Phylogenetic Relationships of Artiodactyls and Cetaceans as Deduced from the Comparison of Cytochrome *b* and 12S rRNA Mitochondrial Sequences

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A data set of complete mitochondrial cytochrome *b* and 12S rDNA sequences is presented here for 17 representatives of Artiodactyla and Cetacea, together with potential outgroups (two Perissodactyla, two Carnivora, two Tethytheria, four Rodentia, and two Marsupialia). We include seven sequences not previously published from Hippopotamidae (Ancodonta) and Camelidae (Tylopoda), yielding a total of nearly 2.1 kb for both genes combined. Distance and parsimony analyses of each gene indicate that 11 clades are well supported, including the artiodactyl taxa Pecora, Ruminantia (with low 12S rRNA support), Tylopoda, Suina, and Ancodonta, as well as Cetacea, Perissodactyla, Carnivora, Tethytheria, Muridae, and Caviomorpha. Neither the cytochrome *b* nor the 12S rDNA genes resolve the relationships between these major clades. The combined analysis of the two genes suggests a monophyletic Cetacea + Artiodactyla clade (defined as "Cetartiodactyla"), whereas Perissodactyla, Carnivora, and Tethytheria fall outside this clade. Perissodactyla could represent the sister taxon of Cetartiodactyla, as deduced from resampling studies among outgroup lineages. Cetartiodactyla includes five major lineages: Ruminantia, Tylopoda, Suina, Ancodonta, and Cetacea, among which the phylogenetic relationships are not resolved. Thus, Suiformes do not appear to be monophyletic, justifying their split into the Suina and Ancodonta infraorders. An association between Cetacea and Hippopotamidae is supported by the cytochrome *b* gene but not by the 12S rRNA gene. Calculation of divergence dates suggests that the Cetartiodactyla could have diverged from other Ferungulata about 60 MYA.

Introduction

Artiodactyla and Cetacea are traditionally assigned to Ungulata *sensu* McKenna (1975), together with the orders Sirenia, Proboscidea, Hyracoidea, Tubulidentata, and Perissodactyla. Close affinities between Artiodactyla and Cetacea were recently suggested by paleontological studies (e.g., Thewissen and Hussain 1993). According to molecular phylogeny, the order Cetacea may be intimately nested within Artiodactyla, arising the question of Artiodactyla monophyly (Irwin, Kocher, and Wilson 1991; Graur and Higgins 1994; Gatesy et al. 1996; Smith et al. 1996; but see Philippe and Douzery 1994 and Hasegawa and Adachi 1996 for alternative views). Living representatives of the order Artiodactyla are placed in three suborders: Ruminantia (cows, deers, and others), Tylopoda (camelids), and Suiformes (pigs and allies). Comparisons of cytochrome *b* DNA provide robust support for a monophyletic tylopod suborder (Douzery 1994, p. 197; Stanley, Kadwell, and Wheeler 1994) but weak support for the monophyly of Ruminantia (Irwin and Arnason 1994). Within the living Suiformes, Simpson (1945) recognized two infraorders, Suina, including the families Suidae (pigs) and Tayassuidae (peccaries), and Ancodonta, represented by the unique family Hippopotamidae (hippos). The infraorder Suina appears to be monophyletic (Gatesy et al. 1996; Randi, Lucchini, and Diong 1996). However, studies by Irwin and Arnason (1994), Gatesy et al. (1996) and Randi, Lucchini, and Diong (1996) indicate that hippopotamids might be more closely related to cetaceans than to Suina (see also Hasegawa and Adachi 1996), thus jeopardizing the concept of Suiformes.

The use of a single gene provides too few informative sites to decipher the bushlike radiations as illustrated by the multifurcations obtained between artiodactyl suborders and cetaceans (Irwin and Arnason 1994; Douzery and Catzeflis 1995). Increasing the number of informative sites can be achieved by combining several sequences (e.g., Miyamoto and Goodman 1986; Arnason and Johnsson 1992; Krettek, Gullberg, and Arnason 1995; d'Erchia et al. 1996). Nevertheless, such an approach may decrease the number of species studied because of limited sequence availability for a given taxon. In this paper, on the one hand, we increase the number of informative sites by combining two complete mitochondrial genes (cytochrome *b* and 12S rRNA) and, on the other hand, we present a taxonomic sampling that includes at least two representatives from all the major artiodactyl lineages (Ruminantia, Tylopoda, Suina, Ancodonta) as well as three representatives (Delphinida, Physeteroidea, and Mysticeti) of the five major Cetacean lineages recovered by Arnason and Gullberg (1996). The impact of species representation on the reliability of the Artiodactyla/Cetacea phylogeny was also addressed by resampling taxa among several putative outgroups.

Materials and Methods

Species Sampling

We sequenced the mitochondrial cytochrome *b* and 12S rRNA from *Hippopotamus amphibius* (hippo), *Hexaprotodon liberiensis* (pygmy hippo), and *Lama guanicoe* (guanaco) and the 12S rRNA from *Camelus bactrianus* (bactrian camel). Taxa included in this study are

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listed in table 1 following the classification of Simpson (1945) and Wilson and Reeder (1993). As shown by Lecointre et al. (1993), species sampling can have a major impact on the inferred phylogeny, particularly with respect to the number of species per taxonomic group. To reduce species sampling biases (Philippe and Douzery 1994; Adachi and Hasegawa 1996), we sampled at least two representatives within Cetacea, each of the Artiodactyla infraorders (or presumed monophyletic groups), and putative outgroups. This constraint led us to (1) omit some taxonomic groups for which at least two distant representatives were not sequenced for both cytochrome *b* and 12S rRNA genes (Primates and Insectivora, for example), and (2) build "chimaerae" between cytochrome *b* and 12S rRNA of two species belonging to the same taxon (see table 1). Thus, the data set included 29 combined sequences (14 Artiodactyla, 3 Cetacea, 2 Perissodactyla, 2 Carnivora, 2 Tethytheria, 4 Rodentia, and 2 Marsupialia).

DNA Sequencing

Tissue samples were obtained from the Collection of Mammalian Tissues from Montpellier (Catzeflis 1991). Samples from *Hippopotamus amphibius, Lama guanicoe,* and *Camelus bactrianus* were provided, respectively, by Diana R. Reynolds (Audubon Zoo, New Orleans, La.), the late Marcel Gallet (Zoo de Lunaret, Montpellier, France), and Françoise Claro (Zoo de Vincennes, Paris, France). DNA extractions from 95% ethanol-preserved tissues were performed according to standard techniques (Sambrook, Fritsch and Maniatis 1989). Hairs from *Hexaprotodon liberiensis* were kindly provided by Françoise Claro, and DNA extraction was performed using Chelex 100 resin (Taberlet et al. 1993).

The complete 12S rRNA gene was PCR-amplified using conserved primers R1 and S2 (Douzery and Catzeflis 1995). The whole cytochrome *b* gene was amplified with the conserved primers L1 (L 5′CGAGATCTGAAAAACCATCGTTG) and H1 (H 5′GGAATTCATCTCTCCGGTTTACAAGAC) located, respectively, in the flanking $tRNA^{Glu}$ and $tRNA^{Thr}$ genes. Additional internal primers, H10 (H 5′CTGGGGTGTAGTTCTCTGGGTC, position 778 in the human cytochrome *b* sequence) and L8 (L 5′CTGCCATGAGGACAAATATCATT, position 400) were required for *Hexaprotodon* to generate two 800-bp overlapping fragments (L1-H10 and L8-H1).

All PCR-amplified molecules were cloned in the pGEM-T vector system 1 (Promega), and the bacterial strain *Escherichia coli* JM 109 was used for transformation. Purified plasmids from positive clones were sequenced following the T7 DNA polymerase protocol (Pharmacia) with PCR and internal primers. At least three individual clones were sequenced for both strands, and the consensus sequence is reported in cases of disagreement between clones. Samples of the recombinant plasmids with the inserted cytochrome *b* and 12S rRNA molecules are available on request from E.D. or C.M. The new sequences have been deposited at EMBL database with accession numbers Y08808–Y08814.

Sequence Alignments

Sequences were aligned by hand with ED editor (MUST package: Philippe 1993). For the cytochrome *b* sequences, gaps were introduced in species lacking the 3′ extension found in rodents and marsupials (Irwin, Kocher, and Wilson 1991; Ma et al. 1993), as well as between codons 325 and 326, due to the 3-bp insertion in the *Loxodonta* sequence (Irwin, Kocher and Wilson 1991). The four new 12S rRNA sequences were aligned according to a previous alignment based on a collection of mammalian sequences (Douzery and Catzeflis 1995; Springer and Douzery 1996) taking into account the secondary structure (i.e., gaps were mainly placed in loops and excluded from stems).

Phylogenetic Reconstructions

The phylogenetic signal in our sequence matrix was tested using the g_1 statistic test based on the skewness of tree-length distributions (Hillis and Huelsenbeck 1992). g_1 was estimated by generating 10^5 trees from the complete data set of 29 taxa with the random-trees option in PAUP version 3.1.1 (Swofford 1993).

All analyses of cytochrome *b* sequences were based on conservative nucleotide substitutions as defined in Arnason and Gullberg (1996). Owing to lower substitution rates in stems than in loops of the 12S rRNA (Miyamoto et al. 1994; Douzery and Catzeflis 1995; Springer and Douzery 1996), sequence analyses were based on transversions only in the single-stranded regions and on both transitions and transversions in stems. Indels were omitted because some hypervariable regions are difficult to align across divergent taxa. The same weight was given to stem and loop regions in the 12S rRNA, as well as to transitions and transversions in both genes.

Phylogenetic trees were reconstructed using distance and parsimony methods. The neighbor-joining method (NJ; Saitou and Nei 1987) was applied on *p*-distances (i.e., the proportion of nucleotide differences between two sequences; Kumar, Tamura, and Nei 1993). Maximum-parsimony (MP) analyses were conducted with PAUP on informative positions only. The robustness of the trees was assessed by the bootstrap method (Felsenstein 1985), using NJ (1,000 replicates in MUST with the NJBOOT program) and MP reconstructions (1,000 replicates of heuristic search, with simple stepwise addition of taxa, TBR branch-swapping and MULPARS options in PAUP). The decay index (DI), defined as the number of extra steps required to collapse the branches (Bremer 1988), was calculated with topological constraints enforced in PAUP. Phylogenetic trees based on amino acid replacements of the cytochrome *b* were constructed using parsimony bootstrap algorithms with PAUP (250 replications of the heuristic search using the PROTPARS step matrix with the options previously described) and the NJ method with NJBOOT (1,000 replicates applied to the Boolean Distance [i.e., any amino acid difference is counted as 1; Philippe 1993]). In all analyses Marsupialia (*Macropus* sp. and *Didelphis virginiana*) were used to root the trees.

Table 1
Systematic Arrangement (following Wilson and Reeder 1993 and Simpson 1945) of the Taxa Considered in this Study and Authors of the Sequences

	12S rRNA	Cytochrome *b*
Class Mammalia		
Infraclass Metatheria (=Marsupialia)		
Family Macropodidae		
*Macropus giganteus**	Douzery and Catzeflis (1995)	
*Macropus rufus**		Arnason (personal communication)
Family Didelphidae		
Didelphis virginiana	Janke et al. (1994)	Janke et al. (1994)
Infraclass Eutheria		
Order Artiodactyla		
Suborder Suiformes		
Infraorder Ancodonta		
Family Hippopotamidae		
Hippopotamus amphibius	This study	This study; Irwin and Arnason (1994)
Hexaprotodon liberiensis	This study	This study
Infraorder Suina		
Family Suidae		
Sus scrofa	Tanhauser (1985)	Irwin, Kocher, and Wilson (1991)
Family Tayassuidae		
Tayassu tajacu	Douzery and Catzeflis (1995)	Irwin, Kocher, and Wilson (1991)
Suborder Tylopoda		
Family Camelidae		
Camelus bactrianus	This study	Stanley, Kadwell, and Wheeler (1994)
Lama guanicoe	This study	This study; Stanley, Kadwell, and Wheeler (1994)
Suborder Ruminantia		
Infraorder Tragulina		
Family Tragulidae		
Tragulus napu	Kraus and Miyamoto (1991)	Irwin, Kocher, and Wilson (1991)
Infraorder Pecora		
Family Antilocapridae		
Antilocapra americana	Kraus and Miyamoto (1991)	Irwin, Kocher, and Wilson (1991)
Family Giraffidae		
Giraffa camelopardalis	Tanhauser (1985)	Irwin, Kocher, and Wilson (1991)
Family Bovidae		
Bubalus bubalis	Miyamoto, Tanhauser, and Laipis (1989)	Chikuni et al. (1995)
Bos taurus	Anderson et al. (1982)	Anderson et al. (1982); Irwin, Kocher, and Wilson (1991)
Capra hircus	Kraus and Miyamoto (1991)	Irwin, Kocher, and Wilson (1991)
Family Cervidae		
*Cervus nippon**		Chikuni et al. (1995)
*Cervus unicolor**	Miyamoto, Kraus, and Ryder (1990)	
*Odocoileus virginianus**	Miyamoto, Kraus, and Ryder (1990)	
*Odocoileus hemionus**		Irwin, Kocher, and Wilson (1991)
Order Cetacea		
Family Delphinidae		
*Stenella attenuata**		Irwin, Kocher, and Wilson (1991)
*Stenella coeruleoalba**	Douzery (1993)	
Family Balaenopteridae		
Balaenoptera physalus	Arnason, Gullberg, and Widegren (1991)	Arnason, Gullberg, and Widegren (1991)
Family Physeteridae		
Physeter macrocephalus	Arnason, Gretarsdottir, and Gullberg (1993)	Arnason and Gullberg (1994)
Order Perissodactyla		
Suborder Hippomorpha		
Family Equidae		
Equus grevyi	Douzery and Catzeflis (1995)	Irwin, Kocher, and Wilson (1991)
Suborder Ceratomorpha		
Family Rhinocerotidae		
*Ceratotherium simum**	Douzery and Catzeflis (1995)	
*Diceros bicornis**		Irwin, Kocher, and Wilson (1991)
Order Proboscidea		
Loxodonta africana	Lavergne et al. (1996)	Irwin, Kocher, and Wilson (1991)
Order Sirenia		
Dugong dugon	Lavergne et al. (1996)	Irwin and Arnason (1994)
Order Carnivora		
Suborder Caniformia		
Family Phocidae		
Phoca vitulina	Arnason and Johnsson (1992)	Arnason and Johnsson (1992)

Table 1
Continued

	12S rRNA	Cytochrome b
Suborder Feliformia		
Family Felidae		
Felis domesticus	Ledje and Arnason (1996)	Ledje and Arnason (1996)
Order Rodentia		
Family Muridae		
Mus musculus	Bibb et al. (1981)	Bibb et al. (1981)
Rattus norvegicus	Galadeta et al. (1989)	Galadeta et al. (1989)
Suborder Hystricognathi		
Family Caviidae		
Cavia porcellus	Frye and Heges (1995)	Ma et al. (1993)
Family Dasyproctidae		
*Myoprocta pratti**		Patton (personal communication)
Family Hydrochaeridae		
*Hydrochaeris hydrochaeris**	Springer (personal communication)	

NOTE.—The following chimaerae were built to use the two genes in combination and the species involved are noted by an asterisk (*) in the table: *Macropus* sp. = *M. giganteus* + *M. rufus*; *Cervus* sp. = *C. unicolor* + *C. nippon*; *Odocoileus* sp. = *O. virginianus* + *O. hemionus*; *Stenella* sp. = *S. coeruleoalba* + *S. attenuata*; Rhinocerotidae = *Ceratotherium simum* + *Diceros bicornis*; Caviomorpha = *Hydrochaeris hydrochaeris* + *Myoprocta pratti*.

The congruence of the cytochrome b and 12S rRNA data sets was tested by the statistical procedure of Farris et al. (1995). The program XARN, kindly provided by J. S. Farris, determines if there is more incongruence between data sets than would be expected by chance alone and gives a significance level for which 5% or lower indicates significant incongruence (Farris et al. 1995).

Resampling Studies

For both genes combined, species resampling was performed on a data set of 29 taxa. Five eutherian lineages falling outside Artiodactyla and Cetacea were resampled: Perissodactyla, Carnivora, Tethytheria, Caviomorpha, and Muridae. In a first test, only one of these five lineages was considered (five combinations of 21 taxa); in a second test, all lineages except one were considered (five combinations of 27 taxa).

Results

Sequence Analysis

Our cytochrome b sequences from *Hippopotamus amphibius* and *Lama guanicoe* differed by 0.8% and 1.1%, respectively, from those published by Irwin and Arnason (1994) and Stanley, Kadwell, and Wheeler (1994). For *Hippopotamus amphibius* the nine differences (six transitions [TI] and three transversions [TV]) occur in third codon positions and only one amino acid change is observed (Phe → Leu in position 149). For *Lama guanicoe,* all differences (10 TI and 3 TV) are synonymous except one in the first codon position (Tyr → His in position 376), and the individual belongs to the genotype B1 defined by Stanley, Kadwell, and Wheeler (1994). These intraspecific cytochrome b divergences fall within the range of variation observed for *Sus scrofa* (Randi, Lucchini, and Diong 1996) and *Ursus arctos* (Talbot and Shields 1996)

The *Hexaprotodon* cytochrome b is 1,140 bp long, terminated by the conventional AGA codon and encoding a 379-amino-acid-long peptide. The overall nucleotide sequence divergence between *Hexaprotodon* and *Hippopotamus* is 7.5% (6.5% TI and 1% TV) for cytochrome b, in contrast to 2% (1.6% TI and 0.4% TV) for 12S rRNA. The *Lama* and *Camelus* sequences differ by 15.4% (12% TI and 3.4% TV) for cytochrome b and by 9.7% (7% TI, 2.3% TV and 0.4% indels) for 12S rRNA .

Phylogenetic Analysis of Cytochrome b and 12S rRNA Sequences

Both genes produced significantly skewed tree-length distributions, with the 12S rRNA having a greater information content ($g_1 = -0.81$, $P < 0.01$) than the cytochrome b ($g_1 = -0.54$, $P < 0.01$). The combination of the two genes also produced a significantly skewed distribution ($g_1 = -0.65$, $P < 0.01$). Thirty-five percent and 36% of the conservative sites were informative for the cytochrome b and the 12S rRNA, respectively. Bootstrap percentages (BPs) and DIs based on parsimony analysis using PAUP are reported in figure 1.

Distance Versus Parsimony Analyses

Regardless of the phylogenetic reconstruction method or of the molecule, eight groups were always found to be monophyletic and supported by 98%–100% BP (fig. 1). These include Pecora, Hippopotamidae, Tylopoda, Cetacea, Tethytheria, Caviomorpha, Muridae, and Eutheria. For these clades, DI varies from +12 to +38 for the cytochrome b and from +9 to +33 for the 12S rRNA, representing on average, 5% and 7% of the number of informative positions, respectively.

For both genes, Suina (*Sus* + *Tayassu*), *Balaenoptera* + *Physeter,* and Perissodactyla are supported by intermediate BP in distance analysis (BP range 71%–99%) but with lower BP (50%–95%) in PAUP analysis. The clustering between Cetacea and Hippopotamidae occurs with both distance (BP = 83%) and PAUP-parsimony (BP = 74%, DI = +8) analyses, but only with the cytochrome b.

Ungulata *sensu* McKenna (1975), here represented by Artiodactyla, Perissodactyla, Cetacea, and Tethytheria, always appear paraphyletic because Carnivora tightly cluster with these orders. The association of Ar-

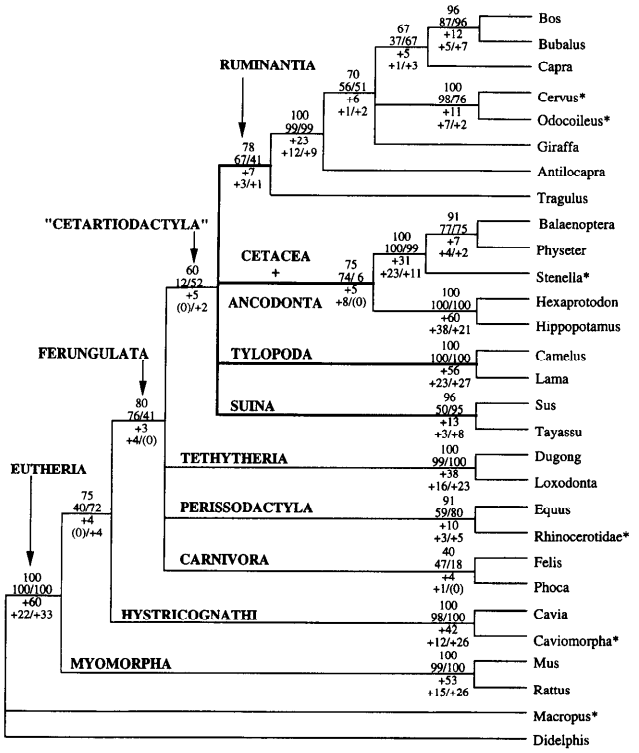

FIG. 1.—Majority-rule consensus tree based on maximum-parsimony analysis (PAUP 3.1.1) of the cytochrome *b* (conservative nucleotide substitutions), the 12S rRNA (transitions and transversions in stems, transversions in loops), and the two genes combined. The bootstrap percentages (BP; after 1,000 replications) and the decay index (DI; number of extra steps to remove a grouping) are indicated above and below branches, respectively. Numbers located on each side of the diagonal (/) line correspond to BP or DI of the cytochrome *b* (on the left) and of the 12S rRNA (on the right), whereas numbers written above indicate BP or DI of the combined data set. Parsimonious trees are 1,845, 1,164, and 3,022 steps long, respectively, for the cytochrome *b*, the 12S rRNA, and the combined genes. Stars indicate taxa for which chimaerae have been built for the analysis of combined genes (see table 1).

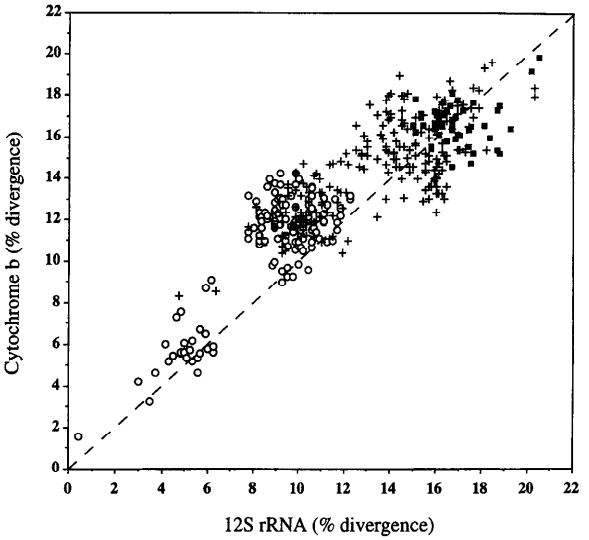

FIG. 2.—Observed sequence divergence of 12S rRNA (transitions and transversions in stems, transversions in loops) versus cytochrome *b* (conservative substitutions) for 406 pairwise comparisons encompassing 29 taxa. Empty circles represent comparisons between Cetacea and Artiodactyla, black stars represent comparisons between Cetacea + Artiodactyla and other eutherian taxa, and black squares represent comparisons between the two Marsupialia and eutherian taxa. The dotted line represents the bisecting line.

tiodactyla, Cetacea, Perissodactyla, and Carnivora is supported in distance analyses of both genes (79% BP with cytochrome *b* and 61% BP with 12S rRNA). However, this cluster is not supported in PAUP analyses (13% with cytochrome *b* and 33% with 12S rRNA).

Amino acid sequence analyses were conducted on a set of 24 species (Pecora was represented by *Bos-Cervus*) representing 119 informative positions. Overall, distance and parsimony provided limited resolution (data not shown). Only eight groups (Hippopotamidae, Tylopoda, Cetacea, Carnivora, Tethytheria, Caviomorpha, Muridae, and Eutheria) received strong BP support in both approaches, whereas the monophyly of Pecora, Ruminantia, Suina, Perissodactyla, and Rodentia is not supported at the 50% level. Hippopotamidae and Cetacea cluster together in parsimony (67% BP) and in distance (55% BP) analyses.

Cytochrome b *Versus 12S rRNA*

In figure 2, transversions in loops and transitions + transversions in stems of the 12S rRNA are plotted versus conservative substitutions of the cytochrome *b* for all 406 pairwise comparisons between 29 taxa. The correlation between the two matrices was significant ($r = 0.86$; Mantel-$t = 7.29$, $P < 10^{-4}$; Sokal and Rohlf 1995, pp. 813–819), indicating that both genes diverged similarly. Comparisons between representatives of Cetacea and Artiodactyla are still linear (fig. 2, empty circles). For the most divergent comparisons, i.e., those involving *Macropus* and *Didelphis* (fig. 2, black squares), the graphic comparison suggests that conservative substitutions of the cytochrome *b* reach mutational saturation relative to the 12S rRNA.

Despite their similar global behavior, the two genes differ in their phylogenetic content. For example, there is no 12S rRNA support for the monophyly of Ruminantia (*Tragulus* does not cluster with pecorans), of Carnivora, or of Hippopotamidae + Cetacea. Many interfamilial or intraordinal relationships are equally well supported by both genes independently, but the relationships between the major ordinal clades are poorly resolved.

Combined Analysis of the Two Genes

Testing Data Heterogeneity

Because combining heterogeneous data may lead to erroneous estimates of phylogeny, the question arises of whether or not to combine data sets (Bull et al. 1993; Miyamoto and Fitch 1995; Huelsenbeck, Bull, and Cunningham 1996). Total congruence between the cytochrome *b* and 12S rRNA of 29 taxa is here indicated by the XARN program ($\alpha = 100\%$). When the clades supported by 100% BP are represented by only one species (i.e., *Bos, Tragulus, Balaenoptera, Stenella, Hippopotamus, Sus, Camelus, Dugong, Phoca, Equus, Cavia, Mus, Macropus*), the data still remain congruent ($\alpha =$

75%) despite some local discrepancies observed in the branching pattern given by each gene. Consequently, a combined analysis of both genes is justified.

Phylogenetic Relationships

The combination of the cytochrome *b* and 12S rRNA for 29 taxa represents 2,003 nucleotide sites (analyzed with NJBOOT; data not shown). Among them, 706 sites are phylogenetically informative, and their MP analysis is presented in figure 1. This combined data set improves robustness for clades supported by intermediate BP in the separate analyses of the two genes. Suina, *Balaenoptera* + *Physeter,* and Perissodactyla are supported by BP of >90% in both approaches. Ruminantia is supported by 78% BP in parsimony and 95% BP in distance, despite the low support given by the 12S rRNA. For these groups, the DI varies from +7 (Ruminantia) to +13 (Suina). On the other hand, Carnivora are the only group for which the combined data set does not improve BP.

In contrast to separate analysis (data not shown), the combined data set suggests a close relationship between Artiodactyla and Cetacea (defined as "Cetartiodactyla," see discussion), but with poor support (60% BP, DI = +5 with MP; 68% BP with distance). Within this clade, the sister taxon relationship of Hippopotamidae and Cetacea is still recovered, but the relationships between Ruminantia, Tylopoda, Suina, and the Hippopotamidae + Cetacea clade are not resolved (fig. 1). Tethytheria, Perissodactyla, and Carnivora, along with the Cetartiodactyla clade, form a monophyletic group referred to as Ferungulata (80% BP, DI = +3 with MP; 92% BP with distance). Finally, the monophyly of Rodentia is either weakly supported (64% BP with distance) or not supported with PAUP, since Muridae (excluding Caviomorpha) forms the sister taxon to all other eutherian mammals (75% BP, DI = +4: fig. 1).

Resampling Study

To test the robustness of the association between Artiodactyla and Cetacea, we resampled among the five major lineages that appeared to be external to the clade (i.e., Perissodactyla, Carnivora, Tethytheria, Caviomorpha and Muridae). The effects of resampling either one or four of the five lineages were tested both with parsimony analysis with PAUP and with distance analysis with NJBOOT (table 2). Support for Cetartiodactyla is not affected when four of five lineages are analyzed (54%–66% BP range in MP analysis for five combinations of 27 taxa). When only one lineage is considered (five combinations of 21 taxa), the Cetartiodactyla clade is not supported with Perissodactyla (BP = 42% with MP and distance), but support increases when the selected taxonomic group is far from Cetartiodactyla (up to 100% with Muridae only). On the other hand, the node Ancodonta + Cetacea is less sensitive to species representation and is supported with moderate BP (71%–88%) in all resamplings, with the exception of Perissodactyla alone in MP analysis (BP = 52%). This analysis confirms Lecointre et al.'s (1993) observation that the impact of species sampling within a given clade on BP values is localized on its neighboring nodes.

Table 2
Bootstrap Support (%) According to Different Taxonomic Samplings for the Clades Artiodactyla + Cetacea and Ancodonta + Cetacea Based on Conservative Substitutions of Cytochrome *b* and 12S rRNA Combined

Taxa Considered	Analysis	Artiodactyla + Cetacea	Ancodonta + Cetacea
All 29 taxa	NJ	68	82
	MP	60	75
27 taxa: except Perissodactyla	NJ	93	77
	MP	64	84
27 taxa: except Carnivora	NJ	68	82
	MP	66	74
27 taxa: except Tethytheria	NJ	45	83
	MP	55	74
27 taxa: except Caviomorpha	NJ	67	83
	MP	54	72
27 taxa: except Muridae	NJ	68	80
	MP	57	71
21 taxa: with Perissodactyla only	NJ	42	77
	MP	42	52
21 taxa: with Carnivora only	NJ	77	73
	MP	59	71
21 taxa: with Tethytheria only	NJ	100	71
	MP	85	77
21 taxa: with Caviomorpha only	NJ	100	75
	MP	98	74
21 taxa: with Muridae only	NJ	100	80
	MP	100	88

NOTE.—NJ: neighbor joining (1,000 replications with NJBOOT in MUST). MP: maximum parsimony (1,000 replications with PAUP).

Divergence Dates

Divergence dates between major clades of interest were tentatively estimated using the molecular clock model calibrated by the date of 70 Myr (late Cretaceous) for the split between Carnivora and Perissodactyla + Artiodactyla (Garland et al. 1993). Divergence dates were deduced from branch lengths on the NJ tree reconstructed with the distance of Tamura and Nei (1993) from all conservative substitutions (2003 positions) of the combined two genes. The molecular clock model is derived from Bailey et al. (1991), which takes into consideration local variations of the rates of evolution (i.e., the age of a dichotomy serves as the reference time to estimate the divergence of the subsequent split). The distance between two taxa is the sum of the stem length plus the average of the terminal branches. Divergence dates obtained from this local clock model are compared with paleontological estimates for a number of clades (table 3). General agreement is observed except for the divergence of Pecora (30.7 vs. 20 Myr) and Ruminantia (49.8 vs. 38 Myr).

Discussion

In the present study, relationships among Artiodactyla and Cetacea were addressed with special attention to species representation in that all the major clades of

Table 3
Estimation of the Divergence Dates Between Lineages Using a Local Molecular Clock Model (see text) for Cytochrome *b* and 12S rRNA in Combination and Paleontological Estimations

Clade	Cytochrome *b* and 12S rRNA[a]	Paleontological Divergence
Hippopotamus/Hexaprotodon	5.7 MYA	4–6 MYA[b]
Cetacea	31.5 MYA	35 MYA[c]
Cetacea/Ancodonta	53 MYA	Unknown
Ruminantia	49.8 MYA	38 MYA[d]
Pecora	30.7 MYA	20 MYA[d]
Sus/Tayassu	42.8 MYA	45 MYA[e]
Lama/Camelus	20.5 MYA	17.5 MYA[d]
Cetartiodactyla	60.5 MYA	Unknown
Equus/Diceros	49.1 MYA	56 MYA[d]
Felis/Phoca	61.9 MYA	58 MYA[d]

[a] Calculation based on a reference date of 70 MYA for the split between Carnivora and Perissodactyla + Artiodactyla (Garland et al. 1993).
[b] Pickford (1993).
[c] Barnes, Domning, and Ray (1985).
[d] Garland et al. (1993).
[e] Sudre (personal communication).

interest are now represented: Ruminantia, Suina, Ancodonta, Tylopoda, and Cetacea. Moreover, the choice of a minimum of two distant representatives per order/suborder allows one to reduce the problem of long branch attraction (Felsenstein 1978). Strong support was found for a dozen major clades which are unambiguously recovered in all analyses including distance or parsimony approaches of separate and combined data sets. We then focused on relationships between major clades that are more widely debated.

Artiodactyla + Cetacea Relationships

The combination of the cytochrome *b* and 12S rRNA genes suggests the monophyletic association of five major Cetacea and Artiodactyla extant lineages: Ruminantia, Cetacea, Ancodonta, Tylopoda, and Suina (fig. 1). The existence of this clade, here called "Cetartiodactyla," is in agreement with previous studies involving various species and gene sampling (Irwin, Kocher, and Wilson 1991; Graur and Higgins 1994; Gatesy et al. 1996). Despite the comparison of more than 2,000 aligned mitochondrial sites, support for Cetartiodactyla is weak (60% BP, DI = +5) but remains stable after resampling among the five external eutherian lineages (table 2). The Cetartiodactyla interrelationships are not fully resolved, but Artiodactyla appear to be paraphyletic because of the association between Cetacea and Ancodonta. We note that the monophyly of Artiodactyla would require 2, 12, and 9 extra steps respectively, for the 12S rRNA, the cytochrome *b,* and the two combined genes.

All these data suggest that several consecutive splits, Cetartiodactyla versus Perissodactyla, Carnivora, and Tethytheria and the subsequent divergences of Ruminantia, Cetacea/Ancodonta, Tylopoda, and Suina, occurred in a very short period of time, more than 60 MYA (table 3).

A mitochondrial and nuclear combined analysis of the cytochrome *b,* 12S rRNA, β- and κ-casein genes for 16 taxa (10 Artiodactyla, 2 Cetacea, 1 Perissodactyla, 1 Carnivora, and 2 muroid rodents as outgroup: Gatesy et al. 1996; this study) reinforces the support for the Ancodonta + Cetacea and Cetartiodactyla clades (distance BPs are 95% and 81%, respectively). Moreover, Perissodactyla appears to be the sister group of Cetartiodactyla (distance BP = 90%), in contrast to a Perissodactyla/Carnivora association suggested by comparison of complete mitochondrial genomes (Xu, Janke, and Arnason 1996).

Paraphyly of the Suiformes

The paleontological record indicates a divergence time of 4–6 Myr between the two hippopotamid genera (Pickford 1993), in agreement with our estimation of 5.7 Myr (table 3). The low divergence (2%) for the 12S rRNA is close to the difference observed for the same molecule between the two genera of extant elephants who diverged 5 MYA (Lavergne et al. 1996).

Hippopotamidae traditionally are included in the suborder Suiformes (Simpson 1945) with Suidae (pigs) and Tayassuidae (peccaries). Our molecular data do not recover such a relationship in any of the analyses performed as previously stated by Irwin and Arnason (1994) and Gatesy et al. (1996). Suiformes seems to be paraphyletic, and the number of extra steps required to constrain Suiformes' monophyly are 11, 5, and 12, respectively, for the cytochrome *b,* the 12S rRNA, and the two combined genes. Moreover, no exclusive amino acid synapomorphy was found for the Suiformes clade among 199 mammal amino acid sequences of cytochrome *b.* The maximum-likelihood study of Hasegawa and Adachi (1996) conducted on protein sequences (cytochrome *b,* hemoglobins α and β) indicates that Suiformes (*Sus* and *Hippopotamus*) monophyly is supported by only 20% BP (total of trees 8, 10, and 15 in their Table 3).

Concerning morphological data, Hippopotamidae could have originated either from the extinct artiodactyl family Anthracotheriidae (Gentry and Hooker 1988) or from an Old World tayassuid stock (Pickford 1989). Our results do not support the hypothesis of a sister group relationship between Tayassuidae and Hippopotamidae, in agreement with the morphological analysis of a fossil peccary (Ducrocq 1994). Molecular studies then fully justified the distinction between the infraoders Suina (Suidae and Tayassuidae) and Ancodonta (Hippopotamidae, including also fossil Anthracotheriidae) defined by Simpson (1945). The molecular estimate of the age of the *Sus/Tayassu* split is 43 Myr, in agreement with the paleontological estimate of 45 Myr (table 3). Moreover, the Suina/Ancodonta divergence remotes to the emergence of the five cetartiodactyl clades (60 MYA according to our estimations).

Relationships Between Ancodonta (Hippopotamidae) and Cetacea

Our study supports the association between Hippopotamidae and Cetacea (75% BP, DI = +5 for the two genes combined), in agreement with results based on cytochrome *b* and milk casein sequences (Irwin and

Arnason 1994; Arnason and Gullberg 1996; Gatesy et al. 1996; Randi, Lucchini, and Diong 1996). The signal for the Cetacea/Hippopotamidae grouping is clearly brought by the cytochrome *b* gene (74% BP, DI = +8), whereas the 12S rRNA does not support this association (6% BP, DI = 0). This result is consistent with the maximum likelihood study of Hasegawa and Adachi (1996), which indicates that only cytochrome *b* (80% BP), not hemoglobins α and β (17% and 23% BP, respectively), supports the Cetacea/*Hippopotamus* grouping. This association is also characterized by a nearly exclusive synapomorphic replacement at position 243 in the cytochrome *b* sequence (the two hippopotamids and 35 cetaceans have a threonine), which is shared by only 11 distantly related mammals (six primates, four rodents, and one sirenian) out of 199 comparisons including representatives of 11 eutherian orders. Finally, three genes (the mitochondrial cytochrome *b* and two nuclear milk caseins) indicate an association between Cetacea and Hippopotamidae while three others (the mitochondrial 12S rRNA and two nuclear hemoglobins) do not support such a relationship. More must be known about the sequence–function relation of the cytochrome *b* and the two caseins to choose between common ancestry and a potential functional convergence as an adaptation to aquatic environment (see Gatesy et al. 1996). Our molecular data suggest 53 MYA for the divergence between Cetacea and Ancodonta, which is not in conflict with the paleontological record since the oldest cetacean, *Pakicetus,* is dated from 52 MYA (Thewissen 1994).

Other Relationships Among Artiodactyla and Cetacea

Some molecular studies (Graur and Higgins 1994; Smith et al. 1996) suggest an association between Ruminantia and Cetacea, but neither Suiformes nor Tylopoda were simultaneously examined. In our survey, such an association is not seen in majority-rule consensus trees nor as an alternative hypothesis (data not shown). Thus, the inclusion of representatives of all major taxonomic groups (Ruminantia, Tylopoda, Suina, Ancodonta, Cetacea) appears to be an essential factor for addressing such difficult relationships as those between and among Artiodactyla and Cetacea.

The Tylopoda clade appears to be well supported, but no clear association is suggested between Tylopoda and other Artiodactyla suborders. Among Tylopoda, the divergence between *Lama* and *Camelus* is estimated to be around 20 MYA according to our local molecular clock. This is earlier than some estimations based on paleontological data that range from 10 to 14 MYA (Pickford, Morales, and Soria 1995; J. Morales, personal communication). Garland et al. (1993) proposed a more ancient origin for these two genera (i.e., 17.5 MYA), yet this remains slightly younger than our estimation.

Although the suborder Ruminantia was not supported by the 12S rRNA, the combined data set gives some support for Ruminantia monophyly (78% BP, DI = +7). Within Pecora, both genes support the monophyly of Cervidae and Bovidae but fail to resolve the interrelationships between Cervidae, Bovidae, and Giraffidae. The molecular divergence date estimated for Pecora is 30.7 MYA, which is much earlier than the paleontological date of 20 MYA, although the pecoran origin could be more ancient (Garland et al. 1993).

Eutherian Relationships

Our data support the inclusion of Cetacea in the Ferungulata, defined by Simpson (1945) as the association of Artiodactyla, Perissodactyla, Carnivora, Tethytheria, Hyracoidea, and Tubulidentata. Conversely, the Ungulata *sensu* McKenna (1975), which would contain Artiodactyla, Cetacea, Perissodactyla, Tethytheria, Hyracoidea, and Tubulidentata, appear to be paraphyletic due to the close association between Carnivora and the first four orders (fig. 1). Because hyraxes and aardvarks were not included in this study, further tests of the two hypotheses (Simpson 1945; McKenna 1975) should include sequences of Hyracoidea and Tubulidentata representatives.

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