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Mitogenomic analysis of Chinese snub-nosed monkeys: Evidence of positive selection in NADH dehydrogenase genes in high-altitude adaptation

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ABSTRACT

Chinese snub-nosed monkeys belong to the genus *Rhinopithecus* and are limited in distribution to six isolated mountainous areas in the temperate regions of Central and Southwest China. Compared to the other members of the subfamily Colobinae (or leaf-eating monkeys), these endangered primates are unique in being adapted to a high altitude environment and display a remarkable ability to tolerate low temperatures and hypoxia. They thus offer an interesting organismal model of adaptation to extreme environmental stress. Mitochondria generate energy by oxidative phosphorylation (OXPHOS) and play important roles in oxygen usage and energy metabolism. We analyzed the mitochondrial genomes of two Chinese snub-nosed monkey species and eight other colobines in the first attempt to understand the genetic basis of high altitude adaptation in non-human primates. We found significant evidence of positive selection in one Chinese snub-nosed monkey, *Rhinopithecus roxellana*, which is suggestive of adaptive change related to high altitude and cold weather stress. In addition, our study identified two potentially important adaptive amino acid residues (533 and 3307) in the *NADH2* and *NADH6* genes, respectively. Surprisingly, no evidence for positive selection was found in *Rhinopithecus bieti* (the other Chinese snub-nosed monkey analyzed). This finding is intriguing, especially considering that *R. bieti* inhabits a higher altitudinal distribution than *R. roxellana*. We hypothesize that a different adaptive genetic basis to high altitude survival exists in *R. bieti* from those seen in other mammals, and that positive selection and functionally associated mutations in this species may be detected in nuclear genes related to energy and oxygen metabolism. More information on the structure, function, and evolution of mitochondrial and nuclear genomes in Chinese snub-nosed monkeys is required to reveal the molecular mechanisms that underlie adaptations to high altitude survival in non-human primates.

1. Introduction

Snub-nosed monkeys are enigmatic and threatened primates that belong to the subfamily Colobinae (Kirkpatrick, 1998; Ren et al., 1998). The snub-nosed monkey genus *Rhinopithecus* is comprised of four distinct allopatric species (Groves, 2001): *Rhinopithecus brelichi* (the gray snub-nosed monkey), *Rhinopithecus bieti* (the black snub-nosed monkey), *Rhinopithecus roxellana* (the golden snub-nosed monkey) and *Rhinopithecus avunculus* (the Tonkin snub-nosed monkey). With the exception of *Rhinopithecus avunculus*, which is distributed in low-medium altitude subtropical forests in northwestern Vietnam (<1200 m above sea level; Boonratana and Le, 1998), *Rhinopithecus* species are endemic to temperate areas of China and inhabit six isolated mountainous regions at high altitude (Kirkpatrick, 1998). For example, *R. bieti* ranges in the Himalayas of southwestern China in altitudes as high as 3400–4600 m where temperatures drop below freezing, making it the non-human primate with the highest known altitudinal distribution (Kirkpatrick et al., 1998). Compared with other colobines and most primates, Chinese snub-nosed monkeys are therefore better adapted to high altitude and display a remarkable ability to tolerate low temperatures and hypoxia. They offer an interesting organismal model of adaptation to extreme environmental stress.

Despite substantial work on the physiological, morphological and behavioral characters of snub-nosed monkeys, little research has examined potential adaptations to high altitude survival in these species, or in any other non-human primate, at the molecular level. It is therefore interesting and necessary to investigate the key genes for aerobic metabolic pathways to understand the molecular mechanisms that underlie their adaptations to cold stress and hypoxia.

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Mitochondria, as the "energy factories" of animals, generate energy by oxidative phosphorylation (OXPHOS) (Luo et al., 2008) and play important roles in oxygen usage and energy metabolism (Xu et al., 2007). Earlier studies have revealed a substantially greater amount of mitochondria in the cells of species native to high altitude environments compared to those that occupy lowland habitats. This is believed to be related to the fact that cold temperature and low oxygen pressure are the two most remarkable characters of highland areas that affect animal physiology (Luo et al., 2008; Ning et al., 2010), and high concentrations of mitochondria are needed to increase oxygen utilization and produce more energy to improve aerobic metabolic fitness. Hence, the mitochondrial genome, which encodes for 13 essential OXPHOS system proteins (7 subunits of the NADH dehydrogenase complex, the cytochrome *b* subunit of the cytochrome bc_1 complex, 3 subunits of the cytochrome *c* oxidase, and 2 subunits of ATP synthase) (Saraste, 1999; Lopez-Barneo et al., 2001; da Fonseca et al., 2008) represents a particular useful genetic marker for investigating the molecular basis of organismal adaptation to high altitude environments. Indeed, several mtDNA analyses have detected signatures of adaptive evolution in the cytochrome *c* oxidase genes of plateau pikas, camelids and Tibetan antelope (Xu et al., 2005; Luo et al., 2008; Di Rocco et al., 2009), the NADH dehydrogenase genes of Tibetan horses (Xu et al., 2007; Ning et al., 2010), the cytochrome *b* gene of alpacas (da Fonseca et al., 2008), and the ATP synthase genes of Caprini antelope (Hassanin et al., 2009). In the present study, we sequenced and compared mitochondrial genome sequences of two Chinese snub-nosed monkey species (*R. bieti* and *R. roxellana*) with those of their closest low altitude living relative (*R. avunculus*) and other colobines in the first investigation of adaptation to high altitude survival in non-human primates. Given that positive selection has been found in the mitochondrial genes of other high altitude adapted mammals (see above), we hypothesize that signatures of adaptive evolution will be found in OXPHOS system related mitochondrial genes of the high altitude living Chinese snub-nosed monkeys when compared to their lower altitude living relatives.

2. Methods

2.1. DNA samples and sequence determination

Currently, complete mitochondrial genome sequences of snub-nosed monkey species that live at high altitude are only available for *R. roxellana* (NC_008218; Sterner et al., 2006). We thus sequenced the mitochondrial genome of the highest altitude living snub-nosed monkey species (*R. bieti*) from an individual collected from Lanping district (3500 m) in Yunnan Province, China and the mitochondrial genome of a lower altitude living snub-nosed monkey (*R. avunculus*) collected from Eastern Vietnam (750 m) for comparison. In addition to the mitochondrial genomes of the *Rhinopithecus* species, those of seven other lower altitude living colobine genera available in GenBank were also included in the analyses (*Pygathrix nemaeus*, NC_008220; *Presbytis melalophos*, NC_008217; *Nasalis larvatus*, NC_008216; *Semnopithecus entellus*, NC_008215; *Procolobus badius*, NC_008219; *Trachypithecus obscurus*, NC_006900; and *Colobus guereza*, NC_006901; Raaum et al., 2005; Sterner et al., 2006). The taxonomic and geographical information for these colobines as well as their mitochondrial genome GenBank accession numbers are provided in Table 1.

For *R. bieti* and *R. avunculus* samples, we extracted total DNA from frozen tissues using a standard proteinase K, phenol/chloroform extraction (Sambrook et al., 1989). Complete mitochondrial genomes were then amplified in 4 overlapping segments using the Long and Accurate PCR™ Kit (Takara Biotechnology Co., Ltd). The primer information is listed in Table 2. These long-PCR primers were designed using conserved mtDNA regions found within Colobinae mitochondrial genome sequences available in GenBank. PCR amplification was carried out using the following parameters: 94 °C hot

Table 1
Species used in this study. **1** — Oates et al. (1994); Nijman and Meijaard (2000); Bennett and Sebastian (1988); Yeager (1989); Napier and Napier (1985). **2** — Lippold (1977, 1998). **3** — Li et al. (2003); Ren et al. (2000); Tan et al. (2007). **4** — Li et al. (2008); Kirkpatrick et al. (1998). **5** — Boonratana and Le (1998). **6** — Brandon-Jones (1999); Kawamura (1979). **7** — Gupta and Chivers (1999); Bernstein (1967); Curtin and Chivers (1978). **8** — Koenig et al. (1997); Sugiyama (1976); Oppenheimer (1977); Molur et al. (2003). **9** — Fashing and Oates (in press); Oates (1977). **10** — Ting (2008).

	Scientific name	Common name	Distribution and habitat	Altitude range	Accession nos.
Asian colobines	*Nasalis larvatus* (Oates et al., 1994; Nijman and Meijaard, 2000; Bennett and Sebastian, 1988; Yeager, 1989; Napier and Napier, 1985)	Proboscis monkey	Borneo, SE Asia — lowland rainforest	0–350 m	NC_008216
	Pygathrix nemaeus (Lippold, 1977, 1998)	Red-shanked douc	Mainland SE Asia — primary evergreen forest	0–2000 m	NC_008220
	Rhinopithecus roxellana (Li et al., 2003; Ren et al., 2000; Tan et al., 2007)	The golden snub-nosed monkey	Qinling, Sichuan/Gansu and Shennongjia Mountains in China — montane, temperate forest	2000–3400 m	NC_008218
	Rhinopithecus bieti (Li et al., 2008; Kirkpatrick et al., 1998)	The black snub-nosed monkey	Himalayan Mountain of southwestern China — montane, temperate forest	3400–4600 m	HM125579 (this study)
	Rhinopithecus avunculus (Boonratana and Le, 1998)	The Tonkin snub-nosed monkey	Vietnam — subtropical rainforest	200–1200 m	HM125578 (this study)
	Presbytis melalophos (Brandon-Jones, 1999; Kawamura, 1979)	Mitered leaf monkey	Malay Peninsula, Sumatra, and western Borneo — tropical lowland and montane rainforest	0–2500 m	NC_008217
	Trachypithecus obscurus (Gupta and Chivers, 1999; Bernstein, 1967; Curtin and Chivers, 1978)	Dusky or spectacled leaf monkey	SE Asia — tropical lowland and montane rainforest	Unknown, low-medium altitudes	NC_006900
	Semnopithecus entellus (Koenig et al., 1997; Sugiyama, 1976; Oppenheimer, 1977; Molur et al., 2003)	Hanuman langur	Himalayan foothills and throughout India — extreme variety of habitats	0–4000 m	NC_008215
African colobines	*Colobus guereza* (Fashing and Oates, in press; Oates, 1977)	*Guereza colobus*	West Central — East Africa, tropical lowland to montane rainforest	200–3300 m	NC_006901
	Procolobus badius (Ting, 2008)	Western red colobus	West African Guinean primary rainforest	Unknown, low-medium altitudes	NC_008219