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Phylogeny of pikas (Lagomorpha, *Ochotona*) inferred from mitochondrial cytochrome *b* sequences

Yidong NIU[1, 2, 3], Fuwen WEI[1*], Ming LI[1], Xiaoming LIU[1, 2] and Zuojian FENG[1]

[1] *Institute of Zoology, Chinese Academy of Sciences, 25 Beisihuanxi Road, Haidian, Beijing 100080, China; e-mail: weifw@panda.ioz.ac.cn*

[2] *Graduate School of the Chinese Academy of Sciences, Beijing, 100039, China*

[3] *Gynecological Oncology Center, People's Hospital, Peking University, 11 Xizhimen Southstreet, Beijing 100044, China; e-mail: guineapig07@yahoo.com.cn*

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Abstract. Phylogenetic relationships of 27 species within the genus *Ochotona* were reconstructed through mitochondrial cytochrome *b* gene. Maximum parsimony, neighbor-joining and maximum likelihood analysis strongly indicated five major species groups: the northern group, the surrounding Qinghai-Tibet Plateau group, the Qinghai-Tibet Plateau group, the Huanghe group, and the Central Asia group. The northern group is composed of *O. alpina*, *O. hyperborea*, *O. pallasi*, *O. princeps*, and *O. collaris*. The surrounding Qinghai-Tibet Plateau group includes *O. macrotis*, *O. roylei*, *O. ladacensis*, *O. rutila*, *O. erythrotis*, *O. gloveri*, *O. brookei*, *O. muliensis*, *O. iliensis*, *O. himalayana*, *O. koslowi*, *O. forresti*, and *O. rufescens*. The Qinghai-Tibet Plateau group contains *O. curzoniae*, *O. thibetana*, *O. cansus*, *O. annectens*, *O. nubrica*, *O. daurica*, and *O. thomasi*. The Huanghe group and the Central Asia group comprise only one species, *O. huangensis* and *O. pusilla*, respectively. Our data did not support the previous subgeneric classification. The phylogenetic trees suggested that divergences of the five groups occurred in the Early Pleistocene (about 2.8 Myr ago), and that the differentiation of the surrounding Qinghai-Tibet Plateau group, the Qinghai-Tibet Plateau group, and the Huanghe group was closely related to the uplifting of the Qinghai-Tibet Plateau and the radiation prompted by environmental changes could play a major role in these groups. Due to the relatively stable environments, however, differentiations were not so strong within the northern group and the Central Asia group, which had never invaded the Qinghai-Tibet Plateau.

Key words: pika, mitochondrial DNA, cytochrome *b*, phylogenetic relationship, phylogeny

Introduction

The extant pikas are endemic to the Holarctic realm and consist of a monotypic genus, *Ochotona*. The species of *Ochotona* in Eurasia occur in open Gobi, at alpine heights above forest levels in the western Chinese highlands (Qinghai-Tibet Plateau) and the Himalayas, as well as at their vicinities. The species in America are only distributed in the northwest. Most pikas live at high altitudes (over 3000 meters above sea level), some even at heights of about 6000 meters (Feng et al. 1986). Although pikas are distributed in broad areas and occur almost exclusively in remote settings, all species of *Ochotona* show remarkable homogeny in general morphology, so that the number of recognized species and phylogenetic relationships among pikas are not stable throughout. To date, the number of recorded species varies from 18 to 29 (Feng & Zheng 1985, Smith et al. 1990, Wilson & Reeder 1993, Gong et al. 2000, Wang 2003).

*Corresponding author

The phylogeny of pikas is also questionable. Luo & Feng (1981) first described the phylogeny of genus *Ochotona* using fossil data, but their phylogenetic tree was not accurate and the relationships of many species were unresolved. Weston (1982) made a comprehensive effort to examine the relationship of the genus *Ochotona*, but Smith et al. (1990) pointed out that her revision was a phonetic analysis and not useful for inferring phylogenetic relationships. Subsequent studies were limited by either small number of species or sampling bias (Yu 1997, Yu et al. 1992, 1996, 1997). Later on, Yu et al. (2000) reported the phylogeny of genus *Ochotona* using mtDNA sequences. In their study, a total of 19 species were analyzed and divided into three groups, and they recommended that *Ochotona* could be divided into three subgenera (Yu et al. 2000). However, many known species were not examined in their study, so it could not provide a full phylogenetic relationships among pikas, and their deduction on evolutionary processes of *Ochotona* is also incomprehensive.

In order to resolve the phylogenetic issues, we employed sequence data from the mitochondrial cytochrome *b* gene, which is adequate for studying taxa at low taxonomic levels like intrageneric or intraspecific relationships (Hillis et al. 1996). In this study, a 402 base-pair region of the cytochrome *b* protein coding gene of 27 species was compared and analyzed.

Material and Methods

Sampling: A total of 35 specimens (32 dried skins and 3 muscles conserved in 95% ethanol) representing 17 species were sampled, of which fifteen species (28 samples) were collected from the Animal Museum of Institute of Zoology (AMIZ), Chinese Academy of Sciences (CAS), and the other two species (7 samples) were offered by the Canadian Museum of Nature (CMN). All the samples investigated are listed in Table 1.

DNA Isolation, amplification and sequencing: Total genomic DNA was isolated by standard protocols and improved methods (Sambrook et al. 1989, Walsh et al. 1991), then genomic DNA was purified with UNIQ–5 Column DNA Gel Extraction Kit (Sangon Inc., Shanghai, China) and stored at –20°C. The published primers were used (Kocher et al. 1989, Irwin et al. 1991, Hillis et al. 1996, Yu et al. 2000). PCR amplification was performed in a 50μl reaction volume mixture, which contained 10 mM Tris-HCl (pH9.0), 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, each dNTP at 200 μM, 1.25 U of *Taq* DNA polymerase, each primer at 200 nM, and 3–6 μl of the purified DNA as a template. The cycling parameters were initial denaturation at 95°C for 10 min (1 cycle); denaturation at 94°C for 1 min; annealing for 1.5 min at 45°C–52°C; extension at 72°C for 1.5 min (42 cycles), and a final extension at 72°C for 10 min (1 cycle). If the quantities of PCR products were not enough for sequencing, the second-round amplification was employed, which was carried out as above except that the reaction volume was added to 100μl and the annealing temperature was increased by 5°C–10°C (Thomas et al. 2000). PCR products were purified by EZ Spin Column PCR Product Purification Kit (Sangon Inc., Shanghai, China). The purified PCR products were directly sequenced using Big Dye Terminator Sequencing Kit (Perkin Elmer, Norwalk, Connecticut) with a DNA sequencer (Model 377; Applied Biosystems, Inc., Foster City, California) with the same primers as used for PCR amplification from both directions.

Data analyses: Amplified fragments were assembled manually based on the overlapping regions and checked with the sequences deposited in GenBank (Table 2). The alignments

Table 1. Summary of the samples investigated in present study.

Species	Sex	ID number	Place of collection	Date	Museum	Type of samples
O. alpina*	♂	00861	Yichun, Hewilongjiang, China	1953	AMIZ[§]	Dried skin
O. alpina	♂	00862	Yichun, Hewilongjiang, China	1953	AMIZ	Dried skin
O. brookei brookei*	♂	19277	Yushu, Qinghai, China	1960	AMIZ	Dried skin
O. brookei brookei	♀	19278	Yushu, Qinghai, China	1960	AMIZ	Dried skin
O. brookei caplloceps*	♀	26852	Zogang, Tibet, China	1976	AMIZ	Dried skin
O. collaris*	♂	33698	Yukon, Canada	1964	CMN[¶]	Dried skin
O. collaris	♀	35324	Yukon, Canada	1966	CMN	Dried skin
O. collaris	♀	35328	Yukon, Canada	1966	CMN	Dried skin
O. collaris	♂	44999	Yukon, Canada	1977	CMN	Dried skin
O. curzoniae*	♀	Ho1	Madoi, Qinghai, China	2000	AMIZ	Muscle in alcohol
O. curzoniae	♀	Ho2	Madoi, Qinghai, China	2000	AMIZ	Muscle in alcohol
O. curzoniae	♀	Ho3	Madoi, Qinghai, China	2000	AMIZ	Liver in alcohol
O. erythrotis*	♀	18912	Delhi, Qinghai, China	1960	AMIZ	Dried skin
O. gloveri*	♂	19370	Dardo, Sichuan, China	1961	AMIZ	Dried skin
O. gloveri	♀	19372	Dardo, Sichuan, China	1961	AMIZ	Dried skin
O. hyperborea*	♀	03343	Ergun, Inner Mongolia, China	1954	AMIZ	Dried skin
O. iliensis*	♀	27125	Borohoro Shan, Xinjiang, China	1985	AMIZ	Dried skin
O. ladacensis*	♀	26968	Datanggula mountain, Qinghai, China	1967	AMIZ	Dried skin
O. macrotis macrotis*	♀	22009	Mongolküre, Xinjiang, China	1978	AMIZ	Dried skin
O. macrotis macrotis	♀	22010	Bay, Xinjiang, China	1977	AMIZ	Dried skin
O. muliensis*	♀	14723	Muli, Sichuan, China	1959	AMIZ	Dried skin
O. muliensis	♂	14724	Muli, Sichuan, China	1959	AMIZ	Dried skin
O. nubrica lama*	♀	27130	Zanda, Tibet, China	1976	AMIZ	Dried skin
O. nubrica lhasaensis*	♀	26804	Damxung, Tibet, China	1975	AMIZ	Dried skin
O. nubrica lhasaensis	♂	26800	Damxung, Tibet, China	1975	AMIZ	Dried skin
O. pallasi pricei*	♂	26615	Beitashan Mountain, Xinjiang, China	1976	AMIZ	Dried skin
O. pallasi pricei	♀	26617	Beitashan Mountain, Xinjiang, China	1976	AMIZ	Dried skin
O. princeps*	♀	08939	British Columbia, Canada	1928	CMN	Dried skin
O. princeps	♀	16668	British Columbia, Canada	1939	CMN	Dried skin
O. princeps	♂	16724	British Columbia, Canada	1964	CMN	Dried skin
O. pusilla*	♀	28352	Former Soviet Union	1940	AMIZ	Dried skin
O. rutila*	♂	28350	Former Soviet Union	1959	AMIZ	Dried skin
O. rutila	♀	28351	Former Soviet Union	1960	AMIZ	Dried skin
O. huangensis*	♀	26966	Xunhua, Qinghai, China	1983	AMIZ	Dried skin
O. huangensis	♂	26967	Xunhua, Qinghai, China	1983	AMIZ	Dried skin

[§] Animal Museum of Institute of Zoology, Chinese Academy of Sciences; [¶] Canadian Museum of Nature;
* indicates that the sequences of these specimens were analyzed and deposited in GenBank.

for sequences were produced with the multiple alignment program Clustal W 1.81 (Thompson et al. 1994) and corrected for obvious alignment errors by hand. Additionally, the protein coding sequence was used to verify the alignments. The published sequences of pikas were employed. Sequences from *Oryctolagus cuniculus* (Genbank Accession No.U07566) and *Pentalagus furnessi* (Genbank Accession No.AB058606) were used as outgroups.

Table 2. The GenBank accession numbers of sequences analyzed in present study.

Species	GenBank Accession Number	Species	GenBank Accession Number
O. alpina1	AY056605	***O. daurica daurica***2	AF273011
O. brookei brookei1	AY056600	***O. daurica bedfordi***2	AF273000
O. brookei caplloceps1	AY191825	***O. erythrotis***2	AF272999
O. collaris1	AY056608	***O. forresti***2	AF272998
O. curzoniae1	AF432908	***O. himalayana***2	AF272997
O. erythrotis1	AY056606	***O. huangensis***2	AF272995
O. gloveri1	AY056602	***O. hyperborea***2	AF272994
O. iliensis1	AY191824	***O. koslowi***2	AF272993
O. ladacensis1	AY056609	***O. ladacensis***2	AF272992
O. macrotis macrotis1	AY191820	***O. macrotis wollastoni***2	AF273010
O. muliensis1	AF421884	***O. nubrica nubrica***2	AF272991
O. nubrica lama1	AY191823	***O. pallasi***	AF272996
O. nubrica lhasaensis1	AY191822	***helanshanensis***2	AF272990
O. pallasi pricei1	AY056607	***O. pallasi sunidica***2	AF272989
O. princeps1	AY056604	***O. princeps***2	AF272988
O. pusilla1	AF260744	***O. roylei***2	AF272986
O. rutila1	AF515733	***O. thibetana***2	AF272987
O. huangensis1	AY191821	***O. thomas***2	AF176578
O. hyperborea1	AY056603	***O. collaris***3	AF176581
O. annectens2	AF273008	***O. curzoniae***3	AF176582
O. alpina2	AF273009	***O. hyperborea***3	AF176579
O. cansus cansus2	AF273006	***O. princeps***3	AF176580
O. cansus morosa2	AF273007	***O. thibetana***3	NC_003033
O. cansus stevensi2	AF273005	***O. collaris***4a	AF348080
O. curzoniae2a	AF273004	***O. collaris***4b	AJ132206
O. curzoniae2b	AF273002	***O. rufescens***5	AB053257
O. curzoniae2c	AF273001	***O. hyperborea***6	

Numbers following the species names indicate that the sequence data were cited from different authors: 1. Niu et al. (present study); 2. Yu et al. (2000); 3. Hafner D.J. et al. (1999); 4. Lin Y et al. (2002); 5. Barome P.O. et al. (1999); 6. Takaki M. et al. (2001).

Tree length distribution skewness computed from ten thousand random trees (g_1 statistics: Hillis & Huelsenbeck 1992) was used for assessing character covariance in the data set. Parsimony analyses were carried out using heuristic search options in PAUP 4.0 (Swofford 1998) by weighting transition and transversion equally, and bootstrap values were computed from 1000 replications. Distance analyses were carried out with the MEGA 2.1 (Kumar et al. 2001) and genetic distances were estimated with the Kimura two–parameter model, and neighbor-joining trees were constructed by including all of transitions and transversions. Bootstrap and interior branch test were carried out from 1000 replications respectively. Maximum-likelihood (ML) tree was calculated via quart puzzling searches in PAUP 4.0 with the Hasegawa-Kishino-Yano (HKY) model (Hasegawa et al. 1985).

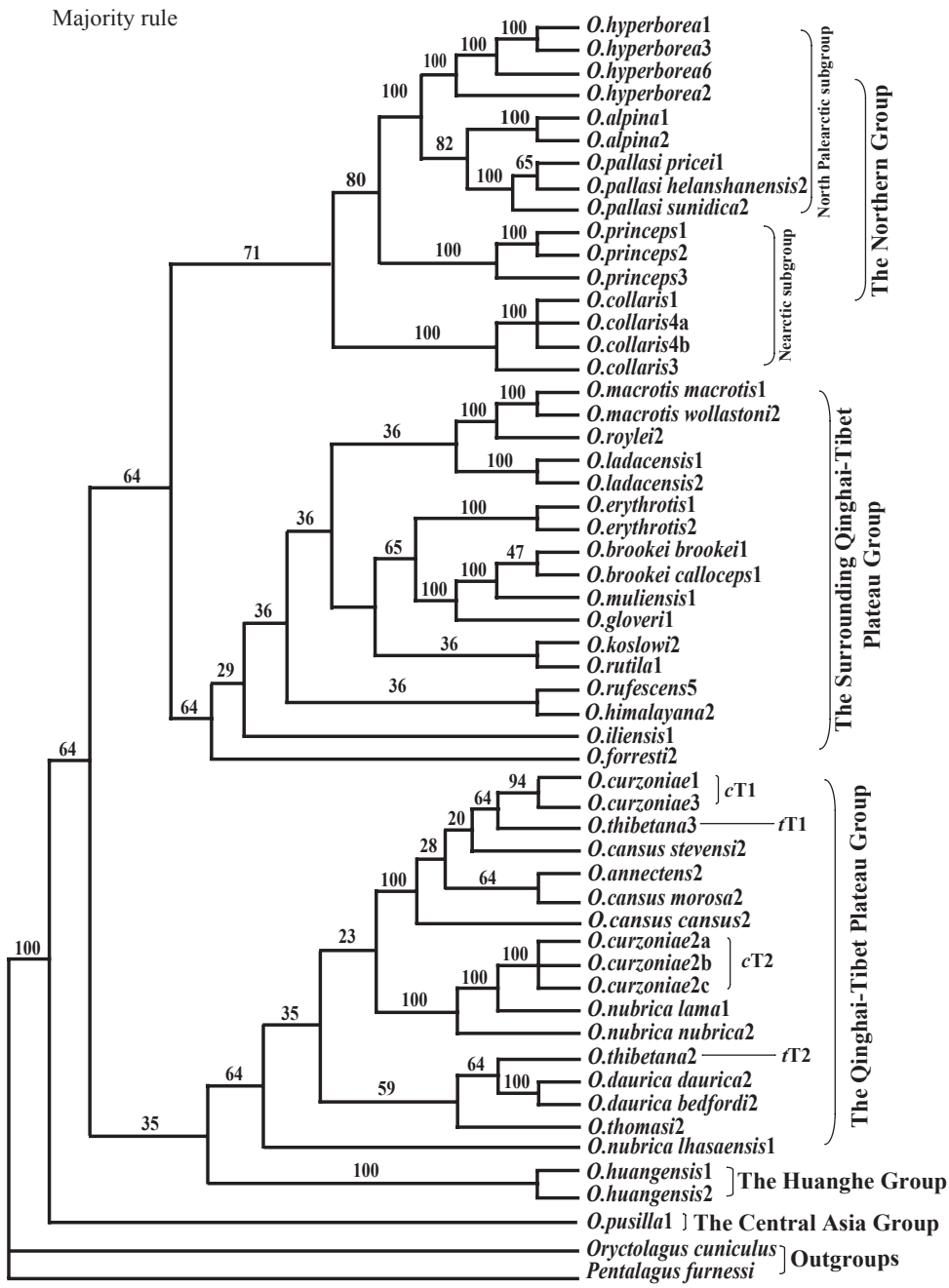


Fig.1. Bootstrap 50% majority-rule consensus parsimony tree derived from heuristic search with 727 steps (C.I.=0.344; R.I.=0.574). Bootstrap analysis consisted of 1000 replications and numbers above the branches are bootstrap values. Numbers following the species names indicate that the sequence data were cited from different authors (please refer Table 2).

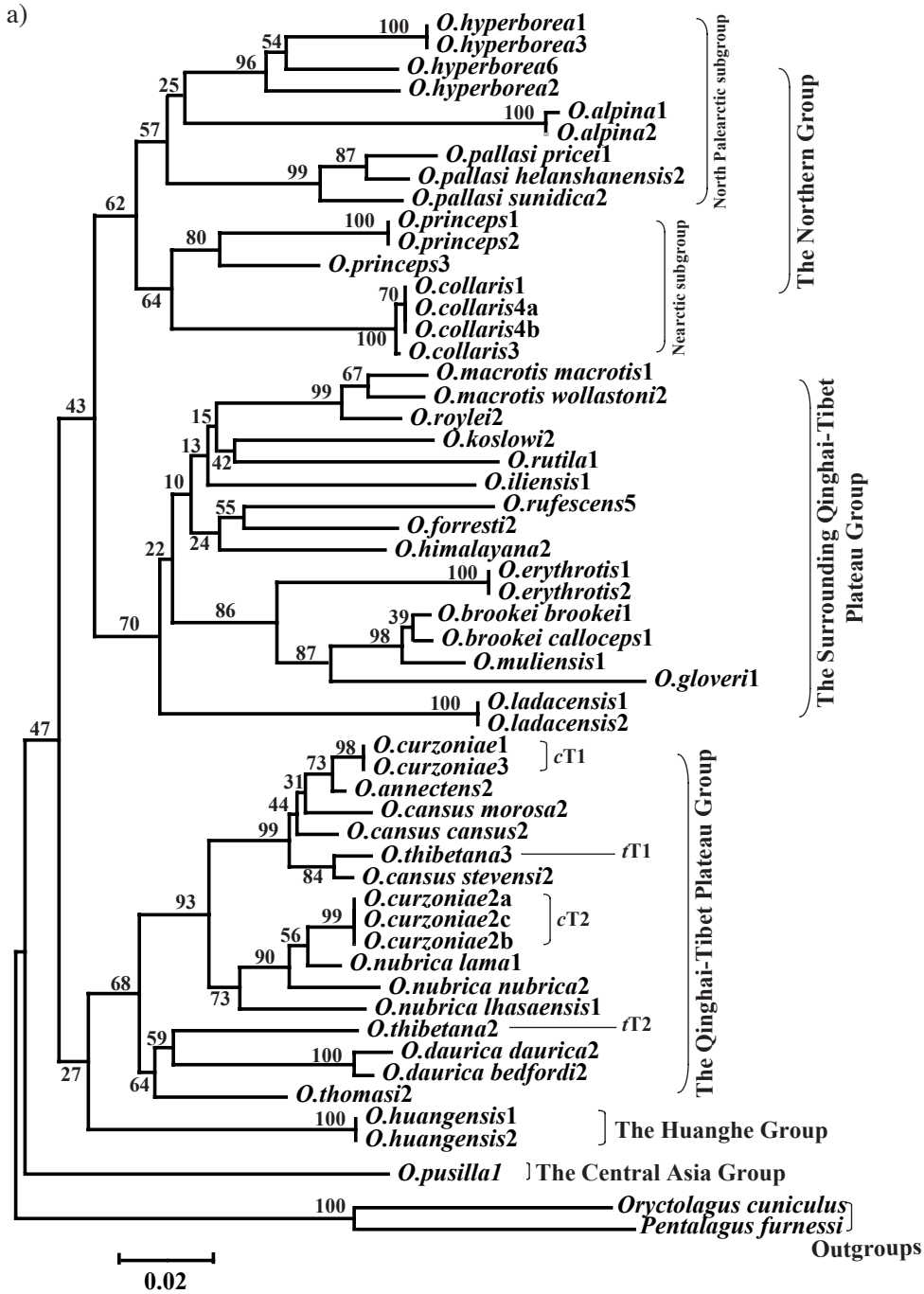
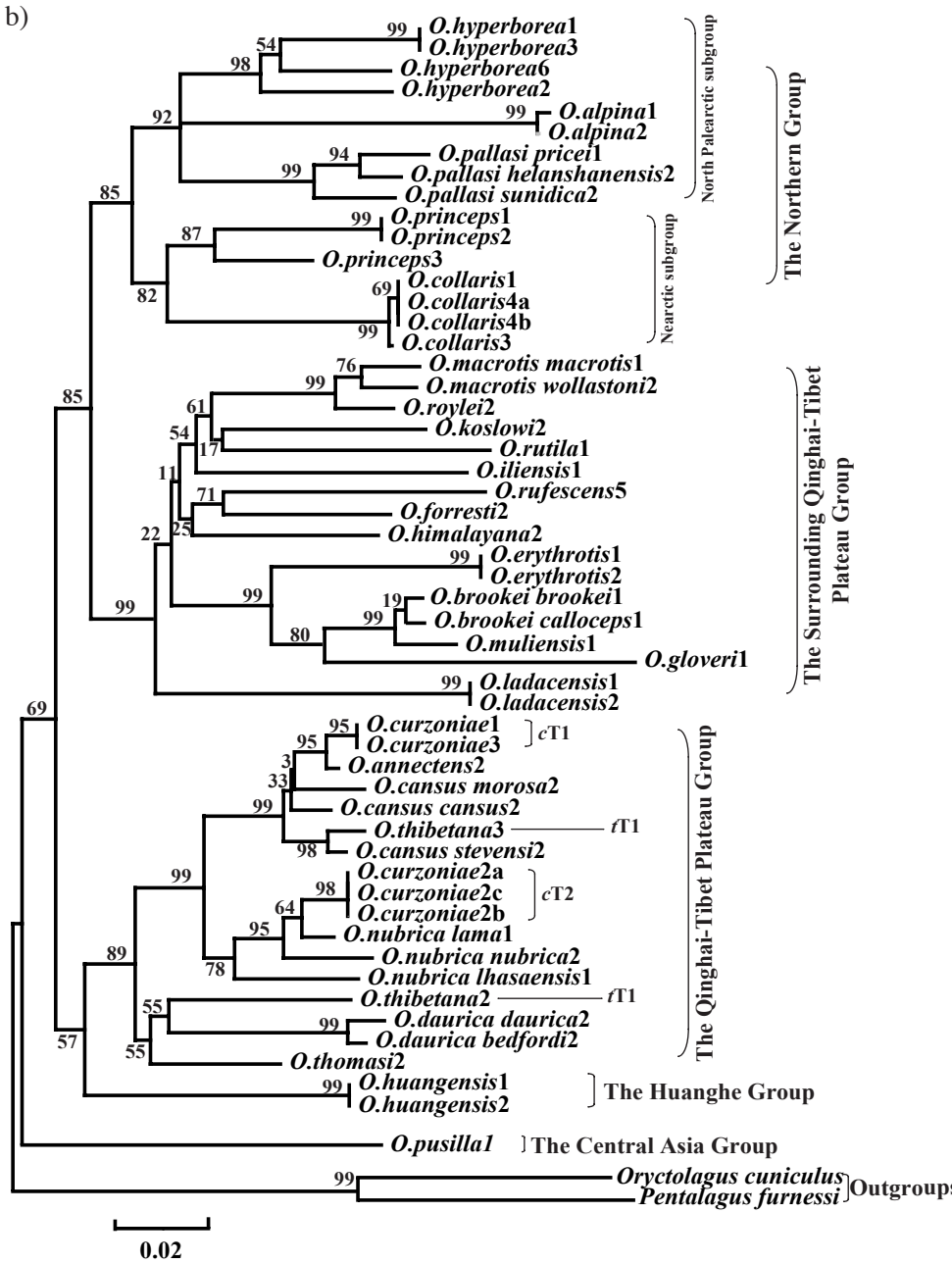


Fig. 2. Neighbour-joining distance trees with distances estimated by the Kimura two-parameter methods. Scores derived from bootstrap analysis (a) and interior branch test (b) with 1000 replications respectively are shown above the branches.


b)
O.hyperborea1
O.hyperborea3
O.hyperborea6
O.hyperborea2
O.alpina1
O.alpina2
O.pallasi pricei1
O.pallasi helanshanensis2
O.pallasi sunidica2
North Palearctic subgroup
O.princeps1
O.princeps2
O.princeps3
O.collaris1
O.collaris4a
O.collaris4b
O.collaris3
Nearctic subgroup
The Northern Group
O.macrotis macrotis1
O.macrotis wollastoni2
O.roylei2
O.koslowi2
O.rutila1
O.iliensis1
O.rufescens5
O.forresti2
O.himalayana2
O.erythrotis1
O.erythrotis2
O.brookei brookei1
O.brookei calloceps1
O.muliensis1
O.gloveri1
O.ladacensis1
O.ladacensis2
The Surrounding Qinghai-Tibet Plateau Group
O.curzoniae1
O.curzoniae3
cT1
O.annectens2
O.cansus morosa2
O.cansus cansus2
O.thibetana3
tT1
O.cansus stevensi2
O.curzoniae2a
O.curzoniae2c
O.curzoniae2b
cT2
O.nubrica lama1
O.nubrica nubrica2
O.nubrica lhasaensis1
O.thibetana2
tT1
O.daurica daurica2
O.daurica bedfordi2
O.thomasi2
The Qinghai-Tibet Plateau Group
O.huangensis1
O.huangensis2
The Huanghe Group
O.pusilla1
The Central Asia Group
Oryctolagus cuniculus
Pentalagus furnessi
Outgroups
0.02

Puzzling scores were calculated by 1000 replications. Topologies obtained with parsimony, distance, and ML analyses were tested by the Kishino-Hasegawa's test (Kishino & Hasegawa 1989).

Results

Because no products were produced for some samples with primers L15136, H15537, L15408, H15870 and L15574, only two pairs of primers L14724/H14994 and L14944/H15149 were chosen to amplify two overlapping fragments (270bp and 205bp) of cytochrome *b* gene for each sample. A total of 402bp sequences were obtained and no intraspecific variation was observed among pikas except for *O. curzoniae* and *O. thibetana*, in which two types of sequences were found respectively, but no changes occurred in each type. Each sequence codes 134 amino acids, and the initial codon is ATG. Analyses of the nucleotide composition revealed the fewest guanines (13.9%) among the four nucleotides. The degree of bias is dependent upon the codon position: 21.6% G in the first position, 16.4% in the second position, and 3.8% in the third position; the first position is rich in adenines (29.9%), the second position is rich in thymines (38.0%), and the third position is rich in cytidines (47.1%). The base composition bias for each position is 0.0665, 0.1720, and 0.4200, which is similar to the reported values for other mammal groups (Irwin et al. 1991, Matthee & Robinson 1999, Halanych & Robinson 1999). There are 220 conserved sites and 182 variable sites, of which 156 are parsimony informative. The sequence differences among pikas average 14.0%. When all positions were considered, the estimated transition/transversion yielded a value of 2.1:1. The distribution of 10^4 random trees is strongly left skewed (g_1= –0.4185; P<0.01), indicating that the data set contains more significant signal than random (Hillis & Huelsenbeck 1992).

Transition/transversion ratios for each codon position and all positions when plotted against Kimura two-parameter (Hassanin & Douzery 1999) distances indicate that nucleotide substitutions in both the 1st and the 3rd codon positions are not saturated, and that the same substitution model appears in both positions. Therefore, all codon positions were included in this study, avoiding loss of phylogenetic information. The most-parsimony heuristic search with all characters weighted equally resulted in forty trees, and a 50% majority-rule consensus tree was obtained (Fig. 1; steps= 727, C.I.= 0.344, R.I.= 0.574). Topology of the consensus tree consists of five major species groups supported by bootstrap values of 64-100%. The first group (the northern group) is composed of *O. alpina*, *O. hyperborea*, *O. pallasi*, *O. princeps*, and *O. collaris*. The second group (the surrounding Qinghai–Tibet Plateau group) includes *O. macrotis*, *O. roylei*, *O. ladacensis*, *O. rutila*, *O. erythrotis*, *O. gloveri*, *O. brookei*, *O. muliensis*, *O. iliensis*, *O. himalayana*, *O. koslowi*, *O. forresti*, and *O. rufescens*. The third group (the Qinghai-Tibet Plateau group) contains *O. curzoniae*, *O. thibetana*, *O. cansus*, *O. annectens*, *O. nubrica*, *O. daurica*, and *O. thomasi*. And each of the rest groups, the forth group (the Huanghe group) and the fifth group (the Central Asia group), comprises only one species, *O. huangensis* and *O. pusilla*, respectively. Distance analyses also reconstructed the identical or almost identical topology with respect to the arrangement of most species (Fig.2). The bootstrap values are in the same range as those from the parsimony analyses. The maximum-likelihood tree obtained from a transition/transversion ratio of 2.0 (kappa=3.99) and gamma shape parameter of 1.67 (Fig.3; quartet examined= 341055, puzzling steps= 1000) shares the identical or almost

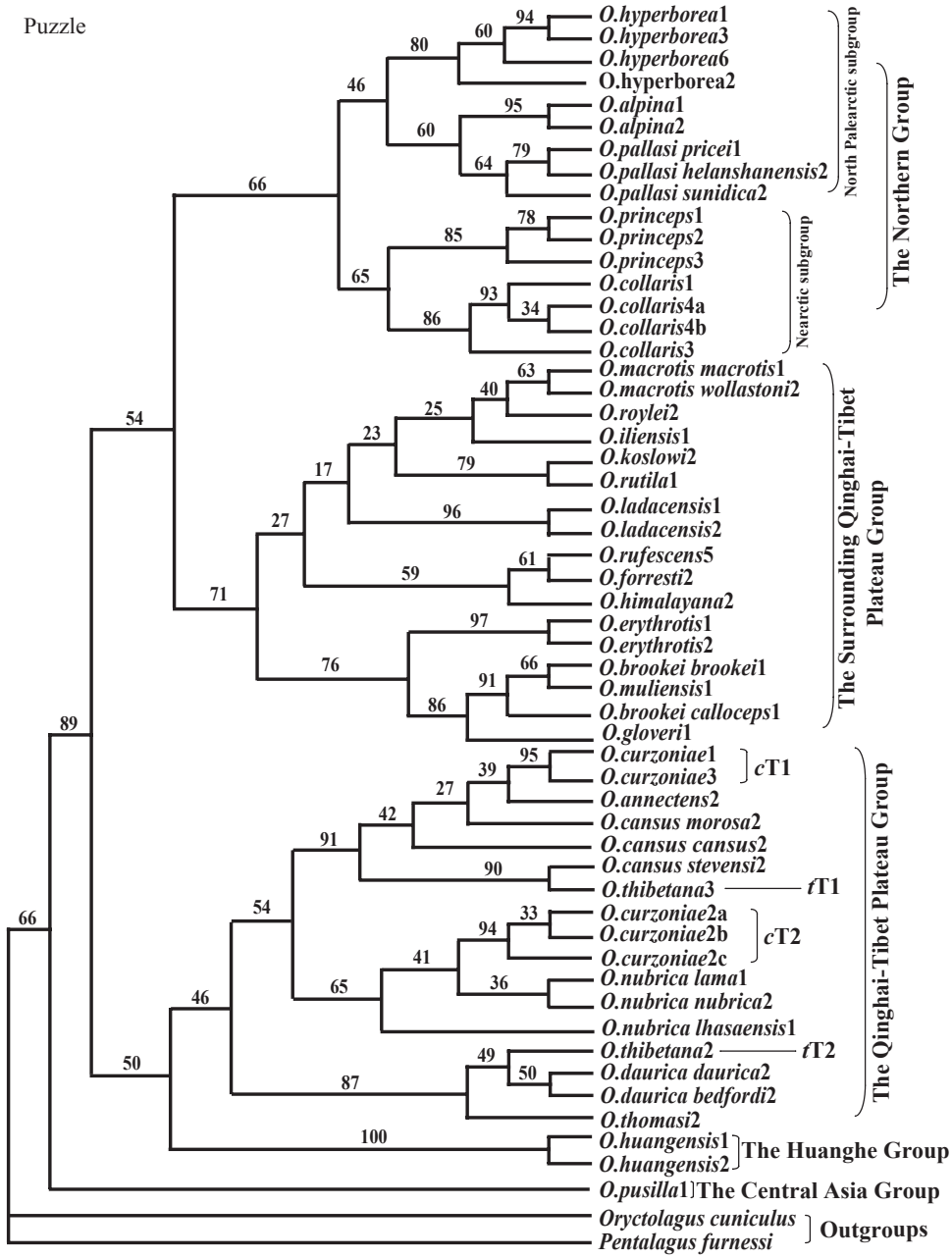


Fig. 3. Maximum-likelihood tree obtained using quartet-puzzling analysis with a 2:1 transition: transversion ratio (Kappa=3.99; number of quartets to examine=341055; number of puzzling steps=1000). Numbers above the branches are puzzling scores.

identical topology of MP and NJ trees, in spite of the Qinghai-Tibet Plateau group supported by low puzzling score (46%). The different topologies of MP, NJ and ML trees were tested by the Kishino-Hasegawa's test (Kishino & Hasegawa 1989), and no significant differences appear among those trees.

In our study, relationships among the five groups are also determined: the Central Asia group is placed on the basis of all trees, and constructs sister group with four other groups supported by bootstrap values of 66–100%; the northern group and the surrounding Qinghai–Tibet Plateau group reconstruct a sister group in all analyses supported by bootstrap values of 43–64 %; the Qinghai-Tibet Plateau group and the Huanghe group always construct a sister group by low bootstrap values of 27–35%.

In the northern group, two subgroups, the North Palearctic subgroup (including *O. alpina*, *O. hyperborea*, and *O. pallasi*) and the Nearctic subgroup (including *O. collaris* and *O. princeps*), are recognized in both NJ and ML puzzling trees (Figs 2, 3); in the surrounding Qinghai–Tibet Plateau group, a subgroup composed of *O. erythrotis* and the *O. gloveri*-like taxa (including *O. gloveri*, *O. brookei* and *O. muliensis*) is found in all analyses and supported by bootstrap values of 65–86 % (Figs 1, 2a); and in the Qinghai-Tibet Plateau group, three subgroups are observed in both NJ and ML puzzling trees (Figs 2, 3), however, a subgroup constructed by *O. nubrica lhasaensis* is only observed in MP analyses (Fig.1).

Discussion

Subgeneric classification: Although subgeneric classification is still under discussion, two subgenera – *Ochotona* and *Pika*, have been accepted by many authors (Ellerman & Morrison-Scott 1951, Luo & Feng 1981, Feng & Zheng 1985, Niu et al. 2001), and also supported by mitochondrial DNA restriction-site analyses (Yu at al. 1996, 1997). However, sequence data inferred from cytochrome *b* and ND 4 genes did not agree with the previous views. Three subgeneric classifications (referring to the northern group, the mountain group, and the shrub-steppe group) were recommended (Yu et al. 2000), which is distinct from Allen's three subgenera (*Ochotona*, *Pika*, and *Ogotoma*) (Allen 1938).

In this study, most species of pikas recorded were examined, and five groups were recognized. All of the species in the northern group are members of the subgenus *Pika* (incisive and palatal foramina separated from each other), and those in the Qinghai-Tibet Plateau group, Huanghe group and the Central Asia group belong to the subgenus *Ochotona* (incisive and palatal foramina form a pear-shaped or triangle opening). The members in the surrounding Qinghai-Tibet Plateau group belong to either the subgenus *Ochotona* (*O. macrotis*, *O. roylei*, *O. iliensis*, *O. koslowi*, *O. forresti*, *O. himalayana* and *O. rufescens*) or the subgenus *Pika* (*O. erythrotis*, *O. gloveri*, *O. brookei*, *O. muliensis*, *O. ladacensis* and *O. rutila*) respectively. In this group, the incisive and palatal foramina of *O. erythrotis* and *O. ladacensis* are not separated completely and connected to each other (Feng et al. 1986, Yu et al. 2000). Therefore, our result does not support the previous subgeneric classification. It is believable that a limited number of species examined led to the incorrect results in Yu et al.'s research (2000). However, we do not suggest that one may classify the five groups as five subgenera because of the limitation in length of sequences, number and type of genes used in this study, and lacking of morphological evidence.

Phylogenetic relationships among pikas: The northern group consists of five species, and can be divided into two subgroups, North Palearctic and Nearctic subgroup. The former

is distributed at high latitudes, and occupy rock-talus areas (*O. alpina* and *O. hyperborea*) or intermediate areas between talus and steppe (*O. pallasi pricei*), but the latter is a group of typical rock and talus-dwelling pikas (Smith et al. 1990). Comparative cytogenetic analysis also revealed the high level chromosomal divergence between North Palearctic (the group with low diploid chromosome number: 2N=38-42) (Vorontsov & Ivanitskaya 1973) and Nearctic species (the group with high diploid chromosome number: 2N=68) (Hsu & Benirschke 1971, Raush & Ritter 1973).

The surrounding Qinghai-Tibet Plateau group comprises a group of rock-talus-dwelling species (*O. macrotis*, *O. roylei*, *O. illiensis*, *O. himalayana*, *O. rutila*, *O. erythrotis*, *O. gloveri*, *O. brookei* and *O. muliensis*) and intermediate types between talus and steppe dwelling (*O. ladacensis*, *O. rufescens*, *O. forresti*, and *O. koslowi*) (Feng et al. 1986, Smith et al. 1990, Yu et al. 1992). *O. roylei* and *O. macrotis* form a sister taxon, in which sequence divergence is only 2.3–3.6%. While *O. erythrotis* and the *O. gloveri*-like taxa form a subgroup and the topologies of this subgroup are stable in all analyses. The average divergence is 0.095% (0.072–0.138%) between *O. erythrotis* and the *O. gloveri*-like taxa, but only 0.058% (0.008–0.105%) within the *O. gloveri*-like taxa. Our data strongly imply that these four species originated from a common ancestor, and that differentiation has occured recently. The sister taxa pair between *O. ladacensis* and *O. koslowi* (Yu et al. 2000) is not supported by our study. Additionally, there are two recently described new species *O. gaoligongensis* (Wang et al. 1988) and *O. nigritia* (Gong et al. 2000). Although *O. gaoligongensis* has been examined by ND 4 gene, the phylogenetic status is still not clear (Yu 1997). According to the original descriptions, the distribution and habitat of both species are very similar to those within the surrounding Qinghai-Tibet Plateau group. Therefore, we infer that both species could be included in the surrounding Qinghai-Tibet Plateau group, but further evidence is needed in both morphology and molecular studies in the future.

The phylogenetic relationships within the Qinghai-Tibet Plateau group are very complicated. Two types of *O. curzoniae* can be recognized in the phylogenetic trees: Type 1(*c*T1) collected from Madoi (*O. curzoniae*1) and Gonghe (*O. curzoniae*3) of Qinghai province are placed together with *O. annectens* and *O. cansus* (bootstrap values of 99–100%), while Type 2 (*c*T2) collected from Gangcha and Reshui (*O. curzoniae*2a-2c) of Qinghai province (Yu et al. 2000) are assembled with *O. nubrica* (bootstrap values of 73–100%). Sequence divergence is 0 within the same type, but 7.5% between the two types. The difference also occurs in *O. thibetana* (*t*T1: *O. thibetana*3 and *t*T2: *O. thibetana*2). We do not know whether the differences resulted from exact genetic divergence or from incorrect identification of species because specimens used by other authors cannot be checked by us. Therefore, further studies are needed. During the last decade, phylogeny of 14 species derived from morphological studies suggested that the steppe-dwelling species (*O. curzoniae* and *O. daurica*) and the shrub-dwelling species (*O. huangensis*, *O. thibetana*, *O. cansus*, and *O. thomasi*) were very distinct, and constructed the base and top branches of the phylogenetic tree respectively (Yu et al. 1992). However, subsequent molecular data indicated that relationships between the steppe and shrub species were very close (Yu 1997, Yu et al. 1996, 1997, 2000). In this study, three subgroups are observed within the shrub-steppe dwellers (1. *O. curzoniae c*T1, *O. cansus*, *O. annectens* and *O. thibetana t*T1; 2. *O. curzoniae c*T2 and *O. nubrica*; 3. *O. daurica*, *O. thibetana t*T2 and *O. thomasi*), except that the shrub-dweller *O. huangensis* constructs a distinct group (The Huanghe

group), and the topology is stable in terms of methods. Our result indicates that these species have a common ancestor, and the differences of habitat choice reflect the adaptability to environment changes, which are considered as a major element in evolutionary processes of pikas (Yu 1997, Yu et al. 1992, 1996, 1997, 2000).

Similar to some species within the Qinghai-Tibet Plateau group, the steppe pika *O. pusilla* is a characteristic burrowing steppe-dweller (Smith et al. 1990). Our data suggested that *O. pusilla* is very distinct. It is located at the base of phylogenetic trees, and represents an ancient species.

Evolutionary development of pikas: If the estimate of divergence rate of 2–5%/Myr (mean 3%/Myr) for cytochrome *b* gene of mammalian species (Irwin et al. 1991) is true for pikas, the five major splits took place in the Early Pleistocene (2.8 Myr ago). The two subgroups in the northern group diverged about 2.0 Myr ago, and divergence between the two Nearctic species occurred about 1.6 Myr ago. Within the surrounding Qinghai–Tibet Plateau group, the *erythrotis*/*gloveri*-like taxa subgroup, the *roylei*/*macrotis* sister taxon and other species diverged from each other about 2.2 Myr ago. The divergence among the three subgroups in the Qinghai–Tibet Plateau group occurred about 1.6–1.1 Myr ago. The estimated divergent times are consist with the historical episodes of geology (Shotwell 1956, Guthrie & Matthews 1971, Guilday 1979, Zheng et al. 1985, Mead 1987, Erbajeva 1988, 1994, Tong et al. 1995, Nesin & Nadachowski 2001), therefore, it is confirmed that a continuous and explosive radiation within genus *Ochotona* took place during the Early Pleistocene.

Environmental changes can often produce strong selective pressures that can result in rapid morphological diversification (Huntley & Webb 1989). During the Pleistocene, *O. pusilla* diversified considerably and inhabited the vast plains of Europe and Asia, spreading widely with the steppe. However, during postglacial time until the Holocene, the reestablishment of forest and grassland restricted the range of *O. pusilla*. Recently, it only inhabited the steppe areas of Central Asia (Erbajeva 1988, 1994, Erbajeva et al. 2001). On the basis of fossil remains, the structure of cheek teeth, and number of chromosomes, *O. pusilla* is often considered as a relic of the Late Pliocene (Erbajeva 1994), which is also supported by our data.

Due to the same diploid number of chromosomes and the similar archaic feature in teeth morphology, the Nearctic pikas are considered to be much closer to *O. pusilla*. There is a hypothesis that the ancient *pusilla*-like taxon probable ancestral form of *O. pusilla*, *O. princeps* and *O. collaris* migrated from Asia to the North America at the end of Late Pliocene and the beginning of Pleistocene, and became distributed widely to southeastern America (Erbajeva 1994, 1998). However, our data does not support this hypothesis. It is well accepted that the Nearctic pikas are dispersal events, which might have happened during the Pliocene through the Beringian Bridge (Kurten & Anderson 1980). The sequence data imply that the Nearctic pikas split during the Early Pleistocene. Geographic study also indicates that separation between *O. princeps* in the south and *O. collaris* in the north is probably the result of the Wisconsinan glaciation (Guthrie 1973). To date, *O. princeps* consists of 36 subspecies, which may be due to the strong isolation of these post-Pleistocene events (Guilday 1979, Hall 1981, Smith & Weston 1990). However, speciation events within the North Palearctic subgroup are few because of its comparatively stable habitats (Yu et al. 2000).

Our study also supports the hypothesis that the differentiation of genus *Ochotona* in the Palearctic Region was closely related to the gradual upliftings of the Qinghai-Tibet Plateau

(Yu et al. 1992b, 1996, 1997, 2000). Geological studies indicate that three large-scale upliftings of the Qinghai-Tibet Plateau occurred strongly and frequently from 3.4 Myr ago to 1.6 Myr ago, accompanied by the large glaciers in the Northern Hemisphere. However, glaciers developed only in major mountain chains, and no unified ice sheet covered the Qinghai-Tibet Plateau during the Quaternary Ice Age (Dong et al. 1995, Fang et al. 1995, Li & Shi 1995, Li et al. 1995), so a large-scale biotic extinction did not happen, and ancestral pikas survived. Species of the surrounding Qinghai-Tibet Plateau group, the Qinghai-Tibet Plateau group, and the Huanghe group are just distributed in the Qinghai-Tibet Plateau and adjacent mountains. Obviously, differentiations of the three groups are closely related to the uplifting of the Qinghai-Tibet Plateau and subsequent climatic changes. The species within the surrounding Qinghai-Tibet Plateau group are typical plateau and high altitude adapted species, which have undergone a rapid radiation since the Early Pleistocene (about 2.2 Myr ago). The molecular data also indicates that radiation of this group may be multi-diverse. The species within the Qinghai-Tibet Plateau group are young and the dominant inhabitants on the plateau. As previously reported (Yu et al. 2000), the steppe dwellers and shrub dwellers are intercrossed within the group of the phylogenetic trees (Figs 1, 2, 3), and the sequence divergence between them is very low, but that between the steppe dwellers or the shrub dwellers are high. This result implies that the divergence event between the two dwellers may have happennes recently. The morphological similarities within steppe dwellers or shrub dwellers was probably due to convergent evolution, apparently because the morphological characters have tracked the environment and resulted in adaptive modification in structure that increase the probability of survival (Yu et al. 1997). The convergent event may also have happened between the Huanghe group and the Qinghai-Tibet Plateau group, now that the former shares the identical habitat with the shrub dwellers of the latter.

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