Development Tortoise shell PCR Detection Kit and Evaluation Parameter of the Kit

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Abstract. Tortoise shell is the dry breastplate and carapace of the testudinidae animal turtle [Chinemys reevesii (Gray)]. Traditional identification methods can't meet the requirements of quality control of Tortoise shell market. We developed a detection kit for Tortoise shell DNA and to investigate the kit's index including specificity, sensitivity, repeatability and stability. The DNA detection kit was assembled by optimized salting out reagent and multiplex PCR reagent. The mtDNA of Tortoise shell was extracted by the kit, the multiplex PCR technique was carried out to authenticate samples of Tortoise shell. The value of mtDNA extracted by the kit was 1.80±0.05.The result of multiplex PCR indicated that two distinct bands at 335bp and 410bp were shown on the agarose gel electrophoresis in genuine Tortoise shell , but there was no band in fake samples. The specificity of the kit was 100%. The detection limit of the kit was 0.025g of each sample. The kit was effective after being frozen-thawed for 20 times, repeatability test indicated the same result through three times. The Tortoise shell DNA detection kit has properties with high specificity, good sensitivity and stability. So it is suitable for the rapid and accurate detection of Tortoise shell.

Introduction

At present, there are more than 240 kinds of turtles animals in the world as we known. Our country existed 31 turtle, belonging to 5 families and 18 genera. Tortoise shell taste salty, bitter and slightly cold. It can kidney strong bone, nourishing blood bushing and solid after the collapse. With functions of night sweats, dizzy, bones and muscles impotent soft by more, etc[1]. Tortoise shell contains animal glue, hornof protein, fat, collagen， calcium, phosphorus, zinc, copper, strontium and other elements. So Tortoise shell is commonly used precious medicinal materials. In recent years, the market demand of tortoise shell is increased. But the tortoise record in pharmacopoeia has low reproduction rate and slowly growth speed. Lead to tortoise shell market confusion and directly affect tortoise shell safety and efficacy of the clinical application.

Mitochondrial DNA (mtDNA) is the ideal object to study the animal origin, evolution and genetic analysis as a core of genetic material. It is used as the most commonly molecular markers. “China pharmacopoeia”(2010) contains the agkistrodon and the zaocys dhumnade PCR identification method, provides a new molecular biology thought identification for traditional Chinese medicine. Our team researched tortoise shell PCR identification method since 2006, extracted the tortoise shell mtDNA, used PCR and multiplex PCR technology identified tortoiseshell successfully, developed the tortoise shell DNA testing kits on the basis and evaluated the kit. Now the report is as follows.

Materials and Instrument

A Materials

Authentic tortoise shell (labeled ZPGJ - 1, ZPGJ - 2, provided by China Offices Shall of

Pharmaceutical and Biological Products, batch number: 121494-201102) as the kit of positive reference substance, 10 samples of tortoise shell identified by JiLin Province Food and Drug Inspection and Appraisal, the six genuine samples and the four fake samples.

B Reagent

10 mmol·L-1 Tris-HCl(pH=8.0), 10 mmol·L-1 Na2EDTA(pH=8.0), 10% SDS(Beijing Dingguo biotechnology company), 50 mmol·L-1 NaCl, Isopropyl alcohol, Proteinase K(20μg/mL), 2×Taq PCR Master Mix(Shanghai Tiangen biotech company).

C Instrument

H-2050R low-temperature high-speed centrifuge (Centrifuge instrument co., LTD of Xiangyi, Changsha), PCR System 9700 gene amplification (American ABI company), DYY-8B voltage steady flow electrophoresis apparatus (Beijing Liuyi instrument factory), UV WHITE-2020D UV gel imaging analyzer (American Biorad company).

Methods

A Composition of DNA kit

Dosage of kit for 20 times detection, made up of DNA extraction and PCR amplification system.

Preparation of DNA extraction reagent

With "P" means "bottle", the DNA extraction reagent made up of P1 - P6.

Preparation of PCR reaction system

Compound reaction system (25.0μL/PCR) : 2 x Taq PCR Master Mix 12.5μL; Cytochrome b (cyt b) upstream and downstream primers each 1μL; Cytochrome C oxidase subunit I (CoI) upstream and downstream primers each 1μL; Add sterilization double evaporate water to 23.0μL; Add 2.0μL waiting for samples, positive and negative reference substance.

PCR Amplification conditions

Amplification program for 94℃ modified 5 min, then 30 cycle, condition of 94℃ modified 30s, 61 ℃ annealing 30s, 72℃ extending 30s, the last extending 72℃ 10 min.

B The kits detect tortoise shell samples

Extraction of DNA from tortoise shell with the Kit

Samples from 1g tortoise shell tripsis to 1mm3, weight 0.1g samples and add P1 500μL, P2 30μL, P3 15μL(after blending in 56℃ water bath oscillation 16-18h. Remove and add P4 500μL, gently oscillation 10min, 4℃ 11000r·min-1×10min centrifuge, take the supernatant and add isovolumetric P5, place 1h at -20℃. Remove and centrifuge 4℃ 11000r·min-1×10min, abandon the supernatant, add 70% alcohol 500μL to precipitate fully flush, 4℃ 11000r·min-1×10min centrifuge, precipitation dry at room temperature. Add P6 80μL dissolve DNA, as a template for PCR reaction.

Measurement the Concentration and Purity of DNA Extract

Take DNA extract 3μL, measured A260 and A280 with ultramicro ultraviolet spectrophotometer at 260 nm and 280 nm absorbance, calculated DNA purity and concentration determined by the ratio of A260 / A280.

PCR Detection

Add DNA extract, positive control and negative control liquid 2μL to the PCR reaction tube respectively. Place the centrifugal tube into PCR instrument, set the PCR reaction parameters, it should be tested within 1h after PCR. Pick up amplification reaction tube liquid 15μL~20μL point in the hole of 2% agarose gel with GelRed dye, 10 v/cm electrophoresis, put the gel at uv analyzer on observation, take pictures.

C Evaluation parameter of he kit

Specificity Extraction of three authentic tortoiseshell and three falsify tortoise shell identified by JiLin Province Food and Drug Inspection and Appraisal tested by kit method respectively.

Sensibility Randomly selected one authentic tortoiseshell, set sample as 1 times the gradient decreasing to 0.003g, take DNA extract 2μL for PCR detection, tested by kit method respectively.

Repeatability Randomly selected two authentic tortoiseshell and one falsify tortoise shell inspection by kit method respectively under the condition of the same laboratory, by the same

experimenter repeat 3 times.

Stability Randomly selected a kit from -20℃, dissolved under room temperature, then placed -20℃, frozen respectively after 1, 5, 10 and 20 times, test DNA extraction, negetive and positive reference substance within the kit.

Results

A Detection purity and concentration of tortoise shell extract sample mtDNA by kit

Purity of the sample mtDNA was 1.80±0.05 extracted by kit, the concentration was 1.90μg/L. It showed that the DNA samples have no protein and RNA pollution extracted by kit (table 1).

Tab.1 Purities and concentration of 12 *Tortoise shell* samples

Number	Size (g)	Genuine or Counter fact	A260	A280	purity	C/μg·L^{-1}
ZPGJ-1	5	Genuine	0.378	0.209	1.81	1.89
ZPGJ-2	5	Genuine	0.376	0.211	1.78	1.88
JLGJ-1	5	Genuine	0.382	0.215	1.78	1.91
JLGJ-2	5	Genuine	0.379	0.213	1.78	1.90
JLGJ-3	5	Genuine	0.380	0.209	1.82	1.90
JLGJ-4	5	Genuine	0.378	0.211	1.79	1.89
JLGJ-5	5	Genuine	0.382	0.213	1.79	1.91
JLGJ-6	5	Genuine	0.380	0.210	1.81	1.90
JLGJ-7	5	Counter fact	0.377	0.212	1.78	1.89
JLGJ-8	5	Counter fact	0.381	0.208	1.83	1.91
JLGJ-9	5	Counter fact	0.379	0.214	1.77	1.90
JLGJ-10	5	Counter fact	0.383	0.211	1.82	1.92

B Tortoiseshell mtDNA PCR products agarose gel electrophoresis

After specific pairs of primers PCR amplification, three authentic tortoise shell mtDNA specific bind with cyt b and CoI primers, amplification products appear two clear bands in 335 bp and 410 bp, and consistent with positive reference substance location, negative reference substance and three falsify tortoise shell without amplification band (Figure 1).

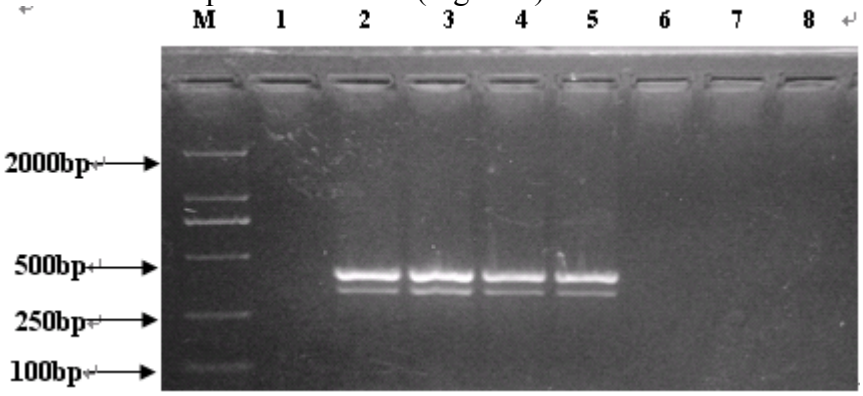

Fig.1 Agarose gel electrophoresis of the *Tortoise shell* sample by kit
M: DL2000 DNA Marker; 1: negative control ;2: positive control;
3:JLGJ-1; 4:JLGJ-2; 5:JLGJ-3;6:JLGJ-7;7: JLGJ-8; 8:JLGJ-9

C Evaluation parameters of the kits

Specificity Tortoise shell samples test by kiti dentified by JiLin Province Food and Drug Inspection and Appraisal, all authentic tortoise shell appear two clear bands in 335 bp and 410 bp, and consistent with the positive control, and all falsify tortoise shell and negative control don't appear band, kit appraisal results and JiLin Province Food and Drug Inspection and Appraisal method identification results are identical, kit specificity is 100% (Figure 1).

Sensibility The results showed that tortoise shell sample is 0.025g still can amplify the target two

bands in 335 bp and 410 bp by the method of kit (Figure 2).

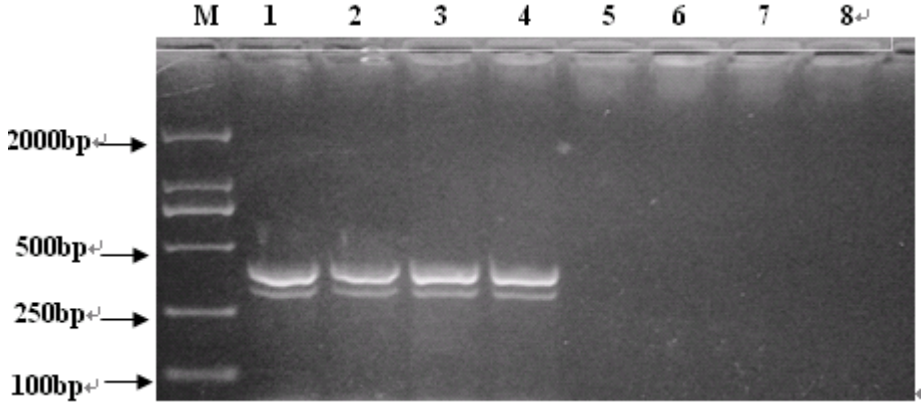

Fig. 2 Agarose gel electrophoresis of the *Tortoise shell* in the kit assay sensibility
M:DL2000 DNA Marker; 1: positive control; 2-7: weight of the *Tortoise shell* samples is 0.1g-0.003g;8: negative control

Repeatability Two authentic tortoise shell and one falsify tortoise shell tested 3 times repeatly, authentic tortoise shell appear two clear bands in 335 bp and 410 bp, and consistent with the positive control, and falsify tortoise shell and negative control don't appear band, explaining kit has good repeatability(Figure omit).

Stability Kit dealt with 1, 5, 10, 20 times respectively by repeated freezing and thawing can still extract tortoise shell DNA and amplify the target bands in 335 bp and 410 bp, show that kit has a good stability (Figure 3).

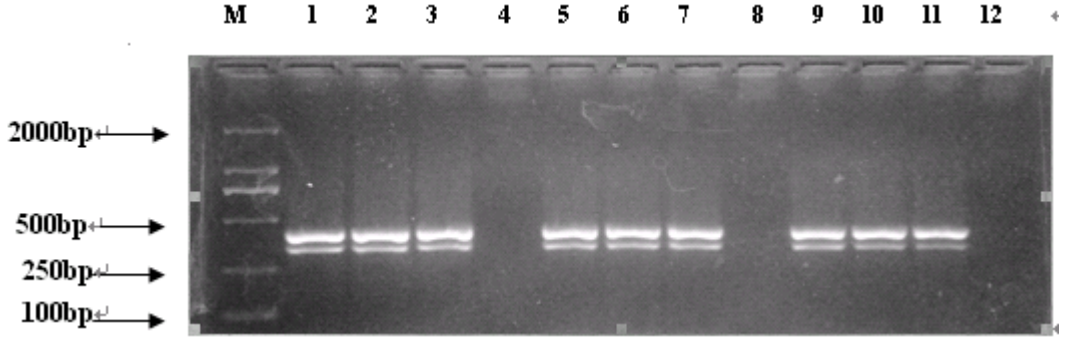

Fig. 3 Agarose gel electrophoresis of the *Tortoise shell* in the kit assay stability
M: DL2000 DNA Marker;1、5、9-the result of testing positive control after frozen-thawed of the kit for 5、10、20 times respectively; 4、8、12- the result of testing negative control after frozen-thawed of the kit for 5、10、20 times respectively; 2、6、10- indicated the result of testing JLGJ-4 by kit after frozen-thawed of the kit for 5、10、20 times respectively;3、7、11- indicated the result of testing JLGJ-5 by kit after frozen-thawed of the kit for 5、10、20 times respectively

Discussion

Traditional Chinese medicine of tortoise shell quality evaluation method is mainly appearance, microstructure and physical and chemical identification, etc. Traditional appearance identification is the main basis of breastplate and carapace properties such as shape, color, smell and texture characteristics [2], the method is simple, quick, but the lack of clear boundaries. Microscopic identification method is the magnification characteristic of tortoise shell texture and plaques, applicable to the broken appearance of tortoise shell, but this method standard has not yet mature. Physical and chemical identification of tortoise shell is analysis it's containing in the main chemical composition or the characteristic components through physical or chemical methods, in order to achieve the purpose of its authenticity identification [3], but tortoise is very similar to the chemical composition, so it brought difficulties for the physical and chemical identification of tortoise shell.

Tortoise shell dentification method in China pharmacopoeia(2010) is thin layer chromatography (TLC). TLC method can contain multiple samples and more information in a chromatography plate, it has the dual role of isolation and identification. Hanying Gu[4] identified Ann cloth armor, red mud ear tortoise shell, the Burmese tortoise shell, the flower tortoise shell and authentic tortoise shell using TLC, chromatogram shows that five kinds of tortoise shell contains the free amino acids composition is very similar, it is difficult to distinguish between significantly. Thus, although TLC method can effectively analysis, identify relevant component of tortoise shell, but can't infer that tortoise shell source of species.

Tortoise shell species differences in the traceability is genotype differences, namely differences in DNA sequence. Therefore, to identify tortoise shell by comparing the difference between the different species tortoiseshell gene sequences become a reliable new method. At present, the DNA molecular diagnostic techniques used in traditional Chinese medicine identification has achieved great development[5,6]. “China pharmacopoeia”(2010) contains the agkistrodon and the zaocys dhumnade PCR identification method[7], based on the above technical support, our team develop tortoiseshell DNA testing kits, including two system of DNA extraction and PCR amplification, can identify different species of tortoise shell one step.

We found that different manufacturers, different batches reagent can affect the result of the extraction of tortoise shell DNA after repeated experiments, thus, the DNA extraction kit unify reagent, avoiding the different sources, different batches of the unknown impact of factors on the experiment. In addition, the kit step is simple to extract the tortoise shell mtDNA, the DNA extraction process can be completed in 2h remove water bath time, shortens the appraisal cycle. Finally, DNA purity can reach 1.80±0.05 by kit, shows that this method to extract DNA was of high purity, is advantageous to the subsequent experiments.

Multiplex PCR technology is used in kit, pairs of specific primers design to aim at Cytb and COI gene of the mtDNA, has higher specificity, can successful identify the tortoise shell included in pharmacopoeia and other species of tortoise shell[8]. Annealing temperature determine success or failure of experiment by pairs of primers for PCR amplification, choose temperature at 61℃ after repeated experiments, authentic tortoiseshell appear two clear bands in 335 bp and 410 bp at this annealing temperature.

Our team can fast identify 10 kinds of Chinese medicinal materials using polymerase chain reaction. Mingcheng Li[9, 10] studied mink heart mtDNA characteristics, and established the mink heart DNA fingerprint characteristic appraisal method; Yujuan Gu[11]identified pilose antler application RAPD. Shuai Wang[12]established ginseng directly amplified fragment length polymorphism DNA fingerprint.

Tortoiseshell DNA testing kit developed by our team has the following advantages: first, the kit identification is realized by using one-step process from extraction to amplification, shorten the time, can identify a large number of samples be in a short period of time; Second, the kits parameters show that, this kit has the very good specificity, sensitivity, repeatability and stability; Third, operation can be realized the real experimental process standardization, automation, simple operation with the kit manual in strict accordance, the intuitive judgment, can effectively alleviate the lack of professional identification. Based on the above characteristics, the kit can be widely used in drug manufacturers and drug inspection agency.

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