**Figure 2.** Amplification Plot and Melt Curve for Specificity Test.

Abbreviations: N, Negative control; TC, *Trionycis Carapax*; TCP, *Testudinis Carapax et Plastrum*; ZCG, vinegar-quenched TCP; ZCHG, fake vinegar-quenched TCP made from *Mauremys sinensis*; ZCBX, fake vinegar-quenched TCP made from *Trachemys scripta*; ZCE, fake vinegar-quenched TCP made from *Chelydra serpentina*; ZCB, vinegar-quenched TC; ZCF, fake vinegar-quenched TC made from *Apalone ferox*.

ranging from 10 to 1×10^7 copies/ μL . The sensitivity of the method was investigated under optimized reaction conditions.

Repeatability Assay

The DNA template from different species was used for the repeatability test to evaluate the variation within and between groups at the time of measurement. For intragroup repeatability, three replicate samples were tested in the same run. In addition, the DNA was tested in three independent runs for intergroup repeatability. The repeatability and stability of the method were measured by coefficient of variation (CV).

Application of Method to Commercial Products

Seventeen batches of commercial vinegar-quenched TCP and 19 batches of commercial vinegar-quenched TC were subjected to the newly developed qPCR assay for the purpose of species identification. Accordingly, genuine products were differentiated from counterfeits and adulterants.

Results

Calibration Curve

As shown in Figure S2, the calibration curve of PS was drawn as $y = -3.090x + 35.08$ ($r^2 = 0.9941$, $\text{eff}\% = 110.6$), and that of CR was drawn as $y = -3.203x + 36.58$ ($r^2 = 0.9956$, $\text{eff}\% = 105.2$), indicating a good linear relationship in the concentration ranging from 1×10^2 to 1×10^7 copies/ μL . Moreover, negative controls should have a $C_t > 38$. The limits of quantification of the DNA template from *C. reevesii* and *T. sinensis* were both 1×10^2 copies/ μL .

Specificity Assay

All the sequences of DNA amplicons and their homology rates against the available corresponding gene sequences are presented in Table S3. An investigation of the specificity of these primer sets was performed on a $10 \text{ ng}/\mu\text{L}$ DNA template of self-made vinegar-quenched samples. It showed that strong

fluorescent signals were achieved after qPCR of the DNA samples from their corresponding species, and no measurable amplification was observed for the negative control and other species DNA. On the other hand, a single peak was shown in all melt curves without a visual primer dimer (Figure 2). The cycle number and Ct value of the amplification plot and Tm of the melt curves obtained in the specificity assay were summarized in Table 4. In addition, the Tm of DNA amplicons was in line with the theoretical parameters, suggesting that the specific primers used were reliable and feasible. These results demonstrated that the method has good specificity and

could be employed to identify the species of vinegar-quenched TC or vinegar-quenched TCP regardless of their destroyed morphological characteristics.

Sensitivity Assay

The sensitivity of each primer set against their corresponding species was investigated on the DNA template extracted from the self-made sample, and a series of concentrations ranging from 10 to 1×10^7 copies/ μL were subjected to the qPCR amplification. As shown in Figure 3, a clear fluorescence

Table 4. Specificity Assay Results of Amplification Plot and Melt Curve.

	Species	Primer	Cycle Number	Ct Value	Tm ($^{\circ}\text{C}$)
Vinegar-quenched <i>Testudinis Carapax</i> et <i>Plastrum</i>	<i>Chinemys reevesii</i>	CR	30	20.32	75.47
	<i>Mauremys sinensis</i>	MS	31	19.67	78.97
	<i>Trachemys scripta</i>	TS	31	19.16	79.86
	<i>Chelydra serpentina</i>	CS	33	17.74	79.12
Vinegar-quenched <i>Trionyx Carapax</i>	<i>Trionyx sinensis</i>	PS	28	19.03	80.91
	<i>Apalone ferox</i>	AF	30	21.62	79.71

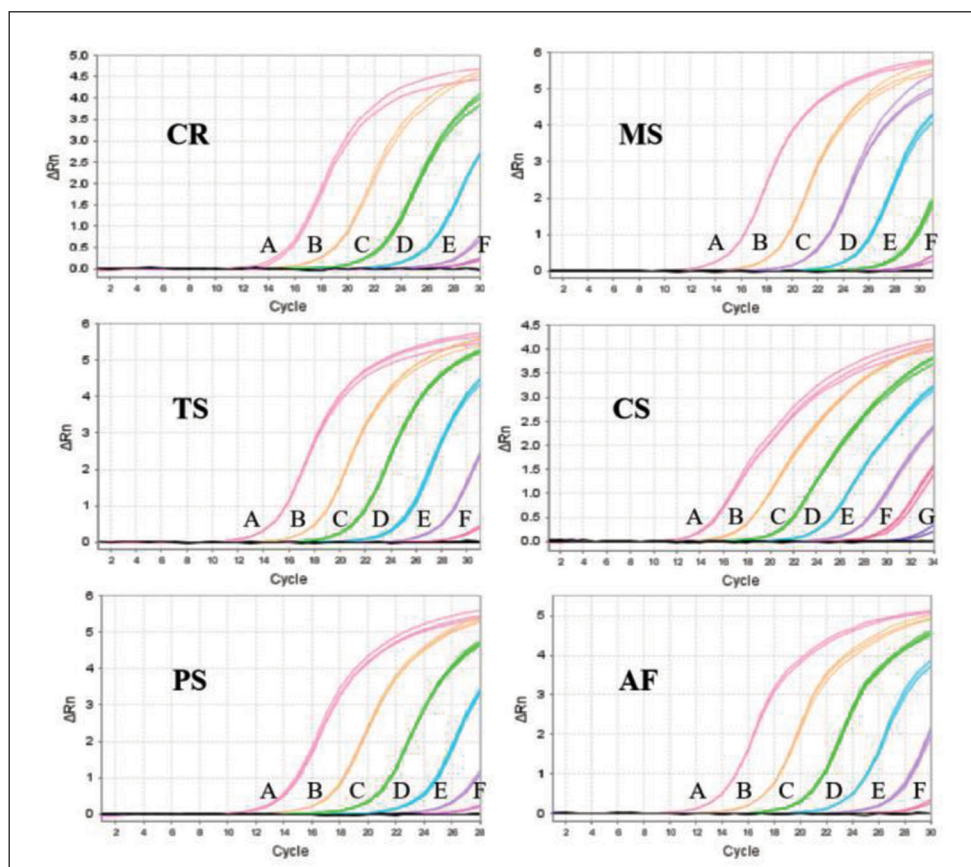


Figure 3. Sensitivity Test of qPCR Assay ($n = 3$). (A: 1×10^7 copies/ μL ; B: 1×10^6 copies/ μL ; C: 1×10^5 copies/ μL ; D: 1×10^4 copies/ μL ; E: 1×10^3 copies/ μL ; F: 1×10^2 copies/ μL ; G: 10 copies/ μL).

Abbreviations: qPCR, Quantitative polymerase chain reaction.

signal was detected after the amplification against CR, TS, MS, AF, and PS when the DNA concentration of these samples was 1×10^2 copies/ μ L or above. Furthermore, CS was even able to generate a fluorescence signal against 10 copies/ μ L of the *C. serpentina* DNA template. The results demonstrated that the qPCR assay was highly sensitive and could be used to detect trace amounts of DNA from six Chelonia species.

Repeatability Assay

Consequently, the intragroup CV for six species ranged from 0.03% to 0.64%, and the intergroup CV varied from 0.10% to 0.79% (Table 5). Both their CVs were less than 1%, indicating the high accuracy and good repeatability of the qPCR method.

Application of Methods for Species Identification of Commercial Products

Eleven batches of vinegar-quenched TCP, including CG1–CG4 and CG7–CG13 have been amplified with the primer CR solely. The results suggested the presence of *C. reevesii* but not the other three fake species in these samples, and accordingly, they were authenticated to be genuine. However, another two batches (CG5 and CG6) have been amplified with the primers CR and TS or MS, indicating the presence of *T. scripta* in CG5 and *M. sinensis* in CG6 in addition to *C. reevesii*, and hence they were both authenticated to be adulterants. Another four batches (CG14–CG17) were not amplified with the primer CR; instead, they were amplified with the primer TS and MS or TS or CS. The results implied the lack of *C. reevesii* in these four products, and therefore

Table 5. Repeatability Tests of the qPCR Assay ($n = 3$).

Code	Intragroup Repeatability		Intergroup Repeatability	
	Average Ct $\pm$ SD	CV (%)	Average Ct $\pm$ SD	CV (%)
CR	28.33 $\pm$ 0.070	0.25	28.02 $\pm$ 0.222	0.79
TS	30.43 $\pm$ 0.194	0.64	30.67 $\pm$ 0.225	0.74
MS	29.17 $\pm$ 0.097	0.33	29.17 $\pm$ 0.029	0.10
CS	32.95 $\pm$ 0.022	0.07	32.91 $\pm$ 0.099	0.30
PS	26.26 $\pm$ 0.084	0.32	26.31 $\pm$ 0.136	0.52
AF	28.52 $\pm$ 0.009	0.03	28.39 $\pm$ 0.097	0.34

Abbreviations: qPCR, Quantitative polymerase chain reaction; CV, coefficient variation.

Table 6. Species Identification in Commercial Vinegar-quenched *Testudinis Carapax et Plastrum*.

Code	CR	MS	TS	CS	Copies/ μ L	Genuine	Counterfeit	Adulteration
CG1	26.31 $\pm$ 0.093	–	–	–	103.21	+	–	–
CG2	26.90 $\pm$ 0.072	–	–	–	103.02	+	–	–
CG3	28.59 $\pm$ 0.098	–	–	–	102.49	+	–	–
CG4	29.76 $\pm$ 0.087	–	–	–	102.13	+	–	–
CG5	28.72 $\pm$ 0.190	–	29.30 $\pm$ 0.148	–	102.45	–	–	–
CG6	29.75 $\pm$ 0.081	29.72 $\pm$ 0.184	–	–	102.13	–	–	–
CG7	28.80 $\pm$ 0.103	–	–	–	102.43	+	–	–
CG8	29.68 $\pm$ 0.053	–	–	–	102.15	+	–	–
CG9	26.33 $\pm$ 0.033	–	–	–	103.20	+	–	–
CG10	28.39 $\pm$ 0.033	–	–	–	102.56	+	–	–
CG11	27.92 $\pm$ 0.064	–	–	–	102.70	+	–	–
CG12	27.04 $\pm$ 0.205	–	–	–	102.98	+	–	–
CG13	29.90 $\pm$ 0.026	–	–	–	102.09	+	–	–
CG14	–	29.11 $\pm$ 0.044	24.70 $\pm$ 0.184	–	–	–	+	–
CG15	–	–	28.57 $\pm$ 0.027	–	–	–	+	–
CG16	–	–	–	30.93 $\pm$ 0.116	–	–	+	–
CG17	–	–	30.43 $\pm$ 0.162	–	–	–	+	–

Table 7. Species Identification in Commercial Vinegar-quenched *Trionycis Carapax*.

Code	PS	AF	Copies/ μ L	Genuine	Counterfeit	Adulteration
CB1	23.44 $\pm$ 0.177	28.94 $\pm$ 0.077	10 ^{3.77}	–	–	+
CB2	24.82 $\pm$ 0.020	–	10 ^{3.32}	+	–	–
CB3	24.32 $\pm$ 0.167	–	10 ^{3.48}	+	–	–
CB4	25.86 $\pm$ 0.043	–	10 ^{2.98}	+	–	–
CB5	25.64 $\pm$ 0.106	–	10 ^{3.06}	+	–	–
CB6	26.06 $\pm$ 0.127	–	10 ^{2.92}	+	–	–
CB7	23.31 $\pm$ 0.017	–	10 ^{3.81}	+	–	–
CB8	24.64 $\pm$ 0.224	–	10 ^{3.38}	+	–	–
CB9	25.10 $\pm$ 0.042	–	10 ^{3.23}	+	–	–
CB10	26.04 $\pm$ 0.085	–	10 ^{2.93}	+	–	–
CB11	26.50 $\pm$ 0.099	–	10 ^{2.78}	+	–	–
CB12	26.80 $\pm$ 0.092	–	10 ^{2.68}	+	–	–
CB13	23.96 $\pm$ 0.028	–	10 ^{3.60}	+	–	–
CB14	24.32 $\pm$ 0.076	–	10 ^{3.48}	+	–	–
CB15	24.25 $\pm$ 0.037	–	10 ^{3.50}	+	–	–
CB16	22.82 $\pm$ 0.035	–	10 ^{3.97}	+	–	–
CB17	24.59 $\pm$ 0.090	–	10 ^{3.39}	+	–	–
CB18	25.78 $\pm$ 0.014	–	10 ^{3.01}	+	–	–
CB19	24.56 $\pm$ 0.190	–	10 ^{3.40}	+	–	–

they were authenticated to be counterfeit (Table 6). On the other hand, 18 out of 19 batches of Vinegar-quenched TC have been amplified uniquely with the primer PS. The results showed the presence of *T. sinensis* but not *A. ferox* in these samples, so they were authenticated to be genuine. However, one batch (CB1) was amplified with the primers PS and AF. It suggested the presence of *A. ferox* in addition to *T. sinensis* in this batch, and accordingly, CB1 was authenticated to be adulterant (Table 7).

Discussion

In this study, six species-specific primers were designed, and a new qPCR method was developed for the identification of vinegar-quenched TCP and vinegar-quenched TC. The calibration curves of PS and CR indicated a good linear relationship in the concentration ranging from 1×10^2 to 1×10^7 copies/ μ L ($r^2 > 0.99$), which could realize the quantitative detection of the original species DNA in these two animal-derived tonics. In the optimized qPCR conditions, the specificity assay showed that the strong fluorescent signals were only achieved after the amplification of the DNA samples from their corresponding species, and high accuracy was verified by sequencing the DNA amplicon obtained from the specific amplification. Moreover, the detection limits of the DNA template from *C. reevesii*, *T. scripta*, *M. sinensis*, *A. ferox*, and *T. sinensis* were all 1×10^2 copies/ μ L, while

C. serpentina was at 10 copies/ μ L, and the CV was all were less than 1% in the repeatability assay. These results demonstrated that the novel qPCR method had good performance in terms of specificity, sensitivity, and repeatability. Consequently, we effectively identified the authenticity of 36 batches of commercial products made from these two tonics by different manufacturers. Among 17 batches of commercial vinegar-quenched TCP, 64.7% (11/17) were considered genuine, 11.8% (2/17) as adulterant, and 23.5% (4/17) as counterfeit. In addition, 94.7% (18/19) were considered genuine and 5.3% (1/19) were adulterants among 19 batches of commercial vinegar-quenched TC. Compared with previous studies, the designed species-specific primers can usefully and accurately detect the species of these two highly processed products, and the qPCR method enables more rapid quantification and performs better for authentication purposes.

In summary, the results above indicated that there are some adulterants and counterfeits in the commercial vinegar-quenched TCP and vinegar-quenched TC, so it is necessary to establish a proper method to detect and quantify their DNA components. In our study, the species-specific primers could rapidly and reliably identify six *Chelonia* species of highly processed products. Real-time PCR has crucial advantages in the identification of Chinese medicines, but it usually requires highly specialized equipment and a long analysis cycle. Therefore, the combination of nucleic acid detection and other cutting-edge technologies will be further studied. For instance, to enhance the detection limitation and achieve absolute DNA

quantitation, droplet digital PCR could be employed, which could be appropriate for the identification of highly processed Chinese medicine or Chinese Patent Medicine. Furthermore, to achieve efficient on-site identification of commercial Chinese medicine products, visual and more rapid detection methods could be developed, such as loop-mediated isothermal amplification (Jawla et al., 2021; Zhao et al., 2022).

Conclusion

In this study, we established a reliable and convenient qPCR method to detect the DNA from *C. reevesii*, *T. sinensis*, and their substitutes for the authentication of commercial Vinegar-quenched TC and Vinegar-quenched TCP products. It was well demonstrated for its stronger species specificity, higher sensitivity, and shorter time, in addition to its desired applicability to adulteration tests in commercial products. In particular, the method performed well in the assay of counterfeits of vinegar-quenched TC and vinegar-quenched TCP products. The newly proposed method could be employed as a routine detection means for the authenticity identification of these highly processed medicines, which will help ensure safety and efficacy in clinical practice and protect fair trade.

Abbreviations

AF-F: *Apalone ferox* forward primer; AF-R: *Apalone Ferox* reverse primer; BLASTn: basic local alignment search tool nucleotide; CR-F: *Chinemys reevesi* forward primer; PS-F: *Trionyx sinensis* forward primer; PS-R: *Trionyx sinensis* reverse primer; TCP: *Testudinis Carapax et Plastrum*; TCM: traditional Chinese medicine; TC: *Trionycis Carapax*.

Declaration of Conflicting Interests

The authors declare no conflict of interest.

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Statement of Informed Consent and Ethical Approval

Necessary ethical clearances and informed consent was received and obtained respectively before initiating the study from all participants.

Summary

A new qPCR method was developed to detect DNA components of vinegar-quenched TCP and vinegar-quenched TC, based on simple sequence repeat differential genes of animal-derived mitochondria.

Six species-specific primer reactions were designed and confirmed to have high specificity and repeatability. The limit of detection was above 1×10^2 copies/ μ L. There was a good linearity in the concentration ranging from 1×10^2 to 1×10^7 copies/ μ L. In particular, the newly method performed well in the assay of counterfeits of vinegar-quenched TC and vinegar-quenched TCP products.

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