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TITLE

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SHORT TITLE

Phylogeny and cranial evolution in pitheciids and *Aotus*

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ABSTRACT

Pitheciids, one of the major radiations of New World monkeys endemic to South and Central America, are distributed in the Amazon and Orinoco basins, and include *Callicebus*, *Cacajao*, *Chiropotes* and *Pithecia*. Molecular phylogenetics strongly support pitheciid monophyly, while morphological analyses infer a range of phylogenies including a sister relationship between *Aotus* and *Callicebus*. We collected geometric morphometric cranial data from pitheciids and *Aotus*, and used cranial data for distance-based phylogenetic analysis and tests of phylogenetic signal. Phylogenetic analyses of pitheciids were repeated with *Lagothrix*, *Callimico* and *Saimiri* outgroups for Procrustes shape with and without *Aotus* based on the whole cranium and six anatomical regions. All phylogenetic signal tests were significant, and tree lengths were shortest and had the least morphological change over the phylogeny for Procrustes residuals from the cranial base and palate. The majority of phylogenetic analyses of Procrustes shape for pitheciids without *Aotus* supported the molecular phylogeny, and with *Aotus* included the majority inferred an *Aotus*-*Callicebus* clade, although three analyses with *Callimico* as outgroup supported the molecular phylogeny. The morphological similarity of *Aotus* and *Callicebus* is likely a mix of plesiomorphy, allometry and homoplasy, and future phylogenetic inference of living and extinct platyrrhine taxa should consider the impact of these factors alongside outgroup selection and cranial region.

Key words: allometry; homoplasy; geometric morphometrics; platyrrhines

INTRODUCTION

The pitheciids (family Pitheciidae; parvorder Platyrrhini) are one of the three major adaptive radiations of primates endemic to South and Central America, and recent molecular analyses estimate the pitheciid clade split from the atelids and cebids around 25 million years ago (MYA) [Perelman et al., 2011; Wilkinson et al., 2011; Jameson Kiesling et al., 2015]. The extant pitheciids are split into two subfamilies: Callicebinae for the smaller-bodied, frugivorous titi monkeys (*Callicebus*), and Pitheciinae (the pitheciins), the larger-bodied, specialized seed predators that includes sakis (*Pithecia*), bearded sakis (*Chiropotes*), and uacaris (*Cacajao*).

Pitheciids are distributed in the Amazon and Orinoco basins, inhabit a range of habitats, are arboreal and have a mixed locomotor repertoire [Kinzey, 1997; Norconk 2011]. The smallest pitheciids belong to the genus *Callicebus*, with body masses of around 1kg, and the largest pitheciid is the moderately sexually dimorphic *Cacajao,* with mean male body masses around 3.1 – 3.5 kg, depending on species, and females are about 20% smaller [Ford & Davis, 1992; Smith & Jungers, 1997]. *Callicebus* and *Pithecia* have a relatively small brain size compared to *Cacajao* and *Chiropotes*, which are both highly encephalized [Isler et al., 2008; Hartwig et al., 2011]. The *Callicebus* diet is primarily frugivorous with some seed consumption, whereas *Cacajao*, *Chiropotes* and *Pithecia* are predominantly seed predators [Norconk et al., 2009]. Seed predation involves sclerocarpic foraging and morphological adaptations to access hard, thick fruits from which seeds are extracted, chewed and swallowed [Kinzey & Norconk, 1990, 1993; Kinzey, 1997].

Monophyly of *Cacajao*, *Chiropotes* and *Pithecia* have been acknowledged in all major primate taxonomic classifications [Kinzey, 1992; Rosenberger et al., 1996]. Morphology-based phylogenetic analyses of platyrrhines have also supported a pitheciin clade with *Cacajao*-*Chiropotes* sister to *Pithecia* [Rosenberger, 1984; Ford, 1986; Kay, 1990, Horovitz, 1999]. However, the systematics of the family are not entirely straightforward. In particular, the relationship with the nocturnal *Aotus* is controversial and there have been debates over the position of *Callicebus*. An *Aotus*-*Callicebus* clade distantly related to the pitheciins has been suggested [Ford, 1986], and *Aotus*-*Callicebus* has been placed as sister to the pitheciins [Rosenberger, 1984]. Alternatively, *Callicebus* has been inferred as the basal-most platyrrhine [Kay, 1990], or sister only to pitheciins [Horovitz, 1999].

Morphology and molecules appear to tell different stories with respect to *Callicebus* and *Aotus*. Platyrrhine molecular phylogenetic data strongly support a pitheciid clade with *Callicebus* basal-most and a sister relationship between *Pithecia* and *Cacajao*-*Chiropotes*, and *Aotus* more closely related to *Cebus*-*Saimiri* and callitrichines than it is to *Callicebus* or the pitheciids [Fig. 1: Wildman et al., 2009; Jameson Kiesling et al., 2015; Schneider & Sampaio, 2015]. Despite the molecular data, *Aotus* and *Callicebus* have similar body masses of around 1kg, are both primary frugivores with tall thin incisors and high temporomandibular joints, are socially monogamous, have small group sizes, and low sexual dimorphism [Kinzey, 1997; Rosenberger & Tejedor, 2013]. The two taxa are sympatric in parts of Peru, and resource competition could be avoided through the evolution of nocturnal behaviour in *Aotus* and reliance on alternative secondary dietary resources [Norconk et al., 2009]. The morphological and behavioural similarities of *Aotus* and *Callicebus* have led some researchers to consider them closely-related sister taxa [Rosenberger, 1981, 1984, 1992, 2002; Kinzey, 1992; Rosenberger et al., 2009; Rosenberger & Tejedor, 2013]. Nonetheless, the two groups have some major biological differences, primarily because the nocturnal and cathemeral activity of *Aotus* is unique among platyrrhines, resulting in its distinctive very large orbits [Kinzey, 1997], and *Aotus* has a wider distribution across Central and South America than pitheciids [Kinzey, 1997].

While both morphological and molecular data provide important information about evolutionary biology, molecular phylogenetics have become ubiquitous as they tend to be more robust and reliable approximations of evolutionary relationships [Scotland et al., 2003]. Morphological datasets generally contain hundreds of characters or anatomical landmarks, whereas next-generation DNA and genome sequencing creates datasets with tens to hundreds of thousands of characters per species for use in phylogenetic inference [Yang & Rannala, 2012]. These large molecular datasets use sophisticated statistics and models of evolution, and combined with increased number of independent traits used, provide a clear advantage over morphology-based analyses [Whelan et al., 2001]. However, molecular phylogenies can vary due to differences between gene trees and species trees, the source of DNA (e.g. nuclear or mitochondrial genomes) and use of coding or non-coding regions, variation in rates of evolution, homoplasy, incomplete lineage sorting, and introgression amongst other factors [Degnan & Rosenberg, 2009, Davalos et al., 2012]. They will not invariably recover the 'correct' relationship, and as Perez & Rosenberger [2014] point out, major disparities are still evident in relationships recovered for platyrrhines. Although there are discrepancies in the position of *Aotus* in relation to callitrichines and *Cebus*-*Saimiri*, on balance it is likely the molecular phylogenetic separation of *Aotus* and *Callicebus* is accurate.

This separation of *Aotus* from the pitheciids in turn suggests the proposed morphological affinity of *Aotus* and *Callicebus* reflects either homology and retention of ancestral platyrrhine plesiomorphic traits or homoplasy and convergence between the two taxa, but not evidence of recent common ancestry. As molecular studies indicate the two groups last shared a common ancestor approximately 25 million years ago [Perelman et al., 2011; Wilkinson et al., 2011; Jameson Kiesling et al., 2015], it raises important research questions applicable to platyrrhines and the palaeontological study of primates more generally. What factors influenced *Aotus* and *Callicebus* convergence or lack of divergence from the common ancestral form? If *Aotus* had gone extinct 1 million years ago and was only known from the fossil record, given its social, ecological and biological similarities with *Callicebus*, would the two groups be erroneously classified as closely related sister taxa? Given that recoverable DNA is absent from most fossil taxa, resolving the "tree of life" of both extant and extinct taxa will require sound and reliable phylogenetic inference using morphology [Wiens, 2004].

The development of geometric morphometric methods has provided new opportunities for quantification and statistical analysis of morphology [Adams et al., 2004] which can be applied to analyse morphological and phylogenetic relationships. Previous morphological analyses that recovered a close sister relationship between *Aotus* and *Callicebus* were based on character-state and cladistic techniques despite high levels of homoplasy across the platyrrhine clade and most characters showing parallel evolution [Lockwood, 1999; Kay et al., 2008]. In contrast, several large-scale studies of primates demonstrated geometric morphometric data, with its ability to capture small yet significant shape variation, may find greater congruence between molecular and morphological phylogenies [Lockwood et al., 2004; Cardini & Elton, 2008b]. A major benefit of geometric morphometric methods is the ability to separate size from shape, which can be used to investigate allometry, the study of size and its consequences, particularly the relationship between body size and traits including morphology, diet, behaviour, and ecology [Gould, 1966; Cheverud, 1982; Fleagle, 1995; Mitteroecker et al., 2013]. Interspecific allometry –size-related differences between adults of different species [Martin, 1990; Fleagle, 1995] – is important for pitheciid evolution, as the largest taxon *Cacajao* is approximately three times larger than the smallest taxa *Callicebus*; the similarities in body mass between the latter and *Aotus* could explain their morphological and behavioural similarities.

Additionally, a combined geometric morphometric and modular approach to phylogenetic inference using cranial variation can highlight which regions are congruent, and incongruent, with molecular phylogenetic results. Modularity involves interaction and co-variation between traits/variables in a shared region that are partially independent, with modules partially distinct from each other in structure and function [Klingenberg, 2008]. If modules of the cranium reflect alternative functional, developmental and evolutionary roles, the pattern of similarity and utility of modules for accurate phylogenetic inference should vary [Wood & Lieberman, 2001; Harvati & Weaver, 2006]. It is unlikely a single cranial anatomical region will accurately infer phylogenetic relationships for all primate clades [von Cramon-Taubadel, 2014], creating the need to investigate each group individually. By examining whether molecular clades are consistently inferred in some regions of the cranium compared to others, the most informative regions may be targeted for phylogenetic reconstructions in fossil taxa, provided appropriate specimens are available for study.

An important concept for understanding the relationship between molecular and morphological evolution is the phylogenetic signal, where closely related taxa will be phenotypically more similar to each other than either is to more distantly related taxa, whereas a weak phylogenetic signal occurs when taxa are more similar to distant relatives or similarity is distributed randomly across the phylogeny [Blomberg et al., 2003, Klingenberg & Gidaszewski, 2010, Kamilar & Cooper, 2013]. The phylogenetic signal can also be considered a statistical measure of the non-independence of trait similarity shared by taxa due their phylogenetic relationships [Revell et al., 2008]. A strong phylogenetic signal is predicted under a Brownian motion model of evolution, while the strength of phylogenetic signal is phenotype and phylogeny dependent and can be lowered by adaptation, measurement error of traits, and error in phylogenetic topology and branch lengths [Blomberg & Garland, 2002, Kamilar & Cooper, 2013]. The phylogenetic signal of primates across a range of phenotypic traits has provided insight into their evolution [Kamilar & Cooper, 2013], and comparative study and quantification of which areas of morphology have stronger or weaker phylogenetic signals can suggest which areas will be informative for phylogenetic inference and help inform our understanding cranial evolution in groups of interest.

In this paper, we examine the evolutionary relationships and phylogenetic signal of pitheciids and *Aotus* based on geometric morphometric data from the cranium. We test two primary hypotheses – [1] there is a phylogenetic signal in the pitheciid cranium, and a particular cranial region and outgroup will find greater congruence between morphological and molecular phylogenies; [2] that phylogenetic analysis of geometric morphometric data will differentiate between *Aotus* and *Callicebus* and find little support for an *Aotus*-*Callicebus* clade.

METHODS

This research complied with the American Society of Primatologists Principles for the Ethical Treatment of Primates, protocols of the appropriate Institutional Animal Care Committee, and legal requirements of each country housing collections.

Morphometric data, consisting of sixty-three 3D anatomical landmarks quantifying morphological variation in the cranium (Table I) were collected from museum collections for *Callicebus cupreus*, *Callicebus hoffmannsi*, *Callicebus moloch*, *Callicebus torquatus*, *Cacajao calvus*, *Cacajao melanocephalus, Chiropotes satanas*, *Pithecia pithecia*, *Pithecia monachus*, *Aotus azarae*, *Aotus lemurinus*, *Aotus vociferans*, *Aotus trivirgatus*, and outgroup taxa *Callimico goeldii*, *Lagothrix lagotricha* and *Saimiri sciureus* (Table II). Museum specimens were originally wild caught except for *Callimico goeldii* specimens that were all captive. Despite the large number of pitheciid species recognized in recent taxonomic classifications, adequate sample sizes are difficult to obtain from museum collections. The 3D anatomical landmarks were analysed with geometric morphometric methods (GMM) that measure and preserve the geometry of structures being studied by removing non-biological variation in scale, orientation and position of landmarks [Rohlf & Slice, 1990; Adams et al., 2004]. The GMM methods used Generalised Procrustes Analysis (GPA), which has the highest accuracy of available superimposition methods in estimating mean shape, lowest error estimates, and greatest power to test for differences in mean shape between taxa [Gower, 1975; Goodall, 1991; Rohlf, 2000a,b, 2003]. Procrustes shape coordinates describing shape are distinct from the measure of size, centroid size, the square root of summed squared distances between landmarks and their centroid [Mitteroecker et al., 2013] are produced following GPA.

Geometric morphometric analysis was carried out in MorphoJ v1.06 (University of Manchester, Manchester, UK; http://www.flywings.org.uk/morphoj_page.htm). Centroid size, the square root of the sum of squared distances of landmarks from the centroid, is the measure of size provided by GMM [Zelditch et al., 2004]. MorphoJ allows geometric morphometric data to be mapped onto a phylogeny, in this case based on molecular phylogenetic relationships of pitheciids with and without *Aotus*, using squared-change parsimony to examine and quantify the phylogenetic signal. The phylogenetic signal will be strongest when closely related taxa are phenotypically more similar to each other and occupy similar morphometric space compared to more distantly related taxa [Klingenberg & Gidaszewski, 2010]. This approach quantifies tree length based on the total sum of squared change along all landmark coordinates and branches of the phylogeny, providing a single measure of morphological change over the phylogeny provided, and morphometric data with a stronger phylogenetic signal will have less shape change across the branches of the phylogenetic tree and shorter tree lengths, whereas morphometric data with a lower phylogenetic signal will exhibit greater morphological change along branches of the phylogeny and have longer tree lengths [Klingenberg & Gidaszewski, 2010]. The measurement of the phylogenetic signal uses permutations to test the null hypothesis of no phylogenetic signal by resampling taxa, recalculating tree length, and providing a P value for the proportion of resampled datasets with a shorter or equal tree length compared to the original dataset [Klingenberg & Gidaszewski, 2010]. If the null hypothesis of no phylogenetic signal is true, the permutation test that randomly swaps the morphometric values at the tip of the phylogeny should not alter tree length and morphological change compared to the original data, while the tree length would increase if the permutation acted on morphometric data with a phylogenetic signal. Different phylogenetic signal results are best considered comparatively where the same phylogeny and alternative shape data, or alternative phylogenies and the same shape data, are used.

The phylogenetic signal in both shape (based on Procrustes coordinates) and size (based on log centroid size) were analysed with and without *Aotus* included, and no outgroup, requiring separate input phylogenies to quantify the phylogenetic signal based on the molecular analyses of all platyrrhines. These phylogenies, based on relationships supported by multiple molecular phylogenetic studies had *Aotus* sister to pitheciids, within which *Callicebus* is basal-most and *Pithecia* is sister to *Cacajao*-*Chiropotes*, and for analyses of just pitheciids the same phylogenetic relationships with *Aotus* removed [Perelman et al., 2011; Jameson Kiesling et al., 2015; Schneider & Sampaio, 2015]. As neither Perelman and colleagues [2011] nor Jameson Kiesling and colleagues [2015] used the neighbor-joining method for phylogenetic inference, for consistency we accessed their publically available molecular datasets and ran neighbor-joining in PAUP 4 (Sinauer Associates, Sunderland, Massachusetts, USA; http://paup.sc.fsu.edu/), which supported the previously described pitheciid relationships and placement of *Aotus* within cebids. Considering the species-level relationships within *Callicebus* and *Aotus* are not fully resolved, the relationships within each genus were treated as unresolved polytomies.

Euclidean morphological distances were used for phylogenetic construction using neighbor-joining in the Neighbor module of Phylip 3.6 (University of Washington, Seattle, Washington, USA; http://evolution.genetics.washington.edu/phylip.html). Neighbor-joining constructs a phylogeny with a stepwise additive method based on a divisive cluster algorithm that minimizes overall branch length, is statistically consistent, inferring the correct evolutionary tree when distances accurately reflect phylogeny, assumes distances between two taxa are equal to the distance between each respective group and a shared node, and roots the tree using an outgroup taxa [Saitou & Nei, 1987; Kuhner & Felsenstein, 1994; Yang, 2006].

Selection of outgroup taxa can impact phylogenetic inference of morphology [e.g. Bjarnason et al., 2011, 2015], and although a plesiomorphic fossil platyrrhine taxa would make an ideal outgroup, in the absence of an adequately large sample size of specimens, using geometric morphometric data for fossil taxa is difficult due to increased error rates in estimating mean shape with low sample sizes [Cardini & Elton, 2008b], and distortion to fossil specimens can require considerable virtual reconstruction [e.g. Zollikofer et al., 2005, Spoor et al., 2015]. As two of the five major extant platyrrhine clades, pitheciids and *Aotus*, are ingroup taxa, one outgroup was sampled from each of the three remaining clades, with phylogenetic inference repeated using an atelid, callitrichine and cebine outgroup. The atelid *Lagothrix lagotricha* was selected as it is likely the closest to the ancestral atelid phenotype and least derived extant group in that clade [Rosenberger & Strier, 1989, Bjarnason et al., 2015], and *Callimico goeldii* has lost multiple typically callitrichine traits in morphology and reproduction and likely acquired secondarily derived traits similar to the ancestral platyrrhine [Martin, 1992, Pastorini et al., 1998, Scott, 2015]. As allometry and the size of outgroups, and its impact on phylogenetic inference, is of interest [Bjarnason et al., 2011], we selected outgroups that were considerably larger (*Lagothrix lagotricha*) and smaller (*Callimico goeldii*) than ingroup taxa, in addition to a third outgroup (*Saimiri sciureus*) that is derived in morphology but shares ancestral platyrrhine body size with *Aotus* and *Callicebus* [Ford & Davis, 1992].

Statistical support for clades was quantified using a jack-knife method where phylogenetic analysis and Procrustes superimposition was repeated with each landmark removed, with percentage clade support the number of times a clade was present in each phylogenetic analysis, and results were collated using the Consensus module in Phylip [Felsenstein, 2005]. Majority consensus trees were drawn using TreeView (University of Glasgow, Glasgow, UK; https://www.ctu.edu.vn/~dvxe/Bioinformatic/Software/Rod%20Page/treeview.html) and TreeGraph 2 (University of Münster, Münster, Germany; http://treegraph.bioinfweb.info/Download). As with the tests of a phylogenetic signal, the neighbour-joining phylogenetic analysis was repeated to include pitheciids only, and with pitheciids and *Aotus* as ingroup taxa.

Tests for phylogenetic signal and neighbour-joining phylogenetic analysis were all repeated with morphometric data from the whole cranium, and hypothesized modules within the cranium. Cranial modules of the orofacial and neurocranium are recognized with further subdivision into the face, palate/oral, nasal, zygomatic, cranial base and cranial vault [Cheverud, 1982; Hallgrimsson et al., 2004], in addition to larger modules for the chondrocranium of the cranial base and dermatocranium of the face and cranial vault based on mode of ossification [Hallgrimsson et al., 2004; Cardini & Elton, 2008a]. Cardini & Elton [2008a] have shown sampling error becomes high in modules with low numbers of landmarks, and we are unable to analyse orbit and zygomatic modules in our cranial dataset due to the low number of landmarks. Modules of the cranial vault and palate region had too few landmarks to be analysed as individual modules, but were combined with the face and cranial base in a series of landmark combinations. Overall, seven regions were analysed: the cranium (landmarks 1-63), face (landmarks 1-15), face and palate (landmarks 1-15, 30-38), face and cranial vault (landmarks 1-26, including landmarks 17-19 from the zygomatic arch), cranial base (landmarks 40-63), cranial base and vault (landmarks 16, 20-26, 40-63), and cranial base and palate (landmarks 30-63, including landmark 39 that falls between regions).

RESULTS

The measures of phylogenetic signal for Procrustes coordinates and log centroid size, without and with *Aotus*, are presented in Table III based on tree length and a permutation test of significance. The permutation test of significance takes morphometric values at the tip of a phylogeny and randomly swaps them, which will have no effect on tree length if there is no phylogenetic signal, but will be significantly different to the tree length from the original data if a phylogenetic signal is present- our results show a phylogenetic signal is present for all iterations, rejecting the null hypothesis there is no phylogenetic signal in cranial data. Tree length quantifies the combined morphological change across all branches of a phylogeny, with lower tree lengths signifying less morphological change and a stronger phylogenetic signal, and larger tree lengths involving greater morphological change and a weaker phylogenetic signal. For each cranial region in pitheciid analyses without *Aotus*, log centroid size tree lengths were longer than for Procrustes coordinates with the exception of the cranial base and palate. For pitheciid analyses including *Aotus*, tree lengths were longer than for analyses without *Aotus* as expected considering the increased taxa sampling, and for each cranial region the tree lengths from Procrustes coordinates were longer than for log centroid size except for the cranial base and palate, and face and palate. For shape coordinates, for pitheciids both with and without *Aotus*, the region with the strongest phylogenetic signal, shortest tree lengths and least morphological change across the phylogeny was the cranial base and palate, followed by the cranium, cranial base and vault, cranial base, face and cranial vault, face, and the weakest phylogenetic signal was in the face and palate.

The results of neighbour-joining phylogenetic analysis are provided at the genus level as majority consensus trees (Figs. 2-3) and jack-knife clade support (Tables IV-V) for pitheciids with and without *Aotus* included as ingroup taxa. Phylogenetic analysis of pitheciids-only

(Fig. 2 and Table IV) supported the molecular phylogeny with *Cacajao*-*Chiropotes* sister to *Pithecia* and *Callicebus* basal-most in eleven of twenty-one analyses, supported a dichotomy between *Callicebus*-*Pithecia* and *Cacajao*-*Chiropotes* in nine analyses, and *Callicebus* sister to *Cacajao*-*Chiropotes* and *Pithecia* basal-most in one analysis.

Phylogenetic analyses of pitheciids with *Aotus* (Fig. 3 and Table V) supported an *Aotus*-*Callicebus* clade in sixteen of twenty-one analyses. Eleven analyses placed *Cacajao*-*Chiropotes* basal-most and *Pithecia* sister to *Aotus*-*Callicebus*, and three analyses inferred *Aotus*-*Callicebus* basal-most and *Pithecia* sister to *Cacajao*-*Chiropotes*. A further three analyses inferred *Cacajao*-*Chiropotes* sister to *Pithecia* in a clade with *Aotus*, and *Callicebus* basal-most, and one analysis inferred a dichotomy between *Aotus*-*Callicebus* and *Cacajao*-*Chiropotes* with *Pithecia* basal-most. Pitheciid monophyly and the molecular phylogeny with *Cacajao*-*Chiropotes* sister to *Pithecia*, *Callicebus* within the pitheciids and *Aotus* basal-most was inferred for three analyses with *Callimico* as outgroup.

DISCUSSION

Phylogenetic analysis of pitheciid cranial variation confirms the first hypothesis of the presence of a phylogenetic signal, with a complex mix of congruence between molecular and morphological phylogenies depending on ingroup taxa, outgroup selection and cranial region. However, considering the majority of phylogenies constructed including pitheciids and *Aotus* inferred an *Aotus*-*Callicebus* clade, we reject the second hypothesis that phylogenetic analysis of geometric morphometric data would differentiate between the two taxa in the majority of analyses, and support earlier findings of a morphological affinity between *Callicebus* and *Aotus* [e.g. Rosenberger, 1984, 2002; Kinzey, 1992; Rosenberger et al., 2009; Rosenberger & Tejedor, 2013].

Rosenberger & Tejedor [2013] view the similarity of *Aotus* and *Callicebus* as phylogenetic, and propose that long-branch attraction in molecular phylogenetics has mis-placed *Aotus* outside of the pitheciids. However, there are a number of other evolutionary scenarios that could explain similarities between *Aotus* and *Callicebus*: (a) *Aotus* and *Callicebus* have maintained plesiomorphic primitive ancestral traits in size, morphology and behaviour, for over 25 million years; (b) *Aotus* and *Callicebus* have undergone major homoplasy, whereby similarity shared by taxa is not due to common ancestry [Lockwood & Fleagle, 1999], and converged upon the same size, morphology and behaviour via convergence in similar ecological and social environments; or (c) a complex mix of the two, with a combination of ancestral and convergent traits.

Interpretation of the early platyrrhine fossil record is important for considering the extent of plesiomorphy and homoplasy found in *Aotus* and pitheciids, although the topic is contentious. The long lineage hypothesis considers extant platyrrhines a more ancient radiation and positions early fossil taxa such as *Tremacebus* and *Soriacebus* within clades alongside extant groups [e.g. Rosenberger et al., 2009, Rosenberger, 2010], whereas the layered hypothesis views extant clades and fossil taxa descended from the crown group common ancestor as a more recent radiation and places several of the earliest platyrrhine fossil taxa outside the crown group as stem platyrrhines [e.g. Kay, 1990, 2015, Kay et al., 2008]. Both hypotheses require extensive homoplasy [Rosenberger 2002, Kay & Fleagle 2010], but differ in an important interpretation of living and fossil groups fundamental to understanding the similarity of *Aotus* and *Callicebus*. The long lineage hypothesis views seed predation in *Soriacebus* as providing an ecophylogenetic link to pitheciids and traits in orbit morphology in *Tremacebus* and *Aotus* are due to shared ancestry [Rosenberger, 2010], indicating traits connecting *Aotus* and *Callicebus* are similarly derived and phylogenetic. In contrast, the layered hypothesis views *Tremacebus* and *Soriacebus* as stem platyrrhines rather than close relatives of *Aotus* and pitheciids [Kay et al., 2008, Kay, 2015], with many similarities between stem and crown groups primitive traits, indicating *Aotus* and *Callicebus* shared traits are ancestral for platyrrhines.

With debate still ongoing over the long lineage and layered hypotheses, we propose the molecular phylogenetic separation of *Aotus* and *Callicebus* is accurate and that a mix of plesiomorphy, allometry and homoplasy combines to drive morphological and behavioural similarity rather than recent common ancestry. While *Aotus* and *Callicebus* may retain the plesiomorphic platyrrhine body size [Ford & Davis, 1992] alongside several other ancestral traits, the callitrichine-like body size of the earliest platyrrhine fossil *Perupithecus* [Bond et al., 2015] suggests a smaller ancestral body size and convergent size evolution in *Aotus* and *Callicebus*, although that interpreation depends on whether *Perupithecus* belongs to a crown or stem group and is representative of the platyrrhine common ancestor. Whether shared body size is ancestral or derived in *Aotus* and *Callicebus*, it seems probable they will share other plesiomorphic traits, yet homoplasy remains a pervasive evolutionary reality [Kay & Fleagle, 2010]. Platyrrhine morphological characters are known to have high levels of homoplasy [Lockwood, 1999], nearly all phylogenetically informative traits from the platyrrhine cladistic analysis of Kay and colleagues [2008] showed some parallel evolution, and due to the high levels of homoplasy morphological characters can be used in support of most phylogenetic relationships [Kay, 2015]. As homoplasy is widespread in the platyrrhine clade, allometry is a particularly powerful intrinsic factor in morphological homoplasy [Lockwood & Fleagle, 1999; Kay & Fleagle, 2010], and post-cranial traits shared by *Aotus* and *Callicebus* have been linked to parallel evolution [Lockwood, 1999], it is likely some of the traits shared by *Aotus* and *Callicebus* are due to homoplasy.

The body size similarity and allometric link between *Aotus* and *Callicebus* contributes to shared morphological similarity, but a key factor in morphology-based phylogenetic inference is also the allometric relationship between outgroup and ingroup taxa. This issue has been previously highlighted in hominoids, where allometric scaling and cranial shape linked to brain size in *Hylobates* and *Homo* complicate accurate phylogenetic inference [Creel, 1986; Bjarnason et al., 2011]. The phylogenetic analyses of pitheciids including *Aotus* with *Saimiri* as outgroup inferred an *Aotus*-*Callicebus* clade in all seven analyses, and *Aotus*, *Callicebus* and *Saimiri* share a similar body size. Using the much larger-bodied *Lagothrix* outgroup supported *Aotus*-*Callicebus* in six of seven analyses, whereas the smaller-bodied *Callimico* outgroup inferred *Aotus*-*Callicebus* in two analyses, and the molecular phylogeny in three. This does not mean using a smaller-bodied outgroup will reduce the influence of allometry on all morphology-based phylogenetic analyses as it will be dependent up the allometric relationships within the ingroup, as in Old World monkeys [e.g. Gilbert & Rossie, 2007; Gilbert et al., 2009] and between ingroup and outgroup taxa, and the issue remains pertinent for accuracy of phylogenetic inference and study of primate groups.

The relative lack of support for a monophyletic pitheciid clade when *Aotus* is included in analyses contrasts with the eleven analyses that support the molecular phylogenetic relationships when only pitheciid cranial data is analysed. This reflects the evolution of multiple traits including morphological adaptations, diet, and relative brain size, which broadly follow a morphocline, with *Callicebus* expressing a relatively ancestral or primitive phenotype, *Pithecia* an intermediate or partially derived condition, and *Cacajao* and *Chiropotes* sharing a derived phenotype [Kinzey, 1992]. For example, in cranial morphology the differentiation in phylogenetic analysis between *Callicebus* and the pitheciins *Cacajao*, *Chiropotes* and *Pithecia* reflects the latter as specialized sclerocarpic foragers with incisor and canine adaptations and enlarged temporalis and masseter muscles able to generate high-forces to open hard-tusked fruits [Kinzey & Norconk, 1990, 1993; Kinzey, 1992, 1997]. Allometry also helps maintain a phylogenetic signal with inference of the smallest lineage *Callicebus* basal-most and a sister relationship between the two largest genera, *Chiropotes* and *Cacajao*. The choice of outgroup is clearly also important, as six of seven phylogenetic analyses with *Callimico* inferred the pitheciid molecular phylogeny, whereas six of seven analyses using *Saimiri* as outgroup inferred a dichotomy including a *Pithecia*-*Callicebus* clade not supported by molecular phylogenetics.

From our data, all cranial regions had a phylogenetic signal, but there were clear differences in tree lengths for different regions. The region with the strongest phylogenetic signal, the cranial base and palate, had a tree length one third of the tree length for the region with the weakest phylogenetic signal, the face and palate, meaning there has been greater morphological change over the phylogeny in the face and palate. The maintenance of a stronger phylogenetic signal in cranial base morphology has been hypothesized as due to strong genetic control and a role in multiple functional systems compared to the more plastic face that is shaped by environmental factors [e.g. Olson, 1981; Lieberman et al., 1996; Lieberman, 1997]. However, Revell and colleagues [2008] cautions against linking strong and weak phylogenetic signals with concepts of conserved or plastic traits, as an array of evolutionary processes and rates of evolution can create a similar phylogenetic signal, and very similar processes can lead to varied phylogenetic signals.

While the region of the cranial base and palate has the strongest phylogenetic signal of the regions investigated here in pitheciids and *Aotus*, the phylogenetic signal in phenotypic traits will likely vary dependent on the taxonomic and phylogenetic level [Kamilar & Cooper, 2013], and no single cranial region will maintain the strongest phylogenetic signal across all primates [von Cramon-Taubadel, 2014]. It is worth considering an additional issue; how a region can have a strong phylogenetic signal, yet phylogenetic inference based on data from that region often fails to support evolutionary relationships strongly supported by molecular data. For our three regions with the strongest phylogenetic signal, the cranial base and palate, cranium, and cranial base and vault, phylogenetic inference that included pitheciids and *Aotus* inferred non-molecular clades in each analysis using *Lagothrix* and *Saimiri* outgroups, but inferred the molecular phylogeny in all three analyses with *Callimico* as outgroup. This suggests the presence of a strong phylogenetic signal is not, of itself, enough to find congruence between molecular and morphological phylogenies, but as has been shown in other primate groups [e.g. Bjarnason et al., 2011, 2015] methodological decisions such as outgroup selection and rooting are integral to using a strong phylogenetic signal for accurate phylogenetic inference.

To return to one of our orginal questions, if *Aotus* was known only from the fossil record and included in a phylogenetic analysis with pitheciids, it would probably be erroneously classified as sister to *Callicebus* – our study, in common with several others demonstrates the morphological similarity between the two taxa despite their deep divergence. This morphological connection is likely to be a mix of the retention of ancestral platyrrhine traits and convergence, both with a link to allometry and similar dietary niches, body mass and cranial form in *Aotus* and *Callicebus*. By considering the effects of allometry, outgroup selection and modularity on phylogenetic analysis alongside the benefits of including fossil taxa, combined datasets, molecular scaffolds and character weighting, it should be possible to have greater confidence in assessing phylogenetic relationships and derived similarity in the platyrrhine fossil record than appears initially from the *Aotus*-*Callicebus* example.

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Table I list of cranial anatomical landmarks

1. Piriform aperture nasospinale
2. Piriform aperture point of greatest width
3. Piriform aperture meeting of nasal and maxilla
4. Piriform aperture rhinion, most anterior midline
5. Nasion suture meeting of fronto nasals
6. Glabella midline point on frontal between supraorbital ridges
7. Supraorbital superior
8. Frontomalare orbitale
9. Frontomalare temporal
10. Zygo-max superior
11. Zygo-max inferior
12. Zygomatic foramen inferior
13. Infraorbital foramen inferior
14. Lacrimal duct fossa bottom
15. Optic foramen most medial
16. Upper posterior maxilla
17. Maximum point of curvature on upper zygomatic
18. Zygo-temp superior
19. Zygo-temp inferior
20. Meeting point of sphenoid and zygomatic
21. Meeting point of sphenoid, parietal and zygomatic process of temporal
22. Midpoint between glabella and bregma

23. Bregma
24. Midpoint between bregma and lambda
25. Lambda
26. Asterion
27. Auditory meatus anterior
28. Auditory meatus posterior
29. Auditory meatus inferior
30. Incisor I1 septum
31. Canine septum
32. Premolar P2 septum
33. Molar M1 septum
34. Midpoint of septum at end of dentition
35. Incisive foramen posterior
36. Meeting point of maxilla and palatine
37. Palatine foramen posterior/lateral
38. Max curvature of posterior edge of palatine
39. Nasal spine midpoint where wings split
40. Midpoint between basisphenoid and basioccipital
41. Petrous apex meeting point of petrous, basiosphenoid and basioccipital
42. Foramen lavelli
43. Meeting point of petrous, sphenoid and zygomatic process of temporal
44. Petrous greatest central projection
45. Stylomastoid foramen
46. Jugular foramen distal

47. Jugular foramen medial
48. Carotid foramen anterior
49. Midpoint between basion and basisphen-basioccipital
50. Basion anterior
51. Occipital condyle anterior apex
52. Occipital condyle posterior midpoint
53. Hypoglossal canal
54. Opisthion posterior
55. Midway between opisthion and inion
56. Inion
57. Greatest curvature on posterior zygomatic process of temporal
58. Temporal meeting point between sphenoid and zygomatic process of
59. Tip of post glenoid process
60. Deepest point within mandibular fossa
61. Articular eminence medial
62. Articular eminence midpoint
63. Articular eminence lateral

Table II Pitheciid and outgroup taxa sample sizes for phylogenetic analyses

Taxa	Sample size		
Ingroups	**Female**	**Male**	**Pooled**
Aotus azarae	10	6	16
Aotus lemurinus	10	10	26
Aotus vociferans	10	10	20
Aotus trivirgatus	11	13	24
Callicebus cupreus	9	10	19
Callicebus hoffmannsi	10	9	19
Callicebus moloch	15	13	28
Callicebus torquatus	9	12	21
Cacajao calvus	10	13	23
Cacajao melanocephalus	17	13	30
Chiropotes satanas	9	14	23
Pithecia pithecia	10	12	22
Pithecia monachus	13	14	27
Outgroups			
Callimico goeldii	11	11	22
Lagothrix lagotricha	10	10	20
Saimiri sciureus	33	15	48

Table III Test of phylogenetic signal as measured by tree length (total amount of shape change across all phylogenetic branches) and statistical significance (comparing tree length for original data against permutation with random swapping of values) for Procrustes coordinates and log centroid size of pitheciids without and with *Aotus*

	Pitheciid without *Aotus*				**Pitheciid with *Aotus***			
	Procrustes coordinates		Log centroid size		Procrustes coordinates		Log centroid size	
	Tree length	***P***	**Tree length**	***P***	**Tree length**	***P***	**Tree length**	***P***
Cranial base	0.0130	<0.0001	0.0337	<0.001	0.0190	<0.0001	0.0413	<0.0001
Cranial base & palate	0.0079	<0.001	0.0367	<0.001	0.0101	<0.0001	0.0450	<0.0001
Cranial base & vault	0.0107	<0.0001	0.0412	<0.001	0.0156	<0.0001	0.0489	<0.0001
Cranium	0.0102	<0.0001	0.0351	<0.001	0.0153	<0.0001	0.0408	<0.0001
Face	0.0244	<0.001	0.0225	<0.001	0.0343	<0.0001	0.0229	<0.0001
Face & cranial vault	0.0137	<0.0001	0.0339	<0.001	0.0204	<0.0001	0.0364	<0.0001
Face & palate	0.0253	<0.0001	0.0316	<0.001	0.0363	<0.0001	0.0322	<0.0001

Table IV Jack-knife clade support for phylogenetic analysis of Procrustes shape of pitheciids.

Cranial region	Cranium			Face			Face & palate			Face & cranial vault			Cranial base			Cranial base & vault			Cranial base & palate		
Outgroup	*Callimico*	*Lagothrix*	*Saimiri*	*Callimico*	*Lagothrix*	*Saimiri*	*Callimico*	*Lagothrix*	*Saimiri*	*Callimico*	*Lagothrix*	*Saimiri*	*Callimico*	*Lagothrix*	*Saimiri*	*Callimico*	*Lagothrix*	*Saimiri*	*Callimico*	*Lagothrix*	*Saimiri*
Molecular clades																					
Cacajao	100	100	100	100	86.6	100	100	95.8	100	100	100	100	100	100	100	100	100	100	100	100	100
Callicebus	100	100	100	93.3	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Pithecia	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Cacajao-Chiropotes	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	87.5	100	100	100
Cacajao-Chiropotes -Pithecia	100	92	<20	100	<20	<20	100	79.2	100	100	<20	<20	<20	100	<20	100	<20	<20	38.2	100	<20
Non-molecular clades																					
Pithecia-Callicebus	<20	<20	100	<20	86.6	100	<20	20.8	<20	<20	80.8	100	100	<20	100	<20	<20	100	61.8	<20	100
Cacajao-Chiropotes -Callicebus	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	19.2	<20	<20	<20	<20	<20	87.5	<20	<20	<20	<20

Table V Jack-knife clade support for phylogenetic analysis of Procrustes shape of pitheciids and *Aotus*.

Cranial region	Cranium			Face			Face & cranial vault			Face & palate			Cranial base			Cranial base & vault			Cranial base & palate		
Outgroup	***Callimico***	***Lagothrix***	***Saimiri***	***Callimico***	***Lagothrix***	***Saimiri***	***Callimico***	***Lagothrix***	***Saimiri***	***Callimico***	***Lagothrix***	***Saimiri***	***Callimico***	***Lagothrix***	***Saimiri***	***Callimico***	***Lagothrix***	***Saimiri***	***Callimico***	***Lagothrix***	***Saimiri***
Molecular clades																					
Aotus	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Cacajao	100	100	100	100	93	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Callicebus	100	100	100	100	100	100	100	96	100	100	100	100	100	100	100	100	100	100	100	82	100
Pithecia	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Cacajao-Chiropotes	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Cacajao-Chiropotes -Pithecia	100	<20	<20	<20	<20	<20	100	46	100	100	<20	<20	<20	<20	<20	100	<20	<20	100	94	100
Cacajao-Chiropotes -Pithecia-Callicebus	100	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	100	<20	<20	100	<20	<20
Non-molecular clades																					
Aotus-Callicebus	<20	100	100	<20	100	100	42	100	100	100	100	100	96	100	100	<20	100	100	<20	<20	100
Aotus-Callicebus -Pithecia	<20	97	100	<20	93	100	<20	54	<20	<20	100	100	88	100	100	<20	<20	100	<20	<20	<20
Aotus-Cacajao -Chiropotes-Pithecia	<20	<20	<20	100	<20	<20	54	<20	<20	<20	<20	<20	<20	<20	<20	<20	97	<20	<20	88	<20

Figure Legends

Figure 1 Platyrrhine genus-level molecular phylogenetic relationships

Figure 2 Consensus genus-level phylogenetic relationships inferred from pitheciid analyses without *Aotus*. (a) Face, and the face and cranial vault with *Lagothrix* as outgroup, the cranial base and palate for both *Callimico* and *Saimiri* outgroups, and the cranium, face, face and cranial vault, cranial base, cranial base and vault for *Saimiri* as outgroup. (b) Molecular phylogeny for the face and palate with all three outgroups, from the cranium, and cranial base for both *Lagothrix* and *Callimico* outgroups, for the cranial base and palate with *Lagothrix* as outgroup, and the face, face and cranial vault, and cranial base and vault for *Callimico* as outgroup. (c) Cranial base and vault data with *Lagothrix* outgroup.

Figure 3 Consensus genus-level phylogenetic relationships inferred from Procrustes shape for pitheciid and *Aotus* analyses. (a) Face and cranial vault with *Callimico* outgroup, and cranial base and palate, and face and palate for *Saimiri* outgroup. (b) Cranial base for all three outgroups, cranium, face, and face and cranial vault for *Lagothrix* and *Saimiri* outgroups, face and palate for *Lagothrix* outgroup, and cranial base and vault for *Saimiri* outgroup. (c) Face, and face and palate for *Callimico*, and cranial base and palate for *Lagothrix* outgroup. (d) Cranial base and vault with *Lagothrix* outgroup. (e) Cranium, cranial base and palate, and cranial base and vault for Callimico outgroup, and congruent with the molecular phylogeny.

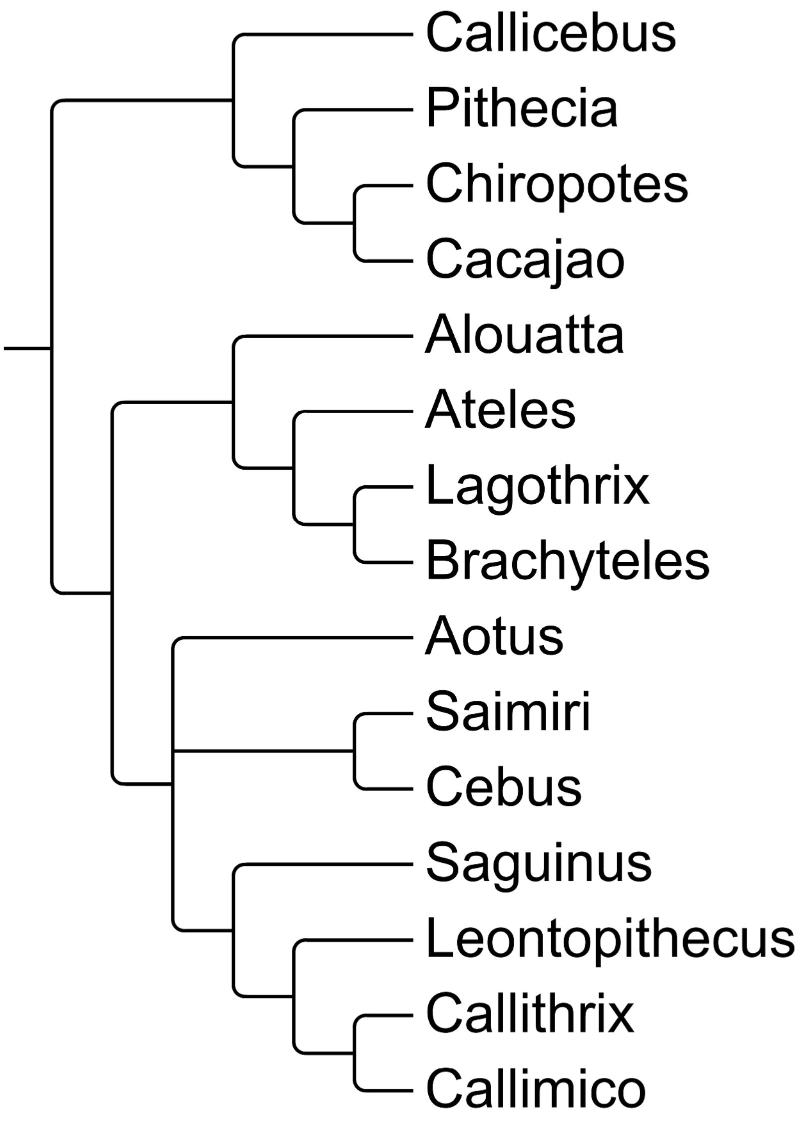

Figure 1 Platyrrhine genus-level molecular phylogenetic relationships
Figure 1

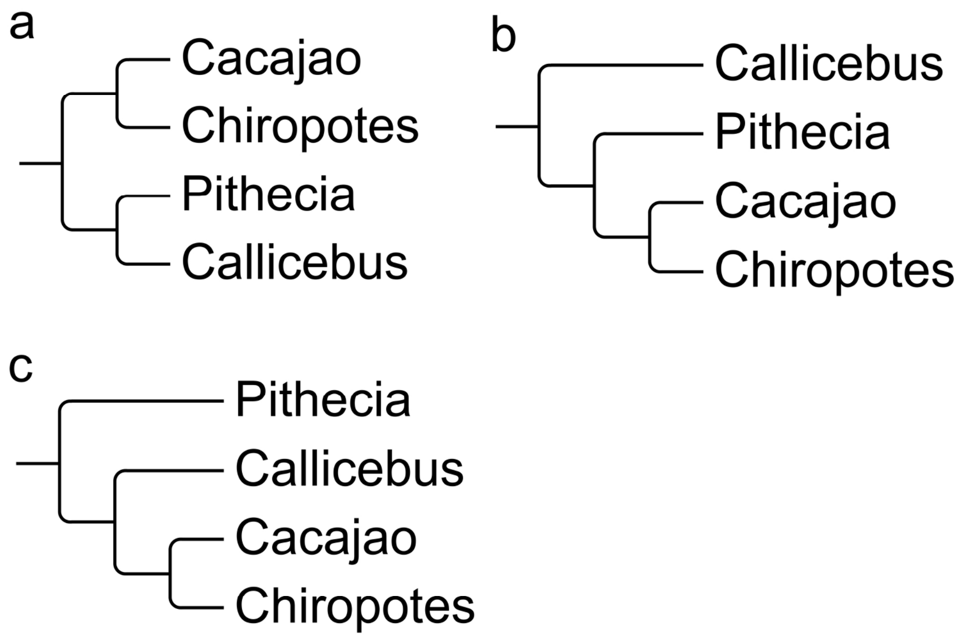

Figure 2 Consensus genus-level phylogenetic relationships inferred from pitheciid analyses without Aotus. (a) Face, and the face and cranial vault with Lagothrix as outgroup, the cranial base and palate for both Callimico and Saimiri outgroups, and the cranium, face, face and cranial vault, cranial base, cranial base and vault for Saimiri as outgroup. (b) Molecular phylogeny for the face and palate with all three outgroups, from the cranium, and cranial base for both Lagothrix and Callimico outgroups, for the cranial base and palate with Lagothrix as outgroup, and the face, face and cranial vault, and cranial base and vault for Callimico as outgroup. (c) Cranial base and vault data with Lagothrix outgroup.
Figure 2
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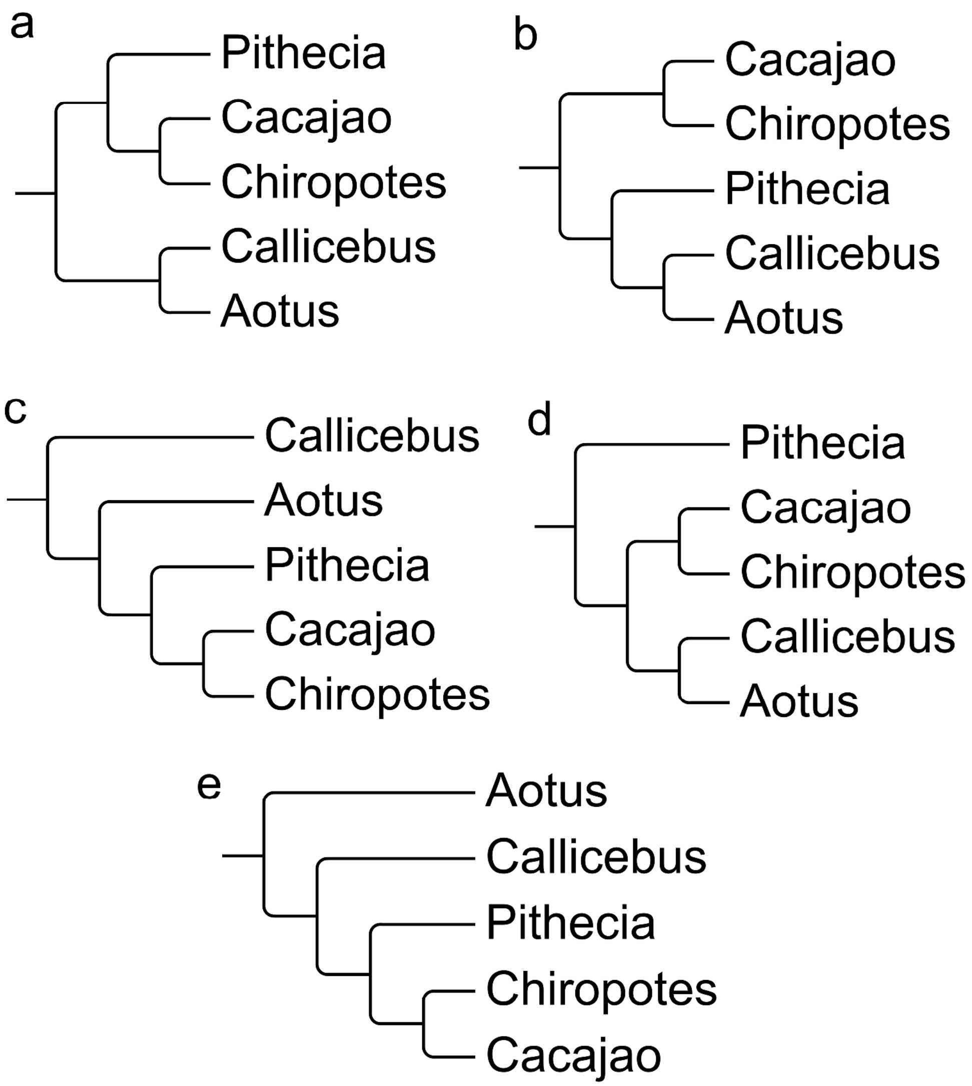

Figure 3 Consensus genus-level phylogenetic relationships inferred from Procrustes shape for pitheciid and Aotus analyses. (a) Face and cranial vault with Callimico outgroup, and cranial base and palate, and face and palate for Saimiri outgroup. (b) Cranial base for all three outgroups, cranium, face, and face and cranial vault for Lagothrix and Saimiri outgroups, face and palate for Lagothrix outgroup, and cranial base and vault for Saimiri outgroup. (c) Face, and face and palate for Callimico, and cranial base and palate for Lagothrix outgroup. (d) Cranial base and vault with Lagothrix outgroup. (e) Cranium, cranial base and palate, and cranial base and vault for Callimico outgroup, and congruent with the molecular phylogeny.
Figure 3