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The evolution of armadillos, anteaters and sloths depicted by nuclear and mitochondrial phylogenies: implications for the status of the enigmatic fossil *Eurotamandua*

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The mammalian order Xenarthra (armadillos, anteaters and sloths) is one of the four major clades of placentals, but it remains poorly studied from the molecular phylogenetics perspective. We present here a study encompassing most of the order's diversity in order to establish xenarthrans' intra-ordinal relationships, discuss the evolution of their morphological characters, search for their extant sister group and specify the timing of their radiation with special emphasis on the status of the controversial fossil *Eurotamandua*. Sequences of three genes (nuclear exon 28 of the Von Willebrand factor and mitochondrial 12S and 16S rRNAs) are compared for eight of the 13 living genera. Phylogenetic analyses confirm the order's monophyly and that of its three major lineages: armadillos (Cingulata), anteaters (Vermilingua) and sloths ('Tardigrada', renamed in 'Folivora'), and our results strongly support the grouping of hairy xenarthrans (anteaters and sloths) into Pilosa. Within placentals, Afrotheria might be the first lineage to branch off, followed by Xenarthra. The morphological adaptative convergence between New World xenarthrans and Old World pangolins is confirmed. Molecular datings place the early emergence of armadillos around the Cretaceous/Tertiary boundary, followed by the divergence between anteaters and sloths in the Early Eocene era. These Tertiary dates contradict the concept of a very ancient origin of modern xenarthran lineages. They also question the placement of the purported fossil anteater (*Eurotamandua*) from the Middle Eocene period of Europe with the Vermilingua and instead suggest the independent and convergent evolution of this enigmatic taxon.

Keywords: molecular phylogeny; Xenarthra; Pilosa; Von Willebrand factor; local molecular clocks; *Eurotamandua*

1. INTRODUCTION

The mammalian order Xenarthra (ex Edentata) (see Glass 1985) is composed of three major lineages corresponding to three distinct living morphotypes: armadillos (Cingulata: Dasypodidae), anteaters (Vermilingua: Myrmecophagidae) and sloths (Bradypodidae and Megalonychidae). Xenarthrans include 30 living species classified in 13 genera, almost all of which are endemic of South and Central America, the nine-banded armadillo (*Dasypus novemcinctus*) being the only one to occur in North America. Extant species provide only a glimpse of xenarthran past diversity since 218 fossil genera are recognized (McKenna & Bell 1997). This order was still greatly diversified no later than 10 000 years ago, when the majority of genera became extinct possibly because of human impact (Patterson & Pascual 1972; Fariña 1996).

The radiation of Xenarthra occurred between the Palaeocene and Eocene periods in an epoch when South America was isolated from other continental masses (Patterson & Pascual 1972). Their fossil record is almost restricted to the American continent (Carroll 1988) with three striking exceptions: a 'primitive edentate' (*Ernanodon antelios*) from the Upper Palaeocene era of China (Ding 1979), a purported anteater (*Eurotamandua joresi*) that was discovered from the Middle Eocene era of Europe (Storch 1981) and an unidentified Eocene sloth from Antarctica (Vizcaíno & Scillato-Yané 1995). These unexpected reports complicated the reconstruction of xenarthran biogeographical history and raised doubts about their confinement to the New World. More specifically, the affinities of *Eurotamandua* with Xenarthra are still highly debated (Gaudin 1999; Rose 1999).

Despite highly specialized and distinct morphologies of the three lineages, the monophyly of the order Xenarthra is classically recognized (McKenna & Bell 1997). All living and fossil xenarthrans exhibit a dental reduction by the loss of enamel, reaching its paroxysm in anteaters which totally lack teeth (Carroll 1988). The name 'Edentata' has been used to recall this evolutionary trend, but it leads to confusion because it originally referred to the grouping of New World xenarthrans and Old World pholidotans (pangolins), which are also lacking teeth (Glass 1985). One exclusive morphological synapomorphy of Xenarthra is the presence of 'xenarthry', which is constituted by additional atypical articulations between vertebrae (Engelmann 1985; Patterson *et al*. 1992; Rose &

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Emry 1993; Gaudin 1999). From the molecular point of view, xenarthran monophyly is strongly supported by a deletion of three consecutive amino acids in the eye lens αA-crystallin protein that is unique among eutherian mammals that have been studied (De Jong *et al*. 1985; Van Dijk *et al*. 1999).

The order Xenarthra is of crucial importance in understanding mammalian phylogenetics. Following McKenna's (1975) assumption that Xenarthra represents the sister group to all other living eutherians, thereby named Epitheria, numerous molecular workers have used its representatives to root eutherian phylogenies (see e.g. Allard *et al*. 1996). However, Gaudin *et al*. (1996) emphasized the weakness of the morphological synapomorphies defining epitherians. The comparison of complete mitochondrial genomes suggested a close relationship between Xenarthra and either Ferungulata (Cetartiodactyla, Perissodactyla and Carnivora) (Arnason *et al*. 1997) or Afrotheria (Proboscidea, Hyracoidea, Sirenia, Macroscelidea, Chrysochloridae and Tenrecidae) (Waddell *et al*. 1999*a*,*b*). Recently, nuclear DNA sequences showed that Xenarthra represents one of the four major clades of placentals (Madsen *et al*. 2001; Murphy *et al*. 2001).

The intra-ordinal relationships within Xenarthra and their timing remain unclear. Morphological data suggest the grouping of anteaters and sloths into Pilosa, a clade that is defined by the replacement of the carapace by a coat (Engelmann 1985; Patterson *et al*. 1992; McKenna & Bell 1997). However, the possibility of an early emergence of anteaters from the ancestral xenarthran stock is suggested by studies on fossils (Carroll 1988), the ear region (Guth 1961) and the cephalic arterial pattern (Bugge 1979). From the molecular perspective, the first immunological (Sarich 1985) and protein (De Jong *et al*. 1985) studies, as well as the analysis of partial mitochondrial 12S and 16S rRNA genes (Höss *et al*. 1996), did not clarify intraxenarthran relationships. Data from the mitochondrial NADH dehydrogenase 1 (ND1) gene favoured the grouping of armadillos and sloths to the exclusion of anteaters (Cao *et al*. 1998), in contrast to the eye lens αA-crystallin, which provided support for a monophyletic Pilosa, but suggested the paraphyly of armadillos (Van Dijk *et al*. 1999). Most recently, in an analysis of a dataset including five xenarthran genera, Murphy *et al*. (2001) presented a tree with strong support for Pilosa, but with no statistical analysis of xenarthran issues or estimates of dates for the various splitting events within the order.

In order to understand xenarthran evolution better, we present here a study encompassing most of the order's diversity based on three genes already used for exploring mammalian phylogenetics: the exon 28 of the Von Willebrand factor (vWF), which is a single-copy nuclear marker and the mitochondrial 12S and 16S rRNAs (e.g. Höss *et al*. 1996; Montgelard *et al*. 1997; Huchon *et al*. 1999). The aims of this study are (i) to investigate the intra-ordinal relationships of Xenarthra using a larger taxonomic diversity than in previous molecular studies, (ii) to discuss the evolution of morphological characters in light of the phylogeny obtained, and (iii) to specify the chronology of their radiation in relation to South American biogeography, with special reference to the occurrence of the purported fossil anteater *Eurotamandua* in Europe.

2. MATERIAL AND METHODS

(a) *Taxonomy*

Throughout the text, we have introduced 'Folivora' for the clade containing the sloths, because the usual terms of 'Phyllophaga' (e.g. Engelmann 1985) or 'Tardigrada' (e.g. Patterson *et al*. 1992) have both referred to different groups of protostomians. Etymologically, Folivora means leaf eater and is the Latin correspondence for 'Phyllophaga'.

(b) *Data acquisition*

Xenarthran samples preserved in 95% ethanol were stored in the collection of mammalian tissues from the Institut des Sciences de l'Evolution de Montpellier (Catzeflis 1991). Total DNAs were extracted for *Chaetophractus villosus* (larger hairy armadillo), *Cabassous unicinctus* (southern naked-tailed armadillo), *D. novemcinctus* (nine-banded armadillo), *Cyclopes didactylus* (pygmy anteater), *Tamandua tetradactyla* (collared anteater), *Myrmecophaga tridactyla* (giant anteater), *Bradypus tridactylus* (pale-throated three-toed sloth) and *Choloepus didactylus* (southern two-toed sloth).

Polymerase chain reaction (PCR) amplifications of the complete 12S rRNA gene were conducted following Hassanin & Douzery (1999). The 16S rRNA fragment (722 bp) for *Myrmecophaga* was amplified with the primers R9 (5′-CGCCTGTTTACCAAAAACATC-3′) (direct) and S1 (5′-TTATGCAATTACCGAGATCTGCCA-3′) (reverse) and cloned using the pCR^{TM} 2.1 plasmid vector and *Escherichia coli* strain INVαF′ (original TA cloning kit, Invitrogen, San Diego, CA, USA). Two overlapping fragments (V1/W2 of 907 bp and V2/W1 of 962 bp) of the vWF exon 28 of sloths and armadillos were amplified according to Huchon *et al*. (1999). Three overlapping fragments (V1/W11 of 398 bp, V8/W10 of 504 bp and V9/W12 of 523 bp) were amplified for anteaters with the following direct (V) and reverse (W) primers: V8 (5′-GGAGCTGCGGCGCRTCGCCAGCCAGGTGAA-3′), V9 (5′-ACGAGATCATCAGCTACCTCTGYGRCCTG-3′), W10 (5′-TGTTGAAGCCGGCCTCGCCGATCCTGTC-3′), W11 (5′-CCGAAGACCTGGAACAGCGTGTAC-3′) and W12 (5′-TGAGGGCCCACRCCRATGGG-3′). PCR products were purified from 1% agarose gels using Amicon Ultrafree-DA columns (Millipore, Bedford, IN, USA) and sequenced on both strands using manual (Thermo Sequenase cycle kit, Amersham, Pharmacia Biotech, Pisccataway, MD, USA) and automatic sequencing (Big Dye Terminator cycle kit) on an ABI 310 (PE Applied Biosystems, Warrington, UK). Three distinct clones were sequenced for the partial 16S rRNA of *Myrmecophaga*. The sequences have been deposited in the European Molecular Biology Laboratory under accession numbers AJ278151–AJ278161 and AJ297939 (electronic Appendix A, available on The Royal Society's Web site). Two already published vWF sequences have been updated (*C. villosus* (AF076480) and *B. tridactylus* (U31603)).

(c) *Taxonomic sampling*

Since species sampling has been shown to have a major impact on phylogenetic reconstruction (Philippe & Douzery 1994), we chose a dataset encompassing a wide range of ordinal mammalian diversity. In order to reduce potential long-branch attraction artefacts, whenever possible we analysed at least two species per placental order. The sequences of two marsupials from Australia (*Macropus giganteus*) and America (*Didelphis virginiana*) were used for rooting eutherian trees unambiguously.

Table 1. Indices of robustness for the nodes of the Xenarthra subtree obtained for 12S rRNA, partial 16S rRNA, vWF exon 28 (codon positions 1 plus 2) and a combination of the three genes with three methods of phylogenetic reconstruction (minimum evolution (distance), maximum parsimony and maximum likelihood).

(Reliability percentages (RP) after quartet puzzling and the number of exclusive (non-homoplasic) synapomorphies (ES) observed for the vWF exon 28 amino acid dataset are also indicated for each node. The position and nature of these exclusive synapomorphies are indicated in electronic Appendix B. BSI, Bremer support indices; ME, bootstrap percentages obtained after 1000 replications of minimum evolution on paralinear log-det distances; MP, bootstrap percentages obtained after 1000 replications of standard parsimony; ML, bootstrap percentages obtained after 300 replications of maximum likelihood; RP, reliability percentages obtained with TREE-PUZZLE after 10 000 quartet puzzling steps. A dash indicates that the node does not appear in the corresponding majority rule consensus bootstrap tree.)

	12S rRNA				partial 16S rRNA				vWF (1 plus 2)				combination				vWF	
	BSI	ME	MP	ML	BSI	ME	MP	ML	BSI	ME	MP	ML	BSI	ME	MP	ML	RP	ES
Cingulata (armadillos)	25	100	100	100	4	100	94	96	10	100	99	99	36	100	100	100	99	2
Cabassous plus *Chaetophractus*	0	75	—	—	1	85	61	48	2	92	67	50	1	83	61	60	90	2
Vermilingua (anteaters)	3	88	73	96	4	99	80	84	11	100	99	100	16	97	100	100	98	3
Tamandua plus *Myrmecophaga*	22	100	100	100	3	100	92	87	32	100	100	100	44	100	100	100	99	5
Folivora (sloths)	15	100	99	100	5	100	95	95	34	100	100	100	33	100	100	100	99	6
Pilosa (anteaters plus sloths)	3	78	53	75	2	73	43	59	4	97	89	93	6	87	88	97	93	2
Xenarthra	3	43	29	48	0	50	24	62	17	100	100	100	27	100	100	100	93	3

Thirty-five mammalian genera were considered for the three genes. All sequences' accession numbers are listed in electronic Appendix A.

(d) *Sequence alignment and phylogenetic analyses*

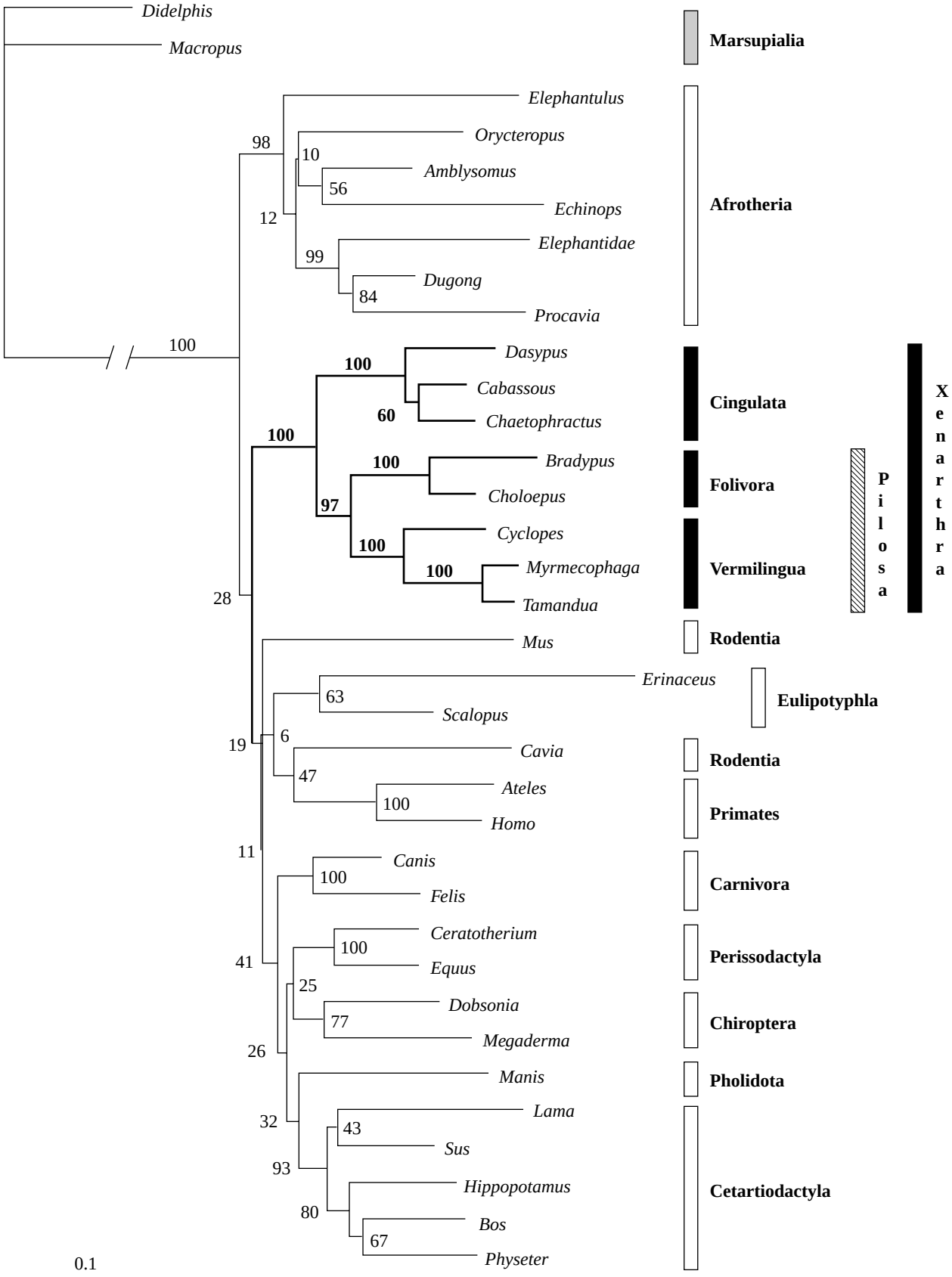
Sequences were manually aligned with the ED editor of the MUST package (Philippe 1993). The alignment of vWF exon 28 sequences started at position 38 of the human sequence and was 1233 bp long, and non-sequenced positions were coded as missing data. Indels and hypervariable zones of the 12S and 16S rRNAs were removed from subsequent analyses. Alignments are available upon request to the first author.

Phylogenetic reconstructions were conducted with PAUP* 4.0b4a (Swofford 1998), TREE-PUZZLE 4.02 (Strimmer & Von Haeseler 1996) and PAML 3.0b (Yang 2000). Distance analyses used the minimum evolution criterion with tree bisection–reconnection (TBR) branch swapping on a neighbour-joining tree based on paralinear log-det distances with constant site removal. Maximum parsimony analyses consisted of heuristic searches using 100 random addition sequence replicates with TBR branch swapping. Maximum likelihood (ML) analyses with PAUP* were made in two steps in order to accelerate computation times. First, maximum likelihood parameters were estimated using a heuristic search with nearest-neighbour interchange branch swapping on a neighbour-joining starting tree. Second, a new search was conducted with random addition of sequences and TBR branch swapping using the previously estimated parameters. Substitution rates were described by the general time reversible (under PAUP* and PAML) and Jones–Taylor–Thornton (JTT) (under TREE-PUZZLE and PAML) models of sequence evolution for nucleotides and amino acids, respectively. The substitution rate heterogeneity for DNA and vWF protein among sites was described by a gamma distribution with eight categories (Γ_8) (Yang 1996).

The robustness of the different nodes was estimated by bootstrap percentages (BP) after 1000 replications for minimum evolution and maximum parsimony with TBR branch swapping and after 300 replications using previously estimated parameters for maximum likelihood with neighbour-joining starting trees and 1000 TBR rearrangements per resampling. Reliability percentages for the protein data set were obtained after 10 000 quartet puzzling steps with TREE-PUZZLE. Bremer support indices, i.e. the number of extra steps required to break the corresponding node under the maximum parsimony criterion, were computed with PAUP*. Log-likelihoods of alternative topologies were compared by the maximum likelihood test of Kishino & Hasegawa (1989) implemented in PAML 3.0b. Three different GTR + Γ_8 models were assumed for the combination of the three genes.

(e) *Molecular datings*

The presence of local molecular clocks (Yoder & Yang 2000) was tested for different data sets and molecular dates were calculated by PAML 3.0b using branch lengths of the maximum likelihood tree with local clocks. Taxa were removed one by one until acceptance by the likelihood ratio test of the hypothesis of three local clocks: one for paenungulates, one for perissodactyls and one for the remaining species. Three independent paleontological calibration points were taken into account in order to check for molecular datings with cross-calibrations (Huchon *et al.* 2000): (i) 55 million years (Myr) for the split between *Ceratotherium* and *Equus* (Waddell *et al.* 1999*b*), (ii) 60 Myr for the split between *Procavia* and *Elephas* (Gheerbrant *et al.* 1996), and (iii) 63 Myr for the Arctocyonia–Mesonychia divergence (Gingerich & Uhen 1998), represented here by the *Sus* (domestic pig)–*Physeter* (sperm whale) split. In order to discuss our molecular dates in the light of geochronology, we have used the geological time-scale compiled for the Tertiary era by McKenna & Bell (1997).


Didelphis
Macropus
Marsupialia
Elephantulus
Orycteropus
Amblysomus
Echinops
Elephantidae
Dugong
Procavia
Afrotheria
98
10
56
12
99
84
100
Dasypus
Cabassous
Chaetophractus
Cingulata
100
60
100
Bradypus
Choloepus
Folivora
100
97
Cyclopes
Myrmecophaga
Tamandua
Vermilingua
100
100
Pilosa
Xenarthra
28
Mus
Rodentia
Erinaceus
Scalopus
Eulipotyphla
63
19
6
Cavia
Rodentia
47
Ateles
Homo
Primates
100
11
Canis
Felis
Carnivora
100
Ceratotherium
Equus
Perissodactyla
41
25
Dobsonia
Megaderma
Chiroptera
77
26
Manis
Pholidota
Lama
Sus
Cetartiodactyla
32
43
93
Hippopotamus
Bos
Physeter
80
67
0.1

3. RESULTS

(a) *Phylogenetic results*

(i) *Nuclear exon 28 of vWF*

Due to significant base composition heterogeneity (evaluated by a χ^2 test in TREE-PUZZLE) for numerous taxa (including *Dasypus*, *Chaetophractus*, *Choloepus*, *Tamandua* and *Myrmecophaga* within Xenarthra), third codon positions were excluded from all subsequent analyses. All phylogenetic reconstructions provided strong support for most nodes involving xenarthrans (table 1): (i) the monophyly of Xenarthra, (ii) the monophyly of each of its three lineages, i.e. armadillos, anteaters and sloths, (iii) the grouping of anteaters and sloths into a clade called Pilosa, and (iv) the close relationship within anteaters between *Myrmecophaga* and *Tamandua*—the subfamily Myrmecophaginae—to the exclusion of *Cyclopes*. Within armadillos, the grouping of *Cabassous* with *Chaetophractus* to the exclusion of *Dasypus* received mixed support (table 1). Phylogenetic analyses on vWF amino acids yielded high support for all nodes within Xenarthra and provided several exclusive amino acid substitutions supporting them (table 1 and electronic Appendix B).

(ii) *Mitochondrial 12S and 16S rRNAs*

All nodes involving xenarthrans were retrieved by phylogenetic analyses using 12S and 16S rRNAs (table 1), with high support (the monophyly of Cingulata, Vermilingua, Folivora and Myrmecophaginae), moderate support (the grouping of anteaters and sloths) or low support (the monophyly of the order). One conflict arose from 12S rRNA relationships within armadillos (table 1): minimum evolution favoured the grouping of *Cabassous* with *Chaetophractus*, in contrast to maximum parsimony and maximum likelihood, which suggested a sister group relationship between *Cabassous* and *Dasypus*.

(iii) *Combination of the three genes*

The maximum likelihood phylogram obtained with the combined nuclear and mitochondrial data sets (2107 bp) is presented in figure 1. Concatenation resulted in a general increase in the robustness indices (table 1). Xenarthra, Pilosa, Cingulata, Folivora, Vermilingua and Myrmecophaginae were clearly monophyletic ($BP_{ML} > 97$) and the grouping of the armadillos *Cabassous* and *Chaetophractus* to the exclusion of *Dasypus* was favoured ($BP_{ML} = 60$).

(iv) *Tests of alternative hypotheses*

The three alternative branching patterns connecting armadillos, anteaters and sloths were compared by Kishino & Hasegawa (1989) likelihood tests. Pilosa appeared to be the most likely branching pattern in all cases. Alternative hypotheses placing either anteaters or sloths as a sister group to the remaining xenarthrans involved a drop in log-likelihoods that is significant when the three markers are combined (table 2). With regard to Xenarthra sister-group relationships, their emergence second amongst placentals, after Afrotheria, was the most likely hypothesis in the vWF and combination cases. Alternative hypotheses, i.e. Xenarthra as a sister group of either remaining placentals (Epitheria) or Afrotheria or Ferungulata, all appeared less likely when the three markers were combined as they involved a drop in log-likelihood by approximately one standard error (table 2). The morphological hypothesis of 'Edentata' (Xenarthra plus Pholidota) was significantly rejected in all cases (table 2).

(b) *Molecular timings*

Local clocks were used in the maximum likelihood datings because they represent a compromise between setting a single substitution rate among all lineages (global clock constrained) and independent rates for each branch (no clock constrained). Amino acid substitutions in vWF satisfied the hypothesis of three local clocks for a reduced data set of 18 taxa, including Xenarthra, Paenungulata, Perissodactyla and Cetartiodactyla (*Lama* was excluded due to a fast rate of evolution) with the two marsupials as the outgroup (likelihood ratio test between non-clock and locally clock-like tree, $2\Delta\ln L = 27.2$ with 19 degrees of freedom and $p = 0.10$). Using the perissodactyl, cetartiodactyl and paenungulate calibration points, we obtained average substitution rates of 0.160, 0.194 and 0.205% amino acid/Myr per lineage, respectively, in the vWF protein (figure 2). Cross-calibration comparisons between these three points showed that the cetartiodactyl and paenungulate points were compatible, with the latter yielding the youngest ages, whereas the perissodactyl point provided the oldest dates.

Within placentals, the separation between Afrotheria (here represented by two paenungulates) (figure 2) and the remaining groups was estimated to have occurred 122 Myr ago (range of datings depending on the choice of calibration points 115–148 Myr ago), whereas Xenarthra diverged from Cetartiodactyla plus Perissodactyla some 106 Myr ago (range 100–128 Myr). Our estimates then placed the xenarthran radiation, which corresponds to the split between Cingulata and Pilosa, at *ca.* 63 Myr (figure 2), around the Cretaceous/Tertiary (K/T) boundary (range 59–76 Myr). Within Xenarthra, the following splits have been dated: (i) Vermilingua versus Folivora 54 Myr ago in the Early Eocene period (range

Figure 1. Maximum likelihood phylogram obtained using the combination of complete mitochondrial 12S rRNA, partial 16S rRNA and positions 1 and 2 of the nuclear vWF exon 28 (2107 sites of which 1150 variable). The GTR model was used (rate matrix, A–C 5.28, A–G 11.33, A–T 4.86, C–G 3.05, C–T 23.03 and G–T 1.00) and between-site variation following a gamma distribution (eight categories) of shape $\alpha = 0.44$ with $\theta = 16\%$ of invariable sites. Branch lengths are proportional to the estimated number of substitutions per site and that leading to the outgroup has been reduced by one half. Xenarthrans are connected by bold lines. The node labels are bootstrap percentages obtained after 300 maximum likelihood replications. Note that the combination representing *Elephantidae* is a chimera between *Elephas maximus* (Asian elephant) for 12S rRNA and vWF and *Loxondonta africana* (African elephant) for 16SrRNA. Parameters for 12S rRNA: 838 sites with 485 variable, rate matrix A–C 3.80, A–G 10.32, A–T 4.67, C–G 0.41, C–T 28.00 and G–T 1.00, $\theta = 5\%$ and $\alpha = 0.30$. Parameters for 16S rRNA: 447 sites with 194 variable, rate matrix A–C 31.84, A–G 33.33, A–T 20.59, C–G 0.64, C–T 123.70 and G–T 1.00, $\theta = 34\%$ and $\alpha = 0.41$. Parameters for vWF (codon positions 1 plus 2): 822 sites with 494 variable, rate matrix A–C 2.27, A–G 6.66, A–T 1.48, C–G 2.64, C–T 5.90 and G–T 1.00, $\theta = 12\%$ and $\alpha = 0.77$.

Table 2. Results of Kishino & Hasegawa (1989) likelihood tests of alternative topologies computed for the nuclear vWF (codon positions 1 plus 2), the mitochondrial 12S and 16S rRNAs and their combination.

($\delta = \Delta\ln L$/s.e. refers to the ratio between the difference in log-likelihood ($\Delta\ln L$) of two phylogenetic hypotheses and its standard error (s.e.). p refers to the one-tailed significance level of the Kishino & Hasegawa (1989) test as performed in PAML (an asterisk means that it is significant at the 5% level).)

phylogenetic *a priori* hypotheses	12S rRNA			partial 16S rRNA			vWF (1 plus 2)			combination		
	$-\ln L$	δ	p	$-\ln L$	δ	p	$-\ln L$	δ	p	$-\ln L$	δ	p
intraxenarthra relationships												
Vermilingua plus Folivora (= Pilosa)	11 216.44	best	—	4602.55	best	—	8569.98	best	—	24 587.14	best	—
Vermilingua plus Cingulata	11 223.96	1.26	0.10	4605.04	0.93	0.18	8576.64	1.35	0.09	24 603.76	2.06	0.02*
Folivora plus Cingulata	11 223.93	1.25	0.11	4604.51	0.58	0.28	8576.42	1.26	0.10	24 603.14	1.92	0.03*
Xenarthra sister group relationships												
Xenarthra plus Pholidota ('Edentata')	11 225.16	1.84	0.03*	4619.23	2.54	< 0.01*	8585.99	1.86	0.03*	24 620.34	3.04	< 0.01*
Xenarthra plus Epitheria	11 216.94	0.44	0.33	4602.55	best	—	8574.29	1.09	0.14	24 591.23	1.07	0.14
Xenarthra second offshoot	11 216.46	0.08	0.47	4604.30	0.75	0.23	8569.98	best	—	24 587.14	best	—
Xenarthra plus Afrotheria	11 216.94	0.44	0.33	4604.13	0.62	0.27	8574.29	1.09	0.14	24 591.31	1.11	0.13
Xenartha plus Ferungulata	11 216.44	best	—	4609.68	0.99	0.16	8572.31	0.41	0.34	24 594.88	0.95	0.17

51–65 Myr), (ii) *Dasypus* versus other armadillos 33 Myr ago in the Early Oligocene period (range 31–40 Myr), followed by *Chaetophractus* versus *Cabassous* 21 Myr ago in the Early Miocene period (range 20–25 Myr), (iii) pygmy anteater versus other anteaters 38 Myr ago in the Late Eocene period (range 35–45 Myr), followed by giant versus collared anteaters 13 Myr ago in the Middle Miocene period (range 12–15 Myr), and (iv) *Bradypus* versus *Choloepus* 18 Myr ago in the Early Miocene period (range 16–21 Myr).

Datings were also conducted with the three concatenated markers at the DNA level, assuming three independent maximum likelihood models and using the same set of 18 species (likelihood ratio test, $2\Delta\ln L = 28.8$ and $p = 0.07$). The paenungulate and the cetartiodactyl points yielded similar dating estimates between them (data not shown) and also similar dating estimates relative to vWF amino acids. For example, within Xenarthra, the first (armadillos versus others) and second (anteaters versus sloths) splits are estimated to be 57–69 Myr and 50–60 Myr old at the DNA level, respectively, against 59–63 Myr and 51–54 Myr for vWF amino acids (figure 2). The perissodactyl calibration point provided the oldest dating estimates and was not compatible with the other two points: it estimated the Hyracoidea/Proboscidea split to have occurred 118 Myr ago, against 60 Myr for palaeontology.

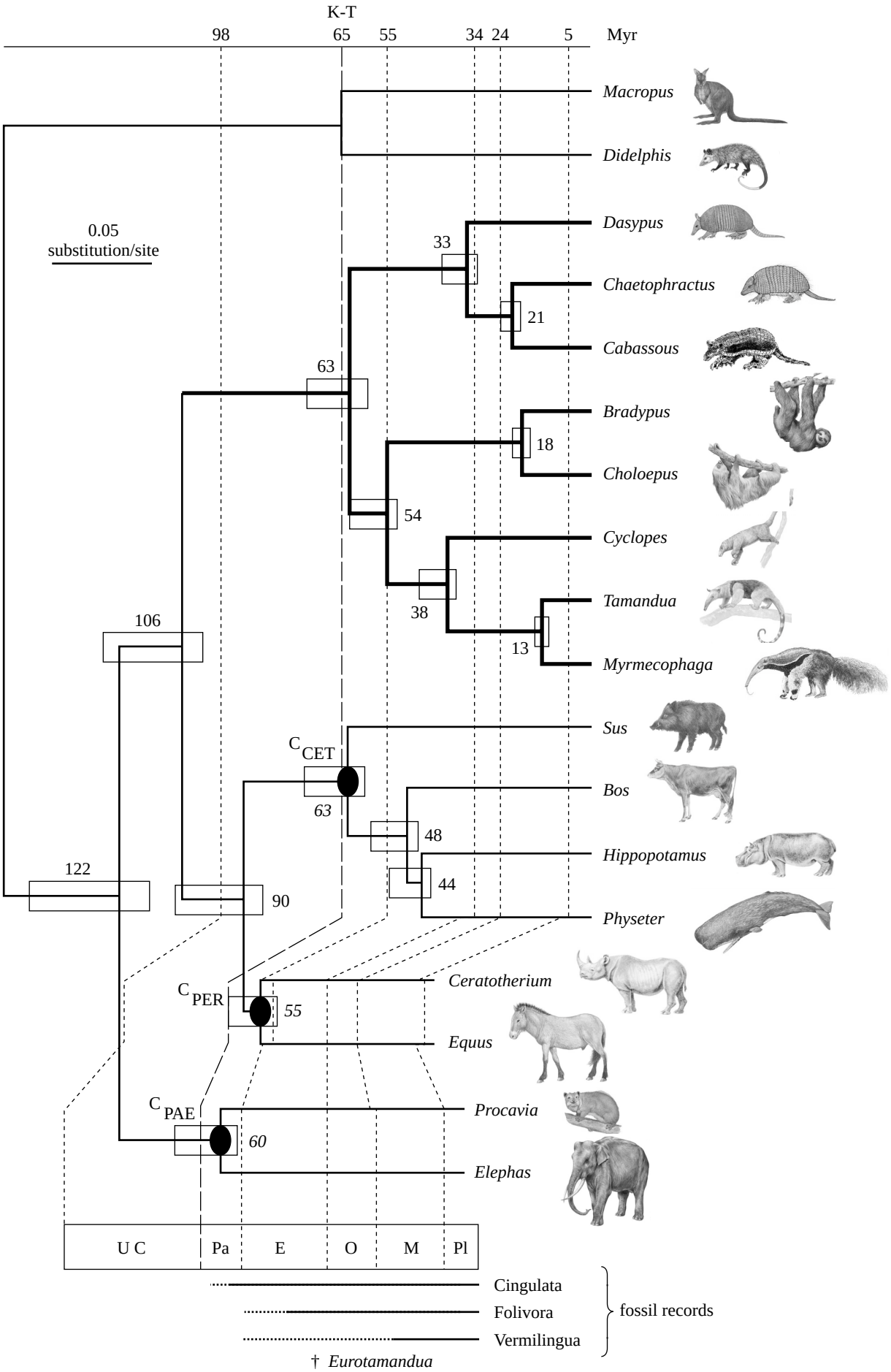
4. DISCUSSION

(a) *Molecular systematics and character evolution in Xenarthra*

(i) *Xenarthra monophyly*

The Xenarthra monophyly is unambiguously supported in all reconstructions by the vWF alone or in combination with the 12S and 16S rRNAs (table 1). The single common ancestry of the eight xenarthrans studied here is also defined by three exclusive vWF amino acid replacements (table 1 and electronic Appendix B), which should be added to the unique derived deletion in the αA-crystallin known for seven xenarthrans (Van Dijk *et al*. 1999). The monophyly of Xenarthra is also supported by morphological characters such as accessory articulations between

Figure 2. Molecular estimates of divergence dates for the xenarthran radiation based on a reduced dataset of vWF amino acids (415 sites of which 319 variable). The JTT model of protein evolution and a gamma distribution (eight categories) ($\alpha = 0.94$) were used as likelihood assumptions. The maximum likelihood phylogram constrained to be clock-like was obtained with three local clocks: one for the branch leading to perissodactyls (*Ceratotherium* and *Equus*) (rate 0.09), one for the branch leading to paenungulates (*Procavia* and *Elephas*) (rate 0.45) and one for the remaining branches (default rate 1.00). Divergence dates (in Myr) are indicated and were deduced from three calibration points: C_{PER} (55 Myr) (split between *Ceratotherium* and *Equus*), C_{PAE} (60 Myr) (split between *Procavia* and *Elephas*) and C_{CET} (63 Myr) (split between *Sus* and *Bos* plus *Hippopotamus* plus *Physeter*). Rectangles at nodes represent the range of dates estimated using the three calibration points. Youngest and oldest estimates are provided by the paenungulate and perissodactyl points, respectively. Intermediate values are given by the cetartiodactyl point and are indicated near the nodes. Cross-calibration results: (i) the perissodactyl split is estimated to have occurred either 43 (calibration by C_{PAE}) or 46 (calibration by C_{CET}) Myr ago, (ii) the paenungulate split is estimated to have occurred either 64 (calibration by C_{CET}) or 77 (calibration by C_{PER}) Myr ago, and (iii) the cetartiodactyl split is estimated to have occurred either 59 (calibration by C_{PAE}) or 76 (calibration by C_{PER}) Myr ago. The split between *Macropus* and *Didelphis* has not been dated because arbitrary dates can be obtained by assuming different relative rates on the two sides of the root (Yoder & Yang 2000). The time-scale is given above the tree. The main geological epochs are indicated and delimited by dashed lines: UC, Upper Cretaceous. Tertiary: Pa, Palaeocene; E, Eocene; O, Oligocene; M, Miocene; Pl, Plio-Pleistocene. The Cretaceous/Tertiary boundary (K/T) is represented by the vertical striped line. Fossil records of Xenarthra (*Eurotamandua* excluded) are figured by continuous lines below the tree. Broken lines indicate gaps in the fossil record suggested by our molecular estimates.


K-T
98
65
55
34
24
5
Myr
0.05
substitution/site
Macropus
Didelphis
Dasypus
Chaetophractus
Cabassous
Bradypus
Choloepus
Cyclopes
Tamandua
Myrmecophaga
Sus
Bos
Hippopotamus
Physeter
Ceratotherium
Equus
Procavia
Elephas
33
21
63
18
54
38
13
106
122
90
C CET
63
48
44
C PER
55
C PAE
60
U C
Pa
E
O
M
Pl
Cingulata
Folivora
Vermilingua
fossil records
† Eurotamandua

vertebrae ('xenarthry'), ischiosacral fusion, with a secondary reversal in *Cyclopes* probably related to its strictly arboreal life style (Gaudin & Branham 1998) and dental simplification by the loss of enamel (Engelmann 1985). These characteristics are generally thought to reflect adaptation towards fossoriality and myrmecophagy (Carroll 1988; Gaudin 1999).

(ii) *Xenarthra intra-ordinal relationships*

The monophyly of Cingulata, Vermilingua and Folivora finds strong support from vWF and mitochondrial rRNAs sequence comparisons. It is also corroborated by numerous morphological characters such as, for example, the modification of dermal ossicles into articulate plates for Cingulata, the total absence of teeth for Vermilingua and the presence of paired perforations of the centra in lumbar vertebrae for Folivora (Engelmann 1985).

Our data, including all anteater and sloth genera, provide strong support for the clade Pilosa (tables 1 and 2), thereby extending the results of Madsen *et al*. (2001) and Murphy *et al*. (2001). This arrangement is in agreement with studies of the ear region (Patterson *et al*. 1992) and other morphological characters that provided remarkable synapomorphies for Pilosa such as, for example, the interruption of the zygomatic arch or the intrapelvian location of the testes (Engelmann 1985). These results contrast with those of Guth (1961), Bugge (1979), Carroll (1988) and Cao *et al*. (1998) who claimed that anteaters are the sister group of the remaining xenarthrans. In the light of our results, it is likely that earlier studies of the ear region (Guth 1961) and cephalic arterial pattern (Bugge 1979) may have been misled by the extreme specialization of the skull towards myrmecophagy in anteaters. Reanalysis of ear characters (Patterson *et al*. 1992) supported the Pilosa hypothesis, and the character pointed to by Bugge (1979) for the grouping of armadillos and sloths (i.e. the median course of the internal carotid artery) is likely to be symplesiomorphic. Carroll (1988) suggested an early emergence of anteaters by emphasizing the fact that no fossil anteaters were found to have retained dermal ossicles, unlike some mylodontid sloths, which possess these relicts of the cingulate-like xenarthrans' ancestor. The fossil record of anteaters is particularly scarce (Gaudin & Branham 1998) and our results showing that anteaters are the sister group of sloths suggest that fossil anteaters with armour relicts remain to be found. Thus, if fossils are taken under consideration, the replacement of the ancestral carapace by a coat could not be considered as a synapomorphy defining Pilosa. However, this evolutionary trend has led to a discrete pattern in living xenarthrans where armoured xenarthrans (armadillos) have retained the ancestral carapace and hairy xenarthrans (anteaters and sloths) share the possession of a true coat. Our conclusions also contradict those of Cao *et al*. (1998) based on the mitochondrial ND1 gene, which moderately supports a close relationship between the armadillo *Cabassous* and the sloth *Bradypus* to the exclusion of the anteater *Tamandua*. This result may be due to the reduced taxonomic sampling and/or to the particular behaviour of this fast-evolving molecule, which seems to be subject to convergence (Cao *et al*. 1998).

Our results confirm the classical arrangement within Vermilingua by favouring the grouping of the strictly terrestrial *Myrmecophaga* and semi-arboreal *Tamandua* into the subfamily Myrmecophaginae, to the exclusion of the strictly arboreal *Cyclopes*. Such a relationship is corroborated by numerous myological (Reiss 1997) and morphological (Gaudin & Branham 1998) characters.

The phylogeny of Cingulata is still poorly understood from a morphological point of view (Patterson *et al*. 1989). Our results suggest the grouping of *Cabassous* (Priodontini) and *Chaetophractus* (Euphractini) to the exclusion of *Dasypus* (Dasypodini), in contrast to Engelmann (1985) who claimed a close relationship between *Dasypus* and *Cabassous*. Our results are in agreement with a recent study of sperm morphology and morphometry (Cetica *et al*. 1998) showing that *Cabassous* and *Chaetophractus* have rather similar spermatozoa. However, the discrepancies existing between vWF, 12S and 16S rRNA suggest that additional genes for a greater taxonomic diversity are needed in order to resolve relationships within armadillos further.

(b) *Searching for the Xenarthra sister group*

The vWF and mitochondrial rRNA genes do not provide a clear response to the difficult question of the position of Xenarthra within Eutheria. However, our analyses including eight armadillo, anteater and sloth genera suggest that Xenarthra may constitute the second offshoot of the placental tree, Afrotheria being the first one to branch off (figure 1 and table 2). This is in agreement with the results obtained by Madsen *et al*. (2001) and Murphy *et al*. (2001) on four and five genera, respectively. Alternative affinities of Xenarthra with either epitherians, afrotherians or ferungulates appear less likely (table 2), but cannot be significantly rejected. Sequencing of additional molecular markers in order to provide an increased number of phylogenetically informative positions concomitant with adequate taxonomic diversity of Xenarthra is needed in order to resolve their position within the eutherian tree further. It is interesting to note that the classical hypothesis of 'Edentata' (Novacek 1992) is significantly rejected in all cases (table 2). Together with other molecular results (De Jong 1998; Madsen *et al*. 2001; Murphy *et al*. 2001) this confirms that morphological similarities between xenarthrans and pangolins are a spectacular example of convergence in relation to myrmecophagy (Bugge 1979; Rose & Emry 1993).

(c) *Molecular timing of the xenarthran radiation*

With vWF amino acids, the three calibration points, i.e. cetartiodactyls, perissodactyls and paenungulates, are reciprocally compatible in the local molecular clocks analysis (figure 2). One should note that setting 63 Myr for the split between *Bos* (rather than *Sus*) and *Physeter* would involve 85 Myr for the split between *Procavia* and *Elephas*, i.e. a divergence time 25 Myr older than the paleontological one. This is the reason why our molecular datings tend to yield younger dates than previously published for the *Hippopotamus*/*Physeter* split, 41–53 Myr (figure 2) versus 53–54 Myr (Montgelard *et al*. 1997; Ursing & Arnason 1998). However, our datings of deeper splits become more compatible with other proposed dates because of increasing intervals between the dates obtained from the three different calibration points. For example, our estimates of the xenarthran separation from other placentals (100–128 Myr) (figure 2) are in the range of those of Springer (1997) and Waddell *et al*. (1999*b*).

The xenarthran radiation, which corresponds to the emergence of armadillos, is estimated here to have occurred close to the K/T boundary (59–76 Myr) and the separation between anteaters and sloths during the Palaeocene era (51–65 Myr). These results are younger than other molecular estimations yielding *ca.* 80 Myr for the radiation of xenarthran families (reviewed in Bromham *et al.* 1999). Indeed, immunological comparisons suggest *ca.* 80 Myr (Sarich 1985) and partial 12S and 16S rRNA gave the same result (Höss *et al.* 1996), and Cao *et al.* (1998) even suggested an interval of 65–130 Myr based on the deep divergences they observed in the ND1 gene. These different molecular dates suggest that breaking long isolated branches through increased taxonomic sampling within Xenarthra might have modified the divergence time estimates.

Our estimates obtained after extensive taxonomic sampling within Xenarthra are compatible with paleontological data. The first armadillo scutes are from the Late Palaeocene era (*ca.* 58 Myr) of Brazil (Scillato-Yané 1976), the first sloth remains are from the Middle Eocene era of Antarctica (Vizcaíno & Scillato-Yané 1995) and the first apparition of undoubted myrmecophagid anteaters is from the Early Miocene era of Patagonia (Carlini *et al.* 1992). Long gaps in the fossil record are clearly not unusual and our dating of the origin of anteaters and sloths (around the Palaeocene/Eocene limit) suggests a gap of *ca.* 30 Myr in the South American fossil record of anteaters and emphasizes its incompleteness. Regarding Myrmecophagidae, the very ancient origin of the pygmy anteater (*Cyclopes*) explains its very divergent morphology shaped by *ca.* 30 Myr of arboreal life style, since arboreality is considered ancestral for anteaters (Gaudin & Branham 1998). The separation between the two modern sloths, which are unknown as fossils (Patterson & Pascual 1972), is dated by our data at *ca.* 18 Myr (end of the Early Miocene era). Two-toed sloths (*Choloepus*) and three-toed sloths (*Bradypus*) have been placed into two distinct families (Megalonychidae and Bradypodidae, respectively) on the basis of their numerous morphological differences and a presumably diphyletic origin (Webb 1985). Our estimation is slightly younger than the 25 Myr of Sarich (1985) but confirms their considerable divergence. It suggests that their apparent external similarities are the result of paralellism and that arboreality may have evolved at least twice within Folivora (Höss *et al.* 1996; Greenwood *et al.* 2001). The quite ancient dates obtained for the armadillos' radiation strengthen the fact that Cingulata contains strikingly divergent taxa, a result coherent with the marked differences observed in the structure of their spermatozoa (Cetica *et al.* 1998). Finally, it is noteworthy that numerous cladogenic events within Xenarthra seem to occur close to transitions between epochs of the chronostratigraphic scale (figure 2). Since those transitions are defined by dramatic environmental and climatic changes (Pascual & Ortiz Jaureguizar 1990), such a synchrony suggests a major role of paleobiogeographic changes in the diversification of xenarthrans.

(d) Eurotamandua: *helping to solve the enigma?*

Our molecular estimates of the divergence dates for armadillos, anteaters and sloths raise the question of the occurrence of purported fossil xenarthrans outside of South America. Indeed, if the presence of a sloth in the Eocene era of Seymour Island is compatible with land connections between Antarctica and the Patagonian province until the Oligocene era (Vizcaíno & Scillato-Yané 1995), the occurrences of *Ernanodon* in the Palaeocene era of China (Ding 1979) and *Eurotamandua* in the Middle Eocene era (45 Myr) of Europe (Storch 1981) are more difficult to explain since South America was an island in these epochs. The true belonging of *Ernanodon* and *Eurotamandua* to Xenarthra has been widely debated (Rose & Emry 1993; Gaudin 1999). Actually, *E. antelios* seems likely to be assigned to a group of endemic Chinese mammals whose morphological similarities with Xenarthra would reflect convergent adaptations to fossorial habits (Rose & Emry 1993; Gaudin 1999). However, there is no consensus about the status of *E. joresi* (Storch 1981). Vermilinguan affinities of *Eurotamandua* have been suggested (Storch 1981; Storch & Habersetzer 1991), but several of the most crucial features for assessing its phylogenetic position remain equivocal (Gaudin & Branham 1998; Gaudin 1999; Rose 1999). If *Eurotamandua* belongs to Vermilingua, our results dating the origin of Vermilingua as being well-nested in the Tertiary era (between 54 and 38 Myr) (figure 2) again raise the question of how an anteater reached Europe at a time when South America was isolated from all other continental masses? The only possible response is dispersal, which seems to be very unlikely in the present state of our knowledge of paleobiogeography based on plate tectonic models. Thus, our results, in contrast to Höss *et al.* (1996), cast doubt on the true belonging of *Eurotamandua* to Vermilingua. They suggest that the striking morphological resemblances between this taxon and Myrmecophagidae might be the result of adaptative convergence towards fossoriality and ant feeding. This example once again underlines how morphological adaptation to similar ecological niches could be positively misleading in terms of phylogenetic signal.

The xenarthran status of *Eurotamandua* was also widely questioned and this fossil was interpreted as either a distinct family level lineage that cannot be classified within the Xenarthra (Szalay & Schrenk 1998) or a Pholidota (Shoshani *et al.* 1997) or a sister group to Pilosa (Gaudin & Branham 1998). However, the evidence for the presence of true xenarthry in *Eurotamandua* is far from convincing (Rose & Emry 1993; Gaudin & Branham 1998; Rose 1999). Based on an exhaustive and detailed study of the evolution of xenarthrous vertebrae, Gaudin (1999) concluded that there is at present little evidence for a close phylogenetic relationship between true xenarthrans and *Eurotamandua*. Moreover, in a study of forelimbs from a second specimen, Rose (1999) argued for a close relationship of *Eurotamandua* and Palaeanodonta (a group of extinct fossorial mammals with reduced dentitions). Our results placing the origin of Pilosa in the Early Tertiary era contradict those of Gaudin & Branham (1998), but our dating of the xenarthrans' origin into the Cretaceous era (between 106 and 63 Myr) (figure 2) leave open the possibility that *Eurotamandua* could be a basal member of Xenarthra. Only the discovery of additional specimens will provide us with a better understanding of the evolutionary affinities of this enigmatic fossil.

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