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Original Article

Identification of *Plastrum Testudinis* used in traditional medicine with DNA mini-barcodes

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ABSTRACT

The carapace of the tortoise *Chinemys reevesii* is an ingredient of "Guijia", a traditional Chinese medicine. However, *C. reevesii* is difficult to raise in aquaculture and is rare in the wild. Counterfeit tablets are made from carapaces of other species. In addition to *C. reevesii*, other species including *Mauremys sinensis*, *Indotestudo elongate* and *Trachemys scripta* have been used in *Plastrum Testudinis* as well. After processing, these carapaces are difficult to identify on the basis of morphological characteristics, which impedes law enforcement. Our study used DNA barcoding technology to identify *C. reevesii* and its substitutes. We extracted concentrated genomic DNA for PCR amplification. Based on the analysis of 61 full-length COI sequences, we designed four pairs of mini-barcode primers: Tu-A, Tu-B, Tu-C and Tu-D. The Tu-B primers sequenced genomic DNA with a success rate of 76.47%, and the Tu-D primers sequenced genomic DNA with a success rate of 88.24%. The identification efficiency of these two mini-barcodes was 70.59% and 64.71%, and the overall identification efficiency was approximately 76.47%. Similarly, a set of mini barcode systems was generated, which may provide an effective and low-cost method for the identification of authentic tortoise shells.

Introduction

As a widely used Chinese medicine, Carapax et *Plastrum Testudinis* refers to processed medicinal tablets that are made from the carapace of *Chinemys reevesii*, which are members of the Testudinidae family (Chinese Pharmacopoeia, 2015). Carapax et *Plastrum Testudinis* is principally derived from *C. reevesii* bred by aquiculture in Zhejiang, Guangdong and other provinces in China, in which carapaces are stripped following capture and dissection. "Guijia" is a unique Chinese medicine that has been used for thousands of years. According to ancient records and modern literature, the shells in clinical use are derived from *C. reevesii* (*Mauremys reevesii* Gray, 1831). Yifei Zhong et al. showed that Nephrokeli, a Chinese herbal formula, which contains "Guijia", may effectively treat IgA nephropathy through the regulation of the sphingosine-1-phosphate pathway (Zhong et al., 2015). Nevertheless, to reduce costs and increase profits, counterfeit tablets are produced using carapaces of other species and obtained from merchants in restaurants, resulting in mixed qualities, of which the safety and effectiveness cannot be guaranteed (Yang, 2001; Yi, 2010). Additionally, the carapaces of some endangered species serve in collections and are traded in illegal markets, which can elude government regulation (Wu, 2012). Considering the protection for rare species and the standard regulation of Chinese herbs, more research is required regarding identification approaches for Testudinidae animal carapaces.

Currently, the universal DNA barcodes used in animal species identification are COI and Cytb. COI is a mitochondrial DNA fragment 650 bp in length (Hebert et al., 2003). A wealth of literature has shown that COI barcodes can precisely identify various animal species. However, for older samples or low-quality DNA, amplifying the intact COI sequence using normal primers is challenging and hinders the identification of Chinese herbal medicines (Chen et al., 2015). In our study, PCR amplification of the Cytb gene was unsuccessful in most of the turtle samples, which may be due to the low homology among turtle species. In 2006, the Canadian scholar

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Hebert invented DNA mini-barcode technology (Hajibabaei et al., 2006a,b) to identify species in museum collections. This method allowed for species identification by using 200 bp sequences from COI barcodes. In recent years, this technique has played a significant role in the food industry (Ward et al., 2009; Shokralla et al., 2015) and for the identification of TCM (Dong et al., 2014; Chen et al., 2015).

In 2009, Lee et al. applied mini-barcoding using the cytb gene as a DNA marker to identify turtle shell samples (Lee et al., 2009). However, the success rates of PCR amplification in our samples using the same mini barcodes were low. In recent studies, COI sequences have been frequently used because mtDNA is more conservative than nuclear DNA, such that universal primers are easier to design. In this study, we designed miniature barcode primers based on COI sequences, and the optimized sequences were selected for the identification of shell fragments.

Materials and methods

Sample collection

Tortoise sample collection occurred over two months (October–November 2015) from the following three provinces in China: Jiangsu Province, Zhejiang Province and Guangzhou Province. A total of forty samples were collected (Table 1), all of which were obtained from aquatic product markets in eight cities and delivered to the laboratory located in Beijing. To investigate the intra- and interspecific genetic distances between these tortoise species, 22 sequences were downloaded from NCBI (https://www.ncbi.nlm.nih.gov) (Table 2). For the analysis of mini-barcode primers with commercial products, a total of seventeen turtle shell products were obtained from TCM markets (Table 3). All samples were identified by Prof. Yang, director of the Chinese Medicine Identification Institution, Beijing University of Chinese Medicine. Tortoises were bred in fish tanks, and blood was collected and prepared for DNA extraction.

DNA extraction

DNA from blood samples was extracted using a whole blood genomic DNA isolation kit (Biomed, China). Minced shell samples were decalcified with EDTA solution followed by DNA extraction using the tissue/cell genomic DNA extraction kit (Biomed, China). Each sample was run in triplicate, and three adsorption columns were eluted with molecular biology grade water (50 μl) to increase the template DNA concentration for PCR.

DNA barcode library construction

PCR was performed in a 25 μl reaction volume containing 10× PCR Buffer (Mg^{2+} plus) (2.5 μl), dNTPs (0.4 mM each), forward and reverse primers (0.2 μM each), template DNA and Taq Polymerase (0.1 U). PCR amplifications were performed in a PCR machine (Bio-Rad Laboratories, TX, USA) utilizing the thermal cycling parameters described in Table 4. The sequence of the PCR products was determined using a 3730×l DNA Analyzer (Shanghai Sangon Biological Technology Co. Ltd., China). The obtained forward and reverse sequences were aligned using Contig Express software (909 Third Avenue, #6260, New York, NY 10150, USA), such that the accurate barcode sequence of each sample was acquired. Then, prepared primers were added to the DNAMAN software (Lynnon Biosoft, USA) for comparison. Next, an analysis of differences among the various species' COI barcode sequences was made. Finally, the construction of a genetic NJ clustering tree was performed by MEGA5, Molecular Evolutionary Genetics Analysis version 5.0 (Tamura et al., 2011).

PCR primer design and analysis

We compared the full-length COI barcodes of all samples and analyzed the highly conserved sites in turtle species. In addition, we considered the overall physical structure

Table 2
Details of 22 sequences downloaded from NCBI.

No.	Species and accession	No.	Species and accession
1	*Mauremys mutica* KP751968	12	*Podocnemis unifilis* JF802204
2	*Mauremys mutica* AF348260	13	*Podocnemis unifilis* KC751491
3	*Geoemyda spengleri* HQ329679	14	*Platysternon megacephalum* GQ867677
4	*Geoemyda spengleri* AY562184	15	*Platysternon megacephalum* GQ867679
5	*Cyclemys dentata* JX455823	16	*Indotestudo elongata* DQ080043
6	*Cyclemys dentata* JN582334	17	*Indotestudo elongata* DQ656607
7	*Cuora mouhotii* EF011474	18	*Cuora galbinifrons* AF348266
8	*Cuora mouhotii* DQ659152	19	*Cuora galbinifrons* AY357742
9	*Cuora amboinensis* AY357738	20	*Chelydra serpentina* KU985806
10	*Cuora amboinensis* FJ763736	21	*Chelydra serpentina* LC145070
11	*Pelomedusa subrufa* AF039066	22	*Danio rerio* NC002333

Table 3
Details of seventeen turtle shell samples.

No.	Morphological identification of species	No.	Morphological identification of species
1	*Mauremys reevesii*	10	*Trachemys scripta*
2	*Mauremys reevesii*	11	*Mauremys sinensis*
3	*Mauremys mutica*	12	*Mauremys sinensis*
4	*Mauremys reevesii*	13	*Mauremys sinensis*
5	*Mauremys reevesii*	14	*Cyclemys dentata*
6	*Mauremys reevesii*	15	*Mauremys reevesii*
7	*Indotestudo elongata*	16	*Mauremys sinensis*
8	*Cyclemys dentata*	17	*Mauremys reevesii*
9	*Mauremys sinensis*		

Table 1
Details of 40 turtle samples used for the full-length COI barcode construction.

Species	Sample quantity	Species	Sample quantity
Mauremys reevesii	13	*Podocnemis unifilis*	1
Mauremys mutica	1	*Pelomedusa subrufa*	1
Geoemyda spengleri	1	*Trachemys scripta*	7
Cyclemys dentata	1	*Platysternon megacephalum*	2
Cuora mouhotii	2	*Indotestudo elongata*	2
Mauremys sinensis	5	*Cuora galbinifrons*	1
Cuora amboinensis	2	*Chelydra serpentina*	1

Table 4
PCR amplification and sequencing primers used for DNA mini-barcoding of the *Plastrum Testudinis* samples.

Primer set	Primer name	Direction	Primer sequence	Barcode length (bp)	Annealing temp. (°C)
COI	COI-F COI-R	Forward Reverse	GGTCAACAAATCATAAAGATATTGG TAAACTTCAGGGTGACCAAAAAATCA	691	45
Mini_Tu-A	MA-F MA-R	Forward Reverse	GTGACCAAAAAATCAGAA ATTAATATAAAATCCCCAGCC	215	49
Mini_Tu-B	MB-F MB-R	Forward Reverse	ATGTTAATTGCTGTGGTG ATAAGCTTCTGGCTTCTACC	215	56
Mini_Tu-C	MC-F MC-R	Forward Reverse	GGTATGACCATGAAGAAAA TGGCACCTTATAATTTTG	171	47
Mini_Tu-D	MD-F MD-R	Forward Reverse	TAGTTAGATCTACAGAGGCG GGTCATACCTATTATAATCGG	109	56

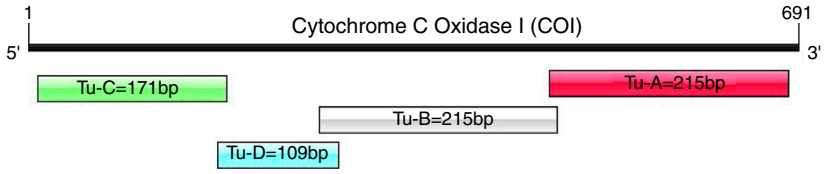

Fig. 1. Schematic representation of regions amplified by the mini-barcode primers designed in this study, shown within the standard COI barcode region.

characteristics and used these data to design four mini-barcode primer sets using online primer design software (http://www.yeastgenome.org/cgi-bin/web-primer – Stanford University) (Table 4). For further testing with the *Plastrum Testudinis* samples, the relative locations of the primers (compared to the COI barcodes) are shown in Fig. 1. All barcodes prepared for screening were subjected to gradient PCR (annealing temperature 45–60 °C). According to the results of agarose gel electrophoresis, the optimum annealing temperature was determined (Table 4). The selected mini-barcode samples matched with the shell DNA templates and were amplified. Using sequence accuracy and identification efficiency as parameters, the best mini-barcode was selected for shell sample identification.

Results and discussion

Genomic DNA of *Plastrum Testudinis* from TCM markets was severely degraded because of processing prior to sale and from preservation over a long period under conditions of high temperature and humidity. The cells in tortoise plastrons are surrounded by bone tissues of high density, which protects the DNA from degradation. In this study, although DNA extraction was improved, only 4 of 17 *Plastrum Testudinis* samples from TCM markets had template DNA concentrations exceeding 2.5 ng/μl, and the success rate for COI barcoding with PCR amplification was 0%. In conclusion, COI barcoding cannot be used for the identification of *Plastrum Testudinis*.

In total, 61 sequences from fourteen tortoise species were obtained, including forty from PCR and 21 from GenBank. All sequences were trimmed to 691 bp and assembled as a dataset of full-length COI barcodes. After alignment, the results showed that 353 locations were conserved, and 338 were polymorphic. The average GC content was 43.5%.

Intraspecific and interspecific genetic distances among the 61 COI sequences of the full-length barcodes are shown in Table 5. More specifically, intraspecific distance was minor, and averaged 1.12%, whereas interspecific genetic distance averaged 19.28%. All fourteen species clades in the NJ phylogenetic tree were supported with 99% bootstrap values (Fig. 2). Compared to other species, the intraspecific genetic distance in turtles is large, indicating high variation. Although only fourteen species from eight families and twelve genera were collected in this study, we observed greater intraspecific genetic distances than in birds, fish, insects and other animals because turtles comprise a large family (Hebert et al., 2004; Hajibabaei et al., 2006a,b; Ward et al., 2006). Therefore, the complete COI barcodes can be used successfully for turtle identification among the fourteen species in this study.

Using the modified DNA extraction method, DNA extraction was conducted with seventeen shell samples purchased from the market. Four pairs of mini-barcoding sequences were amplified by PCR, and the sequencing success rate was recorded. Tu-B products were sequenced successfully at a rate of 76.47% in thirteen samples, and Tu-D had a success rate of 88.24% in fifteen samples. Tu-A and Tu-C were eliminated due to sequencing failure.

To determine the discrimination ability of the four mini-barcoding sequences, data analysis was conducted after the Tu-B sequences were trimmed to 215 bp and the Tu-D sequences were trimmed to 109 bp. The average intra- and interspecific distances of the Tu-B dataset were 0.54% and 18.80%, respectively, whereas those of the Tu-D dataset were 0.51% and 18.60%, respectively (Tables 6 and 7). These distances were both close to that of the 691-bp data set.

The results shown in Table 8 reveal that the efficiency of identification of Tu-B was 70.59%, Tu-D was 64.71%, and the overall identification efficiency was 76.47%. Because all the samples of tortoise shells were purchased from the TCM market, samples were selected that had been stored for a long time but whose textural characteristics were still in existence. Therefore, the results of the morphological identification were reliable. In samples 3, 6, and 7, the results of the mini-barcoding identification were inconsistent with the morphological identification. It is possible that errors in DNA sequencing from amplified intraspecific differences led to the deviation of the identification results.

In China, the identification system includes two barcodes for *Mauremys reevesii* that qualify shell species by Pharmacopeia, and the identification success rate was 100%. Through comprehensive analysis, turtle species such as *Indotestudo elongata* and *Mauremys sinensis* can also be successfully identified. For the identification of *Trachemys scripta*, the two groups of barcodes were unable to successfully amplify the samples. Nonetheless, these mini-barcodes

Table 5
Pairwise genetic distances between and within fourteen *Plastrum Testudinis* species based on full-length COI barcodes.

Species	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]	[14]
[1] *Trachemys scripta*	0.035													
[2] *Podocnemis unifilis*	0.252	0.003												
[3] *Platysternon megacephalum*	0.248	0.288	0.005											
[4] *Pelomedusa subrufa*	0.326	0.263	0.349	0.007										
[5] *Mauremys sinensis*	0.163	0.251	0.228	0.297	0.004									
[6] *Mauremys reevesii*	0.160	0.243	0.244	0.278	0.049	0.016								
[7] *Mauremys mutica*	0.177	0.252	0.224	0.280	0.091	0.077	0.001							
[8] *Indotestudo elongata*	0.177	0.234	0.247	0.294	0.158	0.151	0.160	0.006						
[9] *Geoemyda spengleri*	0.177	0.239	0.247	0.310	0.145	0.149	0.146	0.174	0.001					
[10] *Cyclemys dentata*	0.172	0.229	0.218	0.294	0.123	0.116	0.118	0.164	0.151	0.037				
[11] *Cuora mouhotii*	0.187	0.254	0.222	0.292	0.101	0.092	0.102	0.154	0.151	0.133	0.013			
[12] *Cuora galbinifrons*	0.174	0.273	0.240	0.284	0.098	0.096	0.093	0.164	0.158	0.130	0.079	0.009		
[13] *Cuora amboinensis*	0.203	0.253	0.231	0.330	0.111	0.119	0.124	0.176	0.182	0.139	0.100	0.106	0.019	
[14] *Chelydra serpentina*	0.183	0.233	0.248	0.263	0.194	0.189	0.187	0.197	0.183	0.182	0.193	0.204	0.201	0.002

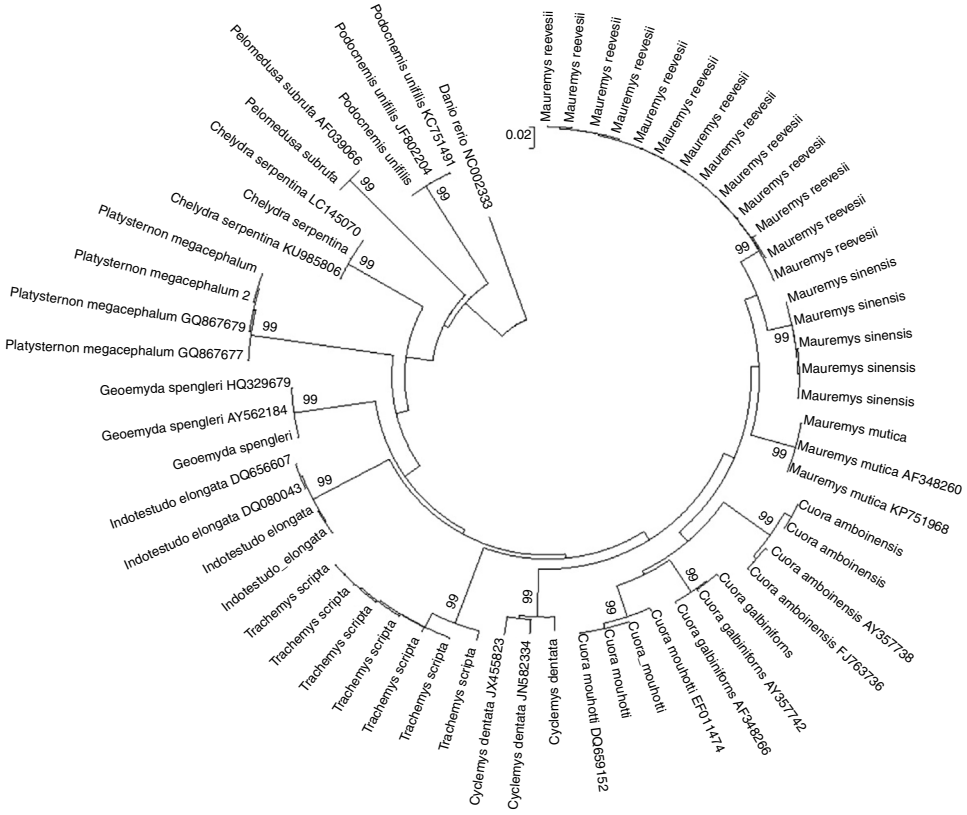

Fig. 2. Neighbor-joining (NJ) tree of full-length COI sequences of from fourteen tortoise species.

Table 6
Pairwise genetic distances between and within fourteen *Plastrum Testudinis* species based on the Mini_Tu-B barcode.

Species	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]	[14]
[1] *Trachemys scripta*	0.011													
[2] *Podocnemis unifilis*	0.309	0.000												
[3] *Platysternon megacephalum*	0.251	0.326	0.000											
[4] *Pelomedusa subrufa*	0.291	0.223	0.334	0.000										
[5] *Mauremys sinensis*	0.174	0.278	0.209	0.266	0.003									
[6] *Mauremys reevesii*	0.179	0.257	0.235	0.239	0.033	0.001								
[7] *Mauremys mutica*	0.210	0.268	0.231	0.240	0.089	0.074	0.000							
[8] *Indotestudo elongata*	0.198	0.243	0.235	0.265	0.132	0.127	0.128	0.000						
[9] *Geoemyda spengleri*	0.190	0.273	0.252	0.257	0.150	0.128	0.142	0.157	0.000					
[10] *Cyclemys dentata*	0.199	0.237	0.235	0.288	0.128	0.111	0.116	0.135	0.156	0.032				
[11] *Cuora mouhotii*	0.191	0.264	0.207	0.267	0.096	0.085	0.090	0.112	0.138	0.128	0.005			
[12] *Cuora galbinifrons*	0.169	0.296	0.232	0.285	0.094	0.094	0.099	0.122	0.148	0.132	0.058	0.003		
[13] *Cuora amboinensis*	0.198	0.271	0.233	0.315	0.095	0.105	0.115	0.138	0.166	0.127	0.105	0.099	0.020	
[14] *Chelydra serpentina*	0.157	0.289	0.225	0.266	0.207	0.182	0.181	0.217	0.161	0.171	0.180	0.193	0.206	0.000

Table 7
Pairwise genetic distances between and within fourteen *Plastrum Testudinis* species based on the Mini_Tu-D barcode.

Species	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]	[14]
[1] *Trachemys scripta*	0.006													
[2] *Podocnemis unifilis*	0.266	0.000												
[3] *Platysternon megacephalum*	0.247	0.290	0.003											
[4] *Pelomedusa subrufa*	0.279	0.203	0.299	0.000										
[5] *Mauremys sinensis*	0.164	0.294	0.218	0.270	0.002									
[6] *Mauremys reevesii*	0.170	0.300	0.236	0.240	0.058	0.001								
[7] *Mauremys mutica*	0.180	0.280	0.228	0.232	0.091	0.077	0.000							
[8] *Indotestudo elongata*	0.158	0.210	0.242	0.218	0.147	0.132	0.122	0.000						
[9] *Geoemyda spengleri*	0.190	0.261	0.252	0.244	0.164	0.180	0.163	0.170	0.000					
[10] *Cyclemys dentata*	0.175	0.249	0.249	0.253	0.117	0.126	0.137	0.138	0.170	0.036				
[11] *Cuora mouhotii*	0.192	0.293	0.212	0.235	0.112	0.083	0.107	0.143	0.181	0.143	0.002			
[12] *Cuora galbinifrons*	0.155	0.301	0.212	0.264	0.092	0.101	0.087	0.147	0.180	0.137	0.082	0.003		
[13] *Cuora amboinensis*	0.203	0.314	0.207	0.282	0.082	0.101	0.106	0.168	0.201	0.127	0.092	0.087	0.018	
[14] *Chelydra serpentina*	0.171	0.267	0.234	0.233	0.166	0.171	0.165	0.186	0.175	0.146	0.165	0.166	0.161	0.000

Table 8
Identification results of 17 turtle shell samples by the Tu-B and Tu-D barcodes.

Sample information		DNA mini-barcoding results			
		Tu-B		Tu-D	
No.	Morphological identification of species	Similarity	BLAST identification[1]	Similarity	BLAST identification[1]
1	*Mauremys reevesii*	98.62%	*Mauremys reevesii*	95.83%	*Mauremys reevesii*
2	*Mauremys reevesii*	98.62%	*Mauremys reevesii*	95.34%	*Mauremys reevesii*
3	*Mauremys mutica*	90.50%	*Cuora amboinensis*	90.30%	*Mauremys mutica*
4	*Mauremys reevesii*	98.62%	*Mauremys reevesii*	91.63%	*Mauremys reevesii*
5	*Mauremys reevesii*	98.62%	*Mauremys reevesii*		Failed
6	*Mauremys reevesii*	96.85%	*Mauremys reevesii*	86.13%	*Cuora amboinensis*
7	*Indotestudo elongata*	96.33%	*Indotestudo elongata*	85.04%	*Cuora amboinensis*
8	*Cyclemys dentata*	90.28%	*Cyclemys dentata*		Failed
9	*Mauremys sinensis*		Failed	97.46%	*Mauremys sinensis*
10	*Trachemys scripta*		Failed		Failed
11	*Mauremys sinensis*	95.96%	*Mauremys sinesis*	95.04%	*Mauremys sinensis*
12	*Mauremys sinensis*		Failed	96.20%	*Mauremys sinensis*
13	*Mauremys sinensis*		Failed	93.56%	*Mauremys sinensis*
14	*Cyclemys dentata*	90.83%	*Cyclemys dentata*		Failed
15	*Mauremys reevesii*	93.78%	*Mauremys reevesii*	96.23%	*Mauremys reevesii*
16	*Mauremys sinesis*	97.73%	*Mauremys sinesis*	95.80%	*Mauremys sinesis*
17	*Mauremys reevesii*	98.17%	*Mauremys reevesii*	94.58%	*Mauremys reevesii*

[a] BLAST results report the top bit score hit.
Green cells indicate that the identification results by the mini barcodes were correct, red cells indicate they were incorrect, and yellow cells indicate PCR failure.

could be an effective way to identify degraded *Plastrum Testudinis* samples from which the full-length COI barcodes are harder to recover.

Conclusions

The optimization of DNA extraction, including DNA from shells, was achieved despite serious degradation. Additionally, the COI barcode database of common turtle species in China was established. Two COI-based DNA mini-barcodes were selected for turtle species identification. We developed a mini barcode system for turtle species identification, by which identification success of shells reached 76.47%. The sample identification success rate of genuine Chinese tortoise shells (conforming to the Chinese Pharmacopeia) peaked at 100%. Our results provide an effective and low-cost method to identify authentic tortoise shells. Additionally, this research contributes to the safety and effectiveness of Chinese herbal medicine and to the protection of this rare turtle.

Author contributions

Conceived and designed the experiments: MYC and CSL. Performed the experiments: MYC, XYL, and JM. Sample collection: MYC, ML, and XNL. Wrote the paper: MYC and JM. Edited the manuscript: MYC and XH.

Conflicts of interest

The authors declare no conflicts of interest.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

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