Ancient DNA from giant extinct lemurs confirms single origin of Malagasy primates

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The living Malagasy lemurs constitute a spectacular radiation of >50 species that are believed to have evolved from a common ancestor that colonized Madagascar in the early Tertiary period. Yet, at least 15 additional Malagasy primate species, some of which were relative giants, succumbed to extinction within the past 2,000 years. Their existence in Madagascar is recorded predominantly in its Holocene subfossil record. To rigorously test the hypothesis that all endemic Malagasy primates constitute a monophyletic group and to determine the evolutionary relationships among living and extinct taxa, we have conducted an ancient DNA analysis of subfossil species. A total of nine subfossil individuals from the extinct genera *Palaeopropithecus* and *Megaladapis* yielded amplifiable DNA. Phylogenetic analysis of cytochrome *b* sequences derived from these subfossils corroborates the monophyly of endemic Malagasy primates. Our results support the close relationship of sloth lemurs to living indriids, as has been hypothesized on morphological grounds. In contrast, *Megaladapis* does not show a sister-group relationship with the living genus *Lepilemur*. Thus, the classification of the latter in the family Megaladapidae is misleading. By correlating the geographic location of subfossil specimens with relative amplification success, we reconfirm the global trend of increased success rates of ancient DNA recovery from nontropical localities.

Madagascar | phylogeny | subfossil | historical biogeography

At least 15% of living primate species are endemic to Madagascar, an island that comprises <0.4% of Earth's land surface area. These primates are considered to belong to a single clade (1–4) that descends from a single common ancestor that colonized Madagascar from Africa sometime in the early Tertiary period (5–7). They represent a remarkable array of primate life histories and morphologies and, given our knowledge of their phylogenetic unity and biogeographic history, can be considered a definitive example of Darwinian radiation in geographic isolation. Yet, there is also a considerable number of extinct primates from Madagascar, all identified from subfossil remains. More than 16 species (8) from at least seven genera have been recovered, predominantly from Holocene sites throughout Madagascar (9). Although now extinct, the fact that at least some species existed as recently as 500 years ago (10) and all but the genus *Babakotia* are known to have existed within the past 2,000 years (11) indicates that they were the evolutionary contemporaries of the living lemurs. What is not clear is whether their phylogenetic relationships to the living lemurs challenge our biogeographic hypotheses of Malagasy primate evolution. If we were to discover that any or all of the extinct Malagasy primates fall outside of the lemuriform clade, then we would have to revise our hypothesis of a single primate colonization of Madagascar.

The phylogenetic relationships and behavioral ecology of the subfossil lemurs have been studied extensively by using morphological characters (12–26). As a result, there are numerous hypotheses to be tested with genetic data. As illustrated in Fig. 1, morphological data indicate that there were once two species

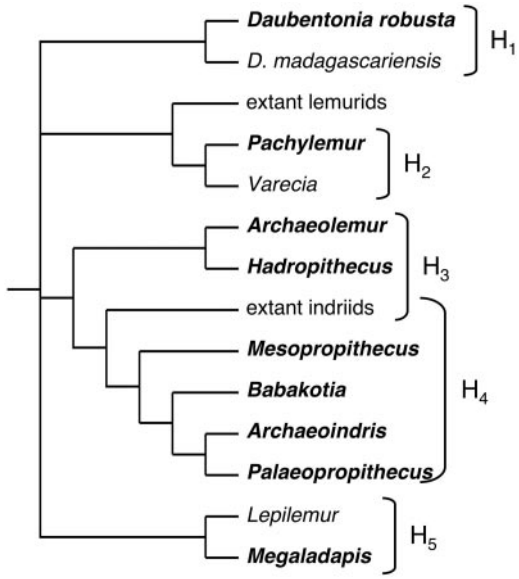

Fig. 1. Morphological hypotheses of extinct and extant Malagasy primates.

of *Daubentonia* (the aye-aye), with the extinct form *Daubentonia robusta* being considerably larger than the extant form *Daubentonia madagascariensis*. Otherwise, they are very similar morphologically (21) and should thus be considered sister taxa [hypothesis (H) 1]. Similarly, the extinct genus *Pachylemur* shows many detailed morphological similarities to the extant genus *Varecia*, leaving little doubt that they were closely related sister taxa (27) (H2). This morphological relationship has also been confirmed by a study of ancient DNA (aDNA) (28). The relationships of the remaining extinct taxa are less clear, due in large part to the many unique and derived features of their cranial and postcranial anatomy. Two extinct genera, *Archaeolemur* and *Hadropithecus*, are clearly allied to each other, with both showing detailed similarities of cranial and postcranial characteristics. Postcranially, both show specializations for terrestrial locomotion, and both are acknowledged to be the most terrestrial of any Malagasy primate, extant or extinct. It is also generally accepted that the two genera together are most closely related to the indriids among living lemurs (29) (H3), although there is some developmental evidence linking them to the family

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Abbreviations: H*n*, hypothesis *n*; AFDIL, Armed Forces DNA Identification Laboratory.

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Table 1. Subfossil samples examined

Species	Code	Locality	AS	Age, years before present	AL	SL	Location of sample analysis		
							NU	YU	AFDIL
Megaladipis	UA 4823	Bevoha	+	1,930 ± 63	161	397		X	X
Megaladapis	UA 5181	Anavoha (Beloha)	+	ND	137	90		X	X
Megaladapis	UA 4543	Bevoha	+	ND	137	90			X
Megaladapis	UA 5482	Anavoha (Beloha)	+	1,308 ± 34	161	499		X	
Megaladapis	UA 4821	Unknown	+	1,476 ± 135	137	168			X
Megaladapis	UA 5476	Unknown	−						X
Megaladapis	UA 4822	Bevoha	+	1,730 ± 192	161	319	X		X
Megaladapis	UA 4566	Bevoha	−						X
Palaeopropithecus	UA 4466	Anavoha (Beloha)	+	1,486 ± 76	161	499		X	X
Palaeopropithecus	9-M-352	Ankilitelo	−					X	
Palaeopropithecus	AM 6184	Ankazoabo	+	1,148 ± 162	161	380	X		X
Palaeopropithecus	UA 4513	Unknown	−				X		
Palaeopropithecus	Unknown	Unknown	+	ND	161	292	X		
Archaeolemur	BSM 1995	Belo-sur-Mer	−	2,000 ± 60				X	X
Archaeolemur	BSM 1996	Belo-sur-Mer	−					X	
Archaeolemur	UA 6808	Ankarana	−						X
Archaeolemur	DUPC 10858	Antsiroandoha	−				X		
Archaeolemur	DUPC 10871	Antsiroandoha	−				X		
Archaeolemur	AM6358	Lamboharana	−				X		
Archaeolemur	DUPC 10863	Antsiroandoha	−						X
Archaeolemur	DUPC 11745	Unknown	−						X
Hadropithecus	UA 5173	Belo-sur-Mer	−					X	
Propithecus	DUPC 3704	Anjohibe	+		156	176			X
Propithecus	DUPC 6852	Anjohibe	+		137	90			X

Radiocarbon ages with 2-Σ (95%) confidence intervals were determined by accelerated mass spectrometry at the Rafter Radiocarbon Laboratory, Institute of Geological and Nuclear Sciences Limited, New Zealand. AS, amplification success (+, success; −, failure); AL, maximum length of amplifiable DNA, including primers; SL, sequence length; ND, no data available; NU, Northwestern University; YU, Yale University.

Lemuridae (19). The giant "sloth lemurs" (*Mesopropithecus*, *Babakotia*, *Archaeoindris*, and *Palaeopropithecus*) have also been allied to the indriids (14, 17, 30) (H4). Although there are numerous cranial characters to support this hypothesis, the sloth lemurs are otherwise highly derived, with detailed postcranial specializations for methodical suspensory locomotion, most reminiscent of those seen in xenarthran sloths (17). Finally, the genus *Megaladapis* also shows unusual postcranial specializations, in this case for vertical climbing and cautious above-branch quadrapedalism (31), with certain cranial characteristics that appear to ally it with the extant genus *Lepilemur* (H5). This latter hypothesis was long ago formalized by placing *Lepilemur* within the family Megaladapidae (32). Of the five hypotheses described above, H5 has been the one most rigorously addressed with aDNA methods, although the results have been contradictory. In one study, aDNA data provided support for the affinity of *Megaladapis* and *Lepilemur* (33), although another found no support for such a relationship (34, 35). In the study described here, we employ aDNA techniques and phylogenetic methods to examine the evolutionary relationships among living and extinct Malagasy primates, with an eye toward testing the morphological hypotheses outlined above. Our primary objective is to determine whether the extinct species belong to the same clade as the living species, thereby allowing an additional test of the hypothesis that non-human primates colonized Madagascar only once.

Materials and Methods

Data Collection. A total of 25 samples of subfossil lemurs belonging to the genera *Palaeopropithecus*, *Hadropithecus*, *Archaeolemur*, *Megaladipis*, and *Pachylemur* were subjected to aDNA extraction (Table 1). Upon successful amplification, radiocarbon ages were determined by accelerated mass spectrometry. Two subfossil specimens of the extant genus *Propithecus* were included as a positive control of our methods. The geographic collecting localities of all subfossils are shown in Fig. 2. Samples were analyzed at one or more laboratory sites, including Northwestern University Medical School (Chicago), Yale University, and the Armed Forces DNA Identification Laboratory (AFDIL) (Table 1). In all cases, DNA extractions were conducted in state-of-the-art clean-room facilities. The Yale clean room comprises three chambers: an antechamber for the donning of lab coat, gloves, goggles, boots, and other clean-room garb; an extraction room containing a fume hood, independent water supply, and other general lab equipment wherein DNA extractions are performed; and a room containing a laminar flow hood for PCR setup before the addition of template. All chambers are under positive air pressure and are routinely decontaminated by overhead UV irradiation. A handheld, high-speed rotary sanding and cutting tool (Dremel, Racine, WI) was used to clean the outer surface of the subfossil and to cut a small fragment for DNA extraction. This fragment (weighing between 0.5 and 1 g) was thoroughly washed in ultra-pure water and later soaked in 2% bleach for 5 min and washed again thoroughly in water. After drying, samples were powdered in a cryogenic impact grinder (CertiPrep 6750 Freezer/Mill, Spex, Metuchen, NJ) following the manufacturer's instructions. The powdered sample was placed inside a dispodialyzer (molecular weight cutoff of 10,000; Harvard Apparatus) with 0.5 M EDTA (pH 8) and was subjected to decalcification in 200 ml of 0.5 M EDTA for 3–4 days. The decalcified sample was digested in 4 ml of extraction buffer (10 mM Tris·HCl/100 mM NaCl/0.5% SDS) with 100 μl of 20 mg/ml proteinase K at 55°C for 12–24 h. After the decalcification step, the sample was extracted twice with phenol-chloroform and then concentrated in a Centricon-30 microconcentrator (Amicon). This DNA extraction protocol (35, 36) was

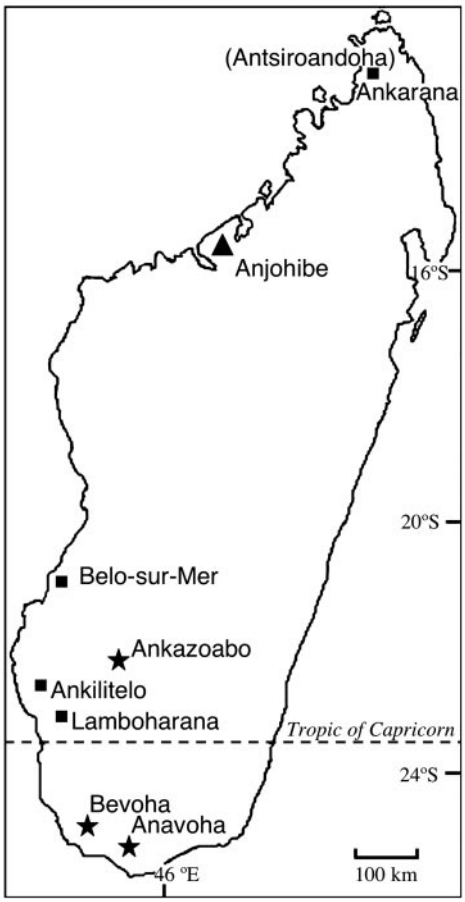

Fig. 2. Map of Madagascar showing the approximate location of subfossil sites sampled in this study. Stars indicate sites from which extinct taxa yielded amplifiable DNA. The triangle indicates the site from which subfossils of extant *Propithecus* were collected.

later modified for some samples, eliminating the use of dispodialyzers in the decalcification step.

To prevent crosscontamination, each sample was subjected to the above procedure independent of the other samples of the same species, with a gap of 1 week between extractions and with an intervening decontamination of the facility. A negative extraction control was always included with each extraction set. No DNA from any extant mammal, other than that from the subfossil *Propithecus* positive controls, has ever been extracted in the clean-room facility. To verify authenticity through interlaboratory reproduction of results, many samples were also extracted, amplified, and sequenced at the AFDIL. The AFDIL is a dedicated aDNA facility with strict separation of pre- and post-PCR laboratories and with high air-exchange entry vestibules and separate ventilation systems. Bone sanding, cleaning, and grinding were performed in specially designed vented hoods, and extraction and amplification setup were performed in separate laminar flow hoods. The AFDIL performs high-volume forensic mtDNA testing of degraded human skeletal remains and has rigorous standards of forensic quality control for aDNA typing (37). However, no non-human primate DNA had been handled in the AFDIL before this work.

Between six and seven primer pairs were used per species to assemble a contiguous sequence of up to 499 bp from the cytochrome *b* gene (Table 2). Primers were designed to target overlapping DNA fragments of 120–160 bp (Fig. 3). A typical PCR mix of 50 μl of volume, consisting of 1 unit of AmpliTaq Gold with 1× buffer (Applied Biosystems), 0.2 mM dNTPs (New England Biolabs), a 0.4 μM concentration of each primer, 2 μl of 4 μg/ml BSA (Roche), and water was prepared in the PCR setup room of the aDNA laboratory. Tubes were then transferred to the extraction room, where 2 μl of DNA extract or water (for negative controls) was added. After the addition of template, tubes were securely closed and transported to the main laboratory to be placed in the thermocycler. The cycling parameters were as follows: initial denaturation for 12 min at 94°C; 50 cycles of 94°C for 30 sec, 50°C for 20 sec, and 72°C for 30 sec; and a final extension at 72°C for 10 min. At least two PCRs and one extraction negative were included in every PCR. Often, the first PCR did not yield a visually detectable band on the agarose gel. In such cases, a second PCR was set up under the same conditions, with 2 μl of PCR product from the first PCR as the template. Each segment of the concatenated sequence (Fig. 3) was PCR-amplified at least twice independently. All PCR products generated at Yale were cloned by using a TOPO cloning kit (Invitrogen). For each cloned product, between three and five positive clones were picked, and the inserts were sequenced directly by using the manufacturer-supplied T3 and T7 primers. Cloned sequences were then compared with each other to detect evidence of sequence heterogeneity that might be expected with inadvertent coamplification of nuclear pseudogenes (i.e., nuclear mtDNA sequences) (38, 39).

Table 2. PCR and sequencing primers used

Primer name	Primer sequence
L14761	GAACACCAATGAMCAAYATYCGA
H14851	AKRGCTARGCAGGCYCCTAGTAGG
MegaL45	CAATTCATTTATCCATCTGC
MegaH14901	TGTRTCTGCYGTRTARTGTATTGC
L14839	TCTAATATCTCCTCATGATGAA
H14954	TCAGCCGTAGTTTACRTCWCGGCA
L14924	GCAATACACTAYACAGCAGAYACA
H15021b	CCGAYGTGRATRAATARGCATA
L15171	CATGAGGACAAATATCATTCTGAGG
MegL15136	AGTAATAGCCACAGCCTTTATAGG
H15261	AAGAATCGTGTYAGKGTRGCTT
MegL15003	TTATCCGCTATCTCCGCTCAAACG
MegH15114	CCTATAAAGGCTGTGGCTATTAC
MegL15083	TGGTTCACATACCTTATCAGAAACC
MegH15171	TAGATTTGTAATTACTGTGGCGCC
L15007	CGCTAYCTYCACGCCAAYGGAGCA
L15154	ATAGGRTATGTYCTYCCATGAGGA
H15113	TATCCTATRAATGCTGTKGCTATTAC
PalH14859	CAATTTGGAGAGCTAAACAAGCTCC
PalH15032	GTATATTCCACGACCTACGTGGACG
PalL15094	CATTATCAGAAACCTGAAATATTGG
PalH15191	AATGTATGGGATTGCTGAGAGTAGG

Sequence Analysis. Sequences of extinct lemurs were aligned by eye compared with a 550-bp cytochrome *b* alignment from extant lemurs. All sequences have been deposited with GenBank (see Table 3, which is published as supporting information on the PNAS web site, for GenBank accession numbers). Three different phylogenetic methods (maximum parsimony, maximum likelihood, and a Bayesian approach) were used to determine the evolutionary relationship among living and extinct Malagasy primates. First, an unweighted parsimony tree was derived through a heuristic search with 100 replicates of the random addition option in PAUP* (40). This tree was used to estimate the transition-transversion ratio, gamma shape parameter, and base frequencies under the maximum likelihood criterion. The selected model [Hasegawa, Kishino, Yano (HKY) 85 + I + Γ), along with the estimated parameters, was used to derive likelihood trees through a heuristic search with 100 replicates of the random addition option in PAUP*. A weighted

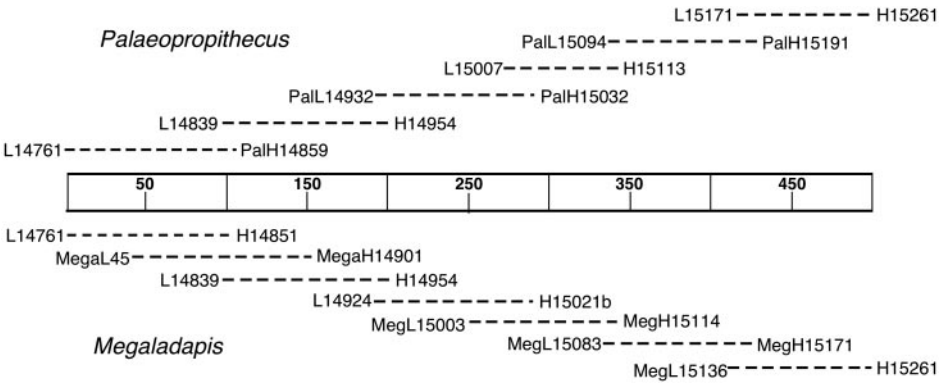

Fig. 3. The locations of various overlapping cytochrome *b* primers. Primer sequences are given in Table 2.

parsimony search in which transversions were given a 10× weight relative to transitions was performed with 100 replicates of the random addition option. Nonparametric bootstrap support was determined with 1,000 replicates of the bootstrap by using 10 replicates of the random addition option under the parsimony criterion and with 1,000 fast stepwise bootstrap replicates under the likelihood criterion. Bayesian phylogenetic analysis was conducted with MRBAYES 3.0 (41) by using the HKY 85 $\Gamma + \Gamma$ model with uniform priors. Three independent Bayesian analyses were initiated with random starting trees for 10×10^6 generations with four chains. Markov chains were sampled at intervals of 100 generations, thinning the data to 100,000 samples. All sample points before the stage when the Markov chain reached a stable likelihood value were discarded as burn-in. The remaining trees were imported into PAUP* to generate a majority-rule consensus tree and to derive posterior probabilities for each node.

The program MACCLADE (42) was used to infer the amino acid sequences and to build a minimal constraint tree consistent with the morphological hypotheses. A constrained search was conducted in PAUP* to generate the null tree. To determine whether the optimal tree is significantly better than the null tree, 100 replicates of a parametetric bootstrap test were performed. To this end, we conducted the Swofford–Olsen–Waddell–Hillis (SOWH) test under the likelihood criterion, exactly as described by Goldman *et al.* (43), by using the program SEQ-GEN (44) to generate the replicate datasets. The Shimodaira–Hasegawa test as implemented in PAUP* was carried out to determine whether the unconstrained topologies recovered under the various methods of analysis are significantly different from each other. The amino acid sequences were examined to test for the possibility that we were inadvertently amplifying nuclear mtDNA sequences, as might be revealed by the appearance of missense or nonsense mutations.

Results

Only 11 of 25 subfossil samples yielded amplifiable DNA (Table 2). Successful samples from the extinct taxa are dated to be between 1,148 and 2,000 years old. Dates were not obtained for the two *Propithecus* specimens. Notably, all of the subfossils of extinct lemurs that yielded amplifiable DNA were collected from sites from the southern part of Madagascar (Fig. 2). Seven of these eight subfossils are from the sites Bevoha and Anavoha, both of which lie outside the tropical zone. This result reconfirms the global trend of increased success rates of aDNA recovery as we move from the tropics (36, 45, 46) toward the poles. For all samples, the average amplicon length was between 120 and 160 bp, including the primers. Longer amplification products were impossible to achieve, as is typical of authentic aDNA (47). Consistency among multiple PCR replicates, sequence homogeneity among replicate clones, and lack of missense or nonsense mutations indicate that we successfully amplified the mtDNA cytochrome *b* gene. Sequence comparison and phylogenetic analysis of subfossil specimens of *Propithecus* indicate their authenticity, thus serving as a positive control of our methods. More importantly, the fact that DNA has been amplified and sequenced from multiple individuals of both *Palaeopropithecus* and *Megaladapis* and that many of these sequences have been confirmed in two independent laboratories provides assurance that we are analyzing the endogenous DNA of extinct lemurs.

Despite the relatively limited amount of sequence data analyzed, all phylogenetic methods found support for the monophyly of living and extinct lemurs with high bootstrap and posterior probability support (Fig. 4). All methods also retrieved the major clades among the lemurs, except for the weighted parsimony tree wherein the family Cheirogaleidae is not recovered as monophyletic. The evolutionary interrelationships among the major clades of lemurs are poorly resolved (48), although *Daubentonia* is consistently basal to all other lemur species (Fig. 4). Relevant to the subfossil taxa, all trees support the branching of *Palaeopropithecus* with the indriid clade, as suggested by the morphological data (14, 35). In contrast, the genera *Megaladapis* and *Lepilemur* were never recovered as sister taxa. Rather, all tree-building methods, except weighted parsimony, support the branching of *Megaladapis* with the family Lemuridae. The likelihood scores of the parsimony ($\ln L = -6501.215$), likelihood ($\ln L = -6497.232$), and Bayesian ($\ln L = -6499.139$) trees were significantly higher than the likelihood score of the null tree ($\ln L = -6506.624$), wherein *Megaladapis* and *Lepilemur* were constrained to be sister taxa ($P < 0.05$, parametric bootstrapping). Conversely, the various molecular trees were not significantly different from each other ($P > 0.05$, Shimodaira–Hasegawa test).

Discussion and Conclusions

Despite determined effort, we were only able to amplify and sequence endogenous DNA from two of the extinct subfossil taxa, *Megaladapis* and *Palaeopropithecus*. For those species for which we could not obtain DNA (see Table 3), the critical factor may have been geographic locality and/or preservational condition of the specimen. Numerous reports in the aDNA literature describe the potentially damaging effects of moisture, UV irradiation, and heat on DNA survival, with the combination of these agents being most severe in the tropics. As detailed in Fig. 2, all of the samples for which DNA retrieval was successful in this study were collected from subtropical localities. The only samples to yield DNA from tropical localities were the two individuals of the extant genus *Propithecus* that were used as positive controls. Thus, the results of our study contribute to the mounting evidence that suggests that prospects for aDNA studies from the tropics are less promising than those from higher latitudes (36, 45, 46).

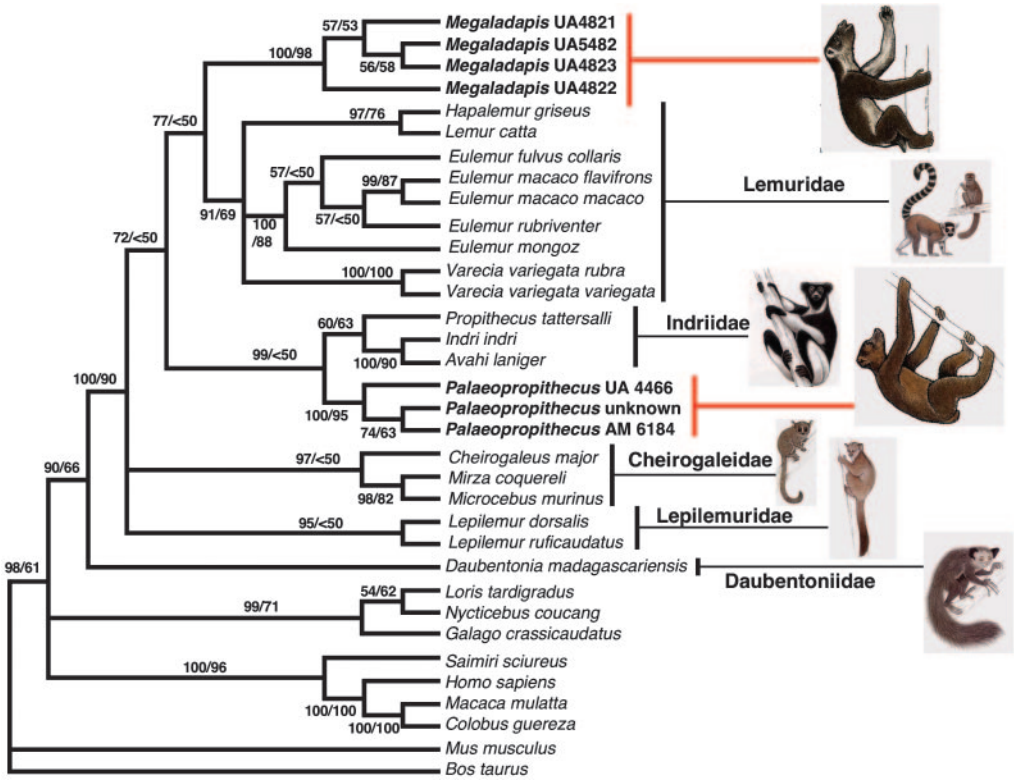

Fig. 4. Bayesian tree of 550-bp alignment of variable-length cytochrome *b* sequences from extinct and extant lemurs. Tree is a 50% majority-rule consensus of 99,500 trees (mean ln $L = -6499.641$). Multiple analyses converged on similar log-likelihood scores and reached stable likelihood value at no later than 50,000 generations. The initial 50,000 samples were discarded as burn-in, leaving a total of 99,500 sample points. The overall tree topology from Bayesian analysis is similar to those from likelihood and parsimony analyses (data not shown). The numbers indicate posterior probability scores and parsimony bootstrap supports. Illustrations are by Stephen D. Nash (copyright 1996, Stephen D. Nash/Conservation International).

Of the five phylogenetic hypotheses described in the introduction, H1 and H2 have been essentially confirmed by morphological data. The level of morphological detail supporting the sister-group relationships between *D. robusta* and *D. madagascariensis* (H1) and between *Pachylemur* and *Varecia* (H2) leaves virtually no doubt that the relationships are real (21, 27). H3, H4, and H5 are considerably less certain. Fortunately, the morphological similarities between *Archaeolemur* and *Hadropithecus* (H3), among the sloth lemurs (H4), and among the species contained within genus *Megaladapis* (H5) clearly indicate that each comprises a monophyletic group. This result then allows us to test the higher-level relationships of the subfossil clades to the living lemurs, even when only a single genus or species from each can be characterized, as has been the case with our study.

Unfortunately, we were not able to amplify DNA from any of the *Archaeolemur* or *Hadropithecus* samples available to us and, thus, are unable to shed additional light on H3. For H4 and H5, however, our study has yielded robust results. In all phylogenetic analyses, under any of the optimality criteria or weighting regimes tested, *Palaeopropithecus* groups with living species of the family Indriidae. This result, therefore, offers strong support for H4. In contrast, *Megaladapis* was never found to form a sister-group relationship with genus *Lepilemur*. Furthermore, all statistical comparisons of the null hypothesis (H5) with the optimal genetic trees indicate that they differ significantly, with the genetic hypothesis of nonmonophyly being strongly preferred. We take these results as evidence for rejecting H5 and, consequently, for removing genus *Lepilemur* from the family Megaladapidae. The most important conclusion to be drawn from our study, however, is that the phylogenetic placement of subfossil lemurs adds additional support to the hypothesis that non-human primates colonized Madagascar only once. We hasten to acknowledge that the limited taxonomic success of our study leaves open the possibility that data from additional taxa will overturn this increasingly robust hypothesis. Even so, the synthetic consideration of long-standing morphological analyses, combined with the genetic analyses presented herein, makes this possibility doubtful.

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