

A NEW WORLD MONKEY MICROSATELLITE (AP74) HIGHLY CONSERVED IN PRIMATES

AP74, un microsatélite de Monos del Nuevo Mundo altamente conservado en Primates

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ABSTRACT

Given their great variability, microsatellites or STRs became the most commonly used genetic markers over the last 15 years. The analysis of these markers requires minimum quantities of DNA, allowing the use of non invasive samples, such as feces or hair. We amplified the microsatellite Ap74 in blood and hair samples in order to analyze the levels of genomic conservation among a wide range of primates including: *Lemur catta*, *Alouatta caraya*, *Ateles belzebuth*, *Ateles chamek*, *Pan troglodytes*, *Papio* sp., and *Homo sapiens*. In all cases we obtained amplification products that exhibited similar size both in monkeys and human (oscillating between 126 and 176 bp), except in the lemur where the detected fragment presented a size of approximately 1000 bp. The analysis of the nucleotide sequences permitted the evaluation of the molecular modifications experienced during the evolutionary process in primates.

Key words: Primates, microsatellite markers, PCR amplification, non-invasive sampling, genomic conservation, genetic variability.

RESUMEN

Dado su alta variabilidad, los microsatélites o STR se convirtieron en los marcadores genéticos más ampliamente utilizados en los últimos 15 años. El análisis de estos

marcadores requiere una mínima cantidad de ADN, permitiendo el uso de muestras no invasivas, tales como pelos o heces. Con el objetivo de analizar niveles de conservación genómica, amplificamos el microsatélite Ap74 en muestras de pelo y sangre de un amplio rango de primates incluyendo: *Lemur catta*, *Alouatta caraya*, *Ateles belzebuth*, *Ateles chamek*, *Pan troglodytes*, *Papio* sp., y *Homo sapiens*. En todos los casos obtuvimos productos de amplificación que exhibieron un tamaño similar (oscilando entre 126 y 176 pb), con excepción del lémur, donde el fragmento detectado presentó un tamaño de aproximadamente 1000 pb. El análisis de las secuencias nucleotídicas nos permitió evaluar las modificaciones moleculares ocurridas durante el proceso evolutivo en primates.

Palabras clave: Primates, marcadores microsatélites, amplificación por PCR, muestreo no-invasivo, conservación genómica, variabilidad genética.

INTRODUCTION

Molecular techniques have enlarged the underlying knowledge about the evolutionary process. Microsatellites, also called short tandem repeats (STRPs), are useful genetic markers for the study of the demographic structure and the phylogenetic history of the populations. These markers are scattered throughout the vertebrate's genome and are highly polymorphic, varying in the number of motifs repetition. As they generally seem to be free of selective constraints, it is evident that this extensive degree of genetic variability requires a high underlying mutation rate (Schlötterer, 2000; Ellegren, 2004). The analysis of these markers is based on the polymerase chain reaction (PCR) and requires minimum quantities of DNA, allowing the use of non invasive samples, such as feces or hair (Frantzen *et al.*, 1998; Taberlet *et al.*, 1999; Oklander *et al.*, 2004). Since their flanking sequences are highly conserved, the employment of heterologous primers allows the detection of diverse sequences conserved across related species.

Since the first phylogenetic analysis employing microsatellites as a new tool was published fifteen years ago, the information on the evolution and molecular dynamics of these markers in different species is constantly increasing (Rogers *et al.*, 1995; Menotti-raymond and O'Brien, 1995; Clisson *et al.*, 2000; Neff and Gross, 2001; Lathuilliere *et al.*, 2001; Oklander *et al.*, 2006; Ruiz García *et al.*, 2005; Ruiz García *et al.*, 2006; Ruiz García *et al.*, 2007).

Microsatellites isolated for humans allowed the amplification of sequences in several species of nonhuman primates including apes, baboons, macaques, and few plathyrrine monkeys (Blanquer-Maumont and Crouau-Roy, 1995; Garza *et al.*, 1995; Coote and Bruford, 1996; Kayser *et al.*, 1996; Ellsworth and Hoelzer, 1998; Rogers *et al.*, 2000; Goossens *et al.*, 2000; Nair *et al.*, 2000; Smith *et al.*, 2000). Conservation of the sequences among groups of taxa is limited, since numerous substitution events and/or insertion/deletion (that don't always happen at random) do occur, and they can modify the molecular structure from regions near to the microsatellites (Clisson *et al.*, 2000; Ruiz García, 2005). The goal of this study was to evaluate the level of conservation of one microsatellite, isolated for a New World Monkey, among many primate species.

MATERIALS AND METHODS

DNA was extracted from samples of two origins: a) blood samples conserved in FTA (Whatman) of the species *Alouatta caraya* (ACA, of a wild specimen from Isla Brasilera, Corrientes, Argentina, within the natural distribution of the species), *Ateles belzebuth* (ABE, in captivity at the Zoo of Buenos Aires, Argentina), *Ateles chamek* (ACH, in captivity at the Zoo of Buenos Aires, Argentina) and *Homo sapiens* (HSA, control sample taken from a GIBE member), following the protocol provided by the supplier and b) hair, from specimens in captivity at the Zoo of La Plata (Buenos Aires, Argentina), of *Lemur catta* (LCA), *Pan troglodytes* (PTR) and *Papio* sp. (PSP), using standard phenol-chloroform extraction followed by purification and concentration of the extracted DNA from hair by means of columns Microcon YM-100 (Millipore).

We amplified one microsatellite isolated from *Alouatta palliata* (AP74). Primers used were: (5'-TGCACTCATCTCTTTCTCTG-3') and (5'-CATCTTTGTTTTCC TCATAGC -3'; Ellsworth and Hoelzer, 1998). DNA amplifications were performed in a total volume of 25 µl (20 mM Tris-HCl, 50 mM KCl, and 1.5 mM MgCl₂, 0.2 mM each dNTP, 1 U Taq DNA Polymerase (Tandil), 4 pmol of each primer and 1 sheet of FTA or 1 ml of the extracted DNA). PCR reaction consisted on 35 cycles of 1 min at 95 °C, 1 min to 52 °C, and 1 min 30 s at 72 °C. PCR products were run in 2% agarose gels, dyed with ethidium bromide and sequenced by means of the method Big dye terminator system (Applied Biosystems). Sequences were analyzed in an automatic secuenciator (ABI Prism 310). The DNA sequences obtained were aligned using Clustal W and the Neighbour-joining analysis was made using MEGA version 3.0 (Kumar *et al.*, 2004).

RESULTS

We obtained amplification products of similar size (between 126 and 176 bp) in all the analyzed primates, with the exception of *Lemur catta*, which presented a size of approximately 1000 pb (Fig. 1). We successfully sequenced the fragments obtained for all the analyzed species.

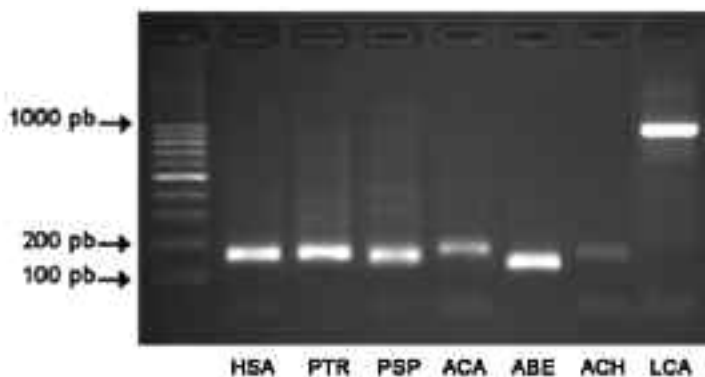


Figure 1: Amplification products of AP74 in 7 different Primate species (HSA: *Homo sapiens*, PTR: *Pan troglodytes*, PSP: *Papio* sp., ACA: *Alouatta caraya*, ABE: *Ateles belzebuth*, ACH: *Ateles chamek*, LCA: *Lemur catta*).

The analysis of the sequences among these species showed a high conservation of the flanking regions, in contrast with the great variation found in the number of repetitions of the microsatellite (CA; Fig. 2). Among New and Old World Primates, inserts, transitions and transversions were observed in the later region of the microsatellite. Also, punctual substitutions were detected in species from the same genus (*Ateles*) and in species phylogenetically closer as *Homo sapiens* and *Pan troglodytes* that were absent in the rest of the primates DNA studied (Fig. 2). *Lemur catta* showed only limited alignment with the other DNA sequence in the 3' extreme. The DNA sequence detected for *Homo sapiens* was searched in Gene Bank database and was located in chromosome 4 (clone RP11-752D24), among bases 169871 and 170030.

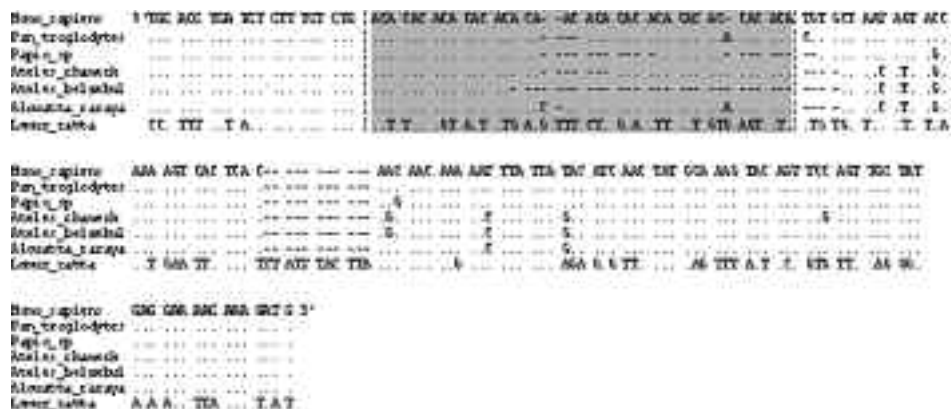


Figure 2: Multiple Sequence Alignment were conducted using MEGA version 3.0. Bold: Primer sequences, Gray: repetitive area of the microsatellite, the points are conserved bases across primate species and the dashes are gaps.

With the differences found among species we made a Neighbor-joining analysis based on TrN+G genetic distances using MEGA version 3.0 (Kumar *et al.*, 2004). The DNA sequence used as reference was the one published for *Alouatta palliata* (NCBI - U36400) that possesses a great number of undetermined bases. Given this fact, this DNA sequence was not included in the neighbor joining analysis. A consensus parsimony cladogram (PAUP*, Swofford, 2002; not shown) was fully concordant with the NJ phenogram. The robustness of the nodes was determined by means of a bootstrap analysis of 1000 replicates. The support of the node among monkeys of the New and Old World was of 96%, between *Homo sapiens* and *Pan troglodytes* of 62% and among species of the same genus of 66% (Fig. 3). The differences found in the repeat area of the microsatellite were not considered informative since it is ignored whether the number of repetitions is species-specific or it possesses intraspecific variation.

DISCUSSION

Many are the mechanisms and strategies that superior organisms present as frequent and characteristic of the specio-genetic process (for a review see Sobel *et al.*, 2009 and

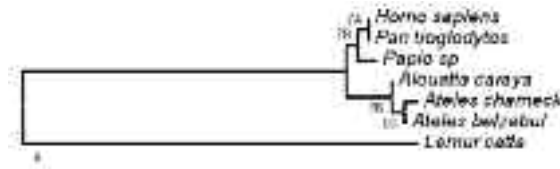


Figure 3: Neighbor-joining tree of AP74 sequence without the repeat area of the microsatellite. Branch length distances are shown on each corresponding branch.

references therein). It cannot be said that there is a unique mechanism. Initially, in order to understand the dynamic of speciation at the population level, molecular genetics appeared to be a more informative tool than classical genetics (morphological data through chromosomal rearrangements).

In vertebrates, and particularly in primates, phylogenetic and phylogeographic studies were initiated (both in Old and New World Primates) recurring to mitochondrial DNA analysis, considering that these genetic markers could be studied at the population level in order to implement the right policies for the conservation of the current species (Collins *et al.*, 2000; Ascunce *et al.*, 2003a; Ascunce *et al.*, 2007). In New World Primates, COII cytochrome analysis constituted an important tool to perform phylogenetic inferences (Ascunce *et al.*, 2003b; Ascunce *et al.*, 2003c), as well as cytochrome b was informative for a better understanding of the speciation process associated to geographic distribution in several species (Lavergne *et al.*, 2003).

However, the appearance of microsatellite studies allowed to enlarge and deepen the knowledge about the changes that took place at genomic level and that are related to species' evolutionary processes. In Primates, species-diagnostic microsatellites loci were used to determine hybrid status in two species of neotropical monkeys from an area of sympatry in Mexico where they have not achieved complete isolation (Cortes Ortiz *et al.*, 2007). Another interesting case was referred employing DNA microsatellites analyzing gene diversity and bottleneck events in different neotropical monkey populations with conservation purposes (Ruiz Garcia *et al.*, 2006; Ruiz Garcia *et al.*, 2007).

In this contribution, the study of sequences flanked by conserved ones allowed us to evaluate molecular modifications during the evolutionary process in primates. Since the fragments obtained for species extremely distanced as *Homo sapiens* and *Alouatta caraya* possess an extremely similar size it was necessary to analyze the composition of sequences in order to identify the substitution insertion or deletion events. The results obtained for the sequence analysis confirmed the evolutionary conservation of this locus in the Order Primates.

Generally crossed amplifications between species is successful at the Genus or Family level and less frequently at the Order level (Clisson *et al.*, 2000; Ruiz Garcia *et al.*, 2006; Ruiz Garcia *et al.*, 2007). Such is the case of Coote and Bruford, 1996, who could not amplify microsatellites isolated from humans in Prosimian and New World primates. In the present contribution, the marker AP74 allowed the amplification of sequences in several species of the Primates Order.

The microsatellite AP74 was located to chromosome 4, but it was not possible to perform an assignment to its correspondent chromosome band (Genbank). Considering

that in genomic conservation analysis regions and bands of the human karyotype have been established as highly conserved among New World and Old World Primates (Weinberg *et al.*, 2005; Stanyon *et al.*, 2008 and references therein) we can suggest that the sequences referred in this contribution could be located on one of the following chromosomes of each analyzed species: *Pan troglodytes*: PTR3 (Jauch *et al.*, 1992); *Papio* sp.: PSP 6 (Rogers *et al.*, 2000); *A. caraya*: ACA4q, ACA 9 and ACA22qter (de Oliveira *et al.*, 2002); *Ateles chamek*: ACH2q12-q14, ACH8p and ACH15 (Ruiz-Herrera *et al.*, 2005); *Ateles belzebul*: ABE2q12-q14, ABE9p and ABE15 (García *et al.*, 2002); *Lemur catta*: LCA5pprox, LCA4p, LCA19ter, LCA23, LCA26 (Warter *et al.*, 2005).

Previous studies have shown that alterations in the base composition of the repetitions is an important component of the variation between individuals and species (Garza *et al.*, 1995; Crouau-Roy *et al.*, 1996). This study was carried out with only one sample by species, therefore the obtained number of repetitions of the reason CA is not necessarily characteristic of each species. Further studies with more individuals are needed to determine if indeed what is reported in the present study occurred at population level. In this manner we will be evaluating the levels of variation within and between populations in order to clarify the possible genomic conservation as well as the phylogenetic reconstruction in these primate taxa.

Our study contributed to the permanent evaluation and correction of the phylogenetic history of the Primate species. Furthermore, new studies involving microsatellites sequence analysis across related species will allow further implementation of these markers in evolutionary studies.

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All research reported in this manuscript has met the appropriate national and institutional guidelines for the legal acquisition and use of laboratory animals and authorized study of wild animals. The authors also adhered to the Guide for Care and Use of Experimental Animals as promulgated by the Canadian Council of Animal Care and to the American Society of Primatologists (ASP) Principles for the Ethical Treatment of Non Human Primates. All authors approved the submission of the manuscript and have no conflicts of interest regarding its publication. Our gratitude to Dr. Daniel Corach for allowing us to perform the studies in his laboratory. We thank the authorities of the institutions and the veterinarians of the Zoo of La Plata and the Zoo of Buenos Aires for their assistance in the sampling of the animals.

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