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Mammalian Mitochondrial DNA Evolution: A Comparison of the Cytochrome *b* and Cytochrome *c* Oxidase II Genes

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Abstract. The evolution of two mitochondrial genes, cytochrome *b* and cytochrome *c* oxidase subunit II, was examined in several eutherian mammal orders, with special emphasis on the orders Artiodactyla and Rodentia. When analyzed using both maximum parsimony, with either equal or unequal character weighting, and neighbor joining, neither gene performed with a high degree of consistency in terms of the phylogenetic hypotheses supported. The phylogenetic inconsistencies observed for both these genes may be the result of several factors including differences in the rate of nucleotide substitution among particular lineages (especially between orders), base composition bias, transition/transversion bias, differences in codon usage, and different constraints and levels of homoplasy associated with first, second, and third codon positions. We discuss the implications of these findings for the molecular systematics of mammals, especially as they relate to recent hypotheses concerning the polyphyly of the order Rodentia, relationships among the Artiodactyla, and various interordinal relationships.

Key words: Cytochrome *b* — Cytochrome *c* oxidase II — Mammals — Mitochondrial DNA — Molecular evolution

Introduction

The mitochondrial cytochrome *b* (COB) and cytochrome *c* oxidase subunit II (COII) genes have been used in several recent molecular systematic studies of rodents (DeWalt et al. 1993; Ma et al. 1993; Thomas and Martin 1993), ungulates (Irwin et al. 1991; Irwin and Wilson 1992; Miyamoto et al. 1994), marine mammals (Irwin and Arnason 1994), primates (Ruvolo et al. 1991; Disotell et al. 1992; Adkins and Honeycutt 1994), and eutherian mammal orders (Adkins and Honeycutt 1991, 1993; Honeycutt and Adkins 1993). The COB gene, in particular, has been used extensively in the investigation of systematic relationships among vertebrates, and the COII gene studies have focused primarily on primates and their presumed relatives. In this paper we examine patterns of nucleotide sequence variation in the COB and COII genes, with an emphasis on determining relationships within and among mammalian orders. New nucleotide sequence data for the COII gene are combined with existing information from both COB and COII, and the similarities and differences among resultant gene phylogenies are evaluated. In addition, rates of nucleotide substitutions, differences in codon usage, and base composition bias are examined for both genes, using representative taxa from the mammalian orders Rodentia and Artiodactyla.

Materials and Methods

Mitochondrial Genes Examined. A total of 35 COB genes and 37 COII genes were examined for six orders of eutherian mammals as well as

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one metatherian order (Table 1). In the case of the new COII sequences reported in this paper, mitochondrial DNA (mtDNA) was isolated using cesium chloride/propidium iodide gradient centrifugation (Brown 1980), and the entire COII gene was amplified with primers H8320 and L7553 (see Adkins and Honeycutt 1994) by the polymerase chain reaction (PCR) using the parameters 95°C denaturation (1 min), 45°C annealing (1 min), and 72°C extension (1.25 min) for 30 cycles. The amplified COII genes were either sequenced directly using single-stranded PCR products (Allard et al. 1991a) or the double-stranded product was ligated into the pBluescript plasmid and then sequenced. In both cases sequencing followed that of Kraft et al. (1988). Because of the inherent error rate of *Taq* polymerase (Saiki et al. 1988; Tindall and Kunkel 1988; Keohavang and Thilly 1989), at least two clones were sequenced for each taxon. In two cases (*Geomys, Perognathus*) a single sequence discrepancy was found. A third clone was sequenced in each case, and the base present in two clones was assumed to be correct.

Data Analysis

Phylogenetic analyses were performed by two methods—maximum parsimony as implemented by PAUP 3.1 (Swofford 1993) and neighbor-joining (Saitou and Nei 1987) as implemented by the MEGA program (Kumar et al. 1993). Maximum-parsimony analyses were conducted using both equal and unequal character weighting. Equal weighting consisted of all substitutions regardless of codon position being given equal weight. Unequal weighting schemes included the use of transversions only as well as a procedure whereby differential weights were assigned to each codon position (e.g., transversions only at third position, all substitutions at second position, and all substitutions at first position with changes involving leucine at the first position recoded as Y, the generic symbol for a pyrimidine). Neighbor-joining analyses were conducted using pairwise distance estimates based on several models (Jukes and Cantor 1969; Kimura 1980; Tajima and Nei 1984; Tamura and Nei 1993). In addition, gamma distances were estimated using MEGA (Kumar et al. 1993) for all the above models except Tajima and Nei (1984). We chose to examine relationships using these various distances in an effort to circumvent assumptions specific to any one model (e.g., rate of nucleotide substitution assumed to be the same for all sites, all nucleotide frequencies equal to 0.25, and no transition bias).

Support for individual nodes on a phylogenetic reconstruction was evaluated using both the bootstrap option (Felsenstein 1985) and the Bremer support index (Bremer 1988), the number of extra steps required to break up a clade. Tests for rate heterogeneity among divergent taxa were evaluated using a relative rate test (Mindell and Honeycutt 1990). Codon usage and base composition values were obtained by the sequence analysis program MacVector 3.5 (International Biotechnologies, Inc.) and MEGA (Kumar et al. 1993).

Results and Discussion

Interordinal Phylogenetic Comparisons

A total of 18 species of eutherian mammals were used in the interordinal comparisons, with a marsupial outgroup (either *Didelphis* or *Monodelphis*). These 18 species represented six eutherian orders including (1) Primates (*Homo* and *Galago*); (2) Carnivora (*Phoca*); (3) Lagomorpha (*Oryctolagus*): (4) Cetacea (*Balaenoptera*); (5) Rodentia (*Geomys, Cratogeomys, Sciurus, Cavia, Mus, Rattus,* and either *Hystrix* or *Georychus*); and (6) Artiodactyla (*Bos, Capra, Odocoileus, Antilocapra, Sus,* and either *Cervus* or *Dama*).

Table 1. Specimens examined

Taxa	COB[a]	COII[a]
Infraclass Eutheria		
Order Artiodactyla		
Family Antilocapridae		
Antilocapra americana	10	13
Family Bovidae		
Bos gaurus		18
Bos grunniens		18
Bos indicus		18
Bos javanicus		18
Bos taurus	3	3
Boselaphus tragocamelus		13
Bubalus depressicornis		18
Capra hircus	10	13
Damaliscus dorcas		18
Gazella spekei		18
Syncerus c. caffer		18
Syncerus c. nanus		18
Tragelaphus imberbis		18
Ovis aries	10	
Family Camelidae		
Camelus dromedarius	10	
Family Cervidae		
Cervus unicolor		13
Dama dama	10	
Odocoileus heminous	10	
Odocoileus virginianus		18
Family Giraffidae		
Giraffa camelopardalis	10	
Family Tragulidae		
Tragulus napu	10	
Family Suiidae		
Sus scrofa	10	18
Tayassu tajacu	10	
Order Carnivora		
Phoca vitulina	5	5
Order Cetacea		
Balaenoptera physalus	4	4
Order Lagomorpha		
Oryctolagus cuniculus	9	17
Order Primates		
Family Galagidae		
Galago crassicaudatus	16	
Galago senegalensis		1
Family Hominidae		
Homo sapiens	2	2
Order Rodentia		
Suborder Sciurognathi		
Family Geomyidae		
Cratogeomys c. castanops	7	18
Cratogeomys c. tamaulipensis	7	
Cratogeomys fumosus	7	
Cratogeomys goldmani	7	
Cratogeomys gymnurus	7	
Cratogeomys merriami	7	
Geomys bursarius	7	18
Pappogeomys bulleri	7	
Family Heteromyidae		
Perognathus flavus		18
Family Sciuridae		
Marmota flaviventris	15	
Sciurus caroliensis	15	18
Spermophilus columbianus	15	
Spermophilus lateralis	15	
Spermophilus richardsoni	15	
Spermophilus tridecemlineatus	15	

Table 1. Continued

Taxa	COB[a]	COII[a]
Family Muridae		
Acomys willsoni		18
Apodemus sylvaticus		18
Malacothrix typica		18
Meriones shawi		18
Microtus pennsylvanicus		14
Mus domesticus	6	6
Peromyscus banderanus		18
Rattus norvegicus	8	8
Suborder Hystricognathi		
Family Bathyergidae		
Georychus capensis		18
Family Caviidae		
Cavia apera		18
Cavia porcellus	12	
Family Hystricidae		
Hystrix africaeaustralis	12	
Infraclass Metatheria		
Didelphis virginiana		11
Monodelphis domestica	12	

[a] Numerical designations for sequences used: 1. Adkins and Honeycutt 1991. 2. Anderson et al. 1981. 3. Anderson et al. 1982. 4. Arnason et al. 1991. 5. Arnason and Johnsson 1992. 6. Bibb et al. 1981. 7 DeWalt et al. 1993. 8. Gadaleta et al. 1989. 9. Irwin and Arnason 1994. 10. Irwin et al. 1991. 11. Janke et al. 1994. 12. Ma et al. 1993. 13. Miyamoto et al. 1994. 14. Pumo et al. 1992. 15. Thomas and Martin 1993. 16. Anne Yoder, pers comm. 17. Genbank X64107. 18. This paper

The resultant COB and COII gene phylogenies, derived from the maximum parsimony and neighbor-joining analyses, had several features in common regardless of the weighting scheme and distance estimate chosen (Fig. 1 and Table 2). These features are pertinent to several recent issues concerned with ordinal-level relationships among eutherian mammals. First, several recent molecular studies (Graur et al. 1991; Li et al. 1992; Graur 1993; Ma et al. 1993), using both amino acid and nucleotide sequence data for a limited number of taxa, have not supported the monophyly of the order Rodentia, with the hystricognath rodents (especially the South American caviomorphs) representing a separate lineage from the sciurognath rodents (e.g., mice, rats, squirrels, etc.). We attempted to avoid the pitfalls of many earlier studies by increasing the number of rodent taxa in the analyses. The COB and COII gene phylogenies, however, have complicated the issue of rodent monophyly even further, with as many as four independently evolving lineages observed (Fig. 1). These molecular findings are totally incongruent with the morphological data, which strongly support rodent monophyly (Allard et al. 1991b; Luckett and Hartenberger 1993).

Second, the monophyly of the order Artiodactyla was not supported by either gene. The COII gene placed the order Cetacea closer to ruminants than *Sus*, a nonruminant artiodactyl, and several COB gene analyses revealed a sister-group relationship between cetaceans and *Sus* (Fig. 1 and Table 2). Both genes also suggested a sister-group relationship between the order Carnivora and the Artiodactyla/Cetacea clade, which included *Sus*. These findings are congruent with several independent studies including a detailed analysis by Graur and Higgins (1994) that examined both nuclear and mitochondrial genes and an examination of several complete mitochondrial genomes of mammals (Arnason and Johnsson 1992; Honeycutt and Adkins 1993). Although the placement of Carnivora is somewhat incongruent with morphological evidence, the association of the cetaceans as sister to ruminant artiodactyls to the exclusion of nonruminants is not contradicted by morphological evidence (Honeycutt and Adkins 1993; Graur and Higgins 1994).

Third, recent morphological evidence (Novacek 1992; Luckett and Hartenberger 1993) and at least one molecular study of mitochondrial genes from four orders of mammals (Pesole et al. 1991) support a monophyletic Glires, a superorder containing Rodentia and Lagomorpha (rabbits). The COB and COII gene trees do not support these findings and are consistent with a large number of other molecular studies that find no support for the monophyly of Glires (reviewed by Honeycutt and Adkins 1993). In fact, several of these molecular studies, including one total evidence tree for COB and COII (transversion only analysis), support a closer relationship between rabbits and primates. The difference between these findings and the study of Pesole et al. (1991) could relate to the number of taxa examined and the methods of analysis chosen.

The COB and COII gene phylogenies also revealed somewhat different relationships among several taxa, and the differences depended upon the gene, character weighting scheme, and method of analysis chosen (Fig. 1 and Table 2). For example, the COB gene did not support the monophyly of the Artiodactyla family Cervidae (represented by *Dama* and *Odocoileus*), whereas the COII gene (represented by *Cervus* and *Odocoileus*) did. Most analyses (equal weighting, transversion only, and neighbor joining) of the COB gene failed to support monophyly of Primates, while the COII gene consistently supported monophyly. The COB gene supported the monophyly of the rodent suborder Hystricognathi (represented by *Cavia* and *Hystrix*) and the COII gene (represented by *Cavia* and *Georychus*) did not. A total evidence approach also was used to evaluate phylogenetic relationships. In this approach both genes were combined, using the taxa held in common, in an effort to use an unpartitioned set of synapomorphies (Kluge 1989; Eernisse and Kluge 1993). The overall results from this analysis were similar to those seen for the two genes analyzed separately, with neither gene having a stronger influence over the other (Table 2). However, unlike the two genes analyzed separately, the monophyly of the artiodactyl/cetacean clade (most analyses), rodent superorder Hystricognathi, and order Primates was supported. The monophyly of Cervidae was not supported. Again,

Table 2. Phylogenetic relationships derived from either neighbor joining (NJ) using Kimura (1980) corrected distances or maximum parsimony (MP) using total substitutions with equal weighting (equal), transversions only (TV), and differential weighting (unequal) at each codon position

	MP equal		MP TV only		MP unequal		MP total[b]			NJ	
Phylogenetic conclusions[a]	COII	COB	COII	COB	COII	COB	Equal	TV	Unequal	COII	COB
Primate monophyly	+	−	+	−	+	+	+	+	+	+	−
Rodent monophyly	−	−	−	−	−	−	−	−	−	−	−
Hystricognathi	−	+	−	+	−	+	+	+	+	−	+
Sciurognathi	−	−	−	−	−	−	−	−	−	−	−
Muridae	+	+	+	+	+	+	+	+	+	+	+
Geomyidae	+	+	+	+	+	+	+	+	+	+	+
Artiodactyl monophyly	−	−	−	−	−	−	−	−	−	−	−
Bovidae	+	+	+	+	+	+	+	+	+	−	+
Cervidae	+	−	+	−	+	−	?	−	−	+	−
Sus Divergent	+	+	+	+	+	+	+	+	+	+	+
Cetacean sister group											
Ruminants only	+	−	+	−	+	−	?	+	+	+	−
Carnivores and artiodactyls	−	−	−	−	−	−	?	−	−	−	−
Sus	−	−	−	+	−	+	?	−	−	−	+
Carnivora	−	+	−	−	−	−	?	−	−	−	−
Carnivora sister group											
Antilocapra	+	−	−	−	−	−	−	−	−	−	−
Cetacea	−	+	−	−	−	−	?	−	−	−	−
Artiodactyla and Cetacea	+	−	?	+	+	+	?	+	+	−	+
Sus	−	−	−	−	−	−	?	−	−	+	−
Lagomorpha sister group											
Rodentia	+/−	+/−	?	−	+/−	−	+/−	−	+/−	−	−
Primates	−	−	−	+/−	−	+	−	+	−	−	+/−

[a] The plus (+) means relationship supported, minus (−) indicates no support, +/− implies a relationship with some but not all taxa in an order, and ? indicates that the relationship is unresolved

[b] Each gene was analyzed separately and combined as total evidence (MP total)

the order Rodentia was found to be polyphyletic (four independently evolving lineages), with the geomyoid genera *Geomys* and *Cratogeomys* being the most divergent eutherian taxa (Fig. 1).

Intraordinal Phylogenetic Comparisons

Using the assumption that the order Rodentia and the Cetacea/Artiodactyla clade represent monophyletic groups, relationships among respective rodent and artiodactyl taxa were reexamined. Two approaches were used in this investigation. First, phylogenetic analyses on the same rodent and artiodactyl taxa, examined in the interordinal comparisons, were conducted using both maximum parsimony (an exhaustive search with either equal or unequal weighting) and neighbor joining. These analyses were done for each gene separately as well as the two genes combined. Second, patterns of variation in artiodactyl and rodent COB and COII genes were examined in reference to an independent phylogeny derived from an examination of other characters, including molecular and morphological (Figs. 2 and 3). For each codon position the retention index (Farris 1989), mean number of steps (average number of changes along each branch), and number of positions that were potentially informative in a cladistic sense (positions exhibiting at least two states with at least two taxa possessing each of the alternate states) were estimated by fitting characters on the reference phylogeny. The retention index was selected because it is insensitive to the number of taxa in a data set (Archie 1989) yet provides a measure of homoplasy. Although the COB and COII data sets for artiodactyls and rodents were not identical in terms of taxa examined, the taxa used in the comparisons spanned approximately the same range of divergence times (Table 3, Figs. 2 and 3) and represented an overall increased number from those examined in the phylogenetic analyses. We realize that relationships involving some taxa in these phylogenies may be equivocal but the overall patterns observed for each order and gene were not influenced, to any large extent, by swapping individual branches in the tree topologies.

The COB and COII gene trees revealed problems similar to those seen in the interordinal comparisons (Fig. 4; see Table 1 for groups examined). In the case of rodents, the COII gene tree was less congruent with morphological data than that seen for COB. For example, the COB gene supported the monophyly of the suborder Hystricognathi (*Cavia* and *Hystrix*) and a sister-group relationship between the *Sciurus* and the *Geomys/Cratogeomys* clade (Fig. 4A). Both of these results are congruent with most ideas based on morphology. The COII gene tree did

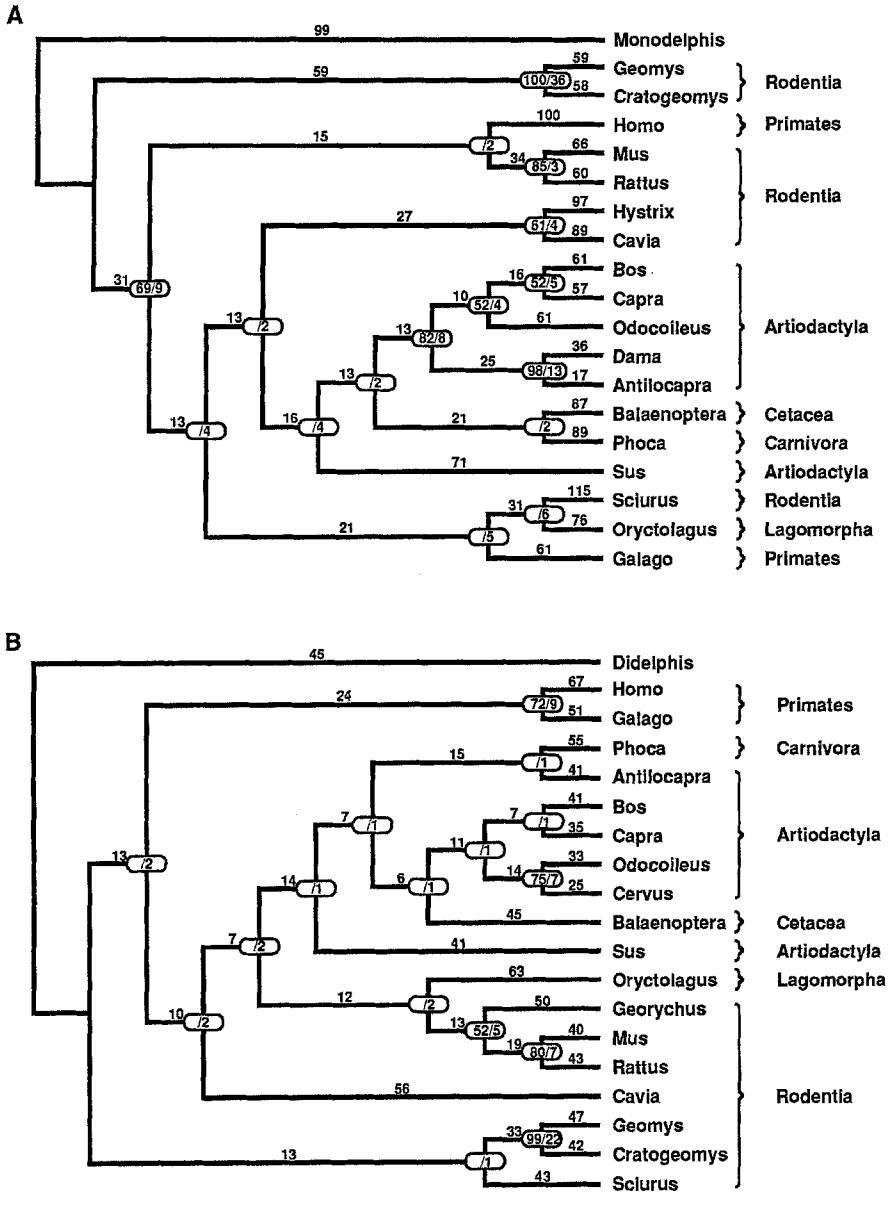

Fig. 1. Phylogenetic relationships among eutherian mammals based on maximum parsimony (**A**) of the COB gene (length = 1,540, retention index 0.311) and (**B**) COII gene (length = 2,557, retention index = 0.322). Both trees were derived using equal weighting of total substitutions. Trees were constructed using 100 replications of the heuristic tree-bisection-reconnection search in PAUP, with the addition of taxa randomized. Inferred nucleotide changes are shown along each branch, and bootstrap values 50% or greater (derived from 100 replicates) are enclosed in *circles* with the Bremer support indices separated from these values by a *diagonal line* (/).

not support either of these results, even when maximum parsimony with unequal character weighting was used (Fig. 4C). Neighbor-joining (not shown) also did not alter these results. The total evidence analysis for rodents was identical to the COB gene tree, and none of the analyses including the total evidence tree supported a monophyletic Sciurognathi (Fig. 4E). While the COII gene tree provided less resolution among the rodents, the COB gene tree provided less resolution for relationships among artiodactyls and cetaceans. The monophyly of Cervidae was not supported by the COB gene, and the placement of cetaceans relative to ruminants was unresolved (Fig. 4B). Both the total evidence and COII gene trees, however, favored a monophyletic Cervidae and a sister-group relationship between Cetacea and ruminants, with *Sus* being basal (Fig. 4D and F).

The level of homoplasy for both the COII and COB genes differed with respect to codon position and the taxa examined (Table 4). Rodents showed a higher level of homoplasy at the third position of the COII gene than that seen for artiodactyls, as well as an overall higher number of phylogenetically informative sites at the second position. Both orders showed a low retention index at the first position in COII. Artiodactyls showed a higher level of homoplasy at all positions in the COB gene than seen with rodents, especially at the first and second codon positions. The pattern revealed by artiodactyls and rodents did not change when the topologies in Figs. 2 and 3 were modified by rearranging individual lineages. This suggests that minor modifications of the accepted phylogeny do not alter the level of homoplasy seen for each gene tree relative to the presumed species trees. There are at least two explanations for these results, and they are not mutually exclusive. First, some of the differences in overall homoplasy observed between rodents and artiodactyls may be the result of differences

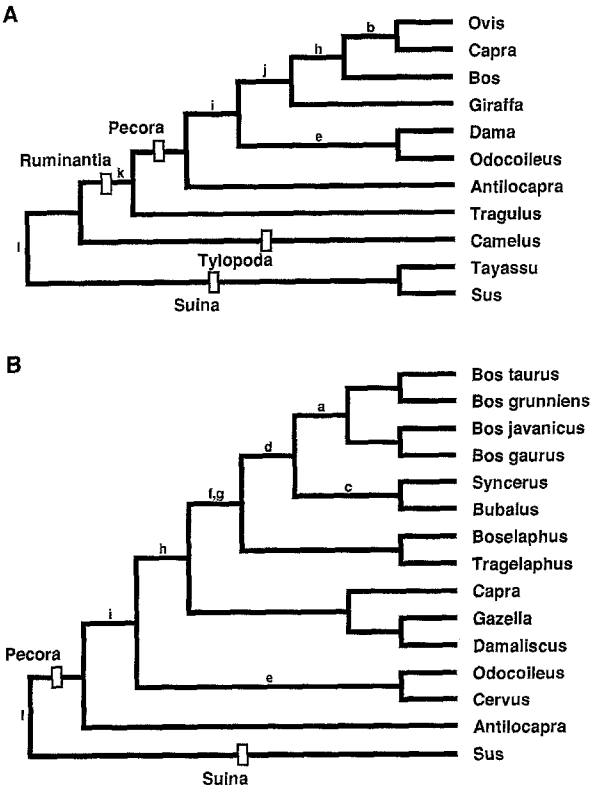

Fig. 2. Reference phylogenies for artiodactyls used to evaluate homoplasy in the **(A)** COB and **(B)** COII genes. These phylogenies are based on several molecular and morphological studies including Janis and Scott (1987), Kraus and Miyamoto (1991), Allard et al. (1992), Gentry (1992), and Miyamoto et al. (1993, 1994). The letters (*a–l*) on branches refer to the divergence dates shown in Table 3.

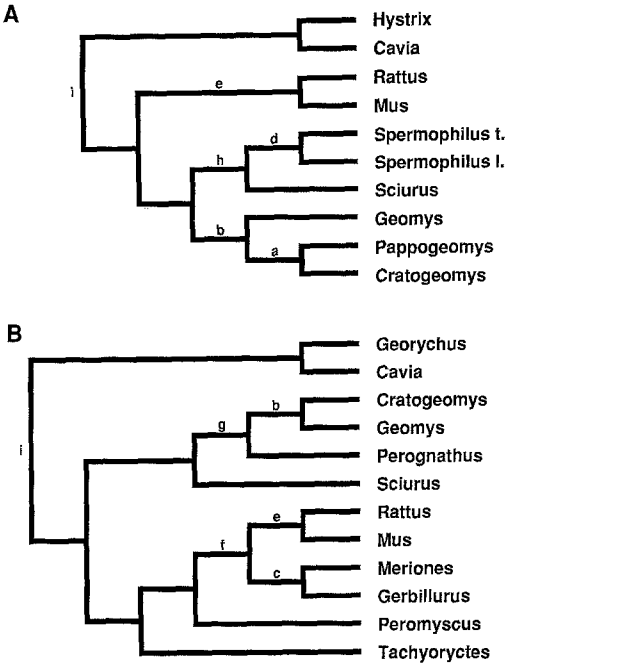

Fig. 3. Reference phylogenies for rodents used to evaluate homoplasy in the **(A)** COB and **(B)** COII genes. These phylogenies are based on current ideas of rodent relationships. (See references in Carleton and Musser 1984; Luckett and Hartenberger 1985; Catzeflis et al. 1992, 1993.) The letters (*a–i*) on branches refer to the divergence dates in Table 3.

in the average divergence time separating taxa used in each phylogeny. In this case closely related taxa would show less homoplasy than more divergent taxa, especially with respect to the degree of saturation effects at third position and the overall amount of change at the first two positions. This cannot totally explain the observations because the patterns of change at the three codon positions for both COB and COII differ within orders. Second, the different patterns of homoplasy may be the result of different levels of selective constraints on the artiodactyl and rodent COII and COB genes. This partially explains differences between genes within orders. The phylogenetic differences observed for the two genes may be the net result of the overall patterns of homoplasy and number of informative characters at each codon position seen for rodent and artiodactyl COB and COII gene evolution.

Processes of Molecular Evolution

Transition/Transversion Ratios

A bias toward transitions over transversions, especially at lower levels of divergence, has been observed for mammalian mtDNA (Brown et al. 1982; Aquadro et al. 1984). Nevertheless, as indicated in a recent comparison of the 12S rRNA gene in primates, rodents, and artiodactyls, the transition/transversion ratio relative to genetic divergence can vary among orders (Allard and Honeycutt 1992). A comparison of transition/transversion ratios relative to Jukes and Cantor (1969) distances between pairs of taxa revealed differences among rodent and artiodactyl COII and COB genes. At similar levels of COB gene divergence, rodents showed a higher transition/transversion ratio than that seen in artiodactyls, whereas artiodactyls revealed a higher ratio with the COII gene. The ratio stabilized in both orders at approximately 20% to 30% divergence for COB gene and 20% divergence for the COII gene. The cause of the observed taxonomic differences in transition/transversion ratios for these two genes is unknown. Either different mutation biases exist in regions under different structural constraint or a highly biased substitution process, in part due to selective constraints on the molecule, may be partially responsible (Allard and Honeycutt 1992). The evaluation of homoplasy found for each gene relative to an accepted phylogeny of rodents and artiodactyls (Table 4) did reveal substitution differences among the three codon positions, and these differences are suggestive of variation in selective constraint.

Tests for Rate Heterogeneity

Rate heterogeneity in terms of nucleotide substitutions and amino acid replacements has been found for both nuclear and mitochondrial genes in mammals, sug-

Table 3. Approximate divergence dates for rodents and artiodactyls

Comparison	Node[a]	Divergence time (Myr)[b]	Reference
Rodentia			
Pappogeomys/Cratogeomys	a	4 (3–5)	Russell (1968b)
Geomys/Cratogeomys	b	6 (5–7)	Russell (1968a)
Gerbillurus/Meriones	c	6	Catzeflis et al. (1993)
Spermophilus t./S. l.	d	10 (9–12)	Black (1963)
Mus/Rattus	e	11 (10–12)	Catzeflis et al. (1992)
Gerbillinae/Murinae	f	15–23	Catzeflis et al. (1992)
Geomys/Perognathus	g	30	Green and Bjork (1980)
Sciurus/Spermophilus	h	30	Black (1963)
Hystricognathi/Sciurognathi	i	55	Flynn et al. (1986)
Artiodactyla			
Bos taurus/Bos gaurus	a	2	Pilgrim (1947)
Capra/Ovis	b	5 (4–10)	Savage and Russell (1983)
Bubalus/Syncerus	c	5	Savage and Russell (1983)
Bos/Syncerus	d	10	Savage and Russell (1983)
Odocoileus/Cervus & Dama	e	10.5 (9–12)	Miyamoto et al. (1990)
Bos/Boselaphus	f	15	Savage and Russell (1983)
Bos/Tragelaphus	g	20	Savage and Russell (1983)
Bos/Capra	h	20	Savage and Russell (1983)
Bovidae/Cervidae	i	25	Savage and Russell (1983)
Bovidae/Giraffidae	j	25	Savage and Russell (1983)
Pecorans/*Tragulina*	k	45	Kraus and Miyamoto (1991)
Ruminantia/*Sus*	l	55 (50–65)	Savage and Russell (1983)

[a] Nodes are identified on Figs. 2 and 3

[b] Date not in parentheses used for plots in Fig. 5

gesting a lack of a global molecular clock for mammals (Wu and Li 1985; Britten 1986; Li et al. 1987; Bulmer et al. 1991; Holmes 1991; Ma et al. 1993; Martin and Palumbi 1993; Adkins and Honeycutt 1994; Irwin and Arnason 1994). As suggested earlier, excessive rate heterogeneity may influence phylogenetic results. The new sequence data presented here allow for a more extensive examination of rate heterogeneity within and between several orders of mammals, especially the orders Rodentia and Artiodactyla. Two approaches were used to examine rate differences for the COII and COB genes. First, both interordinal and intraordinal pairwise comparisons were made, using either total substitutions or third position transversions, and deviations from rate homogeneity were tested using the relative rate test of Mindell and Honeycutt (1990). Second, the rate of nonsynonymous and synonymous substitutions for both artiodactyl and rodent COB and COII genes was determined in reference to known divergence times.

The relative rate tests revealed several cases of rate heterogeneity, especially with respect to total substitutions (Table 5). First, primates had a faster rate of total substitutions in the COII gene relative to all orders, and the primate COB gene was faster relative to muroid rodents (*Mus*) and carnivores (*Phoca*). Within the order Primates the rate of total substitutions for both the COII and COB genes was higher in anthropoid primates (represented by *Homo*) in comparison to prosimian primates (represented by *Galago*). Although there is little nucleotide sequence data for the primate COB gene, these results suggest that the overall pattern of primate COB gene evolution may be similar to that seen for primate COII. As suggested in previous studies, the rate increase in the COII gene observed in primates may relate to an increased rate in the nuclear cytochrome *c* gene of primates, and it now appears that the COB gene, which also interacts with cytochrome *c,* demonstrates a somewhat similar pattern to COII (Ma et al. 1993; Adkins and Honeycutt 1994; unpublished data). Second, within artiodactyls the genus *Bos* (cow) revealed a slower rate of total substitutions than that seen in several other lineages, and unlike the correlation between body size and rate of COB evolution observed by Martin and Palumbi (1993), the *Bos* rate was slower than that of the whale. In addition, both the COII and COB genes revealed a slower rate of total nucleotide substitutions in the artiodactyl genus *Antilocapra* relative to cervids. Finally, most studies of rodent nuclear gene evolution have suggested rate homogeneity among rodent lineages (Bulmer et al. 1991; O'hUigin and Li 1992). Nevertheless, both the COII and COB genes revealed a faster rate of total substitutions in geomyoid rodents, represented by *Geomys* and *Cratogeomys,* than that seen for muroid rodents, *Mus* and *Rattus,* confirming the observations of DeWalt et al. (1993) based on COB.

In addition to the relative rate tests, substitution rate differences for the COB and COII genes were examined by plotting the number of substitutions at both synonymous and nonsynonymous sites over a range of divergence times for pairs of artiodactyl and rodent taxa. Both the COB and COII genes of rodents in comparison to artiodactyls showed a higher rate of substitution at syn-

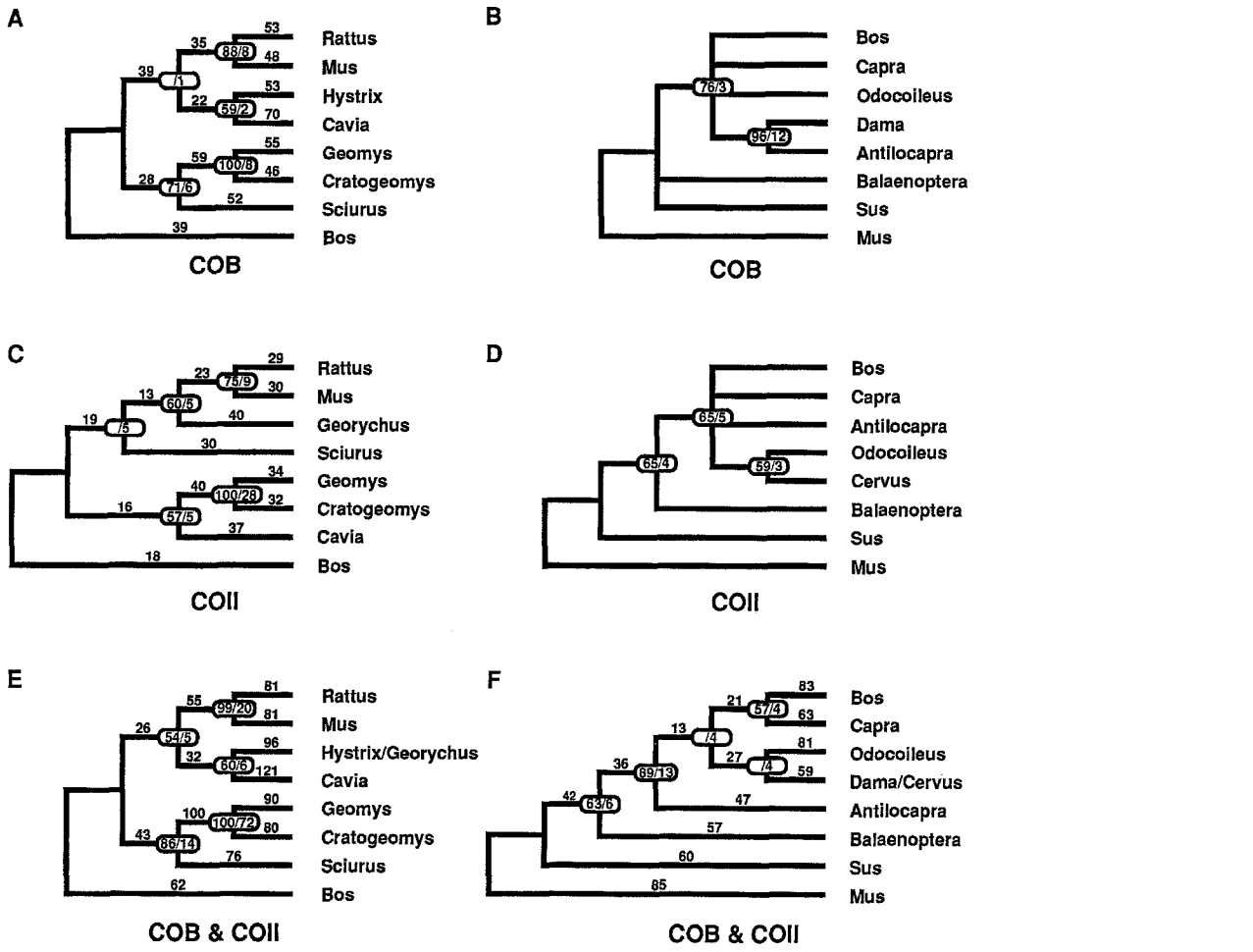

Fig. 4. Maximum parsimony trees derived using the exhaustive search option in PAUP. Trees were constructed using total substitutions and equal weighting for the COB genes of **(A)** rodents (length = 1,005, retention index = 0.379) and **(B)** artiodactyls (length = 622, retention index = 0.351) and the COII genes of **(C)** rodents (length = 590, retention index = 0.378) and **(D)** artiodactyls (length = 382, retention index = 0.314). Both genes were combined to construct a total evidence tree for **(E)** rodents (length = 1,600, retention index = 0.374) and **(F)** artiodactyls (length = 1,020, retention index = 0.313). Numbers along branches represent inferred nucleotide changes. Trees **B** and **D** represent strict consensus trees derived from three and four equally parsimonious trees, respectively. Bootstrap values 50% or greater (based on 100 replicates) are *encircled* and separated from the Bremer support indices by a *diagonal (/) line.*

Table 4. Homoplasy at different codon positions for artiodactyl and rodent COII and COB genes in reference to the phylogenies in Figs. 2 and 3

	First position			Second position			Third position		
Comparison	RI[a]	Informative positions[b]	Mean no. steps	RI[a]	Informative positions[b]	Mean no. steps	RI[a]	Informative positions[b]	Mean no. steps
COII									
Artiodactyla	0.30	31	3.32	—	0	—	0.41	167	2.76
Rodentia	0.35	63	3.01	0.67	15	2.33	0.23	190	4.09
COB									
Artiodactyla	0.33	57	2.74	0.31	18	2.39	0.28	248	3.29
Rodentia	0.56	104	2.53	0.78	43	1.65	0.34	284	3.34

[a] Retention index

[b] Only positions informative in a cladistic sense are considered

onymous and nonsynonymous sites (Fig. 5). This result is similar to that found for synonymous and nonsynonymous rates in the nuclear genes of rodents and artiodactyls (Li et al. 1987; Bulmer et al. 1991). In the case of the COII gene, synonymous sites were saturated in rodents, and this observation is consistent with the high levels of homoplasy at the third position of the COII gene found in rodents relative to artiodactyls (Table 4).

Table 5. Results of relative rate tests

Comparison	Total substitutions[a]		Third position transversions	
	COII	COB	COII	COB
Rodentia[b]				
Mus/Geomys	0.0176*	0.0546*	0.4495	0.1041
Artiodactyla[c]				
Bos/Balaenoptera	0.4668	0.0469*	0.1802	0.3285
Bos/Tragulus	—	0.0503*	—	0.4050
Bos/Camelus	—	0.0049*	—	0.0251*
Antilocapra/Dama	—	0.0363*	—	0.5000
Antilocapra/Cervus	0.0535*	—	0.4159	—
Primates[d]				
Homo/Galago	0.0310*	0.0000*	0.3518	0.1841
Interordinal[e]				
Homo/Bos	0.0006*	0.0992	0.3101	0.5000
Homo/Mus	0.0001*	0.0005*	0.1108	0.2376
Homo/Phoca	0.0018*	0.0419*	0.0103*	0.4234
Homo/Lagomorpha	0.0033*	0.0686	0.0556*	0.2773
Galago/Mus	0.0095*	0.0624	0.3974	0.2810
Galago/Bos	0.0323*	0.3406	0.1528	0.4602
Lagomorpha/*Mus*	0.1649	0.0312*	0.4478	0.0878
Lagomorpha/*Geomys*	0.4722	0.5000	0.5531	0.0335*
Phoca/Bos	0.4063	0.3514	0.0178*	0.3718
Phoca/Mus	0.2262	0.0540*	0.1358	0.3439

[a] Probability values obtained using the binomial distribution of Mindell and Honeycutt (1990), with asterisk (*) indicating significant or nearly significant values at p = .05.
[b] *Cavia* used as outgroup
[c] *Phoca* used as outgroup
[d] *Bos* used as outgroup
[e] *Didelphis* or *Monodelphis* used as outgroup

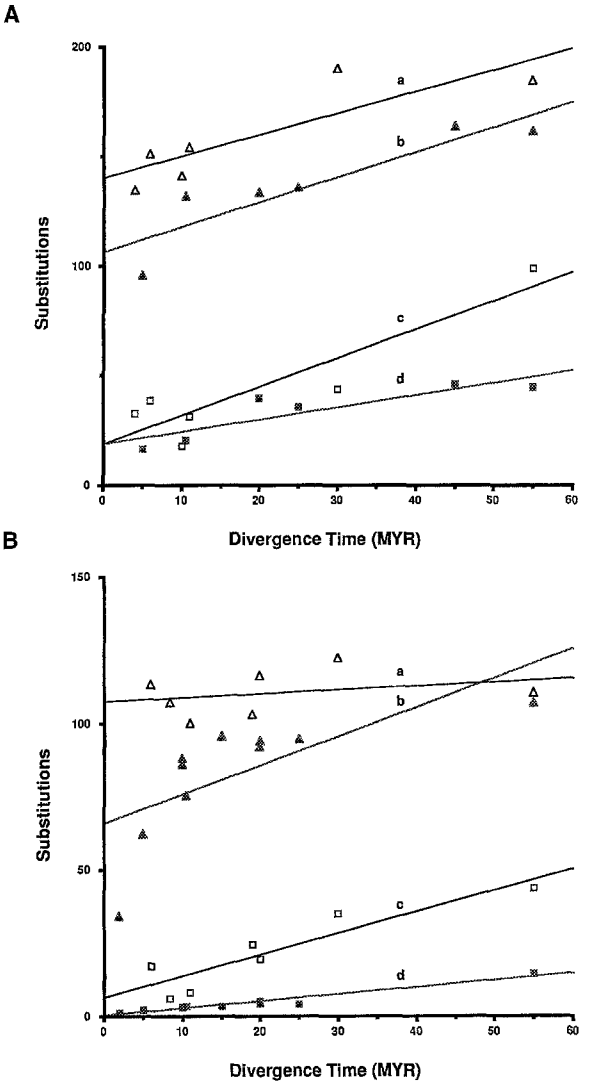

Fig. 5. Plots of nucleotide substitutions for the **(A)** COB and **(B)** COII. Substitutions at both **(a,b)** synonymous and **(c,d)** nonsynonymous sites for pairwise comparisons were plotted relative to divergence time. Each *point* represents the average of all possible pairwise comparisons, with the divergence times for these comparisons shown in Table 3. *Open and closed triangles* represent synonymous substitutions in rodents and artiodactyls, respectively. *Open and closed squares* represent nonsynonymous substitutions for rodents and artiodactyls, respectively.

The COB gene of both rodents and artiodactyls demonstrated a higher rate of nonsynonymous substitutions than the COII gene, and as can be seen in Table 4, the overall number of informative sites at the first, second, and third positions is higher in the COB gene of both rodents and artiodactyls.

Base Composition and Codon Usage

Base composition at each of three codon positions was calculated from the nucleotide sequence data (all rodents and artiodactyls in Table 1), and the index of compositional bias (Irwin et al. 1991), which measures deviation from an equal (25%) frequency of each nucleotide, was estimated. In general, both the rodent and artiodactyl COII and COB genes showed a similar pattern, with base composition bias being greater at the third and lowest at the first codon positions (Table 6). At the second position of both the COB and COII genes, there is a bias toward thymine at the expense of guanine, and in the COII gene adenine is somewhat higher than cytosine, with the opposite observed for the COB gene. The COB gene shows an overall higher level of composition bias at the third codon position, and in both genes the greatest asymmetry is between adenine (high) and guanine (low). In addition, the frequency of cytosine at the third position of COII is somewhat lower than that observed for COB. The overall level of heterogeneity in base composition was higher at the third position and lowest at second position, as can be seen by the increased level of coefficients of variation associated with estimates of base frequencies. The increased heterogeneity has a taxonomic basis in that individual lineages and groups of taxa differ in base composition, with rodents demonstrating a greater amount of heterogeneity. These increased levels of heterogeneity, as a result of taxonomic variation at the third codon position in the COB and COII genes, are similar to patterns reported for primate COII (Adkins and Honeycutt 1994) and ungulate COB (Irwin et al. 1991), and one would expect to see this

Table 6. Base composition at first, second, and third codon positions

	First codon position				Second codon position				Third codon position			
Comparison[a]	A	C	G	T	A	C	G	T	A	C	G	T
COB												
Artiodactyla[b]	0.295	0.258	0.220	0.218	0.200	0.241	0.135	0.416	0.434	0.358	0.043	0.157
CV[c]	4.60	4.91	4.27	5.45	1.82	2.99	1.95	2.01	4.77	6.91	37.32	19.08
Index[d]		(0.077)				(0.227)				(0.395)		
Rodentia	0.280	0.248	0.215	0.252	0.204	0.239	0.139	0.415	0.422	0.306	0.032	0.237
CV	3.35	4.09	2.91	3.47	2.30	2.68	3.16	2.40	11.05	12.46	37.02	18.49
Index		(0.046)				(0.222)				(0.321)		
COII												
Artiodactyla	0.296	0.249	0.235	0.220	0.267	0.242	0.114	0.377	0.468	0.234	0.085	0.213
CV	2.16	3.96	2.41	5.04	0.59	1.05	1.01	0.50	4.25	13.02	26.65	14.87
Index		(0.061)				(0.192)				(0.291)		
Rodentia	0.298	0.237	0.244	0.220	0.269	0.239	0.114	0.378	0.443	0.247	0.050	0.260
CV	3.26	7.34	4.15	6.85	1.93	3.68	2.39	1.53	7.58	20.49	51.66	13.86
Index		(0.064)				(0.196)				(0.271)		

[a] All taxa listed in methods section were used
[b] Mean frequency for all taxa
[c] Coefficient of variation
[d] Index of compositional bias (Irwin et al. 1991)

trend continue as more mammalian orders are examined in detail.

As expected, the frequency of codon usage among twofold and fourfold degenerate codons corresponded to the observed base composition bias seen at the third codon position (Table 7). For instance, codons with guanine at the third position of both the COB and COII genes were used less frequently than adenine. In addition to the similarities in codon usage observed for rodent and artiodactyl COB and COII, the two orders differed with respect to codon usage at several amino acids. A nonrandom usage of synonymous codons has been noted for many organisms (Ikemura 1985; Sharp et al. 1988), and several models have considered genetic drift, selection, and fluctuating mutation bias as explanations for this observation (Li 1987; Shields 1990). In the case of the mammalian COB and COII genes, it is unclear what processes are involved. There appears to be no general pattern across all taxa for a particular gene, and therefore, an argument invoking selection would seem unlikely.

Conclusions

The detail examination of COII and COB gene variation in mammals reveals several examples of incongruence involving different gene trees and comparisons of gene trees with accepted species trees. If one assumes that a single "true phylogeny" exists and that mitochondrial genes are linked and inherited as a single locus, how can the incongruence seen between the gene phylogenies of COB and COII as well as among phylogenies derived from independent characters be explained? In some cases, such as that seen for the placement of cetaceans as sister to ruminant artiodactyls, there may be strong molecular evidence with little morphological evidence contradicting the gene tree results. In other cases (e.g., the monophyly of rodents), an explanation for such differences may require a detailed examination of both molecular and morphological character evolution. The COB and COII gene data provide evidence in support of this idea. For example, heterogeneity in both the overall rate of nucleotide substitutions and the types of substitutions allowed (transition/transversion ratios, base composition, codon usage) has been demonstrated in the detailed comparisons of both genes and orders. This heterogeneity probably explains many of the differences in homoplasy associated with the first, second, and third codon positions seen for the two genes (Table 4), and in the case of mammalian ordinal relationships, a consideration of total evidence did not resolve some discrepancies. As has been suggested by several authors (Felsenstein 1978; Holmes 1991; Irwin et al. 1991; Sidow and Wilson 1991; Honeycutt and Adkins 1993; Miyamoto et al. 1994), rate heterogeneity and differences in the pattern of nucleotide substitutions between taxa and genes may affect phylogeny reconstruction, especially among divergent taxa. If the order Rodentia is monophyletic, then this heterogeneity may well account for the distribution of several divergent rodent lineages throughout the eutherian mammal phylogeny. Finally, it is clear from this study that a detailed phylogenetic examination, using a larger number of taxa within orders of mammals and several orthologous genes, has the potential to provide insight into both the relationships among eutherian mammals and the evolution of mammalian genes. Nevertheless, the overall

Table 7. Codon usage for COII and COB genes of artiodactyls and rodents, reflecting the frequency of all possible bases at the third codon position[a]

Amino acid	Rodent COII				Artiodactyla COII				Rodent COB				Artiodactyla COB			
	A	G	C	T	A	G	C	T	A	G	C	T	A	G	C	T
Phe			0.52	0.48			0.53	0.47			0.58	0.42			0.68	0.32
Leu	0.67	0.07	0.07	0.19	0.69	0.12	0.05	0.14	0.69	0.04	0.13	0.14	0.63	0.07	0.22	0.08
Ile			0.40	0.60			0.43	0.57			0.49	0.51			0.62	0.38
Met	0.83	0.17			0.77	0.23			0.87	0.13			0.80	0.20		
Val	0.48	0.07	0.19	0.26	0.43	0.08	0.26	0.23	0.50	0.03	0.26	0.21	0.49	0.07	0.33	0.11
Ser	0.54	0.03	0.22	0.21	0.50	0.04	0.21	0.25	0.58	0.02	0.20	0.20	0.53	0.03	0.28	0.16
Pro	0.53	0.02	0.18	0.27	0.58	0.05	0.23	0.14	0.56	0.02	0.23	0.19	0.64	0.03	0.23	0.10
Thr	0.54	0.02	0.19	0.25	0.62	0.08	0.19	0.11	0.50	0.03	0.26	0.21	0.62	0.02	0.25	0.11
Ala	0.25	0.01	0.32	0.42	0.49	0.09	0.18	0.24	0.36	0.02	0.38	0.24	0.57	0.02	0.28	0.13
Tyr			0.49	0.51			0.48	0.52			0.55	0.45			0.62	0.38
His			0.61	0.39			0.62	0.38			0.61	0.39			0.75	0.25
Gln	0.92	0.08			0.88	0.12			0.91	0.09			0.84	0.16		
Asn			0.55	0.45			0.64	0.36			0.65	0.35			0.76	0.24
Lys	0.95	0.05			0.75	0.25			0.94	0.06			0.88	0.12		
Asp			0.59	0.41			0.54	0.46			0.67	0.33			0.75	0.25
Glu	0.86	0.14			0.79	0.21			0.83	0.17			0.92	0.08		
Cys			0.54	0.46			0.79	0.21			0.79	0.21			0.89	0.11
Trp	0.91	0.09			0.93	0.07			0.97	0.03			0.97	0.03		
Arg	0.47	0.04	0.27	0.22	0.73	0.04	0.06	0.17	0.81	0.02	0.12	0.05	0.88	0.02	0.06	0.04
Ser			0.80	0.20			0.81	0.19			0.70	0.30			0.76	0.24
Gly	0.35	0.16	0.33	0.16	0.42	0.15	0.29	0.14	0.50	0.09	0.23	0.18	0.60	0.10	0.25	0.05

[a] Termination codons are not included

model of gene evolution, in terms of rates and the distribution of substitutions allowed, may be more complex than previously anticipated.

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