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On the phylogenetic position of a rare Iberian endemic mammal, the Pyrenean desman (*Galemys pyrenaicus*)

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Abstract

The nucleotide sequences of the complete mitochondrial genome and nine partial nuclear genes of the Pyrenean desman (*Galemys pyrenaicus*) were determined in order to establish the relative phylogenetic position of this species at different taxonomic levels within the placental tree. Phylogenetic relationships of desman within the family Talpidae were inferred based on complete mitochondrial cytochrome *b* gene nucleotide sequence data. The Pyrenean desman was unambiguously recovered as sister group of the Russian desman (*Desmana moschata*) confirming the monophyly of the subfamily Desmaninae. However, phylogenetic relationships among major lineages within the Talpidae could not be confidently resolved. Phylogenetic analyses based on mitochondrial (at the amino acid level) and nuclear (at the nucleotide level) sequence data sets confidently placed desman within the Eulipotyphla (that also included moles, shrews, and hedgehogs), and partially recovered placental interordinal relationships. The monophyly of Laurasiatheria (including Eulipotyphla, Chiroptera, Carnivora, Pholidota, Perissodactyla, and Cetartiodactyla) was strongly supported. Mitochondrial amino acid sequences of Pholidota (pangolins) were found to bias phylogenetic inferences due to long-branch attraction effects. A Bayesian inference based on a combined mitochondrial and nuclear data set without Pholidota arrived at an almost fully resolved tree that supported the basal position of Eulipotyphla within Laurasiatheria.

Keywords: Talpidae; Eulipotyphla; Laurasiatheria; Mitochondrial genome; Nuclear genes

1. Introduction

The Pyrenean desman *Galemys pyrenaicus* (E. Geoffroy Saint-Hilaire, 1811) is an endemic mammal species of the Iberian Peninsula that is presently considered as vulnerable in the 2004 IUCN Red List of Threatened Species (http://www.redlist.org). Until recently, the Pyrenean desman was found at both slopes of the Pyrenees, as well as at the Cantabrian, Iberian and Central mountain ranges. However, its distribution has significantly shrunk during the last decades due to habitat loss and fragmentation (Castien and Gosálbez, 1992; Gonzalez-Esteban et al., 2002). This rare species lives associated to aquatic habitats, and exhibits a highly specialized morphology with a characteristic bi-lobed snout adapted to capturing insects, as well as paddle-like hind feet, and an extremely long and laterally compressed tail adapted to swimming and diving (Palmeirim and Hoffman, 1983).

Abbreviations: IUCN, International Union for the Conservation of Nature; dNTP, deoxyribonucleoside triphosphate; Numts, nuclear pseudogene copies of mitochondrial genes; ORF, open reading frame; *ADORA3*, adenosine A3 receptor gene; *ADRB2*, beta-2 adrenergic receptor gene; *APP*, amyloid beta precursor protein gene; *ATP7A*, Menkes disease ATPase 7 alpha polypeptide gene; *CREM*, cAMP responsive element moderator gene; *EDG1*, endothelial differentiation sphingolipid G-protein coupled receptor 1 gene; *PLCB4*, phospholipase C beta 4 gene; *RAG1*, recombination activating gene 1; *RAG2*, recombination activating gene 2; *cytb*, mitochondrial cytochrome *b* gene; MP, maximum parsimony; ME, minimum evolution; BI, Bayesian inference; AIC, Akaike information criterion; GTR, General time reversible model; TVM, transversional model; mtREV, mitochondrial reversible model; Tv, transversion; Ts, transition; *ND*, mitochondrial NADH dehydrogenase gene; *CO*, mitochondrial cytchrome oxidase gene; CSB, conserved sequence block; ETAS, extended termination-associated sequences; BPP, Bayesian posterior probability; BP, Bootstrap proportions.

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Table 1
Primers used to sequence the complete desman mitochondrial genome (see Fig. 1 to trace fragments along the genome)

Fragment	Primer name	Sequence	PCR length (bp)
1	L1091[a]	5′-AAAAAGCTTCAAACTGGGATTAGATACCCCACTAT-3′	1620
	Gale 16SR1	5′-GAGACAGTTAAACCCTCGTGT-3′	
2	16Sar-L[b]	5′-CGCCTGTTTATCAAAAACAT-3′	596
	16Sbr-H[b]	5′-CCGGTCTGAACTCAGATCACGT-3′	
3	Gale 16SF	5′-TAATAATTTCGGTTGGGGTGA-3′	1098
	Gale ND1R	5′-GGGGCTCGGTTTGTTTCTGC-3′	
4	Gale ND1F	5′-GCCCTACGAGCTGTAGCMCAAAC-3′	1392
	Gale ND2R	5′-GTTGTATGTCAAGATAGTGGC-3′	
5	Gale ND2F	5′-CTAACATGACAAAAAATTGCCCC-3′	866
	Gale ASNR	5′-GCCAGTTGATTAGGGTATTTAGCTG-3′	
6	Gale WF	5′-TAACTTAGACCAAGAGCCTTCAAAGC-3′	1814
	Amp-P3 R[c]	5′-GCTTCTCARATAATAAATATYAT-3′	
7	Amp-P4 F[c]	5′-GGMTTTATTCACTGATTYCC-3′	862
	MNCN-COIIR[d]	5′-TAYTCATAGCTTCAGTACCA-3′	
8	Gale COIIF	5′-GACGCAACATCACCTATTATAGAAG-3′	909
	Gale ATP6R	5′-AAAGAGGCGAATAAATTTTCGTTCAT-3′	
9	Gale LysF	5′-GCGTTAACCTTTTAAGTTAAAG-3′	2130
	Gale ArgR	5′-AAAACAAATGATTTCGACTCATTA-3′	
10	Gale COIII F	5′-AGCTCCATTCACCATTGCAGATG-3′	2130
	Gale ND4R	5′-GTAGGTGGTAAGGCTAGG-3′	
11	Gale ND4F	5′-CCTAGCCAACTCAAACTACGARCG-3′	1979
	Gale ND5R	5′-TTCCTAGTAGCAGTCGTTTAATTGG-3′	
12	Gale ND5F	5′-CAACTRTTYATTGGCTGAGAAGG-3′	967
	Gale ND5R	5′-TTCCTAGTAGCAGTCGTTTAATTGG-3′	
13	Gale ND5F	5′-TCGTATACCAACGCCTGAGCCCT-3′	1418
	Gale CytbR	5′-TTACTGATGAGAAGGCTGTTATGG-3′	
14	Gale CytbF	5′-CAAACATCTCATCATGATGRAA-3′	1050
	Gale Cytb2R	5′-TGTTTTCTATAATGCTTGCTAGTGG-3′	
15	L15774[a]	5′-TGTAAACGACGGCTAGTACATGAAATGGGAGGACAACCAGT-3′	600
	H16498[a]	5′-CATCTGGTTCCCAGTTCAGG-3′	
16	Gale loopF	5′-ACTACCATCTCACGTGAAATC-3′	1240
	Amp-12S R[c]	5′-TCGATTATAGAACAGGCTCCTCT-3′	

[a] Kocher et al. (1989).
[b] Palumbi et al. (1991).
[c] San Mauro et al. (2004).
[d] Zardoya (Unpublished data).

The Pyrenean desman is classified within the subfamily Desmaninae (Family Talpidae). The only other extant species included within this subfamily is the Russian desman, *Desmana moschata* that occurs in southwest Russia (Palmeirim and Hoffman, 1983; Hutterer, 1993). The striking distribution of the desmans into two widely separated geographical areas is likely linked to the reduction and isolation of cold areas after the last glaciation. In contrast to the specialized semiaquatic life style of the Desmaninae, the other two subfamilies of moles i.e., Uropsilinae and Talpinae are ambulatory, semi-fossorial or fossorial (Hutchinson, 1968; Yates and Moore, 1990; Hutterer, 1993; Whidden, 2000; Shinohara et al., 2003; Motowaka, 2004). The Uropsilinae includes only one genus (*Uropsilus*), and occurs in Asia (Hutterer, 1993; Motowaka, 2004). The Talpinae includes 14 genera that are classified into five tribes: Condylurini from North America, Scalopini from Eurasia and North America, Scaptonychini from Eurasia, Urotrichini from Eurasia and North America, and Talpini from Eurasia (Hutterer, 1993; Grenyer and Purvis, 2003; Motowaka, 2004). The phylogenetic relationships of desmans within Talpidae are elusive. The highly distinct morphology of desmans has complicated the finding of synapomorphies with other members of the Talpidae (Yates and Moore, 1990; Whidden, 2000; but see Grenyer and Purvis, 2003; Motowaka, 2004). On the other hand, only four recent molecular studies (Douady et al., 2002b; Douady and Douzery, 2003; Shinohara et al., 2003, 2004) using sequence data included desmans but none of them was specifically focused on elucidating their relative phylogenetic position.

Recent phylogenetic studies (Murphy et al., 2001a,b; Douady et al., 2002b, 2004; Roca et al., 2004) based mainly on nuclear gene sequence data clustered moles into a new order termed Eulipotyphla (Waddell et al., 1999), which also included shrews (Soricidae), hedgehogs (Erinaceidae), and solenodons (Solenodontidae) (Murphy et al., 2001b; Douady et al., 2002b; Roca et al., 2004). Eulipotyphla was placed together with carnivores, pangolins, bats, cetartiodactyls and perissodactyls into a new superordinal clade called Laurasiatheria (Waddell et al., 1999; Madsen et al., 2001; Murphy et al., 2001b; Douady et al., 2002b). In contrast, phylogenetic analyses based on complete mitochondrial genomes initially rejected the monophyly of Eulipotyphla by placing the hedgehog in a basal position of the placental tree, and the remaining Eulipotyphla taxa in a more derived position (Cao et al., 2000; Mouchaty et al., 2000a,b; Nikaido et al., 2001; Arnason et al., 2002). However, the

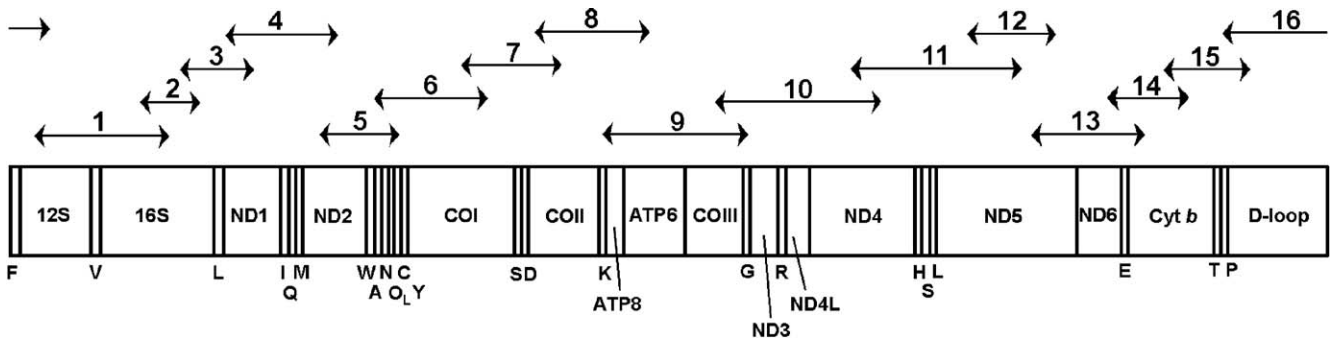

Fig. 1. Gene organization, and sequencing strategy for the mitochondrial genome of the Pyrenean desman. Arrowheaded segments denote the localization of the fragments amplified by PCR with each pair of primers (see Table 1 for the primer DNA sequence association with each fragment).

hedgehog mitochondrial sequence data (Krettek et al., 1995) exhibited extremely fast rates of evolution, and it was later demonstrated that the basal position of hedgehog was an artifact due to long-branch attraction by outgroup taxa (Waddell et al., 2001), and could be partly corrected using a more dense taxon sampling (Lin et al., 2002). Furthermore, a maximum likelihood analysis using an appropriate substitution model that corrected for site-heterogeneity was able to recover hedgehogs as members of the Eulipotyphla based on mitochondrial protein sequences (Nikaido et al., 2003). These results reconciled mitochondrial evidence with nuclear (Murphy et al., 2001b; Douady et al., 2002b; Roca et al., 2004) and morphological (MacPhee and Novacek, 1993; McKenna and Bell, 1997; Grenyer and Purvis, 2003) evidences.

Although there is recent wide agreement on the monophyly of both Eulipotyphla (Douady et al., 2002b, 2004; Lin et al., 2002; Nikaido et al., 2003; Roca et al., 2004) and Laurasiatheria (Waddell et al., 1999; Madsen et al., 2001; Murphy et al., 2001b; Lin et al., 2002; Nikaido et al., 2003), the exact phylogenetic position of Eulipotyphla is not fully resolved (Narita et al., 2001). Although this order has been mostly placed as sister group of all other Laurasiatheria (Murphy et al., 2001b; Lin et al., 2002; Nikaido et al., 2003), in some instances it is recovered as sister group of Chiroptera (e.g. Cao et al., 2000; Madsen et al., 2001; Narita et al., 2001; Nikaido et al., 2001). Furthermore, another related open question is the relative phylogenetic position of the order Perissodactyla within Laurasiatheria either as sister group of Cetartiodactyla (Euungulata hypothesis) or of Carnivora + Pholidota (Zooamata hypothesis) (Waddell et al., 1999). Many authors (Douady et al., 2002b; Lin et al., 2002; Nikaido et al., 2003) agree that further resolution among competing phylogenetic hypotheses on laurasiatherian intrarelationships partly relies on a more thorough taxon sampling of Eulipotyphla, a group that was underrepresented in early phylogenetic studies based on molecular data.

Here, we present the complete mitochondrial genome sequence as well as nucleotide sequence data from nine nuclear genes of *G. pyrenaicus*. We performed a multigene approach because it has been shown that phylogenies based on different partitions can be most confident in having resolved branches (Springer et al., 1999). We conducted different phylogenetic analyses based on the new sequence data with the main aim of clarifying the relative phylogenetic positions of desmans within Talpidae and Eulipotyphla, and of Eulipotyphla within Laurasiatheria. In particular, we wanted to test whether adding a novel non-fast evolving species of Eulipotyphla into the phylogenetic analyses would improve mitochondrial support of the monophyly of this order.

2. Materials and methods

2.1. DNA extraction, PCR amplification, and sequencing

Muscle tissue was obtained from a dead specimen of *G. pyrenaicus* from Navarra (Spain), which was preserved in 96% ethanol. Total DNA was isolated with a standard phenol/chloroform extraction procedure followed by ethanol precipitation (Sambrook et al., 1989). A suite of 32 primers (Table 1) was used to amplify by PCR, contiguous and overlapping fragments that covered the entire mitochondrial genome (Fig. 1). PCR amplifications were conducted in 25 μl reactions containing 67 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.4 mM of each dNTP, 2.5 μM of each primer, template DNA (10–100 ng), and Taq DNA polymerase (1 unit, Biotools; Madrid, Spain), using the following cycling conditions: an initial denaturing step at 94 °C for 5 min; 35 cycles of denaturing at 94 °C for 30 s, annealing at 50–58 °C for 30 s, and extending at 72 °C for 90 s; and a final extending step of 72 °C for 10 min. PCR products were purified by ethanol precipitation, and sequenced directly using the corresponding PCR primers in automated DNA sequencers (ABI PRISM 3100 and 3700) using the BigDye Deoxy Terminator cycle-sequencing kit (Applied Biosystems; Foster City, CA), and following manufacturer's instructions. The sequences obtained averaged 900 bp in length, and each sequence overlapped the next contig by about 150 bp. In no case were differences in sequence observed between the overlapping regions. The possibility of having amplified nuclear pseudogene copies of mitochondrial genes (Numts) was fully discarded based on the absence of discrepancy between overlapping PCR fragments, and by checking for unexpected stop codons within open reading frames (ORFs) or significant changes in base composition among PCR fragments.

Fragments of the nuclear adenosine A3 receptor (*ADORA3*), beta-2 adrenergic receptor (*ADRB2*), amyloid beta precursor protein (*APP*), Menkes disease ATPase 7 alpha polypeptide (*ATP7A*), cAMP responsive element moderator (*CREM*),

endothelial differentiation sphingolipid G-protein coupled receptor 1 (*EDG1*), and phospholipase C beta 4 (*PLCB4*) genes were PCR amplified and sequenced using primers published by Murphy et al. (2001a). Portions of the nuclear recombination activating genes 1 and 2 (*RAG1* and *RAG2*) were PCR amplified and sequenced with primers described in Teeling et al. (2000). PCR and sequencing conditions were as described above.

All new nucleotide sequences reported in this paper have been deposited in GenBank under accession numbers AY833410–AY833418 (nuclear genes) and AY833419 (mitochondrial genome).

2.2. Phylogenetic analyses

Four sequence data sets were analyzed in this study: (1) Mitochondrial cytochrome *b* (*cytb*) data set: the nucleotide sequences of the *cytb* gene from the 19 species of Talpidae listed in Appendix 1; (2) Mitochondrial data set: a concatenated data set including the deduced amino acid sequences of all 13 mitochondrial protein-coding genes from the 39 species listed in Appendix 1; (3) Nuclear data set: a concatenated data set including nucleotide sequences of nine nuclear genes from the 39 species listed in Appendix 1; and (4) Combined data set: the deduced amino acid sequences of all 13 mitochondrial protein-coding genes, and the nucleotide sequences of nine nuclear genes from 15 laurasiatherian taxa were combined into a single data set.

The nucleotide sequences of the mitochondrial *cytb* gene, the deduced amino acid sequences of each of the 13 complete mitochondrial protein-coding genes, and the nucleotide sequences of each of the nine partial nuclear genes were aligned separately, using the default parameters of CLUSTAL X version 1.81 (Thompson et al., 1997). Alignments (available from the authors upon request) were subsequently revised by eye in an effort to maximize positional homology. The construction of the *cytb*, mitochondrial, nuclear, and combined data sets was after excluding gapped positions.

Four commonly used methods of phylogenetic inference, namely maximum parsimony (MP, Fitch, 1971), minimum evolution (ME, Rzhetsky and Nei, 1992), maximum likelihood (ML, Felsenstein, 1981), and Bayesian inference (BI, Huelsenbeck et al., 2001) were applied to the *cytb*, mitochondrial, and nuclear data sets, respectively. MP and ME analyses were performed with PAUP* version 4.0b10 (Swofford, 2002), with 10 random addition sequences and TBR branch swapping. MP analyses of the *cytb* and nuclear data sets used 6:1 and 3:1 Transversion (Tv):Transition (Ts) weighting schemes, respectively (estimated based on the corresponding empirical Ts:Tv ratios). ME analyses of amino acid and nucleotide sequences used mean character and ML distances, respectively. ML analyses were performed using Phyml version 2.4.4. (Guindon and Gascuel, 2003). The best-fit models of nucleotide and amino acid replacement were selected according to the Akaike Information Criterion (AIC) using ModelTest version 3.6 (Posada and Crandall, 1998) and Prottest version 1.2.7 (Abascal et al., 2005). The evolutionary models selected for the *cytb*, nuclear and mitochondrial data sets were GTR+Γ, TVM+I+Γ, and mtmam+I+Γ, respectively. Support of the recovered MP, ME, and ML trees was evaluated with non-parametric bootstrap proportions (BPs-500 pseudoreplicates).

BI was performed using MrBayes version 3.0b4 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) with four simultaneous chains, each of 10^6 generations, sampled every 100 generations. Trees sampled before the cold chain reached stationarity (as judged by plots of ML scores) were discarded as "burn-in". Runs were repeated twice. We used the mtREV24+I+Γ, GTR+Γ, and the GTR+I+Γ models for the mitochondrial, *cytb* and nuclear data sets, respectively (the best-fit models according to the AIC that are available in MrBayes). Support of the recovered BI trees was evaluated with Bayesian posterior probabilities (BPPs).

The Laurasiatheria data set that combined mitochondrial amino acid and nuclear nucleotide sequences was analyzed only with BI (Ronquist and Huelsenbeck, 2003). We applied the mtREV24+I+Γ and GTR+I+Γ models to the amino acid and nucleotide partitions, respectively. Run conditions were as described above.

3. Results

3.1. Main features of the mitochondrial genome of *G. pyrenaicus*

The length of the complete L-strand nucleotide sequence of the Pyrenean desman mitochondrial genome is 16,510 nucleotides. The overall base composition of the L-strand is A: 34.54%, C: 23.15%, G: 13.58%, and T: 28.73%. As in other vertebrates, two rRNA, 22 tRNA, and 13 protein-coding genes were identified in the new mitochondrial genome. The organization of the desman mitochondrial genome conforms to the consensus gene order of other eutherian mitochondrial genomes (Fig. 1). All protein-coding genes use ATG as start codon except *ND2* that uses ATA, *ND3* that starts with ATC, and *ND5* that begins with ATT. Most desman open reading frames end with TAA (*COI*, *COII*, *ATPase 8*, *ND4L*, *ND5*, and *ND6*), one ends with AGA (*cytb*), and the rest have incomplete stop codons, either T (*ND2*, *COII*, *ND3*, and *ND4*) or TA (*ND1* and *ATPase 6*), which may be likely completed by post-transcriptional polyadenylation (Ojala et al., 1981).

The control region of the desman mitochondrial genome is 1020 nucleotides in length, and it does not contain any repeat. As in other mammals (Sbisa et al., 1997), a highly conserved central domain was recognized in the desman mitochondrial control region. The conserved sequence block CSB-1 (Walberg and Clayton, 1981) and the extended termination-associated sequences ETAS-2 (Sbisa et al., 1997) were unambiguously identified in the 3′ and 5′ ends of the control region, respectively. In addition, putative CSB-2 and CSB-3 motifs that shared limited sequence similarity to consensus sequences (Sbisa et al., 1997) were tentatively found in the 5′ end of the control region. No ETAS-1 (Sbisa et al., 1997) was determined in the desman mitochondrial control region. The origin of light-strand replication was identified in a cluster of five tRNA genes (WANCY region) between *tRNA-Asn* and *tRNA-Cys* genes. It folds into a

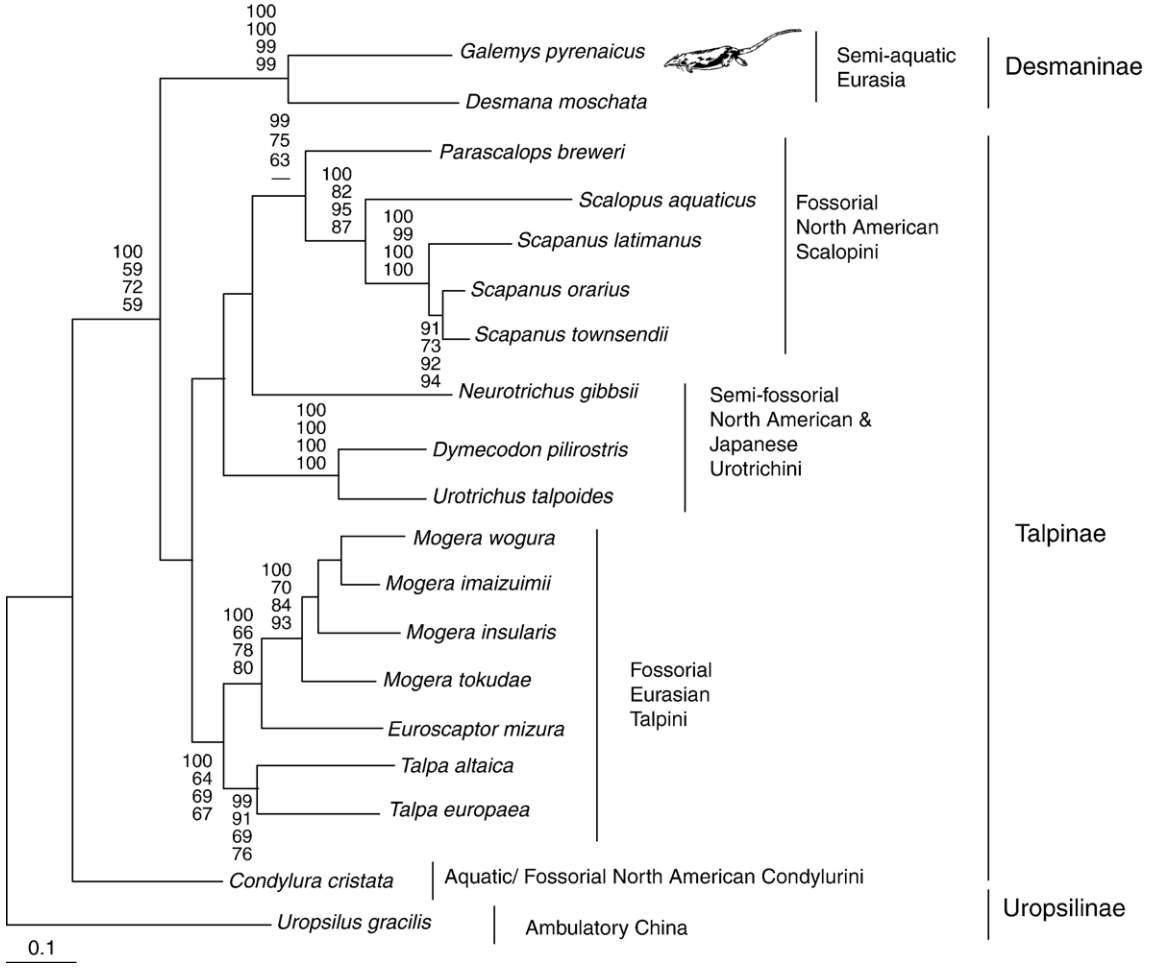

Fig. 2. Phylogenetic relationships (BI phylogram) of Talpidae based on complete mitochondrial cytochrome *b* gene sequence data. Numbers represent support for BI (BPPs above 95%), ME (BPs above 50%), MP (BPs above 50%), and ML (BPs above 50%) from top to bottom. Hyphens indicate support values below 50% BPs.

stem-loop secondary structure like in most vertebrate mitochondrial genomes, and exhibits a poly-T loop. The desman mitochondrial genome is extremely compact with only 11 non-coding intergenic spacers, most 1–3 bp long.

3.2. Phylogenetic relationships within the Talpidae

The phylogenetic position of the desman within the Talpidae was assessed based on the complete mitochondrial *cytb* gene nucleotide sequences of 19 taxa representing the main lineages within the family. A member of the subfamily Uropsilinae (Chinese shrew-like moles) was used as outgroup. An alignment of 1140 positions was produced. No gaps were postulated, 662 positions were invariant, and 399 sites were parsimony informative. The tree inferred using BI is shown in Fig. 2. The North American aquatic/fossorial star-nosed mole *Condylura cristata* was recovered as the sister group of the remaining Talpidae. The Pyrenean desman was recovered as sister group of the Russian desman with a high BPP value (Fig. 2). Fossorial moles were grouped into two distinct and unrelated clades, one corresponding to the Eurasian Talpini, and the other to the North American Scalopini. Semi-fossorial shrew moles were recovered into two distinct lineages, one that grouped Japanese species together, and another including the only North American species (Fig. 2). Phylogenetic relationships among the main lineages of Talpidae were largely unresolved. MP, ME and ML phylogenetic analyses arrived at identical 50% majority-rule bootstrap consensus trees (Fig. 2). Phylogenetic inference based on the *cytb* data set excluding third codon positions (that are prone to saturation) did not achieve better resolution of Talpidae relationships (not shown).

3.3. Phylogenetic position of desmans within Eulipotyphla, and Laurasiatheria intrarelationships

The deduced amino acid sequences of all 13 mitochondrial protein-coding genes of 39 mammals were combined into a single data set that produced an alignment of 3993 positions. A total of 249 gapped positions were excluded from further phylogenetic analyses. Of the remaining positions, 1599 were constant, and 1719 were parsimony informative. The best tree that was recovered using BI is shown in Fig. 3. The phylogenetic position of the desman within the Eulipotyphla was highly supported (100% BPP), as well as its sister group relationship with the moles (100% BPP) to the exclusion of the shrews (Fig. 3). Hedgehogs were recovered as the most basal lineage of

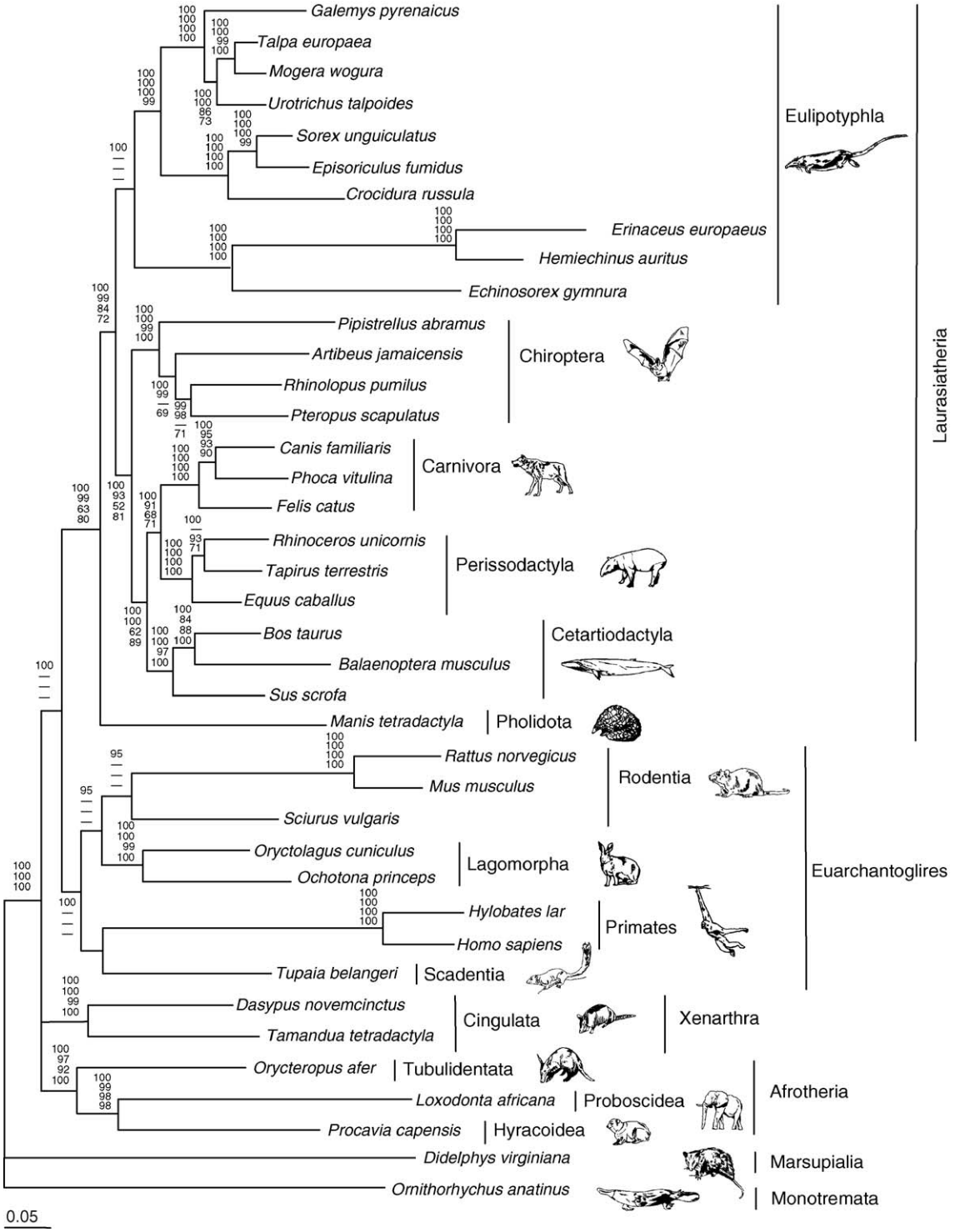

Fig. 3. Phylogenetic relationships (BI phylogram) of Eutheria inferred from a single concatenated data set of the deduced amino acid sequences of all 13 mitochondrial protein-coding genes. Numbers represent support for BI (BPPs above 95%), ME (BPs above 50%), MP (BPs above 50%), and ML (BPs above 50%) from top to bottom. Hyphens indicate support values below 50% BPs.

Eulipotyphla, although with lower statistical support (88% BPP). The monophyly of the different eutherian orders was recovered with high BPP values. Afrotheria, Euarchontoglires, and Laurasiatheria superordinal clades also received maximal BPP statistical support (Fig. 3). Within Laurasiatheria, the order Pholidota exhibited a relatively long branch, and was recovered as the most basal lineage of the clade (Fig. 3). Chiroptera was recovered as sister group to a clade including Perissodactyla +Carnivora, and Cetartiodactyla (100% BPP). The 50% majority-rule bootstrap consensus trees recovered with MP, ME and ML based on the same data set supported essentially the same intraordinal groupings (see bootstrap values in Fig. 3)

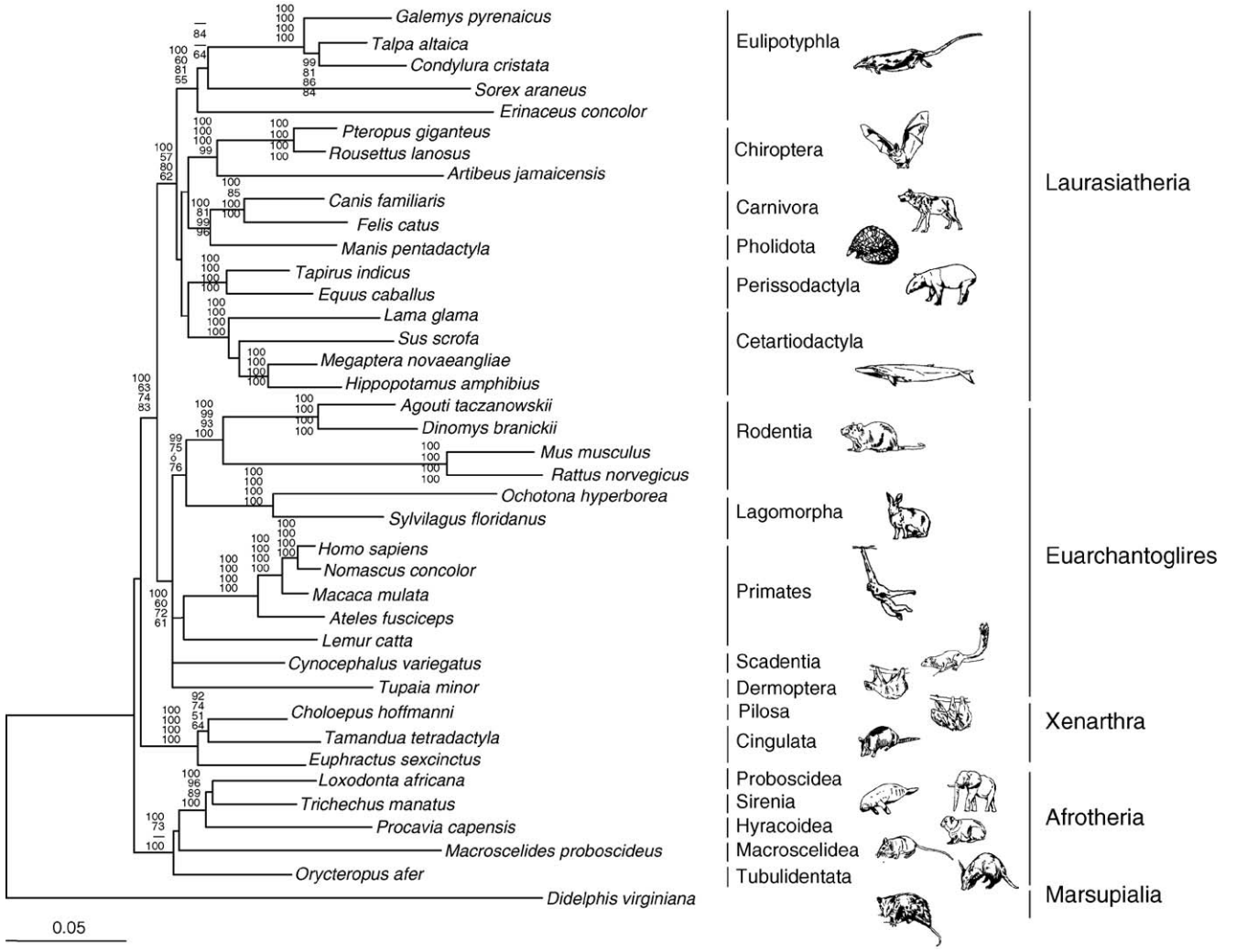

Fig. 4. Phylogenetic relationships (BI phylogram) of Eutheria inferred from a single concatenated data set of partial nucleotide sequences of nine nuclear genes. Numbers represent support for BI (BPPs above 95%), ME (BPs above 50%), MP (BPs above 50%), and ML (BPs above 50%) from top to bottom. Hyphens indicate support values below 50% BPs.

whereas interordinal relationships were largely unresolved. The only striking intraordinal difference was that Eulipotyphla was not recovered as a monophyletic group because of the relative position of hedgehogs as sister group of all the remaining placentals (not shown).

The nucleotide sequences of nine nuclear genes of 39 species representing the 18 main mammalian orders were combined into a single data set that produced an alignment of 5614 positions. A total of 1816 gapped positions were excluded from further phylogenetic analyses. Of the remaining, 1696 positions were invariant, and 1513 were parsimony informative. The recovered best tree based on BI is depicted in Fig. 4. The desman was recovered with high statistical support as a member of the Talpidae within the Eulipotyphla, which also included shrews and hedgehogs (Fig. 4). Four main superordinal clades i.e. Laurasiatheria, Xenarthra, and Afrotheria were recovered with maximal BPP values. Within Laurasiatheria, Pholidota was recovered as sister group of Carnivora with maximal BPP support. Moreover, both groups were recovered as sister group of Chiroptera to the exclusion of Perissodactyla and Cetartiodactyla, but without statistical support (Fig. 4). The 50% majority-rule bootstrap consensus trees recovered with MP, ME, and ML based on the same data set supported the same intraordinal groupings as well as the monophyly of Laurasiatheria, Xenarthra, and Afrotheria (see bootstrap values in Fig. 4). However, interordinal relationships within these four main clades were largely unresolved. The only difference that received bootstrap support was a sister group relationship of *Sorex* and *Erinaceus* in the MP analysis (not shown).

In order to further resolve phylogenetic relationships within Laurasiatheria, both the deduced amino acid sequence of all 13 mitochondrial protein-coding genes, and the nucleotide sequences of nine nuclear genes of 15 taxa representing the main orders of Laurasiatheria were combined into a single data set, and analyzed only with BI. In six instances, we had to artificially make chimeric sequences because either the mitochondrial or the nuclear counterparts were missing. In five of the cases (*Talpa*, *Sorex*, *Pteropus*, *Manis*, and *Tapirus*), the procedure only required mixing sequences of species within the same genus whereas in one case (mitochondrial *Balaenoptera*

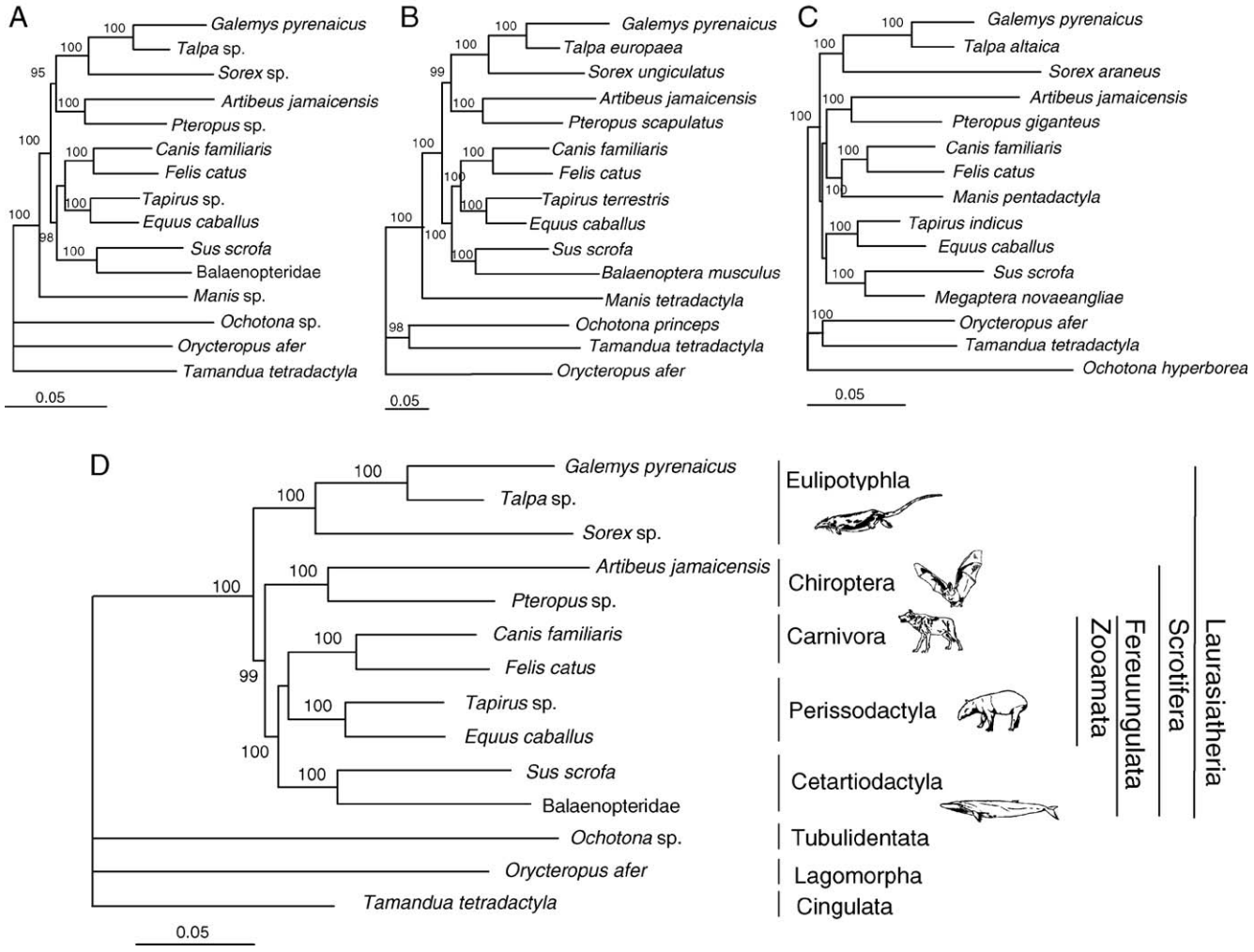

Fig. 5. Phylogenetic relationships (BI phylograms) of Laurasiatheria. A) Tree inferred from a combined mitochondrial and nuclear data set. B) Tree inferred based only on mitochondrial data. C) Tree inferred based only on nuclear data. D) Tree inferred from a combined mitochondrial and nuclear data set without Pholidota. Numbers represent support for BI (BPPs above 95%).

+nuclear *Megaptera*) the mixed sequences belonged to species from the same family (Balaenopteridae). A final alignment of 9605 positions was gathered (3991 amino acids+5614 nucleotides). After gapped positions were removed, a total of 8238 positions (3773 amino acids+4465 nucleotides) were used in subsequent phylogenetic analyses. The number of invariant and parsimony informative sites was 4919 (2225 amino acids+2694 nucleotides) and 1927 (1079 amino acids+848 nucleotides), respectively. The BI tree that was recovered based on the combined data set is shown in Fig. 5A. The monophyly of each laurasiatherian order was recovered with maximum BPP values, but interordinal relationships lacked maximal statistical support. The order Pholidota was recovered as the most basal lineage within Laurasiatheria. Chiroptera and Eulipotyphla were recovered as sister group taxa. Perissodactyla was placed as sister group of Carnivora to the exclusion of Certartiodactyla (Fig. 5A). The same topology was obtained when the BI was based only on mitochondrial amino acid data, but in this case all nodes received maximal statistical support (Fig. 5B). BI based only on nuclear data arrived at a distinct topology (Fig. 5C). The most striking difference was the relative position of Pholidota (pangolins) that was recovered with high statistical support as sister group of Carnivora. Other interordinal relationships lacked support (Fig. 5C). Finally, BI was performed based on the combined data set without Pholidota. The recovered tree was almost fully resolved (Fig. 5D). Eulipotyphla was recovered with high statistical support as the sister group of the remaining Laurasiatheria. Chiroptera was the next lineage that branched off the tree. In a more derived position, the Perissodactyla were recovered as the sister group of Carnivora to the exclusion of the cetartiodactyla, but without statistical support.

4. Discussion

The nucleotide sequences of the complete mitochondrial genome, and nine partial nuclear genes of the Pyrenean desman were determined anew. The desman mitochondrial genome showed typical organization and main features of other mammal mitochondrial genomes. The new sequence data was used to place the desman within the Talpidae and the Eulipotyphla, and

to resolve among competing hypotheses on the relative phylogenetic position of Eulipotyphla within Laurasiatheria.

The recovered molecular phylogeny based on *cytb* gene is the first showing a close phylogenetic relationship of *Desmana* and *Galemys*, and confirms morphological evidence that grouped both genera within the subfamily Desmaninae (Hutchinson, 1968; Hutterer, 1993; Whidden, 2000; Grenyer and Purvis, 2003; Motowaka, 2004). Morphology-based phylogenies generally place the Desmaninae as the sister group of the Talpinae (Hutterer, 1993; Whidden, 2000; Grenyer and Purvis, 2003; Motowaka, 2004; but see Hutchinson, 1968; McKenna, 1975). According to our tree, however, *Condylura* (Talpinae) is the sister group of the remaining ingroup taxa (other Talpinae+Desmaninae), which are grouped together in a clade that receives moderately high statistical support (Fig. 2). *Condylura* is a rather enigmatic talpid from North America with both aquatic and fossorial lifestyles that has its own tribe (Condylurini) (Shinohara et al., 2003; Motowaka, 2004). Even though its precise phylogenetic position is rather contentious from a morphological point of view (Hutchinson, 1968; Yates and Moore, 1990; Whidden, 2000; Grenyer and Purvis, 2003; Motowaka, 2004), our results need to be taken with caution since the phylogenetic performance of the *cytb* gene is rather low. Interestingly, similar phylogenetic analyses based on *cytb* gene also recovered *Desmana* in a derived position within the Talpidae (Shinohara et al., 2003). Although recent studies (Douady et al., 2002b; Douady and Douzery, 2003) based on several nuclear genes and only four Talpidae also recovered the desman in a derived position, our phylogenetic analyses based on the nuclear data set recovered the desman as sister group of *Condylura*+*Talpa* with strong support (see below), in agreement with morphological evidence (Hutchinson, 1968; Whidden, 2000; Grenyer and Purvis, 2003; Motowaka, 2004). Unfortunately, and because of the general lack of resolution of the *cytb*-inferred tree (Shinohara et al., 2003), we cannot conclude whether the fully fossorial lifestyle derived from semi-fossorial habits, and whether it had one or several origins (Shinohara et al., 2003; Motowaka, 2004). The exclusion of the fast evolving third codon positions of *cytb* did not achieve better resolution. More mitochondrial and nuclear sequence data needs to be analyzed to settle this question (Shinohara et al., 2004).

The rate of evolution is much higher in mitochondrial than in nuclear protein-coding genes, and at deeper levels of divergence the former show clear patterns of saturation at third codon positions (Waddell et al., 2001). Protein sequences have more states and a slower substitution rate than DNA sequences. Hence, the amino acid sequences are less prone to saturation effects (Waddell et al., 2001), and in principle may contain more phylogenetic information (but see Simmons et al., 2004). As a result, and in order to maximize phylogenetic information and performance of analyzed genes, phylogenetic inferences of deep relationships based on mitochondrial protein coding genes are often performed at the amino acid level (e.g. Cao et al., 2000; Waddell et al., 2001; Nikaido et al., 2003) whereas those based on nuclear protein coding genes are generally conducted at the nucleotide level (e.g. Madsen et al., 2001; Murphy et al., 2001a; Douady et al., 2004). Moreover, using both types of genes at different levels does not preclude their combination since one of the most promising advantages of Bayesian methods of phylogenetic inference is the possibility of reconstructing phylogenies from combined amino acid and nucleotide partitions (Ronquist and Huelsenbeck, 2003).

Phylogenetic analyses based on the mitochondrial (Fig. 3) and nuclear (Fig. 4) data sets confidently placed the desman (subfamily Desmaninae) within the Talpidae (Hutterer, 1993), as sister group of moles of the subfamily Talpinae (Urotrichini + Talpini in the mitochondrial-based tree; Condylurini +Talpini in the nuclear-based tree) (Grenyer and Purvis, 2003; Motowaka, 2004). Mitochondrial evidence strongly supports shrews (Soricidae) as sister group of the Talpidae (e.g. (Lin et al., 2002; Nikaido et al., 2003) to the exclusion of hedgehogs (Erinaceidae), a hypothesis also supported by several morphological studies (e.g. (Gregory, 1910; Campbell, 1939; Butler, 1988; Grenyer and Purvis, 2003). The BI and ME trees based on the nuclear data set also recovered this relationship, although without statistical support in the former case. Instead, the MP tree based on the nuclear data set strongly supported a sister group relationship of Soricidae and Erinaceidae, the most favored hypothesis in other studies also based on nuclear evidence using different methods of phylogenetic inference (MP, ME, BI) (Miyamoto and Goodman, 1986; Murphy et al., 2001a,b; Douady et al., 2002a, 2004; Douady and Douzery, 2003; Roca et al., 2004). Disagreement between nuclear and mitochondrial evidence may be due to bias in the latter because of the atypical erinaceid amino acid sequences (Cao et al., 2000; Waddell et al., 2001; Nikaido et al., 2003). Nevertheless, this phylogenetic question remains open. All phylogenetic analyses based on nuclear data strongly supported the monophyly of Eulipotyphla (including moles, shrews, and hedgehogs) (Murphy et al., 2001b; Douady et al., 2002b; Roca et al., 2004) whereas such hypothesis was only supported by mitochondrial data with BI analyses (Fig. 2) that used a fair representation of Eulipotyphla taxa (Lin et al., 2002), and evolutionary models that take into account among-site rate heterogeneity (Nikaido et al., 2003). MP, ME, and ML phylogenetic analyses based on mitochondrial data failed to cluster hedgehogs within Eulipotyphla. Instead, hedgehogs were placed at the base of the placental tree (e.g. Cao et al., 2000; Mouchaty et al., 2000b; Arnason et al., 2002) likely due to a long-branch attraction effect (Waddell et al., 2001; Lin et al., 2002; Nikaido et al., 2003).

Bayesian analyses of Laurasiatheria relationships (Fig. 5) based on nuclear and mitochondrial sequence data disagreed on the recovered topologies, and in particular on the relative phylogenetic position of Pholidota (pangolins). In the mitochondrial-based phylogeny, *Manis tetradactyla* exhibits a relatively long branch (Arnason et al., 2002), and it may be spuriously pulled down to the base of the Laurasiatheria clade. In the nuclear-based phylogeny, *Manis pentadactyla* is placed as sister group of Carnivora with strong support (Murphy et al., 2001b; Douady et al., 2002b). The different phylogenetic signals

cancel out reciprocally in the combined analysis of mitochondrial and nuclear sequence data, which fails to recover interordinal relationships. On the other hand, phylogenetic inferences based on complete mitochondrial genome sequence data with a more dense taxon sampling are able to partially counteract the long-branch attraction effect, and recover Pholidota as sister group of Carnivora, although without statistical support (Arnason et al., 2002). The sequencing of complete mitochondrial genomes from other species of Pholidota is needed to further assess the relative phylogenetic position of this order.

If Pholidota is excluded from the combined analysis, an almost fully resolved tree (>95% BPP in all nodes except Perissodactyla+Carnivora) is recovered. This tree (Fig. 5C) is our best hypothesis for the phylogenetic relationships within Laurasiatheria (excluding Pholidota). The Eulipotyphla are recovered as sister group of the remaining laurasiatherian taxa, as previously suggested (Waddell et al., 1999, 2001; Murphy et al., 2001b; Douady et al., 2002b). This result is in agreement with phylogenetic reconstructions based on mitochondrial (Fig. 3) and nuclear (Fig. 4) data sets that have a more thorough sampling of both Laurasiatheria and other placentals. The remaining Laurasiatheria form a clade that was also recovered in previous studies (Murphy et al., 2001b; Arnason et al., 2002; Douady et al., 2002b), and was termed Scrotifera because all members of this group share a postpenile scrotum (Waddell et al., 1999). This clade is also known as Variamana (Springer et al., 2003). Within Scrotifera, bats are recovered as sister group of Fereuungulata (Carnivora+Perissodactyla+Cetartiodactyla) (Waddell et al., 1999). This group was also recovered by Arnason et al. (2002) who called it Cetferungulata. The alternative hypothesis of a sister group relationship between Chiroptera and Eulipotyphla (Mouchaty et al., 2000a,b; Nikaido et al., 2000, 2001; Madsen et al., 2001) that would form a clade termed Insectiphillia (Waddell et al., 2001) is only strongly supported by mitochondrial evidence alone (Fig. 5B). However, this result does not hold when a more thorough sampling of both Laurasiatheria and other placentals (Fig. 3) is considered, and instead the Fereuungulata is strongly supported. Nuclear evidence alone (Figs. 4 and 5C) supported an alternative hypothesis (Chiroptera as sister group of Carnivora+Pholidota), although without statistical support. Within Fereuungulata, our tree recovers the clade termed Zooamata (Carnivora +Perissodactyla) (Arnason et al., 1997, 2002; Cornelli and Ward, 2000; Mouchaty et al., 2000a,b; Murphy et al., 2001a; Robinson-Rechavi and Graur, 2001), but this relationship is not statistically supported, and the alternative hypothesis, i.e. Euungulata (Perissodactyla+Cetartiodactyla) cannot be discarded. In fact, Zooamata was strongly supported by mitochondrial data alone (Figs. 3 and 5B) whereas Euungulata was supported by nuclear data alone (Figs. 4 and 5C), although without statistical support.

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Appendix A

Newly obtained sequences of Pyrenean desman (*Galemys pyrenaicus*) were analyzed together with sequences from the following mammalian species:

A.1. Cytochrome b data set

Family Talpidae: Subfamily Talpinae, *Condylura cristata* (star-nosed mole; AB076810), *Dymecodon pilirostris* (lesser Japanese shrew-mole; AB076830), *Euroscaptor mizura* (Japanese mountain mole; AB076828), *Mogera imaizumii* (lesser Japanese mole; AB037609), *Mogera insularis* (insular mole; AB037606), *Mogera tokudae* (Tokuda's mole; AB037607), *Mogera wogura* (greater Japanese mole; AB037646), *Neurotrichus gibbsii* (American shrew-mole; AB076821), *Parascalops breweri* (hairy-tailed mole; AB076808), *Scalopus aquaticus* (eastern mole; AB076809), *Scapanus latimanus* (broad-footed mole; AB076813), *Scapanus orarius* (coast mole; AB076815), *Scapanus townsendii* (Townsend's mole; AB076818), *Talpa altaica* (Siberian mole; AB037602), *Talpa europaea* (European mole; AB076829), *Urotrichus talpoides* (greater Japanese shrew mole; AB076832); Subfamily Desmaninae, *Desmana moschata* (Russian desman; AB076836); Subfamily Uropsilinae, *Uropsilus gracilis* (gracile shrew mole; AB076699); Family Soricidae: Subfamily Crocidurinae, *Crocidura dsinezumi* (Dsinezumi shrew; AB076837), *Suncus murinus* (house shrew; AB033610); Subfamily Soricinae, *Sorex unguiculatus* (long-clawed shrew; AB061527).

A.2. Mitochondrial data set

Order Monotremata, *Ornithorhynchus anatinus* (platypus; NC_000891); Order Didelphimorphia, *Didelphis virginiana* (North American opossum; NC_001610); Order Xenarthra: *Dasypus novemcinctus* (nine-banded armadillo; NC_001821), *Tamandua tetradactyla* (southern tamandua; NC_004032); Order Insectivora: *Talpa europaea* (European mole; NC_002391), *Mogera wogura* (Japanese mole; NC_005035), *Urotrichus talpoides* (Japanese shrew mole; NC_005034), *Sorex unguiculatus* (long-clawed shrew; AB061527), *Crocidura russula* (white-toothed shrew; NC_006893), *Episoriculus fumidus* (Taiwan brown-toothed shrew; NC_003040), *Hemiechinus auritus* (long-eared hedgehog; NC_005033), *Echinosorex gymnura* (moonrat; NC_002808), *Erinaceus europaeus* (western European hedgehog; NC_002080); Order Tubulidentata: *Orycteropus afer* (aardvark; NC_002078); Order Hyracoidea: *Procavia capensis* (cape rock hyrax; NC_004919); Order Proboscidea: *Loxodonta africana* (African savanna elephant; NC_000934); Order Rodentia: *Rattus norvegicus* (Norway rat; NC_001665), *Sciurus vulgaris* (Eurasian red squirrel; NC_002369), *Mus musculus* (house mouse; AJ489607); Order Lagomorpha: *Oryctolagus cuniculus* (rabbit; AJ001588), *Ochotona princeps* (American pika; NC_005358); Order Scadentia:

Tupaia belangeri (northern tree shrew; NC_002521); Order Primates: *Hylobates lar* (common gibbon; NC_002082), *Homo sapiens* (human; NC_001807); Order Chiroptera: *Pipistrellus abramus* (Japanese house bat; NC_005436), *Rhinolophus pumilus* (Okinawa least horseshoe bat; NC_005434), *Pteropus scapulatus* (little red flying fox; AF321050), *Artibeus jamaicensis* (Jamaica fruit-eating bat; NC_002009); Order Carnivora: *Canis familiaris* (dog; NC_002008), *Phoca vitulina* (harbor seal; NC_001325), *Felis catus* (cat; NC_001700); Order Perissodactyla: *Rhinoceros unicornis* (greater Indian rhinoceros; NC_001779), *Tapirus terrestris* (Tapir; NC_005130), *Equus caballus* (horse; NC_001640); Order Artiodactyla: *Sus scrofa* (pig; NC_000845), *Bos taurus* (cow, AY526085); Order Cetacea: *Balaenoptera musculus* (blue whale; NC_001601); Order Pholidota*: Manis tetradactyla* (long-tailed pangolin; NC_004027).

A.3. Nuclear data set

Order Didelphimorphia, *Didelphis virginiana* (North American opossum; ADORA 3: AY011189; ADRB2: AY059673; APP: AY059678; ATP7A: AY011375; CREM: AY011620; EDG1: AY011685; PLCB4: AY011743; RAG1: AY011864; RAG2: AY059713); Order Xenarthra: *Choloepus hoffmanni* (Hoffmann's two-fingered sloth; ADORA 3: AY011191; ADRB2: AY011252; APP: AY011313; ATP7A: AY011377; CREM: AY011622; EDG1: AY011687; PLCB4: AY011745; RAG1: AY011685; RAG2: AY011920), *Euphractus sexcinctus* (six-banded armadillo; ADORA 3: AY011193; ADRB2: AY011254; APP: AY011315; ATP7A: AY011379; CREM: AY011624; EDG1: AY011688; PLCB4: AY011747; RAG1: AY011867; RAG2: AY011922), *Tamandua tetradactyla* (southern tamandua; ADORA 3: AY011195; ADRB2: AY011256; APP: AY011317; ATP7A: AY011381; CREM: AY011626; EDG1: AY011690; PLCB4: AY011749; RAG1: AY011869; RAG2: AY011924); Order Insectivora: *Talpa altaica* (Siberian mole; ADORA 3: AY011198; ADRB2: AY011259; APP: AY011320; ATP7A: AY011384; CREM: AY011629; PLCB4: AY011752; RAG1: AY011872; RAG2: AY011927), *Condylura cristata* (star-nosed mole; ADORA 3: AY011199; ADRB2: AY011260; APP: AY011321; ATP7A: AY011385; CREM: AY011630; EDG1: AY011693; PLCB4: AY011753; RAG1: AY011873; RAG2: AY011928), *Sorex araneus* (European shrew ADORA 3: AY011200; ADRB2: AY011261; APP: AY011322; ATP7A: AY011386; CREM: AY011631; EDG1: AY011694; PLCB4: AY011754; RAG2: AY011929), *Erinaceus concolor* (eastern European hedgehog; ADORA 3: AY0111197; ADRB2: AY011258; APP: AY011319; ATP7A: AY011383; CREM: AY011628; EDG1: AY011692; PLCB4: AY011751; RAG1: AY011871; RAG2: AY011926); Order Tubulidentata: *Orycteropus afer* (aardvark; ADORA 3: AY011206; ADRB2: AY011266; APP: AY011329; ATP7A: AY011392; CREM: AY011638; EDG1: AY011701; PLCB4: AY011761; RAG1: AY011878; RAG2: AY011935); Order Hyracoidea: *Procavia capensis* (cape rock hyrax; ADORA 3: AY011203; ADRB2: AY011263; APP: AY011325; ATP7A: AY011389; CREM: AY011634; EDG1: AY011697; PLCB4: AY011757); Order Macroscelidea: *Macroscelides proboscideus* (short-eared elephant shrew; ADORA 3: AY011205; ADRB2: AY059674; APP: AY011327; ATP7A: AY059682; CREM: AY011636; EDG1: AY011699; PLCB4: AY011759; RAG1: AY011876; RAG2: AY011933); Order Proboscidea: *Loxodonta africana* (African savanna elephant; ADORA 3: AY011204; ADRB2: AY011264; APP: AY011326; ATP7A: AY011390; CREM: AY011635; EDG1: AY011698; PLCB4: AY011758; RAG1: AY011875; RAG2: AY011932); Order Sirenia: *Trichechus manatus* (Caribbean manatee; ADORA 3: AY011202; ADRB2: AY011262; APP: AY011324; ATP7A: AY011388; CREM: AY011633; EDG1: AY011696; PLCB4: AY011756; RAG1: AY011874; RAG2: AY011931); Order Rodentia: *Rattus norvergicus* (Norway rat; ADORA 3: AY011212; ADRB2: AY011271; APP: AY011335; ATP7A: AY011398; CREM: AY011644; EDG1: AY011707; PLCB4: AY011767; RAG1: AY011884; RAG2: AY011941), *Agouti taczanowskii* (mountain paca; ADORA 3: AY011220; ADRB2: AY011281; APP: AY011344; ATP7A: AY011407; CREM: AY011654; EDG1: AY011715; PLCB4: AY011777; RAG1: AY011894; RAG2: AY011951), *Dinomys branickii* (pacarana; ADORA 3: AY011219; ADRB2: AY011280; APP: AY011343; ATP7A: AY011406; CREM: AY011653; PLCB4: AY011776; RAG1: AY011893; RAG2: AY011950), *Mus musculus* (house mouse; ADORA 3: AY011211; ADRB2: AY011270; APP: AY011334; ATP7A: AY011397; CREM: AY011643; EDG1: AY011706; PLCB4: AY011766; RAG1: AY011883; RAG2: AY011940); Order Lagomorpha: *Ochotona hyperborea* (northern pika; ADORA 3: AY011222; ADRB2: AY011283; APP: AY011346; ATP7A: AY011409; CREM: AY011655; EDG1: AY011717; PLCB4: AY011779; RAG1: AY011896; RAG2: AY011953), *Sylvilagus floridanus* (eastern cottontail; ADORA 3: AY011221; ADRB2: AY011282; APP: AY011345; ATP7A: AY011408; EDG1: AY011716; PLCB4: AY011778; RAG1: AY011895; RAG2: AY011952); Order Scadentia: *Tupaia minor* (pygmy tree shrew; ADORA 3: AY011224; ADRB2: AY011285; APP: AY011348; ATP7A: AY011411; CREM: AY011657; EDG1: AY011719; PLCB4: AY011781; RAG2: AY011955); Order Primates: *Ateles fusciceps* (brown-headed spider monkey; ADORA 3: AY011227; ADRB2: AY011287; APP: AY011351; ATP7A: AY011414; CREM: AY011660; EDG1: AY011721; PLCB4: AY011784; RAG1: AY011889; RAG2: AY011958), *Homo sapiens* (human; ADORA 3: AY011231; ADRB2: AY011291; APP: AY011354; ATP7A: AY011418; CREM: AY011664; EDG1: AY011725; PLCB4: AY011788; RAG1: AY011903; RAG2: AY011962), *Nomascus concolor* (crested gibbon; ADORA 3: AY011229; ADRB2: AY011289; ATP7A: AY011416; CREM: AY011662; EDG1: AY011723; PLCB4: AY011786; RAG1: AY011901; RAG2: AY011960), *Macaca mulatta* (rhesus monkey; ADORA 3: AY011228; ADRB2: AY011288; APP: AY011352; ATP7A: AY011415; CREM: AY011661; EDG1: AY011722; PLCB4: AY011785; RAG1: AY011900; RAG2: AY011959), *Lemur catta* (ring-tailed lemur; ADORA 3: AY011225; ADRB2: AY011286; APP: AY011349; ATP7A: AY011412; CREM: AY011658; EDG1: AY011720; PLCB4: AY011782; RAG1: AY011898; RAG2: AY011956); Order Dermoptera: *Cynocephalus variegatus* (Malayan flying

lemur; ADORA 3: AY011223; ADRB2: AY011284; APP: AY011347; ATP7A: AY011410; CREM: AY011656; EDG1: AY011718; PLCB4: AY011780; RAG1: AY011897; RAG2: AY011954); Order Chiroptera: *Artibeus jamaicensis* (Jamaican fruit-eating bat; ADORA 3: AY011232; ADRB2: AY011292; APP: AY011355; ATP7A: AY011419; CREM: AY011665; EDG1: AY011726; PLCB4: AY011789; RAG1: AY011904; RAG2: AY011963), *Pteropus giganteus* (Indian flying fox; ADORA 3: AY011233; ADRB2: AY011293; APP: AY011356; ATP7A: AY011420; CREM: AY011666; EDG1: AY011727; PLCB4: AY011790; RAG1: AY011905; RAG2: AY011964), *Rousettus lanosus* (long-haired rousette; ADORA 3: AY011234; ADRB2: AY011294; APP: AY011357; ATP7A: AY011421; CREM: AY011667; EDG1: AY011728; PLCB4: AY011791; RAG1: AY011906; RAG2: AY011965); Order Carnivora: *Canis familiaris* (dog; ADORA 3: AY011249; ADRB2: AY011309; APP: AY011372; ATP7A: AY011436; CREM: AY011682; EDG1: AY011741; PLCB4: AY011805), *Felis catus* (cat; ADORA 3: AY011246; ADRB2: AY011306; APP: AY011369; ATP7A: AY011433; CREM: AY011679; EDG1: AY011738; PLCB4: AY011802; RAG1: AY011915; RAG2: AY011977); Order Perissodactyla: *Equus caballus* (horse; ADORA 3: AY011243; ADRB2: AY011303; APP: AY011366; ATP7A: AY011430; CREM: AY011676; EDG1: AY011735; PLCB4: AY011799; RAG1: AY011912; RAG2: AY011974), *Tapirus indicus* (Asiatic tapir; ADORA 3: AY011245; ADRB2: AY011305; APP: AY011368; ATP7A: AY011432; CREM: AY011678; EDG1: AY011737; PLCB4: AY011801; RAG1: AY011914; RAG2: AY011976); Order Artiodactyla: *Hippopotamus amphibius* (hippopotamus; ADORA 3: AY011238; ADRB2: AY011298; APP: AY011361; ATP7A: AY011425; CREM: AY011671; EDG1: AY011731; PLCB4: AY011794; RAG1: AY011909; RAG2: AY011969), *Sus scrofa* (wild boar; ADORA 3: AY011241; ADRB2: AY011301; APP: AY011364; ATP7A: AY011428; CREM: AY011674; EDG1: AY011733; PLCB4: AY011787; RAG1: AY059705; RAG2: AY011972), *Lama glama* (llama; ADORA 3: AY011239; ADRB2: AY011299; APP: AY011362; ATP7A: AY011426; CREM: AY011672; PLCB4: AY011795; RAG1: AY011910; RAG2: AY011970); Order Cetacea: *Megaptera novaeangliae* (humpback whale; ADORA 3: AY011236; ADRB2: AY011296; APP: AY011359; ATP7A: AY011423; CREM: AY011669; EDG1: AY011729; PLCB4: AY011792; RAG1: AY011908; RAG2: AY011967); Order Pholidota: *Manis pentadactyla* (Chinese pangolin; ADORA 3: AY011251; ADRB2: AY011311; APP: AY011374; ATP7A: AY011438; CREM: AY011684; EDG1: AY011742; PLCB4: AY011807; RAG1: AY011919; RAG2: AY011981).

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