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Ancient single origin for Malagasy primates

(primate origins/cytochrome *b*/molecular evolution)

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ABSTRACT **We report new evidence that bears decisively on a long-standing controversy in primate systematics. DNA sequence data for the complete cytochrome *b* gene, combined with an expanded morphological data set, confirm the results of a previous study and again indicate that all extant Malagasy lemurs originated from a single common ancestor. These results, as well as those from other genetic studies, call for a revision of primate classifications in which the dwarf and mouse lemurs are placed within the Afro-Asian lorisiforms. The phylogenetic results, in agreement with paleocontinental data, indicate an African origin for the common ancestor of lemurs and lorises (the Strepsirrhini). The molecular data further suggest the surprising conclusion that lemurs began evolving independently by the early Eocene at the latest. This indicates that the Malagasy primate lineage is more ancient than generally thought and places the split between the two strepsirrhine lineages well before the appearance of known Eocene fossil primates. We conclude that primate origins were marked by rapid speciation and diversification sometime before the late Paleocene.**

Although strepsirrhine (we use the term strepsirrhine to define the living tooth-combed primates, their immediate ancestor, and all of its descendants) primates comprise more than one-third of the living members of the primate order, there is no current consensus concerning their phylogeny, classification, or time of divergence. Phylogenetic debate centers around two groups of Malagasy lemurs, the mouse and dwarf lemur group (family Cheirogaleidae) and the aye-aye (family Daubentoniidae). Morphologists inferred from the basicranial anatomy of the cheirogaleids that these animals are actually members of the Afro-Asian loris group (1, 2). This hypothesis was widely accepted and reflected in the majority of modern primate classifications (3–6). Cladistic studies of DNA sequences (7–9) have failed to support the lorisiform association, however, and have found instead that cheirogaleids belong within a Malagasy primate clade, thereby agreeing with an early synthetic view (10) and with genetic distance studies (11–13). The unusual morphological specializations of the aye-aye (e.g., ever-growing rodent-like incisors, clawed digits, and an extremely elongated middle finger) have made it difficult to place within strepsirrhine phylogeny also. One morphology-based hypothesis claims that the aye-aye comprises a monotypic sister group to all remaining strepsirrhines (14, 15); another holds that the phylogenetic position of the aye-aye is indeterminate relative to all other primates (16). DNA sequence studies have likewise given conflicting results. A study of mitochondrial DNA placed the aye-aye at the base of the strepsirrhine clade (7), whereas a study of nuclear DNA placed it securely with the other Malagasy primates (9).

The resolution of these controversies is important for our understanding of both primate phylogeny and historical biogeography. If Malagasy primates are not monophyletic, there must have been at least two primate colonizations of Madagascar. It has even been suggested that there were as many as three colonizations of ancestral Malagasy primates (5, 17). This seems improbable on geological grounds; Africa (the closest continental landmass) and Madagascar have been separated by a deep oceanic rift for at least 150 million years (18). Thus, it seems surprising that primates managed to cross this sea barrier even once, yet their presence on Madagascar indicates that they must have done so. The question remains, could this unlikely event have occurred more than once?

Another area of debate concerns strepsirrhine fossil affinities and the timing of strepsirrhine divergence. Numerous studies have recognized a special relationship between lemuriforms and the extinct Eocene adapiforms. Again, however, there is little agreement among the various studies. Analyses of the dentition have concluded either that the adapiforms consist of nested clades that originated within the lemuriform radiation (17) or that adapiforms as a whole represent the sister group to the lemuriforms (19, 20). Analyses of the wrist (21, 22) and ankle (21, 23) regions have concluded that lemuriforms evolved directly from adapiform ancestors. These hypotheses have different implications for the timing of lemuriform (and thus strepsirrhine) divergence. The Schwartz and Tattersall (17) hypothesis suggests that lemuriform evolution began by the late Paleocene, whereas the Beard and Godinot (22) hypothesis implies that independent evolution did not begin until the late Eocene—a difference of ≈20 million years.

Our study builds on a previous analysis of strepsirrhine phylogeny (24) by nearly doubling the DNA sequence data to comprise the entire cytochrome *b* gene. Also, we present results from a morphological analysis in which nonoverlapping characters from the two most comprehensive studies of strepsirrhine anatomy to date were combined (24, 25). We also employ distance methods in the analysis of the DNA sequence data to compare rates of molecular evolution and to estimate divergence times within the Strepsirrhini.

MATERIALS AND METHODS

The morphological data set contains cranial and postcranial characters from both the hard and soft anatomy, including those cranial characters which have been previously cited as evidence of cheirogaleid–lorisiform affinities. The entire 1140-bp mitochondrial cytochrome *b* gene was amplified via PCR and directly sequenced. In all cases, both strands were sequenced at least twice from different double-stranded PCR amplification products. Thus, we are confident that we have analyzed the homologous mitochondrial DNA gene sequences

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Abbreviations: mya, million years ago.

Data deposition: The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U53569–U53582).

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for all taxa, despite reports of mitochondrial-like nuclear pseudogenes (26, 27). The sequences were easily aligned by eye (due to lack of insertions or deletions) and analyzed along with homologous sequences for *Homo* (28) and numerous nonprimate outgroups (29–31). The morphological and molecular data sets were analyzed both separately and together with maximum parsimony using the branch-and-bound option of PAUP (32). It is well-established that transitions outnumber transversions during the evolution of mitochondrial DNA (33), although depending on the phylogenetic depth of comparison, the apparent ratio will vary due to saturation effects (34). Accordingly, the effect of differential weighting of transversions was explored. Molecular characters were weighted equally, transversions were weighted 10 times more than transitions, or transitions were weighted zero (i.e., transversion-only weighting). Two different weighting regimes were used in the combined analysis: one in which all characters were equally weighted and another in which the morphological characters were weighted 4 times more than the molecular characters to adjust for the 4-fold excess of phylogenetically informative molecular characters. The relative strengths of the phylogenetic hypotheses were tested by bootstrapping (35).

The molecular data were also analyzed with distance methods. Cytochrome *b* sequences were corrected for multiple substitutions with the Kimura two-parameter model (36), which allows for a frequency difference between transitions and transversions, and also with a maximum likelihood model (37), which allows for differential transition/transversion ratios as well as for different frequencies of the four nucleotides. Transition/transversion ratios of 5:1 and 10:1 were employed with both correction methods. Two data sets were examined: one which includes all characters (and thus all three codon positions) and one which includes only third codon positions. The Fitch–Margoliash tree-building algorithm (38), as implemented in the PHYLIP (39) program, was used to construct trees and estimate branch lengths. Distance analysis thus allowed a test of the maximum parsimony results, while branch lengths were compared to test for variation in rates of evolution among and between the strepsirrhine and anthropoid taxa.

RESULTS

The parsimony analyses unanimously demonstrate the monophyly of the Malagasy primates. Nonetheless, there are subtle topological discrepancies between the molecular and morphological trees (Fig. 1 *a* and *b*). In all molecular analyses, the cheirogaleids are placed securely within the Malagasy primate clade, internal to the aye-aye. It is noteworthy, however, that even though the bootstrap value supporting the cheirogaleids internal position is high (97–99%), the value supporting the entire clade, and thus the aye-aye's position, is low (<50–61%). In the morphological analyses, the bootstrap value supporting the monophyly of the entire clade is again low (57%), but in this case, the aye-aye is shown in an internal position with high bootstrap support (86%). In the combined, equal-weighting analysis (Fig. 1c), tree topology is almost identical to that from the molecular analysis, but the bootstrap value in support of Malagasy primate monophyly has risen from 61% to 70%. When the morphological characters are weighted 4 times more than the molecular characters, tree topology (data not shown) is similar to the morphological tree, except that lorisiforms are shown to be monophyletic and *Daubentonia* becomes the basal Malagasy primate lineage. Most notably, the bootstrap value supporting Malagasy primate monophyly jumps from 57% to 88%.

Regardless of the correction method, transition/transversion ratio, or codon set employed, distance trees also indicate Malagasy primate monophyly. In the case of the Kimura two-parameter correction of third position sites, however, internal branching order within the lemuriform clade is only loosely congruent with that obtained in the parsimony analysis. This failure probably relates to that correction method's inability to account for the extreme base-compositional bias that is typical of mammalian cytochrome *b* third position sites (31). With the maximum likelihood correction, the third-

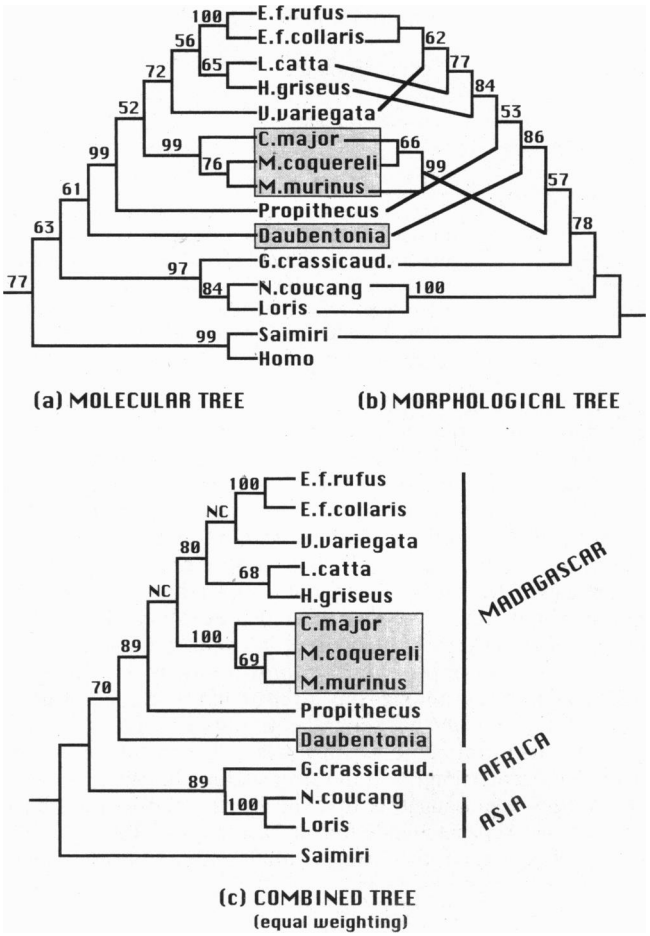

FIG. 1. Comparison of molecular, morphological, and combined hypotheses of strepsirrhine phylogeny. Numbers represent bootstrap values from 100 replications of the random-addition option (10 repeats per bootstrap replicate); NC (no confidence) indicates nodes with bootstrap values ≤50%. Dwarf lemur group and aye-aye are highlighted. Taxonomic descriptors are as follows: E.f.rufus, *Eulemur fulvus rufus* (the red-crowned lemur); E.f.collaris, *Eulemur fulvus collaris* (the collared lemur); L.catta, *Lemur catta* (the ring-tailed lemur); H.griseus, *Hapalemur griseus* (the gray bamboo lemur); V.variegata, *Varecia variegata* (the ruffed lemur); C.major, *Cheirogaleus major* (the greater fat-tailed dwarf lemur); M.coquereli, *Mirza coquereli* (Coquerel's dwarf lemur); M.murinus, *Microcebus murinus* (the mouse lemur); Propithecus, *Propithecus tattersalli* (the golden-crowned sifaka) in the molecular study and *Propithecus verreauxi* (Verreaux's sifaka) in the morphological analysis; Daubentonia, *Daubentonia madagascariensis* (the aye-aye); G.crassicaud., *Galago crassicaudatus* (the greater bush-baby); N.coucang, *Nycticebus coucang* (the slow loris); Loris, *Loris tardigradus* (the slender loris); Saimiri, *Saimiri sciureus* (the squirrel monkey); and Homo, *Homo sapiens sapiens* [human (28)]. (*a*) Molecular tree based on 457 phylogenetically informative characters of the 1140 bp of the primate mitochondrial cytochrome *b* gene. Bootstrap values are from analysis in which transversions were weighted 10 times more than transitions (consistency indices are not reported in PAUP for weighted analyses). The molecular tree was rooted with sequences for mouse (29), rat (30), camel, pig, spinner dolphin, and zebra (ref. 31; data not shown). (*b*) Morphological tree based on 125 morphological and behavioral characters (24, 25); consistency index = 0.592; retention index = 0.656. *Eulemur fulvus* clade lacks bootstrap value because characters examined were identical between subspecies. (*c*) Combined molecular and morphological analysis in which all characters were equally weighted; consistency index = 0.447; retention index = 0.418.

Table 1. Average cytochrome *b* genetic distances for strepsirrhine clades

	All positions		Third positions only	
	Kimura two-parameter	Maximum likelihood	Kimura two-parameter	Maximum likelihood
Primate node	0.168	0.169	1.87	1.70
Strepsirrhine node	0.163	0.165	1.65	1.67
	(97.0%)	(97.6%)	(88.2%)	(98.2%)
Lemuriform node	0.159	0.145	1.23	1.45
	(94.6%)	(85.8%)	(65.8%)	(85.3%)
Lorisiform node	0.135	0.136	1.36	1.48
	(80.4%)	(80.5%)	(72.7%)	(87.1%)
% SD	9.5	9.6	21.0	4.9

Inferred per site, per lineage nucleotide substitutions for 1140-bp primate cytochrome *b* genes, averaged through clades. Distances were calculated with PHYLIP (36) using Kimura two-parameter (31) and maximum likelihood (32) corrections incorporating a 10:1 transition/transversion ratio. Top row of table indicates the total, averaged distance from each strepsirrhine taxon to the ancestral primate node. Percentages (in parentheses) express branch lengths as proportions of the average total distance to primate node. Percent SD reflects variation in branch lengths within the Strepsirrhini; note high value for Kimura two-parameter correction of third position data.

position tree is almost perfectly congruent with the maximum parsimony tree, thus indicating that the third position data contain phylogenetic signal. Two additional results emerge from the distance analyses. First, although there is a significant rate differential between anthropoids and strepsirrhines in the analysis of all positions (with anthropoid cytochrome *b* sequences evolving up to 1.7 times faster than strepsirrhine sequences), the rates among the strepsirrhine lineages are nearly equal, thus demonstrating the existence of a "local clock" (40). The rate differential between anthropoids and strepsirrhines disappears in the third-positions-only analysis, and again, rates among the strepsirrhines are equivalent. Second, the branch that separates the Strepsirrhini from the primate ancestral node is only a small percentage of the averaged branch lengths of the strepsirrhine lineages. In other words, this result suggests that the living strepsirrhines have evolved as a separate clade for the vast majority (88.2–98.2%) of the time since the strepsirrhine and anthropoid stem lineages diverged (Table 1).

DISCUSSION

With the inclusion of our data from cytochrome *b*, it can be emphatically stated that genetic evidence supports the hypothesis that cheirogaleids fall within a Malagasy lemur clade (7–9, 11–13, 41, 42). Some of these same molecular (9, 11, 12) and karyological (41, 42) studies also support the placement of the aye-aye as the most basal taxon of a monophyletic Malagasy primate lineage. Only one molecular study (7) of the mitochondrial cytochrome oxidase subunit II gene contradicts our results by placing the aye-aye as the sister species to other strepsirrhines. The authors of that study recognize, however, that their results regarding the aye-aye are weak. Moving the aye-aye into the lemuriform clade adds only three steps to their most parsimonious cladogram of 568 steps. Given the strength of the combined morphological and DNA sequence results reported here, and their congruence with other molecular data sets, it is surprising that there are only a few candidate morphological synapomorphies of the Malagasy lemur clade and that most of these characters are not universally distributed. The paucity of morphological synapomorphies may in fact be symptomatic of the ancient, explosive radiation of Malagasy primates that is suggested by the molecular data. A possible exception, indicated by this study and by Martin (43), is the subtympanic extension of the middle ear cavity. Due to its distribution in Eocene primates, however, this character has been assumed to be primitive within the strepsirrhines (44).

The estimate of a relatively very short branch separating strepsirrhines from other primates is consistent with other studies in which branch lengths were compared (11, 12). Consequently, we would like to be able to calibrate the cytochrome *b* tree with a fossil divergence date to estimate the paleontological age at which the strepsirrhine clade and its subclades began independent evolution. Currently, however, there is no consensus of opinion on the timing of primate divergence. Proposed times for the origin of the primate clade range from 80 million years ago (mya; ref. 45) to 70 mya (46) to 63 mya (47), a discrepancy of 17 million years. Given this discrepancy and the limitations of the statistical methods for estimating lineage endpoints (48, 49), we take a conservative approach for calibrating the cytochrome *b* clock and use the most recent of these estimates to represent the time of strepsirrhine/anthropoid divergence. Using the branch lengths derived from the maximum likelihood correction of third position sites (which, of the two data sets, is most likely to reflect the neutral mutation rate), we estimate the divergence between lemuriforms and lorisiforms to have occurred 62 mya (Fig. 2); Malagasy primates reached Madagascar and began their radiation by 54 mya (the earliest Eocene). These dates are surprisingly early and have implications for traditional views of primate evolution. Accordingly, it is appropriate to evaluate these results in the light of paleontological evidence.

Virtually all living strepsirrhines possess a unique, complex organization of the anterior dentition that is commonly called the toothcomb. The criterion of parsimony thus indicates that the ancestral strepsirrhine also possessed this character. The toothcomb does not appear in the fossil record until the middle Miocene, however, by which time lorises and galagos had fully diversified. Given the proposed phylogeny and estimated divergence dates, toothcombed primates must have existed considerably earlier than the fossil record has thus far revealed. A question then arises: why, if tooth-combed primates originated by the early Eocene, have we not discovered evidence of this in the fossil record? One pertinent consideration is the geographic location wherein primitive strepsirrhines were likely to have evolved. Living primates are monophyletic, and it is unlikely that the clade as a whole originated on Madagascar. Thus, the split between strepsirrhines and haplorhines must have occurred elsewhere. In fact, there is ongoing debate as to whether primates arose in Africa (50, 51) or in Asia (52). Because there are two lorisiform lineages in Africa (bush babies and true lorises) and only one in Asia (true lorises), an African origin for that clade is most parsimonious. Also, the paleocontinental configurations of Africa, India, and Asia in the early Eocene (53–55) make it extremely improbable that a lemuriform progenitor could have survived an ocean voyage from Asia to Madagascar. The phylogenetic and paleocontinental evidence therefore indicate that the initial split

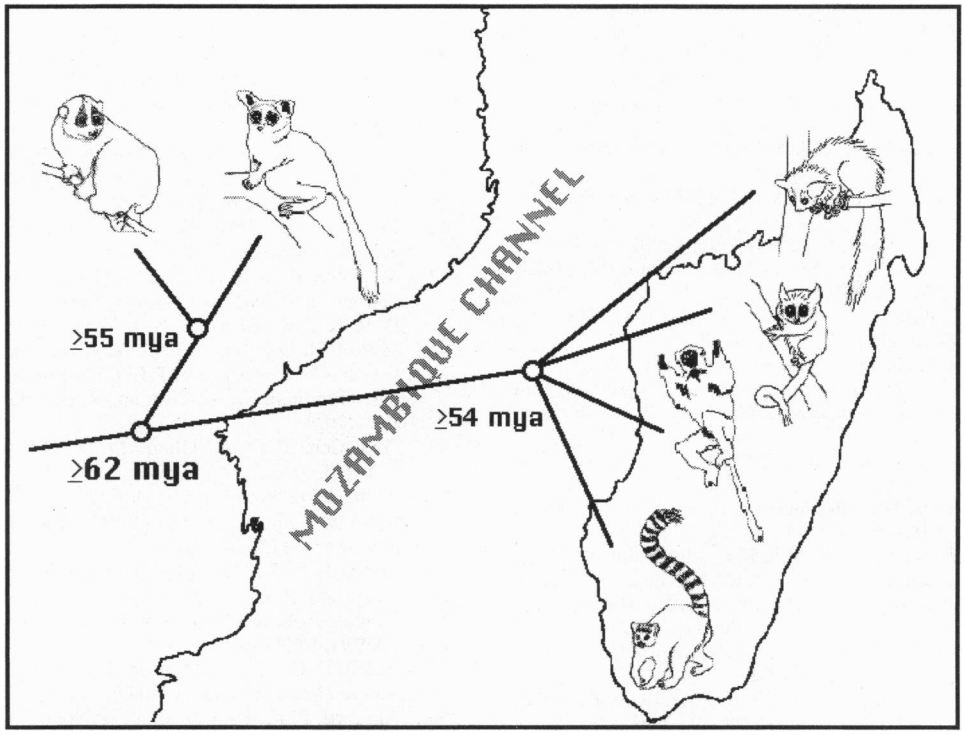

FIG. 2. Summary representation of the timing and pattern of strepsirrhine cladogenesis. Dates are based on a calibration point of 63 mya for the divergence of strepsirrhines and anthropoids, and maximum-likelihood corrected branch lengths of third position sites of the mitochondrial cytochrome *b* gene (Table 1).

between lorisiforms and lemuriforms would have occurred in Africa, followed by an eastward migration of lemurs to Madagascar. This hypothesis agrees with similar inferences based on recent fossil finds (51).

Positing an ancient African origin for the Strepsirrhini may thus offer partial explanation for the virtual absence of toothcombed primates in the fossil record. Only a few years ago, all of the oldest primates had been recovered from either North America or Europe, and none were older than the earliest Eocene. And, although a diverse primate fauna from the Oligocene of Northern Africa is known, it has yielded very few strepsirrhines (56). A number of remarkable discoveries in the past 5 years, however, have increased our appreciation of the diversity and antiquity of the primate radiation. Beard *et al.* (52) have revealed the presence of a rich and diverse primate fauna in China from the middle Eocene, thus expanding our notions of archaic primate geographic distribution. But the oldest known fossil primate was recently unearthed in northern Africa (57), and the Paleocene site from which this fossil was recovered shows evidence of a monodirectional northward migration of eutherian mammals out of Africa (58). Unfortunately, there is an otherwise complete dearth of African Paleocene primates and other eutherian mammals, thus diminishing the probability that basal strepsirrhines will be revealed.

As discussed above, most authorities have considered the extinct adapid and omomyid lineages to be either primitive sister groups of, or fundamentally ancestral to, the basal lineages of living primates. An ancient origin for the strepsirrhines requires, however, that lemurs and lorises diverged before what is known of the Eocene primate radiation, thus implying that the adapiform and omomyid lineages evolved in parallel to the radiation of the living lineages [an idea that has been previously suggested by Martin (45)]. This in turn indicates that early primate evolution was characterized by rapid divergence and diversification that nearly simultaneously (in geological terms) produced both the fossil and the living lineages, sometime before the late Paleocene.

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