Polymorphic Variants of the Mitochondrial Cytochrome b gene (CYB) in the Ecuadorian population

PAZ Y MIÑO C[1], TAPIA A[2], ARÉVALO M[1], MUÑOZ MJ[1], LLUMIPANTA W[2], OLEAS DE LA CARRERA G[1], SÁNCHEZ ME[1]

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[1] Instituto de Investigaciones Biomédicas. Universidad de las Américas. Quito Ecuador. cpazymino@yahoo.com

[2] Escuela de Biología y Química. Universidad Central del Ecuador. Quito – Ecuador.

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The mitochondrial genome is widely used in evolutionary and phylogenetic investigations of human populations, and its molecular information, obtained from the study of its genes, has become a valuable tool in the study of human migrations around the world, supporting available anthropological data. During this research, a segment of 560 pb of the CYB (cytochrome b) gene was analyzed through a PCR-SSCP test, to determine the polymorphic variability in 108 unrelated Ecuadorian individuals belonging to four ethnic groups (Mestizo, Native American, Afrcan-American and Cholos). We report the following variants and frequencies: G15043A (0.57), T15115C (0.06), C15040T (0.32) and A14926G (0.05). The obtained results contribute evidence of human migrations in the New World, and support existing anthropological data.

Introduction

During the past decades, the mitochondrial genome has showed increasing importance in studies of human genetic variation, hereditary diseases and aging (Clayton 2000; Naviaux 2000; Wallace 1999). Because of mtDNA small size (16 kb), ubiquitous presence and its known complete sequence (Anderson *et al*. 1981), many polymorphisms and mutations have been reported (Andreu *et al.* 1999; Naviaux 2000). Over one thousand variants have been found only in the coding region of the mtDNA, and a similar number is reported in the control region (Brandon *et al*. 2005). The origin of modern humans has also been addressed using mitochondrial sequences, due to its more rapid evolution (Ingman *et al.* 2000). Some of its recent applications include forensic and population profiling using highly polymorphic mitochondrial segments (Holland and Parsons 1999).

The Human Mitochondrial Genome Database (MITOMAP) has reported 134 polymorphic variants of the CYB gene (Brandon *et al*. 2005). Many of the variants are accumulated in the interdomains, and frequently in positions that do not affect the redox activity of the Q0 center of the protein (Howell 1993). The sequence of the CYB is widely used in systematic and molecular diversity studies in vertebrates at many taxonomic levels (Farias *et al*. 2001). This gene is one of the most extensively sequenced (Johns and Avise 1998) and which variation has been used to elucidate population structure and intraspecific diversity (Esposti *et al.* 1993; Castresana 2001).

The rapid evolution of the CYB has been associated to the high mutation rate for mitochondrial genome. Nevertheless, CYB shows a significant challenge because of its extensive variation in the sequence, and the co-amplification with nuclear pseudo genes (Zhang and Hewitt 1996). Although, HVI/HVII is probably the most polymorphic segment of human mtDNA (Budowle *et al*. 1999), the CYB variation is useful for many population analyses.

Some particularities of CYB sequences had been reported, and include heterogeneous substitutions, transition/transversion rates and synonymous substitutions (Krajewski and King 1996). Conserved in this gene belong to functional domains (Howell 1989; Howell 1993; Iirwin *et al.* 1991; Krajewski and King 1996).

Cytochrome b protein has five highly conserved intermebranal regions associated to quinol-oxidative function (Howell 1989, 1993). Most of the variability observed in the CYB, belongs to transmembranal domains with substitutions that keep hydrophobic amino acids on the same sites (Esposti *et al.* 1993; Krajewski and King 1996).

Figure 1. Geographic distribution of the four polymorphic variants found in this study. Locality 1: G15043A variant; Locality 2: T15115C variant; Locality 3: C15040T variant and Locality 4: A14926G variant.

The Ecuadorian population has a highly diverse profile of ethnic groups based on a panmictic inbreeding, with a mestizo majority (i. e. mixture of Hispanic and Native American ancestry). Several papers have been published on polymorphic variants for nuclear loci in the Ecuadorian population (Paz-y-Miño and Leone 2002; Paz-y-Miño *et al*. 2002). These showed distinctive frequencies of previously reported variants and unknown single-nucleotide polymorphisms (SNPs). In this context, Genetics Anthropology has become a valuable tool in performing forensic identification, matrilineal analyses and surveys on human migrations (Arango and Luque, 2005).

The purpose of this study is to analyze the genetic variability of the CYB gene among the Ecuadorian population, and to compare its polymorphic variation with those reported in other worldwide populations. These results contribute evidence of human migrations to Ecuador in accordance with current anthropological data. Furthermore, it provides new evidence on human settlement in the New World, marking the utility of Anthropological Genetics in human population studies all around the world.

Materials and methods

Population sample and DNA extraction

A total of 108 non-related healthy individuals belonging to four Ecuadorian ethnic groups were analyzed: mestizo (n=27), Native American (n=27), Afro-American (n=27) and cholos (n=27). Each individual is an Ecuadorian citizen from diverse backgrounds. The geographic location of each group is shown in Figure 1. From each individual, 5 ml of peripheral blood was sampled in EDTA tubes, and complete genomic DNA was obtained using a salting out procedure according with Maniatis *et al.* (1989). One segment of 560 bp of the human mitochondrial CYB gene was amplified and sequenced with the following set of primers: CB1L- CCATCCAACATCTCAGCATGATGAAA y CB2H CCTCAGAATGATATTTGTCCTCA (Kocher *et al*. 1989, Irwin *et al*. 1991). The PCR reactions were set up in 40 µl final volumes and included 100 ng of template DNA, 0.5 mM of dNTPs, 2.5 mM MgCl2, 10 pM of each primer and 0.5 U Taq DNA polymerase (Promega Corp., Madison, Wisconsin). PCR reactions were performed in a MJ-Research PTC-200 thermal cycler (MJ-Research Inc., Watertown, Massachusetts). Using the following parameters: initial 91-94 °C denaturation (5 min.) followed by 38 cycles 91-94 °C denaturation (45 sec), 45-50 °C annealing (60 sec.) and 72 °C extension (90 sec.). Each reaction was finished with a 72 °C final extension step for 8 minutes. The quality of the amplified fragments

were estimated after running 4 µl of double-stranded PCR product in 1% agarose gel in TBE 1X for 30 min at 90 volts.

Strand Conformation Polymorphism (SSCP) analyses and sequencing

From each successful reaction, usually 8-10 µl were used for conformation analyses. 4 µl the SSCP dye (bromophenol blue 0.25%, xylene cyanol 0.25% and sucrose 40%) were added to each amplified fragment to a final volume 12-14 µl. This mixture was denatured at 95°C for 5 minutes and subsequently cooled on ice. The gels were made using native acrylamide with a proportion 49:1 of acrylamide to bis-acrylamide as described elsewhere (Leone *et al*. 1998) with a gel concentration of 8.5%. All electrophoretic runs were done using 0.5X TBE buffer and carried out at room temperature (18-20 °C). The gel was ran for 30 minutes, previous to the sample loading. The electrophoretic migration was carried out to constant power of 400W during 15 hours. The variants were recognized after silver gel staining using the standard protocol (Maniatis *et al.* 1989). Once identified, variants were sequenced and compared with the revised Cambridge Reference Sequence (rCRS) to corroborate the corresponding polymorphism. All variants have been deposited in the GenBank under accession numbers EF090398, EF090399, EF090400 and EF090401.

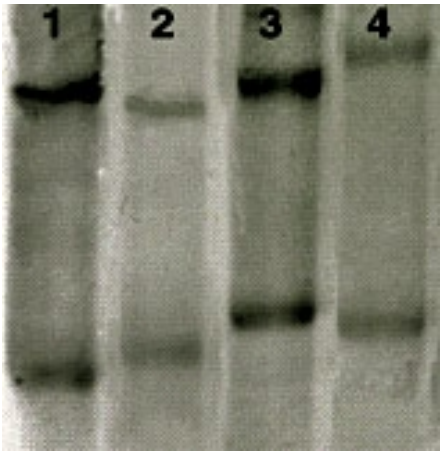

Figure 2. Polymorphic variants found by means of SSCP analyses in a 560 bp fragment of the CYB gene. The sequences of the variants were compared with rCRS. 1 = G15043A variant; 2 = T15115C variant; 3 = C15040T variant and 4 = A14926G variant.

Results

SSCP results

We found four polymorphic variants whose SSCP-electrophoretic profiles are shown in Figure 2. They consist of four identical transitions at nucleotide positions G15043A (Variant 1), T15115C (Variant 2), C15040T (Variant 3) and A14926G (Variant 4), respectively, according to the sCRS (Brandon *et al*. 2005). This segment includes the end of N-terminus, A, B and partial C domain and ab and bc interdomains. The frequencies of each variant are shown in Table 1 and all the frequencies obtained for each population are shown in Table 2.

G15043A and C15040T variants are the most frequent, standing up for 89 percent of all individuals. T15115C and A14926G variants have the lowest frequency with the 11 percent of all individuals. G15043A is assumed to belong to the wild type. All variants are reported for the first time in the Ecuadorian population and could be included among those reported by the Human Genome Database (MITOMAP) for the CYB gene.

Table 1. Frequency of polymorphic variants in 108 non-related Ecuadorian individuals

Population	n	Variant	Frequency	E.D.
Mestizos	27	G15043A	0.57	0.039
Negros	27	T15115C	0.06	0.049
Otavaleños	27	C15040	0.32	0.215
Cholos	27	A14926G	0.05	0.06

Table 2. Frequency of polymorphic variants in non-related Ecuadorian populations

Population*	G15043A	T15115C	C15040	A14926
Mestizos	0.57	0.01	0.05	-
Negros	0.15	0.06	0.10	-
Otavaleños	0.15	0.05	0.32	-
Cholos	0.05	-	-	0.05

*n=108

Variants and Haplotypes

G15043A variant

G15043A transition is the most frequent variant of all surveyed individuals. Because of its high frequency (0.57), this variant belongs to the Ecuadorian wild type population, widely distributed among mestizo population. This variant is extensively reported all around the world and it is included in the haplotypes I, E22i, J, N, M*, D, G, and C at the following locations: León (Spain), AF382007 (Maca-Meyer *et al*., 2001); Caucasus, AY195769 (Mishmar *et al*. 2003); Finland, AY339497.2 (Moilanen *et al*. 2003); India, AY714041 (Palanichamy *et al*. 2004); Italy, AY963586 (Bandelt 2005); Europe, DQ112809; Africa, DQ112927 and Asia, DQ112928 (Kivisild *et al.* 2006) and Asia (Kong *et al.* 2006).

T15115C variant

T15115C transition has been reported in African and African-American populations. This variant belongs to haplotypes L1b and A4L1B2 at following locations: Ibo (Africa), AF346986 (Ingman *et al*. 2000); Mauritania, AF381994 (Maca-Meyer *et al*. 2001); San (Africa) AY195783 (Mishmar *et al*. 2003); Dominican Republic, DQ112690, Burkina Faso, DQ112727 and West Asia (Pakistan), DQ112881 (Kivisild *et al*. 2006) and United States of America (Hernnstadt 2002). This variant presents a low frequency in our population (0.06) and belongs to the Afro-American population, an ethnic group with less than 5 percent of Ecuador's total population.

C15040T variant

C15040T transition belongs to the haplotypes N* and M* and is distributed in the Australian native population, DQ404440 (Van Holst Pellekaan *et al*. 2006) and China, AY255154 (Kong *et al*. 2003, 2006). This variant has a frequency of 0.32 and it belongs to the Native American population from Otavalo town, the second ethnic majority of Ecuador (30 percent).

A14926G variant

The fourth variant is the A14926G transition that belongs to the haplotypes U and Nib. This variant has been reported in Jordan AF382000, AF381999 (Maca-Meyer *et al*. 2001). In our study, it registers comparable frequencies (0.05) to that found for the T5115C mutation. The A14926G variant has been found exclusively in the Cholos population.

Discussion

PCR-SSCP is an innovative method for detecting mutations and polymorphisms (Gu *et al*. 1998; Hayashi 1999). Variants and mutations of human mitochondrial DNA have been detected by SSCP with high sensitivity (Tawata *et al*. 1997; Paz-y-Miño *et al*. 2002). The resolution obtained by our SSCP analyses was optimal, and it clearly showed sequence variants due to different conformation mobility in polyacrylamide gels. Additionally, our results did not show heteroplasmic individuals or any concomitant pseudo gene amplification that would make it difficult to attribute it to a particular variant. The mitochondrial genome presents a high level of inter-individual variability as a result of limited repair, superoxide attack, inaccurate replication and matrilineal inheritance (Lynch 1996). Human mtDNA dynamics determines a high occurrence of single nucleotide polymorphisms (SNPs) (Naviaux 2000). Functional and selective significance of these mitochondrial SNPs are comparable to other nuclear variants (Tanaka *et al.* 2000).

The four variants reported in this study could be included in the 134 reported within this gene (Brandon *et al*. 2005). These variants correspond to synonymous transitions that are reported for the first time in the Ecuadorian population. Sbisa *et al*. (1997) mentions that most of the variation in the CYB gene accumulates on the identical positions whose evolution rate is more than 16 times faster than that of the non synonymous. The CYB presents the rate of higher substitution for the identical positions, while the genes ND3 and ATP8 present the lowest rates (Saccone *et al*. 1999).

The replication of mtDNA suggested more mutation pressure upon the CYB as a result of its close position to the origin of replication of the light-strand (Ori-L) (Fuku *et al*. 2002). However, strong selection on mutations that could alter cytochrome b protein function (Esposti *et al*. 1993) limits its variation and most of the polymorphisms are identical without a net phenotypic effect (Andreu *et al*. 1999). The four variants could be assigned to different haplotypes and support a widespread distribution of these polymorphisms all around the world. The G15043A variant constitutes the wild type due to its high frequency (0.57) in our population. This is the variant with the most widespread distribution, specially focused in the Indo-European area (Maca-Meyer *et al*. 2001; Mishmar *et al*. 2003; Moilanen *et al*. 2003; Palanichamy *et al*. 2004 ; Bandelt 2005 ; Kivisild *et al*. 2006 ; Kong *et al*. 2006). This variant is assigned to the mestizo population which represents the majority of Ecuador's total inhabitants (65 percent). This ethnic group is the result of a mixture between Spaniards and Amerindians since the Spanish Conquer in 1,492, which could explain the widespread distribution of the variant in our ancestral population.

Other variant with a high frequency (0.32) is the C15040T transition found in Native American population (Otavalo town). This variant has been reported in Australia and China (van Holst Pellekaan *et al*. 2006; Kong *et al.* 2003, 2006). According to anthropological data, Amerindians constitute a mixture of ethnic groups that came from different locations in Asia through human migrations (Salazar 1995). A Polynesian origin of the Native Americans has also been stated (Rivet 1908, 1976; Mendes Correa 1928), which could explain the existence of this variant both in Australian aboriginal people and in our group of study. However, Salazar (1995) has criticized this hypothesis due to the difficulty of such human migrations from Oceania suggesting instead only an Asian origin.

The other two variants with the lower frequency are: Afro-American (0.06) and Cholos (0.05). In the first case, the Afro-American population constitutes an ethnic group assigned to the L haplotype with a widespread distribution in different localities of Africa. It has also been reported in people from the Dominican Republic (Kivisild *et al*. 2006) and African-Americans from the United States (Hernnstadt *et al*. 2002). The origins of this ethnic group in Ecuador are apparently well-elucidated since the trade of slaves between 16th and 20th centuries (Estupiñán Tello 1983) permitted the distribution of this variant in our population.

Finally, the Cholos population exhibits the G14926A transition that has been reported in people from Jordan (Middle East) (Maca-Meyer *et al.* 2001). A tentative hypothesis could be stated because of the recent migration of Jewish and Lebanese earlier in the 20th century to our country (Almeida 1997). It is barely understood how these variants have dispersed in our population. For this reason, it is a priority to associate both anthropological and molecular data in order to elucidate the origins of Ecuadorian and other new world's populations. The information gathered from all these sources will allow us to be more conclusive in an explanation of the high polymorphic variation of Ecuadorian population. The present study remarks the usefulness of modern Genetics tools like Anthropological Genetics in population studies. Due to the wide range of information that it provides regarding human migrations all around the world and its concordance with other anthropological data, this discipline has become a mandatory research technique used in most of phylogenetic and evolutionary studies on population researches.

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Resumen

El genoma mitocondrial es ampliamente utilizado en investigaciones filogenéticas y evolutivas de las poblaciones humanas y la información molecular obtenida a partir de la investigación de sus genes se ha convertido en una importante herramienta para el estudio de las migraciones humanas, respaldando la información antropogenética disponible. En el presente trabajo se analizó un segmento de 560 pares de bases del gen Citocromo B (CYB) mediante la técnica de PCR-SSCP para determinar su variabilidad polimórfica en 108 individuos ecuatorianos no emparentados pertenecientes a cuatro grupos étnicos (Mestizos, Nativos Americanos, Afro Americanos y Cholos). Se reportan las siguientes variantes y frecuencias: G15043A (0.57), T15115C (0.06), C15040T (0.32) y A14926G (0.05). Los resultados obtenidos aportan con evidencia sobre las migraciones humanas en el Nuevo Mundo y respaldan la información antropológica existente.

Palabras clave: Citocromo B (CYB), Genoma Mitocondrial, PCR-SSCP, Variantes Polimórficas

Variantes Polimórficas del Gen Mitocondrial Citocromo b (CYB) en la Población Ecuatoriana