

Evolution of the Primate Cytochrome *c* Oxidase Subunit II Gene

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Abstract. We examined the nucleotide and amino acid sequence variation of the cytochrome *c* oxidase subunit II (COII) gene from 25 primates (4 hominoids, 8 Old World monkeys, 2 New World monkeys, 2 tarsiers, 7 lemuriforms, 2 lorisiforms). Marginal support was found for three phylogenetic conclusions: (1) sister-group relationship between tarsiers and a monkey/ape clade, (2) placement of the aye-aye (*Daubentonia*) sister to all other strepsirrhine primates, and (3) rejection of a sister-group relationship of dwarf lemurs (i.e., *Cheirogaleus*) with lorisiform primates. Stronger support was found for a sister-group relationship between the ring-tail lemur (*Lemur catta*) and the gentle lemurs (*Hapapapua*). In congruence with previous studies on COII, we found that the monkeys and apes have undergone a nearly two-fold increase in the rate of amino acid replacement relative to other primates. Although functionally important amino acids are generally conserved among all primates, the acceleration in amino acid replacements in higher primates is associated with increased variation in the amino terminal end of the protein. Additionally, the replacement of two carboxyl-bearing residues (glutamate and aspartate) at positions 114 and 115 may provide a partial explanation for the poor enzyme kinetics in cross-reactions between the cytochromes *c* and cytochrome *c* oxidases of higher primates and other mammals.

Key words: Cytochrome *c* oxidase subunit II — Primates — Evolutionary rates — Systematics

Introduction

The dimeric cytochrome *c* oxidase complex spans the inner mitochondrial membrane with the majority of the protein exposed to the inner matrix of the mitochondrion. Each monomer of cytochrome *c* oxidase is composed of three subunits (I–III) encoded by the mitochondrial genome and at least four subunits encoded by the nucleus. Cytochrome *c* oxidase plays a critical role in the terminal oxidative step of energy metabolism by catalyzing the transfer of electrons from reduced cytochrome *c* (a nuclear protein) to oxygen to form water. In addition, it acts as a charge transfer system and a proton pump, resulting in an ion gradient across the mitochondrial membrane that drives the synthesis of ATP in oxidative phosphorylation (Capaldi 1990).

The molecular evolution of the mammalian cytochrome *c* oxidase complex is interesting for several reasons. First, a considerable amount of information exists for the low-resolution structure of cytochrome *c* oxidase, especially as it relates to potentially important amino acid residues associated with cytochrome *c* binding, electron transfer, and secondary structure (Capaldi et al. 1983; Capaldi 1990). Second, as revealed by high-resolution restriction-site mapping of the mitochondrial genome, primates have a higher level of variation than rodents in one particular mitochondrial subunit, cytochrome *c* oxidase subunit II, or COII (Ferris et al. 1981; Cann et al. 1984). Comparisons of nucleotide and amino acid sequence variation have confirmed a higher level of variation and rate of amino acid replacement in primates relative to rodents for COII (Brown and Simpson 1982; Ramharack and Deeley 1987). Third, the increased amino acid replacement rate

seen for COII for the primate lineage is coincident with an increase in amino acid replacements in primate cytochrome *c* relative to other mammals (Baba et al. 1981; Goodman 1981; Cann et al. 1984), and these two proteins interact during electron transport. Fourth, cross-reactions of cytochromes *c* and cytochrome *c* oxidases between anthropoids (monkeys and apes) and either prosimian primates or nonprimates exhibit much lower enzyme kinetics than do cross-reactions among the latter two groups (Osheroff et al. 1983). This observation is interesting in light of the differing rates of amino acid replacement seen for COII and cytochrome *c* in anthropoids relative to nonprimates. Finally, recent studies have used the COII gene to address higher-level relationships among primate lineages and their relatives (Adkins and Honeycutt 1991, in press; Ruvolo et al. 1991; Disotell et al. 1992), so a more detailed study including consideration of structural and functional constraints is warranted.

In this paper we investigate the overall pattern of nucleotide and amino acid sequence variation in the primate COII gene with an emphasis on comparisons involving haplorhine primates (tarsiers, monkeys, and apes) and strepsirhines (lorises, bushbabies, and lemurs). Three major questions will be addressed: (1) Where throughout the primate radiations did the rate increase in amino acid replacements occur? (2) Can patterns of amino acid replacements be related to the secondary structure model of COII, and are there any important changes that may relate to functionally important regions of COII/cytochrome *c* interaction? (3) What relationships are implied among the primates based on the COII data?

Materials and Methods

Genomic DNA was isolated by phenol-chloroform extraction (Maniatis et al. 1982) from white blood cells, liver, spleen, kidney, or brain of *Daubentonia madagascarensis* (Duke University Primate Center [DUPC] identification number 6201m), *Hapalemur griseus* (DUPC #1310), *Lemur macaco macaco* (DUPC #4539m), *Varecia variegata rubra* (DUPC #6302m), *Cheirogaleus medius* (DUPC #3615m), *Propithecus tattersalli* (DUPC #6198f), and *Nycticebus coucang* (DUPC #981f). DNAs from *Alouatta palliata* (#604) and *Tarsius syrichta* were gifts from Drs. Don Melnick and Morris Goodman, respectively. Sequences taken from previously published sources were *Homo sapiens* (Anderson et al. 1981), *Pan paniscus*, *Gorilla gorilla*, *Hylobates syndactylus*, *Macaca fascicularis*, *Cercopithecus aethiops* (Ruvolo et al. 1991), *Papio anubis*, *Papio hamadryas*, *Theropithecus gelada*, *Cercocebus galeritus*, *Mandrillus leucophaeus*, *Macaca mulatta* (Disotell et al. 1992), *Lagothrix lagothrica*, *Tarsius bancanus*, *Lemur catta* (Adkins and Honeycutt in press), and *Galago senegalensis* (Adkins and Honeycutt 1991).

The entire COII gene was amplified (Table 1) with primers H8320 and L7553 by the polymerase chain reaction (PCR) using the parameters 95°C denaturation (1 min), 45°C annealing (1 min), and 72°C extension (1.25 min) for 30 cycles. Double-stranded PCR products were ligated into either the plasmid pCR (Invitrogen) or the plasmid

Table 1. Oligonucleotides for amplification and sequencing of the cytochrome *c* oxidase subunit II gene

Primer ^a	Sequence
L7553	5'-AACCATTTCATAACTTTGTCAA-3'
L7784	5'-CAAGAAGTAGAAACAGTATGAAC-3'
L7900	5'-AAGACAATAGGCCACCAATGATAC-3'
L8075	5'-GAAGACGTCCTACACTCATG-3'
H7847	5'-GGGTTATTAATTTTCATCTAT-3'
H7966	5'-CGGAGTTCCTCGTTTGTAGGTC-3'
H8169	5'-CCACAGATTTTCAGAGCATTG-3'
H8320	5'-CTCTTAATCTTTAACTTAAAAG-3'

^a Names of oligonucleotides indicate the heavy (H) or light (L) strand and the position of the 3' end of the oligonucleotide according to the numbering of the human (Anderson et al. 1981) mtDNA sequence. Primer L7553 is located in the gene for tRNA^{Asp} and primer H8320 is located in the gene for tRNA^{Lys}.

pBluescript (Stratogene). The latter plasmid was modified to contain thymine overhangs at blunt 3' ends by digestion with *EcoRV* and incubation with dTTP and *Taq* polymerase (Perkin-Elmer). Plasmids were sequenced by the technique of Kraft et al. (1988) with various combinations of the primers in Table 1. Selection of primers for sequencing was dependent on sequence similarity at the priming site. Because of the inherent error rate of *Taq* polymerase (Saiki et al. 1988; Tindall and Kunkel 1988; Keohavang and Thilly 1989), at least two separate clones were sequenced for each taxon. No sequence discrepancies were found between clones.

Phylogenetic analyses were performed by two methods—maximum parsimony as implemented by PAUP 3.0n (Swofford 1990) and neighbor-joining (Saitou and Nei 1987) as implemented by the program Neighbor81 in version 3.4 of PHYLIP (Felsenstein 1992). For the neighbor-joining analyses pairwise distances between taxa were adjusted by the Jukes and Cantor (1969) or maximum-likelihood (using a transition:transversion ratio of 10:1; Felsenstein 1981) methods of the program Dnadist of PHYLIP, version 3.4. The random trees option of PAUP was used to calculate tree distribution skewness for the maximum parsimony analyses, and the bootstrap (Felsenstein 1985) option was used to evaluate the degree to which the COII sequences supported individual nodes. Published (Adkins and Honeycutt 1991) nucleotide sequences of COII from a tree shrew (*Tupaia glis*, order Scandentia) and a colugo (*Cynocephalus variegatus*, order Dermoptera) were used as outgroup references. Although the exact placement of these two orders relative to Primates remains controversial (Novacek 1989; Adkins and Honeycutt 1991; Ammerman and Hillis 1992; Bailey et al. 1992a; Stanhope et al. 1992), there is sufficient evidence to suggest that they are related closely enough to Primates to serve as useful outgroups. The test of Williams and Goodman (1989) was used to assess the strength of relationships among taxa in the family Lemnidae. Codon usage and base composition values were generated by the sequence analysis program MacVector 3.5 (International Biotechnologies, Inc.).

Results and Discussion

Patterns of Nucleotide Substitutions

Base Composition

The nucleotide sequences of the COII gene in 4 hominoids, 8 Old World monkeys, 2 New World mon-