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Variance of molecular datings, evolution of rodents and the phylogenetic affinities between Ctenodactylidae and Hystricognathi

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The von Willebrand factor (*vWF*) gene has been used to understand the origin and timing of Rodentia evolution in the context of placental phylogeny. *vWF* exon 28 sequences of 15 rodent families and eight non-rodent eutherian clades are analysed with two different molecular dating methods (uniform clock on a linearized tree; quartet dating). Three main conclusions are drawn from the study of this nuclear exon. First, Ctenodactylidae (gundis) and Hystricognathi (e.g. porcupines, guinea-pigs, chinchillas) robustly cluster together in a newly recognized clade, named 'Ctenohystrica'. The Sciurognathi monophyly is subsequently rejected. Pedetidae (springhares) is an independent and early diverging rodent lineage, suggesting a convergent evolution of the multiserial enamel of rodent incisors. Second, molecular date estimates are here more influenced by accuracy and choice of the palaeontological temporal references used to calibrate the molecular clock than by either characters analysed (nucleotides versus amino acids) or species sampling. The caviomorph radiation at 31 million years (Myr) and the pig–porpoise split at 63 Myr appear to be reciprocally compatible dates. Third, during the radiation of Rodentia, at least three lineages (Gliridae, Sciuroidea and Ctenohystrica) emerged close to the Cretaceous–Tertiary boundary, and their common ancestor separated from other placental orders in the Late Cretaceous.

Keywords: phylogeny; Rodentia; Ctenodactylidae; Hystricognathi; von Willebrand factor (vWF); molecular dating

1. INTRODUCTION

Molecular phylogenies are commonly used to reconstruct the evolutionary history of living taxa, and to provide divergence date estimates through the use of the molecular clock (Zuckerkandl & Pauling 1965). However, broad discrepancies exist between palaeontological and molecular dates, especially those involving mammals (review in Bromham *et al*. 1999). Among placentals, rodents with their great fossil and extant diversities appear as a model group to understand the variance between dates derived from fossils and sequences. Molecular studies usually make the palaeontological dates for the origin of rodent clades older than about 25–55 million years (Myr) (e.g. Janke *et al*. (1997) and Kumar & Hedges (1998) versus Hartenberger (1998)), but they mostly restrict Rodentia to the mouse, the rat and the guinea-pig. These species were shown to have faster rates of sequence evolution (e.g. Graur *et al*. 1991; Huchon *et al*. 1999), and it is known that contrasted substitution rates can severely affect divergence estimates. Rodents are also the most diversified mammals—they include about half of the extant species—and their biodiversity cannot be summarized by only three taxa, all of which are laboratory bred.

Understanding the timing of evolution involves the deciphering of the phylogeny. Unfortunately, the Rodentia phylogeny is a famous battlefield among and between molecular and morphological approaches (e.g. Hartenberger 1985; Graur *et al*. 1991; Nedbal *et al*. 1996; Reyes *et al*. 1998; Huchon *et al*. 1999; Bentz & Mongelard 1999). Palaeontological contributions suggest that one of the oldest recognized rodent groups is the Ctenodactyloidea. Earliest ctenodactyloids were an important component of the Asian Palaeogene fauna since the Early Eocene, and their presence is well documented all over the fossil record from Asia to Africa (Wang 1997). Today, ctenodactyloids are represented by five North African species of medium-sized rodents, adapted to dry climate and desert landscape: the gundis.

Because of their ancient origin, Ctenodactylidae might be sister to all other living rodents (e.g. Hartenberger 1985). A long-standing classification divides Rodentia between Hystricognathi Tullberg, 1899, and Sciurognathi, based on the plane of insertion of the lower incisors, and also the crested molars, the subplacenta, and the fibrovascular ring (Luckett & Hartenberger 1993). Despite the fact that Ctenodactylidae display the sciurognath state, extant taxa have been brought together with either Hystricognathi (e.g. porcupines, chinchillas, guinea-pigs) (Bryant & McKenna 1995), or Hystricognathi+Pedetidae (springhares) (Flynn *et al*. 1986; Martin 1993, 1995). Molecular data do not help to clarify the phylogenetic status of gundis, especially because Pedetidae and Ctenodactylidae have never been studied together (e.g. Matthee & Robinson 1997). Ctenodactylidae appear to be either the sister clade of Sciuroidea (Sciuridae + Aplodontidae: squirrels, marmots and mountain beavers) in mitochondrial 12S ribosomal RNA sequence analyses (Nedbal *et al*. 1996), or an early offshoot among Rodentia together with the hystricognaths, after comparison of nuclear (globin) protein sequences (Beintema *et al*. 1991). Li *et al*. (1992) reanalysed the latter data set, adding αA-crystalline

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amino-acid (AA) sequences, and concluded that gundis may be an independent eutherian lineage.

Such scarce and conflicting studies need to be evaluated with an independent data set. The von Willebrand factor (*vWF*), a single copy nuclear gene, has recently proven to be a complementary nuclear alternative to mitochondrial markers to reconstruct placental as well as rodent phylogeny (Porter *et al*. 1996; Stanhope *et al*. 1998; Huchon *et al*. 1999). Nucleotide and AA sequences from the exon 28 of the *vWF* are here analysed for a wide taxonomic sample including rodent and other mammalian species to address the following questions.

(i) What is the variance of the molecular dates derived from different approaches? Two statistical methods that evaluate divergence times and manage evolutionary rate heterogeneities are compared. The first involves the calculation of a molecular clock on a set of sequences evolving with a statistically homogeneous rate (Takezaki *et al*. 1995). The second—quartet dating—allows rate differences and provides date estimates in a maximum-likelihood (ML) framework, using a fully resolved quartet of species of which ages of the two most recent common ancestors are independently known (Rambaut & Bromham 1998).
(ii) When did the radiation of Rodentia families occur? A larger taxonomic diversity among rodents is included to study more than the two rodent lineages usually considered in previous molecular studies (the murid and the caviomorph) and to improve the temporal estimates of the divergences.
(iii) What is the phylogenetic position of Ctenodactylidae and its consequence(s) for understanding rodent evolution? To have a better sampling at the superfamily level, the vWF database has been increased with seven new Rodentia sequences including Ctenodactylidae, Pedetidae and additional hystricognath representatives (Bathyergoidea, Chinchilloidea and Octodontoidea).

2. MATERIAL AND METHODS

(a) *DNA sequencing of* vWF *exon 28*

Nucleotide sequences of the *vWF* exon 28 were obtained as described in Huchon *et al*. (1999) with slight modifications (direct sequencing of most polymerase chain reaction products; [α-^{33}P]ddNTP and new internal primers use), for one representative member of three hystricognath superfamilies and three sciurognath families: (i) Bathyergoidea: *Bathyergus suillus* Schreber, 1782 (large cape dune mole-rat, Bathyergidae; EMBL accession number AJ238384); (ii) Chinchilloidea: *Chinchilla lanigera* Molina, 1782 (long-tailed chinchilla, Chinchillidae; AJ238385); (iii) Octodontoidea: *Octodon lunatus* Osgood, 1943 (degú de la Costa, Octodontidae; AJ238386); (iv) Ctenodactylidae: *Ctenodactylus vali* Thomas, 1902 (gundi; AJ238387) and *Massoutiera mzabi* Lataste, 1881 (AJ238388); (v) Pedetidae: *Pedetes capensis* Forster, 1778, subspecies *surdaster* (springhare; AJ238389); and (vi) Muridae: *Mus musculus* Linnaeus, 1758 (domestic mouse; AJ238390).

(b) *Phylogenetic reconstructions*

The new sequences were aligned with 31 placentals and one marsupial orthologue available under accession numbers L16903, M25851, S78431, U31603–U31604, U31607, U31609–U31611, U31613–U31614, U31621–U31622, U97534, X63820, AF004285, AF061060, AF061062, AF061064, AF076480 and AJ224661–AJ224675. Gaps were coded as missing data.

Nucleotide level ML reconstructions were conducted with the complementary use of PAUP 4.0b2 (Swofford 1998) and PUZZLE 4.0 (Strimmer & Von Haeseler 1996), respectively, under the GTR and TN93 models of sequence evolution. PUZZLE 4.0 was used for AA level ML analyses under the JTT model. Substitution rate heterogeneities were always described by a fraction of sites allowed to be invariable, and a gamma distribution of parameter α with eight rate categories. Standard maximum-parsimony (MP) and distance (neighbour-joining (NJ)) analyses were also conducted with PAUP 4.0b2.

Robustness of the nodes was assessed by (i) reliability percentages (RP) under ML after 100 000 quartet puzzling steps; (ii) bootstrap percentages (BP) under ML after 100 replicates (with NJ starting trees, NNI branch swapping, and model parameters fixed to values estimated from the original data), and under MP and NJ after 1000 replicates; and (iii) Bremer's support indices (BSI) under MP calculated after enforcement of topological constraints. Alternative phylogenetic hypotheses were compared by the Kishino & Hasegawa (1989) test implemented in PUZZLE 4.0.

(c) *Molecular datings*

We attempted to date the splits between (i) Ctenodactylidae and Hystricognathi; (ii) Gliridae, Sciuroidea and Ctenodactylidae + Hystricognathi; and (iii) Rodentia superfamilies and placental orders. Our large taxonomic sampling allowed us to consider eight groups of calibrating taxa (table 1), i.e. taxa for which a palaeontological divergence date has been suggested. However, heterogeneity of *vWF* evolutionary rates among rodent and other placentals precluded the use of a uniform molecular clock to date cladogenesis events. Two approaches have been used to account for rate heterogeneities and estimate divergence dates.

(i) *Approach using linearized (clock-like) trees*

Takezaki *et al*. (1995) implemented the two-cluster and branch-length tests in the LINTRE package (http://www.bio.psu.edu/people/faculty/nei/lab) to identify fast- or slow-evolving sequences that should be discarded to obtain a linearized tree (i.e. a tree which satisfies the clock hypothesis). Starting from the 39-taxa matrix, fastest- and slowest-rate species were removed following three requirements: (1) reaching a global homogeneous rate of evolution for all sequences, i.e. the *U*-statistics for the two-cluster and branch-length tests were not significant at the 5% level (Takezaki *et al*. 1995); (2) including one rodent (calibration points (1)–(5): table 1) and one non-rodent (points (6)–(8)) pair of species for which a palaeontological divergence date was available; and (3) keeping the largest taxonomic diversity, i.e. at least ten species representing five placental orders. Clock tests were conducted with the TN93 and the 'amino' distances with gamma rates, and with NJ trees. When a subset of sequences matched the previous requirements, the clock option of PUZZLE 4.0 yielded a clock-like (linearized) tree—without any *a priori* phylogenetic assumption—and estimated ML length and standard errors of branches. For each calibrating taxon, and on each linearized tree, a molecular clock was calculated. This is a bidirectional approach allowing inferences of earlier and older dates relative to the calibration point.

Table 1. *The eight groups of taxa used to calibrate the phylogenetic reconstructions*

(The dates selected were the first palaeontological occurrence of the oldest lineage involved in the calibration point.)

calibration points	age (Myr)	comments	reference
(1) *Massoutiera–Ctenodactylus*	9.7–13.0	divergence between their two fossil lineages *Irhoudia* and *Africanomys*[a]	Jaeger (1977)
(2) radiation of Caviomorpha[b]	31–37	first caviomorph fossil in the Tinguiririca[c]	Wyss *et al.* (1993)
	24.5–29	identification of all South American rodent families in the Deseadan[c]	Walton (1997)
(3) *Mus–Rattus*	12–14	age of the *Potwarmus–Antemus: Progonomys* lineage	Jacobs & Downs (1994)
(4) *Glis–Dryomys*	31	first identifications of Leithiinae lineages[d]	Hartenberger (1994)
(5) Sciuridae–Aplodontidae	37	sciurid identifications since the Late Eocene	McKenna & Bell (1997)
(6) *Canis–Felis*	65	first occurrence of viverravines (*Felis* lineage)	Garland *et al.* (1993, p. 289)
(7) *Equus–Ceratotherium*	56	equoids and tapiroids at the start of the Eocene	Garland *et al.* (1993, p. 290)
(8) Cetacea–Artiodactyla	63	Mesonychia–Arctocyonia divergence, represented by the *Phocoena* (porpoise)–*Sus* (pig) split	Gingerich & Uhen (1998)

[a] Indirect extrapolation from fauna compositions.
[b] Chosen at 31 ±6 Myr.
[c] South American land mammal ages.
[d] Divergence might be younger because the Leithiinae monophyly is not robustly established (Bentz & Montgelard 1999).

(ii) *Approach using quartet dating*

Rambaut & Bromham (1998) developed an ML approach for nucleotide sequences (the quartet dating) in the QDate 1.1 program (http://evolve.zoo.ox.ac.uk/qdate) to estimate divergence dates between two monophyletic groups with two different rates of evolution (each clade including two taxa whose splitting date is known). It gives dates older than the two calibration dates: this is a unidirectional (ascending) procedure. According to this method, it was possible to set all pairs of calibrating taxa (table 1) into fully resolved quartets (see §3(a), figure 1). To make comparable the results from clock-like trees and quartet datings, the latter approach was conducted with the GTR model constrained to TN93 (all parameters were estimated with PUZZLE 4.0). Some quartets were discarded when (i) likelihood ratio tests evidenced rate heterogeneity inside pairs of sequences; (ii) confidence intervals of an estimated date included one of the two calibration dates (for instance, all quartets involving Muridae).

3. RESULTS

(a) *Phylogenetic reconstructions*

The total, variable, and phylogenetically informative numbers of sites were respectively 1239, 857 and 659 for the complete alignment of 39 *vWF* nucleotide sequences. When all codon positions were analysed together, *Dipus*, *Chaetophractus*, *Lepus* and *Phocoena* vWF exon 28 deviated from base composition homogeneity at the 1% level of a χ^2-test. As the bias was located on third codon positions, these sites were excluded from all subsequent ML phylogenetic and dating analyses.

ML analyses of the DNA sequences indicated that afrotherians (golden mole and elephant) branched off first within placentals. Next branchings involved xenarthrans, lagomorphs, primates, and then a clade containing carnivores, cetartiodactyls, perissodactyls and chiropterans. This clade was a sister group of the rodent lineages, which clustered in a monophyletic group (figure 1). The five rodent clades identified were Pedetidae (springhares), Myodonta Schaub, 1955 (i.e. Dipodidae (jerboas) and Muridae (mice, rats and mole-rats)), Gliridae (dormice), Sciuroidea and a highly supported clade including Ctenodactylidae (gundis) and Hystricognathi (figure 1). The interrelationships between rodent and placental clades were not robustly supported, and will not be discussed in this paper (for details, see, for example, Huchon *et al.* 1999).

The molecular affinities between ctenodactylids and hystricognaths were strongly evidenced by all phylogenetic analyses, and they involved the recognition of the paraphyly of Sciurognathi, as gundis display the sciurognath condition (figure 1). Conversely, the monophyly of Hystricognathi was strongly suggested as Hystricidae (Old World porcupines here represented by *Trichys*), Bathyergidae (mole-rats) plus Thryonomyoidea (cane-rats), and Caviomorpha clustered together. New World hystricognaths (caviomorphs) were also clearly monophyletic, with Octodontoidea (degus) sister to Erethizontoidea (New World porcupines), Chinchilloidea (chinchillas), and Cavioidea (guinea-pigs and agoutis) (figure 1). Protein sequences did not deviate from AA composition homogeneity, and yielded similar phylogenetic results (not shown).

(b) *Test of alternative phylogenetic hypotheses for gundis and springhares*

To test the degree of support of the Ctenodactylidae + Hystricognathi clade, we compared the log-likelihood of 945 trees describing all possible bifurcating relationships between six rodent lineages (Pedetidae, Myodonta, Gliridae, Sciuroidea, Ctenodactylidae and Hystricognathi). Three reasons led us to assume the monophyly of rodents during the Kishino–Hasegawa tests: (i) Rodentia monophyly was weakly suggested by the highest-likelihood topology (figure 1); (ii) on the morphological grounds, alternative phylogenetic positions for gundis have been proposed only within rodents; and (iii) ML computing time limitations prompted us to reduce the huge number of trees describing all possible relationships between the 14 rodent and other placental clades. Relative to the

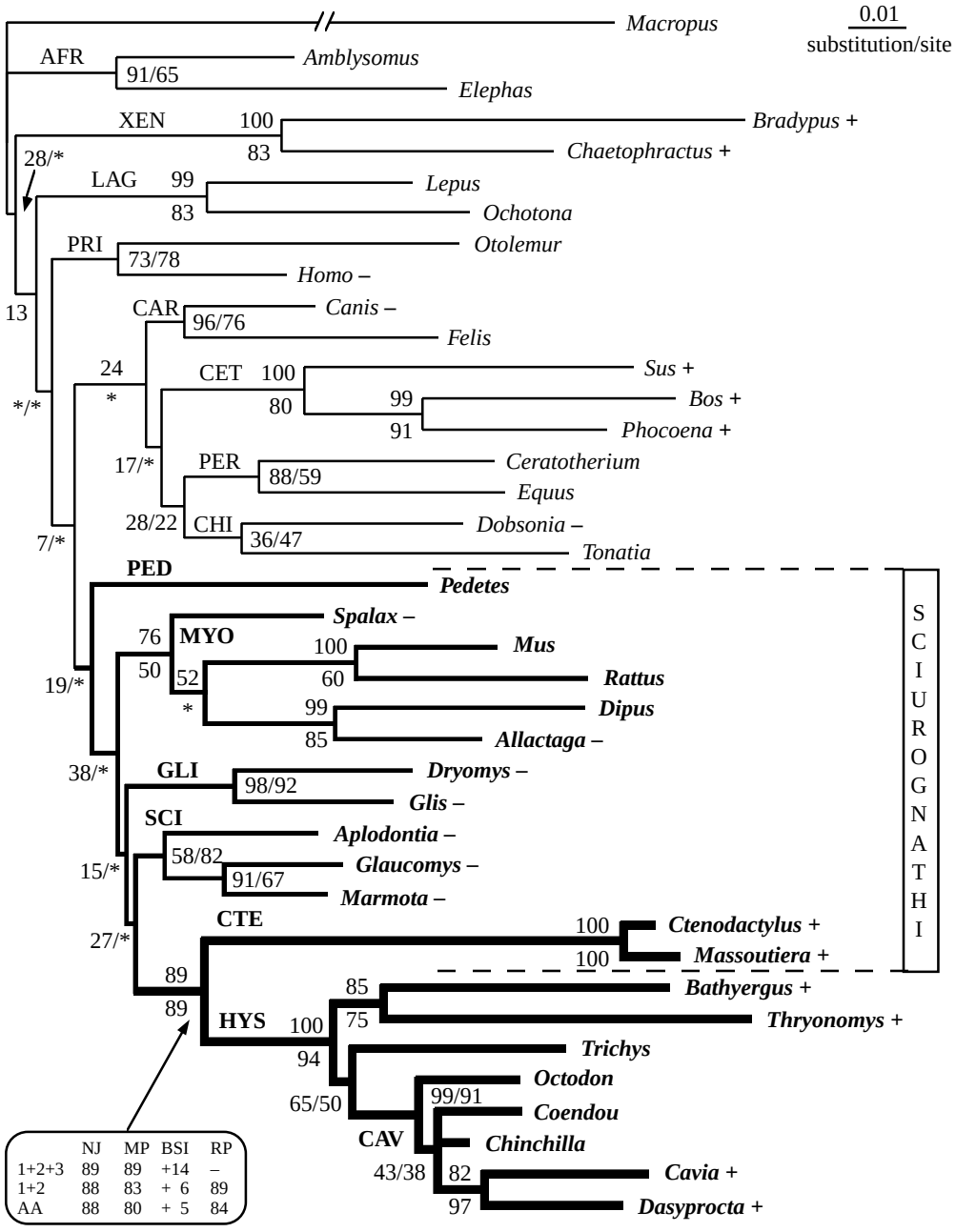

Figure 1. ML phylogram (lnL = −8577.13) of first and second codon positions of 38 placental *vWF* exon 28 nucleotide sequences, and rooted by a marsupial. The GTR model was used (rate matrix: 2.12, 6.18, 1.08, 2.57, 4.74 and 1.00 for respectively A–C, A–G, A–T, C–G, C–T and G–T substitutions), with 41.49% of invariable sites, and site rates following an eight-categories gamma distribution of shape $\alpha = 0.41$. ML BP and quartet puzzling RP are given, respectively, above and below branches (or left and right from the slash). An asterisk indicates that the corresponding node is not supported by analyses. The robustness of the 'Ctenohystrica' clade—i.e. the crown group including all extant Ctenodactylidae and Hystricognathi—has been measured by bootstrap under distance (NJ) and MP, by BSI, and by ML quartet puzzling (RP), on three data matrices (all codon positions: 1 + 2 + 3; first and second positions: 1 + 2; AAs). Branch lengths are proportional to the estimated number of substitutions per site, and the one leading to *Macropus* has been reduced by one-half. Fast- and slow-evolving taxa relative to the average (i.e. branch-length tests conducted on nucleotide and AA sequences are significant at $p < 0.01$) are indicated by plus (+) and minus (−), respectively. Systematic groups are connected by thin branches for non-rodent placentals: Afrotheria (AFR), Xenarthra (XEN), Lagomorpha (LAG), Primates (PRI), Carnivora (CAR), Cetartiodactyla (CET), Perissodactyla (PER) and Chiroptera (CHI); by thicker branches for Pedetidae (PED), Myodonta (MYO), Gliridae (GLI) and Sciuroidea (SCI); and by the thickest branches for Ctenodactylidae (CTE), Hystricognathi (HYS) and Caviomorpha (CAV). Sciurognathi is paraphyletic due to the sister-group relationship of Ctenodactylidae with Hystricognathi.

topology of figure 1, we forced the monophyly of Muridae (*Spalax*, *Mus* and *Rattus*), and collapsed into multifurcation all nodes supported by BP less than 50.

For nucleotides, the 105 trees clustering Ctenodactylidae with Hystricognathi displayed the 105 best log-likelihoods. The 840 remaining trees—which did not support the monophyly of gundis plus hystricognaths—were less likely than the best tree: Kishino–Hasegawa *p*-values (p_{K-H}) ranged from 6.5 to 0.9%. For AAs, 136 trees were not significantly worse than the best tree, including 55 trees that did not establish the monophyly of Ctenodactylidae+ Hystricognathi ($20.5 > p_{K-H} > 3.8\%$). The corollary of these results was the strong rejection of Sciurognathi monophyly ($2.0 > p_{K-H} > 1.0\%$ for nucleotides; $2.4 > p_{K-H} > 0.4\%$ for AAs). It was noteworthy that all trees showing Ctenodactylidae sister to all other rodents were statistically less likely than the best tree ($2.4 > p_{K-H} > 0.9\%$ for DNA; $5.0 > p_{K-H} > 0.5\%$ for protein).

Concerning the phylogenetic position of springhares, all trees showing Pedetidae sister to Ctenodactylidae ($3.0 > p_{K-H} > 0.9\%$ for DNA; $6.6 > p_{K-H} > 0.3\%$ for protein), or Pedetidae sister to Hystricognathi ($3.6 > p_{K-H} > 1.7\%$ for nucleotides; $3.1 > p_{K-H} > 0.6\%$ for AAs) were statistically less likely than the highest-likelihood tree. However, trees clustering Pedetidae with (Ctenodactylidae + Hystricognathi), or trees showing (Ctenodactylidae + Hystricognathi) sister to all other rodent clades, were not significantly worse. It should be noted that the difference of statistical level for the rejection of alternative hypotheses by the Kishino–Hasegawa test between nucleotide (first and second codon positions) and AA matrices probably reflected the greater number of characters of the former (826, 466 and 315 total, variable and informative sites versus 413, 298 and 214).

(c) *Dates of divergence during the evolution of rodents and relative to other placentals*

(i) *Dating using a uniform clock on linearized trees*

Branch-length tests indicated that more than half of the *vWF* exon 28 evolved with nucleotide and AA rates significantly different from the average ($p < 0.01$), and identified ten slow-evolving, and 11 fast-evolving sequences (figure 1; the 17 remaining sequences were called medium-evolving). Unfortunately, the slowest-rate species (e.g. glirids, sciurids, *Spalax*, *Homo* and *Canis*) did not fit the above-mentioned requirements for being kept in linearized trees (see § 2(c)(i), criteria (2) and (3)), and were discarded, together with their corresponding calibration points ((4)–(6): table 1). For the fast- and medium-evolving sequences, the calibrating pairs of taxa were, respectively, *Ctenodactylus–Massoutiera* and *Sus–Phocoena*; *Mus–Rattus* and *Equus–Ceratotherium*.

The linearized topologies (not shown) displayed most of the rodent and placental clades into the same multifurcation, thereafter referred to as the 'placental radiation'. On these clock-like trees, molecular date uncertainties were introduced by standard errors on ML branch lengths, and ranged from 1.0 to 12.2 Myr. The use of nucleotide or AA sequences led to very similar dates, except for the split calibrated by the Caviomorpha radiation (table 2). On the contrary, molecular date estimates strongly depended on the palaeontological calibrations (table 2). First, the fossil record possessed its own uncertainty (e.g. the *Ctenodactylus–Massoutiera* split was dated between 9.7 and 13.0 Myr). This led to a large variance in molecular dates: the palaeontological uncertainty covers 3.3 Myr for *Ctenodactylus–Massoutiera*, but this induced a 13.0–43.8 Myr molecular difference (table 2: AAs). A small uncertainty on a recent calibration point had obviously its strongest impact on the deepest nodes. Second, the choice of the calibration point to date one given splitting event introduced large discrepancies. For example, the use of *Massoutiera–Ctenodactylus* at 13.0 Myr or *Sus–Phocoena* at 63.0 Myr involved date differences ranging from 23.3 Myr (27.7–51.0 Myr: Caviomorpha radiation) to 79.0 Myr (93.7–172.7 Myr: placental radiation) (table 2: fast AA sequences).

The dates provided by the *Mus–Rattus* calibration point were always too young relative to the fossil record. For example, it placed the *Equus–Ceratotherium* split near the end of Early Miocene. Conversely, the oldest estimates were derived from the *Ctenodactylus–Massoutiera* (13.0 Myr) and caviomorph radiation calibration points (i.e. the placental radiation was settled in the Jurassic (146–208 Myr)). The *Sus–Phocoena* calibration point gave dates in good agreement with the fossil record for Ctenodactylidae and Caviomorpha, but it pushed back in the Cretaceous (65–146 Myr) the divergence between Ctenodactylidae and Hystricognathi (in the Campanian (72–83 Myr)), and the placental radiation (in the Cenomanian (92–96 Myr)).

(ii) *Dating using quartet analyses*

Quartet dating results will be interpreted in two ways: (1) the lower (upper) limit of confidence intervals over all quartets conservatively indicates the minimum (maximum) age for the split between the two pairs of calibrating taxa considered, but is strongly dependent on the reliability of the youngest (oldest) quartet; (2) the median of all quartet dating estimates integrates the information brought by all quartets.

As for the former approach, quartet dating results were strongly influenced by the accuracy of calibration points. When the calibrating points used to date the Ctenodactylidae–Hystricognathi split were respectively made older by 3.3 Myr (*Ctenodactylus–Massoutiera*: table 1) and 6.0 Myr (caviomorph radiation), the differences in the estimated dates for the same quartet of species ranged from 23.6 to 38.5 Myr; the 95% confidence intervals lower limits and the median of all quartet estimates moved from 57.0 and 89.8 Myr (figure 2*b*) to 74.8 and 117.9 Myr (figure 2*a*). The sampling of species also impacted on the divergence dates: for example, all quartets involving the pair of gundis systematically provided the oldest estimates. Some quartets gave paradoxical dates, as the split between two closely related taxa was older than the one between more distantly related taxa: the 95% confidence interval of *Cavia–Octodon*/*Ctenodactylus–Massoutiera* was 74.8–135.9 Myr, versus 50.6–74.8 Myr for the quartet *Cavia–Octodon*/*Marmota–Aplodontia*.

Concerning Gliridae, Sciuroidea, and Ctenodactylidae + Hystricognathi, quartet datings indicated that the minimum age for their radiation was in the Middle Eocene (41.5 Myr), although with a median close to the Cretaceous–Tertiary boundary (74.4 Myr: figure 2*c*) in the Campanian (72–83 Myr). We noted that the two quartets involving the slowest-rate rodents—glirids and

Table 2. *Molecular dates with a uniform molecular clock after tree linearization, based on four rodent and two placental calibration points*

(Two sets of *vWF* exon 28 sequences are used: one fast evolving[a], and one medium evolving[b], for either first and second codon positions (1 + 2) or AA. Standard errors based on ML branch lengths are indicated between parentheses for all dates. n.p., dating was not possible because the calibrating species do evolve at a significantly different rate relative to the pair of taxa for which the age of divergence should be estimated. In the linearized trees, the placental radiation refers to the superimposition of the radiation of Rodentia and the radiation of other placentals.)

		estimated molecular dates (Myr)								
		Massoutiera–Ctenodactylus		Caviomorpha radiation			Ctenodactylidae + Hystriocognathi		*Mus–Rattus*	
calibrating points	date (Myr)	1 + 2 fast	AA fast	1 + 2 medium	AA medium	AA fast	1 + 2 fast	AA fast	1 + 2 medium	AA medium
Massoutiera–Ctenodactylus	9.7	9.7 (2.8)	9.7 (2.8)	n.p. —	n.p. —	38.0 (2.0)	97.3 (6.0)	99.6 (6.7)	n.p. —	n.p. —
Massoutiera–Ctenodactylus	13.0	13.0 (3.7)	13.0 (3.7)	n.p. —	n.p. —	51.0 (2.6)	130.7 (8.0)	133.4 (9.0)	n.p. —	n.p. —
Mus–Rattus	14.0	n.p. —	n.p. —	7.0 (1.6)	5.0 (1.1)	n.p. —	n.p. —	n.p. —	14.0 (2.4)	14.0 (1.8)
Caviomorpha radiation	31.0	n.p. —	7.9 (2.2)	31.0 (6.9)	31.0 (6.9)	31.0 (1.6)	n.p. —	81.1 (5.5)	62.0 (10.6)	86.9 (11.0)
Equus–Ceratotherium	56.0	n.p. —	n.p. —	22.9 (5.1)	14.9 (3.3)	n.p. —	n.p. —	n.p. —	45.9 (7.9)	41.9 (5.3)
Sus–Phocoena	63.0	7.4 (2.1)	7.0 (2.0)	n.p. —	n.p. —	27.7 (1.4)	74.6 (4.6)	72.4 (4.9)	n.p. —	n.p. —

		estimated molecular dates (Myr)							
		Equus–Ceratotherium		*Sus–Phocoena*		placental radiation			
calibrating points	date (Myr)	1 + 2 medium	AA medium	1 + 2 fast	AA fast	1 + 2 medium	AA medium	1 + 2 fast	AA fast
Massoutiera–Ctenodactylus	9.7	n.p. —	n.p. —	82.4 (5.9)	86.6 (6.7)	n.p. —	n.p. —	122.9 (4.2)	128.9 (3.8)
Massoutiera–Ctenodactylus	13.0	n.p. —	n.p. —	110.4 (7.9)	116.1 (9.0)	n.p. —	n.p. —	164.7 (5.6)	172.7 (5.1)
Mus–Rattus	14.0	17.1 (2.7)	18.7 (1.8)	n.p. —	n.p. —	34.6 (1.4)	32.3 (1.3)	n.p. —	n.p. —
Caviomorpha radiation	31.0	75.7 (12.2)	116.1 (11.5)	n.p. —	70.6 (5.5)	153.5 (6.2)	200.9 (8.0)	n.p. —	105.0 (3.1)
Equus–Ceratotherium	56.0	56.0 (9.0)	56.0 (5.5)	n.p. —	n.p. —	113.5 (4.6)	96.9 (3.9)	n.p. —	n.p. —
Sus–Phocoena	63.0	n.p. —	n.p. —	63.0 (4.5)	63.0 (4.9)	n.p. —	n.p. —	94.0 (3.2)	93.7 (2.7)

[a] Species in common for fast data sets are *Macropus*, *Bos*, *Phocoena*, *Sus*, *Cavia*, *Dasyprocta*, *Trichys*, *Ctenodactylus* and *Massoutiera*. Species restricted to the DNA or protein sets are respectively *Dipus*, *Galago*, *Tonatia*, *Lepus* and *Elephas*; versus *Coendou*, *Octodon*, *Bradypus*, *Chaetophractus*, *Galago* and *Felis*.

[b] Species in common for medium data sets are *Macropus*, *Octodon*, *Trichys*, *Mus*, *Rattus*, *Dipus* and *Pedetes*. Species restricted to the DNA or protein sets are respectively *Coendou*, *Chaetophractus*, *Galago*, *Tonatia* and *Elephas*; versus *Chinchilla*, *Allactaga*, *Homo*, *Tonatia*, *Lepus*, *Ochotona*, *Equus* and *Ceratotherium*.

sciuroids—yielded the two youngest ages for the radiation of rodent superfamilies. Removal of these two marginally distributed quartets showed that the minimum divergence age for glirids, sciuroids, gundis and hystricognaths was in the Early Eocene (50.7 Myr). Quartet comparisons involving two rodents versus two other placentals mostly provided older dates, suggesting a minimum age in the Palaeocene (59.3 Myr) for the radiation of rodents relative to other placentals, and a median at 108.9 Myr (figure 2*d*).

4. DISCUSSION

(a) *Causes of the variance of molecular dates*

Comparison of two *vWF* exon 28 sequences of *Rattus norvegicus* (accession U50044 versus AJ224673) and *Mus musculus* (U27810 (laboratory strain) versus AJ238390 (wild caught)) revealed 0.3–0.9% divergence (mainly third codon position transitions) corresponding to sequencing errors and intraspecific variations. We neglected the impact of these polymorphisms relative to

other sources of dating error (for an extended framework, see Waddell *et al*. (1999)). Four other causes of the variance of molecular divergence dates for placentals are here examined for a nuclear marker. Our conclusions are drawn from a single exon, and need to be evaluated by the study of longer sequences and congruence between multiple independent genes.

(i) *Characters analysed*

Clock-like trees reconstructed from nucleotide and AA sequences give similar divergence dates, reflecting the strong correlation between pairwise *p*-distances computed on the two first codon positions and AAs. One exception occurs when the calibration point is the Caviomorpha radiation (table 2). Owing to their simultaneous appearance in the fossil record, we considered that the four caviomorph superfamilies diverged at the same time, whereas this remains a crude approximation (e.g. the divergences *Coendou–Octodon* and *Coendou–Cavia* are not superimposed: figure 1). Moreover, differences in date estimates may have been introduced by the fact that caviomorphs exhibit contrasted *vWF* substitution rates (figure 1): each set of homogeneously evolving nucleotide or AA sequences therefore contains different sets of caviomorph species (table 2).

(ii) *Species sampling*

The latter example illustrates that taxonomic sampling has an impact on tree linearization dates, but also on quartet datings. Two closely related sequences from the same clade will lead to similar estimates: the divergence date for the quartet *Aplodontia–Marmota* and *Coendou–Cavia* is 69 Myr, and becomes 72 Myr when we substitute *Glaucomys* for *Marmota* and *Dasyprocta* for *Cavia*. More distantly related sequences can lead to more contrasted dates: *Aplodontia–Marmota* and *Octodon–Cavia* gives 61 Myr, whereas it is 88 Myr when *Marmota* is replaced by *Glaucomys*, and *Cavia* by the slower-rate *Chinchilla*. Both methods are similarly influenced by the Ctenodactylidae calibration point, which always provides the oldest dates (table 2, and quartet results not shown).

(iii) *Uncertainty on palaeontological splitting dates*

Molecular dates computed by the two methods are highly sensitive to the uncertainties of palaeontological calibrations (table 2, figure 2*a*,*b*). Date variations induced by uncertainties in the fossil record can be four times higher than those induced on linearized trees by branch-length standard errors. Moreover, standard error of the palaeontological estimates are often not available (e.g. Garland *et al*. 1993; or the A/C-60 in Arnason *et al*. (1996)). A fortunate exception is the likelihood estimation of the divergence time of Cetacea versus mesonychians based on numerous independent fossil sites (Gingerich & Uhen 1998). Additional accurate palaeontological calibrations with their standard errors are required for a realistic comparison between fossil and molecular dates.

(iv) *Choice of the calibration points*

Most of the molecular dates in the literature are inferred from a single calibration point which is then extended to various distantly related lineages (e.g. Kumar & Hedges 1998). However, the use of a first calibration point (e.g. *Mus–Rattus*) on linearized trees may lead to a divergence date for a second pair of calibrating taxa (e.g. *Equus–Ceratotherium*) which conflicts with the palaeontological estimate (table 2: 17.1–18.7 against 56 Myr). Two explanations can be given to such discrepancies. First, the acceptance of the hypothesis of a constant evolutionary rate by the two-cluster test (Takezaki *et al*. 1995) would reflect a more similar mean rate in all lineages than a regularly ticking molecular clock, particularly if the linearized trees include several isolated branches. This phenomenon might be strongly marked in trees reconstructed from fast-rate sequences. Because of higher probabilities of substitution on long branches, changes in the rate of molecular evolution are more likely to occur in fast- rather than in slow- or medium-evolving sequences. Second, the incompleteness of fossil records might be more likely to be responsible for inaccuracies in calibration points (e.g. Kumar & Hedges 1998), even for well-accepted divergence dates like the Artiodactyla–Cetacea split (Arnason *et al*. 1996; Gingerich & Uhen 1998; but see Waddell *et al*. 1999).

We therefore recommend use of at least two calibration points and cross-validation of them, rather than focusing on a calibration date deemed to be especially reliable. For example, 'cross-calibration' comparisons suggest that the most compatible calibrating taxa with our fast *vWF* AA data are the caviomorph radiation at 31.0 Myr and *Sus–Phocoena* at 63.0 Myr. They, respectively, suggest 70.6 ± 5.5 Myr for the Cetartiodactyla divergence, and 27.7 ± 1.4 Myr for the Caviomorpha radiation (table 2). On the contrary, the improbability of the *Mus–Rattus* split at 14 Myr is suggested by its incompatibility with other calibration points, despite a well-documented fossil record for these murids (Jacobs & Downs 1994). The regression method may also be used to compute one consensus molecular clock from several calibration points (Springer 1997), but it would be better to include cross-validated calibrations to reduce dating errors, especially when reference dates are dissimilar (e.g. 63 and 31 Myr as opposed to 63 and 56 Myr).

(b) *Timing of the Rodentia evolution*

A Palaeocene age (65–55 Myr) is suggested by fossil data for the Rodentia radiation (Hartenberger 1998), whereas published molecular data indicate 98–125 Myr for the divergence between hystricognaths or sciurognaths relative to other eutherians (Janke *et al*. 1997; Kumar & Hedges 1998). Quartet dating based on nuclear *vWF* sequences (figure 2*c*) conservatively supports a radiation of Gliridae, Sciuroidea and Ctenodactylidae+Hystricognathi older than 41.5 Myr (Middle Eocene) or 50.7 Myr (Early Eocene, when the two slowest-rate quartets are excluded), though the median of the quartet distribution suggests that the divergence of these three clades may be as old as the Late Cretaceous (74 Myr). This suggests that fossils belonging to the stem group leading to glirids, sciurids and ctenodactylids + hystricognaths should be sought for in the Palaeocene, and might equally well be discovered in the Late Cretaceous. One should note that quartet results with the youngest calibrations are paradoxical as the Ctenodactylidae–Hystricognathi split is estimated to occur on average before the Rodentia radiation (figure 2*b* versus 2*c*). These inferred divergences which are too old

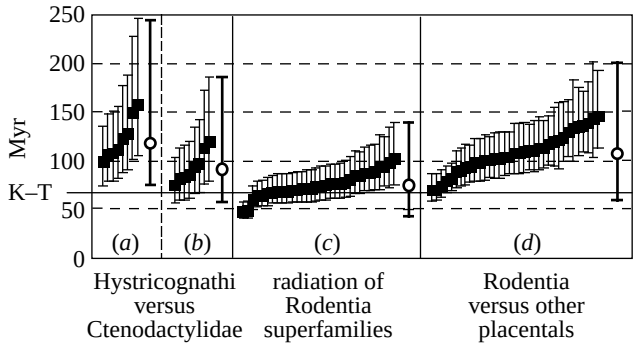

Figure 2. ML date estimates (black squares) and their 95% confidence intervals (bars) for divergences between Ctenodactylidae and Hystricognathi (*a*,*b*), Rodentia superfamilies Gliridae, Sciuroidea and Ctenodactylidae + Hystricognathi (*c*), and Rodentia versus other placentals (*d*). Quartets results are represented in ascending order. Medians of the 8, 8, 32 and 35 quartet dating estimates as well as the lower and upper limits of the 95% confidence intervals are indicated by empty circles and thick bars: 117.9 Myr (74.8–245.7), 89.8 Myr (57.0–186.2), 74.3 Myr (41.5 or 50.7–140.5, depending on the inclusion or exclusion of the two quartets involving sciuroids and glirids, the slowest-rate rodents), and 108.9 Myr (59.3–201.6), for respectively (*a*), (*b*), (*c*) and (*d*). The continuous line indicates the Cretaceous–Tertiary (K–T) limit at 65 Myr. The Ctenodactylidae–Hystricognathi divergence date was estimated with: (*a*) *Ctenodactylus–Massoutiera* at 13.0 Myr, combined to caviomorph diversification at 31.0 Myr; (*b*) *Ctenodactylus–Massoutiera* at 9.7 Myr, combined to caviomorph diversification at 24.0 Myr. Values have been computed by the quartet dating method (QDate 1.1 program by Rambaut & Bromham (1998)).

could result from an inaccurate interpretation of the Ctenodactylidae fossil record, combined with an insufficiently low number of analysed quartets (8 versus 32).

Concerning the split between rodents and other placentals, AAs clock-like trees suggest a Cretaceous radiation (table 2), at either 93.7 Myr (s.e. = 2.7; *Sus–Phocoena* calibration), or 96.9 Myr (s.e. = 3.9; *Equus–Ceratotherium* calibration), or else 105.0 Myr (s.e. = 3.1; Caviomorpha calibration), and conform to quartet dating which gives a median of 108.9 Myr (figure 2*d*). Each of the first two calibration points has been thought to be reliable (Arnason *et al*. 1996; Waddell *et al*. 1999), and here provides congruent dates between medium- and fast-evolving sequences, and between the two dating methods. This result contradicts the hypothesis of an Early Tertiary radiation of placental orders, and confirms previous molecular observations (reviewed in Bromham *et al*. 1999).

Quartet-dating analyses support a younger radiation of Gliridae, Sciuroidea and Ctenodactylidae + Hystricognathi relative to the split between Rodentia and other Placentalia (figure 2*c* versus 2*d*). However, our phylogenetic analyses on a nuclear marker weakly discriminate between both events (figure 1), though the highest-likelihood phylogram is congruent with mitochondrial and retroposon data (affinities between *Glis* and *Cavia*: Reyes *et al*. (1998); between glirids, sciurids, hystricids and caviids: Kramerov *et al*. (1999)). This low branching resolution may reflect either (i) the limited length of the *vWF* marker (466 first and second variable codon positions), and/or (ii) the superimposition of the rapid rodent cladogenesis on the bush-like radiation of placental orders (figure 1; Huchon *et al*. 1999), as illustrated by a partial overlap of quartet-dating confidence intervals (figure 2*c*,*d*).

(c) *Consequences of the phylogenetic position of Ctenodactylidae*

Our analyses strongly support a sister-clade relationship between Ctenodactylidae and Hystricognathi (figure 1), and reject the possibility that the Ctenodactylidae alone might be the earliest branching among rodents (e.g. Hartenberger 1985). They also invalidate the classification that divides Rodentia into reciprocally monophyletic Sciurognathi and Hystricognathi. To account for the *vWF* phylogenetic results, we suggest the following taxonomy for extant taxa.

(i) The suborder 'Ctenohystrica' is defined as a crown group, that is, the least-inclusive clade including all extant Ctenodactylidae and Hystricognathi. The name comes from the Greek 'cteno' (comb), alluding to the comb-like stiff bristles on the feet of Ctenodactylidae, and from 'hystrica' (porcupine), referring to one of the most typical Hystricognathi rodents. Two exclusive molecular synapomorphies across 38 placental *vWF* sequences define Ctenohystrica at the AA level: Lys, Arg, Ile or Ser replaced by Gln (position 198 of the AA sequence of the human *vWF* exon 28), and Glu by Gln or Arg (415). Five anatomical synapomorphies might also define Ctenohystrica: auditory bulla with weakly developed septae, vagina with a closure membrane, penis with a sacculus urethralis, presence of a scapuloclavicularis muscle, malleus and incus fused (Luckett & Hartenberger 1985, pp. 351, 459, 695).
(ii) Hystricognathi is considered as an infraorder.
(iii) Sciurognathi does not apply to a natural group.

The identification of the Ctenohystrica clade does have one consequence on the interpretation of the evolution of the incisor enamel microstructure, a character which has been thought reliable to infer rodent phylogeny. First ctenodactyloids are recorded since the Early Eocene (54.8–49.0 Myr) (e.g. McKenna & Bell 1997, p. 187; Hartenberger 1998), and possess incisors with a plesiomorphic pauciserial enamel (Martin 1993). A transitional state from pauciserial to multiserial microstructure is then evidenced in Middle Eocene (49–37 Myr) fossils (Martin 1993). The study of extant rodents show that Ctenodactylidae share a derived multiserial enamel with Hystricognathi and Pedetidae, whereas all other families display a uniserial enamel. Hystricognathi and Pedetidae might therefore branch within Ctenodactyloidea (Martin 1993, 1995; but see Flynn *et al*. 1986; Bryant & McKenna 1995; Wang 1997). However, *vWF* topologies clustering Pedetidae with either Ctenodactylidae or Hystricognathi are always significantly rejected ($p_{K-H} < 3.6\%$ for DNA; $p_{K-H} < 6.6\%$ for protein).

Pedetidae actually appears to be one major lineage, of the same importance as Gliridae, Myodonta, Sciuroidea and Ctenohystrica, and might be the earliest offshoot among rodents (figure 1). In this case, it would indicate that the multiserial enamel state appeared at least twice

independently during the evolution of rodents, once in gundis and hystricognaths, and once in springhares. Alternatively, we cannot rule out the possibility that Pedetidae is sister to Ctenohystrica (see § 3(b)). In that case, and because quartet datings conservatively indicate that the split between Ctenodactylidae and Hystricognathi is older than 57 Myr (figure 2*b*), it would involve development of multiserial enamel microstructure long before the Eocene, conflicting with the timing of enamel state transition documented by Martin (1993).

The Ctenohystrica monophyly suggests some new perspectives concerning taxonomic sampling in phylogenetic studies involving Rodentia. Species sampling in further morphological and molecular evolutionary studies might benefit from the simultaneous consideration of hystricognaths and ctenodactylids. It is also expected that molecular date estimates will be improved by the choice of several cross-validated calibration points, by an increase in the number and accuracy of palaeontological data, and by development of new dating methods, allowing variation of evolutionary rates (Sanderson 1997; Thorne *et al*. 1998).

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