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Phylogenomic Reconstruction of Sportive Lemurs (genus *Lepilemur*) Recovered from Mitogenomes with Inferences for Madagascar Biogeography

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Abstract

The family Lepilemuridae includes 26 species of sportive lemurs, most of which were recently described. The cryptic morphological differences confounded taxonomy until recent molecular studies; however, some species' boundaries remain uncertain. To better understand the genus *Lepilemur*, we analyzed 35 complete mitochondrial genomes representing all recognized 26 sportive lemur taxa and estimated divergence dates. With our dataset we recovered 25 reciprocally monophyletic lineages, as well as an admixed clade containing *Lepilemur mittermeieri* and *Lepilemur dorsalis*. Using modern distribution data, an ancestral area reconstruction and an ecological vicariance analysis were performed to trace the history of diversification and to test biogeographic hypotheses. We estimated the initial split between the eastern and western *Lepilemur* clades to have occurred in the Miocene. Divergence of most species occurred from the Pliocene to the Pleistocene. The biogeographic patterns recovered in this study were better addressed with a combinatorial approach including climate, watersheds, and rivers. Generally, current climate and watershed hypotheses performed better for western and eastern clades, while speciation of northern clades was not adequately supported using the ecological factors incorporated in this study. Thus, multiple mechanisms likely contributed to the speciation and distribution patterns in *Lepilemur*.

Subject area: Molecular systematic and phylogenetics
Key words: ancestral range reconstruction, divergence dating, ecological vicariance analysis, primate, SEEVA

Madagascar is home to unique adaptive radiations of vertebrates that are among the most threatened in the world (Schwitzer et al. 2015). Framing the distributions of these many endemic species in a biogeographic context has been complex and contentious (Martin 1972; Goodman and Ganzhorn 2004; Wilmé et al. 2006; Yoder and Nowak 2006; Pearson and Raxworthy 2009). A large plateau (central highland, CH hereafter) running nearly the entire length of the island separates the lowland forests in an east-west orientation (Martin 1972; Yoder and Nowak 2006). Additionally, habitat types are fairly specialized across the periphery of the island, likely leading to the small, restricted ranges observed in many fauna today (Raxworthy et al. 2003; Garbutt 2007; Mittermeier et al. 2010). Several hypotheses have been proposed to explain the distribution of areas of endemism across the island, with multiple scenarios identifying rivers as significant barriers to gene flow, hereafter the river barrier hypothesis (Martin 1972; Craul et al. 2007). Two models also incorporate climatic factors, the retreat–dispersion hypothesis (referred to hereafter as the watershed hypothesis, Wilmé et al. 2006) and the current climate hypothesis (Pearson and Raxworthy 2009).

The watershed hypothesis combines the locations of rivers with Quaternary climate shifts to document zones of speciation/endemism (Wilmé et al. 2006). The current climate hypothesis (Pearson and Raxworthy 2009) identified 14 climatic regions from clustering analysis of 19 bioclimatic variables. Pearson and Raxworthy (2009) compared the distribution of a variety of vertebrates (lemurs, leaf and day geckos, and chameleons) and recovered a complex pattern; however, there was more evidence for the watershed or both hypotheses in the evaluated lemurs than the current climate hypothesis alone. Pearson and Raxworthy's (2009) results, however, were only tested on 1 genus of nocturnal lemurs (2 species of *Microcebus*) and 12 species of diurnal lemurs (spanning multiple genera).

The lemurs of Madagascar (Order Primates; Suborder Strepsirrhini) include 5 families and over 100 named species (Tattersall 2007; Thalmann 2007; Mittermeier et al. 2008, 2010; Thiele et al. 2013). Many of these represent the most endangered primates in the world and are 100% endemic to the region (Schwitzer et al. 2015). The decrease in Madagascar's forest cover, documented across forest types (Green and Sussman 1990; Harper et al. 2007; Kull 2012), is fragmenting many of the lemurs' already restricted ranges. Here, we focus on *Lepilemur*, the only extant genus in the family Lepilemuridae (Karanth et al. 2005; Kistler et al. 2015) that is distributed in nearly all forested regions of the island (Lei et al. 2008; Mittermeier et al. 2010), to assess historical patterns of movement across Madagascar and the possible impact of climatic variables on speciation.

The nocturnal sportive lemurs were originally classified as 2 species: *Lepilemur mustelinus* from the eastern rain forests, and *Lepilemur ruficaudatus* from the western and southern dry forests (Schwarz 1931; Hill 1953). The taxonomy of the genus has been revised repeatedly over the past half century (Petter and Petter-Rousseaux 1960; Rumpler and Albignac 1975; Petter et al. 1977; Tattersall 1982; Jenkins 1987; Groves 2001). The most recent revisions using molecular, cytogenetic, and/or morphological data have identified the cryptic diversity of this genus which has expanded to 26 species (Andriaholinirina et al. 2006; Louis et al. 2006; Rabarivola et al. 2006; Craul et al. 2007; Lei et al. 2008; Ramaromilanto et al. 2009). This marked increase is attributed to comprehensive sampling across the entire range of the genus, as well as the utilization of molecular tools that are well suited to detecting cryptic biodiversity (Louis et al. 2006; Craul et al. 2007). However, since researchers have used different combinations of mitochondrial DNA (mtDNA) sequence fragments (Delpero et al. 2001; Pastorini et al. 2003; Andriaholinirina et al. 2006; Rabarivola et al. 2006; Craul et al. 2007), direct comparisons between the various data sets cannot be performed.

Previous research (Louis et al. 2006; Ranaivoarisoa et al. 2013) sequenced the mitochondrial control region for a large number of individual sportive lemurs, but did not recover strong statistical support at many nodes delimiting species level divergences. Complete mitochondrial genomes or mitogenomes have been shown superior to individual genes (or few genes) in resolving the phylogenetic history of closely related or widely distributed species (Raaum et al. 2005; Yu et al. 2007; Matsui et al. 2009; Chan et al. 2010; Matsudaira and Ishida 2010; Bjork et al. 2011; Knaus et al. 2011; Finstermeier et al. 2013; Pozzi et al. 2014; Di Fiore et al. 2015; Hofman et al. 2015; Liedigk et al. 2015; Louis and Lei 2016; Hawkins et al. 2016). Here, we seek to explore the utility of complete mitogenome sequences to fully resolve the relationships of the sportive lemurs. We elucidate the evolution of possible biogeographical areas to compare to centers of endemism predicted by the river barrier, watershed and current climate hypotheses and assess the potential impact of the climatic variables that underscore many species distribution models.

Based on previous research on *Lepilemur* (Andriaholinirina et al. 2006; Louis et al. 2006; Craul et al. 2007; Lei et al. 2008; Ramaromilanto et al. 2009; Lei et al. 2010), we hypothesize that the sportive lemurs will show one or both of the following biogeographic patterns: 1) deep east-west and north-south splits with the central highlands forming an ancient barrier to dispersal (as predicted by Martin 1972, specifically in *Microcebus*, *Lepilemur*, *Avahi*, and *Hapalemur*); and 2) genetic isolation across major rivers, which have previously been identified as important barriers to lemurs (Louis et al. 2006; Craul et al. 2007). We estimated divergent dates between species in our evaluation of biogeographic processes, and extracted climatic data associated with precise geographic locations to identify correlations with phylogenetic splits.

Materials and Methods

Sample Collection

A total of 409 *Lepilemur* individuals were captured from 1999 to 2009 by field crews investigating the biodiversity and biogeography of lemurs through the Madagascar Biodiversity Partnership (www.madagascarpartnership.org, Louis et al. 2006; Lei et al. 2008; Ramaromilanto et al. 2009). Previous research (Louis et al. 2006) confirmed the species designation of several hundred *Lepilemur* collected from over 35 sites across Madagascar. Of the 409 sampled individuals, mitogenomes from 33 sportive lemurs representing at least 1 individual per species were sequenced in this study (Figure 1; Table 1). Samples were selected to span nearly all geographic regions across Madagascar (Figure 1). Due to the cost and computational requirements for mitogenome sequencing and analysis, we only included the aforementioned individuals based on locations which have been previously characterized with mitochondrial sequencing. With data from these, along with 1 published mitochondrial genome of *Lepilemur hubbardorum* (Lei et al. 2010) and 2 of *L. mustelinus* (Kistler et al. 2015), our study included all currently recognized *Lepilemur* species (a total of 36 individuals' mitogenomes) based on the taxonomy of Hoffmann et al. (2009). The sportive lemurs investigated were wild-caught and immobilized with a CO_2 projection rifle or blowgun with 10 mg/kg of Telazol (Fort Dodge Animal Health, Fort Dodge, IA). Four 2.0 mm tissue biopsies and 1.0 cc per kilogram of whole blood were collected during field surveys in Madagascar

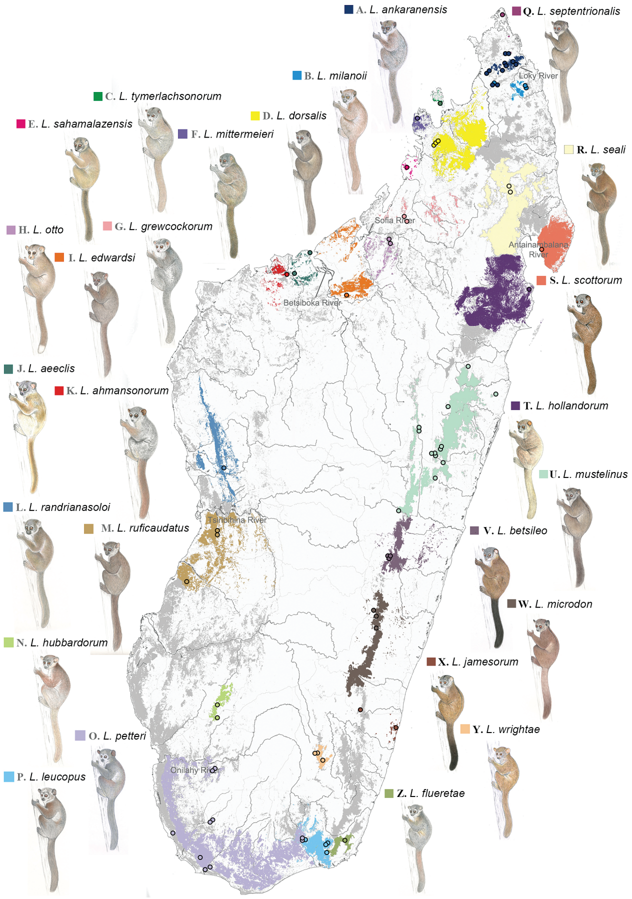

Figure 1. Distribution map of the sportive lemurs (genus *Lepilemur*) of Madagascar. Colored regions define the spatial distribution of each species based on molecular data and existing forest cover. Circles represent field collection sites where multiple animals may have been sampled. Stars represent individuals sequenced in this study. See online color version of this figure at: jhered.oxfordjournals.org.

(Louis et al. 2006; Lei et al. 2008; Ramaromilanto et al. 2009) and immediately stored in room temperature storage buffer (Seutin et al. 1991). Genomic DNA was extracted from the samples using a whole genome amplification kit (GE Healthcare, Piscataway, NJ).

All collection and export permits were obtained from Madagascar National Parks, formerly Association Nationale pour la Gestion des Aires Protégées (ANGAP), and the Ministère de l'Environnement, de l'Ecologie, de la Mer et des Forêts. Samples were imported to the United States under the Convention on International Trade in Endangered Species (CITES) Appendix I permits from the US Fish and Wildlife Service. Capture and sampling procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Omaha's Henry Doorly Zoo and Aquarium under IACUC #12–101. All animal handling followed guidelines by the American Society of Mammalogists (Sikes et al. 2011).

PCR Amplification and DNA Sequencing

Mitochondrial genome sequences of sportive lemurs were amplified with sets of species-specific primers described in Lei et al. (2010). In order to avoid problems associated with amplifying the nuclear insertions of mtDNA (Raaum et al. 2005), the whole mtDNA was amplified in 7 overlapping PCR fragments, described in detail in the Supplementary Materials.

DNA Sequence Alignment

DNA sequences were analyzed using Sequencher v5.1 (Gene Codes Corporation, Ann Arbor, MI). The locations of protein-coding and rRNA genes were confirmed through BLAST comparisons of GenBank sequences from *L. hubbardorum* (Lei et al. 2010). The tRNA genes were identified with ARWEN v1.2 (Laslett and Canback 2008) and verified by comparing GenBank tRNA gene sequences from *L. hubbardorum* (Lei et al. 2010).

Thirty-six complete mitochondrial genomes of *Lepilemur* individuals (33 generated here and 3 previously published) and mitogenomes of individuals from 7 additional lemur species and 5 lorisoid species (Table 1; Kistler et al. 2015) were aligned utilizing MAFFT with the default parameters (Katoh et al. 2002). Initial sequence comparisons and measures of variability were performed using MEGA v6.0 (Tamura et al. 2013). The control region was not included in any analyses since this region is too variable for interspecific comparisons (Krause et al. 2008; Chan et al. 2010). We employed 30 sequence data partitioning schemes as described in Supplementary Table S1. Additional details regarding alignment of coding genes, overlapping sequence removal, and removal of poorly aligned regions are detailed in the Supplementary Materials. A master alignment of 14 755 bp total was created by concatenating the above described alignments, which was equivalent to approximately 87% of the mitochondrial genome.

Data Partitioning Scheme

The program PartitionFinder V1.10 was utilized to select the best model of nucleotide substitution and the best partitioning scheme for our data in the phylogenetic analyses (Lanfear et al. 2012). The second-order Akaike Information Criterion (AIC) was used because it corrects for small sample sizes and converges on the AIC with large datasets. Initially, 39 different data blocks were defined (12 first codon position, 12 second codon position, 12 third codon position, 12S rRNA gene, 16S rRNA gene, and tRNA genes). The "greedy" algorithm (heuristic search) was implemented to search for the best-fit scheme. Additionally, the best-fit schemes selected by PartitionFinder were compared to some of the most frequently used partition schemes in mitochondrial phylogenomics (Supplementary Tables S1 and S2; Pozzi et al. 2014). The analysis with the lowest score was identified as the optimal partitioning scheme.

Phylogenetic Analyses

Maximum likelihood (ML) analyses were run for each partition scheme utilizing RAxML v8.0.0 (Stamatakis 2014). One thousand replications of rapid bootstrapping were implemented to evaluate nodal support. Bayesian inference (BI) analyses for each partition scheme were conducted using MrBayes v3.2.5 (Ronquist et al. 2012). Four simultaneous Markov Chain Monte Carlo (MCMC) runs with 4 chains each and 20 000 000 generations were performed under the models suggested by PartitionFinder. For every 2000 generations, the tree with the best likelihood score was saved. The first 10% of generations were discarded as burn-in, leaving 10 000 trees per run. Convergence was assessed by checking whether the effective sample sizes of parameters exceeded 200 utilizing Tracer v1.6 (Rambaut et al. 2014). After checking convergence of the 4 replicates we combined the trees generated from different runs with LogCombiner v1.8.2, from which a phylogram calculated by TreeAnnotator v1.8.2 (part of the BEAST package, Drummond et al. 2012). The marginal likelihood scores for both ML and BI analyses were compared to evaluate the relative support for competing

Table 1. Samples analyzed in the present study

Catalogue Number	Sample Locality/Publication	Scientific name	Common name	Accession No.
ANAL5	Analamerana	*Lepilemur ankaranensis*	Ankarana sportive lemur	HQ171056
AND6.3	Andohahela	*Lepilemur fleuretae*	Fleurete's sportive lemur	HQ171057
AND65	Andohahela	*Lepilemur leucopus*	White-footed sportive lemur	HQ171058
ANK16	Ankarafantsika	*Lepilemur edwardsi*	Milne-Edwards' sportive lemur	HQ171059
ANT5.2	Antafondro	*Lepilemur dorsalis*	Gray-backed sportive lemur	HQ171060
BEMA7.7	Tsingy de Bemaraha	*Lepilemur ruficaudatus*	Red-tailed sportive lemur	HQ171061
BEMA7.9	Tsingy de Bemaraha	*Lepilemur ruficaudatus*	Red-tailed sportive lemur	HQ171062
BEZ7.20	Beza Mahafaly	*Lepilemur petteri*	Petter's sportive lemur	HQ171063
BIBO7.1	Ambodimahabibo	*Lepilemur otto*	Otto's sportive lemur	HQ171064
DAR5.1	Daraina	*Lepilemur milanoii*	Daraina sportive lemur	HQ171065
FAN6.1	Fandriana	*Lepilemur betsileo*	Betsileo sportive lemur	HQ171066
FARY5.1	Sahafary	*Lepilemur septentrionalis*	Sahafary sportive lemur	HQ171067
DVA8.2	Ampasindava	*Lepilemur mittermeieri*	Mittermeier's sportive lemur	HQ171068
DVA8.3	Ampasindava	*Lepilemur mittermeieri*	Mittermeier's sportive lemur	HQ171069
GAR1	Manongarivo	*Lepilemur dorsalis*	Gray-backed sportive lemur	HQ171070
HAZO5.6	Ihazofotsy	*Lepilemur leucopus*	White-footed sportive lemur	HQ171071
HIH7.4	Anjiamangirana	*Lepilemur grewcockorum*	Grewcock's sportive lemur	HQ171072
JAM4.8	Anjahamena	*Lepilemur aeeclis*	Antafia sportive lemur	HQ171073
JAR3.46	Anjanaharibe-Sud	*Lepilemur seali*	Seal's sportive lemur	HQ171074
KAL7.4	Kalambatritra	*Lepilemur wrightae*	Wright's sportive lemur	HQ171075
KIBO22	Tsiombikibo	*Lepilemur ahmansonorum*	Ahmanson's sportive lemur	HQ171076
KIR6.5	Kirindy	*Lepilemur randrianasoloi*	Randrianasolo's sportive lemur	HQ171077
KMTEA7.5	Kirindy Mitea	*Lepilemur randrianasoloi*	Randrianasolo's sportive lemur	HQ171078
LAZA5.1	Sahamalaza	*Lepilemur sahamalazensis*	Sahamalaza sportive lemur	HQ171079
LOKO4.2	Lokobe	*Lepilemur tymerlachsonorum*	Nosy Be sportive lemur	HQ171080
M104B	Manombo	*Lepilemur jamesorum*	James' sportive lemur	HQ171081
MAR1	Mariarano	*Lepilemur edwardsi*	Milne-Edwards' sportive lemur	HQ171082
MAS6.12	Masoala	*Lepilemur scottorum*	Scott's sportive lemur	HQ171083
MIT16	Antrema	*Lepilemur aeeclis*	Antafia sportive lemur	HQ171084
NARA8.5	Mananara-Nord	*Lepilemur hollandorum*	Holland's sportive lemur	HQ171085
RANO234	Ranomafana	*Lepilemur microdon*	Small-toothed sportive lemur	HQ171086
TAK7.13	Ifotaka Classified Forest	*Lepilemur petteri*	Petter's sportive lemur	HQ171087
TVY7.120	Kistler et al. 2015	*Lepilemur mustelinus*	Weasel sportive lemur	KJ944247
VEV7.7	Vevembe	*Lepilemur jamesorum*	James' sportive lemur	HQ171089
ZAH21	Kistler et al. (2015)	*Lepilemur mustelinus*	Weasel sportive lemur	KJ944256
ZOMB6.3	Lei et al. (2010)	*Lepilemur hubbardorum*	Hubbard's sportive lemur	HM070254
AB371086	Matsui et al. (2009)	*Eulemur fulvus*	Common brown lemur	AB371086
AB371087	Matsui et al. (2009)	*Eulemur fulvus mayottensis*	Mayotte's lemur	AB371087
AB371088	Matsui et al. (2009)	*Eulemur macaco*	Black lemur	AB371088
AM905040	Arnason et al. (2008)	*Eulemur mongoz*	Mongoose lemur	AM905040
AJ421451	Arnason et al. (2002)	*Lemur catta*	Ring-tailed lemur	AJ421451
NC004025	Arnason et al. (2002)	*Lemur catta*	Ring-tailed lemur	NC004025
AB371089	Matsui et al. (2009)	*Varecia variegata*	Black and white ruffed lemur	AB371089
AB286049	Matsui et al. (2007)	*Propithecus coquereli*	Coquerel's sifaka	AB286049
AB371085	Matsui et al. (2009)	*Daubentonia madagascariensis*	Aye-aye	AB371085
AM905039	Arnason et al. (2008)	*Daubentonia madagascariensis*	Aye-aye	AM905039
AB371092	Matsui et al. (2009)	*Galago senegalensis*	Northern lesser bushbaby	AB371092
AB371093	Matsui et al. (2009)	*Otolemur crassicaudatus*	Thick-tailed bushbaby	AB371093
NC 002765	Arnason et al. (2000)	*Nycticebus coucang*	Slow loris	NC 002765
AB371094	Matsui et al. (2009)	*Loris tardigradus*	Slender loris	AB371094
AB371095	Matsui et al. (2009)	*Perodicticus potto*	Potto	AB371095
AB371090	Matsui et al. (2009)	*Carlito syrichta*	Philippine tarsier	AB371090
NC_002811	Schmitz et al. (2002)	*Tarsius bancanus*	Western tarsier	NC_002811
AB371091	Matsui et al. (2009)	*Saimiri sciureus*	Common squirrel monkey	AB371091
NC_002763	Arnason et al. (2000)	*Cebus albifrons*	White-fronted capuchin	NC_002763
NC_001992	Arnason et al. (1998)	*Papio hamadryas*	Hamadryas baboon	NC_001992
NC_005943	Gokey et al. (2004)	*Macaca mulatta*	Rhesus monkey	NC_005943
NC_002764	Arnason et al. (2000)	*Macaca sylvanus*	Barbary ape	NC_002764
NC_002082	Arnason et al. (1996)	*Hylobates lar*	Common gibbon	NC_002082
NC_002083	Xu and Arnason (1996)	*Pongo abelii*	Sumatran orangutan	NC_002083
NC_001646	Horai et al. (1995)	*Pongo pygmaeus*	Bornean orangutan	NC_001646
NC_001645	Horai et al. (1995)	*Gorilla gorilla*	Gorilla	NC_001645
NC_001644	Horai et al. (1995)	*Pan paniscus*	Pygmy chimpanzee	NC_001644
NC_001643	Horai et al. (1995)	*Pan troglodytes*	Common chimpanzee	NC_001643
NC_012920	Anderson et al. (1981)	*Homo sapiens*	Human	NC_012920

partition models (Supplementary Table S1, and additional details in the Supplementary Materials). Tree topologies were visualized with PAUP* 4.0b10 (Swofford 2001).

In order to evaluate the unexpected phylogenetic position of *Lepilemur microdon*, *Lepilemur ahmansonorum*, and *Lepilemur wrightae* based on their geographic distribution (Results section), the program CONSEL (Shimodaira and Hasegawa 2001) was used to calculate the approximately unbiased (AU), Shimodaira–Hasegawa (SH), and Kishino–Hasegawa (KH) tests (Kishino and Hasegawa 1989; Shimodaira and Hasegawa 1999, 2001; Shimodaira, 2002). These tests use the log-likelihood of site-patterns of the trees estimated with PAUP* 4.0b10 (Swofford 2001).

Divergence Date Estimation

The 33 newly generated *Lepilemur* mitogenomes plus 13 published lemur mitogenomes were combined with 19 additional primate mtDNA genomes (Table 1; Anderson et al. 1981; Horai et al. 1995; Arnason et al. 1996, 1998, 2000, 2002, 2008; Xu and Arnason 1996; Schmitz et al. 2002; Gokey et al. 2004; Matsui et al. 2007, 2009). Divergence dating was estimated using a molecular clock approach and several fossil calibration points commonly used in primate studies (detailed in the Supplementary Materials). Divergence times were estimated using the Bayesian approach implemented in the program BEAST v1.8.2 (Drummond et al. 2012). A strict molecular clock was rejected in the molecular clock test performed in MEGA v6.0 (Tamura et al. 2013), so we used an uncorrelated lognormal relaxed clock in all divergence time estimations. Under this model, rates were allowed to vary among branches without *a priori* assumption of autocorrelation between adjacent branches (Drummond et al. 2006).

The model GTR + I + G was utilized in BEAST with the Yule speciation prior. The hierarchical phylogenetic model (HPM) was implemented to estimate variability between and across data partitions for each gene simultaneously (Suchard et al. 2003). The HPMs were utilized to reduce variability in estimates for phylogenetic parameters of individual partitions providing a framework for assessing overall tendencies (Suchard et al. 2003; Edo-Matas et al. 2011). The HPM was implemented in BEAST using three independent MCMC searches of 200 million generations each, with the posterior sampled every 20000 generations. The posterior distributions of parameters, including the tree, were approximated by sampling from 3 independent MCMC analyses, and samples from the posterior were drawn every 1000 steps over a total of 30 million steps per MCMC run following a discarded burn-in of 3 million steps. The independent analyses were compared to assess convergence in Tracer v1.6 (Rambaut et al. 2014) after excluding the first 5 million generations as burn-in, and were combined for subsequent estimations of the parameters. The effective sample size (ESS) for each parameter was greater than 200; therefore, an appropriate number of steps were discarded as burn-in. Subsequently, the sampling distributions were combined (25% burn-in) using LogCombiner (part of the BEAST package, Drummond et al. 2012). Finally, TreeAnnotator from the BEAST package (Drummond et al. 2012), was used to calculate the maximum-clade-credibility tree topology and visualized in FigTree v1.3.1 (Rambaut 2014).

Trees were annotated according to the eight biogeographic regions detailed in Martin (1972) and modified in Pastorini et al. (2003) and Louis and Lei (2016). In this study, we use the same geographic designations with slight modifications: eastern 1 (E1), eastern 2 (E2), western 1 (W1), western 2 (W2), northern (N), northwest 1 (NW1), northwest 2 (NW2), and the central highlands (CH).

Ancestral Range Evolution Analysis

Ancestral range reconstruction was performed to estimate the biogeographic history of all species of *Lepilemur* using the software LaGrange v.20130526 (Ree and Smith 2008). The analysis used modern distribution information (including the 8 biogeographic regions detailed above) and the whole mitogenome tree. A PhyML tree was used as the input, with the following parameters, GTR substitution model, 500 bootstrap replicates, and optimized for topology/length/rate, with a NNI topology search (Guindon and Gascuel 2003; Guindon et al. 2010) as implemented through Geneious v8.18 (Biomatters, Auckland, New Zealand). The root age was not estimated, the adjacency matrix was left to default, a maximum of three biogeographic regions were allowed, and all range combinations were allowed. Dispersal constraints were left to default, and the rates of dispersal and extinction were estimated. The LaGrange analysis was performed on a reduced dataset as the outgroup taxa were primarily used for fossil calibration points in order to recover divergence dating estimates. The only outgroups in the PhyML tree were *Daubentonia madagascariensis* (GenBank Accession #AM905039) and *Eulemur fulvus* (GenBank Accession #AB371086). The topology of the PhyML tree remained the same as the BEAST analysis, with similar branch lengths. Also, we did not use this ancestral reconstruction for inferring divergence dating, so an ultrametric tree was not supplied. We thus deferred to the BEAST analysis for divergence estimates, and the LaGrange analysis to infer ancestral ranges only.

Climate Variables and Divergence

Collection localities for 409 *Lepilemur* wild-captured individuals were mapped using Esri's ArcGIS10.2.2. Data for 19 BioClim variables (Hijmans et al. 2005) and elevation were extracted for each of the mapped points using the values to points function in ArcGIS. The highest resolution data were used, 30 arc-seconds corresponding to about 1 km^2 coverage. The environmental variable "precipitation seasonality (coefficient of variation)" was discounted for this analysis as it is expressed as a percentage, but many values exceeded 100%. This problem has been reported previously, and is mostly restricted to coastal and island areas (O'Donnell and Ignizio 2012). Individuals with identical collection localities were excluded from the dataset, a total of 98 records. The resulting climate and elevational data for the remaining 311 individuals were used as input for a spatial ecology and ecological vicariance analysis using SEEVA v1.01 (Struwe et al. 2011). The SEEVA software utilizes field collection coordinates, environmental data and a user specified phylogenetic tree to identify correlations between ecological data and evolutionary relationships (Struwe et al. 2011). The tree used for ancestral area reconstruction was also used for the SEEVA analysis to perform ecological comparisons between only Malagasy taxa and comparisons made only between the *Lepilemur* sister clades.

The null hypothesis for SEEVA is that there is no significant difference between states for sister groups, but significant divergence indicates that phylogenetic and ecological splits are correlated (Struwe et al. 2011). An index of divergence (D) ranging from 0 to 1 and Fisher's exact test were calculated independently for each variable at every node for all 19 variables. Although many of these environmental variables are correlated, an assumption of independence is not a requirement of SEEVA (Heiberg and Struwe 2012). *D* values, which are independent of sample size, exceeding 0.75 and accompanied by a Bonferroni corrected *P*-value corresponding to an experiment-wise error rate of 0.01 were considered significant for this study (Struwe et al. 2011; Schulte et al. 2015). These thresholds for significance were highly conservative.

A rudimentary test of the watershed and current climate hypotheses was done using them as variables in the SEEVA analysis and their respective biogeographic zones as character states. The biogeographic zone of each locality was extracted using the georeferenced TIFs from Pearson and Raxworthy (2009). The river barrier hypothesis was not tested as a similar mapping tool was not available. For the watershed hypothesis, high indices of divergence at phylogenetic splits for sister species distributed in centers of endemism were viewed as support. Lower *D* values for sister species distributed in retreat–dispersion watersheds would also be support. For the current climate hypothesis, high divergence between sister species in different climate clusters were perceived as support.

Results

Mitochondrial Genome Sequences and Phylogeny

Thirty-three novel sequences of the complete mitochondrial DNA genome were evaluated for 24 sportive lemur species (Table 1). The addition of the previously published *L. hubbardorum* and 2 *L. mustelinus* mitogenomes brought the total to 36 individual genomes from 26 nominal species. The general characteristics of these sportive lemur complete mtDNA genomes are reported in Supplementary Tables S3 and S4.

Partitioning had little effect on the overall tree topology as seen when comparing the tree topologies from different partition schemes (Supplementary Materials). A similar topology with strong bootstrap and posterior probability values were obtained in both ML and BI analyses, respectively, for the phylogenetic trees (Figure 2). Both ML and BI methods revealed 25 well-supported terminals, with one clade containing *Lepilemur dorsalis* and *Lepilemur mittermeieri* (with bootstrap and PP values 100% and 1.0, respectively). Taxa clustered into 4 geographic regions: E1 & E2 clade, W1 & W2, N & NW1 with the exception of *L. ahmansonorum*, and NW2 with the exception of *L. microdon*.

We performed additional analyses via CONSEL on species relationships that did not fit biogeographic expectations. *Lepilemur ahmansonorum* is geographically proximate to *Lepilemur randrianasoloi* and *Lepilemur aeeclis*, but all 3 tests (AU, KH, and SH) rejected grouping *L. ahmansonorum* with *L. randrianasoloi* or *L. aeeclis* (Supplementary Table S5; *P* < 0.001). Despite *L. microdon* being geographically close to *Lepilemur betsileo*, *Lepilemur jamesorum*, and *Lepilemur wrightae*, all three tests (AU, KH, and SH) rejected grouping *L. microdon* with the aforementioned species (Supplementary Table S5; *P* < 0.001). All 3 tests (AU, KH, and SH) also rejected grouping *L. wrightae* with *L. leucopus*, *L. jamesorum*, or *L. microdon* (Supplementary Table S5; *P* < 0.001). For comparison, we verified other possible sister groupings that were supported geographically, such as between *Lepilemur seali* and *Lepilemur scottorum*. Although *L. seali* is geographically close to *L. scottorum*, all 3 tests rejected grouping *L. scottorum* with *L. seali*, but favored the sister relationship between *L. seali* and its other nearest neighbor *Lepilemur hollandorum* (*P* < 0.001). See Supplementary Table S5 for other tests of sister relationships.

Molecular Estimates of Divergence Dates

The divergence of the Lepilemuridae from Lemuridae was estimated at 30.43 (26.12–34.9) million years (Myr), followed by an east-west division within Lepilemuridae 15.12 (12.89–17.71) Myr with the exception of the eastern species *L. microdon* (Figure 3; Table 2).

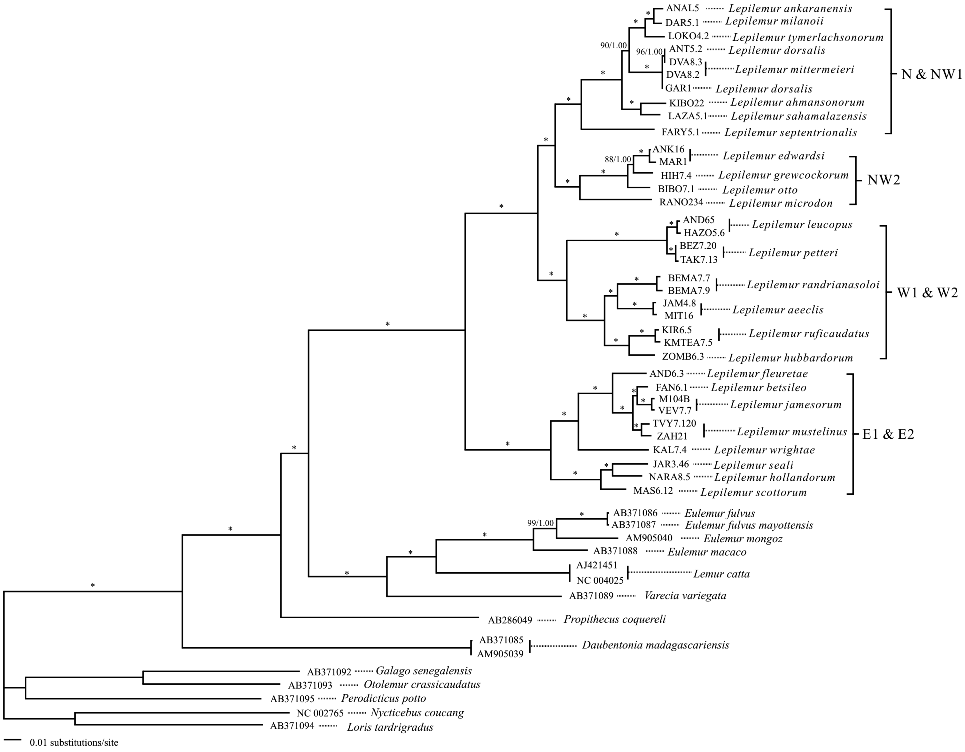

Figure 2. Phylogenetic relationships between *Lepilemur* species inferred from the Maximum Likelihood and Bayesian approaches of complete mitochondrial genome sequences from 36 sportive lemur individuals with 15 outgroup taxa. Numbers on branches represent bootstrap support values followed by posterior probability support values. Nodes that were found with maximum support values by both phylogenetic methods (ML, BS = 100; Bayesian inference, PP = 1.00) are labeled with an asterisk.

Lepilemur microdon was more closely related to the NW1 clade than others in the geographically proximate eastern clade. The initial split within the western group (W1 & W2, NW1 and the predominately N, NW2) was estimated to be 9.07 (7.85–10.46) Myr, with subsequent branching of the NW1 and N, NW2 clades 7.61 (6.47–8.78) Myr. Extant putative species originated between 5.66 and 0.61 Myr. The phylogenetic analyses could not recover the *L. dorsalis*/*L. mittermeieri* clade as reciprocally monophyletic.

Ancestral Range Evolution Analysis

The ancestral range reconstruction portrays the proportion of the range relative to the percentage of the pie chart for each node (Figure 4). Here, we include pie charts representing at least 80% of the reconstruction results. According to our reconstructions, the genus *Lepilemur* may have arisen in the eastern side of Madagascar (potentially including the CH), with the major separation during the Miocene between the E-W divisions. After the initial movement from E1 and E2 into W2, a group of *Lepilemur* moved back to E2 (node 9), and possibly across the CH toward NW2 (the clade containing *Lepilemur microdon/otto/edwardsi/grewcockorum*; nodes 15–16). This movement north via either the west coast or central highlands is further supported by the *Lepilemur septentrionalis* clade (sister to the *L. microdon* group) being found in N, and *Lepilemur ahmansonorum/sahamalazensis* distributed in W1. NW1 is occupied by *Lepilemur tymerlachsonorum* whose current range includes evergreen, humid forest, with another wave of movement back to E1 in *Lepilemur milanoii/ankaranensis* and into forests which are currently deciduous and seasonally dry, more climatically similar to the ancestral NW2 and W1 regions (Figure 4, node 23). Finally, the *L. mittermeieri/dorsalis* complex, currently found in humid evergreen forest, is derived from founders distributed in E1/N/NW1. All other closely related *Lepilemur* species were within the same or proximate biogeographic zones.

Climatic Variables and Niche Divergence

SEEVA was utilized to assess the strength of ecological associations with phylogenetic splits. Eighteen environmental variables plus altitude were analyzed for 35 phylogenetic nodes, resulting in 665 comparisons. Comparisons at nodes between individuals of the same species were excluded, leaving a total of 24 nodes and 63 significant comparisons (Bonferroni corrected $P \leq 0.0006$, $D \geq 0.75$). Numerous precipitation and temperature variables were associated with diversification within the following biogeographic zones: E1, E2, W1, W2, and NW. However, there is very little support for climatic variables or altitude being strongly associated with the diversification of species in the N and NW1 biogeographic zones. See Supplementary Tables S6 and S7 for SEEVA results.

We used SEEVA to assess correlations of phylogenetic splits between sister species with the biogeographic zones identified in the current climate and watershed hypotheses. The current climate hypothesis was favored twice and the watershed hypothesis was favored 3 times. Both hypotheses were supported at 4 nodes, and neither hypothesis was supported at an additional 4 nodes (Supplementary Table S9). The island of Nosy Be was not included in either hypothesis, thus node 23 where *Lepilemur tymerlachsonorum* splits from its sister group could not be assessed.

Discussion

Phylogeographic Patterns across *Lepilemur* and Divergence Estimates

The phylogeographic patterns described here generally corroborate those presented in other genetic reconstructions of the family Lepilemuridae (Andriaholinirina et al. 2006; Louis et al. 2006; Craul et al. 2007, 2008; Lei et al. 2008; Ramaromilanto et al. 2009).

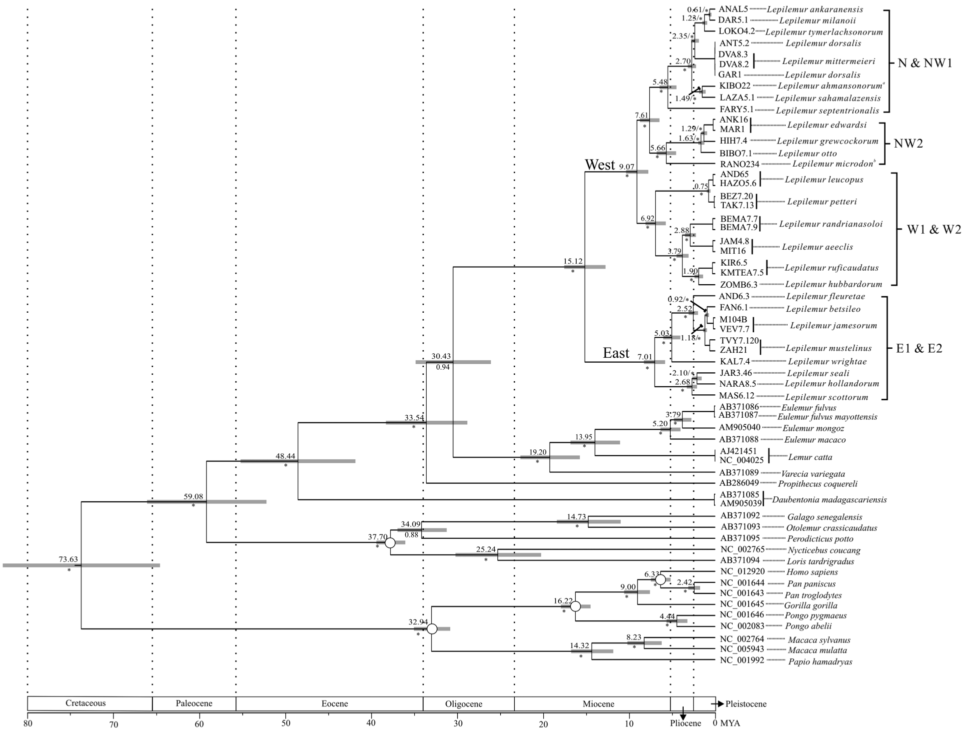

Figure 3. Phylogram used to estimate divergence dates for primates based on complete mitochondrial genome sequences from 36 sportive lemur individuals with 15 outgroup taxa based on a Bayesian relaxed molecular clock analysis. The gray bars display the 95% HPD (highest probability density) interval of node ages. Open circles indicate nodes where fossil evidence was used to calibrate the chronogram. a and b is geographically from W1 and E1.

Table 2. Estimated dates based on mtDNA heavy chain combined sequence data in this study and primate divergence times estimated in recent studies

	Date estimates from previous studies (Myr; TMRCA [95% HPD])								
Branch pairs	This study	1	2	3	4	5	6	7	8
Haplorhini–Strepsirrhini	73.63 (63.68–84.68)	77.5 (67.1–97.7)	78.8 (69.9–88.4)	63.7 (58.3–68.7)	76.0 (69.3–82.5)	87.2 (75.9–98.6)	67.8 ± 8.1	66.2 (61.0–73.5)	74.1 (68.2–81.2)
Strepsirrhini	59.08 (52.23–66.14)	57.1 (49.4–71.4)	67.1 (60.2–74.5)	51.6 (47.7–55.7)	64.5 (57.2–71.7)	68.7 (58.8–76.6)	54.2 ± 5.1	56.9 (50.5–64.1)	66.3 (61.1–72.8)
Lemuriformes	48.44 (42.74–55.85)	40.9 (35–51.0)	59.6 (53.3–66.7)	32.4 (28.6–33.6)	55.3 (47.7–63.0)	58.6 (38.6–76.8)	50.0 ± 6.3	47.1 (40.1–53.6)	43.5 (37.5–50.1)
Lemuridae	19.20 (16.05–22.99)	N/A	29.8 (24.6–36.6)	21.3 (17.8–24.9)	26.1 (20.0–32.6)	26.2 (16.0–35.6)	20.7 ± 5.8	18.1 (14.9–21.5)	31.5 (25.4–38.0)
Lorisiformes	37.70 (36.9–40.29)	N/A	39.5 (38.0–41.8)	37.5 (36.9–38.7)	35.4 (28.5–43.1)	40.3 (35.2–45.6)	34.7 ± 4.0	34.5 (30.2–39.0)	40.3 (37.1–46.3)
Hominoidea–Cercopithecoidea	32.94 (29.95–34.20)	30.5 (26.9–36.4)	23.9 (23.1–25.9)	29.3 (28.0–30.0)	30.5 (25.8–35.3)	31.6 (25.7–37.9)	25.1 ± 5.5	31.9 (28.3–35.7)	32.1 (29.4–33.8)
Pongo–Gorilla + Pan + Homo	16.22 (14.52–18.00)	18.3 (16.3–20.8)	18.6 (17.1–20.5)	15.9 (13.7–18.3)	15.8 (13.3–17.9)	16.5 (13.5–19.7)	15.1 ± 4.0	15.1 (12.7–17.6)	17.3 (16.0–18.0)
Gorilla–Pan + Homo	9.00 (7.54–10.57)	8.6 (7.7–8.2)	9.6 (8.7–10.0)	10.7 (10–11.9)	8.4 (6.6–10.3)	8.3 (6.6–10.1)	8.0 ± 2.8	8.4 (6.9–9.9)	10.6 (10.0–11.7)
Pan–Homo	6.33 (5.19–7.50)	6.6 (6–7)	7.1 (6.5–8.2)	8.1 (6.5–9.7)	6.2 (4.7–7.8)	6.6 (5.4–8.0)	6.7 ± 2.3	6.0 (5.0–7.1)	7.7 (6.7–8.8)
Pongo abelii–Pongo pygmaeus	4.44 (3.27–5.72)	N/A	N/A	4.7 (3.2–6.1)	4.7 (3.5–6.0)	1.3 (0.6–2.2)	1.3 ± 1.4	4.0 (2.8–5.4)	4.2 (3.4–5.1)
Pan troglodytes–Pan paniscus	2.42 (1.79–3.20)	N/A	2.7 (2.2–3.4)	2.8 (1.8–3.9)	3.0 (2.1–4.1)	2.2 (1.3–3.2)	1.6 ± 1.5	2.2 (1.5–2.9)	3.0 (2.4–3.7)
Indriidae–Lepilemuridae + Lemuridae	33.54 (28.93–38.43)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Lepilemuridae–Lemuridae	30.43 (26.12–34.90)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Lepilemur	15.12 (12.89–17.71)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
E1 & E2	7.01 (5.85–8.35)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
NW2	5.66 (4.62–6.89)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
N & WN1	5.48 (4.54–6.53)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
W1 & W2	6.92 (5.74–8.13)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
NW2-N & WN1	7.61 (6.47–8.78)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
W1 & W2-NW2 + N & NW1	9.07 (7.85–10.46)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

1. Steiper and Young (2006); 2. Fabre et al. (2009); 3. Chatterjee et al. (2009); 4. Matsui et al.(2009); 5. Perelman et al. (2011); 6. Springer et al. (2012); 7. Finstermeier et al. (2013); 8. Pozzi et al. (2014).

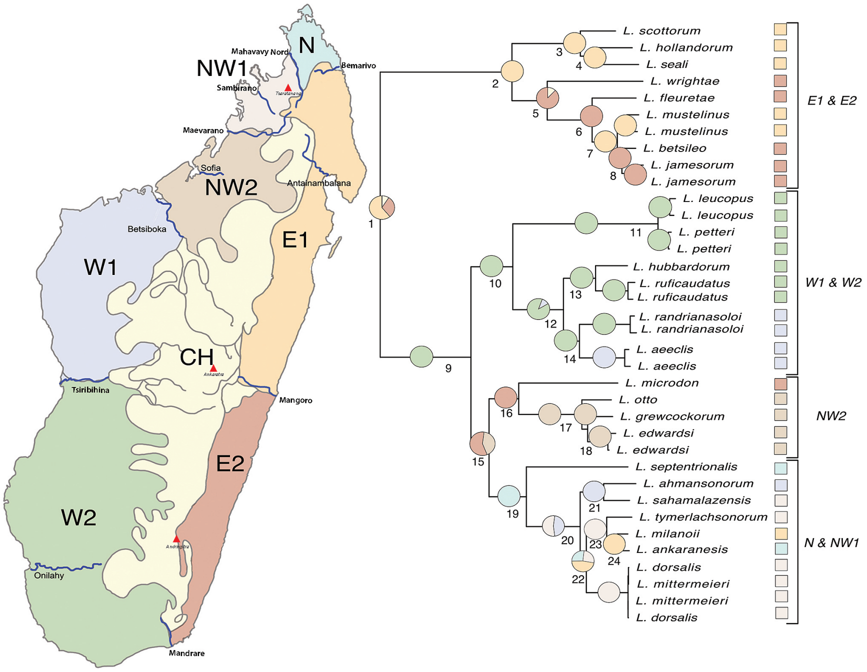

Figure 4. Ancestral range reconstructions of Lepilemur with a map of Madagascar depicting a modified version of Martin's (1972) biogeographic zones. Pie charts represent the percentage of the history corresponding to the various biogeographic zones. See online color version of this figure at: jhered.oxfordjournals.org.

In particular, all studies recovered an early east-west split across Madagascar around 15 Myr (mid-Miocene). This divergence date pattern is not unique to *Lepilemur*, but also found to loosely correlate to the split between *Prolemur simus* and *Hapalemur*, and some of the generic level divisions within the Family Cheirogaleidae (Herrera and Dávalos 2016), implying a lemur-wide diversification event. Radiations in other Malagasy taxa also occurred at this time, such as in dicotyledons (*Impatiens*; Janssens et al. 2009). These events correspond to the end of the Miocene Climatic Optimum, a period of global cooling and species turnover (e.g., Böhme 2003; You 2010).

The average age of extant putative *Lepilemur* species is 2.47 Myr (not including the *L. mittermeieri*/*L. dorsalis* clade), with some species diverging as early as 5.66 Myr (*L. microdon*), (Figure 3); however, most splits occurred closer to the Pliocene-Pleistocene boundary (2.9 to 0.7 Myr). All of these Lepilemuridae terminal clades correspond to species identified in recent phylogenies (Delpero et al. 2001; Andriaholinirina et al. 2006; Louis et al. 2006; Rabarivola et al. 2006; Craul et al. 2007; Lei et al. 2008; Ramaromilanto et al. 2009) with one exception. We found no evidence to support the separation of *L. dorsalis* from *L. mittermeieri*. Sampling from the syntypes (1868.9.7.4[a]; 1868.9.7.5; British Natural History Museum) of *L. dorsalis* should be done to establish the validity of *L. mittermeieri* as a species or a synonym of *L. dorsalis* (Groves CP, unpublished data). Also, additional analyses of nuclear DNA could provide insight into the *L. dorsalis*/*L. mittermeieri* complex, to determine if the pattern observed here is solely from only sequencing mitochondrial DNA prior to revising the taxonomy. Further investigation adding nuclear loci to a *Lepilemur* phylogeny would allow for the most comprehensive molecular characterization of this genus.

Biogeography across Madagascar

The central highlands has been suggested to act as a strong, although not absolute (Yoder and Heckman 2006; Craul et al. 2008), east-west barrier to gene flow (Martin 1972). Our study corroborates this with the divergence of the eastern and western Lepilemuridae lineages at about 15.12 Myr during the Middle Miocene Climate Optimum (You 2010) when grasslands were expanding across Africa (Kürschner et al. 2008). Although the grasslands in Madagascar were thought to be of anthropogenic origin, it has been argued that they are also the result of a post-Miocene savanna proliferation (Bond et al. 2008). It is possible that at this time the expansion of the grasslands of the central highlands may have dissected the ancestral distribution of the genus.

As the grasslands expanded, the central highlands experienced the majority of their uplift, which occurred mostly within the last 10 million years (Roberts et al. 2012). The central highlands creates a rain shadow on its western side resulting in evergreen, humid forest in the east and seasonally dry to arid conditions in the west (Logan 1968). Thus, this evolutionary split was marked by an ecological shift with significant divergence indices for numerous temperature variables and precipitation levels during the colder, drier months of the austral winter (see Figure 4, Node 1; Supplementary Table S6).

The phylogenetic position of *L. microdon* challenges the east-west biogeographic division among sportive lemurs. This species is found in the east (E2), in and around Ranomafana National Park, and is a close genetic affiliate of *L. grewcockorum*, *L. otto*, and *L. edwardsi*, all species that are endemic to the northwest (NW2, Figure 4). Given that the NW2 group is closely related to other western clades, it appears that the migration occurred from west to east at some time between the split of *L. microdon* from the rest of the NW2 group (ca. Miocene-Pliocene boundary) and the diversification of *L. otto* and *L. grewcockorum*/*L. edwardsi* (ca. 1.6 Myr) (Figure 4). Riparian forest corridors along the tributaries of the Betsiboka River may have acted as a link between the 2 regions during past climatic conditions in a manner similar to that described in the watershed hypothesis (Wilmé et al. 2006).

The central highlands do not extend to the northern tip of the island, removing this as a possible mechanism driving speciation in the north and northwest clades, although there are numerous mountains (Figure 4). In the N and NW1 clade, there is little support for ecological divergence based on the abiotic variables assessed in this study (nodes 19–24, Supplementary Tables S6 and S7). Thus, the current climate hypothesis does not explain *Lepilemur* distributions in northern Madagascar and/or Worldclim climate data are not sufficiently refined to test it. In climate class I (Pearson and Raxworthy 2009), which nearly entirely overlays regions N and NW, 6 *Lepilemur* species occur (Figure 4, Supplementary Table S8). This contradicts Kamilar and Muldoon (2010), who found that closely related lemur species tend to occupy different climatic niches, but they did not sample sister species. In particular, there are no significant comparisons with high *D*-values at the split between *L. septentrionalis* from its sister group in contrast to the split of *L. microdon* from its sister group, although both species are of similar antiquity dating to the Miocene-Pliocene boundary (Figure 4, Nodes 16 and 19). It has been suggested that trait divergence should swamp conservatism as phylogenetic distance increases (Svensson 2012), but this is not the case in the N and NW1 biogeographic regions within the genus *Lepilemur*. This supports vicariant mechanisms of speciation, other than ecological, such as the watershed and large river hypotheses (Craul et al. 2007; Wilmet et al. 2014; this study). In partially sympatric species, *L. ankaranensis* and *L. milanoii* (Salmona et al. 2014), other mechanisms promoting speciation should be considered such as learned mate preferences (Svensson 2012).

These data suggest that in certain biogeographic regions, niche conservatism is strong despite the global climatic shifts that may have been occurring; nevertheless, climate-driven hypotheses may be applicable to the western biogeographic regions. Climate change during the Pleistocene is not well known for Madagascar and difficulties arise when attempting to infer historical patterns from other regions (Wilmé et al. 2006). However, splits between sister species in the W1 and W2 clade have dates of divergence (2.88, 1.90, and 0.75 Myr; Figure 3) coinciding with shifts in African climate change (2.8, 1.7, and 1.0 Myr) as summarized in deMenocal (2004). Also, evolutionary landmarks with similar dates were noted in bovids on the African mainland (2.7 to 2.5, 1.8, and 0.7 Myr) (deMenocal 2004). The coinciding steps in *Lepilemur* and bovid evolution suggest that models of paleoclimate change for subequatorial mainland Africa, which was also undergoing uplift and possibly changing orographic precipitation patterns at the same time as Madagascar (Paul et al. 2014), may be suitable to western Madagascar. This should be tested with comparisons to evolution in other Malagasy taxa and more comprehensive sampling of western *Lepilemur* populations.

The SEEVA analysis offers a statistical test to identify geographic regions where different hypotheses may be favored. Using this approach, we found support in multiple clades for both the climate and watershed hypotheses (Supplementary Table S9). Thus, we concur with Pearson and Raxworthy (2009) in that a single scenario does not adequately predict biogeographic patterns throughout Madagascar.

Although, we could not assess the river barrier hypothesis in a similar manner, Craul (2008) demonstrated the value of rivers in delineating zones of endemism in northern Madagascar. An exception to rivers as a major driver of speciation in Lepilemuridae is *L. seali*, which was identified by Craul et al. (2008) as having "jumped" the Antainambalana River (and possibly, the Rantabe River, even further south), resulting in populations of the same species on both sides of a large river. It is possible that *L. seali* was able to traverse the Antainambalana River at higher elevations if suitable forest habitat persisted at the headwaters (Craul et al. 2008). Additionally, it is possible that climatic conditions changed at some point to allow populations of *L. seali* to cross the river (prolonged drought, for instance). A similar example is *L. milanoii* whose range is bisected by the Loky River (Louis et al. 2006). The presence of these species on both sides of large rivers does not discount the apparent importance of riverine barriers as drivers of sportive lemur speciation; however, it does underscore the potential complexity in biogeographic and evolutionary patterns in the family Lepilemuridae (Figures 2–4). For additional examples on exceptions to the river barrier hypothesis see the supplementary materials.

While all lemurs in Madagascar face the threat of extinction, some species within the *Lepilemur* represent the most endangered primates in the world (Mittermeier et al. 2008, 2010; IUCN 2015), and this study in combination with various others provide baseline data for groups seeking to understand these increasingly threatened animals. Finer scale environmental data, the addition of vegetation data, as well as more extensive sampling may provide greater insight on biogeographic patterns in *Lepilemur* and the complex factors driving speciation in Madagascar.

Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

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Conflict of Interest

The authors declare no conflict of interest.

Data Accessibility

All mitogenomes generated here can be found on GenBank under the following accession numbers: HQ171056–HQ171089.

All alignments, tree files, and partitioning information have been placed on Dryad at: doi: http://dx.doi.org/10.5061/dryad.g4760.

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