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Research Paper

A complete mitochondrial genome sequence of Asian black bear Sichuan subspecies (*Ursus thibetanus mupinensis*)

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We obtained the complete mitochondrial genome of *U.thibetanus mupinensis* by DNA sequencing based on the PCR fragments of 18 primers we designed. The results indicate that the mtDNA is 16 868 bp in size, encodes 13 protein genes, 22 tRNA genes, and 2 rRNA genes, with an overall H-strand base composition of 31.2% A, 25.4% C, 15.5% G and 27.9% T. The sequence of the control region (CR) located between tRNA-Pro and tRNA-Phe is 1422 bp in size, consists of 8.43% of the whole genome, GC content is 51.9% and has a 6bp tandem repeat and two 10bp tandem repeats identified by using the Tandem Repeats Finder. *U. thibetanus mupinensis* mitochondrial genome shares high similarity with those of three other Ursidae: *U. americanus* (91.46%), *U. arctos* (89.25%) and *U. maritimus* (87.66%).

Key words: *Ursus thibetanus mupinensis*; mitochondrial genomes; sequencing; sequence analysis

1. Introduction

Mitochondria are vital subcellular organelles, responsible for the oxidizing reaction of the tricarboxylic acid cycle, the electron transfer and the energy metabolism in cells and have an independent genetic material called the mitochondria genome (mtDNA). Mammalian mtGenomes exist as closed circular strands and have a set of 13 protein-coding genes (NADH-ubiquinone oxidoreductase chain 1, 2, 3, 4L, 4, 5, 6, cytochrome c oxidase subunit 1, 2 and 3, ATP synthase F0 subunit 8 and ATP synthase F0 subunit 8), two rRNA genes (12s RNA and 16s RNA), and a full set of 22 tRNAgenes. The two strands that make up the genome are commonly known as the heavy strand (H-strand) and the light strand (L-strand) because of molecular weight differences as a result of different base compositions. Of the 13 protein-coding genes, 12 are on the H-strand and only one is on the L-strand. Noncoding regions are mainly limited to areas called the D-loop, thought to have functional roles in replication and transcription, and origin of replication of the L-strand (OL, thought to have a functional role in replication) [1]. The gene order is also highly conserved among most vertebrates [2]. During the last decades, mitochondrial genome sequence and gene arrangement comparisons were employed as powerful new tools for resolving ancient phylogenetic relationships [3, 4-8].

The black bear (*Ursus thibetanus mupinensis*) is listed in "The Convention on International Trade in Endangered Species of Wild Fauna and Flora "(CITES) the appendix I species, the national 2 levels of key protections wild animals and "China Red Data Book of Endangered Animals" V species [9]. There have been researches for mitochondria genome of *Ursidae*. Delisle and Strobeck obtained mitochondrial genome sequences of the Americas black bear (*Ursus americanus*), polar bear (*Ursus maritimus*) and brown bear (*Ursus arctos*) in 2002 [10]. Despite some studies of Asian black bear mitochondria individual genes such as the Cytb gene, there are no reports about the mitochondrial genome of the black bear, which live within the boundaries of Sichuan. In the current study, we report the complete mitochondrial genome sequence from a single Asian black bear Sichuan subspecies *Ursus thibetanus mupinensis*.

2. Materials and Methods

Skeletal muscle tissue samples of the Asian black bear Sichuan subspecies (*Ursus thibetanus mupinensis*) were obtained from black bears collected from Sichuan Province Traditional Chinese Medicinal Materials Company Dujiangyan Raising Deer Field.

DNA Extraction

Tissue sample was preserved in 70% ethanol until analysis. Total DNA was extracted from approximately 3×3×3 mm of the tissue using the conventional proteinase K/phenol/chloroform method [11] with some modifications as described by Masuda and Yoshida [12].

Primers Design, Polymerase Chain Reaction, Cloning of PCR Products and Sequencing

A series of primers were designed (Table 1), based on conserved regions identified from an alignment of published complete mitochondrial genomes

from carnivores by Primer Premier 5.0: *Ursus americanus* (American black bear) (GenBank accession No. AF303109), *Ursus maritimus* (polar bear) (GenBank accession No. AF303111) and *Ursus arctos* (brown bear) (GenBank accession No. AF303110) [10]. PCR was using rTaq DNA polymerase (TAKARA) with 1–10μl DNA extracts in a total volume of 50μl.

According to PCR reaction condition exists difference because G, C and A, T basis Content and the base number of primers is different, we lead primers to be divided into 4 sets, the reaction condition of the same of set consistent. And established temperature steps a degree circulation in each set. It set circumstance with the reaction condition for corresponding describe respectively in table 2.

Table 1 Primers designed with Primer Premier 5.0 for amplifying the complete mitochondrial genome of *U.thibetanus mupinensis*

No.	Forward Primer	Reverse Primer	Product length (bp)	Forward Primer Sequence (5′-3′)	Reverse Primer Sequence (5′-3′)
1	1-25	1072-1095	1095	GATCACACATAACTGTGGTGTCATG	CGGAGACTTACATGTGTAATCTTG
2	1036-1059	3622-3646	2611	TAAAGGTTTGGTCCTAGCCTTCCC	AAGCCCTGTCTCTTGGGCAGTATTG
3	2857-2882	3824-3849	993	AGATTAAAAGAAGTAAAAGGAACTCG	GGCCCTACAATGTTTGGTCCTTTACG
4	3725-3752	4657-4681	957	ATGTTTATAATTAACACTATCTCACTAG	TTATATTTGGGGGGGAATGCTTGCT
5	4181-4205	5207-5230	1050	GTCCTACTAATGAATGGCTCATTCG	CAGAAGTGGAAGGGGGATAGGCCG
6	4852-4878	5928-5953	1101	ATACCCCGAAAATGTTGGTTTATCCCC	GGTCTTTTTAGCCTAAATCTCTAGTC
7	5560-5583	6487-6514	955	CCACAACAACACTATCACTGTCCC	GATTATCACGAATGCATGGGCAGTTACG
8	6286-6310	7834-7854	1568	CAGTCTAGTGCTTTTATCAGCCATT	AGTATAACGTAAGCGGGTTCT
9	7202-7224	8188-8209	1008	GGTATAGATGTCGACACACGAGC	GCCGGCAAGATGGTTCATACCG
10	7958-7981	8728-8748	791	CTTTGTCAGGGTTAAATTATAGGT	GGAGAAGTCTGCATTCTCAGT
11	8671-8694	9588-9612	942	CCTCAATACTATAAAATCATTGAG	GTTTGGTGAGTCATTAGGTGTTATC
12	9588-9610	10434-10457	870	GATAACACCTAATGACTCACCAA	GTTGATTGTTTCTTTCTGGATTAC
13	10405-10427	11150-11178	774	CAATTGACTTCCAATCAATTAGC	ATTTTTAGCATTGTAAGAG
14	11147-11172	12522-12548	1402	AATCTCTTACAATGCTAAAAATTATC	AAACTATATTTACAGTAAATGGGCCCC
15	12196-12215	12947-12971	776	GGTCTACAAACACTCCTTCC	AGCTTAGAGTTAGCTTTAGGGTTTG
16	12703-12722	15134-15158	2456	CAAAAAATTGGTGCAACTCC	GTTTTTCGGATGTTGGTCATTAAGG
17	15139-15161	16261-16278	1140	ATGACCAACATCCGAAAAACCCA	TCTTCATTTTGAGAGGTT
18	16194-16215	158-183	858	GCTAGCCTCCATCCTCTACTTC	CCCGTAACCATTGACTGAATAGCCCC

Table 2. Reaction condition of PCR for 18 pairs primerse (Group 1 primers to have: 2, 5, 7, 9, 18; Group 2 primers to have: 1, 3, 4, 6, 8, 10,12,15; Group 3 primers to have: 11,14,16; Group 4 primers to have: 13,17)

Reaction condition	Group 1	Group 2	Group 3	Group 4
Denaturation temperature	94℃	94℃	94℃	94℃
Time	5min	5min	5min	5min
Cycleindex	1	1	1	1
Denaturation temperature	94℃	94℃	94℃	94℃
Time	30s	30s	30s	30s
Annealing temperature	60-50℃	58-48℃	55-46℃	49-40℃
Time	30s	30s	30s	30s
Primer extension temperature	72℃	72℃	72℃	72℃
Time	90s	90s	90s	90s
Cycleindex for each annealing temperature	2	2	2	2
Denaturation temperature	94℃	94℃	94℃	94℃
Time	30s	30s	30s	30s
Annealing temperature	55℃	52℃	49℃	45℃
Time	30s	30s	30s	30s
Primer extension temperature	72℃	72℃	72℃	72℃
Time	90s	90s	90s	90s
Cycleindex	30	30	30	30
Preservation temperature	4℃	4℃	4℃	4℃

The PCR products were fractionated on 1.5% (*W*/*V*) agarose gel, and selected bands were purified using a gel extraction kit (Sangon, Shanghai, China). The purified PCR products were ligated into the pMD18-T vector (TakaRa) and transformed into JM109 competent cells. Bacteria were grown in LB-ampicillin agar. Cloned PCR products were sequenced by BGI Life Tech Co., Ltd.

Sequence Analysis

The gene sequences of *U. thibetanus mupinensis* mitochondrial genome were identified by sequence comparison with published Carnivora gene sequences and similarity analysis using DNASTAR. Start and stop codons were used to help defining the sequences of protein coding genes. Percentage of GC base component and Base skew overall were calculated to analyze the genome characters. Tandem repeat was found by Tandem Repeats Finder [13].

3. Results and Discussion

General features of *U.thibetanus mupinensis* mitochondrial genome

The complete mtDNA sequence has been determined for *U. thibetanus mupinensis* mtDNA (GenBank accession no. DQ402478). The mtDNA is 16 868 bp in size, shorter than those of *U. maritimus* and *U. arctos*, which are 17017bp and 17020bp in length, yet longer than that of *U. americanus* which is 16841bp in length. The size differences result from different lengths of the control region among these species. It encodes 13 protein genes, 22 tRNA genes, and 2 rRNA genes (Fig 1). Eight tRNA genes and one protein gene are located on the light strand. The overall base composition of *U. thibetanus mupinensis* mtDNA for the H-strand is: A, 31.2%; C, 25.4%; G, 15.5%; T, 27.9%. Guanine (G) is the rarest nucleotide and GC content is 40.9%. Nucleotide composition analysis reveals that the *U. thibetanus mupinensis* genome is biased towards AT rich; such an AT content is lower than that of *U. americanus* and higher than those of *U. maritimus* and *U. arctos*. The gene content and gene order of *U. thibetanus mupinensis* mtDNA is typical for vertebrates [14]. With respect to the length of intergenic spacers and overlaps, *U. thibetanus mupinensis* has a rather compact genome similar to the other three *Ursidae* (Table 3 and Table 4). The *U.thibetanus mupinensis* mitochondrial genome shares high similarity with those of the other three *Ursidae*: *U. americanus* (91.46%), *U. arctos* (89.25%) and *U. maritimus* (87.66%).

Vertebrate animal mitochondrial genomes deviate from a random usage of nucleotide. Saccone et al. (1999) [14] used the formula, base-skew= (A-T/A+T) or (G-C/G+C), to evaluate the degree of the base bias, and found all the values of GC-skew were negative while all the values of AT-skew were positive in amniotes [14]. The base bias overall (GC=-0.242; AT=0.056) of the *U.thibetanus mupinensis* is most similar to that of *U. arctos* (GC=-0.238; AT=0.053) (Table 4).

Table 3 Components of *U.thibetanus mupinensis* Mitochondrial Genome

Gene	Nucleotide number	Start codon	Stop codon	Size (bp)	aa	Strand[1]
Control region	1-967			967		-
tRNA Phe	968-1035			68		H
12S rRNA	1036-2000			956		H
tRNA Val	2001-2066			66		H
16S rRNA	2067-3646			1580		H
tRNA Leu	3648-3722			75		H
NADH1	3725-4681	ATG	TAA	957	318	H
tRNA Ile	4680-4749			70		H
tRNA Gln	4747-4819			73		L
tRNA Met	4821-4889			69		H
NADH2	4890-5933	ATA	TAG	1044	347	H
tRNA Trp	5932-5998			69		H
tRNA Ala	6007-6075			69		L
tRNA Asn	6076-6148			73		L
Origin of L-strand replication	6149-6181			35		-
tRNA Cys	6182-6248			67		L
tRNA Tyr	6248-6315			68		L
COXI	6317-7861	ATG	TAA	1545	514	H
tRNA Ser	7858-7929			72		L
tRNA Asp	7934-8000			67		H
COXII	8001-8684	ATG	TAA	684	227	H
tRNA Lys	8688-8755			68		H
ATPase8	8758-8961	ATG	TAA	204	67	H
ATPase6	8919-9599	ATG	TAA	681	226	H
COXIII	9599-10382	ATG	Taa	784	260	H
tRNA Gly	10383-10451			69		H
NADH3	10452-10798	ATC	TAa	347	115	H
tRNA Arg	10799-10867			69		H
NADH4L	10868-11164	ATG	TAA	297	98	H
NADH4	11158-12535	ATG	Taa	1378	458	H
tRNA His	12536-12604			69		H
tRNA Ser	12605-12663			59		H
tRNA Leu	12664-12733			70		H
NADH5	12734-14562	ATT	TAa	1829	609	H
NADH6	14538-15065	ATG	TAA	528	175	L
tRNA Glu	15066-15134			69		L
Cytochrome b	15139-16278	ATG	AGA	1140	379	H
tRNA Thr	16279-16348			70		H
tRNA Pro	16349-16413			65		L
Control region	16414-16868			455		-

[1] H=heavy strand;L=light strand

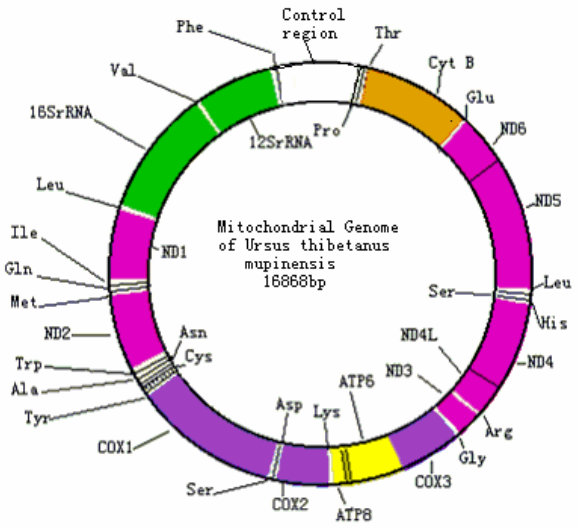

Figure 1. The structure and annotation of the *Ursus thibetanas mupinensis* mitochondrial genome. (16S: 16S ribosomal RNA gene; 12S: 12S ribosomal RNA gene; ND1: NADH dehydrogenase subunit 1 gene; ND2: NADH dehydrogenase subunit 2 gene; ND3: NADH dehydrogenase subunit 3 gene; ND4L: NADH dehydrogenase subunit 4L gene; ND4: NADH dehydrogenase subunit 4 gene; ND5: NADH dehydrogenase subunit 5 gene; ND6: NADH dehydrogenase subunit 6 gene; COX1: Cytochrome c oxidase subunit 1 gene; COX2: Cytochrome c oxidase subunit 2 gene; COX3: Cytochrome c oxidase subunit 3 gene; ATP8: ATP synthase F0 subunit 8 gene; ATP6: ATP synthase F0 subunit 6 gene; Cyt B: Cytochrome b gene; Phe: Phenylalanine tRNA gene; Val: Valine tRNA gene; Leu: Leucine tRNA gene; Ile:Isoleucine tRNA gene; Gln: Glutamine tRNA gene; Met: Trp: Tryptophan tRNA gene; Ala: Alanine tRNA gene; Tyr: Tyrosine tRNA gene; Ser: Serine tRNA gene; Asn: Asparagine tRNA gene; Cys: Cysteine tRNA gene; Asp: Aspartic acid tRNA gene; Lys: Lysine tRNA gene; Gly: Glycin tRNA gene; Arg: Arginine tRNA gene; His: Histidine tRNA gene; Glu: Glutamic acid tRNA gene; Thr: Threonine tRNA gene; Pro: Proline tRNA gene)

Protein coding genes

The mtDNA encodes 13 protein genes. Eight tRNA genes and one protein gene are located on the light strand [3]. We found the direction or the encoding-strand selection of the genes of *U.thibetanus mupinensis* is identical to the typical vertebrates (Table 3). Among 13 protein genes of *U.thibetanus mupinensis*, ten use ATG as start codon; NADH2, NADH3 and NADH5 use ATA, ATC and ATT as start codon, respectively. Some of these 13 protein genes are terminated with incomplete stop codons: NADH5 and NADH3 are terminated with TA; COXIII (cytochrome c oxidase subunit III) and NADH4 are terminated with T; the rest are terminated with TAA and AGA (Table 1). All these start codons and stop codons are almost the same with those of *U. americanus*, *U. arctos* and *U. maritimus* except that *U. americanus*, *U. arctos* and *U. maritimus*'s ND5 use ATC as start codon, *U. arctos* and *U. maritimus*'s ND4L use GTG as start codon and *U. arctos* and *U. maritimus*'s ATP8 and COX2 are terminated with TAG. Presumably, these incomplete stop codons are accommodated post-transcriptionally in the mRNA maturation process, *i.e.* polyadenylation [15]. The 13 protein genes' nucleotide sequence and amino acid sequence of *U.t. mupinensis* mitochondrial genome shares high similarity with those of the other three *Ursidae*: *U. americanus* (88.7% to 93.7% and 80.6% to 100%), *U. arctos* (84.3% to 92.9% and 83.6% to 98.98%) and *U. maritimus* (84.8% to 93.4% and 82.1% to 98.98%) (Table 5).

Table 4. Comparisons of mitochondrial genome features in four *Ursidae*

Genome character	Black bear	American black bear	Polar bear	Brown bear
GC base component	40.9%	40.6%	41.3%	41.3%
Base skew overall	GC=-0.242 AT=0.056	GC=-0.235 AT=0.048	GC=-0.235 AT=0.053	GC=-0.238 AT=0.053

Table 5. The 37 gene's similarity comparison at the nt and aa level from between *U.thibetanus mupinensis* mtDNA with *Ursus Americanus*, *Ursus maritimus* and *Ursus arctos*

Gene	*Ursus Americanus* (American black bear)		*Ursus maritimus* (polar bear)		*Ursus arctos* (brown bear)	
	nt	aa	nt	aa	nt	aa
tRNA Phe	89.71%		88.24%		88.24%	
12S rRNA	95.85%		95.45%		95.13%	
tRNA Val	96.97%		95.45%		92.42%	
16S rRNA	96.01%		94.75%		95.25%	
tRNA Leu	97.33%		100%		100%	
NADH1	92%	97%	90%	97%	90%	97%
tRNA Ile	98.57%		98.57%		98.57%	
tRNA Gln	97.26%		97.26%		97.26%	
tRNA Met	98.55%		97.10%		100%	
NADH2	93%	96%	91%	95%	91%	96%
tRNA Trp	92.54%		95.52%		95.59%	
tRNA Ala	100%		97.10%		97.10%	
tRNA Asn	97.26%		97.26%		98.63%	
tRNA Cys	100%		100%		100%	
tRNA Tyr	100%		92.65%		95.59%	
COXI	92.4%	97.5%	90.7%	97.7%	90.3%	96.9%
tRNA Ser	94.44%		97.22%		97.22%	
tRNA Asp	100%		100%		100%	
COXII	93.7%	98.2%	89.2%	96.5%	89.0%	96.0%

Gene	*Ursus Americanus* (American black bear)		*Ursus maritimus* (polar bear)		*Ursus arctos* (brown bear)	
	nt	aa	nt	aa	nt	aa
tRNA Lys	95.59%		100%		98.53%%	
ATPase8	88.7%	80.6%	84.3%	83.6%	84.8%	82.1%
ATPase6	90.0%	100%	88.8%	94.3%	89.1%	94.3%
COXIII	91.2%	98.1%	92.9%	98.9%	93.4%	98.5%
tRNA Gly	91.30%		89.86%		91.30	
NADH3	91%	96%	93%	97%	93%	97%
tRNA Arg	92.75%		92.75%		94.20%	
NADH4L	89%	100%	89%	98.98%	88%	98.98%
NADH4	91%	94%	89%	94%	89%	95%
tRNA His	94.20%		95.65%		95.65%	
tRNA Ser	96.61%		96.61%		94.92%	
tRNA Leu	98.57%		97.14%		97.14%	
NADH5	90%	95%	90%	93%	90%	93%
NADH6	90%	94%	90%	94%	90%	95%
tRNA Glu	95.65%		97.10%		95.65%	
Cytochrome b	90.7%	96.3%	90.9%	94.7%	89.8%	94.7%
tRNA Thr	81.43%		75.71%		75.71%	
tRNA Pro	78.46%		75.38%		75.38%	

RNA genes

There are 22 tRNA genes identified in the *U. thibetanus mupinensis* genome, with length ranges from 59 to 75 bp. The 12S rRNA and 16S rRNA genes are 965 bp and 1 580bp in length, respectively. These are typical for mammalian mitochondrial genomes [16-19]. The 24 RNA genes of *U.t. mupinensis* mitochondrial genome shares high similarity with those of the other three *Ursidae*: *U. americanus* (78.46% to 100%), *U. arctos* (75.38% to 100%) and *U. maritimus* (75.38% to 100%), too (Table 5).

Control region

The stem-and-1oop structure of origin for the putative light-strand replication of *U.thibetanus mupinensis* located between tRNA-Asn and tRNA-Cys, is 35bp in size, which is the same size and location detected in *U. americanus*, *U. arctos* and *U. maritimus*. The sequence of the control region (CR) of *U. thibetanus mupinensis* located between tRNA-Pro and tRNA-Phe is 1422 bp in size and consists of 8.43% of the whole genome. This percentage is slightly lower than that of *U. maritimus* (9.25%) and *U. arctos* (9.27%), and higher than that of *U. americanus* (8.28%). Its GC content is 51.9%, which is lower than *U. americanus*'s (52.6%) and higher than *U. maritimus*'s (48.6%) and *U. arctos*'s (48.2%). By using the Tandem Repeats Finder [14], we have found a 6bp tandem repeat (ACGTGT) and two 10bp tandem repeats (ACGTGTACGT and TACGTGTACG). But *U. maritimus* and *U. arctos* contain two 10bp tandem repeats (*U. maritimus* and *U. arctos* have the same two 10bp tandem repeats which are CGTACGCATA and CGCACGTGTA) and no 6bp tandem repeat, while *U. americanus* contains a 6bp tandem repeat (ACGTGT) and no two 10bp tandem repeats. The sequence of control region of *U.thibetanus mupinensis* shares 90% identity with that of *U. americanus*, 71% with that of *U. arctos*, and 68% with that of *U. maritimus*.

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Conflict of interest

The author has declared that no conflict of interest exists.

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