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Ancient DNA in subfossil lemurs: methodological challenges and their solutions

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Abstract

We present preliminary results from an ongoing study of ancient DNA in Madagascar's subfossil lemurs. These animals, though extinct, are the evolutionary contemporaries of the living lemurs. Any phylogenetic study that focuses only on the extant Malagasy primates is therefore incomplete. Here, we present a cytochrome *b* phylogeny that includes sequences for *Palaeopropithecus* and *Megaladapis*. We also discuss the various methodological challenges that we have faced in acquiring these sequences along with our solutions for overcoming them. In addition to serving as a report of primary data, we hope that this contribution will serve as a guide for other projects facing similar challenges. Our analyses suggest that *Palaeopropithecus* is sister to the living indrids, as predicted by morphological studies. Contrary to morphological data, however, *Megaladapis* appears to belong to an independent lemuriform lineage rather than form a clade with *Lepilemur*. Our results are subject to further testing as longer sequences and additional subfossil taxa are added to the study.

Introduction

Within the past 2,000 years, approximately 25 species of large-bodied vertebrates went to extinction on the island of Madagascar (Burney and MacPhee, 1988); the vast majority of these were primates. As many as 16 species of primates from at least 7 genera are known from subfossil remains, many of which have been discovered only recently (Godfrey et al., 1990; Jungers et al., 1991; Simons et al., 1992; Simons et al., 1995b). Although extinct, the fact that at least some species existed as recently as 500 years b.p. (Godfrey et al., 1997, chapter 8) indicates that in an evolutionary sense, subfossil lemurs should be considered contemporaries of the living lemurs. When both the extinct and extant forms are considered, lemuriforms span more than four orders of magnitude in body size, exhibit every possible activity cycle, display at least three different types of social system, consume almost every possible food type, and show a remarkable array of locomotor patterns (Richard and Dewar, 1991; Godfrey et al., 1997; Simons, 1997). Of course, these ecological and behavioral categories must be inferred from the skeletal and dental remains of the subfossils rather than observed directly. Even so, numerous functional-morphological studies have revealed that the subfossil taxa were even more remarkable in their diversity than the living lemurs. For instance, *Megaladapis* has been likened as an ecological analog more to the koala than to any other primate (Walker, 1974; Jungers, 1980). *Palaeopropithecus* has been likened to an arboreal sloth (MacPhee et al., 1984) whereas *Archaeoindris* has been described as a ground sloth analog (Jungers, 1980; Vuillaume-Randriamanantena, 1988). Early classifications based on craniodental evidence of *Archaeolemur* and *Hadropithecus* placed them as monkeys (Major, 1896; Standing, 1908) and subsequent postcranial studies have found them to be remarkably terrestrial for strepsirrhine primates (Walker, 1974), thus strengthening the monkey analogy.

Although morphological data have been employed to identify the phylogenetic relationships of the subfossil lemurs, these studies have been handicapped by the homoplasy

that is typical of endemic Malagasy primates (Eaglen, 1980). Even so, morphological data have provided a number of discrete phylogenetic hypotheses. Current understanding of subfossil lemur phylogeny holds that a number of the subfossil genera (*Palaeopropithecus*, *Mesopropithecus*, *Babakotia*, and *Archaeoindris*) comprise a monophyletic family Palaeopropithecidae that is in turn the sister group of the extant Indridae with the Archaeolemuridae as sister of the combined palaeopropithecid-indrid clade (Jungers et al., 1991; Simons et al., 1992). The genus *Megaladapis* is proposed to be the sister taxon to the extant genus *Lepilemur* (Schwartz and Tattersall, 1985), *Daubentonia robusta* is assumed to be sister to the smaller, living *D. madagascariensis* (Simons, 1994), and *Pachylemur* has been determined to be most-closely-related to the extant genus *Varecia* within the Lemuridae (Seligsohn and Szalay, 1974; Crovella et al., 1994). Figure 1 summarizes these hypotheses as a best estimate of subfossil, relative to extant, lemuriform relationships. Ideally, we would like to employ genetic data to test this morphology-based phylogeny.

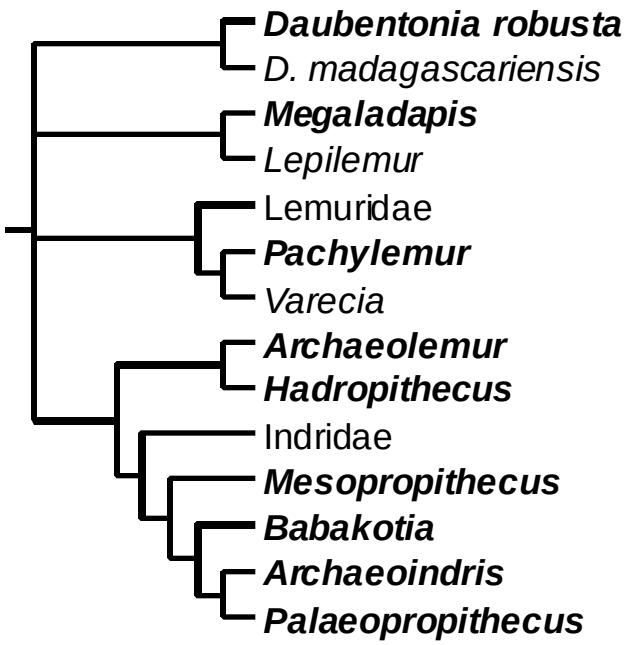

Figure 1: Summary estimate of subfossil lemur phylogenetic relationships based on published morphological analyses (subfossils are depicted in bold italics)

Recent molecular-phylogenetic investigation of the extant lemurs indicates that they are a monophyletic assemblage and are thus the product of a single colonization of Madagascar (Adkins and Honeycutt, 1994; Yoder, 1994; Porter et al., 1995; Yoder et al., 1996a; Porter et al., 1997; Yoder, 1997). These phylogenetic results are compatible with geological data that show

that Madagascar has existed as an isolated refugium for the duration of eutherian mammal evolutionary history (Krause et al., 1997). At present, Madagascar lies approximately 300 miles to the east of Africa at the narrowest point of the Mozambique Channel and is otherwise completely isolated from other significant landmasses. Moreover, there has been a deep oceanic rift separating Madagascar from Africa for at least the past 150 million years (Rabinowitz et al., 1983) for which changing sea levels would have had little effect. Thus, Madagascar's separation significantly predates the first appearance of any of the modern mammalian lineages in the fossil record. Phylogenetic analysis of the subfossil lemurs is therefore critical, not only for understanding the array of morphological and ecological adaptations that they display, but also for further testing the hypothesis that primates colonized Madagascar only once.

Within the past decade or so, the Polymerase Chain Reaction (PCR) technique has been employed to access DNA from extinct organisms, with variable success. PCR is a biochemical technique whereby free nucleotides, DNA primer sequences, and a polymerase (*Taq*) are employed to amplify DNA in vitro. A solution containing these ingredients, along with target DNA, is taken through a series of thermal cycles whereby copies of the target DNA are manufactured exponentially. Theoretically, one can begin with a single target DNA molecule and end with millions of copies that can then easily be sequenced with routine methods. Just as there have been spectacular successes using this technique (Krings et al., 1997), there also have been equally spectacular failures (Woodward et al., 1994). The failures relate directly to the universal challenges of ancient DNA (aDNA) studies: aDNA is always fragmented, usually in sizes from 100-500 bp (Hoss et al., 1996), and in low copy number. In both empirical efforts, and in tests of amino acid racimization (which occurs at rates nearly equivalent to DNA degradation), workers have found that age is not the fundamental determinant of DNA preservation (Beraud-Colomb et al., 1995; Bailey et al., 1996; Hoss et al., 1996; Poinar et al., 1996), thus suggesting that environmental factors play a key role. Of critical importance to DNA preservation is that the specimen be protected from the ravages of U.V. irradiation, hydrolysis, and oxidation

(Lindahl, 1993; Lindahl, 1995). Empirical results also indicate that cool environments are more likely to yield specimens for which DNA is amplifiable (Poinar et al., 1996; Yang et al., 1996) and that the degree of histological preservation can be an indicator of DNA preservation (e.g., Herrmann and Hummel, 1994). Nonetheless, even in ideal preservation conditions (such as cool, dry caves), there are theoretical limits to DNA preservation (Lindahl, 1993). Thus far, there have been no repeatable studies to demonstrate that aDNA older than 100,000 years can be recovered.

Fortunately for the subfossil lemur problem, most of the known specimens are less than 25,000 years old, with the majority being considerably younger (Simons et al., 1995a). Furthermore, a significant percentage of subfossil lemur specimens were (and continue to be) collected from cave sites in Madagascar which, in addition to being cool, are also dark and often dry, thereby affording at least some protection against hydrolysis and U.V. irradiation. Certainly, the majority of subfossil lemur teeth appear to be well-preserved at the macroscopic level, though detailed histological analysis has not been performed. Given the advantages of recent extinction, favorable environmental conditions, and good histological preservation, aDNA studies offer real hope for the reconstruction of subfossil lemur evolutionary history. Even so, the challenges are significant and cannot be underestimated.

Ancient DNA Challenges

The two greatest challenges for the recovery and characterization of DNA from extinct organisms is that aDNA is fragmented, whether from 4-, 100-, or 13,000-year-old samples, and it is typically in very low copy number (Paabo, 1993). Both qualities relate to the fact that DNA is chemically fragile. Once the mechanism of DNA repair in the living organism is turned off with death, DNA quickly degenerates and is modified by breakage due to loss of bases and/or is rendered inaccessible to enzymatic amplification due to crosslinkage. These characteristics conspire to introduce contamination as a continual nightmare for aDNA researchers (Cimino et

al., 1990; Handt et al., 1994; Hardy et al., 1994; Schmidt et al., 1995; Zischler et al., 1995; Austin et al., 1997). Although there have been published reports of aDNA of astonishing antiquity (Poinar et al., 1994; Woodward et al., 1994), all of the sequences reported from samples more than a million years old are thought to derive from contamination (Zischler et al., 1995; Austin et al., 1997; Gutierrez and Marin, 1998). In any PCR amplification, the DNA polymerase will always favor intact contaminating DNA to the desired aDNA. Contamination usually stems from two possible sources: 1) human genomic DNA from handling and preparation of ancient samples and 2) PCR amplicons from one's own lab. One particularly problematic and unexpected source of contamination has been human mitochondrial DNA (mtDNA)-like nuclear pseudogenes. A notorious example of the misleading effects of these contaminants concerns the report of DNA sequences from Cretaceous bone fragments (Woodward et al., 1994; Allard et al., 1995; Hedges and Schweitzer, 1995; Henikoff, 1995; Zischler et al., 1995). The most abundant and insidious source of contaminants comes from homologous PCR amplicons, however. One aerosol droplet of a robust PCR reaction has been estimated to contain as many as 10,000 copies of the target DNA sequence (Sykes, 1993) and thus PCR products can quickly cover lab surfaces, clothes, reagents, etc.

Damaged DNA can also result in amplification of artifact sequences. If aDNA is poorly represented in the PCR reaction, modern DNA of any abundance can compete for primer annealing, even if the PCR primers are well-designed and specific to the target DNA. Mis-priming of modern DNA, combined with weak priming of target DNA, can result in the generation of artifact sequences early in the reaction that can then be exponentially amplified in subsequent PCR cycles (Huang and Jeang, 1994). If such is the case, at best, one ends up with a heterogeneous population of amplicons --- some representing the desired target and some representing contaminants or artifacts.

The problems described above can be compounded or (even worse) amplification made impossible by the co-purification of PCR inhibitors. One of the many consequences of the organic decay process is that soil-derived degradation products (collectively known as humus) can become associated with the subfossil specimen. These products often act as strong inhibitors of *Taq* polymerase (Tuross, 1994). Another cause of inhibition results when cross-links between reducing sugars and amino groups occur as a consequence of the Maillard reaction (Poinar et al., 1998). Maillard products can usually be identified by a brownish tint in the DNA extract and by their blue fluorescence under UV light when the DNA extract is run through an agarose gel (Paabo, 1990). Although lack of PCR amplification can relate to numerous characteristics of aDNA, inhibition can be unambiguously identified. If adding an aliquot of aDNA extract to a positive control (i.e., "spiking") prevents amplification, then polymerase inhibition is immediately confirmed.

Finally, primer design is critical to the success of an aDNA project. Whereas modern DNA can be amplified in long sections, with primers conveniently designed to anneal to conserved regions of the genome, aDNA must be amplified in short segments due to its fragmentary nature. Not only does this substantially increase the cost per base pair sequenced, it introduces numerous challenges for primer design. Because one must design primers more frequently, perhaps every 50-100 bases versus every 500-1000 bases with modern DNA, one must always confront the possibility that the aDNA primers will either be too specific (if they are designed from the sequences of a single organism) or that they will be too general (amplifying numerous organisms, but poorly). Moreover, due to the demand to design many primers, and often in non-ideal regions of the genome, it is unavoidable that relative efficiency of individual primer pairs will vary. Whereas certain primer pairs will be capable of amplifying few (or even single) molecules, others will be far less reliable (Krings et al., 1997). The issue of primer design is a particular challenge for the subfossil lemur problem. There are very few primers that can be designed to recognize the full array of subfossil lemur diversity because the lemuriform radiation

is quite old, dating to at least the middle Eocene (Yoder et al., 1996a; Porter et al., 1997), and because many of the primary lemuriform lineages have been evolving independently of one another for most of this time period (Yoder, 1997).

Materials and Methods

As this project's ultimate goal, individuals for at least one representative, for at least one species, for each of the nine genera (*Archaeolemur*, *Archaeoindris*, *Babakotia*, *Daubentonia*, *Hadropithecus*, *Megaladapis*, *Mesopropithecus*, *Pachylemur*, and *Palaeopropithecus*) will be sampled to yield cytochrome *b* and cytochrome oxidase subunit II (COII) sequences. Subfossil teeth and bone fragments are being sampled, with teeth preferred for reasons described in the following section. Teeth are surface decontaminated with a 5% household bleach solution for 10 minutes. Bone fragments, when used, are UV irradiated for fear that bleach can penetrate the bone, thereby destroying endogenous DNA. After surface decontamination, specimens are reduced to powder in either a counter-top coffee grinder or in a Spex freezer mill.

DNA is extracted using a phenol/chloroform (PCI) protocol, modified from several published protocols (Hagelberg and Clegg, 1991; Richards et al., 1995; Bailey et al., 1996; Parr et al., 1996; Lalueza et al., 1997). Negative extraction controls (i.e., water is substituted for bone or tooth material) are always included with each set of extractions. After PCI extraction, DNA is collected via filtration with Centricon-50's (Amicon) rather than via ethanol precipitation. DNA is then amplified with PCR that employs *Taq* Gold (PE Applied Biosystems) in order to achieve a "hot start" (i.e., DNA is completely denatured before primer annealing and extension can occur) which improves specificity and sensitivity (Paabo, 1990). Sequencing of the PCR products is accomplished with cycle sequencing using a dye terminator sequencing kit (PE Applied Biosystems). Sequences are then edited and compiled with AutoAssembler 1.3.0 (PE Applied

Biosystems). A sequence is not accepted as accurate until both strands have been sequenced from at least two independent PCR reactions.

For this report, sequences were analyzed with maximum parsimony using the program PAUP* (Swofford, 1998). Heuristic searches were conducted with 100 replicates of the random addition option and all other options set by default. Relative support for internal nodes was estimated using the bootstrap (Felsenstein, 1985). For all bootstrap tests, 100 replicates were run with the random addition option selected from the heuristic search menu. For all analyses, a step matrix was imposed in which transversions received a weight of ten and transitions received a weight of one (10X weighting). Analyses of two different character sets were conducted: one in which all sites were included and one in which only third position sites were considered. For both types of analysis, three different samples of taxa were considered: one in which all taxa were included, one that excluded *Megaladapis* and one that excluded *Palaeopropithecus*. The rationale for character weighting and taxon sample is discussed in the Phylogenetic Results and Discussion section.

Ancient DNA Solutions

Like all ancient DNA studies, this study faces the challenges of maximizing DNA yield while also minimizing contamination and inhibition, of designing primers that are both specific to and universal for the organisms under investigation, and of designing primers that are efficient and can be placed at intervals such that target sequences are within the range expected for DNA that is severely fragmented. Fortunately, subfossil lemur specimens are large enough, abundant enough, and recent enough so that these challenges are not insurmountable. The methods by which we have overcome many of the obstacles are reviewed below with the hope that this contribution will serve as a guide for other projects facing similar challenges.

Table 1. Methods employed for verifying aDNA authenticity.

1) All preparation of subfossils performed in "clean" facility.
2) Contamination control measures (bleach; UV irradiation; protective clothing).
3) Analyze multiple individuals per taxon when possible.
4) Perform multiple DNA extractions per individual.
5) Perform multiple PCR amplifications per extraction.
6) Amplification and sequencing strategy that allows for overlapping sequences.
7) Obtain sequences for two genes (cytochrome *b* and COII).
8) Always include negative extraction and PCR controls.
9) Replicate results in two independent laboratories.

Minimizing Contamination

This study has taken extreme measures (summarized in Table 1) to control for contamination and to authenticate the sequenced DNA as intrinsic to the subfossil samples. Two modes of sequence authentication have been completed. First, different specimens of two taxa (*Palaeopropithecus* and *Megaladapis*) have been independently processed (decontaminated, extracted, amplified, and sequenced) in two labs (ADY's at NU and TJP's at AFDIL) and have yielded either identical or highly similar sequences. This strategy follows the idea that if specimens of the same species, processed in different labs, show closely-related sequences, it will strengthen the argument that they are of authentically-ancient origin (Handt et al., 1994). Second, some of the specimens have been divided and processed in parallel between the two labs. This strategy follows the idea that material taken from the same specimen and processed in two labs should yield identical sequence. In all cases, subfossil samples and extracts are never exposed to post-PCR facilities. For both labs, primers and all other reagents are shipped directly from the respective manufacturers. This circumvents the possibility that contamination can be transmitted from one lab to the other via reagents. Final verification of sequences is determined by phylogenetic analysis (DeSalle and Grimaldi, 1994; Handt et al., 1994; Richards et al., 1995; Young et al., 1995). Such analysis, for example, could have readily identified the Cretaceous bone-fragment sequences as spurious in that the reported sequences would have grouped closely with humans rather than with birds, reptiles, or non-human mammals.

Maximizing DNA yields

We are focusing on subfossil lemur teeth as the primary source of DNA for this study. Several workers have found that DNA is less fragmented and can be more readily amplified in teeth than in bone (Ginther et al., 1992; DeGusta et al., 1994; Zierdt et al., 1996), probably due to the higher concentration of hydroxyapatite which binds and thus preserves DNA (DeGusta et al., 1994). The fact that there are literally thousands of subfossil lemur teeth is therefore a distinct advantage of this project. mtDNA is the molecule of choice for this and other aDNA studies due to the fact that most cells possess multiple mitochondria but only a single nucleus. For every single-copy nuclear gene within a given cell, there will be approximately a 1000-fold excess of mitochondrial genes. Given the fact that only very small amounts of DNA can typically be recovered from ancient specimens, one has a far greater chance of recovering a mitochondrial gene than a nuclear gene via PCR. Two methods of DNA extraction are commonly employed in aDNA studies. The first is a modified silica protocol (Boom et al., 1990; Hoss and Paabo, 1993; Hoss, 1994). The silica method is considered desirable for two reasons: 1) silica beads, in the presence of salt, act as "DNA magnets" and for that reason are highly efficient in binding even minute quantities of DNA and 2) because the silica beads bind only DNA, all other potential co-purifiers are washed away, leaving behind only purified DNA. Also, it has been recently determined that guanidine thiocyanate (a component of the silica protocol) provides greater yields relative to number of years post-mortem than does EDTA (Tuross, 1995). The second method is a modified PCI protocol (Hagelberg and Clegg, 1991; Richards et al., 1995; Bailey et al., 1996; Parr et al., 1996).

Early efforts in this project focused on the silica method, with zero success. Although subsequent experience indicates that the lack of success might relate more to primer issues than to low DNA yield, we soon moved on to the PCI method. Originally, we employed a PCI

extraction method that did not include an EDTA decalcification stage. DNA yields were quite low, however (80 ul. of extract from 1-3 grams of tissue, of which 10 ul. were necessary for a 50 ul. reaction). In an effort to increase DNA yields, we modified the protocol to introduce an EDTA decalcification step as is commonly employed in other aDNA protocols. This we achieved with dialysis, rather than washing, as one of us (TJP) has found significant loss of DNA with the latter. We have also found that full agitation of the sample during tissue digestion and cell lysis is important in that it allows for nearly full digestion of tooth and/or bone powder, thus further increasing DNA yields. Finally, we employ Centricon 50's (Amicon) to concentrate the DNA rather than the more typically-employed Centricon 100's. This modification is due to the fact that the membrane pore size is smaller with the former and thus will retain shorter fragments of DNA than will the latter.

Minimizing PCR Inhibition

One immediate and unfortunate consequence of enhanced DNA yields is that the very same extraction modifications that are favorable to DNA retention are also favorable to the co-purification of PCR inhibitors . As we progressively enhanced DNA yields, we noted a trend for the resulting extractions to show the brownish tinge associated with the probable co-purification of Maillard products. Moreover, we observe a correspondence between the degree of brownish discoloration of the extract and the degree of blue "haze" under UV illumination at a position roughly corresponding to 500 bp reported by Paabo (1990) to be indicative of the co-purification of Maillard products. When these characteristics are observed, PCR amplification is always diminished or prevented entirely. Also, by using the positive-control spike test in these cases, we have confirmed PCR inhibition.

Several methods for overcoming inhibition have been employed by us with variable success: 1) simple dilution of the sample, in some cases up to 1:50, 2) Nested PCR, 3) the

addition of 12.5 units of *Taq* Gold ("Max Taq"), and 4) isopropanol precipitation (Hanni et al., 1995). Of the four methods, we prefer the dilution technique. Nested PCR can be problematic in that it requires a starting PCR product long enough to permit a second PCR with at least one internal primer --- something that is often not possible with highly fragmented DNA. "Max Taq" is not always effective but is always costly. And contrary to the Hanni et al. (1995) report, isopropanol precipitation did not eliminate inhibitors in the three samples attempted. In summary, inhibition has been problematic though not insurmountable and we will continue our efforts to enhance DNA yields and thus overcome inhibition through dilution.

Balancing Primer Sensitivity and Specificity

In the initial stages of this project, we attempted to amplify a 198 bp (plus primer sequences) target of the cytochrome *b* gene --- a target size that is well within the expected limits of aDNA fragment lengths. Aside from a single bout of contamination, however, we had no success in amplifying this fragment. It was not until we reduced our target length to 98 bp (plus primer sequences) that we were first able to amplify and sequence authentic subfossil lemur DNA. These and subsequent experiments have confirmed that typical subfossil lemur DNA is even more fragmented than it is for many other extinct organisms of Holocene age (Stone and Stoneking, 1993; Cooper, 1994; Hagelberg et al., 1994; Yang et al., 1996). Thus, a significant emphasis on primer design has been introduced to the subfossil lemur project.

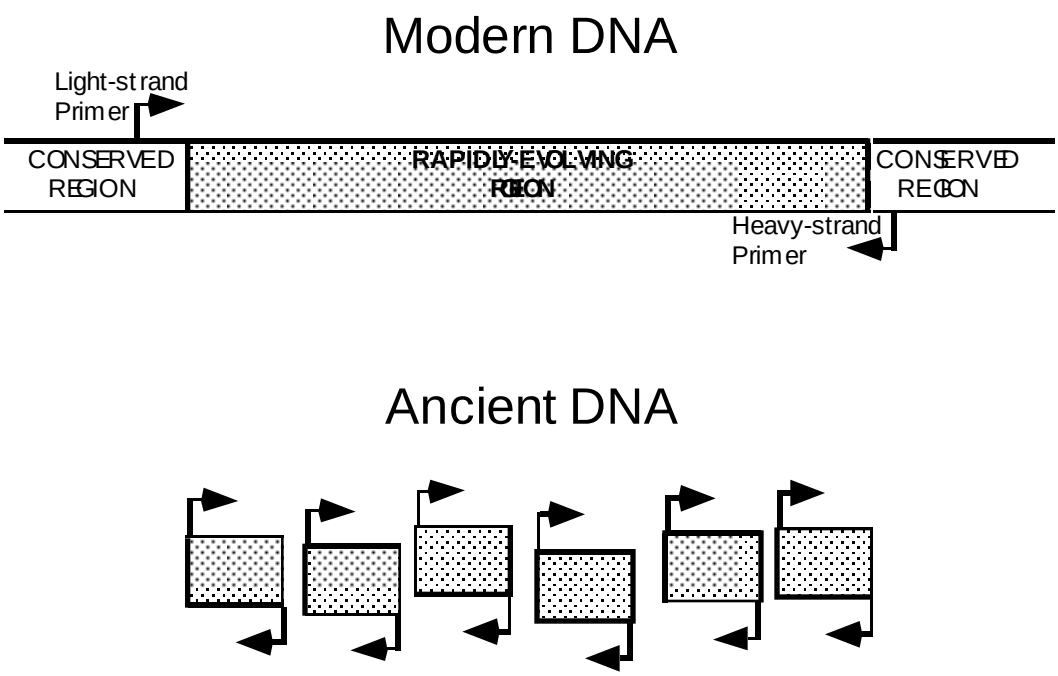

Figure 2: Comparison of PCR amplification and sequencing possibilities for modern DNA versus ancient DNA. Drawing on top represents long strand of modern DNA wherein only two primers are necessary to amplify and sequence entire region of interest (rapidly-evolving region). Drawing below indicates that because ancient DNA is so fragmented, twelve primers are required to amplify and sequence the equivalent target stretch of DNA sequence.

The combined challenges of working within a diverse evolutionary radiation and of targeting a rapidly-evolving region of the total genome constrain our ability to design efficient primers. For a study that focuses on extant organisms, these competing constraints can easily be overcome by designing primers such that they lie within conserved regions of the mitochondrion (e.g., the tRNA regions) but frame and amplify the more variable and informative regions (Figure 2). Because of the extreme degree of fragmentation in the subfossil lemurs, however, we do not have this luxury. Our primers must be designed such that the target sequences are no more than 150 base pairs (bp) in length. Moreover, the primers must be placed such that the amplicons, when sequenced and aligned, overlap to form a continuous stretch of at least 500 bp for each gene (as an example, see Figure 3). We have been forced to design primers in genomic regions that are too variable to allow for universal amplification of the lemuriform radiation. Thus, it is frequently the case that universality must be sacrificed for primer sensitivity.

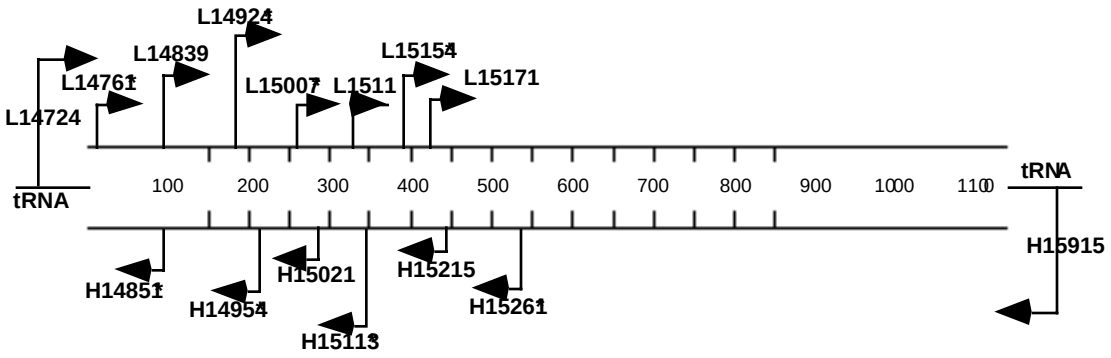

Figure 3: Example of PCR and sequencing strategy used in this study. Figure is map of cytochrome b gene with primer positions indicated by arrows. Primers are lettered to indicate light (L) and heavy (H) strands and numbered to match Anderson et al. (1981) sequence. In extant lemurs, entire cytochrome b gene can be amplified and sequenced with two primers (L14724 and H15915). In subfossil lemurs, up to thirteen primers are required to amplify and sequence only half of the gene (approximately 550 bp). Primers shown with asterisk (*) were designed to prevent amplification of human sequences.

Figure 4 illustrates this effect in extant lemuriforms for four different primer pairs designed to amplify portions of the cytochrome *b* gene. The figure demonstrates that whereas a primer pair combination might be quite sensitive in one taxon, it may be functional yet inefficient in another taxon, and completely non-functional in yet another. For example, primer pair L14839-H14954 (Fig. 4b) amplifies its target with great efficiency in *Propithecus* and *Cheirogaleus*, with only moderate efficiency in *Eulemur*, and with none in *Daubentonia* and *Lepilemur*. The results are even less encouraging for certain other primer pairs (e.g., Fig. 4c). Given that we don't know a priori within which, if any, of the living lemuriform lineages the extinct lineages belong, early efforts towards amplification must of necessity be experimental.

Phylogenetic Results and Discussion

Thus far, we have been successful in obtaining intrinsic DNA sequences for three subfossil genera: *Palaeopropithecus*, *Megaladapis*, and *Archaeolemur*. From *Palaeopropithecus* we have obtained cytochrome *b* sequence from two individuals, from *Megaladapis* three individuals, and from *Archaeolemur* one individual. We have also obtained COII sequence from one *Megaladapis* individual. In this report, we present the phylogenetic analysis of cytochrome

b sequences for one *Palaeopropithecus* (AM 6184; Ankazoabo) and one *Megaladapis* (UA 4822 - AM 6479; Bevoha) as these individuals have thus far yielded the most complete sequences.

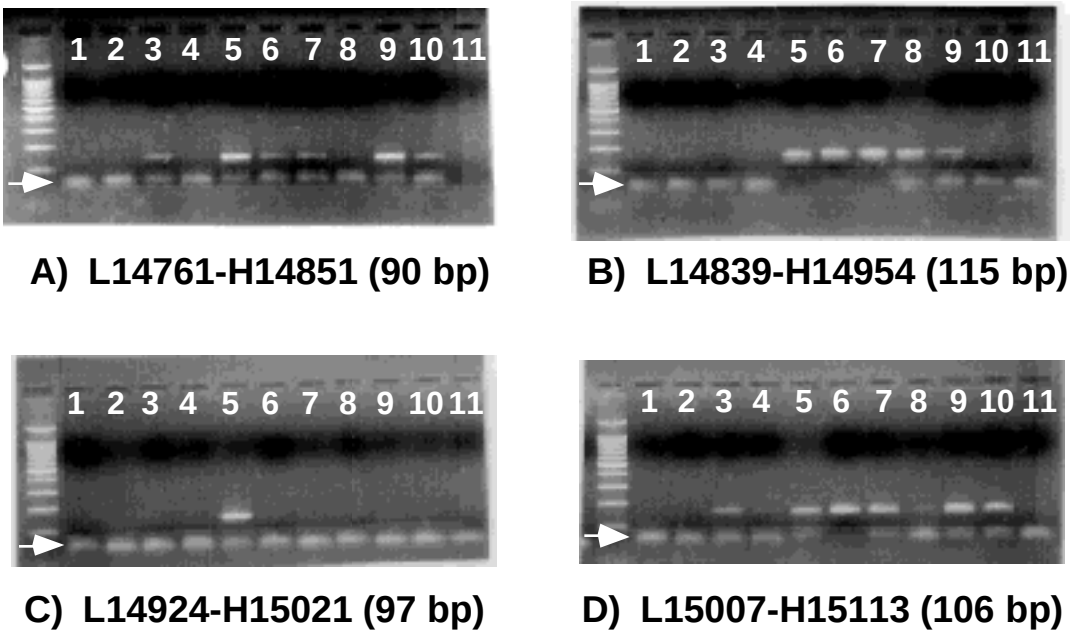

Figure 4: Comparison of agarose gels for which cytochrome b PCR amplicons have been run out for four different primer pairs. Primers are lettered and numbered as in Fig. 3. Length of target sequence is shown in parentheses. Lanes contain amplicons from the following starting templates: 1) Daubentonia (100 pg/ul), 2) Daubentonia (10 pg/ul), 3) Lepilemur (100 pg/ul), 4) Lepilemur (10 pg/ul), 5) Propithecus (100 pg/ul), 6) Propithecus (10 pg/ul), 7) Cheirogaleus (100 pg/ul), 8) Cheirogaleus (10 pg/ul), 9) Eulemur (100 pg/ul), 10) Eulemur (10 pg/ul), 11) PCR negative control. Figure compares performance of primer pairs in different lemuriform lineages as well as sensitivity of primers (i.e., all taxa are tested at two different DNA concentrations). White arrows indicate primer dimer.

The subfossil sequences were analyzed in a data matrix that contains numerous lemuriform taxa, representing all five lemuriform families, and three lorisiform outgroups. This matrix contains an alignment of the first 550 bp of the cytochrome *b* gene (Anderson #'s 14747 - 15296; Anderson et al., 1981). Of the two subfossils, *Palaeopropithecus* has the most complete sequence with 508 bp of the possible 550 sites. *Megaladapis* is less complete with only 350 bp. Regions of the gene that contain missing sequence for *Palaeopropithecus* do not overlap with regions where *Megaladapis* is missing sequence due to the fact that there was differential primer failure in the two taxa (much as is illustrated for extant taxa in Fig. 4).

All Sites (Transversions Weighted 10X)

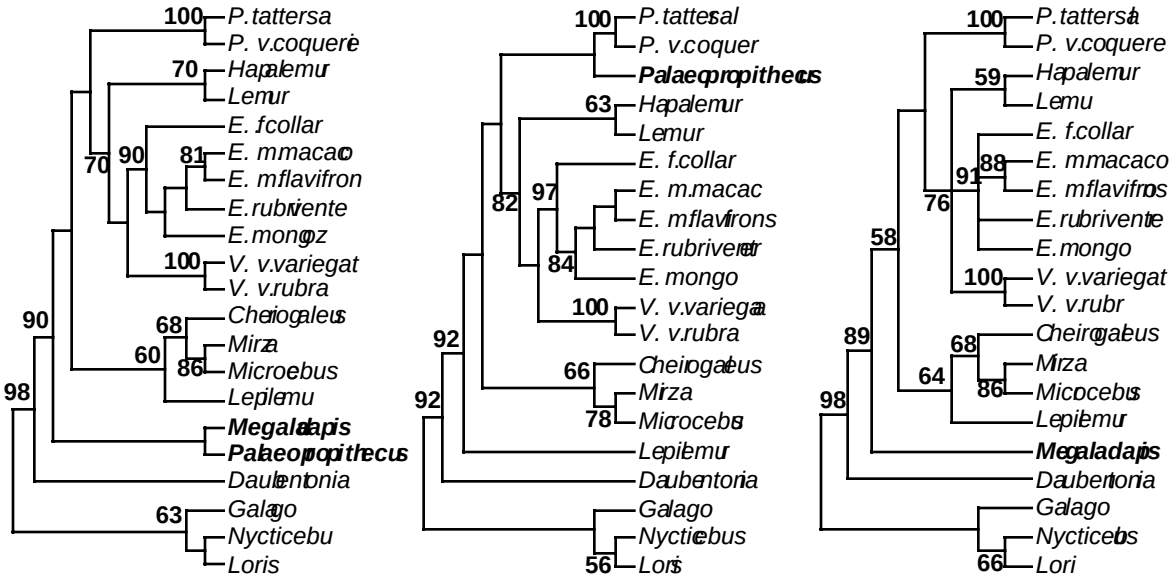

Figure 5: Parsimony analyses of 550 bp of the cytochrome b gene for two subfossil taxa, various extant lemuriforms, and three lorisiform outgroups. All sites were included in analysis and transversions were weighted ten times transitions. A) All study taxa included in analysis, B) Megaladapis excluded, and C) Palaeopropithecus excluded. Numbers on branches represent bootstrap support for node above that branch. Lack of a number indicates bootstrap support less than 50%. Subfossil taxa are in bold font.

Figure 5 illustrates the most parsimonious arrangement of the ingroup taxa wherein all sites are analyzed with a 10X weighting of transversions. This weighting scheme has been empirically determined to be appropriate and typically more informative for strepsirrhine cytochrome *b* sequences than is equal weighting, especially for small data sets (Yoder et al., 1996b). When all taxa are included in the analysis, a surprising arrangement of subfossil taxa is suggested; *Palaeopropithecus* and *Megaladapis* are shown to form a clade that is basal to all lemuriforms except *Daubentonia* (Fig. 5a). This result is surprising in that it has not been anticipated by any of the morphological data. One possible interpretation of this result is the unsettling possibility that the two taxa are joined because they are similar due to contamination. There is much evidence to prove that this is not the case. First, the bootstrap value in support of this clade is less than 50%, indicating that the grouping is very weak. Second, pairwise distance

analysis shows that the two sequences are 5.5% divergent which is well within the range for other intergeneric comparisons (e.g., *Propithecus* and *Varecia* are 4.6% divergent). If shared contamination was the explanation for the grouping, we would expect the sequences to be identical. Third, as discussed above, multiple individuals have been sequenced for both subfossil taxa, and in two different labs, with the result that intra-genus sequences are either identical or nearly so.

Another explanation for the putative *Palaeopropithecus*/*Megaladapis* clade is that it is real. Perhaps, contrary to all predictions based on morphological data, these two taxa are more closely related to each other than either is to other lemuriform taxa. We suggest that this is unlikely. Certainly, the morphological data in support of a close evolutionary relationship between *Palaeopropithecus* and living indrids is persuasive (Jungers et al., 1991; Simons et al., 1992) A more likely explanation for the association of the two subfossil taxa is that they, like extant lemuriform family lineages (Yoder, 1997), began independent evolution many millions of years ago. In other words, it is likely that they are long branches (sensu Felsenstein, 1978), and thus, long-branch attraction my be playing a role. This problem is probably exacerbated by the fact that the data set is small and that both taxa are missing a subset of the data. To tease apart the potential effects of long-branch attraction and sampling error, we conducted two similar analyses in which one or the other subfossil taxon was removed from the analysis (Figs. 5b and c). It is interesting to note that in the absence of the other subfossil, both genera fall in very different parts of the lemuriform radiation. As predicted by the morphological data, *Palaeopropithecus* is placed as the basal member of an indrid clade (Fig. 5b), but contrary to the morphological data, *Megaladapis* appears to be an independent lemuriform lineage (Fig. 5c) rather than sister to *Lepilemur*.

Another possible explanation for the seemingly spurious results in Figure 5a is that codon sites under selection could be biasing the results. Empirical exploration of protein-coding

mitochondrial genes in the Strepsirrhini suggests that this may be a factor (Yoder et al., 1996b; Stanger-Hall and Cunningham, 1998). To investigate this possibility, we omitted first and second position sites (wherein 96% and 100% respectively of nucleotide substitutions will yield amino acid replacements; Li and Graur, 1991) and analyzed third position sites only (wherein only about 30% of nucleotide substitutions yield an amino acid replacement). Again, transversions were weighted 10X and analyses were run for all taxa, all taxa minus *Megaladapis*, and all taxa minus *Palaeopropithecus*. Figure 6 illustrates the results. In this case, there is perfect agreement between the complete analysis (Fig. 6a) and both subset analyses (Figs. 6b and c) with regard to the placement of the subfossil taxa. In all three analyses, as with the taxon-subset analyses of the complete data set (Figs. 5b and c), *Palaeopropithecus* joins the indrid clade and *Megaladapis* is basal to other lemuriforms except *Daubentonia*.

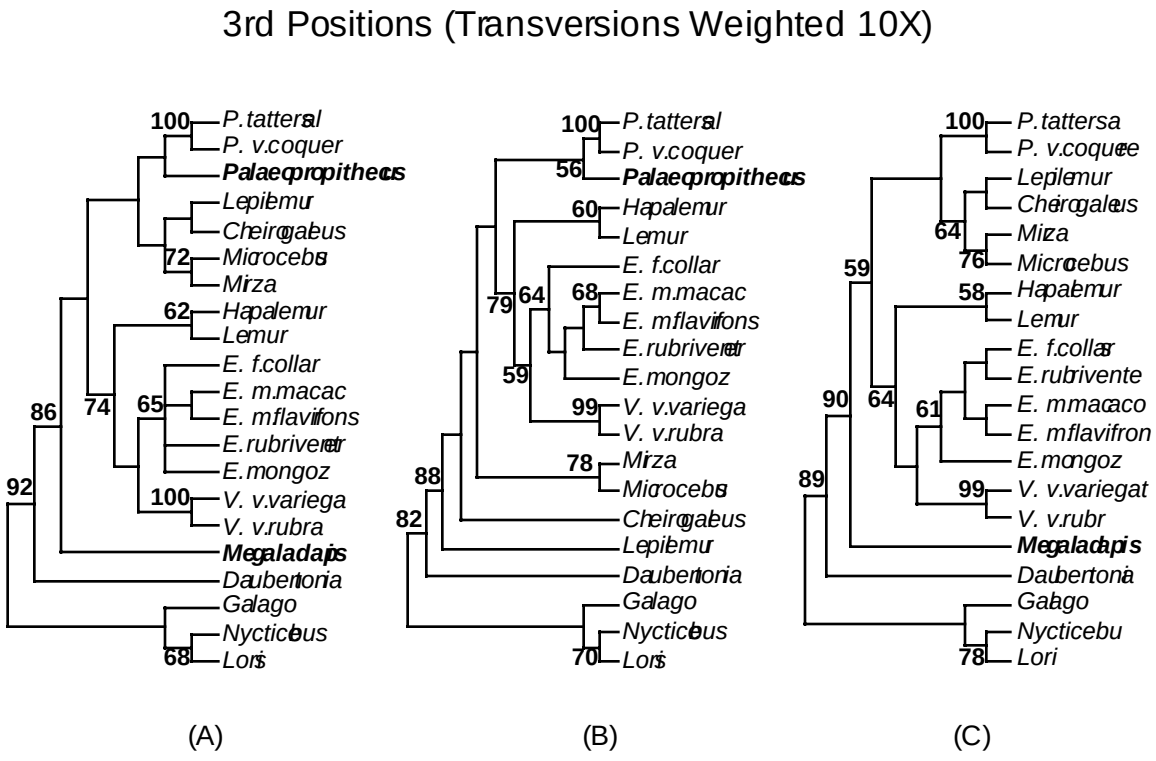

Figure 6: Parsimony analyses of same region of the cytochrome b gene analyzed in Fig. 5 except that first and second position sites are excluded. As in Fig. 5 analysis, transversions were weighted ten times transitions. A) All study taxa included in analysis, B) Megaladapis excluded, and C) Palaeopropithecus excluded. Numbers on branches represent bootstrap support for node above that branch. Lack of a number indicates bootstrap support less than 50%. Subfossil taxa are in bold font.

Conclusions

The most significant conclusion reached by this study is that intrinsic DNA, though highly fragmented, exists in sufficient quantities to be extracted, amplified and sequenced in many if not all subfossil lemur taxa. The phylogenetic analyses presented here should nonetheless be considered preliminary due to the small data set analyzed. In addition to the surprising placement of the two subfossil taxa in Figure 6a, it is notable that certain other lemuriform taxa (e.g., *Lepilemur*) tend to be variably placed in the analyses depending on taxon and character sample. In fact, with regard to the two subfossil taxa, it may be seen as significant that their relative placement can be said to be more stable than some of the extant taxa for this small data set. Thus, the conclusion that *Palaeopropithecus* joins the indrid clade and *Megaladapis* is another independent long branch within the lemuriform radiation, and not sister to *Lepilemur*, may ultimately be born out by future analyses. It is our intent that these future analyses will enjoy the benefits of longer DNA sequences and larger subfossil-taxon samples.

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