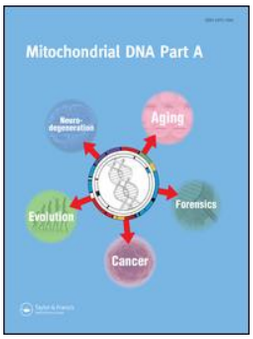

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FULL LENGTH RESEARCH PAPER

Phylogenetic analysis of the endangered takin in the confluent zone of the Qinling and Minshan Mountains using mtDNA control region

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Abstract

The takin (*Budorcas taxicolor*) is an Endangered ungulate. We analyzed the variation within mtDNA control region sequences of takin populations in the Qinling Mountains, the Minshan Mountains and the confluence of these two mountain ranges. We did not find any shared haplotypes among the populations. We observed apparent variation in the control region length among the three populations, and independent population expansions in the late of Pleistocene, which suggests these populations may have independent evolutionary histories. We found only one haplotype, and the lowest measures of genetic diversity ($h = 0$; $\pi = 0$) in the population from the confluent zone, which suggests populations in the confluent zone may have grown from small founder populations and gene flow with other populations has ceased. Based on their phylogenetic relationships, we concluded that the takin population in the confluent zone was in the same clade as the Tangjiahe population, which suggests that these takin populations are Sichuan takin (*Budorcas taxicolor tibetana*).

Keywords

D-loop region, genetic diversity, genetic structure, population history

History

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Introduction

Various genetic markers are used to study the genetic diversity and the evolutionary history of Endangered animals in conservation genetics, including microsatellite, MHC, and mitochondrial DNA (mtDNA) (Sunnucks, 2000; Ujvari & Belov, 2011; Wan et al., 2004). MtDNA is maternally inherited genetic marker and does not exhibit recombination (Harrison, 1989). The mtDNA control region (CR) sequence is a highly variable region, and the mutation rate is estimated to be several times higher than that of the remainder of the mtDNA genome (Aquadro & Greenberg, 1983; Sigurðardottir et al., 2000). In addition, studies indicate that the mtDNA CR sequence is a reliable tool to study the relationships among different populations (Wilkinson & Chapman, 1991). Therefore, the CR sequence is an effective marker to resolve problems of genetic relationships below the rank of species; for instance, phylogenetic analysis or genetic diversity studies of goats (Kang et al., 2011), horses (Bigi et al., 2014), Japanese Sika deer (*Cervus nippon*), Saudi native chicken strains (Yacoub & Fathi, 2013), *Brachymystax lenok tsinlingensis* (Liu et al., 2014), etc.

The takin (*Budorcas taxicolor*) is an Endangered member of the subfamily Caprinae (Wu, 1990). This species has four subspecies, out of which two are endemic to China: the golden takin (*Budorcas taxicolor bedfordi*) which mainly dwells in the Qinling mountains, and the Sichuan takin (*Budorcas taxicolor tibetana*) with a distribution that covers portions of the Minshan, Qionglai, Xiangling and Liangshan Mountains (Wu, 1990). The golden takin's color varies from creamy white to golden yellow, while the Sichuan takin is darker, from yellowish grey to reddish brown (Li et al., 2003). The takin populations in the Maozhai and Qingmuchuan (MZ-QMC; within the confluent zone of the Minshan and Qinling Mountains) Nature Reserves were first recorded as golden takin according to the geographical distribution of populations (Wu, 1986). However, other researchers have suggested that the takin populations in the MZ-QMC Nature Reserves are in fact Sichuan takin on the basis of morphological characteristics (Li et al., 2003; Zeng et al., 2005). This controversy may be resolved using methods from molecular biology.

Here, the genetic diversity and phylogenetic structure of the takin, based on mtDNA CR sequences, were measured in the populations found in the confluent zone of the Qinling and Minshan Mountains (MZ-QMC), then compared with those of populations from the central Qinling Mountains and from the eastern area of the Minshan Mountains. Our aim was to improve our understanding of the level of genetic diversity in the small, isolated MZ-QMC population, and to provide genetic evidence that can definitively identify the subspecies status of takin in the confluent zone for the first time.

Methods

Sample collection

Samples were collected via non-invasive sampling methods from 2006 to 2009. Sampling sites were selected from three different nature reserves: MZ-QMC, Foping Nature Reserve (FP; in the Qinling Mountains), and Tangjiahe (TJH; in the Minshan Mountains) Nature Reserve (Figure 1). We ensured a minimum distance between sampling locations to avoid counting the same

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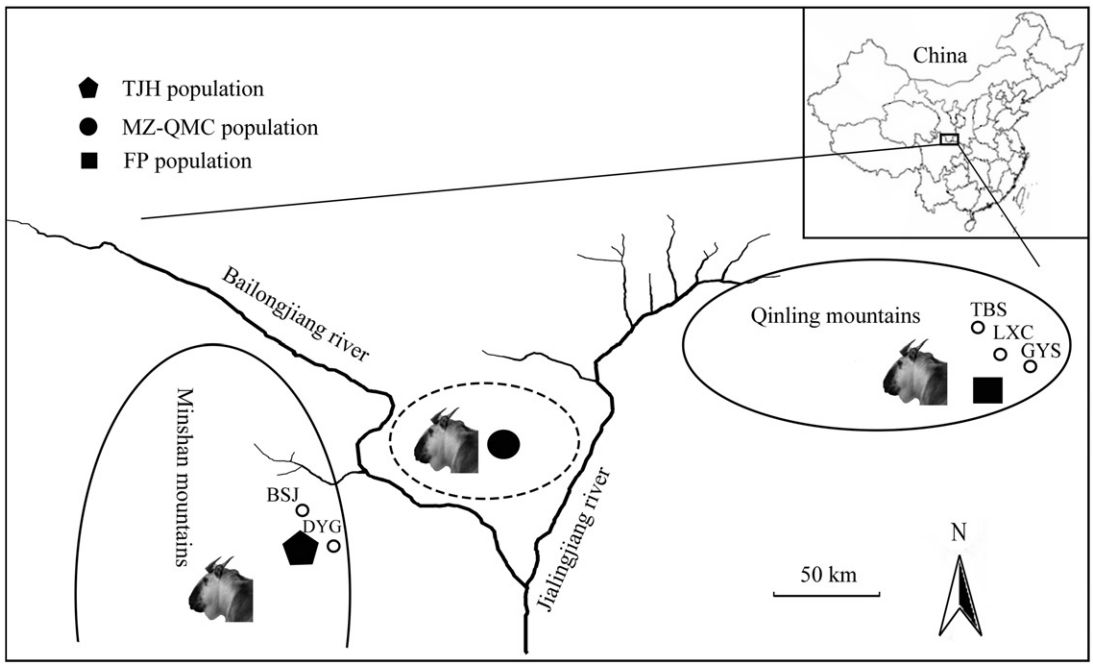

Figure 1. The distribution of takin in the Minshan Mountains, Qinling Mountains and the confluent zone. The takin distribution regions are denoted with semiellipse or ellipse, and the dashed ellipse indicates the confluent zone. The empty circles denote neighboring nature reserves. TJH, Tangjiahe Nature Reserve; MZ-QMC, Maozhai and Qingmuchuan Nature Reserves; FP, Foping Nature Reserve; BSJ, Baishuijiang Nature Reserve; DYG, Dongyanggou Nature Reserve; TBS, Taibaishan Nature Reserve; LXC, Laoxiancheng Nature Reserve; GYS, Guanyinshan Nature Reserve.

Table 1. Summary information on the samples of takin used in this study.

Mountain range	Sampling site	Type of sample (*n*)
Minshan	TJH	Faeces (17), Muscle (3)
The confluent zone of Qinling and Minshan	MZ-QMC	Faeces (13)
Qinling	FP	Skin (23)

individuals repeatedly. Our samples included feces ($N = 40$), muscles ($N = 3$) and museum skins ($N = 23$) (Table 1).

Laboratory methods

Genomic DNA in the fecal samples was extracted using the CTAB method based on previous work (Zhang et al., 2006). Total genomic DNA in the fresh muscle tissue and the museum skins was extracted using standard methods (Sambrook, 1989). We designed a set of primers to amplify the partial overlapping sequences and obtain the complete mtDNA CR sequences. Three pairs of primers were designed. The primers for the first hypervariable region are: L15383 (forward), 5′-CCT CAC CAT CAA CAC CCA A-3′ and H16096 (reverse), 5′-GAG AAA TCC CTG CCA AGC-3′. Moreover, two pairs of primers were designed to amplify the second hypervariable sequences: primers L15993 (forward), 5′-TAA AGT CAA ATC TAT CCT CGT C-3′ and H186 (reverse), 5′-TGC TTG ATA CCT GCT CCT-3′ for the FP takin population; the primers L15966 (forward), 5′-TTC CCT GCT TGA ATC GTA-3′ and H186 (reverse), 5′-TGC TTG ATA CCT GCT CCT-3′) for the MZ-QMC and TJH takin populations.

The reaction (PCR) mixes were performed in 50 µl of total volume containing 20 ng of template DNA (0.5–1 µl), 20 µM of each primer (1 µl), 2.5 mM of each dNTP (4 µl), 10 × PCR buffer with $MgCl_2$ (5 µl), 5 U/µl Taq DNA polymerase (0.25 µl), and sterile water to dilute to the final volume. The cycling profile began with 1 cycle of DNA denaturation at 94 °C for 4 min, followed by 34 cycles of sequence amplification, each with a denaturation step at 94 °C for 45 s, annealing at 47–51 °C (48 °C for pairs of L15383 and H 16096, 51 °C for L15993 and H186, 47 °C for L15996 and H186) for 45 s, and extension at 72 °C for 5 min. The products were electrophoresed through 2% agarose gel with Ethidium bromide and inspected under UV light to confirm the success of the amplification procedure. Afterwards, the products were purified by a Cycle-pure kit (Omega, Guangzhou, China) and sequenced by the ABI 377 automated Sequencer (Invitrogen Co. limited, Shanghai, China). Then, the sequences were ascertained by comparison with published sequences of the target animal. Negative and positive controls, respectively, were adopted to avoid error. Finally, to ensure the accuracy of the results, haplotypes were defined when we found them in different individuals or the same haplotype was found in all cases when we completed the entire experimental procedure independently three times.

Statistical methods

We used Tandem Repeats Funder Program to find the tandem repeat sequences in the takin mtDNA CR sequences (Benson, 1999). In addition, we identified conserved sequence boxes (CSB) in accordance with the positions in other mammals (Saccone et al., 1991; Southern et al., 1988). We detected termination association sequences (TAS) according to the sequences in humans and mice (Doda et al., 1981).

The sequences were aligned with Clustal X2 (Larkin et al., 2007) and refined manually. The software MEGA 5 was used to calculate average nucleotide distance using the Kimura 2-parameter model (Tamura et al., 2011). Standard haplotype diversity (h) and nucleotide diversity (π) within populations were estimated using DnaSp 5 (Librado & Rozas, 2009). The hierarchical analysis of molecular variance (AMOVA) was performed in Arlequin 3.5 (Excoffier et al., 2005). For the

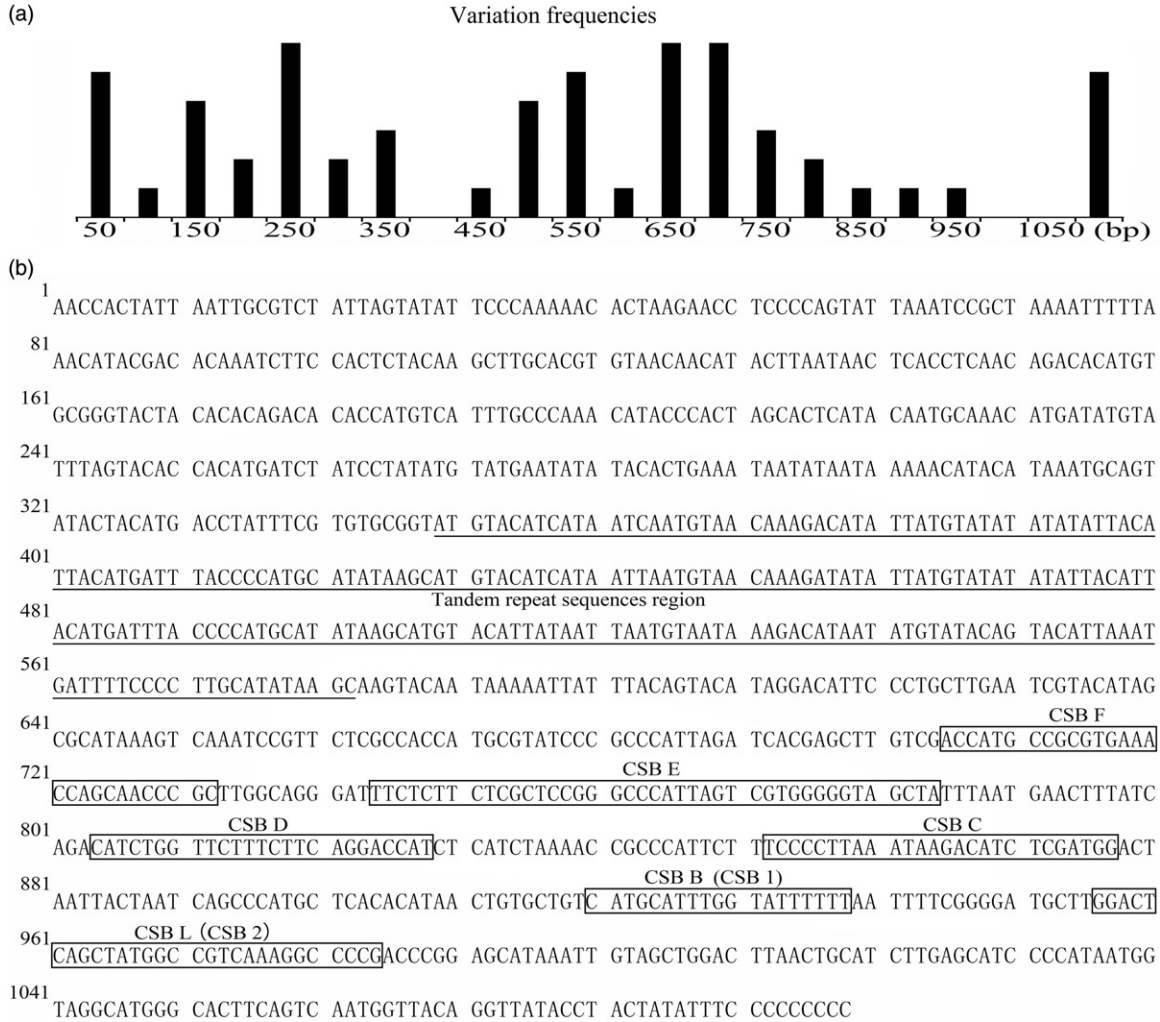

Figure 2. The nucleotide sequence variation of the takin CR sequence. (a), the variation frequencies of every 50 bp CR sequences. (b), the CR sequence (JQ229952.1) analysis of the takin. The tandem repeat sequences region is underlined, and CSB are denoted with simple rectangle boxes.

AMOVA analysis, 1000 permutations with the Kimura 2-parameter distance method were used to calculate the genetic structure of populations. The extent of genetic differentiation (fixation index, F_{ST}) of subpopulations was calculated in Arlequin 3.5 (Excoffier et al., 2005). A Mantel test was performed to test the correlation between ongoing gene flow and geographical distances with 10,000 permutations (Mantel, 1967). Geographical distances were measured by the direct distance between two sample sites using Google Earth. Mismatch distribution was analyzed in DnaSp 5, and the raggedness statistic, Ramos-Onsins and Rozas statistic (R_2 statistic), were calculated simultaneously (Librado & Rozas, 2009). In addition, DnaSp 5 was also used to calculate the values of Tajima's D, Fu and Li's D, Fu and Li's F and Fu's Fs statistic (Librado & Rozas, 2009).The neighbor-joining (NJ) and Bayesian inference (BI) trees were constructed to detect the phylogenetic relationship of all haplotypes, and the relationship between the haplotypes and the other goat species. The NJ tree was constructed in MEGA 5 using Kimura 2-parameter distance model, and the reliability of the phylogeny was tested by bootstrapping with 10,000 permutations (Tamura et al., 2011). The BI tree was constructed in Mrbayes 3.2 (Ronquist & Huelsenbeck, 2003), and Mrmodeltest 2.3 was used to find the best substitution model (Nylander, 2004). The run length in Markov chain Monte-Carlo analysis was 10,000,000 generations. The sample frequency was 1000, and the first 25% of samples were discarded as burn-in.

Results

CR characteristics

The mtDNA CR sequences in the TJH population were 1098 bp long. In contrast, the complete CR sequences in the MZ and FP populations were 1018 and 1016 bp long, respectively. Our study showed that the takin also has two hypervariable regions within the mtDNA CR sequences (Figure 2a). We found six CSBs in the CR sequence of the takin, CSB B (CSB 1), CSB C, CSB D, CSB E, CSB F and CSB L (CSB 2), and a tandem repeat sequences region which included three repeats in the left region of the CR (Figure 2b). In addition, there were three TASs in the tandem repeat sequences region (TAS1, TAS2 and TAS3), and five pairs of hairpin sequences (TACAT and ATGTA) in the tandem repeat region (Figure 3). Finally, we found an insertion of nucleotides (TA) in the first repeat and a deletion of nucleotides (TA) in the third repeat (Figure 3).

Population history

For the neutrality tests, Tajima's D, Fu and Li's D, Fu and Li's F, and Fu's Fs tests were all negative in the TJH and FP populations (Table 2), which indicates that the TJH and FP populations are not at equilibrium. Selection pressure, past bottleneck or population expansion could explain this result (Loaiza et al., 2010). We also found a relatively low value of the R_2 and Raggedness statistics in

our study (Table 2). We used $t = \tau/2u$ to calculate the time since population expansion, where $u = \mu NT$, μ is the mutation rate of CR sequence, N is the number of base pairs of the CR sequences, and T is the generation time of the takin (Loaiza et al., 2010). The mutation rate of 10^{-7} site/year was used to calculate the time since expansion (Henn et al., 2009), and the generation time of the takin is 10 years (Wu, 1990). We observed that the time since population expansion was 57,969 years (0.058 Mya) ago in the TJH population and 62,524 years (0.062 Mya) ago in the FP population.

We found unimodal curves in the TJH and FP populations by mismatch distribution analysis (Figure 4), which also denotes

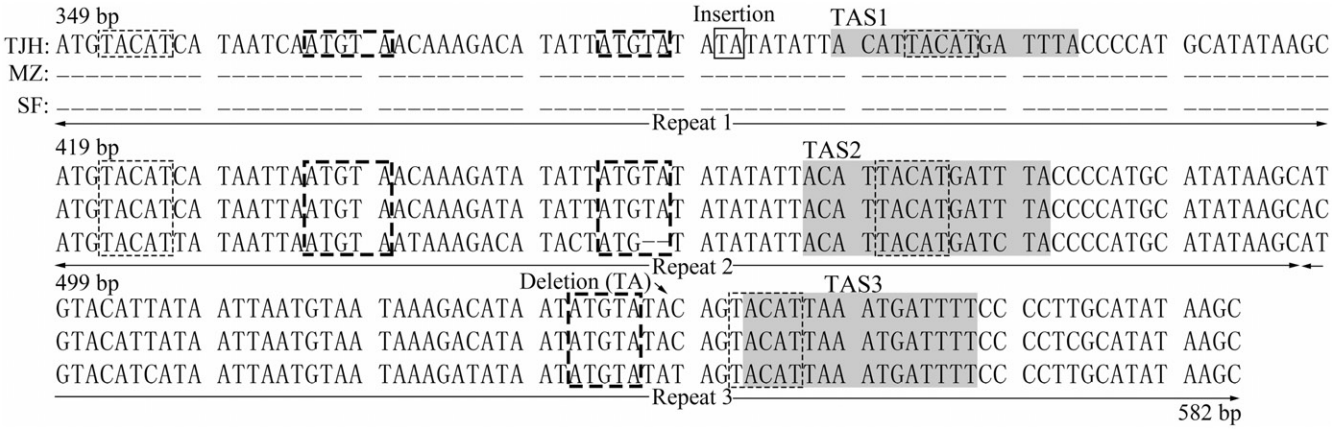

Figure 3. The tandem repeat sequences variation (marked under sequences alignment with underline) of the three isolated takin populations. TJH, Tangjiahe population; MZ (MZ-QMC), Maozhai-Qingmuchuan populations; SF, Foping population. Hyphens (-) indicate deletion compared with the TJH population. Simple and bolded dashed boxes indicate paired hairpin sequences (TACAT and ATGTA). TAS (termination association sequences) are marked with a gray background.

Table 2. Neutrality tests of takin populations.

Population	*D* (*p* value)	*D** (*p* value)	*F** (*p* value)	*F*s (*p* value)	R_2	r
TJH	−1.16 (>0.10)	−1.56 (>0.01)	−1.67 (>0.01)	−2.05 (>0.05)	0.10	0.14
MZ-QMC	NA	NA	NA	NA	NA	NA
FP	−1.08 (>0.10)	−1.28 (>0.10)	−1.41 (>0.10)	−5.08 (<0.01)	0.08	0.04

D, Tajiama'D; *D**, Fu and Li's *D*; *F**, Fu and Li's *F*; *F*s, Fu's *F*s statistic; r, Raggedness statistic; R_2, Ramos-Onsins and Rozas, R_2 statistic; NA, no values. We did not analyze the data in the MZ-QMC population which was found only one haplotype.

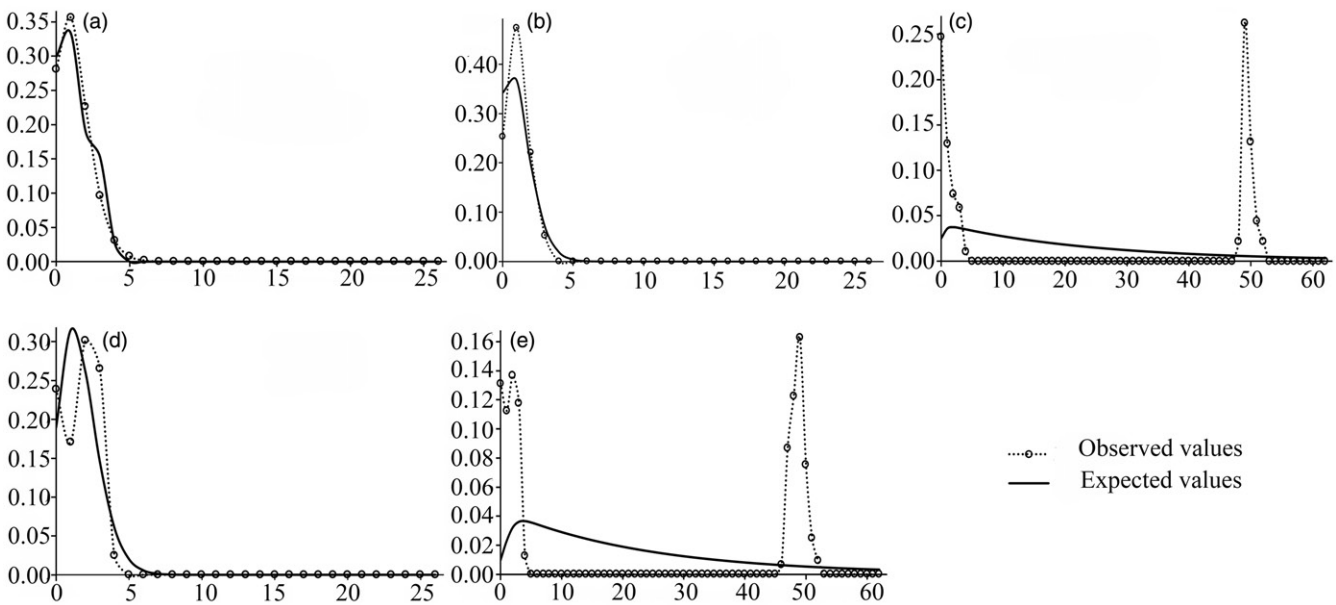

Figure 4. Mismatch distribution (pairwise nucleotide site differences) analysis of the takin populations. (a), samples from the FP population; (b), samples from the TJH population; (c), samples from the MZ-QMC and FP populations; (d), samples from the MZ-QMC and TJH populations; (e), samples from the MZ-QMC, FP and TJH populations.

population expansion of the FP and TJH populations (Rogers & Harpending, 1992). We tried to detect the mismatch distribution of the MZ-QMC population (only one haplotype) by comparing the samples with the other two populations. If the MZ-QMC population has the same population history as the other populations, the mismatch distribution curve (samples with MZ-QMC and the other populations) may be the same as the mismatch distribution curves (samples only from the other populations) of the other populations. Our results showed that the MZ-QMC population has a different population history than both the TJH and FP populations (Figure 4).

Table 3. Genetic diversity of takin population. *h*, haplotype diversity; π, nucleotide diversity.

Population	Sample sizes	Haplotypes	Transition	Tranversion	*h*	π
TJH	20	6	4	2	0.75	0.00098
MZ-QMC	13	1	0	0	0.00	0.00000
FP	23	9	5	2	0.70	0.00130

Genetic diversity

Overall, the samples contained 16 (H01–H16) mtDNA CR haplotypes (Accession No. in Genbank, JQ229951–JQ229966) which included 59 mutation sites in total. However, we did not find any haplotypes shared among the takin populations. The haplotypes in the TJH population were H01, H02, H03, H05, H06 and H07, and the haplotypes in the FP population were H08, H09, H10, H11, H12, H13, H14, H15 and H16. We found only one haplotype in the MZ-QMC population (H04). Our results showed that the nucleotide ratio of A + T was higher than that of G + C (A + T = 61.48%, C + G = 38.52%). We observed that the average nucleotide distance of all individuals was 7.3%, whereas we found low average nucleotide distance within each population (TJH 0.1%, FP 0.1%, MZ-QMC 0.0%). In addition, the MZ-QMC population had the lowest haplotype diversity ($h = 0$) and nucleotide diversity ($\pi = 0$) compared to the other two populations (Table 3).

Phylogeographic structure

The data from AMOVA revealed significant genetic differentiation among the three takin populations. We calculated both the intrapopulation and interpopulation variance (Table 4). *F*-statistic showed the fixation index (F_{ST}) between the

Table 4. AMOVA of CR sequences for the three takin populations.

Source of variation	df	Sum of squares	Variance components	Percentage of variation	Fixation indices	*p* Value
Among populations	2	8.94	0.23 Va	44.75	0.45	0.00 ± 0.00
Within populations	53	15.06	0.28 Vb	55.25		
Total	55					

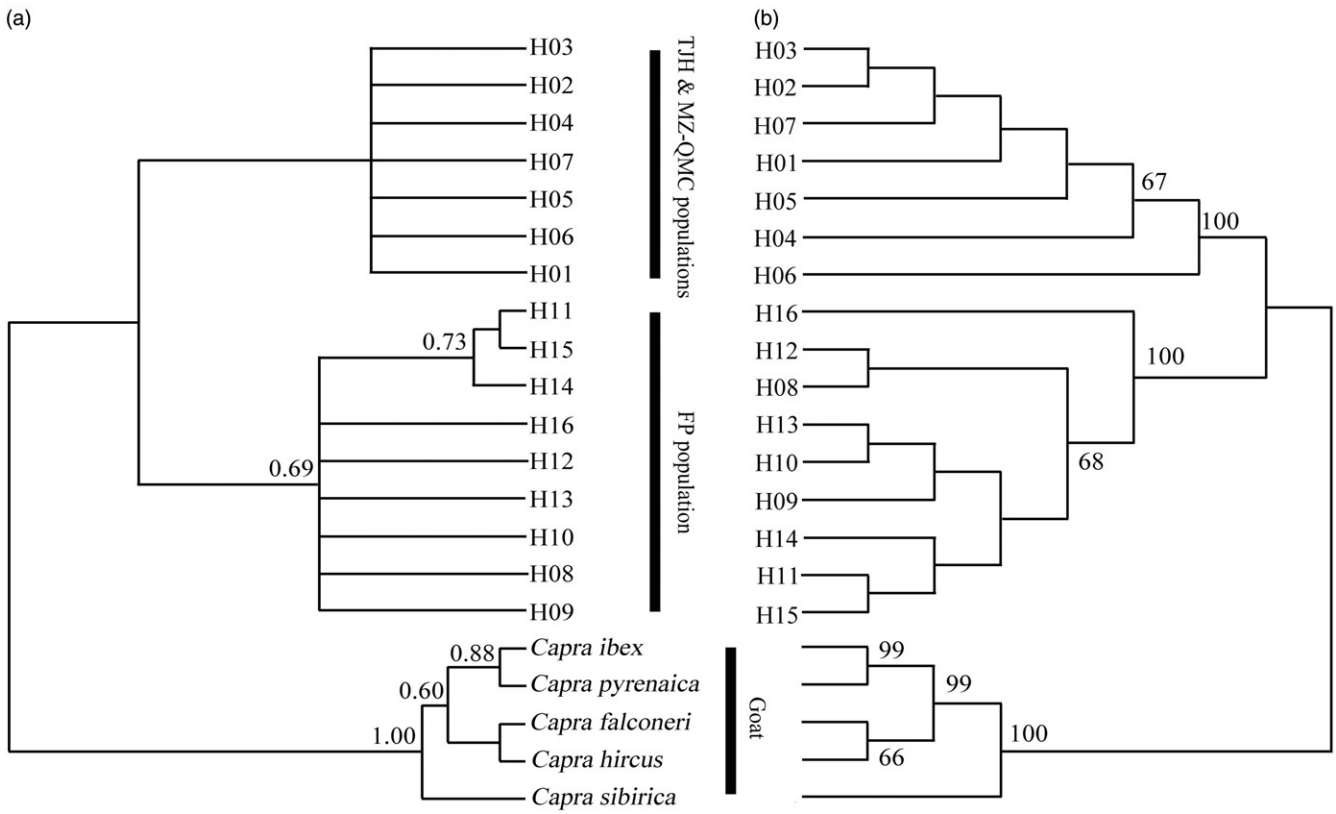

Figure 5. Evolutionary relationship of the haplotypes of the takin populations. (a), BI tree; (b), NJ tree. Goat species: *Capra hircus* (AB004081), *C. pyrenaica* (NC_020625), *C. falconeri* (NC_020622), *C. sibirica* (NC_020626) and *C. ibex* (NC_020623).

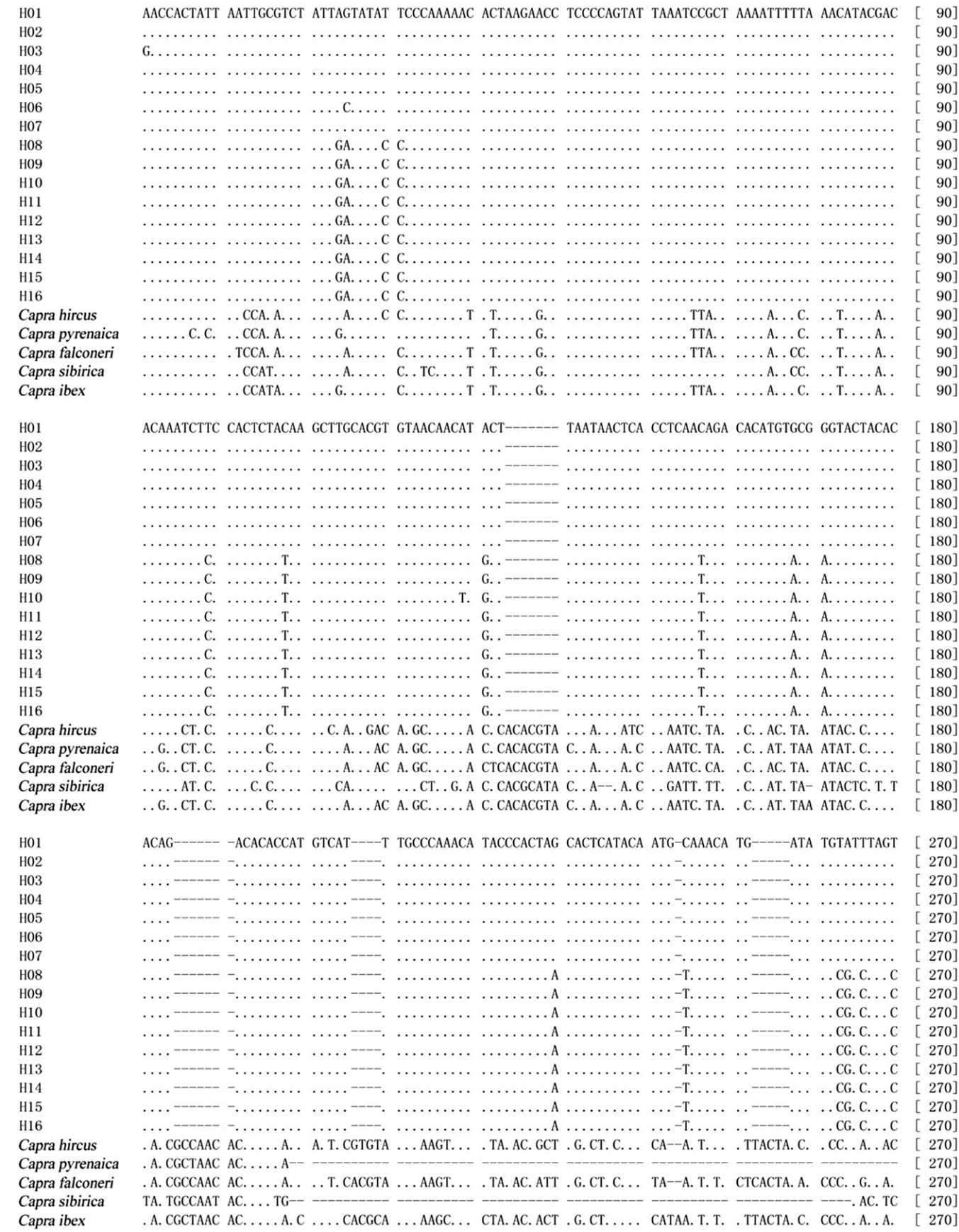

Figure 6. The D-loop sequence alignment between takin and the other goat species. Small dot (.) indicates the same nucleotide as the first sequence, and the short dash (–) indicates a gap in the alignment. The sequences of goat species are the same as Figure 5.

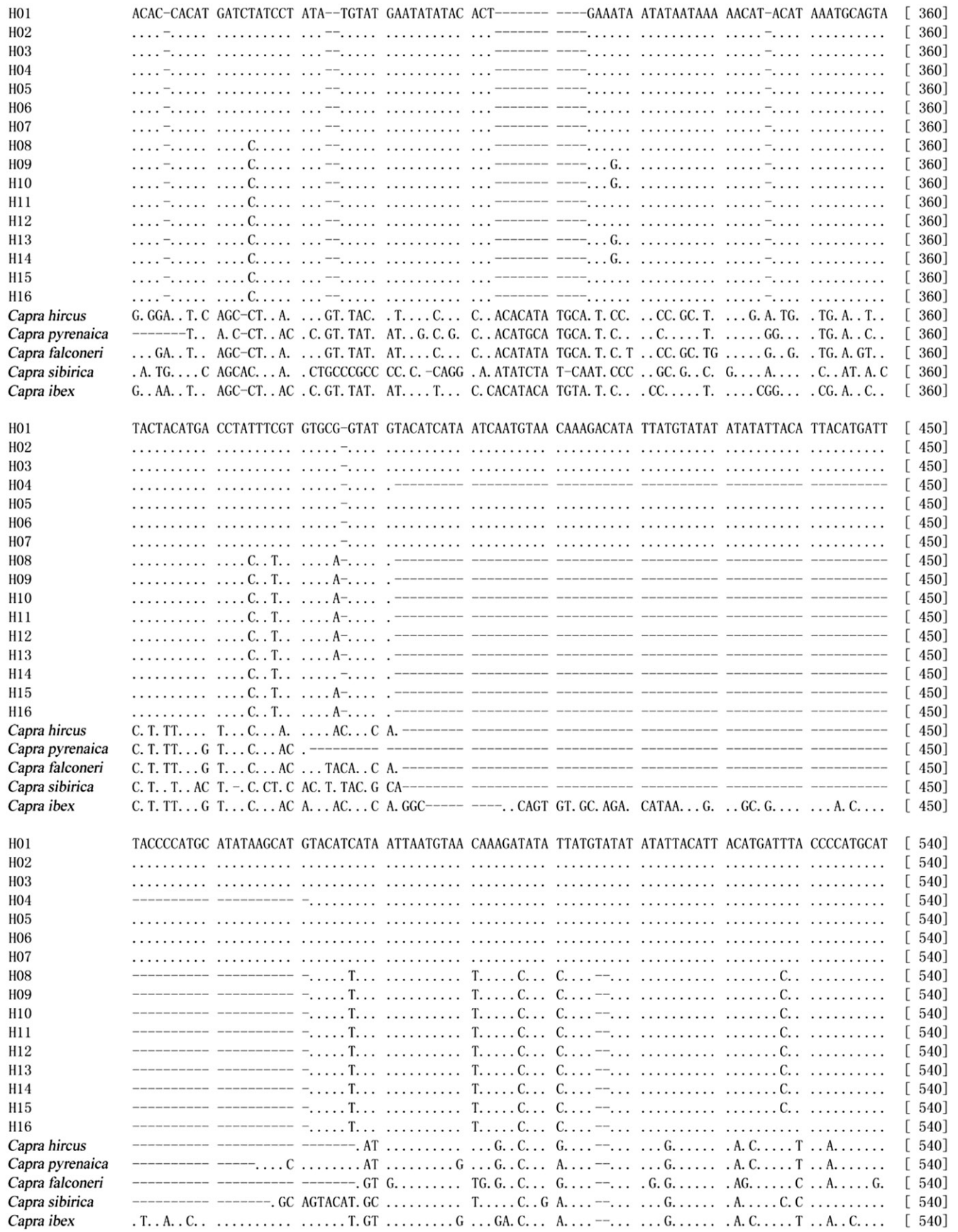

Figure 6. Continued.

```
H01              AT-AAGCATG TACATTATAA TTAATGTAAT AAAGACATAA TATGTATACA GTACATTAAA TGATTTTCCC CTTGCATATA AGCAAGTACA [ 630]
H02              ..-..... .......... .......... .......... .......... .......... .......... .......... .......... [ 630]
H03              ..-..... .......... .......... .......... .......... .......... .......... .......... .......... [ 630]
H04              ..-.....C. .......... .......... .......... .......... .......... .......... ..C..... .......... [ 630]
H05              ..-..... .......... .......... .......... .......... .......... .......... .......... .......... [ 630]
H06              ..-..... .......... .......... .......... .......... .......... .......... .......... .......... [ 630]
H07              ..-.....G.. .......... .......... .......... .......... .......... .......... .......... [ 630]
H08              ..-.....C.... .......... .....T.... ........T. .......... .......... .......... .......... [ 630]
H09              ..-.....C.... .......... .....T.... ........T. .......... .......... .......... .......... [ 630]
H10              ..-.....C.... .......... .....T.... ........T. .......... .......... .......... .......... [ 630]
H11              ..-.....C.... .......... .....T.... ........T. .......... .......... .......... .......... [ 630]
H12              ..-.....C.... .......... .....T.... ........T. .......... .......... .......... .......... [ 630]
H13              ..-.....C.... .......... .....T.... ........T. .......... .......... .......... .......... [ 630]
H14              ..-.....C.... .......... .....T.... ........T. .......... .......... .......... .......... [ 630]
H15              ..-.....C.... .......... .....T.... ........T. .......... .......... .......... .......... [ 630]
H16              ..-.....C.... .......... .....T.... ........T. .......... .......... .......... .......... [ 630]
Capra hircus     ..T.....C. .....C.GT. .......... ..G......G ........TT .......... C...C....T .A........ ....T...T. [ 630]
Capra pyrenaica  ..-.....C. .....C.AT. ........GC ..G..... ........T. .......... C........A .A........ ....T..... [ 630]
Capra falconeri  ..T.....C. ........GT. ........G. .........G G.......TC .......... .......... .A........ ....T..... [ 630]
Capra sibirica   ..-....CC. .....C.AT. .......... ..G..... ........T. .......... .......... .A........ ....T..... [ 630]
Capra ibex       ..-..... .......GT. ........GC ..GA..... ........T. .......... C........A .AC..... ....T..... [ 630]

H01              ATAAAAATTA TTTACAGTAC ATAGGACATT CCCTGCTTGA ATCGTACATA GCGCATAAAG TCAAATCCGT TCTCGCCACC ATGCGTATCC [ 720]
H02              .......... .......... .......... .......... .......... .......... .......... .......... .......... [ 720]
H03              .......... .......... .......... .......... .......... .......... .......... .......... .......... [ 720]
H04              .......... .......... .......... .......... .......... .......... .......... .......... .......... [ 720]
H05              .......... .......... .......... .......... .......... .......... .......... .......... .......... [ 720]
H06              .......... .......... .......... .......... .......... .......... .......... .......... .......... [ 720]
H07              .......... .......... .......... .......... .......... .......... .......... .......... .......... [ 720]
H08              ..........C..T..... .......... T.......AG .......... .......... .......TA. C....T.... .......... [ 720]
H09              ..........C..T..... .......... T.......AG .......... .......... .......TA. C....T.... .......... [ 720]
H10              ..........C..T..... .......... T.......AG .......... .......... .......TA. C....T.... .......... [ 720]
H11              ..........C..T..... .......... T.......AG .......... .......... .......TA. C....T.... .......... [ 720]
H12              ..........C..T..... .......... T.......AG .......... .......... .......TA. C....T.... .......... [ 720]
H13              ..........C..T..... .......... T.......AG .........G .......... .......TA. C....T.... .......... [ 720]
H14              ..........C..T..... .......... T.......AG .......... .......... .......TA. C....T.... .......... [ 720]
H15              ..........C..T..... .......... T.......AG .......... .......... .......TA. C....T.... .......... [ 720]
H16              ..........C..T..... .......... T.......AG .......... .......... .......TA. C....T.... .......... [ 720]
Capra hircus     ..G-CTTC...CGG..... ....T..... TTACTG.AT. T.......... ..A....G.. ........A. ...T.T..A. .......... [ 720]
Capra pyrenaica  G.G-TTTCC...G..... ....T..... TTACTGCAT. C.......... ..A...G... ........A. ........A. ....A..... [ 720]
Capra falconeri  ..T-TCCC...AA..... ..G.T..... TTGCTG.ATG T.......... ..A....G.. .......T.. C..T.T..A. ....A..... [ 720]
Capra sibirica   G.T-T.CC...A..... .......... TTACTGCAT. T.......... ..A...G... ........A. .....T..A. .......... [ 720]
Capra ibex       G..-TCT.C. .CA..... ..G.T..... TTACTGCATG T.......... .TA...G.G. ........A. ...T....A. ....A..... [ 720]

H01              CGCCCATTAG ATCACGAGCT TGTCGACCAT GCCGCGTGAA ACCAGCAACC CGCTTGGCAG GGATTTCTCT TCTCGCTCCG GGCCCATTAG [ 810]
H02              .......... .......... .......... .......... .......... .......... .......... .......... .......... [ 810]
H03              .......... .......... .......... .......... .......... .......... .......... .......... .......... [ 810]
H04              .......... .......... .......... .......... .......... .......... .......... .......... .......... [ 810]
H05              .......... .......... .......... .......... .......... .......... .......... .......... .......... [ 810]
H06              .......... .......... .......... .......... .......... .......... .......... .......... .......... [ 810]
H07              .......... .......... .......... .......... .......... .......... .......... .......... .......... [ 810]
H08              ..T...C... ..........AC..... .......... .......... .......... .......... .......... .........A [ 810]
H09              ..T...C... ..........AC..... .......... .......... .......... .......... .......... .........A [ 810]
H10              ..T...C... ..........AC..... .......... .......... .......... .......... .......... .........A [ 810]
H11              ..T...C... ..........AC..... .......... .......... ....A..... .......... .......... .........A [ 810]
H12              ..T...C... ..........AC..... .......... .......... .......... .......... .......... .........A [ 810]
H13              ..T...C... ..........AC..... .......... .......... .......... .......... .......... .........A [ 810]
H14              ..T...C... ..........AC..... .......... .......... ....A..... .......... .......... .........A [ 810]
H15              ..T...C... ..........AC..... .......... .......... ....A..... .......... .......... .........A [ 810]
H16              ..T...C... ..........AC..... .......... .......... .......... .......... .......... .........A [ 810]
Capra hircus     ..T...C... ...T..... .......... .......... .......... ....CC.... .......... .......... .........A [ 810]
Capra pyrenaica  T.T..... .......... .......... .......... .......... ....C..... ....CC.... .......... .........A [ 810]
Capra falconeri  ..T..... .......... .......... .......... .......... .......... ....CC.... .......... .........A [ 810]
Capra sibirica   ..T...C... .......... .......... .......... .......... .......... ....CC.... .......... .......CGA [ 810]
Capra ibex       ..T...C... .......... .......... .......... .......... .......... ....CC.... .......... .........A [ 810]
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Figure 6. Continued.

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H01              CCGTGGGGGT AGCTATTTAA TGAACTTTAT CAGACATCTG GTTCTTTCTT CAGGACCATC TCATCTAAAA CCGCCCATTC TTTCCCCTTA  [ 900]
H02              T......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 900]
H03              T......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 900]
H04              T......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 900]
H05              T......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 900]
H06              T......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 900]
H07              T......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 900]
H08              T......... .......... .......... .......... .......... ....G..... .......... .......... .....T....  [ 900]
H09              T......... .......... .......... .......... .......... ....G..... .......... .......... .....T....  [ 900]
H10              T......... .......... .......... .......... .......... ....G..... .......... .......... .....T....  [ 900]
H11              T......... .......... .......... .......... .......... ....G..... .......... .......... .....T....  [ 900]
H12              T......... .......... .......... .......... .......... ....G..... .......... .......... .....T....  [ 900]
H13              T......... .......... .......... .......... .......... ....G..... .......... .......... .....T....  [ 900]
H14              T......... .......... .......... .......... .......... ....G..... .......... .......... .....T....  [ 900]
H15              T......... .......... .......... .......... .......... ....G..... .......... .......... .....T....  [ 900]
H16              T......... .......... .......... .......... .......... ....G..... .......... .......... .....T....  [ 900]
Capra hircus     .......... .......... .......... .......... .......... ....G..... ...C...... T......C.. ..C..T....  [ 900]
Capra pyrenaica  TT........ .......... .......... .......... .......... ....G..... ...C...G.. T.........  .........  [ 900]
Capra falconeri  .T........ .......... ....T..... .......... .......... ....G..... .......... T......C.. .....T....  [ 900]
Capra sibirica   T......... .......... ....T..... .......... .......... ....G..... .......... T......C.. .....T....  [ 900]
Capra ibex       TT........ .......... ....T..... .......... .......... ....G..... .......... T......C.. ..C.......  [ 900]

H01              AATAAGACAT CTCGATGGAC TAATTACTAA TCAGCCCATG CTCACACATA ACTGTGCTGT CATGCATTTG GTATTTTTTA ATTTTCGGGG  [ 990]
H02              .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 990]
H03              .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 990]
H04              .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 990]
H05              .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 990]
H06              .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 990]
H07              .......... .......... .......... .......... .......... .......... .......... .......... ..........  [ 990]
H08              .......... .......... .......... .......... .......... .......... ...A...... .......... ..........  [ 990]
H09              .......... .......... .......... .......... .......... .......... ...A...... .......... ..........  [ 990]
H10              .......... .......... .......... .......... .......... .......... ...A...... .......... ..........  [ 990]
H11              .......... .......... .......... .......... .......... .......... ...A...... .......... ..........  [ 990]
H12              .......... .......... .......... .......... .......... .......... ...A...... .......... ..........  [ 990]
H13              .......... .......... .......... .......... .......... .......... ...A...... .......... ..........  [ 990]
H14              .......... .......... .......... .......... .......... .......... ...A...... .......... ..........  [ 990]
H15              .......... .......... .......... .......... .......... .......... ...A...... .......... ..........  [ 990]
H16              .......... .......... .......... .......... .......... .......... ...A...... .......... ..........  [ 990]
Capra hircus     .......... .......... ....G..... .......... .......... .......... ...A...... .......... ..........  [ 990]
Capra pyrenaica  .......... .......... ..G.G..... .......... .......... .......... .......... ..........  .C...T....  [ 990]
Capra falconeri  .......... .......... ....G..... .......... .......... .......... ...A...... .......... ..........  [ 990]
Capra sibirica   .......... .......... ....G..... .......... .......... .......... ...A...... .......... .....T....  [ 990]
Capra ibex       .......... .......... ..G.GG.... .......... .......... .......... ...A...... .......... .C...T....  [ 990]

H01              -ATGCTTGGA CTCAGCTATG GCCGTCAAAG GCCCCGACCC GGAGCATAAA TTGTAGCTGG ACTTAACTGC ATCTTGAGCA TCCCCATAAT  [1080]
H02              -......... .......... .......... .......... .......... .......... .......... .......... ..........  [1080]
H03              -......... .......... .......... .......... .......... .......... .......... .......... ..........  [1080]
H04              -......... .......... .......... .......... .......... .......... .......... .......... ..........  [1080]
H05              -......... .......... .......... .......... .......... .......... .......... .......... ..........  [1080]
H06              -......... .......... .......... .......... .......... .......... .......... .......... ..........  [1080]
H07              -......... .......... .......... .......... .......... .......... .......... .......... ..........  [1080]
H08              -......... .......... .......... .......... .......... .......... .......... .......... ..........  [1080]
H09              -......... .......... .......... .......... .......... .......... .......... .......... ..........  [1080]
H10              -......... .......... .......... .......... .......... .......... .......... .......... ..........  [1080]
H11              -......... .......... .......... .......... .......... .......... .......... .......... ..........  [1080]
H12              -......... .......... .......... .......... .......... .......... .......... .......... ..........  [1080]
H13              -......... .......... .......... .......... .......... .......... .......... .......... ..........  [1080]
H14              -......... .......... .......... .......... .......... .......... .......... .......... ..........  [1080]
H15              -......... .......... .......... .......... .......... .......... .......... .......... ..........  [1080]
H16              -......... .......... .......... .......... .......... .......... .......... .......... ..........  [1080]
Capra hircus     -......... .......... ......TG.. .......... .......... .......... .......... .......... ..........  [1080]
Capra pyrenaica  -......... .......... ......TG.. ....T..... .......G.. .......... .......... .C........ ..........  [1080]
Capra falconeri  -......... .......... ......TG.. .......... .......G.. .......... .......... .......... ..........  [1080]
Capra sibirica   G......... .......... ......TG.. .......... .......G.. .......... .......... .......... ..........  [1080]
Capra ibex       -......... .......... ......TG.. ....T..... .......G.. .......... .......... .......... ..........  [1080]
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Figure 6. Continued.

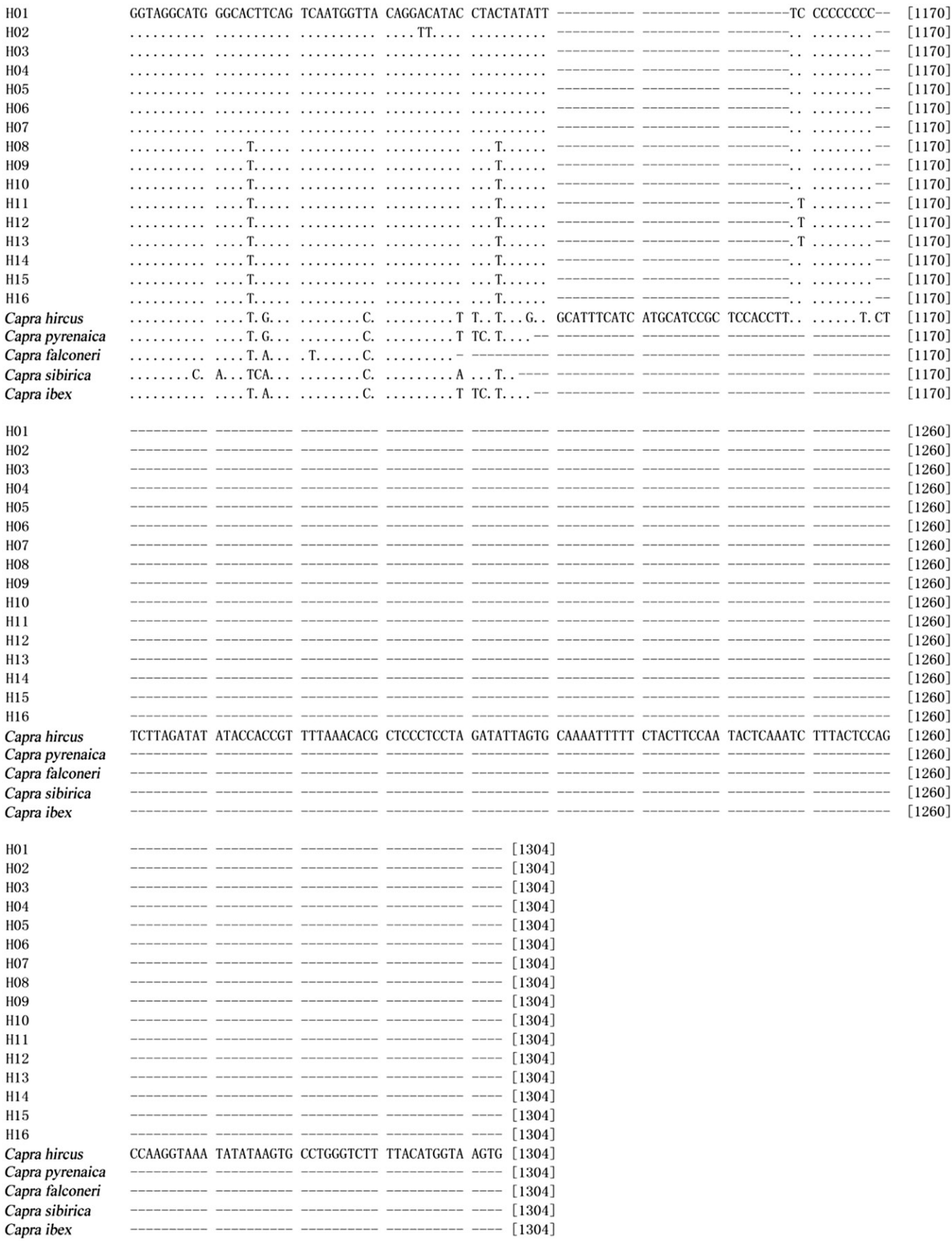

Figure 6. Continued.

MZ-QMC and TJH populations was the same as that between the MZ-QMC and FP populations ($F_{ST} = 0.57$, $p = 0.00$), and the fixation indexes were higher than that between the FP and TJH populations ($F_{ST} = 0.26$, $p = 0.00$). A Mantel test detected no positive correlation between the genetic distance and the geographical distance ($R = -0.79$, $p = 1.00$), which suggests there is presently no ongoing gene flow among the subpopulations (Beebee & Rowe, 2004). The phylogenetic relationship of all haplotypes suggests that two phylogenetic clades were observed in our study (Figure 5). The first clade included all haplotypes in the TJH (H01, H02, H03, H05, H06 and H07) and MZ-QMC (H04) populations. The second clade included all haplotypes in the FP population (H08, H09, H10, H11, H12, H13, H14, H15, and H16). In addition, the phylogenetic analysis and the alignment sequences suggest there are considerable differences among goat species and the takin D-loop sequences (Figure 6).

Discussion

Many genetic markers provide useful tools for conservation genetics; in the takin, microsatellite has been used (Zhang et al., 2008). However, the difficulties of obtaining high-quality DNA from takin populations in the wild limit the types of markers that can be used. Therefore, we used mtDNA CR sequences to study the genetic variation of the takin populations in the FP, TJH and MZ-QMC Nature Reserves.

Our results suggest that the characteristics of the takin CR sequence have the same structure as other mammals (Saccone et al., 1991; Southern et al., 1988). The several tandem repeat sequences and several TASs may contribute to the regulation of mtDNA genomic repetition (Brown et al., 1996; Faber & Stepien, 1998). We found five pairs of hairpin sequences (Figure 3) in the tandem repeat sequences region, which may contribute to the stable secondary structure of CR (Liu et al., 2014). Moreover, we did not find CSB 3, which has been observed in some other vertebrates (Saccone et al., 1991), in the takin CR.

We observed apparent variation in the length of the takin mtDNA CR in the different geographical populations. The length variation of the CR may be attributed to slippage mispairing when mtDNA replicates its genome (Faber & Stepien, 1998), which would make the length of the CR vary in different populations (Wilkinson & Chapman, 1991). The CR is usually elongated by repeating a certain unit of the tandem repeat sequences (Faber & Stepien, 1998; Liu et al., 2014; Wilkinson & Chapman, 1991). Previous studies suggest that ovine CR lengths (*Ovis canadensis canadensis*) range from 1031 to 1333 bp based on the varied number of 75 bp tandem repeat sequences in the CR (Hiendleder et al., 2002). The population in TJH had the longest CR sequences (1098 bp), which contained three tandem repeat sequences. The populations in MZ-QMC (1016 bp) and FP (1018 bp) had shorter CR sequences, which contained only two tandem repeat sequences (Figure 3). These results suggest different geographical populations of takin may have their own evolutionary histories, which caused different CR lengths.

Our study suggests that population expansion may be attributed to a warmer and more humid stage in the late Pleistocene. The advance and retreat of the Pleistocene glacier influenced faunal expansion and contraction by climate oscillation (Yu et al., 2013; Zhao et al., 2012). Many studies suggest the fauna in the eastern flanks of the Tibetan Plateau had a population expansion in the last inter-glaciation or late Pleistocene, e.g. the Red Knobby Newt (*Tylototriton shanjing*) (0.5–0.07 Mya) (Yu et al., 2013), blood pheasant (*Ithaginis cruentus*) (0.246–0.021 Mya) (Zhan et al., 2011), and giant panda (0.005 Mya) (Chen et al., 2013). Our results indicate that expansion of the takin occurred 0.058 Mya ago in the TJH population and 0.062 Mya ago in the FP population, corresponding to the time of inter-glaciation or late Pleistocene (Yu et al., 2013; Zhan et al., 2011). Therefore, different takin populations may have experienced different population histories in the late Pleistocene. The MZ-QMC population had the lowest genetic diversity compared to the other two populations ($h = 0$, $\pi = 0$) and only one haplotype was observed, which may be attributed to a small number of individuals in the founder population. Takin populations have recently suffered from extensive hunting and habitat loss, though this situation was alleviated after nature reserves were established (Chakravarti, 1999; Peery et al., 2012). Tangjiahe Nature Reserve is located in the eastern part of the Minshan Mountains, contiguous with Baishuijiang Nature Reserve in the north and Dongyanggou Nature Reserve in the east (Figure 1). A similar situation is found in the Foping Nature Reserve, located in the heart of the Qinling Mountains with takin populations also distributed in neighboring nature reserves, such as Taibaishan, Laoxiancheng, and Guanyinshan (Figure 1) (Wu, 1990; Zeng et al., 2005). Thus, the takin populations in TJH and FP are able to exchange genes with surrounding populations. However, the MZ-QMC population has been isolated from other populations by a natural barrier (Wei & Hu, 1993). The MZ-QMC population is separated from Qinling populations by the Jialingjiang River and its tributaries in the east, and the population is also isolated from Minshan populations by the Bailongjiang River and its tributaries in the west (Zeng et al., 2005). Therefore, limited gene flow and random genetic drift could have led to low genetic diversity, similar to what has been found in the toad (*Rhinella ornata*) (Dixo et al., 2009), field mouse (*Apodemus speciosus*) (Sato et al., 2014), edible dormouse (*Glis glis*) (Fietz et al., 2014) and Africa wild dog (*Lycaon pictus*) (Leigh et al., 2012).

The takin population in the MZ-QMC Nature Reserve has been regarded as the same subspecies as the FP population (*B. t bedfordi*) according to its geographic distribution (Wu et al., 1983). However, Zeng et al. (2005) identified it as a population of Sichuan takin (*B. t. tibetana*) based on morphological characteristics. Our phylogenic relationship analysis indicated that all individuals were partitioned into just two clades, one includes both the MZ-QMC and TJH populations (*B. t. tibetana*), and the other included only the FP population (*B. t bedfordi*). Therefore, the takin population in the MZ-QMC is more likely to be the Sichuan takin (*B. t. tibetana*).

Conclusions

We observed that the three geographical populations have different evolutionary histories. We also found that the MZ-QMC population is the Sichuan takin (*B. t. tibetana*), as demonstrated by phylogenetic relationships. Our work using the mtDNA CR is the first step towards studying the genetic diversity and the phylogeographic relationships of the takin. Our conclusions should be validated by collecting more samples and using more genetic markers.

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Declaration of interest

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