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ORIGINAL PAPER

Rapid evolution of cytochrome c oxidase subunit II in camelids (*Tylopoda, Camelidae*)

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Abstract Within Cetartiodactyl species, both New and Old World camelids are uniquely adapted to the extremely hot and dry climates of African-Asian territories and to the high altitude cold and hypoxic environment of the whole Andean area. In order to investigate the potential association between these particular adaptations and mitochondrial aerobic energy production, we examined the camelid genes of cytochrome c oxidase subunits I, II, and III and the replacement of amino acids inferred. We found that all subunits had undergone a number of replacements in sites otherwise conserved in other cetartiodactyls. Changes of COXI and COXIII were mainly located in the transmembrane helices of proteins. For COXII, although most of the changes did not occur in sites directly involved in electron transfer, a shift of D by T at 115 position of Old World camelid might modify electrostatic interactions with cytochrome c. COXII also showed an increased relative evolutionary rate respect to other cetartiodactyls compared.

Keywords Camelidae · Extreme climate adaptation · Mitochondrial DNA · Cytochrome coxidase · Amino acid replacement · Increased relative rate

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Introduction

Cytochrome c oxidase (COX) is a multimeric complex involved in the terminal oxidative step of energy metabolism by catalyzing the transfer of electrons from reduced cytochrome c (a nuclear protein) to oxygen. In vertebrates, COX comprises 13 protein subunits, three of which (COXI, COXII, and COXIII) are encoded in the mitochondrial genome and the remaining ten in the nucleus (Capaldi, 1983). COXI and COXII, forming the catalytic core, are involved in electron transport whereas COXIII is believed to play a structural or regulatory role (Brunori et al., 1987, Haltia et al., 1991, Tzukihara, 1996). Mitochondrial proteins have attracted the interest of scientists specially since the demonstration of an evolutionary rate acceleration of COX occurred during the radiation of anthropoid primates (Adkins and Honeycutt, 1994; Schmidt et al., 2004). These findings were assigned to an adaptive process related to the emergence of a larger energy-dependent neocortex in anthropoid lineages (Grossman et al., 2001). In line with these observations, Mishmar et al., (2003) reported striking differences in the ratio of nonsynonymous/synonymous nucleotide changes in mtDNA genes among human populations from different geographic latitudes and speculated that these differences might reflect human adaptation to increasingly colder climates since they left Africa. On the other hand, a recent report of Xu et al. (2005) suggests that nonsynonymous mutations of the COXI gene may have functional implications in the high altitude adaptation of native mammals of the Tibetan Plateau. Moreover, in order to adapt to high altitude environments with low atmospheric oxygen pressure, some mammalian species may have developed strategies that still persist in animals born and living at sea level. Accordingly, studies involving fetuses from highland and lowland mammals such as llama (*Lama glama*) and sheep, showed a

hypometabolic response to acute hypoxia in llama (Llanos et al., 2000).

Among Cetartiodactyl species, Camelidae family offers an interesting model of physiological adaptation to extreme environmental conditions. Camelids were originated in the plains of North America continent around 40–45 million years (MY) ago. During late Pliocene, 3 MY before present, ancestral camelid forms migrated to the Old World through the Bering strait and to South America by crossing Panama land bridge. Old World species evolved to the present camels, bactrian and dromedary, becoming uniquely adapted to the hot and dry climates of African-Asian territories (Franklin, 1982). In South America, camelid species spread along the Andean rangeland from the Peruvian Puna to Tierra del Fuego and onto the pampas of South America, developing physiological adaptations to high altitude hypoxic environments, extremely cold temperatures and scarce food supply. On these bases, both camelid lineages have become interesting taxa for the study of mtDNA-encoded COX subunits and the functional implications in these adaptations.

In this work we propose to analyse the amino acid replacement of COXI, COXII and COXIII genes with the purpose of investigating the potential role of mtDNA in the adaptability of Camelidae species to environmental conditions such as hot and desert dryness and cold hypoxic altitudes. We also tested COXII evolutionary relative rate compared with other Cetartiodactyl species living under different conditions.

Materials and methods

Genomic DNA from each South American camelid (SAC) species, guanaco, vicuña, llama and alpaca, and each of Old World camels, bactrian and dromedary, was isolated from blood by the procedure described elsewhere (Bustamante et al., 2002). COXI, COXII and COXIII genes were PCR amplified in the four SAC species. COXII gene from Old World bactrian and dromedary camels was also PCR amplified. The primers used were as follow: COXI Fw 5′-GAACTTGCAATTCAATGTGT-3′, Rv 5′-AAAGTCATAGTGGCTATGGG-3′; COXII Fw 5′-CAAGG TTAAATTATAGGTGA-3′, Rv 5′-AAAAGGTTAACGCTG ACTAG-3′; COXIII Fw 5′- TACTTACAATTCTCGAATTT GCCG-3′, Rv 5′-TATCCTTTTTCCGGGTCTCT-3′, designed on a complete sequence of alpaca mitochondrial genome (Acc. AJ566364). Each PCR reaction included 15 ng of genomic DNA, 1X PCR-buffer (10 X buffer 500 mM KCl, 200 mM Tris-HCl pH 8.4), 2.5 mM MgCl2, 0.2 mM dNTPs, 0.4 U Taq polymerase (Invitrogen, Carlsbad, CA, USA) and 1 μM of each primer, in 50 ul. Cycling profile included an initial denaturation step at 94°C for 3 min, followed by 35 cycles of 1 min at 94°C, 1 min at 50–54°C, 1 min at 72°C, and a 10 min final extension at 72°C. Afterwards, PCR product was purified by QIAquick PCR-purification kit (QIAGENE Inc.,Valencia, CA, USA) and sequenced on both strands with Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequencing reactions were analyzed using an Automated 3730 DNA analyzer (Applied Biosystems, Foster City, USA).

Obtained sequences from each species were deposited in GenBank under the following accession numbers: *Lama guanicoe*: COXI, DQ534053; COXII, AY994139; COXIII, DQ539397; *Lama glama*: COXI, DQ534054; COXII, AY994143; COXIII, DQ539398; *Lama pacos*: COXI, DQ534055; COXII, AY994145; COXIII, DQ539399; *Vicugna vicugna*: COXI, DQ534056; COXII, AY994144; COXIII, DQ539400, C*amelus bactrianus:* COXII, AY994147, *Camelus dromedarius:* COXII: AY994148.

Data analysis

Nucleotide and deduced amino acid sequences were aligned using the Clustal X (Thompson et al., 1997) software and compared with the GeneBank available sequences of other cetartiodactyl species, namely: *Sus scrofa* (AF034253), *Bos taurus* (J01394), *Ovis aries* (NC001941), and *Balaenoptera musculus* (X72204). Relative rate test of amino acid replacements was performed on COXII protein data by means of Tajima test (Tajima, 1993) with MEGA3 software (Kumar, 2004). Perissodactyl *Equus caballus* sequence (AY584828) was used as outgroup. COXII was modeled using as template bovine heart cytochrome c oxidase (*PDB code locz*) (Fig. 1) and the program SCWRL (Canutescu et al., 2003).

Fig. 1 Monomeric structure of the cytochrome c oxidase model obtained from the homology modeling. Dark grey indicates COXII while light grey shows the rest of the subunits comprising the protein

Table 1 Characteristics of COXI, II, and III genes from Camelidae

Gene	Length (bp)	Start codon	Stop codon	Amino acid number
COX I	1545	ATG	TAG	514
COX II	684	ATG	TAA	227
COX III	784	ATG	Taa	261

Results

Mitochondrial COX genes

The general features of camelid mitochondrial COXI, II, and III genes are shown in Table 1. The same as for most mammals, the three genes showed methionine as start codon (ATG). Contrarily, each gene exhibited different stop codon. While COXII ended in TAA, COXI exhibited an unusual TAG codon and COXIII an incomplete stop codon T which is probably completed post-transcriptionally by polyadenylation (Ojala, 1981).

The amino acid sequence deduced from DNA sequences from each of the three genes was identical in the four SAC species. The alignment of the amino acid sequence of COXI from cow, sheep, pig, whale and SAC showed 7% variable positions, mainly located in the COOH terminal end. Both COXII and COXIII showed higher variation, with 12.3% polymorphic sites dispersed throughout the molecule, although in COXII they were slightly weighted toward the COOH terminus. From the total substitutions found in the three camelid proteins, 21 occurred in sites which in the compared Cetartiodactyl species are conserved (Table 2). From these changes, almost 50% correspond to COXII, and 33% and 19% to COXI and COXIII, respectively.

The high number of amino acid replacements found in COXII encouraged us to investigate whether this is a particular feature of South American camelids or if it is shared by all Camelidae members, extending the sequencing of COXII gene to Old World camels bactrian and dromedary. The alignment confirmed that changes at residues 27, 30, 170, and 182 are exclusive for the four South American camelids, and the remaining six are shared by all Camelidae here analysed. In addition, while the dromedary has changed S to P in the amino acid 126, the two Old World camels showed a shift of D to T in the 115 position (Fig. 2).

Relative rate test of COXII evolution

The possibility that in Camelidae Family the COXII protein evolves at rates different from those observed for other cetartiodactyl species was further tested by the pairwise relative rate test of Tajima, (1993) using the horse sequence as outgroup (Table 3). Results indicate significantly high chi-square values for the comparison among the six Camelidae species with cow, pig, and whale sequences. Additionally, comparisons with sheep showed higher relative rates though not statistically different except for dromedary which resulted significantly higher than sheep. These results indicate that COXII protein evolves faster in all Camelidae species respect to the other Cetartiodactyl species.

Discussion

In the present study we sequenced the three mitochondrial subunits of the cytochrome c oxidase gene from Camelidae species as a first attempt to understand evolutionary factors that may be involved in the adaptation of these species to the

Table 2 Comparison of amino acid (AA) variations in the three COX subunits between South American camelids (SAC) and other cetartiodactyls

COXI				COXII				COXIII			
AA #[a]	SAC	C/P/S/W[b]	TM Helix[c]	AA #[a]	SAC	C/P/S/W[b]	TM Helix[c]	AA #[a]	SAC	C/P/S/W[b]	TM Helix[c]
393	L	F	X	2	P	A	–	37	Y	H	–
394	I	V	X	22	Y	H	I	84	V	I	III
405	I	L	–	27	A	T	I	95	S	T	III
406	D	N	–	30	V	I	I	191	S	G	VI
413	Q	H	XI	100	I	M	–				
456	V	M	XII	155	T	S	–				
472	V	I	XII	170	V	L	–				
				175	V	I	–				
				182	V	T	–				
				221	E	K	–				

[a] Position of the aminoacid within the polypeptide.
[b] C = cow; P = pig; S = sheep; W = whale.
[c] Specific transmembrane domain of the individual protein.

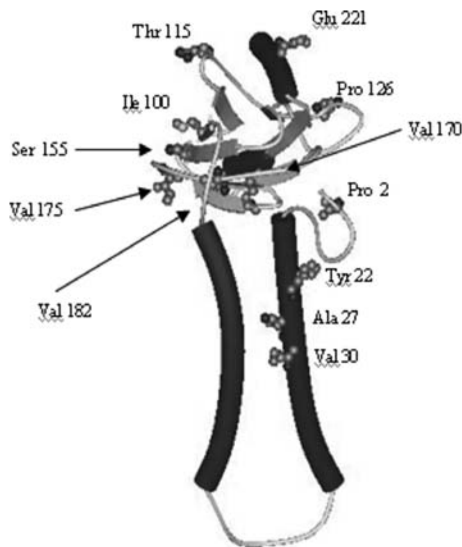

Fig. 2 Cartoon representation of the COXII tertiary structure from camelids. The 12 residues shown in ball and stick style are those found in both South American and Old World camelids

extreme environmental conditions under which they become adapted.

COXI is the largest mitochondrial subunit containing 12 transmembrane helices (Tzukihara, 1996). Non-synonymous mutations of the COXI gene affecting amino acids between positions 400–500 have been recently associated with the adaptation of two native mammals to the high altitude hypoxic environment of the Tibetan Plateau (Xu et al., 2005). In agreement with these results we found 7 amino acid changes

Table 3 Tajima's relative rates test for amino acid substitution in SAC and other Cetartiodactyls

Species 1	Species 2	$m1$	$m2$	χ^2
SAC	blue whale	12	4	4**
SAC	sheep	13	5	3.56
SAC	pig	14	3	7.12*
SAC	cow	15	3	8*
Bactrian	blue whale	12	4	4**
Bactrian	sheep	13	5	3.56
Bactrian	pig	14	3	7.12*
Bactrian	cow	15	3	8*
dromedary	blue whale	13	4	4.76**
dromedary	sheep	14	5	4.26**
dromedary	pig	15	3	8*
dromedary	cow	16	3	8.89*
blue whale	sheep	5	5	0
blue whale	pig	6	3	1
blue whale	cow	6	2	2
sheep	pig	7	4	0.82
sheep	cow	5	1	2.67
pig	cow	4	3	0.14

*Level of significance: 1%.
**Level of significance: 5%.

(Table 2) that besides to be exclusive for the SAC species are also located within the region that contains transmembrane helices X, XI and XII (Tsukihara, 1996). Since four of these changes are conservative, we assume that they do not affect protein function and probably result neutral for COXI interactions with other subunits. Other amino acid replacements observed in this region comprise more stringent substitutions like those from F to L in 393 position, from N to D in 406, and from H to Q in 413 that modify the amino acid size, charge or hydrophobicity, and may therefore have functional significance.

Compared to COXIII of other cetartiodactyls, the sequence of the SAC protein presents two conservative substitutions at helix III that interact with helices IV and V of the COXI subunit. We also observed a less conservative replacement from H to Y at 37 position, and one non-conservative change from G to S in 191 amino acid, which is located in the transmembrane helix VI known to contribute with helices V and VII to the stability of the enzyme molecule. Moreover, COXIII does not play a direct role in energy conversion but it is involved in the assembly and stabilization of the COX complex.

COXII is the most variable mitochondrial subunit observed in this study (Fig. 2). It contains two transmembrane helices (Tsukihara, 1996), being helix I the one that interacts with the nuclear encoded COXVIc subunit. Respect to the compared cetartiodactyls species, the four SAC showed two conservative amino acid substitutions: from T to A in 27 amino acid and I to V in 30 amino acid, one less conservative substitution from H to Y at 22 position, and one non-conservative substitution from K to E in 221 amino acid. The latter change is of questionable importance because it is within the carboxyl terminal region known to be hypervariable in all eutherians (Adkins et al., 1996). Substitutions of amino acids 22 and 221 are shared by both South American and Old World camelids, suggesting that they stem from the last common Camelidae ancestor. Interestingly, more recent changes from D to T at 115 position is present in bactrian and dromedary individuals while that from S to P at 126 residue is only present in dromedary samples. The substitution in 115 amino acid may be of significant importance since it is adjacent to 114 residue and both of these sites have been implicated in the electrostatic interaction with cytochrome c. In most organisms, one or both of these positions have negatively charged amino acid except for the Catharrine primates in which they have been replaced by small uncharged glycine residues (Ramharack and Deeley, 1987). Further findings on glycine uniformity at 114 and 115 positions in the Catharrine lineage suggest that aspartate or glutamate to glycine replacements in these primates have been positively selected and preserved by purifying selection (Grossman et al., 2001). In line with these speculations and since COXII is essential for the cytochrome c oxidase function, we cannot rule out

that a change at 115 position such as the one observed in Old World camels may have some effect on the electron transfer activity. On the other hand, changes found in mitochondrial COXII from South American camelids, although not encompassing residues associated to the electron transfer, could modify the interaction with nuclear and mitochondrial subunits. In fact, COX assembly in mammals has been shown to be very sensitive to small changes in amino acid sequences not necessarily involved in the catalytic function (Barrientos et al., 2001).

In primates, COXII has been identified as a particularly rapidly evolving mitochondrial subunit, and extensive comparisons of both nucleotide and amino acid sequences evidenced that this high rate of evolution occurred during the radiation of anthropoid primates (Adkins and Honeycutt, 1994; Schmidt et al., 2005). In the present investigation, we assessed the amino acid relative rate of evolution using Tajima's test (1993) which considers only those changes that are unique for each lineage. Additionally, this test allows relative rate assays, independently of the divergence time of the species involved in the comparison. Evidence from this test indicates that all Camelidae members have a significant higher rate in the amino acid replacement relative to other cetartiodactyls compared. Whether this increase in the evolutionary rate of COXII was associated with the adaptive evolution of the protein or a relaxation of its functional constraints, remains to be investigated.

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