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Cytochrome b gene selection of subterranean rodent Gansu zokor *Eospalax cansus* (Rodentia, Spalacidae)

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ABSTRACT

We analyzed the molecular selection of a typical subterranean rodent Gansu zokor (*Eospalax cansus*) combined with mitochondrial cytochrome b (cytb) and D-loop sequences. A total of 121 samples (from 12 sites belonging to 3 main geographical units: U1, U2, and U3) were sequenced, from which 40 cytb haplotypes and 53 D-loop haplotypes were identified. The results of our tests from two site-specific models (M2a and M8) and one branch-site model (Clade model C) suggest that the evolution of cytb in Gansu zokor is chiefly governed by purifying selection. Moreover, the distribution of non-synonymous mutations and the tests by Clade model C indicated the cytb in U3, where the zokors were confronted with more severe hypoxia because of higher altitude and a colder, drier climate, were facing a higher purifying selection pressure than those in U1 and U2.

1. Introduction

The mitochondrial cytochrome b (cytb) gene encodes an integral membrane protein component of the cytochrome bc1 complex, which catalyzes the redox transfer of electrons from ubiquinone to cytochrome c in the mitochondrial electron transport chain (Iwata et al., 1998). As the efficiency of the electron transport chain governs key aspects of aerobic energy metabolism, several investigators have suggested that human cytb gene may be involved in physiological adaptation to different thermal environments (Mishmar et al., 2003; Ruiz-Pesini et al., 2004). The cytb gene has been popularly used as a genetic marker in phylogenetics as well as in population genetics for decades; however, very little attention has been paid to the evolution in wild animals in different environments. Gering et al. (2009) used likelihood-based comparisons of synonymous and non-synonymous substitution rates to test for evidence of divergent selection between high- and low-altitude haplogroups of deer mice (*Peromyscus maniculatus*). Although there was no evidence for different rates of non-synonymous substitution in high- and low-altitude haplogroups in the study, their novel analyses provided a good example for testing molecular evolution in different environments.

The mitochondrial control region (D-loop), which is suggested to be the most variable part of the mtDNA, is another frequently used mtDNA marker, especially for intraspecific analysis. Most importantly, the D-loop is believed to be selectively neutral (Howell et al., 2007). Hence, the D-loop could be used as a backdrop to test the selection pressure of the encoding gene although most of the available related methods for selection analysis are based on the encoding gene itself (Gering et al., 2009 and also see below).

Gansu zokor (*Eospalax cansus*) is a typical subterranean rodent species endemic to the Loess Plateau in China (Li, 1989). There are three main geographical units of Gansu zokor in the plateau: the Shaanxi unit (U1), the Ningxia unit (U2), and the Qinghai unit (U3) (Table 1, Fig. 1). The three distribution regions of Gansu zokor have very similar latitude from east to west; however, altitude increases by nearly 2000 m. Hypoxia has been viewed as one of the most important environmental stresses on subterranean rodents (Nevo, 1999; Begall et al., 2007). Since the inspired oxygen pressure falls roughly linearly with altitude (Peacock, 1998), the hypoxia stress increases with increasing altitude. This makes Gansu zokor a good candidate for studying molecular evolution of the cytb gene across altitudinal gradients. Moreover, other climatic variables like temperature and rainfall, which might influence the molecular evolution of mitochondrial cytb in subterranean rodents (Nevo et al., 1999), also vary greatly among the three geographic regions. The objective in this study was, combining with cytb and D-loop, to test whether there were differences of molecular selection of the cytb gene in different altitudes and with different climatic variables.

2. Materials and methods

2.1. Sampling information

A total of 121 individuals were sampled from 12 sites belonging to 3 units: U1, including Ganquan (GQ), Yanchang (YC), Luochuan

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Table 1
Locality information (geographical unit, U1-U3; *N*, number of samples in U1, U2 and U3; longitude, LO/N°; latitude, LA/E°; altitude, AL/m; AT, annual temperature/°C; AP annual precipitation/mm), sample size (N), and cytb haplotype (C1-C40) and D-loop haplotype (D1-D53) distribution of the zokor samples from 12 sites.

	Site	*N*	LO	LA	AL	AT	AP	Cytb haplotypes (frequency)	D-loop haplotypes (frequency)
U1	GQ	8	109.3445	36.2804	1091	9.5	520.03	C1(4), C2(2), C3(2)	D1(4), D2(2), D3(2)
	YC	8	109.8382	36.5750	1016	9.3	490.32	C4(7), C5(1)	D4(1), D5(6), D6(1)
	LC	9	109.5657	35.8357	1293	8.9	595.33	C5(7), C6(2)	D7(6), D8(1), D9(2)
	ZN	12	108.5210	35.4315	1607	7.8	675.02	C7(7), C8(1), C9(2), C10(1), C11(1)	D10(7), D11(1), D12(2), D13(1), D14(1)
U2	GY	14	106.1040	35.9402	2358	6.0	453.52	C12(4), C13(5), C14(2), C15(1), C16(1), C17(1)	D15(4), D16(5), D17(2), D18(1), D19(1), D20(1)
	XJ	11	105.7586	35.9354	2046	5.2	460.69	C18(5), C19(5), C20(1)	D21(5), D22(2), D23(3), D24(1)
	JY	10	106.2719	35.6248	2024	5.6	536.74	C21(5), C22(1), C23(2), C24(2)	D25(5), D26(1), D27(2), D28(2)
	TW	6	105.2011	35.3489	2144	5.6	544.4	C25(4), C26(2)	D29(1), D30(1), D31(2), D32(2)
U3	HZ	10	102.1965	36.7039	2880	2.1	364.94	C27(3), C28(6), C29(1)	D33(3), D34(2), D35(1), D36(4)
	LD	11	102.6376	36.5818	3000	2.1	376.09	C30(5), C31(3), C32(2), C33(1)	D37(1), D38(2), D39(3), D40(1), D41(3), D42(1)
	MH	11	102.7278	36.0485	2670	3.5	425.34	C34(4), C35(1), C36(3), C37(2), C38(1)	D43(2), D44(1), D45(3), D46(2), D47(2), D48(1)
	PA	11	101.9672	36.3743	2750	3.2	382.81	C39(4), C40(7)	D49(1), D50(2), D51(5), D52(1), D53(2)

(LC), and Zhengning (ZN); U2, including Guyuan (GY), Xiji (XJ), Jingyuan (JY) and Tongwei (TW); U3, including Huzu (HZ), Ledu (LD), Minhe (MH) and Pingan (PA) (Table 1; Fig. 1). Muscle tissue samples were collected from each individual and preserved in 95% alcohol.

The longitude (LO), latitude (LA), and altitude (AL) at each sampling site were recorded using an Etrex GPS monitor (Garmin Ltd., Taipei, Taiwan). The annual temperature (AT) and annual precipitation (AP) were extracted from two data products provided by the Environmental & Ecological Science Data Center for West China, National Natural Science Foundation of China (http://westdc.westgis.ac.cn), using Spatial Analysis Tools in ArcGIS 9.3 (Environmental Systems Research Institute; The AT and AP data products were the average values from 1961 to 1990 and from 1971 to 2000, respectively).

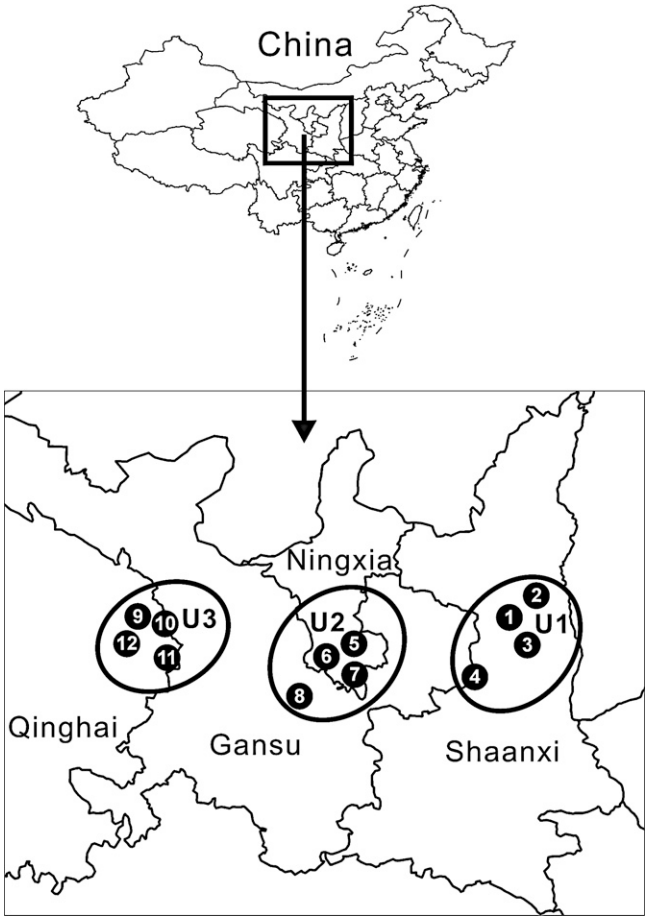

Fig. 1. Geographic distribution of Gansu zokor samples used in the present study. The number with filled-in circles 1–12 represents GQ, YC, LC, ZN, GY, XJ, JY, TW, HZ, LD, MH, and PA, respectively.

2.2. DNA extraction, amplification and sequencing

Total DNA was extracted using standard methods for animal tissue (Sambrook and Russell, 2001). The complete sequence of the cyt b gene was amplified via a polymerase chain reaction (PCR) using the primer pairs L14724 (5′-CGA AGC TTG ATA TGA AAA ACC ATC GTT G-3′) and H15917 (5′-CGG AAT TCC ATT TTT GGT TTA CAA G-3′) (Zhou et al., 2004). Partial D-loop sequence was amplified using the primer pairs D-loopF (5′-GAG GCC AAC CAG TTG AAC ACC C-3′) and D-loopR (5′-ATA AGG CCA GGA CCA AAC CT-3′) with references of the *Spalax ehrenbergi* mitochondrial genome (accession No. AJ416891) and the study of Yang et al. (2009).

PCR amplifications were performed in total reaction volumes of 50 μL, containing 10 mM Tris–HCl (pH 8.3), 1.5 mM MgCl2, 50 mM KCl, 100 μM of each dNTP, 0.25 μM of each primer (synthesized by Sangon), 0.5 μl (about 50 ng) of template DNA, and 2.5 U Taq DNA polymerase (Sangon, China). The reaction mixtures were denatured at 95 °C for 5 min and subjected to 35 cycles of 45 s at 95 °C, 1 min at 54 °C, 1.5 min at 72 °C, and a final extension step of 7 min at 72 °C. PCR products were purified using a CASpure PCR Purification Kit, following the manufacturer's recommended protocol (Casarray, Shanghai, China). Purified DNA products were sequenced in both directions with the PCR primers on an ABI 373 automated sequencer.

2.3. Molecular analysis

The genetic variation analyses of cytb and D-loop were analyzed separately. Sequences were aligned using CLUSTAL X (Thompson et al., 1997), with the default settings, and refined manually. The basic polymorphism information (variable site, haplotype distribution, etc.) was determined using DnaSP (version 4.0; Rozas et al., 2003).

Positive selection sites were detected using codeml program in PAML (Phylogenetic Analysis by Maximum Likelihood) program package version 4.4a (Yang, 2007). Following the suggestions in the User Guide of PAML program package, the site models M2a (selection) and M8 (beta&ω) with the Bayes Empirical Bayes algorithm were used. The methods use the nonsynonymous/synonymous substitution rate ratio (ω) as a measure of selective pressure at the protein level, with $\omega > 1$ indicating positive selection.

The Clade model C in codeml program allowed three classes of sites: site class 0 included codons that were subject to stringent functional constraints with $0 < \omega 0 < 1$ estimated from the data; site class 1 included codons that were unconstrained with $\omega_1 = 1$ fixed, and; site class 2 allowed selective pressure differing in different

parts of a phylogeny (for more than two clades), with ω_2 estimated as a free parameter. This provided a useful method to compare selection pressures among different zokor units. Since the haplotypes formed three monophyletic clades (see below), belonging to each of the three geographic regions, we used Clade model C to compare the ω values among three clades. In order to avoid the probable effects brought on by complicated models, a neighbor-joining Kimura 2-Parameters tree generated by MEGA (Kumar et al., 2008) was used as the tree structure file required by Clade model C; the U1, U2, and U3 were marked as #1, #2 and #3, respectively.

We also tested the selection pressure variation among three units based on the polymorphism (haplotype diversity of DNA/amino acid sequences) and mutation site information. The null hypothesis was: a unit confronted with higher selective pressure would have a lower ratio of amino acid/DNA polymorphism as well as a lower ratio of nonsynonymous/synonymous nucleotide mutations. The haplotype diversity based on cytb DNA (Hcd), cytb amino acid (Hca), and D-loop DNA (Hdl) sequences of each unit were determined using Arlequin (version 3.5; Excoffier and Lischer, 2010). The non-synonymous mutations of cytb (NSc) and the synonymous mutations of cytb (Sc) and D-loop (Sdl) sequences of the individuals were identified using MEGA: in each site, the majority nucleotide was defined as a wild type, while the minority was defined as a mutant type. The numbers of mutant types of the three units were then counted and compared with each other using crosstab analysis (chi-square test) in SPSS 15.0.

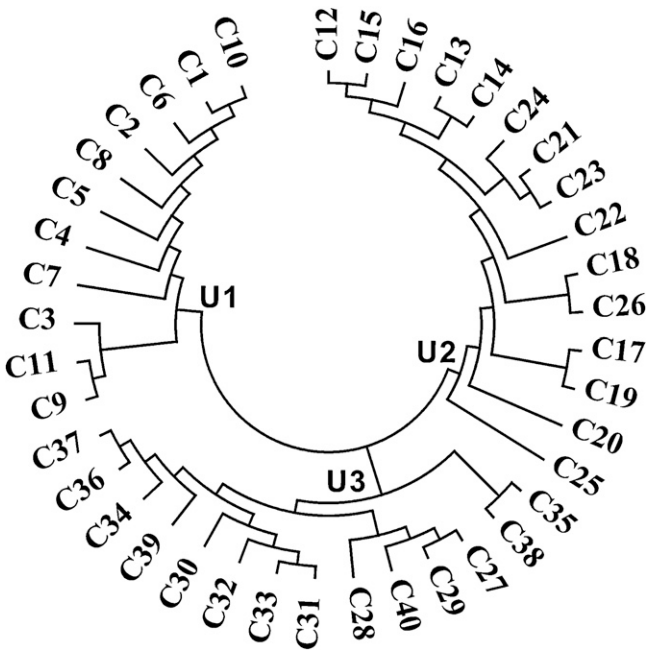

Fig. 2. Un-rooted neighbor-joining tree of the forty cytochrome b haplotypes based on Kimura 2-parameter distance method.

3. Results

The length of the complete cytb gene and partial D-loop were 1140 bp and 700 bp, respectively; there were no insertions or deletions in both of the two segments. The aligned sequence matrix of cytb gene and D-loop contained 111 and 143 variable sites, respectively. Forty cytb haplotypes (deposited in GenBank under Accession No. GQ244363 – GQ244402) and fifty-five D-loop haplotypes (GenBank under Accession No. JF797233 – JF797285) were identified.

The phylogenetic structure of the forty cytb haplotypes (belonging to three distinct clades) was shown in Fig. 2. The M2a and M8 models indicated that the amino acid site 117 was under positive selection, however, with low confidence levels ($P > 0.05$). Parameter estimates under Clade Model C with $k = 3$ site classes showed a larger set of sites (94.575%) evolving under strong purifying selection ($\omega = 0.02989$) and a small set of sites (5.425%) evolving under divergent selective pressures, with a very strong purifying selection in the U3 (ω_2) clade but weaker purifying selection in the U1 (ω_0) and U2 (ω_1) clades (Table 2).

The haplotype diversity (Hdl, Hcd, and Hca) and mutations (Sdl, Sc, and NSc) values were listed in Table 3. A total of 16 non-synonymous mutant codon sites were detected from cytb sequences, and 12 amino acid types were involved in these mutations. Seven sites occurred in U1, eight sites occurred in U2, while only two occurred in U3 (Table 4). Crosstab analysis indicated that the ratio of NSc/Sc of U3 (6/359) is significantly ($\chi_1^2 = 7.69$, $P < 0.01$) smaller than that of U2 (30/547) but is smaller than that of U1 (36/1226) without significance ($\chi_1^2 = 1.65$, $P = 0.135$). The ratio of NSc/Sdl of U3 (6/965) is significantly ($\chi_1^2 > 14.50$, $P < 0.001$) smaller than that of U1 (36/1233) and U2 (30/1017).

4. Discussion

In contrast to the purported evidence for positive selection on mitochondrial proteins in humans and other nonhuman mammals (see the reviews in Gering et al., 2009), results of our tests suggest that the evolution of cytb in Gansu zokor is chiefly governed by purifying selection. Interestingly, however, we detected different ω values in site class 2 among the three regions at the same time, i.e., a stronger purifying selection in the U3 clade (ω_2) than in the U1 (ω_0) and U2 (ω_1) clades (Table 2).

There were similar DNA sequence-based haplotype diversity values (Hdl and Hcd) among the three units, however, the amino acid sequence-based haplotype diversity of U3 was lower than half of those of U1 and U2. Additionally, the distribution of non-synonymous mutant codon sites was rather disproportional: the U3 had only two non-synonymous sites, which was merely a quarter of that in U1 and U2. Moreover, as mentioned above, the crosstab analysis showed that the ratio of non-synonymous/synonymous mutations of U3 was smaller than those of U1 and U2. These results again indicated that zokors in U3 had confronted an obviously higher purifying selection pressure than those from U1 and U2.

The subterranean ecotopes of zokors as well as other subterranean rodents are hypoxic and hypercapnic (reviewed in Nevo (1999) and Begall et al. (2007)). Furthermore, the inspired oxygen pressure falls linearly with altitude (Peacock, 1998) and, subsequently, the hypoxia stress increases with increasing altitude. The Mann–Whitney test showed that U3 had a higher mean altitude than U1 and U2 ($N = 8$, $P < 0.05$), indicating that zokors in U3 were confronted with severer hypoxia stress. Also, the Mann–Whitney test showed that the temperature and precipitation values in U3 was much lower than the other two regions ($N = 8$, $P < 0.05$). Hence, zokors in U3 needed to spend more energy than their U1 and U2 counterparts to deal with the cold weather (Zeng et al., 1984) and dry hard soil (Wang et al., 2000). We hypothesize that the increased hypoxia stresses and the severely cold and arid conditions in U3 caused local zokors to have stronger purifying selection in the cytb gene than the other two units.

Rather than the selection pressure indices used in this study, previous studies on *Spalax ehrenbergi* superspecies, another group of subterranean rodents, directly used genetic diversity (heterozygosity, heterogeneity, haplotype diversity, etc.) distribution to test the animal's adaptation to regions with different climatic conditions. Their results showed that the genetic diversity increased with climatic unpredictability toward the arid regions (Nevo et al., 1994, 1999; Reyes et al., 2003; Karanth et al., 2004). Nevo et al. (1999)

Table 2
Parameter estimates for the cytochrome b gene of Gansu zokor (SC, site class; ω_0, U1 clade; ω_1, U2 clade; ω_2, U3 clade; for details of the parameters please see the manual of PAML version 4.3).

Model	Parameters estimated	Positive site
M2a ($k = 3$)	P: 0.99197, 0.00260, 0.00542 ω: 0.04043, 1.00000, 1.00000	117 V, $P = 0.542$
M8 ($k = 11$)	$P_0 = 0.99999$, $P = 0.32304$, $q = 6.07335$, $P_1 = 0.00001$, $\omega = 1.00000$	117 V, $P = 0.666$
Clade model C ($k = 3$)	SC: 0, 1, 2 P: 0.94575, 0.00000, 0.05425 ω_0: 0.02989, 1.00000, 0.45794 ω_1: 0.02989, 1.00000, 0.41763 ω_2: 0.02989, 1.00000, 0.00010	–

Table 3
Haplotype diversity and mutations based on zokor individuals in each of the three geographical units U1-U3 (Hdl, haplotype diversity of D-loop DNA sequence; Hcd, cytb DNA sequence; Hca, haplotype diversity of cytb amino acid sequence; Sdl, synonymous mutations of D-loop; Sc, synonymous mutations of cytb; NSc, non-synonymous mutations of cytb).

Unit	Hdl	Hcd	Hca	Sdl	Sc	NSc
U1	0.908 ± 0.024	0.880 ± 0.026	0.607 ± 0.0561	1233	1226	36
U2	0.945 ± 0.015	0.932 ± 0.015	0.739 ± 0.0839	1017	547	30
U3	0.961 ± 0.012	0.924 ± 0.016	0.256 ± 0.0769	965	359	6

Table 4
Distributions (frequency) of non-synonymous mutations in each of the three geographical units (U1-U3) based on 121 cytochrome b sequences (WA, wild type amino acid; MA, mutant type amino acid; WC, wild type codon; MC, mutant type codon).

Site	WA	MA	WC	MC	U1	U2	U3
16	H	Q	CAT/CAC	CAA	0	7	0
39	V	I	GTC	ATC	2	0	0
81	Y	H	TAC	CAC	0	1	0
109	Y	N	TAT	AAT	2	0	0
117	V	I	GTT	ATT	1	2	0
122	T	A	ACT	GCT	0	0	3
173	A	S	GCT	TCT	0	1	0
194	I	V	ATT	GTT	2	0	0
214	D	N	GAC	AAC	2	0	0
230	L	M	CTA	ATA	0	5	0
234	I	T	ATC	ACC	2	0	0
306	M	T	ATA	ACA	0	2	0
348	I	T	ATC	ACC	0	0	3
349	I	M	ATC	ATA	0	7	0
372	L	M	CTA	ATA	0	5	0
376	I	M	ATC/ATT	ATA	25	0	0

hypothesized that two selective regimes had directed cytochrome b evolution in the *S. ehrenbergi* superspecies: the populations in continuously hypoxic range were mainly governed by purifying selection, while those in climatically variable regions were affected by diversifying selection. For the three regions where the zokors were sampled in the present study, the environmental conditions in the sampling sites within each region did not differ too much from each other (see Table 1). Also, the climatic conditions generally changed synchronously among different regions (Wang et al., 2004). Hence, the continuously opposite selective stresses which increase from east to west, rather than their unpredictability within local regions, constitute the key selective forces on zokors. We suggest this is the main reason that the zokors are chiefly governed by purifying selection (with different intensities in different regions) although further studies are still needed to verify this hypothesis.

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