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A Composite Molecular Phylogeny of Living Lemuroid Primates

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Key Words
Lemurs · mtDNA · Nuclear DNA · Radiation · Molecular phylogeny · Madagascar

Abstract
Lemuroid phylogeny is a source of lively debate among primatologists. Reconstructions based on morphological, physiological, behavioural and molecular data have yielded a diverse array of tree topologies with few nodes in common. In the last decade, molecular phylogenetic studies have grown in popularity, and a wide range of sequences has been brought to bear on the problem, but consensus has remained elusive. We present an analysis based on a composite molecular data set of approx. 6,400 bp assembled from the National Center for Biotechnology Information (NCBI) database, including both mitochondrial and nuclear genes, and diverse analytical methods. Our analysis consolidates some of the nodes that were insecure in previous reconstructions, but is still equivocal on the placement of some taxa. We conducted a similar analysis of a composite data set of approx. 3,600 bp to investigate the controversial relationships within the family Lemuridae. Here our analysis was more successful; only the position of *Eulemur coronatus* remained uncertain.

Introduction

The lemuroid radiation on Madagascar is the most diverse and extensive of all extant primate radiations. Up until 1,000 years (or possibly only a few hundred years [Godfrey and Jungers, 2002]) ago, it comprised more than 30% of living primate genera, assigned to 7 easily distinguishable families. Lemuroid body sizes span the entire range observed among extant primates, from the smallest (approx. 30 g) to the largest (approx. 197,500 g) [Godfrey et al., 1995; Rowe, 1996], and diets, locomotor specializations and social organization vary accordingly [Fleagle, 1999].

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Table 1. Number of nucleotide, protein and popset (population study data sets) records for each lemuroid taxon deposited in NCBI (updated March 2005)

Taxa	Nucleotide	Protein	Popset
Strepsirhini	6,030	2,239	89
Daubentoniidae	45	41	8
Cheirogaleidae	2,586	498	19
Allocebus	6	6	0
Cheirogaleus	81	72	6
Microcebus	2,478	400	12
Mirza	16	18	3
Phaner	5	2	0
Megaladapidae	509	226	5
Lepilemur	507	225	5
Indriidae	255	114	12
Avahi	36	11	2
Indri	5	4	1
Propithecus	214	99	11
Lemuridae	1,708	1,025	58
Eulemur	873	599	18
Hapalemur	195	172	10
Lemur	452	167	42
Varecia	188	87	19

Attempts to understand the history of this radiation have been severely hampered by the complete absence of any primate fossil record on Madagascar prior to 26,000 years ago [Simons et al., 1995]. Thus, the only route open to investigators is through reconstructions of phylogenetic relationships among the living and recently extinct taxa. Much endeavour has been directed towards the end of generating a reliable phylogeny for the Lemuroidea, drawing on data from morphology, physiology, behaviour and molecular genetics [see DelPero et al., 2001, for a review], but consensus remains elusive. Almost all possible relationships have been proposed, and most nodes have been contested.

During the last decade, nucleotide sequencing has become increasingly popular, and the amounts of genetic data stored in public databases have grown enormously [e.g. Adkins and Honeycutt, 1994; Yoder, 1994; Porter et al., 1995; Yoder, 1996; Yoder et al., 1996a, b; Porter et al., 1997; Yoder, 1997; Arnason et al., 1998; Goodman et al., 1998; Stanger-Hall and Cunningham, 1998; Yoder and Irwin, 1999; Wyner et al., 2000; DelPero et al., 2001; Pastorini et al., 2001, 2003; Yang and Yoder, 2003; Poux and Douzery, 2004; Roos et al., 2004; Yoder and Yang, 2004]. By August 2004, when we began this project, nearly 4,000 sequences for strepsirhine species had been deposited in the National Center for Biotechnology Information (NCBI) database, and in the course of the following year, the data set expanded by another 2,000 records (an updated number of records is listed in table 1). Some species (e.g. *Lemur catta, Microcebus murinus*) have been particularly well studied because of their use as outgroups or as examples of ancestral primates in studies of molecular evolution; others, by contrast, (e.g. indriids and megaladapids) have been relatively neglected.

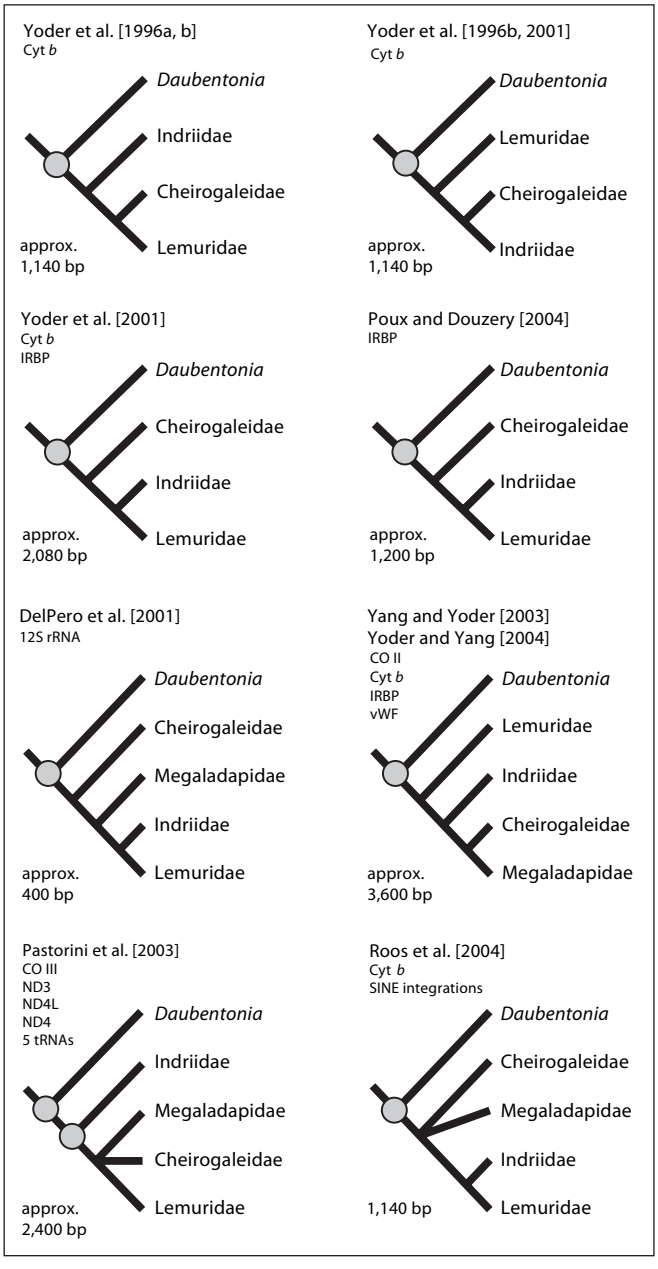

Fig. 1. Phylogenetic relationships among lemuroid families recovered from recent molecular studies. For each tree, the genes analysed, the total number of base pairs and the reference are shown. Grey circles at internal nodes indicate strong bootstrap support (>95).

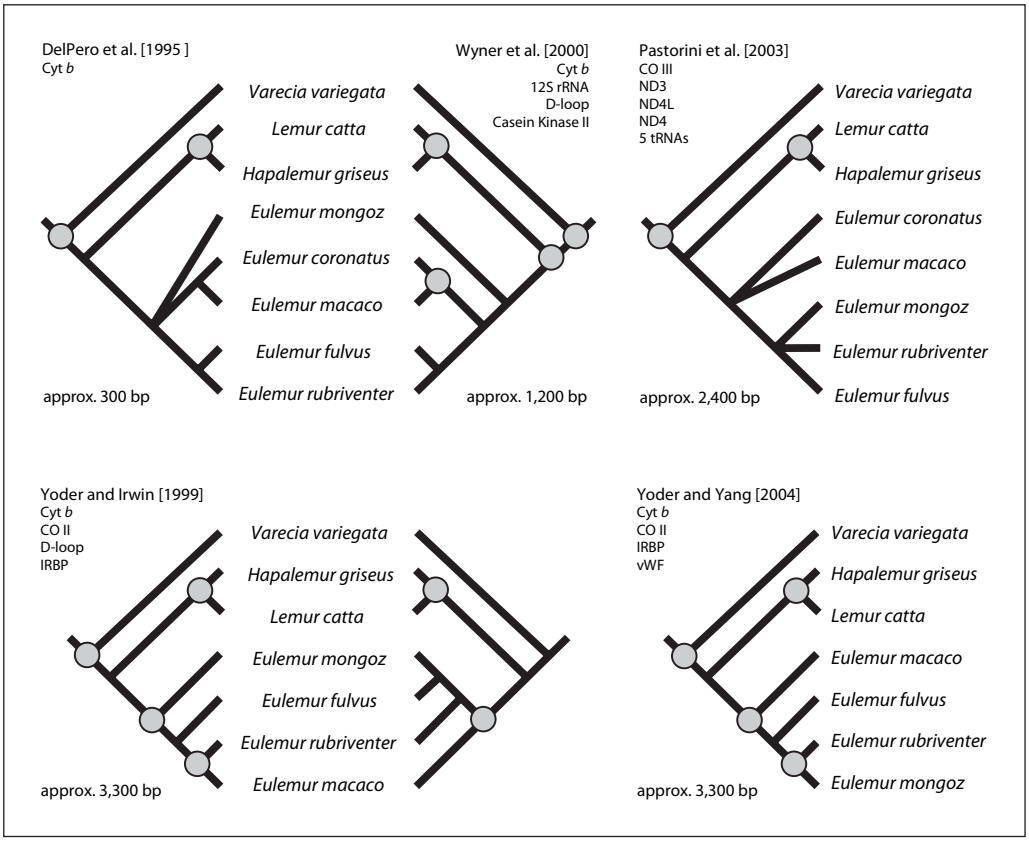

Fig. 2. Evolutionary trees for the family Lemuridae resulting from recent molecular analyses. For each tree, the genes analysed, the total number of base pairs and the reference are shown. Grey circles at internal nodes indicate substantial bootstrap support (>95).

Figure 1 summarizes the results of molecular investigations of lemuroid phylogeny that have been published to date. The studies have yielded inconsistent reconstructions of relationships among the 5 extant lemuroid families, and the internal nodes have generally been weakly supported. Similar problems have been encountered with investigations of relationships within the families, particularly for the Lemuridae. Figure 2 details the lemurid phylogenetic relationships hypothesised by various authors.

In this investigation, we mined the NCBI sequence database to compile the largest molecular data set currently available for the living Lemuroidea. The rationale behind combined analyses is that each gene has a particular window of phylogenetic applicability, and combination of different genes should allow for more accurate analysis at several phylogenetic levels concurrently [Kluge, 1989]. In choosing our sequences, we attempted to maximize both the numbers of genes and numbers of taxa, provided that at least 1 species for each family could be included. The final data

Table 2. Genes, sequence lengths and references for the lemuroid data set

Genes	bp	Reference
Mitochondrial sequences		
Pastorini's markers	2,389	Pastorini et al. [2003]
COIII (CDS partial)	53	
tRNA-Gly	71	
ND3 (complete CDS)	348	
tRNA-Arg	71	
ND4L (complete CDS)	290	
ND4 (complete CDS)	1,374	
tRNA-His	70	
tRNA-Ser	65	
tRNA-Leu	47	
Cytochrome *b* (complete CDS)	1,140	Yoder et al. [1996]
Cytochrome oxidase II (complete CDS)	684	Adkins and Honeycutt [1994] Yang and Yoder [2003]
12S rRNA (partial)	386	DelPero et al. [2001]
Nuclear sequences		
IRBP exon I (partial CDS)	938	Yoder and Yang [2004]
vWF (intron 11)	838	Yoder and Yang [2004]
Total bp analysed	6,375	

Alignments are available from the authors.

set included almost 6,400 bp, and 11 Malagasy lemuroid species. Additionally, we performed a similar analysis for the most highly contested lemuroid family, the Lemuridae (true lemurs). The lemurid data set comprised almost 3,600 bp, and 15 species and subspecies.

Materials and Methods

Samples, Sequences and Data Sets

Nucleotide sequences representing 1 Asian and 11 Malagasy strepsirhine taxa were selected. The data set included at least 1 representative from each of the 5 extant families: *Daubentonia madagascariensis,* 2 species from the family Cheirogaleidae *(Microcebus murinus* and *Mirza coquereli),* 1 representative of Megaladapidae *(Lepilemur ruficaudatus),* 1 member of the Indriidae *(Propithecus verreauxi)* and 5 species from the family Lemuridae *(Varecia variegata, Lemur catta, Hapalemur griseus, Eulemur fulvus, Eulemur mongoz and Eulemur macaco). Nycticebus coucang* was used as the out-group taxon for phylogenetic reconstructions.

The combined data set comprises both mitochondrial and nuclear sequences (table 2). The mitochondrial sequences include 5 complete protein coding genes, 5 tRNAs and a fragment of the 12S rRNA gene, while the nuclear counterpart is made up of the IRBP (exon I) and the von Willebrand factor (intron 11) genes. When more than 1 haplotype was found per taxon, we calculated the consensus sequence, which was then used in the analyses. In total, approx. 6,400 bp were analysed. To test for significant heterogeneity in phylogenetic signal among the sequences/

Table 3. Genes, sequence lengths and references for the lemurid data set

Genes	bp	Reference
Mitochondrial sequences		
Pastorini's markers	2,389	Pastorini et al. [2003]
COIII (CDS partial)	53	
tRNA-Gly	71	
ND3 (complete CDS)	348	
tRNA-Arg	71	
ND4L (complete CDS)	290	
ND4 (complete CDS)	1,374	
tRNA-His	70	
tRNA-Ser	65	
tRNA-Leu	47	
Cytochrome *b* (partial CDS)	180	DelPero et al. [1995] Montagnon [unpubl.]
D-loop	276	Wyner et al. [2000]
12S rRNA (partial)	231	Wyner et al. [2000] DelPero et al. [2001]
Nuclear sequences		
Casein kinase II (intron)	507	Wyner et al. [2000]
Total bp analysed	3,583	

Alignments are available from the authors.

genes investigated, we performed a partition homogeneity test [Farris et al., 1994] with 100 replicates on the combined data sets including all partitions, using PAUP* version 4.0b10 [Swofford, 2003].

On a finer taxonomic scale, we constructed a second data set comprising 15 taxa of the family Lemuridae, to investigate the highly controversial relationships within the genus *Eulemur*. The following taxa were included: the 2 subspecies of *Varecia variegata (Varecia variegata variegata* and *Varecia variegata rubra), Hapalemur simus, Lemur catta, Eulemur coronatus, Eulemur mongoz, Eulemur rubriventer,* 2 subspecies of *Eulemur macaco (Eulemur macaco macaco* and *Eulemur macaco flavifrons)* and 6 subspecies/species of the *Eulemur fulvus* group *(Eulemur fulvus fulvus, Eulemur fulvus rufus, Eulemur fulvus sanfordi, Eulemur fulvus albocollaris, Eulemur fulvus collaris* and *Eulemur fulvus albifrons).* The combined data set is reported in table 3, and contained 3,583 bp. We performed a similar analysis using the coding sequence of the entire cytochrome *b* gene, but had to exclude the taxa *Eulemur fulvus fulvus, Eulemur fulvus sanfordi* and *Eulemur fulvus albocollaris,* for which only incomplete sequences were available in the NCBI database. In both of these analyses of lemurid relationships, *Daubentonia madagascariensis* served as the out-group.

Phylogenetic Analyses

Phylogenetic reconstructions were conducted using several tree-building methods with different analytical approaches.

Parsimony analyses were carried out treating all characters as equally weighted, as well as by applying different weighting schemes to evaluate the effects of the transition/transversion

bias present in the data set, including a transversion-only (Tv-only) weighting scheme. An additional analysis was also performed for the lemuroid data set in which third codon positions were excluded altogether. Gaps were treated as missing data. All parsimony analyses were conducted using the exhaustive search strategy and branch supports were evaluated using 1,000 bootstrap replicates. A tree was constructed by stepwise clustering of the Tamura-Nei genetic distances [Tamura and Nei, 1993] using the neighbour-joining method that allows different evolutionary rates of change [Saitou and Nei, 1987]. The most appropriate model of nucleotide evolution for maximum likelihood and Bayesian analyses was estimated using the likelihood ratio test criterion as implemented in Modeltest 3.06 [Posada and Crandall, 1998] and MrModelTest 2.0 [Nylander, 2004]. Bayesian inferences were performed using MrBayes 3.01 [Huelsenbeck et al., 2001]. Bayesian analysis searches for the best set of trees that is consistent with a given model of sequence evolution and the data set under investigation. The consensus of this set of trees is used to estimate a posteriori probabilities for node support, which can be taken as an equivalent of bootstrap values. The Bayesian a posteriori probabilities represent the probability of the data, given the hypothesis, instead of representing the probability of the hypothesis, given the data, as in the case of maximum likelihood. Analyses were conducted using Metropolis coupling with four incrementally heated Markov chains (MC3, default heating parameter). Chains were run for 1×10^6 generations, and sampled every 100.

Maximum likelihood and maximum parsimony analyses were conducted using PAUP* version 4.0b10 [Swofford, 2003]. Neighbour-joining trees were obtained using MEGA 3.1 [Kumar et al., 2004]. Alternative topologies were compared using the Shimodaira-Hasegawa (SH) test statistic [Goldman et al., 2000; Shimodaira and Hasegawa, 1999], as implemented in PAUP*, with a fully optimised resampling estimated log-likelihood (option Fullopt) and 1,000 bootstrap replicates.

Results

Lemuroid Data Set

The partition homogeneity test justified combining the data from different genes by revealing no significant conflict between sequences. Of 6,375 bp, 3,700 characters were constant, 975 variable characters were parsimony uninformative, and 1,700 were parsimony informative. The best fitting model proved to be the general time-reversible model with estimated base frequencies (A = 0.29200, C = 0.28590, G = 0.18240, T = 0.23970), proportion of invariable sites (I = 0.4322), and among-site rate heterogeneity following a gamma distribution (shape parameter $\alpha = 0.9554$).

All of the analyses yielded one of two remarkably similar topologies that were strongly supported at most of the internal nodes (fig. 3). Each parsimony analysis yielded a single most parsimonious tree, but different weighting schemes gave different topologies.

In both topologies, *Daubentonia* emerged as the most basal divergence among the in-group taxa. The two topologies differed, however, by two arrangements in the pattern of branching among the remaining families. The first of these differences concerns the position of the indriids (here represented by *Propitheus verreauxi*). Maximum likelihood, Bayesian inference and the Tv-only parsimony strongly supported indriids as the most divergent lineage after *Daubentonia,* while neighbour-joining and the other parsimony analyses recovered a sister group relationship between indriids and lemurids. The bootstrap values for this indriid-lemurid sister group, however, were lower than the support obtained for the early divergence of Indriidae found in the other tree. The second discrepancy concerns the branching pattern of megaladapids and cheirogaleids. In the maximum likelihood, Bayesian infer-

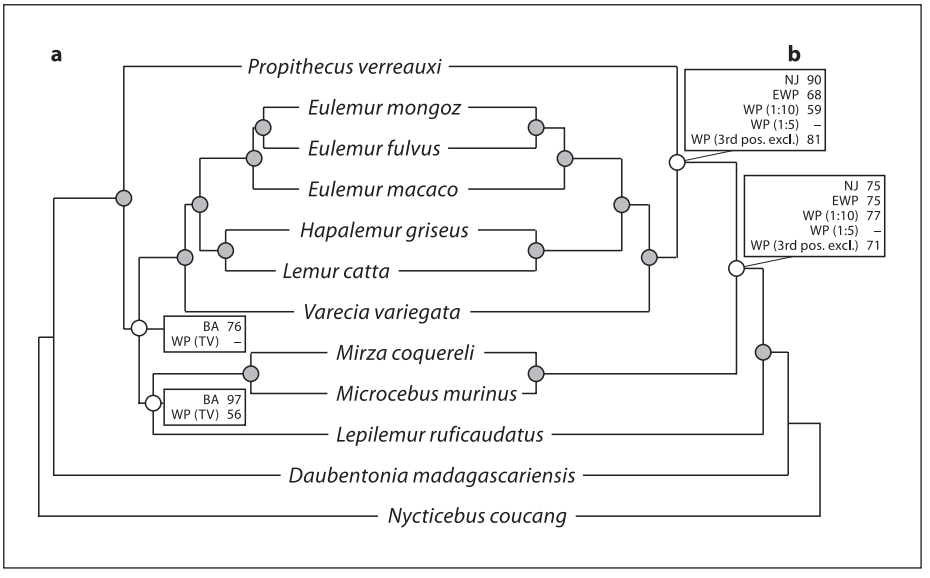

Fig. 3. Phylogenetic reconstructions obtained from the analyses of the lemuroid data set. **a** Topology resulting from maximum likelihood (–ln L = 33,624.775), Bayesian analysis (BA) and Tv-only parsimony [WP (Tv); tree length = 1,818; CI = 0.67; RI = 0.55]. **b** Tree deriving from neighbour-joining (NJ) analysis, equally weighted parsimony (EWP; tree length = 6,219; CI = 0.58; RI = 0.42), 1:5 weighted parsimony [WP (1:5); tree length = 13,502; CI = 0.63; RI = 0.49], 1:10 weighted parsimony [WP (1:10); tree length = 22,602; CI = 0.65; RI = 0.52] and most parsimonious tree excluding 3rd codon positions [WP (3rd pos. excl.); tree length = 2,572; CI = 0.65; RI = 0.49]. Grey circles at internal nodes indicate bootstrap support and Bayesian values >95; for the white circles, the support for the node from each analysis is indicated if >50.

ence and Tv-only parsimony trees, Megaladapidae and Cheirogaleidae formed a clade (strongly supported by Bayesian analysis) that is sister to the Lemuridae, while neighbour-joining and the remaining parsimony analyses showed an early divergence of megaladapids that was strongly supported, followed by the less strongly supported divergence of the cheirogaleids.

Comparison of the two alternative topologies using the SH test revealed no significant differences between the trees [tree 3A: –ln L (likelihood) = 33,624.775; tree 3B: –ln L = 33,630.938; difference in tree likelihood –ln L = 6.162, p = 0.18].

Lemurid Data Set

Once again, two topologies were found using the different methods of reconstruction, and these shared most of their nodes with very high statistical support (fig. 4). *Varecia* was consistently the first lemurid lineage to emerge, followed by the *Lemur catta*/*Hapalemur* clade. The only topological difference between the two trees is in the position of *Eulemur coronatus,* which was placed either as the sister taxon to *Eulemur macaco* (using maximum likelihood, Bayesian inference, neighbour-joining, and differentially weighted parsimony) or as the sister species to the clade

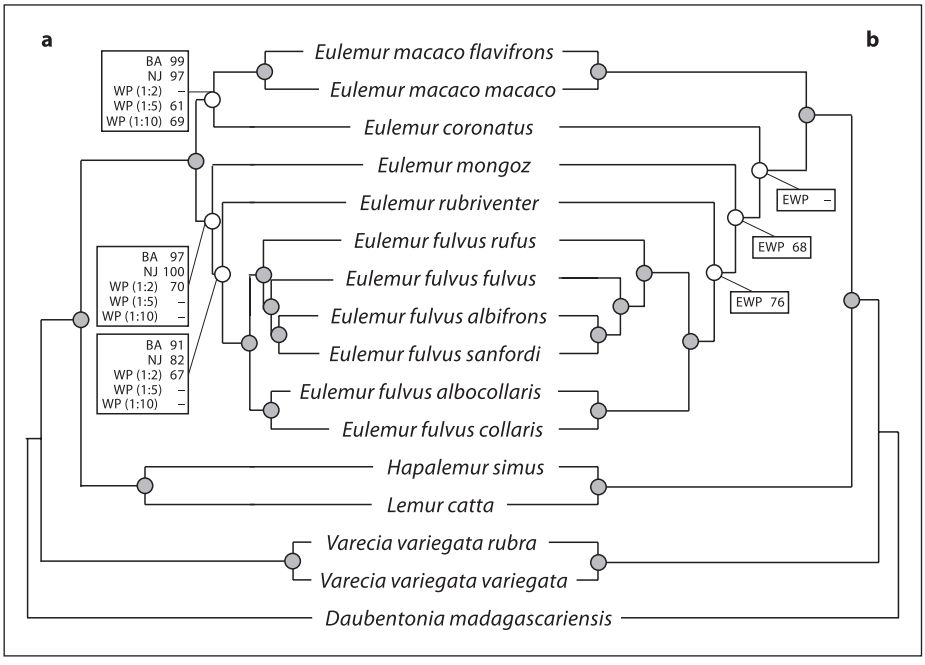

Fig. 4. Phylogenetic reconstructions obtained from the analyses of the lemurid data set. **a** Topology resulting from maximum likelihood (–ln L = 14,947.421), Bayesian analysis (BA), neighbour-joining analysis (NJ), 1:2 weighted parsimony [WP (1:2); tree length = 2,910; CI = 0.70; RI = 0.65], 1:5 weighted parsimony [WP (1:5); tree length = 4,530; CI = 0.76; RI = 0.70] and 1:10 weighted parsimony [WP (1:10); tree length = 7,216; CI = 0.80; RI = 0.74]. **b** Tree deriving from equally weighted parsimony (EWP; tree length = 2,369; CI = 0.65; RI = 0.63). Grey circles at internal nodes indicate bootstrap support and Bayesian values >95; for the white circles, the support for the node from each analysis is indicated if > 50.

comprising *Eulemur mongoz, Eulemur rubriventer* and *Eulemur fulvus* (using equally weighted parsimony). However, it should be stressed that the latter hypothesis does not have significant bootstrap support. The rest of the branching pattern is invariable and strongly supported in most of the analyses. As in the case of the lemuroid analysis, the p value associated with the SH test was not significant (p = 0.13), indicating that the tree in figure 4b (–ln L = 14,953.069) was not significantly worse than the ML/BA tree (–ln L = 14,947.421).

Analysis of the taxon-reduced data set, which included the complete cytochrome *b* coding sequence, but excluded 3 subspecies of *Eulemur fulvus (Eulemur fulvus fulvus, Eulemur fulvus sanfordi* and *Eulemur fulvus albocollaris),* yielded similar but not identical results. Maximum likelihood, Bayesian inference and neighbour-joining analyses once again yielded the tree depicted in figure 4a, while differentially weighted and equally weighted parsimony analyses all produced the topology shown in figure 4b. In both cases, this smaller data set resulted in slightly lower bootstrap values at the critical nodes.

Discussion

To our knowledge, the lemuroid data set analysed here is the largest in terms of numbers of characters analysed, for which at least 1 lemuroid species per family has been included. Many previous attempts to reconstruct lemuroid phylogeny have ended with a lament concerning the considerable amounts of homoplasy present in all data sets. Despite this ubiquitous homoplasy, our combined data set seems to have consolidated many of the nodes that were insecure in previous analyses.

Daubentonia was always the basal in-group taxon to diverge. This is a result that has been obtained by several previous studies [Yoder, 1994; Porter et al., 1995; Yoder et al., 1996a, b; Porter et al., 1997; Yoder, 1997; Goodman et al., 1998; Stanger-Hall and Cunningham, 1998; DelPero et al., 2001; Pastorini et al., 2003; Yang and Yoder, 2003; Roos et al., 2004; Yoder and Yang, 2004]. In one of our two topologies (fig. 3a), Indriidae was the second lineage to emerge after *Daubentonia*. Schwartz and Tattersall [1985] identified structural similarities between the teeth and skulls of daubentoniids and indriids that led them to hypothesise a sister taxon relationship between the groups. A phylogeny based on figure 3a would imply that such shared characters may be plesiomorphic for the lemuroids. Alternatively, the phylogeny depicted in figure 3b, which is also indicated by one SINE integration [Roos et al., 2004], implies that such similarities are homoplastic.

The position of Megaladapidae is also equivocal in these reconstructions. Different analytical methods placed it in different relationships with the remaining lemuroid families (fig. 3a, b), and both topologies showed strong support for its respective positions (either as the sister taxon to the cheirogaleids, or as the second lineage to emerge after *Daubentonia*). Megaladapidae is the most neglected family from the point of view of molecular analyses, and yet its position is crucial. Inclusion or omission of this family is the key that defines the range of possible topologies for lemuroid phylogeny (fig. 1). Finally, our results indicate that the cheirogaleids are either the third lineage to diverge in the lemuroid radiation, or they are the sister taxon to the megaladapids. There is no evidence to support a position as the sister taxon to Lemuridae [Rumpler and Dutrillaux, 1990; Yoder, 1996a, b] (fig. 1).

Our phylogenetic reconstruction provides some insight into the nature of the ancestral lemuroid. The analyses presented here indicate that cheirogaleids do not make a suitable model for the ancestral lemuroid, because in both trees they appear nested within the lemuroid radiation. It is thus likely that this family consists of taxa with derived features. This scenario was also indicated by some previous analyses [Yoder et al., 1996a, b; Pastorini et al., 2003; Yang and Yoder, 2003; Yoder and Yang, 2004] but is opposed to others that have been proposed [Purvis, 1995].

The composite molecular data set also prompts some questions concerning the tempo and mode of the lemuroid radiation on Madagascar. All molecular studies of lemuroids to date report uncertainties at deeper levels of the phylogeny. This kind of problem almost invariably means that there are short internodes (or very few data points) between the more ancient divergences. The question is: is this just an indicator of the limits to molecular resolution, or does it imply something fundamental about the rates of the processes that marked the lemuroid radiation? The fact that it may imply a rapid rate of early divergence among Lemuroidea is suggested by the paucity of synapomorphic SINE insertions that could allow the unambiguous grouping of the different family lineages [Roos et al., 2004]. Alternatively, it is also plau-

sible that the evolutionary signal at these deep levels is simply being hidden by the homoplastic noise that is inherent in sequence data, and particularly in mitochondrial genes. One way to overcome the ubiquitous homoplasy that bedevils sequence data may be through the more widespread use of genomic elements that have the capacity to yield a more stable evolutionary signal, such as SINEs [Roos et al., 2004] or other transposable elements.

Finally, with respect to the analysis of the Lemuridae data set, *Eulemur coronatus* was missing from some previous analyses [Yoder and Irwin, 1999; Yoder and Yang, 2004] but turned out to be the most interesting taxon in terms of its ability to change the *Eulemur* tree topology, as well as the level of support for the internal nodes. Most of our analyses supported a sister group relationship between *Eulemur coronatus* and *Eulemur macaco* (as was also suggested by Wyner et al. [2000]). On the other hand, the arrangement of the *Eulemur fulvus* complex was a secure topology with no ambiguity, which should allow the development of some interesting phylogeographic and biogeographic reconstructions [Pastorini et al., 2003].

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