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Molecular Systematics of Pikas (Genus *Ochotona*) Inferred from Mitochondrial DNA Sequences

Ning Yu,*,†,‡,[1] Changlin Zheng,† Ya-Ping Zhang,* and Wen-Hsiung Li‡

*Key Laboratory of Cellular and Molecular Evolution, Kunming Institute of Zoology, The Chinese Academy of Sciences, Kunming 650223, China; †Northwest Plateau Institute of Biology, The Chinese Academy of Sciences, Xining 810001, China; and ‡Department of Ecology and Evolution, University of Chicago, 1101 East 57th Street, Chicago, Illinois 60637

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The phylogenetic relationships among worldwide species of genus *Ochotona* were investigated by sequencing mitochondrial cytochrome *b* and ND4 genes. Parsimony and neighbor-joining analyses of the sequence data yielded congruent results that strongly indicated three major clusters: the shrub–steppe group, the northern group, and the mountain group. The subgeneric classification of *Ochotona* species needs to be revised because each of the two subgenera in the present classification contains species from the mountain group. To solve this taxonomic problem so that each taxon is monophyletic, i.e., represents a natural clade, *Ochotona* could be divided into three subgenera, one for the shrub–steppe species, a second for the northern species, and a third for the mountain species. The inferred tree suggests that the differentiation of this genus in the Palearctic Region was closely related to the gradual uplifting of the Tibet (Qinghai-Xizang) Plateau, as hypothesized previously, and that vicariance might have played a major role in the differentiation of this genus on the Plateau. On the other hand, the North American species, *O. princeps*, is most likely a dispersal event, which might have happened during the Pliocene through the opening of the Bering Strait. The phylogenetic relationships within the shrub–steppe group are worth noting in that instead of a monophyletic shrub-dwelling group, shrub dwellers and steppe dwellers are intermingled with each other. Moreover, the sequence divergence within the sister taxa of one steppe dweller and one shrub dweller is very low. These findings support the hypothesis that pikas have entered the steppe environment several times and that morphological similarities within steppe dwellers were due to convergent evolution.

INTRODUCTION

The pikas are one of the most complex and problematic groups in mammalian systematics. Living pikas are distributed only in the Northern Hemisphere and form a single genus, *Ochotona*, within the Family Ochotonidae, Order Lagomorpha. All species in this genus are remarkably homogeneous in morphology and body mass. They occupy two different habitat types: the burrowing steppe, shrub, and forest dwellers and the nonburrowing talus dwellers. A few are intermediate species—those burrowing species that sometimes occupy a talus or rock habitat (Kawamichi, 1971; Formozov, 1981; Smith, 1981, 1988). Talus-dwelling pikas are relatively asocial and comparatively long lived and have relatively stable low population densities and low fecundity rates. In contrast, burrowing pikas normally are highly social and short lived and may have high but fluctuating population densities and high fecundity rates (Smith *et al.*, 1990). The only distinctive morphological difference between these two groups appears to be that the vibrissae are longer in talus-dwelling pikas, whereas the claws are straighter and more powerful in burrowing pikas (Fedosenko, 1974; Formozov, 1981). The many morphological similarities among forms and the difficult access to their habitats have been the major obstacles to their systematics. To date, however, most of the studies on pika systematics have been based on morphological characteristics (Allen, 1938; Ellerman and Morrison-Scott, 1951; Corbet, 1978; Feng and Zheng, 1985; Smith *et al.*, 1990; Yu and Zheng, 1992a,b; Yu *et al.*, 1992; Wilson and Reeder, 1993), and only two molecular studies have been conducted, which were restricted to restriction site analysis of mitochondrial DNA (mtDNA) in a few species (Yu *et al.*, 1996, 1997).

The taxonomic status of some forms proposed in previous studies has been confirmed by molecular data (Yu *et al.*, 1997). *O. daurica* and *O. curzoniae*, two meadow–steppe burrowing forms, for example, were once regarded as conspecific, but were later treated as sibling species (Corbet, 1978; Feng and Zheng, 1985;

[1] To whom correspondence should be addressed at Northwest Plateau Institute of Biology, The Chinese Academy of Sciences, Xining 810001, China. Fax: (773) 702-9740. E-mail: ningyu@midway.uchicago.edu.

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TABLE 1

Major Disagreements in the Taxonomy of *Ochotona* in Recent Revisions

Taxon	Yu *et al.* (1997)	Willson and Reeder (1993)	Yu *et al.* (1992)	Corbet and Hill (1991)	Smith *et al.* (1990)	Feng and Zheng (1985)
O. annectens	Subspecies of *O. daurica*	Subspecies of *O. daurica*	Subspecies of *O. daurica*	Subspecies of *O. daurica*	Subspecies of *O. daurica*	Subspecies of *O. daurica*
O. cansus	*O. cansus* (including *morosa*)	*O. cansus* (including *morosa*)	*O. cansus*	—[a]	*O. cansus* (including *morosa*)	*O. cansus*
O. huangensis	*O. huangensis*	Subspecies of *O. thibetana*	*O. huangensis*	—	Subspecies of *O. thibetana*	Subspecies of *O. thibetana*
O. hyperborea	—	*O. hyperborea*	*O. hyperborea*	*O. hyperborea*	*O. hyperborea*	Synonym of *O. alpina*
O. macrotis	—	*O. macrotis*	*O. macrotis*	Synonym of *O. roylei*	*O. macrotis*	*O. macrotis*
O. nubrica	—	*O. nubrica*	*O. nubrica*	Synonym of *O. thibetana*	*O. nubrica*	Synonym of *O. thibetana*
O. thibetana	*O. thibetana*	*O. thibetana* (including *huangensis*)	*O. thibetana* (including *morosa*)	*O. thibetana*	*O. thibetana* (including *huangensis*)	*O. thibetana* (including *nubrica, huangensis, morosa*)

[a] —, not studied.

Smith *et al.*, 1990; Weston, 1982; Yu *et al.*, 1992). *O. cansus* and *O. thibetana* are similar in morphology and habitat (both being shrubland, burrowing species); so, there has been a debate on the taxonomic position of the form *cansus*. Our mtDNA restriction site data supported the view that these four forms are separate species (Yu *et al.*, 1996, 1997). The mtDNA restriction site data also supported the views that *huangensis* is a distinct species and that *morosa* is a subspecies of *O. cansus* rather than *O. thibetana*. However, the systematic positions of some forms within the *cansus/thibetana* complex and some forms within the *curzoniae/daurica* complex continue to be controversial (Table 1). *O. annectens* was originally published as a new species by Miller (1911). However, Howell (1929) placed it as a subspecies in *O. daurica* and this opinion was followed by all subsequent studies. *O. nubrica* was described as an independent species by Thomas (1922), but was later listed as a subspecies of *O. thibetana* by Allen (1938), Ellerman and Morrison-Scott (1951), and Feng and Zheng (1985). Smith *et al.* (1990) and Yu and Zheng (1992a), however, considered it to be a distinct species. The *hyperborea/alpina* complex is another group whose systematics remain unclear, because the form *hyperborea* has been included in *O. alpina* by many authors (Vinogradov and Argyropulo, 1941; Argyropulo, 1948; Grueev, 1964; Corbet, 1978; Honacki *et al.*, 1982; Weston, 1982; Feng and Zheng, 1985). Some authors (Argyropulo, 1948; Gureev, 1964; Corbet, 1978) also included the American pika, *O. princeps*, in *O. alpina*. Recent studies, however, treated these three forms as separate species (Weston, 1981; Corbet and Hill, 1986; Smith *et al.*, 1990; Wilson and Reeder, 1993).

Another obstacle to the phylogenetic analysis of the relationships among pikas has been limited availability of specimens, especially tissue samples. Although Weston's (1982) numerical approach was a comprehensive effort, she did not examine all of the specimens used in her revision. Smith *et al.* (1990) pointed out that Weston's revision was a phenetic analysis and not useful for inferring phylogenetic relationships. Later, a phylogenetic tree of 14 species in China was derived from 22 morphological characters using a cladistic analysis (Yu *et al.*, 1992). In that study, the shrub-dwelling species *O. huangensis, O. thibetana, O. cansus,* and *O. thomasi* formed one clade, while the steppe-dwelling species *O. curzoniae* and *O. daurica* appeared to be most closely related to each other. A phylogeny recently reconstructed from mtDNA restriction site data (Yu *et al.*, 1997) suggested a picture of the systematics of pikas very different from that of previous studies. In particular, traditionally regarded sibling species *cansus/thibetana* and *curzoniae/annectens* (*daurica*) were suggested to have been derived from different maternal lineages. However, this study included only 6 species; so, more molecular work remains to be done.

In addition to systematics, it is interesting to know the evolutionary history of this genus. The pikas are a novel group that was clearly differentiated from the other lagomorphs as early as the Oligocene (Dawson, 1967). At present, most pikas are restricted to Asia, where there are 24 species. Only 2 species occur in North America. Among the species in Asia, 18 are concentrated in the Tibet (Qinghai-Xizang) Plateau and adjacent areas. Yu *et al.* (1992) proposed that the diversification of pikas in China was correlated with the uplifting of the Tibet Plateau. Earlier, two separate studies using different techniques indicated that the major adaptive patterns within the genus (i.e., species occupying distinctively different habitats) have evolved several times (Vorontsov and Ivanitskaya, 1973; Weston, 1982). Yu *et al.* (1997) also pointed out that *O. curzoniae* and *O. annectens* (*O. daurica*) were derived from different maternal lineages.

The purposes of this study were to estimate the phylogenetic relationships among pika species throughout the world, to clarify some long-standing taxonomic problems, and to test the hypothesis concerning the correlation between diversification of pikas and geological events. Previous studies have demonstrated that among the 13 mitochondrial DNA protein-coding genes, NADH dehydrogenase subunits ND4, ND5, and ND2, cytochrome *b*, and COI are good phylogenetic performers in recovering the true phylogenetic relationships among distant taxa (Zardoya and Meyer, 1996). Because the evolutionary rates of cytochrome *b* (Irwin *et al.*, 1991) and ND4 (Cracraft and Helm-Bychowski, 1991; Arevalo *et al.*, 1994; Forstner *et al.*, 1995; Wang *et al.*, 1997) seem appropriate for investigating phylogenetic relationships of *Ochotona*, we chose these two genes for our study.

MATERIALS AND METHODS

Specimens Examined

A total of 32 specimens representing 19 species and 23 forms were used in this study. Nine species (11 forms) were successfully collected from the field (tissue samples) and the other 10 species (12 forms) were collected from museum specimens. The samples used in this study are listed in Table 2. The sequence from rabbit (*Oryctolagus cuniculus*) (Gissi *et al.*, 1998; GenBank Accession No. AJ001588) was used as an outgroup.

DNA Extraction, Amplification, and Sequencing

Genomic DNA was extracted from frozen tissues according to standard techniques (Sambrook *et al.*, 1989). In the majority of museum specimens, DNA extraction from skin was performed using Chelex 100 resin (Walsh *et al.*, 1991). Later, the tissue protocol of the QIAmp Tissue Kit (Qiagen Inc., Chatsworth, CA) was used for the following species: *O. alpina, O. himalayana, O. koslowi, O. ladacensis, O. roylei,* and *O. thomasi.*

The entire cytochrome *b* gene and the mtDNA fragment between $tRNA^{Arg}$ and $tRNA^{Leu}$, which encompasses the ND4L and ND4 genes, were amplified from tissue samples. The PCR conditions used were as follows: 94°C for 1 min, 42°C (or 50°C) for 1 min, 72°C for 1.5 min for 40 cycles using the combination of the following two primer pairs: L14724 with H15915 (Kocher *et al.*, 1989) for cytochrome *b* and Arg2 with Leu for the ND4L/ND4 fragment (Table 3). The internal sequencing primers were designed based on the sequences obtained. Four primers (L15136, H15608, H15274, and L15408) and seven primers (L10136, H10602, H11037, L11237, H11389, ND4 and ND4 R) were used, respectively, to obtain the complete sequences of cytochrome *b* and ND4 genes from tissue samples. Internal primers were designed to amplify short fragments (~300 bp) from museum specimens based on the sequences from seven species for which frozen tissues were available. Table 3 lists the PCR and sequencing primers. Annealing locations for each primer are shown in Fig. 1. Negative controls were included in all PCR experiments to ensure that the products obtained were not contaminated with foreign DNA. Any reactions containing PCR products in the negative controls were discarded. PCR products were purified using either low-melting agarose gel (Sambrook *et al.*, 1989) or the Wizard PCR Preps DNA Purification Resin (Promega).

Purified PCR products were sequenced with the DyeDeoxy Terminator Cycle Sequencing Kit (Perkin–Elmer), following the supplied protocol modified by half reactions (total reaction volume = 10 μl), or the Big Dye Terminator Cycle Sequencing Kit (Perkin–Elmer). Cycle sequencing products were purified and then electrophoresed on a 4.25% polyacrylamide gel using an ABI 377 automated sequencer.

Sequence and Phylogenetic Analysis

Partial sequences were assembled manually based on overlapping regions and later checked using the PCGENE 6.60 program (Bairoch, 1991).

The two genes were initially analyzed separately, because different genes may have different evolutionary pathways (Hillis, 1987; Miyamoto and Fitch, 1995). Tree length distribution skewness computed from 10,000 random trees (g1 statistics; Hillis and Huelsenbeck, 1992) and permutation tail probability (PTP; Faith and Cranston, 1991) were used for assessing character covariance in the data sets. Then, a combined data analysis was conducted.

The maximum-parsimony analysis was carried out using PAUP* (Version 4.0b2; Swofford, 1999) and the majority-rule consensus tree was obtained from 100 bootstrap replicates using a heuristic search, with rabbit as the outgroup. Neighbor-joining trees with Kimura's two-parameter model were computed, and 500 bootstrap replicates were conducted to assess the statistical confidence of each node, using MEGA 1.02 (Kumar *et al.*, 1993).

RESULTS

Initially, we sequenced four individuals of *O. curzoniae* from three localities, two individuals of *O. cansus cansus* from one locality, and six individuals of *O. hyperborea* from one locality. The average percentage divergence for *O. curzoniae* was very low (0.63% for cytochrome *b* and 0.68% for ND4) and no variation was detected between the two *O. c. cansus* sequences and among the *O. hyperborea* sequences. Therefore, we sequenced only one individual from each of the remaining taxa studied.

TABLE 2

Tissue and Skin Used in the Present Study

Species	ID number	Museum[a]	Sex	Collection locality	Habitat	Collection date	Sample type
O. alpina	0053	NWBI	♂	Buhasi, Xinjiang	Rock and talus, nonburrowing	8/31/75	Skin
O. annectens	94J005	NWBI	♀	Gangcha, Qinghai	Steppe, burrowing	12/8/94	Tissue
O. c. cansus	93J019	NWBI	♀	Menyuan, Qinghai	Shrub, burrowing	7/13/93	Tissue
O. c. cansus	93J020	NWBI	♂	Menyuan, Qinghai	Shrub, burrowing	7/13/93	Tissue
O. c. stevensi	93J003	NWBI	♂	Kangding, Sichuan	Shrub, burrowing	5/17/93	Tissue
O. c. morosa	93J008	NWBI	♀	Mountain Taibai, Shaansi	Shrub, burrowing	6/24/93	Tissue
O. curzoniae	94J003	NWBI	♂	Gangcha, Qinghai	Steppe, burrowing	12/8/94	Tissue
O. curzoniae	94J012	NWBI	♀	Reshui, Qinghai	Steppe, burrowing	7/26/95	Tissue
O. curzoniae	93J035	NWBI	♀	Gangcha, Qinghai	Steppe, burrowing	8/6/93	Tissue
O. curzoniae	93J039	NWBI	♂	Gangcha, Qinghai	Steppe, burrowing	8/6/93	Tissue
O. d. daurica	87-185	AI	♀	Abageqi, Inner Mongolia	Steppe, burrowing	8/28/87	Skin
O. d. bedfordi	N5	NWBI	♂	Youyu, Shanxi	Steppe, burrowing	6/16/63	Skin
O. erythrotis	93J043	NWBI	♂	Xunhua, Qinghai	Rock, nonburrowing	9/4/93	Tissue
O. forresti	97J034	KAI	♂	Mountain Gaoligong, Yunnan	Forest belt, burrowing	10/13/97	Tissue
O. himalayana	070	AI	♂	Zhangmo, Xizang	Rocky habitats	7/7/75	Skin
O. huangensis	93J046	NWBI	♂	Xunhua, Qinghai	Shrub, burrowing	9/9/93	Tissue
O. hyperborea	96J013	NWBI	♂	Wudalianchi, Helongjing	Rock, nonburrowing	9/5/96	Tissue
O. hyperborea	96J014	NWBI	♂	Wudalianchi, Helongjing	Rock, nonburrowing	9/6/96	Tissue
O. hyperborea	96J015	NWBI	♂	Wudalianchi, Helongjing	Rock, nonburrowing	9/7/96	Tissue
O. hyperborea	96J016	NWBI	♂	Wudalianchi, Helongjing	Rock, nonburrowing	9/9/96	Tissue
O. hyperborea	96J017	NWBI	♂	Wudalianchi, Helongjing	Rock, nonburrowing	9/9/96	Tissue
O. hyperborea	96J018	NWBI	♂	Wudalianchi, Helongjing	Rock, nonburrowing	9/9/96	Tissue
O. koslowi	840200	XBDS	♀	Nuoqiang, Xinjiang	Alpine measow, burrowing	7/19/84	Skin
O. ladacensis	74082	NWBI	♀	Ritu, Xizang	Barren places, burrowing	7/9/74	Skin
O. macrotis	74141	NWBI	♂	Geer, Xizang	Rock, nonburrowing	8/21/74	Skin
O. nubrica	74166	NWBI	♂	Geer, Xizang	Scrub, burrowing	8/27/74	Skin
O. roylei	74175	NWBI	♂	Gujin, Xizang	Rock, nonburrowing	8/9/73	Skin
O. pallasi sunidica	87-212	AI	♀	Dongwuqi, Inner Mongolia	Intermediate, rock[b]	8/31/87	Skin
O. p. helanshanensis	02	AI	♀	Mountain Helan, Ningxia	Intermediate, rock	5/31/85	Skin
O. princeps	97J036	KAI	♂	Lemhi Mt. Range, Falls, Idaho, USA	Rock, nonburrowing	7/97	Tissue
O. thibetana	93J001	NWBI	♀	Kangding, Sichuan	Meadow, burrowing	5/17/93	Tissue
O. thomasi	71090	NWBI	♂	Jiuzhi, Qinghai	Shrub, burrowing	8/17/71	Skin

[a] NWBI, Northwest Plateau Institute of Biology, the Chinese Academy of Sciences. AI, Institute of Zoology, the Chinese Academy of Sciences. XBDS, Xinjiang Institute of Epidimology. KAI, Kunming Institute of Zoology, the Chinese Academy of Sciences.

[b] Habitat preference of *O. pallasi* is intermediate between burrowing and talus-dwelling pikas; it may inhabit either open desert or rocky areas. Pallas's pikas typically burrow in steep cliff faces (Allen, 1938; Ognev, 1940; Ma *et al.*, 1987).

The complete cytochrome *b* and ND4 gene sequences were obtained from 30 individuals (representing 18 species and 22 forms) and 23 individuals (representing 16 species and 19 forms), respectively. Because some of the primers did not work for some of the museum skins, only partial sequences were obtained from these species. Specifically, partial cytochrome *b* gene sequences (946–963 bp) were obtained from 2 forms (*O. d. daurica* and *O. macrotis*) and partial ND4 gene sequences (820–1267 bp) were obtained from 5 forms (*O. macrotis, O. d. daurica, O. d. daurica, O. pallasi sunidica,* and *O. roylei*).

TABLE 3

Primers Used for Amplification and Sequencing

	Sequence (5′ to 3′)
L14724	cga agc ttg ata tga aaa acc atc gtt g
H15915	cgg aat tcc att ttt ggt tta caa gac
H15608	gga tgg agc gga gga tgg c
H14994	agg tag cgg ata att cag cc
L14944	gtc acc cac att tgc cga ga
H15274	agg gtg gct ttg tct act ga
L15136	ggc tac gtc cta cca tga gg
H15537	ctg ggg aga aat aag act a
L15408	gca gac aaa atc ccc ttc ca
H15870	atg ctt cgt tgt ttt gat gt
L15574	gca tac gcc atc ctc cgc tc
L15574b	gca tat gcc atc ctt cgt tc
ARG2[a]	act caa aaa gga cta gaa tga
ARG1	atg gat taY cca taa tta tca
Leu[a]	cat tac ttt tac ttg gat ttg cac ca
L10115	tcc aat aca tac ggc ata gac ta
ND4 R[a]	ata ctc ata ccY ctg acc tg
L10136	gtc cac aac cta aat ctc ct
H10139	agg gat gat gag ttt tag cat
H10434	tag tat cga gat gaa cag ttt tt
L10327	acc taa act tct cac taa tat t
H10602	tag ggt gta gaa tag gaa gt
H10602b	tag ggt gta aaa tag gaa at
L10557	atc ccc aca ctc atc att at
L10557b	atc cct aca ctc att atc at
H10861	ata tcc ccc tag ttt tag ta
H10861b	gta tcc gcc cag ttt tag ca
ND4[a]	tga cta cca aaa gct cat gta gaa gc
H11037	ggc tga cgg atg agt atg cg
L11273	cct tcc atc aac ctR atY gga g
H11389	ata tag agg gtg taa agg gc
L10979	tga ggc ata gtt ata aca ag
H11245	gct agt agt cat cat gcg gc
L11181	aca ata atc cta gcc cga gg
L11181b	aca ata atc tta gcc cgc gg
H11414	ttt tcc tcg ttg ggt tgt gg
L11328	aac ctc aca atc atc cta gc
L11328b	aac ttc aca att atc tta Rca gg
H11640	gca gga gtt agc agt ctc tg

[a] Provided by Dr. Wen Wang.

The cytochrome *b* gene is 1140 nucleotides long, coding for 379 amino acids. The stop codon from all taxa examined is AGG. All the sequences obtained follow the pattern of compositional bias common in mammalian cytochrome *b* gene (Irwin *et al.*, 1991). There is a low proportion of guanines (12.4–13.6%) (and correspondingly a high proportion of adenines, 26.3–28.9%) in the overall composition of cytochrome *b* gene, especially at the third codon positions (2.1–5.3%), but least so at the first positions (21.4–23.0%). Observed substitutions are not evenly distributed, but most abundant in third positions and least abundant in second positions. The ND4 gene (1383 bp) consists of 460 codons plus an AGA stop codon, though there is usually no stop codon in the ND4 gene from other mammals. The same compositional bias as in cytochrome *b* gene was observed.

Both the cytochrome *b* and ND4 data yielded significant PTP values of 0.001 (999 replicates), which indicates a significant character covariance in the data sets (Faith and Cranston, 1991). The distribution of 10,000 random trees is strongly left skewed ($g1 = -0.5122$ in cytochrome *b* and $g1 = -0.5330$ in ND4; $P < 0.01$ for both), indicating that the data set is significantly structured and that relatively few alternative solutions exist near the optimal solution compared to elsewhere in the distribution. This, in turn, is an indication that the data set contains strong phylogenetic signals and has a strong correlation among characters beyond that expected at random (Hillis and Huelsenbeck, 1992).

A parsimony analysis was conducted using PAUP* (4.0b2) with rabbit as an outgroup; 355 parsimony-informative sites were obtained from the cytochrome *b* sequences, and 471 from the ND4 gene sequences. The analysis suggested three major clusters for both the cytochrome *b* and the ND4 data sets. The neighbor-joining analysis with Kimura's two-parameter model using MEGA (1.02) also resulted in a similar topology with the same three major clusters for each data set. These results are largely compatible. Because both cytochrome *b* and ND4 genes are mitochondrial protein-coding genes, a combined data approach is appropriate (Doyle, 1992; Huelsenbeck *et al.*, 1996). The combined data set should reinforce the phylogenetic signals presented in the smaller data sets.

When the parsimony and neighbor-joining methods were applied to the combined data set, both gave the same three major clusters as obtained from each separate data set (Fig. 2). The first cluster (the shrub–steppe group) is composed of shrub and steppe burrowing species: *O. daurica*, *O. annectens*, *O. curzoniae*, *O. nubrica*, *O. cansus*, *O. thibetana*, and *O. thomasi*. The second cluster (the northern group) contains *O. hyperborea*, *O. alpina*, *O. pallasi*, and *O. princeps*. The third cluster (the mountain group) includes seven species: *O. erythrotis*, *O. forresti*, *O. koslowi*, *O. ladacensis*, *O. macrotis*, *O. roylei*, and *O. himalayana*. The three clusters were supported by high bootstrap values, that is, ≥98%, except for the northern group (86%) and the mountain group (69%) in the parsimony analysis (the left tree in Fig. 2). An identical or almost identical topology within each cluster was obtained by both methods. However, we cannot determine which two of the three clusters have a closer relationship because the basal relationships of the tree are different for the two different methods of analysis. Furthermore, the position of *O. huangensis* was uncertain: for the neighbor-joining method, it was a sister taxon of the shrub–steppe group, whereas for the parsimony method, it joined the base of the tree.

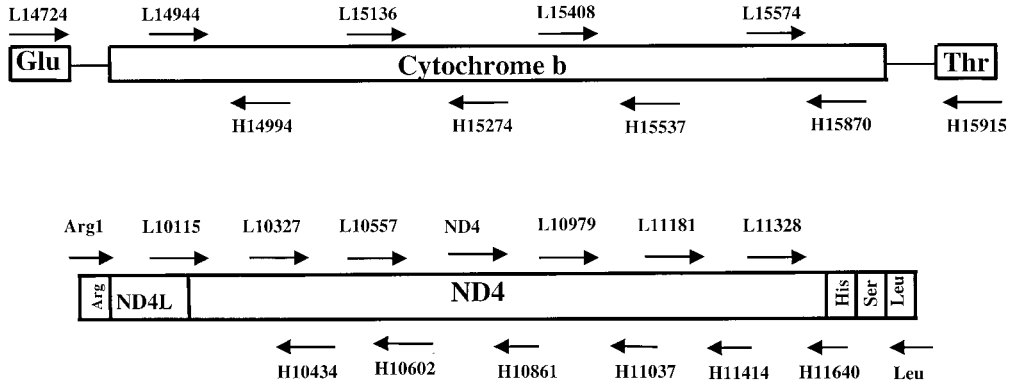

FIG. 1. Primer annealing locations for the mitochondrial cytochrome *b* and ND4L-ND4 DNA regions.

DISCUSSION

Subgeneric Classification

Some authors still insist on subgenera classification, though most of the recent treatments have ceased to do so. Ellerman and Morrison-Scott (1951), Feng and Zheng (1985), and Yu *et al.* (1992) recognized two subgenera (*Pika* and *Ochotona*) based on whether or not the incisive and palatal foramina were separated. However, the present study does not support this classification. The trees that we constructed consist of three clusters (Fig. 2). All of the species in the shrub–steppe group belong to the subgenus *Ochotona* (the incisive and palatal foramina form a pear-shaped or triangle opening) and those in the northern group are members of the subgenus *Pika* (incisive and palatal foramina separated from each other). However, although most of the species within the mountain group are in the subgenus *Ochotona*, *O. erythrotis* and *O. ladacensis* are in the subgenus *Pika* because their incisive and palatal foramina are not completely separated but connected to each other. Therefore, according

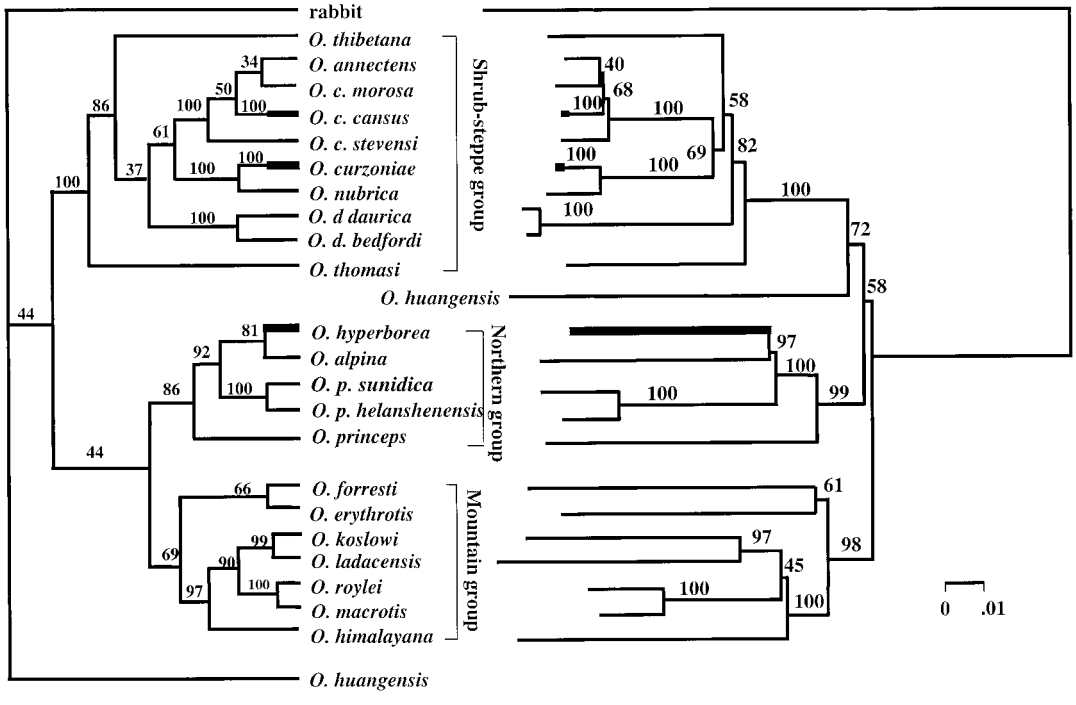

FIG. 2. Left tree: the consensus parsimony tree derived using PAUP* Ver 40.b2 heuristic search based on mitochondrial cytochrome *b* and ND4 DNA sequence data. All nucleotide substitution characters were specified as unordered and given an equal weight. Right tree: the neighbor-joining (NJ) tree constructed using Kimura's two-parameter model in MEGA (1.02) based on both genes. Bootstrap analysis consisted of 100 (parsimony) and 500 (NJ) replications. The bootstrap value of each branch is indicated above the branch. The thick lines indicate multiple individuals from the same species. The GenBank accession numbers are AF272986–AF273011 and AF273110–AF273135 for cytb and ND4 genes, respectively.

to Fig. 2, both of the proposed subgenera are not monophyletic groups, i.e., not natural clades. However, one may classify the three groups in Fig. 2 as three subgenera.

Phylogenetic Relationships among Pikas

A phylogeny of 14 species derived from morphological characters suggested that the shrub-dwelling species *O. huangensis, O. thibetana, O. cansus,* and *O. thomasi* formed a clade, the rock-talus-dwelling species *O. himalayana, O. iliensis, O. macrotis,* and *O. roylei* formed another cluster, and the steppe-dwelling species *O. curzoniae* and *O. daurica* were at the base of the tree (Yu *et al.,* 1992). However, an analysis of mitochondrial DNA restriction site data did not support this hypothesis (Yu *et al.,* 1997). The present study strongly suggests that the shrub dwellers and the steppe dwellers form a clade, i.e., the shrub–steppe group (Fig. 2).

The phylogenetic relationships within the shrub–steppe group are interesting. Instead of the shrub-dwelling species forming a monophyletic group, they are intermingled with steppe-dwelling species. The sequence divergence between the sister taxa of one steppe dweller and one shrub dweller is very low. For instance, *O. curzoniae* is a typical steppe dweller, whereas *O. nubrica* is a shrub habitant. They form a sister group with 100% of the bootstrap replicates, regardless of the gene or method used. The sequence divergence is only 3.2% for cytochrome *b* and even smaller for ND4 (1.4%). The sister taxa of *O. annectens* and *O. cansus* also show a low divergence.

The northern group consists of four species, three from Asia, which are distributed at high latitudes (42° N or further north), and one from the New World. Within this cluster, *O. hyperborea* is a sister taxon of *O. alpina* and these two species therefore form a pair of sibling species. American pika, *O. princeps,* was at the base of this clade. The topology was stable in terms of genes and methods.

The remaining seven species constitute the mountain group. Within this group, *O. ladacensis* is a sister taxon of *O. koslowi,* while *O. macrotis* and *O. roylei* form another sister taxa pair. The divergence between the latter sibling species is only 3.8% for both genes.

The Systematic Position of Some Forms

The number of recognized species varies among the numerous morphological studies in the past century because of the many morphological similarities. Forms considered as separate species by some authors were included in a single species by others. Therefore, much of the discussion below focuses on which of these taxonomic conflicts can be resolved by our sequence data.

First, our sequence data support many of the proposals based on morphological studies (Feng and Kao, 1974; Feng and Zheng, 1985; Smith *et al.*, 1990; Yu and Zheng, 1992a,b) and mitochondrial DNA restriction site data (Yu *et al.*, 1997). The form *huangensis* was originally described as an independent species but was later recognized as a subspecies of *O. thibetana* by Allen (1938) and by many other authors (Ellerman and Morrison-Scott, 1951; Feng and Kao, 1974; Weston, 1982; Feng and Zheng, 1985; Smith *et al.*, 1990). Quantitative morphological studies revealed significantly different skull characteristics in *huangensis* and *O. thibetana,* suggesting that *huangensis* is an independent species (Yu and Zheng, 1992b). This conclusion was later supported by mitochondrial DNA restriction site data (Yu *et al.*, 1997). The present study clearly indicates that *O. huangensis* is an independent species. The sequence divergence and the topology of the trees (Fig. 2) also strongly indicate that *O. cansus* and *O. thibetana* are independent species, not sibling species. The same comment applies to *O. curzoniae* and *O. daurica.* The form *morosa* should be recognized as a subspecies of *O. cansus,* rather than a subspecies of *O. thibetana* (Fig. 2).

Second, the sequence divergence of cytochrome *b* genes was 22.7–27.8% between rabbit and pikas, 7.2–17.7% between well-accepted species, and below 2.9% within species; that of ND4 genes was 25.6–30.4% between rabbit and pikas, 6.5–19.2% between species, and below 1.7% within species. These levels of divergence can help us clarify some of the ongoing debate concerning the systematic position of the forms within the *thibetana/cansus* complex, the *curzoniae/daurica* complex, and the *hyperborea/alpina* complex.

O. nubrica, in the *thibetana/cansus* complex, was originally described as a distinct species (Thomas, 1922). Subsequent authors placed it as a synonym of *O. thibetana* or *O. roylei* (Table 1). In the present study, the sequence divergences in cytochrome *b* between *nubrica* and *O. thibetana* or *O. roylei* were 9.0% (7.2% for ND4) and 15.7% (16.3% for ND4), respectively, which are at the between-species divergence level. The topology of Fig. 2 also supports *O. nubrica* as a distinct species. This conclusion is consistent with the results from the morphological studies of Smith *et al.* (1990) and Yu and Zheng (1992a).

O. annectens was proposed as a new species by Miller (1911). However, Howell (1929) placed it as a subspecies in *O. daurica* apparently because Miller (1911) mentioned that *annectens* was very similar to *O. daurica* in morphology, except for the larger auditory bullae. This opinion was followed by all of the authors subsequent to this study. The sequence divergence between *annectens* and *O. daurica* is 9.1% for cytochrome *b* and 8.1% for ND4. However, it diverged from *O. cansus* by only 1.8% on the average. It is a sister taxon to the latter within the shrub–steppe group, with 100% bootstrap replicates for both genes. Note that there are detectable differences between *annectens* and *O. cansus* in both morphology and ecology. *O. annectens* has a

wider zygomatic width and inhabits steppe rather than shrub. Therefore, it is treated here as a separate species.

Northern pika *O. hyperborea* has been placed in *O. alpina* by many authors (Vinogradov and Argyropulo, 1941; Argyropulo, 1948; Gureev, 1964; Corbet, 1978; Honacki *et al.*, 1982; Weston, 1982; Feng and Zheng, 1985). Argyrpulo (1948), Gureev (1964), and Corbet (1978) also regarded the American pika *O. princeps* as conspecific with *O. alpina*. However, authors who have investigated *hyperborea* and *O. alpina* commented on their noticeable differences throughout their zone of sympatry (Ognev, 1940; Sokolov and Orlov, 1984). The specimens that we have examined also show distinguishable morphological characteristics in the two forms. As to the taxonomic status of *O. princeps*, Weston (1981) found that there was a significant morphological difference between the North American forms and the Asian forms and therefore regarded *O. princeps* as independent. Recent revisions (Corbet and Hill, 1986; Smith *et al.*, 1990; Wilson and Reeder, 1993) also regarded them as distinct species. In addition, the diploid chromosome number is 40 in *O. hyperborea*, 42 in *O. alpina* (Vorontsov and Ivanitskaya, 1973), and 68 in *O. princeps* (Hsu and Benirschke, 1971). In the present study, the sequence divergence between *hyperborea* and *O. alpina* is 12.1% for cytochrome *b* and 7.3% for ND4. The divergence between *O. princeps* and *O. alpina* is even higher, 15.7 and 13.2%, respectively, which are at the level of species divergence. Therefore, our sequence data strongly support these three forms as distinct species.

O. helanshanensis, a recently described new species, was also in this complex. The sequence divergence between *O. helanshanensis* and *O. pallasi* is 4.3 and 3.5% for the cytochrome *b* and ND4 genes, respectively, which are between the species level and the subspecies level. Is it a subspecies of *O. pallasi* or a distinct species? Further studies are needed to answer this question.

O. macrotis and *O. roylei* were regarded as conspecific by Argyropulo (1948), Gureev (1964), and Corbet (1978). Although the divergence between them is not high, 3.8% for both genes, they are considered as separate based on previous morphological studies. Kawamichi (1971) observed that *macrotis* generally dwells in arid alpine zones, whereas *roylei* dwells in relatively humid forest zones. The vertical segregation of the two species is apparent, *macrotis* occupying the upper part, 4000–4200 m, and *roylei* occupying the lower part, 2800–4150 m. They probably contact each other at 4000–5630 m. In fact, Abe (1971), Kawamichi (1971), and Mitchell (1978, 1981) observed the two species in the region of their sympatry.

Evolutionary Processes

Ochotonidae are endemic to the modern Holarctic Region. Most of the species are confined to either high latitudes or high altitudes. The earliest emergence of *Ochotona* in the fossil record seems to be the late Miocene in central Asia (Dawson, 1967). At that time, there was distinct zoogeographic differentiation of small mammals between southern and northern China (Qiu, 1996). The fossil record indicates that pikas occurred only in north Asia and never in south Asia. Fossil remains of *O. daurica* have been reported from many sites in China from the Pliocene to the Holocene (Yue and Xue, 1996; Tong *et al.*, 1995). *O. alpina* and *O. koslowi* were found in the middle of the Pleistocene deposit in China (Tong *et al.*, 1995). Fossil pikas in the Palearctic Region were typical dry–cool weather animals, while those in America were typical rock-dwelling species. *Ochotona* first appeared in North America during the Hemphillian land mammal age (late Pliocene) (Mead, 1987). Fossils of *O. princeps* have been reported in the literature from 44 sites from the Late Pleistocene and the Holocene records (Hafner, 1993).

If the estimate of divergence rate in the cytochrome *b* gene (10%/Myr for silent substitution; Irwin *et al.*, 1991) as determined for other mammalian species is true for pikas, the mountain group, the northern group, and the shrub–steppe group diverged from each other about 2.4 Myr ago; these three lineages are close to a trichotomy, with an average synonymous divergence of 0.24 substitutions per synonymous site. *O. princeps* split from the northern group about 2.1 Myr ago. The divergence between the *cansus/annectens* clade and the *nubrica/curzoniae* clade happened about 1.2 Myr ago. The divergence time estimates are consistent with the historical episodes of geologic and climatic changes.

The tree in Fig. 2 might imply that the differentiation of this genus in the Palearctic Region was closely related to the gradual uplifting of the Tibet Plateau, as hypothesized previously (Yu *et al.*, 1992). Geological studies indicated that the uplift events of the Tibet Plateau occurred more strongly and frequently since 3.4 Myr ago. The first large-scale uplifting of the Tibet Plateau happened about 3.4 Myr ago, which was accompanied by the largest glaciers in the Northern Hemisphere. After that, it strongly uplifted again about 2.5 Myr ago. The Asian monsoons began to establish and the Tibet Plateau monsoons appeared stable. The largest ice age occurred throughout the Northern Hemisphere. During this period, glaciers developed in the major mountain chains of the Tibet Plateau (Dong *et al.*, 1995; Fang *et al.*, 1995; Shackleton *et al.*, 1984). The third phase of uplifting occurred about 1.6 Myr ago. This event led to a colder, drier climate, followed by an increase in the contrast of relief over short distances and the formation of the modern water

system. Among the extensive uplifts of the Tibet Plateau, one at 1.2 Myr ago was a turning point for climatic changes. The uplifted Tibet Plateau then formed an effective barrier to the penetration of moist air currents from the Indian Ocean and the Pacific Ocean. A rain shadow effect by the Himalaya massif resulted in a drier climate, leading to the formation of the modern desert. The faunas of arid adaptation, therefore, evolved in northern China. The flourishing of Ochotonidae in Asia with a simultaneous decline in Europe during the Neogene were possibly the results of such a trend (Qiu, 1996).

Geological studies also demonstrated that a unified ice sheet did not cover the Tibet Plateau during the Quaternary Ice Age (Li and Shi, 1995; Li *et al.*, 1995); so, a large-scale biotic extinction was unlikely. Ancestral pikas may have been typical of arid, cold-adapted steppes, but now they inhabit various environments. As mentioned above, with the uplifting of the Tibet Plateau, the environment altered frequently, which provided a good opportunity for the differentiation within the genus. The modern distribution of the genus is that the majority of the species are distributed in the Tibet Plateau and adjacent mountains. The present study shows that these species are clustered into two groups, the mountain group and the shrub–steppe group, which implies that vicariance might have played a major role in the differentiation of this genus on the Tibet Plateau. Environmental changes (climatic fluctuations) can often produce strong selective pressures that can result in rapid morphological diversification (Huntley and Webb, 1989). In contrast, speciation events of the Asian forms in the northern group were few because of their comparatively stable habitats. The American pika, *O. princeps,* in the northern group, however, consists of 36 subspecies (Hall, 1981), which occupy a discontinuous range in isolated populations of alpine areas. Obviously, *O. princeps* was a dispersal event, which might have happened during the Pliocene through the opening of the Bering Strait (Kurten and Anderson, 1980). Morphological diversification within *O. princeps* appears to have occurred rapidly following the colonization of new habitats. The common ancestor of the northern group was rock dwelling, and all descendants except *O. pallasi pricei* retained this character.

The phylogeny of the six pika species derived from mtDNA restriction site data (Yu *et al.*, 1997) is consistent with the present study if only six species are considered. The present sequence data do not support the phylogeny derived from morphological characters (Yu *et al.*, 1992). A plausible interpretation of the conflict is that morphological similarities among species might be due to convergent evolution. Ecological (Smith, 1988; Smith *et al.*, 1990) and karyological (Vorontsov and Ivanitskaya, 1973) information support this conclusion. A karyological study of eight taxa of pikas (*O. pusilla, O. rutlia, O. macrotis, O. rufescens, O. daurica, O. alpina, O. hyperborea,* and *O. pallasi pricei*) suggested that the steppe-dwelling pikas (*pusilla, daurica,* and *pricei*) might have entered the steppe environment three independent times (Vorontsov and Ivanitskaya, 1973). Weston (1982) suggested that habitat disruption and subsequent reinvasion could have been a common occurrence for the ochotonids. Our data suggest that pikas invaded the steppe environment more than three times (*pallasi, daurica, curzoniae,* and *annectens*).

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