Molecular Evolutionary Dynamics of Cytochrome *b* in Strepsirrhine Primates: The Phylogenetic Significance of Third-Position Transversions

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DNA sequences of the complete cytochrome *b* gene are shown to contain robust phylogenetic signal for the strepsirrhine primates (i.e., lemurs and lorises). The phylogeny derived from these data conforms to other molecular studies of strepsirrhine relationships despite the fact that uncorrected nucleotide distances are high for nearly all intrastrepsirrhine comparisons, with most in the 15%–20% range. Cytochrome *b* sequences support the hypothesis that Malagasy lemuriforms and Afro-Asian lorisiforms each comprise clades that share a sister-group relationship. A study (Adkins and Honeycutt 1994) of the cytochrome *c* oxidase subunit II (COII) gene placed one Malagasy primate (*Daubentonia*) at the base of the strepsirrhine clade, thereby suggesting a diphyletic Lemuriformes. The reanalysis of COII third-position transversions, either alone or in combination with cytochrome *b* third-position transversions, however, yields a tree that is congruent with phylogenetic hypotheses derived from cytochrome *b* and other genetic data sets.

Introduction

The evolutionary history of the primate suborder Strepsirrhini (i.e., Madagascar lemuriforms and Afro-Asian lorisiforms) has been the subject of numerous DNA-based phylogenetic studies during the past few years (Jung, Crovella, and Rumpler 1992; Crovella, Montagnon, and Rumpler 1993; Adkins and Honeycutt 1994; Yoder 1994; Del Pero et al. 1995; Porter et al. 1995; Yoder et al. 1996). Consequently, a robust phylogeny for strepsirrhine primates is emerging after more than 2 decades of controversy. The controversy has focused in particular on the position of the mouse and dwarf lemur group (family Cheirogaleidae) and on the position of the aye-aye (genus *Daubentonia*). Several morphological studies concluded that the cheirogaleids actually belong within the lorisiform clade (Szalay and Katz 1973; Tattersall and Schwartz 1974; Cartmill 1975). Morphological studies of *Daubentonia* have been less consistent in their conclusions, finding the aye-aye to be either a highly derived member of the Malagasy primate family Indridae (Schwartz 1986), the basal-most branch of the strepsirrhines (Groves 1990), or unclassifiable in relation to other living primates (Oxnard 1981). Because three of the four morphology-based hypotheses suggest that lemuriforms are either para- or diphyletic, the systematics of the Strepsirrhini is of considerable biogeographic interest. If the lemuriforms are not monophyletic, at least two primate migrations between Africa and Madagascar must be invoked to explain current strepsirrhine distributions (Charles-Dominique and Martin 1970). The combined consideration of paleogeographic (Rabinowitz, Coffin, and Falvey 1983; Patriat and Achache 1984) and primate paleontological (Gingerich 1990; Martin 1993; Gingerich and Uhen 1994) data, however, indicate that multiple migrations would have been highly unlikely.

Genetic studies, for the most part, concur with the biogeographic data in finding that Malagasy primates are monophyletic. It is certainly true that virtually every relevant genetic study has placed the cheirogaleids securely within the lemuriform radiation (Dene et al. 1976; Bonner, Heinemann, and Todaro 1980; Rumpler et al. 1983; Dutrillaux 1988; Rumpler et al. 1988; Koop et al. 1989; Crovella, Montagnon, and Rumpler 1993; Adkins and Honeycutt 1994; Yoder 1994; Porter et al. 1995; Yoder et al. 1996). Even so, many anthropologists have been reluctant to relinquish the morphological hypothesis of cheirogaleid/lorisiform affinities. Nearly all of the primate classifications published post-1970 have placed the cheirogaleids within the lorisiforms (Szalay and Delson 1979; Schwartz 1986; Andrews 1988; Fleagle 1988), although there are notable exceptions (e.g., Martin 1990). In fact, if the genetic data have been at all ambiguous, it is with regard to the placement of *Daubentonia* (Adkins and Honeycutt 1994; Yoder 1994).

The analysis of the mitochondrial sequences covered in this paper is the continuation of an ongoing study of strepsirrhine interrelationships. An earlier report described the phylogenetic analysis of the first 700 bases of the 5′ end of cytochrome *b* (Yoder 1994). These preliminary sequences were analyzed separately and in combination with a large morphological data set. The separate analysis of the cytochrome *b* sequences indicated poor support for a number of the clades that were well resolved in the combined analysis. Most significantly, the partial-gene data could not confidently resolve the position of *Daubentonia* nor could they resolve strepsirrhine monophyly. When the complete gene sequences are analyzed (Yoder et al. 1996), however, they support both strepsirrhine and lemuriform monophyly. This result suggests that the previous lack of resolution was a consequence of sampling error (i.e., not enough sequence) rather than a more general failure of cytochrome *b*'s resolving power. The question of cytochrome *b*'s resolving power is nonetheless controversial due to

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Table 1
List of Primates Included in Cytochrome *b* Study

Taxonomic Designation	Common Name	Specimen #
Anthropoidea		
Homo sapiens	Human	GenBank #J01415
Saimiri sciureus	Squirrel monkey	ISIS 8445f; Tulsa Zoo
Lorisiformes		
Galagonidae		
Galago crassicaudatus	Greater bush baby	DUPC #unknown
Loridae		
Loris tardigradus	Slender loris	DUPC #1966m
Nycticebus coucang	Slow loris	DUPC #1942f
Lemuriformes		
Daubentoniidae		
Daubentonia madagascariensis	Aye-aye	DUPC #6262f
Cheirogaleidae		
Cheirogaleus major	Greater dwarf lemur	DUPC #639m
Mirza coquereli	Coquerel's dwarf lemur	DUPC #384f
Microcebus murinus	Gray mouse lemur	DUPC #846f
Indridae		
Propithecus tattersalli	Golden-crowned sifaka	DUPC #6196m
Lemuridae		
Varecia variegata rubra	Red-ruffed lemur	DUPC #5874f
Hapalemur griseus	Gentle lemur	DUPC #6043m
Lemur catta	Ring-tailed lemur	DUPC #5738m
Eulemur fulvus collaris	Collared lemur	DUPC #561m
Eulemur fulvus rufus	Red-fronted lemur	DUPC #6341f

NOTE.—DUPC, Duke University Primate Center; f, female; m, male.

recent criticism of its usefulness as a phylogenetic marker (e.g., Graybeal 1993; Meyer 1994). Several characteristics of cytochrome *b*—and other mitochondrial genes—can present obstacles for phylogenetic algorithms. Some of these obstacles are unequal base frequencies (Lockhart et al. 1994), rate inequalities (Felsenstein 1978), third-position saturation (Meyer 1994), and insufficient variation at replacement sites (Graybeal 1993). Despite these problems, it would be premature to discount cytochrome *b* as a potentially powerful phylogenetic tool. Of all the mitochondrial genes, none has been more widely used for the purpose of phylogenetic reconstruction (e.g., Irwin, Kocher, and Wilson 1991; Crozier and Crozier 1992; Graybeal 1993; Ma et al. 1993; Thomas and Martin 1993; Honeycutt et al. 1995). The advantages of abundant comparative sequence data and well-characterized gene function and protein structure therefore enhance its utility for evolutionary investigations. It is not surprising, then, that cytochrome *b* has been preferred to other, potentially suitable, molecular phylogenetic markers.

We examine the potentially problematic characteristics of the gene (e.g., base frequency differentials, codon position attributes, evolutionary rates) and their effects, if any, on phylogenetic resolution of strepsirrhine primates. In addition to testing the hypothesis of lemuriform monophyly, there are other phylogenetic questions pertinent to strepsirrhine systematics addressed by this study: (1) Is the family Cheirogaleidae monophyletic? (2) Do *Lemur catta* and *Hapalemur* form a clade, as has been recently proposed (Groves and Eaglen 1988; Simons and Rumpler 1988)? (3) Is there a monophyletic Lemuridae? (4) Are the slow lorises (family Loridae) monophyletic? By comparing the cytochrome *b* gene tree to those derived from other genetic markers (Dene et al. 1976; Dutrillaux 1988; Adkins and Honeycu 1994; Porter et al. 1995), we define areas of strepsirrhi phylogeny that are well supported. The phylogene tree is also employed to investigate primate cytochro *b* evolution at the amino acid level to facilitate the co parison of our results with those from other studies (e. Howell 1989; Irwin, Kocher, and Wilson 1991; Ma al. 1993; Ballard and Kreitman 1994; Jermiin et 1994; Honeycutt et al. 1995).

Materials and Methods

DNA Sources

DNA samples were extracted from tissues (liv spleen, kidney, muscle) of animals that died of natu causes (table 1). With the exception of *Saimiri sciure* (ISIS 8445f), all samples originated from the Duke U versity Primate Center. Total genomic DNA was extra ed with a standard phenol/chloroform technique after gesting overnight in an SDS-based extraction buff Outgroup sequences were acquired from GenBank und the following accession numbers: human—J01415 (A derson et al. 1981), mouse–J01420 (Bibb et al. 198 rat—J01436 (Koike et al. 1982), dolphin—X5629 camel—X56281, pig—X56295, and zebra—X56282 (win, Kocher, and Wilson 1991).

DNA Amplification and Sequencing

The polymerase chain reaction (PCR), using pri ers L14724 and H15915 (table 2), was employed to ge erate double-stranded template of the entire cytochro *b* gene (1,140 bp encoding 380 amino acids). Sequenc were generated with a combination of manual and a tomated methods. For manually generated sequenc double-stranded PCR product was purified using eith

Table 2
Oligonucleotide Primers Used to Generate Cytochrome *b* Sequences

Name of Primer	Sequence (5′ to 3′)
L14724*	CGA AGC TTG ATA TGA AAA ACC ATC GTT G
L14901	CAA ATC ATC ACA GGA CTA TT(C,T) (C,T)TA GC
L14979*	GAC GTA AAT TAC GGC TGA AT
L15171	CAT GAG GAC AAA TAT CAT TCT GAG G
L15375	GAA (A,T)CA GGA TC(A,T) AA(C,T) AAC CCA (C,T)(C,T)A GG
L15429	CAC CCT TAC TAC ACA ATC AAA GA
L15615	CGA TC(C,T) AT(C,T) CC(C,T) AAT AAC CTA GGA GG
H15915*	AAC TGC AGT CAT CTC CGG TTT ACA AGA C
H15677	GGT CGG AAT (A,G)T(C,T) AT(A,G) CTT (C,T)GT TGT TT
H15494	ATA ATT GTC TGG GTC GCC TAG
H15260	GCG AAG AAT CG(A,T) GT(T,G) AG(T,G) GT(A,G) GCT TT
H15149*	TCA GAA TGA TAT TTG GCC TCA
H14972	GC(A,G) TG(A,G) AG(A,G) TA(A,G) CG(A,G) ATG ATT CAG CC

NOTE.—Primers marked with an asterisk were taken from Irwin, Kocher, and Wilson (1991, table 1). All other primers were designed by A.D.Y. Nucleotides in parentheses indicate degenerate sites; "L" indicates light strand, "H" indicates heavy strand; numbers correspond to Anderson et al. (1981) sequence.

Centricon 100, Microcon 100 (Amicon), or Wizard Preps (Promega) to remove excess primers and unincorporated nucleotides. The purified double-stranded product was then used as starting template for an asymmetric amplification in which one primer was in limiting quantities. This single-stranded product was then sequenced with various primers (table 2) using conventional dideoxy chain termination. Automated sequences resulted from two different sets of amplification primers (L14724–H15260 and L15171–H15915) which were then cycle-sequenced using a dye terminator sequencing kit (Applied Biosystems, Foster City, Calif.). After purification with Centrisep columns (Princeton Separations, Adelphia, N.J.), sequencing reactions were analyzed by gel electrophoresis with an Applied Biosystems automated DNA sequencer model 373A. These sequences were edited and compiled with AutoAssembler 1.3.0 (Applied Biosystems). The complete gene sequences are the consensus of at least two different double-stranded PCR amplification reactions for which both strands were sequenced and are available from GenBank under accession numbers U53569–U53582.

Although there have been numerous reports of mitochondrial-like DNA sequences appearing in the nuclear genome (e.g., Collura and Stewart 1995; Zischler et al. 1995), presumably due to introgression of mtDNA into the nucleus, we are confident that the sequences reported here represent the functional mitochondrial cytochrome *b* gene. Sequences were repeatedly confirmed with different PCR amplification reactions using three different PCR primer pairs, were unambiguous (i.e., double bands were rare or nonexistent), and were confirmed for both strands. Moreover, the sequences do not have the characteristics of nuclear pseudogenes; all sequences show a complete lack of insertions or deletions and patterns of nucleotide substitution are consistent with those typical of protein-coding genes. It does appear, however, that one or more nuclear cytochrome *b* pseudogenes co-amplify with the mitochondrial copy in *Tarsius bancanus* and in *T. syrichta* (unpublished data). Consequently, the sequences for these taxa are not reported, nor are they included in the phylogenetic analysis.

Data Analysis

The primate sequences, along with the nonprimate outgroup sequences, were easily aligned by eye due to the lack of insertions and deletions. PAUP 3.1.1 (Swofford 1993) was employed for all parsimony analyses. Heuristic searches were conducted with 100 replicates of the random addition option and all other options were set by default. The random trees option was used to estimate the g1 value (Hillis and Huelsenbeck 1992). 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. Maximum-likelihood trees were estimated with fastDNAml 1.0 (Olsen et al. 1994) formatted for the Power Macintosh by D. Gilbert. The DNAdist program in the PHYLIP 3.5p package (Felsenstein 1993) was used to create a distance matrix under a maximum-likelihood correction (Felsenstein 1981) and a transition-to-transversion ratio of 10:1; a least-squares tree was then constructed with the FITCH81 (Fitch and Margoliash 1967) algorithm in which the input order of taxa was randomized and 10 global rearrangements were executed. One hundred bootstrap trials were conducted following the procedure outlined in the PHYLIP manual. MacClade 3.0.4 (Maddison and Maddison 1992) was employed to convert nucleotide sequences into amino acid sequences and for all analyses of character evolution.

Results and Discussion

Genetic Distances

Pairwise genetic distances are sometimes used to judge a gene's potential for phylogenetic signal. In fact, much of the criticism of cytochrome *b* and other mitochondrial markers relates to the large genetic distances that are typically assumed to indicate saturation and, thus, nonutility of these genes. Authors (e.g., Meyer 1994) have suggested that an early observation of high

genetic divergence (≥15%) might be sufficient to disqualify a gene from further analysis. In bufonid frogs, Graybeal (1993) found that at nucleotide distances between 15% and 20%, amino acid sequences are nearly invariant even though silent sites are saturated. Thus, in that study, nucleotide distances within this range were symptomatic of a situation in which only two types of characters exist: highly constrained (and thus noninformative) coding sites or wildly homplasious (and thus noninformative) neutral sites. This result therefore emphasizes the point that nucleotide distances are an incomplete measure of a gene's potential resolving power (Graybeal 1993).

Table 3 indicates that the uncorrected pairwise nucleotide distances among the primates are quite high. In the comparison of any lemur to any loris, the uncorrected nucleotide distance is at least 21%. This figure increases to over 25% for comparisons of strepsirrhines and anthropoids, whereas the distance between any of the nonprimate outgroups and any primate is either the same as or lower than the anthropoid–strepsirrhine comparisons. These results suggest the troubling possibility that cytochrome *b* is reaching saturation at the level of lemuriform–lorisiform divergence and will thus be uninformative at this or deeper phylogenetic levels. Moreover, most of the intertaxonomic distances among the strepsirrhines are within the 15%–20% presumed to indicate saturation of third-position sites (Meyer 1994). The amino acid distances are on average 1.7 times lower than nucleotide distances, but are not dramatically low as in bufonid frogs (Graybeal 1993).

Codon Positions

Base composition for each codon position was averaged over the primates included in this study. Like other mammals (Irwin, Kocher, and Wilson 1991), the primates show a fairly evenly distributed base composition at first-position sites, a marked preference for T at second-position sites, and a significant underrepresentation of G at third-position sites. Thus, cytochrome *b* in primates shows the light-strand bias against G that is typical of animal mtDNA. These patterns of base compositional asymmetry can create problems for phylogenetic analysis. The extreme underabundance of one character state increases the tendency for these sites to saturate prematurely (Irwin, Kocher, and Wilson 1991; Meyer 1994). Also, a skewed mutation bias can violate the assumptions of parsimony (Perna and Kocher 1995). If a mutation bias exists that maintains G's at a much lower frequency than A's, the likelihood of an A-to-G transition will be much lower than the likelihood of a G-to-A transition. Unless otherwise instructed, parsimony algorithms weight these events equally. This shortcoming can be overcome with differential weighting and/or through the use of alternate tree-building algorithms that take starting base composition into account (e.g., maximum likelihood). Relative base frequencies among the primates are nearly uniform, however, with second-position sites showing the least intertaxonomic variation (mean standard deviation = 0.75) and third-position sites showing the greatest

Table 3
Uncorrected Nucleotide Distances (Above the Diagonal) and Amino Acid Distances (Below the Diagonal) for Cytochrome *b*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1. *E. f. collaris*		0.042	0.152	0.139	0.168	0.168	0.183	0.197	0.172	0.228	0.219	0.240	0.211	0.270	0.251	0.234	0.244	0.232	0.219	0.220	0.246
2. *E. f. rufus*	0.011		0.154	0.135	0.161	0.170	0.192	0.201	0.169	0.218	0.222	0.234	0.209	0.271	0.250	0.234	0.243	0.222	0.227	0.225	0.248
3. *Hapalemur*	0.071	0.066		0.129	0.175	0.166	0.196	0.197	0.178	0.225	0.225	0.246	0.230	0.278	0.267	0.255	0.252	0.238	0.227	0.222	0.250
4. *Lemur*	0.063	0.061	0.037		0.175	0.171	0.183	0.192	0.171	0.218	0.222	0.247	0.214	0.270	0.251	0.248	0.236	0.231	0.235	0.225	0.239
5. *Varecia*	0.100	0.095	0.097	0.089		0.185	0.205	0.214	0.178	0.228	0.215	0.235	0.204	0.275	0.258	0.243	0.247	0.236	0.244	0.229	0.237
6. *Cheirogaleus*	0.095	0.095	0.076	0.084	0.132		0.156	0.173	0.184	0.213	0.225	0.227	0.213	0.275	0.269	0.238	0.237	0.225	0.237	0.226	0.238
7. *Mirza*	0.100	0.100	0.100	0.105	0.137	0.071		0.179	0.189	0.236	0.241	0.243	0.232	0.275	0.279	0.238	0.236	0.229	0.246	0.231	0.250
8. *Microcebus*	0.113	0.113	0.100	0.100	0.126	0.079	0.097		0.196	0.239	0.241	0.242	0.225	0.266	0.281	0.244	0.245	0.242	0.239	0.241	0.246
9. *Propithecus*	0.089	0.087	0.084	0.074	0.103	0.097	0.108	0.108		0.211	0.221	0.225	0.217	0.275	0.259	0.238	0.236	0.231	0.232	0.208	0.245
10. *Daubentonia*	0.145	0.145	0.150	0.139	0.161	0.161	0.176	0.161	0.147		0.219	0.234	0.224	0.268	0.239	0.249	0.230	0.225	0.246	0.225	0.248
11. *Galago*	0.137	0.139	0.139	0.142	0.161	0.155	0.171	0.147	0.147	0.147		0.206	0.193	0.263	0.248	0.237	0.226	0.218	0.235	0.208	0.233
12. *Nycticebus*	0.145	0.150	0.150	0.158	0.168	0.158	0.166	0.158	0.163	0.171	0.118		0.179	0.282	0.257	0.244	0.239	0.225	0.234	0.235	0.242
13. *Loris*	0.163	0.161	0.163	0.161	0.171	0.158	0.166	0.166	0.158	0.166	0.113	0.124		0.246	0.250	0.224	0.207	0.208	0.232	0.217	0.230
14. *Saimiri*	0.211	0.218	0.226	0.224	0.232	0.229	0.266	0.226	0.224	0.208	0.218	0.226	0.221		0.261	0.256	0.266	0.258	0.285	0.248	0.269
15. *Homo*	0.187	0.197	0.203	0.203	0.205	0.211	0.218	0.216	0.208	0.200	0.200	0.218	0.224	0.208		0.255	0.248	0.258	0.286	0.268	0.268
16. Mouse	0.184	0.187	0.179	0.182	0.192	0.184	0.192	0.192	0.184	0.192	0.168	0.195	0.176	0.232	0.221		0.164	0.210	0.227	0.218	0.241
17. Rat	0.176	0.179	0.166	0.174	0.192	0.176	0.179	0.182	0.171	0.174	0.150	0.179	0.166	0.229	0.221	0.068		0.217	0.234	0.209	0.226
18. Pig	0.161	0.158	0.155	0.161	0.182	0.158	0.171	0.176	0.158	0.161	0.155	0.158	0.158	0.237	0.226	0.147	0.155		0.209	0.188	0.193
19. Camel	0.142	0.147	0.139	0.150	0.174	0.145	0.161	0.166	0.150	0.161	0.145	0.166	0.174	0.237	0.221	0.158	0.145	0.103		0.197	0.217
20. Zebra	0.129	0.129	0.132	0.134	0.137	0.145	0.139	0.153	0.118	0.139	0.134	0.147	0.139	0.218	0.200	0.150	0.139	0.103	0.103		0.210
21. Dolphin	0.174	0.182	0.184	0.179	0.189	0.176	0.192	0.187	0.168	0.174	0.176	0.192	0.184	0.221	0.218	0.184	0.187	0.129	0.121	0.126	

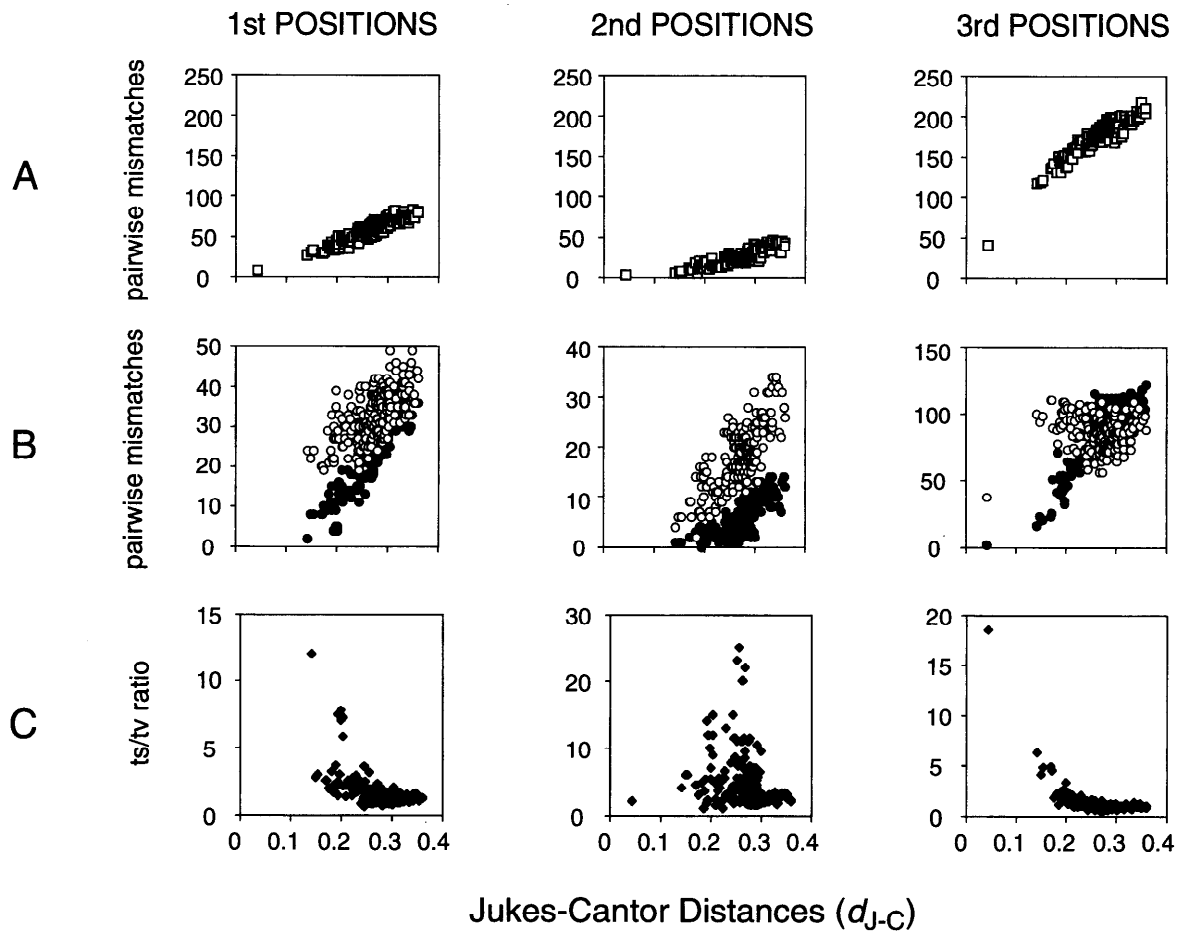

FIG. 1.—Codon position comparisons of (*A*) overall rates of nucleotide accumulation, (*B*) rates of transition (open circle) and transversion (closed circle) accumulation, and (*C*) transition to transversion ratios against total Jukes-Cantor corrected pairwise distances. Note: there are no first- or second-position transversions for intraspecific *E. fulvus* comparison.

amount of variation (mean standard deviation = 3.33). These values are comparable to those found within ungulates (Irwin, Kocher, and Wilson 1991) and correlate with the degree of selective constraint acting on the sites. In all taxa, taxon-specific patterns of base frequency coincide with the averaged relative frequencies except for third-position sites in four taxa. In *Homo, Galago, Daubentonia,* and *Propithecus,* C's outnumber A's as the most numerous base; the opposite pattern is seen in all other primate taxa. This raises the possibility that these four taxa might be erroneously associated in phylogenetic analyses (Lockhart et al. 1992).

In addition to base-compositional effects, overall rate of evolution and the relative abundance of transitions and transversions can vary by codon position. Rapidly evolving characters (e.g., transitions, third positions), due to their higher substitution rate, may be preferentially subject to homoplasy due to multiple substitutions. Conversely, characters that are under intense selection (e.g., second positions) might be so constrained that they are not informative (as discussed in Graybeal 1993), or those substitutions that do occur might reflect similar selective constraints rather than common history (Naylor, Collins, and Brown 1995). Figure 1 illustrates substitution patterns in more detail. For each codon position, overall substitution rate (row A), relative abundance of transitions and transversions (row B), and transition/transversion ratio (ts/tv ratio—row C) are plotted against Jukes-Cantor corrected genetic distances ($d_{J\text{-}C}$). Genetic distance is employed as a surrogate for time since cladogenesis because the strepsirrhine fossil record is too poor to allow for a more accurate measure. Pairwise comparisons with low $d_{J\text{-}C}$ values are therefore assumed to be more recently diverged than those with high values.

Figure 1*A*, in which pairwise mismatches for each codon site are plotted on the ordinate, shows that third-position sites evolve most rapidly and second-position sites evolve most slowly, thus indicating an inverse relationship between amino acid constraint and evolutionary rate. Nonetheless, first positions are evolving considerably more rapidly than second positions even though their assumed amino acid constraints are roughly equivalent (i.e., 96% vs. 100% of nucleotide substitutions will yield amino acid replacements [Li and Graur 1991]). Substitutions appear to have accumulated linearly for all three codon classes throughout the taxonomic range included in this study. When transitions and transversions are individually graphed (fig. 1*B*), however, it becomes clear that at least some portion of third-

Table 4
Tajima's (1993) Relative Rate Test of Class 1 and Class 2 Substitutions in Primate Cytochrome *b* Sequences

Species			Class 1			Class 2		
1	2	3	m1	m2	χ^2	m1	m2	χ^2
Lemur	*Saimiri*	Pig	73	80	0.32	35	61	7.04**
Lemur	*Homo*	Pig	70	72	0.03	29	58	9.67**
Daubentonia	*Saimiri*	Pig	72	85	1.08	33	58	6.87**
Daubentonia	*Homo*	Pig	62	69	0.37	32	61	9.04**
Galago	*Saimiri*	Pig	69	71	0.03	27	65	15.70***
Galago	*Homo*	Pig	67	66	0.01	18	64	25.80***
Lemur	*Saimiri*	Mouse	76	67	0.57	37	59	5.04*
Lemur	*Homo*	Mouse	72	57	1.74	30	56	7.86**
Daubentonia	*Saimiri*	Mouse	80	74	0.23	39	53	2.13
Daubentonia	*Homo*	Mouse	74	66	0.46	36	53	3.25
Galago	*Saimiri*	Mouse	74	67	0.35	30	56	7.86**
Galago	*Homo*	Mouse	76	69	0.34	24	54	11.54***
Lemur	*Galago*	*Saimiri*	64	62	0.03	30	29	0.02
Lemur	*Galago*	*Homo*	69	71	0.03	33	29	0.26
Daubentonia	*Galago*	*Saimiri*	71	62	0.61	30	34	0.25
Daubentonia	*Galago*	*Homo*	63	78	1.60	34	30	0.25

Note.—Number of unique nucleotide characters calculated from sites in which only two nucleotide types are present in three-way comparison. m1, number of sites unique to sequence in column 1 when compared to sequences in columns 2 and 3 (where sequence 3 is the outgroup). m2, defined identically but with respect to sequences in columns 1 and 3.

* Significant at the 0.05 level.

** Significant at the 0.01 level.

*** Significant at the 0.001 level.

position sites (transitions) have reached saturation by $d_{J\text{-}C} = 0.1$. First and second positions do not show this pattern, although at the highest values of $d_{J\text{-}C}$, first-position transition and transversion substitutions are converging in frequency. Thus, based on these graphs alone, one would expect to see apparent ts/tv ratios that converge on 1.0 for first and third positions and that steadily increase in magnitude for second positions as $d_{J\text{-}C}$ gets larger. In the case of first and third positions, this prediction is upheld (fig. 1*C*), although the pattern is less consistent in first than in third positions. Second positions, on the other hand, do not show a clear relationship between ts/tv ratio and $d_{J\text{-}C}$, particularly for $d_{J\text{-}C}$ values between 0.2 and 0.3.

These analyses, taken together, reveal characteristics of each codon class that might affect its phylogenetic signal. For example, third-position sites are clearly the most rapidly evolving, thus suggesting that they could be more liable to homoplasious change than either first or second positions. In fact, similar observations in other phylogenetic studies have been taken as evidence that third positions should be omitted or down-weighted. A closer look at the substitutional patterns in this study, however, indicates that third positions have not yet reached saturation, at least not for transversions. The differential rates of evolution also have obvious consequences for the number of potentially informative characters provided by each codon position. For the primates, there are 103, 48, and 302 phylogenetically informative characters for first, second, and third positions respectively (453 for the complete data set). These different patterns of character change presumably reflect the interaction between mutation bias and functional constraint operating on the sites. Whereas all second-position substitutions will result in amino acid replacements, only 30% of third-position substitutions will. Even though we might consider both third-position transversion and all second-position changes to be "conservative" (i.e., relatively free from the effects of saturation), the degree to which they reflect phylogeny on one hand and selection on the other might be quite different.

Evolutionary Rates

If taxa are evolving at vastly different rates, this can adversely effect parsimony analysis (Felsenstein 1978). To investigate the possibility that the primate taxa included in this study are evolving at different rates, we conducted relative rate tests (Tajima 1993) in which two exemplar lemuriforms (genus *Lemur* and genus *Daubentonia*) and an exemplar lorisiform (genus *Galago*) were individually compared to both anthropoid taxa relative to two nonprimate outgroups. The two lemuriforms were also compared to the lorisiform relative to the anthropoid taxa. In addition to addressing the fundamental question of rate differentials and their potential impact on phylogenetic resolution, these comparisons were designed as a preliminary test of the hypothesis that lemuriforms show a retarded rate of molecular evolution compared to all other primates (Bonner, Heinemann, and Todaro 1980). These tests were conducted separately for class 1 (i.e., third positions) and class 2 (i.e., first and second positions) sites (Hasegawa, Kishino, and Yano 1985) to distinguish between possible mutation and selection effects on evolutionary rates. Table 4 summarizes the results. For class 1 sites, there are no significant rate differentials for any of the comparisons. For class 2 sites, however, all but one of the strepsirrhine–anthropoid comparisons indicate that anthropoids are evolving significantly faster than strepsirrhines. Thus, if rate effects are to be problematic for this study, they could only be manifest for class 2 sites. No significant rate

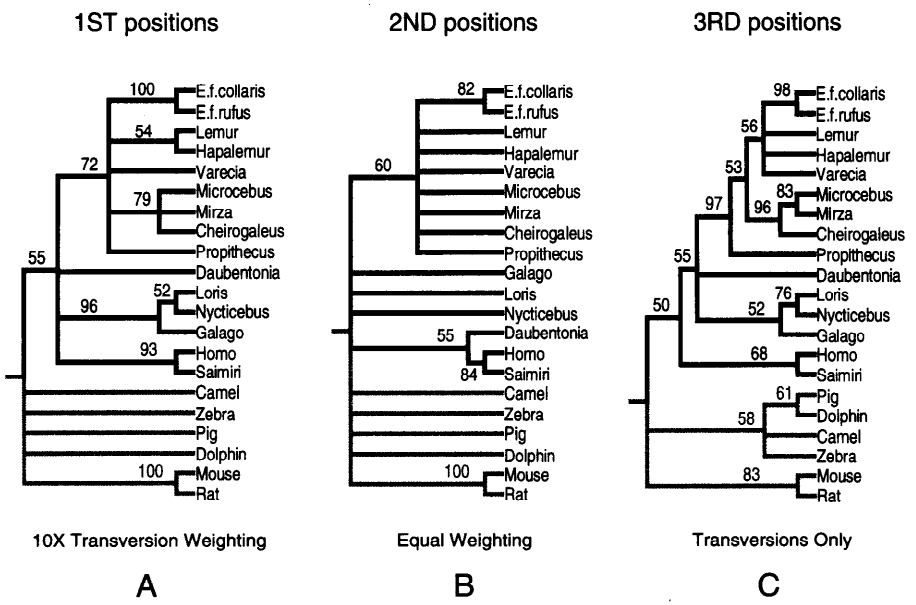

FIG. 2.—Comparison of 50%-majority-rule bootstrap parsimony trees generated for (*A*) first-, (*B*) second-, and (*C*) third-position data sets. The weighting scheme described below each tree yields the greatest number of resolved nodes for that data set. Weighting schemes tested were equal weighting, transversions weighted 10 times more than transitions, and transitions weighted zero (i.e., transversions only). Numbers represent bootstrap values from 100 replicates. Outgroup branches are shaded in gray.

differential is apparent between either of the lemuriforms and the lorisiform. For cytochrome *b* then, it does not appear that rates of lemuriform molecular evolution are uniquely slow.

Phylogenetic Analysis

Parsimony analyses for each codon position were conducted as a further exploration of that position's inherent phylogenetic signal. A total of nine analyses were performed in order to test different transversion : transition weighting schemes (1:1; 10:1; 1:0) for each position. Figure 2 illustrates the individual first-, second-, and third-position parsimony trees in which the weighting scheme that yielded the highest number of resolved nodes for each data set was employed. The third-position character set yielded the most highly resolved tree, with decreasing levels of resolution in the first- and second-position trees, respectively. The g1 values for each data set (−0.478, −0.412, and −0.501 for first, second-, and third-positions respectively), all of which are significant at the 0.01 level (Hillis and Huelsenbeck 1992), also indicate the stronger signal contained in the third-position data set (the more negative the g1, the stronger the signal). The strength of the g1 statistic correlates with the number of phylogenetically informative characters within each codon class. Thus, any analysis of cytochrome *b* data for these taxa that excluded or underweighted third positions would consequently ignore most of the phylogenetic information contained in the complete data set. With only one exception among the three trees, all resolved nodes agree with accepted ideas of mammalian phylogeny (Irwin, Kocher, and Wilson 1991; Catzeflis, Aguilar, and Jaeger 1992; Porter et al. 1995). The exception concerns the placement (albeit with weak support) of *Daubentonia* with the anthropoid primates in the second position tree, thus indicating a possible molecular convergence between anthropoid and *Daubentonia* cytochrome *b*.

Recent experimental explorations of the strengths and weaknesses of various phylogenetic algorithms (Huelsenbeck 1995) have shown that their success varies in accordance with the evolutionary parameters (e.g., branch lengths, rates of evolution) affecting the sequences. If unequal base composition, character saturation, differential rates of evolution, or any other impediments to phylogenetic reconstruction have adversely affected the cytochrome *b* data set, contradictory results from the various algorithms might be expected. Conversely, congruence could suggest that the data contain a consistent phylogenetic signal. Accordingly, we conducted maximum-parsimony, maximum-likelihood, and distance analyses of the complete data set. The results of the three analyses are in near perfect agreement (fig. 3). The single exception pertains to the placement of *Propithecus* in the maximum-likelihood tree. Otherwise, each algorithm found support for monophyly of the taxa Primates, Strepsirrhini, Lemuriformes (including *Daubentonia* and Cheirogaleidae), Cheirogaleidae, Lemuridae, Lorisiformes, and Loridae. Moreover, the resolution of the outgroup taxa conforms to results from previous phylogenetic studies (Irwin, Kocher, and Wilson 1991; Catzeflis, Aguilar, and Jaeger 1992).

Table 5 describes the effects of differential character weighting on the strength of resolution in parsimony analyses (as revealed by bootstrap analysis) for each of the named or controversial primate clades. Four weighting regimes were tested: all characters weighted equally (I); transversions weighted 10 times more than transitions to reflect the 10:1 ratio of transitions to transversions that is typical of mitochondrial DNA (Yang 1994) (II); transversions weighted one and transitions

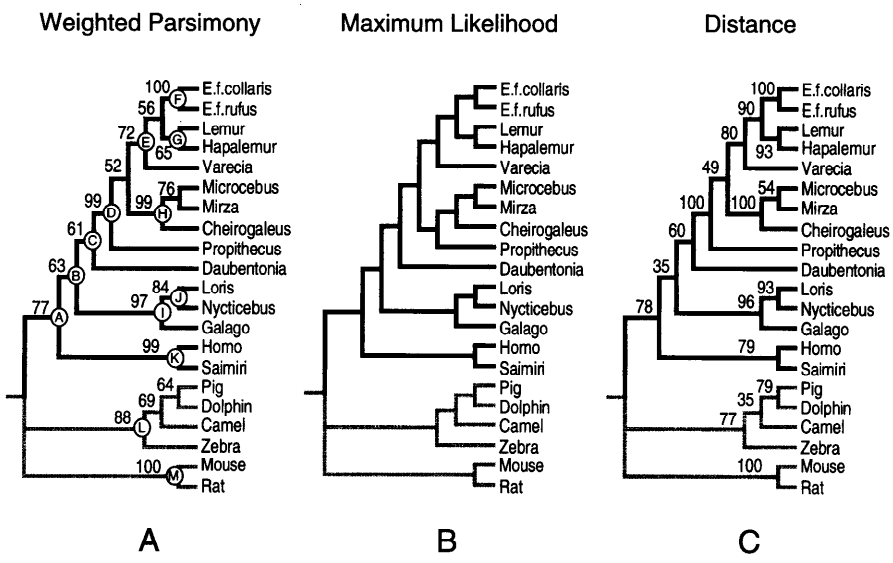

FIG. 3.—Comparison of phylogenetic analyses of complete data set. (*A*) Parsimony analysis in which transversions were weighted 10 times more than transversions, (*B*) maximum-likelihood analysis in which transition/transversion ratio was specified as 10:1, and (*C*) least-squares analysis of maximum-likelihood corrected distances with transition/transversion ratio specified as 10:1. Tree was constructed with Fitch-Margoliash (1967) algorithm. Numbers represent bootstrap values from 100 replicates. Letters on parsimony tree (*A*) correspond to named nodes in table 5. Outgroup branches are shaded in gray.

weighted zero (III); and codon positions weighted as the inverse of the relative number of phylogenetically informative characters, standardized to third positions (3:6:1–IV). The last weighting regime was employed to test the prediction that the differing degrees of phylogenetic resolution apparent in figure 2 are strictly a reflection of the number of phylogenetically informative characters contained within each codon data set. In other words, it is possible that the second-position data set yields the least resolved tree simply because it contains the fewest number of phylogenetically informative characters.

The results of the weighting experiment indicate that the universal weighting of transversions over transitions (either 10:1 or 1:0) provides the best and most uniform support for all nodes. These two weighting regimes were the only two of the four to produce a single most-parsimonious tree that is entirely congruent with traditional, and several other genetic (e.g., Dene et al. 1976; Dutrillaux 1988; Rumpler et al. 1988; Porter et al. 1995), hypotheses of primate phylogeny (fig. 3*A*). Weighting regime I produces three equally most parsimonious trees, the strict consensus of which is similar to the transversion-weighted tree except for the placement of *Varecia* and *Propithecus*. With all characters weighted equally, *Propithecus* is placed as the sister taxon to the cheirogaleids and *Varecia* is placed as the sister taxon to the cheirogaleids plus *Propithecus* plus the remaining lemurids. Furthermore, the relative branching order of the cheirogaleid genera is unresolved. The tree resulting from weighting regime IV agrees with weighting regime I in the placement of *Varecia* and *Propithecus,* but also places the anthropoid primates as sister group to *Daubentonia* within the Strepsirrhini. This unlikely association is supported by a surprisingly high bootstrap value (86%).

Table 5
Bootstrap Values for Named Nodes in Parsimony Analysis

Node	I	II	III	IV
A. Primates	61	77	75	85
B. Strepsirrhini	NC	63	65	NC
C. Lemuriformes	NC	61	58	NC[a]
D. Lemuriformes (except *Daubentonia*)	97	99	99	99
E. Lemuridae	NC	72	68	NC
F. *Eulemur fulvus*	100	100	100	100
G. *Lemur/Hapalemur*	76	65	60	75
H. Cheirogaleidae	94	99	99	88
I. Lorisiformes	72	97	96	83
J. Loridae	73	84	78	NC
K. Anthropoidea	79	99	97	99
L. Ungulates	69	88	83	86
M. Rodents	100	100	100	100

NOTE.—I = all characters equally weighted; II = transversions weighted 10 times more than transitions; III = transversions only (transitions weighted zero); IV = codon positions weighted as the inverse of relative number of phylogenetically informative sites, standardized to third positions (3:6:1); NC = no confidence (for bootstrap values ≤50%).

[a] Weighting scheme IV produced a node in which *Daubentonia* is the sister group to the anthropoids with 86% bootstrap support.

The Mitochondrial Tree

The mitochondrial genome is inherited as a single nonrecombining locus. All mitochondrial genes therefore share a single evolutionary history and should theoretically produce identical phylogenetic trees for a given taxonomic sample. Previous studies have indicated, however, that congruence is not the rule (Cao et al. 1994; Miyamoto et al. 1994; Honeycutt et al. 1995; Sullivan, Holsinger, and Simon 1995) but can sometimes be attained through appropriate character weighting in a parsimony analysis (Miyamoto et al. 1994). In this study, transversion weighting (fig. 3*A*) produces a cytochrome *b* gene tree that is by and large congruent with

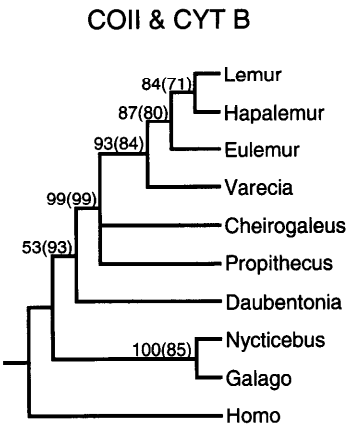

FIG. 4.—Weighted parsimony tree for combined COII and cytochrome *b* data. First number on branch represents bootstrap value from transversion weighting of complete data set. Number in parentheses represents bootstrap value from transversion weighting of third positions only (i.e., class 1 sites).

the transversion-weighted COII gene tree published by Adkins and Honeycutt (1994, their fig. 3*B*). The important exception concerns the placement of the morphologically problematic *Daubentonia*. Whereas cytochrome *b* places it at the base of a Malagasy primate clade, thereby supporting a monophyletic Lemuriformes, COII places it at the base of the strepsirrhine clade, thereby implying a diphyletic Lemuriformes. The Kishino and Hasegawa (1989) test of the variance of log likelihood differences (as implemented in the DNAML program of the PHYLIP package) indicates that the difference between these two trees is not significant. Nonetheless, the phylogenetic and biogeographic implications are important enough to warrant concern over this discrepancy.

In an effort to resolve the disagreement, we combined the cytochrome *b* and COII data for overlapping taxa and conducted two weighted parsimony analyses. In the first analysis, all transversions were weighted one with transitions weighted zero (as in Adkins and Honeycutt 1994, their fig. 3*B*) and in the second analysis, the same weighting was employed but for third positions only. Both analyses yielded the same tree topology (fig. 4) but with differing bootstrap support. Most notably, the support for a Malagasy primate clade, including *Daubentonia,* increases from 53% (with all transversions) to 93% (with third-position transversions only). Because third-position transversions form a subset of total transversions, this result suggests a conflict between class 1 and class 2 sites with regard to *Daubentonia*'s placement. It appears that there may be functional constraints acting at the amino acid level that contribute to this conflict for COII and for cytochrome *b,* both of which are links in the same electron transport chain (Cann, Brown, and Wilson 1984). When class 2 sites are omitted from the analysis, the combined mitochondrial data yield a well-resolved phylogeny in which all but one node is well supported, indicating that the mitochondrial genome contains robust phylogenetic signal in support of lemuriform monophyly. Moreover, the separate bootstrap analyses of the two genes (not shown) indicates that most of the support comes from the COII gene.

Evolution of the Protein

Cytochrome *b* is a mosaic of evolutionarily plastic and conserved regions and there appears to be a significant correlation between the location and type of protein function and the degree of amino acid conservation (Howell 1989; Irwin, Kocher, and Wilson 1991; Crozier and Crozier 1992; Esposti et al. 1993; Ma et al. 1993; Krajewski and King 1996). The transmembrane regions are relatively unconstrained by specific protein functions (but see Naylor, Collins, and Brown 1995), whereas portions of the outer membrane and inner membrane segments perform essential biological roles. For the most part, cytochrome *b* in primates fulfills expectations of the effects of functional constraint on amino acid evolution. As in other mammals (Irwin, Kocher, and Wilson 1991; Ma et al. 1993), the transmembrane regions are more variable than the functionally active regions, with transmembrane substitutions primarily involving exchanges between hydrophobic amino acids. There are also frequent changes from either alanine, leucine, or isoleucine to the polar amino acid threonine within the transmembrane regions. In agreement with previous studies, transmembrane regions VI and IX are the most divergent segments of the gene, with $\geq$ 43% of sites changing two or more times. It is conspicuous, however, that transmembrane region III is one of the most conserved segments of the entire protein.

By mapping the amino acid changes for primates on to the parsimony tree in figure 3*A,* we calculate that 238 sites (62.6%) are invariant, 49 (12.9%) change only once, and 93 (24.5%) change two or more times. Of these changes, we observe a replacement of cysteine by threonine at position 70 in *Saimiri* and *Homo.* This replacement had previously been reported as unique to humans (Ma et al. 1993) but now seems instead to represent an anthropoid synapomorphy. There are four additional amino acid replacements that are unique to anthropoids within the taxa included in this study at positions 123, 180, 219, and 258. As with position 70, three of these replacements involve changes to threonine. Within the primates, *Daubentonia* and anthropoids share a unique character state at positions 42, 121, 194, and 234. Only one of these, however, is a two-state character. Although these similarities are suggestive of a molecular convergence and a possible explanation of the association of these two taxa in some of the parsimony analyses, the removal of the associated nucleotides has minimal impact on the phylogenetic analysis.

Conclusions

Despite the demonstrated base compositional asymmetries, saturation of third-position transitions, and the rate differential between strepsirrhine and anthropoid class 2 sites, cytochrome *b* has proven to be an appropriate phylogenetic marker for resolving strepsirrhine relationships. Even though observed genetic distances for intrastrepsirrhine comparisons are quite high, most of

the fundamental clades within the Strepsirrhini are resolved consistently and with reasonably high levels of confidence. This result suggests that the relationship between genetic distance and phylogenetic signal is more complex than can be assessed by simple pairwise measures. In agreement with immunodiffusion (Dene et al. 1976), DNA hybridization (Bonner, Heinemann, and Todaro 1980), nuclear DNA sequence (Koop et al. 1989; Porter et al. 1995), and cytogenetic (Dutrillaux 1988) studies, cytochrome *b* recovers a monophyletic order Primates, suborder Strepsirrhini, and infraorders Lemuriformes and Lorisiformes. The possibility raised in an earlier section, that *Homo, Galago, Daubentonia,* and *Propithecus* might be falsely attracted in a parsimony analysis due to their similar third-position base composition, has not affected the phylogenetic outcome. It is also notable that the complete gene sequences are better able to resolve fundamental clades than are the previously reported partial gene sequences (Yoder 1994).

Cytochrome *b* is at its most informative for more recently evolved clades within the fundamental lineages. Regardless of the phylogenetic algorithm or weighting strategy employed, there is robust support for a monophyletic *Eulemur fulvus* and families Lemuridae, Cheirogaleidae, and Loridae. A *Hapalemur/Lemur* clade is also well supported, especially by the distance analysis. The notable deficiencies of cytochrome *b* in this study are the weak support of *Daubentonia* at the base of the Lemuriformes and the ambiguous placement of *Propithecus* within the Malagasy primate radiation. Nevertheless, combined cytochrome *b* and COII third-position transversions give robust support to *Daubentonia*'s placement and this result is confirmed by several other genetic studies (Dene et al. 1976; Dutrillaux 1988; Rumpler et al. 1988; Porter et al. 1995). The precise phylogenetic position of *Propithecus,* however, has proven to be problematic for genetic markers in addition to cytochrome *b* (Adkins and Honeycutt 1994; Porter et al. 1995) and may in fact be an example of taxon sampling error (i.e., *Propithecus* is but a single representative of the family Indridae).

Our results draw a more general conclusion regarding cytochrome *b*'s usefulness as a phylogenetic marker: an initial observation of high intertaxonomic genetic distances should not necessarily condemn further plans for sequencing and phylogenetic analysis. For the taxonomic sample reported here, even with intertaxonomic distances that are often within the 15%–20% range disputed by Meyer (1994) and found to be problematic by Graybeal (1993), cytochrome *b* demonstrates substantial ability to recover well-corroborated phylogenetic relationships. In our estimation, this potential was at least partially revealed by the analysis of third-position transversions. Although third positions as a class are often down-weighted due to their high substitution rate, we found that they in fact contain the preponderance of phylogenetic signal for the primates and outgroups included in this study. It is certainly true that the phylogenetic depth for which these characters will be informative is not limitless. Nonetheless, for the range in which they are informative, they appear to be less susceptible to the effects of differential rates of taxonomic evolution and selective constraints than are class 2 sites. Thus, in addition to ascertaining total genetic distance as a preliminary test of a gene's phylogenetic utility, estimates of third-position saturation also seem advisable.

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LITERATURE CITED

ADKINS, R. M., and R. L. HONEYCUTT. 1994. Evolution of the primate cytochrome *c* oxidase subunit II gene. J. Mol. Evol. **38**:215–231.

ANDERSON, S., A. T. BANKIER, B. G. BARRELL et al. (14 co-authors). 1981. Sequence and organization of the human mitochondrial genome. Nature **290**:457–465.

ANDREWS, P. 1988. A phylogenetic analysis of the primates. Pp. 143–175 *in* M. J. BENTON, ed. The phylogeny and classification of the tetrapods. Clarendon Press, Oxford.

BALLARD, J. W. O., and M. KREITMAN. 1994. Unraveling selection in the mitochondrial genome of *Drosophila.* Genetics **138**:757–772.

BIBB, M. J., R. A. VAN ETTEN, C. T. WRIGHT, M. W. WALBERG, and D. A. CLAYTON. 1981. Sequence and gene organization of mouse mitochondrial DNA. Cell **26**:167–180.

BONNER, T. I., R. HEINEMANN, and G. J. TODARO. 1980. Evolution of DNA sequences has been retarded in Malagasy primates. Nature **286**:420–423.

CANN, R. L., W. M. BROWN, and A. C. WILSON. 1984. Polymorphic sites and the mechanism of evolution in human mitochondrial DNA. Genetics **106**:479–499.

CAO, Y., J. ADACHI, A. JANKE, S. PAABO, and M. HASEGAWA. 1994. Phylogenetic relationships among eutherian orders estimated from inferred sequences of mitochondrial proteins: instability of a tree based on a single gene. J. Mol. Evol. **39**:519–527.

CARTMILL, M. 1975. Strepsirhine basicranial structures and the affinities of the Cheirogaleidae. Pp. 313–354 *in* W. P. LUCKETT and F. SZALAY, eds. Phylogeny of the primates: a multidisciplinary approach. Plenum Press, New York.

CATZEFLIS, F. M., J.-P. AGUILAR, and J.-J. JAEGER. 1992. Muroid rodents: phylogeny and evolution. TREE **7**:122–126.

CHARLES-DOMINIQUE, P., and R. D. MARTIN. 1970. Evolution of lorises and lemurs. Nature **227**:257–260.

COLLURA, R. V., and C.-B. STEWART. 1995. Insertions and duplications of mtDNA in the nuclear genomes of Old World monkeys and hominoids. Nature **378**:485–489.

CROVELLA, S., D. MONTAGNON, and Y. RUMPLER. 1993. Highly repeated DNA analysis and systematics of the Lemuridae, a family of Malagasy prosimians. Primates **34**:61–69.

CROZIER, R. H., and Y. C. CROZIER. 1992. The cytochrome *b* and ATPase genes of honeybee mitochondrial DNA. Mol. Biol. Evol. **9**:474–482.

DEL PERO, M., S. CROVELLA, P. CERVELLA, G. ARDITO, and Y. RUMPLER. 1995. Phylogenetic relationships among Malagasy lemurs as revealed by mitochondrial DNA sequence analysis. Primates **36**:431–440.

DENE, H., M. GOODMAN, W. PRYCHODKO, and G. W. MOORE. 1976. Immunodiffusion systematics of the primates: the Strepsirhini. Folia Primatol. **25**:35–61.

DUTRILLAUX, B. 1988. Chromosome evolution in primates. Folia Primatol. **50**:134–135.

ESPOSTI, M. D., S. DE VRIES, M. CRIMI, A. GHELLI, T. PATARNELLO, and A. MEYER. 1993. Mitochondrial cytochrome *b*: evolution and structure of the protein. Biochim. Biophys. Acta **1143**:243–271.

FELSENSTEIN, J. 1978. Cases in which parsimony or compatibility methods will be positively misleading. Syst. Zool. **27**: 401–410.

———. 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. J. Mol. Evol. **17**:368–376.

———. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution **39**:783–791.

———. 1993. PHYLIP (phylogeny inference package). Department of Genetics, University of Washington, Seattle.

FITCH, W. M., and E. MARGOLIASH. 1967. Construction of phylogenetic trees. Science **155**:279–284.

FLEAGLE, J. G. 1988. Primate adaptation and evolution. Academic Press, New York.

GINGERICH, P. D. 1990. African dawn for primates. Nature **346**: 411.

GINGERICH, P. D., and M. D. UHEN. 1994. Time of origin of primates. J. Hum. Evol. **27**:443–445.

GRAYBEAL, A. 1993. The phylogenetic utility of cytochrome *b*: lessons from bufonid frogs. Mol. Phylogenet. Evol. **2**: 256–269.

GROVES, C. P., and R. H. EAGLEN. 1988. Systematics of the Lemuridae (Primates, Strepsirhini). J. Hum. Evol. **17**:513–538.

GROVES, G. C. 1990. Primate evolution. W. W. Norton, New York.

HASEGAWA, M., H. KISHINO, and T. YANO. 1985. Dating of the human–ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. **22**:160–174.

HILLIS, D. M., and J. P. HUELSENBECK. 1992. Signal, noise, and reliability in molecular phylogenetic analyses. J. Hered. **83**:189–195.

HONEYCUTT, R. L., M. A. NEDBAL, R. M. ADKINS, and L. L. JANECEK. 1995. Mammalian mitochondrial DNA evolution: a comparison of the cytochrome *b* and cytochrome *c* oxidase II genes. J. Mol. Evol. **40**:260–272.

HOWELL, N. 1989. Evolutionary conservation of protein regions in the protonmotive cytochrome *b* and their possible roles in redox catalysis. J. Mol. Evol. **29**:157–169.

HUELSENBECK, J. P. 1995. Performance of phylogenetic methods in simulation. Syst. Biol. **44**:17–48.

IRWIN, D. M., T. D. KOCHER, and A. C. WILSON. 1991. Evolution of cytochrome *b* in mammals. J. Mol. Evol. **32**:128–144.

JERMIIN, L. S., D. GRAUR, R. M. LOWE, and R. H. CROZIER. 1994. Analysis of directional mutation pressure and nucleotide content in mitochondrial cytochrome *b* genes. J. Mol. Evol. **39**:160–173.

JUNG, K. Y., S. CROVELLA, and Y. RUMPLER. 1992. Phylogenetic relationships among lemuriform species determined from restriction genomic DNA banding patterns. Folia Primatol. **58**:224–229.

KISHINO, H., and M. HASEGAWA. 1989. Evaluation of the maximum likelihood estimates of the evolutionary tree topologies from sequence data, and the branching order in Hominoidea. J. Mol. Evol. **29**:170–179.

KOIKE, K., M. KOBAYASHI, K. YAGINUMA, M. TAIRA, E. YOSHIDA, and M. IMAI. 1982. Nucleotide sequence and evolution of the rat mitochondrial cytochrome *b* gene containing the ochre termination codon. Gene **20**:177–185.

KOOP, B. F., D. A. TAGLE, M. GOODMAN, and J. L. SLIGHTOM. 1989. A molecular view of primate phylogeny and important systematic and evolutionary questions. Mol. Biol. Evol. **6**:580–612.

KRAJEWSKI, C., and D. G. KING. 1996. Molecular divergence and phylogeny: rates and patterns of cytochrome *b* evolution in cranes. Mol. Biol. Evol. **13**:21–30.

LI, W.-H., and D. GRAUR. 1991. Fundamentals of molecular evolution. Sinauer, Sunderland, Mass.

LOCKHART, P. J., C. J. HOWE, D. A. BRYANT, T. J. BEANLAND, and A. W. D. LARKUM. 1992. Substitutional bias confounds inference of cyanelle origins from sequence data. J. Mol. Evol. **34**:153–162.

LOCKHART, P. J., M. A. STEEL, M. D. HENDY, and D. PENNY. 1994. Recovering evolutionary trees under a more realistic model of sequence evolution. Mol. Biol. Evol. **11**:605–612.

MA, D.-P., A. ZHARKIKH, D. GRAUR, J. L. VANDEBERG, and W.-H. LI. 1993. Structure and evolution of oppossum, guinea pig, and porcupine cytochrome *b* genes. J. Mol. Evol. **36**:327–334.

MADDISON, W. P., and D. R. MADDISON. 1992. MacClade, analysis of phylogeny and character evolution. Sinauer, Sunderland, Mass.

MARTIN, R. D. 1990. Primate origins and evolution: a phylogenetic reconstruction. Princeton University Press, Princeton.

———. 1993. Primate origins: plugging the gaps. Nature **363**: 223–234.

MEYER, A. 1994. Shortcomings of the cytochrome *b* gene as a molecular marker. TREE **9**:278–280.

MIYAMOTO, M. M., M. W. ALLARD, R. M. ADKINS, L. L. JANECEK, and R. L. HONEYCUTT. 1994. A congruence test of reliability using linked mitochondrial DNA sequences. Syst. Biol. **43**:236–249.

NAYLOR, G. J. P., T. M. COLLINS, and W. M. BROWN. 1995. Hydrophobicity and phylogeny. Nature **373**:565–566.

OLSEN, G. J., H. MATSUDA, R. HAGSTROM, and R. OVERBEEK. 1994. fastDNAml: a tool for construction of phylogenetic trees of DNA sequences using maximum likelihood. Comput. Appl. Biosci. **10**:41–48.

OXNARD, C. E. 1981. The uniqueness of *Daubentonia*. Am. J. Phylogenet. Anthropol. **54**:1–21.

PATRIAT, P., and J. ACHACHE. 1984. India-Eurasia collision chronology has implications for crustal shortening and driving mechanisms of plates. Nature **311**:615–621.

PERNA, K., and T. KOCHER. 1995. Unequal base frequencies and the estimation of substitution rates. Mol. Biol. Evol. **12**:359–361.

PORTER, C. A., I. SAMPAIO, H. SCHNEIDER, M. P. C. SCHNEIDER, J. CZELUSNIAK, and M. GOODMAN. 1995. Evidence on primate phylogeny from ϵ-globin gene sequences and flanking regions. J. Mol. Evol. **40**:30–55.

RABINOWITZ, P. D., M. F. COFFIN, and D. FALVEY. 1983. The separation of Madagascar and Africa. Science **220**:67–69.

RUMPLER, Y., J. COUTURIER, S. WARTER, and B. DUTRILLAUX. 1983. Chromosomal evolution in Malagasy primates. Cytogenet. Cell Genet. **36**:542–546.

RUMPLER, Y., S. WARTER, J. J. PETTER, R. ALBIGNAC, and B. DUTRILLAUX. 1988. Chromosomal evolution of Malagasy

lemurs XI. Phylogenetic position of *Daubentonia.* Folia Primatol. **50**:124.

SCHWARTZ, J. H. 1986. Primate systematics and a classification of the order. Pp. 1–42 *in* D. R. SWINDLER and J. ERWIN, eds. Comparative primate biology. Alan R. Liss, New York.

SIMONS, E. L., and Y. RUMPLER. 1988. Eulemur: new generic name for species of *Lemur* other than *Lemur catta.* C. R. Acad. Sci. Paris **307**:547–551.

SULLIVAN, J., K. E. HOLSINGER, and C. SIMON. 1995. Among-site rate variation and phylogenetic analysis of 12S rRNA in sigmodontine rodents. Mol. Biol. Evol. **12**:988–1001.

SWOFFORD, D. L. 1993. PAUP: phylogenetic analysis using parsimony. Smithsonian Institution, Washington, D.C.

SZALAY, F. S., and E. DELSON. 1979. Evolutionary history of the primates. Academic Press, New York.

SZALAY, F. S., and C. C. KATZ. 1973. Phylogeny of lemurs, galagos and lorises. Folia primatol. **19**:88–103.

TAJIMA, F. 1993. Simple methods for testing the molecular evolutionary clock hypothesis. Genetics **135**:599–607.

TATTERSALL, I., and J. H. SCHWARTZ. 1974. Craniodental morphology and the systematics of the Malagasy lemurs (Primates, Prosimii). Anthropol. Pap. Am. Mus. Nat. Hist. **52**: 141–192.

THOMAS, W. K., and S. L. MARTIN. 1993. A recent origin of marmots. Mol. Phylogenet. Evol. **2**:330–336.

YANG, Z. 1994. Maximum likelihood phylogenetic estimation from DNA sequences with variable rates over sites: approximate methods. J. Mol. Evol. **39**:306–314.

YODER, A. D. 1994. Relative position of the Cheirogaleidae in strepsirhine phylogeny: a comparison of morphological and molecular methods and results. Am. J. Phys. Anthropol. **94**: 25–46.

YODER, A. D., M. CARTMILL, M. RUVOLO, K. SMITH, and R. VILGALYS. 1996. Ancient single origin of Malagasy primates. PNAS **93**:5122–5126.

ZISCHLER, H., H. GEISERT, A. VON HAESELER, and S. PAABO. 1995. A nuclear "fossil" of the mitochondrial D-loop and the origin of modern humans. Nature **378**:489–492.

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