NOT ONE, BUT MULTIPLE RADIATIONS UNDERLIE THE BIODIVERSITY OF MADAGASCAR'S ENDANGERED LEMURS

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Abstract

Lemurs are a well-known example of adaptive radiation. Since colonizing Madagascar, more than 100 extant lemur species have evolved to fill the variety of ecological niches on the island. However, recent work suggests that lemurs do not exhibit one of the hallmarks of adaptive radiations: explosive speciation rates that decline over time. We test this idea using a phylogenomic dataset with broad taxonomic sampling of lemurs and their sister group, the lorisiforms of Asia and continental Africa. We find higher rates of speciation in Madagascar's lemurs compared to lorisiforms and we confirm that lemurs did not experience an "early burst" of speciation after colonizing Madagascar. Instead, we identify three independent bursts of speciation approximately 15 million years ago that underly much of today's lemur diversity. We demonstrate that the lemur clades with exceptionally high diversification rates have higher rates of introgression. This suggests that hybridization in these primates is not an evolutionary dead-end, but a driving force for diversification. Considering the conservation crisis affecting strepsirrhine primates, with approximately 95% of species being threatened with extinction, this phylogenomic study offers a new perspective for explaining Madagascar's exceptional primate diversity and reveals patterns of speciation, extinction, and gene flow that will help inform future conservation decisions.

Keywords:

Angwantibos, Divergence Times, Diversification Rates, Galagos, Gene Flow, Hybridization, Lemurs, Lorises, Madagascar, Phylogenomics, Pottos, Primates, Speciation, Strepsirrhini, Systematics

INTRODUCTION

The lemurs of Madagascar (Strepsirrhini: Lemuriformes and Chiromyiformes) are a fascinating case study in evolutionary biology. They are exceptionally diverse—representing more than 15% of all living primate species—yet all members of the clade live on an island representing < 1% of Earth's land area.[1] After colonizing Madagascar, lemurs evolved to fill a wide range of ecological niches, from the smallest primate species in the world—the arboreal mouse lemurs (*Microcebus*)—to recently extinct terrestrial species as large as female gorillas (*Archaeoindris*). Given their exceptional phenotypic and ecological diversity, lemurs are often highlighted as an example of adaptive radiation.[2] However, other classic examples of adaptive radiation like Darwin's finches from the Galápagos Islands and cichlids from Lake Victoria are characterized not only by their phenotypic diversity, but also by a pattern of rapid or explosive speciation followed by a decrease in speciation rate over time as niches became filled.[3,4] Recent work has suggested that the diversification of lemurs did not follow this "early burst" pattern,[5,6] raising the question: How did speciation rates in lemurs change over time? And, if they did not follow a typical pattern of adaptive radiation, then how did the exceptional diversity of lemurs arise?

Lemurs and their sister group Lorisiformes (collectively known as the "wet-nosed primates," infraorder Strepsirrhini) together form an excellent comparative system for understanding how evolutionary dynamics in different geographical regions can produce drastically different levels of species diversity. The lorisiform primates, which occur in Asia and continental Africa, include galagos, pottos, angwantibos, and lorises, all of which are nocturnal and elusive. While they exhibit several interesting morphological adaptations—e.g., they include the only venomous primates (*Nycticebus* and *Xanthonycticebus*)—the lorisiforms are less diverse than lemurs overall, both phenotypically and in terms of species diversity.[5,7] As a result, they have been comparatively neglected in scientific literature.[8] Here, we use a phylogenomic dataset to reconstruct the evolutionary history of Strepsirrhini, providing a framework for evaluating if lemurs diversified according to the classic adaptive radiation model and whether their rates of diversification differed from those of lorisiforms. Given abiding uncertainty about phylogenetic relationships within these groups, we also consider the possibility that introgressive hybridization has introduced conflicting genealogical histories across the genome. Additionally, we test for a relationship between introgression and the rate of diversification, providing insights into an

ongoing question in evolutionary biology, i.e., whether hybridization restricts or promotes the formation of new species.[9–11]

RESULTS AND DISCUSSION

A phylogenomic tree of strepsirrhine primates

Using a phylogenomic dataset comprising 334 nuclear loci with an average length of 3,339 base pairs (bp; range: 158–6,985 bp; total concatenated alignment length: 1,108,850 bp), we estimated a phylogenetic tree of Strepsirrhini that includes all strepsirrhine genera and 71% of all currently recognized species (50% of all lorisiform species and 79% of all lemur species). After assessing the impacts of missing data (Supplementary Methods, Fig. S1), we used two different species-tree inference approaches (see Materials and Methods). Both analyses recovered Madagascar's lemurs (infraorders Chiromyiformes and Lemuriformes) as a monophyletic group sister to all strepsirrhine species from Asia and continental Africa (infraorder Lorisiformes) and well-supported clades representing each strepsirrhine family (Fig.1, Figs. S1, S2).

Our nuclear dataset confirms that the family Lorisidae [the 'slow-climbing' angwantibos (*Arctocebus*), pottos (*Perodicticus*), and lorises (*Loris*, *Nycticebus*, and *Xanthonycticebus*)] and the family Galagidae [the 'fast-leaping' galagos and bushbabies (*Euoticus*, *Galago*, *Galagoides*, *Otolemur*, *Paragalago*, and *Sciurocheirus*)] are reciprocally monophyletic, resolving a longstanding debate (Fig. 1).[8,12–14] Some previous genetic studies have recovered a paraphyletic Lorisidae (with galagids sister to angwantibos/pottos; see also our mitochondrial results below), leading some authors to conclude that traits associated with slow climbing evolved in parallel.[8,15] Although our study does not support parallel evolution, our molecular analyses do recover a relatively short internode suggesting that adaptations to slow climbing evolved rapidly (Fig. 2).

In lemurs, a major area of phylogenetic disagreement has been the placement of the family Indriidae [the woolly lemurs (*Avahi*), sifakas (*Propithecus*), and indri (*Indri*)]. Some studies have placed indriids sister to the family Lemuridae [the true lemurs (*Eulemur*), bamboo lemurs (*Hapalemur* and *Prolemur*), ring-tailed lemur (*Lemur*), and ruffed lemurs (*Varecia*)];[16,17] while other studies have placed indriids sister to the Cheirogaleidae + Lepilemuridae clade [the mouse

Haplorhini (outgroups)

Total number of species sampled: 104/147

Carlito syrichta
Saimiri sciureus
Macaca mulatta
Gorilla gorilla
Homo sapiens
Pan troglodytes

Lorisiformes

Lorisidae
Arctocebus calabarensis
Perodicticus ibeanus
Perodicticus potto
Loris tardigradus
Xanthonycticebus pygmaeus
Nycticebus coucang
Nycticebus kayan

7/16

Galagidae
Euoticus elegantulus
Galagoides demidoff
Galagoides thomasi
Sciurocheirus alleni
Otolemur garnettii
Otolemur monteiri
Paragalago granti
Paragalago cocos
Paragalago zanzibaricus
Galago matschiei
Galago moholi
Galago senegalensis

12/22

Chiromyiformes

Daubentoniidae
Daubentonia madagascariensis

1/1

Lemuriformes

Lemuridae
Varecia rubra
Varecia variegata
Lemur catta
Prolemur simus
Hapalemur occidentalis
Hapalemur gilberti
Hapalemur griseus
Hapalemur meridionalis
Eulemur mongoz
Eulemur rubriventer
Eulemur coronatus
Eulemur flavifrons
Eulemur macaco
Eulemur albifrons
Eulemur sanfordi
Eulemur rufifrons
Eulemur fulvus
Eulemur rufus
Eulemur cinereiceps
Eulemur collaris

20/22

Indriidae
Indri indri
Avahi cleesei
Avahi unicolor
Avahi betsileo
Avahi laniger
Avahi meridionalis
Avahi peyrierasi
Propithecus edwardsi
Propithecus diadema
Propithecus perrieri
Propithecus coronatus
Propithecus verreauxi
Propithecus coquereli
Propithecus tattersalli

14/19

Lepilemuridae
Lepilemur seali
Lepilemur wrightae
Lepilemur betsileo
Lepilemur mustelinus
Lepilemur septentrionalis
Lepilemur ankaranensis
Lepilemur milanoii
Lepilemur sahamalazensis
Lepilemur ahmansonorum
Lepilemur dorsalis
Lepilemur tymerlachsoni
Lepilemur edwardsi
Lepilemur grewcockorum
Lepilemur otto
Lepilemur leucopus
Lepilemur petteri
Lepilemur hubbardorum
Lepilemur ruficaudatus
Lepilemur aeeclis
Lepilemur randrianasoloi

20/26

Cheirogaleidae
Phaner furcifer
Cheirogaleus sibreei
Cheirogaleus medius
Cheirogaleus shethi
Cheirogaleus major
Cheirogaleus crossleyi
Cheirogaleus andysabini
Cheirogaleus grovesi
Allocebus trichotus
Mirza coquereli
Mirza zaza
Microcebus ravelobensis
Microcebus griseorufus
Microcebus manitatra
Microcebus ganzhorni
Microcebus murinus
Microcebus boraha
Microcebus gerpi
Microcebus marohita
Microcebus simmonsi
Microcebus tanosi
Microcebus mittermeieri
Microcebus lehilahytsara
Microcebus myoxinus
Microcebus rufus
Microcebus tavaratra
Microcebus arnholdi
Microcebus mamiratra
Microcebus margotmarshae
Microcebus berthae
Microcebus sambiranensis

31/41

Bootstrap support

○	<75
●	>75 - <95
[N/A]	>95

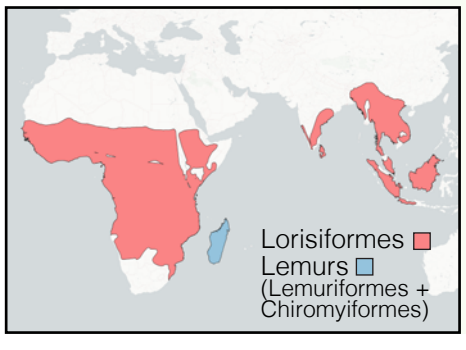

Figure 1. A species tree of strepsirrhine primates estimated using SVD-Quartets. Node support values were estimated using 1000 bootstrap replicates. Nodes with > 95% bootstrap support are not labeled, while nodes with 75-95% support and < 75% support are indicated by gray and white circles, respectively. Precise bootstrap support values for all nodes are shownin Fig. S2. Branch lengths are not scaled. Infraordinal names are shown in black bars, and family names are shown in different colors matching the silhouette image of a representative species to the right of the tree. Silhouettes were obtained from PhyloPic.org and are credited to T.M. Keesey, R. Lewis, Maky, R.D. Sibaja, and G. Skollar. The numbers next to each silhouette indicate the number of species sampled from each family as a fraction of the total number of described species in the family. Inset map shows the combined distributions of all lorisiform and lemur species in red and blue, respectively. Distribution maps were obtained from the IUCN Red List spatial database.

lemurs (*Microcebus*), fork-marked lemurs (*Phaner*), dwarf lemurs (*Allocebus*, *Cheirogaleus*, and *Mirza*), and sportive lemurs (*Lepilemur*)];[18,19] and still other studies have recovered indriids as the sister group to all of these.[20,21] Our study confirms the placement of Indriidae as sister to Cheirogaleidae + Lepilemuridae, although bootstrap support for this node was 89%, which was lower than any other family-level relationship on the tree and may be due to an ancient history of introgression (Fig. 1; discussed below).

The timing of the colonization of Madagascar

We calibrated the strepsirrhine phylogeny using a Bayesian approach with soft constraints on six primate fossils with known occurrence dates (Fig. 2A, Fig. S4, Table S1).[22] This analysis dated the split between lorisiforms and lemurs at 80 MYA [95% confidence interval (C.I.) = 70.4–88.6 MYA] and the crown diversification of lemurs at 66.8 MYA [95% C.I. = 56.3–76.5 MYA]. Those two intervals represent the upper and lower bounds on the colonization of Madagascar, assuming that all lemurs originated from a single colonization event. However, a recent study of African fossils potentially related to the aye-aye suggests that Chiromyiformes and Lemuriformes may have still colonized Madagascar independently,[23] and we cannot rule out this possibility. We also note that our dates are relatively ancient compared to previous studies, which have more often supported a colonization event during the Paleogene (66-50 MYA),[14,16,24–27] and we acknowledge that our divergence times were affected by the presence of reticulation (see results below).[28] Regardless of the specific dates, we can be confident that lemurs would have colonized Madagascar well after it split from the African continent approximately 170 MYA.[29]

Multiple radiations within Madagascar's lemurs

Lemurs are often cited as a classic example of adaptive radiation. However, recent studies have proposed that lemurs did not follow an early-burst pattern typical of other adaptive radiations.[5,6] Using metrics based on lineages-through-time (LTT) plots, our data confirmed a clear and significant pattern of increasing diversification rates toward the present in lemurs without a subsequent decline (Pybus and Harvey's $\gamma = 4.02$, p-value 0.0001). In contrast, lorisiform diversification rates were constant through time ($\gamma = 0.15$, p-value 0.8963; Fig. 2B, Fig. S5). This

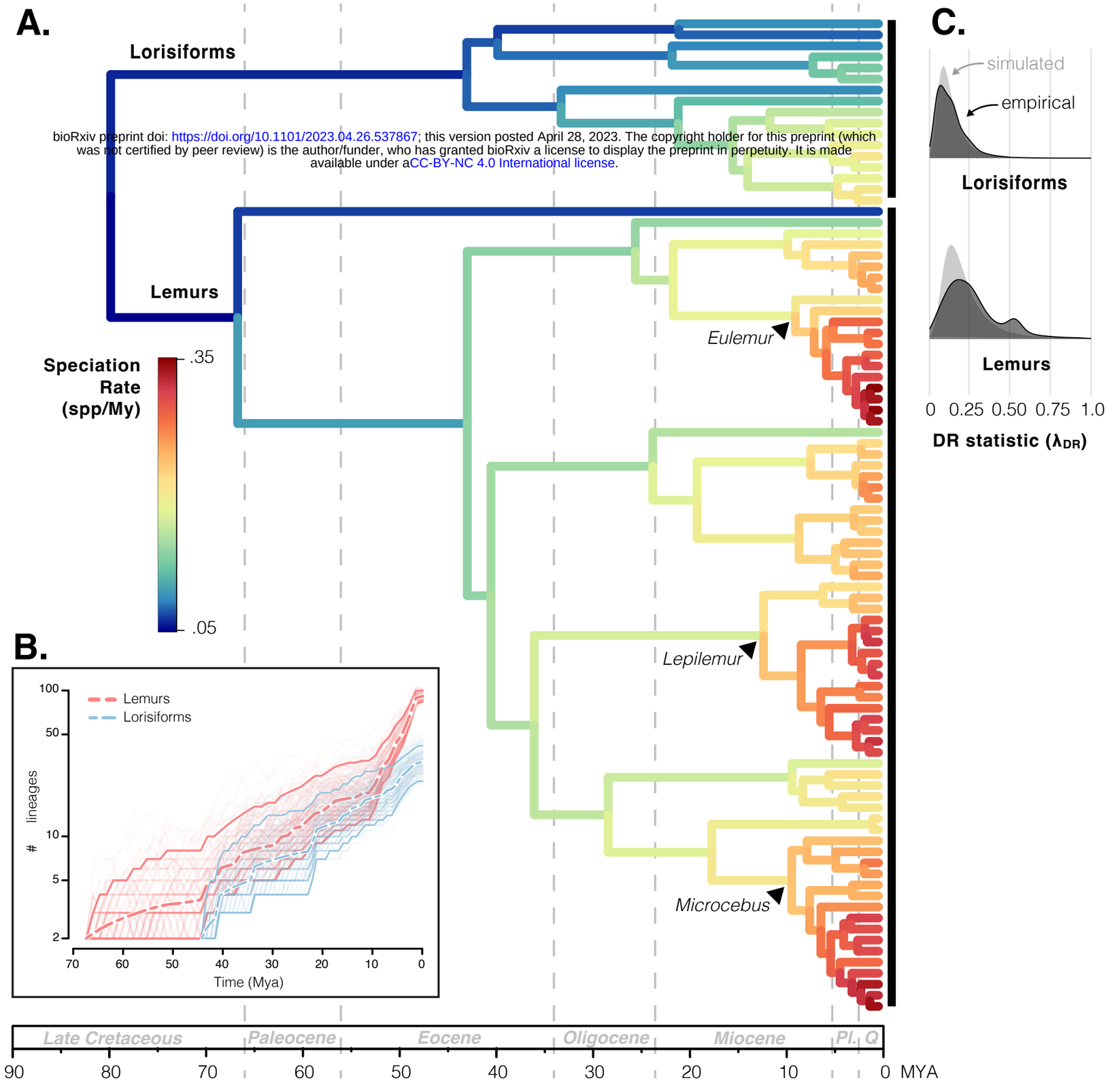

Figure 2. Variation in diversification rate (DR) over time and across strepsirrhine phylogeny. (A) An extant time-calibrated phylogeny of Strepsirrhini, with branches colored according to speciation rates (species per million years) estimated in CLaDs. Full results of this analysis including tip labels can be found in Fig. S7, and similar results estimated by MiSSE can be found in Fig. S6. Dashed vertical lines distinguish geological epochs (Pl. = Pliocene, Q. = Quaternary). A fully detailed time-calibrated phylogeny showing tip labels, node confidence intervals, and outgroups is shown in Fig. S4. Three genera with particularly high speciation rates (*Eulemur*, *Lepilemur*, and *Microcebus*) are indicated on the tree with black arrows and are discussed in the text. (B) Lineages-through-time plots of lemurs (dark gray) and lorisiforms (light gray). We accounted for incomplete taxonomic sampling by estimating a set of 1000 trees (translucent solid lines) where missing taxa were assigned to the correct genus then stochastically resolved using TACT. Solid opaque lines represent the 95% highest posterior density, while the dashed lines represent the consensus tree for each group. Note that the y-axis representing the number of lineages is log-transformed. (C) DR variation in Lorisiformes and lemurs, visualized as the distribution of tip DR values for all species within each taxon. Dark gray distributions represent the empirical tip-DR values across the set of 1000 stochastically resolved trees, while light gray distributions represent simulated tip-DR values, in which trees of the same species richness as each family were simulated using a rate-constant birth-death model.

was supported by a further analysis of tip diversification rates (λ_{DR}),[30] which recovered a distribution of λ_{DR} values in lorisiforms that were not significantly different from expectations under a pure-birth model (Fig. 2C). Meanwhile, the empirical lemur λ_{DR} distribution was bimodal, suggesting that some lemur branches exhibit higher rates of speciation than expected. To further explore variation in macro-evolutionary rates of speciation, we used two model-based approaches: MiSSE, which models diversification as a function of one or more hidden states;[31] and ClaDS, which allows diversification rates to vary across all branches on the phylogeny.[32] MiSSE results strongly supported at least two hidden states with different net diversification rates (Fig. S6), and both analyses highlighted a concentration of increased speciation rates in three lemur genera that originated in the last 15 million years: *Microcebus*, *Lepilemur*, and *Eulemur* (clades dominated by red branches on Fig. 2A, Figs. S6,S7).

Overall, these analyses suggest that lemurs might be better characterized not as a single adaptive radiation, but as an amalgam of several distinct radiations on a background of relatively steady, gradual evolution. Multiple independent bursts of speciation occurred quasi-synchronously, leading to the proliferation of a large proportion of the diversity in lineages, phenotypes, and ecologies that we see today. We estimate that these radiations began ~15 MYA, although we note that other studies have recovered more recent ages using analyses based on mutation rates[33,34] and our divergence time estimates may have been influenced by the presence of reticulation as well as incomplete lineage sorting (ILS) in these rapid radiations.[28] Overall, these results greatly reshape our understanding of how the extraordinary biological diversity of lemurs evolved. In the future, more analyses should link our findings to other defining characteristics of adaptive radiations like morphological and ecological diversity. Even if lemurs as a whole do not meet the contemporary diversification criteria of adaptive radiations (i.e., an early burst of speciation followed by a gradual decline in diversification rates),[3] it is also worth noting that many early writers on adaptive radiation did not include explosive speciation as a defining feature,[35–37] or they acknowledged that similar patterns of ecological adaptation might proceed gradually.[38]

Increasing rates of diversification in lemurs over time may be driven by any combination of biotic (e.g., resource competition and ecological specialization) and abiotic (e.g., landscape and

climate) factors.[39,40] One known driver of increased speciation rates in mammals is increasing topological complexity due to uplift.[41] On Madagascar, uplift was continuous until ~10 MYA, when the mountains reached their current elevations;[42] thus, it is possible that increasing topological complexity on Madagascar set the stage for increasing speciation rates toward the present. One line of evidence supporting this hypothesis is that lemur species are tightly linked to specific watersheds that were shaped by these mountains.[43] High rates of diversification, particularly in cases of island colonization, are also thought to be linked to the availability of diverse ecological niches. However, the niche space that lemurs encountered upon colonizing Madagascar was likely more homogeneous than it is today. Palaeoclimatic reconstructions estimate that neither the eastern rainforests nor the western spiny forests existed until after the K-P boundary (66 MYA), and the intensification of seasonal rainfall, including Indian Ocean monsoons, did not begin until the Miocene (~23 MYA).[6,44] As a group, primates generally live in warm, tropical habitats, yet the climate during the Paleogene was more arid and cool than it is today. All of this suggests that ecological niche space for lemurs has increased over time, offering one explanation for the delayed increase in speciation rates after the colonization of Madagascar. Overall, we echo other researchers that the diversification of lemurs is likely the result of multiple complex processes leading to speciation.[39,40]

Rapid diversification in lemurs relative to their neglected sister group

Previous molecular evidence suggests that lorisiforms have experienced lower rates of diversification relative to lemurs.[5] However, most studies to date have suffered from poor species-level sampling, and several new species have been identified in recent years, making lorisiform taxonomy and diversification a subject of continuing discussion.[8,45,46] Our macroevolutionary rate analyses (Fig. 2A, Figs. S6, S7) estimated a moderate rise in diversification rate within the family Galagidae (specifically the genera *Galago* and *Paragalago*), but these were still lower overall than most lemuriform clades. These results were concordant with our analyses of λ_{DR}, which estimated median values to be almost twice as high in Lemuriformes relative to Lorisiformes: 0.24 species per million years (My) compared to 0.11 species/My, respectively (Fig. 2C, Table S2). It is certainly worth noting, however, that whereas lemurs occur singularly on an island, the evolution of lorisiforms has played out over continental scales where several of the niches occupied by lemurs have been occupied by competing species

not found on Madagascar (e.g., there are no diurnal lorisiforms perhaps due to competition with catarrhine primates).

Importantly, we made the decision to focus our analyses on extant species because there are no known primate fossils from Madagascar older than the Holocene. A consequence of using time trees with only extant taxa is that—because lineages that originated recently have had less time to go extinct—there can be a bias toward increased rates of speciation closer to the present, particularly in methods that are based on LTT trends.[47] Nonetheless, we note that our focus here was on *relative* rates of speciation (how fast lemurs radiated relative to lorisiforms, and how quickly specific clades radiated relative to background rates). Previous research has found that, although the absolute rates of speciation and/or diversification might be inaccurate when extant-only time trees are used, relative differences in state-dependent rates can still be estimated with high confidence.[48]

Signatures of introgression in Madagascar's lemurs

Considering the historical difficulty in resolving strepsirrhine phylogeny, it seems likely that a variety of biological processes, including ILS and introgression, have left signatures in the genome that deviate from the true history of speciation.[49] As a first step toward assessing whether introgression has been present in the evolutionary history of strepsirrhines, we compared the phylogeny generated from our nuclear dataset to a mitochondrial phylogeny (Fig. 3). While we acknowledge that our nuclear dataset is more likely to reflect the true species tree compared to mitochondrial data, topological differences between these two trees can be helpful for identifying candidate regions of the tree that have experienced introgression.[50] In this case, we observed three topological differences between these trees (Fig. 3): (1) in the mitochondrial phylogeny the genera *Perodicticus* and *Arctocebus* formed a clade with galagids, rendering Lorisidae non-monophyletic; (2) Indriidae was sister to all other lemuriform families in the mitochondrial phylogeny; and (3) the genus *Hapalemur* was sister to *Lemur* in the mitochondrial phylogeny, as opposed to *Prolemur* in the nuclear phylogeny.

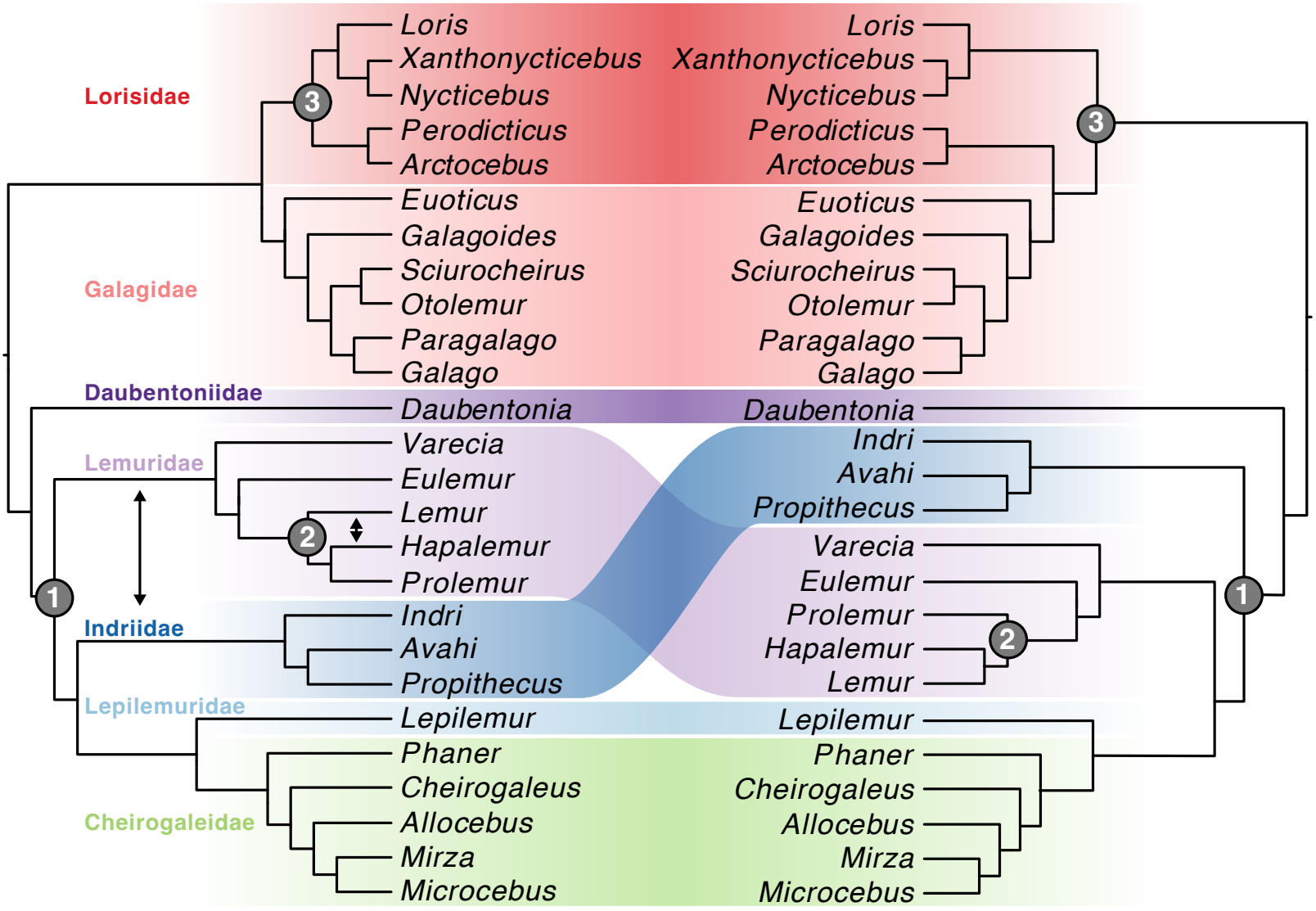

Figure 3. Discordance between nuclear (left) and mitochondrial (right) phylogenies of strepsirrhine genera. Both phylogenies were estimated using IQ-TREE with all available individuals, then species-level branches were manually collapsed to visualize one branch per genus. Fully detailed phylogenies from these analyses are available as Figs. S9 and S10. The nuclear and mitochondrial trees are discordant in three locations labeled with gray numbered circles. Black arrows on the nuclear phylogeny indicate two locations where gene flow was inferred using D-statistics. Families are colored to match Fig. 1.

To explicitly test whether introgression caused these topological differences (labeled with gray circles on Fig. 3), we used D-statistics (see Materials and Methods).[51] These analyses recovered significant signatures of introgression between (a) the family Lemuridae and the family Indriidae (D-statistic = 0.085, p-value = 0.012), and (b) the genus *Lemur* and the genus *Hapalemur* (D-statistic = -0.11, p-value = 0.003; black arrows on Fig. 3). This indicates that an ancient history of introgression likely contributed to topological uncertainty in these regions of the phylogeny. We did not observe signatures of introgression within the family Lorisidae (all p-values > 0.05; Table S3).

At shallower taxonomic levels we also saw uncertain relationships (low or moderate node support) within five lemuriform genera: *Eulemur*, *Propithecus*, *Lepilemur*, *Cheirogaleus*, and *Microcebus*. One possible reason for low node support could be introgression. To test this hypothesis, we estimated phylogenetic networks for each of the five genera. In all five analyses a model with at least one reticulate branch (H = 2–5) was highly supported (Fig. 4, Fig. S8). This suggests that introgression has been prevalent in the evolutionary history of all five of these genera and has likely been an additional source of genealogical conflict beyond ILS. This result highlights an important consideration for phylogeneticists: if we continue to use species-tree models that only account for ILS as a source of gene tree heterogeneity across the genome, larger genomic datasets will never result in 100% node support for branches affected by a history of introgression.

Present-day gene flow is a topic of keen interest for lemur biologists, as there are several documented zones of current hybridization.[52] Our phylogenomic evidence expands our understanding of the history of hybridization in lemurs by showing that introgressive hybridization is not merely a recent phenomenon but has been a pervasive force throughout the evolutionary history of lemuriforms. Indeed, we identified introgression during the early divergence of families ~40 MYA (between Lemuridae and Indriidae), during the divergence of genera ~10 MYA (between *Lemur* and *Hapalemur*), and among species in the same genus within the last 10 million years (Figs. 3, 4).

A correlation between introgression and speciation rate

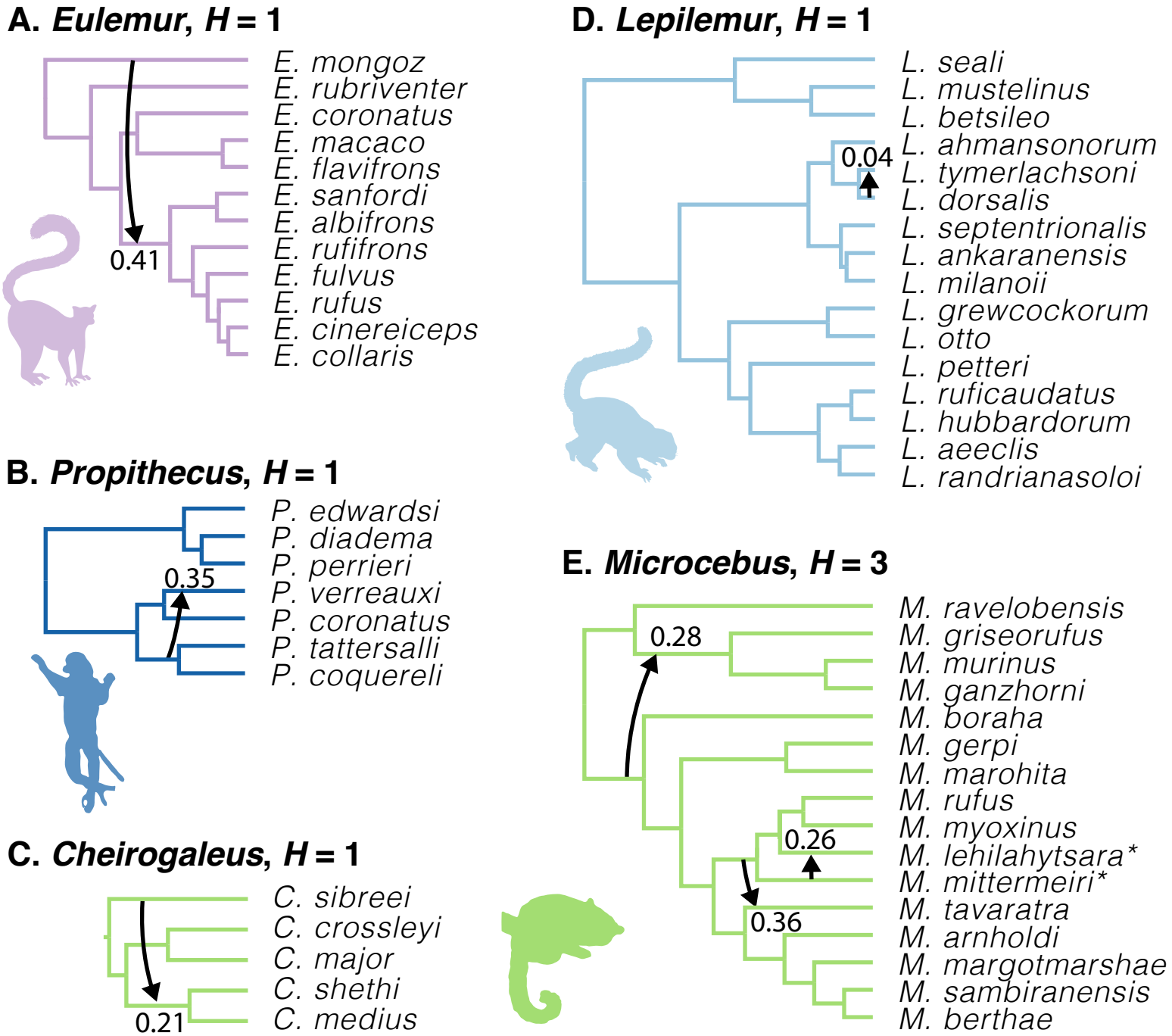

Figure 4. Phylogenies with reticulate relationships estimated by PhyloNet for five strepsirrhine genera (A-E) that had poorly resolved nodes (bootstrap support values < 75%) in our species tree analyses (Fig. 1). Arrows indicate the reticulation events (H) and are labeled with the estimated inheritance probabilities. Note that several models received similar support (Fig. S8), so we are showing the models with the lowest H among the well-supported models. Colors and silhouette images for each genus match the family-level formatting from Fig. 1. *Note that a recent paper[33] proposed synonymizing M. mittermeieri and M. lehilahytsara as a single species; however, we treated these as distinct species as our data did not support a sister relationship.

One interesting finding from recent studies using phylogenetic network-based approaches has been that, counterintuitively, some of the most species-rich clades have experienced the highest amounts of introgression.[e.g., 11] To understand whether there is a correlation between speciation rates and introgression in our system, we used a model-testing approach based on the hidden-state dependent speciation and extinction (HiSSE) model.[53] We tested five different models of diversification (Table S4), in which each species was scored as "hybridizing" or "non-hybridizing", based on this study as well as an extensive literature review (Table S5). Both conservative and liberal scoring systems were tested (see Materials and Methods) and in both cases, the top-ranking model of diversification was a binary-state speciation and extinction model; i.e., a model in which diversification rate is tightly correlated with whether or not the species hybridizes (Table S4). Hybridizing species were estimated to have a median net diversification rate that was approximately twice as high as non-hybridizing species (Table 1).

Table 1. Mean diversification rates [speciation (λ), extinction (μ), and net diversification (λ-μ)] estimated using a state-dependent speciation and extinction modeling framework in HiSSE. The results shown were estimated from the top-ranking models using the full phylogeny. Model scores and weights from all variations of this analysis are provided in Table S4. Species were coded as "hybridizers" or "non-hybridizers" based on literature review. The conservative coding scheme classified species as hybridizers only if there was documentation of known hybridization in the wild, whereas the liberal coding scheme also classified species as hybridizers if there was documentation of introgressed genetic material and/or hybridization in captivity (Table S5).

	Conservative Coding Scheme		**Liberal Coding Scheme**	
	Non-Hybridizers	**Hybridizers**	**Non-Hybridizers**	**Hybridizers**
Net Diversification Rate	2.72	5.08	2.00	5.23

This work contributes to a growing body of evidence that hybridizing species can experience accelerated rates of diversification.[9,11] Our results run counter to the still prevailing idea among vertebrate biologists that hybridization erodes species diversity by generating offspring with poor fitness or by homogenizing gene pools during the divergence process.[54] However, it is important to note that our data do not allow us to disentangle cause and effect of this correlation: is introgression merely a byproduct of rapid speciation, resulting from an insufficient amount of time for reproductive barriers to evolve? Or does hybridization itself drive rapid speciation? One

mechanism by which hybridization can promote speciation is through the process of reinforcement, or the accumulation of reproductive barriers through selection against hybrids.[10] Alternatively, a combinatorial view of speciation posits that hybridization might fuel rapid diversification by shuffling old genetic variants or introducing novel alleles to new populations.[9,55] This is a fruitful area of research, and we can point to the three genera (*Eulemur*, *Microcebus*, and *Lepilemur*) that we identified with high diversification rates as well as high levels of introgression, which serve convenient jumping-off points for future studies.

Considerations for strepsirrhine conservation

Strepsirrhine primates are in the midst of a biodiversity crisis, with approximately 95% of species being threatened with extinction and 90% experiencing population declines.[56,57] From a conservation perspective our findings provide several important advances. First, they point to species and clades that are most prone to hybridization and gene flow. For some taxa gene flow can be a positive force by introducing new genetic variation and adaptive genes, while in others hybridization can lead to genetic swamping and speciation reversal.[58] Conservation practitioners will need to evaluate instances on a case-by-case basis to determine how best to preserve unique genetic variants while also maintaining population sizes and health. Second, this study provides a robust phylogenetic framework that future researchers can use to place new species as they continue to be identified. It is worth noting that strepsirrhine taxonomy is a moving target and some groups have received greater taxonomic attention than others;[8,59] unfortunately we were unable to account for undescribed species or taxonomic biases in this study.

Our results also reiterate that certain branches on the strepsirrhine tree are evolutionarily significant, i.e., lineages that diverged a long time ago and that perform important ecosystem functions, but now contain few living species. Examples include the monotypic genera *Lemur*, *Indri*, and—as an extreme example—the lone member of Chiromyiformes, the aye-aye (*Daubentonia*). Our results showed that these lineages are even older than previously recognized and have experienced slower rates of evolution relative to other lemurs. Finally, our study provides a nuanced perspective on the often-neglected lorisiforms, which are difficult to sample and are therefore underrepresented in strepsirrhine research (including the present study, which included 50% taxonomic sampling of lorisiforms compared to 71% of lemur species). Major

effort will be needed to understand lorisiform distributions, taxonomy, population sizes, and diversity moving forward.

MATERIALS AND METHODS

Data generation

We sequenced DNA from 129 individuals obtained as frozen tissues from a variety of sources including museum collections, the Duke Lemur Center, and the German Primate Center (Table S6). All samples were obtained as part of previous studies and collected under appropriate permits issues by the local governments (e.g., Madagascar, Kenya, Tanzania) and approved by ethics boards for animal welfare (IACUC or equivalent) by the institutions involved in the study. Genomic DNA was extracted from frozen tissues using a Qiagen DNEasy Blood and Tissue kit (Qiagen, Inc.) and double-stranded DNA in each extraction was quantified using a Qubit fluorometer (Invitrogen, Inc.). Where DNA quantities were very low, we used a Repli-G whole-genome amplification kit (Qiagen, Inc.) to increase the amount of DNA prior to library preparation. DNA samples were transported to Florida State University to undergo library preparation, Anchored Hybrid Enrichment (AHE)[60], and sequencing on an Illumina NovaSeq instrument. We supplemented our dataset by extracting the same AHE loci from previously published whole genome data from 34 individuals (Table S7, and see Supplementary Methods), including five outgroup taxa from the suborder Haplorrhini. See Supplementary Methods for a detailed description of library preparation, sequencing, quality control, assembly, and alignment protocols, which ultimately produced individual sequence alignments for each locus.

Phylogenetic analysis

We estimated species trees using two different approaches: (1) an alignment-based analysis in the program SVDquartets[61] and (2) a gene-tree-based analysis in the program ASTRAL.[62] Both are coalescent programs that use quartet scores to select the best species-tree topology. We ran SVDquartets in PAUP*[63] using a concatenated sequence file as input. We used multilocus bootstrapping and the evalq=all setting, which specifies that all quartets should be evaluated, and designated the five haplorrhine species as the outgroup. Finally, we ran ASTRAL with default settings using individual gene trees from each locus as input. These gene trees were generated from individual sequence alignments for each locus using RAxML-ng[64] under the GTR model.

Estimation of divergence times

Estimation of divergence times with MCMCTree[65] requires an input phylogeny with branch lengths in coalescent units. To obtain this phylogeny, we performed a maximum-likelihood phylogenetic analysis in the program IQ-TREE[66], using the concatenated dataset pruned to include only taxa and loci with < 50% missing data (see Supplementary Methods for a description of how we analyzed the effects of missing data) and only one individual per species (the individual with the lowest proportion of missing data). This analysis was constrained to the topology of the species tree previously identified and was conducted with 1000 bootstrap replicates. The best-fit tree estimated by IQ-TREE (Fig. S9) and the concatenated sequence file were then used as the input to MCMCTree in the program PAML[67] to estimate divergence times. Six nodes on the phylogeny were time-calibrated using dates based on the fossil record (Table S1).[22] We used the approximate likelihood method, which first estimates branch lengths and a likelihood surface using maximum likelihood, then generates an approximation of divergence times in a second step. We used the independent rates model and default settings for parameters and priors in both steps. We conducted two independent Markov chain Monte Carlo (MCMC) runs with 10,000 iterations of burn-in followed by 25,000 generations sampling every 50 generations. We confirmed convergence and stationarity of the MCMC chain using Tracer,[68] and verified that effective sample sizes were > 200.

Macroevolutionary rates of speciation

To generate lineages-through-time (LTT) plots, we used our time-calibrated phylogeny as input to the ltt function in the R package phytools.[69] To visualize potential variation in these plots that might be caused by incomplete taxonomic sampling, we also generated a suite of 1000 trees using the program TACT[70] to stochastically add all missing species to the proper genera on the time-calibrated phylogeny, and estimated an LTT plot for each tree. Finally, we used the mccr function in phytools to estimate Pybus and Harvey's γ[71] while accounting for incomplete taxon sampling.

Macroevolutionary rates were estimated using two different methods: (1) the missing state speciation and extinction model (MiSSE)[31], and (2) the cladogenetic diversification rate shift

model (ClaDS).[32] The first method, MiSSE, belongs to a speciation and extinction set of models[53,72,e.g., 73,74] and reconstructs diversification rates as a function of one or more hidden states. We performed this analysis in R using the package hisse, setting the estimated proportion of extant species sampled in the phylogeny (*f*) to 0.71.[53] We tested five models which varied in the number of hidden states, from one to five, each with an associated turnover rate and extinction fraction. The top-ranking model was selected using the Akaike Information Criterion (Table S8) and this model was then used to reconstruct rates across the trees using the function MarginReconMiSSE (Fig. S6). The second macroevolutionary rate analysis, ClaDS, is a Bayesian approach that allows shifts in diversification rate to occur at each branching event on the phylogeny.[32] We fitted the ClaDS2 model, which assumes varying extinction rates and constant turnover, using the R package RPANDA with $f = 0.71$.[75] We ran three chains for 100,000 generations, each with a thinning of 500, and visualized the estimated rates on the phylogeny using the function plot_ClaDS_phylo (Fig. S7).

We also estimated speciation rates using a simple summary statistic, the tip diversification rate (λ_{DR}), which reflects the weighted inverse of phylogenetic branch lengths leading to each tip. [30] The λ_{DR} metric was calculated for each tip across all 1000 phylogenies that were estimated previously using TACT. We also simulated λ_{DR} distributions expected under a homogeneous birth-death process in order to identify specific regions of the tree with higher or lower empirical speciation rates than expected, following the procedure outlined in [76]. Tree simulations and calculation of λ_{DR} metrics were performed using a custom R code (Supplementary Materials).

Comparison of mitochondrial and nuclear datasets

Mitochondrial sequences were captured as off-target reads in our sequencing protocol and were harvested from our raw sequence data (forward and reverse fastq.gz files) using the program MitoZ with the "Chordata" clade setting.[77] This pipeline retrieved mitochondrial sequence data in 104 individuals. Sequences were assembled and aligned using Geneious [78] and a mitochondrial phylogeny was estimated using the IQ-TREE web server[79] with default settings, allowing the substitution model to be ascertained automatically (Fig. S10). Genera were collapsed into single branches in the main text for visualization purposes (Fig. 3).

Tests of hybridization and gene flow

We used variations of Patterson's D-statistic[80] to test whether the three topological differences observed between the nuclear and mitochondrial datasets could be attributed to introgression. These analyses require a rooted tree with four[51] or five[81] tips, and are used to test for introgression among non-sister ingroup taxa. For Topological Difference #1 (Fig. 3) we considered the four-taxon tree (Daubentoniidae,(Lemuridae,(Indriidae,Cheirogaleidae+Lepilemuridae))), where Cheirogaleidae and Lepilemuridae were combined to represent a single taxon. For topological difference #2 we considered the four-taxon tree (*Varecia*,(*Lemur*,(*Prolemur*,*Hapalemur*))) (note that the genus *Eulemur* was excluded for computational efficiency). Finally, for topological difference #3 we considered the symmetrical five-taxon tree (*Otolemur*,((*Loris*,*Nycticebus*),(*Arctocebus*,*Perodicticus*)). Note that during the course of this research a new genus was introduced for the species *Nycticebus pygmaeus* (*Xanthonycticebus*);[46] these two genera form a clade and were grouped together as *Nycticebus* in this analysis. In traditional ABBA-BABA tests each tip represents a species; however, in this case we used the function CalcPopD in the R package evobiR[82] to calculate Patterson's D for all possible four-species combinations within the above tree constraints. In all datasets we used concatenated fasta alignment files containing all loci and all taxa within the relevant clades. To reduce computational burden, we pruned individuals that had > 20% missing data and removed all nonvariant sites, and we used the jackknife method to estimate statistical significance using 100 replicates and a block size of 5,000 bp. Because the third topological difference is a symmetrical four-taxon clade, a standard ABBA-BABA test was not appropriate. Instead, we used a variation of the D-statistic called exhaustive D_{FOIL}[81] to test for introgression among non-sister lineages as well as their ancestral lineages. This test was conducted using ExDFOIL (https://github.com/SheaML/ExDFOIL, accessed November 2022).

We also used the program PhyloNet[83] to test for introgression in each genus that had topological uncertainty (low node support) in our species-tree analyses (i.e., *Cheirogaleus, Eulemur*, *Lepilemur*, *Microcebus*, and *Propithecus*). For each genus, we first calculated sets of gene trees (one for each locus), using RaxML-ng[64] with the ultrafast bootstrapping method and the GTR model of substitution. This set of gene trees was then used as input to PhyloNet using the

maximum pseudo-likelihood approach to estimate quartet counts under models that varied in the number of hybridization events (*H*), which we allowed to vary from zero to five. To choose the correct *H* for each analysis, we visualized the log-likelihood scores for each analysis and used the lowest value of *H* beyond which little improvement in likelihood was observed (Supplementary Fig. 8).

Finally, to understand whether there was a correlation between speciation rates and hybridization, we used an approach similar to Patton et al. 2020[11], who applied the hidden-state speciation and extinction (HiSSE) trait-dependent diversification model.[53] For this analysis, each species was scored as "hybridizing" or "non-hybridizing" based on this study as well as an extensive literature review using the search engine Google Scholar, where the species name was paired with the words "hybrid" and "introgress" and their structural variants (e.g., "hybridize" and "introgression"; Table S5). We generated two scoring systems: (1) a conservative system, where species were only classified as "hybridizing" if there was documentation of that species hybridizing in the wild, and (2) a liberal system, where species were classified as "hybridizing" if there was any documentation of that species hybridizing in the wild or captivity, or if any previous study had found evidence of gene flow using phylogenetic or population genetic analyses, or if that species was a descendant of a reticulate branch leading to 3 or fewer tips in our PhyloNet analysis (Fig. 4E). We evaluated a total of five competing models using the hisse package in R[53], ranging from a null character-independent model with a single diversification rate, to a full character-dependent HiSSE model accounting for hidden states. Parameter values were estimated from the top-ranking models, which were selected using the Akaike Information Criterion. We applied this model-testing framework to both the liberal and conservative coding schemes using the whole dataset (lemurs + lorisiforms), and to both coding schemes again with lorisiforms removed. Finally, to understand the influence of unsampled taxa where hybridization status is unknown, we re-ran all of those tests again using two different values for the sampling parameter *f*: one in which all unsampled taxa on the phylogeny are assumed to hybridize [*f* = c(0.71, 1)], and one in which all unsampled taxa on the phylogeny are assumed not to hybridize [*f* = c(1, 0.71)]. The top-ranked models in these analyses were either the BiSSE model (a character-dependent model without hidden states) or the dull null model (Table S4).

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Competing interests

Authors declare that they have no competing interests.

Data and materials availability

All DNA sequence data have been deposited in the NCBI SRA under BioProject ID PRJNA957840. Analytical files including scripts, code, and input files have been deposited on K.M.E.'s GitHub page: https://github.com/keverson25/StrepsirrhineAHEs.

SUPPLEMENTARY MATERIALS

Materials and Methods

Figs. S1-S16

Tables S1-S8

SUPPLEMENTARY FIGURE CAPTIONS

Figure S1. A visualization of the amount missing data in our nuclear dataset. Each row in the matrix represents an individual and each column represents a locus. Cells are colored according to the amount of missing data for in each alignment, along a gradient from blue (0% missing data) to red (100% missing data). The effects of missing data on our phylogenetic analyses were explored (see Supplementary Methods and Figs. S11-S16). Family names for groups of individuals are shown to the left of the matrix.

Figure S2. A species tree of strepsirrhine primates estimated using SVDQuartets. Node support values were estimated using 1000 bootstrap replicates. Branch lengths are not scaled.

Figure S3. A species tree of strepsirrhine primates estimated using ASTRAL. Node support values represent support for the quadripartition, or the fraction of induced quartet trees in the input set of gene trees that are also in the species tree. Branch lengths are not scaled.

Figure S4. The extant time-calibrated phylogeny of Strepsirrhini, as estimated using MCMCTree. Black squares indicate the nodes that were calibrated to real time using fossil evidence (Table S1). The median age of each node is shown, as well as purple bars indicating the 95% highest posterior density intervals.

Figure S5. (A) Lineages-through-time plots for lemurs (blue) and lorisiforms (red) using only the taxa that were sampled in this study. Arrows in (B) and (C) show the observed values of Pybus and Harvey's γ for lemurs and lorisiforms, respectively. The vertical bars show the expected distribution of γ for trees of the same species richness generated under a pure-birth speciation model. A significant p-value indicates that the observed γ is significantly different from expectations, as calculated using a Monte Carlo constant rates test implemented using the function ltt in the R package phytools.

Figure S6. An extant time-calibrated phylogeny of Strepsirrhini, with branches colored according to speciation rates (species per million years) estimated in MiSSE. The inset graph shows the corrected Akaike Information Criterion (AICc) score for models containing between one and five hidden states. A model with two hidden states was best supported.

Figure S7. An extant time-calibrated phylogeny of Strepsirrhini, with branches colored according to speciation rates (species per million years) estimated in CLaDs. The inset graph shows the mean speciation rate through time for lemurs (blue) and lorisiforms (red), as estimated using CLaDs. The dashed lines represent the mean speciation rate across all trees, while the solid opaque lines represent the 95% confidence intervals.

Figure S8. Model test results for the PhyloNet analyses of five lemur genera. For each genus, models containing between zero and five reticulations (*H*) were explored. In the main text (Fig. 4), we showed the models (indicated by red circles) with low –(log likelihood) scores that did not substantially improve at higher values of *H*.

Figure S9. The phylogeny used as input for the divergence time analysis in MCMCTree. This phylogeny was estimated using the partitioned nuclear dataset in IQTree, pruned to include only one individual from each species and only individuals with <20% missing data.

Figure S10. A mitochondrial phylogeny estimated using IQTree. Node support values are from 1000 ultrafast bootstrap replicates (before the slash) 1000 replicates of the Shimodaira-Hasegawa approximate likelihood ratio test (SH-aLRT; after the slash). Tip labels correspond to the individuals listed in Tables S7 and S8. Note that only 104 individuals had sufficient mitochondrial data to be harvested from our sequencing runs.

Figure S11. A nuclear phylogeny estimated using all individuals and all loci in IQTree. Node support values are from 1000 ultrafast bootstrap replicates are shown. Tip labels correspond to the individuals listed in Tables S7 and S8. Figures S11-S16 were visualized to explore the effects of missing data.

Figure S12. A nuclear phylogeny estimated in IQTree using all individuals but dropping the 37 loci that failed to sequence in lorisiforms and outgroups. Formatting follows Fig. S11.

Figure S13. A nuclear phylogeny estimated in IQTree using all loci and all individuals with <50% missing data. Formatting follows Fig. S11.

Figure S14. A nuclear phylogeny estimated in IQTree using all individuals with <50% missing data and dropping the 37 loci that failed to sequence in lorisiforms and outgroups. Formatting follows Fig. S11.

Figure S15. A nuclear phylogeny estimated in IQTree using all loci and all individuals with <20% missing data. Formatting follows Fig. S11.

Figure S16. A nuclear phylogeny estimated in IQTree using all individuals with <20% missing data and dropping the 37 loci that failed to sequence in lorisiforms and outgroups. Formatting follows Fig. S11.

SUPPLEMENTARY TABLE CAPTIONS

Table S1. Calibrations used on our divergence-time analysis, based on Pozzi & Penna 2022. MCMCTree uses soft minimum and maximum bounds, shown in columns two and three in millions of years (Ma). Columns four and five provide a list of the fossils that have been used to justify those bounds. Nodes are indicated by black squares in Supplementary Fig. S4.

Table S2. Mean tip diversification rates (λ_{DR}) for each strepsirrhine species. We accounted for incomplete taxonomic sampling by estimating a set of 1000 trees where missing taxa were assigned to the correct genus then stochastically resolved using TACT. The λ_{DR} values shown were averaged across all 1000 trees.

Table S3. D-statistics and p-values resulting from the D_{FOIL} analysis, which tested for introgression among members of the family Lorisidae. In all analyses, *Otolemur* was used as the outgroup.

Table S4. Model test results from the HiSSE analysis. We explored models that were character-dependent and character-independent, and models that did or did not include hidden states. We further explored the effects of coding scheme (conservative vs. liberal, see Methods) and the effect of including or excluding lorisiforms. Finally, we explored the effect of assuming that all unsampled taxa hybridize or do not hybridize, or that unsampled taxa hybridize at the same frequency as sampled taxa (the default). In all analyses, the top-ranking model was that with the lowest corrected Akaike Information Criterion (AICc) score, or the greatest proportion of model weight.

Table S5. Coding schemes used for the HiSSE analyses, with the relevant literature supporting these classifications. A code of zero indicates a non-hybridizing species while a code of one is used for hybridizing species. The conservative coding scheme considers hybridizers to be only species where active hybridization has been documented in the wild. The liberal coding scheme expands on this definition to include species where introgression has been inferred using genetic data, or where species have been observed to hybridize in captivity.

Table S6. Information on all samples with new sequence data generated for this study. The first column contains the sample IDs and the second column contains the source of the tissue for sequencing. Columns 3-5 contain the taxonomic information about each sample. The final four columns contain information about the sequencing run.

Table S7. Previously published genomes added to our nuclear dataset. Note that only the homologous loci from our nuclear dataset were extracted from these individuals.

Table S8. Model test results from the HiSSE analysis. We explored models containing between one and four diversification rates across the tree. The top-ranking model (a model with two rates) had lowest corrected Akaike Information Criterion (AICc) score and the greatest proportion of model weight.