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Ancient DNA of the extinct Jamaican monkey *Xenothrix* reveals extreme insular change within a morphologically conservative primate radiation

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Abstract. The insular Caribbean until recently contained a diverse mammal fauna containing four endemic platyrrhine primate species, which all died out during the Holocene. Previous morphological studies have attempted to establish how these primates are related to fossil and extant platyrrhines, whether they represent ancient or recent colonists, and whether they constitute a monophyletic group. These efforts have generated multiple conflicting hypotheses, from close sister-taxon relationships with several different extant platyrrhines, to derivation from a stem platyrrhine lineage outside the extant Neotropical radiation. This diversity of opinion reflects the fact that Caribbean primates were morphologically extremely unusual, displaying numerous autapomorphies and apparently derived conditions present across different platyrrhine clades. Here we report the first ancient DNA data for an extinct Caribbean primate: a limited-coverage entire mitochondrial genome and seven regions of nuclear genome for the most morphologically derived taxon, the Jamaican monkey *Xenothrix mcgregori*. We demonstrate that *Xenothrix* is part of the existing platyrrhine radiation rather than a late-surviving stem platyrrhine, despite its unusual adaptations, and falls within the species-rich but morphologically conservative titi monkey clade (Callicebinae) as sister to the newly recognized genus *Cheracebus*. These results are not congruent with previous morphology-based hypotheses, and suggest even morphologically conservative lineages can exhibit phenetic plasticity in novel environments like those found on islands. *Xenothrix* and *Cheracebus* diverged *c.*11 Ma, but primates were present in the Caribbean since 17.5–18.5 Ma, indicating that Caribbean primate diversity was generated by multiple over-water colonizations.

Keywords: biogeography, *Callicebus*, extinct mammal, island evolution, phylogeny, platyrrhine

Significance statement. Until recently the Caribbean contained a remarkable evolutionary radiation of mammals, including several highly unusual primates; the oddest was the Jamaican monkey *Xenothrix*. Unfortunately all of these primates are now extinct, and efforts to reconstruct their evolutionary history have had to use limited morphological information from incomplete subfossils. Despite generally poor preservation of DNA in ancient tropical samples, we extracted the first ancient DNA from an extinct Caribbean primate, which reveals that instead of being distantly related to living Neotropical monkeys, *Xenothrix* is actually an extremely unusual titi monkey that underwent major body-plan modification after colonizing an island environment. The date of the split between *Xenothrix* and other titi monkeys also reveals that primates colonized the Caribbean more than once.

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INTRODUCTION

Islands are the home of spectacular evolutionary novelty, and have long acted as ‘natural laboratories’ that have inspired evolutionary thinking (*1-3*). For example, the biota of the insular Caribbean has been extensively studied to test competing hypotheses for island colonization by vicariance, land bridges, or over-water dispersal, and to reconstruct ecological drivers and evolutionary dynamics of morphological differentiation under novel environments (*2*, *3*). Insular taxa frequently exhibit unusual morphologies that differ markedly from continental taxa (*4*), which can represent either evolutionary responses to unique ecological conditions on islands, or “ancestral” traits of ancient lineages with relict distributions (*5*, *6*). Morphological characters have been of limited usefulness for reconstructing evolutionary histories of many morphologically unusual island taxa, and the advent of molecular phylogenetic methods has overturned morphology-based hypotheses about the affinities of several insular lineages (*7-9*).

Most island systems have experienced high levels of human-caused biodiversity loss (*6*), and many unusual insular taxa are now extinct and represented only by incomplete subfossil remains. In the absence of molecular analyses, such taxa can remain evolutionarily enigmatic, often with multiple competing non-congruent phylogenetic hypotheses derived from restricted morphological datasets (*10*, *11*). Improved molecular sampling of extinct taxa is necessary to resolve these conflicts and reconstruct the evolution of insular biotas through time, and distinguish between morphologies representing adaptive responses to island environments versus those representing “primitive” traits lost from continental representatives of diversifying clades. However, molecular study of extinct species from tropical islands is limited by preservation of DNA, which is greatly reduced by the high thermal age represented by hot, humid tropical conditions (*12*, *13*).

Oceanic-type (non-continental) islands have rarely been colonized by terrestrial mammals, limiting investigation of evolutionary patterns and processes in one of the best-studied animal groups. The insular Caribbean is remarkable in this context, as it contained a diverse late Quaternary terrestrial mammal fauna including lipotyphlan insectivores, rodents, sloths and primates. However, most of these species disappeared during the world's largest postglacial mammal extinction event, associated with arrival of human colonists from the mid-Holocene onwards, which led to complete loss of several Caribbean mammal groups, including all the endemic primates (*6*, *14*).

Primates of the Caribbean. The oldest Caribbean primate, *Paralouatta marianae*, is known from an astragalus dated to *c.*17.5-18.5 Ma (Early Miocene) based on associated invertebrates and sequence stratigraphy at Domo de Zaza, central Cuba. This fossil provides an earliest constrained age for regional presence of primates (*15*). Recent discovery of a tick in mid-Tertiary amber, containing blood cells similar to those of primates but not other Caribbean mammals, has been interpreted as evidence of possible primate occurrence on Hispaniola from at least 15 Ma and possibly 30-45 Ma (*16*). All other Caribbean primates (*Antillothrix bernensis* and *Insulacebus toussaintiana* from Hispaniola, *Paralouatta varonai* from Cuba, and *Xenothrix mcgregori* from Jamaica; *6*, *17*, *18*) are known from late Quaternary cave deposits. Several taxa persisted into the Holocene and were contemporaneous with prehistoric human settlers (*6*, *14*). *Xenothrix* was apparently the last surviving Caribbean primate: a direct AMS date of 1,477±34 BP gives an estimated last-occurrence date of *c.*900 BP (*19*), and European accounts of primate-like animals from Jamaica suggest possible historical survival (*20*).

An outstanding aspect of Caribbean primates is their morphological uniqueness. All were clearly platyrrhines, but they exhibit features and character combinations that are rare or absent in living taxa. Uniqueness is particularly noteworthy in *Xenothrix*, described as "the most enigmatic of all South American fossil monkeys" (*21*) (Fig. 1). *Xenothrix* lacks third molars, potentially representing a derived resemblance to callitrichids (marmosets). However, dental reduction in callitrichids is possibly associated with body size reduction (*22*), whereas *Xenothrix* was comparable in size to the much larger *Cebus* (capuchins). Another highly unusual autapomorphy of *Xenothrix* is size disproportion of cheekteeth, with the first molars much larger than the second (*17*). Other features that, in combination, differentiate *Xenothrix* from other platyrrhines exist in the shape of the mandible, size of orbit, and volume of maxillary sinuses (*23*). The postcranial morphology of *Xenothrix* is comparably unusual, revealing it was a slow-moving arboreal quadruped, a locomotory adaptation unique in recent platyrrhines (*20*). Other Caribbean monkeys exhibit similarly distinctive characters (e.g., evidence of semiterrestriality in *Paralouatta varonai*), which further complicates morphological phylogenetic analysis (*24*).

Colonization history and evolutionary affinities of Caribbean primates. Using morphology to reconstruct Caribbean primate evolutionary history has been challenging because of their biological distinctiveness and the paucity of their remains. These factors have led to widely diverging hypotheses regarding their origin, colonization and diversification, particularly for *Xenothrix* (Fig. 2). Debate has focused on three related questions: [**1**] Do *Xenothrix* and other Caribbean taxa fall within the living platyrrhine radiation, or do they represent an older lineage of late-surviving stem platyrrhines? [**2**] If they are part of the modern radiation, which platyrrhine clade are they most closely related to? [**3**] Do different endemic Caribbean primates represent a monophyletic clade?

Williams and Koopman (*17*) only classified *Xenothrix* as a non-callitrichid platyrrhine when describing the taxon. Hershkovitz (*25*) suggested it was not closely related to living platyrrhines and placed it in its own family, Xenotrichidae. Rosenberger (*26*, *27*) considered it was most closely related to *Aotus* (night monkeys) because both taxa exhibited enlarged orbits and broadened upper incisors. In their description of new *Xenothrix* material, MacPhee and Horovitz (*23*) concluded that *Xenothrix* exhibited no derived characters in common with *Aotus*, but was instead closely allied with callicebines (titi monkeys) on the basis of several derived craniodental traits. All callicebines were then referred to the single genus *Callicebus*; however, recent molecular analysis recognises three clades within *Callicebus sensu lato* which diverged during the Miocene, and which have been elevated to distinct genera (*Callicebus*, *Cheracebus*, *Plecturocebus*) (*28*, *29*). More recently, geometric morphometric analysis of extant and fossil platyrrhines suggested that *Xenothrix* could represent an ancient lineage that diverged before the radiation of crown platyrrhines (*30*). Combined molecular-morphological analysis of extant and fossil platyrrhines also suggested that *Xenothrix* and other Caribbean monkeys were late-surviving stem platyrrhines, although this was based on a restricted character dataset with limited support values (*31*).

Several authors have considered that Caribbean primates form a monophyletic group, with suggested synapomorphies including a shared enlarged nasal fossa in *Xenothrix* and *Paralouatta*, and shared unique tooth morphology in *Xenothrix* and *Insulacebus* (*18*, *23*). This clade has been proposed as the sister group of *Callicebus sensu lato* (*23*), or all crown platyrrhines (*31*). Conversely, the marked variation in morphological features between different taxa has led other authors to interpret their diversity as indicating multiple mainland lineages, reflecting separate colonizations at different times or a single multi-lineage colonization (*27*).

Study overview and aims. In this study, we employ aDNA techniques (Next Generation Sequencing (NGS) techniques combined with target capture enrichment) and phylogenetic methods to investigate evolutionary relationships between extinct Caribbean primates and extant platyrrhines. Our objectives are to evaluate the relationship of *Xenothrix* to mainland platyrrhine taxa, to reconstruct its phylogenetic history and the dynamics of its morphological evolution, and to date the divergence from its closest living relatives to determine whether Caribbean primates belong to one or more independently colonizing clades.

RESULTS

Screening results indicated poor survival of endogenous DNA in the two late Holocene *Xenothrix* samples used in this study. The sample with the highest amount of endogenous DNA (AMNH 268010) was used for target capture enrichment. This technique greatly increased endogenous DNA recovery, with almost 20 times more reads mapped to the mitochondrial genome (*SI Appendix, Table S6*). This permitted recovery of a limited-coverage entire mitochondrial genome, along with seven regions of the nuclear genome. The whole mitochondrial genome was used in preliminary analysis, to determine the affinities of *Xenothrix* to extant platyrrhine genera. To include a wider range of extant species for which only reduced sequence data were available, notably multiple representatives of all three newly recognized callicebine genera, a reduced dataset of two mitochondrial genes and one nuclear gene were then used in final species-level analysis. In tests of alternative tree topologies, AU p-values were <0.5 for all phylogenetic hypotheses previously suggested for *Xenothrix* (*SI Appendix, Table S4*). We recovered convergent Maximum Likelihood (ML) and Bayesian phylogenies for both genus-level and species-level trees (Figs 3-4; *SI Appendix, Fig. S3*). Our dated phylogeny shows that *Xenothrix* falls within the group of taxa formerly classified as *Callicebus sensu lato*. More specifically, it resolves as sister to the recently erected genus *Cheracebus*, with a mean estimated divergence date between *Xenothrix* and *Cheracebus* of *c.*11 Ma (95% highest posterior density [HPD], 5.2-14.9 Ma).

DISCUSSION

In this study, we were able to extract and sequence the first ancient genomic sequence data from an extinct Caribbean primate, despite adverse preservational conditions that greatly reduce likelihood of DNA preservation in subfossil samples from tropical environments. The results of our molecular phylogenetic analysis of *Xenothrix* are not congruent with any phylogenetic hypothesis previously proposed using morphological data, providing an important and unexpected new understanding of the evolutionary history and affinities of this enigmatic extinct animal. It is not a stem-group platyrrhine, an outlier within New World monkeys, a close relative of *Aotus* or callitrichids, or sister to the entire callicebine radiation, as previously suggested, but is instead nested within the callicebine radiation and sister to the recently described genus *Cheracebus*.

Morphological versus molecular phylogenies for Caribbean primates. Disparities between morphological and molecular phylogenetic reconstructions are not unusual in platyrrhine taxonomy. Morphology-based analyses have often suggested a close relationship between *Aotus* and callicebines (*23*, *32*, *33*), but molecular studies group callicebines within Pitheciidae and *Aotus* with Callitrichidae and Cebidae (*34*, *35*). Partition homogeneity analysis has demonstrated that phylogenetic analyses of platyrrhines, and specifically those including Caribbean primates, recover different results using craniodental versus postcranial data (*31*), suggesting that phylogenetic hypotheses based on restricted morphological character datasets available for extinct species are not robust and must be interpreted with care. Most previous morphological hypotheses have also relied upon taxonomy that is inconsistent with more recent platyrrhine molecular phylogenies (*28*, *29*). These considerations have obvious implications for the explanatory value of morphology-only data for Caribbean primates.

Primate insular evolution and morphological conservatism. The main morphological differences among living callicebines relate to pelage characteristics and body size, and craniodental and other skeletal characters exhibit little variation across the subfamily (*28*). Extant callicebines are therefore remarkably conservative morphologically compared to other platyrrhine lineages (*30*, *36*, *37*), which makes the peculiar mixture of features in *Xenothrix* evolutionarily unexpected. How can this be accounted for?

Two contrasting modes of speciation are likely to have driven evolution in *Xenothrix* and mainland Callicebinae. Barriers to gene flow created by river systems (*38*) and Pleistocene climate refugia (*39*) are considered primary factors responsible for generating the high primate species diversity found today in the Neotropics, including the diversity observed within *Callicebus*, *Cheracebus* and *Plecturocebus*, which are thought to have diversified primarily through sequential “jump dispersal” across rivers (*29*). Although mainland callicebine populations are separated geographically, they inhabit relatively similar environments and occupy comparable niches, an ecological context likely to be associated with little morphological divergence over time. Conversely, colonization of Jamaica by a callicebine lineage may have led to ecological release in a novel environment containing vacant niches, which was associated with equivalent divergence in primate morphospace. Caribbean islands apparently lacked medium-sized frugivores before the arrival of primates (*40*), and the unique morphological traits exhibited by *Xenothrix* may be associated with adaptation to this new niche. Geographic isolation of other lineages in island ecosystems has resulted in comparably unusual morphologies, drastic size changes, and accelerated evolution (*4*, *41*, *42*), such that a lineage's potential for phenetic plasticity when exposed to novel environments cannot be predicted on the basis of past morphological conservatism within more homogeneous systems.

Characteristic evolutionary patterns representing adaptations to insular environments are also seen in other primates. Famously, the extinct insular hominin *Homo floresiensis* exhibits morphological divergence from mainland Asian and African hominins consistent with the general "island rule", whereby larger-bodied lineages decrease in body size and smaller-bodied lineages increase in body size following isolation on islands (*4*, *43*). Macaques have also colonized multiple oceanic-type insular environments, and a series of morphological differences are exhibited between island and mainland populations including divergence in body size and tail length (*43*-*45*). Our study provides further evidence of island evolution causing radical morphological changes over relatively short geological timeframes in an insular primate. However, apart from the recently extinct subfossil lemurs of Madagascar (*40*), there are no examples of primates in Quaternary island faunas exhibiting the extreme level of adaptation shown by *Xenothrix*, perhaps making it easier to understand how morphological and molecular analyses can arrive at markedly different conclusions about the evolutionary history of this unusual extinct primate.

Colonization and evolutionary history of Caribbean primates. Our estimated divergence date between *Xenothrix* and *Cheracebus* suggests that the ancestral *Xenothrix* lineage colonized Jamaica during the late Middle Miocene *c*.11 Ma, with an upper 95% HPD of 14.9 Ma. This estimated divergence considerably postdates the geological formation of the Greater Antilles as oceanic-type islands, and also the hypothesized existence of a subaerial landspan connecting these islands to South America during the Eocene-Oligocene transition (*46*), indicating that primates must have arrived via over-water dispersal, in contrast to some other components of the Caribbean Neogene mammal fauna (*13*). This hypothesized colonization mechanism for *Xenothrix* is consistent with the present-day distribution of its extant sister genus *Cheracebus*, the northernmost callicebine genus, which occurs across northern South America into the Orinoco region of Venezuela (*28*, *29*).

The oldest known Caribbean primate, *Paralouatta marianae*, comes from sediments dated to 17.5-18.5 Ma (*15*). It therefore pre-dates our oldest estimate for *Xenothrix*–*Cheracebus* divergence by at least 2.6 Ma. This indicates that at least two colonizations of the insular Caribbean by primates occurred at different times during the Neogene. The extinct Caribbean primate assemblage therefore cannot be monophyletic, contrary to earlier morphology-based hypotheses (*23*). This discovery matches the evolutionary history of several other Quaternary Caribbean vertebrate groups (e.g., leptodactylid frogs, mabuyid skinks, megalonychid sloths, Lesser Antillean oryzomyine rice rats), which have been shown to comprise multiple distantly related lineages representing separate colonizations from mainland South America (*12*, *47*, *48*).

Our findings are also consistent with previous hypotheses about the origins and evolution of other components of Jamaica's vertebrate fauna. The Jamaican Quaternary fauna is biogeographically distinct, lacking several groups that characterize other major Caribbean islands (e.g., megalonychid sloths, solenodonotan insectivores), and showing the greatest avifaunal species-level endemism for any Caribbean island (*49*). Other vertebrate groups known from both Jamaica and elsewhere in the insular Caribbean also have different colonization histories. Molecular evidence supports inclusion of all Jamaican *Anolis* species in a monophyletic clade, whereas *Anolis* diversity elsewhere across the Caribbean was generated by two separate colonizations (*50*). Oryzomyine rice rats were formerly present on both Jamaica and the Lesser Antilles, but whereas Lesser Antillean rice rats comprise two distantly related clades that colonized from northern South America (*12*), the now-extinct Jamaican rice rat *Oryzomys antillarum* represents a separate colonization that probably occurred over-water from Central America (*51*). The distinct evolutionary history of Jamaica's fauna probably reflects both geographic distance from other islands and the major marine barrier represented by the deep Cayman Trough, which likely hindered dispersal between Jamaica and other Caribbean islands even during periods of low sea-level (*52*).

Ancient DNA analysis reveals that the morphologically aberrant extinct Caribbean primate *Xenothrix* falls within the otherwise morphologically conservative callicebine radiation, and while we cannot yet identify sister taxa of extinct primates from Cuba and Hispaniola, our findings indicate that the Caribbean primate assemblage cannot represent a within-Caribbean evolutionary radiation resulting from a single over-water dispersal. These findings provide crucial insights into the evolutionary history and affinities of island platyrrhines, and have important implications for reconstructing the evolution of both Neotropical primates and Caribbean mammal faunas across space and time.

METHODS

Data collection. Two subfossil specimens identified morphologically as *Xenothrix mcgregori* (*20*) in the American Museum of Natural History (AMNH), from Somerville Cave, Jamaica, were subjected to sampling for aDNA extraction. One specimen, a femur (AMNH 268003), has previously given a direct AMS date of 1,477±34 cal BP (*19*). The other specimen, a proximal ulna (AMNH 268010), has not been dated directly but is suspected to be similar in age.

Extractions and NGS library-builds took place in a dedicated aDNA laboratory at the Natural History Museum, London. DNA was extracted using protocols from ref. (*53*). Single-index DNA libraries were built following protocols from ref. (*54*). Libraries were screened for endogenous DNA using the Illumina MiSeq 500. In-solution, hybridisation-capture enrichment kits (MYcroarray, Ann Arbor) were applied. Baits were designed from the whole mitochondrial genome and five nuclear genes available on NCBI database Genbank for callicebines (*SI Appendix, Table S1*). These reference sequences were chosen on the basis of previous suggestions that *Xenothrix* may be most closely related to callicebines (*23*).

Sequence analysis. Raw data were analysed in CLC Workbench software v.8 (CLC Bio-Qiagen, Aarhus, Denmark). Reads were paired, merged, and trimmed of adapters using default settings. To reduce potential for ascertainment bias during sequence assembly, reads were mapped to a range of 20 reference sequences for the whole mitochondrial genome and each nuclear gene targeted. The set of reference sequences included platyrrhines and three outgroups: *Homo sapiens, Macaca fuscata*, *Pan troglodytes* (*SI Appendix, Table S2*). Mapping parameters were as follows: Length fraction: 0.8, Similarity fraction: 0.8. More reads mapped to callicebine reference sequences than to other reference sequences, with the highest amount of reads mapping to *Cheracebus lugens* (*SI Appendix, Fig. S2, Table S7*).

Xenothrix sequence data were then aligned to 14 callicebine species and using *Saimiri sciureus*, *Cebus albifrons*, *Pithecia pithecia*, *Chiropotes israelita* and *Cacajao calvus* as outgroup taxa, using ClustalW (*55*) implemented in Geneious v.8.0.5 (*56*). Alignments of each gene were concatenated using Seaview v.4 (*57*). Phylogenetic relationships were estimated using Maximum Likelihood (ML) and Bayesian methods, with DNA substitution models chosen for the partitioned dataset using PartitionFinder (*58*) (*SI Appendix, Table S3*). A ML tree with bootstrap support values was generated using RAxML v.8 (*59*) implemented in CIPRES Science Gateway v.3.3 (*60*). Bayesian trees were constructed using MrBayes (*61*) with four chains (three heated, one cold) run for 1×10^6 generations, sampling every 1×10^3 generations with a burn-in of 250 trees. Tests of alternative topologies suggested by previous studies (Fig. 2) were conducted by submitting sitewise log-likelihood values from RAxML v.8 (*59*) to CONSEL (*62*), to calculate p-values for each tree topology using AU tests (*SI Appendix, Table S4*).

Phylogeny and diversification times were simultaneously assessed under an uncorrelated relaxed lognormal molecular clock in BEAST v.1.8.3 (*63*). Best-fit evolutionary models were chosen in PartitionFinder as in previous phylogenetic analyses. A Yule model of speciation was used; the birth-death model was run for comparison and generated identical topology. Prior distributions on two nodes were set using two fossil calibration points: Cebidae (12.5 Ma), Pitheciidae (15.7 Ma) (*SI Appendix, Table S5*). To provide an ingroup calibration point, a further prior distribution was set for the divergence between Callicebinae and Pitheciinae following the estimate in ref. (*28*) (95% HPD, 15.8-22.6 Ma), using tmrca for soft upper and lower bounds. The analysis was run for 25 million generations, sampling every 1000 generations. Tracer v.1.6.0 (http://beast.bio.ed.ac.uk/Tracer) was used to access convergence and effective sample size for all parameters after a burn-in of 10%. A maximum credibility tree was generated in TreeAnnotator v.1.8.3 (*63*), using trees sampled in the prior distribution.

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Figure Legends

Fig. 1. Upper dentitions of platyrrhine monkeys, comparing (**A**) the most complete known skull of *Xenothrix mcgregori*, preserving P3-M2 (AMNH 268006), (**B**) copper titi monkey, *Plecturocebus cupreus* (AMNH 34636), and (**C**) Azara's night monkey, *Aotus azarae* (AMNH 94133) (scale = 1 cm). Important morphological features of *Xenothrix*: [**1**] two rather than three molars (differs from all known platyrrhines except non-*Callimico* callitrichines); [**2**] swollen cusps on molars (resembling pitheciids in general, including callicebines); [**3**] third premolar is premolariform (specifically resembling callicebines among pitheciids); [**4**] incisor alveoli indicate that incisors were probably primitively slender (not expanded as in *Aotus*).

Fig 2. Five alternative tree topologies illustrating previously proposed phylogenetic hypotheses about the evolutionary affinities of *Xenothrix*. **H1**: Genus-level tree with *Xenothrix* as sister to *Callicebus* within Pitheciidae (*23*). **H2**: Genus-level tree with *Xenothrix* as sister to *Aotus* within Cebidae (*27*). **H3**: Genus-level tree with *Xenothrix* as sister to *Aotus* within Pitheciidae (*27*). **H4**: Genus-level tree with *Xenothrix* falling outside all extant platyrrhine families (*31*). **H5**: Species-level tree with *Xenothrix* as sister to all recently recognized callicebid genera (*23*, *28*).

Fig 3. Genus-level Maximum Likelihood phylogeny generated using whole mitochondrial genomes and produced in RAxML, using data sequenced in this study for *Xenothrix* and data for 15 other primate genera from Genbank, and with *Macaca fuscata* selected as outgroup. Node values represent bootstrap support (100 replicates).

Fig 4. Time-calibrated phylogeny showing estimated divergence dates for *Xenothrix*, 14 other callicebine species, and five other platyrrhine genera. Estimates of median divergence dates are shown in red above nodes. Node bars indicate 95% highest posterior density values. Branch values represent posterior probabilities.