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

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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 lemurs, 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 (Burney 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 loss is 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 (Jernvall 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 sequences 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 mapped 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