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Divergence dates for Malagasy lemurs estimated from multiple gene loci: geological and evolutionary context

ANNE D. YODER* and ZIHENG YANG†
*Department of Ecology and Evolutionary Biology, Yale University, 21 Sachem Street, New Haven, CT 06520, USA, †Department of Biology, University College London, Darwin Building, Gower Street, London WC1E 6BT, UK

Abstract

The lemurs of Madagascar are a unique radiation of primates that show an extraordinary diversity of lifestyles, morphologies and behaviours. However, very little is known about the relative antiquity of lemuriform clades due to the lack of terrestrial fossils for the Tertiary of Madagascar. Here, we employ a Bayesian method to estimate divergence dates within the lemuriform radiation using several unlinked gene loci and multiple fossil calibrations outside the lemuriform clade. Two mitochondrial genes (cytochrome oxidase II and cytochrome *b*), two nuclear introns (transthyretin intron 1 and von Willebrand factor gene intron 11) and one nuclear exon (interphotoreceptor retinoid binding protein, exon 1) are used in separate and combined analyses. The genes differ in taxon sampling and evolutionary characteristics but produce congruent date estimates. Credibility intervals narrow considerably in combined analyses relative to separate analyses due to the increased amount of data. We also test the relative effects of multiple vs. single calibration points, finding that, when only single calibration points are employed, divergence dates are systematically underestimated. For the mitochondrial DNA data set, we investigate the effects of sampling density within the mouse lemur radiation (genus *Microcebus*). When only two representative species are included, estimated dates throughout the phylogeny are more recent than with the complete-species sample, with basal nodes less affected than recent nodes. The difference appears to be due to the manner in which priors on node ages are constructed in the two analyses. In nearly all analyses, the age of the lemuriform clade is estimated to be approximately 62–65 Ma, with initial radiation of mouse lemurs and true lemurs (genus *Eulemur*) occurring approximately 8–12 Ma. The antiquity of the mouse lemur radiation is surprising given the near uniform morphology among species. Moreover, the observation that mouse lemurs and true lemurs are of similar ages suggests discrepancies in rates of morphological, behavioural and physiological evolution in the two clades, particularly with regard to characteristics of sexual signalling. These differences appear to correlate with the nocturnal vs. diurnal lifestyles, respectively, of these two primate groups.

Keywords: Bayes method, combined data analysis, divergence times, Madagascar, molecular clock, primates

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Introduction

There is presently an enormous appetite in the biological community for methods that will allow for accurate estimation of organismal divergence dates. This appetite stems from the understanding that precise estimates of clade ages permit ecological and evolutionary investigation on a scale not allowed by relative (i.e. hierarchical) age estimates. For example, only absolute dates of divergence among lineages will allow investigators to draw firm conclusions about the historical effects of climatological and/or geological conditions on patterns of speciation and geographical distribution among organisms. Absolute age estimates can also permit more subtle measures such as the estimation of rates of morphological and molecular

Correspondence: Anne D. Yoder, Department of Ecology and Evolutionary Biology, Yale University, PO Box 208105, New Haven, CT 06551, USA. Fax: 203-432-5176;
E-mail: anne.yoder@yale.edu

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evolution and their fit to the predictions of ecological and evolutionary theory.

Although methods for such estimation are still in their infancy, tremendous strides have been made within the past several years. Bayesian (Thorne *et al*. 1998; Kishino *et al*. 2001; Thorne & Kishino 2002) and likelihood (Yoder & Yang 2000; Yang & Yoder 2003) methods have been developed and modified such that they can account both for violations of the molecular clock and for uncertainties of the fossil record by incorporating multiple calibration points within a single analysis. Most recently, these methods have been further refined to incorporate data partition heterogeneity in molecular evolutionary parameters (Thorne & Kishino 2002; Yang & Yoder 2003). The latter innovation allows the investigator to combine different gene loci in a single analysis whilst accounting for their idiosyncratic patterns of evolutionary change. Thus, we predict that the next decade will witness an explosion of empirical studies that utilize an explicit temporal framework for investigating both evolutionary and ecological phenomena, much as the refinement and improved understanding of phylogenetic methods (Hillis *et al*. 1994) has done for evolutionary and other biological studies over the past decade.

The unique flora and fauna of Madagascar represent ideal candidates for this invigorated investigative energy. Madagascar is legendary as a natural evolutionary 'laboratory' but, for biologists interested in events that occurred during the Cenozoic (e.g. the evolution of placental mammals), a significant impediment exists in that there is no terrestrial vertebrate fossil record for Tertiary. Thus, there are no external criteria for placing temporal constraints on the evolutionary events of interest. Investigators, therefore, have no choice but to rely on molecular phylogenetic data and on fossil calibrations from outside the Malagasy clades. For those of us with particular interest in the Malagasy lemurs (Order Primates), this is further hampered by the notoriously poor sampling of the primate fossil record (Martin 1993; Tavaré *et al*. 2002). Indeed, a comparative investigation of the effects of a variety of single calibration points on date estimates confirmed the expectation that the primate fossil record tends to give systematic underestimates of divergence times (Yoder & Yang 2000). In that study, the primate calibration consistently underestimated the ages of the two other calibration points utilized by the study. It is worth emphasizing, however, that when that study was conducted, available methods of analysis allowed the use of only a single calibration point in any given analysis, therefore hampering our ability to incorporate the available fossil evidence in an integrated fashion.

All of this has changed with the Bayesian and likelihood methods described above. We expect that the shortcomings of any single calibration point will be ameliorated by other, hopefully more precise, calibrations. Thus, the analytical landscape has changed dramatically and investigators have begun to exploit these methods accordingly (e.g. Springer *et al*. 2003). The ability to incorporate rate variation, both among lineages and loci, and to incorporate fossil record uncertainties has already had an impact on our understanding of placental mammal evolution in Madagascar. A recent investigation demonstrated that at least two of the four clades of Malagasy mammals, the primates and carnivorans, colonized Madagascar independently and at vastly different times, most probably by over-water dispersal (Yoder *et al*. 2003). Presumably, similar analyses will be applied to the other two mammalian clades, rodents and lipotyphlan insectivores, so that we may finally have a comprehensive view of the timing and mechanisms by which placental mammals came to inhabit this remote island. Such data will allow detailed investigation within both historical and temporal constraints, allowing investigators to determine the precise time frame within which the traits of interest have evolved.

Our focus here is to take a comprehensive *in situ* view of lemuriform evolution within Madagascar. We employ the most sophisticated methods currently available for estimating divergence times throughout lemuriform phylogeny. These age estimates are then placed as much as possible within the context of geological and climatological events, with an eye towards identifying possible correlations between biotic and abiotic phenomena. We also note that two of the more diverse species radiations within the Lemuriformes, the mouse lemurs (genus *Microcebus*) and the 'true' lemurs (genus *Eulemur*), appear to be of similar age. This invites speculation that the differential effects of nocturnality (in the mouse lemurs) and diurnality (in the true lemurs) have affected rates of change in the morphological, acoustic and olfactory traits related to mate recognition.

Materials and methods

Sequence data

We used four genetic data sets: one mitochondrial DNA (mtDNA) data set comprised of the complete cytochrome oxidase II and cytochrome *b* genes; two nuclear introns [transthyretin intron 1 (TR) and von Willebrand factor gene intron 11 (vWF)] and one nuclear exon [interphotoreceptor retinoid binding protein, exon 1 (IRBP)]. Primer sequences for amplifying each gene are given in Table 1. Extraction, amplification and sequencing conditions were as reported in Yoder & Irwin (1999). Protein-coding sequences (the mtDNA and IRBP data sets) were aligned by eye and noncoding sequences (TR and vWF) were aligned with CLUSTALW and adjusted by eye. The sequence alignments for each gene are available in TreeBASE under Accession no. SN1625.

The three nuclear markers, TR, vWF and IRBP, are located on chromosomes 18, 12 and 10, respectively in humans and are thus assumed to be independently segregating

Table 1 Primers employed to amplify gene loci

Primer name	Gene	Sequence 5′–3′	Reference
L7553	COII	AACCATTTCATAACTTTGTCAA	(Adkins & Honeycutt 1994)
H8320	COII	CTCTTTAATCTTTAACTTAAAAG	(Adkins & Honeycutt 1994)
L14724	Cytochrome *b*	CGAAGCTTGATATGAAAAACCATCGTTG	(Irwin *et al.* 1991)
L15171	Cytochrome *b*	CATGAGGACAAATATCATTCTGAGG	(Yoder *et al.* 1996b)
H15506	Cytochrome *b*	AGTGGRTTRGCTGGTGTRTARTTGTC	(Yoder *et al.* 1996b)
H15915	Cytochrome *b*	AACTGCAGTCATCTCCGGTTTACAAGAC	(Irwin *et al.* 1991)
p217	IRBP (exon 1)	ATGGCCAAGGTCCTCTTGGATAACTACTGCTT	(Stanhope *et al.* 1992)
p379	IRBP (exon 1)	CCTCGCCTGGTCATCTCCTATGAGCCCAGCAC	(Stanhope *et al.* 1992)
m1426	IRBP (exon 1)	CAGGTAGCCCACATTGCCTGGCAGCAC	(Stanhope *et al.* 1992)
m1531	IRBP (exon 1)	CGCAGGTCCATGATGAGGTGCTCCGTGTCCTG	(Stanhope *et al.* 1992)
F635	Transthyretin (intron 1)	TGCCTCGCTGGACTGGTATT	(Flynn & Nedbal 1998)
R1628	Transthyretin (intron 1)	GACAGCATCTAGAACTTTGACCAT	(Flynn & Nedbal 1998)
vWF-10	vWF (intron 11)	GAGCTGGATGTCCTGGCCATCCATGGCAAC	(Chaves *et al.* 1999)
vWF-8	vWF (intron 11)	GAGTGCCTTGTCACTGGTCATCCCACTTCAA	(Chaves *et al.* 1999)

COII, cytochrome oxidase II; IRBP, interphotoreceptor retinoid binding protein; vWF, von Willebrand factor.

in all taxa examined. Taxon sampling is virtually complete for the mtDNA and IRBP data, intermediate for vWF and poor for TR. GenBank Accession nos for all genes and taxa are given in Table 2. For the mtDNA data, we examined two taxon samples within the mouse lemur radiation: one that contains multiple individuals for each of the nine recognized species of *Microcebus*, yielding a total of 25 individuals, and another wherein we sampled only one individual for one species each of the 'northern' and 'southern' mouse lemur clades identified in Yoder *et al*. (2000). We refer to the former as the 'complete' taxon sample and the latter as the 'two-species' taxon sample. Data sets also differ in the relative amounts of missing data in the alignments. The polymerase chain reaction amplification was unproblematic for the mtDNA and IRBP data but was more difficult for both vWF and TR. Thus, the latter two data sets contain significantly more missing data than the former, just as they contain numerous indels of differing lengths due to their nonprotein-coding status. Finally, it is notable that the data sets do not contain a uniform outgroup. *Didephis virginiana* was employed for the mtDNA and IRBP data sets, *Mus* for the vWF data set and *Scalopus* for the TR data set (Table 2).

Methods of analysis

The Bayes method was used to estimate divergence dates while accounting for stochastic changes in evolutionary rate over time (Thorne *et al.* 1998; Thorne & Kishino 2002). Program packages written by Jeff Thorne were used to perform Markov chain Monte Carlo (MCMC) calculation. The procedure involves two steps. The first step is to use the ESTBRANCHES program to obtain maximum likelihood estimates of branch lengths for the ingroup rooted tree and to calculate their approximate variance–covariance matrix. This step requires input of parameter values in the substitution model. We used the F84 + G model and estimated parameters using the BASEML program in the PAML package (Yang 1997). The model accounts for the transition/transversion rate ratio κ and uses five site classes under the discrete-gamma model of rates for sites (Yang 1994). The ESTBRANCHES program also requires an outgroup to locate the root of the ingroup tree. The second step is to run the Markov chain to approximate the posterior distributions of rates and times. This is achieved using the program DIVTIME5B for one gene and MULTIDIVTIME for multiple genes or site partitions. The latter program can accommodate differences in the substitution parameters among genes or site partitions.

We analysed the four loci separately and in two combined analyses. The two combined analyses were of the three nuclear loci in combination and of all four loci in combination. For protein-coding genes (mtDNA and IRBP), the three codon positions were always treated as different partitions, with their heterogeneity accounted for (Table 3). Thus, the combined analyses account for heterogeneity both among codon positions and among gene loci. A single underlying history was assumed for all analyses (Fig. 1) but, given the differing taxon samples available for each marker, different user trees were employed for individual data sets (Fig. 2). Thus, the phylogeny that serves as the basis for branch length estimation is supplied by the investigator, rather than being generated by the ESTBRANCHES program. For the combined analyses, sequences from the individual locus data sets were trimmed such that each taxon in the combined analysis was represented by at least two loci. This approach was taken to maximize taxon coverage while minimizing the effects of missing data in the combined analysis. The resulting taxon sample in the combined analyses is illustrated in Fig. 3.

Table 2 GenBank Accession numbers for sequences employed in this study

	mtDNA				
Taxa	COII	Cytochrome *b*	IRBP	vWF	Transthyretin
M. ravelobensis	AF285493; AF285496	AF285529; AF285532	—	—	—
M. sambiranensis	AF285518; AF285520	AF285554; AF285556	—	—	—
M. tavaratra	AF285497; AF285498	AF285533; AF285534	—	—	—
M. myoxinus	AF285499; AF285503	AF285535; AF285539	—	—	—
M. berthae	AF285504; AF285507	AF285540; AF285543	—	—	—
M. murinus	AF321177; AF285527; AF285525; AF321179	AF285563; AF285561; AF285564; AF285566	AF081054	—	—
M. griseorufus	AY167064; AF321181	AF285567; AF285568	—	—	—
M. 'rufus1'	AF285508; AF285515; AF285513; AF285511	AF285544; AF285551; AF285549; AF285547	—	—	—
M. 'rufus2'	AF285516; AF285517	AF285552; AF285553	—	—	—
Mirza	AY321460	U53571	AY434080	AY434036	—
Cheirogaleus	L22775	U53570	AF271421	AY434037	AY434064
Lepilemur	AY321459	AY321456	AY434081	AY434039	—
Propithecus	L22782	U53573	AF081053	AY434038	—
E. f. collaris	AF081041	U53576	AF081059	AY434041	AY434067
E. f. albifrons	AF081043	AF081048	AF081061	AY434040	AY434061
E. f. rufus	AF081042	U53577	AF081060	AY434042	AY434065
E. mongoz	AF081045	AF081051	AF081064	AY434044	—
E. rubriventer	AF081046	AF081052	AF081065	AY434045	—
E. m. flavifrons	AF081044	AF081050	AF081063	AY434043	AY434066
E. m. macaco	L22777	AF081049	AF081062	—	AY434068
Lemur	L22780	U53575	AF081058	AY434046	AY434060
Hapalemur	L22778	U53574	AF081057	AY434047	AY434059
V. v. rubra	L22785	U53578	AF081055	AY434048	AY434063
V. v. variegata	AF081040	AF081047	AF081056	—	AY434062
Daubentonia	L22776	U53569	AF271422	AY434049	AY434069
Nycticebus	U53580	U53580	AF271419	AY434050	—
Loris	AY321458	U53581	AF271418	AY434051	—
G. moholi	—	—	AF271415	AY434053	—
G. demidoff	AY44077	AF271411	AF271416	AY434052	—
Callithrix	AY44078	AY34079	AY434083	AF092828	AY434071
Saguinus	—	—	AY434082	AY434054	AY434070
Cebus	—	—	—	AF092821	—
Colobus	—	—	AY434084	AF092829	AY434074
Macaca	U38272	M74005	AY434085	AY434057	AY434075
Cercopithecus	—	—	AY434087	AY434055	AY434073
Erythrocebus	—	—	AY434086	AY434056	AY434072
Pongo	D38115	D38115		AF092833	—
Gorilla	D38114	D38114	AY434088	AY434058	—
Pan	D38113	D38113	—	—	—
Homo	J01415	J01415	J05253	AC00656	AC079096
Felis	U20753	U20753	Z11811	—	AF039724
Canis	U96639	U96639	AY170074	—	AF039732
Ursus	AF303109	AF303109	—	—	—
Bos	J01394	J01394	M20748	—	—
Hippopotamus	U07565	U07565	AF108837	—	—
Physeter	AJ277029	AJ277029	U50818	—	—
Balaenoptera	X61145	X61145	U50820	—	—
Tapirus/Rhinocerus	X97336	X97336	AF179294	—	—
Equus	X79547	X79547	U48710	—	—
Outgroup	*Didelphis*	*Didelphis*	*Didelphis*	*Mus*	*Scalopus*
	Z29573	Z29573	Z11814	www.ensembl.org	AY434076

mtDNA, mitochondrial DNA; COII, cytochrome oxidase II; IRBP, interphotoreceptor retinoid binding protein; vWF, von Willebrand factor.

Table 3 Molecular evolutionary parameters estimated by maximum likelihood for each gene locus

Gene	Position	L	π_T	π_C	π_A	π_G	κ	α	Substitution rate	Tree length
mtDNA*	1	608	0.22	0.26	0.29	0.23	3.19	0.30	0.21	14.53
	2	608	0.40	0.25	0.22	0.13	3.03	0.17	0.09	14.11
	3	608	0.21	0.35	0.40	0.05	21.02	1.06	4.49	13.12
IRBP	1	315	0.10	0.28	0.22	0.40	1.70	0.61	0.07	18.02
	2	315	0.31	0.23	0.26	0.20	1.29	0.44	0.04	16.80
	3	315	0.10	0.42	0.09	0.39	4.33	1.71	0.21	20.04
vWF	—	936	0.23	0.27	0.19	0.31	1.44	2.29	0.11	16.62
TR	—	901	0.32	0.19	0.31	0.18	1.35	3.12	0.14	8.28

*Parameters estimated from the 'two species' data set for mitochondrial DNA (mtDNA). IRBP, interphotoreceptor retinoid binding protein; vWF, von Willebrand factor; TR, transthyretin.

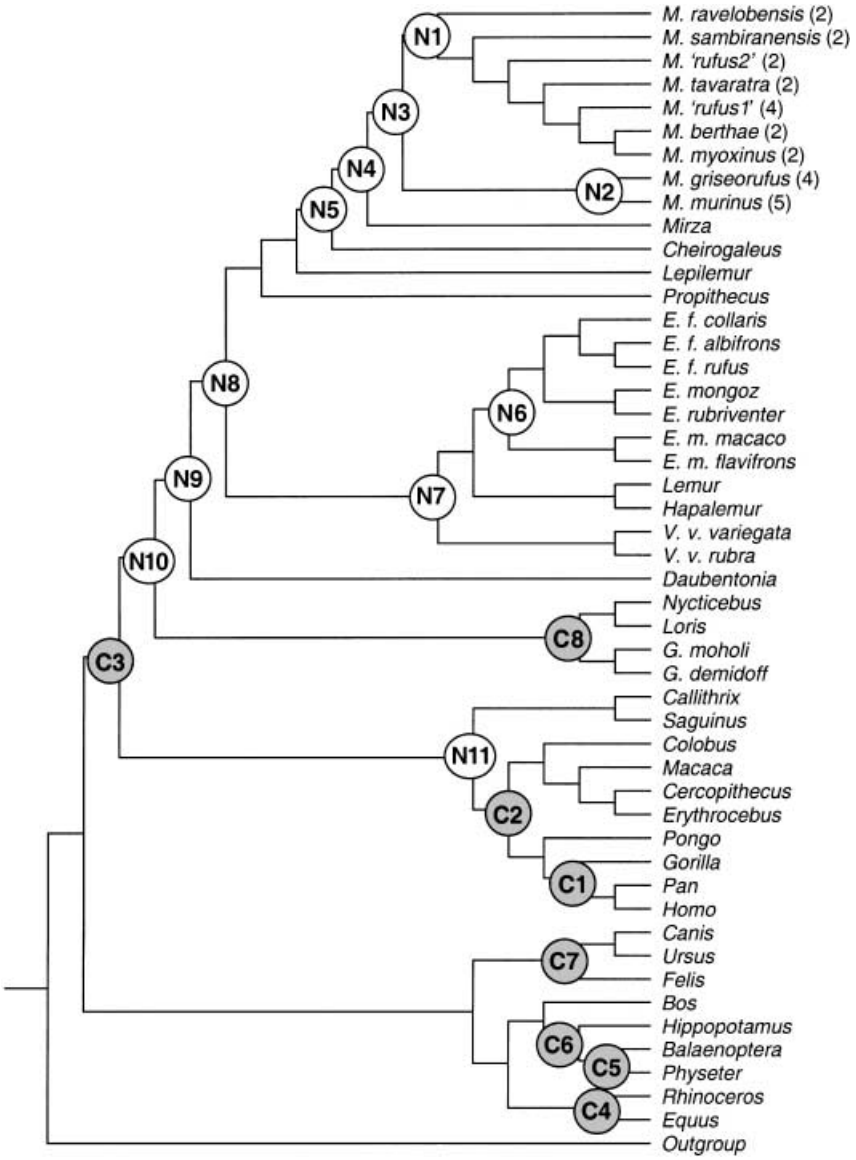

Fig. 1 Assumed phylogenetic tree for all ingroup taxa represented in this study. Some taxa (e.g. *Ursus*) are represented by only one gene locus and are, therefore, not represented in the combined analyses. C1–C8 are nodes for which information from the fossil record was employed as calibrations. Calibration bounds are given in Materials and methods. N1–N11 are nodes of interest for which estimated dates are presented in Table 4.

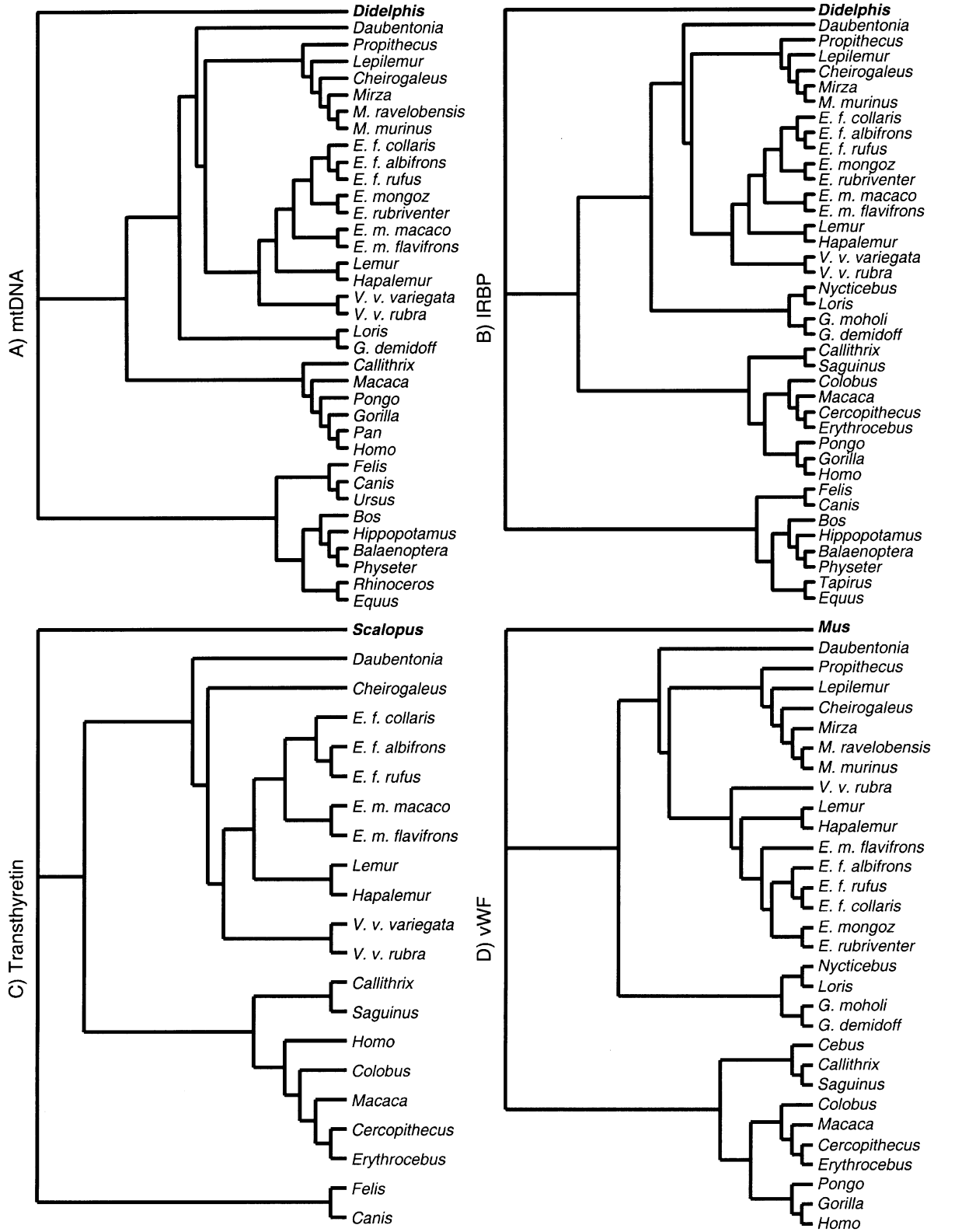

Fig. 2 User trees for analyses of four gene loci: (A) mitochondrial DNA (mtDNA) (cytochrome oxidase II, cytochrome *b*); (B) interphotoreceptor retinoid binding protein (IRBP), exon 1; (C) transthyretin intron 1 and (D) von Willebrand factor (vWF), intron 11. Trees illustrate precise taxon sample for individual analyses (columns 2–5 in Table 4). The outgroup species is *Didelphis virginiana* for the mtDNA, IRBP genes, *Scalopus* for transthyretin and *Mus* for vWF.

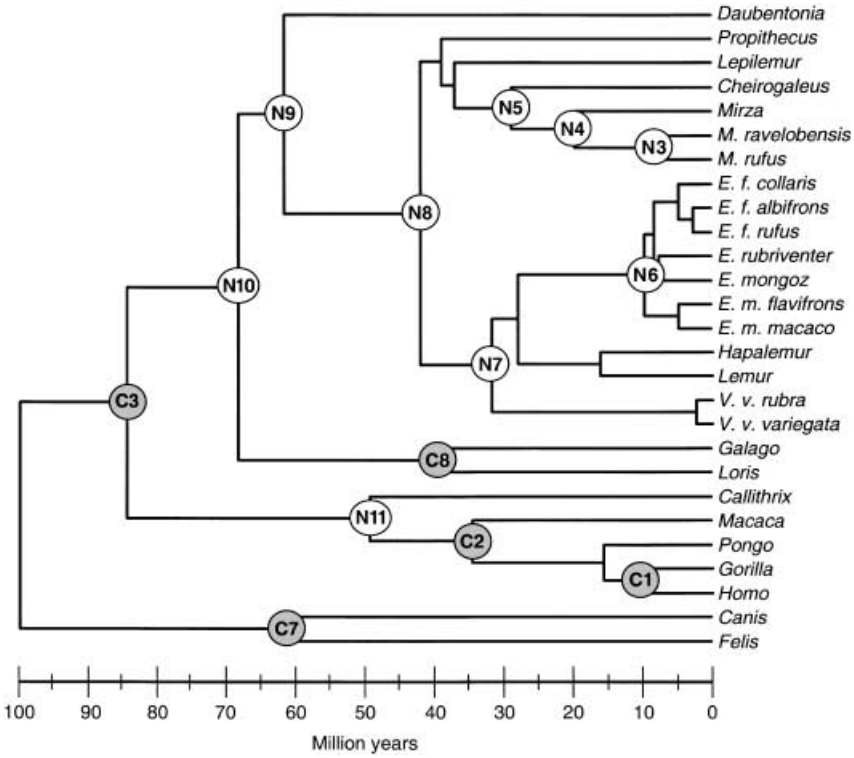

Fig. 3 Rooted ingroup tree assumed in combined analyses (columns 6 and 7 in Table 4). Branches are drawn to reflect divergence times estimated in 'all genes' combined analysis (column 7 in Table 4). Estimated nodes and calibrated nodes are indicated as in Fig. 1.

Eight nodes in the master tree (Fig. 1) were calibrated with age ranges estimated from the fossil record. The MCMC programs also require specification of prior distributions for the age of the root node and the substitution rate at the root. These were specified as in Yang & Yoder (2003). The prior for divergence times is specified using a recursive procedure (Kishino *et al*. 2001), starting from the root and moving towards the tips. A gamma density is used for the age of the root. Each path from an ancestral node to the tip is then broken into random segments, corresponding to branches on the path, by using a Dirichlet density with equal probabilities. Fossil calibration information is incorporated as upper and lower bounds on the node age, equivalent to specifying a uniform distribution between the bounds. These calibrations act as prior information in the Bayes framework by constraining node ages. Seven of these calibrations (C1–C7) were used in previous studies (Yang & Yoder 2003; Yoder *et al*. 2003), while an additional calibration (C8) is novel to this study. Node calibrations are: C1, the divergence between gorilla and human at 8–12 Ma (Shoshani *et al*. 1996); C2, the divergence between monkeys and apes at 32–38 Ma (Shoshani *et al*. 1996; Yoder & Yang 2000); C3, the basal radiation of primates at 63–90 Ma (Martin 1993; Gingerich & Uhen 1994; Tavaré *et al*. 2002); C4, horse and other perrisodactyls at 50–58 Ma (Prothero & Schoch 1989; Janis *et al*. 1998); C5, toothed and baleen whales at 33–40 Ma (Thewissen 1994); C6, whales and hippopotamus at 51–60 Ma (Thewissen 1994); C7, canids and felids at 45–65 Ma (Flynn 1996) and C8, between slow lorizes and galagos at 38–42 Ma (Seiffert *et al*. 2003). Several analyses were conducted to test the effects of the calibration priors on the posterior mean estimates and credibility intervals. One analysis was run wherein bounds for all calibration nodes were narrowed by 20% and another wherein they were expanded by 20%. To test the impact of the basal primate calibration (C3), which is the broadest calibration interval, spanning 27 Myr between upper and lower bounds, one analysis was run wherein the calibration bounds were narrowed by 15 Myr to 75–87 Ma. Two analyses were run to test the consequences of employing only a single calibration, one in which a single primate calibration (C1) was employed and another in which a single outgroup calibration (C7) was employed.

For all MCMC analyses, we took 20 000 samples after the burn-in, sampling every five generations. Burn-in was designated at 20 000 samples. The algorithm was run at least twice from different random starting points to test for convergence. Convergence on the target distribution was also tested more formally using a variety of methods. The methods of Raftery & Lewis (1992) and Geweke (1992) were implemented in the program BOA 1.0.1 (Bayesian Output Analysis), available from Brian Smith (http://www.public-health.uiowa.edu/boa/), running in the statistics package R (http://lib.stat.cmu.edu/R/CRAN/). The Raftery & Lewis (1992) convergence diagnostic is appropriate for the analysis of individual chains. It tests for convergence to the stationary

Table 4 Posterior means and 95% credibility intervals for divergence times

Node	mtDNA (3 partitions) Complete	2 species	TR	vWF	IRBP (3 partitions)	3 nuclear (5 partitions)	All genes (8 partitions)
N1) Northern clade	10.0 (6.3, 15.2)	NA	NA	NA	NA	NA	NA
N2) Southern clade	8.8 (5.3, 13.6)	NA	NA	NA	NA	NA	NA
N3) *Microcebus*	12.0 (7.8, 17.9)	9.0 (5.2, 14.0)	NA	8.7 (3.7, 16.5)	NA	NA	8.9 (5.5, 13.2)
N4) *Microcebus/Mirza*	24.2 (16.8, 33.4)	20.5 (13.5, 29.2)	NA	19.3 (10.8, 30.5)	22.8 (11.3, 36.6)	19.1 (12.3, 27.6)	19.9 (14.6, 26.1)
N5) Cheirogaleidae*	31.8 (23.4, 41.6)	26.7 (18.8, 36.4)	NA	31.3 (19.9, 44.2)	30.9 (19.2, 44.7)	30.6 (22.4, 39.7)	29.0 (22.7, 35.9)
N6) *Eulemur*	8.4 (5.3, 13.4)	6.6 (3.8, 11.1)	7.0 (2.1, 15.7)	14.6 (7.8, 24.2)	14.6 (7.8, 23.7)	11.1 (7.0, 16.4)	9.7 (6.5, 13.7)
N7) Lemuridae	35.9 (27.0, 46.3)	33.1 (24.2, 43.8)	32.1 (20.4, 46.5)	33.4 (22.4, 46.2)	26.4 (16.3, 39.1)	31.1 (23.2, 39.8)	31.9 (25.6, 38.8)
N8) Internal lemuriform	46.7 (36.9, 57.5)	43.8 (33.8, 54.9)	NA	45.7 (33.5, 58.5)	45.1 (32.4, 60.1)	42.2 (33.5, 51.3)	42.3 (35.4, 49.5)
N9) Lemuriformes	67.1 (56.8, 77.2)	65.7 (54.8, 76.3)	54.9 (41.7, 70.6)	62.8 (51.3, 74.4)	60.2 (46.2, 75.1)	65.0 (56.3, 73.7)	62.0 (57.9, 73.0)
C8) Lorisiformes (38–42 Ma)	40.0 (38.1, 41.9)	39.9 (38.1, 41.9)	NA	39.7 (38.1, 41.8)	39.6 (38.1, 41.8)	39.5 (38.1, 41.8)	39.1 (38.0, 41.5)
N10) Strepsirrhini	72.9 (64.0, 82.0)	72.4 (63.2, 81.7)	NA	70.7 (60.3, 81.6)	70.1 (56.8, 83.8)	74.9 (66.1, 83.0)	68.5 (61.3, 75.4)
C1) Human/gorilla (8–12 Ma)	10.9 (8.9, 12.0)	10.7 (8.6, 12.0)	NA	10.1 (8.1, 11.9)	9.4 (8.0, 11.7)	9.6 (8.1, 11.8)	10.2 (8.2, 11.9)
C2) Monkey/ape (32–38 Ma)	34.0 (32.1, 37.5)	34.2 (32.1, 37.6)	35.2 (32.2, 37.9)	34.7 (32.1, 37.8)	34.7 (32.1, 37.8)	34.2 (32.1, 37.5)	34.7 (32.1, 37.8)
N11) Anthropoidea	61.8 (51.0, 73.8)	62.2 (50.5, 75.1)	43.5 (36.6, 51.5)	42.0 (35.4, 49.9)	49.8 (37.6, 64.3)	42.9 (37.8, 48.7)	49.4 (43.5, 55.7)
C3) Basal primate (63–90 Ma)	85.9 (78.4, 89.9)	85.9 (78.1, 89.8)	70.0 (63.2, 84.0)	85.9 (78.1, 89.8)	85.4 (75.4, 89.8)	82.5 (73.0, 89.6)	84.9 (76.9, 89.8)
C7) Canid/felid (45–65 Ma)	58.1 (48.9, 64.6)	57.9 (48.6, 64.6)	55.7 (45.8, 64.5)	NA	57.2 (46.4, 64.6)	60.1 (50.3, 64.8)	61.4 (54.0, 64.9)
C6) Whale/hippo (51–60 Ma)	56.0 (51.4, 59.8)	56.0 (51.4, 59.8)	NA	NA	54.6 (51.1, 59.5)	NA	NA
C5) Whale/whale (33–40 Ma)	34.8 (33.1, 38.7)	34.9 (33.1, 38.9)	NA	NA	35.3 (33.1, 39.4)	NA	NA
C4) Perrisodactyla (50–58 Ma)	53.0 (50.1, 57.5)	53.0 (50.1, 57.4)	NA	NA	54.6 (50.4, 57.9)	NA	NA

NA, not applicable if relevant taxa were missing from analysis. Node numbering refers to Fig. 1; C1–C8 are calibration nodes, for which the prior bounds on divergence dates are shown. Analysis of data of multiple partitions (genes or codon positions) accounted for differences in evolutionary parameters among partitions (Table 2).
*Basal taxon of family Cheirogaleidae (*Phaner furcifer*) is not included in this study; this age estimate is certain to be an underestimate.
mtDNA, mitochondrial DNA; TR, transthyretin; vWF, von Willebrand factor; IRBP, interphotoreceptor retinoid binding protein.

distribution and reports the minimum number of iterations needed to achieve stationarity. When convergence of the mean of some function of the sampled parameters is of interest (e.g. posterior mean age estimates), the Geweke (1992) diagnostic compares the performance of multiple individual chains. This test is conservative in that it cannot prove that convergence has been obtained but can readily detect cases wherein convergence has not been achieved.

Results and Discussion

Congruence of age estimates derived from independent loci

Estimated divergence dates for separate analysis of each locus, and for the two combined analyses, are given in Table 4. The posterior mean age estimates across analyses are notably congruent despite the obvious differences in

Table 5 Effects of calibration priors on all genes (8 partition) combined analysis

Node	Narrowed (20%)	Expanded (20%)	Narrowed primate	Single primate	Single outgroup
N3) *Microcebus*	8.7 (5.4, 13.1)	8.9 (5.5, 13.4)	8.7 (5.4, 12.9)	6.9 (3.8, 11.2)	6.2 (3.6, 10.0)
N4) *Microcebus*/*Mirza*	19.5 (14.3, 25.7)	20.0 (14.5, 26.4)	19.5 (14.4, 25.6)	15.8 (10.1, 23.4)	14.2 (9.5, 20.4)
N5) Cheirogaleidae*	28.5 (22.2, 35.3)	29.1 (22.7, 36.2)	28.4 (22.1, 35.0)	22.7 (15.1, 32.8)	20.3 (14.3, 28.2)
N6) *Eulemur*	9.5 (6.6, 13.2)	9.8 (6.4, 13.7)	9.6 (6.5, 13.7)	7.6 (4.7, 11.9)	6.7 (4.3, 10.1)
N7) Lemuridae	31.4 (25.4, 38.1)	32.2 (25.8, 39.4)	31.6 (25.4, 38.3)	24.9 (16.9, 36.0)	22.1 (15.5, 30.4)
N8) Internal lemuriform	41.6 (34.9, 48.7)	42.7 (35.3, 50.3)	41.6 (34.9, 48.5)	33.0 (22.7, 46.9)	29.4 (21.2, 39.6)
N9) Lemuriformes	61.0 (54.2, 67.6)	62.7 (54.9, 70.5)	61.0 (54.4, 67.4)	48.7 (34.1, 67.8)	43.4 (32.3, 57.3)
C8) Lorisiformes	39.4 (38.5, 41.2)	38.8 (37.5, 41.6)	39.1 (38.0, 41.4)	22.8 (14.5, 33.7)	20.2 (13.7, 28.4)
N10) Strepsirrhini	67.6 (60.9, 73.9)	69.3 (61.4, 77.0)	67.4 (61.2, 73.7)	53.0 (37.2, 73.8)	47.4 (35.5, 62.2)
C1) Human/gorilla	10.2 (8.7, 11.4)	10.4 (7.9, 12.4)	10.2 (8.2, 11.9)	9.5 (8.1, 11.8)	7.7 (4.7, 11.9)
C2) Monkey/ape	34.7 (32.6, 37.3)	34.7 (31.7, 38.2)	34.6 (32.1, 37.7)	29.2 (21.4, 39.7)	25.2 (17.7, 34.9)
N11) Anthropoidea	48.9 (43.2, 54.7)	49.9 (43.6, 56.5)	48.8 (43.2, 54.8)	41.3 (29.9, 56.1)	36.2 (26.4, 48.4)
C3) Basal primate	83.4 (76.3, 87.3)	86.5 (77.2, 92.6)	83.2 (76.8, 86.9)	71.9 (52.6, 96.6)	64.8 (50.9, 82.4)
C7) Canid/felid	59.8 (52.8, 62.9)	63.2 (55.4, 66.9)	61.3 (53.8, 64.9)	58.8 (40.5, 83.1)	53.3 (45.4, 64.1)

Narrowed (20%), original calibration bounds were narrowed by 20% for all calibration points; Expanded (20%), original calibration bounds were expanded by 20% for all calibration points; Narrowed primate, basal primate calibration (C3) bounds were narrowed to 75–87 Ma; Single primate, only gorilla/human calibration (C1) was employed in analysis; Single Outgroup, only canid/felid calibration (C7) was employed in analysis.

evolutionary properties across loci (Table 3). Evolutionary parameters are also notably distinct among codon positions in the protein-coding genes. As might be predicted, the α parameter, as well as κ and the average substitution rate, are considerably higher for third positions than for first and second positions. Additional discrepancies are observed in comparisons across markers, with the two intron markers vWF and TR showing the highest α values, and thus the lowest among-site rate variation, among all data partitions (Table 3). Clearly, analyses that can simultaneously integrate and accommodate these differences are to be preferred (Yang & Yoder 2003).

The posterior estimates were found to be highly similar between runs, indicating convergence. Figure 4 shows the states of divergence time estimates for the lemuriform clade (N9) over the MCMC iterations in four independent chains, which suggests that convergence is achieved extremely rapidly after only several hundred iterations. More iterations are needed for some other variables to converge (results not shown). The convergence diagnostics of Geweke (1992) and Raftery & Lewis (1992) indicate that the standard chains described above (20 000 samples after the burn-in, sampling every five generations) were more than adequate to achieve stationarity on the target distribution. The comparison of posterior with prior estimates reveals significant differences (e.g. the difference between 31.9 and 42.7 Ma for N7 in the 'all genes' combined analysis), thereby indicating that the sequence data have a major impact on the estimation of divergence dates.

We examined the sensitivity of the posterior estimates to prior ages at the calibration nodes. Narrowing or expanding the bounds at all calibration nodes by 20% had little

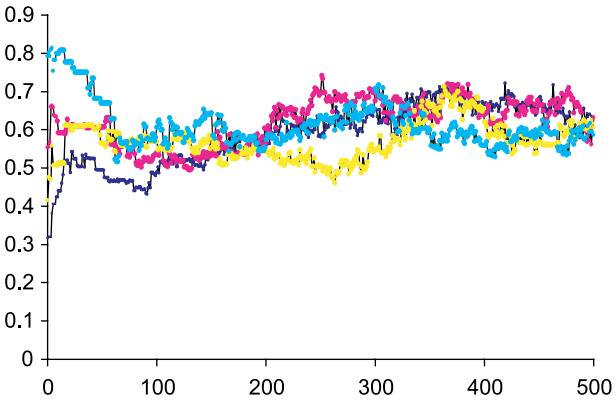

Fig. 4 Convergence of the Markov chain Monte Carlo algorithm in the combined analysis of all genes using eight site partitions [Table 4, All genes (8 partitions)]. The age of the root of the lemuriform clade (N9) is plotted against the iterations for four chains started from different places. 1.0 time unit on the y-axis represents 100 Ma.

effect on the posterior mean estimates but affected the credibility intervals for all estimated nodes (Table 5, columns 1 and 2). For example, the original prior bounds for C2 were between (32, 38) and the posterior interval was (32.1, 37.8). When the prior bounds were shrunk by 20% the prior bounds became (32.6, 37.4) and the posterior credibility interval became (32.6, 37.3) (compare Table 4, column for '8 partitions' with Table 5, column 2). Similarly, the prior bounds after a 20% expansion became (31.4, 38.6), with which the posterior interval became (31.7, 38.2) (compare Table 4, column for '8 partitions' with Table 5, column 3). The narrowing of the basal primate calibration bounds had

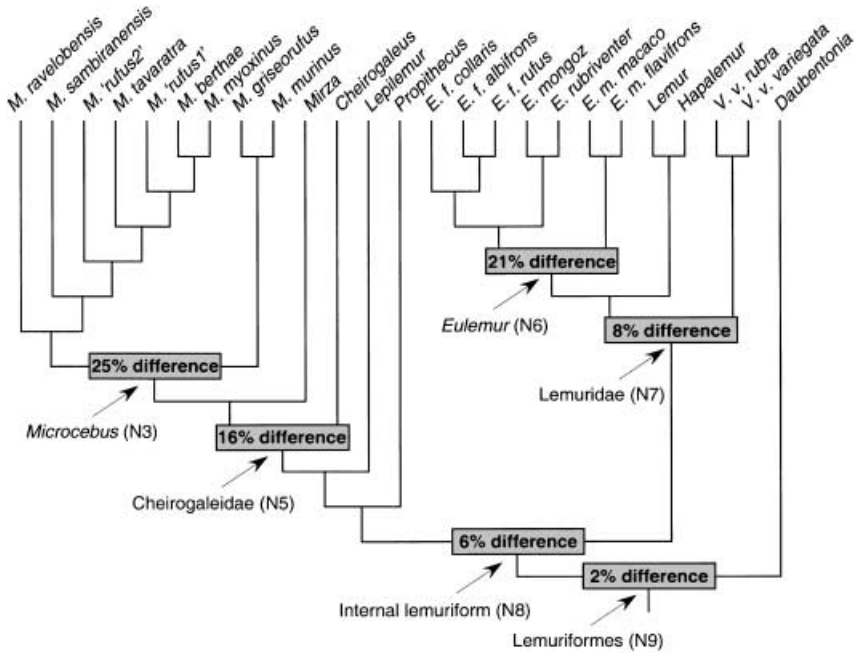

Fig. 5 Relative impact of 'complete' vs. 'two-species' taxon samples for mitochondrial DNA data set on date estimates. Ages from 'complete' taxon sample are consistently older than those estimated from 'two-species' taxon sample. Age differential is 3 years on average for all nodes. Thus, relative impact on age estimation is greatest for recent nodes and least for oldest nodes.

a negligible effect on the posterior mean age estimates or on credibility intervals (Table 5, column 3). As might be expected, the largest effects were observed when only a single calibration was employed. The effects were quite dramatic both in the case when a single primate calibration (C1) was employed and when a single outgroup calibration (C7) was employed. Without exception, divergence dates were estimated to be more recent than when all calibrations were used and credibility intervals were notably expanded. Given that this is also true for the otherwise calibrated nodes, for which abundant fossil information is available to confirm their antiquity, we interpret these results as strong evidence favouring the use of multiple calibration points when accurate age estimates are the goal. A particularly compelling case in point is the underestimation of the lorisiform divergence age (C8). Recent fossil discoveries by Seiffert *et al*. (2003) have confirmed that the basal divergence between galagos and lorizes was under way by at least 38–40 Ma. Therefore, the single calibration tests can be said unequivocally to underestimate the age of this node by at least 15 Myr (Table 5, columns 4 and 5).

Another discrepancy in age estimates is observed in the comparison of the 'complete' and 'two species' taxon samples for the mtDNA (Table 4, columns 1 and 2). In this case, we know that all sampling and data conditions are identical between the two analyses except for the density of taxon sampling within the mouse lemurs. The age estimates for lemuriform clades (N3–N9) differ on average by about 3 Myr, with the complete taxon sample consistently yielding older ages. Although the difference is minor for the deeper nodes in the tree, the impact becomes more severe as one progresses to the tips of the tree (Fig. 5). What is only a 2% differential at the base of the lemuriform radiation becomes a 25% differential for the mouse lemur radiation. We suspect that the effect is due to the prior of node ages used in the analysis. The model of Thorne & Kishino (2002) specifies the prior for divergence times by breaking down the path from a tip of the tree to the root (or ancestral node) into identically distributed segments. For example, in the complete data set, the path from *Microcebus murinus* to the root is broken into 12 segments/branches (see Fig. 1), with uniformly distributed lengths. Such a prior tends to push divergences within mouse lemurs and within the true lemurs to unrealistically old ages. Indeed, the prior mean ages for N3 and N9 are 8.1 and 58 Myr for the two-species data set while, for the complete data set, they are 39.7 and 65.5 Myr. The prior for divergence times seems to have an undue influence on the posterior estimates. We suggest that the estimates under the two-species data set are more reliable as the prior is more realistic. Depending on the desired level of precision required of the analysis, there is thus an alarming potential for unacceptable error due to sampling bias for recent evolutionary radiations. For example, if an investigator seeks to test for congruence of the mouse lemur radiation with a climatological event that occurred at precisely 9 Ma, then the potential error is unacceptably high. If, on the other hand, an investigator wishes to know if the mouse lemur radiation began in the Pleistocene, then either estimate would be sufficient to reject the Pleistocene speciation hypothesis.

When all calibrations are employed in the analyses, there is remarkable congruence in posterior mean date estimates across loci and analyses, despite the observed differences in molecular evolutionary properties and despite

concerns relating to the different sampling properties of each data set. For example, ages for nodes N4, N5, N6, N7 and N8 range from 19.1 to 24.2 Ma, 26.7 to 31.8 Ma, 6.6 to 14.6 Ma, 26.4 to 35.9 Ma and 42.2 to 46.7 Ma, respectively. Moreover, this congruence holds even in the face of different outgroup sampling for three of the four genetic loci. Our results, therefore, suggest that the analyses are robust to outgroup choice. There are, however, several notable exceptions to the overall pattern of congruence. For example, the estimated age of the anthropoid clade, containing monkeys, apes and humans, is markedly older for the mtDNA analyses (61.8–62.2 Ma) than for the nuclear and/or combined analyses. Also, the age of the lemuriform and the basal primate radiation is estimated by the TR data to be considerably more recent than the ages estimated by other loci. Presumably, this latter discrepancy relates to the fact that poor taxon sampling for this gene did not allow us to employ either the slow loris/galago divergence (Seiffert *et al*. 2003) or the gorilla/human divergence (Shoshani *et al*. 1996), both of which are arguably among the most precise and accurate available within the primate radiation. As demonstrated in Yang & Yoder (2003), both proximity to and accuracy of fossil calibrations can have a significant impact on the reliability of estimated node ages. Nodes that are in close proximity to accurate calibrations are most likely to be accurately estimated, just as nodes that are proximal to inaccurate calibrations will likewise be inaccurately estimated.

In discussing age estimates in the subsequent discussion, we refer primarily to the posterior mean point estimates. It should be noted, however, that the 95% credibility intervals (CI, shown in parentheses in Table 4) typically indicate a considerable amount of uncertainty regarding the point estimates. For example, the IRBP posterior mean estimate for the age of the lemuriform clade is 60.2 Ma, although the 95% CI indicates that this date could plausibly lie anywhere within an approximately 29-Myr interval. The reader should, therefore, keep this uncertainty in mind. That being said, it is to be noted that CIs narrow considerably in the two combined multilocus analyses (the 'three nuclear' genes and the 'all genes' analyses), due to the increased amount of data in these analyses. Furthermore, given the observed congruence among posterior estimates from these loci of differing evolutionary properties, we suspect that additional data would further substantiate the divergence times estimated here.

Age of lemuriform clades in geological context

Numerous phylogenetic studies have shown that the > 40 species of Malagasy lemurs constitute a single clade, all descending from a single common ancestor (e.g. Yoder 1994; Goodman *et al*. 1998; Pastorini 2000). Furthermore, it appears that this ancestor colonized Madagascar via waif dispersal from Africa (Yoder *et al*. 1996a, 2003; Martin 2000). Thus, Madagascar has served as the arena wherein the lemuriform radiation took place and, as such, commands special attention as an evolutionary laboratory for generating primate diversity. Madagascar is the world's fourth largest island, stretching 1600 km north–south from approximately 12 to 26° S. It is 600 km wide, at its widest point, yielding a total landmass of about 0.75×10^6 km^2. Due to its large surface area, and its varied assortment of microclimates and habitats, it is often referred to as a mini-continent (e.g. Tyson 2000; de Wit 2003). Much of Madagascar's ecological variation relates to its sharply asymmetrical topography. The eastern edge, where it was once conjoined with India, is ruggedly mountainous, abruptly rising from the Indian Ocean to attain elevations of 2000 m and is characterized by moist evergreen rainforest. Altitudes gradually diminish to sea level in the west, where the vegetation is predominated by dry deciduous forest. There, rainfall is sharply lower, with the extreme southwest receiving rainfall of less than 35 cm/year. The intervening central plateau is comprised primarily of depauperate grassland. Madagascar's varied terrain and climates, aided and abetted by its long-term geographical isolation, may well have contributed to its apparent capacity for generating biodiversity in some organismal groups. It is worth remembering, however, that Madagascar's geological and climatological conditions have not been static, evolving themselves just as has its biota. Until 160 Ma, Madagascar was contiguous with Gondwana and did not achieve true isolation until *c.* 88 Ma when it separated from India. Throughout this period, and well into the Cenozoic, Madagascar's geographical position has progressively shifted, as have the positions of the other Gondwana fragments. All of these events have had profound effects on the biotic and abiotic conditions in Madagascar. Therefore, the potential exists for reciprocal illumination when lemuriform divergence dates are examined within the framework of Madagascar's geological and climatological evolution.

In this study, we have sampled representatives of each of the five primary lineages (*sensu* Yoder 1997) within the lemuriform radiation. Thus, we are able to estimate the age of all of the fundamental divergence events within the clade. Moreover, we have extensive interspecies level sampling within two of these lineages, the cheirogaleids (dwarf and mouse lemurs) and the lemurids (true lemurs). Although we lack comparable taxon sampling for other speciose lineages, namely *Lepilemur*, the phylogenetic coverage within the lemuriform clade is sufficient to take a comprehensive view of this remarkable evolutionary radiation, asking if there are indications of fit between divergence ages and significant climatological events in Madagascar. Observed correlations between divergence age and geological conditions can, therefore, be interpreted as tentative hypotheses to be subjected

to further external testing. For clarity of discussion, and because we believe on first principles that the 'all genes' combined analysis is likely to yield the most accurate divergence ages, we will limit the following discussion to these age estimates (illustrated in the last column in Table 4 and in Fig. 3). We begin our discussion at the base of the lemuriform clade, working our way to the tips.

The initial radiation of lemuriform primates (N9; Fig. 3) is estimated to have occurred approximately 62 Ma, near the onset of the Tertiary. This is a surprisingly ancient date, as it precedes the appearance of euprimates in the global fossil record. Indeed, if we were to base our judgements of primate antiquity on a strict interpretation of the known fossil record, this estimate of lemuriform antiquity would be considered incredible. Instead, increasing numbers of primatologists and palaeontologists concur that the fossil record is far too scant and 'frighteningly incomplete' (Fleagle 2002) to impose strict limits on our interpretation of the temporal context for primate evolution. Therefore, our finding that the ancestral lemuriform had colonized Madagascar by the earliest Tertiary is not particularly controversial for its temporal implications. It is somewhat remarkable, however, that this unique event occurred at a time of virtually unprecedented geological and climatological upheaval. It is now widely accepted that the Cretaceous-Tertiary (K/T) boundary was contemporaneous with a massive bolide impact and extensive volcanism that, in turn, led to global mass extinctions and extremely rapid faunal turnover (Kerr 1996; Macleod *et al*. 1997; Mukhopadhyay *et al*. 2001; Beerling *et al*. 2002) although the relative impact that these events had on the biota of the southern hemisphere continues to be debated (Johnson 1993; Vajda *et al*. 2001). Thus, the idea that a presumably small and struggling founding population of ancestral lemurs was able to take hold and flourish in Madagascar during this period of geological upheaval is notable. In fact, lemurs may well have been among the first pioneers of the apparent faunal turnover of Malagasy vertebrates at the K/T boundary suggested by the fossil record (Krause *et al*. 1997b, 1999; Rogers *et al*. 2000). Madagascar's relative global position at this time may have factored into this somewhat unlikely colonization event. In the early parts of the Palaeocene, the island was far enough south such that it lay in the path of prevailing winds which would have favoured waif dispersal of small vertebrates from Africa to Madagascar (Krause *et al*. 1997a).

Our analyses indicate that the next divergence event within the lemuriform radiation (N8) did not occur until approximately 43 Ma, more than 20 Myr after the inferred colonization of Madagascar. This result is surprising in that it implies that there were only two lemuriform lineages existing within Madagascar for this protracted evolutionary period. In fact, we question the accuracy of this result, not calling into question the accuracy of the time interval, but rather we suspect the possibility that unrecorded lineage extinctions may explain this apparent lineage diversification vacuum. It is possible that certain of the giant 'subfossil' lemuriforms, all of which succumbed to extinction during the Holocene, will ultimately be shown to radiate from the inordinately long branch subtended by nodes N9 and N8, although early indications from ancient DNA analysis of *Megaladapis* and *Palaeopropithecus* do not as yet bear out this prediction (Yoder *et al*. 1999). As for fit with the palaeoclimatological features of the geological period, spanning all of the Palaeocene and much of the Eocene, it is reasonable that this might have been a time of relative evolutionary stasis. Although the Palaeocene from 59 to 50 Ma was a period of pronounced global warming, followed by a 16-Myr period of global cooling (Zachos *et al*. 2001), Wells (2003) has suggested that Madagascar was confined to low latitudes during this period, which should have kept it largely or entirely within the 'desert belt' wherein the island would have experienced extensive dry conditions. This is in distinct contrast to its present state wherein the eastern escarpment is markedly wet and characterized by succulent rainforest habitat.

The long period of apparent evolutionary stasis ends in the late middle Eocene. It appears that the lemuriforms began their radiation in earnest at approximately 42 Ma, with the common ancestor of all extant lineages except *Daubentonia* occurring at this point. Notably, this is contemporaneous with the initial radiation of lorisiform primates in Africa (Seiffert *et al*. 2003) and with the divergence of the five major clades of squirrels (Mercer & Roth 2003). From this node (N8), the following major divergence events, yielding the indriid, lepilemurid, cheirogaleid and lemurid lineages, occurred within a period of approximately 10 Myr. Indeed, this relative acceleration in lineage diversification coincides precisely with that portion of the lemuriform clade that has proven problematic with regard to phylogeny estimation (Yoder *et al*. 1996a; Yoder 1997; Stanger-Hall & Cunningham 1998). It also coincides with the 10-Myr interval that has been described as 'the most significant episode of climatic change and extinction since the end of the Cretaceous, with the exception of the Palaeocene/Eocene boundary event' (Berggren & Prothero 1992, p. 1). This period saw major changes in global climate and ocean circulation, reflected in notable turnovers in both marine and terrestrial biota, and with a major extinction event taking place at around 40–41 Ma. The latter date is nearly coincident with the time that we infer lineage diversification to have accelerated within the lemuriforms.

From this period of accelerated diversification, it appears that speciation events within the lemuriforms continue at a relatively vigorous pace for much of the Oligocene and Miocene periods. Notably, both the cheirogaleid and lemurid radiations have their origins at very near the Eocene/Oligocene boundary. The lemurid radiation (N7)

is estimated at 31.9 Ma, while the cheirogaleid radiation (N5) is identified at 29.0 Ma (Table 4). It is important to stress here, however, that, for the cheirogaleids, the estimated age is certain to be an underestimate given that the basal lineage of that clade, *Phaner furcifer* (Pastorini *et al*. 2001), was not sampled by our study. In all likelihood, the ages of these two radiations are even more similar than indicated in Table 4 and the coincidence with the Eocene/Oligocene boundary may well be significant. There is ample evidence that this geological period saw dramatic climatic changes, both globally and in Madagascar. Globally, there is an abrupt 'aberration', defined as 'brief (~10^3–10^5 year) anomalies that stand out well above "normal" background variability' (Zachos *et al*. 2001; p. 690), at approximately 34.0 Ma with dramatic cooling and the sudden appearance of large continental ice sheets on Antarctica. This is generally recognized as the most significant cooling event of the entire Eocene–Oligocene period, with a concomitant change in the composition of land floras dating to about 33.5 Ma (Berggren *et al*. 1992). The cooling would have been especially felt in the southern hemisphere with the establishment of significant ice sheets on Antarctica and with the development of the cool circum-Antarctic oceanic circulation created by the separation of Antarctica and Australia (Leclaire 1974). Somewhat paradoxically, this period of marked global cooling coincides with the time that Madagascar would have passed north of 30° S latitude to enter the warmer climates of the subtropical zone (Smith *et al*. 1994). In so doing, it entered the Trade Wind zone of the Indian Ocean and, as that happened, the prevailing winds should have set up the principle climatological conditions that persist to this day. Then, as now, India would have cleared the north-centre of the Indian Ocean, thus exposing Madagascar's eastern shores. Given that modern Indian Ocean circulation derives fairly logically and simply from the present geographical configurations of surrounding land masses and that these geographical configurations were essentially established at the onset of the Oligocene (Smith *et al*. 1994), it is reasonable to hypothesize that present-day climatological conditions were similarly established in the early Oligocene (Wells 2003). The eastern on-shore breezes would have conspired with the elevated and abrupt topology of Madagascar's east coast to sequester the moisture carried across the Indian Ocean by the trade winds. This, therefore, was putatively the precursor of the rain shadow effect presently operating and supporting the eastern rainforests, although palaeobotanical data are as yet unavailable to confirm this hypothesis. Until such data are available, additional phylogeny-based divergence time estimates, particularly of the eastern Madagascar plant communities, may serve as additional tests of the hypothesis.

It appears that the majority of lemuriform species presently extant in Madagascar had their origins prior to the late Miocene. Although species level sampling is somewhat sparse in our study, with only about half of recognized species represented, we have appropriate taxonomic and gene sampling to allow for a multilocus estimate of the antiquity of the *Eulemur* radiation at approximately 9.7 Ma. For the genus *Microcebus*, we have a complete species-level sample but only for the mtDNA and for one nuclear (vWF) locus. The mtDNA age estimates of 12.0–9.0 Ma and the vWF estimate of 8.7 Ma for the initial diversification of *Microcebus* species are notably congruent with the 'all genes' combined estimate of 9.7 Ma for *Eulemur*, although they are considerably older than the 8.4–6.6 Ma mtDNA estimates. In the more directly comparable 'all genes' combined analysis, the ages of the two clades differ by less than 1 Myr. Although additional genetic sampling is required for more detailed comparisons, it seems clear that neither clade is younger than the late Miocene, thus neither group fits with the Pleistocene speciation models that have been proposed for so many other groups from a variety of geographical localities (Alemseged 2003; Cardini 2003; Eastwood & Hughes 2003; Veith *et al*. 2003). In summary, we estimate that the preponderance of lineage diversification within both the cheirogaleid and lemurid clades, from their basal divergence up through the most recent speciation events, occurred predominantly in the Miocene, a period of strong and rapid (100–400 kyr) cycles of global climatic fluctuations (Zachos *et al*. 2001).

Implications of age estimates for speciation mechanisms in lemurs

Unless and until we know the precise age of an evolutionary radiation, it is impossible to quantify absolute rates of evolution for an organismal trait, be it morphological, behavioural, genetic or physiological and until we are able to place individual radiations in a comparative hierarchical framework, we cannot possibly identify rates of relative change in these same traits. Ideally then, we wish to examine patterns of character transformation within a comparative framework wherein history is controlled (i.e. the traits are examined within a clade) and clade ages are known. We are approaching such a state for comparisons among and between *Eulemur* and *Microcebus* species, thus finding ourselves in a radically different analytical framework than that articulated by Tattersall & Sussman (1998) at which time we had 'no idea whatever about the timescale on which the most recent round of speciations occurred in Madagascar' (p. 386).

Given current taxonomy, these two genera are the most speciose among all of the lemuriforms (with the possible exception of genus *Lepilemur*), with *Eulemur* presently comprising at least five species, and *Microcebus* comprising at least eight species. The species count for *Eulemur* has remained nearly stable for at least the past 20 years (Tattersall 1982) while that for *Microcebus* has changed dramatically in

the past several years. With increased field and morphometric scrutiny (Schmid & Kappeler 1994; Zimmermann *et al*. 1998; Rasoloarison *et al*. 2000), coupled with molecular phylogenetic investigation (Yoder *et al*. 2000, 2002), a remarkable amount of evolutionary diversity has been revealed among the mouse lemurs. Ostensibly, the differences in taxonomic stability relate to the fact that the various *Eulemur* species are readily identifiable with reference to their variety of colouration patterns and other morphological features, while *Microcebus* species are not. Given that we now infer that their temporal origins are so nearly contemporaneous, why should rates of apparent morphological evolution have been markedly more rapid in one genus than in the other? We suspect that the answer relates to the fact that *Eulemur* is primarily diurnal while *Microcebus* is strictly nocturnal.

For mammals, visual signals will be most efficiently transmitted and received by day and other signals, such as acoustic or olfactory, will be required for nocturnal signalling. Numerous studies have investigated the olfactory and auditory communication among mouse lemurs, finding that the signals communicated are both powerful and nuanced (Perret & Schilling 1995; Perret 1996; Zimmermann *et al*. 2000; Zimmermann & Hafen 2001). Although the evidence is presently limited, there are indications that olfactory and auditory signalling in *Eulemur* is weaker and less informative than it is for *Microcebus*. The opposite is true for visual signalling, however. Whereas there is no sexual dichromatism in mouse lemur species, it is pervasive among *Eulemur*, with all five named species showing marked patterns of differential colouring between males and females (for detailed illustrations of these differences, see Mittermeier *et al*. 1994). The sensory drive hypothesis predicts that mate choice criteria will tend to mirror the signal transmission favoured in a given environment (Endler 1992; Jones 1997). Thus, the idea that activity patterns (diurnal vs. nocturnal in this case) will influence signalling and that sensory signalling will influence the evolution of mate choice criteria can potentially be applied to the comparison of rates of character change in *Eulemur* and *Microcebus*. Furthermore, these correlations may ultimately provide a powerful explanatory framework for determining why mouse lemur species are 'cryptic', *sensu* Jones (1997).

The comparisons are preliminary and based on incomplete data. In other words, our understanding of gender- and species-specific *Microcebus* vocalization patterns and olfactory signalling is based on directed investigations that have simply not been conducted for *Eulemur*. Thus, it is conceivable that similar nuances exist within the auditory and chemosensory signals of *Eulemur* but have simply not been described. Nonetheless, given our present state of knowledge, the observed patterns are compatible with the predictions of the sensory drive hypothesis. As would be predicted, mate choice criteria appear to have evolved in the directions favoured by sensory characteristics, with male-specific olfactory and auditory signals having been enhanced in the nocturnal species (*Microcebus*) and visual signals having been enhanced in the primarily diurnal species (*Eulemur*). Also, as has been observed in numerous other studies of closely related species radiations (Jones 1997; Bromham *et al*. 2002; Losos & Miles 2002), it appears that rates of morphological, behavioural and genetic divergence have been only loosely correlated during and after speciation in these two groups of lemurs.

Conclusions

In this study, we have employed several genetic markers of different evolutionary properties and sampling densities for estimating divergence dates in an insular radiation of primates. The markers differ qualitatively in the density of taxon sampling, amount of missing data and in the phylogenetic proximity of the outgroup to the ingroup. Despite these discrepancies, we observe a remarkable congruence of date estimates across loci and analyses. This leads us to conclude that Bayesian methods are capable of revealing the underlying historical signal contained within a given data set, despite rate heterogeneity among sites and lineages and despite a variety of vagaries in sampling design. Even so, although congruence among markers is reassuring, only congruence with external data can ultimately confirm accuracy. In our case, we had the good fortune to have the age of at least one node (the divergence between lorizes and galagos, C8 in Figs 1 and 3) confirmed by an important fossil discovery (Seiffert *et al*. 2003) during the course of our analysis. In a previous study (Yang & Yoder 2003), Bayesian estimates from a combined mitochondrial analysis indicated that the lorisiform divergence occurred approximately 40.5 Ma. This estimate was remarkable in that it was nearly twice as old as then-current palaeontological estimates but has now been shown to be perfectly congruent with the revised palaeontological estimate of 38–42 Ma (Seiffert *et al*. 2003). Thus, a single palaeontological discovery has increased our confidence in the methods and has provided valuable calibration information for the subsequent analyses presented herein.

Our study, therefore, represents the most confident estimates yet available for clade ages within the Malagasy lemuriforms. In turn, this has allowed us to look for correlations with the geological and climatological record in the interest of identifying the potential effects of climate on patterns of lineage diversification in these primates. Needless to say, these correlations must be viewed as tentative, given the imprecision in our age estimates (as indicated by the wide credibility intervals) and for the rather broad geological periods within which we have drawn our comparisons. Another outcome of the study has been our ability to compare the ages of the two most speciose radiations

within the five primary lemuriform lineages. Contrary to expectations that might arise from morphological comparisons alone, we find that these two groups, the mouse lemurs (*Microcebus*) and true lemurs (*Eulemur*), are of surprisingly similar age. In turn, these age estimates have allowed us to ask questions concerning rates and degree of change in the sexual signalling characteristics typical of the two groups. We find, as might be expected (Endler 1992; Jones 1997; Boughman 2002), that nocturnal species have emphasized olfactory and auditory signalling over visual signalling, with the reverse being true of the predominantly diurnal species. Increased amounts of genetic and fossil data and further development of the methods will permit more refined tests of the evolutionary correlations described above.

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Anne Yoder has long been interested in Madagascar as a natural laboratory for generating vertebrate diversity. She has used phylogenetic methods to reconstruct the evolutionary history of lemurs and other Malagasy vertebrates. Ziheng Yang is primarily interested in developing models and methods for phylogenetic applications and for understanding the mechanisms of molecular evolution. They enjoy combining their respective empirical and theoretical skills to address issues of primate evolution.
