Mol Biol Rep (2009) 36:871–878
DOI 10.1007/s11033-008-9257-9

The complete mitochondrial genome structure of snow leopard *Panthera uncia*

Lei Wei · Xiaobing Wu · Zhigang Jiang

Received: 22 October 2007 / Accepted: 10 April 2008 / Published online: 23 April 2008
© Springer Science+Business Media B.V. 2008

Abstract The complete mitochondrial genome (mtDNA) of snow leopard *Panthera uncia* was obtained by using the polymerase chain reaction (PCR) technique based on the PCR fragments of 30 primers we designed. The entire mtDNA sequence was 16 773 base pairs (bp) in length, and the base composition was: A—5,357 bp (31.9%); C—4,444 bp (26.5%); G—2,428 bp (14.5%); T—4,544 bp (27.1%). The structural characteristics [0] of the *P. uncia* mitochondrial genome were highly similar to these of *Felis catus*, *Acinonyx jubatus*, *Neofelis nebulosa* and other mammals. However, we found several distinctive features of the mitochondrial genome of *Panthera unica*. First, the termination codon of COIII was TAA, which differed from those of *F. catus*, *A. jubatus* and *N. nebulosa*. Second, $tRNA^{Ser\ (AGY)}$, which lacked the ''DHU'' arm, could not be folded into the typical cloverleaf-shaped structure. Third, in the control region, a long repetitive sequence in RS-2 (32 bp) region was found with 2 repeats while one short repetitive segment (9 bp) was found with 15 repeats in the RS-3 region. We performed phylogenetic analysis based on a 3 816 bp concatenated sequence of 12S rRNA, 16S rRNA, ND2, ND4, ND5, Cyt *b* and ATP8 for *P. uncia* and other related species, the result indicated that *P. uncia* and *P. leo* were the sister species, which was different from the previous findings.

L. Wei · X. Wu (✉)
Anhui Provincial Key Lab of the Conservation and Exploitation of Biological Resources, College of Life Sciences, Anhui Normal University, Wuhu 241000, China
e-mail: wuxb@mail.ahnu.edu.cn

Z. Jiang
Laboratory of Animal Ecology and Conservation Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100080, China
e-mail: jiangzg@ioz.ac.cn

Keywords *Panthera uncia* · Complete mitochondrial genome · Phylogenetic analyses

Introduction

The content of the animal mitochondrial genome, including 13 protein-coding genes, two rRNA genes, and 22 tRNA genes, as well as a control region, is highly conserved among most vertebrates [1]. Complete mitochondrial (mt) DNA sequence data contain information for study of molecular dynamics, comparative of genetics and evolution of species [2–4]. Many vertebrate complete mtDNA genomes have been sequenced since the human mitochondrial genome was sequenced [5]. These mitochondrial genome data have promoted research on molecular issues of animal evolution [6]. The partial comparisons of mtDNA at the population and species level were employed to resolve ancient phylogenetic relationships [7, 8]. Nevertheless, the use of complete mtDNA is essential for phylogenetic study because using different individual mtDNA gene might provide different answers to phylogenetic questions [9–11]. Thus, comparing complete animal mitochondrial genome sequences is now common for phylogenetic reconstruction and as a model for genome evolution. Not only were they much more informative than shorter sequences of individual genes for inferring evolutionary relatedness, but these complete animal mitochondrial genome data also provided sets of genome-level characteristics, such as the relative arrangements of genes, which could be especially powerful in inferring phylogenetic relationship [12].

Thirty-eight cat species of Felidae were generally divided into the pantherine, domestic cat, and ocelot lineages [7, 13]. The pantherine lineage is consisted of five big cats of the genus *Panthera* and many median-sized cat species. The lineage is the most recently evolved within 1 ~ 8MYA [14] and the

largest felid group which has demonstrated great confusion in their taxonomy and phylogeny [15–17]. So far, the complete mtDNA sequences of only three feline species (*Felis catus*, *Acinonyx jubatus*, *Neofelis nebulosa*) have been reported [18–20]. But the data were not enough to explain the phylogenetic relationships of Felidae. In present study, we sequence the complete mitochondrial genome of *P. uncia* and compare it with the mtDNAs of other feline species. It will contribute to a further understanding the evolution and phylogeny of Felidae to append the complete mitochondrial genome of *P. uncia* to the databases.

Materials and methods

DNA samples, extraction and PCR amplification

Skin of *P. uncia* was collected from Ningguo, Anhui Province, China. The sample was stored at −80°C in Animal Conservation Biology Laboratory, College of Life Sciences, Anhui Normal University.

Mitochondrial DNA of *P. uncia* was extracted from a piece of skin tissue using GENMED mtDNA Extraction Kit. Two major steps, isolation of mitochondria and mitochondrial DNA extraction, were included (GENMEN Scientifics Inc., USA). According to the instruction of Extraction Kit, the step of crushing of skin tissue was carried out under ice-bath condition. Other extraction steps were performed at 4°C. Moreover, as compared the mtDNA sample with the total DNA by agarose gel electrophoresis, no interference from the total DNA was observed. Based on the complete mtDNA sequences of *F. catus* (NC_001700), *A. jubatus* (AY463959), *N. nebulosa* (DQ257669) and some partial sequences of *P. tigris* (DQ151550), we successfully designed 30 pairs of primers for amplifying the complete mitochondrial genome sequences of *P. uncia* using Oligo 6.0 [21] (Table 1). PCR reactions were performed in the MJ

Table 1 Primers designed for amplifying and sequencing the complete mitochondrial genome of *P. uncia*

Primer name	Forward primer sequence (5′→3′)	Reverse primer sequence (5′→3′)	Annealing temperature
U1-F/R	TGAAAATGCCTAGATGAG	ATCTTCTGGGTGTAAGCC	54.0°C
U2-F/R	AATATGTACAYACCGCCCGTC	ATTACGCTACCTTYGCACG	54.5°C
U3-F/R	AACTCGGCAAACACAAGCC	TCGTTCAACTAGGGTTAGG	55.0°C
U4-F/R	TCAGAGGTTCAATTCCTC	TAGGATTAGGTTCGATTCC	54.0°C
U5-F/R	CAAGYATCCCACCTCAAAC	CAGCCTATATGGGCGATTG	55.0°C
U6-F/R	CCTACTCCTAACAATATCC	AGCAGTCCCTACTATACC	56.0°C
U7-F/R	CAGTCTAATGCTTACTCAGC	AGTATGCTCGTGTGTCTAC	55.0°C
U8-F/R	ATCGTCACCTACTACTCC	GTGGTCGTGRAAGTGTAG	55.0°C
U9-F/R	TGGTTTCAAGCCAATGCC	GATGTATCTAGTTGTGGC	54.0°C
U10-F/R	GTTCTTGAATTAGTYCCCC	GTTATAAGGAGGGCTGAAAG	53.0°C
U11-F/R	TGCTGTAGCCCTAATCCAA	TGTCTGTTTGTGAGGCTC	56.0°C
U12-F/R	TATGAGTGCGGATTTGACCC	CGTTCCGTTTGATTACCTC	53.0°C
U13-F/R	TCTAGTAGGCTCACTACC	GGTTCCTAAGACCAATGGA	52.0°C
U14-F/R	GAACTGCTAATTCATGCCTC	GTAGAAAYGCGAGGTAAG	53.0°C
U15-F/R	GCTATCTGTGCTCTCACAC	ARTAAGAGTARGCTGAGGG	54.0°C
U16-F/R	TGAGCCAAAARTCCGCATC	GTGCCAAAGTTTCATCAYG	53.5°C
U17-F/R	CCCTCAGAATGATATTTGTCCTA	TGAGATCTGAAAAACCATCGTTG	53.0°C
U18-F/R	GCTCCTACACCTTCTCAG	GCACAGTATGGGTATATG	56.0°C
U19-F/R	TCAAGGAAGAAGCAACAGCC	GGTCATAGCTGAGTCATAGC	52.0°C
U20-F/R	ACTGTGGTGTCATGCATTTGG	GACTCATCTAGGCATTTTCAG	55.0°C
U21-F/R	GTCTCTCATTCTATTTATCGGGTC	GGGAATAATGCCTGTTGGT	53.0°C
U22-F/R	CGAGACATTATCCGAGAAA	TTCAGTTCACTCTAGTCCTT	52.0°C
U23-F/R	CACGAGAAAACGCCTAAT	GACCCAGAGCACATCAATAA	53.0°C
U24-F/R	ACCACCAGCCACAATCAAA	TGGATCGGAGGATTGCGTAT	52.0°C
U25-F/R	TCCAGGTCGGTTTCTATCTA	TAGGATGGGTGCTGTGATGAAT	53.1°C
U26-F/R	CTCTAAGTAAGCCCTATA	GCATGGGCAGTAACTACTA	55.0°C
U27-F/R	ACACCTATTCTGATTCTTCG	GAGAATTAAGATGATGGCTGGT	54.0°C
U28-F/R	TCAAGCCAATACCATAACCACT	TCCTATTATTGTTGGGGTA	53.0°C
U29-F/R	ACATGCCACAGTTAGATAC	TTTGAGTGATAGAAGGCCCAGA	53.0°C
U30-F/R	CCACTGCCATACTCATACCAAT	GTCTTTTGGTAGTCACAGGT	54.0°C

Note: Y = C or T, R = A or G, K = G or T, M = A or C, respectively

Model PTC-200 thermal cycler with the following conditions: 95°C for 5 min, 30 cycles of 94°C for 50 s, 52–56°C for 1 min, 72°C for 1 min; 72°C for 10 min. Each reaction included 19 μl sterile distilled water, 3 μl 10× PCR Buffer, 2 μl dNTP (2.5 mM), 2 μl $MgCl_2$ (25 mM), 1 μl of each primer (10 μM), 1 unit of Taq DNA polymerase (Promega) and 1 μl of template for a total reaction volume of 30 μl. The resultant PCR fragments were electrophoresed on a 1% agarose gel.

DNA sequencing, alignment and analyses

The DNAs were purified from excised pieces of gel using DNA Gel Extraction Kit (Axygen) for sequencing on an automatic DNA sequencer (Applied Biosystems) from both strands with the primer walking method. Then, the sequences of *P. uncia* were submitted for BLAST searching [22] to ensure that the required sequences had been amplified. Nucleotide sequences were analyzed with CLUSTAL X program (1.8) [23] and refined by visual inspection. Manual adjustments of protein-coding nucleotide sequences alignments were facilitated through translation to amino acid sequences. The locations of 13 protein-coding genes were identified using software SEQUIN (Version 5.35, 2004) and 2 ribosomal RNA genes were determined by comparing the corresponding sequence of *F. catus* and homologous sequence of other Felidae mtDNAs. The tRNA genes were identified using software tRNA Scan-SE 1.21 (http://lowelab.ucsc.edu/tRNAscan-SE) with their cloverleaf secondary structure and anticodon sequences presumed using DNASIS (Version 2.5, Hitachi Software Engineering). The complete nucleotide sequence of mitochondrial genome of *P. uncia* was submitted to GenBank with the accession number EF551004.

Molecular phylogenetic analyses

In order to clarify the phylogenetic relationship of *P. uncia*, we constructed the phylogenetic tree based on the concatenated 7 mitochondrial genes of 10 species with the data set obtained from GenBank (Table 2). *Canis familiaris* was used as the outgroup. The data set were subjected to two different methods of phylogenetic reconstruction: maximum likelihood (ML) and Bayesian inference (BI) methods. The ML analyses were performed using PAUP*4.0b10 [24], the best-fit model of DNA substitution was estimated using ModelTest ver. 3.6 [25, 26] and a general-time-reversible + gamma + invariant (GTR + I + G, G = 1.8671, I = 0.5549) model proposed under AIC consideration. MrBayes ver. 3.0b4 [27] was used for BI analyses. For BI analyses, the following settings were applied: number of Markov chain Monte Carlo (MCMC) generations = 400,000 and sampling frequency = 1000. For the Bayesian analyses, we used posterior probabilities as indicators of node confidence. Because these represent the true probabilities of the clades [28], probabilities >95% were considered to be significant [29]. The robustness of ML trees topologies was tested using bootstrap analyses [30] with 1,000 replicates [31]. We considered topologies with bootstrap values >70%to be sufficiently supported, and those with values between 50 and 70% to be weakly supported [32].

Results and discussion

Complete mitochondrial genome content and structure

The numbering and determination of the gene boundaries within the mitochondrial genome were performed on the

Table 2 Species in phylogenetic analyses

Scientific name	12S rRNA	16S rRNA	ND2	ND4	ND5	Cyt *b*	ATP8
Panthera uncia	EF551004	EF551004	EF551004	EF551004	EF551004	EF551004	EF551004
Panthera pardus	AM779888	AF006443	AY634383	AY634395	AF006444	EF437590	DQ899943
Panthera tigris	DQ151550	DQ151550	DQ151550	AY634396	AF006460	EF437588	DQ899931
Panthera onca	AY012151	AF006441	AY634391	AY634403	AF006442	EF437582	DQ899924
Panthera leo	S9300	AF006457	AY170043	AY634398	AF006458	S79302	DQ899945
Neofelis nebulosa	DQ257669	DQ257669	DQ257669	DQ257669	DQ257669	DQ257669	DQ257669
Acinonyx jubatus	AY463959	AY463959	AY463959	AY463959	AY463959	AY463959	AY463959
Puma concolor	U33495	AF006455	AY634392	AY634404	AF006456	AY598487	AY598483
Lynx lynx	D28891	AF006413	AY634389	AY634401	AF006414	AY773083	AY598471
Felis catus	NC_001700	NC_001700	NC_001700	NC_001700	NC_001700	NC_001700	NC_001700
Canis familiaris	U96639	U96639	U96639	U96639	U96639	U96639	U96639

basis of mitochondrial genome of *F. catus* [18]. The mitochondrial genome organization of *P. uncia* was shown in Fig. 1. The entire complete nucleotide sequence of mitochondrial genome of *P. uncia* was 16 773 bp in length containing 13 protein-coding genes, 12S and 16S ribosomal RNA genes, 22 transfer RNA genes, as well as noncoding regions (including the control region), the base composition was as follows: A 5 357 bp (31.9%), C 4 444 bp (26.5%), G 2 428 bp (14.5%), T 4 544 bp (27.1%); A + T 9 901 bp (59%), G + C 6 872 bp (41%). [0] The sequence showed a higher homology[0] compared to *F. catus*, *A. jubatus* and *N. nebulosa*.

Protein-coding genes

All the 13 protein-coding open reading frames (ORF) were included in mitochondrial genome of *P. uncia* with the same organization and no major rearangements, which were found in other mammalian mitochondrial genomes as well [6, 33, 34]. The longest one was ND5 gene (1821 bp), while the shortest one was ATP8 gene (204 bp). All the protein-coding genes were encoded in H-strand except ND6, in L-strand. ND2 was started with ATC, [0] which was different from *F. catus*, *A. jubatus*, and *N. nebulosa*. ND3 and ND5 were started with ATA; and the rest of them were started with ATG. These characteristics of the initial codons were homologous to most vertebrate mitochondrial protein-coding genes [35]. The termination codons were TAA, except Cyt *b* ended with AGA, ND3 ended with TA and others ended with single T, which were also homologous to those of most mammals. However, the termination codon of COIII was TAA, which was different from *F. catus*, *A. jubatus* and *N. nebulosa*.

It was known that imcomplete termination codons (T or TA) were completed by PolyA of the 3' end of mRNA after transcription [36]. Thus, the incomplete termination codons in the mtDNA of *P. uncia* should be terminated with UAA. It was noticeable that the termination codons of the Cyt *b* genes in *F. catus*, *A. jubatus*, *N. nebulosa* and *P. uncia* were all AGA. The Cyt *b* genes were terminated with AGA, which seemed to typically occur in most mammals, such as *Canis* [6], pig [33], and rabbit [34], and was distinctive from other animals, such as amphibians [37, 38], reptiles [39–41], Aves [42] and Nematoda [43], which often had Cyt *b* genes terminated with T, TA, TAA, or even with no termination codons at all. Such situations did not happen in other protein-coding genes, the starting and termination codons of which often appeared undiversified among species.

In addition, coding sequences overlapped between ATP8 and ND6, ND4 and ND4L, and ND5 and ND6 genes, respectively. The light strand encodes the ND6 gene and the tRNAs Glutamine, Alanine, Aspargine, Cysteine, Tyrosine, Serine UCN, Glutamic acid and Proline. The pattern of codon usage in the *P. uncia* mitochondrial genome follows the preference patterns observed in other mammalian mtDNA sequences.

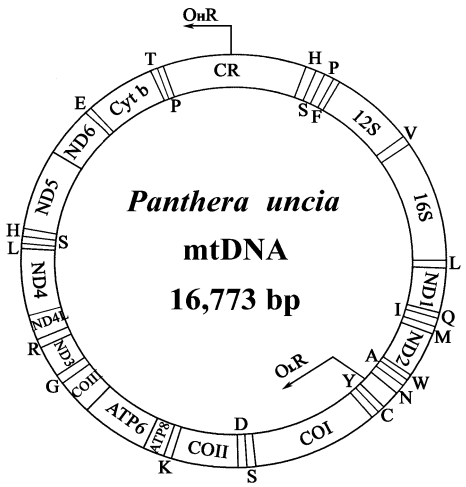

Fig. 1 Complete mitochondrial (mt)DNA organization of *P. uncia*. Gene abbreviations used were 12S, 12S rRNA; 16S, 16S rRNA; ND1–6, NADH dehydrogenase subunits 1–6; COI–III, cytochrome oxidase subunits I–III; ATP6 and ATP8, ATPase subunits 6 and 8; cyt b, cytochrome b; Transfer (t)RNA genes were identified by single letter of amino acid codon. O_HR and O_LR stand for the heavy-strand replication origin and the light-strand replication origin, respectively. Except for ND6, all protein genes were H-strand-encoded

Ribosomal and transfer RNA genes

Located between $tRNA^{Phe}$ and $tRNA^{Val}$, the 12S rRNA gene of *P. uncia* was 960 bp, the same length as of *F. catus* and *A. jubatus*, 2 bp longer than of *N. nebulosa*. But it was more conserved than that of *F. catus* and *A. jubatus*, and only 71 sites were variable, accounting for 7.6%. The 16S rRNA gene of *P. uncia*, located between $tRNA^{Val}$ and $tRNA^{Leu\ (UUR)}$, was 1 580 bp and was 6, 8 and 7 bp longer than the ones in *F. catus*, *A. jubatus* and *N. nebulosa*, respectively. The 16S rRNA gene was much more conserved than the 12S rRNA gene, with only 7.1% of sites varying from *F. catus* and *A. jubatus*.

A total of 22 tRNAs were found in the mtDNA of *P. uncia*, including $tRNA^{Leu\ (UUR)}$, $tRNA^{Leu(CUN)}$, $tRNA^{Ser\ (UCN)}$, and $tRNA^{Ser\ (AGY)}$. The typical length of tRNA genes was about 59–75 bp in verebrates [18, 19]. In the mtDNA genomes of *P. uncia*, the shortest tRNA was $tRNA^{Ser(AGY)}$ (59 bp) and the longest one was $tRNA^{Asn}$ (73 bp). All the tRNAs in this genome could be folded into a cloverleaf secondary structure as in most other mammals, $tRNA^{Phe}$ (Fig. 2a) was a good example while $tRNA^{Ser(AGY)}$ (Fig. 2b) was an exception which missed the "DHU" arm [5, 9, 44].

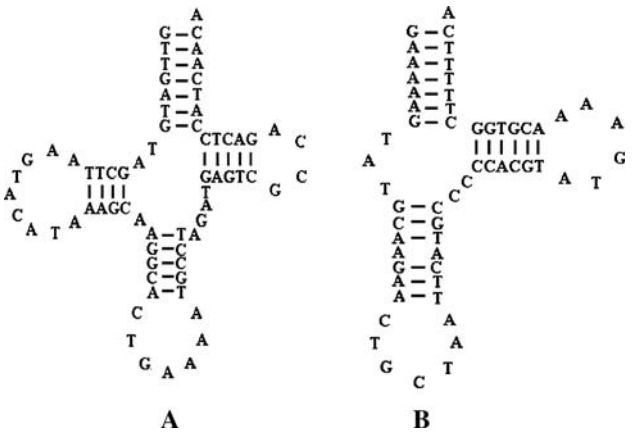

Fig. 2 (**a**) The secondary structure of $tRNA^{Phe}$, (**b**) The secondary structure of $tRNA^{Ser\ (AGY)}$

Non-coding region

The control region (CR) of *P. uncia* was located between $tRNA^{Pro}$ and $tRNA^{Phe}$, and contains only promoters and regulatory sequences for replication and transcription, but no structural genes. Tandem repeats generally occur in this region. Jae-Heup et al. [45] studied the control region of *Panthera* species and divided it into 3 parts: the left domain, the central conserved region (CCR), and the right domain. Hypervariable segment (HVS)-1 and repetitive sequence (RS)-2 were in the left domain, whereas RS-3 and HVS-2 were in the right domain. Conserved sequence block (CSB)-2 and (CSB)-3 were in HVS-2, (CSB)-1 was in CCR, which was located between RS-2 and RS-3 (Fig. 3). By comparing the CR sequence of *P. uncia* with those of *F. catus*, *A. jubatus* and *N. nebulosa* [18, 20, 45], we identified a central conserved region, a left domain with HVS-1 and RS-2 at the 5′ end, and a right domain with HVS-2 and RS-3 at the 3' end of the control region. It was notable that the length of RS-3 in *P. uncia* was much shorter than those in *F. catus*, *A. jubatus* and *N. nebulosa.* Yet, the length of HVS-1 in *N. nebulosa* was much shorter than those in *F. catus*, *A. jubatus* and *P. uncia*. These results explained why the size of the control region of *P. uncia* was much smaller than those of *F. catus* and *A. jubatus* but was nearly equal to *N. nebulosa*, as well as why the complete mtDNA of *P. uncia* was shorter than those of *F. catus* and *A. jubatus* but was similar to *N. nebulosa*. In the mtDNA control region, RS-2 consisted of long repetitive motifs that were 80 bp in *A. jubatus* [46], whereas RS-3 consisted of short repeat units (usually 6–10 bp). In this study, by comparing *F. catus*, *A. jubatus* and *P. tigris* [47], one long repetitive segment (5′-TGTATATCGTGCATTAA TCGCTAGTCCCCATG-3′) was found in RS-2 with 2 repeats. The RS-3 sequence of *P. uncia*, which was composed of short repetitive segments, was compositionally similar to those of the other big cats. One example was that the segment 5′-ACACGTACA-3′ repeated 15 times. There were two major opinions about the biological function of repetitive segments. Some reports indicated that they had regulative function in the course of replication and transcription of mtDNA [48, 49]; however, Casane et al. [50] thought that repetitive segments seemed to have no important biological function, but just trifle sequences; the function of the repetitive segments needs further research. In addition, CSB-3 was considered to be conserved in the control region of *P. uncia* with no variable sites found. Jae-Heup et al. [45] studied this region in several species of *Panthera* genus, and only one variable position was found in *P. tigris*, while 4 variable position was found in *N. nebulosa* [20].

Phylogeny of *P. uncia*

The ML and Bayesian methods of phylogenetic analyses yielded a tree of same topology (Fig. 4). The 10 cat species used in phylogenetic analyses were divided into two supported major groups. Group I comprised 6 species belonging to the genus *Panthera*. *N. nebulosa* occupied the most basal position the branch of the tree, followed by *P. tigris*, *P. onca*, *P. pardus*, and then last two most recently diverged sister species *P. leo* and *P. uncia*. Group

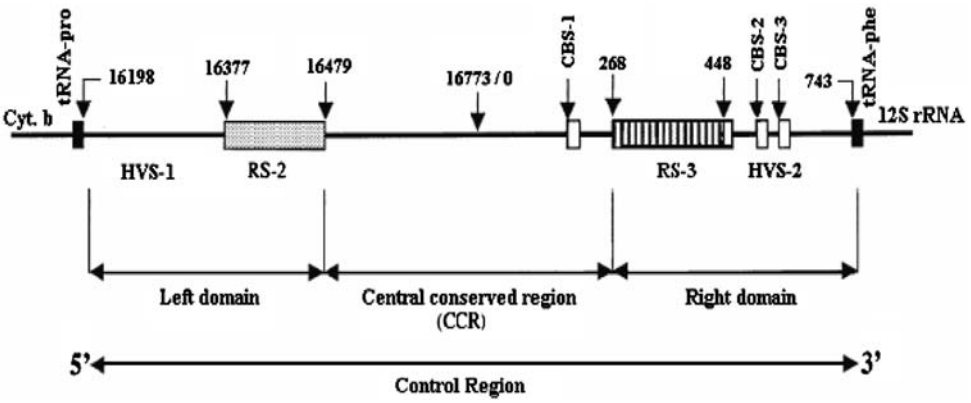

Fig. 3 Scheme of the *P. uncia* mitochondrial control region, showing conserved blocks, the location of repetitive sequences (RS), and other defined domains

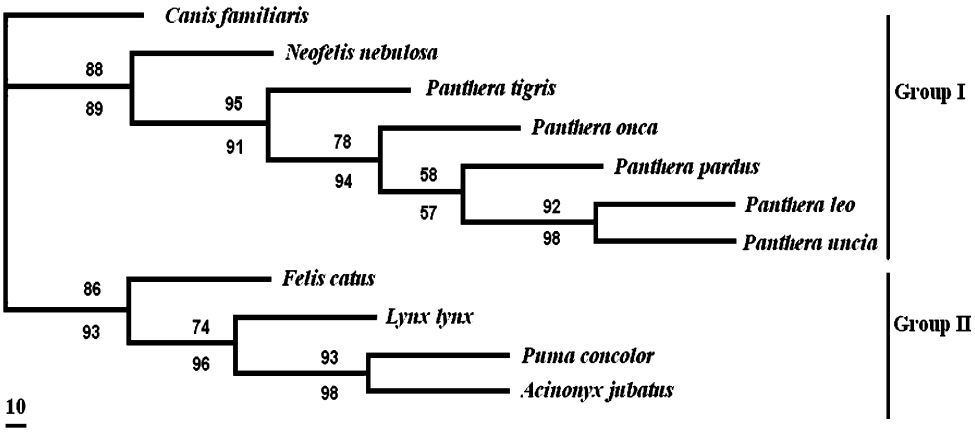

Fig. 4 Phylogenetic tree based on analyses of concatenated 7 mitochondrial gene segments (12S rRNA + 16S rRNA + ND2 + ND4 + ND5 + Cyt *b* + ATP8). Analyses of ML and Bayesian produced the same topology tree and bootstrap values were above branches, Bayesian probabilities were below branches

II contained 3 pantherine cats and domestic cat. *Panthera* was composed of five recognized extant species (*P. tigris*, *P. uncia*, *P. pardus*, *P. leo*, *P. onca*) and *N. nebulosa* in recent studies of molecular phylogeny [17, 51], however, interspecific relationships within the genus have been equivocal probably due to extremely recent speciation (1 ~ 2MYA; [52, 53]). In this paper, our analyses indicated that *N. nebulosa* was included as the most based member within *Panthera* genus (ML, 88%; Bayesian, 98%). *P. tigris* was the sister taxon to the other members of *Panthera*, within the clade containing the other members of the genus (ML, 95%; Bayesian, 91%). The position of *P. onca* was clearly upheld in ML and Bayesian analyses (ML, 78%; Bayesian, 94%), but *P. pardus* was weakly supported as the sister taxon to *P. leo* and *P. uncia* in ML (58%) and Bayesian trees (57%). The most interesting and novel finding of this study was that *P. uncia* and *P. leo* was probably sister species, this relationship was supported by ML bootstrap values and Bayesian probabilities based on 7 combined genes (92%, 98% support in ML bootstrap values and Bayesian posterior probability). Many biologists have studied the evolution and phylogeny of *P. uncia*, but they had different findings based on different methods in previous studies. *P. uncia* was placed as the most based taxon in genus *Panthera* by Johnson and O'Brien [15] using partial 16S rRNA and ND5 genes. Mattern and McLennan [54] obtained the same result based on partial 12S and 16S rRNA, ND5, and Cyt *b* genes with morphological and karyological characters. Jae-Heup et al. [45] suggested that *P. uncia* was the closest relative of *P. tigris* based on complete mtDNA control region. *P. uncia* has also been alternatively hypothesized as the sister species of *P. tigris* based on analysis of chemical signals [55] and combined nuclear DNA (combined 19 autosomal, five X-linked, six Y-linked = 18,853 bp) [17]. However, Li et al. [16] suggested that *P. uncia* and *P. pardus* were sister species based on combined six genes (ND2, ND4, ND5, cytb, 12S and 16S rRNA) and three nuclear DNAs (β-fibrinogen, IRBP and TTR) analyses. Buckley-Beason et al. [56] thought that *P. uncia* was the sister taxon to the other three *Panthera* species (*P. pardus*, *P. onca*, *P. tigris*) based on analysis of combined mtDNA (ATP8, Cyt *b*, ND5 and control region) and nuclear gene segments (ATP-7A, BGN, HK1, IDS and PLP). Our phylogenetic analyses using seven mtDNA genes sequences strongly supported the close affinity between *P. uncia* and *P. leo*. In conclusion, although precise relationships among the genus *Panthera* were not well resolved in this study, the combined mtDNA data, however, provided insightful understanding of *Panthera*'s evolution.

Aknowledgements This work was supported by grants from Knowledge Innovation Project of Chinese Academy of Sciences (No. CXTDS2005–4), National Natural Science Foundation of China (NSFC, No.30470244), Research Fund for the Doctoral Program of Higher Education (20070370002), the Fund for Leading Scientist of Science and Technology in Anhui and Key Laboratory of Biotic Environment and Ecological Safety in Anhui Province, China.

References

1. Boore JL (1999) Animal mitochondrial genomes. Nucleic Acids Res 27(8):1767–1780
2. O'Brien SJ (1994) Genetic and phylogenetic analyses of endangered species. Annu Rev Genet 28:467–489
3. D'Erchia AM, Gissi C, Pesole G, Saccone C, Arnason U (1996) The guinea-pig is not a rodent. Nature 381:597–600
4. Penny D, Hasegawa M (1997) The platypus put in its place. Nature 387:549–550
5. Anderson S, Bankier AT, Barrell BG, de Bruijn MH, Coulson AR, Drouin J, Nierlich DP, Roe BA, Sanger FH, Smith J, Staden R, Young IG (1981) Sequence and organization of the human mitochondrial genome. Nature (London) 290:457–465
6. Kim KS, Seong EL, Ho WJ, Ji HH (1998) The complete nucleotide sequence of the domestic dog (*Canis familiariz*) mitochondrial genome. Mol Phylogenet Evol 10:210–220
7. Janczewski DN, Modi WS, Stephens JC, O'Brien SJ (1995) Molecular evolution of mitochondrial 12S RNA and cytochrome *b* sequencse in the Pantherine lineage of Felidae. Mol Biol Evol 12:690–707

8. Vila C, Savolainen P, Maldonado JE, Amorim IR, Rice JE, Honeycutt RL, Crandall KA, Lundeberg J, Wayne RK (1997) Multiple and ancient origins of the domestic dog. Science 276:1687–1689
9. Arnason U, Johnsson E (1992) The complete mitochondrial DNA sequence of the harbor seal, *Phoca vitulina*. J Mol Evol 34: 493–505
10. Cao Y, Adachi J, Janke A, Paabo S, Hasegawa M (1994) Phylogenetic relationships among eutherian orders estimated from inferred sequences of mitochondrial proteins: instability of tree based on a single gene. J Mol Evol 39:519–527
11. Xu X, Arnason U (1996) The mitochondrial DNA molecule of Sumatran orangutan and a molecular proposal for two (Bornean and Sumatran) species of orangutan. J Mol Evol 43:431–437
12. Boore JL, Macey JR, Medina M (2005) Sequencing and comparing whole mitochondrial genomes of animals. Methods Enzymol 395:311–348
13. Masuda R, Lopez JV, Pecon Slattery J, Yuhki N, O'Brien SJ (1996) Molecular phylogeny of mitochondrial cytochrome b and 12S rRNA sequences in the Felidae: ocelot and domestic cat lineages. Mol Phylogenet Evol 3:351–365
14. Pecon SJ, Johnson WE, Goldman D, O'Brien SJ (1994) Phylogenetic reconstruction of South American felids defined by protein electrophoresis. J Mol Evol 39:296–305
15. Johnson WE, O'Brien SJ (1997) Phylogenetic reconstruction of the felidae using 16SrRNA and NADH-5 mitochondrial genes. Mol Evol 44(1):98–116
16. Li Y, Zhang YP (2005) Phylogenetic studies of pantherine cats (Felidae) based on multiple genes, with novel application of nuclear β-fibrinogen intron 7 to carniores. Mol Phyl Evol 35: 483–495
17. Johnson WE, Eizirik E, Slatter JP, Murphy WJ, Antunes A, Teeling E, O'Brien SJ (2006) The late miocene radiation of modern Felidae: a genetic assessment. Science 311:73–77
18. Lopez JV, Cevario S, O'Brien SJ (1996) Complete nucleotide sequences of the Domestic cat (Felis catus) mitochondrial genome and a transposed mtDNA tandem repeat (Numt) in the nuclear genome. Genomics 33:229–246
19. Burger PA, Steinborn R, Walzer C, Petit T, Mueller M, Schwarzenberger F (2004) Analysis of the mitochondrial genome of cheetahs (*Acinonyx jubatus*) with neurodegenerative disease. Gene 338:111–119
20. Wu XB, Zheng T, Jiang ZG, Wei L (2007) The mitochondrial genome structure of the clouded leopard (*Neofelis nebulosa*). Genome 50(2):252–257
21. Rychlik W, Rychlik P (2000) Oligo Primer Analysis Software. Version6.01. Molecular Biology Insights, Inc., Cascade, Colorado
22. Altschul SF, Madden TL, Schäer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402
23. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The clustal_X windows interface: xexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25(24):4876–4882
24. Swofford DL (2003) PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4.0b10. Sinauer Associates, Sunderland, Massachusetts.
25. Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. Bioinformatics 14:817–818
26. Posada D, Buckley TR (2004) Model selection and model averaging in phylogenetics: advantages of the AIC and Bayesian approaches over likelihood ratio tests. Syst Biol 53:793–808
27. Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17(8):754–755
28. Rannala B, Yang Z (1996) Probability distribution of molecular evolution trees: a new method of phylogenetic inference. J Mol Evol 43:304–311
29. Leaché AD, Reeder TW (2002) Molecular systematics of the eastern fence lizard (*Sceloporus undulatus*): a comparison of parsimony, likelihood, and Bayesian approaches. Syst Biol 51:44–68
30. Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791
31. Hedges SB (1992) The number of replications needed for accurate estimation of the bootstrap *P* value in phylogenetic studies. Mol Biol Evol 9:366–369
32. Huelsenbeck JP, Hillis DM (1993) Success of phylogenetic methods in the four-taxon case. Syst Biol 42:247–264
33. Ursing BM, Arnason U (1998) The complete mitochondrial DNA sequence of the pig (Sus scrofa). J Mol Evol 47:302–306
34. Gissi C, Gullberg A, Arnason U (1998) The Complete mitochondrial DNA sequence of the Rabbit (*Oryctolagus cuniculus*). Genomics 50:161–169
35. Xu X, Gullberg A, Arnason U (1996) The complete mitochondrial DNA (mtDNA) of the donkey and mtDNA comparisons among four closely related mammalian species-pairs. J Mol Evol 43(5):438–446
36. Ojala D, Monotoya J, Attardi G (1981) tRNA punctuation model of RNA processing in human mitochondria. Nature (London) 290:470–474
37. Sumida M, Kanamori Y, Kaneda H, Kato Y, Nishioka M, Hasegawa M, Yonekawa H (2001) Complete nucleotide sequence and gene rearrangement of the mitochondrial genome of the Japanese pond frog *Rana nigromaculata*. Genes Genet Syst 76:311–325
38. Sano N, Kurabayashi A, Fujii T, Yonekawa H, Sumida M (2004) Complete nucleotide sequence and gene rearrangement of the mitochondrial genome of the bellring frog, *Buergeria buergeri* (family Rhacophoridae). Genes Genet Syst 79(3):151–163
39. Wu XB, Wang YQ, Zhou KY, Zhu WQ, Nie JS, Wang CL (2003) Complete mitochondrial DNA sequence of Chinese alligator, *Alligator sinensis*, and phylogeny of crocodiles. Chin Sci Bull 48(19):2050–2054
40. Janke A, Arnason U (1997) The complete mitochondrial genome of Alligator mississippiensis and the separation between recent archosauria (birds and crocodiles). Mol Biol Evol 14:1266–1272
41. Janke A, Erpenbeck D, Nilsson M, Arnason U (2001) The mitochondrial genomes of the iguana (*Iguana iguana*) and the caiman (*Caiman crocodylus*): implications for amniote phylogeny. Proc Biol Sci 268:623–631
42. Sun Y, Ma F, Xiao B, Zheng JJ, Yuan XD, Tang MQ, Wang L, Yu YF, Li QW (2004) The complete mitochondrial genomes sequences of Asio flammeus and Asio otus and comparative analysis. Sci China Ser C Life Sci 47(6):510–520
43. Hu M, Chilton NB, Gasser RB (2003) The mitochondrial genome of Strongyloides stercoralis (Nematoda)—idiosyncratic gene order and evolutionary implications. Int J Parasitol 33:1393–1408
44. Frazer-Abel AA, Hagerman PJ (1999) Determination of the angle between the acceptor and anticodon stems of a truncated mitochondrial tRNA. J Mol Biol 85(2):581–593
45. Jae-Heup K, Eizirik E, O'Brien SJ, Johnson WE (2001) Structure and patterns of sequence variation in the mitochondrial DNA control region of the great cats. Mitochondrion 14:279–292
46. Freeman AR, Machug DE, McKeown S, Walzer C, McConnell DJ, Bradley DG (2001) Sequence variation in the mitochondrial DNA control region of wild African cheetahs (*Acinonyx jubatus*). Heredity 86:355–362
47. Cracraft J, Feinstein J, Vaughn, Helm-Bychowski K (1998) Sorting out tigers (*Panthera tigris*): mitochondrial sequences, nuclear inserts, systematics, and conservation genetics. Anim Conserv 1:63–74

48. Delarbre C, Rasmussen AS, Arnason U, Gachelin G (2001) The complete mitochondrial genome of the hagfish *Myxine glutinosa*: unique features of the control region. J Mol Evol 53:634–641
49. Delport W, Ferguson JW, Bloomer P (2002) Characterization and evolution of the mitochondrial DNA control region in hornbills (Bucerotiformes). J Mol Evol 54:794–806
50. Casane D, Dennebouy N, de Rochambeau H, Mounolou JC, Monnerot M (1997) Nonneutral evolution of tandem repeats in the mitochondrial DNA control region of Lagomorphs. J Mol Biol Evol 14:779–789
51. Bininda-Emonds OR, Gittleman JL, Purvis A (1999) Building large trees by combining phylogenetic information: a complete phylogeny of the extant Carnivora (Mammalia). Biol Rev 74:143–175
52. Kurten B, Anderson E (1980) Pleistocene mammals of North America. Columbia University Press, New York, pp 108–118
53. Wayne RK, Benveniste RE, Janczewski DN, O'Brien SJ (1989) Molecular and biochemical evolution of the Carnivora. In: Gittleman JL (ed) Carnivore behaviour, ecology and evolution. Chapman and Hall, London, pp 465–494
54. Mattern MY, McLennan DA (2000) Phylogeny and speciation of felids. Cladistics 16:232–253
55. Bininda-Emonds OR (2001) The utility of chemical signals as phylogenetic characters: an example from the felidae. Biol J Linn Soc 72:1–15
56. Buckley-Beason VA, Johnson WE, Nash WG, Stanyon R, Menninger JC, Driscoll CA, Howard J, Bush M, Page JE, Roelke ME, Stone G, Martelli PP, Wen C, Ling L, Duraisingam RK, Lam PV, O'Brien SJ (2006) Molecular evidence for species-level distinctions in clouded leopards. Curr Biol 16(23):2371–2376