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Mitochondrial genome analysis of *Ochotona curzoniae* and implication of cytochrome *c* oxidase in hypoxic adaptation

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ABSTRACT

Pikas originated in Asia and are small lagomorphs native to cold climates. The plateau pika, *Ochotona curzoniae* is a keystone species on the Qinghai–Tibet Plateau and an ideal animal model for hypoxic adaptation studies. Altered mitochondrial function, especially cytochrome c oxidase activity, is an important factor in modulation of energy generation and expenditure during cold and hypoxia adaptation. In this study, we determined the complete nucleotide sequence of the *O. curzoniae* mitochondrial genome. The plateau pika mitochondrial DNA is 17,131 bp long and encodes the complete set of 37 proteins typical for vertebrates. Phylogenetic analysis based on concatenated heavy-strand encoded protein-coding genes revealed that pikas are closer to rabbit and hare than to rat. This suggests that rabbit or hare would be a good control animal for pikas in cold and hypoxia adaptation studies. Fifteen novel mitochondrial DNA-encoded amino acid changes were identified in the pikas, including three in the subunits of cytochrome c oxidase. These amino acid substitutions potentially function in modulation of mitochondrial complexes and electron transport efficiency during cold and hypoxia adaptation.

1. Introduction

Pikas are small, non-hibernating mammals (order Lagomorpha) that belong to the family Ochotonidae. Pikas are native to cold climates and considered to have a common Asiatic origin. Most pika species are restricted to Asia (Yang et al., 2008), with only three extant pika species living outside of Asia including the American pika (*Ochotona princeps*), the collared pika (*Ochotona collaris*) inhabiting the mountains in western North America (Table 1) and the steppe pika (*Ochotona pusilla*) dwelling west of the Ural mountains in Europe. A majority of the Asian pikas, both in number and in species, are distributed in the Qinghai–Tibet Plateau and surrounding areas (Niu et al., 2004), demonstrating that pikas are particularly adapted to survive on the highest plateau of the world. The remarkable environment of the Qinghai–Tibet Plateau is associated with cold and hypoxia, and under these extreme environmental conditions, survival requires a modified and adapted energy metabolism. The plateau pika (*O. curzoniae*) is a well-known keystone species of the Qinghai–Tibet Plateau ecosystem (Smith and Foggin, 1999) and is considered an ideal animal model for cold and hypoxia adaptation studies (Ge et al., 1998; Zhao et al., 2004). Plateau pikas are native to the Qinghai–Tibetan plateau region and live in remote mountain areas at extremely high altitudes, generally 3000–6000 m above sea level. The plateau pika has evolved to tolerate hypoxia and low temperature with a markedly high resting metabolic rate (RMR), non-shivering thermogenesis (NST) and a high ratio of oxygen utilization (Li et al., 2001). This adaptation includes morphological changes such as loss of hypoxic pulmonary vasoconstriction, right ventricle hypertrophy and thinner-walled pulmonary arterioles (Ge et al., 1998). At the molecular level, accelerated evolutionary rates have been observed in specific stress-response proteins, including leptin, potentially induced by cold and hypoxia (Yang et al., 2008). Furthermore, high altitude pikas have dramatically higher levels of total LDH (lactate dehydrogenase) activity when compared to low altitude congeners (Sheafor, 2003).

Natural selection, imposed by hypoxia and cold, can have profound effects on energy generation and expenditure strategies in animals. For example, two of the most notable responses or defense mechanisms to oxygen deprivation are significant down-regulation of energy turnover (Hochachka, 1986) and

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Table 1
Comparisons of the biological characteristics of the lagomorphs included in this study

	O. curzoniae (plateau pika)	*O. collaris* (collared pika)	*O. princeps* (American pika)	*O. cuniculus* (rabbit)	*L. europaeus* (hare)
Type locality	district of Chumbi, Chumbi valley, Tibet, China	about 322 km south of Font Yukan, Alaska near the head of the Tanana river	rocky mountains	In Europa Australia	Restricted by Trouessart to Poland
Distribution	Tibet, adjacent Gansu, Qinghai, Sichuan (China); Sikkim (India) and E Nepal	WC Mackenzie, S Yukon, NW British Columbia (Canada); SE Alaska (USA)	Mountains of W North America from C British Columbia (Canada) t0 to N New Mexico, Utah, C Nevada, and EC California (USA)	W and S Europe through the Mediterranean region to Morocco and N Algeria; original post-Pleistocene range probably limited to S France, Iberia and N W Africa, but late late-Pleistocene records occur from Ireland to Italy, Hungary and even W Siberia	Open woodland, steppe and sub-desert. From S Sweden and Finland to Britain, through Europe, to W Siberian lowlands; south to N Israel, N Syria, N Iraq, the Tigris-Euphrates valley and W Iran; SE border of range from S Caspian sea south to Persian Gulf
Altitude level	4500 m	1070 m	3350 m		
Habitat	Rock dwellers: rocky scree on mountains (including these three species) Burrowing forms: alpine meadow, steppe, semi-desert			Wide ranging. From desert, mountain forest, tropical rain forest, arctic tundra, swamp, tall grassland, agricultural landscapes	
Longevity	Rock dwellers: up to 7 years (including these three species) Burrowing forms: up to 3 years, most live only 1 year			Average less than 1 year in the wild. Maximum 12 years	
Size	13–20 cm 0.1–0.2 kg			30–40 cm 1.5–2 kg	52–60 cm 3.2–3.9 kg

up-regulation of the efficiency of ATP production. Meanwhile, coping with coldness includes enhanced generation of heat and reduced production of ATP (Coskun et al., 2003; Wallace et al., 2003). Mammalian cells undergo oxidative stress due to hypoxia and generate increased levels of reactive oxygen species (ROS) (Dirmeier et al., 2002). In contrast, cold adaptation is associated with loosely coupled mitochondria and decreased ROS production (Wallace, 2005). Mitochondria, the "energy factories" of animal cells, generate energy by oxidative phosphorylation (OXPHOS). This process involves oxidation of hydrogen to generate ATP and water. Electrons from hydrogen atoms flow down the electron transport chain (ETC), located in the inner mitochondrial membrane, through complex I (NADH dehydrogenase) or II (succinate dehydrogenase) to CoQ10, then complex III (cytochrome *bc1* complex), cytochrome *c*, and complex IV (cytochrome *c* oxidase) to "half" an oxygen atom to produce H_2O (Wallace, 2005). Energy released from electron transport is used to pump protons from the mitochondrial matrix to the inner membrane space. This creates an electrochemical gradient, which is utilized by complex V (ATP synthase) to generate ATP. In addition to ATP synthesis, mitochondria also generate heat to maintain body temperature. The efficiency OXPHOS determines the balance between ATP and heat generation, which are mutually exclusive processes. Tightly coupled OXPHOS results in maximum ATP and minimum heat generation, whereas loosely coupled mitochondria generate more heat but potentially at the expense of ATP production (Wallace, 2005). The latter is preferable in cold weather and important for survival in freezing environments. This is supported by the fact that the mitochondrial DNA (mtDNA) encoded ATP6 protein, which regulates ATP production, is hypervariable in humans in the arctic (Mishmar et al., 2003).

Mammalian mitochondrial genomes include 37 genes and their products are necessary for OXPHOS, electron transport and mitochondrial protein synthesis (Boore, 1999). Pikas tolerate extreme hypoxia and cold environments well, and are therefore expected to show elevated rates of mtDNA evolution and altered OXPHOS efficiencies. Currently, complete mitochondrial genome sequences are only available for the two American species *O. princeps* and *O. collaris* (Lin et al., 2002). The goal of this study was to determine the mitochondrial genome of the classical plateau pika *O. curzoniae* and to identify any novel amino acid changes that could contribute to altered OXPHOS and adaptation. In addition, we used the mitochondrial genome data for phylogenetic affinity analysis, establishing a comprehensive molecular phylogenetic framework for pikas and other mammals. This phylogenetic analysis can assist in the selection of control animals for studies of hypoxia and cold adaptation.

2. Methods

2.1. Animal sampling and DNA preparation

Three male plateau pikas (160–180 g) were captured near the Kunlun Mountain in the Qinghai Province (4500 m). The pikas were sacrificed by decapitation and dissected on the spot. Livers were rapidly collected and frozen in liquid nitrogen. Genomic DNA was extracted from 100 mg liver using the Omega DNA extraction kit (OMEGA, Atlanta, GA). All procedures involving animals were in accordance with the China Practice for the Animal Care.

Table 2
Primers for amplifying the complete mitochondrial genome of *Ochotona curzoniae*

No.	Position as mapped on the American pika mitochondrial genome	primer(5′ → 3′)
1	19	AAAGCAAAGCACTGAAAAATG
	1932	ATGCTAGAGGTGATGTTTTTG
2	1878	AAGGAAAGATTAAAAGGAGT
	3725	CTATTATTTACTCTATCAAAG
3	3720	AGTTACTTTGATAGAGTAAA
	5249	TTCTTACCAAGCCCTGAGGT
4	5134	AATACCCTAATCAACTGGCTTCAATCTA
	7333	GTCTTCATAGTCGGTATATTCATAGCTTCA
5	7309	CAATGATTGAAGCTATGA
	9178	GCTATGAAGAATGTTGA
6	9091	CAACAAGCCCTACTAAT
	10,696	TTTGGATAACTAGGAAG
7	10,600	TAAACGCAGGAACCTACTT
	11,710	GGTTCCTAAGACCAACGGA
8	11,624	AAGTATGCAAGAACTGCTAA
	13,563	GTGATTGAGATTACTCGTGG
9	13,376	TATTCAGCCTTAATACTGCAACCTCTACCC
	15,884	GCCCTGAAGTAAGAACCAGATGCCAG
10	15,787	TCTACCATCCTCCGTGAAACC
	173	GTGTGCTTATTACCCGCTCCT

2.2. Complete mitochondrial genome amplification and sequencing

The complete mitochondrial genome of the plateau pika was amplified using 10 primer pairs (listed in Table 2). The primers were designed using conserved mtDNA regions of the American pika (NC_005358) and the collared pika (NC_003033), both congeners for the plateau pika. PCR reactions were performed in a 50 μl volume with 50 ng templates DNA, 5.0 μmol dNTP, 20 pmol of the forward and reverse primers, 2.5 U Ex-Taq DNA polymerase enzymes (Takara), and corresponding Taq buffer supplemented with 75 μmol $MgCl_2$. A touch-down procedure was used for the amplification, which included 94 °C 5 min, (94 °C 45 s, 60 °C 30 s, and 72 °C 3 min) × 15 cycles (−1 °C/cycle), and (94 °C 45 s, 50 °C 30 s, and 72 °C 3 min) × 20 cycles, followed by final incubation step at 72 °C for 8 min. PCR products were purified with the QIAquick PCR purification kit (QIAGEN, Valencia, CA) and sequenced

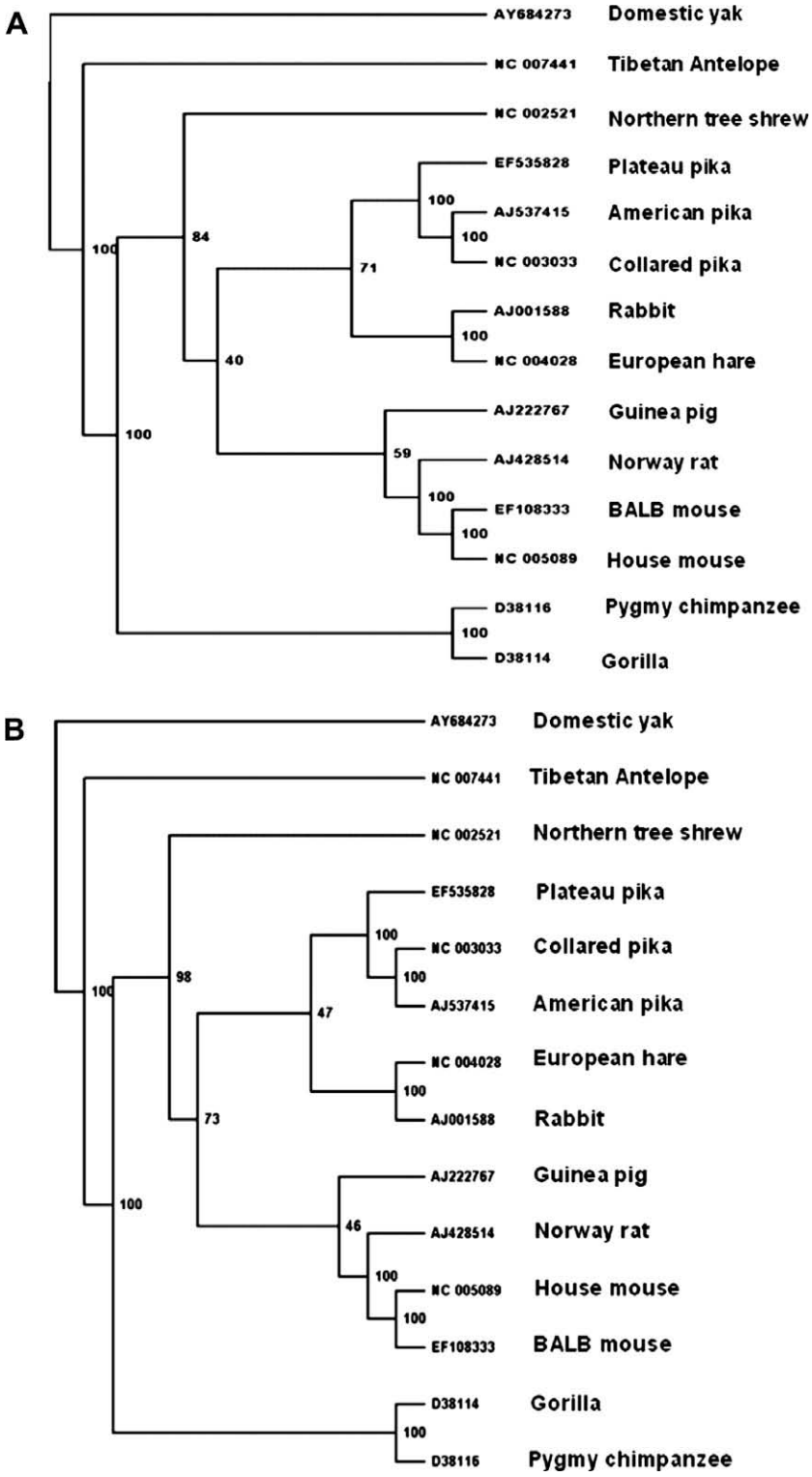

Fig. 1. Phylogenetic trees based on the 12 concatenated heavy-strand encoded protein-coding genes by ML (A) and NJ (B) algorithms. Percentage bootstrap values are shown on interior branches with 1000 replicates. The domestic yak was set as the outgroup in the analysis.

at least three times with an ABI Prism 3730 DNA sequencer (Applied Biosystems, Foster City, CA) at the Shanghai Sangon Biological Engineering Technology & Services CO. DNA sequence assembly, alignment and mitochondrial gene annotation were conducted using DNASTAR SeqMan and Vector NTI 8.0.

2.3. Phylogenetic analyses

To further confirm the evolutionary relationships among the different pikas, and to investigate phylogenetic affinities between pikas and other Qinghai–Tibet Plateau autochthons as well as common laboratory animals, the concatenated amino acid sequences of 12 heavy-strand encoded proteins of domestic yak (AY684273, outgroup), Tibetan antelope (NC_007441), rabbit (AJ001588), hare (NC_004028), rat (AJ428514), guinea pig (AJ222767), BALB mouse (EF108333), American pika (NC_005358), collared pika (NC_003033), plateau pika (EF535828, this study), gorilla (D38114), pygmy chimpanzee (D38116), northern tree shrew (NC_002521), and house mouse (NC_005089) were used to construct phylogenetic trees. The mammals of rabbit, hare, rat, guinea pig, BALB mouse, gorilla, pygmy chimpanzee, northern tree shrew and house mouse were selected to find the good control animals for pikas in cold and hypoxia adaptation studies. The American pika and collared pika were selected to find the phylogenetic relationship between the pikas. The reason of selecting domestic yak Tibetan antelope was they were the common Qinghai-Tibet Plateau autochthons animals. Multiple alignments of the concatenated sequences were generated with ClustalX_1.81. Phylogenetic analysis was done in Phylip3.66 using both the maximum likelihood (ML) and neighbor joining (NJ) algorithms with 1000 bootstrap replicates.

3. Results

3.1. General features of the plateau pika mitochondrial genome

Ochotona curzoniae mtDNA is 17,131 bp in length, 650 and 163 bp longer than mtDNA from *O. princeps* and *O. collaris*, respectively. The size differences are explained by the extended control region of *O. curzoniae*. The plateau pika mitochondrial genome comprises the set of 37 genes generally found in metazoans. The genes are arranged in the typical order for circular mammalian mitochondrial genomes. The total nucleotide composition for the plateau pika mtDNA is 31.02% A, 29.65% T, 13.46% C, and 36.04% G. The high A+T content is characteristic of vertebrate mitochondrial genomes.

3.2. Features of the plateau pika mtDNA-encoded proteins

The plateau pika mtDNA encodes proteins that are predicted to be similar to the corresponding mammalian orthologs. Of the 13 protein-coding genes, ten use ATG as start codon. The NADH dehydrogenase subunit 2 and 5 (ND2 and ND5) start with ATC, and the ND3 utilizes ATT as its translation initiation codon. Eight of the 12 protein-coding genes on the H-strand terminate with TAA or TAG; AGG is predicted to terminate translation of the cytochrome *b* (CYTB) gene. Cytochrome oxidase subunit 3 (COX3), ND3 and ND4 appear to use a template encoded "T", which can be converted to the complete stop codon TAA by polyadenylation. ND6 resides on the L-strand and has AGA as its stop codon. The 12S and 16S ribosomal RNA genes in the plateau pika mtDNA are 964 bp and 1567 bp, respectively. The *O. curzoniae* mitochondrial tRNA genes vary in length from 59 to 75 bp and employ the anti-codons typical of vertebrate mt-tRNAs.

3.3. The non-coding region

The longest non-coding region of the *O. curzoniae* mtDNA is 1667 bp in length and is in-between tRNA-Pro and tRNA-Phe. This size is shorter than the non-coding regions of rabbit (1800 bp) and hare (2295 bp), but longer than that of *O. princeps* (1057 bp) and *O. collaris* (1540 bp). The light-strand origin of replication is predicted to be between positions 5164 and 5198.

3.4. Phylogenetic analysis

To determine the phylogenetic relationship among pikas and the evolutionary position of pikas relative to closely related mammals, a phylogenetic tree was constructed using the concatenated amino acid sequences of the 12 heavy-strand encoded protein-coding genes. The light-strand encoded ND6 gene was excluded from the analysis since it deviates markedly in nucleotide and amino acid composition from the other protein-coding genes (Arnason et al., 2002; Nikaido et al., 2001). From this dataset, a similar topology was recovered with both the ML and NJ analyses (Fig. 1). The three pikas form a single clade (Family Ochotonidae), with the plateau pika (Subgenus Ochotona) positioned as the basal lineage to sister taxa of the North American pikas *O. princeps* and *O. collaris* (Subgenus Pika). The closest relatives of the pikas are rabbit and hare (Family Leporidae), representing the same order Lagomorpha as the pikas. The rodents, including guinea pig, Norway rat, BALB mouse and house mouse, are clustered with the lagomorphs.

Table 3
Pika-specific amino acid substitutions in mitochondrial DNA encoded proteins

Gene	Position	Domain	Pikas	Other mammals	Change in polarity and charge
CYTB	299	Helix	A	L	Both non-polar, hydrophobic
COX1	122	Mitochondrial intermembrane topological domain	V	A	Both non-polar, hydrophobic
	146	Transmembrane domain IV (helix)	A	T	Polar, hydrophilic → non-polar, hydrophobic
	408	Transmembrane domain XI (helix)	V	T	Polar, hydrophilic → non-polar, hydrophobic
COX2	47	Mitochondrial matrix topological domain	T	S	Both polar, hydrophilic
ND2	39	N/A	S	A	Non-polar, hydrophobic → polar, hydrophilic
	231	N/A	A	S	Polar, hydrophilic → non-polar, hydrophobic
	321	N/A	N	K	Positively charged → no charge
ND3	27	N/A	T	L	Non-polar, hydrophobic → polar, hydrophilic
	28	N/A	Y	N	Both polar, hydrophilic
ND5	235	N/A	A	S	Polar, hydrophilic → non-polar, hydrophobic
	552	N/A	T	L	Non-polar, hydrophobic → polar, hydrophilic
	588	N/A	S	F	Non-polar, hydrophobic → polar, hydrophilic
	590	N/A	T	S	Both polar, hydrophilic
ND6	6	N/A	L	F	Both non-polar, hydrophobic

These amino acids are conserved 100% in the 11 non-pika mammalian species included in the phylogenetic analysis, as well as in humans.

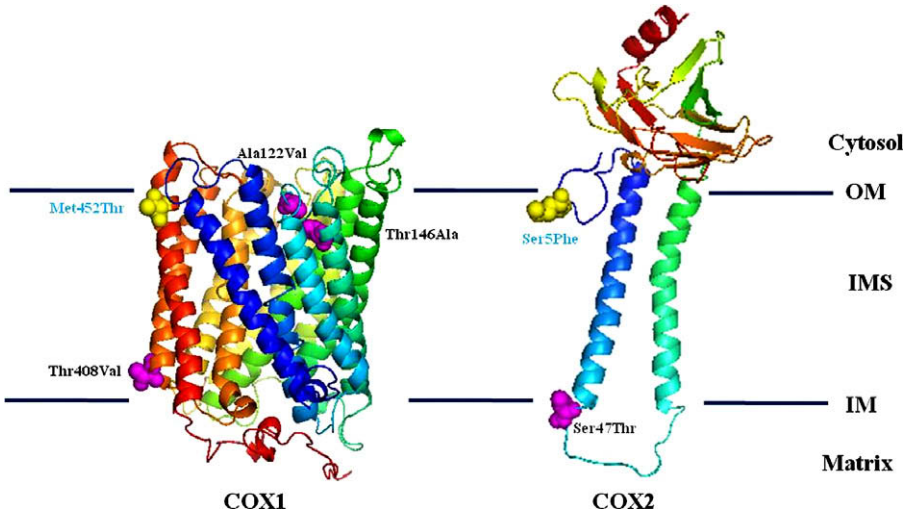

Fig. 2. Cartoon drawings of the cytochrome c oxidase submit I and II and pika-specific (magentas) or plateau pika-specific (yellow) amino acid substitutions. OM, IMS, and IM indicate mitochondrial outer membrane, intermembrane space and inner membrane, respectively. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

3.5. Novel amino acid changes in the pikas

In the mtDNA-encoded proteins of the three pikas, there are 186 intrageneric amino acid variations, representing about 5% of all the 3789 positions. Fifteen pika-specific amino acid variants were identified; these amino acids are 100% conserved in the 11 non-pika mammalian species included in the phylogenetic analysis, as well as in humans. The affected pika genes include CYTB, COX1, COX2, ND2, ND3, ND5, and ND6 (summarized in Table 3 and shown in Fig. 2).

4. Discussions

In this study, the complete nucleotide sequence of the plateau pika mitochondrial genome was determined. This was done as part of an endeavored to understand the genetic basis of hypoxia and cold adaptation of high altitude native fauna. The plateau pika mitochondrial genome comprises the complete set of 37 genes typical of mammalian mtDNAs.

Phylogenetic analysis based on the concatenated heavy-strand encoded protein-coding genes suggests that the plateau pika is closer to its American congeners than to rabbit and hare. Plateau pika has long been considered as an ideal animal model for studies of hypoxia adaptation. However, the Wistar rat has been used as a controversial control animal in *O. curzoniae* studies. Based on our results, it is clear that rabbit or hare would be better control animals from a molecular evolutionary perspective.

Pikas are thought to have separated from rabbits and hares in the late Eocene, around 35–38 million years ago. Pikas quickly differentiated into many forms and became particularly diverse during the Miocene. The first pika appeared in central Asia in the middle Oligocene and spread to North America and Europe in the Pliocene (about 5–1.8 million years ago) (Dawson, 1967; Mead, 1987). The uplift of the Qinghai–Tibet Plateau 3.4–1.6 million years ago resulted in harsh environmental changes, which created a strong selective pressure for survival (Yang et al., 2008). The organisms native to this region, including the pikas, were thus exposed to a hypoxic environment over an evolutionary time frame and developed a set of novel metabolic reactions that help maintaining ATP synthesis under limited oxygen availability. Mitochondria are essential for metabolism and energy production, and are therefore predicted to account for some of the adaptations to hypoxia in this environment. However, few studies have focused on understanding the contribution of mitochondria to hypoxia adaptation. It has been shown that state 3 and 4 respiration of plateau pika liver mitochondria is significantly higher than that of several small control mammals (Li et al., 2001). In addition, increased citrate synthase activity was observed in high altitude pikas, consistent with greater mitochondrial density, (Sheafor, 2003). An increased mitochondrial number is thought to be advantageous under hypoxic conditions, as it enhances the cells' ability to utilize oxygen.

In this study, fifteen novel amino acid changes were identified in the pika mtDNA-encoded proteins. These changes are in components of the electron transport chain and potentially have functional implications. Previous studies have implicated the respiratory chain in both cold and hypoxia signaling in eukaryotes (Chandel et al., 1998; Kwast et al., 1999; Poyton, 1999). In cold environments, a less efficient OXPHOS is preferred as it results in maximum heat generation and minimum ATP and ROS production (Wallace, 2005). When mammalian cells are exposed to reduced oxygen concentrations, they experience oxidative stress leading to increased production of ROS and expression of SOD1. The mitochondria-generated ROS can stabilize HIF-1α, which is an important regulator of hypoxia-induced genes (Brunelle et al., 2005; Dirmeier et al., 2002; Magalhaes et al., 2005; Mansfield et al., 2005). Furthermore, mitochondria produce increased amounts of NO under hypoxia. Cytochrome c oxidase has been identified as the mitochondrial enzyme that reduces NO_2^- to NO, which induces expression of nuclear hypoxic genes, possibly via a pathway that involves protein nitration (Castello et al., 2006). Therefore, it seems possible that modification of the structure and/or activity of cytochrome *c* oxidase, or complex IV of the respiratory chain contributes to hypoxia adaptation. Furthermore, since oxygen is the ultimate electron acceptor, resulting in the production of H_2O in a process that is catalyzed by cytochrome *c* oxidase, modifications in cytochrome *c* oxidase activities are expected to cope with reduced oxygen supply. The nuclear transcription factor HIF-1 regulates the replacement of the cytochrome *c* oxidase complex subunit, COX4, and therefore the efficiency of mitochondrial respiration under hypoxic conditions (Fukuda et al., 2007). Although the mtDNA-encoded cytochrome *c* oxidase subunits are the most conserved genes in mitochondrial genomes, we have identified three pika-specific amino acid changes in COX1 and one in COX2 (Table 3). In COX1, the amino acid substitutions are located in either the

intermembrane topological domain (position 122) or the transmembrane domains (positions 146 and 408) (Tomizaki et al., 1999; Tsukihara et al., 1996). mtDNA-encoded COX1-3 constitute the catalytic core of complex IV. The crystal structure of the bovine heart cytochrome c oxidase has revealed that COX4 interacts with both COX1 and COX2 (Tsukihara et al., 1996) and that COX1–COX4 interaction is the first step in complex IV assembly (Nijtmans et al., 1998). The two amino acid substitutions in the transmembrane helices of COX1 involve a change from polar, hydrophilic to non-polar, hydrophobic residues, implying that these substitutions are functionally significant. The pika-specific amino acid at position 47 in COX2 is in the mitochondrial matrix topological domain. We speculate that these amino acid substitutions change the structure of COX1 and COX2, thereby modifying the conformation of complex IV and altering the function of the cytochrome c oxidase. This would impact mitochondrial NO production and subsequently, hypoxic signaling. In addition, one amino acid change in CYTB (component of complex III) and ten changes in the mtDNA-encoded subunits of the NADH dehydrogenase (complex I) were mapped in the pikas. Although the roles of complex I and III in adaptation to cold or hypoxia remain unclear, these amino acid changes may have functional implications and thus be part of the adaptive response.

The plateau pikas live in an extremely hypoxic environment relative to its congeners. Therefore, we compared amino acid substitutions in *O. curzoniae* and the two North American species. A single plateau pika-specific amino acid change was identified in COX1 and COX2 (Fig. 2). At position 452, in the transmembrane domain XII of COX1, the plateau pika has a threonine residue while its congeners have a methionine. At position 5 in the N-terminus of COX2, which is part of the mitochondrial intermembrane topological domain, a serine to phenylalanine change is present in the plateau pika. These amino acid substitutions potentially are significant and contribute to the hypoxia adaptation of *O. curzoniae*. Furthermore, these changes could explain the mitochondrial genome-mediated cytochrome *c* oxidase subunit modifications that enhance mitochondrial respiration during hypoxia and contribute to HIF-1 modulated complex IV subunit swapping.

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