Molecular Evolution of the Nuclear von Willebrand Factor Gene in Mammals and the Phylogeny of Rodents

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Nucleotide sequences of exon 28 of the von Willebrand Factor (vWF) were analyzed for a representative sampling of rodent families and eutherian orders, with one marsupial sequence as outgroup. The aim of this study was to test if inclusion of an increased taxonomic diversity in molecular analyses would shed light on three uncertainties concerning rodent phylogeny: (1) relationships between rodent families, (2) Rodentia monophyly, and (3) the sister group relationship of rodents and lagomorphs. The results did not give evidence of any particular rodent pattern of molecular evolution relative to a general eutherian pattern. Base compositions and rates of evolution of vWF sequences of rodents were in the range of placental variation. The 10 rodent families studied here cluster in five clades: Hystricognathi, Sciuridae and Aplodontidae (Sciuroidea), Muridae, Dipodidae, and Gliridae. Among hystricognaths, the following conclusions are drawn: a single colonization event in South America by Caviomorpha, a paralogy of Old World and New World porcupines, and an African origin for Old World porcupines. Despite a broader taxonomic sampling diversity, we did not obtain a robust answer to the question of Rodentia monophyly, but in the absence of any other alternative, we cannot reject the hypothesis of a single origin of rodents. Moreover, the phylogenetic position of Lagomorpha remains totally unsettled.

Introduction

The order Rodentia represents almost half of the mammalian diversity, with more than 2,000 species organized into 29 families (Wilson and Reeder 1993). Rodents include animals with very different morphologies, life histories, population structures, and demographic variables. Because of this diversity, rodents are a key taxon for the study of biogeography, ecology, and even patterns of DNA evolution. Numerous studies have focused only on mice and rats, but little is known about the overall rodent diversity. The Rodentia phylogeny is still controversial and provides an illustration of the conflicts between morphological and molecular approaches.

At the subordinal level, Brandt (1855) used the insertion pattern of masseter muscles to define myomorph, sciuromorph, and hystricomorph rodents, but this myological character has been shown to be homoplastic (Hartenberger 1985; Nedbal, Honeycutt, and Schlitter 1996). Tullberg (1899) used the plane of incisor insertion to split rodents into Sciurognathi and Hystricognathi, and this is the current classification (Wilson and Reeder 1993). Whereas Sciurognathi may be paraphyletic, Hystricognathi is recognized as monophyletic and includes Old World phiomorphs and New World caviomorphs. The lack of straightforward, nonhomoplastic morphological characters to define suprafamilial clades among rodents is explained by the bushlike radiation of rodent families associated with convergent adaptations in similar environments (Jaeger 1988; Hartenberger 1996).

Key words: evolution, phylogeny, mammals, rodents, Lagomorpha, von Willebrand Factor (vWF).

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Molecular investigations of the relationships between rodent families focused on three mitochondrial genes: 12S rRNA (Catzeflis et al. 1995; Nedbal, Honeneycutt, and Schlitter 1996), cytochrome oxidase II (Adkins, Honeycutt, and Disotell 1996), and cytochrome b (Matthee and Robinson 1997). Despite the use of ribosomal or protein-encoding mitochondrial markers, a general lack of phylogenetic resolution was observed, which could be the consequence of the rapid evolutionary rate of the mitochondrial genome. To explore the rodent phylogeny, the use of single-copy nuclear markers, e.g., exonic regions of the von Willebrand Factor gene (vWF; Porter, Goodman, and Stanhope 1996) or the lecithin cholesterol acyltransferase gene (LCAT; Robinson et al. 1997), appeared to be inescapable alternatives to mitochondrial studies.

The human vWF gene consists of a single functional nuclear copy with 52 exons spanning 180 kb on chromosome 12 (Mancuso et al. 1989). Exon 28 is the longest, with 1,379 bp corresponding to amino acids 463–921 of the mature subunit. The vWF gene codes for a plasma glycoprotein, constituted by multimerized 270-kDa subunits and produced by megakaryocytes and endothelial cells. It plays two major roles in haemostasis: it mediates platelet adhesion to damaged vessels and stabilizes blood coagulation factor VIII in the circulation (review in Sadler 1991).

Recently, Porter, Goodman, and Stanhope (1996) suggested that vWF exon 28 could be a powerful tool with which to investigate the eutharian phylogeny. In their analysis, the monophyly of three rodent species (Mus, Spalax, and Dasyprocta) was highly supported, but their location in the placental bushlike radiation remained unresolved (Porter, Goodman, and Stanhope 1996; Springer et al. 1997b). The large database now available for vWF exon 28 calls for further studies, especially for the exploration of the rodent diversity, together with the identification of a much-needed marsupial outgroup. Three interdependent questions are therefore asked: (1) What are the relationships between the main rodent families? (2) Is Rodentia a monophyletic order? (3) What are the closest living relatives of rodents?

To explore the Rodentia phylogeny in the context of eutharian evolution, we sequenced vWF exon 28 for representative rodent and lagomorph species and for a marsupial outgroup. The molecular evolution of this newly described phylogenetic marker is considered, and its contribution to the rodent phylogeny is evaluated at both familial and ordinal levels.

Materials and Methods
Species Sampling

We increased the mammalian data matrix of vWF sequences of Porter, Goodman, and Stanhope (1996) and Springer et al. (1997b) with 12 new rodent sequences, 2 new lagomorph sequences, and 1 new marsupial sequence (table 1). For each Sciurognathi family considered (Muridae, Dipodidae, Aplodontidae, Sciuridae, Gliridae), we analyzed, whenever possible, two species from different subfamilies in order to reduce a potential long-branch attraction phenomenon (Felsenstein 1978). For Hysterichognathi, whose subfamilial diversity is less important, we studied one species for two Phiomophia and three Caviomorpha families. In order to test for the Glires concept, three lagomorph sequences were considered: two Leporidae and one Ochotonidae. The sequence of a marsupial (the eastern gray kangaroo, Macropus giganteus) was determined in order to root the eutharian tree without ambiguity.

DNA Sequencing of vWF Exon 28

Tissue samples came from the Collection of Preserved Mammalian Tissues of the Institut des Sciences de l’Evolution de Montpellier (France) (Catzeflis 1991). Taxonomy, origins, and references of the tissues are shown in table 1. DNA extraction from the 95% ethanol-preserved tissues was performed according to Sambrook, Fritsch, and Maniatis (1989). The main part of exon 28 from the vWF gene (1,265 bp) was amplified with primers V1 (5'–TGTCAACCTCACCTGTAAGCCTG-3') and W1 (5'–TGCAGGACCAGGTCAGGAGCCTCTC-3'), constructed from the alignment of human, murine, canine, and bovine ortholog sequences. PCR reactions were performed using the following parameters: one cycle of 95°C denaturation (5 min), 50–60°C annealing (1 min), 72°C extension (2 min); 25 cycles of 95°C denaturation (45 s), 50–60°C annealing (45 s), 72°C extension (2 min); one cycle of 72°C final extension (15 min). A minimum of two independent PCR products were pooled and fragments were purified on a NuSieve (FMC Bioproduct) agarose gel (1% in 1× TAE). PCR products of the expected size (1,314 bp) were excised, purified with the Wizard® PCR preps DNA purification kit (Promega), and cloned in the pCR® 2.1 plasmid vector. The Escherichia coli strain INVaF was used for transformations according to the Original TA cloning kit (Invitrogen). Recombinant plasmids were sequenced using the dyeoxy chain termination method (Sanger, Nicklen, and Coulson 1977) with [α35S]dATP and the T7Sequencing® mixes kit (Pharmacia Biotech). Sequencing of vWF inserts was conducted on both strands with the V1 and W1 external primers, and with internal primers V2 (5'–CCCTCAGAGCTGCGGCGCAT-3'), W2 (5'–AGGTCATGCGCTGATCACT-3'), V3 (5'–TCCATGTATTTCCGATGTGCT-3'), W3 (5'–GTGTAATCGTGTTACCTGTTGCTC-3'), V4 (5'–AAAGTCGGCCCTGAACAAACAA-3'), W4 (5'–GCCCTTCAGGGGGTGTGCTT-3'), and W5 (5'–GGAGCCCTCCAGGAGA-3'). Internal primers were determined from the alignment of Porter, Goodman, and Stanhope (1996). For each species, a minimum of two clones was sequenced. The procedure was slightly modified for Dryomys nitedula and Allactaga elater. Fragments of 1,300 bp were reamplified with pairs of external and internal primers, and smaller overlapping fragments V1/W2 (903 bp), V2/W1 (992 bp), or V4/W1 (713 bp) were cloned and sequenced according to the protocol previously described. The sequence of M. giganteus was obtained by direct sequencing of the two overlapping V1/W2 and V2/W1 reamplified PCR frag-


Table 1
Taxonomic Frame Following Wilson and Reeder (1993) for the Rodent, Lagomorph, and Marsupial Taxa Studied

<table>
<thead>
<tr>
<th>Rodentia Sciurognathi</th>
<th>Muridae</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Murinae</td>
</tr>
<tr>
<td></td>
<td><em>Rattus norvegicus</em> (Norway rat); Yale Medical School; F. Catzeffis (T1413)/AJ224673; this paper</td>
</tr>
<tr>
<td></td>
<td><em>Mus musculus</em> (domestic mouse); BALB/C strain/U27810; Nichols et al. (1994)</td>
</tr>
<tr>
<td></td>
<td>Spalacinae</td>
</tr>
<tr>
<td></td>
<td><em>Spalax polonicus</em> (Ukrainian blind mole-rat); origin unknown/ U31621; Porter, Goodman, and Stanhope (1996)</td>
</tr>
<tr>
<td></td>
<td>Dipodidae</td>
</tr>
<tr>
<td></td>
<td>Dipodinae</td>
</tr>
<tr>
<td></td>
<td><em>Dipus sagitta</em> (northern three-toed jerboa); Caucasus, Georgia; P. Gambarian (T869)/AJ224665; this paper</td>
</tr>
<tr>
<td></td>
<td>Allactaginae</td>
</tr>
<tr>
<td></td>
<td><em>Allactaga elater</em> (small five-toed jerboa); Turbat Jam, Iran; M. Zadé (T1045)/AJ224661; this paper</td>
</tr>
<tr>
<td></td>
<td>Sciuridae</td>
</tr>
<tr>
<td></td>
<td>Sciurinae</td>
</tr>
<tr>
<td></td>
<td><em>Marmota monax</em> (woodchuck); Yukon, Canada; R. S. Hoffmann (T137)/AJ224671; this paper</td>
</tr>
<tr>
<td></td>
<td>Petauristinae</td>
</tr>
<tr>
<td></td>
<td><em>Glaucomys volans</em> (eastern flying squirrel); Audubon Zoo, New Orleans, Louisiana; R. M. Zink and D. Reynolds (T1110)/ AJ224667; this paper</td>
</tr>
<tr>
<td></td>
<td>Aplodontidae</td>
</tr>
<tr>
<td></td>
<td><em>Aplodontia rufa</em> (mountain beaver); Washington; D. Nolta (T1462)/AJ224662; this paper</td>
</tr>
<tr>
<td></td>
<td>Gliridae</td>
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<tr>
<td></td>
<td>Glirinae</td>
</tr>
<tr>
<td></td>
<td><em>Glis glis</em> (fat dormouse); Forel, Switzerland; F. Catzeffis (T1220)/AJ224668; this paper</td>
</tr>
<tr>
<td></td>
<td>Leithiinae</td>
</tr>
<tr>
<td></td>
<td><em>Dryomys nitedula</em> (forest dormouse); Caucasus, Georgia; M. Baskevich (T768)/AJ224666; this paper</td>
</tr>
<tr>
<td></td>
<td>Rodentia Hystricognathi</td>
</tr>
<tr>
<td></td>
<td>Thryonomyidae (Phiomorpha)</td>
</tr>
<tr>
<td></td>
<td><em>Thryonomys swinderianus</em> (cane rat); Koubatchi, Congo; L. Granjon (T809)/AJ224674; this paper</td>
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<tr>
<td></td>
<td>Hystricidae (Phiomorpha)</td>
</tr>
<tr>
<td></td>
<td><em>Trichys fasciculata</em> (long tailed porcupine); Sabah, Borneo; R. Stuebing (T726)/AJ224673; this paper</td>
</tr>
<tr>
<td></td>
<td>Erethizontidae (Caviomorpha)</td>
</tr>
<tr>
<td></td>
<td><em>Coendou melanurus</em> (prehensile tailed porcupine); Kourou, French Guiana; J.-C. Vié (T1559)/AJ224664; this paper</td>
</tr>
<tr>
<td></td>
<td>Caviidae (Caviomorpha)</td>
</tr>
<tr>
<td></td>
<td><em>Cavia porcellus</em> (guinea pig); breeding colony; P. Perret (T1575)/AJ224663; this paper</td>
</tr>
<tr>
<td></td>
<td>Dasypodictidae (Caviomorpha)</td>
</tr>
<tr>
<td></td>
<td><em>Dasyprocta leporina</em> (agouti); origin unknown/U31607; Porter, Goodman, and Stanhope (1996)</td>
</tr>
<tr>
<td></td>
<td>Lagomorpha</td>
</tr>
<tr>
<td></td>
<td>Leporidae</td>
</tr>
<tr>
<td></td>
<td><em>Lepus crawshayi</em> (hare); Yono Féré, Senegal; J.-M. Duplantier and L. Granjon (T480)/AJ224669; this paper</td>
</tr>
<tr>
<td></td>
<td>Oryctolagus (rabbit); origin unknown/U31618; Porter, Goodman, and Stanhope (1996)</td>
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<tr>
<td></td>
<td>Ochotonidae</td>
</tr>
<tr>
<td></td>
<td><em>Ochotona princeps</em> (pika); Albuquerque, New Mexico; T. Yates (T1691)/AJ224672; this paper</td>
</tr>
<tr>
<td></td>
<td>Diprotodontia</td>
</tr>
<tr>
<td></td>
<td>Macropodidae</td>
</tr>
<tr>
<td></td>
<td><em>Macropus giganteus</em> (eastern gray kangaroo); Zoo La Palmyre, France; T. Petit (T727)/AJ224670; this paper</td>
</tr>
</tbody>
</table>

**Note:** The following information is also provided: latin name (common name), origin of the animal, name of the tissue collector (catalog number in the collection of mammalian tissues of the Institut des Sciences de l’Evolution de Montpellier; Catzeffis 1991)/EMBL Data Bank accession numbers for the sequences of the exon 28 of the von Willebrand Factor gene, and reference of the corresponding works.

Sequence Alignment

Sequences were aligned by hand with the ED editor (MUST package; Philippe 1993). The alignment was unequivocal; a single insertion of one codon was required for the two Gliridae species, and a deletion of one codon was introduced for the *Macropus* sequence. In the subsequent analyses, gaps were coded as missing data.

Phylogenetic Reconstructions

Data were analyzed by distance, maximum-parsimony (MP) and maximum-likelihood (ML) methods. The neighbor-joining method (NJ; Saitou and Nei 1987) was performed on Tamura and Nei (1993) distances with the NJ program (MUST package; Philippe 1993). MP analyses were conducted with PAUP 3.1.1 (Swofford 1993). Heuristic searches were realized with the tree bi-section-reconnection (TBR) branch-swapping option and 100 random-addition sequences. ML trees were constructed with MOLPHY 2.3b3 (Adachi and Hasegawa 1996). Direct sequencing was realized with [α-33P]ddNTP and using the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham).
1996b) and with the quartet puzzling procedure of Strimmer and von Haeseler (1996) using PUZZLE 4.0.
The Tamura and Nei (1993) model of sequence evolution was used to describe the substitution rate heterogeneities (Yang 1996). Because of the computation time limitations of MOLPHY 2.3b3, orders represented by only one species were discarded (Artiodactyla, Dermoptera, Pholidota, and Xenarthra), as were primates and carnivoires, which were represented only by two closely related taxa. All rodent and lagomorph species were kept with two pe-rirossoactyls, two bats (the Microchiroptera Tomatia and the Megachiroptera Dobsonia), two divergent Paenun-gulata sensu lato (Amblysomus and Elephas) and the kangaroo (Macropus). The MOLPHY 2.3b3 search for the best dichotomous ML tree for this limited data set of 25 species was realized in four steps: (1) a constrained topology was defined (i.e., the quartet puzzling tree; see below and fig. 2), with unresolved relationships (multifurcations) left free to vary; (2) an exhaustive search was done with the ProtML program, and the best 1,000 amino acid trees were retained by the approximate-likelihood criterion; (3) the log-likelihood of these 1,000 starting trees was computed with NucML for each codon position separately; and (4) the log-likelihoods were summed up over the three positions with TotalML.
The robustness of trees was assessed by bootstrap percentages (BP; Felsenstein 1985) after 1,000 replications for NJ and MP. In the case of ML, reliability percentages (RP; Strimmer and von Haeseler 1996) estimated the occurrence of the nodes in the quartet puz-zling trees after 10,000 puzzling steps. Bremer’s (1988) support indices (BSIs) were calculated on the most parsimonious trees with enforcement of topological constraints. Likelihoods of alternative topologies were compared with MOLPHY 2.3b3 (Adachi and Hasegawa 1996b). According to Kishino and Hasegawa (1989), an alternative hypothesis was rejected when lnL > 1.96 SE, where lnL is the difference between the log-like-lihoods of the best and the evaluated trees, and SE is the standard error of this difference.

Homoplasy and Saturation Analysis
Pairwise numbers of observed nucleotide differ-ences were plotted against changes inferred by MP for each of the six types of substitutions at the three codon positions (Hassanin, Lecointre, and Tiller 1998). Each of the 18 resulting plots (obtained through the “homoplasy matrix” option of PAUP 3.1.1) was characterized by the slope (S) of the linear regression between observed and inferred mutational events and by the consistency index (CI; excluding uninformative characters) of the corresponding most-parsimonious trees. The character-state changes were then weighted according to the product CI × S of the homoplasy and saturation parameters in the weighted MP (MPw) reconstructions (cited in Hassanin, Lecointre, and Tiller 1998).

Relative-Rate Test
The two-cluster test and the branch-length test (Takezaki, Rzhetsky, and Nei 1995) were conducted with the LINTRE package (http://www.bio.psu.edu/People/Faculty/Nei/Lab/) to evaluate the clocklike behavior of the data. The Tamura and Nei (1993) and amino acid distances with gamma rates were, respectively, applied on nucleotide and amino acid matrices (the rate heteroge-neity parameter α was estimated by PUZZLE 4.0). The topology used for the tests was the MPw tree (see below and fig. 1).

For comparisons between lineages, relative-rate tests were conducted with RRTree, version 0.5 (Robinson et al. 1998; ftp://pbiol.univ-lyon1.fr/pub/datasets/MBE98/), which improves the test of Wu and Li (1985) by taking into account several sequences per lineage under focus, and introducing a topological weighting. Relative-rate tests were performed for different taxonomic levels (supra- and infrafamilial comparisons), and the ML tree computed by quartet puzzling was chosen as the reference phylogeny (see below and fig. 2). In each case, the outgroup chosen was diversified, slow-evolv-ing, and closely related to the ingroup. Such properties are more prone to reveal a rate heterogeneity (Robinson et al. 1998). Relative-rate tests on nucleotide sequences were performed on the proportions of synonymous (Ks) and nonsynonymous (Ks) substitutions.

Results
Nucleotide and Amino Acid Characteristics of vWF Exon 28

Nucleotides
The third codon position of exon 28 exhibited the highest variability, the highest bias against transversions, and the lowest substitution rate heterogeneity (table 2). The strongest bias in nucleotide composition was also found at the third position of the codon (table 2). By comparison with the human sequence, a standard used for isochore studies (e.g., Robinson, Gautier, and Mouchiroud 1997), eutherians grouped in three catego-ries following their G+C content at the third codon po-sition (GC3): (1) ΔGC3 (i.e., GC3human − GC3others) ranged from 0 to +10% for Chiroptera, Artiodactyla, Xenarthra, Pholidota, Dermoptera, Leporidae, Thryonomyys, Dipus, and the two Gliridae; (2) ΔGC3 ranged from 0 to −10% for Perissodactyla, Carnivora, Theri-theria, Ochotonidae, the two Sciuridae, the Aplodontidae, Al-actaga, and the two porcupines; (3) ΔGC3 exceeded −10% for Cavia, Dasypodidae, three Muridae, and the four Paenungulata s.l. (Procavia, Orycteropus, Ele-phantulus, Amblyomus). Relative to the placental or-thologs, the marsupial (M. giganteus) vWF sequence showed a strongly deviating ΔGC3 (+23%).

Amino Acids
The vWF amino acid composition indicated that exon 28 encodes a hydrophobic region, and the most frequent amino acids were valine (11.1%), leucine (8.7%), arginine (7.9%), serine (7.9%), and glutamine (7.2%). Variable sites of the polypeptide were mainly
Phylogenetic Reconstructions

Equally Weighted Maximum Parsimony

MP analyses based on equal weighting of each nucleotide substitution strongly supported the monophyly of representative species of the three Rodentia families Dipodidae, Sciuridae, and Gliridae (fig. 1; BP > 95% and BSI > +11). Lower support was evidenced for the three Muridae species (*Mus, Rattus*, and *Spalax*; BP = 73% and BSI = +5).

vWF exon 28 provided marked support for several superfamilial clades: Sciuroidea (Sciuridae + Aplodontidae; BP = 86% and BSI = +5), Caviomorpha (BP = 99% and BSI = +14), Caviomorpha + Hystricidae (BP = 90% and BSI = +9), and Hystricomorpha (the latter two taxa plus Thryonomyidae; BP = 100% and BSI = +17). Within caviomorphs, *Coendou* (Erethizontidae) emerged first relative to the two other South American taxa. The two Phiomorpha representatives (*Thryonomys* and *Trichys*) constituted a paraphyletic assemblage. Five rodent lineages (Dipodidae, Muridae, Sciuroidea, Hystricognathi, and Gliridae) were therefore clearly defined (fig. 1), and relationships between them were not resolved. Rodentia appeared to be monophyletic, but the support was very weak (BP = 33% and BSI = +2). Lagomorpha was monophyletic (BP = 98% and BSI = +14), and the two Leporidae clustered together (BP = 100% and BSI = +25) with respect to the Ochotonidae species. Negligible support was observed for the monophyly of Glires (i.e., Rodentia + Lagomorpha). Basal relationships between eutherian orders received only
very weak support, except for a clade comprising X.
Carnivora, Perissodactyla, Pholidota, and Chiroptera, which was defined by a high BSI (+7) but a low BP (48). Other relationships concerning placental orders (e.g., Chiroptera or Paenungulata) were similar to those described by Porter, Goodman, and Stanhope (1996) and Springer et al. (1997).

To check if one of the 38 placental vWF sequences behaved randomly (see, e.g., Sullivan and Swofford 1997), 100 random sequences were generated using MacClade 3.04 (Maddison and Maddison 1992), constraining each codon position to respect the mean placental vWF base composition. Due to computing time limitations, 100 MP analyses were run, each with one random sequence, and BPs after 100 replications were approximated without branch swapping. Most of the random sequences weakly clustered with either the canine, and Chiroptera, which was defined by a high BSI (+7) but a low BP (48). Other relationships concerning placental orders (e.g., Chiroptera or Paenungulata) were similar to those described by Porter, Goodman, and Stanhope (1996) and Springer et al. (1997).

Table 2

<table>
<thead>
<tr>
<th>Position 1</th>
<th>Position 2</th>
<th>Position 3</th>
<th>All Positions</th>
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<td>31.0</td>
<td>8.6</td>
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<tr>
<td>% C</td>
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</tr>
<tr>
<td>% G+C</td>
<td>63.3</td>
<td>38.7</td>
<td>78.2</td>
</tr>
</tbody>
</table>

Table 2 Mean Base Composition and Number of Sites Compared (total, variable, and phylogenetically informative) for von Willebrand Factor Exon 28 Sequences of 1 Marsupial and 38 Placentals at Each Codon Position and for the Whole Molecule

Number of sites

- Total: 412, 412, 412, 1236
- Variable: 255, 210, 392, 857
- Informative: 167, 128, 341, 636
- Ti/Tv: 1.39, 2.38, 4.26, 2.44
- \( \alpha \) parameter: 0.48, 0.36, 2.25, 0.52

Maximum Likelihood

The ML tree obtained with the PUZZLE 4.0 program is presented in figure 2. All the nodes that were strongly supported in the MP tree were retrieved, and the five rodent clades and the other placental orders emerged in the same multifurcation.

The highest-likelihood tree computed with 25 species showed the monophyly of rodents but not that of Glires (fig. 3). Among rodents, Myodonta (Muridae + Dipodidae) was monophyletic, and sciuroidea clustered with glires. Moreover, the relationships between placental orders were depicted by very short branches, and 720 were significantly worse relative to the best tree (fig. 3). Among the 280 remaining trees, Rodentia as well as Myodonta appeared to be monophyletic in the 100 best phylograms, and the Glires monophyly was not statistically rejected.

The Alternatives to the Best Trees

Only two main alternatives to Rodentia monophyly were retrieved by both NJ and MP bootstrap analyses as well as in the 280 most likely trees: (1) Lagomorpha clustered with either Glires, Dipodidae or both; (2) Muridae or Myodonta, and then the other rodent clades, were sister group to all other placentals (i.e., the eutherian tree was rooted by Macropus on the Muridae or Myodonta nodes).

Fig. 2—Maximum-likelihood phylogram (lnL = -17,831.8) calculated by quartet puzzling after 10,000 replicates (PUZZLE 4.0 program) on 38 placental DNA sequences of vWF exon 28 and rooted by the marsupial sequence. Reliability percentages are reported near the corresponding nodes, and nodes supported by less than 50% are collapsed into multifurcations. Branch lengths are proportional to the estimated number of substitutions per site. The tree star presentation has been adopted to highlight the superimposition of the bushlike radiation of rodent families and placental orders. All rodent taxa branches are thickened, and the arrow is directed toward the outgroup (Macropus giganteus).
The highest-likelihood tree (fig. 3) was then used as the reference topology to investigate all 105 relationships connecting the five hystricognaths. Only two topologies did not exhibit significantly worse log-likelihood: (1) the Phiomorpha and Caviomorpha monophilies (+0.67 SE) and (2) the exchange of the relative positions of Trichys and Thryonomys (+0.55 SE).

Reconstructions on Amino Acids

Plots of the number of observed aa differences against inferred substitutions did not reveal that variable regions were experiencing multiple aa replacements or saturation (data not shown). For this reason, all sites were kept in the phylogenetic reconstructions. Amino acid analyses showed congruent but more unresolved results (table 3), and did not support the clade comprising Xenarthra, Pholidota, Chiroptera, Carnivora, Perissodactyla, and Artiodactyla.

Altogether, the results obtained with standard MP and ML on nucleotide and aa matrices were congruent with those obtained with weighted MP and NJ (except for weak Sciuroidea support with the distance approach) (table 3).

Relative-Rate Tests

A branch length test indicated that half of the 39 nucleotide and amino acid sequences evolved with an evolutionary rate significantly different from the average ($P < 0.05$). A two-cluster test showed that, for both data sets, five robustly supported nodes exhibited significantly different rates in their descent lineages. Mus and Rattus, Thryonomys, Felis, Procavia, and Elephantulus, respectively, evolved faster than Spalax, the remaining hystricognaths, Canis, Dugong, and the other paenungulates s.l.

To identify taxa involved in the vWF substitution rate heterogeneities, relative-rate tests were conducted

---

**Table 3**

Indices of Robustness for Various Nodes of the Phylogenetic Trees Reconstructed from DNA and Amino Acid vWF Exon 28 sequences Using Maximum-Parsimony (MP), Distance, and Maximum-likelihood (ML) Analyses

<table>
<thead>
<tr>
<th>Clades</th>
<th>NUCLEOTIDE ANALYSIS</th>
<th>AMINO ACID ANALYSIS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BSI</td>
<td>MP</td>
</tr>
<tr>
<td>Murinae</td>
<td>+29</td>
<td>100</td>
</tr>
<tr>
<td>Muridae</td>
<td>+5</td>
<td>73</td>
</tr>
<tr>
<td>Dipodidae</td>
<td>+22</td>
<td>100</td>
</tr>
<tr>
<td>Myodonta</td>
<td>+3</td>
<td>38</td>
</tr>
<tr>
<td>Sciuridae</td>
<td>+11</td>
<td>95</td>
</tr>
<tr>
<td>Sciuroidea</td>
<td>+5</td>
<td>86</td>
</tr>
<tr>
<td>Gliridae</td>
<td>+18</td>
<td>99</td>
</tr>
<tr>
<td>Caviidae + Dasyproctidae</td>
<td>+10</td>
<td>98</td>
</tr>
<tr>
<td>Caviomorpha + Hystricidae</td>
<td>+14</td>
<td>99</td>
</tr>
<tr>
<td>Caviomorpha + Hystrixidae</td>
<td>+9</td>
<td>90</td>
</tr>
<tr>
<td>Hystricognathi</td>
<td>+17</td>
<td>100</td>
</tr>
<tr>
<td>Rodentia monophyly</td>
<td>+2</td>
<td>33</td>
</tr>
<tr>
<td>Rodentia + Lagomorpha</td>
<td>12</td>
<td>25</td>
</tr>
</tbody>
</table>

Note.—Bremer support indices (BSIs), i.e., numbers of extra steps required to break the corresponding nodes under the MP criterion, are first given. Then, bootstrap percentages computed after standard MP (i.e., with equal weighting), MP weighted by consistency indices and slopes of saturation profiles (MP$_{w}$, for DNA analysis only), and neighbor-joining (NJ) on Tamura and Nei (1993) distances with gamma rates ($n = 0.52$ for DNA and 0.64 for amino acids) are reported. Reliability percentages deduced from the quartet puzzling ML method are also given. A dash indicates that the node does not appear in the corresponding majority-rule consensus bootstrap tree.
with each of the 15 placental clades (cf. fig. 2) against the remaining groups. Whereas neither Ks nor amino acid comparisons showed significant contrast, Ks comparisons evidenced marked differences in evolutionary rates: Sciuroidea were slowly evolving (P < 0.01), and Hystricidae and Xenarthra were fast-evolving (P < 0.05). Muridae did not have a particularly fast rate of evolution. Differences in behavior between Ks and Ks could reflect the fact that synonymous substitutions saturated at the ordinal level for eutherians. The lack of significant results at the amino acid level could also result from the lower number of sites analyzed.

Relative-rate tests were then performed within each rodent family, with the slow-evolving sciuroids as outgroup. Comparisons within murids confirmed that Spalax evolved more slowly than did Mus and Rattus (P < 0.05 for Ks comparisons; P < 0.01 for Ks comparisons). Within hystricognaths, significant Ks comparison involved a fast-evolving Thryonomys relative to Coendou (P < 0.05). Then, Thryonomys appeared to be the fastest-evolving hystricognath, as evidenced in the two-cluster test (see also branch length in figs. 2 and 3). Neither DNA comparisons within sciuroids, gliroids, or dipodids nor amino acid comparisons with each rodent family were significant.

Discussion

vWF Exon 28 Structure and Function

Medical studies revealed that two different bleeding disorders result from point mutations in two regions of exon 28 (review in Ginsburg and Sadler 1993). IIb mutations are responsible for spontaneous platelet aggregation and occur between aa 497 and 698. This region includes a disulphide loop between Cys(509) and Cys(695) that could contain the sites involved in vWF ability to react with platelets (e.g., Matsushita and Sadler 1995). IIa mutations occur between Gly(742) and Glu(875) and decrease the half-life of the vWF protein in blood circulation (Berkowitz et al. 1987). Our vWF sequence comparisons across mammals suggest that: (1) the cysteine loop and the Ila mutations domain and its surrounding sites are characterized by a high conservation of aa sites, in agreement with medical works which suggest a major functional role of these two regions; and (2) the short Cys(695)–Pro(709) domain, including part of the IIb region, is more variable, although it has been suggested that it may play an important role in vWF adhesion to platelets. The latter observation agrees with the results of Matsushita and Sadler (1995), which moderate the functional importance of the aa 695–709 domain.

Evolution of vWF Exon 28 in Mammals: Are Rodents a Molecular Exception?

It has been suggested that some rodents have many molecular peculiarities relative to other eutherians. For example, Muridae possess a more homogeneous genome base composition than do phylogenetically distant placental species which represent the “general mammalian pattern” (Mouchiroud and Bernardi 1993; Sabeur et al. 1993; Robinson, Gautier, and Mouchiroud 1997). It has also been claimed that rodents have a fast-evolving genome because they have short generation times, small adult body sizes, and/or high metabolic rates (Wu and Li 1985; Catzeflis et al. 1987; Ohta 1993; Li et al. 1996). These results, however, were deduced from a restricted set of murid species (mouse, rat, hamster).

Base Composition

The GC3 composition of vWF (ranging from 69% to 90% in eutherians) indicates that this gene belongs to the richest class of isochores in the genome. This class is characterized by the most contrasted differences between murid and human base compositions (Mouchiroud and Gautier 1990). Comparison of hominid (82% GC3), murid (69%–70%), and cavimorph (69%–77%) vWF sequences shows that their GC3 compositions agree with the hypothesis of a GC homogenization, and/or reordering, in murine and guinea pig genomes (Robinson, Gautier, and Mouchiroud 1997). One should also note that some paenungulates show GC3 contents similar to those of murids. Generalizations are thus premature, as our taxonomic sampling indicates that in the same rodent family, two different genera can have strikingly contrasted base compositions; among Dipodidae, ΔGC3 is −5.8% for Dipus versus +5.7% for Allactaga. The same contrast in ΔGC3 values is observed for some eutherian orders, such as Lagomorpha (Ochotona +2.6%, Lepus −7.8%).

Molecular Evolutionary Rates

Relative-rate tests indicate that vWF exon 28 of rodents is not especially fast-evolving compared with those of other eutherian species. Faster-evolving clades (Hystricognathi), as well as slower-evolving clades (Sciuroidea), are evidenced among rodents. Moreover, animals with relatively similar life history traits, such as South American porcupines (Coendou) and African cane rats (Thryonomys), exhibit significantly different rates of evolution. These results weaken the hypothesis suggesting that rates of molecular evolution are mainly correlated to generation times (e.g., Li et al. 1996), and they suggest that other parameters, such as metabolic rates and paleodemographic evolutions, may be acting (Martin and Palumbi 1993; Eastal and Collet 1994; Douzery, Lebreton, and Catzeflis 1995; Matthee and Robinson 1997).

Within rodents, murid sequences are not especially fast-evolving, and this finding contrasts with other nuclear and mitochondrial studies (e.g., Catzeflis et al. 1987; Philippe 1997). This result is probably the consequence of the use of a “topology-weighting” procedure in the computation of the relative-rate test (Robinson et al. 1998). Spalax is a slow-evolving genus, and its lineage has a topological weight of one-half among the three Muridae sequences. However, the mouse and the rat remain fast-evolving species relative to Spalax. It is also noteworthy that Thryonomys is a particularly fast evolving species. Such a fast rate of evolution for the cane rat was also reported after mitochondrial cytochrome b studies (Matthee and Robinson 1997). Those
observations may suggest a correlation between rates of evolution of nuclear and mitochondrial genomes.

In conclusion, our observations indicate that rodent biodiversity cannot be summarized by just a few exemplar sequences. When all available taxonomic diversity is considered, rodents do not appear to have a particular pattern of evolution, as they are included in the range of the eutherian diversity. We here caution that these inferences are derived from a single nuclear gene, and we do not generalize our results to the whole genome.

Phylogenetic Relationships Between Rodentia Families

Phylogenetic analyses of the vWF DNA sequences evidence 5 distinct clades among the 10 rodent families studied: Hystricognathi, Sciuroidea (Sciuridae and Aplodontiidae), Muridae, Dipodidae, and Gliridae. The interrelationships between these five clades are not robustly resolved (figs. 1–3). Such a lack of phylogenetic resolution between Rodentia families has also been observed with mitochondrial 12S rRNA (Nedbal, Honeycutt, and Schlitter 1996) and nuclear LCAT comparisons (Robinson et al. 1997).

Hystricognathi

Hystricognathi is the best-supported clade in the present analyses. Comparison of 38 placental vWF amino acid sequences and 1 marsupial vWF amino acid sequence indicates that hystricognaths are defined by six exclusive synapomorphic replacements of residues: Q by S (position 626, referring to the complete vWF human sequence), K by Q (position 662), L by H (position 699), E by A (position 762), R by Q (position 815), and H by G (position 873).

Among hystricognaths, living Caviomorpha are restricted to South America, where they suddenly appear in the fossil record at the Late Eocene–Early Oligocene transition, between 37.5 and 31.5 MYA (Wyss et al. 1993). The biogeographical origin of caviomorphs relative to phiomorphs remains controversial. Either the hystricognath radiation took place in North America (Wood 1985), with subsequent colonization of South America by caviomorphs and Africa by phiomorphs, or South American caviomorphs originated from an African phiomorph stock (Lavocat 1969). These two hypotheses, respectively, involve either the monophyly of Phiomorpha (here, *Thryonomys* + *Trichys*) or their paraphyly.

Phylogenetic analyses of vWF data evidence an unexpected result: the sister group relationship between Old World porcupines (Hystricidae) and Caviomorpha (table 2). This is the consequence of the basal position of Thryonomyidae among hystricognaths and could be the result of a long-branch attraction phenomenon (Felsenstein 1978), as the cane rat (*Thryonomys*) exhibits the fastest-evolving and richest GC3 hystricognath sequence. Alternative topologies are not significantly different from an ML point of view: log-likelihoods of trees either clustering *Thryonomys* with Caviomorpha or clustering *Thryonomys* with *Trichys* are, respectively, 0.55 SE and 0.67 SE ($P > 0.50$) lower than that of the best tree (which groups *Trichys* with Caviomorpha; fig. 3). Our results therefore favor the hypothesis of Lavocat (1969) but cannot statistically reject the Wood (1985) hypothesis. Current paleontological evidence assumes an African origin of Caviomorpha before 37 MYA (Lavocat 1969; Wyss et al. 1993), while extant Thryonomyidae and Hystricidae are located, respectively, in Africa and in Asia plus Africa. Identification of a Caviomorpha + *Trichys* clade (table 2 and figs. 1–3, but see above) suggests an African location of Old World porcupines ancestors, possibly at the end of the Eocene. The latter observation agrees with Jaeger, Denys, and Coif-fait (1985), even if available hystricid fossils are recorded only in South Asia Miocene deposits. One should also note that our comparisons include representatives of only two of the four Phiomorpha families.

Improving the taxonomic diversity for phiomorphs, i.e., adding Petromuridae and Bathyergidae taxa, could help to break the long Thryonomyidae branch, to assess the question of Phiomorpha monophyly, and to test for the phylogenetic affinities of Hystricidae.

South American porcupines (Erethizontidae) are, from a morphological point of view, a rogue taxon. Despite their extant American location, these porcupines are sometimes considered a distinct caviomorph lineage, possibly grouped with Old World porcupines (e.g., Lavocat and Parent 1985), or considered the deepest originating hystricognath family (e.g., Woods and Herman-son 1985; Bryant and McKenna 1995). All of the 102 topologies involving the paraphyly of caviomorphs are significantly less likely than the three showing their monophyly ($SE = 1.97–4.09$, $P < 0.05$). Therefore, in the highest-likelihood vWF phylogenies, (1) erethizontids are true caviomorphs; (2) *Coendou* is a sister group to *Cavia*/Dasyprocta; (3) “porcupines” are paraphyletic.

Sciuridae and Aplodontiidae

The Aplodontiidae are currently represented by a single species highly adapted to a semifossorial way of life. Morphological studies conflict about Aplodontiidae relationships, because extant mountain beavers mix autapomorphic and plesiomorphic traits (e.g., Bugge 1985). MP and ML analyses of vWF characters strongly cluster Aplodontiidae with Sciuridae into a Sciuroidea clade (table 3). This agrees with conclusions based on mitochondrial data (Nedbal, Honeycutt, and Schlitter 1996) and with some morphological plus paleontologi-cal analyses (review in Luckett and Hartenberger 1985).

Muridae and Dipodidae

Support for a Myodonta clade (Muridae + Dipod-idae) is provided by studies on morphological, myolog-ical, and embryological (review in Luckett and Harten-berger 1985) and molecular retroposon (Serdobova and Kramerov 1998) characters. Conversely, 12S rDNA and vWF sequence data weakly support the monophyly of Myodonta (Nedbal, Honeycutt, and Schlitter 1996; table 2). This weak support could be the result of a deep divergence between the two clades (possibly as old as the Late Eocene; Flynn, Jacobs, and Lindsay 1985) com-
bined with contrasted base compositions between murid and dipodid DNA sequences.

**Gliridae**

The Gliridae were traditionally grouped with Muridae and Dipodidae because they show a myomorph pattern, but it has been inferred from paleontological data that gliroids actually display a pseudomyomorph pattern (Vianey-Liaud 1985). Other analyses clustered Gliridae either with Sciuridae (Bugge 1985; Lavocat and Parent 1985) or with Hystriocnathi (Reyes, Pesole, and Saccone 1998 [sciurids were not included in their mitochondrial DNA study]). The vWF data suggest the grouping of Gliridae with Sciuroidea (fig. 3). However, there is no significant difference in likelihood among the three alternatives: log-likelihoods of trees displaying the Gliiridae/Hystriocnathi and Gliidae/Myodonta associations are, respectively, 0.12 SE and 0.69 SE ($P > 0.49$) lower than that of the best ML tree and its Gliiridae/Sciuroidea node (fig. 3). Gliridae vWF polypeptides are also characterized by an insertion of one Pro or Tyr residue between human positions 706 and 707, and this character could allow identification of the gliroid sister clade among other rodent families.

**Rodentia in the Context of the Eutherian Ordinal Radiation**

**The Question of Rodentia Monophyly**

Rodentia appeared monophyletic in the MP (fig. 1) and ML (fig. 3) phylogenetic trees, but the support indices were very weak (table 2). The same results were obtained with 12S rRNA sequences (Nedbal, Honeycutt, and Schlitter 1996). Conversely, Luckett and Hartenberger (1993) listed seven exclusive morphological synapomorphies to define Rodentia. The fossil record indicates that an explosive radiation took place just after the emergence of the order and produced an average number of 2.8 families per million years (Hartenberger 1996). These subsequent radiations probably precluded the accumulation of synapomorphies in molecular markers, and consequently, phylogenetic analyses cannot robustly resolve the questions of rodent monophyly and most of relationships between rodent families. The discrepancy between the numbers of morphological and molecular synapomorphies defining Rodentia remains a conundrum.

In this study, the increased taxonomic diversity for rodents leads to one conclusion drawn by Porter, Goodman, and Stanhope (1996). In their vWF analysis, the monophyly of Rodentia was highly supported, but only three representatives were included: *Dasyprocta* (Caviomorpha), *Mus*, and *Spalax* (Muridae). Our results indicate that their high support for rodent monophyly was based on homoplastic changes. More generally, given the tremendous Rodentia biodiversity, phylogenetic conclusions concerning the order cannot be inferred without ambiguity with only three taxa.

A key point in favor of rodent monophyly is that there is no obvious alternative against it. Moreover, vWF alternatives are not equivalent to those proposed by Graur, Hide, and Li (1991), D’Erchia et al. (1996), or Reyes, Pesole, and Saccone (1998) (but see Philippe 1997; Sullivan and Swofford 1997; Halanych 1998). To discover which rodent lineage may emerge first among placentals, we constrained each rodent clade of the best tree (fig. 3) to be the sister group of the remaining placentals. All resulting trees had significantly lower log-likelihoods, except when Hystriocnathi or Gliridae were displaced. Contrasting with results derived from complete mitochondrial genome comparisons (D’Erchia et al. 1996; Reyes, Pesole, and Saccone 1998), the topology was significantly worse when Muridae alone (i.e., without the other rodents) or Myodonta alone were moved at the base of the tree.

One should also note that vWF alternatives cluster species having extreme GC levels. These alternatives could be the consequence of sensitivity to base composition of reconstruction methods (e.g., Galtier and Gouy 1995). Hence, we consider that the traditional hypothesis of Rodentia monophyly is not yet rejected—nor confirmed—by comparative molecular approaches.

**The Bushlike Radiation of Placental Orders**

The comparison of 39 vWF sequences of placentals and a marsupial illustrates the rapid cladogenesis of Rodentia families and its superimposition on the bushlike radiation of placental orders (fig. 2). The additional sampling of Lagomorpha biodiversity did not allow us to identify the sister clade of rabbits, hares, and pikas among eutherians. Although Luckett and Hartenberger (1993) found evidence of 12 morphological and embryological synapomorphies for Glires, no strong vWF signal was found for the association of Lagomorpha with Rodentia (table 3).

Other eutherian relationships obtained with exon 28 of the vWF gene were previously discussed by Porter, Goodman, and Stanhope (1996). Moreover, the presence of the marsupial sequence allowed investigation of the phylogenetic position of Xanarthra. It was repeatedly proposed that armadillos, anteaters, and sloths may represent the oldest extant eutherian order (Novacek 1992), and xanarthran sequences were commonly used to root molecular trees (e.g., Porter, Goodman, and Stanhope 1996; Madsen et al. 1997; Springer et al. 1997b). Recent molecular studies suggested that Xanarthra could cluster with a Ferungulata clade including Cetartiodactyla, Carnivora, and Perissodactyla (Arnason, Gullberg, and Janke 1997) or with Paenungulata *s.l.* (Springer et al. 1997a). Our analysis of mammalian vWF sequences reveals that among eutherians, the xanarthran sequence repeatedly clusters with random sequences (see Results). Consequently, its relative phylogenetic position could be the result of random behavior. Phylogenetic conclusions about the order Xanarthra will be doubtful without additional sampling within its four families.

**Conclusion**

Our results strengthen the importance of considering taxonomic diversity before reaching molecular conclusions in such a difficult area of phylogeny as the evolution of rodents. First, it appears that mouse and rat
sequences do not adequately represent the rodent or even the murid pattern of evolution. Second, embracing several well-chosen representatives helps to avoid biased phylogenetic conclusions (Lecointre et al. 1993; Philippe and Douzery 1994; Adachi and Hasegawa 1996a).

Further research in the field of rodent phylogeny will identify the total number of major extant clades through the sampling of additional families (e.g., Ctenodactylidae, Castoridae, Pedetidae, Bathyergidae) and test morphological and paleontological hypotheses. From this perspective, exon 28 of the vWF gene appears to be a promising phylogenetic tool, which has already answered questions on hystriognath evolution and revealed molecular signatures for some clades.

The improvement of the taxonomic diversity has not permitted us to solve the basal dichotomies within rodents, probably because of their bushlike radiation. Such a tremendous radiation (as documented by paleontology; Hartenberger 1996) may be at the limit of the resolving power of sequence comparisons. Other molecular approaches, such as the analysis of repeated sequences in various parts of the genome (Shimamura et al. 1997; Verneau, Catzeflis, and Furano 1997; Serdobova and Kramerov 1998), should be investigated to resolve the radiation of rodent families.

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