Molecular Relationships and Classification of Several Tufted Capuchin Lineages (*Cebus apella*, *Cebus xanthosternos* and *Cebus nigritus*, Cebidae), by Means of Mitochondrial Cytochrome Oxidase II Gene Sequences

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Key Words

Cebus apella · *Cebus nigritus* · *Cebus xanthosternos* · Molecular phylogeny and phylogeography · Mitochondrial cytochrome oxidase II gene · Colombia · Peru · Bolivia · French Guyana · Brazil · Argentina · Paraguay

Abstract

The morphological systematics of the tufted capuchins is confusing. In an attempt to clarify the complex systematics and phylogeography of this taxon, we provide a first molecular analysis. We obtained mitochondrial cytochrome oxidase II (*mtCOII*) gene sequences from 49 tufted capuchins that had exact geographic origins from diverse lineages in Colombia, Peru, Bolivia, French Guyana, Brazil, Argentina and Paraguay and that belonged to clearly recognized morphological taxa. This project had 4 main findings: (1) we determined 2 established and related taxa in the northern Amazon River area, which we named *C. a. apella* and *C. a. fatuellus*. *C. a. apella* is distributed from French Guyana until, at least, the Negro River in the northern Brazilian Amazon, whereas *C. a. fatuellus* is distributed throughout the Colombian Eastern Llanos and the northern Colombian Amazon. We also determined 2 other southern *C. apella* taxa, which we named *C. a. macrodon* and *C. a. cay*. *C. a. macrodon* has a western and southern Amazon distribution, while *C. a. cay* has a more southern distribution outside the Amazon basin. (2) In the upper Amazon basin, there is a unique lineage (*C. a. macrocephalus*) with 1 widely distributed haplotype. The 4 morphological subspecies (*C. a. maranonis*, *C. a. macrocephalus*, *C. a. peruanus*, *C. a. pallidus*), and maybe a fifth unknown subspecies, described in this area were molecularly undifferentiated at least for the mitochondrial gene analyzed. (3) Our molecular analysis determined that 1 individual of *C. robustus* fell into the lineage of *C. a. macrocephalus*. Therefore, this form does not receive any spe-

cific name. (4) The animals classified a priori as *C. nigrinus* and *C. xanthosternos* (because of their morphological phenotypes and by their geographical origins) were clearly differentiated from the other specimens analyzed with the molecular marker employed. Therefore, we consider that these 2 lineages could be assigned the status of full species following the biological species definition. (5) In 2001, Groves described 4 tufted capuchin species (*C. apella*, *C. libidinosus*, *C. nigrinus* and *C. xanthosternos*), while Silva Jr. determined 7 species (*C. apella*, *C. macrocephalus*, *C. libidinosus*, *C. cay*, *C. nigrinus*, *C. robustus* and *C. xanthosternos*). The tests of Swofford-Olsen-Waddell-Hillis, of Shimodaira and Hasegawa and of Templeton did not fit with either of these two classificatory schemes, although Groves' scheme was better with regard to our data than that of Silva Jr. (6) All the temporal splits among the tufted capuchin taxa studied were estimated to have occurred during the last phase of the Pleistocene by using the ρ statistic applied to the median joining haplotype network.

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Introduction

If the tufted capuchins [*Cebus (Sapajus) apella*, Linnaeus, 1758] are considered as a single species, it has the largest geographical distribution of all the New World primate species. Its range extends from the eastern Andes cordillera in Colombia (but with a population in the upper Magdalena River basin within the Colombian departments of Huila and Tolima) to 27° south within the southern Brazilian states, as well as inhabiting the Misiones, Salta and Jujuy provinces of Argentina.

The taxonomy of the tufted capuchin is extremely confusing and is as diverse as the authors that have worked on the subject. Elliot [1913] and Hershkovitz [1949, 1955] each recognized 12 species of tufted capuchins, with Cabrera [1957] being the first author to include all the tufted capuchin taxa in a unique species, *C. apella*, with 11 subspecies. Hill [1960] also suggested a single unique species, *C. apella*, but with 16 subspecies. The distinctive morphological features of tufted capuchins across their range are described in Rylands et al. [2005]. Torres de Assumpcao [1983, 1988] identified phenotypic differences between tufted capuchins from each of 5 distinct geographic regions. She also identified an extensive sixth area (that included central and northeastern Brazil) where there was more phenotypic variation in the animals inhabiting this area compared to the other 5 areas because of different selective habitat pressures, extensive hybridization with different surrounding forms or less genetic speciation differentiation within this sixth area. Torres de Assumpcao [1983, 1988] did not assign any specific or subspecific nomenclature to the tufted capuchins of these geographic areas. Groves [2001] determined 4 tufted capuchin species (*C. apella*, *C. libidinosus*, *C. nigrinus* and *C. xanthosternos*) and 14 subspecies, whereas Silva Jr. [2001] distinguished between tufted and untufted capuchins, assigning the former to the genus *Sapajus*, Kerr, 1792, and including 7 species (*S. apella*, *S. macrocephalus*, *S. libidinosus*, *S. cay*, *S. nigrinus*, *S. robustus* and *S. xanthosternos*) [see also Fragaszy et al., 2004]. He assigned untufted capuchins (which included the species *C. albifrons* and *C. capucinus*) to the genus *Cebus*. The inclusion of the *apella* taxonomic complex in the genus *Sapajus* has most recently been supported by Lynch-Alfaro et al. [2012], based on morphological, genetic, behavioral, ecological and biogeographic evidence.

However, given that *Sapajus* has yet to gain widespread acceptance and further evidence is required before we feel confident adopting this taxonomic scheme, in this paper, we continue to assign the tufted capuchins to the genus *Cebus*.

To date, no molecular studies have been conducted to help shed light on the complex systematic of the tufted capuchins suggested by morphological studies. In this paper, we present to our knowledge the first attempt to resolve the systematic and phylogeography of the tufted capuchins using molecular analysis, specifically by using the mitochondrial cytochrome oxidase II (*mtCOII*) gene from wild-living samples representing most of the commonly recognized subspecies. Nevertheless, the *mtCOII* gene, like other mitochondrial genes, poses several problems for primate phylogeny including heterogeneity in base composition at each codon position, different transition and transversion substitution rates and third-codon position saturation. However, Ascunce et al. [2003] showed that at an intrageneric or intraspecific level, the sequences of this gene are phylogenetically informative, although not at a higher taxonomic level. Nonetheless, extreme care should be taken when using a unique mitochondrial gene for resolving taxonomic problems because gene trees do not necessarily correspond well with species trees. Species can diverge simultaneously with a pair of mitochondrial haplotypes or they can diverge after a pair of haplotypes diverged. However, it is possible that some time after a population divides, a new haplotype may appear in the gene tree. A migrant could carry the new haplotype to the other population and, finally, the new haplotype is lost in one population and the ancestral haplotype is lost in the other. Therefore, if we use the gene tree to estimate genetic heterogeneity and the divergence time for the species tree, the species will appear to have diverged more recently than they really did [Freeman and Heron, 1998]. Additionally, mitochondrial data show only the evolution of the female lineages, and this could miss hybridization events between close species when males are the gene flow vectors ('mitochondrial capture' [Burrell et al., 2009]). Although our study is limited by this fact, it is the first use of a molecular approach to elucidate the phylogeny of the tufted capuchins. It may provide a useful reference point for future studies regarding the phylogeny of the tufted capuchins.

In this paper, we use the biological species concept (BSC) to define different species. For this, we have employed reciprocal monophyly and significantly differentiated clusters with Bayesian assignation procedures and genetic heterogeneity statistics, bootstrapping and a posteriori significance tests. This way of defining a species is disputed by some authors. However, we prefer this definition of species, using traditional genetic data such as those employed in this study, to other definitions such as the phylogenetic species concept (PSC), which we will comment on in the discussion. Additionally, we defined subspecies as those individuals which were restricted to a determined geographic distribution, or ecogeographic region. They had certain characteristic phenotypes and were tested for uniqueness using phylogenetic and population genetics methods. We considered as subspecies those groups which were closely related genetically to other groups of other geographic distributions.

In this paper we have the following specific aims: (1) to determine how many tufted capuchin molecular lineages are found in the northern Amazon; (2) to determine how many molecular lineages are currently present in the Peruvian Amazon, and to assess the correspondence between the subspecies indicated by morphological studies (*C. a. maranonis*, *C. a. macrocephalus*, *C. a. peruanus*, *C. a. pallidus*, and possibly a fifth unnamed subspecies) and the molecular lineages; (3) to determine if the

4 morphological species described by Groves [2001] or the 7 morphological species described by Silva Jr. [2001] correspond to the tufted capuchin molecular lineages; (4) to determine the temporal splits among these *Cebus* taxa for the *mtCOII* gene.

Material and Methods

We chose 49 *Cebus* samples that had precise geographical origins and clearly described phenotypes from Colombia, Peru, Brazil, French Guyana, Bolivia, Argentina and Paraguay.

The DNA samples from hair and blood were obtained from animals found alive or dead in diverse Indian and 'colonos' communities. We also requested permission to collect biological materials from either carcasses or live animals that were already present in the community. We sampled small pieces of tissue (muscle) or teeth from hunted animals that were discarded during the cooking process, or hairs with bulbs plucked from live pets. Communities were visited only once, all sample donations were voluntary, and no financial or other inducement was offered for supplying specimens for analysis. All the pets and the hunted animals analyzed were obtained by the Indian communities at a maximum of 15 km from the community. These sampling procedures complied with all the protocols approved by the Ethical Committee of the Pontificia Universidad Javeriana (No. 45684) and the laws of the Ministerio de Ambiente, Vivienda y Desarrollo Territorial (R 1256) from Colombia. This research adhered to the stipulations set by the American Society of Primatologists.

The exact geographic origins of these individuals and their classification within a morphological taxon are shown in figure 1 and table 1. In Colombia, a total of 15 samples were sequenced, 14 from the Eastern Llanos and the northern Colombian Amazon and 1 from the southern Colombian Amazon. Eight individuals were sequenced from Peru. Ten Bolivian individuals, 7 Brazilian individuals, 3 Paraguayan individuals, 2 Argentine individuals and 4 individuals from French Guyana were also sequenced. We selected 2 sequences of *Saimiri boliviensis boliviensis*, 1 sequence of *Saimiri macrodon*, 2 sequences of *Aotus nigriceps*, 1 sequence of *Aotus nancymaae*, 2 sequences of *Aotus vociferans* and 5 *Cebus albifrons* individuals (4 *versicolor* from Antioquia, Colombia, and 1 *aequatorialis* from Jama, Ecuador) as outgroups.

Molecular Analyses *mtCOII* Gene

The DNA, from teeth, bone, muscle or skin, was extracted using the phenol-chloroform procedure [Sambrook et al., 1989], while DNA samples from hair and blood were extracted with 10% Chelex resin [Walsh et al., 1991]. The primers employed to amplify (polymerase chain reaction, PCR) the *mtCOII* gene (located in the lysine and asparagine tRNAs) were L6955 (5'-AACCATTTCATAACTTTGTCAA-3') and H7766 (5'-CTCTTAATCTTAACTTAAAAG-3') [Ashley and Vaughn, 1995; Collins and Dubach, 2000; Ruiz-García et al., 2012]. The PCRs were performed in a 50- μ l volume with reaction mixtures including 4 μ l of 10 $\times$ buffer, 6 μ l of 3 mM MgCl₂, 2 μ l of 1 mM dNTPs, 2 μ l (8 pmol) of each primer, 2 units of Taq DNA polymerase, 13.5 μ l of H₂O and 2 μ l (20–80 ng/ μ l) of DNA. PCR reactions were carried out in a BioRad thermocycler. The temperatures employed were as follows: 95°C for 5 min, 35 cycles of 45 s at 95°C, 30 s at 50°C and 30 s at 72°C and a final extension time for 5 min at 72°C. All amplifications, including positive and negative controls, were checked in 2% agarose gels, using the molecular weight marker ϕ X174 DNA digested with *HindIII* and *HinfI*. Those samples that amplified were purified using membrane-binding spin columns (Qiagen). The double-stranded DNA was directly sequenced in a 377A (ABI) automated DNA sequence. The samples were sequenced in both directions using the BigDye™ kit and all the samples were repeated to ensure sequence accuracy. The sequences obtained were deposited in GenBank (Bankit: 1334566-13334615).

It is possible that some of the sequences obtained represent numts (mitochondrial DNA fragments inserted into the nuclear genome) rather than true mtDNA [Chung and Steiper, 2008]. However, we note that all amino acid translations of the sequences obtained showed the presence of initial start and terminal stop codons and the absence of premature stop codons.

Fig. 1. Map that depicts the collection sites of *C. apella* and related taxa sampled.

Protein translation was also checked to evaluate the possible presence of numts. Nevertheless, observed mutations were synonymous changes, suggesting that there were no numts in the sequences obtained. Additionally, one would expect to see a signal relating to numts in the DNA chromatogram. When the ratio of nuclear to mitochondrial genomes per cell is around 1/1,000 [Takamatsu et al., 2002], the proportion of numts and real mitochondrial sequences is expected to be amplified. If the potential amplified numts were highly differentiated from the real mitochondrial gene or included some insertions or deletions, many double peaks would be expected in the chromatogram and this was not observed in our analysis. However, the possibility of numt amplifications cannot be completely ruled out, and this must be taken into consideration when interpreting the results.

Data Analyses

Genetic Diversity and Heterogeneity Analyses

The statistics used to determine the genetic diversity within the diverse tufted groups detected were: the number of polymorphic sites (S), the haplotypic diversity (H_d), the nucleotide diversity (π), the average number of nucleotide differences (K) and the θ statistic by sequence.

Two kinds of procedures were carried out to estimate genetic heterogeneity and theoretical gene flow estimates among the diverse tufted capuchin lineages detected by the phylogenetic analyses. The procedures employed were applied to haplotypic frequencies (G_{ST} statistic) and to nucleotide sequences (γ_{ST} , N_{ST} and F_{ST} statistics [Hudson et al., 1992]). To determine possible differences among the putative *C. apella* group pairs considered, we employed exact tests [Raymond and Rousset, 1995] and F_{ST} statistics [Weir and Hill, 2002]. These gene diversity and genetic heterogeneity statistics were undertaken in the programs DNAsp 4.56 and Arlequin 3.1.

Table 1 (continued)

Sampling localities (number of samples)	Geographical area	Groves [2001]	Silva Jr. [2001]	Hershkovitz [1955] for Peru	Cabrera [1957]	Hill [1960]	Morphological description
Santa Rosa de Pando (1)	Bolivia	<i>C. libidinosus</i> <i>pallidus</i>	<i>S. cay</i>	<i>C. a. pallidus</i>	<i>C. a. pallidus</i>	<i>C. a. pallidus</i>	<i>pallidus</i> : a very contrasting black cap with hair partially erect; pelage is light so that black of crown contrasts with body
Cerrado San José (1)							
Chuchini-Ibaré River (1)							
Trinidad-Ibaré River (2)							
Yungas (5)	Bolivia-Argentina						
Novo Airao-Negro River (1)	Brazilian Amazon	<i>C. a. fatuellus</i>	<i>S. apella</i>	–	<i>C. a. apella</i>	<i>C. a. fatuellus</i>	<i>apella</i> : dull brown in color; underside with more reddish color; crown and sideburns black; crown tufts small
Carnopi River (4)	French Guyana	<i>C. a. apella</i>				<i>C. a. apella</i>	
Matto Grosso do Sul (1)	Southern Brazil			–			<i>cay</i> : very pale pelage, often with no dark cap at all
Hernandarias-Alto Paraná River (3)	Paraguay	<i>C. libidinosus</i> <i>paraguayanus</i>	<i>S. cay</i>		<i>C. a. paraguayanus</i>	<i>C. a. cay</i>	
Colatina-Espirito Santo (1)	Brazil	<i>C. nigritus</i> <i>robustus</i>	<i>S. robustus</i>	–	<i>C. a. robustus</i>	<i>C. a. robustus</i>	<i>robustus</i> : very dark wood brown pelage, hands and feet black; face very dark with clear surrounding pelage
Misiones (2)	Argentina	<i>C. nigritus</i> <i>cucullatus</i>	<i>S. nigritus</i>	–	<i>C. a. vellerosus</i>	<i>C. a. nigritus</i>	<i>nigritus</i> : crown not contrasting, cap only slightly darker than the body; pelage of the body very dark wood brown or blackish above
Bahia (2)	Brazil	<i>C. xanthosternos</i>	<i>S. xanthosternos</i>	–	<i>C. a. xanthosternos</i>	<i>C. a. xanthosternos</i>	<i>xanthosternos</i> : crown not contrasting with body, which is brindled reddish above and underside sharply marked golden red

To analyze the number of different taxa in the tufted capuchins surveyed, the method described by Pritchard et al. [2000], extended by Falush et al. [2003a, b, 2007] and developed in the Structure program version 2.3 was employed. This method employs a Markov Chain Monte Carlo procedure and the Gibbs sampler and uses, in this case, the polymorphic nucleotide positions to infer taxa or population assignments of the individuals analyzed. Sequence data were analyzed with Structure [Pritchard et al., 2000], bearing in mind that as the program was not originally created for sequence data, discrimination power may be lower than for other markers such as microsatellites. The model considers K populations, where K , as in this case, may be unknown and the individuals assigned tentatively to one population based on their assignment probability. The posterior K probabilities are calculated assuming uniform prior values of K , in this case between 1 and 12 (USEPOPINFO = 0). The presence of the most likely number of taxa or populations within the data considered is revealed by an increasing likelihood. We employed the linkage model [Falush et al., 2003a], because a mitochondrial sequence is one entirely linked locus with little or no recombination, we also employed allele frequencies correlated among populations and assuming different values of F_{ST} for different populations. Models with informative priors (LOCPRIOR [Hubisz et al., 2009]) have also been employed, with similar results to those reported here. The structure analysis shown was carried out with 500,000 iterations, following a burn period of 50,000 iterations.

Phylogenetic Analyses

The *mtCOII* sequence alignments were carried out manually and with the DNA Alignment program (Fluxus Technology Ltd.), and to reconstruct the possible phylogeography and the phylogeny of the tufted capuchins, several analyses were undertaken. The FindModel program was applied to determine which among 28 different evolutionary nucleotide models was the most probable for the tufted capuchin sequence set.

Some phylogenetic trees were obtained by means of maximum likelihood and maximum parsimony (with tree bisection and reconnection) with the program PAUP*4.0b8.

A Bayesian analysis was performed using a GTR (general time reversible) model of nucleotide substitution with the gamma distributed rate varying among sites, and 9 rate categories (GTR + G) because it was determined to be the better model using the FindModel program. This Bayesian analysis was completed with the BEAST v1.4.8 program [Drummond and Rambaut, 2007]. Two separate sets of analyses were run, assuming a Yule speciation model and a relaxed molecular clock with an uncorrelated log-normal rate of distribution [Drummond et al., 2006]. Results from the two independent runs (50,000,000 generations with the first 5,000,000 discarded as burn-in and parameter values sampled every 1,000 generations) were combined with LogCombiner v1.6.2 software [Drummond and Rambaut, 2007]. Posterior probability values provide an assessment of the degree of support of each node on the tree. The effective sample size for the parameter estimates and convergence were checked using the program Tracer version 1.5 [Rambaut and Drummond, 2007]. The lower and upper 95% highest posterior densities of these parameters as well as the means, geometric means, medians, marginal densities and traces were also estimated with the Tracer v1.5 program [Drummond and Rambaut, 2007]. To determine the reality of the values of these parameters, the autocorrelation tree and effective sample size for parameter estimates were obtained. The final tree was estimated in TreeAnnotator v1.6.2 [Drummond and Rambaut, 2007] and visualized in the FigTree v1.3.1 program [Drummond and Rambaut, 2007].

We tested the hypothesis that tufted capuchins fall into 4 (*C. apella*, *C. libidinosus*, *C. nigritus* and *C. xanthosternos*) [Groves, 2001] or 7 (*C. apella*, *C. macrocephalus*, *C. libidinosus*, *C. cay*, *C. nigritus*, *C. robustus* and *C. xanthosternos*) [Silva Jr., 2001] distinct evolutionary lineages (table 2). We thus performed parametric bootstrapping and a posteriori significance tests (Swofford-Olsen-Waddell-Hillis test, SOWH test [Huelsenbeck and Bull, 1996; Swofford et al., 1996; Goldman et al., 2000]) to compare the clusters of the hypotheses of Groves and Silva Jr. and that obtained in the present work. The Groves and Silva Jr. hypotheses were employed as a model tree for parameter estimation and for generating 100 replicate data sets in the software Seq-Gen 1.2.5 [Rambaut and Grassly, 1997] which presented a uniform base composition. Gold-

Table 2. Different morphological classifications for the tufted capuchins obtained by diverse authors

Elliot [1913]	Cabrera and Yepes [1940]	Cabrera [1957]
<i>C. apella</i>	<i>C. xanthosternos</i>	<i>C. apella</i>
<i>C. fatuellus</i>	<i>C. paraguayanus</i>	<i>C. a. apella</i>
<i>C. f. fatuellus</i>	<i>C. libidinosus</i>	<i>C. a. libidinosus</i>
<i>C. f. peruanus</i>	<i>C. macrocephalus</i>	<i>C. a. macrocephalus</i>
<i>C. macrocephalus</i>	<i>C. frontatus</i>	<i>C. a. margaritae</i>
<i>C. azarae</i>	<i>C. nigrinus</i>	<i>C. a. nigrinus</i>
<i>C. azarae azarae</i>	<i>C. vellerosus</i>	<i>C. a. pallidus</i>
<i>C. azarae pallidus</i>	<i>C. fatuellus</i>	<i>C. a. paraguayanus</i>
<i>C. libidinosus</i>	<i>C. apella</i>	<i>C. a. robustus</i>
<i>C. frontatus</i>		<i>C. a. vellerosus</i>
<i>C. variegatus</i>		<i>C. a. versutus</i>
<i>C. versuta</i>		<i>C. a. xanthosternos</i>
<i>C. cirrifer</i>		
<i>C. crassiceps</i>		
<i>C. caliginosus</i>		
<i>C. vellerosus</i>		
Hill [1960]	Groves [2001]	Silva Jr. [2001]
<i>C. apella</i>	<i>C. apella</i>	<i>S. apella</i>
<i>C. a. apella</i>	<i>C. a. apella</i>	<i>S. macrocephalus</i>
<i>C. a. margaritae</i>	<i>C. a. fatuellus</i>	<i>S. libidinosus</i>
<i>C. a. fatuellus</i>	<i>C. a. macrocephalus</i>	<i>S. cay</i>
<i>C. a. tocantinus</i>	<i>C. a. peruanus</i>	<i>S. nigrinus</i>
<i>C. a. macrocephalus</i>	<i>C. a. tocantinus</i>	<i>S. robustus</i>
<i>C. a. magnus</i>	<i>C. a. margaritae</i>	<i>S. xanthosternos</i>
<i>C. a. juruanus</i>	<i>C. libidinosus</i>	
<i>C. a. maranonis</i>	<i>C. l. libidinosus</i>	
<i>C. a. peruanus</i>	<i>C. l. pallidus</i>	
<i>C. a. pallidus</i>	<i>C. l. paraguayanus</i>	
<i>C. a. cay</i>	<i>C. l. juruanus</i>	
<i>C. a. libidinosus</i>	<i>C. nigrinus</i>	
<i>C. a. robustus</i>	<i>C. n. nigrinus</i>	
<i>C. a. frontatus</i>	<i>C. n. robustus</i>	
<i>C. a. nigrinus</i>	<i>C. n. cucullatus</i>	
<i>C. a. xanthosternos</i>	<i>C. xanthosternos</i>	

Our molecular genetic data were, in particular, contrasted with the classifications of Groves [2001] and Silva Jr. [2001]. The taxonomic review of Groves [2001] is identical for the tufted capuchins to that of Rylands et al. [2000].

man et al. [2000] demonstrated that this procedure can increase power in rejecting the null hypothesis and is better than typical nonparametric tests for comparisons of a posteriori hypotheses. The differences between the log likelihood of the Groves and Silva Jr. hypotheses and the tree herein obtained were compared with the distribution of the differences between each parametric replicate and the tree employed as representative of the Groves and Silva Jr. hypoth-

Table 3. Genetic diversity statistics estimated for total *C. apella* and related lineages, as well as for all the different species and subspecies determined in this study

	S	Eta	NH	H _d	π	K	θ per sequence
Total <i>C. apella</i> and related lineages	78	80	19	0.874 ± 0.037	0.0123 ± 0.003	7.224 ± 3.450	17.931 ± 5.402
<i>C. apella apella</i>	33	33	4	0.900 ± 0.161	0.0269 ± 0.0101	14.600 ± 7.907	15.840 ± 8.228
<i>C. apella fatuellus</i>	1	1	2	0.143 ± 0.119	0.00024 ± 0.0002	0.143 ± 0.219	0.314 ± 0.315
<i>C. apella macrocephalus</i>	4	4	5	0.600 ± 0.127	0.00166 ± 0.00042	0.975 ± 0.698	1.205 ± 0.707
<i>C. apella cay</i>	5	5	3	0.833 ± 0.145	0.00424 ± 0.0025	2.500 ± 1.685	2.727 ± 0.877
<i>C. nigrinus</i>	10	10	2	1.000 ± 0.500	0.01695 ± 0.00847	10.000 ± 7.416	10.000 ± 7.416
<i>C. xanthosternos</i>	1	1	2	1.000 ± 0.500	0.00169 ± 0.00085	1.000 ± 1.000	1.000 ± 1.000

S = Number of polymorphic sites; Eta = number of mutations; NH = number of haplotypes determined; H_d = haplotypic diversity; π = nucleotide diversity; K = average number of different nucleotides within each group analyzed; θ per sequence (= 2N_eμ), with N_e being the effective female population size, and μ being the mutation rate per generation.

Table 4. Diverse genetic heterogeneity statistics (χ^2 , H_{ST}, K_{ST}, K_{ST}*, Z_S, Z_S*, S_{nn}) and their associated probabilities

Genetic differentiation estimated	p	Gene flow	
		statistics	Nm
$\chi^2 = 215.000$, d.f. = 85	0.0000	G _{ST} = 0.3679	0.86
H _{ST} = 0.45572	0.0000	γ _{ST} = 0.7038	0.21
K _{ST} = 0.61974	0.0000	N _{ST} = 0.7154	0.20
K _{ST} * = 0.63861	0.0000	F _{ST} = 0.7127	0.20
Z _S = 182.48264	0.0000		
Z _S * = 4.84192	0.0000		
S _{nn} = 1.00000	0.0000		

All the probability values were significant (* p < 0.00001). Also some gene flow estimates (Nm) and the genetic heterogeneity statistics from which they derived are shown. All the Nm estimates were lower than 1 indicating that genetic isolation exists between the diverse *C. apella* lineages considered.

eses. We also employed the Shimodaira and Hasegawa [1999] test (nonparametric SH test) and the Templeton test [Templeton, 1983].

To estimate possible divergence times among the haplotypes found in the tufted capuchins studied, a median joining network [Bandelt et al., 1999] was constructed by means of the software Network 4.2.0.1 (Fluxus Technology Ltd.). Additionally, the ρ statistic [Morral et al., 1994] was estimated and transformed into years. The standard deviation of ρ was also calculated [Sailard et al., 2000]. The ρ statistic is unbiased and highly independent of past demographic events. Ruvolo et al. [1991] determined a mutation rate of 0.85% per million years per lineage for Hominoidea at the *mtCOII* gene. This represents 1 mutation each 199,402 years. This mutation rate is practically identical to that determined by Ruiz-García and Pinedo-Castro [2010] for *Lago-*

Table 5. Genetic heterogeneity statistic and gene flow (F_{ST} , Nm) among all the pairs of different *C. apella* lineages and populations considered

		Lineage							
		1	2	3	4	5	6	7	8
1	<i>C. a. fatuellus</i>		0.660	0.089	0.014	0.019	0.056	0.006	0.022
2	<i>C. a. apella</i>	0.431**		0.497	0.986	inf.	1.295	0.344	0.439
3	<i>C. a. macrocephalus</i>	0.855**	0.502**		0.331	0.654	0.373	0.025	0.039
4	<i>C. a. (Yungas)</i>	0.973**	0.336**	0.602**		0.000	0.161	0.006	0.053
5	<i>C. a. robustus</i>	0.965	0.000	0.433	1		0.625	0.028	0.417
6	<i>C. a. cay</i>	0.899**	0.279*	0.573**	0.756	0.444		0.048	0.114
7	<i>C. xanthosternos</i>	0.989**	0.592**	0.952**	0.988**	0.946	0.912*		0.982
8	<i>C. nigrinus</i>	0.958**	0.532**	0.927**	0.904**	0.545	0.814*	0.836	

* $p < 0.05$, ** $p < 0.001$. inf. = Infinite.

thrix at the same mitochondrial gene. These authors estimated 1 mutation every 191,000 years at the *mtCOII* gene for *Lagothrix*. Similarly for *Aotus* and other Neotropical primates, Ashley and Vaughn [1995] and Ruiz-García et al. [2010a, b, c, 2012] determined 1 mutation every 199,000 years at this same mitochondrial gene. Therefore, we employed 1 mutation each 195,000 years for the tufted capuchins.

Results

Of all the evolutionary mutation models tested, the model selected was GTR with the AIC = 4,464.204 and LnL = -2,226.102. The homogeneity pattern test indicated no significant difference in mutation rates on different branches of the tree.

Genetic Diversity and Heterogeneity Statistics

For the overall set of tufted capuchins studied, 19 haplotypes were found with $S = 78$, $\pi = 0.0123$ and $K = 7.224$.

Table 3 shows the genetic diversity statistics of each of the different lineages found in this work. The French Guyana and Negro River group showed the highest levels of genetic diversity ($S = 32$; $\pi = 0.0238$; $K = 14$), followed by *C. nigrinus* ($S = 10$; $\pi = 0.017$; $K = 10$), while the Colombian Eastern Llanos group had the lowest genetic diversity ($S = 1$; $\pi = 0.00024$; $K = 0.1429$). Table 4 shows the overall genetic heterogeneity among all the tufted capuchin groups analyzed. All the statistics analyzed yielded significant values. All the gene flow estimates from different statistics presented values lower than 1, which means that gene flow is scarce among the different tufted groups that were analyzed globally. Therefore, these groups are, genetically speaking, different lineages. Table 5 shows the genetic heterogeneity between the pairs of the groups considered. The majority of these statistics was significant providing credibility that most of these groups represented different lineages.

Fig. 2. Assignment analyses with the Structure program. **a** Including the 49 individuals studied. The best analysis was with $K = 5$ populations. Pink = *C. nigrinus* (1); red = *C. a. fatuellus* (2); green = *C. libidinosus pallidus* (3), *C. a. macrocephalus* (4), *C. a. cay* (5) and *C. robustus* (7); blue = *C. a. apella* (6); yellow = *C. xanthosternos* (8). **b** Excluding the most differentiated individual of *C. a. apella* (French Guyana). The best analysis was with $K = 5$ populations. Yellow = *C. nigrinus* (1); blue = *C. a. fatuellus* (2); pink = *C. libidinosus pallidus* (3), *C. a. macrocephalus* (4), *C. a. cay* (5) and *C. robustus* (7); red = *C. a. apella* (6); green = *C. xanthosternos* (8). In both analyses, only the *C. a. apella* individuals showed high probabilities to belong to the *C. a. fatuellus* and *C. a. macrocephalus* taxa. Colors refer to the online version only.

The analysis with the Structure program showed that the highest likelihood value corresponded to $K = 5$ (table 6; fig. 2). *C. nigrinus* and *C. xanthosternos* were completely discriminated in 2 different groups. The third differentiated group contained *C. a. fatuellus* (all the animals from the Colombian Eastern Llanos and northern Colombian Amazon following Hill [1960]). The fourth group consisted of taxa that we assigned a priori to *C. macrocephalus*, *libidinosus pallidus*, *cay* and *robustus* (following the nomenclature of Silva Jr. [2001]). Finally, the fifth group consisted of animals classified a priori as *C. apella apella* because they had the requisite phenotype and were from the geographical area where Linnaeus in 1758 originally described *C. apella*. The animals classified as *nigrinus*, *xanthosternos*, *fatuellus* and *macrocephalus-libidinosus-cay-robustus* showed very high assignment probability to their corresponding groups. However, in the *C. a. apella* group, 1 animal classified morphologically and geographically as *C. a. apella* from the Negro River (Novo Airao, Brazil) showed a high probability of being related to the group of *fatuellus* (90.8%). Also, 3 animals from French Guyana classified as *apella* showed elevated probabilities of membership in 2 other groups. All three of these individuals showed a high probability of being part of the *fatuellus* group (83.8, 51.7 and 83.3%) and also the likelihood of being in

Table 6. Results of the Structure program to determine the greatest likelihood number of different genetic taxa inside the *apella* complex analyzed

NP	ELn	MLn	VLn	Mα
Analysis A				
K = 1	-704.5	-686.8	35.5	–
K = 2	-460.6	-442.0	37.0	0.0425
K = 3	-391.0	-363.6	54.9	0.0289
K = 4	-326.2	-276.4	254.3	0.0309
K = 5	-323.8	-199.1	94.8	<i>0.0281</i>
K = 6	-350.9	-199.4	303.0	0.0277
K = 7	-362.9	-203.3	319.2	0.0269
K = 8	-374.2	-205.7	337.0	0.0266
K = 9	-328.4	-210.0	236.9	0.0264
K = 10	-330.2	-209.6	261.2	0.0272
K = 11	-333.0	-215.2	235.5	0.0261
K = 12	-346.1	-200.1	292.0	0.0268
Analysis B				
K = 1	-552.7	-539.6	26.1	–
K = 2	-367.8	-352.5	30.5	0.0436
K = 3	-360.0	-280.1	159.9	0.0292
K = 4	-265.6	-179.9	171.5	0.0303
K = 5	-177.0	-135.9	74.5	<i>0.0285</i>
K = 6	-188.3	-151.0	82.1	0.0294
K = 7	-181.5	-137.5	88.1	0.0286
K = 8	-195.2	-156.0	78.4	0.0272
K = 9	-189.1	-141.9	94.5	0.0281
K = 10	-190.2	-142.8	94.9	0.0278
K = 11	-189.4	-143.8	91.1	0.0277
K = 12	-210.6	-145.4	130.3	0.0277

The results were obtained for USEPOPINFO = 0, where no prior information is given in the analysis. Analysis A: analysis carried out with the 49 individuals analyzed; the best result was that with K = 5. Analysis B: analysis carried out with 48 individuals, extracting the most divergent animal inside the group of *C. a. apella*. NP = Number of different groups; ELn = estimated Ln probability of the data; MLn = mean value of the Ln likelihood; VLn = variance of the Ln likelihood; Mα = mean value of alpha. The best results are given in italics.

the *macrocephalus-libidinosus-cay-robustus* group (15.6, 8.2 and 15.7% respectively). If we eliminate the most differentiated individual of *C. apella apella* in this analysis because it showed a very divergent sequence, the most probable number of populations was again K = 5: *C. nigritus*, *C. xanthosternos*, *C. a. apella*, *C. a. fatuellus*, and the group of *macrocephalus-libidinosus-cay-robustus*.

Phylogenetic Analyses

The maximum likelihood and the maximum parsimony trees offered the same results with similar bootstrap percentages. We observed that all the samples ana-

lyzed from the Amazon basin (with the exception of that sampled in the Negro River and in the northern Colombian Amazon) conformed to a clear lineage, across the Peruvian, Bolivian and southern Colombian Amazon as well as in the Juruá River in the western Brazilian Amazon. This ensemble represented *C. a. macrocephalus*. All the samples morphologically classified by other authors as *C. a. maranonis*, *C. a. peruanus*, *C. libidinosus pallidus* and *C. libidinosus juruanus* were assigned to this group. The sequence from one *C. robustus* sampled in Espiritu Santo was also found in this cluster. The animals from the Bolivian and northwestern Argentine Yungas also belonged to this cluster, and they showed an elevated bootstrap percentage.

The sequences obtained from samples originating in French Guyana and from the Negro River (*C. apella apella*) as well as those that came from the Colombian Eastern Llanos and northern Colombian Amazon (*C. a. fatuellus*) formed 1 cluster with 2 subclusters well defined, although the genetic heterogeneity between these 2 clusters was small.

The next group comprised all the animals sampled in Mato Grosso do Sul and Paraguay. The bootstrap percentage of this cluster was not so elevated and the group occurred in different positions depending on the different analyses carried out. We named this lineage *C. a. cay* following Hill [1960]. The most divergent taxa were represented by the sequences of *C. nigritus* from Argentina (100% bootstrap) and by the sequences of *C. xanthosternos* (100% bootstrap) from eastern Brazil. Clearly, these forms represented 2 distinct clades from *C. apella* (fig. 2).

The Bayesian tree created with the BEAST program showed that the main groups described in the previous analyses had an elevated posterior probability density mean (fig. 3). This Bayesian tree showed similar results to the previous trees. The most divergent *Cebus* group was *C. nigritus* (with a posterior probability, $p = 1$). The next most divergent group was *C. xanthosternos* ($p = 1$) and the next was the individuals assigned to *C. a. cay* ($p = 0.82$). The next significant cluster was composed of all the other analyzed *C. apella* individuals ($p = 0.93$). This significant cluster was composed of 2 other subgroups. One was the Amazonian *C. a. macrocephalus* group ($p = 0.13$), that had a subcluster, the Bolivian-Argentine Yungas group, with a very elevated posterior probability ($p = 1$) as well as other animals from northern and central Bolivia, Peru and the Brazilian Javari and Juruá rivers, with a very low posterior probability ($p = 0.03$). The other group was composed of 2 northern Amazon River subclusters ($p = 1$; *fatuellus* in the Eastern Colombian Llanos and north of the Colombian Amazon, $p = 0.96$, and *apella* in the northern Brazilian Amazon and French Guyana, $p = 0.47$). Within these 2 last northern Amazon River groups, no genetic structure was observed because the posterior probabilities were very small and not related to the geographic origins of the animals. The *Saimiri* outgroup also showed an elevated posterior probability ($p = 1$) as did the *Aotus* outgroup ($p = 1$).

The results of SOWH, SH and Templeton tests did not support the taxonomic schemes suggested by either Groves or Silva Jr., and maximum parsimony trees were significantly different at the 0.001 level (34,144 and 63,238 log likelihood units, respectively). However, the scheme suggested by Groves (4 species) fitted better with our data than did that of Silva Jr., who proposed 7 species (8,433 log likelihood units, $p < 0.05$).

The median joining network showed 2 noteworthy results (fig. 4). First, the main Amazonian *C. a. macrocephalus* haplotype was the most common haplotype and the one with the most extensive geographic distribution. This haplotype is con-

Fig. 3. Maximum parsimony tree with the *mtCOII* gene sequences of 49 *C. apella* and related taxa. Several sequences of *Saimiri* (*S. boliviensis peruvensis* and *S. macrodon*) and *Aotus* (*A. nigriceps*, *A. nancymae* and *A. vociferans*) were employed as outgroups.

Fig. 4. Bayesian tree with the *mtCOII* gene sequences of 49 *C. apella* and related taxa. Several sequences of *Saimiri* (*S. boliviensis peruvensis* and *S. macrodon*) and *Aotus* (*A. nigriceps*, *A. nancymae* and *A. vociferans*) were employed as outgroups. The numbers on the nodes are those posterior probabilities with the highest values.

nected to other haplotypes discovered in the upper Amazon, as well as to that of the eastern *C. a. robustus* from Espiritu Santo, the main haplotype of *C. a. cay* and the main haplotype of *C. a. apella* from the northern Amazon River. Also, the main *C. a. cay* haplotype is connected with the most divergent haplotypes of *C. nigrinus* and of *C. xanthosternos*. These *nigrinus* and *xanthosternos* haplotypes are probably extremely divergent because the genetic drift was very intense during their speciation processes. The main *C. a. apella* haplotype is highly related with the main haplotype of the Colombian *C. a. fatuellus*. Second, the temporal splits obtained to apply the ρ statistics to the median joining network analysis were as follows: the main Amazonian *C. a. macrocephalus* haplotype diverged $83,571 \pm 41,786$ years ago (YA; $\rho = 0.4286 \pm 0.2143$) from the most divergent upper Amazonian haplotype (presumably also from a *C. a. macrocephalus*), $16,250 \pm 16,250$ YA ($\rho = 0.0833 \pm 0.0833$) from the *C. a. robustus* haplotype, $30,000 \pm 30,000$ YA ($\rho = 0.1538 \pm 0.1538$) from the main *C. a. cay* haplotype, $60,000 \pm 42,426$ YA ($\rho = 0.3077 \pm 0.2176$) from the main French Guyana *C. a. apella* haplotype, $276,250 \pm 62,936$ YA ($\rho = 1.4167 \pm 0.3227$) from the less divergent *C. xanthosternos* haplotype and $357,000 \pm 39,804$ YA ($\rho = 1.8333 \pm 0.2041$) from the less divergent *C. nigrinus* haplotype. Additionally, the main French Guyana *C. a. apella* haplotype diverged from the main Colombian *C. a. fatuellus* haplotype $169,000 \pm 169,000$ YA ($\rho = 0.8667 \pm 0.8667$).

Discussion

Molecular Phylogenetics of the Tufted Capuchins

It is clear that there are 2 closely related clades in the northern Amazon River: *C. a. apella* (from French Guyana to, at least, the Negro River in the northern Brazilian Amazon) and *C. a. fatuellus* (in all the Colombian Eastern Llanos and northern part of the Colombian Amazon), but the two groups are genetically different.

In the Amazon basin (at least, in the western Amazon of Bolivia, Peru, Colombia and Brazil), there is a unique group with one widely distributed haplotype. Therefore, all the *C. apella* samples within this vast area were assigned to *C. a. macrocephalus*, because this seems to be the oldest subspecific name for whatever *C. apella* taxa that resides in this area of South America. However, if the eastern *robustus* form is inside this group, *robustus* (Kuhl, 1820) is older than *macrocephalus* (Spix, 1823), which means that *robustus* takes precedence. Nevertheless, we studied only 1 *robustus* specimen and our result needs to be confirmed by future studies.

Aquino and Encarnación [1994], following a personal communication from Hershkovitz, affirmed that 5 *C. apella* subspecies could be found in Peru: (1) *C. a. maranonis* in the north of the Marañón and Amazon rivers, along both banks of the Huallaga River, and the Pachitea River to the south; (2) *C. a. macrocephalus* on the right bank of the Ucayali River and from the Amazon River in the north to the Alto Purús River in the south; (3) *C. a. peruanus* from the Alto Purús River to the Madre de Dios River and the Inambari River (in the Department of Puno); (4) *C. a. pallidus* (for other authors, *C. libidinosus pallidus*), from the Madre de Dios River to the major part of northern and middle Bolivia, and (5) an unknown possible subspecies in the mountain forests (800–1,000 m above sea level) of the Departments of Huánuco, Pasco and Junín. Our molecular study covered all the geographical range of the 'supposed' first 4 subspecies and the analyzed mtDNA gene detected only 1 *C. apella*

Fig. 5. Median joining haplotype network with the 19 *C. apella* haplotypes found. Blue = *C. nigrilus*; green = *C. xanthosternus*; pink = *C. apella cay*; brown = *C. apella apella*; yellow = *C. apella macrocephalus*; black = *C. apella fatuellus*; red = no discovered intermediate haplotypes. Numbers in red are nucleotide mutant sites. The surfaces of the circles (haplotypes) are proportional to the number of individuals which shared a given haplotype. Colors refer to the online version only.

lineage in the combined areas of the Peruvian Amazon, the northern Bolivian Amazon and the central Bolivian Amazon. Thus, we disagree with Groves' [2001] suggestion that 2 subspecies (*C. a. macrocephalus* and *C. a. peruanus*) exist in the forests of Peru. Silva Jr. [2001] considered *C. a. macrocephalus*, *C. a. peruanus* and *C. libidinosus juruanus* to be a single taxon, *C. macrocephalus*. Our molecular results agree quite well with the fact that all these taxa are a single population, *C. a. macrocephalus*. We did not analyze any individuals of the 'supposed' fifth subspecies. Therefore, one pending analysis could be to add some samples from the Peruvian Departments of Huánuco, Pasco and Junín.

C. libidinosus juruanus was maintained by Rylands et al. [2000] and Groves [2001] as a good taxon. Nevertheless, Silva Jr. [2001] claimed that it belonged to *macrocephalus*. The molecular data revealed, without any doubt, that the animals of the Juruá River belong to *C. a. macrocephalus*. Therefore, the enigma highlighted by Rylands et al. [2005] over the isolation of *C. libidinosus juruanus* from other *libidinosus* subspecies to the north of the Rio Madre de Dios in Bolivia and Peru is now resolved. The *libidinosus* and the *juruanus* taxa seem to be artificial ones and are not supported by this first molecular study.

C. robustus from eastern Brazil seems to be undifferentiated from the *C. a. macrocephalus* individuals existing in the upper Amazon. One possible explanation of this result could be some kind of contamination. However, this sample had

DNA independently extracted and sequenced 3 times and the results were the same each time. Thus, DNA contamination seems not to be a plausible explanation. This could be the first evidence that *C. robustus* is a very recent introgression (or mtDNA introgression) from the aforementioned Amazonian group towards eastern Brazil. If so, this eastern Brazilian tufted capuchin could be named *C. a. robustus* or, perhaps, should not receive any specific name (an eastern population of *C. a. macrocephalus*). We prefer this last possibility. Thus, we negate *C. robustus* as a full species or as a subspecies of *C. nigrilus*. However, more animals of *C. robustus* must be sequenced and analyzed to corroborate our affirmation. But the fact that Kinzey [1982] recorded a specimen from Tomas Gonzaga, near Corinto, as a hybrid of *C. a. robustus* $\times$ *C. a. libidinosus*, and considering that *libidinosus* is probably molecularly undifferentiated from *C. a. macrocephalus*, this could confirm that *C. robustus* is a simple recent eastern extension of *C. a. macrocephalus*. In addition, Amaral et al. [2008], showed that *C. a. cay* presented a very similar karyotype to that of *C. a. robustus*, both having the same 12 synteny blocks with reference to humans and 18 identical conserved segments with regard to *Saguinus oedipus*. Additionally, *cay* and *robustus* shared 2 synapomorphic traits, the association 14/15/14, resulting in a submetacentric chromosome and the pericentric inversion that corresponds to the HSA8b probe. Given that the genetic differentiation between *cay* and *macrocephalus* is very limited, the high similarity between *robustus* and *macrocephalus* is not rare.

The animals sampled in southern Brazil and Paraguay composed 1 gene group in all the analyses carried out. This group was also related to the upper Amazon *C. a. macrocephalus*. Our molecular analysis showed that this gene lineage had its own characteristics but its divergence from other *C. apella* lineages was low. This is additional proof that *libidinosus* is an artificial taxon that does not possess sufficient genetic characteristics to be considered a full species as stated by Cabrera [1917]. We named this lineage *C. a. cay* (= *paraguayanus*). It is unclear whether the northwestern Argentinean *C. apella* population is a southern extension of *C. a. macrocephalus* or a disconnected *C. a. cay* population. Our genetic results showed that the animals from the Bolivian-Argentine Yungas belonged to the western Amazon *macrocephalus* group and not to the *C. a. cay* group. Thus, the affirmation of Avila [2004] that the population of the southern Yungas in Bolivia and northwestern Argentina must be named *C. l. pallidus* (like the northern Bolivian population) and not *C. l. paraguayanus* must be reevaluated. Our molecular study ratified the karyotypic studies of Mudry [1990] and Zunino and Mudry [1993] that determined differences between the Paraguayan and eastern Argentinean population from the southern Bolivian and northwestern Argentinean population.

Our genetic analysis clearly differentiated *C. xanthosternos* from other *C. apella* lineages. In this case, the striking phenotypic differences of this taxon (large body, round head, smooth forehead and crown with short hair – without tufts or lateral crests; large area of yellowish tinge on the forehead and temples; shoulders, front of arms and chest ranging from pale yellow to orange), as described by Rylands et al. [2005] agree quite well with the genetic differences we detected. Also, our genetic analysis agrees with the assertions of Torres de Assumpcao [1983, 1988], Coimbra-Filho [1986], Seuánez et al. [1986], Rylands et al. [1988] and Kierulff et al. [2004] that *C. xanthosternos* and *C. a. robustus* are different lineages, although they are relatively near geographically speaking. Seuánez et al. [1986] determined important dif-

ferences at the chromosomal level between *C. xanthosternos* and other lineages of *C. apella*. It could be interesting to analyze molecularly *C. flavia* (also known as *C. queirozi* [Mendes et al., 2006]), a new species of the *apella* complex, which has been recently rediscovered [Oliveira and Langguth, 2006] from the Pernambuco endemism center (Brazilian states of Alagoas, Pernambuco, Paraíba and Rio Grande do Norte – north of the Sao Francisco River).

Our genetic analysis showed that the two samples from the Iguazú National Park in the Argentinean province of Misiones were differentiated from the other *C. apella* specimens. This result accords quite well with those results obtained by Mudry et al. [1991], who determined that the group they named *C. a. vellerosus* (described by other authors as *C. nigrinus nigrinus* or *C. n. cucullatus*) showed a different G banding for chromosome 11 compared to other *C. apella* populations. Additionally, the C banding in this group showed the loss of the large heterochromatic block on chromosome pair 11 present in other *C. apella* lineages. This taxon also showed 8 small and 8 large acrocentric chromosomes which disagrees with that determined for other *C. apella* groups, which have 7 small and 9 large acrocentric chromosomes [Freitas and Seuánez, 1982; Mantecón et al., 1984; Matayoshi et al., 1986]. Furthermore, chromosomes 17 and 20 showed inconsistent heterochromatic blocks, which are absent in other *C. apella* populations, as well as showing 3 of the small acrocentrics which correspond to pairs 22 and 23 and show a secondary constriction not present in other forms of *C. apella*. These chromosomal characteristics of *C. nigrinus* (= *vellerosus*) clearly contrasted with some of the chromosomal characteristics of *C. a. cay* (= *paraguayanus*) found by Seuánez et al. [1983] and Matayoshi et al. [1986], although their geographical distribution could overlap in some areas of eastern Paraguay and northeastern Argentina. This is similar to the pattern we determined for the *mtCOII* gene. Similarly, Ferrucci et al. [1995] demonstrated that the *HaeIII* enzyme pattern differentiated *nigrinus* and *cay* from Argentina.

The genetic differentiation (and genetic distances) among the diverse tufted capuchin taxa detected in this study (19 haplotypes, $S = 78$, $\pi = 0.0123$ and $K = 7.224$) was substantially lower than those values found for different subspecies of *C. albifrons* with the same molecular marker, although the geographic distribution of *C. albifrons* is more limited than that of the tufted capuchins (for *C. albifrons*, 55 haplotypes, $S = 361$, $\pi = 0.0319$ and $K = 22.068$ [Ruiz-García et al., 2010a]). For instance, by using the Kimura 2-parameter distance, we obtained the following genetic distances for diverse tufted capuchin lineages: *C. a. macrocephalus* versus *C. a. cay* = 0.002, *C. a. macrocephalus* versus *C. a. apella* = 0.007, *C. a. fatuellus* versus *C. a. cay* = 0.007. When the 2 most differentiated tufted capuchins were compared, the genetic distances were higher (*C. a. macrocephalus* vs. *C. xanthosternos* = 0.030 or *C. a. macrocephalus* vs. *C. nigrinus* = 0.035). When the same genetic distance was applied to the diverse gene lineages detected in *C. albifrons*, we obtained the following results: *C. albifrons versicolor* versus *C. albifrons leucocephalus* (recall that these two subspecies were classified as the same subspecies by Groves [2001]) = 0.018, *C. a. versicolor* versus *C. a. cesarae* (also classified as the same subspecies) = 0.024, or *C. a. malitosus* versus *C. a. leucocephalus* = 0.061. If we consider all the studied tufted capuchins as different species (as was proposed most recently by Silva Jr. [2001]), then all the subspecies of *C. albifrons* studied by Ruiz-García et al. [2010a] must be considered new species (*C. malitosus*, *C. cesarae*, *C. versicolor*, *C. leucocephalus*, and so on) because the molecular differences among them are considerably

higher than the majority of the genetic differences found among the tufted capuchin groups analyzed here. For this reason, following Cabrera [1957] and Hill [1960], we reduced most of the tufted capuchin groups studied to *C. apella*, with the exception of *C. nigrurus* and *C. xanthosternus*, which have greater genetic differentiation relative to the other tufted capuchin groups studied as well as some striking morphological differences. Both *C. nigrurus* and *C. xanthosternus* also occupy some restricted habitats in specific geographic areas of eastern and southern Brazil and northeastern Argentina on the boundaries of the southern geographical distribution of the tufted capuchins in South America. We consider that although they could potentially hybridize with other *C. apella* groups, *C. xanthosternus* and *C. nigrurus* could be considered different species from *C. apella* following the BSC. Related to this, Boubli et al. [2012] considered that the different taxa of *C. albifrons* that we molecularly characterized in northern Colombia and in the western Amazon area [Ruiz-García et al., 2010a] should be considered full species following the PSC. These were the same taxa that we maintained as subspecies following the nomenclature of other authors that had previously studied these animals from a morphological perspective. They defined many new species as *C. versicolor*, *C. cesarae*, *C. adustus*, *C. unicolor*, *C. yuracus*, and so on. If we employ the same species criteria, we could define a minimum of 7 or 8 species inside the *apella* complex. It should be noted that there will probably be many more species because we have not included all the possible *apella* taxa in this study. However, we disagree with the conclusions of Boubli et al. [2012] because we have observed fertile hybrids of the supposed different species (*C. versicolor*-*C. cesarae*, *C. versicolor*-*C. leucocephalus*, *C. yuracus*-*C. cuscinus* for instance) in nature as well as observed diverse animals representing these supposed different species living in the same troop in truly natural conditions. Additionally, in the Colombian zoos, there are dozens and dozens of hybrids of these presumed species maintained during many generations in captivity. These hybrids are viable and fertile. Also, the vocalizations and behaviors of the hybrids, their parents, and the hybrids' descendants are the same [Ruiz-García, unpubl. observations]. All of these facts, combined with the consideration that reproductive isolation is a critical criterion in the BSC [Mayr, 1942], make us question whether these different *C. albifrons* populations are really different species. If this is highly plausible for *C. albifrons*, it is even more so for the *apella* complex, where the molecular genetic differences are considerably smaller than in *C. albifrons*. The general consensus among scientists regarding humans is that there is only 1 species (*Homo sapiens*) with no living subspecies, and there is no attempt to apply the PSC indiscriminately. If this is the view for our own primate species, then we must disagree with those authors that apply different species rules for other primate species. Additionally, an indiscriminate application of PSC could generate an unmanageably large number of species (see for instance Garber [2012]). We would be negligent to forget the great number of problems and confusion caused by using primitive typological classifications in the past (see for example the case of the 86 supposed species of brown bears in North America alone [Merriam, 1918]). The primatologists who follow the PSC, upgrade all the primate subspecies to species, but we consider that this is only moving the problem up one level as it obscures the reality of a real evolutionary unit. The BSC should not be ignored just because for some taxa it is not easily translated into an operational definition.

Temporal Splits among Different Tufted Capuchin Taxa

All the splits among tufted capuchins were in the later part of the Pleistocene. The 2 most differentiated lineages, which showed the highest temporal split estimates, were the ancestors of *C. nigrinus* (357,000 YA) and of *C. xanthosternos* (276,000 YA). In the Yarmouthian interglacial period (0.2–0.38 million YA), precipitation increased and the forests expanded [Van der Hammen, 1992]. However, in the Amazon and in the Atlantic forests, there were some dry peaks within the Yarmouthian interglacial period at 0.36, 0.30 and 0.24 million YA. At least twice, these forests were fragmented in the dry periods [Van der Hammen et al., 1991; Van der Hammen, 1992], and these 2 lineages could have been isolated from the main Amazon populations. These events could have prompted the emergence of the ancestors of *C. nigrinus* and *C. xanthosternos*. These climatic shifts were caused by the Milankovitch cycles operating across the Quaternary, with cold and dry phases, potentially generating refuges in the Amazon and in other areas of South America [Kinzey, 1982; Whitmore and Prance, 1987; Haffer, 1997]. Therefore, the Pleistocene forest refugia invoked by Haffer [1969, 1982] could be responsible for a considerable degree of the fragmentation of *C. apella*. The last glaciation (Wisconsin-Würm), from 130,000 to 10,000 YA, had a maximum glacial peak 18,000 YA and could have generated a large fraction of the current *C. apella* lineages. In the lower pleniglacial period, some glacial peaks (with their corresponding dry period) appeared 90,000–70,000 and 60,000–40,000 YA. This could have generated the separation of some diverse haplotypes within the *macrocephalus* lineage (84,000 YA) in the upper Amazon and the separations of the *C. a. apella* ancestral haplotypes (60,000 YA). Another dry peak was reached 33,500–26,500 YA [Liu and Colinvaux, 1985]. The separation of the ancestors of *C. a. cay* could have occurred in this period (30,000 YA). Finally, the upper pleniglacial period, around 22,000–14,000 YA, concomitant with the major cold and dry period (20,000–18,000 YA) [Climap, 1976; Brown, 1982; Van der Hammen et al., 1991; Van der Hammen, 1992], could have separated the *robustus* haplotype (16,000 YA) from *C. a. macrocephalus*, which had expanded widely throughout the Amazon basin. Therefore, the Pleistocene refugia theory could play an important role in the diversification of the tufted capuchins.

In conclusion, other molecular markers, as well as a more extensive and diversified number of samples, are needed to reconstruct the real phylogenetic relationships of the tufted capuchins as well as the real time splits among the different lineages analyzed. However, this was a first molecular genetics attempt to reconstruct the phylogenetics and the phylogeography of the tufted capuchins and thus could be useful for future studies with more molecular markers and more samples representing more taxa. The use of a greater number of molecular markers (and more diversified: nuclear DNA, MHC genes, introns of chromosomes X and Y) along with more representative samples could indicate if the relationships that we showed with the *mtCOII* gene are real or not. Also it is important in the future to carry out additional analyses (such as the application of the Migrate-N program), when the sample sizes within each lineage are enlarged and thus make it possible to analyze the levels of hybridization among different populations of tufted capuchins; this in turn could help to determine which taxa are really significant species or lineages with differentiated evolutionary trajectories.

Acknowledgments

Thanks go to Dr. Diana Alvarez, Pablo Escobar-Armel, Luisa Fernanda Castellanos-Mora and Alexandra Parra for their help in obtaining *C. apella* samples during the last 12 years. Thanks go also to Dr. Benoit de Thoisy, who provided 4 samples from French Guyana and to Dr. Alcides Pissinatti, who provided the 2 samples of *C. xanthosternos*. Many thanks go to the Peruvian Ministry of Environment, to the PRODUCE (Dirección Nacional de Extracción y Procesamiento Pesquero from Peru), the Consejo Nacional del Ambiente and the Instituto Nacional de Recursos Naturales and to the Colección Boliviana de Fauna (Dr. Julieta Vargas) for their role in helping us obtain collection permits in Peru and Bolivia. The first author also thanks the Ticuna, Yucuna, Yaguas, Witoto and Cocama Indian communities in the Colombian Amazon, the Bora, Ocaina, Shipibo-Comibo, Capanahua, Angoteros, Orejón, Yaguas, Cocama, Kishuarana and Alama in the Peruvian Amazon, the Sirionó, Canichana, Cayubaba and Chacobo in the Bolivian Amazon and the Marubos, Matis, Mayoruna, Kanaimari, Kulina, Maku and Waimiri-Atroari communities in the Brazilian Amazon. Dr. Joseph Shostell helped with the English syntax.

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