# Primate Phylogeny, Evolutionary Rate Variations, and Divergence Times: A Contribution From the Nuclear Gene IRBP

Céline Poux[1,2] and Emmanuel J.P. Douzery[1]*

[1]*Laboratoire de Paléontologie, Paléobiologie et Phylogénie-CC064, Institut des Sciences de l'Evolution UMR 5554/CNRS, Université Montpellier II 34095 Montpellier, France*
[2]*Department of Biochemistry, University of Nijmegen, 6500 HB Nijmegen, The Netherlands*

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*ABSTRACT* The first third (ca. 1200 bp) of exon 1 of the nuclear gene encoding the interstitial retinoid-binding protein (IRBP) has been sequenced for 12 representative primates belonging to Lemuriformes, Lorisiformes, Tarsiiformes, Platyrrhini, and Catarrhini, and combined with available data (13 other primates, 11 nonprimate placentals, and 2 marsupials). Phylogenetic analyses using maximum likelihood on nucleotides and amino acids robustly support the monophyly of primates, Strepsirrhini, Lemuriformes, Lorisiformes, Anthropoidea, Catarrhini, and Platyrrhini. It is interesting to note that 1) Tarsiidae grouped with Anthropoidea, and the support for this node depends on the molecular characters considered; 2) Cheirogaleidae grouped within Lemuriformes; and 3) *Daubentonia* was the sister group of all other Lemuriformes. Study of the IRBP evolutionary rate shows a high heterogeneity within placentals and also within primates. Maximum likelihood local molecular clocks were assigned to three clades displaying significantly contrasted evolutionary rates. Paenungulata were shown to evolve 2.5–3 times faster than Perissodactyla and Lemuriformes. Six independent calibration points were used to estimate splitting ages of the main primate clades, and their compatibility was evaluated. Divergence ages were obtained for the following crown groups: 13.8–14.2 MY for Lorisiformes, 26.5–27.2 MY for Lemuroidea, 39.6–40.7 MY for Lemuriformes, 45.4–46.7 MY for Strepsirrhini, and 56.7–58.4 MY for Haplorrhini. The incompatibility between some paleontological and molecular estimates may reflect the incompleteness of the placental fossil record, and/or indicate that the variable IRBP evolutionary rates are not fully accommodated by local molecular clocks. Am J Phys Anthropol 124:1–16, 2004. 

Primates form one of the 18 orders of placental mammals identified by morphology, paleontology (Novacek, 1992a), and molecular data (de Jong, 1998). The extant sister group of primates is not clearly identified. On morphological bases such as ankle structure (Novacek, 1992b, 1994), primates are grouped with Chiroptera (microbats and megabats), Scandentia (tree shrews), and Dermoptera (flying lemurs) into Archonta. Molecular studies suggest the polyphyly of this superorder due to the inclusion of Chiroptera (Adkins and Honeycutt, 1991; Stanhope et al., 1992; Bailey et al., 1992; Ammerman and Hillis, 1992; Allard et al., 1996; Murphy et al., 2001a). After the exclusion of Chiroptera, the phylogenetic relationships between the three remaining suborders (Euarchonta, Waddell et al., 1999) are still debated. Furthermore, in recent molecular studies, primates are either linked with Glires (Madsen et al., 2001), or are not monophyletic (Murphy et al., 2001a; Arnason et al., 2002) due to the inclusion of Dermoptera (but for contrasting results, see Eizirik et al., 2001). The newest survey (Murphy et al., 2001b) displays strong support for primates as sister group of Dermoptera plus Scandentia. This question might be clarified by an extensive taxon sampling of both primates and nonprimate placentals.

Despite the abundance of studies on primates, some questions about their phylogeny remain unanswered. The most debated point is about the evolutionary position of Tarsiiformes. Indeed, primates are divided into two suborders, of which the taxon

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*Correspondence to: Emmanuel J.P. Douzery, Laboratoire de Paléontologie, Paléobiologie Phylogénie-CC064, Institut des Sciences de l'Evolution UMR 5554/CNRS, Université Montpellier II, Place E. Bataillon, 34095 Montpellier Cedex 05 France.
E-mail: douzery@isem.univ-montp2.fr

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content differs according to the phylogenetic position of tarsiers. *Tarsius* shares morphological characters with lemuriforms, lorisiforms, and anthropoids. Consequently, *Tarsius* is either grouped with Malagasy lemuriforms and Afro-Asian lorisiforms into prosimians, while the remaining primates form the simians (Rowe, 1996; Murphy et al., 2001a,b), or *Tarsius* is associated with anthropoids (represented by Afro-Asian catarrhines and South American platyrrhines) to form the haplorrhines (Goodman et al., 1998; Zietkiewicz et al., 1999; Schmitz et al., 2001), while other primates (lemuriforms and lorisiforms) are classified into strepsirrhines. The latter group presents many morpho-anatomical synapomorphies (the dental toothcomb and the laterally flaring talus) and symplesiomorphies (the moist rhinarium, the tapetum, and the bicornuate uterus) (Fleagle, 1999).

Within strepsirrhines, both lorisiforms and lemuriforms appear monophyletic (Yoder et al., 1996a,b; Yoder, 1997; Goodman et al., 1998). In the lorisiform clade, Galagonidae is monophyletic, whereas the status of Loridae is controversial between morphological and molecular studies (Yoder et al., 2001). In the lemuriform clade, each family (Lemuridae, Cheirogaleidae, Daubentoniidae, Indridae, and Lepilemuridae) is well-defined; nevertheless, the relationships among them can be represented as a multifurcation, with different studies suggesting different phylogenetic relationships (for a review of molecular studies, see Yoder, 1997). The phylogenetic status of two families has been highly controversial. First, dwarf and mouse lemurs (Cheirogaleidae) are endemic to Madagascar, and are considered lemuriforms, but the anatomy of their ascending pharyngeal artery would suggest associating them with lorisiforms (Szalay and Delson, 1979; but see Yoder, 1994). Second, the aye-aye (Daubentoniidae: *Daubentonia*) is considered the sister group of either all other lemuriformes (Yoder et al., 1996a, 2003) or strepsirrhines on the basis of morphology (Groves, 1989) and mitochondrial molecules (Adkins and Honeycutt, 1994; Arnason et al., 1998). The latter hypothesis would involve a double event of colonization of Madagascar by lemuriforms, a biogeographic scenario that is refuted by other molecular studies (Yoder, 1994, 1997; Yoder et al., 1996a, 2003).

About platyrrhines (the South American anthropoids), molecular phylogenies are not in agreement with morphological classifications. Platyrrhines (i.e., ceboids) are traditionally divided into the two families Callitrichidae (marmosets and tamarins) and Cebidae (cebids) (Rowe, 1996). Molecular studies tend, however, to demonstrate that cebids are paraphyletic, because the Cebinae subfamily would group with callitrichids (Goodman et al., 1998). The relationships among Pithecidae (e.g., *Pithecia*), Atelidae (e.g., *Ateles*), and Cebidae (Cebinae, e.g., *Cebus* or *Saimiri,* and Callitrichinae, e.g., *Callithrix*) remain unresolved (Goodman et al., 1998; Canavez et al., 1999; von Dornum and Ruvolo, 1999; Schneider, 2000; Schneider et al., 2001). A composite primate phylogeny reconstructed from many independent studies also found different results (Purvis, 1995).

The timing of the evolution of primates is also debated. For example, mitochondrial markers used to assess primate divergence dates display ages far more ancient than the paleontological ones (Arnason et al., 1998, 2000; Yoder and Yang, 2000; vs. Gingerich, 1984; Gingerich and Uhen, 1994; Fleagle, 1999; Rosenberger et al., 1991). Gaps in the fossil record of primates and variation in molecular evolution rates between lineages, e.g., faster rates in anthropoid primates (Adkins and Honeycutt, 1994; Andrews et al., 1998; Andrews and Easteal, 2000; Liu et al., 2001) and slower rates in strepsirrhines (Yoder et al., 1996a), might explain this observation. Because the absence of a global molecular clock still remains one of the most limiting factors of molecular dating, two different approaches were recently proposed and applied to estimate primate speciation ages. Local molecular clocks in a maximum likelihood framework allocate independent substitution rates to groups that evolve at different rates (Yoder and Yang, 2000), and relaxation of the molecular clock is achieved in a Bayesian framework to describe evolutionary rate variation along tree nodes (Thorne et al., 1998; Yoder et al., 2003).

To reconstruct the phylogeny of primates and to estimate their divergence times, we focused on a nuclear marker because of its potential resolving power relative to mitochondrial genes at deep (Springer et al., 2001) and ordinal (DeBry and Sagel, 2001) levels. The nuclear gene for the interstitial retinoid-binding protein (IRBP) encodes a 140-kD protein involved in vision. It passively carries the retinol from photoreceptors to the pigmented epithelium, where it is transformed in retinal (references in Nickerson et al., 1998), but its exact function is not yet well-established. The IRBP gene is exclusively present in the genome of vertebrates (Borst et al., 1989). It is located on human chromosome 10 (Fong et al., 1990) and comprises four exons. In mammals, the first exon is formed by three repeated regions (each corresponding to ca. 300 amino acids) and the beginning of a fourth region (Wagenhorst et al., 1995). The first 1.2 kb of exon 1, which cover the first and half of the second repeat, were sequenced for a variety of placental and marsupial mammals, and are widely used to reconstruct their phylogeny (e.g., Stanhope et al., 1992, 1996; Springer et al., 1997; Jansa and Voss, 2000; DeBry and Sagel, 2001; Madsen et al., 2001; Huchon et al., 2002; Mercer and Roth, 2003). IRBP displays several advantages: 1) it is a single-copy nuclear gene, and no other gene belonging to the same potential family has been identified (Borst et al., 1989); 2) the high degree of divergence between the repeats (up to 60%) avoids confusion between them, and allows us to compare orthologous sequences; 3) the coding nature of the

*TABLE 1. Latin and common names, tissue collectors, and IRBP EMBL-GenBank-DDBJ accession numbers for primate taxa used in this study*

| Latin name | Common name | Collector or reference | Accession no. |
|---|---|---|---|
| Anthropoidea | | | |
| Catarrhini | | | |
| *Hominoidea* | | | |
| *Hylobates lar*[1] | White-handed gibbon | Ulfur Arnason | AJ313478 |
| *Homo sapiens* | Human | Fong et al., 1990 | J05253 |
| *Cercopithecoidea* | | | |
| *Cercopithecus solatus*[1] | Sun-tailed guenon | Jean-Pierre Hugot | AJ313477 |
| *Macaca mulatta*[1] | Rhesus macaque | Dr. M. Brack, Deutsches Primaten Zentrum | AJ313476 |
| Platyrrhini | | | |
| *Ateles paniscus*[1] | Black spider monkey | Jean-François Mauffrey | AJ313474 |
| *Callithrix jacchus*[1] | Common marmoset | Dr. M. Brack, Deutsches Primaten Zentrum | AJ313472 |
| *Cebus apella*[1] | Brown capuchin | Jean-François Mauffrey | AJ313473 |
| *Pithecia pithecia*[1] | White-faced saki | Faune Sauvage (EDF-CNEH) | AJ313475 |
| *Saimiri sciureus* | Common squirrel monkey | Yoder et al., 2001 | AF271424 |
| Tarsiiformes | | | |
| *Tarsius bancanus* | Western tarsier | Yoder et al., 2001 | AF271423 |
| *Tarsius syrichta* | Philippine tarsier | Stanhope et al., 1992 | Z11806 |
| Strepsirrhini | | | |
| Lemuriformes | | | |
| *Lemuroidea* | | | |
| *Lemur catta*[1] | Ring-tailed lemur | Mr. Combes, Zoo Montpellier | AJ313470 |
| | | Yoder and Irwin, 1999 | AF081058 |
| *Hapalemur griseus* | Lesser bamboo lemur | Yoder and Irwin, 1999 | AF081057 |
| *Varecia variegata* | Ruffed lemur | Yoder and Irwin, 1999 | AF081056 |
| *Eulemur mongoz* | Mongoose lemur | Yoder and Irwin, 1999 | AF081064 |
| *Microcebus murinus*[1] | Gray mouse lemur | Noëlle Bons | AJ313469 |
| | | Yoder and Irwin, 1999 | AF081054 |
| *Cheirogaleus major* | Greater dwarf lemur | Yoder et al., 2001 | AF271421 |
| *Propithecus verreauxi*[1] | Verreaux's sifaka | R. Albignac | AJ313471 |
| *Propithecus tattersalli* | Tattersall's sifaka | Yoder and Irwin, 1999 | AF081053 |
| *Daubentonioidea* | | | |
| *Daubentonia madagascariensis*[1] | Aye-aye | Michel Tranier, Muséum National | AJ313468 |
| | | d'Histoire Naturelle; Yoder et al., 2001 | AF271422 |
| | | | AF271422 |
| Lorisiformes | | | |
| *Nycticebus coucang*[1] | Slow loris | Ole Madsen | AJ313467 |
| | | Yoder et al., 2001 | AF271419 |
| *Loris tardigradus* | Slender loris | Yoder et al., 2001 | AF271418 |
| *Perodicticus potto* | Potto | Yoder et al., 2001 | AF271420 |
| *Otolemur crassicaudatus* | Thick-tailed greater bush baby | Stanhope et al., 1992 | Z11805 |
| *Galagoides demidoff* | Demidoff's bush baby | Yoder et al., 2001 | AF271416 |
| *Galago moholi* | Southern lesser bush baby | Yoder et al., 2001 | AF271415 |

[1] Taxa sequenced in present study.

IRBP sequence allows comparison of taxa at different taxon levels, e.g., within mammalian orders (Jansa and Voss, 2000; DeBry and Sagel, 2001), including primates (Yoder and Irwin, 1999; Yoder et al., 2001, 2003); and 4) a large dataset of mammalian sequences is available. Here, we use the nuclear gene for IRBP to study the phylogenetic relationships within all major clades of primates. We provide evidence of marked evolutionary rate differences for this gene in primates, and we apply a maximum likelihood approach based on local molecular clocks to estimate the divergence times of their major clades.

## MATERIALS AND METHODS

### Taxon sampling

We incorporated 25 primate species in our analyses, with at least one representative for each family, except Pongidae (Table 1). We added two marsupials (*Didelphis virginiana:* Virginia opossum, and *Macropus giganteus:* eastern gray kangaroo), and 11 nonprimate placentals sampled from the four placental lineages identified by Murphy et al. (2001a): 1) Afrotheria, represented by *Dugong dugon* (dugong), *Elephantulus rufescens* (rufous elephant shrew), *Loxodonta africana* (African elephant), and *Procavia capensis* (rock hyrax); 2) Xenarthra: *Bradypus tridactylus* (pale-throated three-toed sloth); 3) Euarchontoglires (Euarchonta + Glires), including potential sister groups of primates: Dermoptera (*Cynocephalus variegatus:* Malayan flying lemur), Scandentia (*Tupaia glis:* common tree shrew), Lagomorpha (*Oryctolagus cuniculus:* European rabbit), and Rodentia: (*Mus musculus:* house mouse); and 4) Laurasiatheria, represented by Perissodactyla: *Equus caballus* (horse) and *Tapirus pinchaque* (mountain tapir). This taxonomic sampling is justified by the fact that primates belongs to the unambiguously monophyletic Euarchontoglires (Madsen et al., 2001; Murphy et al., 2001a,b; Poux et al., 2002). Laurasiatherians, the xenarthran, afrotherians, and the marsupials are included as succes-

sively more distant outgroups, and the choice of the laurasiatherian and afrotherian representatives is dictated by their ability to provide paleontological calibration points for molecular dating (see below).

### DNA amplification and sequencing of exon 1 of IRBP

DNA of 12 primate species (Table 1) was extracted from tissue samples in the collection of 95% ethanol-preserved mammalian tissues of the Institut des Sciences de l'Evolution (Catzeflis, 1991).

Nucleotide sequences were obtained for the partial exon 1 of the IRBP gene (1278 bp, corresponding to positions 25–451 of the protein) by polymerase chain reaction (PCR). Two fragments with 300 overlapping base pairs (bp) were amplified: I1/J2 (827 bp) and I2/J1 (931 bp), using primers I1 (5′- ATGGCCAAGGTCCTCTTGGATAACTACTGCTT-3′), J1 (5′-CCACTGCCCTCCCATGTCTG-3′), I2 (5′-ATCCCCTATGTCATCTCCTACYTG-3′), and J2 (5′-CGCAGGTCCATGATGAGGTGCTCCGTGTCCTG-3′).

PCR reactions were performed using the followings parameters: 29 cycles with 94°C denaturation (20 sec), 47°C annealing (30 sec), 68°C extension (2 min), and one final cycle of 68°C extension (10 min). A minimum of two PCR products was pooled and excised from a 1% agarose gel in TAE 1 × buffer, and then purified on Ultrafree-DA Amicon columns (Millipore), and reconcentrated on Microcon filterable columns (Millipore). Manual sequencing was conducted using the dideoxy chain termination method with [$\alpha^{33}$P-ddNTP] and the Thermo Sequenase cycle sequencing kit (Amersham). PCR fragments were sequenced on both strands with I1/J2 and I2/J1 external primers and with internal primers I5 (5′-GCCCTGGACCTCCAGAAGCTGAGGATMGG-3′) and J5 (5′-CARGGTCCAGATCTCYGTGGT-3′).

### Phylogenetic analyses

IRBP sequences were aligned by hand with the ED editor of the MUST package (Philippe, 1993), version 2000. Sites not sequenced for more than 75% of the taxa were removed from subsequent analyses. Other nonsequenced positions and gap sites were coded as missing data. Phylogenetic reconstructions were performed by maximum likelihood (ML) with PAUP* (Swofford, 2001), version 4, beta 8. ML was exclusively used because it is a powerful technique based on explicit models of sequence evolution that allows statistical testing of alternative phylogenetic hypotheses (Whelan et al., 2001).

The ML assumptions included a general time reversible (GTR) model of nucleotide sequence evolution, and an eight-category Gamma distribution ($\Gamma_8$) to describe the substitution rate heterogeneities between sites (Yang, 1996a). Maximum likelihood parameters of the GTR + $\Gamma_8$ model were estimated by PAUP*, and the highest-likelihood topology was identified after an ML heuristic search conducted with a neighbor-joining (NJ) starting tree, and tree bisection-reconnection (TBR) branch swapping. The stability of the nodes was estimated by bootstrap (Felsenstein, 1985), with 500 replicates of heuristic searches (NJ starting trees, ML parameters identically set to their optimal value for each replicate, and TBR branch swapping with limitation to 1,000 rearrangements per replicate). ML analysis of amino acids was conducted with PAML (Yang, 1997), version 3.0d, using nearest-neighbor interchange (NNI) branch swapping.

The choice of the model of DNA evolution was justified because the log-likelihood of the best ML tree estimated by PAUP* increases from $\ln L = -13{,}383.70$ in the HKY model, to $-13{,}383.61$ in the TN93 model ($\delta = 0.09$; $P = 0.67$ for the significance of the more complex model under the likelihood ratio test), $-13{,}372.74$ in the GTR model ($\delta = 10.87$; $P < 0.001$), $-12{,}610.55$ in the GTR + $\Gamma_8$ model ($\delta = 762.19$; $P < 0.001$), and $-12{,}608.53$ in the GTR + $\Gamma_8$ model with a fraction of invariable sites ($\delta = 2.02$; $P = 0.05$). To homogenize analyses between PAUP* and PAML, we used the GTR + $\Gamma_8$ model for all DNA analyses, the fraction of invariable sites being not implemented under PAML. For protein evolution, we used the Jones-Taylor-Thornton (JTT) + $\Gamma_8$ model, with amino-acid frequencies adjusted to the IRBP data set (+ F option).

### Tests of alternative phylogenetic hypotheses

Alternative hypotheses were evaluated in an ML framework, using the nonparametric KH test (Kishino and Hasegawa, 1989), with correction for comparisons of topologies defined a posteriori (KH-SH test) (Shimodaira and Hasegawa, 1999). To take into account the different evolutionary processes of each codon position (in terms of nucleotide frequencies, substitution rates, and rate variation across sites; cf. Table 2), we followed the approach suggested by Yang (1996b). A partitioned likelihood analysis was conducted, with GTR + $\Gamma_8$ model parameters independently estimated for each of the three IRBP codon positions and independence of branch lengths estimated across the three codon positions. To appreciate the phylogenetic content of third codon positions (Yoder et al., 1996b; Yoder and Yang, 2000) and their impact on the acceptance or rejection of evolutionary alternatives, KH-SH tests were performed: 1) on all codon positions, 2) on nucleotides after exclusion of third codon positions, and 3) on amino acids. All tests were performed with PAML 3.0d., with estimation of three sets of base composition, GTR rates, Gamma shape, and branch lengths parameters, one for each codon position.

Alternative phylogenetic hypotheses for *Tarsius* were also evaluated under the parametric SOWH test (Swofford et al., 1996), following the guidelines for the "posPfud" procedure of Goldman et al. (2000): 1) The alternative topology to be tested was a posteriori defined, e.g., *Tarsius* was constrained to be sister group of the Strepsirrhini (the Prosimia topology). 2) The parametric approach was conducted by

*TABLE 2. Molecular characteristics of IRBP exon 1 inferred from partitioned maximum likelihood analysis*[1]

| | IRBP codon positions | | | |
|---|---|---|---|---|
| | First | Second | Third | All |
| Total number of characters | 421 | 421 | 421 | 1,263 |
| Variable characters | 233 | 159 | 389 | 781 |
| Informative characters | 152 | 99 | 338 | 589 |
| %A | 20.4 | 26.1 | 9.8 | 18.8 |
| %C | 29.4 | 24.5 | 40.3 | 31.4 |
| %G | 38.8 | 18.6 | 38.4 | 32.0 |
| %T | 11.3 | 30.8 | 11.4 | 17.8 |
| Relative rate | 1.49 | 1.00 | 5.62 | |
| A ↔ C | 1.25 | 3.20 | 1.37 | 1.56 |
| A ↔ G | 2.97 | 13.22 | 8.18 | 6.30 |
| A ↔ T | 0.87 | 0.94 | 4.73 | 1.35 |
| C ↔ G | 0.58 | 3.19 | 0.49 | 1.04 |
| C ↔ T | 3.36 | 4.69 | 8.38 | 6.27 |
| G ↔ T | 1.00 | 1.00 | 1.00 | 1.00 |
| α (Γ distribution) | 0.72 | 0.37 | 2.98 | 0.53 |

[1] Following parameters are given for each codon position and for their combination: total, variable, and informative number of characters; base composition in percent of A, C, G, and T; relative substitution rate of each partition calculated relative to slowest; rate parameters of GTR model of sequence evolution; and α parameter of Gamma distribution of rate heterogeneity among sites.

simulating 1,000 character matrices under Seq-Gen (Rambaut and Grassly 1997), version 1.2.5, using the Prosimia topology and its optimal likelihood GTR, $\Gamma_8$, and branch length parameters. 3) The log-likelihoods of three topologies (Prosimia ($T_P$), *Tarsius* in basalmost position ($T_B$) among primates, and Haplorrhini ($T_H$)) were computed under the 1,000 simulated matrices, with a full optimization method where ML parameters were estimated from the data. 4) The SOWH test was conducted by comparison against the uncentered distribution of the 1,000 parametric estimates of the difference in log-likelihoods of the best tree ($T_P$, $T_B$, or $T_H$) and that of the alternative tree ($T_P$). 5) The confidence level of the test was obtained by direct comparison of the test statistics with the estimated distribution.

## Local clock analyses

A three-step approach was conducted for molecular dating on DNA characters. First, we detected species or clades that evolved significantly slower or faster than the others by three complementary approaches. The two-cluster (TC) and branch-length (BL) tests from the LINTRE package (Takezaki et al., 1995) respectively examined 1) the hypothesis of equality of the average substitution rate for two clusters that are linked by a given node in the tree, and 2) the deviation from the average of the total branch lengths connecting the root of the ingroup to a given terminal sequence. Relative-rate tests between groups of sequences were conducted with RRTree (Robinson-Rechavi and Huchon, 2000), to take into account the phylogenetic relationships between taxa and to investigate substitution rate differences between clades that are not directly connected in the phylogeny (Robinson et al., 1998). We evaluated rate variations between six placental clades (primates, *Cynocephalus* + *Tupaia,* Glires, Perissodactyla, Paenungulata, and Xenarthra). Rate variations were also explored within primates and involved five clades (Lemuriformes, Lorisiformes, Tarsiiformes, Platyrrhini, and Catarrhini). Dermoptera and Scandentia were chosen as the nearest outgroup which increases the power and accuracy of the relative rate test (Robinson et al., 1998).

Second, we followed the ML local molecular clock approach of Yoder and Yang (2000), because coding regions of the nuclear genome, like IRBP, are often subject to important variations in evolutionary rates among primates (Bailey et al., 1991; Liu et al., 2001). This approach postulates different evolutionary rates for some lineages while assuming (local) rate constancy in others. It represents a compromise between two extreme situations, that either use a global molecular clock (a single substitution rate is imposed for all lineages), or independent rates for each branch (no clock is imposed). In this latter case, molecular dating will be highly sensitive to potential evolutionary rate differences between lineages. Different evolutionary rates were assigned under ML to taxa and clades previously identified to be deviating by the relative rate tests, in order to obtain a tree that satisfies the local clock hypothesis. Likelihood ratio tests (Felsenstein, 1988) were performed to test whether the IRBP nucleotide sequences fit global or local molecular clock hypotheses. One should note that the "ML local clock" approach of Yoder and Yang (2000) substantially differs from the "local clock" approach of Bailey et al. (1991): the former is based on the likelihood criterion to define local constancy of rates, whereas the latter is based on a distance approach and reiterates a calibration of each new local clock, based on the molecular time estimate of the previous (deeper) node.

Third, we used six independent divergence points consistent with the paleontological record in order to calibrate the local molecular clocks. Among the factors influencing the reliability of molecular dating, it was found that one of the most important is the choice of calibration points (Huchon et al., 2000; Yoder and Yang, 2000). Therefore, cross-calibration comparisons should be performed to evaluate the reliability of each calibration point (Huchon et al., 2000). Two calibration points were outside the primates: the radiation of paenungulates ($C_{PAE}$; see Fig. 3) at 55–60 MY (Gheerbrant et al., 1996), and that of perissodactyls ($C_{PER}$) at 55 MY (Garland et al., 1993). Four points were chosen within the primates: the radiation of the order ($C_{PRI}$) at 63 MY (Gingerich, 1984; Gingerich and Uhen, 1994), of Anthropoidea ($C_{ANT}$) at 34 MY (Fleagle, 1999), of Platyrrhini ($C_{PLA}$) at 26 MY (Rosenberger et al., 1991), and of Catarrhini ($C_{CAT}$) at 20–25 MY (Fleagle, 1999). All local clocks, divergence dates, and standard errors were estimated with PAML (Yang, 1997), version 3.0d.

## RESULTS AND DISCUSSION

### Molecular properties of IRBP

The nucleotide composition for the three IRBP codon positions was homogeneous for all taxa except *Macropus* ($P < 0.01$), *Didelphis* ($P < 0.01$), and *Elephantulus* ($P = 0.02$), as estimated by a $\chi^2$ test of deviation from the mean. For this reason, all codon positions were kept in subsequent phylogenetic analyses. The partitioned maximum likelihood analysis yields information about the molecular characteristics of each codon position of IRBP, as presented in Table 2. Third-codon positions are evolving 5.62 faster than second positions. They provide most of the variable sites of the complete alignment, and substitutions occur at nearly all of the third positions, as indicated by the high value of the Γ-distribution parameter. Third positions also display a strong base composition bias against A and T, making this IRBP exon rather G + C-rich (78.7%). Transitions occur more frequently than transversions at all codon positions, and a highly pronounced bias towards A ↔ G transitions is recorded at second positions, as well as a rather high level of A ↔ C and C ↔ G transversions.

### Monophyly of primates and their phylogenetic position within placentals

Because of ML computing time restrictions, the taxonomic sampling was limited to 36 placentals rooted with two marsupials. This included 12 new primate IRBP sequences, and a selection of laurasiatherians and afrotherians restricted to taxa providing calibration points for molecular dating. The ML analysis of all codon positions of this data set highly supports the monophyly of primates (bootstrap percentage (BP) = 92), here represented by an extended taxon sampling and including a variety of other placental representatives (Fig. 1). The IRBP tree also depicts the major placental clades as previously identified by Murphy et al. (2001a,b) and Madsen et al. (2001): Afrotheria (BP = 100), *Bradypus* (representing Xenarthra), Perissodactyla (BP = 100, representing Laurasiatheria, and providing one fossil calibration point; see below), and a clade (BP = 43) containing primates, Dermoptera, Scandentia, and Glires (Rodentia + Lagomorpha). *Equus* plus *Tapirus* branch off at a basal position relative to other placentals, a feature consistent with the observations of DeBry and Sagel (2001) using the same molecule. However, this branching pattern receives no support (BP = 20), and is not in agreement with molecular studies in which Laurasiatheria are connected to Euarchontoglires (Madsen et al., 2001; Murphy et al., 2001a,b). This might be explained by a spurious rooting by the two divergent marsupial sequences. A KH-SH test performed to evaluate both hypotheses displayed no significant difference ($P = 0.23$ for nucleotide sequences). This is why we decided to constrain perissodactyls to branch as the sister clade of Euarchontoglires to calculate divergence dates. The flying lemur (*Cynocephalus*) and the tree shrew (*Tupaia*) cluster together (BP = 63), and are closer to Glires than to primates (BP = 49). This weakly supported grouping is consistent with the phylogeny of DeBry and Sagel (2001) using the same marker, but implies the paraphyly of Euarchonta (primates, Dermoptera, and Scandentia), a group usually considered monophyletic (Madsen et al., 2001; Murphy et al., 2001a,b). IRBP unambiguously suggests the monophyly of primates, but does not answer the question of their sister clade, perhaps because of the limited number of molecular characters and/or poor taxon sampling within Glires.

### Phylogenetic relationships within primates

***IRBP polymorphism between closely related taxa.*** Yoder and Irwin (1999) and Yoder et al. (2001) sequenced a shorter (939 bp) but overlapping part of IRBP for a number of individuals belonging to the same or congeneric species, as sequenced in the present study. This allows one to evaluate the IRBP polymorphism between some pairs of identical and closely related taxa over 939 common positions: *Lemur catta* (0.3% nucleotide divergence), *Microcebus murinus* (2.0%), *Daubentonia madagascariensis* (0.6%), *Nycticebus coucang* (0.6%), and *Propithecus verreauxi*/*P. tattersalli* (0.7%). Some ambiguities, likely due to sequencing errors, in the last 130 bp of the *Microcebus* IRBP sequence of Yoder and Irwin (1999) might explain the high level of intraspecies polymorphism observed in this species. Actually, the removal of this DNA region leads to 0.6% divergence, which conforms to the values (less than 1.0%) observed for the other taxa. This higher nucleotidic divergence could be also explained by a phylogeographic variation in *Microcebus.* Indeed, phylogenetic analysis of mtDNA sequences showed a high diversity in this species (Yoder et al., 2000). Overall, the low IRBP exon 1 polymorphism observed between closely related taxa suggests that the phylogenetic relationships inferred between primate genera (see below) have not been affected by this factor.

***Phylogenetic position of Tarsius.*** The ML analysis of the IRBP sequences recovered several major clades and subclades of primates with high support (Fig. 1): Strepsirhini (BP = 99), Lorisiformes (BP = 100), Lemuriformes (BP = 83), *Tarsius* + Anthropoidea (BP = 88), Anthropoidea (BP = 100), Catarrhini (BP = 100), and Platyrrhini (BP = 100). Interestingly, the IRBP gene suggests the monophyly of Haplorrhini (Tarsiiformes + Anthropoidea).

Whatever the characters used (nucleotides with or without third-codon positions), the two alternative topologies for the branching position of *Tarsius* (i.e., Prosimia and *Tarsius* basal among primates) all involve a decrease in log-likelihood, which is highly significant ($P < 0.001$) under the parametric SOWH test (Fig. 2).

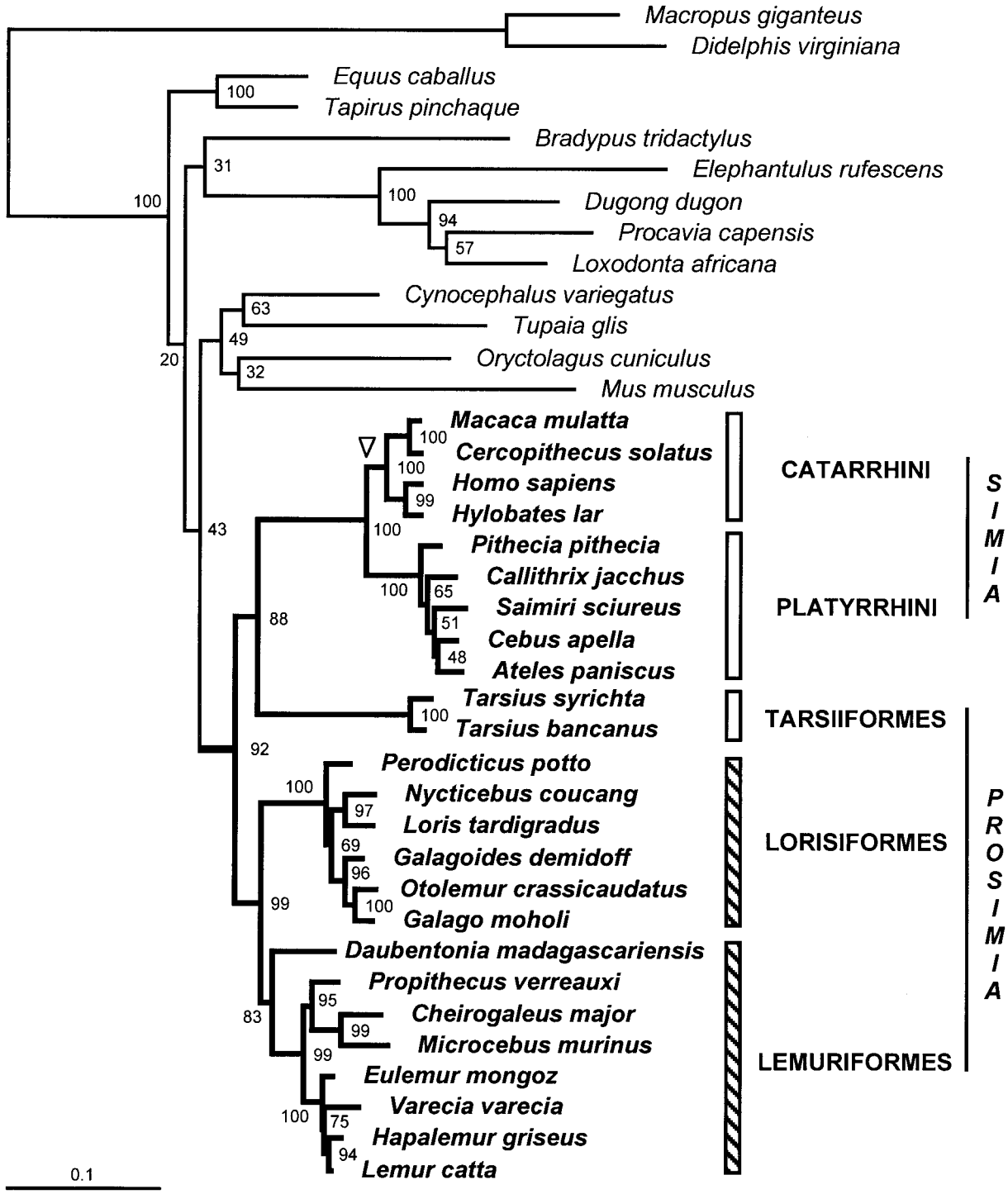


**Fig. 1.** Highest-likelihood phylogram ($-\ln L = 12{,}297.21$) of primate and other placental relationships reconstructed from all codon positions of nuclear gene IRBP. Branch lengths are proportional to number of predicted substitutions per site, under a GTR model with rate matrix (AC; 1.58; AG; 6.27; AT; 1.31; CG; 1.03; CT; 6.23; GT; 1.00), and a Gamma distribution of parameter $\alpha = 0.53$. Branch leading to marsupial outgroup was reduced four times in lengh. Maximum likelihood bootstrap percentages obtained after 500 replicates are indicated at nodes. Open triangle indicates that one insertion of two consecutive codons is diagnostic for monophyly of catarrhines. Open and hatched rectangles indicate Haplorrhini and Strepsirrhini taxa, respectively. Simia is monophyletic, whereas Prosimia is paraphyletic due to position of Tarsiiformes.

Contrasting with these results, the log-likelihood drop for alternatives to the monophyly of Haplorrhini does not reach the significance level of 5% under the nonparametric KH-SH test, neither at the nucleotide nor at the amino-acid level (Table 3). As compared to Haplorrhini, the Prosimia hypothesis is always worse than the unorthodox grouping of *Tarsius* as sister group of all other Primates (i.e., Strep-

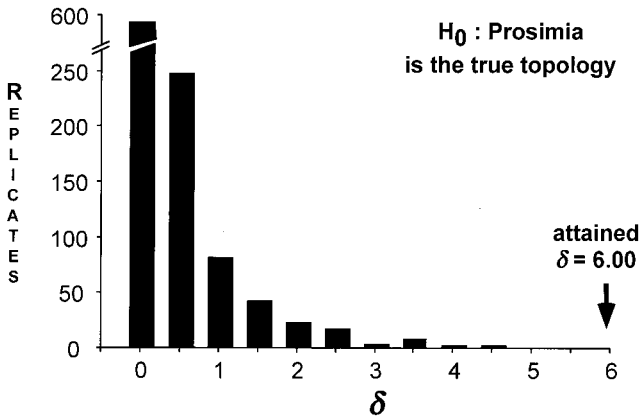


**Fig. 2.** Statistical rejection of Prosimia hypothesis by SOWH test conducted on all codon positions of IRBP. After evaluating 1,000 simulated data sets, greatest difference of log-likelihood between Prosimia and either Haplorrhini or basal *Tarsius* hypotheses was 4.07, which is lower that $\delta = 6.00$ observed on original data set. Prosimia hypothesis is therefore rejected at $P < 0.001$.

sirrhini and Anthropoidea are sister group), and this result is most pronounced when all nucleotide positions are evaluated (e.g., $P = 0.08$ *vs.* 0.18). The difference of behavior between the SOWH and KH-SH tests was previously noted, with the latter being more conservative. This might be explained by the increased power and/or greater reliance on sequence evolution models of parametric tests (Goldman et al., 2000).

The KH-SH tests of alternative phylogenetic hypotheses were conducted under partitioned ML. To compare models with a single character partition or with partitioning of the data according to codon positions, we used the Akaike information criterion (AIC), which should be minimized for the most suitable model. When a single model is defined for IRBP, the best tree (Fig. 1) had a log-likelihood of $\ln L = -12{,}647.16$ (as estimated by PAML), with a total of 82 free parameters (i.e, three independent nucleotide frequencies, five independent GTR rates, one Gamma rate, and 73 [$2 \times 38 - 3$] branches), and this gives an AIC of $2 \times 12{,}647.16 + 2 \times 82 = 25{,}458.32$. When IRBP is partitioned according to codon positions, the highest log-likelihood is $-12{,}295.94$, with $3 \times 82 = 246$ free parameters, and this yields a lower AIC of $2 \times 12{,}295.94 + 2 \times 246 = 25{,}083.88$. Therefore, the three-partition model better describes the IRBP data, and the decrease of log-likelihood observed when the monophyly of Tarsiiformes + Anthropoids is disrupted does not reflect the use of an oversimplified ML model (Whelan et al., 2001).

A critical analysis of IRBP phylograms after ML reconstruction of character-state changes shows that there are 13 synapomorphies supporting the Haplorrhini clade (five on first codon positions (P1), two on second (P2) positions, and six on third (P3) positions), against five (two on P1, two on P2, and one on P1) favoring the basal position of *Tarsius* among primates, and one supporting prosimians on P1. The latter one occurs on a site that, according to the polarization of the character-state change, can support the three alternative hypotheses. This means that this site cannot be used to discriminate between the three alternative branching positions of Tarsiiformes, and this implies that the IRBP gene does not support the monophyly of Prosimia.

The majority of shared derived substitutions for Haplorrhini thus occurred at third positions (most of them being silent), which is likely to explain that both removal of third positions and analysis of amino acids decrease the nonparametric statistical contrast between the three alternative topologies (see Table 3). These results (KH-SH tests, and the distribution of synapomorphies among different codon positions) show that third-codon positions might contain phylogenetic information, as previously suggested (e.g., Yoder et al., 1996b; Yoder and Yang, 2000).

The results obtained with IRBP for the phylogenetic position of *Tarsius* corroborate those obtained through the study of other DNA sequences (Goodman et al., 1998), Alu repeats (Zietkiewicz et al., 1999), SINE insertions (Schmitz et al., 2001), and composite trees (Purvis, 1995). To the contrary, paleontological studies cannot distinguish between three alternative hypotheses: either *Tarsius* is sister group of strepsirrhines, or it branches before the anthropoid-strepsirrhine split, or it forms a trifurcation with anthropoids and strepsirrhines (Gregory, 1910; Simpson, 1945; Shoshani et al., 1996; Fleagle, 1999). Alternative points of view also occur with molecular studies: Murphy et al. (2001a) analyzed about 10 kb from 18 orthologous mitochondrial and nuclear DNA segments, and showed that *Tarsius* clusters with *Lemur* in a Prosimia clade. Either the strong support for Haplorrhini here reflects a gene-sampling artifact because of a peculiar behavior of the IRBP marker, or there is a taxon-sampling artifact in the data matrix of Murphy et al. (2001a) because only one strepsirrhine (*Lemur*) is included. Moreover, analyzing their data sets after removal of mitochondrial genes, as well as those nuclear genes not sequenced for *Tarsius*, severely decreases the support for the prosimian hypothesis (BP drops from 99 to 59; analyses not shown).

***Phylogeny of Anthropoidea.*** Anthropoidea is a robustly supported clade that is subdivided into Catarrhini and Platyrrhini (Fig. 1). Catarrhini is also highly supported, and defined by one diagnostic insertion of six consecutive nucleotides at positions 1111–1116 of the human IRBP. Within catarrhines, the four taxa here included cluster into a cercopithecoid and a hominoid group.

The monophyly of platyrrhines is strongly supported as well (BP = 100). This confirms the occurrence of a unique colonization event of South America by anthropoids, in agreement with Goodman et al. (1998), Canavez et al. (1999), and von Dornum

*TABLE 3. Nonparametric tests of alternative hypotheses based on IRBP nucleotides (with and without third-codon positions) and amino acids for phylogenetic position of* Tarsius, *Cebidae monophyly, and association between Cheirogaleidae and Lorisiformes*[1]

| Phylogenetic hypotheses evaluated | Nucleotides codon positions 1 + 2 + 3 | | | | Nucleotides codon positions 1 + 2 | | | | Amino acids | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $-\ln L$ | Δ | S.E. | $P_{SH}$ | $-\ln L$ | Δ | S.E. | $P_{SH}$ | $-\ln L$ | Δ | S.E. | $P_{SH}$ |
| 1. Position of *Tarsius* | | | | | | | | | | | | |
| *Tarsius* + Anthropoidea (= Haplorrhini) | 12,295.94 | | | | 5,568.88 | | | | 5,724.25 | | | |
| *Tarsius* + Strepsirrhini (= Prosimia) | 12,301.93 | 5.98 | 4.19 | 0.08 | 5,572.92 | 4.04 | 3.58 | 0.13 | 5,726.09 | 1.84 | 3.77 | 0.48 |
| *Tarsius* sister group to all other primates | 12,300.43 | 4.49 | 4.85 | 0.18 | 5,571.00 | 2.12 | 4.51 | 0.32 | 5,724.83 | 0.58 | 4.39 | 0.59 |
| 2. Monophyly of Cebidae | 12,306.18 | 10.24 | 7.16 | 0.08 | 5,576.50 | 7.62 | 5.87 | 0.10 | 5,733.06 | 8.81 | 6.90 | 0.11 |
| 3. Cheirogaleidae + Lorisiformes | 12,350.48 | 54.54 | 15.69 | <0.01 | 5,600.47 | 31.59 | 12.43 | 0.01 | 5,754.44 | 30.19 | 13.36 | 0.02 |

[1] Log-likelihood of each topology ($\ln L$), difference (Δ) relative to highest log-likelihood, its standard error (S.E.), and confidence probability ($P_{SH}$) of Kishino-Hasegawa test with Shimodaira-Hasegawa correction are given.

and Ruvolo (1999), but in contrast with the immunological results of Bauer and Schreiber (1997). Within platyrrhines, the evolutionary relationships are less well resolved (BPs within this clade do not exceed 65), but Cebidae (sensu Goodman et al. 1998), here represented by Callithrichinae (*Callithrix*) + Cebinae (*Cebus* and *Saimiri*), are paraphyletic. Constraining the monophyly of Cebidae involves a severe drop in log-likelihood, and yields a topology which is significantly worse relative to the best one (Table 3, marginal significance of the KH-SH test: $0.08 < P < 0.11$, depending on the characters considered). Moreover, our IRBP phylogeny does not match the traditional morphological view, which groups *Pithecia, Saimiri, Cebus,* and *Ateles* into Cebidae (Rowe, 1996).

The relationships within platyrrhines have already been studied with mitochondrial (Horovitz and Meyer, 1995) and nuclear (Harada et al., 1995; Schneider et al., 1996; von Dormum and Ruvolo, 1999) sequences, without succeeding in resolving their phylogeny. The difficulty in resolving the evolutionary affinities between the different platyrrhine families, and the incongruence of topologies obtained with different markers, could reflect a fast radiation in this group. This is confirmed by two surveys with respectively 6700 and 6723 bp of concatenated genes (Schneider, 2000; Schneider et al., 2001) that cannot solve the phylogenetic relationships between pitheciines, atelines, and cebids, because they appeared almost at the same time.

***Phylogeny of Strepsirhini.*** Strepsirrhine primates constitute a robustly supported clade (Fig. 1; BP = 99). It contains two reciprocally monophyletic groups: Lemuriformes and Lorisiformes, the monophyly of the latter being the most strongly evidenced (BP = 100 vs. 83). The monophyly of lemuriforms confirms the hypothesis of a single migration event of strepsirrhines to Madagascar, in agreement with Yoder et al. (1996a, 2003). Actually, constraining the lemuriform family Cheirogaleidae to branch with lorisiforms is a significantly worse phylogenetic alternative ($P < 0.02$; Table 3). The characters shared by cheirogaleids and lorisiforms (such as the anatomy of their ascending pharyngeal artery) thus appear to be symplesiomorphous for strepsirrhines (Yoder, 1994), or to have been convergently acquired by both groups. Analysis of dental and anatomical characters showed that Cheirogaleidae clusters with Lemuriformes, but not with Lorisiformes (Marivaux et al., 2001), in agreement with the present IRBP data.

Within Lemuriformes, the phylogenetic position of the aye-aye has been controversial. Because of its morphological specializations, *Daubentonia* has sometimes been considered as a basal member of strepsirrhines (Groves, 1989) and even of primates (Oxnard, 1981). However, in the present study, *Daubentonia* branches with strong support as the sister group of all other lemuriforms (BP = 99), as suggested by other molecular studies (Yoder et al., 1994, 1996; Porter et al., 1995) and karyotype comparisons (Rumpler et al., 1988). As for the remaining lemuriform families, IRBP shows that Cheirogaleidae and Lemuridae are well-defined (BP = 99–100). There is a strong signal (BP = 95) to group Indridae (*Propithecus*) with Cheirogaleidae (*Cheirogaleus* and *Microcebus*). This suggests the resolution of the trifurcation between Indridae, Lemuridae, and Cheirogaleidae shown by Porter et al. (1995) and Goodman et al. (1998). It also contrasts with the cytochrome *b* analyses of Yoder et al. (1996a,b), and the study of dental and morpho-anatomical characters of Marivaux et al. (2001) in which Cheirogaleidae and Lemuridae cluster together.

Within Lorisiformes, three nodes are strongly supported: *Nycticebus* + *Loris, Otolemur* + *Galago,* and the monophyly of Galagonidae (BP = 96–100). However, Loridae appear paraphyletic due to the basal position of *Perodicticus.* For a detailed discussion of the conflict between morphological and molecular data for the branching position of *Perodicticus,* one should refer to Yoder et al. (2001). Interesting to note, *Perodicticus* is the slowest-evolving species for IRBP (see below, and Fig. 1). This might explain the instability of its phylogenetic position. Indeed, se-

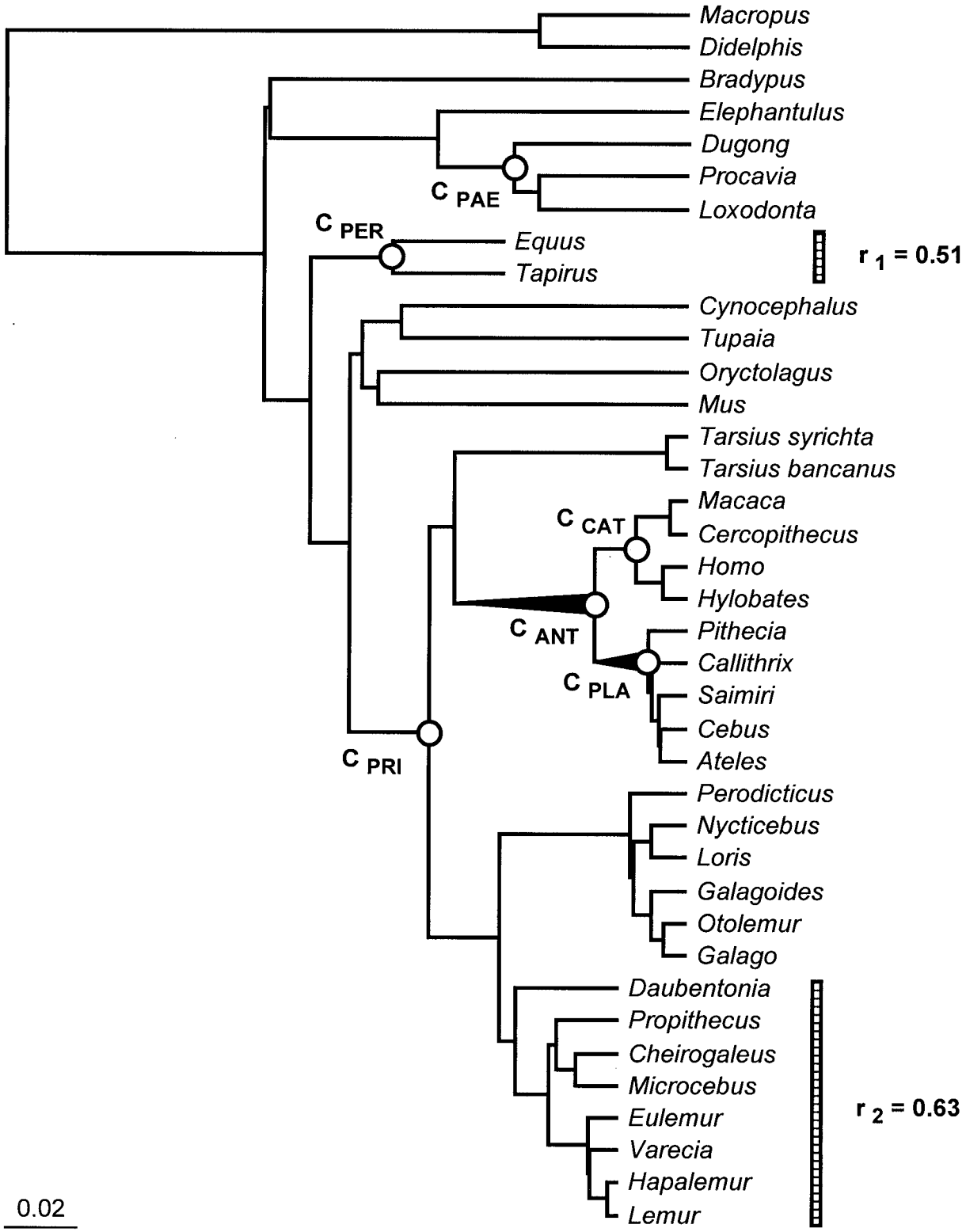


**Fig. 3.** Local clock phylogram used to estimate divergence dates for primates. Topology is derived from highest-likelihood phylogram after exchanging positions of perissodactyls and xenarthrans + paenungulates (see additional details in Monophyly of Primates and Their Phylogenetic Position Within Placentals). Local molecular clocks were defined for Perissodactyla ($r_1 = 0.51$) and Lemuriformes ($r_2 = 0.63$). Local clock $r_1$ (or $r_2$) was defined for all branches connecting most ancient common ancestor of perissodactyls (or lemuriformes) to two (or eight) terminal taxa, i.e., for 3 and 15 branches, respectively. Other branches evolve according to default $r_0 = 1.00$ value. Paleontological calibration points are indicated: $C_{PAE} = 55–60$ MY, $C_{PER} = 55$ MY, $C_{PRI} = 63$ MY, $C_{ANT} = 34$ MY, $C_{CAT} = 20–25$ MY, and $C_{PLA} = 26$ MY. Scale is 0.02 expected nucleotide substitution per site (i.e., 2% substitution). Thick lines correspond to branches subtending clades for which intraprimates cross-calibrations lead to incompatible results (see text for further details).

quences that evolve faster (e.g., Galagonidae, *Loris,* or *Nycticebus*) might cluster together because of long-branch attraction (Felsenstein, 1978): faster evolutionary rates increase the probability of parallel homoplastic substitutions between these sequences, leading to the exclusion of the slower-evolving potto from Loridae.

***Contribution of amino acids.*** The ML analysis of amino acids yields a primates subtree identical to the one inferred from nucleotides (Fig. 1), with three exceptions: 1) *Varecia* is the most basal taxon within Lemuridae, as found by Yoder and Irwin (1999) with combined genetic data; 2) *Perodicticus* is involved in a trifurcation with Galagonidae and the two other Loridae (*Nycticebus* and *Loris*), illustrating the difficulty of molecular data to reveal the monophyly of slow lorises (Yoder et al., 2001); and 3) *Pithecia* branches with *Callithrix,* in disagreement with other studies on nuclear markers (e.g., von Dornum and Ruvolo, 1999). The latter point emphasizes the need of additional taxon sampling for the IRBP exon 1 within platyrrhines in order to stabilize their phylogeny.

### Variations of evolutionary rates between and within clades

The TC, BL, and RRTree tests recorded marked evolutionary rate differences in IRBP sequences from different clades. Below, we report results involving taxa that are consistently identified by all three tests as evolving at a significantly contrasted rate: Perissodactyla and Lemuriformes are the slowest evolving, and Afrotheria is the fastest. The BL test indicates that slower-evolving taxa are all lemuriform and perissodactyl species, whereas faster-evolving taxa are *Elephantulus rufescens* and *Procavia capensis* (confidence probability $P < 0.01$). The TC test indicates that Perissodactyla evolve significantly more slowly than Euarchontoglires ($P < 0.05$), and Afrotheria evolve faster than Xenarthra. Within primates, Lorisiformes appears to evolve faster than Lemuriformes, as already pointed out on the noncoding ε-globin gene by Goodman et al. (1998). Significant differences ($P < 0.01$) were also detected by RRTree tests. Perissodactyla is the slowest-evolving clade, evolving significantly more slowly than Paenungulata. Conversely, Paenungulata evolve faster than Perissodactyla and primates. Within primates, platyrrhines and lemuriforms evolve as fastest and slowest, respectively.

Interestingly, the TC test indicates that Platyrrhini evolve faster than Catarrhini. The deceleration of the evolutionary rate of nuclear markers inside the catarrhine clade is in agreement with the hominoid slowdown hypothesis (Britten, 1986; Li and Tanimura, 1987; Goodman et al., 1989; Koop et al., 1989; Bailey et al., 1991). This hypothesis needs to be confirmed by further analyses, in order to evaluate whether the slowdown indeed took place along the hominoid branches (usually termed "hominoid slowdown") or along the catarrhine branches (as suggested by the IRBP gene), and making the term "catarrhine slowdown" more appropriate.

### Molecular datings using maximum likelihood local clocks

The fossil record for some primate lineages such as strepsirrhines is rather poor, but the following chronological landmarks can be given. The stem group of primates might be represented by insectivore-like placentals that lived 63–65 million years (MY) ago (Gingerich, 1984; Fleagle, 1999). The Platyrrhini-Catarrhini and Cercopithecoidea-Hominoidea splits are estimated at 35 MY and 25 MY, respectively (Fleagle, 1999). The oldest fossils of South American monkeys are dated at 26 MY (Rosenberger et al., 1991), and this estimate fits well with the molecular study of Goodman et al. (1998). Nevertheless, the discovery of a fossil rodent from the beginning of the Oligocene (31.5 MY) in the south of Chile (Wyss et al., 1993) suggests that platyrrhine fossils older than 26 MY might also be found. From the fossil record, the lemuriform-lorisiform divergence is estimated to be 30–40 MY old (Gingerich, 1984; Beard et al., 1988; Martin, 1988, 1990). Calibrated with the fossil record, different molecular markers have been used to assess primate divergence dates. Mitochondrial genes display ages far more ancient than the paleontological ones (Arnason et al., 1998, 2000; Yoder and Yang, 2000). This fact has been explained by the incompleteness of the fossil record of primates, and/or by different rates of molecular evolution between lineages and markers, e.g., faster rates in anthropoid primates (Adkins and Honeycutt, 1994; Andrews et al., 1998; Andrews and Easteal, 2000; Liu et al., 2001). Given these discrepancies between fossil and molecular data, we decided to evaluate the contribution of IRBP exon 1 to assess divergence times between clades.

### Setting local molecular clocks

The above-mentioned IRBP rate heterogeneities between and within lineages call for the use of local molecular clocks. Indeed, the global clock hypothesis is significantly rejected for our IRBP data ($P < 0.01$; Table 4). Thus, different molecular evolutionary rates are allocated by ML to those branches that were previously identified as displaying significantly contrasting rates. To that end, an independent local molecular clock is sequentially assigned to branches that connect slower-evolving taxa (Perissodactyla and Lemuriformes) and faster-evolving taxa (Afrotheria).

For each definition of a given local clock, a gain of log-likelihood is observed relative to the global clock hypothesis, and this increase is more pronounced for perissodactyls than for Afrotheria and Lemuriformes (Table 4). The different values for the local clocks (see Table 4) allow the quantification of the

*TABLE 4. Likelihood ratio tests for presence of global and local molecular clocks in IRBP gene of 38 mammals (including 25 primates) and partitioned according to three codon positions*[1]

| | $-\ln L$ | $2\delta$ | $P$ |
|---|---|---|---|
| No clock (cf. Fig. 1) | 12,297.21 | | |
| Global clock [108] | 12,377.19 | 159.96 | <0.01 |
| $r_1$ = 0.52 (Perissodactyla) [105] | 12,361.82 | 129.22 | 0.05 |
| $r_2$ = 1.43 (Afrotheria) [105] | 12,371.26 | 148.10 | <0.01 |
| $r_3$ = 0.63 (Lemuriformes) [105] | 12,373.08 | 151.74 | <0.01 |
| $r_1$ = 0.51 (Perissodactyla), $r_3$ = 0.63 (Lemuriformes) [102] | 12,356.90 | 119.38 | 0.12 |
| $r_1$ = 0.54 (Perissodactyla), $r_2$ = 1.35 (Afrotheria) [102] | 12,357.29 | 120.16 | 0.11 |

[1] Each line provides log-likelihood ($-\ln L$) that corresponds to definition of a new local clock (i.e., individual substitution rates $r_1$–$r_3$), for a taxon which is indicated between brackets. Twice the difference ($2\delta$) of log-likelihoods between hypothesis with and without clock is also given. $P$ values of likelihood ratio test measure significance of decrease of log-likelihood relative to hypothesis without clock. Number of degrees of freedom between models with and without clock is given in brackets. For example, in the case of global clock, it is equal to 108 = 3 (number of codon partitions) × 79 (5 GTR + 1 Γ + 73 branch length parameters of model without clock) − 3 (number of codon partitions) × 43 (5 GTR + 1 Γ + 37 branch-length parameters of global clock model). All sequences for which a local clock is not defined are assumed to evolve at same default rate $r_0$ = 1.00.

*TABLE 5. Molecular estimates of divergence dates inferred from independent calibration points*[1]

| | Calibration points | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Paenungulata | | Perissodactyla | Primates | Anthropoidea | Catarrhini | | Platyrrhini |
| | [55 Ma] | [60 Ma] | [55 Ma] | [63 Ma] | [34 Ma] | [20 Ma] | [25 Ma] | [26 Ma] |
| Paenungulata | | | 43.6 (4.5) | 42.3 (4.3) | **63.7 (6.5)** | 68.5 (7.0) | 85.6 (8.8) | 118.7 (12.2) |
| Perissodactyla | 69.4 (9.6) | 75.7 (10.5) | | **53.4 (7.4)** | 80.4 (11.2) | 86.4 (12.0) | 108.0 (15.0) | 149.8 (20.8) |
| Primates | 81.8 (7.1) | 89.3 (7.8) | **64.8 (5.6)** | | 94.8 (8.2) | 101.9 (8.9) | 127.4 (11.1) | 176.6 (15.4) |
| Anthropoidea | 29.4 (3.2) | **32.0 (3.5)** | 23.3 (2.5) | 22.6 (2.4) | | **36.5 (4.0)** | 45.7 (5.0) | 63.4 (6.9) |
| Catarrhini | 16.1 (2.3) | **17.5 (2.5)** | 12.7 (1.8) | 12.4 (1.7) | **18.6 (2.6)** | | | 34.7 (4.9) |
| Platyrrhini | 12.0 (1.6) | 13.1 (1.7) | 9.5 (1.2) | 9.3 (1.2) | 13.9 (1.8) | 15.0 (2.0) | 18.7 (2.4) | |
| Lorisiformes | 17.9 (2.3) | 19.5 (2.5) | 14.2 (1.8) | 13.8 (1.8) | 20.7 (2.7) | 22.3 (2.9) | 27.9 (3.6) | 38.6 (5.0) |
| Lemuroidea | 34.4 (5.4) | 37.5 (5.9) | 27.2 (4.3) | 26.5 (4.2) | 39.8 (6.3) | 42.8 (6.8) | 53.5 (8.4) | 74.2 (11.7) |
| Lemuriformes | 51.4 (7.0) | 56.1 (7.7) | 40.7 (5.6) | 39.6 (5.4) | 59.5 (8.2) | 64.0 (8.8) | 80.0 (11.0) | 110.9 (15.2) |
| Strepsirrhini | 59.0 (7.0) | 64.3 (7.6) | 46.7 (5.5) | 45.4 (5.4) | 68.3 (8.1) | 73.4 (8.7) | 91.8 (10.8) | 127.2 (15.0) |
| Haplorrhini | 73.7 (6.8) | 80.4 (7.4) | 58.4 (5.4) | 56.7 (5.3) | 85.4 (7.9) | 91.8 (8.5) | 114.7 (10.6) | 159.1 (14.7) |

[1] Each line corresponds to estimates of divergence ages (with standard errors [S.E.] between brackets) obtained for crown group under focus from several calibration points. Dates that are compatible, given ±1 standard error, are indicated in bold. For example, a perissodactyl calibration point at 55 Ma yields a divergence date for primates at 64.8 Ma (range ± 1 S.E., 59.2–70.4), which is compatible with 63 Ma assumed for their divergence. Estimates are also given of age of first split within strepsirrhines, lorisiforms, lemuriforms, lemuroids, and haplorrhines.

magnitude by which taxa evolve at contrasting rates, and indicate a 2.5–3-fold contrast between the slowest evolving clades of placentals (Perissodactyla, $r_1$ = 0.52; Lemuriformes, $r_3$ = 0.63) and among the fastest (Afrotheria, $r_2$ = 1.43). Such rate variations are not special to IRBP, as they have been evidenced for other nuclear markers and for other clades of placentals (e.g., Huchon et al., 2000, 2002).

Relative to the tree without a clock (Fig. 1), the definition of the perissodactyl local clock leads to a marginal acceptance of this hypothesis by the likelihood ratio test ($P$ = 0.05; Table 4). The highest log-likelihood tree with local clocks is obtained after combining two independent rates (Perissodactyla and Lemuriformes; Table 4). This locally clock-like phylogram with its three clocks ($r_1$, $r_2$, and the default $r_0$ rate) is shown in Figure 3, and represents an explanation of the IRBP data that is not significantly different from the hypothesis without a clock ($P$ = 0.12; Table 4). Therefore, three local clocks were introduced into the model and independently calibrated by six paleontological points (Fig. 3), in order to estimate the age of different splitting events among placentals.

## Molecular estimates of divergence ages

Several conclusions can be drawn from the results of the molecular cross-calibrations between the six fossil references (Table 5). First, there are three pairs of calibration points that are reciprocally compatible, given the standard errors on divergence ages (cf. Table 5):1) primates (63 MY) vs. Perissodactyla (55 MY); 2) Paenungulata (60 MY) vs. Anthropoidea (34 MY); and 3) Catarrhini (20 MY) vs. Anthropoidea (34 MY). The compatibility of the latter two points would suggest that Paenungulata (60 MY) should be compatible with Anthropoidea (34 MY). However, whereas the paenungulate point at 60 MY estimates the Catarrhini split between 15–20 MY (i.e., 17.5 ± 2.5; Table 5), the catarrhine point at 20 MY provides older estimates for the paenungulate split (61.5–75.5 MY, i.e., 68.5 ± 7.0). When other splitting events are considered, the compatible calibration points Paenungulata (60 MY) vs. Catarrhini (20 MY) and Anthropoidea (34 MY) vs. Catarrhini (20 MY) give estimates too old according to the fossil record for the splitting ages outside anthropoids (e.g., a range of 64.3–73.4 MY and 80.4–

91.8 MY for the first splits, respectively, within Strepsirrhini and Haplorrhini; Table 5). Notably, the Anthropoidea (34 MY) vs. Catarrhini (20 MY) points indicate 59.5–64.0 MY for the diversification of Malagasy lemuriforms (Table 5), in agreement with the Bayesian relaxed molecular clock IRBP estimate of Yoder et al. (2003). In contrast, the primates (63 MY) vs. Perissodactyla (55 MY) points suggest the following range of mean divergence ages: 13.8–14.2 MY for Lorisiformes, 26.5–27.2 MY for the radiation of the Lemuroidea, 39.6–40.7 MY for Lemuriformes, 45.4–46.7 MY for Strepsirrhini, and 56.7–58.4 MY for Haplorrhini (Table 5). These divergence times are consistent with those proposed by Goodman et al. (1998) except for Lorisiformes and within anthropoids, but more recent than those found with mitochondrial genes.

To compare with mitochondrial results, Yoder et al. (1996a) proposed 62 MY, 54 MY, and 55 MY, respectively, for the strepsirrhine, lemuriform, and lorisiform radiations, based on the calibration of ML branch lengths of a cytochrome *b* tree. A recent Bayesian age estimate based on the latter marker also suggested a 50–78 MY credibility interval for the diversification of Malagasy primates (Yoder et al., 2003). The difference between cytochrome *b* and IRBP age estimates is especially important for the lorisiforms (13.8–14.2 vs. 55 MY, respectively). Given the possible divergence between lemuriforms and lorisiforms at 30–40 MY, as suggested by the fossil record (Gingerich, 1984; Beard et al., 1988; Martin, 1988, 1990), and the postulated occurrence of lorises in the Late Eocene/Early Oligocene (Simons et al., 1986), the mitochondrial estimate of divergence time appears older than the paleontological one, whereas the nuclear estimate is far too recent. Another study using mitochondrial proteins displayed older divergence dates (Arnason et al., 1998), e.g., 60 MY for the Catarrhini/Platyrrhini split, vs. 22.6–23.3 MY with our IRBP datings. These differences in the estimates derived from nuclear and mitochondrial markers might be the corollary of the greater resolving power of the nuclear DNA at deep phylogenetic levels (Springer et al., 2001), and/or might reflect the use of different dating methods in different studies.

Divergence dates outside anthropoids are apparently overestimated by anthropoid and catarrhine calibration points, whereas the latter two points are underestimated by the primate and perissodactyl calibration points (Table 5). Two nonexclusive possibilities might explain this observation: 1) the IRBP substitution rate increased along the branch leading to the anthropoids; and 2) the split between modern anthropoids ($C_{ANT}$) is more recent than assumed, because the 34-MY-old parapithecids (i.e., the earliest fossil anthropoids) branched off deeper in the haplorrhine subtree. Within anthropoids, the platyrrhine calibration point at 26 MY consistently yields divergence ages too old for the other calibration points (e.g., the primate radiation is estimated to be 176.6 MY old). Conversely, these five other calibration points never correctly estimate the age of the platyrrhine split, leading to dates that are too recent (range, 9.3–18.7 MY). With regard to the far more ancient dates calculated with the platyrrhine calibration point, this might reflect large variations of the IRBP evolutionary rates after the Platyrrhini/Catarrhini split. Assuming the accuracy of the fossil estimates of 20 and 26 MY for the divergence of crown catarrhines and platyrrhines, respectively, we calculated a mean IRBP nucleotide substitution rate of 0.116%/MY for primates evolving with the default clock (Fig. 3; $r_0 = 1.00$), followed by a ca. 1.5-fold increase along the anthropoid and platyrrhine ancestral branches (i.e., 0.162–0.187%/MY), and then a 4.5-fold slowdown among living platyrrhines (to 0.042%/MY). One way to attenuate the magnitude of these substitution rate variations would be to consider that modern platyrrhines did not diversify 26 MY ago (the age of the oldest platyrrhine) but rather 14 MY ago, which would yield rates of 0.075–0.078%/MY before and after the radiation of extant platyrrhine genera, similar to those measured before and after the split of crown catarrhines (i.e., 0.078–0.065%/MY for a split calibrated at 20 MY). Therefore, the age of the most recent common ancestor of extant platyrrhines might be more recent than commonly assumed.

The present study stresses the fact that datings based on local molecular clocks might not be reciprocally compatible, depending on the paleontological calibration points used. This can reflect the incompleteness of the fossil record, and/or indicate that the high variability of molecular evolutionary rates in nucleotide and amino-acid sequences is not fully accommodated by local molecular clocks. Reconciliation between paleontological and molecular estimates of divergence ages in primates may benefit from the discovery of new fossils (e.g., Marivaux et al., 2001) and the sophistication of molecular dating techniques (e.g., Sanderson, 1997; Thorne et al., 1998; Huelsenbeck et al., 2000), in order to better understand how molecular evolutionary rates vary through time.

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