

The Marsupial Mitochondrial Genome and the Evolution of Placental Mammals

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ABSTRACT

The entire nucleotide sequence of the mitochondrial genome of the American opossum, *Didelphis virginiana*, was determined. Two major features distinguish this genome from those of other mammals. First, five tRNA genes around the origin of light strand replication are rearranged. Second, the anticodon of tRNA^{Asp} is posttranscriptionally changed by an RNA editing process such that its coding capacity is altered. When the complete protein-coding region of the mitochondrial genome is used as an outgroup for placental mammals it can be shown that rodents represent an earlier branch among placental mammals than primates and artiodactyls and that artiodactyls share a common ancestor with carnivores. The overall rates of evolution of most of the mitochondrial genome of placentals are clocklike. Furthermore, the data indicate that the lineages leading to the mouse and rat may have diverged from each other as much as 35 million years ago.

THE mitochondrial genome of vertebrates contains the genes for 13 proteins involved in oxidative phosphorylation, 2 ribosomal RNA (rRNA) genes and 22 transfer RNA (tRNA) genes. Except for the gene for the NADH dehydrogenase subunit 6 (*ND6*) and eight tRNAs, all genes are encoded on one strand (the H strand). A non-coding region, responsible for replicational and transcriptional control, contains regions of conserved sequence (WALBERG and CLAYTON 1981; SACCONI *et al.* 1991) as well as regions that differ in length and sequence between species. In marsupials, the arrangement of tRNA genes around the origin of light strand replication is altered (PÄÄBO *et al.* 1991) and an RNA editing process modifies the anticodon of a tRNA transcript (JANKE and PÄÄBO 1993). Here, the complete nucleotide sequence of the American opossum mitochondrial genome is presented. It reveals that the localization of all other genes is identical to that of placentals and that RNA editing seems to be confined to one tRNA.

The complete nucleotide sequence of the opossum was used to clarify the phylogeny of placental mammals, which have been repeatedly used to argue both for differences in evolutionary rates among lineages (WU and LI 1985; LI *et al.* 1987) and for equality of rates (BULMER *et al.* 1991; EASTEAL 1988; LI *et al.* 1990). The phylogenetic relationships among placental mammals are of great relevance for these arguments because they affect the interpretation of the observed sequence differences (EASTEAL 1992). For the elucidation of the mammalian phylogeny, sequences of entire mitochondrial genomes

are available for human (ANDERSON *et al.* 1981), mouse (BIBB *et al.* 1981), cow (ANDERSON *et al.* 1982), rat (GADALETA *et al.* 1989), fin whale (ARNARSON *et al.* 1991) and harbor seal (ARNASON and JOHNSON 1992). The only complete mitochondrial sequences from non-placental vertebrates are from an amphibian (ROE *et al.* 1985) and a bird (DESJARDINS and MORAIS 1990). Since these species have diverged from the mammalian ancestor about 350 and 300 million years ago, respectively (CARROLL 1988), multiple substitutions have erased much of the phylogenetic information on the lineages leading to the outgroups, making phylogenetic inferences difficult. For example, a recent study (ADACHI *et al.* 1993) using the combined protein sequences of all vertebrate mitochondrial proteins failed to resolve the branching order among the human, rodent and cow clades. Placentals and marsupials constitute a monophyletic group, which according to paleontological evidence diverged from a common ancestor early in the Cretaceous some 130 million years ago (CARROLL 1988; NOVACEK 1992). Thus, marsupials constitute the most appropriate outgroup for the placental radiation. The complete sequence of the mitochondrial genome of the North American opossum is here used to elucidate the phylogeny of placentals and to show that the majority of the genes in the mitochondrial genomes of placentals evolve at a constant rate.

MATERIALS AND METHODS

Liver was obtained from a fresh road-killed opossum, *Didelphis virginiana*, found near the University of California at Berkeley campus. Mitochondrial DNA was purified from liver by cesium chloride-gradient centrifugation (LANSMAN *et al.* 1981). Five *EcoRI* fragments (0.8, 3.1, 3.7, 4.2, 5.2 kb,

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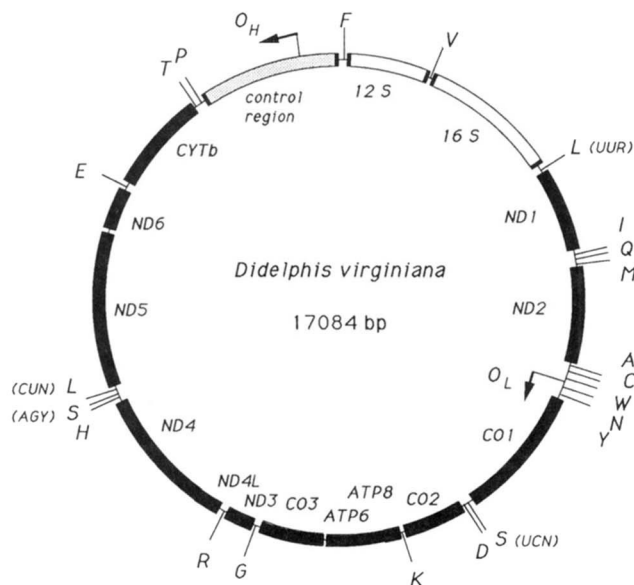


FIGURE 1.—Map of the *D. virginiana* mitochondrial DNA molecule. The location of origins of replication as well as the identity and arrangement of the various genes were determined by comparison of published mammalian sequences. Each tRNA is identified by its one-letter amino acid code. The tRNAs for serine and leucine are further identified by their codon family specificity. The *ATPase6* and *ATPase8* genes overlap by 46 nucleotides.

respectively) were ligated into the vector pBluescript SK⁺ (Stratagene, La Jolla, California) and cloned in the *Escherichia coli* host strain DH5 α . Some fragments were subcloned as *Hind*III fragments. Inserts were sequenced from the vector and by primer walking using the dideoxy chain termination technique. Both strands of the genome were sequenced and all overlaps between clones were confirmed by polymerase chain reaction (PCR) and direct sequencing from the purified mitochondrial DNA preparation.

Sequences were aligned by ESEE (CABOT and BECKENBACH 1989) and phylogenetic analyses were performed using the PAUP (SWOFFORD 1990) and the PHYLIP (FELSENSTEIN 1989) programs as well as programs designed by A.v.H. The alignment and a list of primers used for sequencing are available from A.J.

RESULTS AND DISCUSSION

Genome structure and organization: Like other mammalian mitochondrial genomes the marsupial genome appears to code for 22 tRNAs, 2 rRNAs and 13 proteins. The organization of the opossum mtDNA is shown in Figure 1 and the sequence of the L strand is presented in Figure 2. The length of the molecule is 17,084 bp.

Control region: The opossum control region is 1613 bp long. Three conserved sequence blocks (CSBs) have been identified in placentals (WALBERG and CLAYTON 1981) and two of these (CSBII and CSBIII) can be identified in opossum (Figure 2). By deletion analysis, these regions have been shown to constitute a bipartite recognition element for a RNase involved in processing of the RNA primer for DNA replication (BENNETT and CLAYTON 1990).

Heterologous assays with human enzyme and mouse mitochondrial RNA indicate that essential sequences for substrate recognition are conserved among placentals (BENNETT and CLAYTON 1990). The sequence similarity for this region suggests that the functional conservation extends also to marsupials. Only a tentative identification of CSBI was possible due to limited sequence similarity. CSBI seems to be separated from the other sequence blocks by a region of repeats, as is the case in the rabbit mitochondrial control region (MIGNOTTE *et al.* 1990).

Additional regions of primary sequence conservation have been identified among placentals (SACCONI *et al.* 1991). These regions are located in the left and middle domain of the control region and are partially conserved in the marsupial. In particular, some stretches are highly conserved between placentals and opossum (Figure 2). One of these is located around position 15600 and displays similarity to a sequence that is associated with the termination of displacement loop-synthesis in humans and rodents (WALBERG and CLAYTON 1981).

The opossum control region contains several repeated sequence motifs. One stretch of 25 nucleotides adjacent to the putative termination-associated sequence exists in two almost perfect copies. Furthermore, a region of repeated sequence motifs is located between the putative CSBI and CSBII (positions 16361 to 16613). It is made up exclusively of adenine and thymine residues, that are repeated in two imperfect motifs (AAATA-AAAAAAAA(A)TAAAATT and TA(A)TAATAAAA), with additional As and Ts interjected. The above two motifs are repeated 3 and 12 times, respectively, creating a 253-bp region of As and Ts. A further region of repeats is located between CSBIII and *tRNA^{Phe}*, starting at position 16772. This region contains 8 copies of the motif (A/G)T(A/C)AAATTAT-AAAATTT(T)A(A/G), two of which are partial.

The two regions containing multiple repeated motifs in the opossum mitochondrial genome were amplified from DNA from three animals from Berkeley and Santa Cruz, California, and Sweeny, Texas, as well as from cloned mitochondrial DNA. Both regions vary in length among the animals and two of the animals were heteroplasmic with respect to the number of AT-rich repeats (A. JANKE, unpublished observation). Thus, the copy numbers of these repeats vary both between and within individuals as is the case in other animals displaying repeats in the control region (*e.g.*, SOLIGNAC *et al.* 1983; HAUSWIRTH *et al.* 1984; SOLIGNAC *et al.* 1986; WILKINSON and CHAPMAN 1991; ARNASON and RAND 1992; BROWN *et al.* 1992).

Transfer RNAs: As in other animals, 22 tRNA genes are found in the mitochondrial genome of the opossum. Mitochondrial tRNAs show more structural variation than nuclear tRNAs, particularly in their dihydrouridine (DHU) and T Ψ C loops. The marsupial gene sequence allows the evolutionary timing of some of these structural changes. Unlike the chicken (DESJARDINS and

TATTTTCTACCAAAATCTCACCCCTCATTAAATATATCCCTACTAATAATTTCTCAGTATCACCTCAACCCCTTTTAGGAGGATGAGGAGGGTTAAACCAAAACACAACCTACGAAAAATCCTAGC 4440
I F Y Q I S P S L N M S L L M I L S I T S T L L G G W G G L N Q T Q L R K I L A

ATATTCATCAATTCGCTCATATAGGATGAATAGCCATTATTATCATAATTTATCCACTCACTCACTTCCCTCAACTTAATCTTATATCTGGCCCTACAATTAATATATTTATAGTACTTAA 4560
Y S S I A H M G W M A I I I M I Y P S L T I L N L I L Y L A S T I T M F M V L N

TCAATCATCTTCAACCAAAATCAACTCACTATCCATTTTGAACAAATCTGCTCCAATATAATCATTATTACTCTTACCCCTACTATCACTAGGAGGACTCCACCCCTAACCGGATT 4680
Q S S S T K I N S L S I L W N K S A P N M I I I T L T L L S L G G L P P L T G F

TATACCCAAATGACTTATTTTACAAGAATAAATACTTAATAATAATCCCTCTAGCAATAATACTAGCCTTATCAACTCTACTTAATCTATTTTCTACATACGAATTATCTACTCATC 4800
M P K W L I L Q E L I N F N N I P L A M M L A L S T L L N L F F Y M R I I Y S S

AACCCCTAATATATTTCCATCAATTAATAATACAAAAATCAATGAACCCCTATACTCACATAAAACAATTCACCCATCCCAACTCTAACCATATTTCTTCTTCTTCTCCCTATAAC 4920
T L T M F P S I N N T K M Q W T L Y S H K T I S P I P T L T I I S S L L L P M T

CCCTGTATTCATACCTTATCATAAGAATAACAAGACTTTATCTTGCATCACTCGAACGCAAAATCGAATACTTTAATTAAGCTAAATCTTATTTAATTAAGACTTTGCGAGCTCTATAC 5040
P V F I T L S * *tRNA^{Ala}* <- *tRNA^{Cys}* <-

TACATCTCTGAATTTGCAATTCACATATATTATATACTTCAAAGTCCCAATCATTTAAAGGCTTAGGATTAATATTAGACCAAGGCCCTCAAAGCCCTTAAGCAGGTGATAAACCCCTA 5160
tRNA^{Trp}

GCCTTTGCTAAATGGGCTCTCCCGCGAGGGGGGAGAGGGCGGAGGCCCCCCCGGGGAGGGGGGGTAAAGGGGGGGTGGGGGGGGTACTAAACTGGAGGGTATTTATCCCCTA 5280
stem *loop* *stem* *tRNA^{Asn}* <-

AATCTTAGTAAACAGCTAAGCGCCTGTACATTCGGCTTCAATTTATATGGTAAAAAGAGATTATTATTCTCTGTCTTTGAATTTACAGTTCAATGCTTACCTCAGCCATTTACCTATGT 5400
M *tRNA^{Tyr}* <- *CO1*

TCATCAATCGTTGACTTTTTTCAACTAACCAACAGACATCGGAACACTATACTTACTATTTGGTGCCTGAGCAGGCATAGTTGGCACTGCCCTAAGCTTTTAAATTCGAGCAGAGCTCG 5520
F I N R W L F S T N H K D I G T L Y L L F G A W A G M V G T A L S L L I R A E L

GTCACCAAGGACTTTAATTTGGCGATGATCAAAATTTACAATGTGATCGTAACCGCCCTGCTTTTATTATGATTTTTTTTATAGTAATACCTATTATAATTTGGAGGATTTGGTAATTGAC 5640
G Q P G T L I G D D Q I Y N V I V T A H A F I M I F F M V M P I M I G G F G N W

TTGTCACCTTATAATTTGGAGCTCCTGATATAGCATTCCCCGAATAAATAATAAGCTTCTGATTACTTCTCCCATCATCTCTATTACTATTAGCATCTCAACAATGAAGCAGGAG 5760
L V P L M I G A P D M A F P R M N N M S F W L L P P S F L L L L A S S T I E A G

CTGGAACAGGTTGAACAGTATATCCACCCTTCTGTCGCAACTTAGCCCATGACAGGCGCTTCAGTTGATCTAGCCATCTTTCCCTTCATTTAGCAGGTATCTCTCCATCTAGGGGCTA 5880
A G T G W T V Y P P L A G N L A H A G A S V D L A I F S L H L A G I S S I L G A

TCAATTTTACTACTATATTAATAATAAAACCTCCCGCAATATCAATAACCAAACTCCCTGTTGCTGTCGATCAGTAATAATCAGCAGTATTACTCCTTCTATCTTCCAGTGC 6000
I N F I T T I I N M K P P A M S Q Y Q T P L F V W S V M I T A V L L L L S L P V

TAGCCGAGGAATTAATTAATTAACAGATCGTAATTTAAATACTACTTCTTTGACCCCTGCTGGAGGAGGGGACCAATCTATATCAACATCTATCTGATTTTTTGGTCAACCCAG 6120
L A A G I T M L L T D R N L N T T F F D P A G G G D P I L Y Q H L F W F F G H P

AAGTTTATATTTTAAATTTACCTGGATTCGGTATAATTTCTCATATCGTAACCTATTATTCAGGCAAGAAAGAACCTTTCCGGTTATATAGGAATAGTCTGAGCCATAATATCTATTGGAT 6240
E V Y I L I L P G F G M I S H I V T Y Y S G K K E P F G Y M G M V W A M M S I G

TCTTAGGGTTTATGCTGAGCACATCATATGTTTACAGTAGGCTTAGATGTAGATACAGGCTTATTTTACATCAGCAACAATAATTTAGCCATCCCAACAGGAGTCAAAGTTTTTA 6360
F L G F I V W A H H M F T V G L D V D T R A Y F T S A T M I I A I P T G V K V F

GTGATTAGCCACATTACATGAGGAAATATAAATGATCCCCAGCAATGCTATGAGCCCTAGGATTTATCTTCTGTTTACAATTTGAGGTTCTAACAGGTATCGTACTAGCCAAATTCAT 6480
S W L A T L H G G N I K W S P A M L W A L G F I F L F T I G G L T G I V L A N S

CATTAGATATGACTACAGACATACTACGTAGTACCCATTTCCACTATGTTTATCTATAGTGTCTGATTTGCTATCATGGCGGATTTGTCCTGATTTCCCTTTATTTACAG 6600
S L D I V L H D T Y Y V V A H F H Y V L S M G A V F A I M G G F V H W F P L F T

GTATATGCTTAACGATATATGAGCCAAAATCCACTTCTTTATATATTTGTTAGGAGTAACTTAACATTTTCCCCCAACATTTCTAGGTTTATCTGGCATACCAGCAGTACTCAG 6720
G Y M L N D M W A K I H F F I M F V G V N L T F F P Q H F L G L S G M P R R Y S

ATTATCCAGATGCCATATATGAAATGTTGTTTCAATCGGCTCGTTTATTTCAATTAACAGCTGTGATTTTAAATAGTATTATTTATTTGAGAAGCTTTCGCATCCAAACGTGAAG 6840
D Y P D A Y T M W N V V S S I G S F I S L T A V I L M V F I I W E A F A S K R E *tRNA^{Ser} (UCN)* <-

TACTAGATGTTGAATTAACATAACCAACATGAATGATATACGGATGCCACCTTCTTACCATACATTTGAACAACAGTTTTCATTAAGCCATAATTAAGAAAGGAGGAATGAAC 6960
V L D V E L T T T N I E W L Y G C P P P Y H T F E Q P V F I K A * *tRNA^{Asp}*

CCCCTAAGATTAATTTCAAGTCAATCCCATACCCCTTATGACTTTCTCAAAGATATAGTAAAATTCATTACATAACTTTGCCATAGTTAAATTAAGTTTAACTCTATATATCTT 7080
CO2

AATATGCCCTATCCAATCAACTAGGTTTCCAAGAGCTACATCTCCCTATTATAGAAGAATTTATATACTTTTCATGATCATACATTAATAATTTGATTTCTGATCAGTTCACATAGTATTA 7200
M P Y P M Q L G F Q D A T S P I M E E L M Y F H D H T L M I V F L I S S L V L

TATATATTATCTTATACCTTACTACAAAATTAATCACTACACAGCATTATAGATGCCCAAGAAGTGAACAAATTTGAACAATTTTACCAGCGTAATTCITATCCCTTATGCCCTTCTT 7320
Y I I I L M L T T K L T H T S T M D A Q E V E T I W T I L P A V I L I L I A L P

TCCTTACGAATCTTTACATAATAGATGAAATCTATAATCCTTATCTAACAGTAAAGCAATGGGTCATCAATGATATTGAAGCTATGAGTTACACAGACTATGAAAATTTAATATTCCGAC 7440
S L R I L Y M M D E I Y N P Y L T V K A M G H Q W Y W S Y E F T D Y E N L M F D

TCATACATAATCCCAACCAAGACCTTAGTCTGGGCAACTTCGTTTACTAGAAGTTGATAACCGAATTTCTTCCCAATAGAACTACCAATTCGATGCTAATTTTCATCAGAAGACGTT 7560
S Y M I P T K D L S P G Q L R L L E V D N R I V L P M E L P I R M L I S S E D V

CTCCATGCATGAACAATGCCATCATTAGGCTTAAAAGCAGATGCTATCCAGGGGATTAACCAAAATTTACCTTAACATCATCCCGACCGGGGTGTTTATGGTCAATGTTTCAGAAATC 7680
L H A W T M P S L G L K A D A I P G R L N Q I T L T S S R P G V F Y G Q C S E I *tRNA^{Lys}*

TGTGGTTCAAACCAAGCTTTATGCTTATGCTCAGAAATAGCCCTCAATAATTTTCGAGAAATGATCTTCTATAATGCAATCATTTTGGAGTATTATATATTTAATATAATCGA 7800
C G S N H S F M P I V L E M A S L K Y F E K W S S M M Q

FIGURE 2.—Continued

ATPase β

GACCTAAGAACTCCTCAAATAACCCATGCCTCAACTAAATACTTCAACATGAACCCCTAACCCATTCTACTAATAATTTCCCTATTCTGTATCTATCAACTTAAAAATAAACCAGA 7920
M P Q L N T S T W T L T I S L I I I S L F C I Y Q L K I I N Q

ATPase δ

CATTAATCCAAATCTCTTCAACCGAACAATCAAACCTAACTAAACATACATTACCTTGAGAAAAAATGAACGAAAAATCTATTGCCCCATTCATCACCAACAATTTAGGTAT 8040
T L I Q I T P S T E Q S K L T K H T L P W E K K M w N t E k N i L y F l A p P h F s I s T h P q T q I f L * G I

TACAACACTACCAATTTATTACATTTCCATGTCTTATCCTATCTTCCCAAACGATGATTACCAAATCGAATTCAAATCTTACAATATGATTAATCCGCTTAACTACTAAACAAT 8160
T T L P I I I T F P C L I L S S P K R W L P N R I Q I L Q M W L I R L I T K Q M

AATGACAATGCATAACAACAAGGACGAACCTGAACCTCTAATACTTATATCACTAATTTCTATTTCGCTTCAACTAATTTACTAGGACTTACCATACTCTTTTACACCTACTACACA 8280
M T M H N K Q G R T W T L M L M S L I L F I A S T N L L G L L P Y S F T P T T Q

ACTTTCTATAAATATGGAAATAGCTATCCCATATGAGCAGGAACAGTAATATAGGATTCGAAATAAACCAAAAATATCTCTAGCTCATTTTTTACCTCAAGGTACACCTACTCCTTT 8400
L S M N I G M A I P L W A G T V I M G F R N K P K M S L A H F L P Q G T P T P L

AATCCCAACTATTATTATTGAAACTATTAGCCTATTATTCAACCACTAGCTCTGCGACTGACTAACAGTAATATTACAGCTGGACATCTTTTAACTCATCTTATTGGGTACAGC 8520
I P M L I I I E T I S L F I Q P L A L A V R L T A N I T A G H L L I H L I G S A

TACATTAGCCCTATCTCAATTAGTATAACTGTATCAACTATTACATCTCTATCTCTATTCTCTCACTTCTCGAAATCGCTGTAGCTATAATTCAGCTTACGTATTACCCCTACT 8640
T L A L S S I S M T V S T I T F S I L F L L T L L E I A V A M I Q A Y V F T L L
.CO3

AGTAAGCTTGTATCTACATGATAACTCATAATGACCCACCAAACTCACGCATACCACATAGTTAACCCAAAGCCCATGACCTCTAACAGGAGCTTTATCAGCATTACTATTAACATCAGGC 8760
V S L Y L H D N S * M T H Q T H A Y H M V N P S P W P L T G A L S A L L L T S G

TTAATTATATGATCCACTATAATCTTCTACTCTTATATTATAGGACTAACCACTGCTGCTAACAATATACCAATGATGACGAGATATCATTGAGAAGGCACATTTCAAGGACAC 8880
L I M W F H Y N S S T L I F M G L T T M L L T M Y Q W W R D I I R E G T F Q G H

CACACCCCTGTAGTACAAAAAGGCTTACGATATGGAATAATCTTTTTTCTATCGGAAGCTCTTTTTTATCGGATCTCTCGAGCTTTTTATCATCAAGCTAGCTCCCACTCCA 9000
H T P V V Q K G L R Y G M I L F I L S E V F F F I G F F W A F Y H S S L A P T P

GAATTAGGAGTGTGACCTCCAACAGGTATTCTCCATTGAACCCACTAGAAGTCCCTACTAAATACATCCATCTTCTAGCTCAGGAGTATCTATTACATGAGCACATCACAGC 9120
E L G G C W P P T G I H P L N P L E V P L L N T S I L L A S G V S I T W A H H S

TTAATAGAAGGTAATCGCAAGCAATAATCAAGCTCTCTAATTACAATTTCTTAGGACTTTACTTTACTATTTTACAAGCCATAGAATACTATGAAGCTTCATTACTATCTCAGAC 9240
L M E G N R K Q M I Q A L L I T I S L G L Y F T I L Q A M E Y Y E A S F T I S D

GGAGTATACGGITCGACCTCTTTGTAGCAACAGGTTCCATGGCCTTCATGTTATCATGGATCAACTTTCCTAATGTTTGGCTACTTCGCAATATTTTATCACTTCACTCTACA 9360
G V Y G S T F F V A T G F H G L H V I I G S T F L I V C L L R Q L F Y H F T S T
.tRNA^{Gly}

CATCACTTTGGATTGAAAGCAGCTGCTGTACTGACATTTGTAGATGATGTTGACTTTTCTATATGTGCAATTTACTGATGAGGTTTCATATTTTCTAGTATAATTAGTACTACT 9480
H H F G F E A A A W Y W H F V D V V W L F L Y V S I Y W W G S *
ND3

GATTTCCAATCATTAGTCTGGGTCAAACAGAGAAAAATCAATCTTATCATCACATTAATACAATTCCTTGCTATCCACCATATCATTATCATTGCCCTTTGACTACCTCAA 9600
M I N L I I T L I T N S L L S T I I I I I A F W L P Q

TTATATCTATATTAGAAAAATCAAGTCCCTATGAATGTGGATTTGATCCTTTAGGATCAGCAGGACTACCCCTTTCAATAAAAATTTTCCCTAGTAGCTATTACATTTCTGCTATTGAC 9720
L Y L Y L E K S S P Y E C G F D P L G S A R L P F S M K F F L V A I T F L L F D

CTAGAAATGTCTACTACTACCATTACCATGAGCCATCCAACCTCCCATCCATTTACTACATTAATCTTCTTATTGCTAATTTACTTCTAACAAGTAGGACTAGCATATGAATGA 9840
L E I A L L L P L P W A I Q L P S P F T T L I L S Y C L I M L L T V G L A Y E W
.tRNA^{Arg} ND4

ATCCAAAAGGCTTAGAATGGACTGAATAGGTATTTAATCTAATTAAGATAGTTGATTTGCACTCAACAAATCATGTTTCAATCCATGAACACCTTATAGTATTAATAACTAAACA 9960
I Q K G L E W T E * M V L I K L N

TTATTGTAGCCTTTTACTAGCCCTTTCAGGAGTACTCATTACCGCTCACATCTAATATCAACTTTACTTTGCCCTAGAAGGAATAATACTATCACTATTCAITTTTATAGCAGCAATAA 10080
I I V A F M L A L S G V L I Y R S H L M S T L L C L E G M M L S L F I F M A A M

TTACCCACTTCCACATATTTCAATCTCTATAATACCCTAATTTCTACTCGTATTTTCCGCTTCCGAGCCGGAGTAGGACTAGCCTTACTCGTTCCTACTCTAATACCTATGGTAATG 10200
I T H F H M F S I S M M P L I L L V F S A C E A G V G L A L L V S I S N T Y G N
ND4

ATCAAGTCCAAAACCTTAATTTACTACAATGCTAAAAATCCTATTACCAACACTAATGTTAATCCACTAACCTGACTCTCCAAAAATAAATGATTATGAATCAACACTACAACCTATAG 10320
D Q V Q N L N L L Q M c L * K I L L P T L M L I P L T W L S K N K W L W I N T T T Y S

CCTACTTATAGTATTACTAGCTTACCTATACTATATACCCTATAGATCTAGGATACAACCTTTAATAATTCATCTCCCTAGACTCATTATCAAGTCCACTATTAGTTTTATCTGCTG 10440
L L I S I T S L P M L Y H P M D L G Y N F N N S F S L D S L S S P L L V L S C W

ACTTCTCCACTGATAATTATAGCTAGCCAAAATCATTTAAATAAAGAATCACTTATACGAAAAAACTATATCTAATCTAATAGTTATCCTTCAATCACTCCCTAATTTATGCTTTTAC 10560
L L P L M I M A S Q N H L N K E S L M R K K L Y L T M M V I L Q S S L I I A F T

CTCACTGAACTAATAATTTTACATCTTATCGAACAACCTTTAATCCAACCCCTAATTTATATCACTCGATGAGGCAATCAAAATGAACGATTAACGAGGAATTTACTTCTCTATT 10680
S S E L M M F Y I L F E T T L I P T L I I I T R W G N Q N E R L N A G I Y F L F

TTATACACTAGTAGGACTCTCCACTATTAGTGGCTTTATTAACCTAAACAAAACTTAGAACACTTCATATCCTTATAAACTCTATCTTAATTAACCAATTAACATATACTCTATC 10800
Y T L V G S L P L L V A L L T M N K N L G T L H I L M N S I L I N Q L N Y T L S

CAACTCAACACTATGATATGATGATAAATGCAATTCATAATTAATAACCATATATGGCCCTTCACTTTGATTACCAAAAGCACACGTTGAAGCCCTATGAGGATCTATAGCTTT 10920
N S T L W Y A C M T A F M I K M P L Y G L H L W L P K A H V E A P I A G S M V L

AGCAGCTATCTTACTAAAAATAGGAGGTTACGGAATCATACGAATTTTATTACTGAACTATAACTATACATTTACTTTACCCATTTATTTCTATCCATATGAGGAATAATCAT 11040
A A I L L K L G G Y G I M R I S L F T E P M T M H L L Y P F I I L S M W G M I I

AACAAGCTCAATTTGTATACGACAACAGATCTAAAACTACTAATGCTTACTCATCTGTCAGCCACATAGCCTTAGTTATCATTGCGGCATTAATCAATCAACAACAGTTTTATAGG 11160
T S S I C I R Q T D L K S L I A Y S S V S H I A L V I I A A L I Q S T T S F I G

GGCAACAATCTTATAGTAGCCCATGGACTCACATCTCTATATCTGCTAGCTAATACAATATGAACGAATTCATAGTCGAACAATAATCTAGCAGGAGATTACAACCTTAT 11280

FIGURE 2.—Continued

A T I L I V A H G L T S S I L F C L A N T N Y E R I H S R T I I L A R G L Q L I
 CCTTCCCTTAATAACTACCTGATGACTAACAGCCAGCCTAGCTAACTAGCCTACCTCCAACAATTAACCTATTAGGTGAGTAAATAATTATTACTGCATCCTTTTCATGGCTAAGT 11400
 L P L M T T W W L T A S L A N L A L P P T I N L L G E L M I I T A S F S W S N F
 CTCATCTACTTTTAGGACTAAATACAGTTATTACAGCCTTACTACTATATATACTAATCTCAACAGGTAATTCACACACCACATAACATCCTTATATCCATCATTAC 11520
 S I L L L G L N T V I T A L Y S L Y M L T T S Q R G K F T H H M T S L Y P S F T
 ACGTGAACATATACTTATAACCCCTCATATTATACCATTAATCTTATTATCCCTTAATCCAAAGTATATCTAGGTATAACTTACTGCAAATATAGTTTAAATAAAACATTAGATTGTA 11640
 R E H M L M T L H I M P L I L L S L N P K Y I L G M T Y *
 .tRNA^{His}
 ATCTAAACATAGAAGTTTAAAGCTTCTTATATGCCGAGAATGCATCAAGAACTGCTAATTCATGAACCCATATTTAAACAATATGGCTTCTCACTTTTAAAGGATAGCAGTAATCCATTG 11760
 .tRNA^{Ser} (AGY) .tRNA^{Leu} (CUN)
 .ND5
 GTCTTAGGAACCAAAACTTTGGTGAACCTCCAATAAAAAGTAATTAATTTCAAACTATATCAATTATATCCATTATCTACTAATCTTCCATTATTATATAACCTACTTTCCCT 11880
 I N I S N T M S I M S I I L L I L P L L Y N L L S L
 TAAAAAATTAATTTCCACTATATTGCAAAAACATAATTATACTAGCTTTTATAATAAGCTTACCTTCACTCTACTATTTATATAAAGGCCAAGAATCAATTACTACTGACA 12000
 K K I N F P L Y C K N M I M L A F M M S L P S L L L F M Y K G Q E S I I T N W H
 TTGATCTCTATCCATTCATTAAACATCTCAATAAGTTTAAATAGACTTTTCTCAATATTTTATCCCAATTGCCCTATTGTTACATGAGCAATTTAGAAATTTCCCTATGATA 12120
 W F S I H S F N I S M S F K M D F F S I I F I P I A L F V T W A I L E F S L W Y
 TATACACTCAGACCCTAATATTTCCCAATTTTAAATACCTTATCATCTTTCTCCAAACCATGATTATCTAGTATCAGCAAAACCTATTCAACTATTCATGGATGAGAAGGAGT 12240
 M H S D P N I S Q F F K Y L I I F L L T M I I L V S A N N L F Q L F I G W E G V
 AGGCATATATCTTTTCTACTAATCGGATGATGGTATGGACGATCAGACGCCAACCCGAGCTCTACAGGCTATCCTATATAATCGTATCGGAGACATTGGATTACTCACAATAGC 12360
 G I M S F L L I G W W Y G R S D A N T A A L Q A I L Y N R I G D I G F M L T M A
 CTGACTTATACTAAACTGCAACTCATGAGATCTCCAACACATCTTCTATAAATATACACCCCATGACTATTAGGACTAATTTGCTGCCACAGGAAAATCAGCACAATTTAGCCT 12480
 W L I L N C N S W D L Q H I F S M N M H P I A L L G L I I A A T G K S A Q F S L
 CCACCCATGACTCCCTCAGCCATAGAAGTCCAACCTCCAGTATCAGCTTTACTTCACTCAAGTACTATAGTTGAGCAGGAATTTTTTACTCATCGGATTCATCCAATATTAGAGAA 12600
 H P W L P S A M E G P T P V S A L L H S S T M V V A G I F L L I R F H P M L E N
 TAATAAAACAATACTTACTATTACCCTATGCTTAGGGGCATTAACAACACTATTCACAGCTATATCGCAATATACAAAACGACATAAAAAATGTAGCATTTCCACATCCAGTCA 12720
 N K T M L T I T L C L G A L T T L F T A M C A I M Q N D I K K I V A F S T S S Q
 ACTAGGACTAATAATAGTAACTGTAGGTTTAAATCAACCTCACTTAGCCTTTCTACATATTTGTAAGTACAGCATTCTTCAAGCAATACTATTCTTATGTTCTGGGCTATTATTATAA 12840
 L G L M M V T V G L N Q P H L A F L H I C T H A F F K A M L F L C S G S I I H N
 CTTAAACGACGAACAGACATTGCAAAAATAGGAGGATTTTATACCTTACCATTACATCTCCGCCCTAATAACAGGTAGCCTAGCACTTATAGGTACACCATTCTAGCAGGATT 12960
 L N D E Q D I R K M G G L F Y T L P I T S S A L M T G S L A L M G T P F L A G F
 TTACTCTAAGATTCATTATTGAAGCAATAAACACATCATACACCACTCATGAGCTCTAACAATTAACATAATGCTACATCATTAAACAGCCATTTATAGTCTACGCATTATTATA 13080
 Y S K D S I I E A M N T S Y T N S W A L T I T L I A T S L T A I Y S L R I I Y Y
 CACTTCTTAGGACACCCAGGATTCATAACAATATCCCCCTTAATGAAAATAACCTAACCTTATTAACTTATTATTCGACTAGCACTAGGACTATTTTGGAGGATTCATATTAA 13200
 T L L G H P R F M T M S P L N E N N P N L I N P I I R L A L G T I F A G F M L T
 TACAAATATACCCCTCATACTCTATACAATAACCATGCCAATATTATTAAACAATAAGCCTTAATAGTAACTACAGGACTATAATAGGAATAGAACTTAATCTCTACTAA 13320
 T N M P P S Y S I T M T M P M F I K Q M A L M V T T T G L M M G M E L N S L T N
 TAACTCATAAAATCAATAACCATATAAATCTTAACTATATTAGGATTTTAACTCAAATATACATCGAATACAACCCCTAATAGCCCTTTATAGGTCAACGAATTGCTAC 13440
 K L M K S N N H T N N F L T M L G F Y T Q I M H R M Q P L I S L F M G Q R I A T
 CATACTAATGATATAAAGTATGAAAAGCAGGTCAAAAGGCCAAGTAAATGTTCACTCTAATATATCATCATTAAATTTCTCATCTCAAAAAGGACTAATAAAATGACTTTCT 13560
 M L I D M N W Y E K A G P K G Q A N V H S K L S S L I S S S Q K G L M K M Y F L
 ATCCTTTTAGTTTCTATAATCTTCTATTCTATTACCTAATAACTCTGTTCCGAAGTACCTCTAATACAACATAAATAGTTATGAACAAGATTATCTCTAATAAAGCTAAAGCTCA 13680
 S F L V S M I F I I L F T *
 TCCTCCACAATAATAACTGAGAAACACCATTATAATCTGTCCGACTAACTTGCTCGACAAAATCAATAATTTAATTGCTATGATGATATATACCACTTAGACATAAAATATCA 13800
 TCCTACCTGTAATAATAATACAACAATAACATGATAAAGCTACAACATTTCCAACCTCATGTTTCAGGATACTCTCCGTAGCTATAGCAGTAGTATAACCAAAACTACTAATATACC 13920
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 .tRNA^{Glu} <-
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 . Cyt b
 TTCAACTACAAAATAATGACCAATATCGCAAAACACATCCACTCATAAAAATCATTAAATGATTCATTATGACTACCAACACCATTAACATCTCAGCCTGATGAAATTTGGTTC 14280
 M T N I R K T H P L M K I I N D S F I D L P T P S N I S A W W N F G S
 ACTATTAGGAGTGCCTAATATTCAAATCTCACAGGCTTATTCTTAGCAATACATTATACATCTGATACATTAAACCGCATTTTCATCAGTAGCCCATATTTGCCGAGACGTAACATA 14400
 L L G V C L I I Q I L T G L F L A M H Y T S D T L T A F S S V A H I C R D V N Y
 CGGATGACTTATCCGAAATATCCATGCCAAGGAGCATCTATATCTTATATGCTTTTCTTCTCATGAGGACGAGGAATTTACTATGGATCATATCTTTACAAAGAAAATGAAATAT 14520
 G W L I R N I H A N G A S M F F M C L F L H V G R G I Y Y G S Y L Y K E T W N I
 TGGAGTTATCTACTACTAACAGTTATAGCTACTGCATTGTTGGCTACGTAACCATGAGGACAAAATATCATTTTGAGGCGCAACAGTTATTACTAACCTATTATCTGCCATCCATA 14640
 G V I L L L T V M A T A F V G Y V L P W G Q M S F W G A T V I T N L L S A I P Y
 TATCGAAGTACACTAGTAGAATGAAATTTGAGGAGGATTCTCCGTTGATAAAGCTACACTAACCCGATTTTTGCTTTTCACTTTATTCTTCCATTCACTTTTAGCTATAGTAGTAGT 14760
 I G S T L V E W I W G G F S V D K A T L T R F F A F H F I L P F I I L A M V V V

FIGURE 2.—Continued

TABLE 1

Mean base composition for the mitochondrial protein-coding genes of seven mammalian species

Codon position	A	G	C	T
1st	32.1 ± 0.7	20.7 ± 0.8	24.4 ± 1.6	22.8 ± 1.5
2nd	19.5 ± 0.2	12.2 ± 0.1	26.2 ± 0.7	42.1 ± 0.6
3rd	42.4 ± 3.4	5.0 ± 1.6	31.2 ± 7.0	21.4 ± 5.1

Mean base compositions (%) for the 13 protein-coding genes and standard deviations are given for each of the three codon positions.

ably formed by polyadenylation of the transcript. Two genes (*ATPase 8* and *ND6*) use TAG as termination codons, whereas the AGG and AGA termination codons used in humans (ANDERSON *et al.* 1981) and frog (ROE *et al.* 1985) are not utilized in opossum. In eight cases, ATG is used as translational initiation codons, whereas ATA is used in four cases and ATT in one.

Evolutionary relationships among placental mammals: To elucidate the relationship among placental mammals, we used sequences from six complete mitochondrial genomes: rat, mouse, human, cow, whale and seal. Gaps had to be introduced at a few positions to align the protein-coding genes, especially at the 3'-ends of genes. Positions with gaps and areas where the alignment was ambiguous were excluded from subsequent analyses. The *ND6* gene, encoded on the L strand, differs significantly in its base composition from the other genes and was therefore excluded from the analyses when not otherwise stated. Similarly, tRNA, rRNA and non-coding sequences were excluded to avoid ambiguous alignments and allow for a coherent maximum likelihood analysis.

Base composition: Base composition of nucleotide sequences is known to vary among taxa (*e.g.*, SUEOKA 1988) and this may obscure phylogenetic information. Table 1 shows the mean base composition for the seven mammalian species for each of the three codon positions. Most changes at third codon positions are silent, and this position demonstrates the highest level of compositional bias (MUTO and OSAWA 1987) as reflected by the higher standard deviation at this codon position. As for other mitochondrial genomes (GADELATA *et al.* 1989), guanosine residues (G) are underrepresented on the L strand in the opossum, an observation most apparent at the third codon positions, where the average G content is 5%. Furthermore, the adenosine content of third positions and the thymine content of second positions are high (42%).

To elucidate whether the nucleotide compositions of the protein-coding genes of the different taxa differ, we tested if the nucleotide distribution is homogeneous among the placental taxa by a chi-square test (VON HAESLER *et al.* 1993). Although this test suffers from the drawback that the sequences are not independent, it can be used as a rough guide to detect differences in base composition. The base compositions of the second

TABLE 2

Expected numbers of sequence differences at saturation and observed numbers of differences among seven mammalian taxa

Codon position	Transitions		Transversions	
	Expected	Observed	Expected	Observed
1st	909	284–416	1855	194–591
2nd	999	110–292	1610	58–235
3rd	654	760–924	1855	751–1188

The expected numbers of transitions and transversions at saturation were calculated using the base composition in Table 1 and the method of HASEGAWA *et al.* (1985). In addition, the highest and lowest observed numbers of transitional and transversional differences among the seven taxa are given.

codon positions are homogenous ($P = 0.996$) as are those of the first codon positions ($P = 0.073$). For the first positions, the base composition of the opossum differs significantly from that of the placentals. However, since this affects only the outgroup in the phylogenetic analysis, it is not expected to cause incorrect tree topologies to be inferred. The base composition at third codon positions is highly non-homogeneous among the placentals and is thus unsuitable for phylogenetic reconstruction. When the distribution of purines and pyrimidines at third codon positions is investigated, the inhomogeneity remains. However, this is due to a biased composition of purines and pyrimidines in the human mitochondrial genome. When the human sequence was removed from the analysis, the remaining taxa were homogeneous with respect to their purine-pyrimidine composition at third codon positions. Thus, since only one taxon has a base composition that differs from the other taxa, this is not expected to affect the estimation of the tree topology but only the inferred lengths of branches. Transversions at third codon positions can therefore be used to infer the branching order among placental lineages.

Multiple substitutions: In animal mitochondria, transitions predominate over transversions by a factor of at least 10 (BROWN *et al.* 1982; DESALLE *et al.* 1987). Furthermore, in the genetic code of mammalian mitochondria, transitions at third codon positions do not result in amino acid replacements. As a consequence, the third codon positions evolve several times faster than first and second codon positions, where the majority of changes cause amino acid replacements. Thus, transitions at third codon positions are expected to be particularly prone to losing phylogenetic information due to multiple hit phenomena.

We computed the expected pairwise differences at saturation using the base compositions given in Table 1 and the procedure of HASEGAWA *et al.* (1985). Table 2 gives the expected numbers of transitions and transversions at saturation for the three codon positions. When the observed transitional and transversional differences are compared to the numbers expected at saturation, we found that all

observations at first and second codon positions are well below the expected saturation level. At third codon positions, the transitional differences between all taxa compared are above the expected saturation level whereas for transversions at third codon positions, no value exceeds the saturation level. Thus, transitions at third codon positions are heavily affected by multiple substitutions and should be avoided in phylogenetic analyses of these taxa.

To investigate why the observed numbers of transitional differences at third codon positions are higher than the expected saturation level (Table 2), we calculated how the observed transitions are expected to vary as a function of observed transversions after divergence of two taxa assuming various transition-transversion ratios. Figure 4 shows how the relative number of observed transitions first increases and subsequently decreases as transversions erase the record of previous transitions (BROWN *et al.* 1982). Irrespective of the transition to transversion ratio, the amounts of observed substitutions converge to an equilibrium point when the sequences have been saturated with substitutions. For first and second codon positions, the numbers of transitions and transversions that are observed from the data are far from the saturation point both with respect to transitions and transversions. In contrast, at third codon positions, the numbers of transitions lie above the saturation point whereas transversions have not yet reached saturation. That the observed numbers of transitional sequence differences can exceed the numbers at saturation agrees with a study of mitochondrial sequences for ungulates where sequence differences were plotted against paleontologically inferred divergence times (IRWIN *et al.* 1991). Thus, the transitions at third positions of codons are saturated for all divergences in the phylogeny whereas transversional changes at third codon positions are well suited for phylogenetic inference.

First positions of leucine codons are similar to third codon positions in that they may experience silent transitions (TTR to CTR) (IRWIN *et al.* 1991). Consequently, they are likely to be saturated for deeper divergences among placentals. Therefore, transitions at first positions of leucine codons were excluded from subsequent analyses except where indicated.

Tree reconstruction: Due to their different base compositions, first and second codon positions were independently used to construct phylogenetic trees using the maximum likelihood procedure (FELSENSTEIN 1981). Figure 5 shows the best tree derived from the second codon positions. Maximum parsimony (SWOFFORD 1990) and neighbor-joining (SAITOU and NEI 1987) yielded trees of identical topology. Bootstrap analyses (FELSENSTEIN 1985) for maximum parsimony and neighbor-joining independently showed that all internal branches were seen in more than 95% of 1000 bootstrap replications. Identical results were obtained for first codon positions and for transversions at third codon positions.

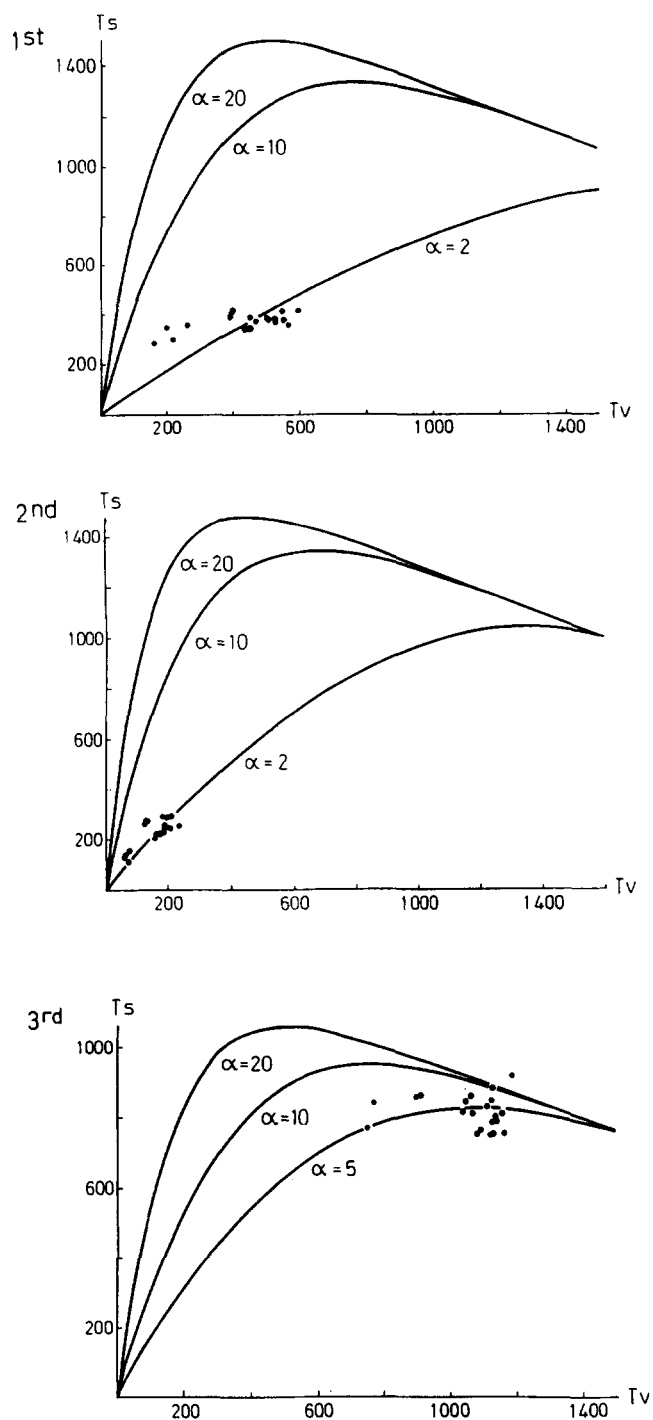


FIGURE 4.—Plot of expected observed transversions (T_v) vs. observed transitions (T_s) assuming various transition-transversion biases (α) calculated according to HASEGAWA *et al.* (1985) and using the the observed base compositions given in Table 1. Dots denote the transitional and transversional differences as observed from the data. The apparent transition bias for first and second codon positions of around 2 may be underestimated due to invariable positions in the sequences which cause the expected numbers of changes to be overestimated.

To evaluate also other possible tree topologies, the taxa were reduced to six by constraining the topologies to those where the mouse and rat are monophyletic.

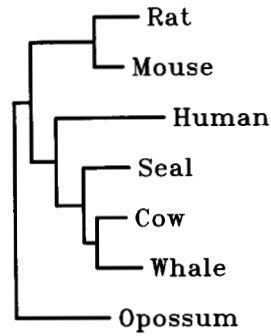


FIGURE 5.—Maximum likelihood tree relating the mitochondrial genomes of six placental mammals and one marsupial mammal to each other. The topology is based on all differences at second codon positions of all protein-coding genes except *ND6*. Trees based on differences causing amino acid replacements at first codon positions and on transversions at third codon positions have identical topologies.

This allowed all possible 105 trees to be evaluated. The likelihoods for the trees based on first and second codon positions which had identical topologies were added and the best tree (Figure 5) was tested against all other topologies according to KISHINO and HASEGAWA (1989). Only one tree was not significantly worse than the tree in Figure 5. This tree differs from the best tree in having the seal as the sister taxon of the cow. The monophyly of ungulates, which is supported by other lines of evidence (PROTHERO *et al.* 1988), is thus the least well supported relationship in this data set.

To further evaluate the robustness of the tree topology to assumptions about the mode of evolution and the monophyly of placentals, we included all nucleotide differences observed at first, second and third codon positions, the gene for *ND6* as well as the homologous sequences of chicken and frog in the analysis. Several maximum likelihood computations with varying values of the transition/transversion ratio and varying substitution ratios of first and second codon positions relative to the third codon positions were performed. In all cases, the same tree topology as in Figure 5 was found (Figure 6), demonstrating the astounding robustness of the result.

Placental phylogeny: The analyses above establish with a high degree of confidence that of the species analyzed here, primates and ungulates are sister taxa and that rodents represent an early divergence among placentals. This is in sharp contrast to the view of placental evolution which regards primates and rodents as sister taxa to the exclusion of ungulates (ROMER 1966; KIELAN-JAWOROWSKA *et al.* 1979; YOUNG 1981; LI *et al.* 1987) but agrees with the opinion of some morphologists (MCKENNA 1975; SZALAY 1977) as well as trees obtained from nuclear-encoded protein genes (EASTEAL 1988, 1990, 1992; LI *et al.* 1990). Thus, both organellar and nuclear molecular data agree in the establishment of rodents as an outgroup to primates and ungulates.

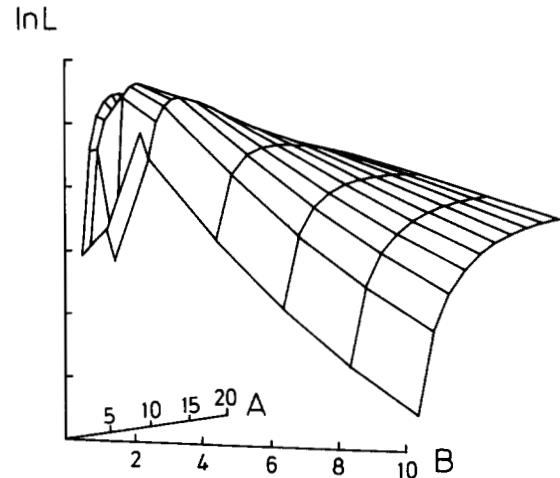


FIGURE 6.—Approximate maximum likelihood surface for different transition/transversion ratios (A) and different ratios of substitution rates for first and second *vs.* third codon positions (B) using all differences at all positions of 13 protein-coding genes from the species used in Figure 5 as well as the frog and the chicken sequences. The maximum value of the surface is in the neighborhood of (1, 5).

In the tree emerging from the mitochondrial data, the seal groups significantly with the cow and the whale thus associating carnivores and ungulates in a monophyletic group. In his classification of mammals, SIMPSON (1945) suggested that carnivores and ungulates were monophyletic and joined them in the group Ferungulata, which, however, did not include whales. The mitochondrial data confirm the view that carnivores and ungulates share a common ancestor and thus that Ferungulata represents a natural taxon to which cetaceans should be added.

Evolutionary rates of protein coding genes: The elucidation of the phylogeny of placentals has important consequences for the understanding of molecular evolution. Using artiodactyls as an outgroup to primates and rodents, it has been argued that the rate of molecular evolution is twice as fast on the lineage to rodents (WU and LI 1985). However, since artiodactyls rather than rodents appear to be the sister taxon of primates, the acceleration on the lineage to rodents may be absent (EASTEAL 1988; GU and LI 1992) or much less pronounced.

To test whether the rate of evolution is similar in various placental lineages, we investigated if the molecular evolution of the 13 protein-coding genes conforms to a molecular clock model. Due to differences in nucleotide composition third positions were excluded from the analyses. Maximum likelihood estimates were computed for the tree structure in Figure 5 under the assumption of identical evolutionary rates in all parts of the tree (clock assumption) as well as without such an assumption. A likelihood ratio test was applied to test whether the non-constrained tree resulted in a significantly better fit to the data. Table 3 shows that the clock assumption cannot be rejected in either first or second codon

TABLE 3
Clocktest for mitochondrial genes

Gene	Codon position	
	1	2
<i>ATP6</i>	10.53	17.54*
<i>ATP8</i>	14.14	2.27
<i>CO1</i>	16.22*	18.90*
<i>CO2</i>	24.09*	35.07*
<i>CO3</i>	10.80	11.05
<i>CYTb</i>	15.23*	24.22*
<i>ND1</i>	3.10	13.04
<i>ND2</i>	1.18	9.25
<i>ND3</i>	11.41	5.88
<i>ND4</i>	16.00*	4.62
<i>ND4L</i>	4.49	11.51
<i>ND5</i>	7.51	8.65
<i>ND6</i>	6.86	7.89

Likelihood ratio statistic for the first and second positions of 13 protein-coding genes. The analysis is based on the tree in Figure 5. Asterisks denote significant deviations from the clock assumption. The genes in boldface are clock-like for both first and second codon positions. The test is based on a chi square distribution with 5 degrees of freedom and a significance level of $\alpha = 0.01$.

positions for eight of the 13 genes. Thus, for the majority of mitochondrial genes a clock assumption is valid. For three of the genes, the clock is rejected for first and second positions. Most strongly, this is the case for the *cytochrome oxidase 2* gene which shows a clear acceleration on the lineage leading to humans. This is in agreement with a previous observation that this gene evolved at an increased tempo during early primate evolution (RAMHARACK and DEELEY 1987). The genes for *cytochrome b* and *cytochrome oxidase 1* also fail to conform to a clock model at first as well as second positions. The former gene has been shown to be accelerated in primates (MA *et al.* 1993; IRWIN and ARNASON 1994) and at least for first codon positions, this is seen also in the current data.

Divergence dates: The eight genes that conform to a clock model within placental lineages (Table 3) were used to estimate the approximate times of divergence of the placental radiation. As a calibration point the divergence between placental and marsupial mammals was used. The paleontological record shows that by the early late Cretaceous, approximately 95 million years ago, the placental and marsupial lineages were well separated (CIFELLI and EATON 1987). Based on this, a date of 130 million years for the marsupial-placental divergence is commonly assumed (CARROLL 1988) and agrees with a date derived from comparisons of globin sequences (AIR *et al.* 1971). When this date is used as a calibration point, the average dates given in Table 4 are obtained. It is worth noting, that even if the calibration date would be shown to be wrong, the relative timing of the divergences would remain the same.

These divergence dates are in general agreement with paleontological data as well as previous molecular investigations with the exception of the divergence between the mouse and rat lineages. Paleontological data

TABLE 4
Tentative dates for divergences among placental lineages

Lineage divergence	Date
Rodents, ferungulates and primates	114 ± 15
Primates, ferungulates	93 ± 12
Carnivores, artiodactyles	55 ± 15
Cow, whale	41 ± 10
Mouse, rat	35 ± 17

Approximate dates of divergence were calculated using 1st and 2nd codon positions of each of the genes shown to evolve in a clock like manner in Table 3. As a calibration point, 130 million years for the divergence between placental and marsupial mammals was used. Means of the dates and one standard deviation are given.

suggest that the mouse and rat lineages diverged 8–12 million years ago (JAEGER *et al.* 1986). A study using quantitative immunological comparisons of albumin have arrived at dates between 20 and 35 million years ago (WILSON *et al.* 1977; SARICH 1985) and studies (*e.g.*, BROWNELL 1983) based on DNA/DNA hybridizations have arrived at a date of 17–25 million years ago. The date inferred from the tree analyses above remains approximately the same when the calculations are based on observed transversions at first and second codon positions. Thus, the divergence between mouse and rat may be substantially older than the current interpretation of the fossil record indicates. In particular, the fossil taxon *Antemus* which is generally thought to represent a common lineage leading to mice and rats (CATZEFLIS *et al.* 1992), may belong to one or the other of these lineages or possibly to neither. Interestingly, the morphological characters used to define the murine group and this fossil have recently been suggested to have evolved convergently among muroid rodents (CHEVRET *et al.* 1993).

It is noteworthy that the times of divergence of the rodent, primate, carnivore and ungulate lineages range from 41 to 114 million years ago. This is in contrast to the traditional view of placental evolution, which assumes a rapid radiation following a presumed catastrophic event causing the extinction of dinosaurs at the end of the Cretaceous. The fact that the mitochondrial sequence data are able to resolve the divergence of the placental groups as well as the tentative dates of these divergences may indicate that the evolution of early placentals was not bush-like but rather might have taken place during several tens of millions of years prior to the Cretaceous-Tertiary boundary.

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