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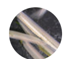

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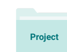

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Authentication of an Animal Crude Drug, Zaocys, by Diagnostic PCR

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A pair of diagnostic primers for distinguishing the Chinese crude drug Zaocys (*Zaocys dhumandes*) from its substitutes was designed based on the sequence data of the original animal of the drug and substitutes. Total DNAs were extracted from genuine crude drug and 5 of its substitutes, as well as from 12 species of original animal of the snake crude drug. Diagnostic PCRs were performed using the primers with these total DNAs as a template, annealing at 60—65 °C. Positive amplifications were obtained from all DNA templates of Zaocys, whereas negative amplifications were obtained from that of others. The results indicate that Zaocys samples could be definitely distinguished from its substitutes by diagnostic PCR, and no incorrect discrimination was found under the same reaction conditions. The advantages of the method in the authentication of crude drugs are also discussed in the present paper.

Key words animal crude drug; diagnostic PCR; Zaocys; diagnostic primer

The application of animal medicinal materials has a long history in China. As one of the common crude drugs, Zaocys is recorded in Pharmacopoeia of the People's Republic of China.[1] Its original animal is a non-poisonous snake of Colubridae, *Zaocys dhumnades*. In the application of Chinese medicine, Zaocys is used to dispel wind, remove obstruction of the collaterals, and relieve spasms and convulsions. However, our previous report indicated that substitutes of the crude drugs taken from some other species of snakes frequently appeared in the market.[2] For the benefit of consumers and the quality evaluation of crude drugs, correct identification of crude drugs is important. Unfortunately, the identification of animal crude drugs is always problematical because their substitutes or adulterants are very similar to the quality goods both in morphological characteristics and in organic or inorganic components, especially when it is cut into pieces. Thus, more advanced measures should be adopted for the identification of Zaocys and other animal crude drugs.

With the rapid development of biological techniques, molecular genetic markers have been introduced into the identification of medicinal materials.[3,4] Several methods, which mainly include random amplified polymorphic DNA (RAPD), arbitrary primed PCR (AP-PCR), restriction fragment length polymorphism (RFLP), PCR-RFLP and DNA sequencing, have been used for the authentication of medicinal materials until now. Although they have proven to be efficient in distinguishing genuine crude drugs from their substitutes or adulterants in previous reports,[5—14] the limitation in the application of these methods is inevitable because fine quality template DNA is necessary in these experiments and they are relatively expensive.[15]

DeSalle *et al.* designed several primers specific for diagnostic nucleotide substitutions for each of three commercial Russian sturgeons to identify the species of caviar.[16] This method was also successfully employed in the identification of Bungarus Parvus and its adulterants using a pair of diagnostic primers specific for certain DNA positions of the original animal, *Bungarus multicinctus*.[17] In the present research, we designed a pair of diagnostic primers based on the sequence data reported previously[11] for PCR identification of the animal crude drug, Zaocys.

MATERIALS AND METHODS

Samples Samples of snake crude drug Zaocys and its substitutes were purchased from markets in 7 cities in China (Table 1). The samples were kept in bottles with silica gel at room temperature for 3 or more years before study. The original animals of the samples were identified according to morphological characters. Tiny dried muscle samples were taken for DNA extraction and molecular identification.

A total of 20 individual original animals involving 12 species of snakes were also collected from Jiangsu and Anhui Provinces, China (Table 2). The specimens were identified according to morphological characters, and some muscle or liver samples were stored at −20 °C for the following research.

DNA Extraction About 0.5 g dried muscle of crude drugs was ground into a powder and transferred into two

Table 1. Samples of Snake Crude Drugs Used in the Study

Name of crude drugs	Name of species	Code	Number	Collection place
Zaocys	*Zaocys dhumnades*	Z	11	Nanjing, Jiangsu; Qizhou, Hubei; Yulin, Guangxi
Substitute of Zaocys	*Elaphe taenirua*	T	3	Changsha, Hunan; Zhangshu, Jiangxi
Substitute of Zaocys	*E. rufodorsata*	R	3	Zhangshu, Jiangxi
Substitute of Zaocys	*E. carinata*	C	3	Qizhou, Hubei
Substitute of Zaocys	*Sinonatrix annularis*	A	3	Zhangshu, Jiangxi
Substitute of Zaocys	*Ptyas korros*	K	3	Qianshan, Fujian

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15 ml centrifugal tubes with 5 ml DNA extraction buffer (sucrose 0.25 M, Tris–HCl 10 mM, Na_2EDTA 10 mM, pH 8.0) in each tube. Following slight mixing, proteinase K and sodium dodecyl sulfate (SDS) were added to a final concentration of 50—150 μg/ml and 0.8% respectively. After incubating them for 4 h at 55 °C and gently mixing them several times during the process, the sample was extracted with phenol–chloroform. Then the super aqueous phase was transferred to a fresh centrifuge tube, and 0.1 volume of 3 M NaAc and 2 volumes of ethanol were added. Later on, the tube was turned over end several times and left at −20 °C over night. DNA was precipitated by centrifugation at 5500 ***g*** for 15 min, and after discarding the supernatant, the pellet was rinsed with 70% ethanol two times and dried at room temperature. Finally, total DNA was redissolved in TE buffer or ddH_2O, and the DNA solution was stored at 4 °C for PCR.

About 0.1 g frozen tissue was cut from the original animals for total DNA extraction. The tissue was put in a stainless mortar with liquid nitrogen in it, ground into powder, and then the powder was transferred to a tube. The remaining steps were the same as those in the DNA extraction of crude drugs.

Primers Our previous study proved that the Cyt *b* gene is a good molecular genetic marker for the authentication of snake crude drugs because its variation among different species is much larger than that among intraspecific individuals.[11] Based on the DNA sequence data of Cyt *b* gene fragments (Accession Number: AF036010—AF036019, AF036021—AF036024 and AF036027—AF036034), we designed a pair of diagnostic primers, ZA-DL1 and ZA-DH1 (Chinese patent No. 99114133.4), which can be employed to amplify a 183 bp segment of the Cyt b gene of *Z. dhumnades* specifically. The universal primers, L14841 (5′-AAAAAAGCTTCCATCCAACATCTCAGCATGATGAAA-3′) and H15149 (5′-AAACTGCAGCCCCTCAGAATGATATTTGTCTCA-3′), designed by Kocher *et al.*,[18] were used to amplify a 308 bp Cyt *b* gene fragment from all templates serving as positive controls. All primers were synthesized by TaKaRa Biotechnology Co.

Table 2. Original Animals of Zaocys and Its Substitutes Used in the Study

Name of species	Code	Number	Localities
Elaphe taeniura	Et	2	Anhui
E. mandarina	Ema	1	Anhui
E. rufodorsata	Er	2	Jiangsu
E. carinata	Ec	2	Anhui
E. bimaculata	Eb	2	Jiangsu
E. schrenckii	Es	1	Jiangsu
Sinonatrix annularis	Sa	2	Jiangsu
Dinodon rufozonatum	Dr	1	Jiangsu
Zaocys dhumnades	Zd	2	Jiangsu
Ptyas korros	Pk	2	Anhui
Rhabdophis nuchalis	Rn	1	No record
Rhabdophis tigrinus	Rt	1	Anhui

PCR Reaction A total of 30 μl PCR reaction mixture was composed of 10 mM Tris–HCl (pH 8.3); 50 mM KCl; 2.5 mM $MgCl_2$; 0.1% Triton X-100; 1 unit of *Taq* DNA polymerase (Promega); 50 μM of each deoxyribonucleoside triphosphate (dNTP) (Sangon); 10 pM of each diagnostic primer; and 10—100 ng of template DNA. Amplification was carried out in a GeneAmp PCR System 2400 (Perkin Elmer) or Mastercycler Gradient (Eppendorf) programmed for predenaturalization of 4 min at 95 °C and 30 cycles of 40 s at 95 °C, 40 s at 50—65°, and 60 s at 72 °C, followed by one cycle of 5 min at 72 °C. PCR product was detected by electrophoresis in 1.5% agarose gel. To find a suitable annealing temperature range for diagnostic PCR, we performed the reactions under several different annealing temperatures. In addition to the negative control, positive controls using universal primers were also performed in the experiments.

RESULTS

Positive controls for all DNA templates, both from original animals and from crude drugs, were performed at 50 °C annealing temperature with the purpose of confirming the quality of DNA templates. A DNA fragment of about 308 bp was amplified from each template (Fig. 1).

In diagnostic PCR for original animals using diagnostic primers, a 183 bp DNA segment was clearly amplified from the template of *Z. dhumnades* at 55 °C annealing temperature, whereas only a faint DNA fragment was obtained from some templates of other species, and no DNA fragment appeared in the application of others under the same conditions. When the annealing temperature rose to 60 °C, the DNA fragment was amplified well from the template of *Z. dhumnades* only, and no DNA fragment appeared in the rest of the amplifications that had a weak band at 55 °C annealing

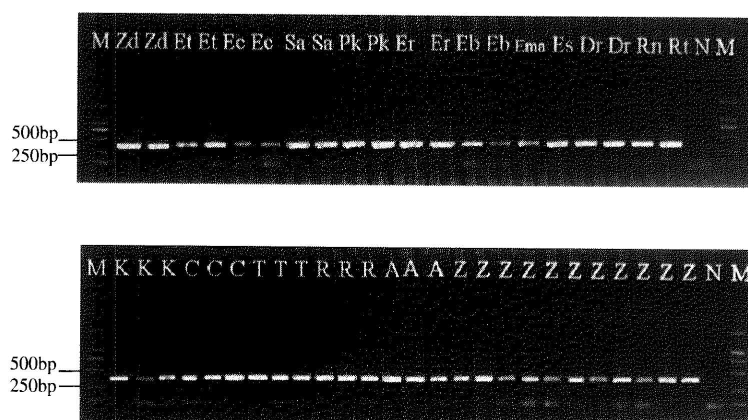

Fig. 1. Positive Controls Annealing at 50 °C for All DNA Templates Resolved by 1.5% Agarose Gel Electrophoresis

The sample codes are shown in Tables 1 and 2. N, negative control; M, DNA molecular marker DL-2000 (TaKaRa Biotechnology Co.).

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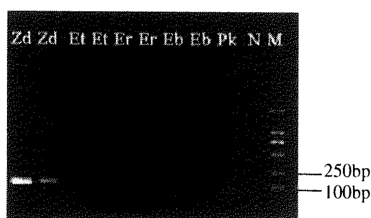

Fig. 2. Diagnostic PCR Annealing at 60 °C for Original Animals Resolved by 1.5% Agarose Gel Electrophoresis

The sample codes are shown in Table 2. N, negative control; M, DNA molecular marker DL-2000 (TaKaRa Biotechnology Co.).

Fig. 3. Diagnostic PCR Annealing at 60 °C for Crude Snake Drugs Resolved by 1.5% Agarose Gel Electrophoresis

The sample codes are shown in Table 1. N, negative control; M, DNA molecular marker DL-2000 (TaKaRa Biotechnology Co.).

temperature (Fig. 2).

Diagnostic PCR for crude snake drugs was also carried out using ZA-DL1 and ZA-DH1 at 55 °C, 60 °C and 65 °C annealing temperatures, respectively. The results indicated that a 183 bp DNA fragment was amplified from all Zaocys templates at the above annealing temperatures. The fragment appeared in some amplifications of DNA template from its substitutes at 55 °C. No DNA fragment was amplified from these templates when the annealing temperature was at 60 °C or above (Fig. 3). These results repeated well.

DISCUSSION

A DNA molecule carries a large amount of information that is unique in each species. Although the DNA of crude drugs is degraded during preparation, transportation and storage of the medicinal materials, enough DNA can still be obtained with more tissue used in DNA extraction. We have reported our studies on the authentication of snake crude drugs by RAPD analysis and DNA sequencing.[11,19] For the purpose of identification, DNA templates extracted from snake crude drugs and some other animal crude drugs were also amplified and sequenced.[8—11,13,14] In addition, PCR-RFLP is another method for the identification of medicinal materials.[12] All these results imply that DNA from crude drugs can serve as templates for PCR, in spite of inhibitors which sometimes exist in DNA solution.[20] However, the reproducibility of RAPD analysis is badly affected by the quality and concentration of template DNA, the ratio of template to primer, and slight fluctuation of reacting components or cycling parameters. As for the PCR-RFLP method, the length of PCR products also confines its utilization, since the number of restriction enzyme sites is limited in the DNA segments between the two PCR primers. Although the sequence analysis of PCR products reveals more variation among species, and has quite good stability of results, the relatively high expense of DNA sequencing and the sensitivity to contamination in the PCR reaction using universal primers obstruct its wide use in the quality control of crude drugs. Therefore, a more reproducible and expedient method is needed to authenticate Chinese medicinal materials.

To develop a more simple and efficient method for the identification of crude drugs, we successfully distinguished Bungarus Parvus from 5 of its adulterants using a pair of highly specialized diagnostic primers for Bungarus Parvus.[17] In the present study, we also designed a pair of diagnostic primers that match exactly with a definite DNA nucleotide sequences of *Zaocys dhumnadus*, and incompletely with the sequences of templates from other species, especially at the 3′ end of the primers. Therefore, the PCR performed with the primers is a site specific PCR. Theoretically, the annealing temperature of diagnostic primers is above 80 °C. It is important for diagnostic PCR that the 3′ end of the primers should be mismatched with the sequences of the substitutes. A high-stringency PCR reaction with primers specific for *Zaocys dhumnadus* will give a positive reaction only from the template DNA of genuine crude drugs and a negative reaction from that of substitutions. In order to verify the quality of templates from the crude drugs, all template DNAs were amplified using universal primers at a 50 °C annealing temperature, and all PCR reactions under this condition gave clear bands on agarose gel electrophoresis. These results proves that the DNA templates used here are suitable for PCR. It also indicates that a particular DNA band should be amplified respectively from these templates using diagnostic primers if the primer can find an appropriate annealing position on the template DNA, since two annealing sites of the primers are located on the 308 bp band. If the diagnostic primer fails to match the template, especially at the 3′ end of primers, the reaction will give out a negative result and no DNA band appear on agarose gel.

The results shown above (Figs. 1—3) were obtained from repeated experiments with same reaction parameters performed independently by two experimenters. Our assumption discussed above is supported by these results. The results show that Zaocys samples could be definitely distinguished from their substitutes by diagnostic PCR using the specific primers annealing at 60—65 °C, and no incorrect discrimination was found at these temperatures. Therefore, the method has high accuracy and excellent reproducibility. In addition, because the diagnostic primers are specific for Zaocys only, and diagnostic PCR is performed under high-stringency conditions, the result will not be influenced by foreign DNA.

Our method reported here has several advantages in the authentication of crude drugs: 1) Only a small quantity of sample is required for examination. 2) The process is simple and costs little. 3) The result will not be affected by contamination in samples and offers an accurate identification with excellent reproducibility. Therefore, we believe the method is practicable in the quality control of Chinese crude drugs.

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