



# Analysis of the cytochrome *c* oxidase subunit II (*COX2*) gene in giant panda, *Ailuropoda melanoleuca*

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**ABSTRACT.** The giant panda, *Ailuropoda melanoleuca* (Ursidae), has a unique bamboo-based diet; however, this low-energy intake has been sufficient to maintain the metabolic processes of this species since the fourth ice age. As mitochondria are the main sites for energy metabolism in animals, the protein-coding genes involved in mitochondrial respiratory chains, particularly cytochrome *c* oxidase subunit II (*COX2*), which is the rate-limiting enzyme in electron transfer, could play an important role in giant panda metabolism. Therefore, the present study aimed to isolate, sequence, and analyze the *COX2* DNA from individuals kept at the Giant Panda Protection and Research Center, China, and compare these sequences with those of the other Ursidae family members. Multiple sequence alignment showed that the

*COX2* gene had three point mutations that defined three haplotypes, with 60% of the sequences corresponding to haplotype I. The neutrality tests revealed that the *COX2* gene was conserved throughout evolution, and the maximum likelihood phylogenetic analysis, using homologous sequences from other Ursidae species, showed clustering of the *COX2* sequences of giant pandas, suggesting that this gene evolved differently in them.

**Key words:** Giant panda; Mitochondrial gene; Energy metabolism; Cytochrome *c* oxidase subunit II; Evolution; Ursidae

## INTRODUCTION

The giant panda, *Ailuropoda melanoleuca*, is known as a “living fossil” and is one of the most endangered species worldwide; its habitat is limited to the remote high mountains of Sichuan, Gansu, and Shanxi provinces in China (Jin et al., 2007). As an ancient species that has been evolving since the fourth ice age, the giant panda is an important model for studying biological and genetic diversity. Despite many giant panda conservation programs established to date, this species still faces extinction owing to its low reproductive rate, limited diet, and habitat destruction (McClure, 1943; Liu et al., 2007; Songer et al., 2012; Fan et al., 2014).

Mitochondrial processes are particularly important in the metabolism of giant pandas as they are involved in converting their characteristic high-fiber, low-energy bamboo diet into cellular energy. The characteristics of the mitochondrial genome, such as its small size and fast evolutionary rate, have led to it becoming ever more popular in phylogenetic, phylogeographic, and evolutionary studies among animals (Zhang et al., 2013). Thus, studying mitochondrial gene structure and function can help in understanding the physiology and evolution of the giant panda. Mitochondrial DNA is divided into coding and non-coding regions. The coding regions contain 37 genes, including 13 protein-coding genes involved in mitochondrial respiratory chains, two ribosomal RNAs (rRNAs), and 22 transfer RNAs (tRNAs). Proteins encoded by mitochondrial genes include cytochrome *b*, cytochrome *c* oxidase subunits I, II, and III, two ATPase subunits ATPase 6 and ATPase 8, and seven NADH subunits ND1, ND2, ND3, ND4, ND5, ND6, and ND4L. The two ribosomal rRNAs, 12S rRNA and 16S rRNA, are encoded by the 12S and 16S rRNA genes. The 22 dispersed tRNAs (TA, TR, TN, TC, TQ, TD, TE, TG, TH, TI, TL1, TL2, TK, TM, TF, TP, TS1, TS2, TT, TW, TY, and TV) are located among the protein-coding and rRNA genes in the mitochondrial genome. The genes for ND6 and eight tRNAs (Glu, Ala, Asn, Cys, Tyr, Ser, Gln, and Pro) are located in a ring of the light strand, and the remaining genes are located in the outer ring of the heavy strand of mitochondrial genome (Ramzan et al., 2010).

Cytochrome *c* oxidase (COX) has thirteen subunits, of which, three (I, II, and III) are encoded by the mitochondrial genes, and ten by the nuclear genes. COX subunit II (COX2) is the terminal enzyme of the mitochondrial electron transport chain, and a rate-limiting enzyme in the electron transfer process; it plays a key role in the cellular energy metabolism (Careau et al., 2008). COX2 acts as a proton pump, and is mainly involved in the transfer of cytochrome *c* to oxygen molecules and catalytic reduction of oxygen molecules from water molecules (Wikström, 1981). The acidification processes that produce ATP are the main source of energy for mammals (Helling et al., 2012). Despite the low energy-intake associated with

the bamboo-based diet of giant panda, it is sufficient for its basic metabolism (Senshu et al., 2007), suggesting that *COX2* plays an important role in converting food to energy. Therefore, in the present study, the *COX2* gene from a giant panda population was amplified, sequenced, and analyzed with respect to its role in energy metabolism and genetic structure.

## MATERIAL AND METHODS

### Ethics statement

All procedures were approved by the Institutional Animal Care and Use Committee of Sichuan Agricultural University, China. All experiments were performed in accordance with the approved guidelines and regulations.

### Sample collection

Venous blood samples were collected from 15 individuals kept at the Giant Panda Protection and Research Center, China, during April and May 2015. All samples were immediately frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  until use.

### DNA extraction and PCR amplification

Total genomic DNA was extracted from blood samples using a QIAamp DNA Blood Mini Kit according to the manufacturer instructions (Qiagen, Hilden, Germany). The quality of genomic DNA was assessed by determining its  $A_{260}/A_{280}$  ratio using a NanoDrop (NanoDrop products, Tattall Building, Wilmington, DE, USA) and its integrity was checked by agarose gel electrophoresis.

Based on the complete mitochondrial genome reference sequence of the giant panda available in GenBank (accession No. NC\_009492.1), two primers were designed to amplify the complete sequence of *COX2* (1178 bp) using the Primer 5.0 software (<http://www.premierbiosoft.com/primerdesign/>): F (5'-GAGAAGTGGCAGTGATTGAGC-3'; annealing temperature  $57.9^{\circ}\text{C}$ ) and R (5'-TGGGGTAGTAAAAGAGGCGAA-3'; annealing temperature  $58.2^{\circ}\text{C}$ ). PCR was performed in a 25- $\mu\text{L}$  reaction volume, comprising 12.5  $\mu\text{L}$  2X TaqMasterMix (BIOMED), 1  $\mu\text{L}$  each primer (10  $\mu\text{M}$ ), 2  $\mu\text{L}$  genomic DNA template (the concentration of the template is 10  $\mu\text{L}$ ), and 8.5  $\mu\text{L}$  ddH<sub>2</sub>O. The following PCR cycling program was used: initial denaturation at  $95^{\circ}\text{C}$  for 5 min; 28 cycles at denaturation at  $95^{\circ}\text{C}$  for 30 s, annealing at  $58.5^{\circ}\text{C}$  for 30 s, and extension at  $72^{\circ}\text{C}$  for 90 s; and a final extension at  $72^{\circ}\text{C}$  for 10 min. The PCR products were subjected to agarose gel (1%) electrophoresis in order to verify their size and integrity, and those presenting the correct size were sequenced by TSINGKE Biological Technology Corporation (Wuhan, China).

### Sequence analysis

The *COX2* nucleotide sequences were submitted to GenBank (accession No. KU961973), edited and assembled using the BioEdit 7.1.11 software (<http://en.bio-soft.net/format/BioEdit.html>), and searched for homology against the NCBI (National Center for Biotechnology Information; <http://www.ncbi.nlm.nih.gov>) nucleotide database using the basic

local alignment search tool (BLAST). Both singleton and parsimony-informative variable sites were determined using the MEGA 5.1 software (Tamura et al., 2013). The number of haplotypes, haplotype diversity, nucleotide diversity, and the average number of nucleotide differences were calculated using DnaSP 4.50.3 (Rozas et al., 2003) program.

## Phylogenetic analysis

The phylogenetic relationships within the giant panda family, Ursidae, were evaluated through a maximum likelihood (ML) tree constructed in PhyML (R package) (Liu and Yu, 2010) and plotted in MEGA 5.1, using the *COX2* sequences obtained in our study along with the 17 sequences of the other Ursidae species retrieved from the NCBI/GenBank database (Table 1). A model test was run before constructing the phylogeny tree. Tajima's D and Fu's FS neutrality tests were performed using MEGA 5.1 to assess signatures of recent historical demographic events. A codon-based Z-Test was performed on the ratio of non-synonymous (dN) to synonymous (dS) substitutions per site (Ramos-Onsins and Rozas, 2002) in order to investigate the type of selection occurring in the giant panda population. A significance level of 0.01 was used in all tests.

**Table 1.** Summary of the giant panda *COX2* sequence and other reference sequences used in the phylogenetic analysis.

Species	Sequence name	Source	GenBank accession No.
<i>Ailuropoda melanoleuca</i>	<i>Ailuropoda melanoleuca</i>	NCBI	gi 148298631
	WJ	This study	KU961973
	XG	(Wolong, Sichuan)	
	ZZ		
	JZ		
	Y2		
	HN		
	YG		
	YM		
	Y4		
	WG		
	WY		
	Y3		
	YN		
YY			
YH			
<i>Arctodus simus</i>	<i>Arctodus simus</i>	NCBI	gi 195661255
<i>Helarctos malayanus</i>	<i>Helarctos malayanus</i>	NCBI	gi 159524371
<i>Melursus ursinus</i>	<i>Melursus ursinus</i>	NCBI	gi 159524390
<i>Tremarctos ornatus</i>	<i>Tremarctos ornatus</i>	NCBI	gi 159524410
<i>Ursus americanus</i>	<i>Ursus americanus haplogroupA-east</i>	NCBI	gb KM257060.1
	<i>Ursus americanus haplogroupA-west</i>	NCBI	gb KM257059.1
	<i>Ursus americanus</i>	NCBI	gi 19343488
<i>Ursus arctos</i>	<i>Ursus arctos</i>	NCBI	gi 19343502
<i>Ursus maritimus</i>	<i>Ursus maritimus</i>	NCBI	gi 19343516
<i>Ursus deningeri</i>	<i>Ursus deningeri</i>	NCBI	gb KF437625.2
<i>Ursus spelaeus</i>	<i>Ursus spelaeus</i>	NCBI	gi 195661114
<i>Ursus thibetanus</i>	<i>Ursus thibetanus ussuricus</i>	NCBI	gi 195933635
	<i>Ursus thibetanus formosanus</i>	NCBI	gi 138892884
	<i>Ursus thibetanus mupinensis</i>	NCBI	gi 120944002
	<i>Ursus thibetanus thibetanus</i>	NCBI	gi 195933649
	<i>Ursus thibetanus</i>	NCBI	gi 159793497

## RESULTS

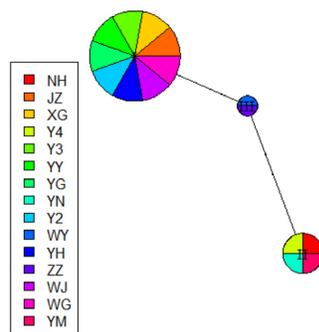
### Nucleotide composition of giant panda *COX2*

The PCR-amplified *COX2* DNA sequences of 15 individuals were 1094 bp in length on average. The BLAST analysis revealed these sequences were closely related to the mitochondrial sequences of giant panda, showing 100% sequence identity with a *COX2* sequence (684 bp; GenBank accession No. DQ093077.1). The sequence analysis of *COX2* DNA revealed an open reading frame encoding 222 amino acids, with an ATG initiation codon, and no introns.

The average nucleotide ratios in the *COX2* gene were: 33.9% for A, 29.5% for T, 15.1% for G, and 21.5% for C; thus, the G + C content was 36.6% and the A + T content was 63.4%, indicating a bias in the *COX2* base content. Sequence alignment revealed three point mutations (363T→C, 382T→C, and 612T→C), all of which were synonymous.

### *COX2* genetic diversity

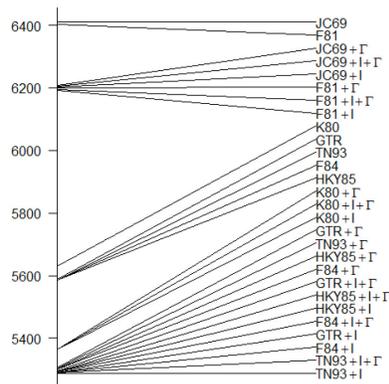
The *COX2* sequences of 15 giant pandas showed a haplotype diversity of 0.567 and a nucleotide diversity of 0.0019, comprising three haplotypes (I, II, and III) defined by the nucleotide sequences TTT, CCC, and TCT respectively. The statistical parsimony network constructed for *COX2* haplotypes showed that 60% of sequences belonged to haplotype I, 27% to haplotype III, and 13% to haplotype II (Figure 1). A negative Tajima's D signifies an excess of low frequency polymorphisms, indicating population size expansion and/or purifying (negative) selection. A positive Tajima's D signifies low levels of both low- and high-frequency polymorphisms, indicating a decrease in population size and/or balancing selection (Hu et al., 2016). Similarly, the data were applied to the Fu's  $F_s$  test. However, Tajima's D and Fu's  $F_s$  values were not found to be significant (1.26946 and 1.610, respectively;  $P > 0.1$ ). We calculated dN and dS of the *COX2* gene; dN > dS indicates a positive selection, whereas dN < dS refers to a purifying selection. The significant dN/dS < 1 (0.038,  $P < 0.01$ ) indicated the presence of more synonymous than non-synonymous changes, suggesting that the *COX2* gene was subjected to negative (conservative) selection in giant pandas.



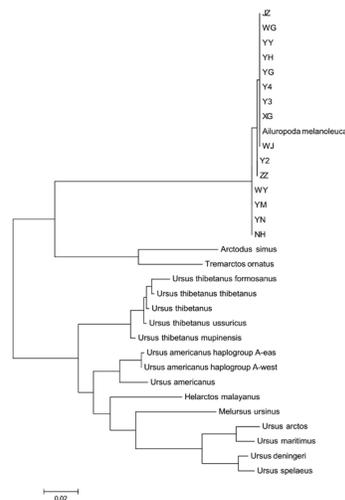
**Figure 1.** Statistical parsimony networks of *COX2* haplotypes. (The study samples NH, JZ, XG, Y4, Y3, YY, YG, YN, Y2, WY, YH, ZZ, WJ, WG, and YM were obtained from Wolong, Sichuan.) Each color represents a different sample. Each circle represents a haplotype. The sizes of circles representing haplotypes reflect the number of sequences that shares a haplotype.

### Phylogenetic analysis of *COX2*

The phylogenetic analysis performed using DNA substitution model showed that the 17 giant panda sequences obtained in the present study were clustered within the Ursidae family (Figure 2), suggesting that the *COX2* gene of giant panda had an evolutionary history distinct from the remaining members of the family. A model test was applied before the phylogeny tree was plotted. All phylogenetic methods make explicit or implicit assumptions about the process of DNA substitution; therefore, it is important to apply a model test, which indicates all phylogenetic analyses depend on their underlying models. In addition, a general time-reversible (GTR) model, GTR +  $\Gamma$ , was found to be ideal for our data. Model testing indicated Tamura and Nei 1933 + 1 model as the best evolutionary model for giant panda *COX2* data (Figure 3).



**Figure 2.** Model tests for data. The models of DNA substitution are: JC69 (Jukes and Cantor, 1969), K80 (Kimura, 1980), F81 (Felsenstein, 1981), HKY85 (Hasegawa et al., 1985), and GTR (Rodríguez et al., 1990).  $\Gamma$  indicates shape parameter of the gamma distribution and I indicates proportion of invariable sites. The Y-axis represents AIC (Akaike Information Criterion). AIC is a way of selecting a model from a set of models.



**Figure 3.** Phylogenetic tree of the *COX2* gene. For abbreviations, see Table 1.

## DISCUSSION

The *COX2* sequences obtained herein were consistent with those within the complete mitochondrial genome of giant panda as reported (Peng et al., 2007) and with those deposited in GenBank, as the BLAST results revealed 100% sequence identity. Although some mutations were detected in the *COX2* sequences, none of those mutations caused changes in the amino acids, and hence the function of COX2 protein. These findings were in agreement with the  $dN/dS < 1$  value obtained from the closely related *COX2* sequences, indicating the conservation of this gene throughout evolution.

Among the three haplotypes found, one had a more recent origin than the other two, suggesting that giant pandas had two colonies with some gene exchange between them, resulting in increased genotype diversity, and ultimately, generation of a new haplotype. This gene exchange counteracted the possible genetic degeneration resulting from population (colony) inbreeding due to accumulation of many harmful genes in homozygous populations (Lacy et al., 1993). The recent haplotype might have been advantageous to giant pandas as it was conserved during evolution.

Mitochondria are the main organelles of glucose metabolism. The Krebs cycle occurs in the mitochondrial matrix, and the electron transport chain and oxidative phosphorylation sites are located in the inner mitochondrial membrane. Mitochondria provide 80% of the energy for cellular activities (Aksenov et al., 1999; Viña et al., 2007). At high cellular energy (ATP/ADP) levels, ATP functions as an allosteric inhibitor of COX activity, resulting in a sigmoidal titration curve of COX activity depending on cytochrome *c* concentration and a Hill coefficient of 2 (Arnold and Kadenbach, 1999). This mechanism explains the role of COX in the regulation of the ATP/ADP ratio and energy production in the cell (Roemgens et al., 2011; Misiak et al., 2010). *COX2* is one of the COX subunits, and some studies have reported that the *COX2* gene structure was not conserved through animal evolution (Kaufmann et al., 1996; Crofford, 1997; Buttar et al., 2002). The changes in *COX2* gene structure leads to changes in gene expression and production of hormones, such as prostaglandins (Rouzer and Marnett, 2009). Nie et al. (2015) showed that *COX2* could regulate thyroid hormone levels and control energy consumption (Nie et al., 2015). Therefore, the conserved structure and function of giant panda *COX2* might be related to the lower energy intake and slower movement of this species than in the other species of Ursidae. The present study showed that *COX2* is a highly conserved gene in the panda group, and could distinguish the giant panda from other species of Ursidae.

Tajima's D can measure the difference between two estimators of the population mutation rate; positive values indicate population bottlenecks, structure, and/or balancing selection, whereas negative values indicate an excess of low-frequency alleles that can result from population expansion or positive selection (Mead et al., 2003). Similarly, Fu's Fs positive and negative deviations indicate distinct demographic and/or selective events. Both neutrality tests were positive, although not significant ( $P > 0.1$ ), suggesting a population bottleneck. However, the significant value of  $dN/dS < 1$ , which indicated that *COX2* was negatively selected (conserved).

Overall, the results of the present study suggested that the *COX2* structure within the Ursidae family was conserved throughout evolution, and that its function as an energy regulator and converter might be fundamental for meeting energy requirements with a bamboo-based diet in giant pandas.

## CONCLUSION

The *COX2* structure has been conserved and positively selected during the evolution of giant pandas. Its efficient function as an energy transducer and regulator could play an important role in meeting energy requirements with a bamboo-based diet in giant pandas, given the low levels of physical activity in this species. However, further gene studies are needed to understand the relationship between gene function and energy intake in giant pandas.

## Conflicts of interest

The authors declare no conflict of interest.

## ACKNOWLEDGMENTS

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