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A primer on the phylogeography of *Lagothrix lagotricha* (*sensu* Fooden) in northern South America

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ABSTRACT

The taxonomic history of the genus *Lagothrix* is complex, with molecular and morphological assessments giving conflicting results for the separation between its taxa. Phylogeographic studies of the most widely distributed species, *Lagothrix lagotricha*, have only been attempted recently and are limited to few individuals per collection site, many of which were captive making their geographical origin dubious. There is debate regarding the possibility of raising subspecies of *Lagothrix lagotricha* to the species level, therefore the geographical origin of samples is particularly relevant. In the present work we revisit the intraspecific phylogeography of *L. lagotricha* from northwestern South America, including the subspecies *L. l. poeppiggi*, *L. l. lagotricha* and *L. l. lugens* (sensu Fooden, 1963), using DNA sequence data from hypervariable region I of the mitochondrial control region (D-loop HVI). Our results suggest a complex picture in which there are well delimited evolutionary units that, nonetheless, do not correlate well with the morphological variation used to support the current delimitation of taxa. Additionally, we corroborate previous results showing a lack of reciprocal monophyly between the putative subspecies of *Lagothrix lagotricha*, and we propose that this may be due to ancestral polymorphism that has been maintained following the recent spread of woolly monkeys throughout the western Amazonian lowlands and into the inter-Andean region of Colombia.

1. Introduction

Lagothrix species, commonly known as woolly monkeys, are large Neotropical frugivores from the subfamily Atelinae distributed across the central and western Amazon basins in Brazil, Bolivia, Peru, Ecuador, western Venezuela, and Colombia, extending north along eastern cordillera of the Andes (Fooden, 1963). Wooly monkeys are important as seed dispersers (Stevenson, 2000, 2007; Stevenson and Guzmán-Caro, 2010), but their large size and specialization as frugivores makes them particularly vulnerable to anthropogenic effects, especially hunting (Peres and Palacios, 2007; Stevenson and Aldana, 2008).

The taxonomic history of the genus is complex. In the first major taxonomic revision of the woolly monkeys, Fooden (1963) recognized two species within the genus, *Lagothrix flavicauda*, the yellow tailed woolly monkey and *Lagothrix lagotricha*, the lowland or common woolly monkey, with four subspecies. Four decades later, Groves (2001) undertook a comparative cladistic analysis of all of the atelids using craniodental morphology and concluded that the yellow-tailed woolly monkey was most closely related to spider monkeys, not to other woolly monkeys. He thus resurrected a former subgenus name, *Oreonax*, for *O. flavicauda*, and raised all of the subspecies of *Lagothrix lagotricha* to species status: *Lagothrix lagotricha*, *L. lugens*, *L. cana*, and *L. poeppigii*.

More recently, the resurrection of *Oreonax* has been shown to be an artifact of sampling choice when replicating the methodology that Groves (2001) employed (Matthews and Rosenberger, 2008). Moreover, molecular analyses of both a large mitogenomic dataset (Di Fiore et al., this issue) and a large suite of nuclear markers (Chaves et al., 2012) have shown that *Lagothrix flavicauda* is indeed the sister group to other woolly monkeys, with a divergence time estimated at roughly 2 Ma, but have not resolved the relationships among the putative *Lagothrix lagotricha* subspecies. One study using the mitochondrial cytochrome oxidase II gene (COII) found a lack of reciprocal monophyly between the currently-recognized subspecies (Ruiz-Garcia and Pinedo-Castro, 2010), and this was corroborated for Colombian *L. l. lugens* and

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L. l. lagotricha using the hypervariable region I of the mitochondrial control region (D-loop HVI), COII, and karyotyping (Botero et al., 2010). Thus, there seems to be little rationale to consider the different forms of lowland woolly monkeys as different species, and we will use Fooden's (1963) subspecific nomenclature for the rest of the paper.

A major limitation of the aforementioned studies is that they were based on either a single or few individuals for each collection locale. In many cases, too, the samples used for analysis came from individuals of unconfirmed origin such as pets or animals hunted for subsistence (Ruiz-Garcia and Pinedo-Castro, 2010), or from captive individuals whose geographical provenience was unknown (Botero et al., 2010). The use of samples with unconfirmed origin complicates the interpretation of results since there is significant phenotypical variability among and within each of the putative subspecies and intermediate phenotypes are common (Defler, 2004). The only phylogenetic study to include wild individuals directly sampled by the researchers included six Colombian populations and confirmed the lack of monophyly between *L. l. lagotricha* and *L. l. lugens* using the D-loop HVI (Botero and Stevenson, 2014). This study also found a population of woolly monkeys that shows the brown phenotype characteristic of *L. l. lagotricha* and that is located within that subspecies' geographical range, but is nonetheless indistinguishable at the molecular level for the D-loop HVI marker from the closest population of *L. l. lugens* included in the study. When this population is considered to be *L. l. lugens*, as its mitochondrial D-loop haplotype implies, Colombian woolly monkeys indeed segregate into two well separated evolutionary units. Botero and Stevenson (2014) thus suggest that these molecularly defined demes might be sufficiently different to be considered subspecies, but that the current taxonomic scheme based on pelage coloration is not appropriate to distinguish them. While these results remain to be corroborated with other molecular markers and are limited to *L. l. lagotricha* and *L. l. lugens*, they suggest a complex picture for the phylogeography of Lagothrix that warrants further investigation.

Our goal in this paper, then, is to expand previous work by revisiting the intraspecific phylogeography of *Lagothrix lagotricha* from northwestern South America, incorporating samples from *L. l. poeppigii*, as well as *L. l. lagotricha* and *L. l. lugens* into the analysis. Samples of the fourth subspecies, *L. l. cana*, which has the largest and southernmost distribution were not available for our analysis.

2. Materials and methods

2.1. Populations and samples included

We included previously published genetic samples from 8 wild populations of woolly monkeys, including two populations of *L. l. lagotricha*, four of *L. l. lugens* (Botero and Stevenson, 2014) and two of *L. l. poeppiggi* (Di Fiore and Fleischer, 2005; Di Fiore et al., 2009) (Fig. 1). We only included populations represented by samples from multiple wild individuals where we were certain of the geographic origin of the samples. Each population location fell within the geographical limits of an ascribed subspecies and showed the characteristic phenotype of that subspecies (Fooden, 1963). However, we assigned samples from the Guaviare population in Colombia as *L. l. lugens* for our analysis, since in a previous study (Botero and Stevenson, 2014) these samples were indistinguishable in terms of their mtDNA haplotypes at the HVI locus from the Macarena, Colombia population of *L. l. lugens*. In the previous studies sampling was performed non-invasively by collecting fecal matter during extended follows of social groups (Botero and Stevenson, 2014; Di Fiore et al., 2009), except for some samples of *L. l. poeppigii*, which were collected using tissue biopsy darting (Di Fiore and Fleischer, 2005). All available samples for each population were included, thus the number of samples reflects the success in DNA extraction and sample collection. No new samples were collected for the present study. The coordinates and sampling sizes for each population are indicated in Table 1. Fig. 1 shows their location.

2.2. Molecular marker

We used a 431 bp fragment of the D-loop HVI as the molecular marker since it was available from the previous study of the *L. l. lagotricha*, *L. l. lugens* (Botero and Stevenson, 2014), and *L. l. poeppiggi* (Di Fiore, 2009) populations. Accession numbers for the sequences in GenBank are given in Table 1.

While the *L. l. poeppiggi* sequences have been used in previous studies (Di Fiore, 2009), they had not been submitted to GenBank previously and conditions for their amplification were not provided in that publication. These sequences were amplified using primers H16340 (5′-CCTGARGTAGGAACCARATG-3′) and L15926 (5′-SAATTACCCCGGYCTTGTAAACC-3′) which amplify a fragment of approximately 610 bp (Collins and Dubach, 2000; Kocher et al., 1989). PCR amplification was performed in 25 μl reactions with final concentrations of 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 0.48 μM of each primer, 1 μl of a 1:10 dilution of DNA template, 1× of PCR reaction buffer, and 0.125 μl of Promega GoTaq® polymerase at 5 U/μl. Cycling conditions consisted of an initial denaturation step of 5 min at 94 °C followed by 40 cycles of 1 min denaturation at 94 °C, 1 min annealing at 55 °C and 1.5 min extension at 72 °C, after which a final extension step of 5 min at 72 °C and a ⩽4 °C incubation step were used to stop the reaction. In cases where the selected primers did not provide a clear band or failed to amplify, 3 alternate PCR reactions were attempted with primer pairs that amplify shorter fragments within the HVI region: H16340 – R264 (5′-ACAACAAGCTTACAAGCAAGTAC-3′), F222 (5′-ATGGATTTACGTGTTAGATGGC-3′) – R423 (5′-AGGCTTTGCCACAAAGTACC-3′), and F373 (5′-AATGCACTAATTACATAGGG-3′) – L15926. For the last fragment, the annealing temperature was reduced to 53 °C, and in all cases the reaction was supplemented with BSA to a final concentration of 1 μg/μl. Note that for the present study we only included sequences for which the complete 431 bp region corresponding to fragment sequenced by Botero and Stevenson (2014) was available. Other details about the *L. l. poeppiggi* sequences have been published elsewhere (Di Fiore, 2009).

Traditionally, woolly monkeys have been thought to be characterized by having philopatric males and dispersing females. However, evidence of male dispersal (Di Fiore et al., 2009; Maldonado and Botero, 2009) and great flexibility in the grouping patterns in some populations has been observed (Stevenson et al. unpublished). If only females disperse, mitochondrial estimates would be an accurate estimate of the total gene flow, whereas if males disperse too, the total gene flow is expected to be greater. As a result, estimates based on mitochondrial markers do not fully represent the patterns of gene flow occurring, but a lower bound to it. Our conclusions should be treated with caution until replicated with nuclear markers.

2.3. Analyses

We used the software DNAsp v5.00.07 (Rozas et al., 2003) to calculate basic descriptive statistics: nucleotide diversity π (Nei and Li, 1979), number of haplotypes and segregating sites per population and per taxa. We then calculated Tajima's D (Tajima, 1989), Fu's F (Fu, 1997), and Ramos-Onsins and Rosas' R2 (Ramos-Onsins and Rozas, 2002) statistics to examine whether, for any of the taxa, the patterns of molecular diversity were suggestive of deviation

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