The Mitochondrial Genome of the Sperm Whale and a New Molecular Reference for Estimating Eutherian Divergence Dates

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Abstract. Extant cetaceans are systematically divided into two suborders: Mysticeti (baleen whales) and Odontoceti (toothed whales). In this study, we have sequenced the complete mitochondrial (mt) genome of an odontocete, the sperm whale (*Physeter macrocephalus*), and included it in phylogenetic analyses together with the previously sequenced complete mtDNAs of two mysticetes (the fin and blue whales) and a number of other mammals, including five artiodactyls (the hippopotamus, cow, sheep, alpaca, and pig). The most strongly supported cetartiodactyl relationship was: outgroup,((pig, alpaca),((cow, sheep),(hippopotamus,(sperm whale,(baleen whales))))). As in previous analyses of complete mtDNAs, the sister-group relationship between the hippopotamus and the whales received strong support, making both Artiodactyla and Suiformes (pigs, peccaries, and hippopotamuses) paraphyletic. In addition, the analyses identified a sister-group relationship between Suina (the pig) and Tylopoda (the alpaca), although this relationship was not strongly supported. The paleontological records of both mysticetes and odontocetes extend into the Oligocene, suggesting that the mysticete and odontocete lineages diverged 32–34 million years before present (MYBP). Use of this divergence date and the complete mtDNAs of the sperm whale and the two baleen whales allowed the establishment of a new molecular reference, O/M-33, for dating other eutherian divergences. There was a general consistency between O/M-33 and the two previously established eutherian references, A/C-60 and E/R-50. Cetacean (whale) origin, i.e., the divergence between the hippopotamus and the cetaceans, was dated to ≈55 MYBP, while basal artiodactyl divergences were dated to ≥65 MYBP. Molecular estimates of Tertiary eutherian divergences were consistent with the fossil record.

Key words: Molecular references — Cetferungulata — Cetartiodactyla — Cetancodonta — Cetacea — Mysticeti — Odontoceti — Sperm whale

Introduction

Molecular dating of evolutionary divergences is dependent on three factors: (i) an established phylogeny, (ii) a reliable paleontological dating of at least one node of the phylogenetic tree, and (iii) testing and correcting for differences in evolutionary rates. In recent years the use of orthologous and comprehensive sets of sequence data, such as complete mitochondrial (mt) genomes, has allowed the examination and resolution of a number of mammalian relationships which could not be resolved by earlier analyses of shorter data sets. While a number of contentious relationships still remain to be examined, a strongly supported phylogenetic framework has been established for many mammalian relationships, thus fulfilling the first requirement for molecular dating. The second requirement is more difficult to achieve, however, due to the incomplete nature of the fossil record (Martin

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1993). Nevertheless, some specific mammalian groups, most notably the cetferungulates (Carnivora, Perissodactyla, Artiodactyla, and Cetacea), do have an extensive fossil record. This makes the cetferungulates an obvious choice for use in estimating mammalian divergence times. The third requirement is achieved through the use of a well-established phylogeny and of an outgroup which is unequivocally located outside the taxa to be analyzed.

Two cetferungulate molecular references have already been established. The first, A/C-60 (Arnason and Gullberg 1996), was established through analyses of complete cytochrome *b* sequences from about 30 cetaceans and a number of artiodactyls. Phylogenetic analyses of these sequences identified five basal lineages of extant cetaceans (one mysticete and four odontocete branches). Even though the analyses did not conclusively resolve the interrelationships among the five lineages, suggesting that their radiation took place within a limited period of time, the best supported tree identified the mysticetes as the sister group of all odontocetes (Arnason and Gullberg 1996). The cetacean paleontological record shows that baleen whales had differentiated already by the early to Middle Oligocene (McKenna and Bell 1997), while well-differentiated odontocete fossils occur in the late Oligocene (Fordyce 1994). In conjunction with molecular data the record suggests that the basal radiation of extant cetaceans took place 32–34 million years before present, MYBP (R.E. Fordyce, personal communication). The molecular distances between cetacean lineages together with those between cetaceans and artiodactyls were used to establish a molecular/paleontological reference point (A/C-60) (Arnason and Gullberg 1996) based on the molecular distances between ruminant **A**rtiodactyla and **C**etacea and their estimated divergence date at 60 MYBP. The second cetferungulate reference, E/R-50 (Xu et al. 1996a; Arnason et al. 1998), was established using the complete mtDNAs of the **E**quidae (horse, donkey) and the **R**hinocerotidae (Indian and white rhinoceroses) and a paleontological dating of 50 MYBP for the intraordinal perissodactyl divergence between the Equidae and the Rhinocerotidae lineages (Hooker 1989; Prothero and Schoch 1989). Consistent with the fossil record, both A/C-60 and E/R-50 give a dating estimate for the origin of eutherians of ≈130 MYBP.

A number of complete cetferungulate mt genomes are now available, including those of two mysticetes (the blue and fin whales). With the addition of an odontocete complete mtDNA, a new molecular reference, O/M-33 (the divergence between **O**dontoceti and **M**ysticeti 33 MYBP), can thus be established for testing other molecular references, using comprehensive sequence data. The data set represents several lineages which experienced different evolutionary radiations during the Tertiary. This has made it possible to examine the suggestion (Alroy 1999; Benton 1999) of a relationship between extensive radiations and an increase in the rate of molecular evolution.

Materials and Methods

The material used (liver) was collected from a male sperm whale at the whaling station in Hvalfjördur, Iceland. An enriched mtDNA fraction was isolated using the procedure of Arnason et al. (1991). This mtDNA was digested separately and/or in combination with the restriction enzymes *Bcl*I, *Bln*I, *Spe*I, and *Xba*I and cloned in M13mp18. The resulting clones covered the regions between position 44 and position 5282 and between 7559 and 9023, in addition to some shorter segments. The remaining regions were PCR-amplified using specific primers containing restriction sites for the enzymes *Eco*RI and *Hind*III and cloned in M13 mp18/19. Sequencing was performed manually (Sanger 1981). The sequences of the PCR-amplified regions represent the consensus of three separate PCR clones. The mtDNA sequence of the sperm whale, with annotations, has been deposited at EMBL under accession number AJ277029. Users of this sequence are kindly requested to refer to this paper, and not to the accession number alone.

Phylogenetic analyses were carried out using three methods: maximum parsimony (MP) (Fitch 1971), neighbor joining (NJ) (Saitou and Nei 1987), and maximum likelihood (ML) (Felsenstein 1981). The programs used to implement these methods were PHYLIP (Felsenstein 1991), MOLPHY (Adachi and Hasegawa 1996a), and PUZZLE version 4.02 (Strimmer and von Haeseler 1996). Analyses were carried out on both the amino acid (aa) and the nucleotide (nt) data sets from the concatenated sequences of the 12 H strand-encoded protein-coding genes. The TN model of nt evolution (Tamura and Nei 1993) was used in the ML analysis and to estimate distances. The mtREV-24 rate matrix of aa sequence evolution (Adachi and Hasegawa 1996b) with and without the assumption of a Γ model of rate heterogeneity (Yang 1994) was used for ML analysis of the aa data set and for distance calculations. The length of the alignments used was 9870 nt or 3290 aa. The nt data set consisted of all changes at first codon positions (except synonymous leucine transitions) and all changes at second codon positions. Analyses included the following taxa: mouse, *Mus musculus* (Bibb et al. 1981); rat, *Rattus norvegicus* (Gadaleta et al. 1989); human, *Homo sapiens* (Arnason et al. 1996a); white-handed gibbon, *Hylobates lar* (Arnason et al. 1996b); hamadryas baboon, *Papio hamadryas* (Arnason et al. 1998); armadillo, *Dasypus novemcinctus* (Arnason et al. 1997); neotropical bat, *Artibeus jamaicensis* (Pumo et al. 1998); mole, *Talpa europaea* (Mouchaty et al. 2000); cow, *Bos taurus* (Anderson et al. 1982); sheep, *Ovis aries* (Hiendleder et al. 1998); pig, *Sus scrofa* (Ursing and Arnason 1998a); alpaca, *Lama pacos* (Ursing et al. 2000); hippopotamus, *Hippopotamus amphibius* (Ursing and Arnason 1998b); fin whale, *Balaenoptera physalus* (Arnason et al. 1991); blue whale, *B. musculus* (Arnason and Gullberg 1993); sperm whale, *Physeter macrocephalus* (present study); harbor seal, *Phoca vitulina* (Amason and Johnsson 1992); gray seal, *Halichoerus grypus* (Arnason et al. 1993a); domestic cat, *Felis catus* (Lopez et al. 1996); horse, *Equus caballus* (Xu and Arnason 1994); donkey, *Equus asinus* (Xu et al. 1996b); Indian rhinoceros, *Rhinoceros unicornis* (Xu et al. 1996a); and white rhinoceros, *Ceratotherium simum* (Xu and Arnason 1997).

Results

The length of the complete mtDNA of the sperm whale is 16,428 nt. The gene order of this sequence conforms to that of other described eutherian complete mtDNAs. All of the protein-coding genes have a methionine start codon (ATG or ATA). Five genes (COII, ATPase8, NADH4L, NADH5, and NADH6) have the stop codon

Table 1. Pairwise distances[a]

	Rno	Mmu	Pha	Hla	Hsa	Dno	Aja	Teu	Fca	Hgr	Pvi	Csi
Rno	Rno	0.0953	0.4229	0.3677	0.3663	0.2836	0.2826	0.2588	0.2685	0.2810	0.2828	0.2588
Mmu	0.0861	Mmu	0.4236	0.3683	0.3683	0.2852	0.2833	0.2561	0.2760	0.2848	0.2865	0.2687
Pha	0.3358	0.3384	Pha	0.2237	0.2250	0.3938	0.3850	0.3678	0.3662	0.3683	0.3670	0.3324
Hla	0.2960	0.2970	0.1935	Hla	0.1153	0.3395	0.3448	0.3214	0.3207	0.3198	0.3192	0.2904
Hsa	0.2948	0.2966	0.1945	0.1058	Hsa	0.3257	0.3292	0.3119	0.3111	0.3092	0.3104	0.2796
Dno	0.2364	0.2370	0.3168	0.2746	0.2648	Dno	0.2309	0.2137	0.2079	0.2050	0.2070	0.1879
Aja	0.2346	0.2347	0.3090	0.2794	0.2684	0.1977	Aja	0.1672	0.1652	0.1677	0.1708	0.1672
Teu	0.2211	0.2179	0.2972	0.2620	0.2562	0.1857	0.1479	Teu	0.1521	0.1481	0.1496	0.1400
Fca	0.2246	0.2298	0.2956	0.2611	0.2538	0.1807	0.1454	0.1384	Fca	0.0983	0.0979	0.1142
Hgr	0.2365	0.2380	0.2956	0.2588	0.2537	0.1772	0.1472	0.1344	0.0901	Hgr	0.0160	0.1195
Pvi	0.2353	0.2369	0.2973	0.2591	0.2524	0.1756	0.1499	0.1333	0.0905	0.0157	Pvi	0.1197
Csi	0.2176	0.2247	0.2710	0.2398	0.2313	0.1634	0.1469	0.1269	0.1044	0.1080	0.1074	Csi
Run	0.2204	0.2240	0.2723	0.2404	0.2357	0.1676	0.1500	0.1299	0.1108	0.1095	0.1105	0.0430
Eas	0.2170	0.2234	0.2768	0.2454	0.2379	0.1653	0.1427	0.1243	0.1041	0.1100	0.1101	0.0717
Eca	0.2215	0.2257	0.2763	0.2451	0.2387	0.1659	0.1450	0.1230	0.1066	0.1137	0.1138	0.0726
Lpa	0.2372	0.2401	0.2964	0.2672	0.2560	0.1870	0.1701	0.1506	0.1344	0.1429	0.1413	0.1248
Ssc	0.2296	0.2320	0.2958	0.2579	0.2514	0.1785	0.1606	0.1389	0.1272	0.1340	0.1317	0.1175
Bta	0.2258	0.2257	0.2857	0.2556	0.2429	0.1709	0.1474	0.1328	0.1179	0.1208	0.1167	0.1067
Oar	0.2234	0.2215	0.2874	0.2566	0.2430	0.1692	0.1522	0.1285	0.1224	0.1168	0.1154	0.1052
Ham	0.2430	0.2441	0.2883	0.2638	0.2551	0.1913	0.1711	0.1647	0.1505	0.1498	0.1491	0.1251
Pma	0.2590	0.2640	0.3097	0.2868	0.2742	0.2081	0.1948	0.1880	0.1796	0.1739	0.1731	0.1603
Bph	0.2496	0.2520	0.3033	0.2741	0.2666	0.1906	0.1843	0.1781	0.1632	0.1564	0.1548	0.1406
Bmu	0.2468	0.2490	0.3051	0.2757	0.2704	0.1908	0.1802	0.1756	0.1639	0.1571	0.1540	0.1383

TAA. The stop codon of the ATPase6 gene is either TAA or TA. If it is TAA, the second A is also the A of the COIII gene iniation codon. Two genes (COI and Cyt *b*) have the stop codon AGA. The remaining five protein-coding genes have an incomplete stop codon (TA or T), with the terminal 3′ nt being contiguous with the 5′ terminal nt of the following tRNA gene. It has been proposed that transcripts from such protein-coding genes contain a stop codon completed by posttranscriptional polyadenylation (Ojala et al. 1981).

The control region of the sperm whale is strikingly different from that of other whales, including the confamilial pygmy sperm whale, *Kogia breviceps.* Complete cetacean control regions of these two physeterids and all extant baleen whales have been described previously (Arnason et al. 1993b) and thus this region of the sperm whale is not discussed here.

As mentioned in the Introduction, the particular aims of the present study were to establish a new molecular reference point (O/M-33) and to test this reference point against the two previously established molecular references, A/C-60 and E/R-50, which are supported by paleontological records predating those of the odontocete and mysticete lineages. In addition, the inclusion of the sperm whale allows further examination of the cetferungulate tree. The tree was rooted using two muroid rodents (the mouse and the rat), which constitute outgroups to all of the other species in the tree as demonstrated by several studies of complete mtDNAs in which the eutherian tree was rooted using marsupials and/or monotremes (Arnason et al. 1997, 1998; Janke et al. 1996, 1997). Several other noncetferungulate taxa which are of par-

ticular importance with regard to discussing the origin of the cetferungulate clade and the estimated divergence times were also included. The aa distances among these taxa, established by both homogeneous and heterogeneous rate models, are shown in Table 1.

Recent phylogenetic analyses (Pumo et al. 1998) of the complete mtDNAs of a neotropical bat, *Artibeus jamaicensis,* and a number of other mammals showed a sister-group relationship between Chiroptera (bats) and Cetferungulata. An even more recent study (Mouchaty et al. 2000) identified a sister-group relationship between the bat and the mole, *Talpa europaea* (Soricomorpha), with this grouping forming the sister group of Cetferungulata. Inclusion of the bat and the mole is thus important to phylogenetic analyses of the cetferungulates and to any examination of the time of origin of Cetferungulata, with the date of the divergence between the bat/mole grouping and the cetferungulates constituting the upper limit for cetferungulate origin.

The phylogenetic relationships among the cetferungulates and other included taxa are shown in Fig. 1 together with the support values for individual branches. Consistent with the analyses of Xu et al. (1996a), the cetferungulates were split into two groups, one consisting of Carnivora and Perissodactyla, the other of Artiodactyla and Cetacea. Within the cetartiodactyls, a basal split was identified between alpaca/pig and the remaining taxa. The sister-group relationship between Suina (pig) and Tylopoda (alpaca) was reconstructed in most analyses but the support for this relationship was markedly lower in the NJ analyses than in the MP and ML/QP ones. The sister-group relationship between the ruminants (cow/

Table 1. Extended

	Run	Eas	Eca	Lpa	Ssc	Bta	Oar	Ham	Pma	Bph	Bmu
Rno	0.2625	0.2545	0.2605	0.2846	0.2756	0.2692	0.2667	0.2942	0.3127	0.3046	0.2977
Mmu	0.2683	0.2636	0.2670	0.2885	0.2790	0.2715	0.2653	0.2957	0.3211	0.3089	0.3018
Pha	0.3355	0.3392	0.3391	0.3700	0.3661	0.3529	0.3534	0.3554	0.3840	0.3790	0.3795
Hla	0.2940	0.2968	0.2971	0.3303	0.3148	0.3122	0.3132	0.3244	0.3530	0.3414	0.3429
Hsa	0.2865	0.2878	0.2891	0.3133	0.3064	0.2942	0.2941	0.3112	0.3355	0.3306	0.3352
Dno	0.1929	0.1876	0.1877	0.2188	0.2048	0.1996	0.1965	0.2234	0.2430	0.2257	0.2242
Aja	0.1713	0.1620	0.1645	0.1952	0.1834	0.1676	0.1728	0.1967	0.2267	0.2149	0.2092
Teu	0.1436	0.1374	0.1357	0.1700	0.1553	0.1510	0.1441	0.1873	0.2171	0.2097	0.2036
Fca	0.1218	0.1120	0.1145	0.1511	0.1420	0.1328	0.1363	0.1705	0.2062	0.1892	0.1865
Hgr	0.1222	0.1207	0.1245	0.1601	0.1492	0.1312	0.1299	0.1703	0.1992	0.1809	0.1764
Pvi	0.1208	0.1206	0.1243	0.1621	0.1515	0.1359	0.1312	0.1708	0.2004	0.1825	0.1798
Csi	0.0458	0.0764	0.0769	0.1421	0.1303	0.1207	0.1180	0.1419	0.1820	0.1631	0.1572
Run	Run	0.815	0.0808	0.1427	0.1355	0.1245	0.1245	0.1422	0.1818	0.1652	0.1613
Eas	0.0756	Eas	0.0134	0.1461	0.1284	0.1179	0.1102	0.1447	0.1832	0.1609	0.1567
Eca	0.0754	0.0126	Eca	0.1430	0.1272	0.1170	0.1109	0.1425	0.1842	0.1628	0.1579
Lpa	0.1252	0.1301	0.1278	Lpa	0.1478	0.1367	0.1418	0.1607	0.1919	0.1762	0.1736
Ssc	0.1221	0.1164	0.1156	0.1316	Ssc	0.1259	0.1289	0.1565	0.1877	0.1670	0.1652
Bta	0.1095	0.1051	0.1041	0.1197	0.1128	Bta	0.0563	0.1296	0.1615	0.1442	0.1388
Oar	0.1113	0.0999	0.0999	0.1255	0.1154	0.0536	Oar	0.1358	0.1740	0.1512	0.1490
Ham	0.1249	0.1292	0.1278	0.1402	0.1386	0.1133	0.1193	Ham	0.1742	0.1485	0.1432
Pma	0.1597	0.1605	0.1615	0.1676	0.1646	0.1426	0.1535	0.1523	Pma	0.1105	0.1098
Bph	0.1427	0.1395	0.1413	0.1502	0.1439	0.1248	0.1312	0.1306	0.0976	Bph	0.0280
Bmu	0.1420	0.1387	0.1400	0.1501	0.1443	0.1223	0.1315	0.1283	0.0979	0.0247	Bmu

[a] Taxa: Rno, rat; Mmu, mouse; Pha, baboon; Hla, gibbon; Hsa, Homo; Dno, armadillo; Aja, bat; Teu, mole; Fca, cat; Hgr, gray seal; Pvi, harbor seal; Csi, white rhinoceros; Run, Indian rhinoceros; Eas, donkey; Eca, horse; Lpa, alpaca; Ssc, pig; Bta, cow; Oar, sheep; Ham, hippopotamus; Pma, sperm whale; Bph, fin whale; Bmu, blue whale.

The upper matrix shows amino acid distances as calculated by the PUZZE program using the mt-REV 24 matrix and the assumption of rate heterogeneity with four classes of variable and one class of constant sites, while the lower matrix shows the amino acid distances under the assumption of rate homogeneity.

sheep) and the hippo/whale grouping was supported in all analyses and the hippopotamus/cetacean lineage received strong support, as did the relationship between the odontocete sperm whale and the two mysticetes.

The relationship between the pig (Suina), the alpaca (Tylopoda) and the other cetferungulates was examined using an exhaustive ML search (Table 2). The best tree, which was the same as that shown in Fig. 1, joined Suina and Tylopoda on a common branch to the exclusion of Cetruminantia ((cow,sheep)(hippo,whales)). The support for this tree was distinctly stronger than that for the second-best tree (which had the Suina as the sister group to the other cetartiodactyls), although the latter could not be statistically refuted. The topology with Tylopoda as the sister group to the other cetartiodactyls was refuted, receiving >2 SE less support than the best tree. The topology remained the same when a Γ model of rate heterogeneity with one class of invariable and four classes of variable sites was used. The estimated α parameter was 0.66 ± 0.03.

Taking 33 MY as the time of the divergence between mysticetes and sperm whales and using the ML distances given in Table 1, the times of various other eutherian divergences were calculated (Table 3). These were then compared to dating estimates based on A/C-60 and E/R-50. It should be noted that the dating estimates shown in Table 3 have been corrected for differences in evolutionary rate between the different lineages. The fast evolu-

Table 2. Statistical analysis of the relationship between pig and alpaca[a]

	$\Delta \ln L$	σ	p_{boot}	Steps	SE
OG,((pig,alpaca),((cow,sheep), (hippo,whales)))	$\langle 40251.1 \rangle$		0.867	$\langle 5706 \rangle$	
OG,(pig,(alpaca,((cow,sheep), (hippo,whales))))	−20.2	±17.0	0.105	+11	±7.4
OG,(alpaca,(pig,((cow,sheep), hippo,whales))))	−31.9	±15.2	0.008	+13	±7.1
OG,(pig,((alpaca,(cow,sheep), (hippo,whales))))	−56.0	±24.4	0.000	+17	±9.8
OG,(alpaca,((pig,(cow,sheep), (hippo,whales))))	−70.1	±25.1	0.001	+23	±9.4
OG,(((pig,alpaca),(cow,sheep)), (hippo,whales))	−39.0	±19.3	0.020	+11	±5.5

[a] The ML analysis was based on the mtREV-24 (Adachi and Hasegawa 1996b) model of aa sequence evolution. The value in angle brackets shows the log-likelihood (ln$L$) value of the best tree. $\Delta \ln L$ indicates the difference in ln$L$ from that of the best tree, followed by the standard deviation, σ (Kishino and Hasegawa 1989), and bootstrap probability, p_{boot}, for this particular topology (Kishino et al. 1990). The number of substitutions for the best tree (in angle brackets) and that of alternative trees relative to the best tree and the number of steps, as well as the corresponding standard error (SE) (Templeton 1983), were calculated by the PROTPARS program, as implemented in PHYLIP.

tionary rate of the cetaceans does not permit uncalibrated use of the new reference or of A/C-60 and E/R-50. According to the homogeneous rate model the evolutionary rate of the whales, assuming a steady evolutionary
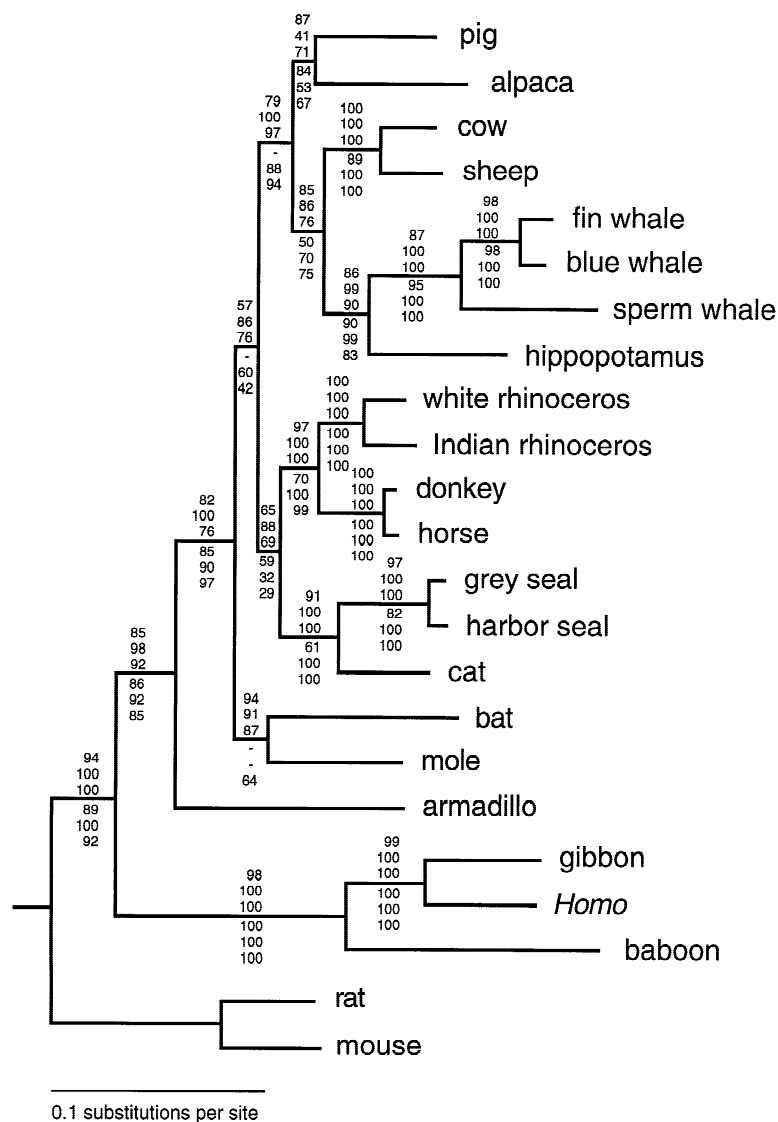
Fig. 1. Phylogenetic relationships among cetferungulates plus a selection of other taxa represented by complete mitochondrial genomes. The tree was reconstructed by ML analysis of the concatenated amino acid (aa) sequences of 12 protein-coding genes. The support values for aa analyses are shown *above* each branch (ML/QP, *top;* NJ, *middle;* MP, *bottom*). The corresponding values for the nucleotide (nt) analyses are given *below* each branch. The nt analyses were based on first (excluding synonymous leucine transitions) plus second codon positions. The ML/QP support values were calculated on the basis of 1000 puzzling steps. The NJ and MP nt values represent 1000 bootstrap replicates, while the corresponding aa values are based on 100 replicates. For scientific names, see Materials and Methods.

rate after the divergence between cetaceans and hippopotamus, is 0.0030 substitution/site per million years. The corresponding rate for A/C-60 (artiodactyl ruminants and cetaceans) is 0.0022, while that for E/R-50 is 0.0015. Indeed, the evolutionary rate of the whales, twice that of the perissodactyls, appears to be the fastest rate yet recognized among mammals.

Discussion

Many molecular analyses have identified a close relationship between artiodactyls and cetaceans (Boyden and Gemeroy 1950; Goldstone and Smith 1966; Goodman et al. 1982; Beintema et al. 1986; Czelusniak et al. 1990; Irwin et al. 1991; Arnason and Johnsson 1992; Janke et al. 1994, 1996, 1997; D'Erchia et al. 1996; Arnason et al. 1991, 1996c, 1997, 1998). Indeed, cetaceans have often even been placed within the artiodactyls, thereby sug-

gesting artiodactyl paraphyly (Beintema et al. 1986; Irwin et al. 1991; Irwin and Arnason 1994; Graur and Higgins 1994; Arnason and Gullberg 1996; Gatesy et al. 1996, 1999; Shimamura et al. 1997; Ursing and Arnason 1998b; Ursing et al. 2000; Kleineidam et al. 1999).

The cytochrome *b* study by Irwin and Arnason (1994) identified a sister-group relationship between cetaceans and the hippopotamus (*Hippopotamus amphibius*), to the exclusion of the other two suiforms included (the pig and the peccary). Thus, in addition to identifying the closest artiodactyl relative of the cetaceans, their study also suggested paraphyly of the Suiformes (pigs, peccaries, and hippopotamuses). Irwin and Arnason's (1994) findings have subsequently been supported by an extended cytochrome *b* analysis of about 40 cetacean and artiodactyl species (Arnason and Gullberg 1996) and by Montgelard et al. (1997), who, in addition to *Hippopotamus amphibius,* also included the pygmy hippo, *Hexaprotodon liberiensis,* thereby splitting the hippopotamid branch. The findings have also been supported in the nuclear data

Table 3. Estimated times of eutherian divergences in million years before present (MYBP)[a]

	O/M-33	ΔR	A/C-60	ΔR	E/R-50	ΔR	Mean ± σ
Edentata vs. Cetferungulata/mole/bat	88.7	1.19	81.3	1.06	93.6	0.84	87.8 ± 6.2
	85.2	*1.23*	*91.4*	*1.08*	*92.6*	*0.80*	*89.7 ± 3.9*
Cetferungulata vs. mole/bat	69.5	1.18	73.6	1.04	78.2	0.68	73.8 ± 4.3
	81.2	*1.26*	*82.3*	*1.19*	*78.0*	*0.77*	*80.5 ± 2.2*
Carniv./Perissodactyla vs. Cetartiodactyla	65.5	1.41	71.1	1.24	70.8	0.81	69.1 ± 3.2
	80.2	*1.59*	*80.2*	*1.36*	*73.6*	*0.74*	*78.0 ± 3.8*
Suidae/Tylopoda vs. Rum./hippo/Cetacea	65.3	1.30	70.7	1.13	64.8	0.74	66.9 ± 3.3
	68.4	*1.42*	*71.4*	*1.14*	*72.1*	*0.71*	*70.6 ± 2.0*
Carnivora vs. Perissodactyla	62.8	1.78	67.1	1.14	67.5	0.92	65.8 ± 2.6
	65.4	*1.82*	*70.0*	*1.49*	*68.6*	*0.91*	*68.0 ± 2.4*
Cow/sheep vs. hippo/Cetacea	59.6	1.36	**60**		62.8	0.73	61.2 ± 2.3
	68.2	*1.48*	**60**		*56.7*	*0.57*	*62.5 ± 8.1*
Bat vs. mole	51.7	1.18	62.7	1.04	68.7	0.68	61.0 ± 8.6
	63.4	*1.26*	*71.9*	*1.10*	*73.9*	*0.77*	*69.7 ± 5.6*
Baboon vs. *Homo*/gibbon	51.8	1.25	51.2	0.58	63.0	0.48	55.3 ± 6.6
	46.1	*1.57*	*49.1*	*0.56*	*59.8*	*0.42*	*51.6 ± 7.2*
Canoidea vs. Feloidea	51.4	1.75	56.9	1.45	47.1	0.75	51.8 ± 4.9
	53.5	*1.82*	*57.3*	*1.49*	*53.3*	*0.86*	*54.7 ± 2.2*
Equidae vs. Rhinocerotidae	52.8	2.20	57.7	1.73	**50**		55.2 ± 3.5
	50.0	*2.11*	*53.9*	*1.74*	**50**		*52.0 ± 2.8*
Hippopotamidae vs. Cetacea	58.6	1.32	53.3	0.86	47.6	0.50	53.2 ± 5.5
	57.6	*1.24*	*58.4*	*0.96*	*57.4*	*0.48*	*57.8 ± 0.5*
Homo vs. gibbon	30.0	1.18	30.1	0.64	34.6	0.54	31.6 ± 2.6
	24.9	*1.42*	*28.7*	*0.62*	*34.0*	*0.4*	*29.2 ± 4.6*
Cow vs. sheep	29.9	1.72	32.9	1.36	32.9	0.91	30.9 ± 1.7
	33.3	*1.97*	*32.8*	*1.49*	*32.2*	*0.90*	*32.8 ± 0.6*
Balaenopteridae vs. Physeteridae	**33**		30.5	0.69	30.0	0.45	30.3 ± 0.4
	33		*27.6*	*0.64*	*32.5*	*0.47*	*30.1 ± 3.5*
White rhinoceros vs. Indian rhinoceros	30.6	2.20	33.5	1.73	29.0	1.00	30.0 ± 2.3
	29.3	*2.14*	*30.1*	*1.72*	*28.9*	*1.00*	*29.4 ± 0.6*
Horse vs. donkey	9.0	2.20	9.8	1.73	8.5	1.00	9.1 ± 0.7
	8.7	*2.16*	*9.1*	*1.78*	*8.5*	*1.00*	*8.8 ± 0.3*
Blue whale vs. fin whale	6.4	1.29	5.9	0.53	7.6	0.45	6.6 ± 0.9
	6.6	*1.27*	*5.4*	*0.49*	*8.5*	*0.48*	*6.8 ± 1.6*

[a] Divergence times were calculated by a method adopted from Li et al. (1987), described by Arnason et al. (1996c, 1998), from amino acid distances shown in Table 1. The splits between Balaenopteridae and Physeteridae at 33 MYBP, ruminant Artiodactyla and Cetacea at 60 MYBP, and Equidae and Rhinocerotidae at 50 MYBP (bold face) were used as calibration points. ΔR indicates the rate difference between individual divergences and the respective reference using the closest possible outgroup. Values in italics are based on distances calculated according to mtREV-24 and the Γ model of rate heterogeneity.

studies by Gatesy et al. (1996, 1999) and Kleineidam et al. (1999). Analyses of the complete mtDNA genome of the hippopotamus yielded a statistically conclusive support for a sister-group relationship between the Cetacea and the Hippopotamidae (Ursing and Arnason 1998b). Inclusion of the sperm whale in the present analyses did not change the sister-group relationship between cetaceans and hippopotamuses. The present analyses have also identified a sister-group relationship between Ruminantia and Cetacea/Hippopotamidae (Cetancodonta), to the exclusion of Tylopoda and Suina.

This study, which includes both the bat and the mole (two lineages which help define the time of origin of the Cetferungulata clade), has allowed further testing of cetferungulate references for molecular dating of evolutionary divergences. The most strongly supported tree, OG,((pig,alpaca),((cow,sheep),(hippo,whales))), joined the pig (Suina) and the alpaca (Tylopoda) on a common branch as the sister group of Ruminantia/Hippopotamidae/Cetacea (Fig. 1, Table 3). This tree was distinctly better than the second-best tree, which had Suina as the sister group of all other artiodactyls/cetaceans. The topology with Tylopoda as the sister group of the other artiodactyls/cetaceans was supported by Gatesy et al. (1999) and Nikaido et al. (1999). However, this relationship remained unsupported in the present study.

Waddell et al. (1999) proposed the term Whippomorpha for the Cetacea/Hippopotamidae grouping. This term is problematic, however, because Hippomorpha is a perissodactyl lineage, unrelated to cetacean and hippopotamid evolution. Montgelard et al. (1997) proposed the now commonly used name Cetferungulata for the grouping of Carnivora, Perissodactyla, Artiodactyla, and Cetacea and used the term Ancodonta for the Hippopotamidae plus the extinct Anthracotheriidae. Consistent with their terminology, we propose the name Cetancodonta

for the cetacean/hippopotamid grouping. This nomenclature avoids the confusion involved in the term Whippomorpha proposed by Waddell et al. (1999).

In 1993, Milinkovitch et al. reported that the odontocete family Physeteridae (sperm whales) and the mysticete family Balaenopteridae (rorquals) were sister groups, to the exclusion of the other odontocetes. These conclusions were supported by Adachi and Hasegawa (1995). The findings of Milinkovitch et al. (1993) were challenged, however, by Arnason and Gullberg (1994, 1996), Ohland et al. (1995) and several other comprehensive studies at the morphological and/or molecular levels (Heyning 1997; Cerchio and Tucker 1998; Messenger and McGuire 1998; Luckett and Hong 1998; Gatesy et al. 1999), none of which identified a sister-group relationship between Mysticeti and Physeteridae. It is of a particular interest to note that both Cerchio and Tucker (1998) and Messenger and McGuire (1998) independently reported concern with the alignments used by Milinkovitch et al. (1993), showing that correction of the alignment erased the support for the balaenopterid-(mysticete)/sperm whale relationship. The establishment of a sister-group relationship between Mysticeti and Odontoceti has particular relevance for the present study, as it allows the use of the data set of any odontocete species to estimate the time of divergence between Mysticeti and Odontoceti.

After calibration for rate differences among lineages the odontocete/mysticete reference point (O/M-33) yielded dating estimates reasonably consistent with those produced using two previously established references, A/C-60 and E/R-50, which are both based on paleontological datings which predate the divergences between mysticetes and odontocetes. A/C-60 is supported by the age (53.5 MY) of the earliest archaeocete fossils (Gingerich et al. 1994; Bajpai and Gingerich 1998), while E/R-50 is defined by the age (early Eocene) of the primordial perissodactyl genus *Hyracotherium* (Hooker 1989; Prothero and Schoch 1989). Table 3 includes the mean values for the estimates of the time of various eutherian divergences. The differences between the mean for rate homogeneity and that for rate heterogeneity were generally limited. This suggests that stochastic extremes related to the use of a single reference point are progressively reduced with the inclusion of additional references.

The use of O/M-33, A/C-60 and E/R-50 placed the divergence between ceropithecoids (baboon) and hominoids (gibbon, *Homo*) at >50 MYBP (Table 3). This is strikingly different from the dating of 30 MYBP allocated to the same divergence by Sarich and Wilson (1967) in their estimates of hominoid divergences. As recently discussed (Arnason et al. 1998), Sarich and Wilson's (1967) dating of this and other calibration points used to propose a Gorilla/Pan/*Homo* divergence at about

5 MYBP is refutable on both paleontological (see Simons 1990; Godinot and Mahboubi 1992) and molecular grounds (Arnason et al. 1996c, 1998).

The phylogenetic relationships reconstructed in this study for other mammalian groups are consistent with previous analyses of complete mtDNAs, including inter alia the position of the edentate armadillo (Arnason et al. 1997), the sister-group relationship between the bat (Pumo et al. 1998) and the mole (Mouchaty et al. 2000), and that between the bat/mole grouping and the cetferungulates (Mouchaty et al. 2000).

McKenna (1987) and McKenna and Bell (1997) proposed the term Epitheria for eutherians other than Xenarthra (Edentata). According to this proposal, the basal eutherian divergence is between Edentata and Epitheria. The nonbasal position of the armadillo (Edentata) among the other eutherians (Arnason et al. 1997) is inconsistent with this distinction. The morphological support for the cohort Epitheria was examined by Gaudin et al. (1996), who concluded that this support was questionable. It should be noted, however, that the phylogenetic conclusions of Gaudin et al. (1996) do differ in some respects from those in this study. Nevertheless, the revised position of Edentata means that all eutherian phylogenies and datings established using edentate rooting should be treated with caution.

Use of the O/M-33, A/C-60, and E/R-50 references gave an estimate of ≤90 MYBP for the time of the split between Xenarthra (Edentata) and the mole/bat/ Cetferungulata clade. This dating is not consistent with that of Kumar and Hedges (1998), who placed edentates basal in the eutherian tree and estimated their origin at ≈130 MYBP. Similarly, Kumar and Hedges (1998) calculated the eutherian origin at 173 MYBP, a dating much older than that commonly recognized by paleontologists (Novacek 1992) or previously estimated by the cetferungulate references A/C-60 and E/R-50 (Arnason et al. 1996c, 1998).

There are several possible explanations for the differences between the present study and that by Kumar and Hedges (1998). First, the calibration point used by Kumar and Hedges (1998) was the divergence between Synapsida and Diapsida 310 MYBP (Benton 1997). The use of such a distant reference point may make it difficult to recognize differences in evolutionary rates among recently diverged lineages. Second, while Kumar and Hedges (1998) stated that they used 658 genes in their analyses, this total number is irrelevant for the analysis of each specific divergence. For example, their estimates of the splits between edentates and other eutherians and that between cetaceans and artiodactyls were calculated using only three genes. It has been shown (e.g., Cao et al. 1994) that small data sets can be subject to pronounced stochastic effects, and for this reason, use of the concatenated sequences of a number of genes is generally ad-

visable (Saccone et al. 1990). Consistent with this, in cases where Kumar and Hedges (1998) analyzed more than a few genes, their results were generally in better agreement with previously published mtDNA findings (e.g., Janke et al. 1994, 1996, 1997; Arnason et al. 1996c, 1997, 1998; Xu et al. 1996; Montgelard et al. 1997). Finally, while Kumar and Hedges (1998) did not discuss this point, it should also be observed that their estimates were based on distance values compiled without the necessary support provided by phylogenetic analysis. This can lead to dating estimates that are inconsistent with phylogenetic findings. Thus, even though the statistical overlap in the calculations did not allow firm conclusions, the analysis of Kumar and Hedges (1998) suggested a closer relationship between pigs and cetaceans than between ruminant artiodactyls and cetaceans. This relationship is unsupported by the present and previous (Ursing and Arnason 1998a, 1998b; Ursing et al. 2000) studies based on phylogenetic analyses of complete mtDNAs as well as studies including nuclear data sets (Gatesy et al. 1999).

The time of the divergence between Arctocyonia + Artiodactyla and the lineage leading to Archaeoceti was recently calculated on the basis of the age (53.5 MY) of fossils of *Himalayacetus subathuensis* (Bajpai and Gingerich 1998). In accord with Van Valen (1996), Bajpai and Gingerich (1998) assumed a sister-group relationship between Mesonychia and Archaeoceti and between Mesonychia and Arctocyonia/Artiodactyla, i.e., that both Archaeoceti and Arctocyonia/Artiodactyla had arisen separately from Mesonychia. On the basis of these assumptions, the authors proposed a date of 62.5–66.4 MYBP for the divergence between the artiodactyl and the cetacean lineages. While this paleontological estimate is reasonably consistent with the molecular dating for the divergence between ruminant artiodactyls and cetaceans (Arnason and Gullberg 1996) (see also Table 3), the phylogenies proposed by Bajpai and Gingerich (1998) are not supported by molecular studies.

The discussion of early eutherian evolution has centered largely around the extensive diversification occurring in the early Tertiary (Alroy 1999, Benton 1999). Benton (1999) has argued that the burst of morphological evolution at the K/T boundary may have been accompanied by a simultaneous acceleration in the rate of molecular evolution and that this acceleration accounts for the early estimates obtained in molecular datings of Cretaceous eutherian divergences. This position is inconsistent with Martin (1993), who provided indirect paleontological evidence suggesting that the ages of eutherian divergences can be much older than indicated by the fossil record.

This study has allowed the comparison and testing of three calibration points, each of which has strong paleontological support. There was a general congruence among the estimates provided by the three references. The calculations were also consistent with paleontology when the estimates could be tested against a well-substantiated fossil record such as the divergence between Canoidea and Feloidea (52–55 MYBP; Table 3) (Flynn and Galiano 1982). The different lineages and calibration points involved in the dating estimates had different patterns of evolutionary radiations in the Tertiary (e.g., Cifelli 1981). Yet the dating estimates are (i) consistent with each other and (ii) consistent with the paleontological record of testable lineages. Thus, the estimates do not suggest that extensive morphological radiations are accompanied by fast molecular evolution, making it difficult to give credence to a scenario in which accelerated and coordinated rates in eutherian evolution at the K/T boundary may have skewed the molecular dating of Cretaceous divergences. Furthermore, as yet there are no cases in which a correlation has been demonstrated between the rates of morphological and molecular evolution (e.g., Albertson et al. 1999; Easteal 1999).

Molecular datings of eutherian divergences have, in many instances, been based on analyses of mtDNAs sequenced in their entirety. Taxon sampling has been limited in some of these studies, emphasizing deep ordinal splits rather than the time and succession of intraordinal Tertiary divergences as elaborated in more taxon-rich studies (Arnason et al. 1996c, 1997, 1998; Janke et al. 1996, 1997; Xu et al. 1996; Reyes et al. 1998; Mouchaty et al. 2000; Ursing et al. 2000). According to molecular estimates, only one eutherian order, Cetacea, has originated in the Tertiary (Arnason and Gullberg 1996). In conjunction with the fossil record the molecular estimates show that the orders Artiodactyla, Carnivora, and Perissodactyla include intraordinal divergences which took place 50–65 MYBP. Thus, accumulation of morphological differences during a period of 50–65 MY may not qualify for distinction at the ordinal level. The existence of 10–20 definable lineages of Cretaceous eutherians is commonly recognized (Novacek 1992; Alroy 1999; Benton 1999). Considering that extant lineages which diverged from each other 50–65 MYBP are still included in the same order, the notion of a pre K/T origin of most eutherian orders is fully compatible with the view of an extensive post-K/T diversification within the 10–20 eutherian lineages identified in the Cretaceous, whether or not these lineages have been given ordinal status in taxonomic schemes.

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