



Explosive Pleistocene range expansion leads to widespread Amazonian sympatry between robust and gracile capuchin monkeys

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ABSTRACT

Aim Capuchin monkey species are widely distributed across Central and South America. Morphological studies consistently divide the clade into robust and gracile forms, which show extensive sympatry in the Amazon Basin. We use genetic data to test whether Miocene or Plio-Pleistocene processes may explain capuchin species' present distributions, and consider three possible scenarios to explain widespread sympatry.

Location The Neotropics, including the Amazon and Atlantic Coastal Forest.

Methods We sequenced the 12S ribosomal RNA and cytochrome *b* genes from capuchin monkey specimens. The majority were sampled from US museum collections and were wild-caught individuals of known provenance across their distribution. We applied a Bayesian discrete-states diffusion model, which reconstructed the most probable history of invasion across nine subregions. We used comparative methods to test for phylogeographic association and dispersal rate variation.

Results Capuchins contained two well supported monophyletic clades, the morphologically distinct 'gracile' and 'robust' groups. The time-tree analysis estimated a late Miocene divergence between *Cebus* and *Sapajus* and a subsequent Plio-Pleistocene diversification within each of the two clades. Bayesian analysis of phylogeographic diffusion history indicated that the current wide-ranging sympatry of *Cebus* and *Sapajus* across much of the Amazon Basin was the result of a single explosive late Pleistocene invasion of *Sapajus* from the Atlantic Forest into the Amazon, where *Sapajus* is now sympatric with gracile capuchins across much of their range.

Main conclusions The biogeographic history of capuchins suggests late Miocene geographic isolation of the gracile and robust forms. Each form diversified independently, but during the Pleistocene, the robust *Sapajus* expanded its range from the Atlantic Forest to the Amazon, where it has now encroached substantially upon what was previously the exclusive range of gracile *Cebus*. The genus *Cebus*, as currently recognized, should be split into two genera to reflect the Miocene divergence and two subsequent independent Pliocene radiations: *Cebus* from the Amazon and *Sapajus* from the Atlantic Forest.

Keywords

Amazon Basin, Atlantic Forest, *Cebus*, Neotropical primates, Platyrrhini, Pleistocene expansion, *Sapajus*, sympatry.

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INTRODUCTION

Capuchin monkeys are widely distributed across Central and South America. Elliot (1913, p. 77) separated them into two groups: those with 'heads without tufts on male' (Group A) and those with 'heads with tufts or ridges on male' (Group B). This turned into a division of so-called 'untufted' and 'tufted' capuchins, adopted by Hershkovitz (1949), Hershkovitz (1955) and Hill (1960), and used ever since. Hershkovitz (1949) recognized four species, one tufted (*Cebus apella*) and three untufted [*Cebus albifrons*, *Cebus capucinus* and *Cebus nigritatus* (= *olivaceus*)]. Not all 'tufted' capuchins actually have hair tufts, however (for example, *Cebus xanthosternos*), and some members of the 'untufted' group (A) have tufts (for example, female *Cebus olivaceus*). What, in fact, best divides these two groups is their anatomy: Elliot's Group A is more slender and gracile compared with Group B, which is more robust in both cranial and postcranial morphology (Silva, 2001). Groves (2001) used coat colour variation and tuft shape to divide the tufted or 'robust' capuchins into four species: *C. apella*, *C. libidinosus*, *C. xanthosternos* and *C. nigritus*. Silva (2001) placed this group in its own subgenus, *Sapajus* Kerr, 1792, with seven species: *Cebus* (*Sapajus*) *macrocephalus* and *C. (Sapajus) apella* in the Amazon, *C. (Sapajus) libidinosus* and *C. (Sapajus) cay* in the Caatinga, Cerrado and Chaco habitats, and *C. (Sapajus) xanthosternos*, *C. (Sapajus) robustus* and *C. (Sapajus) nigritus* in the Atlantic Coastal Forest (Fig. 1b). Marcgrave's capuchin, *Cebus flavius* (Schreber, 1774), also a robust capuchin, was recently rediscovered in north-east Brazil (Mendes Pontes *et al.*, 2006; Oliveira & Langguth, 2006).

Capuchin taxonomy is contentious (Hill, 1960; Rylands *et al.*, 2000), due in part to extreme intraspecific and even within-population variation in phenotypes (Hill, 1960; Torres de Assumpção, 1983; Rylands *et al.*, 2005) and also to extended life history, with delayed maturation and distinct differences in appearance among age and sex classes (Izawa, 1980). All treatments based on morphology, however, recognize the division of robust and gracile forms (Hill, 1960; Rylands *et al.*, 2000; Groves, 2001; Silva, 2001). Here we refer to *Cebus* for the gracile (or untufted) capuchins and *Sapajus* for the robust (or tufted) capuchins, for clarity.

Both forms are found throughout much of the Amazon Basin. Robust species also extend into the dry Caatinga and Cerrado of Brazil, the Atlantic Forest, and as far south as Paraguay and northern Argentina, while the gracile species extend across the Andes in western Ecuador, northern Colombia and north-western Venezuela, into Central America (Fig. 1). The area of sympatry between the robust and gracile forms is strikingly large, exceeding that found in any other genus of Neotropical primate, but there have been no well supported hypotheses to explain the current geographic distribution or extent of sympatry of capuchins. While it has been assumed that capuchins originated in the Amazon (Kinzey, 1982), there has been no attempt to indicate the timing of capuchin invasions, either north-west across the Andes and into Central America, or south-east into the dry Cerrado and the Atlantic Coastal Forest.

Here we develop several hypotheses regarding the chronology and biogeography underlying present-day capuchin distributions. One of the longest-standing explanations is that

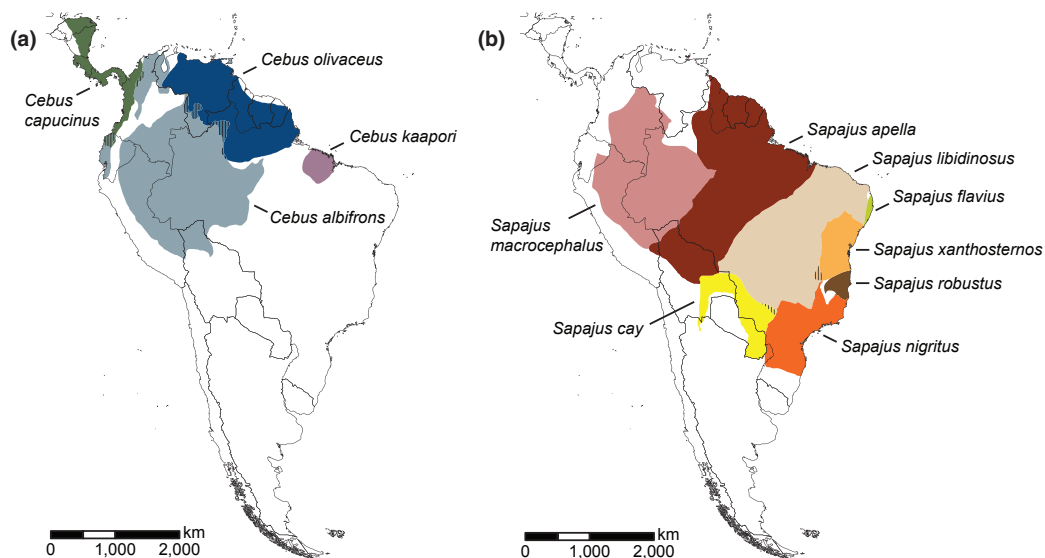


Figure 1 Approximate distribution of (a) *Cebus* and (b) *Sapajus* species in the Neotropics. Species distributions follow IUCN Red List maps (IUCN, 2011), with minor corrections, using the taxonomy of Silva (2001, 2002), with the addition of the newly rediscovered *Sapajus flavius*. Hashes symbolize intrageneric sympatry. Note the extreme overlap between (a) *Cebus* and (b) *Sapajus* species in the Amazon Basin.

they have colonized their present ranges as a result of dispersal from Pleistocene forest refugia (Kinzey, 1982). Diverse lines of evidence suggest that during much of the Pleistocene (1.64 Ma to 10 ka), arid climates prevailed in the Neotropics, although there is also evidence against this conclusion (Knapp & Mallet, 2003). Schneider *et al.* (2001) estimated that divergence within each extant platyrrhine genus occurred sometime during the Plio-Pleistocene, and Kinzey (1982) argued for the importance of Pleistocene forest refugia in influencing distributions of the species seen today.

There have been strong arguments against Pleistocene rain forest fragmentation and refugia in the Amazon and beyond (Colinvaux *et al.*, 2000). Molecular phylogenetic studies of a variety of Amazonian rain forest taxa have indicated that most divergences between sister species of rain forest vertebrates significantly pre-date the Pleistocene (Boubli & Ditchfield, 2000; Moritz *et al.*, 2000; Patton *et al.*, 2000; Salazar-Bravo *et al.*, 2001; Cheviron *et al.*, 2005; Ribas *et al.*, 2005). In their biogeographic analysis of howler monkeys (*Alouatta*) across Central and South America, Cortés-Ortiz *et al.* (2003) found that the earliest split separated *cis*- and *trans*-Andean clades at about 6.8 Ma, and that divergences among clades within the *cis*-Andean region were also relatively old (5.1–2.4 Ma). Collins & Dubach (2000) also found that the four main clades of spider monkeys (*Ateles*) diverged in the Pliocene, around 3.6–3.2 Ma. Chiou *et al.* (2011), however, found that squirrel monkeys (*Saimiri*) diverged in the Pleistocene, with the first split between the clades *Saimiri boliviensis* and *S. sciureus*/*S. oerstedii* at c. 1.5 Ma. Casado *et al.* (2010) gave a provisional estimate of the split between *Sapajus* and *Cebus* as 4.2 Ma, based on their maximum likelihood tree topology for cytochrome *b*, calibrated at 22.0 Ma for the splitting of the Cebidae and a range of 17.0–20.0 Ma for the divergence of capuchin monkeys from squirrel monkeys. In contrast, Chiou *et al.* (2011) estimated the split between capuchins and squirrel monkeys at c. 13.8 Ma. Here we present a formal time scale of divergence among major clades of capuchin monkeys, and use it to identify the major historical events that have shaped their current distributions.

We consider three different models to explain the current distribution of capuchin monkeys and the sympatry of the robust and gracile lineages in the Amazon.

1. 'Out of the Amazon'. The long-standing assumption to explain the observed sympatry of robust and gracile forms is that the Amazon is the centre of origin for all capuchin monkeys, and robust and gracile lineages evolved by allopatric or sympatric speciation in the Amazon Basin. The gracile group subsequently radiated north and the robust radiated south. Predictions of this model include: (1) the Amazon Basin is the ancestral range for capuchin monkeys; (2) the diversification into gracile and robust forms occurred within the Amazon Basin; and (3) a recent invasion of northern South America, Central America, the Cerrado and the Atlantic Forest habitats best explains present-day distributions. Under this hypothesis, we predict that the basal division for capuchins was

north and south of the Rio Amazonas, with (1) gracile forms diversifying north of the Amazon, and later north into the Andes, Central America, and northern Venezuela, as well as south of the Amazon; and (2) the robust form diversifying south of the Amazon, eventually radiating south-east to the Cerrado and Atlantic Forest, as well as north across the Amazon. Note that the lack of samples from south of Rio Amazonas (in Southern Amazonia) limits our ability to test this hypothesis.

2. 'Atlantic versus Amazon'. A second possibility is that vicariance between the Brazilian Atlantic coastal forest and Amazon Basin is the major force shaping capuchin monkey distributions. For many clades of South American mammals and birds, the deepest divergence is found between coastal Brazil and all other lowland tropical forested regions (Patton *et al.*, 2000). Other Neotropical primate genera show this pattern; for example, Tagliaro *et al.* (1997) reported a basal split between Atlantic Forest *Callithrix* and Amazon *Callithrix* (*Mico*) + *Cebuella*. Under this scenario, the robust morphology would be the ancestral condition, and gracile species would have evolved relatively recently from a robust ancestor in the Amazon. Fossil evidence (Kay & Frailey, 1993; Kay & Cozzuol, 2006) has placed the earliest known cebine, †*Acrecebus* (6–9 Ma), as the sister taxon to extant capuchin monkeys [† indicates that the taxon is extinct]. †*Acrecebus*, from Rio Acre in the south-western Amazon Basin, has hyper-robust teeth, with an upper second molar three to four times larger than that found in the average *Cebus capucinus* (see Kay & Cozzuol, 2006). Predictions of the 'Atlantic versus Amazon' model include (1) a basal split within capuchins between robust Atlantic and robust Amazonian forms; (2) paraphyly of robust capuchins, with the robust Atlantic clade as the sister group to the Amazonian gracile + robust clade (i.e. gracile capuchins form a subclade of Amazonian robust capuchins); and (3) relatively recent origin of gracile capuchins.

3. 'Reinvasion of the Amazon'. Under this model, after a basal gracile Amazon–robust Atlantic Forest divergence, the robust Atlantic Forest capuchin evolved in allopatry from the Amazon gracile capuchin, and later re-established sympatry when the robust form expanded across the Cerrado and into the Amazon Basin. Predictions of this model include (1) initial divergence of a robust Atlantic capuchin clade and a gracile Amazon capuchin clade; and (2) robust Amazonian capuchins as a recently evolved subclade nested with the Atlantic Forest robust clade. This type of geographic distribution would be unique among Neotropical primate genera. The high phenotypic diversity within the Atlantic Forest *Sapajus*, in comparison with lower phenotypic diversity across larger regions for the Amazonian *Sapajus* (see Rylands *et al.*, 2005), provides morphological support to the 'reinvasion of the Amazon' hypothesis.

To test these hypotheses, we adopted a comparative statistical approach to construct a time scale for capuchin monkey phylogeographic history. We constructed a molecular phylogeny using mitochondrial DNA sequences (largely from museum specimens) to sample broadly across the ranges of

gracile and robust forms. We time-calibrated this phylogeny, using external calibrations from New World primates, to test whether Miocene or Plio-Pleistocene events underlie capuchin distributions. To infer the phylogeographic history of capuchins and to test models underlying sympatry of gracile and robust forms in the Amazon Basin, we used a recently developed Bayesian phylogeographic approach based on a discrete-states diffusion model (Lemey *et al.*, 2009), a method that allows for inference of spatial history and ancestral location while integrating over uncertainty in topology and other model parameters. We also used coordinate-based likelihood methods to evaluate predictions from the three scenarios to explain sympatry in robust and gracile forms through the use of Bayesian stochastic search variable selection. Our study provides the first general framework for understanding the processes responsible for shaping present-day capuchin monkey distributions.

MATERIALS AND METHODS

Study areas and sample collection

Blood and faecal samples were collected from capuchins in the wild, and tissue and skin samples were collected from museum specimens (Table 1; for more details see Appendix S1 in Supporting Information) of wild-caught animals of known provenance (Fig. 2); some sequences were also downloaded from GenBank. The collection of material from wild monkeys was carried out in accordance with the appropriate collection and export permits in Costa Rica (CONAGEBio, MINAE, Ministry of the Environment and Energy), Ecuador (Ministry of the Environment), Brazil (IBAMA, license no. 005/2005 – CGFAU/LIC) and Argentina (Iguazú National Park), with necessary importation permits from the US Fish and Wildlife Service or Centers for Disease Control, and with animal care and use guidelines. Geographic coordinates were taken from museum databases or from GPS data at field sites. When coordinates were not available on museum specimens, we used locality information from the museum databases to determine approximate coordinates.

DNA extraction, amplification and sequencing

We sequenced portions of two mitochondrial genes for our analysis of platyrrhine divergence times: 769 bp of 12S and 307 bp of cytochrome *b* (cyt *b*). For the subsequent cyt *b* capuchin analysis, we sequenced 946 bp of cyt *b*; for degraded museum samples, this sometimes required the use of multiple overlapping internal sets of primers (Table S1 in Appendix S2). For some samples, we were unable to recover the entire length of the cyt *b* fragment, and the missing bases were treated as missing data. Blood samples were extracted using a QIAgen DNeasy Blood & Tissue Kit, and faecal samples were collected in RNAlater and extracted using a modified QIAgen QIAamp DNA Stool Kit (see Di Fiore *et al.*, 2009 for modifications from manufacturer's suggested protocol).

Museum tissues were extracted in a Chelex solution following the protocol by Barber (2004). To prevent contamination, either museum DNA samples were extracted in a clean room in a building with no polymerase chain reaction (PCR) products (at Washington State University, WSU), or samples were extracted and set up for PCR in a UV-irradiated area where modern samples were never handled, with all reagents sterile and UV irradiated (at University of California, Los Angeles, UCLA). Extracted DNA from museum samples was quantified using a Nanodrop reading. All museum samples were run with negative controls in extractions and PCR reactions, and for a subset of samples we ran multiple extractions, PCRs, and sequencing for the same samples in different laboratories (WSU; UCLA; University of Alaska Museum, University of Alaska Fairbanks) to test for contamination in samples. To safeguard against the inadvertent inclusion of nuclear-derived mitochondrial pseudogenes (numts) in our sequences, we manually aligned the 12S sequences in accordance with the published secondary structural model (see below; but see Olson & Yoder, 2002) and checked the stem-and-loop structure against known sequences; we verified that cyt *b* sequences did not have indels; and we sequenced a full mitochondrial genome for one of the capuchin samples using circular overlapping primer pairs (A.D. & K.L.C., unpublished data).

PCR amplifications at Washington State University were performed in 25 µL reactions [standard reactions include 1.5 µL template, 5 µL 5× GoTaq (Promega, Madison, WI, USA) buffer, 13.8 µL ddH₂O, 1.5 µL MgCl₂ (25 mM), 1.25 µL (10 mM) each of forward and reverse primers, 0.5 µL (8 mM) dNTPs, 0.2 µL GoTaq DNA polymerase (Promega)]. An initial 2 min extension at 95 °C was followed by 38 cycles of 30 s at 95 °C, 1 min at 58 °C and 1 min at 72 °C, with a final 5 min annealing at 72 °C. Touchdown PCR techniques (Don *et al.*, 1991) were used for museum samples that would not amplify by regular protocols.

PCR amplifications at UCLA were performed in 20 µL reactions [standard reactions include 2 µL template, 2 µL 10× AmpliTaqGold (Applied Biosystems, Foster City, CA, USA) buffer, 10.64 µL ddH₂O, 1.2 µL (25 mM) MgCl₂, 0.8 µL (10 mM) each of forward and reverse primers, 1.6 µL (10 mM) dNTPs, 0.8 µL (10 mg mL⁻¹) BSA, 0.16 µL (5 units µL⁻¹) AmpliTaq Gold DNA polymerase (Applied Biosystems)]. The thermal cycling program included 9 min activation at 94 °C, annealing temperature of 56 °C, and a final extension at 72 °C for 10 min.

Template volume, number of cycles, annealing temperature and MgCl₂ concentration varied by primer pair, tissue type and template concentration.

Sequencing was conducted using an Applied Biosystems Prism 373 capillary sequencer at Washington State University, an Applied Biosystems 3730xL DNA Analyzer at Yale University or at New York University, or an Applied Biosystems Automated 3730 DNA Analyzer at Cornell University. We deposited all sequences in GenBank (see Table 1 and Appendix S1 for accession numbers).

Table 1 Specimens of capuchin monkeys used in the cytochrome *b* analysis.

Code	Species	Region	Latitude	Longitude	GenBank	Location	Sample ID
1	<i>Cebus capucinus</i>	Central America	10.35	-85.35	JN409305	UCR	CC55
2	<i>Cebus capucinus</i>	Central America	10.08	-84.47	JN409287	UCR	CC02
3	<i>Cebus capucinus</i>	Central America	9.95	-84.55	JN409288	UCR	CC51
4	<i>Cebus capucinus</i>	Central America	9.78	-84.93	JN409306	UCLA	CU005
5	<i>Cebus capucinus</i>	Central America	9.45	-84.15	JN409307	UCR	CC19
6	<i>Cebus capucinus</i>	Central America	9.73	-82.85	JN409308	WSU	J048
7	<i>Cebus capucinus</i>	Central America	9.48	-79.56	JN409309	USNM	171487
8	<i>Cebus capucinus</i>	Central America	9.07	-79.55	AY065907	GenBank	AY065907
9	<i>Cebus olivaceus</i>	Coastal Venezuela	10.62	-68.41	JN409289	USNM	372765
10	<i>Cebus olivaceus</i>	Coastal Venezuela	10.90	-68.77	JN409310	USNM	443218
11	<i>Cebus olivaceus</i>	Coastal Venezuela	10.66	-62.5	JN409311	USNM	261319
12	<i>Cebus olivaceus</i>	Coastal Venezuela	9.74	-61.42	JN409290	LACM	14378
13	<i>Cebus olivaceus</i>	North Amazon	7.65	-66.17	JN409312	USNM	296608
14	<i>Cebus olivaceus</i>	North Amazon	3.62	-65.68	JN409313	USNM	388187
15	<i>Cebus olivaceus</i>	North Amazon	6.29	-61.32	JN409291	USNM	374796
16	<i>Cebus olivaceus</i>	North Amazon	5.03	-60.95	JN409314	USNM	449466
17	<i>Cebus olivaceus</i>	North Amazon	4.42	-61.58	JN409315	USNM	443211
18	<i>Cebus olivaceus</i>	North Amazon	2.83	-58.95	JN409316	USNM	339662
19	<i>Cebus olivaceus</i>	North Amazon	0.50	-64.00	JN409335	INPA	JPB OLI
20	<i>Cebus olivaceus</i>	North Amazon	-0.96	-62.92	FJ529106	GenBank	FJ529106
21	<i>Cebus albifrons</i>	Coastal Venezuela	10.39	-61.3	JN409317	AMNH	24201
22	<i>Cebus albifrons</i>	North Andes	10.96	-74.79	JN409318	USNM	398449
23	<i>Cebus albifrons</i>	North Andes	9.14	-73.57	JN409292	LACM	27327
24	<i>Cebus albifrons</i>	North Andes	9.20	-72.64	JN409319	USNM	443501
25	<i>Cebus albifrons</i>	North Andes	7.32	-71.96	JN409293	USNM	443629
26	<i>Cebus albifrons</i>	North Amazon	3.17	-65.82	JN409320	USNM	406628
27	<i>Cebus albifrons</i>	North Amazon	2.25	-65.28	JN409321	USNM	406439
28	<i>Cebus albifrons</i>	North Amazon	0.50	-64.00	JN409336	INPA	JPB 100
29	<i>Cebus albifrons</i>	North Amazon	-0.96	-62.92	FJ529109	GenBank	FJ529109
30	<i>Cebus albifrons</i>	West Amazon	-0.70	-76.35	JN409322	NYU	217
31	<i>Cebus albifrons</i>	West Amazon	-4.45	-78.27	JN409294	MVZ	153479
32	<i>Cebus albifrons</i>	West Amazon	-8.67	-72.78	JN409323	MVZ	193676
33	<i>Cebus albifrons</i>	West Amazon	-10.00	-71.02	JN409295	LSUMZ	9922
34	<i>Sapajus macrocephalus</i>	West Amazon	4.15	-73.63	JN409324	USNM	241394
35	<i>Sapajus macrocephalus</i>	West Amazon	3.72	-73.48	JN409296	USNM	397979
36	<i>Sapajus macrocephalus</i>	North Amazon	-3.45	-68.80	JN409325	USNM	518262
37	<i>Sapajus macrocephalus</i>	West Amazon	-7.53	-74.97	JN409326	AMNH	268240
38	<i>Sapajus macrocephalus</i>	West Amazon	-10.00	-71.02	JN409327	LSUMZ	12294
39	<i>Sapajus macrocephalus</i>	West Amazon	-13.27	-74.25	JN409328	LSUMZ	16653
40	<i>Sapajus macrocephalus</i>	North Amazon	-0.96	-62.92	FJ529103	GenBank	FJ529103
41	<i>Sapajus apella</i>	North Amazon	13.48	-17.02	JN409297	USNM	296634
42	<i>Sapajus apella</i>	North Amazon	-2.70	-59.65	JN409329	USNM	547902
43	<i>Sapajus cay</i>	Cerrado	-15.46	-55.75	FJ529053	GenBank	FJ529053
44	<i>Sapajus cay</i>	Paraguay/Argentina	-26.88	-56.88	JN409298	UMMZ	124696
45	<i>Sapajus cay</i>	Paraguay/Argentina	-26.5	-57.73	FJ529076	GenBank	FJ529076
46	<i>Sapajus libidinosus</i>	Cerrado	-15.68	-48.2	JN409299	LACM	27344
47	<i>Sapajus libidinosus</i>	Cerrado	-17.22	-46.87	JN409300	USNM	518369
48	<i>Sapajus xanthosternos</i>	North Atlantic	-15.87	-39.12	JN409330	USNM	518303
49	<i>Sapajus xanthosternos</i>	North Atlantic	-15.14	-39.16	FJ460174	GenBank	FJ460174
50	<i>Sapajus robustus</i>	North Atlantic	-18.58	-39.75	JN409301	USNM	518327
51	<i>Sapajus robustus</i>	North Atlantic	-17.85	-41.50	JN409331	USNM	518429
52	<i>Sapajus robustus</i>	North Atlantic	-21.23	-41.20	JN409302	USNM	518311
53	<i>Sapajus nigrinus</i>	South Atlantic	-21.88	-43.33	JN409332	USNM	518421
54	<i>Sapajus nigrinus</i>	South Atlantic	-23.00	-44.30	JN409333	USNM	518478
55	<i>Sapajus nigrinus</i>	South Atlantic	-21.47	-47.02	JN409303	USNM	518515
56	<i>Sapajus nigrinus</i>	South Atlantic	-20.27	-50.23	JN409304	USNM	518524
57	<i>Sapajus nigrinus</i>	Paraguay/Argentina	-25.70	-54.44	JN409334	U. Montana	GRU3

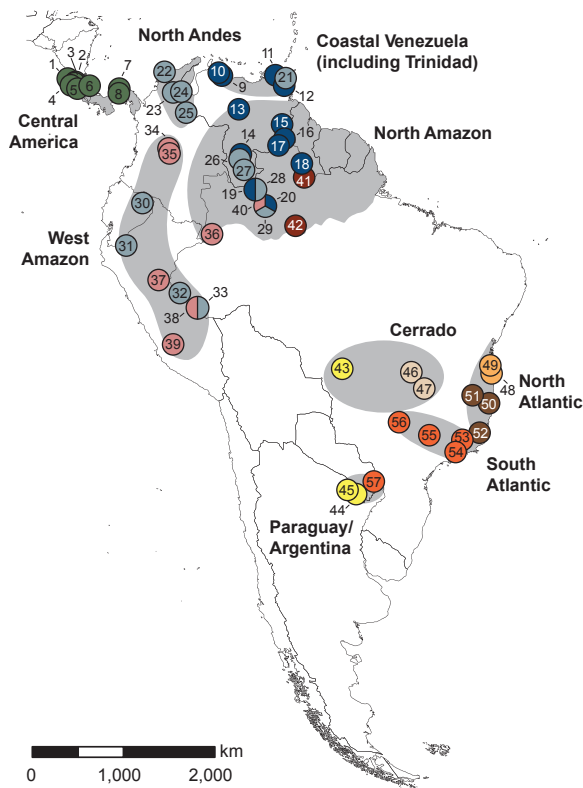


Figure 2 Biogeographic regions and provenance of samples of capuchin monkeys used in this study. Biogeographic regions are described in detail in the Materials and Methods. Sample numbers correspond to samples listed in Table 1, and are coloured based on species identification as in Fig. 1. Note that for samples 8 and 49, the origin is a gross approximation as these are GenBank sequences from captive monkeys, identified by species but with unknown geographic origins.

Phylogenetic analysis

Divergence time analysis of capuchins versus other Neotropical primates

There are no fossils that can be reliably assigned to subclades in capuchin monkeys. To test for the monophyly of the capuchin radiation and to infer the ages of the major splits within capuchin monkeys for Bayesian phylogeographic analysis (see below), we first performed a divergence time study of platyrrhines. We created matrices based on new sequences for 12S and *cyt b* for 30 capuchin samples combined with GenBank and some new sequences for 16 Neotropical primate outgroups (see Appendix S1). We aligned *cyt b* sequences manually using the visual alignment editor Se-AL (Rambaut, 1996). 12S sequences were aligned with reference to a model of secondary structure for the transcribed 12S ribosomal RNA molecule in mammals (Springer & Douzery, 1996) using a text editor. We excluded ambiguously aligned 12S regions from all analyses.

We used BEAUTi (part of BEAST 1.5: Rambaut & Drummond, 2007) to partition the concatenated 12S + *cyt b* data

matrix, specifying independent HKY + G models for 12S stems and loops and *cyt b* codon partitions. This partitioning scheme was favoured over simpler models (unpartitioned, partitioning by gene) by Bayes factor comparisons conducted in TRACER 1.5 (Rambaut & Drummond, 2007). We constrained the ages of five nodes on the basis of Neotropical primate fossil information: a minimum age for the crown Pitheciidae at 15.7 Ma, based on the fossil †*Proteropithecina* (Kay *et al.*, 1998, 1999); a minimum age for crown Atelidae at 12.6 Ma, based on the fossil †*Stirtonia* (Hartwig & Meldrum, 2002); a minimum age of crown Aotinae/Cebinae at 13.0 Ma, based on the fossil †*Aotus dindensis* (see Kay & Fleagle, 2010); a minimum age for crown Callitrichinae at 13.4 Ma, based on †*Patasola* and †*Lagonimico* (see Kay & Fleagle, 2010); and a minimum age on crown Cebinae with †*Neosaimiri* at 12.5 Ma (Rosenberger *et al.*, 1991a,b; Takai, 1994; Hartwig & Meldrum, 2002). We used exponential priors with the estimated initial divergence time of platyrrhines from catarrhines at 34.0 Ma as the upper 95% confidence limit for all splits within the Platyrrhini.

We used BEAST 1.5 (Drummond & Rambaut, 2007) to infer phylogeny and divergence times based on the 12S + *cyt b* data matrix. We ran the Markov chain Monte Carlo (MCMC) analysis for 50 million steps, sampling states every 5000 generations with a random starting tree, birth–death prior on diversification rate, uncorrelated, lognormal relaxed molecular clock, and program default prior distributions on other model parameters. We assessed convergence visually using TRACER 1.5 to plot likelihood scores for all parameters by generation time and by calculating effective sample sizes (ESS). We used TREEANNOTATOR (part of BEAST 1.5) to summarize the sampled trees after discarding initial ‘burn-in’ states.

Discrete-based model of spatial history

We used a recently developed model of diffusion to discrete states to explore the phylogeographic history of capuchin monkeys (Lemey *et al.*, 2009). This approach allows for probabilistic inference of spatial history, historically important migration pathways, and visualization of phylogeographic history while accommodating uncertainty in tree topology and other parameters of the phylogenetic model. The geographic model does not rely on an explicit description of the landscape, but instead explains the distribution of discrete regions in the tips of the phylogeny as an outcome of a diffusion process among them.

We assembled a second *cyt b* data matrix to perform Bayesian phylogeographic analysis with an expanded sample (57) of capuchin monkeys. We assigned each sample to one of nine major geographic regions (Table 1). We divided the Atlantic Forest into North and South Atlantic at 20° S, across Espírito Santo and Minas Gerais, marking the distributional limits of *S. robustus* in the north and *S. nigritus* to the south (Groves, 2001).

The North Atlantic region includes samples of *S. xanthosternos* from southern Bahia, following Kinzey’s (1982) Bahia centre of endemism and Torres de Assumpção’s (1983) Eastern

Bahia centre for capuchins, and *S. robustus* from Rio Doce–Espírito Santo and Minas Gerais, following Kinzey's (1982) Rio Doce centre of endemism and Torres de Assumpção's (1983) Eastern Minas Gerais and Espírito Santo, north of the Rio Doce centre for capuchins (see Rylands *et al.*, 1996, 2005).

The South Atlantic region includes *S. nigrurus* samples from the south-east Atlantic Forest, corresponding to Kinzey's (1982) Paulista centre of endemism and Torres de Assumpção's (1983) coastal areas of São Paulo, Paraná and Santa Catarina centre (Rylands *et al.*, 1996, 2005). The Cerrado region encompasses the dry, open, savanna scrub habitat between the Amazon and Atlantic Forests, also considered a major barrier to dispersal between the two forested areas, and includes samples from both *S. cay* and *S. libidinosus*. The Paraguay/Argentina samples, at the borders of these two countries, represent the southern extreme of robust capuchins' range, and include samples of *S. nigrurus* in the southernmost Atlantic Forest and *S. cay* from the geographically proximate north-eastern edges of the Chaco.

We divided Amazonian samples into two regions. The North Amazon region includes those samples of *Cebus albifrons*, *C. olivaceus*, *Sapajus apella* and *S. macrocephalus* north of Rio Amazonas, considered a major barrier for dispersal, and also includes specimens of *C. olivaceus* in Venezuela up into Llanos habitat. The North Amazon region corresponds, in part, to Torres de Assumpção's (1983) middle and lower Rio Amazonas and the Guianas centre for *Sapajus* (see Rylands *et al.*, 2005). The Western Amazon region includes samples south of the Amazon as well as from western Amazonia in Colombia, Ecuador and Peru. It corresponds to Torres de Assumpção's (1983) Western Amazonia centre for *Sapajus* (Rylands *et al.*, 2005).

The Andes are another major barrier to dispersal, and we have categorized the *C. albifrons* samples found in the valleys and foothills of the Northern Andes as within this separate region. Coastal Venezuelan specimens are those northernmost specimens that have reached Venezuelan shores or are on the coastal island of Trinidad, and include both *C. albifrons* and *C. olivaceus* samples. Central American samples include all the *C. capucinus* samples on the Isthmus of Panama and in Central America, an area isolated from South America before the Isthmus of Panama closed in the late Pliocene.

We used BEAUTI (part of BEAST 1.5) to specify separate HKY + G models for each cyt *b* codon position. This model was preferred by Bayes factor comparison over simpler partitioning schemes (codon positions 1+2, unpartitioned) in preliminary analyses. The discrete-states model does not require an outgroup. However, some node ages in the analysis must be constrained to infer the timing of divergence events. In the absence of a fossil calibration for any divergence within the crown-group for capuchin monkeys, we derived a prior on the age of the split between robust and gracile capuchins from the 95% posterior age distribution inferred from our 12S + cyt *b* platyrrhine divergence time analysis (above).

To infer the migration pathways most important to explaining the distribution of capuchin samples in our study,

we used Bayesian stochastic search variable selection (BSSVS) (Lemey *et al.*, 2009). This approach allows inference of the most probable dispersal pathways among the nine localities. By placing 50% of the prior probability on the minimum number of rate categories necessary to connect all of the geographic regions (eight rates to connect nine regions) with the remaining 50% of the prior evenly distributed over all possible connections among regions, our model favoured a parsimonious reconstruction of phylogeographic history while allowing for the possibility of non-parsimonious (repeated) colonization of regions. We considered non-zero rates with a Bayes factor > 3 as significant. We ran the MCMC analysis for 50 million steps, sampling states every 5000 generations with a coalescent-constant size tree prior, random starting tree, uncorrelated, lognormal relaxed molecular clock and program default prior distributions on other model parameters. We assessed convergence visually using TRACER 1.5 (part of BEAST 1.5) to plot likelihood scores for all parameters by generation time and by calculating ESS, and used TREEANNOTATOR (part of BEAST 1.5) to summarize the sampled trees after discarding initial 'burn-in' states.

Coordinate-based phylogeographic analysis

We tested whether dispersal rates varied among major subclades of capuchin monkeys following a method developed by Lemmon & Lemmon (2008), implemented in the program PHYLOMAPPER 1.0. This likelihood-based approach differs from the discrete-states model (Lemey *et al.*, 2009) in explicitly using coordinate data. We collected geographic coordinates for all specimens from the Bayesian analysis (Table 1) and removed 12 redundant haplotypes (because PHYLOMAPPER does not allow zero length branches – see Appendix S1). We first tested for a significant association between phylogeny and geography using the phylogeographic association test (Lemmon & Lemmon, 2008) with 1000 replicates to generate a null distribution for Ψ , the scaled dispersal parameter. If the empirical value of Ψ fell into the lower 5% tail of the null distribution, we interpreted this as evidence for phylogenetic association (that more closely related individuals were more likely to be geographically close to each other than distantly related individuals).

We also performed two tests of the dispersal rate to evaluate predictions of our biogeographic hypotheses. If the 'out of the Amazon' hypothesis is true, we predict that both robust and gracile radiations began diversifying at the same time, and that each dispersed at relatively equal rates. In this scenario, the gracile clade radiated north from the Amazon, the robust clade south from the Amazon, and so the Atlantic Forest robust, as the most distant dispersal group from the Amazon, might be expected to have the highest dispersal rate.

If the 'Atlantic versus Amazon' hypothesis is true, we predict that: (1) the robust species have been diversifying longer in both the Amazon and the Atlantic, which could explain their large distribution without recourse to rapid dispersal; and (2) the gracile clade, as a recently evolved subclade of the

Amazonian robust capuchins, would have rapidly colonized the Amazon, the northern Andes, northern coast of Venezuela, and Central America. Therefore we would predict a more rapid rate of dispersal for gracile compared with robust capuchins.

Finally, if the 'reinvansion of the Amazon' hypothesis is true, the gracile Amazonian and robust Atlantic Forest forms have been diversifying and dispersing the longest amount of time, but the relatively recent reinvansion and dispersal of robust capuchins throughout the Cerrado and Amazonian habitats actually encompasses the largest area; this would require a more rapid dispersal rate for robust Amazonian capuchins than that for either the gracile Amazonian or the Atlantic Forest robust capuchins.

To distinguish among these three hypotheses for phylogeographic diffusion, we used the dispersal rates test in PHYLOMAPPER (Lemmon & Lemmon, 2008) and compared Akaike Information Criterion (AIC) scores of three models for the dispersal parameter: shared rate among all lineages; separate rates for gracile and robust forms; and separate rates for gracile, Amazonian robust and Atlantic Forest robust forms.

RESULTS

Bayesian phylogeographic analysis

Bayesian analysis of the 12S + cyt *b* data set recovered capuchins as a monophyletic clade, confirmed the gracile and robust forms as reciprocally monophyletic, and placed the age of the split between gracile and robust capuchins at 6.7 Ma (95% highest posterior density 4.1–9.4 Ma). Further details of this analysis are reported in the Supporting Information (Appendices S1–S3).

Visual inspection of MCMC samples from the Bayesian phylogeographic analysis of the cyt *b* data set revealed that the chain appeared to reach stationarity after 10 million generations (ESS values for all parameters > 300 and parameter values appeared to have plateaued).

The cyt *b* maximum clade credibility tree (Fig. 3) recovered a basal split between gracile and robust capuchins that was similar in age and range to the prior (Table 2). This was expected because the prior on the gracile–robust split was the only time-calibrated node in the analysis.

Our results strongly support two major clades in capuchin monkeys: (1) the robust *Sapajus* group, *S. apella*–*S. macrocephalus*–*S. cay*–*S. libidinosus*, *S. nigrinus*, *S. robustus*, *S. xanthosternus* (and presumably *S. flavius*, not sampled here); and (2) the gracile *Cebus* group, *C. albifrons*, *C. capucinus* and *C. olivaceus* (and presumably *C. kaapori*, not sampled here). The estimated robust crown group age (c. 2.7 Ma) was more than a half a million years older than the gracile crown group age (c. 2.1 Ma). Our age of 6.2 Ma for the *Sapajus*–*Cebus* split was older than the 4.2 Ma reported by Casado *et al.* (2010).

Our estimate for the emergence of the robust capuchin crown group (2.7 Ma) is concordant with Casado *et al.*'s (2010) estimate of 3.1 Ma. Our data on *Sapajus* indicate an early diversification among Atlantic Forest clades, and a

relatively rapid dispersal across Atlantic Forest as far south as Argentina. The high phenotypic diversity within and across populations in the Atlantic Forest (Rylands *et al.*, 2005) may in part be a result of repeated events of isolation and recolonization between genetically distinct populations. If we remove the *S. xanthosternus* sample 49 from the tree (downloaded from GenBank, of unknown provenance, and possibly a captive hybrid or an error resulting from contamination), then the resulting pattern suggests that southern *S. nigrinus cucullatus* (as described by Groves, 2001) sampled from Rio de Janeiro, São Paulo, and Iguazú, Argentina (samples 54, 56 and 57, respectively, in Fig. 3), diverged first from the more northern *S. nigrinus nigrinus*–*S. robustus*–*S. xanthosternus* group (samples 48, 50, 51, 52 and 53), and it is this northern *S. nigrinus nigrinus*–*robustus*–*xanthosternus* clade of Atlantic Forest *Sapajus* that is a sister group to the Cerrado and Amazonian *Sapajus*.

Our analyses did not find any distinction between Amazonian *Sapajus apella* and *S. macrocephalus*, and we provisionally suggest that all Amazonian *Sapajus* may be lumped together as one species. We also found that *S. cay* is the sister group to *S. apella/macrocephalus* (as did Casado *et al.*, 2010), and that the Amazonian *Sapajus* may have given rise to *S. libidinosus*, with a secondary Amazonian expansion into the Cerrado.

Our data on the gracile *Cebus* species strongly signal that *C. albifrons* is paraphyletic. The first split of the gracile capuchin radiation is between Amazonian *Cebus* and Venezuelan Coastal/Central American *Cebus* at 2.1 Ma. This split is concordant with present-day evidence of the range for *C. albifrons*, as *C. albifrons* has a disjunct distribution with wide separation between the Amazon and Andes populations (see Fig. 1b). *Cebus olivaceus* (from the Llanos, Guiana Shield, and eastern Venezuelan Coast) appears to be a recent subclade of Amazonian *C. albifrons*, diversifying only 500 ka. One clade of North Andes *C. albifrons* is sister to *C. capucinus*, and another clade of North Andes *C. albifrons* is sister to *C. olivaceus* from Coastal Venezuela + *C. albifrons* from the disjunct island population in Trinidad. Clearly, the gracile capuchin clade is in need of further molecular analysis and taxonomic revision.

Bayes factor tests for significant non-zero rates revealed several historically important dispersal routes for capuchin monkeys (Appendix S4). The present-day distribution of capuchin lineages in the broader Atlantic Coastal Forest reflects a history of dispersal between the South Atlantic and North Atlantic, Cerrado and Paraguay/Argentina regions. The distribution of Amazonian lineages reflects dispersal events between the Northern Amazon and the Western Amazon, Coastal Venezuela, Northern Andes and Central America.

The Bayesian maximum clade credibility (MCC) tree was congruent with the topology from the platyrrhine-wide divergence time analysis (Appendix S3) in identifying a basal split between the reciprocally monophyletic robust and gracile forms. The North Atlantic emerged as the most likely ancestral locality for robust capuchins, with all other Atlantic localities favoured over any Amazonian regions (Fig. 4a). The North Amazon was the most likely ancestral location of gracile forms,



Figure 3 Reconstructed phylogeographic history of capuchin monkeys. Branch colours indicate most probable region inferred under a model of geographic diffusion. Under this model, the modern capuchin radiation most probably originated in Western Amazonia followed by early colonizations of the Northern Amazon by *Cebus* and North Atlantic Forest by *Sapajus*. Colonization of the Cerrado occurred at about 700 ka, and *Sapajus* expanded into the Amazon Basin about 400 ka.

and all Amazonian localities were strongly favoured over any non-Amazon region (Fig. 4b).

The basal split of the clade into robust and gracile forms, at *c.* 6.2 Ma, preceded radiation within each lineage by *c.* 4 Myr. Robust forms quickly dispersed across the North and South Atlantic Forest and as far south as Argentina. However, they remained isolated on the Eastern Coast of South America for about 2 Myr. It was not until about 700 ka that they reached the Cerrado, and they reinvaded the Amazon only within the last 400 kyr.

Gracile forms had colonized the Northern Amazon, Central America and Coastal Venezuela by 1.7–2 Ma. Snapshots of capuchin phylogeographic history visually emphasize the recent expansion of gracile *Cebus* and robust *Sapajus* lineages into much of their present-day range (Fig. 5a–f).

Phylogeographic association

The phylogeographic association tests were highly significant for all subclades analysed ($P < 0.001$ for all groups). Groups

Table 2 Divergence times for major clades within capuchin monkeys in the Neotropics.

Split or clade	Mean age (Ma)	Lower 95% HPD	Upper 95% HPD
<i>Sapajus</i> versus <i>Cebus</i>	6.15	4.21	7.86
Atlantic Coastal Forest <i>Sapajus</i> radiation	2.65	1.57	3.80
Andes and Central American <i>Cebus</i> versus Amazonian <i>Cebus</i>	2.12	1.32	2.98
Atlantic Forest <i>Sapajus</i> versus Atlantic Forest + Amazon <i>Sapajus</i>	1.95	1.15	2.82
Coastal and Andes Venezuelan <i>Cebus</i> versus Colombian Andes and Central American <i>Cebus</i>	1.86	1.13	2.67
West Amazon <i>Cebus albifrons</i> versus Amazon <i>Cebus albifrons</i> + <i>Cebus olivaceus</i>	1.75	1.05	2.49
Costa Rican <i>Cebus capucinus</i> versus Panama <i>Cebus capucinus</i> + Colombian Andes <i>Cebus albifrons</i>	1.61	0.95	2.34
Panama <i>Cebus capucinus</i> versus Colombian Andes <i>Cebus albifrons</i>	1.46	0.83	2.14
Venezuelan Andes <i>Cebus</i> versus Coastal Venezuela <i>Cebus</i>	1.37	0.72	2.08
<i>Cebus olivaceus</i> radiation (Llanos, Amazon, Eastern Venezuela Coast)	0.54	0.24	0.88
Costa Rican <i>Cebus capucinus</i> radiation	0.41	0.13	0.75
<i>Sapajus cay</i> versus Cerrado + Amazon <i>Sapajus</i>	0.40	0.21	0.67
Amazon <i>Sapajus apella/macrocephalus</i> radiation	0.33	0.17	0.51
Cerrado <i>Sapajus libidinosus</i> versus Amazon <i>Sapajus</i>	0.20	0.07	0.35
Coastal Venezuelan <i>Cebus olivaceus</i> versus Amazon/Llanos <i>Cebus olivaceus</i>	0.08	0.02	0.16

HPD, highest posterior density.

analysed included capuchins as a whole, the gracile *Cebus* clade, the robust *Sapajus* clade, and the *S. apella/macrocephalus* clade. The phylogeographic association tests indicated that, for capuchins as a whole, as well as within each subclade analysed, individuals that were more genetically similar were more likely to be geographically close to each other than more genetically divergent individuals.

Variation in dispersal distance

Although robust forms show greater overall dispersal distance than the gracile forms (Table S3 in Appendix S2), model comparison strongly supports the hypothesis that this pattern is driven by high rates of dispersal within the Amazonian robust lineages (three-rate model, Table S3). Under this model, the Amazon robust forms have dispersed more than twice as quickly as either the Atlantic robust or the gracile forms. This result best fits predictions made under the ‘reinvansion of the Amazon’ model, which included relatively slow dispersal rates for gracile capuchins within the Amazon and robust capuchins within the Atlantic Forest, but recent and rapid dispersal by robust capuchins colonizing West and North Amazon regions.

DISCUSSION

Phylogeographic analyses revealed three major results. (1) Capuchin monkeys were found to contain two well supported reciprocally monophyletic clades, corresponding to the morphologically distinct ‘gracile’ or untufted capuchins (*Cebus*) and the ‘robust’ or tufted capuchins (*Sapajus*). (2) The time-tree analysis estimates a late Miocene divergence time (c. 6.2 Ma) between *Cebus* and *Sapajus*, and a late Pliocene diversification within each of the two genera. (3) The current wide-ranging sympatry of *Cebus* and *Sapajus* across much of the Amazon Basin is best explained by the ‘reinvansion of the

Amazon’ hypothesis. Additionally, phylogeographic analyses point to an Amazonian origin for the gracile *Cebus* clade, and an Atlantic Forest or Cerrado origin for the *Sapajus* clade.

Timing of origin of the *Cebus* and *Sapajus* clades

Our molecular data support the classification of capuchin monkeys into two distinct clades, the gracile *Cebus* and the robust *Sapajus*, as suggested by previous morphological studies. All gracile, non-tufted species sampled (*C. capucinus*, *C. albifrons*, *C. olivaceus*) together formed a reciprocally monophyletic clade with respect to robust tufted species sampled (*S. nigritus*, *S. xanthosternos*, *S. robustus*, *S. cay*, *S. libidinosus*, *S. apella*, *S. macrocephalus*). The time-tree analysis estimates a late Miocene divergence time (c. 6.2 Ma) between *Cebus* and *Sapajus*. While *C. kaapori* was not sampled here, Barroso *et al.* (1997) estimated the divergence time between *C. kaapori* and *C. olivaceus* at 0.7 Ma using the nuclear markers IRBP and epsilon globin. This would nest *C. kaapori* within the Amazonian *C. albifrons*–*C. olivaceus* clade if considered in relation to our results, which put this radiation as having begun its diversification at c. 1.8 Ma. The other capuchin taxon not sampled here, the blond capuchin, *S. flavius*, has not been subject to any genetic analyses to date. It has some unusual morphological characteristics that are unique among capuchin monkeys, for example, a completely yellow coat, and a large throat sac in adult males. However, the characteristic large jaw and robust musculature, as well as its location in the extreme northern Atlantic Forest and Caatinga habitats, suggest that it will most likely be nested within, or fall sister to, the other robust capuchin taxa. Genetic analysis of this species would be revealing as to whether it is aligned with *S. libidinosus* (physically and geographically closest) or *S. xanthosternos* (as, for example, *Alouatta belzebul* is to *A. guariba* occurring to the south; see Cortés-Ortiz *et al.*, 2003).

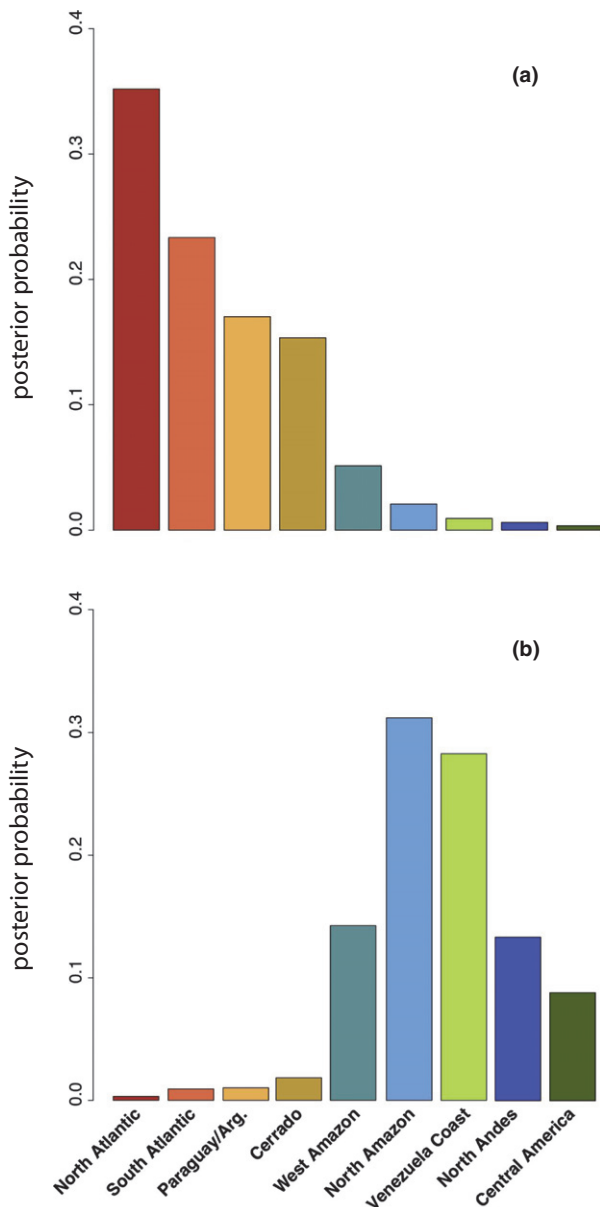


Figure 4 Posterior probability of root states for (a) *Sapajus* and (b) *Cebus* clades. For *Sapajus*, the most probable region of origin is North Atlantic, South Atlantic, or Paraguay/Argentina, and for *Cebus*, the most probable region of origin is the North Amazon or Venezuelan Coast.

'Reinvasion of the Amazon' best explains species distributions

A principal goal of our paper was to distinguish among three hypotheses about the origin of widespread Amazonian sympatry in capuchin monkeys. Our data strongly support the predictions of the 'reinvasion of the Amazon' hypothesis and give no support to those for 'out of the Amazon' or 'Atlantic versus Amazon'. Our data upheld all three predictions of the 'reinvasion of the Amazon' hypothesis: (1) the initial divergence in capuchin monkeys was between a robust Atlantic

Forest clade and a gracile Amazon clade; (2) robust Amazonian capuchins form a recently evolved subclade nested within the Atlantic Forest robust clade; and (3) robust Amazonian capuchins dispersed at a significantly higher rate than either gracile or Atlantic robust capuchins. The current wide-ranging sympatry of *Cebus* and *Sapajus* across much of the Amazon Basin is best explained by a Pliocene (*c.* 2 Ma) diversification of gracile capuchins in the Amazon, and a much more recent (*c.* 700 ka) expansion of Atlantic Forest *S. cay* into the southern Cerrado, followed by a single explosive Pleistocene invasion (*c.* 400 ka) of *S. apella/macrocephalus* across the Amazon, where *Sapajus* is now sympatric with, or has displaced, gracile capuchins across a large portion of their range.

One important point is that our present analysis lacks *Sapajus* samples from the southern bank of the Amazon; an alternative hypothesis remains that *Sapajus* originated here, first invaded the Atlantic Forest, and then later reinvaded the Amazon Basin (to the west and north of the Amazon).

Cebus and *Sapajus* origins and diversification

The timing and estimated geographic locations of ancestral *Cebus* and *Sapajus* populations (Fig. 4a,b) suggest that the initial vicariance may have been caused by the establishment of the Rio Amazonas at *c.* 7 Ma (Hoorn *et al.*, 2010); ancestral *Cebus* populations may have been restricted to the Guiana Shield, whereas the ancestral *Sapajus* populations may have been restricted to the Brazilian Shield. The gracile capuchins that founded present-day *Cebus* populations most likely originated and diversified in the Amazon 2 Ma and quickly radiated north into Coastal Venezuela and Central America, and subsequently into the Northern Andes from both Central America and Coastal Venezuela in multiple independent early Pleistocene dispersal events. The robust capuchins, on the other hand, originated either in the Atlantic Coastal Forest or perhaps further inland in what is now Cerrado, diversified in the Atlantic Forest in isolation from other capuchins throughout the Pliocene, and only in the last 750 kyr expanded out across the southern Cerrado and back into the Amazon.

All gracile capuchins appear to have radiated rapidly from an *albifrons*-like ancestor in the Amazon. The dispersal of *Cebus capucinus* into Central America around *c.* 1.9 Ma occurred later than that of ateline primates into Central America (Collins & Dubach, 2000; Cortés-Ortiz *et al.*, 2003), although it may pre-date the spread of squirrel monkeys into that region (see Appendix S3 for the relatively recent diversification of squirrel monkeys compared with capuchins). Spider monkeys and howler monkeys are believed to have colonized the northern Andes and Central America by 2.7 Ma or earlier. The closure of the Isthmus of Panama occurred around 3.5 Ma (Nores, 2004), but our study places capuchins in Central America by 1.9 Ma, well after this date. This corresponds well with predictions by Ford (2006) based on her analysis of the current distribution of primates in Central

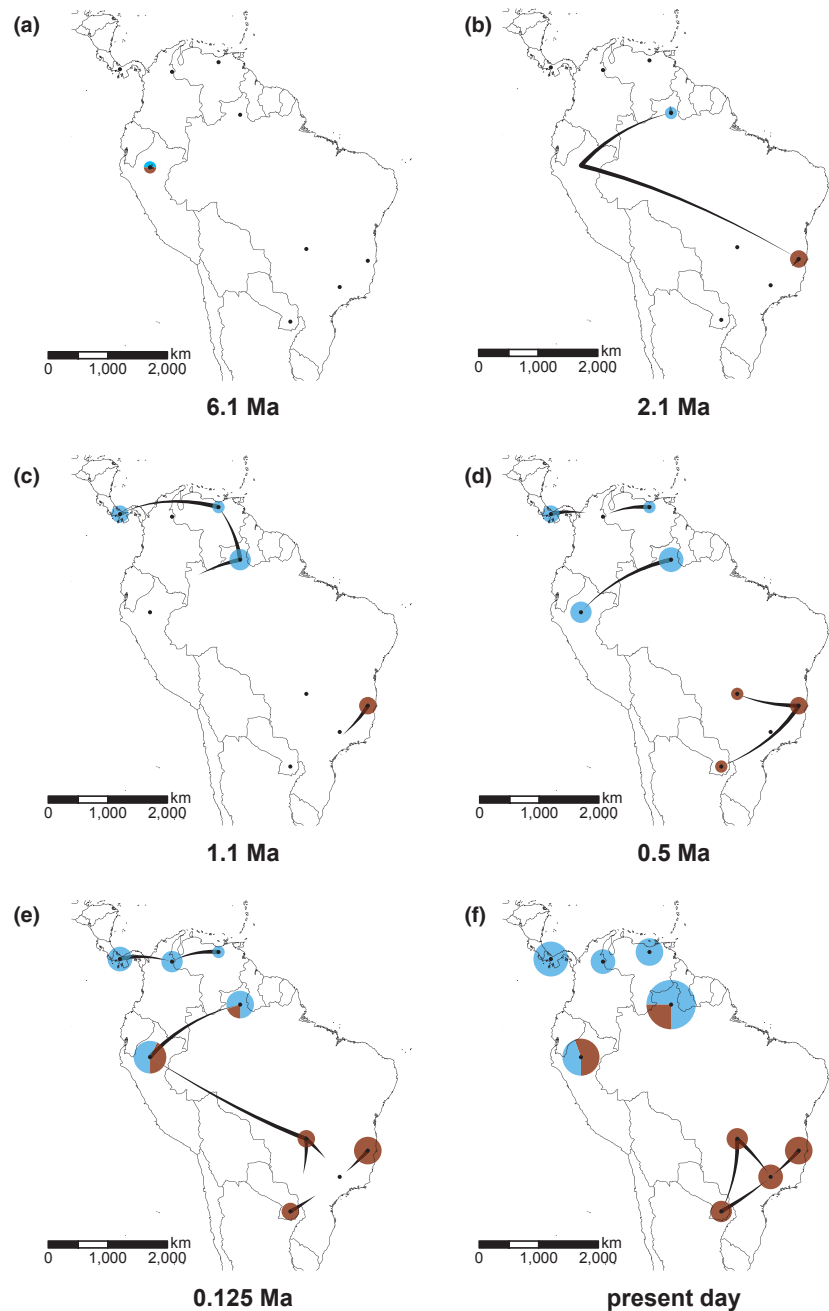


Figure 5 Time slices of capuchin monkey evolutionary history. Circle areas are proportionate to the number of lineages present in locations. Regions are the same as in Fig. 2. (a) 6.1 Ma: the split between gracile and robust capuchins, here depicted as occurring in the West Amazon region. (b) 2.1 Ma: the gracile group begins its radiation in the North Amazon region, and the robust group in the North Atlantic Forest region. (c) 1.1 Ma: North Amazonian capuchins have radiated into Coastal Venezuela and Central America. Robust capuchins begin expanding south from North Atlantic. (d) 0.5 Ma: gracile capuchins expand their range from both the Venezuelan Coast and Central America into the Northern Andes. Robust capuchins have invaded Paraguay/Argentina and the Cerrado from the Atlantic Forest. (e) 0.125 Ma: robust capuchins have invaded the West Amazon and North Amazon. (f) Present: the map reflects present-day capuchin distribution across all regions analysed in this study, including increased dispersal of robust capuchins across the Cerrado and South Atlantic Forest. Redrawn from satellite images from Google Earth based on *BEAST* Phylogeography Visualization (see Lemey *et al.*, 2009).

America; she suggests that *C. capucinus* arrived in Central America in a 'second wave' of primate introductions, at 2 Ma, a time of re-emerging terrestrial connection across the Isthmus of Panama.

From the mid-Miocene to the early Pliocene (*c.* 15–5 Ma), the elevations in the Eastern Cordillera were fairly low, about 40% of present height (Gregory-Wodzicki, 2000). Elevations increased to their present extent in the mid-Pliocene, about

2.7 Ma (Gregory-Wodzicki, 2000). According to Nores (2004), the final phase of the Andean uplift (5–2.7 Ma) would have separated lowland species even if they are now at higher elevations (Nores, 2004). All *trans*-Andean *Ateles* are believed to have crossed over the eastern cordillera of the Andes before the end of uplift of the chain during the late Pliocene, approximately 3 Ma (Collins & Dubach, 2000). However, our results indicate that the gracile *Cebus* populations that inhabit the northern Andes in Venezuela dispersed into these areas between 1.3 and 1.5 Ma, and this region appears to have been colonized independently at this time from capuchin source populations in Central America and Coastal Venezuela. This may mean that gracile capuchin populations have traversed the Andes more than once after the end of the uplift of the chain, when the mountains were already at their full height. In contrast, the distribution of robust *Sapajus* ends at the Andes, all along the Eastern Cordillera (Defler, 2004), suggesting that its much later arrival has, thus far, prohibited its ability to traverse the mountains, creating a limit to its expansion west.

For the robust capuchin clade, Atlantic Forest capuchins (*S. nigratus*, *S. robustus*, *S. xanthosternus*) as a group show the earliest diversification, with the Cerrado, Paraguay/Argentina, and Amazonian robust species (*S. cay*, *S. libidinosus*, *S. apella*, *S. macrocephalus*) clustering as a subclade of one of several lineages within the Atlantic Forest group. A study of the genetic variation among capuchin populations in Argentina suggested that at least two independent radiations of capuchins from the Amazon reached the southernmost part of the group's distribution (Martinez *et al.*, 2002). Our study confirms that *S. nigratus cucullatus* from Argentina and *S. cay* from Paraguay have been evolving independently for over 2 Ma, and that their common ancestor was most likely from the Atlantic Forest. Our analysis revealed one *S. nigratus* haplotype (Fig. 3, sample 53) as sister to the *S. cay/macrocephalus/apella/libidinosus* clade. Another individual with *S. nigratus* morphology and in the *S. nigratus* distribution area had a haplotype that clustered with *S. libidinosus* (Fig. 3, sample 55); this may be the result of introgression at border areas where two morphotypes of *Sapajus* are overlapping in distribution. Nuclear markers would help elucidate the pattern of gene flow or hybridization in these populations.

An expansion of wet forestland in the Pleistocene, uniting the Amazon and Atlantic Forest, may explain the expansion of *Sapajus* from the Atlantic into the Amazon. There is evidence of Pleistocene increases in forest cover across north-eastern Brazil, connecting the Atlantic Forest and the Amazon (Lynch, 1988; De Oliveira *et al.*, 1999; Puerto *et al.*, 2001; Costa, 2003); Por (1992) suggested another major historical pathway connecting the Amazon and Atlantic Forest through a southern route along the Paraná river basin. From our data, the southern route seems more likely for the capuchin expansion into the Amazon. If such a habitat shift made range expansion possible for capuchins, perhaps other primate species and/or non-volant mammals also expanded their ranges at this time. In fact, Costa (2003) provides evidence that three marsupials (*Caluromys lanatus*, *Metachirus nudicaudatus* and *Marmosa*

murina) may have extended their distribution to range from the Atlantic Forest to the Amazon along this route. Further investigation of the timing of divergence and the phylogeography of other mammals inhabiting the Atlantic, Cerrado and Amazon regions could shed more light on this subject.

Importantly, our data suggest that the robust *Sapajus* radiation into the Amazon Forest was the result of a single recent expansion and not multiple waves. One intriguing possibility is that tool use and cultural traditions observed in modern *Sapajus* have also been an important determining factor in their past ability to expand into and across the Cerrado. Compared with most other Neotropical monkeys, *Sapajus* inhabit drier habitats, surviving in marginal Caatinga and Cerrado environments that are harsh even for humans (Mannu & Ottoni, 2009). Present-day *Sapajus* in these dry habitats are known to use tools habitually to break open palm nuts, a behaviour not observed in any other Neotropical monkey (Ottoni & Izar, 2008). It may be that their cultural traditions and their ability to survive in harsh environments gave robust capuchins the ability to enter and traverse drier habitats and eventually expand their range up to the edge of the Amazon Forest. Once back into rain forest habitat, they flourished and rapidly expanded across the entire Amazon Basin.

Taxonomic implications

The deep split between the robust and gracile capuchins, with divergence estimated during the late Miocene (c. 6.2 Ma), is concordant with a separation of each clade into a different subgenus, as suggested by Silva (2001, 2002) based on morphological data; or in our opinion, into two different genera. The age of the split is similar, for example, to that of humans and chimpanzees (estimated at 5–7 Ma). Within Neotropical primates, the age of the split between *Cebus* and *Sapajus* is similar to that between the Amazonian woolly monkeys (genus *Lagothrix*) and the Atlantic Forest muriquis (genus *Brachyteles*), estimated as occurring c. 8.0 Ma using IRBP (Barroso *et al.*, 1997), or c. 10.5 Ma (10.4–10.7) using IRBP and epsilon globin combined (Schrägo, 2007).

Some genera of Neotropical primates are as old as, or older than, the split between *Sapajus* and *Cebus*; for example, the first split in the howler monkeys (*Alouatta*) was estimated at 6.8 Ma by Cortés-Ortiz *et al.* (2003). However, we argue that for *Alouatta* there is no large morphological gap/distinction between the two main clades (*cis*- and *trans*-Andean), there has been more continuous splitting and speciation through time in *Alouatta*, ecological niches are more similar across *Alouatta*, and the species are generally allopatric. There are two divergent radiations of capuchin monkeys, with separate geographic origins, and the two types now overlap geographically and are distinct in their behaviour and morphology. The molecular and biogeographic evidence presented here, and clear differences in morphology, ecology and behaviour, argue strongly for the separation of these capuchin monkeys into two genera, *Cebus* and *Sapajus*.

Implications for future study

The expansion of *Sapajus* into the range of *Cebus* may give insight into evolution in action, with expanding ranges, re-entry into sympatry, competition between gracile and robust forms, and perhaps ultimately competitive exclusion, which may play out differently in different habitat types. Are robust *Sapajus* in the Amazon an invasive threat to gracile *Cebus* biology? What happens when a genetically diverse population is rapidly invaded by a genetically homogeneous population across its range? These are questions for further study; to date, there have been few field studies that examine the behavioural biology of *Cebus* and *Sapajus* in sympatry, but those reports that are available show large variations in interspecific relationships across the different sites studied (Defler, 1982, 1985; Janson, 1986a,b; Youlatos, 1998; Peres & Janson, 1999; Haugaasen & Peres, 2005, 2009). The data presented here provide the first modern evolutionary framework within which to interpret the complex and changing relationships between gracile and robust capuchin monkey populations across the Amazon Basin.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Detailed sample list of platyrrhine sequences for 12S and cytochrome *b*.

Appendix S2 List of primers used for 12S and cytochrome *b* analyses (Table S1), age of focal nodes for the platyrrhine diversification analysis (Table S2) and a comparison of dispersal models (Table S3).

Appendix S3 Platyrrhine diversification analysis with fossil calibrations.

Appendix S4 Significant dispersal rates between major geographic regions.

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