Comparative and population mitogenomic analyses of Madagascar’s extinct, giant ‘subfossil’ lemurs


Abstract

Humans first arrived on Madagascar only a few thousand years ago. Subsequent habitat destruction and hunting activities have had significant impacts on the island’s biodiversity, including the extinction of megafauna. For example, we know of 17 recently extinct ‘subfossil’ lemur species, all of which were substantially larger (body mass ~11–160 kg) than any living population of the ~100 extant lemur species (largest body mass ~6.8 kg). We used ancient DNA and genomic methods to study subfossil lemur extinction biology and update our understanding of extant lemur conservation risk factors by i) reconstructing a comprehensive phylogeny of extinct and extant lemurs, and ii) testing whether low genetic diversity is associated with body size and extinction risk. We recovered complete or near-complete mitochondrial genomes from five subfossil lemur taxa, and generated sequence data from population samples of two extinct and eight extant lemur species. Phylogenetic comparisons resolved prior taxonomic uncertainties and confirmed that the extinct subfossil species did not comprise a single clade. Genetic diversity estimates for the two sampled extinct species were relatively low, suggesting small historical population sizes. Low genetic diversity and small population sizes are both risk factors that would have rendered giant lemurs especially susceptible to extinction. Surprisingly, among the extant lemur species, we did not observe a relationship between body size and genetic diversity. The decoupling of these variables suggests that risk factors other than body size may have as much or more meaning for establishing future lemur conservation priorities.

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Introduction

The paleoecological record of Madagascar demonstrates dramatic alterations in the island’s endemic biodiversity over the last two millennia, concurrent with the arrival and spread of humans (Burney et al., 2004). From pollen data (MacPhee et al., 1985) and the widespread distribution of species-diverse subfossil sites (Crowley, 2010), we can infer that most regions of the island were likely forested or partially wooded, including the vast central plateau that is mostly depauperate today. All endemic animal taxa with body masses >10 kg are now extinct (Crowley, 2010), including up to seven giant ‘elephant bird’ species, two giant tortoises, a horned crocodile, three hippopotamus species, three raptors, a giant fosa (carnivoran), two aardvark-like species (Plesiorycteropus spp.), and 17 species of lemurs. Evidence of habitat modification and tool-assisted butchery (MacPhee and Burney, 1991; Burney, 1999; Perez et al., 2005) suggests that human activities contributed to these extinctions (Burnney et al., 2004; Godfrey and Irwin, 2007; Dewar and Richard, 2012).

Today, Madagascar is considered among the world’s most significant and threatened biodiversity hotspots (Mittermeier et al., 2005), as the surviving endemic fauna continue to face habitat loss and hunting pressures. The rate of forest potentially accelerating (Harper et al., 2007), and many species are at imminent risk of extinction. For example, over 70% of the ~100 extant lemur species are now considered endangered or critically endangered by the International Union for the Conservation of Nature (Davies and Schwitzer, 2013). Future efforts towards the conservation of extant Malagasy species can benefit from evolutionary and demographic comparisons to the extinct subfossil taxa (Dietl and Flessa, 2011), which represent an important record of past human—environment interactions. In this study, we use ancient DNA and genomic methods to study phylogenetic relationships and compare levels of genetic diversity among extinct and extant lemur taxa. We assess the extent to which phylogeny is a useful predictor for lemur extinction risk (Jermall and Wright, 1998), and test the hypothesis that giant subfossil lemurs were characterized by low genetic diversity, a potential indicator of low population size (Frankham, 1996). Large body size is often associated with low population size (Peters, 1983), an important extinction risk factor. Moreover, low genetic diversity itself is also an extinction risk factor (Frankham, 2005), expanding the potential value of this variable for studies of conservation and extinction biology.

Material and methods

Ancient DNA considerations

Ancient DNA analysis is challenged by low endogenous DNA copy number, short fragment lengths, and chemical modifications including a characteristic pattern of damage related to cytosine to uracil deamination at the single-stranded ends of fragments (Briggs et al., 2007). To address the resulting contamination and consensus sequence accuracy concerns, we implemented standard procedures to prevent contamination from modern DNA sources and correct for ancient DNA damage prior to analysis. All DNA extraction and handling prior to library PCR amplification was carried out in dedicated, sterile facilities with positive pressure, HEPA filtered air, stringent decontamination protocols using strong bleach solution, and the use of personal protective clothing. We limited the incorporation of damaged sites into our consensus sequences by hard-masking (i.e., replacing with ‘N’) all sites potentially affected by the characteristic ancient DNA damage pattern of cytosine deamination in single stranded overhangs (each T on the 5’ end and A on the 3’ end) (Briggs et al., 2007), 10–14nt (nucleotides) from fragment ends in all ancient samples, informed by observed nucleotide abundance patterns, prior to final consensus sequence calling (Supplementary Online Material [SOM] Fig. S1). Finally, independent extractions and preparations of the same Palaeopropithecus ingens sample (AM 6184) were performed in clean labs at Pennsylvania State University and the University of Illinois Urbana–Champaign, and sequenced separately. The resulting mtDNA consensus sequences were identical, suggesting that our results are not likely explained by laboratory-specific contamination.

DNA isolation

We isolated DNA from subfossil lemur bone and tooth samples (SOM Dataset S1) using established protocols for ancient DNA recovery from animal hard tissue (Rohland, 2012). We surface-decontaminated samples using a rotary tool or bleach, depending on sample size and integrity, and ground them to a fine powder using a bleach- and heat-sterilized rotary tool, ball mill, or mortar and pestle. At Pennsylvania State University, samples were demineralized and digested overnight in a buffer of 0.25 mg/ml proteinase K, 0.45 M EDTA, 1% Triton-X 100, and 50 mM DTT, followed by in-suspension silica adsorption and spin column recovery of DNA. At the University of Illinois Urbana–Champaign, a buffer comprised of 0.5 M EDTA, 3.33 mg/ml proteinase K, and 10% N-lauryl sarcosine was used to digest hard tissue powder, and silica membrane columns were used to recover DNA.

Library preparation and sequencing

At Pennsylvania State University, we constructed barcoded DNA libraries (DNA fragments from each sample prepared for sequencing on Illumina HiSeq platforms with unique identifiers so that multiple samples could be sequenced simultaneously) using the protocol described by Meyer and Kircher (2010). We independently amplified multiple libraries from each template to increase the proportion of unique molecules sequenced per sample. At the University of Illinois, we used Illumina TruSeq Library Preparation kits. All ancient DNA libraries were sequenced on Illumina HiSeq platforms using 76nt or 101nt paired-end reads (read length). Sequence read data have been deposited in the Sequence Read Archive under SRA Bioproject number PRJNA242738.

Complete mtDNA genomic sequencing

With the goal of recovering whole mitochondrial genome sequences from as many subfossil taxa as possible, we extracted DNA and prepared barcoded sequencing libraries from multiple specimens from each available species (SOM Dataset S1), and sequenced these libraries in parallel on several HiSeq flow cell lanes. We screened sequence reads for endogenous lemur DNA of sufficient quality and quantity for complete mtDNA genome sequencing by using the Burrows-Wheeler Aligner (BWA; Li and Durbin, 2009) to map the reads to the complete mtDNA genomes of various extant lemur species available from GenBank (SOM Dataset S2) and to the mtDNA genomes of extinct lemurs, after they had been assembled for some species (SOM Dataset S1). We used default BWA parameters with the exception of a value of 0.01 for the --n (maxDiff) parameter in order to allow a greater proportion of mismatches, due to evolutionary distance between the subfossil samples and the reference sequences. After mapping, we discarded unmapped reads with length <40nt to prevent off-target mapping of exogenous, short-fragment DNA. For the samples with the highest proportion of endogenous sequence reads for each species, we prepared additional libraries to increase the proportion of
non-redundant reads, and sequenced the combined libraries on one or more lanes of an Illumina HiSeq flow cell. Using shotgun sequencing, we recovered complete or near-complete mitochondrial genome assemblies for *Hadropithecus* *stenognathus*, *Megaladapis edwardsi*, *Pachylemur julii*, and *Palaeopropithecus ingens*. We also identified a second *M. edwardsi* individual, for which the complete mtDNA genome could be obtained by sequencing on only a partial lane. For *Palaeopropithecus maximus*, we did not identify a sample with sufficient proportions of endogenous DNA content for shotgun sequencing, but we used a target capture approach, as described below, based on the *P. ingens* mtDNA sequence, to recover a near-complete mtDNA genome.

Mitochondrial genome enrichment

For the *P. ingens* population study, the *P. maximus* sample, and for a third *M. edwardsi* individual, we used an in-solution biotinylated RNA bait hybridization method (Gnirke et al., 2009) to selectively enrich the DNA libraries for mtDNA fragments. In this method, biotinylated RNA bait molecules complementary to the reference sequence are synthesized and hybridized to the total DNA libraries from each sample. The biotinylated RNA baits and hybridized DNA are bound to streptavidin-coated magnetic beads and immobilized on a magnetic stand, then purified through several washing steps, facilitating magnitudes-scale enrichment of targeted fragments for sequencing. We used our newly-assembled high-coverage mtDNA *P. ingens* and *M. edwardsi* reference sequences (AM 6184 and UA 4822/AM 6479, respectively) to design 100mer biotinylated RNA baits that covered the circularized complete mtDNA genomes with 10nt tiling on one strand, so that each site was covered by 10 unique baits. We used MycroArray’s MyBaits system according to the manufacturer’s protocol, but with twice as many wash steps following hybridization, using up to 1 ug of amplified DNA library in each capture reaction. One or two independent, barcoded libraries from a given sample were included in each capture reaction. We attempted to use this method to collect mtDNA sequence data from the remains of 31 additional *P. ingens* and four additional *M. edwardsi* individuals. We obtained data from 24 of the *P. ingens* samples (excluding the hypervariable region: mean = 6225 bp covered by a minimum of two independent sequence reads, s.d. = 5002 bp; SOM Dataset S1) and one *M. edwardsi* sample (6007 bp excluding the hypervariable region). We included the 21 *P. ingens* samples (including AM 6184) and three total *M. edwardsi* samples with >2500 bp of the non-hypervariable mtDNA genome (*P. ingens* mean = 7842 bp, s.d. = 4806 bp; *M. edwardsi* mean = 12,062 bp, s.d. = 5265 bp) in our genetic diversity analyses.

To confirm that the DNA capture process does not introduce sequence errors, we enriched a set of libraries from the AM 6184 sample for subsequent sequencing. The consensus sequence obtained from the AM 6184 DNA capture was identical to the two mtDNA genome sequences assembled from AM 6184 shotgun sequencing libraries independently constructed at Pennsylvania State University and the University of Illinois Urbana–Champaign.

Assembly and consensus sequence calling

We demultiplexed the Illumina output (i.e., used the barcode information from each library to identify reads from each simultaneously-sequenced sample), trimmed adapter sequences (part of the library construction process) from fragments shorter than the total paired-end read length, and merged overlapping paired-end reads using scripts described by Kircher (2012), enforcing an 11nt overlap for merging and a combined phred quality score of merged pairs >20. For unmerged reads, we trimmed bases downstream of any site with phred quality <20, enforcing a minimum surviving read length of 30nt, and retained for analysis only reads pairs where both reads passed quality filtration. For each sequenced subfossil taxon, we used the Mapping Iterative Assembler (MIA; https://github.com/udo-stenzel/mapping-iterative-assembler) to reiteratively align merged reads to reference sequences from the hypothesized most closely related extant species (for *P. ingens*, *P. maximus*, and *H. stenognathus*: *Propithecus diadema*; for *P. julii* and *M. edwardsi*: *Varecia variegata*). We ran MIA to convergence — until further iterations failed to improve the assembly — using a kmer size of 13 and collapsing redundant reads, called a consensus sequence of minimum 2× coverage using the -f41 output from the ma command, in the MIA package, and then checked and corrected each assembly manually to make any possible improvements. We also implemented a base masking procedure to limit errors from cytosine-to-uracil deamination ancient DNA damage (see ‘Ancient DNA considerations’, above).

For *P. ingens*, we replicated the sequencing and assembly of the AM 6184 sample following independent extraction and library construction at the Pennsylvania State University and University of Illinois Urbana–Champaign clean labs, yielding identical sequences. For all captured *P. ingens* libraries, we used BWA (Li and Durbin, 2009) to align reads to the AM 6184 reference and SAMtools (Li et al., 2009) to identify consensus sequences for each sample. Burrows-Wheeler Aligner mapping was conducted using default parameters, but we discarded all mapped reads shorter than 20 bp to avoid analyzing potentially highly similar, but short sequence fragments from exogenous (e.g., bacterial) sources. Consensus sequence calling was done using the mpileup command, allowing multiple mismatches, and the -q 20 parameter to enforce a minimum mapping quality of 20. We also enforced a minimum 2× non-redundant coverage cutoff for consensus calling. Given its increased diversity and divergence, the mtDNA hypervariable region is susceptible to DNA capture and assembly biases. Specifically, the RNA bait hybridization reaction (Gnirke et al., 2009) favors the recovery of highly complementary molecules over divergent ones, and assembly algorithms likewise have difficulty reconstructing divergent regions in poorly preserved samples. The mtDNA hypervariable region, which can be relatively divergent even among individuals of the same species, was thus excluded from phylogenetic diversity and genetic diversity analyses due to the higher levels of variability in this region and the unavailability of the entire hypervariable region for each subfossil sample, which would otherwise bias the results. All assembled and consensus mtDNA genome sequences have been deposited in GenBank under accessions KJ944173–KJ944258.

Phylogenetic analysis

We aligned the subfossil lemur mitochondrial sequences with representative sequences from all available strepsirrhine genera (SOM Dataset S2), plus selected other primates, using the MAFFT program (Katoh et al., 2002), and extracted heavy-strand coding regions for analysis based on *Propithecus verreauxi* annotations. We
first concatenated these regions to form a single alignment, and analyzed the alignment as a whole. We estimated phylogenies using Bayesian Markov Chain Monte Carlo (MCMC) analysis with BEAST 1.7.5 (Drummond et al., 2012), combining eight independent chains that were run to convergence, and with Maximum Likelihood (ML) analysis implemented in PhyML (Guindon et al., 2010) with 1000 bootstrap replicates. We selected the GTR + γ evolutionary model with four gamma rate categories for both analyses using a likelihood ratio test, after calculating relative model likelihood values using PhyML with no bootstrapping (Guindon et al., 2010). We enforced monophyly among haplorhines in the Bayesian tree to ensure the correct placement of the tarsier sequence, which was incorrectly positioned at the base of the strepsirrhine lineage under ML analysis. The rest of the tree topology was in agreement between the two analyses. To estimate divergence dates within BEAST, we assumed a lognormal relaxed molecular clock (Drummond et al., 2006) with four fossil calibration points across both haplorhine and strepsirrhine primates. For the full set of node age estimates and descriptions of calibration priors, see SOM Fig. S2. We also tested more complex, alternative mtDNA partitioning schemes (i.e., with subsets of the data) with BEAST (SOM Fig. S3), which did not result in any branching order differences or significant divergence date estimate changes from the single partition analysis.

In extinct taxa with incomplete final consensus sequences (SOM Dataset S1), some assembly gaps may occur in regions of divergence from closely related taxa due to inefficient re-iterative assembly to provisional reference sequences, and—in the case of *P. maximus*—due to hybridization bias away from genomic regions dissimilar to the *P. ingens* reference sequence during bait capture (see SOM Fig. S4). We tested the effects of non-random missing data in a series of analyses using modern taxa with simulated missing data. These results suggest that, for subfossil taxa with incomplete mtDNA genome sequences, our mean divergence date estimates may be slightly underestimated, but the effect is expected to be minimal. With extensive artificial masking of modern taxa, we only observed a mean underestimate of 12% in the most extreme scenario, and we observed complete overlap of the 95% highest posterior density (HPD) of divergence dates across all simulations (SOM Fig. S4).

In order to i) test for proper implementation of Bayesian priors driving the analysis, ii) check for unexpected joint priors imposing undue influence on the results through the interaction of explicit priors, and iii) ensure that the tree topology and interpretations are data-driven rather than based solely on prior constraints, we repeated the BEAST analysis with an empty dataset, sampling only from the Bayesian priors invoked in the analysis. Using Tracer (http://beast.bio.ed.ac.uk/tracer), we observed convergence of model parameters on reasonable values free of obvious influence from artifactual joint priors. Specifically, we confirmed that parameter traces converged to sampling from finite ranges of local values rather than approaching extremely large or small means, and checked for effective sample size (ESS) values of at least 200, signaling that all model parameters (e.g., base frequencies, substitution rates, site heterogeneity statistics, tMRCA)s were converging on a congruent set of values (Drummond et al., 2007). We also observed no narrow constraint of parameters for which we had not specified non-default priors, such as calibration points. Finally, whereas the prior and posterior traces were identical in the empty dataset (as expected), they differ dramatically in our main analysis, and an MCC tree summarized from the empty dataset contained no phylogenetic information beyond the monophyletic grouping of the haplorhines and MRCA values on calibrated nodes reflecting our fossil calibrations. We therefore concluded that our priors were not prescriptive of the observed results, but integrated appropriately with our mitogenomic data.

**Modern sequences and genetic diversity analysis**

In addition to the *P. ingens* and *M. edwardsi* population sequence data obtained by the DNA capture approach described above, we obtained complete mtDNA genomes for population samples of eight extant lemur species. DNA was extracted using a standard phenol/chloroform method from blood and tissue samples from wild-caught individuals (SOM Fig. S5; SOM Dataset S3). Collection and export permits were obtained from Madagascar National Parks, formerly Association Nationale pour la Gestion des Aires Protégées, and the Ministère des Eaux et Forêts of Madagascar. Samples were imported to the USA under requisite CITES permits from the U.S. Fish and Wildlife Service. Capture and sampling procedures were approved by the Institutional Animal Care and Use Committee of Omaha’s Henry Doorly Zoo and Aquarium (#12-101). Complete mtDNA genome sequences were obtained for *aye–aye* (*Daubentonia madagascariensis*) by aligning shotgun sequence data from a previous study (Perry et al., 2013) to an *aye–aye* complete mtDNA genome reference sequence (GenBank NC_010259.1) using default alignment parameters in BWA (Li and Durbin, 2009), and consensus sequences were called using SAMtools (Li et al., 2009). Sequence data were generated for indri (*Indri indri*), diadem sifaka (*Propithecus diadema*), and Milne-Edwards’ sifaka (*Propithecus edwardsi*) by PCR amplification and Sanger sequencing. For black and white ruffed lemur (*V. variegata*), red-bellied lemur (*Eulemur rubriventer*), ring-tailed lemur (*Lemur catta*), and weasel sportive lemur (*Lepilemur mustelinus*), we PCR and Sanger sequenced one individual per species, then selected long-range PCR primers to amplify two overlapping mtDNA fragments from all remaining individuals. These PCR products were combined, sheared using the Covaris Model S2, prepared as barcoded Illumina sequencing libraries at Pennsylvania State University as described above, and sequenced in parallel on an Illumina MiSeq. Reads were aligned to the corresponding reference sequence using default alignment parameters in BWA (Li and Durbin, 2009), and SAMtools (Li et al., 2009) was used to obtain individual consensus sequences. Overlapping primer-binding regions were hard-masked in consensus mtDNA sequences. All PCR and Sanger sequencing primers are provided in SOM Dataset S4, and complete mtDNA sequences have been deposited in GenBank under accessions KJ944173–KJ944258. The non-hyprervariable region sequences for each species were aligned using ClustalW with default parameters (Chenna et al., 2003). Genetic diversity estimates were obtained by comparing, for each intraspecific pair of sequences with >1000 aligned bp in common, the number of aligned nucleotide positions that were different between the two sequences to the total number of aligned nucleotide positions, and then computing the average proportional difference across all analyzed pairs for each species. This analysis was performed in the R statistical environment (R Core Team, 2013).

**Radiocarbon dating**

Of the subfossil lemur dates reported in SOM Dataset S1, all but three are from Crowley (2010). The methods used to prepare and date the *P. ingens* sample from Mikoboka Plateau, Cave #12 (DPC 24136; radiocarbon lab number CAMS 148398) are identical to those reported in Crowley (2010). The radiocarbon date for the *P. ingens* specimen AM 5814 is from Karanth et al. (2005). The *P. ingens* specimen from Ankilitelo, 97-M-352, has not been dated directly, but all other mammal samples (including subfossil lemur) that have been recovered from this site and radiocarbon dated have yielded dates of 600–500 calendar years before present (BP).
(Simons et al., 1995; Simons, 1997; Muldoon et al., 2009; Muldoon, 2010). Therefore, we inferred a date of 550 BP for this sample. We note, however, that recently-published calibrated dates from this site for six subfossil bird specimens range from approximately 13000 to 300 BP (Goodman et al., 2013). Thus, the 550 BP estimate for the Ankiliteo specimen should be treated cautiously. Mean calibrated dates reflect the weighted mean of the calibrated date distribution for each sample, assuming the SHCal04 Calibration Curve (McCormac et al., 2004) in OxCal 4.2 (Bronk Ramsey, 2009). The 95.4% calibrated date ranges and weighted means for each sample are reported in SOM Dataset S1. We note that since our P. ingens samples are unevenly distributed across space and time (e.g., the six oldest samples are all from a single site, Taolambiba), we did not attempt to use these data to estimate temporal population size changes, for example with a Bayesian skyline/skyride analysis (e.g., Minin et al., 2008).

Data accessibility

Demultiplexed Illumina sequence read data for the subfossil lemur shotgun and DNA capture analyses and the extant lemur long-range PCR mtDNA enrichment analyses have been deposited in the NCBI Sequence Read Archive under SRA Bioproject number PRJNA242738. All extant and complete subfossil mtDNA consensus sequences generated as part of this study have been annotated and deposited in GenBank (Accession nos. KJ944173–KJ944258). The bait library sequences based on our new P. ingens and M. edwardsi mtDNA reference sequences and used in the DNA capture experiments, incomplete subfossil mtDNA consensus sequences, the intra-species sequence alignment files used in genetic diversity analyses, and the BEAST xml file, which contains the multi-species alignments used for phylogenetic analysis and the model inputs have been deposited in the Dryad digital repository at http://dx.doi.org/10.5061/dryad.32n76.

Results

Phylogenetic relationships and divergence dates

DNA preservation in skeletal remains recovered from tropical and sub-tropical latitudes (e.g., Madagascar) is typically poor (Reed et al., 2003; Letts and Shapiro, 2012). Because of this issue and the limitations of PCR-based methods, previous ancient DNA-based studies have been unable to fully resolve evolutionary relationships between extinct and extant lemurs. First, based on a DNA–DNA hybridization approach, Crovella et al. (1994) concluded that extinct Pachylemur and extinct Varecia were sister taxa, a clade that had been supported by dental (Seligsohn and Szalay, 1974) but not postcranial (Shapiro et al., 2005) morphology. However, the methods used in that study are simply not reliable for ancient DNA. Next, using PCR and Sanger sequencing methods on mitochondrial DNA (mtDNA), Montagnon et al. (2001) identified a Megaladapis/Leptilemur clade, consistent with craniodental-based phylogenies (Tattersall and Schwartz, 1974; Wall, 1997). Studies by Yoder, Karanth, Orlando and colleagues (Yoder et al., 1999; Karanth et al., 2005; Orlando et al., 2008) called that result into question, instead suggesting a sister taxon relationship for Megaladapis and extinct Lemuridae to the exclusion of Leptilemur. However, due to the availability of only several hundred bp of Megaladapis mtDNA sequence, their results were not robustly supported. Yoder, Karanth, and colleagues (Yoder et al., 1999; Karanth et al., 2005) also identified a relationship between the extinct Palaeopropithecus and the extinct Indriidae, and Orlando et al. (2008) recovered a sister relationship between extinct Archaeolemur and Hadropithecus (the Archaeolemuridae), in both cases matching morphological assessments (Orlando et al., 2008). However, the Orlando et al. (2008) molecular phylogeny also suggested (with low support) that the Archaeolemuridae and Palaeopropithecus form a clade to the exclusion of the extant Indriidae, which is at odds with morphological phylogenies (Orlando et al., 2008).

Here, we sought to resolve these outstanding phylogenetic questions and for the first time estimate divergence dates for extinct lemur taxa through the application of massively parallel sequencing technology, which has facilitated rapid advances in paleogenomic analysis (Millar et al., 2008; Stoneking and Krause, 2011). In contrast to the studies described above, which were restricted to the analysis of several hundred mtDNA bp per species, we obtained complete or near-complete mtDNA reference genome sequences (mean = 14,470 bp; SOM Dataset S1) from five extinct subfossil lemur species: H. stenognathus (~35 kg), M. edwardsi (~85 kg), P. julyi (~13 kg), P. ingens (~42 kg), and P. maximus (~46 kg) (Jungers et al., 2008). We implemented stringent protocols to ensure ancient DNA authenticity and mitigate potential analytical biases introduced by chemical damage in ancient DNA, including independent laboratory replication, use of dedicated sterile facilities, personal protective clothing, strict decontamination protocols, and masking of potentially damaged bases in sequence data (Material and Methods; SOM Fig. S1). We required that each nucleotide position used in our analyses be covered by at least two independent sequence reads, although high coverage was typically achieved, up to 114 average reads per nucleotide (SOM Dataset S1).

We aligned our new subfossil lemur mtDNA reference sequences for each species with those from extant lemur and other primates to estimate a phylogenetic tree and fossil-calibrated divergence dates. The positions of all lemur taxa were robustly supported and consistent across different phylogenetic methods (Fig. 1). The extinct subfossil taxa are distributed across the lemur phylogenetic tree, supporting the notion that gigantism was not a singular evolutionary event for the Malagasy lemurs (Karanth et al., 2005).

Our analysis strongly supports (i.e., posterior probability = 1; bootstrap support = 100%) a sister taxon relationship between Pachylemur and the extinct genus Varecia (Fig. 1). Likewise, we confirm a close relationship between the extinct Palaeopropithecidae (Palaeopropithecus) and the extant Indriidae, as well as a sister taxon relationship between this clade and the extinct Archaeolemuridae (Hadropithecus), which is counter to the inconclusive molecular phylogeny of Orlando et al. (2008) but consistent with their preferred morphological phylogeny. Finally, in contrast with morphological phylogenies (Tattersall and Schwartz, 1974; Wall, 1997) as well as the Montagnon et al. (2001) ancient DNA result that likely reflects contamination (Yoder, 2001; Orlando et al., 2008), our results strongly indicate shared ancestry for Megaladapis and the extinct Lemuridae. The two most notable phenotypic traits shared by Megaladapis and Lepilemur are the absence of permanent upper incisors and the presence of an expanded articular facet on the posterior face of the mandibular condyle (Tattersall and Schwartz, 1974; Wall, 1997). Lepilemur diets seasonally comprise up to 100% leaves (Thalmann, 2001), and microwear patterns on Megaladapis molars also suggest extensive folivory (Rafferty et al., 2002; Godfrey et al., 2004; Scott et al., 2009). Therefore, rather than reflecting shared ancestry, these phenotypes likely signify convergent evolution to highly folivorous diets and a leaf-cropping foraging method in separate clades descended from ancestors with already-reduced upper incisors.

Genetic diversity

To study genetic diversity, we used a DNA capture protocol (Gnirke et al., 2009) to collect >2500 bp of the non-hypervariable
mtDNA genome from a total of 21 *P. ingens* and three *M. edwardsi* samples. We also sequenced complete mtDNA genomes from modern population samples of eight extant lemur species, including the three largest species but otherwise representing a wide range of body sizes \((n = 9 - 12)\) individuals per species; geographic sampling spreads for each species are similar to that for *P. ingens* (SOM Fig. S5). In total, we analyzed mitochondrial genome data from an unprecedented sample of 107 extinct \((n = 27)\) and extant lemur \((n = 80)\) individuals spanning a wide spatiotemporal range in this study.

Fig. 2A shows mtDNA genetic diversity estimates for the two extinct and eight extant lemur species. The average pairwise proportion of nucleotide differences for both *P. ingens* \((\pi = 0.21\%)\) and *M. edwardsi* \((\pi = 0.16\%)\) were lower than those for any of the eight extant lemur species \((\pi = 0.24\% - 1.29\%)\). Among the extant lemurs, we did not observe an inverse relationship between genetic diversity and body size (Fig. 2B).

The observed difference between the extinct and extant lemurs cannot readily be explained by experimental artifacts such as ancient DNA damage, the availability of only partial mtDNA genome sequences for some samples, or temporal sampling variation. Specifically, while we have taken multiple steps to reduce the likelihood that DNA damage could propagate errors into our subfossil lemur sequences, any persistent errors are expected to inflate the observed diversity, which would be conservative with respect to our result. Furthermore, *P. ingens* genetic diversity remains low under more restrictive minimum mtDNA sequence length cutoffs, and extant species \(\pi\) estimates are virtually unchanged when individual sequences are partially masked to match *P. ingens* patterns of incomplete mtDNA sequences (SOM Fig. S6). Finally, temporal diversity (ca. 3189 - 550 BP for our *P. ingens* samples) could also be expected to inflate observed genetic diversity, but this is contrary to our result. We do observe the highest genetic diversity among our most recent *P. ingens* samples (ca. 1500 - 550 BP; \(n = 8\); \(\pi = 0.35\%\)), which likely reflects our relatively wide geographic sampling for this time period (Fig. 3).

**Discussion**

Our paleogenomic approach has helped us gain insight into communities that no longer exist, but that disappeared so recently that these insights connect directly to the modern conservation problem on Madagascar. Thus, our comparisons between extinct and extant lemurs can potentially help inform future efforts to preserve Madagascar’s remaining biodiversity. Large body size is often, but not always, associated with low population size (Peters, 2008), an important extinction risk variable. The low *P. ingens* mtDNA diversity estimate (Fig. 2A) suggests that the ancestral population size of this large-bodied species \((- 42 \text{ kg}; \text{ Jungers et al., 2008})\) may have been considerably lower than those of many smaller-bodied, extant lemurs. Although the *M. edwardsi* sample size is small \((n = 3)\), our preliminary genetic diversity estimate for this species is consistent with the *P. ingens* result, which was estimated from a larger population sample \((n = 21)\). While mtDNA diversity and female effective population size \((N_e)\) are imperfect proxies for census population size (Frankham, 1996; Bazin et al., 2006; Mulligan et al., 2006), they are much better indicators of this key conservation variable for extinct species than is the density of their recovered skeletal remains. We suggest that low population sizes, in the face of hunting pressure and habitat degradation on Madagascar, may have contributed to the extinctions of megafaunal lemur species.

In contrast, among the extant lemurs, the largest-bodied species are characterized by relatively high levels of genetic diversity (Fig. 2B), suggesting that below a certain size threshold \((\text{i.e.,} - 10 \text{ kg})\), traits other than body mass may ultimately be better predictors of lemur responses to hunting pressure and habitat degradation. Future studies that integrate genetic diversity and body size data with other important conservation variables — for example, geographic species range, dietary preference, and life history pattern — would extend our analysis and have the strongest potential to provide more precise extinction risk profiles for extant species. In this respect, paleogenomics represents the most recent
addition to a broader toolkit that can be used to reconstruct aspects of subfossil lemur demography and behavioral ecology. In particular, analyses of stable isotope ratios and dental histology have already provided important insights into the diets (Crowley et al., 2011, 2012; Godfrey et al., 2011; Crowley and Godfrey, 2013) and life histories (Schwartz et al., 2002, 2005; Catlett et al., 2010; Godfrey et al., 2013) of these taxa, respectively. Paleogenomics complements these methods by offering, for the first time, the ability to track the spatiotemporal course of genetic diversity and population size, which are important components of the overall profile and chronology of extinction events. Integrated and expanded applications of these tools will thus help us continue to advance our understanding of the ongoing history, as well as consequences, of human–environment interactions on Madagascar.

On the strength of new reference mtDNA genome sequences for five subfossil lemur species, our phylogenetic analysis (Fig. 1) has clarified several areas of recent uncertainty concerning the evolutionary relationships of extinct and extant lemur taxa. As previously hypothesized, our results definitively show that large-bodied, extinct lemurs do not belong to a single clade, providing no reason to suspect any phylogenetic restriction to the pattern of potential future lemur extinctions. Therefore, if the factors that drove past extinctions are similar to those acting today, then species relationships should not be given strong consideration as lemur conservation risk factors. This conclusion reinforces previous results based on taxonomic analysis of the conservation statuses of extant lemur species (Jernvall and Wright, 1998).

Additionally, for the first time, we have estimated divergence dates on a comprehensive lemur phylogeny comprised of both extant and extinct taxa (Fig. 1; SOM Fig. S2). These results may provide insight into the possible existence of a Tertiary period mass extinction event and subsequent recovery on Madagascar. Specifically, there is a gap of ~20 million years between a) the initial divergence of the Daubentoniidae from the lineage leading to all other lemurs, and b) the diversification of non-Daubentoniidae lemur taxa. The explosive radiation marked by that diversification, which we estimate to have begun ~31 mya (millions of years ago) (95% HPD: 26.6–35.0 Mya), may have followed a major lemur extinction at the Eocene-Oligocene boundary (~34 Mya) – the ‘Grande Coupure’, or great cut (Stehlin, 1909). This boundary was marked by precipitous global cooling and aridification associated with the initiation of the Antarctic ice-cap buildup (e.g., Zachos and Kump, 2005; Zanazzi et al., 2007; Wade et al., 2012), and was accompanied by a massive turnover and loss of species, including other primates, in other regions of the world (e.g., Hallam and Wignall, 1997; Janis, 1997; Ivany et al., 2000; Seiffert, 2007).

Figure 2. Lemur mtDNA diversity in relation to extinction and body size. A) Comparison of average pairwise genetic diversity (π) estimates from the non-hypervariable mtDNA genome for two extinct and eight extant lemur species. Over 2500 bp of non-hypervariable mtDNA sequence was obtained for each of the 21 P. ingens and three M. edwardsi samples included in the analysis; comparisons were only performed and included in the species average for sample pairs with >1000 bp of overlapping sequence. The phylogeny shown is based on Fig. 1. B) Pairwise genetic diversity (π) in relation to body mass (Smith and Jungers, 1997; Jungers et al., 2008). Including all lemur species, the two variables are not significantly correlated (Pearson correlation; r = –0.45; P = 0.19). Considering the extant lemur species only, the two variables are marginally significantly correlated (Pearson correlation; r = 0.66; P = 0.07). Illustrations by Stephen D. Nash/IUCN/SSC Primate Specialist Group, copyright 2013, used with permission.
While potentially informative fossil deposits from relevant time periods have not yet been discovered on Madagascar, our lemur divergence date estimates provide indirect evidence of a similar Grande Coupure event on this island. This event likely helped to shape the current pattern of lemur species diversity, which unfortunately is now similarly imperiled.

Acknowledgments

Subfossil lemurs were sampled under collaborative agreements between L.R.G., David Burney, William Jungers and the Department of Paleontology and Biological Anthropology at the University of Antananarivo. Other subfossil lemur samples were kindly provided by Gregg Gunnell (Duke Lemur Center, Division of Fossil Primates). This is Duke Lemur Center publication #1269. We thank the Madagascar Biodiversity Partnership for assistance in extant lemur sample collection and field logistics in Madagascar, and the Madagascar National Parks, formerly the Association Nationale pour la Gestion des Aires Protégées, and the Ministère des Eaux et Forêts of Madagascar for sampling permission. The subfossil lemur sampling was supported by a Guggenheim Fellowship to L.R.G. and the National Science Foundation (BCS-0129185 to David Burney, William Jungers, and L.R.G.; BCS-0237388 to L.R.G.). Funding for ancient DNA laboratory work and sequencing analyses and for the extant lemur sample collection was provided by Conservation International, the Primate Action Fund, the Margot Marsh Biodiversity Foundation, and the National Geographic Society, along with logistical support from the Ahmanson Foundation and the Theodore F. and Claire M. Hubbard Family Foundation (all to E.E.L.). We thank Craig Praul and Candace Price from the Pennsylvania State Huck Institutes DNA Core Laboratory for assistance with sequence data collection, and Luca Pozzi for providing an extant lemur *Mirza coquereli* complete mtDNA sequence for our analysis prior to its publication. We thank Stephen D. Nash (Conservation International, IUCN/SSC Primate Specialist Group) for providing the illustrations used in Fig. 2. Emily Davenport, Gregg Gunnell, Jason Hodgson, and Mark Shriver provided helpful comments on an earlier version of the manuscript.

Appendix A. Supplementary material

Supplementary material related to this article can be found online at http://dx.doi.org/10.1016/j.jhevol.2014.06.016.

References