A Mitochondrial Genome Sequence of the Tibetan Antelope (Pantholops hodgsonii)

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To investigate genetic mechanisms of high altitude adaptations of native mammals on the Tibetan Plateau, we compared mitochondrial sequences of the endangered Pantholops hodgsonii with its lowland distant relatives Ovis aries and Capra hircus, as well as other mammals. The complete mitochondrial genome of P. hodgsonii (16,498 bp) revealed a similar gene order as of other mammals. Because of tandem duplications, the control region of P. hodgsonii mitochondrial genome is shorter than those of O. aries and C. hircus, but longer than those of Bos species. Phylogenetic analysis based on alignments of the entire cytochrome b genes suggested that P. hodgsonii is more closely related to O. aries and C. hircus, rather than to species of the Antilocaprinae subfamily. The estimated divergence time between P. hodgsonii and O. aries is about 2.25 million years ago. Further analysis on natural selection indicated that the COXI (cytochrome c oxidase subunit I) gene was under positive selection in P. hodgsonii and Bos grunniens. Considering the same climates and environments shared by these two mammalian species, we proposed that the mitochondrial COXI gene is probably relevant for these native mammals to adapt the high altitude environment unique to the Tibetan Plateau.

Key words: tibetan antelope, mitochondrial genome, adaptation, COXI

Introduction

As the most prominent terrestrial highland on the earth, the Tibetan Plateau enacts great effects on global climate and biosphere. Its fauna and flora prospering on the plateau are constantly challenged by the harsh environment of hypoxia, low temperature, high solar radiation, and lack of biological production. Native animals of the Tibetan Plateau, surviving over thousands of years on the highland, have developed various physiological, behavioral, and morphological strategies to cope with these environmental challenges; some of the changes are certainly attributable to phenotypic plasticity and others are genetic. The genomic mean for discovering inheritable changes in a species is to sequence its genomes, including both nuclear and organellar genomes.

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molecule with a length of about 16 Kb. In general, it contains 13 protein-coding genes, 2 rRNA genes, 22 tRNA genes, and a non-coding control region (2–4). The thirteen proteins encoded by the genome are all related to oxide metabolism.

In the current study, we report a complete mitochondrial genome sequence from a single P. hodgsonii individual and results from comparative analysis in search of genetic outcomes in living under high altitude environments.

Results

General features of the P. Hodgsonii mitochondrial genome

The P. hodgsonii mitochondrial genome is 16,498 bp long, shorter than those of Ovis aries (NC_001941) and Capra hircus (NC_005044), which are 16,616 bp and 16,640 bp in length, respectively, yet longer than that of Bos taurus (NC_001567), Bos indicus (NC_005971), and Bubalus bubalis (NC_006295), which are 16,338 bp, 16,339 bp, and 16,359 bp in length, respectively. The size differences are resulted from different lengths of the control region among these species. Nucleotide composition analysis revealed that the P. hodgsonii mitochondrial genome is biased towards AT (A 33.59%, T 26.87%, G 13.11%, and C 26.41%); such an AT content is lower than those of O. aries and C. hircus. The P. hodgsonii mitochondrial genome encodes 13 proteins, 2 rRNAs, and 22 tRNAs (Figure 1). Eight tRNA genes and one protein gene are located on the light strand (Table 1). And the number of polymorphic sites at protein coding genes and RNA genes in the mitochondrial genomes of P. hodgsonii, C. hircus and O. aries is shown in Table 2.

![Mitochondrial genome of Tibetan antelope](image)

**Fig. 1** Annotation of the Pantholops hodgsonii mitochondrial genome. The original figure was from Bae et al (30). ND1 to ND6 refer to NADH subunits; COXI, COXII, COXIII refer to cytochrome c oxidase subunits; ATP6 and ATP8 refer to ATPase6 and ATPase8; the tRNA genes are denoted by shade and are noted accordingly; Origin refers to Origin of L-strand replication. The ATPase8 and ATPase6 genes are overlapped for 40 bp. Some bases between genes are too few to be denoted in the figure.
### Table 1 Components of *P. hodgsonii* Mitochondrial Genome

| Gene          | Direction | Nucleotide number | Start codon | Stop codon |
|---------------|-----------|------------------|-------------|------------|
| tRNA Phe      | F         | 1–69             |             |            |
| 12S rRNA      | F         | 70–1,026         |             |            |
| tRNA Val      | F         | 1,027–1,093      |             |            |
| 16S rRNA      | F         | 1,094–2,659      |             |            |
| tRNA Leu      | F         | 2,663–2,737      |             |            |
| NADH1         | F         | 2,740–3,694      | ATG         | TAA        |
| tRNA Ile      | F         | 3,696–3,764      |             |            |
| tRNA Gln      | R         | 3,762–3,833      |             |            |
| tRNA Met      | F         | 3,836–3,904      |             |            |
| NADH2         | F         | 3,905–4,946      | ATA         | Taa        |
| tRNA Trp      | F         | 4,947–5,013      |             |            |
| tRNA Ala      | R         | 5,015–5,083      |             |            |
| tRNA Asn      | R         | 5,085–5,157      |             |            |
| Origin of L-strand replication | R | 5,158–5,189 | | |
| tRNA Cys      | R         | 5,190–5,256      |             |            |
| tRNA Tyr      | R         | 5,257–5,324      |             |            |
| COXI          | F         | 5,326–6,870      | ATG         | TAA        |
| tRNA Ser      | R         | 6,868–6,936      |             |            |
| tRNA Asp      | F         | 6,944–7,011      |             |            |
| COXII         | F         | 7,013–7,696      | ATG         | TAA        |
| tRNA Lys      | F         | 7,700–7,767      |             |            |
| ATPase8       | F         | 7,769–7,969      | ATG         | TAA        |
| ATPase6       | F         | 7,930–8,610      | ATG         | Taa        |
| COXIII        | F         | 8,610–9,393      | ATG         | Taa        |
| tRNA Gly      | F         | 9,394–9,462      |             |            |
| NADH3         | F         | 9,463–9,809      | ATA         | Taa        |
| tRNA Arg      | F         | 9,810–9,878      |             |            |
| NADH4L        | F         | 9,879–10,175     | ATG         | TAA        |
| NADH4         | F         | 10,169–11,546    | ATG         | Taa        |
| tRNA His      | F         | 11,547–11,616    |             |            |
| tRNA Ser      | F         | 11,617–11,676    |             |            |
| tRNA Leu      | F         | 11,678–11,747    |             |            |
| NADH5         | F         | 11,748–13,568    | ATA         | TAA        |
| NADH6         | R         | 13,555–14,081    | ATG         | TAA        |
| tRNA Glu      | R         | 14,080–14,148    |             |            |
| Cytochrome b  | F         | 14,153–15,292    | ATG         | AGA        |
| tRNA Thr      | F         | 15,296–15,366    |             |            |
| tRNA Pro      | R         | 15,366–15,431    |             |            |
| Control region|          | 15,432–16,498    |             |            |

### Protein coding genes

There are 13 protein-coding genes in the *P. hodgsonii* mitochondrial genome. Among these genes, eight use ATG as start codon and three (NADH2, NADH3, NADH5) use ATA as start codon. Some of these 13 protein genes are terminated with incomplete stop codons: NADH1, NADH3, and ATP6 are terminated with TA; COXIII (cytochrome c oxidase subunit III), NADH2, and NADH4 are terminated with T; the rest are terminated with TAA and AGA (Table 1). Presumably, these incomplete stop codons are accommodated post-transcriptionally in the mRNA maturation process, i.e. polyadenylation (5).
### Table 2 Number of Polymorphic Sites at Protein Coding Genes and RNA Genes

| Location | Gene    | Number of mutations |
|----------|---------|---------------------|
|          | All     | P. hodgsonii | C. hircus | O. aries |
| 1        | tRNA-Phe| 9            | 4         | 2        | 1       |
| 70       | 12s rRNA| 101          | 39        | 29       | 31      |
| 1,027    | tRNA-Val| 10           | 5         | 3        | 2       |
| 1,094    | 16s rRNA| 160          | 53        | 56       | 45      |
| 2,663    | tRNA-Leu| 4            | 4         |          |         |
| 2,740    | NU1M    | 149          | 53        | 48       | 42      |
| 3,762    | tRNA-Gln (L) | 1 | 1 | | |
| 3,836    | tRNA-Met| 1            | 1         |          |         |
| 3,905    | NU2M    | 168          | 59        | 63       | 43      |
| 4,947    | tRNA-Trp| 3            | 3         |          |         |
| 5,015    | tRNA-Ala (L) | 4 | 1 | 1 | 2 |
| 5,085    | tRNA-Asn (L) | 4 | 2 | 2 | |
| 5,190    | tRNA-Cys (L) | 1 | 1 | | |
| 5,257    | tRNA-Tyr (L) | 4 | 2 | 1 | 1 |
| 5,326    | COXI    | 241          | 100       | 65       | 71      |
| 6,868    | tRNA-Ser (L) | 4 | 1 | | 3 |
| 6,944    | tRNA-Asp| 3            | 1         | 1        | 1       |
| 7,013    | COXII   | 106          | 35        | 36       | 32      |
| 7,700    | tRNA-Lys| 12           | 4         | 3        | 4       |
| 7,769    | ATP8    | 39           | 13        | 12       | 11      |
| 7,930    | ATP6    | 131          | 44        | 45       | 39      |
| 8,610    | COXIII  | 148          | 59        | 56       | 30      |
| 9,394    | tRNA-Gly| 4            | 1         | 2        |         |
| 9,463    | NU3M    | 57           | 25        | 13       | 17      |
| 9,810    | tRNA-Arg| 5            | 1         | 3        | 1       |
| 9,879    | NU4L    | 45           | 10        | 10       | 23      |
| 10,169   | NU4M    | 273          | 101       | 74       | 90      |
| 11,547   | tRNA-His| 8            | 3         | 4        |         |
| 11,617   | tRNA-Ser| 10           | 5         | 2        | 3       |
| 11,678   | tRNA-Leu| 2            | 2         |          |         |
| 11,748   | NU5M    | 365          | 126       | 110      | 121     |
| 13,555   | NU6M (L) | 69 | 19 | 25 | 24 |
| 14,080   | tRNA-Glu (L) | 6 | 3 | 2 | 1 |
| 14,153   | CYB     | 185          | 60        | 62       | 57      |
| 15,296   | tRNA-Thr| 11           | 6         | 4        | 1       |
| 15,366   | tRNA-Pro (L) | 5 | | 4 | 1 |

Number of polymorphic sites at protein coding genes and RNA genes in the mitochondrial genomes of *P. hodgsonii*, *C. hircus* and *O. aries*. The column “All” refers to the number of sites at which all three species are different; the column “*P. hodgsonii*” refers to number of the sites at which *P. hodgsonii* is different from the other two species.

We compared protein sequences between *P. hodgsonii* and other mammalian species. The *P. hodgsonii* CYTB, ND6, ND4L, and COXII bare higher homology to those of *C. hircus* than *O. aries* and other species; Its COXI, COXIII, NU1M, NU2M, NU3M, NU4M, NU5M, and NU6M are most similar to those of *O. aries* than *C. hircus* and other species. Interestingly, ATP8 of *P. hodgsonii* is 93.93% identical to that of *Bos grunniens* whereas it shares much identity with those of other species, including those of *O. aries* (89.39%) and *C. hircus* (83.33%). The nucleotide similarity of these *P. hodgsonii* proteins is
in general higher when compared to O. aries and C. hircus than to other species. AT contents of the P. hodgsonii protein coding genes are higher than those of human and lower than those of mouse and rat (Data not shown).

We analyzed mutations of four protein coding genes in details, including CYTB, COXI, COXII, and COXIII, among O. aries, C. hircus, B. grunniens, P. hodgsonii, and a few other species of the Bovidae family, attempting to predict possible functional implications for mutations discovered from comparative analyses. In the case of COXI, there are five mutations affecting amino acids in P. hodgsonii, four in B. grunniens, three in C. hircus, and none in O. aries, as compared with B. taurus (Table 3). The amino acid changes in P. hodgsonii and B. grunniens occur mostly at sites between 400 and 500 (Table 3, Figure 2), whereas in C. hircus the mutations were found within 150 to 512. The region from 400 to 500 constitutes the transmembrane components X, XI, and XII.

### Table 3 Amino Acid Mutations of the COXI Gene

| Species       | Mutation | E/B | Neighbor rigidity | Rigidity change | Volume change | Charge change | Polarity change |
|---------------|----------|-----|------------------|-----------------|---------------|---------------|-----------------|
| C. hircus     | V155I    | 64  | -0.2351483       | -0.34353        | 26.7          | 0             | 0               |
|               | A308T    | 5   | -0.2854679       | -0.4675         | 27.5          | 0             | 0               |
|               | N512S    | 80  | 2.12195984       | 2.659928        | -114.1        | 0             | -2              |
| P. hodgsonii  | F8Y      | 136 | 0.2337137        | -0.0661         | 3.7           | 0             | 0               |
|               | D407T    | 76  | 0.63863865       | -0.2854679      | 5             | 1             | -1              |
|               | M449V    | 129 | 0.05870691       | 1.199486        | -22.9         | 0             | 0               |
|               | L467V    | 34  | 0.18495316       | 0.683123        | -26.7         | 0             | 0               |
|               | T509V    | 52  | -0.2661298       | -0.22177        | 23.9          | 0             | -1              |
| B. grunniens  | Y440S    | 108 | -0.3272085       | 0.142943        | -193.6        | 0             | 0               |
|               | S441P    | 5   | 0.35454652       | -0.54554        | 112.7         | 1             | 0               |
|               | I453V    | 51  | 0.1775126        | -0.22684        | -26.7         | 0             | 0               |
|               | F470L    | 96  | 0.08862233       | -0.404623       | -23.2         | 0             | 0               |

Amino acid mutations of the COXI gene for C. hircus, P. hodgsonii, and B. grunniens, as compared with B. taurus. The influences of these mutations are predicted by the methods referred from Mirkovic et al (31).
Mitochondrial Genome Sequence of Tibetan Antelope

B. taurus

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| Species          | Sequence  | Length | Identity | Length |
|------------------|-----------|--------|----------|--------|
| B. taurus        | HNPJILLKQGKQMKT EYQGOQGSKFL YNGAQVSK NFSQETQV HAFTQVNT   | 360    |          |        |
| B. bubalis       |           |        |          |        |
| B. grunniensis   |           |        |          |        |
| P. hodgsoni      |           |        |          |        |
| S. scrofa        |           |        |          |        |
| O. aries         |           |        |          |        |
| M. mast. jak     |           |        |          |        |
| C. familiaris    |           |        |          |        |
| E. cabalbus      |           |        |          |        |
| M. musculus      |            |        |          |        |
| C. hircus        |           |        |          |        |
| N. sapiens       |           |        |          |        |
| E. robustus      |           |        |          |        |
| T. rubripes      |           |        |          |        |
| X. laevis        |           |        |          |        |
| S. croc. dilurus |           |        |          |        |
| G. gallus        |           |        |          |        |
| A. albifrons     |           |        |          |        |
| B. rerio         |           |        |          |        |
| T. rubripes      |           |        |          |        |
| B. taurus        | URMFQHT KYAFTQV KSQKVKML KMQVRVMNL KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVKML KSQKVL
Mitochondrial Genome Sequence of Tibetan Antelope

**Fig. 2** Alignment of amino acid sequences of the COXI gene using CLUSTAL W1.83. The species included were Bos taurus (P00396), Bubalus bubalis (YP_087084), Bos grunniens (NP_008636), Ovis aries (P18943), Capra hircus (Q9MIY8), Male musculus (NP_008473), Eschrichtius robustus (NP_944635), Ursus arctos (Q5D15), Ursus maritimus (Q5D1S1), Xenopus laevis (P00389), Shinisaurus crocodilurus (NC_005959), Gallus gallus (P18943), Anser albifrons (NP_777304), Brachydanio rerio (Q9MII8), and Takifugu rubripes (NP_694917). Mutations of *P. hodgsonii* were highlighted as bold letters.

**RNA genes**

There are 22 tRNA genes identified in the *P. hodgsonii* mitochondrial genome, typical for mammalian mitochondrial genomes (6–9). Lengths of these tRNAs range from 65 to 74 bp. Some indels occur in the dihydrouridine and TψC arms (Figure 1). The 12S rRNA and 16S rRNA genes are 957 bp and 1,566 bp in length, respectively. Mitochondrial control region of *P. hodgsonii* is 1,067 bp in length, shorter than that of *O. aries* (1,180 bp), *C. hircus* (1,212 bp), domestic dog (1,270 bp), and domestic horse (1,192 bp), but longer than that of *B. taurus* (910 bp), *B. indicus* (913 bp), *B. bubalis* (910 bp), and *B. grunniens* (894 bp). By using the Tandem Repeats Finder (10), we found a 75-bp tan-
andom repeats that vary among different species: four in O. aries (Ref. 6; another O. aries haplotype in the same study has three repeats), two in C. hircus, and two in P. hodgsonii. The 75-bp repeats appear at the same location for these three species, close to the 5’-end of the last prolinyl tRNA (Pro) gene (Figure 1). Sequence consensuses of these 75-bp repeats are very similar with identities of 86.7% between O. aries and P. hodgsonii, 74.0% between C. hircus and P. hodgsonii, and 76.6% between C. hircus and O. aries. In addition, P. hodgsonii has two additional 25-bp tandem repeats, which have not been found in Bos species. This repeat is inserted at the 3’-end of the P. hodgsonii control region, close to phenyalaninyl tRNA (Phe) gene (Figure 1).

The 75-bp repeat has a lower GC content (25%) than the average of the control region (39%), whereas the 25-bp repeat has slightly higher GC content (41%) than the average. Although we only found two 75-bp-long tandems in control region of the C. hircus mitochondrial genome, there is no loss of length in the C. hircus control region, compared to the same region of O. aries.

Phylogenetic analysis and time of divergence

Complete cytochrome b of P. hodgsonii, O. aries, C. hircus, and other species of the Bovidae family were used to construct phylogenetic trees by using PAUP* (4b10) (11), with Giraffe camelopardalis as the outgroup. Our result indicates that P. hodgsonii is more related to O. aries, C. hircus, and Oreotragus oreotragus (klipspringer) (Figure 3), consistent with previously studies (12, 13). Based on the phylogenetic tree and a substitution rate of 0.056 per site per million years between two mammalian taxa (14), we estimated that P. hodgsonii and O. aries divided about 2.25 million years ago (Table 4).

![Fig. 3 Neighbor-joining tree based on the complete cytochrome b genes of some Bovidae species. The tree was constructed by using PAUP* (4b10), with parameters estimated by MODELTEST3.6 and with Giraffe camelopardalis as the outgroup. Number of bootstrap replication is 1000. Bootstrap values over 50% are shown on the branches.](image)

### Table 4 Molecular Divergence and Estimated Divergence Time Between Native Mammals on the Tibetan Plateau and Their Lowland Relatives

| Species                  | Molecular divergence | Divergence time (million years before presence) |
|--------------------------|----------------------|-----------------------------------------------|
| P. hodgsonii–C. hircus   | 0.124                | 2.22                                          |
| P. hodgsonii–O. aries    | 0.126                | 2.25                                          |
| O. aries–C. hircus       | 0.124                | 2.21                                          |
| B. grummiens–B. taurus   | 0.089                | 1.59                                          |

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Based on complete sequences of mitochondrial cytochrome b and a substitution rate of 0.056 per site per million years between two mammalian taxa.
Positive selection analysis

We used PAML (15) to analyze natural selection on mitochondrial genes of *P. hodgsonii*. The numbers of *N* (nonsynonymous substitutions) and *S* (synonymous substitutions) of each branch were calculated for eight genes that are more than 1,000 bp in length by codeml in PAML package. We found that *N/S* ratios of the COXI gene are significantly higher for the *P. hodgsonii* branch and the *B. grunniens* branch, compared with other branches (Fisher test *P*<0.001; Figure 4). The difference between *N/S* ratios of the *B. grunniens* branch and the *P. hodgsonii* branch is not significant (Fisher test *P*>0.1). These results suggested that the COXI gene has probably undergone positive selection in *P. hodgsonii* and *B. grunniens*. Despite the fact that COXI of *P. hodgsonii* has only three unique amino acid changes, it has other variation shared with one or more other species, which may collectively alter the function of the protein itself or interactions with other components in the mitochondrial respiration system. Experimentation and detailed structural analysis are of essence to pinpoint the relatedness of structure and function underscored by genetic changes. The sequence alignment of a highly variable region of COXI from selected species is shown in Figure 2.

Discussion

In the current study, we sequenced a complete mitochondrial genome from one *P. hodgsonii* individual, as the first attempt in a long-term research effort to understand the genetic basis of hypoxic adaptations of native fauna and flora on the Tibetan Plateau. Similar to other mammalian mitochondrial genomes, the *P. hodgsonii* mitochondrial genome contains 13 protein genes, 2 rRNA genes, 22 tRNA genes, and one control region. Its control region has two 75-bp tandem repeats near the last tRNA gene, whereas *O. aries*, *C. hircus*, and *Bos* species have three/four, two, and none, respectively. Although we only identified two 75-bp repeats in the control region of *C. hircus*, it is still possible that there might have been four units since *C. hircus* mitochondrial genome is very similar to the *O. aries* sequence in length and two of these four repeat units may occur early in time, resulted in poor homology due to mutations over a long decaying period. There are two additional 25-bp tandem repeats near the end of *C. hircus*’s control region, suggesting that short tandem repeats occur frequently among the mitochondrial genomes of *O. aries*, *C. hircus*, and *P. hodgsonii*.

Phylogenetic analysis on cytochrome b genes revealed that *P. hodgsonii* is more closely related with *O. aries*, *C. hircus*, and *O. oreotragus*, rather than other antelope species (the Antilopinae subfamily). Based on a substitution rate of 0.056 per site per million years between two mammalian taxa, we estimated that *P. hodgsonii* and *O. aries* divided about 2.25 million years ago. It was reported previously that climates around the Tibetan Plateau have undergone rapid changes at about 3.6–2.6 million years ago (16, 17). Our results are in close agreement with the proposed climatic changes around the Tibetan Plateau.

Fig. 4 The COXI gene was used to analyze *N/S* ratio of each branch within Artiodactyla mammals. Phylogeny was inferred with PAUP* (4b10). Numbers on the branches are mutation of nonsynonymous/synonymous for protein coding region of the COXI gene, as obtained with codeml in PAML package. * Fisher test *P*-value < 0.001. Take other branches (except *B. grunniens* and *P. hodgsonii* branches) as background for testing. The difference between the *B. grunniens* and *P. hodgsonii* branches is not significant (Fisher test *P*-value > 0.1).
To investigate whether the *P. hodgsonii* mitochondrial genes have been selected under the hypoxic environment of the plateau, we compared functional genes (CYTB, COXI, COXII, and COXIII) encoded in the mitochondrial genomes among *P. hodgsonii* and other distant relatives, including *O. aries*, *C. hircus*, and *B. grunniens*. In the COXI gene, we found that most of the mutations affecting amino acid sequences in *P. hodgsonii* and *B. grunniens* occur at sites between 400 and 500, whereas mutations in *C. hircus* occur at sites between 120 and 512. The region of 400 to 500 contains the transmembrane components X, XI, and XII, suggesting that nonsynonymous mutations unique to the two high altitude-adapted species may have functional implications. Although this process of functional prediction may not be very reliable, our results suggested to the likely targets for the next experimentation to verify the novel conjecture.

Evidence from N/S ratios shows that the COXI gene has more functional mutations in *P. hodgsonii* and *B. grunniens* compared with other mammals (Figure 4), providing further evidence that the COXI gene might have undergone positive selection among the native mammalian species of the plateau. The cytochrome c oxidase is the last step of the electron transport chain. It is consisted of 13 subunits, of which three subunits are encoded by the mitochondrial genome (COXI, II, and III), and ten subunits are encoded by the nuclear genome. Functional core of the enzyme complex is composed of subunits 1, 2, and 3 (18). Numerous studies have shown that some of the subunits of the COX gene have higher nonsynonymous substitution rate in primate than in other animals, such as COXI (19), COXII (20), and the nuclear coded COXIV (21) and COXVII (22). Several pieces of evidence suggest that structure and activity of cytochrome c oxidase may have adaptive changes during physiological hypoxia in mouse and rat cells (23). Expression of the mitochondrial genome encoded subunit COXI can decrease due to hypoxia while the enzyme efficiency remained. It suggested that expression of the COXI gene was regulated by the oxygen content. In addition, significant higher expression of COXI mRNA was observed in mammalian tissues, such as kidney and heart, during hibernation, and the change was not found in eutherian animals (24, 25).

Since *P. hodgsonii* and *B. grunniens* are both well-adapted to the same environment, the Tibetan Plateau, natural selection may have resulted in similar genetic signatures in their genomes, including the nuclear and mitochondrial genomes. Similar seemingly function-associated mutations and dN/dS ratios at COXI of *P. hodgsonii* and *B. grunniens* provided useful clues for further studies and functional confirmations on the role of mtDNA-encoded COX subunits on adaptation of native mammals to the unique Tibetan Plateau.

### Materials and Methods

**DNA extraction and sequencing**

Blood samples of *P. hodgsonii* individuals were collected from the Kekexi1 Natural Reservation in Qinghai Province, China, in December 2004. Samples were stored at 4°C for a few days before whole genomic DNA was extracted from the whole blood with standard salt-extraction method.

PCR primers and sequencing primers were designed based on a sequence of *O. aries* mitochondrial genome (NC_001941; ref. 6). PCR reactions were conducted on a PTC-200 thermal cycler with the following conditions: an initial denaturation step of 95°C (3 min) followed by 34 cycles of 95°C (30 s), 58°C/56°C (30 s), and 72°C (90 s) followed by 72°C for 10 min. PCR products were purified by Montage PCR Cleanup Kit (Millipore, Billerica, USA) and sequenced with ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, USA).

**Sequence analysis**

Base calling was performed with Phred (26) at the lowest Phred quality values control of 20. The sequences were assembled with Phraps (http://www.phrap.org) in a default setting. Sequence contigs were further finished, compared, and annotated in referencing to that of the *O. aries* sequence (NC_001941). Genes of tRNA were defined with tRNAscan-SE 1.2 (http://www.genetics.wustl.edu/eddy/tRNAscan-SE/). Comparative analysis was performed by using BLAST (27) and CLUSTAL W1.83 (28). Tandem repeats were defined with the program Tandem Repeats Finder (10).

**Phylogenetic analysis**

Complete sequences of cytochrome b genes from *P. hodgsonii*, *O. aries*, *C. hircus*, and other selected species of the Bovidae family were used to construct
phylogenetic trees, with G. camelopardalis as an outgroup. The sequences used were from P. hodge-
sonii, O. oreotragus (AF022052), Antilope cervicapra (AF022058), Ourebia ourebi (AF320574), Neotragus
moschatu (AF022069), Madoqua kirki (AF022070), Madoqua guentheri (AF022071), Saiga tatar-
ica (AF064487), Raphicerus sharpei (AF022050), Gazella dama (AF025954), Gazella subgutturosa
(F036282), Raphicerus campestris (AF022068), Raphicerus melanotis (AF022053), Antidorcas mar-
supialis (AF022054), O. aries (NC_001941), C. hircus (NC_005044), Bubalus bubal (NC_006295), B. grun-
niens (NC_006380), B. indicus (NC_005971), B. ta-
rus (NC_006853), and G. camelopardalis (AB001612).

Sequences were aligned with CLUSTAL W1.83 in
default options. Evolutionary models and parameters
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default options. Evolutionary models and parameters
default options. Evolutionary models and parameters
default options. Evolutionary models and parameters
of the aligned sequences were estimated by MODEL-
TEST3.6 (29). Phylogenetic trees were constructed
with the neighbor joining arithmetic method using
PAUP*4b10). Bootstrap analysis of 1,000 replicates
was performed to estimate robustness of the tree.

Analysis of positive selection

Eight protein coding genes with the length of over
1,000 bp were used to calculate N/S in each branch
by using PAML, and phylogenetic trees were con-
structed with PAUP*4b10). The F3X4 model for
codon frequency and free ratio model of \( \omega \) ratio as-
sumption was performed with the genetic codon of
mammalian mitochondrial, and the other parameters
were used as default. The DNA sequences were
aligned as protein sequences. The following sequences
were also included in the analysis from Homo sapi-
ens (X93334), Sus scrofa (NC_000845), Bubalus bubal
(NC_006295), B. granniens (NC_006380), B. indi-
cus (NC_005971), B. taurus (NC_006853), Muntiacus mantjak (AY225986), O. aries (NC_001941), C. hircus
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