Authentication of Chinese Crude Drug, Gecko, by Allele-Specific Diagnostic PCR

Zhongquan Liu[1], Yiquan Wang[1,*], Kaiya Zhou[1], Demin Han[1], Xuegan Yang[1], Xunhong Liu[2]

[1] Institute of Genetic Resources, Nanjing Normal University, Nanjing, China
[2] Department of Chinese Medicinal Materials, Nanjing University of Traditional Chinese Medicine, Nanjing, China

Received: June 15, 2000; Accepted: September 10, 2000

Abstract: **Based on the sequences of the mitochondrial 12S rRNA gene fragment of 17 samples from Gekkonidae, Salamandridae, Agamidae and Hynobiidae, respectively, a pair of allele-specific primers was designed for differentiating the Chinese medicinal material Gecko from its adulterants by PCR. The results of amplification with the primers indicate that amplicons from the templates of *Gekko gecko* were clearly revealed by agarose gel electrophoresis, whereas no evident amplicons were found from other species. The primers were employed to identify crude drug samples from different sources. Among a total of 9 samples, 3 were diagnosed as genuine Gecko. This result is consistent with morphological identification and DNA sequence analyses.**

Gecko is recorded in Pharmacopoeia of the People's Republic of China as dried body of *Gekko gecko* that lives mainly in the tropical region in southern China and southeastern Asia. It is a common Chinese crude drug used mainly to reinforce the function of the lung and the kidney (1). There are a great number of substitutes or adulterants of the Gecko in the market owing to a high demand. The common substitutes were made of *Gekko japonicus* and *G. swinhonis* of Gekkonidae, *Agama himalayna* of Agamidae, *Tylototriton verrucosus* and *T. taliangensis* of Salamndridae (2), (3), (4). The traditional identification of animal crude drugs by morphological characteristics is always problematical because their substitutes or adulterants are very similar to the original goods. The purpose of the present study is to establish a convenient and accurate identification method for the crude drug, Gecko.

A fragment of about 400 bp of the mitochondrial 12S rRNA gene from 7 samples [*G. gecko* (4 individuals), *L. stoliczkana*, *P. cocincinus*, *T. taliangensis*], was amplified and sequenced. The sequences were submitted to GenBank with accession numbers from AF236819 to AF236823. After alignment of these sequences and the sequences of 9 relevant species from a published reference (5), 262 variable sites were found among the thirteen species. According to the sequence pattern of the gene fragment, two haplotypes were found among four *G. gecko* individuals. Sequence diversity was estimated as 5.20% between the two haplotypes of *G. gecko*, 24.13% – 29.69% between *G. gecko* and other species of Gekkonidae, and 31.02% – 38.42% between *G. gecko* and species of other families (Table **1**).

Planta Med 67 (2001) 385 – 387

ISSN: 0032-0943

Table 1 Numbers of transitions/transversions (upper triangle) and the percentage of sequence diversity (lower triangle) of 12S rRNA genes of 13 species of original animals examined with gaps deleted in each pairwise comparison

OTUs	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1 Gg1*		0/0	0/0	18/2	18/2	48/45	47/46	54/46	57/45	51/45	53/51	57/50	58/50	44/46	49/63	67/66	65/72
2 Gg2	0		0/0	18/2	18/2	48/45	47/46	54/46	57/45	51/45	53/51	57/50	58/50	44/46	49/63	67/66	65/72
3 Gg3	0	0		18/3	18/3	48/46	47/47	53/47	56/46	51/46	53/52	58/49	58/49	45/45	49/64	67/65	64/73
4 Gg4	5.20	5.20	5.20		0/0	54/49	54/50	57/51	58/45	56/45	59/50	61/52	62/52	49/48	53/63	68/68	63/74
5 Gg5	5.20	5.20	5.20	0		54/49	54/50	57/51	58/45	56/45	59/50	61/52	62/52	49/48	53/63	68/68	63/74
6 Ce	24.54	24.54	24.87	26.41	26.41		0/3	48/59	51/50	46/51	51/57	51/52	52/52	49/46	54/54	56/66	59/74
7 Cr	24.54	24.54	24.87	26.67	26.67	0.75		47/60	50/52	45/52	50/58	52/51	53/51	49/47	53/55	57/65	60/73
8 Gs	27.17	27.17	27.17	28.72	28.72	28.16	18.16		0/5	29/32	52/53	46/59	47/59	40/53	53/55	62/59	59/69
9 Gh	27.27	27.27	27.27	27.61	27.61	26.93	27.2	1.33		29/33	53/55	43/58	44/58	40/45	55/54	65/59	61/71
10 Gj	25.74	25.74	26.01	27.15	27.15	26.01	26.01	16.49	16.58		48/52	39/55	40/55	40/40	56/52	58/66	59/64
11 Gm	27.81	27.81	28.15	28.31	28.31	28.13	28.13	28.30	29.43	27.40		55/50	56/50	49/53	55/54	67/63	56/79
12 Hb	28.76	28.76	28.76	29.43	29.43	26.61	26.61	27.78	27.01	25.34	27.78		2/0	45/41	55/60	65/60	63/70
13 Hf	29.03	29.03	28.76	29.69	29.69	26.87	26.87	28.04	27.27	25.61	28.04	0.52		47/41	54/60	63/60	63/70
14 Tt	24.13	24.19	24.19	25.46	25.46	24.93	24.93	25.14	23.29	21.86	26.98	22.93	23.47		57/49	57/56	51/68
15 Ta	31.02	31.02	31.30	32.13	32.13	29.92	29.92	30.50	30.70	30.34	30.62	32.21	31.92	29.44		52/53	58/56
16 Ls	37.57	37.57	37.29	38.42	38.42	34.37	34.37	34.78	35.22	35.43	37.14	35.41	34.84	32.10	42.15		54/50
17 Pc	37.85	37.85	37.85	37.85	37.85	36.74	36.74	36.16	36.87	34.45	37.82	37.05	37.05	32.24	32.57	29.21	

*codes are same as described in Table **2**.

In the diagnostic assay, all samples of the 15 species of original animals were amplified using the universal primers L1091 and H1478 for positive controls, and approximately 400 bp fragments were amplified (Fig. **1 A**). However, while using allele-specific primers IG-L03 and IG-H03 for PCR under the same annealing conditions, we could only obtain a 260 bp amplified DNA band from *G. gecko*, and no PCR product from the other animals (Fig. **1 B**). The results were reproducible until the annealing temperature was increased to 60 °C. Similar observations were found for the nine medicinal crude drugs (Fig. **2 A** and **B**).

Although the DNA of crude drugs is usually degraded during preparation, transportation and storage of the medicinal materials (6), in our case, enough DNA could still be obtained from them for authentication. In the present study, DNA from Gecko and its adulterants was amplified and sequenced directly with universal primers. The results of sequence alignment showed that interspecific diversity of a 12S rRNA gene fragment of the samples studied was more evident than the sequence diversity between the two haplotypes of *G. gecko*. So, the genuine Gecko can be clearly distinguished from its adulterants by comparing the DNA sequences. However, DNA sequencing is a time and cost consuming method.

The allele-specific primers we designed complement the corresponding sequence of *G. gecko* perfectly but not that of other species. The templates of all original animals of *G. gecko* and three samples of genuine Gecko could be amplified with the primers and gave amplicons of the expected size, but no evident PCR products in the others. These results are reproducible at annealing temperatures from 55 °C to 60 °C.

With the rapid development of PCR technology, allele-specific diagnostic PCR is widely used for clinical diagnosis. DeSalle et al. reported a PCR method to identify the species of caviar from three commercial Russian sturgeons (7). This method, called diagnostic PCR in our previous reports, was also employed to authenticate crude drugs of snakes and tortoise plastrons (8), (9), (10). After designing a pair of allele-specific primers for a definite species, the process of PCR identification of the species is very simple. We conclude that allele-specific diagnostic PCR is a time-effective and low-cost method for the authentication of Chinese medicinal materials. This method will aid the quality control of Chinese medicinal materials and species identification of any other biomaterials.

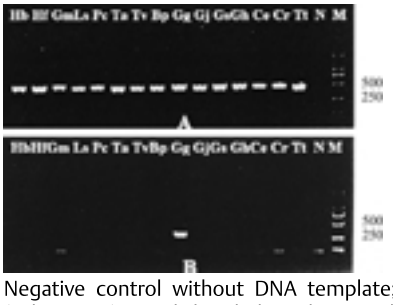

Fig. 1 Agarose gel electrophoresis of PCR products of mtDNA 12S rRNA gene fragment from original animals. **A**: Positive control with primers L1091 and H1478 annealed at 55 °C. **B**: with the primers IG-L03 and IG-H03 annealed at 55 °C. N: Negative control without DNA template; M: DNA marker, DL2000 (Takara Co.). Weak bands less than 100 bp in Gm, Cr, and N lanes are the primer dimers. Codes are same as described in Table **2**.

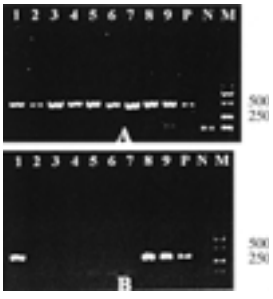

Fig. 2 Agarose gel electrophoresis of PCR products of mtDNA 12S rRNA gene fragment from Chinese crude drugs. **A**: Positive control with primers L1091 and H1478 annealed at 55 °C. **B**: with the primers IG-L03 and IG-H03 annealed at 55 °C. Lane 1 – 9: JD1 – JD9 crude drugs; P: Positive control of original animal *Gekko gecko*, N: Negative control without DNA template; M: DNA marker, DL2000 (Takara Co.).

Materials and Methods

Samples of original animals used to prepare crude drug and its substitutes or adulterants were collected from different localities in China (Table **2**). They were kept at – 20 °C with the

exception of one sample which was taken from a formalin preserved specimen. Voucher specimens have been placed in the collection of Nanjing Normal University (NJNU), China.

Nine samples of chinese crude drugs Gecko used in this study were as follows: five samples (JD1 – 5) were provided by Nanjing University of Traditional Chinese Medicine, two samples (JD6 – 7) obtained from Jiangsu Institute for Drug Control, and two samples (JD8 – 9) purchased from Kunming Crude Drug Store.

Samples of crude drugs were either washed with sterilized water and dried in an oven to remove a possible contamination with foreign DNA, or the superficial part of samples was removed carefully with a sterile knife. The total DNA was isolated from frozen samples and crude drugs using a protocol described in the previous report (10).

A pair of universal primers, L1091 (5′-AAAAAGCTTCAAACTGGGATTAGATACCCCACTAT-3′) and H1478 (5′-TGACTGCAGAGGGTGACGGGCGGTGTGT-3′), were used for the amplification of the 12S rRNA gene fragment, and they were also employed for positive control in subsequent diagnostic PCR. The amplification was carried out in a PTC-200 thermocycler (MJ Research) in a 30 μl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 150 μM of each dNTP, 0.3 μM of each primer (synthesized by TaKaRa Co.), 100 ng template DNA, and 1 U *Taq* DNA polymerase (Promega). The reaction mixtures were denatured at 95 °C for 4 min and subjected to 30 cycles of 40 s at 95 °C, 40 s at 55 °C or 60 °C, 1 min at 72 °C, and a final extension step of 5 min at 72 °C. Amplified products were purified using a DNA purification kit (Shanghai Watson Bioengineering Inc.) according to the manufacturer's instruction, and then sequenced directly with BigDye™ on an ABI PRISM™ 310 Genetic Analyzer (Perkin Elmer).

Based on the obtained DNA sequence data of Gecko and its adulterants, a pair of primers, IG-L03 and IG-H03 (Chinese patent pending), was designed for diagnostic PCR to authenticate Gecko (Fig. **3**). This pair of primers is specific for *G. gecko* only, and amplifies a 260 bp DNA segment from the 12S rRNA gene. The diagnostic PCR was performed under the conditions mentioned above.

Table 2 Samples of original animals used in present study

Code	Species	Voucher no.	Locality
	Gekkonidae		
Gg	*Gekko gecko*	NJNU L960901 ~ 5, NJNU L99Y01 ~ 2	Nanning, Guangxi; Yunnan
Gj	*G. japonicus*	NJNU L9401101 ~ 5	Hefei, Anhui
Gs	*G. swinhonis*	NJNU L950403 ~ 5	Xuzhou, Jiangsu
Gh	*G. hokouensis*	NJNU L9604018 ~ 19	Shaowu, Fujian
Ce	*Cyrtopodion elongatus*	NJNU L9509W01	Tuokexun, Xinjiang
Cr	*C. russowi*	NJNU L9509W002	Kuerle, Xinjiang
Tt	*Teratoscincus toksunisis*	NJNU L961001 ~ 2	Tulufan, Xinjiang
Hb	*Hemidactylus bowingii*	NJNU L950607	Qionghai, Hainan
Hf	*H. frenatus*	NJNU L950628	Leizhou, Guangdong
Gm	*Gehyra mutilatus*	NJNU L950611	Qionghai, Hainan
	Agamidae		
Ls	*Laidaloa stoliczkana*	NJNU L9509001 ~ 2	Kuerle, Xinjiang
Pc	*Physignathus cocincinus*	NJNU L9912001 ~ 2	Yunnan
	Salamandridae		
Ta	*Tylototriton taliangensis*	NJNU U980710 ~ 1	No record
Tv	*T. verrucosus*	NJNU U980054, NJNU U99Y03	Tengchong, Yunnan
	Hynobiidae		
Bp	*Batrachuperus pinchonii*	NJNU U940601 ~ 2	No record

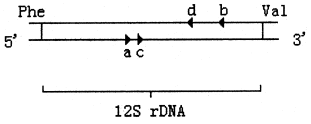

Fig. 3 Schematic organization of mitochondrial 12S rRNA gene. Arrows indicate orientation and approximate position of the primers. a: L1091; b: H1478; c: IG-L03; d: IG-H03. Phe, Val: $tRNA^{Phe}$ and $tRNA^{Val}$ genes, respectively.

Acknowledgements

The authors would like to express their thanks to Prof. P. C. Shaw for his comments and linguistic corrections and to Dr. Gong Dajie and Dr. Jiang Jianping for offering some animal samples. The manuscript has benefited greatly from the constructive comments and helpful suggestions of anonymous referees. This work is supported by National Natural Science Foundation of China (NSFC No. 39870913), a grant from the Jiangsu Educational Committee (No. 98KJB360010) and a grant from the 333 Project of Jiangsu Province, China.

References

[1] The Pharmacopoeia Commission of PRC (ed). Pharmacopoeia of the People's Republic of China. (English edition 1977) Volume 1. Beijing: Chemical Industry Press, 1997: 81
[2] Chen JH, Deng ZJ. The authentication of a few substitutes of *Gekko gecko*. Journal of Chinese Medicinal Materials 1982; 5: 10
[3] Zhang CY. *Gekko gecko*. Journal of Chinese Medicinal Materials 1988; 11: 19 – 22
[4] Liu XH, Wang QG, Chen B. The protein viscosity identification on *Gekko gecko* and its substitutes. Journal of Chinese Medicinal Materials 1992; 15: 20 – 1
[5] Han DM, Zhou KY, Wang YQ. Phylogenetic relationships of ten species of Gekkonid lizards (Gekkonidae: Lacertilia) inferred from mitochondrial 12S ribosomal RNA gene sequences. Acta Zoologica Sinica 2001; (in press)
[6] Wang YQ, Zhou KY, Xu LS, Karl Tsim. The present situation and application of the molecular identification of Chinese medicinal materials. Progress in Pharmaceutical Sciences 1999; 23: 193 – 9
[7] DeSalle R, Birsteln VJ. PCR identification of black caviar. Nature (London) 1996; 381: 197 – 8
[8] Wang YQ, Zhou KY, Xu LS, Xu GJ. Sequencing of Cyt *b* gene fragments and PCR identification of "Jinqian baihuashe" (*Bungarus parvus*) and its adulterants. Acta Pharmaceutica Sinica 1998; 33: 941 – 7
[9] Wang YQ, Zhou KY, Xu LS, Dong Tina TX, Karl WK Tsim. Authentication of an animal crude drug, Zaocys, by diagnostic PCR. Biological & Pharmaceutical Bulletin 2000; 23: 585 – 8
[10] Liu ZQ, Wang YQ, Zhou KY, Yang XG, Cao L, Liu WX. Study on highly specific diagnostic PCR of the traditional Chinese medicine turtle shells and their original animals. Acta Pharmaceutica Sinica 1999; 34: 941 – 5

Prof. Yi-Quan Wang

Institute of Genetic Resources
College of Life Science
Nanjing Normal University
122# Ninghai Road
Nanjing, 210097
China
E-mail: wangyqnj@jlonline.com
Fax: 86-25-3738174