Molecular Authentication of the Animal Crude Drug Sailonggu (Bone of *Myospalax baileyi*)

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Two pairs of allele-specific diagnostic primers (SL1L/SL1H and SL2L/SL2H) for distinguishing the Chinese crude drug Sailonggu (bone of plateau zokor, *Myospalax baileyi*) from its substitutes were designed based on complete sequences of mitochondrial 12S rRNA and cytochrome *b* genes of the original animals of Myospalacinae, bamboo rat *Rhizomys sinensis* and black lipped pika *Ochotona curzoniae*. Total DNA was extracted from crude drug samples and original animals. Allele-specific diagnostic PCRs were performed using these primers with the total DNA as a template annealing at 65 °C. Positive amplifications were obtained from all DNA templates of Sailonggu and *M. baileyi*, whereas negative amplifications resulted from those of other zokors, the bamboo rat and black lipped pika. These results indicate that Sailonggu samples can be definitely distinguished from their substitutes by diagnostic PCR, and no incorrect discrimination was found under the same reaction conditions. Each of the two diagnostic primer pairs can be used to distinguish crude drug Sailonggu from its substitutes or adulterants. The three Sailonggu samples studied were diagnosed as genuine Sailonggu. In addition, the results of sequence alignment and phylogenetic analysis are congruent with that of the allele-specific diagnostic PCR.

Key words crude drug Sailonggu; *Myospalax baileyi*; allele-specific diagnostic PCR; molecular authentication; mitochondrial gene sequence

The application of animal medicinal materials has a centuries-old history in China. Zokors (Myospalacinae) were recorded as a type of animal crude drug in *Bencao gangmu*, a famous work written by Li Shizhen about 400 years ago during the Ming Dynasty, which identified 1892 kinds of medicines. The flesh and bone of the zokors were reported to cure many kinds of inflammation.¹⁾ The dried bone of plateau zokor (*Myospalax baileyi*) is called Sailonggu, and has been used medicinally by local Tibetans in the Qinghai Province in China.²⁾ In the middle of the 1970s, it was found that the Tibetan drug Sailonggu could cure rheumatic diseases and is an ideal substitute for the Chinese crude drug "tiger bone."^{3,4)} Sailong Rheumatic Liquor, made from the bone of plateau zokor, has been produced since 1995 by the Beijing Tongrentang Medicinal Liquor Factory, Beijing Tongrentang Medicine Industry Limited Group.

Plateau zokor (*M. baileyi*) is found in plateau habitats in Qinghai, Gansu and Sichuan Provinces.⁵⁾ However, it shares habitats with the Gansu zokor (*M. cansus*), the bamboo rat (*Rhizomys sinensis*), and several other rodents such as the black lipped pika (*Ochotona curzoniae*). Consequently, Sailonggu is mingled with adulterants in the course of crude drug collection. The identification of animal crude drugs has always been 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 cut into pieces. The mtDNA sequences of 12S rRNA and cytochrome *b* (cyt *b*) genes have been used successfully by Wang and Zhou⁶⁾ as molecular markers in the authentication of a variety of animal crude drugs. These were investigated in the present study in order to develop molecular genetic markers for the identification of Sailonggu.

MATERIALS AND METHODS

Sample Collection Twenty-one individuals, representing seven species of zokors of the genus *Myospalax*, were used in this study (Table 1). One Chinese bamboo rat (*Rhizomys sinensis*) and one black lipped pika (*Ochotona curzoniae*) were also included. The original animals of the samples were identified according to morphological characteristics. The crude drug Sailonggu samples were provided by Beijing Tongrentang Medicinal Liquor Factory, Beijing Tongrentang Medicine Industry Limited Group. All specimens are deposited in the collection of Nanjing Normal University (NNU). Tissue samples derived from muscle are preserved in 95% ethanol.

DNA Extraction Total genomic DNA was extracted from the ethanol preserved muscle samples as described in Sambrook *et al.*⁷⁾ For isolation of total DNA from the crude drug Sailonggu, about 1.0 g bone pieces taken from each sample were washed with water and ddH₂O twice, respectively, and dried at 80 °C. The bone pieces were exposed under a 20 W ultraviolet lamp for 40 min in order to remove *exo*-DNA contaminations. The samples were ground to a fine powder using liquid nitrogen. One milliliter of 0.5 M EDTA (pH 8.0) was added to 0.5 g bone powder in a 1.5 ml Eppendorf tube, and incubated for 72 h at 56 °C, then centrifuged for 5 min at 5000 *g* in 4 °C to change EDTA twice in the course of incubation. The sample was then incubated for 4 h at 65 °C in 0.5 ml of lysis buffer (0.5 M EDTA, 10 mM Tris-HCl (pH 8.5), 0.5% SDS, 200 µg/ml Proteinase K). After digestion, DNA was extracted with phenol and chloroform and precipitated with ethanol. The final volume of the extract varied between 100—120 µl.

PCR and Sequence Analysis Two pairs of primers, L14724 (5'-C GAA GCT TGA TAT GAA AAA CCA TCG TTG-3') and H15917 (5'-C GGA ATT CCA TTT TTG GTT

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Table 1. Samples of Original Animals and Crude Drugs Used in This Study

Species	Code ^{a)}	Locality	Catalogue no.	GenBank Accession no.	
				12S rDNA	Cyt <i>b</i> DNA
<i>Myospalax baileyi</i> 1	4	Xihai, Qinghaihu	NNU M9901	AF326236	AF326255
<i>M. baileyi</i> 2	5	Sangke, Gansu	NNU M9902	AF326235	AF326256
<i>M. baileyi</i> 6	6	<i>ditto</i>	NNU M9915 ^{b)}		
<i>M. baileyi</i> 7	7	Qinghai	NNU M9916 ^{b)}		
<i>M. baileyi</i> 8	8	Sangke, Gansu	NNU M9917 ^{b)}		
<i>M. cansus</i> 1	11	Ledu, Qinghai	NNU M9903	AF326240	AF326260
<i>M. cansus</i> 2		Qinghai	NNU M9904	AF326241	AF326261
<i>M. cansus</i> 3		Guyuan, Ningxia	NNU M 9906	AF326243	AF326263
<i>M. cansus</i> 4		<i>Ditto</i>	NNU M9905		AF326262
<i>M. cansus</i> 5		Qingshui, Gansu	NNU M 9907	AF326242	
<i>M. rothschildi</i> 1	12	Wanyuan, Sichuan	NNU M9912	AF326246	AF326267
<i>M. rothschildi</i> 2		<i>Ditto</i>	NNU M9913	AF326247	AF326268
<i>M. fontanieri</i> 1	15	Chicheng, Hebei	NNU M9908	AF326244	
<i>M. fontanieri</i> 2		<i>Ditto</i>	NNU M 9909		AF326264
<i>M. fontanieri</i> 3		<i>Ditto</i>	NNU M 9910		AF326265
<i>M. fontanieri</i> 4		<i>Ditto</i>	NNU M9911	AF326245	AF326266
<i>M. psilurus</i> 1	14	Daxing, Beijing	NNU M9920	AF326249	AF326270
<i>M. psilurus</i> 2		<i>Ditto</i>	NNU M9921	AF326250	AF326271
<i>M. aspalax</i> 1	16	Balinzuoqi, Inner Mongolia	NNU M9922	AF326251	AF326272
<i>M. aspalax</i> 2		<i>Ditto</i>	NNU M9923	AF326252	
<i>M. rufescens</i>	17	Ningshaan, Shaanxi	NNU M9914	AF326248	AF326269
<i>Rhizomys sinensis</i>	13	<i>ditto</i>	NNU M9925	AF326254	AF326274
<i>Ochotona curzoniae</i>	10	Hudong, Qinghai	NNU M9924	AF326253	AF326273
Sailonggu 1	1		NNU Md9901	AF326238	AF326258
Sailonggu 2	2		NNU Md9902	AF326239	AF326259
Sailonggu 3	3		NNU Md9903	AF326240	AF326257

a) Codes identical to those in Fig. 3. b) For allele-specific diagnostic PCR only.

Table 2. Diagnostic Primers Designed for Amplification and Sequencing of Mt 12S rRNA and Cyt *b* Gene Fragments

Name	Sequence 5'-3'
SL1-L	GAAACAGCAGTGATAAAAAATTAAGCC
SL1-H	CTGGCGGGTAATTTGTTGGAAATT
SL2-L	CATTCTCATCAGTAACCCATATTTGC
SL2-H	ATTCATTCTACTAGGGAGGTTCTCTACA

TAC AAG-3') for complete mitochondrial cyt *b* gene, and L-tRNA^{Phe} (5'-AA AGC AAA GCA CTG AAA ATG-3') and H-tRNA^{Val} (5'-G CTA GGG TTA GTT CAA AGT GTT-3') for complete 12S rRNA gene, were used in the present study. Primer L14724 was cited from Kocher *et al.*,⁸⁾ and the other three were designed by the authors. Two other primer pairs, L1091 (5'-AA AAA GCT TCA AAC TGG GAT TAG ATA CCC CAC TAT-3') and H1478 (5'-T GAC TGC AGA GGG TGA CGG GCG GTG TGT-3'), and L15513 (5'-CT AGG AGA CCC TGA CAA CTA-3')⁸⁾ and H15917, were used in this study. We failed to amplify the complete sequences of the 12S rRNA and cyt *b* genes from the crude drug samples, because the DNA of the crude drug Sailonggu was degraded in varying degrees. Therefore, the primer pair L1091/H1478 was used for the amplification of the 12S rRNA gene fragment to prove the quality of genomic DNA in subsequent diagnostic PCR and in sequencing the crude drug samples. L15513/H15917 was used for amplification and sequencing of the Cyt *b* gene fragments of the crude drug samples.

The amplification was carried out on a PTC-200 thermocycler (MJ Research) in a 30 μ l reaction mixture containing

10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 150 mM of each dNTP, 0.3 mM of each primer (synthesized by TaKaRa), 1.0 μ l (about 100 ng) template DNA, and 1 U *Taq* DNA polymerase (Promega). The reaction mixtures were denatured at 95 °C for 5 min and subjected to 30 cycles of 40 s at 95 °C, 1 min at 55 °C, 1.5 min at 72 °C, and a final extension step of 7 min at 72 °C. Amplified products were purified using WizardTM PCR Prep DNA Kit (Promega) according to the manufacturer's instructions, then the two strands were sequenced directly with PRISMTM BigDye Terminator Ready Reaction Kit (Applied Biosystems) on an ABI 310 Genetic Analyzer (Perkin Elmer).

Data Analysis Sequences of fragments of the two genes were aligned in ClustalX 1.8⁹⁾ and adjusted manually, taking into account the secondary structure of the gene. Relationships among taxa were investigated using analysis of distance data derived from nucleotide substitutions among taxa employing Kimura's two-parameter model.¹⁰⁾ UPGMA analysis of the 12S rRNA and cyt *b* datasets was carried out by Molecular Evolutionary Genetics Analysis (MEGA 2.1).¹¹⁾ Gaps were coded as missing. *Ochotona curzoniae* and *Rhizomys sinensis* were chosen as outgroup taxa. Robustness of the inferred phylogeny was evaluated by bootstrap resampling with 1000 replications.

Allele-Specific Diagnostic PCR Based on sequences of the two mitochondrial DNA genes of 7 species of zokors, *Rhizomys sinensis* and *Ochotona curzoniae*, two pairs of primers, SL1L/SL1H and SL2L/SL2H (Table 2), were designed for diagnostic PCR. Both primer pairs are specific for *M. baileyi* only. The former amplify a 338 bp DNA segment of the 12S rRNA gene, and the latter a 320 bp DNA segment of the Cyt *b* gene, respectively. The reaction system of diag-

Table 3. Variable Sites Found among Partial Sequences of 12S rRNA Gene of the Original Animals and Crude Drug Sailonggu (Dashes Denote Gap)

	3	9	1 5	1 8	1 9	2 0	2 1	2 2	2 5	2 6	2 7	2 8	2 9	3 5	3 7	3 8
<i>M. psilurus</i> 1, 2	T	G	T	A	G	C	A	A	—	—	A	A	C	T	T	C
<i>M. aspalax</i> 1, 2						T	G		—	—						
<i>M. fontanieri</i> 1, 2						T	G		—	—	T	C		A		
<i>M. cansus</i> 1, 2						T	G		—	—	T	C		A		
<i>M. cansus</i> 3, 5						T	G		—	—	T	C		A		
<i>M. rufescens</i>						T	G		—	—	C	C		A		
<i>M. rothschildi</i> 1, 2						T	G		—	—	T	C		A		
<i>M. baileyi</i> 1, 2; Sailonggu 2, 3						T			—	—	T	C		A		T
Sailonggu 1						T			—	—	T	C		A		T
<i>R. sinensis</i>			C			T			C	T	T	T	T	A		T
<i>O. curzoniae</i>	C	A	C	G	A	T		G	A	C		—	T	A	C	T
	3 9	4 0	4 1	6 0	6 2	6 3	9 0	9 6	1 0	1 0	1 0	1 1	1 8	1 2	1 3	1 9
<i>M. psilurus</i> 1, 2	G	C	C	C	C	C	A	T	T	G	T	C	T	G	C	A
<i>M. aspalax</i> 1, 2	A													A		
<i>M. fontanieri</i> 1, 2	A													A		
<i>M. cansus</i> 1, 2	A															
<i>M. cansus</i> 3, 5	A					T										
<i>M. rufescens</i>	A															
<i>M. rothschildi</i> 1, 2	A															
<i>M. baileyi</i> 1, 2; Sailonggu 2, 3	A															
Sailonggu 1	A															
<i>R. sinensis</i>	A		T	A	T			C				A	G	A	T	
<i>O. curzoniae</i>	A	T	T	A		A	G		C	—	C			A		T
	1 4 0	1 4 5	1 5 2	1 5 3	1 5 8	1 6 1	1 6 9	1 8 3	1 8 8	1 9 5	1 9 6	1 9 7	2 0 2	2 0 3	2 0 4	2 0 5
<i>M. psilurus</i> 1, 2	C	T	C	C	T	T	A	C	G	G	T	C	A	C	C	C
<i>M. aspalax</i> 1, 2								T							T	
<i>M. fontanieri</i> 1, 2	T							T					T			A
<i>M. cansus</i> 1, 2	T							T					T			A
<i>M. cansus</i> 3, 5	T							T					T			A
<i>M. rufescens</i>	T							T					T			A
<i>M. rothschildi</i> 1, 2	T							T					T			A
<i>M. baileyi</i> 1, 2; Sailonggu 2, 3								T					T			A
Sailonggu 1	T							T					T			A
<i>R. sinensis</i>	T		T					T	A					A		A
<i>O. curzoniae</i>	T	C		T	C	C	G	T	A	A	A	A	C	G	T	A
	2 0 6	2 0 7	2 1 1	2 1 5	2 1 9	2 2 0	2 2 3	2 2 4	2 2 5	2 2 6	2 2 7	2 2 9	2 3 4	2 5 6	2 5 9	2 6 0
<i>M. psilurus</i> 1, 2	A	T	G	A	C	G	T	—	A	A	C	G	T	T	G	G
<i>M. aspalax</i> 1, 2								—				A				
<i>M. fontanieri</i> 1, 2					T		—	—			T					A
<i>M. cansus</i> 1, 2					T		—	—			T					A
<i>M. cansus</i> 3, 5			A		T		—	—			T					A
<i>M. rufescens</i>					T		—	—			T					A
<i>M. rothschildi</i> 1, 2					T		—	—			T					A
<i>M. baileyi</i> 1, 2; Sailonggu 2, 3					T		—	—			T					A
Sailonggu 1							—	—			T					A
<i>R. sinensis</i>		C	A	C	T			T	T	T		A				A
<i>O. curzoniae</i>	G	C	A	T	T	A	—	—	C	C		A	A	C	A	
	2 6 6	2 6 7	2 6 9	2 7 0	2 8 8	2 8 9	2 9 0	2 9 1	2 9 2	2 9 3	2 9 4	2 9 6	2 9 9	3 0 0	3 0 1	3 0 2
<i>M. psilurus</i> 1, 2	A	A	G	C	A	A	T	T	T	A	G	A	T	—	—	C
<i>M. aspalax</i> 1, 2					T									—	—	T
<i>M. fontanieri</i> 1, 2							C							—	—	T
<i>M. cansus</i> 1, 2						G								—	—	

Table 3. (Continued)

<i>M. cansus</i> 3, 5																—	—	T
<i>M. rufescens</i>							G									—	—	
<i>M. rothschildi</i> 1, 2												G				—	—	T
<i>M. baileyi</i> 1, 2; Sailonggu 2, 3				T		<u>G</u>	A	<u>A</u>								—	—	T
Sailonggu 1						<u>G</u>		<u>A</u>								—	—	T
<i>R. sinensis</i>		C	A		C	T	C		C	T	A	G	C	A		—	—	
<i>O. curzoniae</i>	G				T	T		A	—		A	G	C	A	C			
	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
	0	0	0	1	1	1	1	1	2	2	2	2	2	2	2	3	3	3
	6	8	9	1	2	3	4	5	1	2	4	6	7	8	0	1		
<i>M. psilurus</i> 1, 2	G	T	C	C	C	C	C	A	—	C	G	G	G	G	C	C		
<i>M. aspalax</i> 1, 2		C				T	T		—	T								
<i>M. fontanieri</i> 1, 2				T		T	T		—		A				T	G		
<i>M. cansus</i> 1, 2				T		T	T		—				A		T	G		
<i>M. cansus</i> 3, 5				T		T	T		—				A		T	G		
<i>M. rufescens</i>				T	T	T	T		—				A		T	G		
<i>M. rothschildi</i> 1, 2				T		T	T		—		A		A		T	G		
<i>M. baileyi</i> 1, 2; Sailonggu 2, 3				T		T	T		—		A		A		T	G		
Sailonggu 1				T		T	T		—		A		A		T	G		
<i>R. sinensis</i>		A		T	T	T	T	T	—	G	A	A	A		T	G		
<i>O. curzoniae</i>	A	A	A		T	T	T		T	T	A	A		T	T	A		
	3	3	3	3	3	3	3	3	3	3	3							
	3	4	6	7	7	7	7	8	8	9	9							
	6	5	4	6	7	8	9	5	9	1	3							
<i>M. psilurus</i> 1, 2	A	T	A	C	T	T	A	A	T	T	C							
<i>M. aspalax</i> 1, 2		C			C			G										
<i>M. fontanieri</i> 1, 2				A	C	A		G										
<i>M. cansus</i> 1, 2						A		G										
<i>M. cansus</i> 3, 5						A		G										
<i>M. rufescens</i>						A		G										
<i>M. rothschildi</i> 1, 2						A		G										
<i>M. baileyi</i> 1, 2; Sailonggu 2, 3						A		G										
Sailonggu 1						A		G										
<i>R. sinensis</i>		C	T		A			G		C	T							
<i>O. curzoniae</i>	C	C			A	C	G	G	C	C								
GCTTAGCCGT	AACTTAAGC	AATTAACAAC	AATATCGCCC	GCCAGAGAAC	TACTAGCCAC													
CGCTTAAAC	TCAAAGGACT	TGGCGGTACT	TTATACCTGT	CTAGAGGAGC	CTGTTCTATA													
ATCGATGAAC	CCCGCTACAC	CTTACCACCC	CTTGCTAATT	CAGCCTATAT	ACCGCCATCT													
CCAGCGAACC	CTGTCAAGGA	CCCATAGTGA	GCACAACGAT	TAACCGTAAA	TACGTTAGGT													
CAAGGTGTAG	CCTATGGGGT	GGAAAGCAAT	GGGCTACATT	TTCTAATTTA	GAAAATCACG													
GATCCCCCA	TGAAACTGAG	GGGCCAAGGA	GGATTAGTA	GTAAATTAAG	AATAGAGAGC													
TAAATTGAAC	TTAGCAATAA	AGTATGC																

Mutational sites and specific diagnostic sites (underlined below the capital) used to authenticate crude drug Sailonggu are indicated in the Table, with the 387bp fragment sequence of *M. psilurus* listed below.

nostic PCR is the same as mentioned above. The diagnostic PCR conditions are as follows: denaturing at 95 °C for 4 min, then 30 cycles of 20 s at 95 °C, 25 s at 65 °C, 10 s at 72 °C, and a final extension of 2 min at 72 °C. Then, 5 ml of the reaction mixture was used for electrophoresis in 2.0% TBE agarose gel stained by ethidium bromide, and the results were recorded with a Gel Documentation System (GDS-7600, UVP).

RESULTS

12S rRNA and Cyt *b* Datasets Seventeen complete sequences of 12S rRNA on one hand and cyt *b* on the other for 7 species of zokors and 2 outgroup taxa were obtained. In addition, 3 partial sequences of the two genes, respectively, for crude drug Sailonggu were obtained (see Table 1 for GenBank accession numbers). The aligned partial sequences of

12S rRNA fragments of the original animals and Sailonggu consisted of a total of 393 sites, including 107 variable sites (Table 3). There are two fixed nucleotide substitutions in the 12S rRNA gene (position 289, 291) for all *M. baileyi* and Sailonggu samples, the “A” being substituted for “G,” and “T” for “A,” respectively. The aligned cyt *b* fragments of the original animals and Sailonggu consist of a total of 370 sites, including 170 variable sites (Table 4). There are also two substitutions in the cyt *b* gene (position 79, 334) for *M. baileyi* and Sailonggu, the “C” being substituted for “T,” and “A” for “C,” respectively. Therefore, they were referred to as specific diagnostic genetic markers for identification of the crude drug. The three crude drug samples were determined as qualified Sailonggu, according to these diagnostic markers.

The divergence between any of the three crude drugs and the original animal of *M. baileyi* ranged from 0 to 0.011 in

Table 4. Variable Sites Found among Partial Sequences of Cyt *b* Gene of the Original Animals and Crude Drug Sailonggu (Dashes Denote Gap)

	1	4	7	8	9	10	13	16	17	19	21	22	25	28	31	34	37	46	49
<i>M. psilurus</i> 1	C	C	A	G	C	A	C	A	C	T	A	C	A	A	A	T	C	A	T
<i>M. psilurus</i> 2										C									
<i>M. aspalax</i> 1			G							A	G	T			C				
<i>M. fontanieri</i> 2	T					T			T	A		T			C				
<i>M. fontanieri</i> 3	T					T			T	A		T			C				
<i>M. fontanieri</i> 4	T					T			T	A		T			C				
<i>M. baileyi</i> 1	T			C	G					A					C		T		
<i>M. baileyi</i> 2	T									A					C		T		
Sailonggu 2	T									A					C		T		
Sailonggu 3	T									A					C		T		
Sailonggu 1	T									A					C		T		
<i>M. cansus</i> 1, 2	T	T								A					C		T		
<i>M. cansus</i> 4	T	T								A					C		T		
<i>M. cansus</i> 3	T	T								A					C		T	G	
<i>M. rufescens</i>	T									A		T			C	C	T		
<i>M. rothschildi</i> 1	T									A		T			C	C	T		
<i>M. rothschildi</i> 2	T									A		T			C	C	T		
<i>R. sinensis</i>	T		C		T		T	T	T	C					C	C	T		G
<i>O. curzoniae</i>	T	T	C		C					C			C	G		C	T		
	52	55	58	61	64	67	70	73	74	76	77	82	85	88	91	97	100	103	106
<i>M. psilurus</i> 1	T	C	C	C	C	C	A	C	C	A	C	T	T	C	T	A	A	A	C
<i>M. psilurus</i> 2																			
<i>M. aspalax</i> 1	C	T										A	C	T		T		C	T
<i>M. fontanieri</i> 2		T		T			C	T					C	A			C	T	A
<i>M. fontanieri</i> 3		T		T			C	T					C	A			C	T	A
<i>M. fontanieri</i> 4		T		T			C	T					C	A			C	T	A
<i>M. baileyi</i> 1			T				T		T		T			A	C			T	A
<i>M. baileyi</i> 2			T				T		T		T			A	C			T	A
Sailonggu 2			T				T		T		T			A	C			T	A
Sailonggu 3			T				T		T		T			A	C			T	A
Sailonggu 1		T	T				T		T		T			A	C			T	A
<i>M. cansus</i> 1, 2	C	T	T			T	T		T		T		C	A	C	T	T	T	A
<i>M. cansus</i> 4	C	T	T			T	T		T		T	C		A	C	T	T	T	A
<i>M. cansus</i> 3	C	T	T			T	T		T		T			A	C	T	T	T	A
<i>M. rufescens</i>	C	T	T	T			T		T				C	A			T	C	A
<i>M. rothschildi</i> 1	C	T	T	T			T		T				C	A			T	C	A
<i>M. rothschildi</i> 2			T			T	T	T				C		A		G	T	T	A
<i>R. sinensis</i>			A			T	C	T		C			C	T				T	T
<i>O. curzoniae</i>			A		A		C			C		C	C		C	C	C	C	A
	107	109	113	115	116	118	119	121	124	127	128	132	133	134	135	137	138	143	149
<i>M. psilurus</i> 1	C	A	C	A	A	C	C	C	C	T	C	T	A	A	T	C	A	T	C
<i>M. psilurus</i> 2																			
<i>M. aspalax</i> 1				C		T	T	T		C			C		C	T			
<i>M. fontanieri</i> 2										C			C		C	T			T
<i>M. fontanieri</i> 3										C			C		C	T			T
<i>M. fontanieri</i> 4										C			C		C	T			T
<i>M. baileyi</i> 1									T				C		C	T			T
<i>M. baileyi</i> 2									T				C		C	T			
Sailonggu 2									T				C		C	T			
Sailonggu 3									T				C		C	T			
Sailonggu 1									T				C		C	T			
<i>M. cansus</i> 1, 2							A	A	C				C						T
<i>M. cansus</i> 4							A	A	C				C						T
<i>M. cansus</i> 3			T				A	A	C				T						T
<i>M. rufescens</i>			T					T	C				T			T			T
<i>M. rothschildi</i> 1								T	C				C			T			T
<i>M. rothschildi</i> 2								T	C				T	G		T	G		T
<i>R. sinensis</i>	A	T		T	G		T	A	G		G	C	C		C		G	C	T
<i>O. curzoniae</i>					G		A	A				C	C		C				

Table 4. (Continued)

	1 4 0	1 4 2	1 4 3	1 4 5	1 4 8	1 4 9	1 5 1	1 5 2	1 5 4	1 5 7	1 6 0	1 6 3	1 6 4	1 6 5	1 6 6	1 6 7	1 6 8	1 7 2	1 7 5
<i>M. psilurus</i> 1	C	A	A	A	C	T	A	C	A	T	A	C	G	C	C	C	A	A	C
<i>M. psilurus</i> 2																			
<i>M. aspalax</i> 1					T		T			C									
<i>M. fontanieri</i> 2		T	C					T											T
<i>M. fontanieri</i> 3		T	C					T							A				T
<i>M. fontanieri</i> 4		T	C					T							A				T
<i>M. baileyi</i> 1		C	C		A					C					A				
<i>M. baileyi</i> 2		C	C		A					C									
Sailonggu 2		C	C		A					C									
Sailonggu 3		C	C		A					C									
Sailonggu 1		C			A					C			A						
<i>M. cansus</i> 1, 2		T	T		T	A				C								G	T
<i>M. cansus</i> 4		T	T		T	A				C									T
<i>M. cansus</i> 3		T	T		T	A				C									T
<i>M. rufescens</i>		C	T		A					C			T						
<i>M. rothschildi</i> 1		C	T		A					C			T						
<i>M. rothschildi</i> 2		T	T		A					C			T						
<i>R. sinensis</i>	T		C	C	T				C		C	A	C		T	A	T	C	
<i>O. curzoniae</i>	G	C					C		C	C		A	A	A	A				
	1 7 6	1 7 8	1 8 0	1 8 1	1 8 4	1 8 7	1 9 0	1 9 1	1 9 3	1 9 4	1 9 6	1 9 9	2 0 0	2 0 2	2 0 3	2 0 5	2 0 8	2 1 2	2 1 4
<i>M. psilurus</i> 1	C	A	T	A	T	C	A	A	T	G	C	A	T	C	T	A	C	A	C
<i>M. psilurus</i> 2																			
<i>M. aspalax</i> 1					C						A			T	C				
<i>M. fontanieri</i> 2	A					T			C	A					C	T			
<i>M. fontanieri</i> 3	A					T			C	A					C	T			
<i>M. fontanieri</i> 4	A					T			C	A					C	T			
<i>M. baileyi</i> 1					C					A					C				
<i>M. baileyi</i> 2					C					A					C				
Sailonggu 2					C					A					C				
Sailonggu 3					C					A					C				
Sailonggu 1					C	T			C	A			G		C	G	T		
<i>M. cansus</i> 1, 2					C					A									T
<i>M. cansus</i> 4					C					A									T
<i>M. cansus</i> 3					C	T				A									T
<i>M. rufescens</i>	T				C					A		G			C		T		T
<i>M. rothschildi</i> 1	T				C					A		G			C		T		T
<i>M. rothschildi</i> 2										A				T			T		T
<i>R. sinensis</i>		C	C		C	A	T	C	A	A				T			T		
<i>O. curzoniae</i>	A	G		G	C				C	A								G	T
	2 1 5	2 1 7	2 2 0	2 2 2	2 2 3	2 2 6	2 2 7	2 2 9	2 3 0	2 3 2	2 3 4	2 3 5	2 3 6	2 3 8	2 4 7	2 5 3	2 5 9	2 6 2	2 6 6
<i>M. psilurus</i> 1	T	A	A	G	A	C	C	A	T	C	T	C	C	C	T	A	A	C	C
<i>M. psilurus</i> 2																			
<i>M. aspalax</i> 1					T					T									
<i>M. fontanieri</i> 2	C		C	C	T			C		T	C		T	A	C				
<i>M. fontanieri</i> 3	C			C	T			C		T	C		T	A	C			T	
<i>M. fontanieri</i> 4	C			C	T			C		T	C		T	A	C			T	
<i>M. baileyi</i> 1	C			C	T			C		T	C	T	T	A				T	
<i>M. baileyi</i> 2	C			C	T			C		T	C	T	T	A				T	
Sailonggu 2	C			C	T			C		T	C	T	T	A				T	
Sailonggu 3	C			C	T			C		T	C	T	T	A				T	
Sailonggu 1				C	C					T	C	T		A	C			T	
<i>M. cansus</i> 1, 2			G	C	T					T	C	T	T	A	C			T	
<i>M. cansus</i> 4			G	C	T		T			T	C	T		A				T	
<i>M. cansus</i> 3			G	C	T					T	C	T		A	C			T	
<i>M. rufescens</i>	C			C	T			T		T	C	T		A				T	
<i>M. rothschildi</i> 1	C			C	T			T		T	C	T		A				T	
<i>M. rothschildi</i> 2	C			C	T			C		T	C	T		A		C		T	
<i>R. sinensis</i>	C			C	T	T	T		A		C	A		T	C			A	A
<i>O. curzoniae</i>	C	C		C	C			T	C		C	A			C		C		

Table 4. (Continued)

	2 6 7	2 6 8	2 7 1	2 7 3	2 7 4	2 7 5	2 7 6	2 7 7	2 7 8	2 8 0	2 8 3	2 8 6	2 9 0	2 9 5	2 9 9	3 0 2	3 0 4	3 0 7	3 1 0
<i>M. psilurus</i> 1	A	C	A	T	C	A	T	C	A	C	C	A	C	C	A	A	A	C	C
<i>M. psilurus</i> 2																			
<i>M. aspalax</i> 1												T							
<i>M. fontanieri</i> 2			C	A	T	G						T	C				T		T
<i>M. fontanieri</i> 3			C	A	T	G						T	C				T		T
<i>M. fontanieri</i> 4	T		C	A	T	G	C					T	C				T		T
<i>M. baileyi</i> 1	T							T			T	T	T	T			T		T
<i>M. baileyi</i> 2								T			T	T	T	T			T		T
Sailonggu 2								T			T	T	T	T			T		T
Sailonggu 3								T			T	T	T	T			T		T
Sailonggu 1								T			T		C	T			T		T
<i>M. cansus</i> 1, 2					T							T	C				T		
<i>M. cansus</i> 4					T			T				T	C				T		
<i>M. cansus</i> 3												T	C				T		
<i>M. rufescens</i>												T	C				C		T
<i>M. rothschildi</i> 1					T			T			T	T	C		T		C		T
<i>M. rothschildi</i> 2								T	C		T	T	C		T		C		T
<i>R. sinensis</i>	C	T						T				T	C	G			C	T	T
<i>O. curzoniae</i>											T		C		A	T	C	C	
	3 1 1	3 1 2	3 1 3	3 1 4	3 1 6	3 1 7	3 1 9	3 2 2	3 2 3	3 2 4	3 2 5	3 2 6	3 2 8	3 2 9	3 3 1	3 3 4	3 3 5	3 3 6	3 3 7
<i>M. psilurus</i> 1	C	T	C	A	C	A	T	C	G	T	C	C	A	A	A	A	T	T	A
<i>M. psilurus</i> 2												T							
<i>M. aspalax</i> 1	A	C							A				C						
<i>M. fontanieri</i> 2			T		T			T		G	T		C						
<i>M. fontanieri</i> 3			T		T			T			T		C						
<i>M. fontanieri</i> 4			T		T			T			T		C						
<i>M. baileyi</i> 1					T		C	T	A		T					C			
<i>M. baileyi</i> 2					T		C	T	A		T					C			
Sailonggu 2					T		C	T			T					C			
Sailonggu 3					T		C	T			T					C			
Sailonggu 1					T		C	T			T					C			
<i>M. cansus</i> 1, 2					T		C	T				A							
<i>M. cansus</i> 4					T		C	T				A							
<i>M. cansus</i> 3					T		C	T				A							
<i>M. rufescens</i>			T				C	A			T		G						
<i>M. rothschildi</i> 1			T				C	A			T		G						
<i>M. rothschildi</i> 2			T				C	T		G	T		T						
<i>R. sinensis</i>	T	C				C			A			T	C	T	G				
<i>O. curzoniae</i>	T	C		C				A	A		T		T				A	C	C
	3 3 8	3 3 9	3 4 0	3 4 1	3 4 3	3 4 4	3 4 6	3 4 7	3 4 9	3 5 5	3 5 9	3 6 0	3 6 1	3 6 2	3 6 4	3 6 8	3 6 9	3 7 0	
<i>M. psilurus</i> 1	G	C	C	G	C	A	C	T	A	T	A	T	T	A	A	C	T	C	
<i>M. psilurus</i> 2																			
<i>M. aspalax</i> 1					T					C			C					T	
<i>M. fontanieri</i> 2			T		T		T	C					C			A		A	
<i>M. fontanieri</i> 3			T		T		T	C					C			A		A	
<i>M. fontanieri</i> 4			T		T		T	C					C			A		A	
<i>M. baileyi</i> 1			T		T		T	C					C			A			
<i>M. baileyi</i> 2			T		T		T	C					C			A			
Sailonggu 2			T				T	C		C			C			A			
Sailonggu 3			T				T	C					C			A			
Sailonggu 1			T		T		T	C		C			C			A			
<i>M. cansus</i> 1, 2			T				T	C					C			A			
<i>M. cansus</i> 4			T				T	C					C			A			
<i>M. cansus</i> 3			T				T	C					C			A			
<i>M. rufescens</i>							T	C		C			C			A			
<i>M. rothschildi</i> 1							T	C		C			C			G			
<i>M. rothschildi</i> 2			T				C						C			A		T	
<i>R. sinensis</i>			T		T		A			C		C	C			T		T	
<i>O. curzoniae</i>	T	G		A		C	A	A	T	C	C			C	C	T	G	A	

Table 4. (Continued)

CACCCAGCA	AACCCACTTA	ACACACCACC	ACATATCAAA	CCAGAATGAT	ATTTCCTCTT
CGCCTACGCA	ATCCTACGCT	CTATTCCCAA	TAAACTAGGA	GGAGTCCTAG	CCCTAATCCT
CTCCATTCTA	ATTCTAATCC	TAATACCCCT	ACTACATACA	TCCGCCCAAC	GAAGCCTAAT
ATTTCGCCCCA	ATTGGCCAAT	GCTTATTCTG	AATCTTAGTA	GGAGACCTAT	TCATCCTCAC
ATGAATTGGA	GGACAACCAG	TGGAACACCC	ATTTCATCATC	ATCGGACAAC	TAGCCTCAAT
CATATACTTC	CTCATCATTC	TCGTCTTAAT	ACCATTAGCC	GGCATCTTAG	AAAATAAAAT
TATAAAACTC					

Mutational sites and specific diagnostic sites (underlined below the capital) used to authenticate crude drug Sailonggu are indicated in the Table, with the 370 bp fragment sequence of *M. psilurus* listed below.

Fig. 1. UPGMA Tree for Mitochondrial 12S rRNA Gene Fragments of Sailonggu and Original Animals of Zokors Based on Kimura's 2-Parameter Distance

Bootstrap values <50% were not shown.

Fig. 2. UPGMA Tree for Mitochondrial Cyt *b* Gene Fragments of Sailonggu and Original Animals of Zokors Based on Kimura's 2-Parameter Distance

Bootstrap values <50% were not shown.

the 12S rRNA, and 0.005 to 0.079 in the Cyt *b* gene sequences, respectively. Both are the smallest compared with those between the crude drugs and the original animals of other species of zokors in the present study.

Phylogenetic Analysis UPGMA analysis of the datasets of 12S and cyt *b* gene sequences resulted in very similar topologies (Figs. 1, 2). The original animals of *M. baileyi* and Sailonggu clustered to a clade with a high value of nodal support in both trees. The results of phylogenetic analysis based on the two gene fragments also support that all of the crude drug samples tested are made of the bone of *M. baileyi*.

Diagnostic PCR In the diagnostic assay, using universal primer L1091 and H1478, we amplified a 387 bp fragment of the mitochondrial 12S rRNA gene for positive controls from 16 samples (Fig. 3a). While using the allele-specific primers SL1L/SL1H and SL2L/SL2H for PCR, 338 bp and 320 bp amplified DNA fragments, respectively, were obtained from

Fig. 3. Agarose Gel Electrophoresis of PCR Identification of Original Animals and Crude Drug Sailonggu

a: Positive controls annealing at 55 °C for all DNA templates resolved by 2.0% agarose gel electrophoresis. b: Agarose gel electrophoresis of PCR products of 12S rRNA fragment from original animals, and crude drug Sailonggu samples using primers SL1L and SL1H annealed at 65 °C. c: Agarose gel electrophoresis of PCR products of Cyt *b* fragment from original animals and crude drug Sailonggu samples using primers SL2L and SL2H annealed at 65 °C. N: Negative control without DNA template; M: DNA marker, DL2000 (Takara). 9: including 1 ml templates of No. 4 and 1 ml mixture templates of No. 10, 11 and 12. Other codes are identical to those in Table 1. The sizes of No. 1, 2 and 3 in b look different from each other, but this is due to the higher gel well position for the latter two, respectively.

Sailonggu and original animal *M. baileyi*. No specific PCR product was obtained from the other animals using the allele-specific primers (Figs. 3b,c). These results were reproducible until annealing temperature increased to 68 °C.

DISCUSSION

We tested the possibility of authentication of the crude drug Sailonggu by sequence alignment and phylogenetic analysis. Two diagnostic sites were found in both 12S and cyt *b* for authentication of Sailonggu. However, only a limited number of individuals of *M. baileyi* and other zokor species were sampled in this study. Therefore, sequence alignment will not be an accurate tool for identification of the crude drug before a significant sequence database has been built. Although sequence variations of the 12S and cyt *b* fragments were found among the samples of Sailonggu and the original animal of *M. baileyi*, the UPGMA trees support that all three

crude drug samples are of the genuine drug Sailonggu.

DNA of the crude drugs was degraded during the preparation, transportation and storage of the medicinal materials. However, sufficient DNA was obtained for authentication.^{12–14)} The two pairs of diagnostic primers we designed match exactly with the definite DNA nucleotide sequences of *Myospalax baileyi*, but incompletely with the sequences of templates from other species of zokors. Therefore, the PCR performed with these two pairs of primers is a site specific PCR. Theoretically, the annealing temperature of the 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 or adulterants in order to not amplify their DNA templates under the high temperature condition. A high-stringency PCR reaction with each one pair of primers specific for *M. baileyi* will give a positive result only from the template DNA of genuine crude drugs, and a negative result from that of substitutions. However, a negative result could also be produced from poor quality substitutes or adulterants' DNA, or good quality of genuine drugs' DNA. Therefore, amplification of positive controls using universal primers prior to the diagnostic PCR is necessary. In this study, all template DNAs were amplified using universal primers at 55 °C annealing temperature. All PCR under this condition produced clear bands on agarose gel electrophoresis (Fig. 3). The result indicated that the DNA templates used are suitable for PCR. It also indicated that a particular DNA band should be amplified from these templates using diagnostic primers if the primer can find an appropriate annealing position on the template DNA. On the other hand, if the diagnostic primers fail to match the template, especially at the 3' end of the primers, the reaction will give a negative result. Our results indicate that Sailonggu samples could be definitely distinguished from its substitutes or adulterants by diagnostic PCR using any one pair of the diagnostic primers annealing at 65–68 °C. In addition, the result will not be influenced by *exo*-DNA, because the diagnostic primers are specific for Sailonggu only, and the reaction is performed under high-stringency conditions.

Among the three methods tested, the sequencing method is far more complicated compared with the PCR method, and the phylogenetic analysis method is based on DNA se-

quences. On the other hand, the allele-specific diagnostic PCR method has several advantages: 1) Only a small quantity of sample is required for identification; 2) the process is simple and inexpensive; 3) the result will not be affected by contamination in samples, and offers an accurate identification. Therefore, we believe the PCR method is a useful tool for the quality control of Chinese crude drugs.

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