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Two mitochondrial genes under episodic positive selection in subterranean octodontoid rodents

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ABSTRACT

Tuco-tucos (*Ctenomys*) and related coruros (*Spalacopus*) are South American subterranean rodents. An energetically demanding lifestyle within the hypoxic, underground atmosphere may change the selective regime on oxidative phosphorylation. We examined whether weak and/or episodic positive directional selection affected the evolution of two mitochondrial genes (COX2, CytB), in a background of purifying selection in these lineages. We estimated rates of synonymous (dS) and non-synonymous (dN) substitutions and found: 1) significantly higher dN/dS ratio in subterranean groups relative to non-subterranean related species, and 2) two codons in each gene under episodic selection: 94 and 277 of COX2 and 269 and 307 of CytB.

1. Introduction

Identifying selective pressures on protein-coding genes is central to the goal of characterizing Darwinian processes in evolutionary biology. Following the introduction of computationally tractable codon substitution models (Goldman and Yang, 1994; Muse and Gaut, 1994) nearly two decades ago, there has been sustained interest in using these models to study the past action of natural selection on protein coding genes. Positive selection can be inferred whenever the estimated ratio (ω) of non-synonymous (dN) to synonymous (dS) substitution rates significantly exceeds one (reviewed in Anisimova and Kosiol, 2009; Delport et al., 2009).

Mitochondrial genes have often been assumed to be under strong purifying selection because they encode for proteins involved in oxidative phosphorylation (OXPHOS) that can directly influence metabolic performance. However, it is possible that shifts in the ecology of organisms that imply changes in the metabolic demand may be associated with changes in the selection pressure of the proteins that participate in the biochemical pathways of cellular respiration. Supporting that idea, Andrews et al. (1998) and Adkins and Honeycutt (1994) found that the rate of evolution of the cytochrome b and cytochrome c oxidase subunit II, respectively, was greater in simians than in non-simian mammals, suggesting an event of concerted evolution. Subsequent studies have suggested that despite strong functional constrains, mtDNA may be subject to positive directional selection in cases, for example, of energy demanding lifestyles and/or limited availability of oxygen (see below in this section). None of these cases, however, detected ω greater than one, which would be unequivocal indications of positive selection. However, the early models used to estimate variation in ω among sites in an alignment or among lineages have little power to detect the signature of weak and/or episodic positive selection in a background of strong purifying selection (e.g.: Tomasco and Lessa, 2011). Recently, Murrell et al. (2012) presented a mixed effects model of evolution (MEME), based on the broad class of branch-site random effects phylogenetic methods that allows the distribution of ω to vary from site to site and also from branch to branch at a site.

The cytochrome c oxidase (COX) is a well known multimeric complex involved in the terminal oxidative step of energy metabolism; specifically, it catalyzes the transfer of electrons from reduced cytochrome c (a nuclear-encoded protein) to oxygen and contributes to establishing the proton gradient. Its functional modulation underlies adaptation to high-altitude hypoxia, at least in a Tibetan migratory locust (Zhang et al., 2013). Subunits I, II and III (COX1, COX2, and COX3, respectively) are encoded in the mitochondrial genome and the remaining ten in the nucleus (Capaldi et al., 1983). Mitochondrial subunits of COX have attracted particular interest in the study of molecular adaptations because of an acceleration of their rate of substitution during the radiation of anthropoid primates (Adkins and Honeycutt, 1994; Andrews and Easteal, 2000; Schmidt et al., 2005). These findings were attributed to an adaptive process related to the emergence of a larger, energy-dependent neocortex in anthropoid lineages (Grossman et al., 2001; Schmidt et al., 2005). We have focused on COX2, one of the core catalytic subunits of COX, since it is involved in

Abbreviations: dS, rate of synonymous substitutions; dN, rate of non-synonymous substitutions; dN/dS, ratio between rate of synonymous and non-synonymous substitutions; mtDNA, mitochondrial genome; OXPHOS, oxidative phosphorylation; *COX*, cytochrome c oxidase; *COX1*, cytochrome c oxidase subunit I; *COX2*, cytochrome c oxidase subunit II; *COX3*, cytochrome c oxidase subunit III; *CytB*, cytochrome b.

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electron transfer from cytochrome c and plays a role in substrate/product channeling, and in COX assembly, stability, and regulation. Recent studies have detected positive selection acting on COX2 on taxa known to have experienced significant changes in their metabolic needs, such as in high-performance fish (Dalziel et al., 2006). Among mammals, adaptations in COX2 have been suggested in species adapted to unusual oxygen requirements, like diving in cetaceans, flying in bats, and life at high altitudes (da Fonseca et al., 2008; Luo et al., 2008; Xu et al., 2005), and other extreme environments (Di Rocco et al., 2006; Fontanillas et al., 2005). Finally, Tomasco and Lessa (2011) found increased values of ω in COX2 of subterranean coruros (almost 30×) and tuco-tucos (11×) relative to other, non-subterranean octodontoids.

Cytochrome b (CytB) is a key component of bc1, one of the protein complexes involved in oxidative phosphorylation in the mitochondrial membrane. It has been extensively used for phylogenetic and phylogeographic studies and its role in oxidative phosphorylation is well understood (Saraste, 1999). After the work of Andrews et al. (1998), indirect evidence of the action of positive selection in the evolution of this gene has been reported. Nevo et al. (1999) correlated sequence variation of a portion of the CytB gene with ecological differences among chromosomal races of blind mole-rats (*Spalax ehrenbergi*), although in that case, the direct link with variation in respiratory function is not clear. More recently, Grossman et al. (2004) reviewed these and new results and suggested that the co-adaptation of nuclear and mitochondrial subunits of the electron transport chain proteins during primate evolution has been driven by the metabolic demands associated with an expanding cortex. Additionally, McClellan et al. (2005) found shifts in both the amino acid residues and physicochemical properties influenced by positive selection in cetacean CytB protein relative to that of artiodactyls. Furthermore, da Fonseca et al. (2008) found a wide range of variation in the properties of amino acids at functionally important regions of CytB in species with more specialized metabolic requirements (such as adaptation to unusual oxygen requirements, for example diving in cetaceans, flying in bats, and living at high altitudes in alpacas). Shen et al. (2010) found two sites of CytB that show evidence for significant positive selection on the branch leading to bats. Finally, Da Silva et al. (2009) found a significantly higher estimated ratio (ω) in independent lineages of subterranean rodents with respect to their non-subterranean counterparts, suggesting a link between evolution of this gen and the colonization of hypoxic environment. This was latter confirmed by Tomasco and Lessa (2011) using a set of seven complete mtDNA genomes of octodontoids.

Fossorial rodents constitute an ideal study system to test hypotheses about adaptive evolution driven by important ecological shifts. The subterranean niche is characterized by high levels of carbon dioxide and low levels of oxygen (Buffenstein, 2000) and implies high energy requirements associated to burrowing (Vleck, 1979). So, it is likely that proteins involved in respiration may have evolved under positive directional selection in response to habitat requirements. The sister families Octodontidae and Ctenomyidae provide a unique opportunity to trace the evolution of adaptations related to digging (Lessa et al., 2008). Burrowing for sheltering and rearing is the rule in these rodents but only two extant lineages, *Ctenomys* (tuco-tucos) and *Spalacopus* (monotypic genus, coruro), have recently evolved fully subterranean habits. Phylogenetic relationships among genera are relatively well established (Opazo, 2005; Upham and Patterson, 2012, and references therein), making it possible to trace changes associated to the acquisition of subterranean adaptations along a known phylogeny (Lessa et al., 2008).

In this study we analyze and compare the evolution of CytB and COX2 genes, looking for footprints of positive natural selection linked to the invasion of the subterranean niche. We use an extensive representation of tuco-tuco species and two samples of coruro, and compare them with their non-subterranean octodontoid relatives, using a spiny rat as an outgroup. In a previous study (Tomasco and Lessa, 2011), analyses of complete mitochondrial genomes of seven octodontoid rodents allowed us to suggest that the evolution of mtDNA may have been influenced by episodic positive selection in a background of purifying selection. However, sampling a large number of lineages of particular genes could improve the performance of the tests substantially, even for very similar sequences (e.g.: Anisimova et al., 2001, 2002; Murrell et al., 2012). In this study, we took advantage of the extensive sampling of species and complete CytB sequences available for tuco-tucos (Parada et al., 2011). We added a complete CytB gene sequence of a species of the genus *Aconaemys* (*A. fuscus*), which is closely related to the coruro (Honeycutt et al., 2003; Opazo, 2005; Upham and Patterson, 2012) and an additional sequence of the latter. We generated a comparable data set for COX2 and subjected both datasets to recently developed algorithms to detect episodic selection (Murrell et al., 2012) that allows the distribution of ω to vary across both branches and sites. This algorithm can find signatures of episodic selection even when the majority of lineages are subject to purifying selection, and it is now the recommended procedure to test for positive selection in the Datamonkey server. We found two sites in each gene under positive selection. Of these, three involved subterranean lineages.

2. Materials and methods

2.1. Specimens examined

We used the complete COX2 and CytB sequences of 35 species of octodontoid rodents, including representatives of two related, but independent, subterranean lineages (27 species of tuco-tucos — *Ctenomys* and 2 individuals of the coruro *Spalacopus cyanus*), three non-subterranean allies (*A. fuscus*, *Octodon degus* and *Tympanoctomys barrerae*), and a spiny rat (*Proechimys longicaudatus*, Family Echimyidae) as an outgroup (see Table 1). Species of tuco-tucos (genus *Ctenomys*) were chosen to represent the known diversity of the genus; i.e., 2–3 representatives of each species group and all the divergent species identified by Parada et al. (2011).

2.2. DNA extraction, amplification, sequencing and alignment

Total DNA extractions were made with SDS/proteinase K digestion/NaCl protein precipitation/alcohol precipitation of DNA (modified from Miller et al., 1988) from liver preserved in 95% ethyl-alcohol. The complete COX2 and CytB were amplified using primers MF7766L (5′-tctarytgtggcatttcatta-3′) and MF6Hb (5′-ttyccwcaacayttyytagg-3′), and MVZ05 (Smith and Patton, 1993) and Tuco14A (Wlasiuk et al., 2003), respectively. Amplification was carried out in a total volume of 20 μl containing the following final concentrations of each constituent: 10 μl of DNA (≈0.4 μg/ml) used as a template, 1× Taq Polymerase Buffer, 240 μM of each dNTP, 240 nM of each primer, 2 units of Taq Polymerase and 4 mM of MgCl2. PCR amplifications were performed in a PXE0.2 Thermal Cycler (Thermo-Electron Corporation), by an initial denaturation of 1 min at 94 °C, followed by 30 cycles of 30 s of denaturation at 94 °C, 30 s of annealing at 47 °C and 30 s of extension at 72 °C, and a final extension of 5 min at 72 °C. In each reaction, the corresponding negative control was included. The amplified products were electrophoresed in 0.8% agarose gels (100 V, 20 min), the DNA bands were visualized after EtBr staining under UV light, and expected size was determined in relation to a 100 bp DNA size standard (GIBCO BRL). PCR products were purified and automatic sequencing was done by Macrogen. Inc. (http://www.macrogen.com), under BigDye™ terminator cycling conditions in an ABI 3730xl Sequencer. Before the analyses, sequences were aligned used ClustalX implemented in Mega (Tamura et al., 2011). All new sequences were deposited in GenBank (see Table 1 for information on these and other sequences).