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Secondary Structure and Patterns of Evolution Among Mammalian Mitochondrial 12S rRNA Molecules

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Abstract. Forty-nine complete 12S ribosomal RNA (rRNA) gene sequences from a diverse assortment of mammals (one monotreme, 11 marsupials, 37 placentals), including 11 new sequences, were employed to establish a ''core'' secondary structure model for mammalian 12S rRNA. Base-pairing interactions were assessed according to the criteria of potential base-pairing as well as evidence for base-pairing in the form of compensatory mutations. In cases where compensatory evidence was not available among mammalian sequences, we evaluated evidence among other vertebrate 12S rRNAs. Our results suggest a core model for secondary structure in mammalian 12S rRNAs with deletions as well as additions to the Gutell (1994: *Nucleic Acids Res. 22*) models for *Bos* and *Homo.* In all, we recognize 40 stems, 34 of which are supported by at least some compensatory evidence within Mammalia. We also investigated the occurrence and conservation in mammalian 12S rRNAs of nucleotide positions that are known to participate in the decoding site in *E. coli.* Twenty-four nucleotide positions known to participate in the decoding site in *E. coli* also occur among mammalian 12S rRNAs and 17 are invariant for the same base as in *E. coli.* Patterns of nucleotide substitution were assessed based on our secondary structure model. Transitions in loops become saturated by approximately 10–20 million years. Transitions in stems, in turn, show partial saturation at 20 million years but divergence continues to increase beyond 100 million years. Transversions accumulate linearly beyond 100 million years in both stems and loops although the rate of accumulation of transversions is three- to fourfold higher in loops. Presumably, this difference results from constraints to maintain pairing in stems.

Key words: Mitochondrial 12S rRNA — Secondary structure — Evolutionary rates — Nucleotide substitution patterns — Mammalia

Introduction

Protein synthesis is a cellular process of great complexity that has resisted the elucidation of molecular detail that is available on replication and transcription (Dahlberg 1989). Nevertheless, it has become abundantly clear in recent years that ribosomal RNA (rRNA) has a primary functional role in most if not all stages of protein synthesis, including binding of aminoacyl-tRNA; binding of mRNA; binding of initiation, elongation, and termination factors; and peptide bond formation (Dahlberg 1989; Wool et al. 1990).

Various methods have been used in developing higher-order (i.e., secondary and tertiary) structure models for both tRNA and rRNA. Sequence analysis proved important in the establishment of the cloverleaf secondary structure model for tRNAs (Holley et al. 1965; Madison et al. 1966; RajBhandary et al. 1966; Zachau et al. 1966) and positional covariance evidence provided confirmation for a few higher-order structural features (Levitt 1969). However, the bulk of secondary and tertiary base-pair interactions in tRNAs were demonstrated by

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X-ray crystallography (Kim 1979). After the fact, most of these higher-order interactions have been confirmed by positional covariance (see Gutell et al. 1992, 1994).

In the case of rRNAs, comparative sequence analysis has played a more important role in establishing secondary structure models owing to the difficulty of X-ray crystallography studies on these large RNA species (but see Yonath et al. 1990). Thus, rather detailed higher-order structures for 5S, 16S, and 23S-like rRNAs have now been inferred based primarily on comparative sequence analysis (Fox and Woese 1975; Woese et al. 1980, 1983; Glotz et al. 1981; Stiegler et al. 1981; Zwieb et al. 1981; Gutell et al. 1985). Chemical protection and crosslinking studies, in turn, have played an important role in elucidating functional roles of specific nucleotides in ribosome function for 16S and 23S rRNAs in *E. coli* (e.g., see Dahlberg 1989; Moazed and Noller 1990; Noller 1993).

Among 16S-like rRNAs, the mitochondrial family is the most atypical. A number of stem-loop structures found in *E. coli* 16S rRNA, as well as most other 16S-like rRNAs, are absent from small-subunit mitochondrial rRNAs (Gutell et al. 1985). Mitochondrial rRNAs have also received much less attention in structure–function studies that may clarify the importance of these differences. Nevertheless, Gutell and colleagues (Gutell et al. 1985, 1994; Gutell 1994) have provide secondary structure models for 12S rRNAs in assorted mammals including *Rattus, Bos,* and *Homo.* These models reveal a set of core pairing interactions common to small-subunit mitochondrial rRNAs and other 16S-like rRNAs. Recently, Douzery and Catzeflis (1995) suggested refinements in the Gutell et al. (1985) models for *Rattus* and *Bos.* However, that study only included representatives of nine eutherian orders and one marsupial order and did not include an analysis of compensatory evidence for base-pairing interactions.

In the present study, we have compiled a large number of mammalian 12S rRNA gene sequences, as well as sequences for other vertebrates, to evaluate and refine Gutell's models for mammalian 12S rRNAs and to establish a core set of mammalian base-pairing interactions. In addition to a large number of sequences already available in nucleic acid databases (Anderson et al. 1981, 1982; Bibb et al. 1981; Hixson and Brown 1986; Gadaleta et al. 1989; Kraus and Miyamoto 1991; Arnason et al. 1991, 1993; Allard and Miyamoto 1992; Allard et al. 1992; Arnason and Johnson 1992; Arnason and Gullberg 1993; Douzery 1993; Xu and Arnason 1994; Frye and Hedges 1995; Douzery and Catzeflis 1995; Springer et al. 1995), our study incorporates 11 new sequences that we have obtained for additional taxa. Together, these sequences include a representative monotreme, a diverse assemblage of American and Australian marsupials, and representatives of 14 of 17 orders of placental mammals (sensu Nowak and Paradiso 1983). Thus, this collection is appropriate for defining a core set of base-pairing interactions among mammals. In addition, we have assessed whether or not key sites in the *E. coli* model (e.g., decoding sites, subunit association sites) may also occur in the mammalian 12S model. Finally, we have evaluated patterns of nucleotide substitution in stems and loops in the mammalian 12S rRNA genes based on the findings of our secondary structure model.

Materials and Methods

Mammalian 12S rRNA gene sequences included in our study are given in Table 1. In addition to sequences extracted from GenBank and from the literature, we sequenced the 12S rRNA gene for 11 additional taxa shown in Table 1. We used the polymerase chain reaction (PCR) (Saiki et al. 1988) to amplify the complete 12S rRNA gene with primers described in Springer et al. (1995) and Douzery and Catzeflis (1995). Conditions for amplification are described therein. Sequences were obtained using the dideoxy technique (Sanger et al. 1977) by direct sequencing of PCR products or after cloning PCR products into PCR II (Invitrogen). Sequences for all of the internal primers used in sequencing are given in Springer et al. (1995) and Douzery and Catzeflis (1995).

In all, sequences included in this study are representative of monotremes, all seven orders of marsupials (following Marshall et al. 1990), and 14 of the 17 orders of placental mammals. This ordinal representation is summarized in Table 1. In addition, we divided chiropteran, rodent, and artiodactyl sequences at the subordinal rather than ordinal level because of recent questions about the monophyly of these orders [e.g., see Graur and Higgins (1994) and Philippe and Douzery (1994) for artiodactyls; Graur et al. (1991, 1992), Luckett and Hartenberger (1993), Cao et al. (1994), and Frye and Hedges (1995), for rodents; and Pettigrew (1986, 1994) and Simmons (1994) for chiropterans]. This resulted in a total of 25 orders/suborders represented by our sequences.

In recognizing stem regions that occur among mammalian 12S rRNAs, we started with the Gutell (1994) models for *Bos taurus* (cow) and *Homo sapiens* (human), as well as a more recent model for *Bos* that derives from the Gutell (1994) model (S. Damberger and R. Gutell, unpublished), as a basis for further revision and for identifying a set of core secondary structures that occur across mammalian 12S rRNAs. We also evaluated the putative occurrence of several tertiary interactions for 16S-like rRNAs suggested by Gutell (1994).

Initially, sequences were aligned using CLUSTAL (Higgins and Sharp 1988). Sequence alignments were modified based on Gutell's (1994) models. We then employed a two-step procedure to elucidate core interactions. First, we used the criterion that potential base-pairing must occur in at least the majority of species for at least 75% of the orders/suborders. Stems in the Gutell (1994) and Damberger and Gutell (unpublished) models, as well as bases adjacent to these stems that in some cases were also candidates for base-pairing, were evaluated according to this criterion. In assessing potential base-pairing, we allowed standard Watson-Crick base pairs, as well as U:G-type interactions. Other types of noncanonical pairing (e.g., A:G) were not considered in our analysis although we recognize that some authors (e.g., Gautheret et al. 1994; Gutell et al. 1994) have demonstrated the occurrence of such interactions in some instances.

Next, we searched for compensatory mutations as evidence to validate (or not) these putative stems. Compensatory evidence often occurs in the form of positional covariance, i.e., changes at one position that covary with changes at a complementary position so as to maintain base-pairing. Fox and Woese (1975) suggested that a putative association should be considered ''proven,'' in the absence of negative evidence, if at least two examples of positional covariance occur in the taxonomic group being studied. In addition, certain single-base substitutions (e.g., transitions from U:G to C:G or U:G to U:A) provide

Table 1. 12S rRNA gene sequences included in this study

Class and order	Latin binomial and common name	Accession # and/or reference
Class Mammalia		
Monotremata	*Ornithorhynchus anatinus* (platypus)	U33498; Springer et al. (1995)
Didelphimorphia	*Didelphis virginiana* (opossum)	Z29573; Janke et al. (1994)
Didelphimorphia	*Lutreolina crassicaudata* (opossum)	U33494; Springer et al. (1995)
Paucituberculata	*Caenolestes fuliginosus* (rat opossum)	U61072; this paper
Microbiotheria	*Dromiciops australis* (monito del monte)	U61073; this paper
Peramelina	*Isoodon macrourus* (bandicoot)	U61074; this paper
Dasyuromorphia	*Phascogale tapoatafa* (phascogale)	U33497; Springer et al. (1995)
Notoryctemorphia	*Notoryctes typhlops* (marsupial mole)	U61075; this paper
Diprotodontia	*Macropus giganteus* (grey kangaroo)	X86941; Douzery and Catzeflis (1995)
Diprotodontia	*Phalanger orientalis* (grey cuscus)	U33496; Springer et al. (1995)
Diprotodontia	*Phascolarctos cinereus* (koala)	U61076; this paper
Diprotodontia	*Vombatus ursinus* (common wombat)	U61078; this paper
Primates	*Homo sapiens* (human)	J01415; Anderson et al. (1981)
Primates	*Pongo pygmaeus* (orangutan)	Hixson and Brown (1986)
Rodentia	*Mus musculus* (mouse)	J01420; Bibb et al. (1981)
Rodentia	*Rattus norvegicus* (rat)	X14848; Gadaleta et al. (1989)
Rodentia	*Hydrochaeris hydrochaeris* (capybara)	U61081; this paper
Rodentia	*Cavia porcellus* (guinea pig)	L35585; Frye and Hedges (1995)
Pholidota	*Manis* sp. (pangolin)	U61079; this paper
Xenarthra	*Chaetophractus villosus* (hairy armadillo)	U61080; this paper
Insectivora	*Atelerix albiventris* (hedgehog)	M95109; Allard and Miyamoto (1992)
Insectivora	*Amblysomus hottentotus* (golden mole)	M95108; Allard and Miyamoto (1992)
Insectivora	*Blarina brevicauda* (short-tailed shrew)	M95110; Allard and Miyamoto (1992)
Chiroptera	*Eptesicus fuscus* (brown bat)	U61082; this paper
Chiroptera	*Nyctimene albiventer* (tube-nosed bat)	U61077; Hollar and Springer (unpublished)
Carnivora	*Felis concolor* (mountain lion)	U33495; Springer et al. (1995)
Pinnipedia	*Phoca vitulina* (harbor seal)	X63726; Arnason and Johnsson (1992)
Pinnipedia	*Halichoerus grypus* (grey seal)	X72004; Arnason et al. (1993)
Hyracoidea	*Procavia capensis* (rock hyrax)	U60184; Lavergne et al. (1996)
Hyracoidea	*Dendrohyrax dorsalis* (tree hyrax)	X86941; Douzery and Catzeflis (1995)
Sirenia	*Dugong dugon* (dugong)	U60185; Lavergne et al. (1996)
Sirenia	*Trichechus manatus* (manatee)	U60183; Lavergne et al. (1996)
Proboscidea	*Loxodonta africana* (African elephant)	U60182; Lavergne et al. (1996)
Proboscidea	*Elephas maximus* (Asiatic elephant)	Lavergne et al. (1996)
Cetacea	*Stenella coeruleoalba* (striped dolphin)	X78168, X78169; Douzery (1993)
Cetacea	*Balaenoptera physalus* (fin whale)	X61145; Arnason et al. (1991)
Cetacea	*Balaenoptera musculus* (blue whale)	X72204; Arnason and Gullberg (1993)
Artiodactyla	*Bos taurus* (cow)	J01394; Anderson et al. (1982)
Artiodactyla	*Tragelaphus imberbis* (lesser kudu)	M86493; Allard et al. (1992)
Artiodactyla	*Capra hircus* (domestic goat)	M55541; Kraus and Miyamoto (1991)
Artiodactyla	*Odocoileus virginianus* (white-tailed deer)	M35874; Miyamoto et al. (1990)
Artiodactyla	*Hydropotes inermus* (Chinese water deer)	M35876; Miyamoto et al. (1990)
Artiodactyla	*Antilocapra americana* (pronghorn antelope)	M55540; Kraus and Miyamoto (1991)
Artiodactyla	*Tragulus napu* (mouse deer)	M55539; Kraus and Miyamoto (1991)
Artiodactyla	*Sus scrofa* (pig)	Tanhauser (1985)
Artiodactyla	*Tayassu tajacu* (peccary)	X86944; Douzery and Catzeflis (1995)
Perissodactyla	*Equus grevyi* (zebra)	X86943; Douzery and Catzeflis (1995)
Perissodactyla	*Equus caballus* (horse)	X79547; Xu and Arnason (1994)
Perissodactyla	*Ceratotherium simum* (rhinoceros)	X86942; Douzery and Catzeflis (1995)
Class Aves		
Galliformes	*Gallus gallus* (chicken)	X52392; Desjardins and Morais (1990)
Galliformes	*Coturnix coturnix* (Japanese quail)	X57245; Desjardins and Morais (1991)
Anseriformes	*Anas platyrhunchos* (duck)	L16770; Liu and Lin (unpublished)
Anseriformes	*Cairina moschata* (duck)	L16769; Liu and Lin (unpublished)
Class Reptilia		
Crocodylia	*Alligator mississippiensis* (alligator)	L28074; Hedges (1994)
Sphenodonta	*Sphenodon punctatus* (tuatara)	L28076; Hedges (1994)
Squamata	*Sceloporus undulatus* (lizard)	L28075; Hedges (1994)
Chelonia	*Trachemys scripta* (turtle)	L28077; Hedges (1994)
Class Amphibia		
Anura	*Rana catesbeiana* (frog)	X12841; Nagae et al. (1988)
Anura	*Xenopus laevis* (toad)	X02890; Roe et al. (1985)

Table 1. Continued

Class and order	Latin binomial and common name	Accession # and/or reference
Class Osteichthyes		
Salmoniformes	*Oncorhynchos mykiss* (trout)	L29771; Zardoya et al. (unpublished)
Cypriniformes	*Cyprimus carpio* (carp)	X61010; Chang et al. (1994)
Cypriniformes	*Crossostoma lacustre* (loach)	M91245; Tzeng et al. (1992)
Dipnoi	*Neaceratodus forsteri* (Australian lungfish)	Z21926-7; Hedges et al. (1993)
Dipnoi	*Protopterus* sp. (African lungfish)	Z21923; Hedges et al. (1993)
Dipnoi	*Lepidosiren paradoxa* (S. American lungfish)	Z48715; Hedges et al. (1993)
Coelacanthini	*Latimeria chalumnae* (coelacanth)	Z21921; Hedges et al. (1993)

validation, though less robust, for the maintenance of base-pairing. In cases where stems were invariantly paired, or nearly so, but compensatory evidence was absent, we searched among other vertebrate 12S rRNA sequences for such evidence. Nonmammalian vertebrate 12S rRNA sequences used for this purpose included representatives of the classes Osteichthyes (*Cyprinus, Oncohynchus, Crossostoma, Neoceratodus, Lepidosiren, Protopterus, Latimeria*), Amphibian (*Rana, Xenopus*), Reptilia (*Trachemys, Sphenodon, Scleroporus, Alligator*), and Aves (*Gallus, Coturnix, Anas, Cairina*). In cases where these sequences failed to confirm or repudiate the validity of putative base-pairing interactions, we accepted interactions that are considered proven based on compensatory evidence for other mitochondrial and nonmitochondrial 16S-like sequences (e.g., see Gutell et al. 1994).

Stems were delimited in our model by bilateral bulges of two or more base-pairs. Unilateral bulges were allowed in the context of a single stem.

Base compositions were determined using MEGA 1.01 (Kumar et al. 1993). MEGA was also used to calculate pairwise percent sequence divergence (uncorrected), both for transitions and transversions, after eliminating regions that could not be aligned reliably (see Fig. 3). Divergence time estimates were taken from the literature (see Fig. 4). In plotting percent sequence divergence against divergence time, individual points in Fig. 4 represent means for all relevant, pairwise comparisons, e.g., the eutherian–metatherian divergence values are means based on 407 pairwise comparisons for the 11 metatherians and 37 eutherians.

Multiple regressions were performed using Statistica (Mac) to examine percent divergence, both for transitions and transversions, as a function of (1) divergence time and (2) stem vs loop. Both linear and nonlinear polynomial curve-fitting options were employed.

Results and Discussion

Secondary Structure Model

Figure 1 shows the Damberger and Gutell (unpublished) secondary structure model for *Bos taurus;* also included in this model are tertiary interactions. Our core secondary structure model is illustrated in Fig. 2 using the same *Bos taurus* sequence. In addition, secondary structure interactions are indicated in the multiple alignment shown in Fig. 3, which includes eight representative mammal 12S rRNA gene sequences.

In all, we recognize 40 stems; these are numbered 1–40 in Figure 2. In this multiple alignment (Fig. 3), these stems are depicted as 1 and 1′, 2 and 2′, etc., for canonically paired regions. We also recognize three instances where there are one-base-pair interactions but have not numbered these among the helices (see Fig. 2).

Thirty-four of the 40 stems are supported by at least some compensatory evidence within Mammalia. Additionally, sequences from other vertebrates provide some compensatory evidence for three (2, 29, 32) of the remaining six stems. Thus, only stems 6, 11, and 38 are unsupported by compensatory evidence among vertebrate 12S rRNA sequences. Examples of compensatory changes evidenced by mammalian and other vertebrate 12S sequences are provided in Table 2.

Our core model implies a two-dimensional secondary structure that is very similar to the Gutell (1994) models for *Homo* and *Bos* and especially to the Damberger and Gutell derivation (unpublished) based on these models. There are also differences between our core model and these models. These differences, as well as other aspects of our core model that require further explanation, are outlined below:

1. Stem 3 is one base shorter than in Gutell (1994) and Damberger and Gutell (unpublished). Potential base-pairing occurs in 92% of the orders/suborders at the deleted position, but all of the mutations (i.e., in *Atelerix,* cervids, *Pongo, Nyctimene*) are noncompensatory. Thus, compensatory evidence is completely absent and is clearly outweighed by noncompensatory evidence. In other vertebrates, there is pairing at this position in 15 of 17 taxa, but both of the mutations (*Crossostoma* and *Lepidosiren*) are noncompensatory.
2. In agreement with Damberger and Gutell (unpublished), we recognize a short, unpaired region between stems 7 and 8. In Gutell's (1994) earlier *Bos* model this region is almost entirely paired.
3. Gutell (1994) recognizes a two-base-pair stem between 9′ and 6′ in *Homo* (one base-pair in *Bos;* see Fig. 1). This stem shows potential pairing in all taxa (but no positional covariance) at one position and potential pairing in 64% of the orders/suborders at the second position. Among placentals, there are at least six independent noncompensatory mutations without

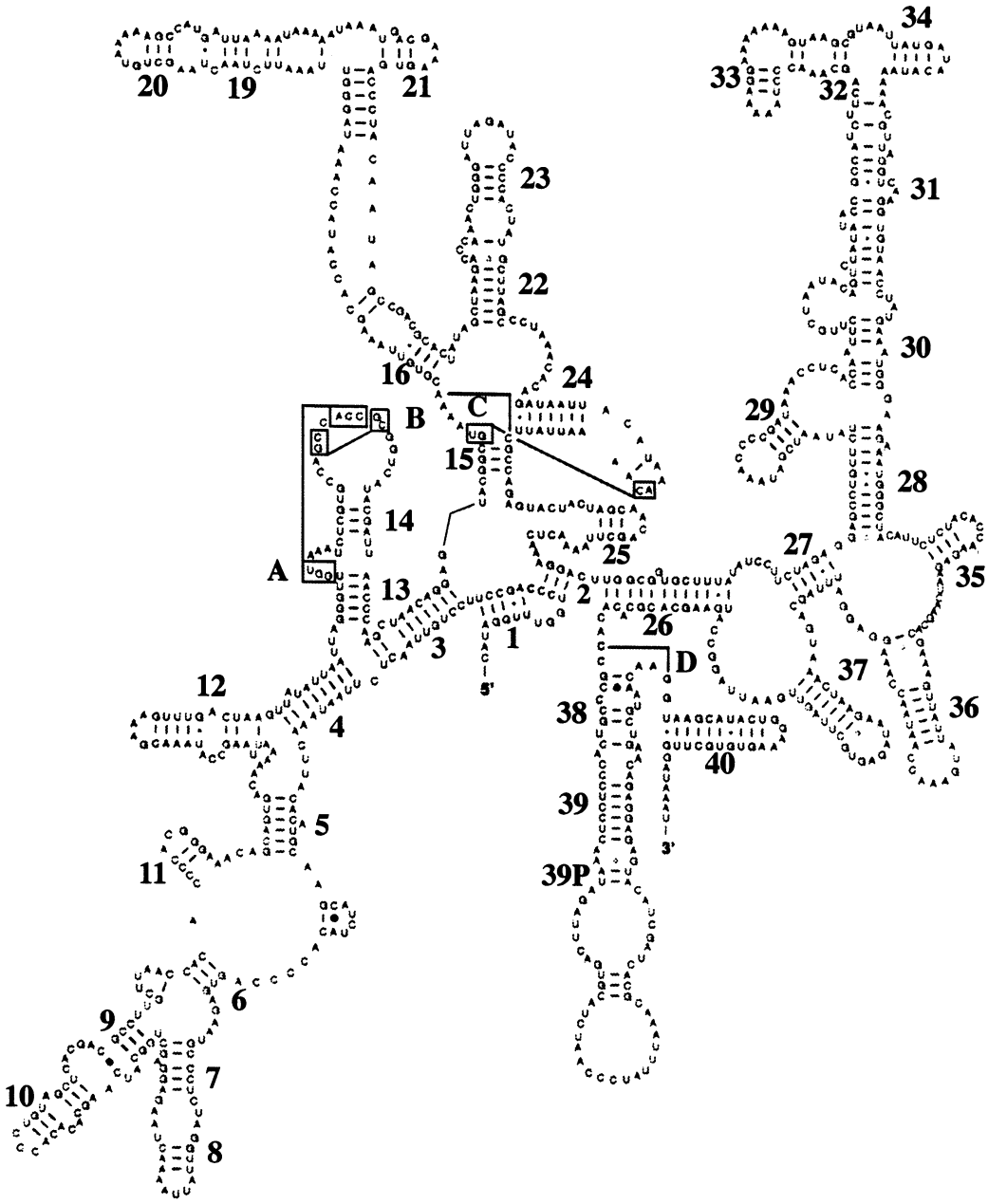

Fig. 1. Secondary structure model of S. Damberger and R. Gutell (unpublished) for *Bos taurus*, which is a derivation of the Gutell (1994) model. Stem *numbers* correspond to our model (see Fig. 2).

any positional covariance. Compensatory vs noncompensatory mutations in marsupials are mixed. We have therefore omitted this stem from the core model because of the marginal level of potential pairing and the prevalence of noncompensatory mutations at one of the two helix positions. However, we allow for a one-base-pair interaction that could be stabilized by base-pairing elsewhere. We also note that a two-base-pair stem apparently holds for other vertebrate 12S sequences where potential pairing occurs in 100% of the taxa examined and there is compensatory evidence in *Latimeria, Coturnix,* and *Rana.*

4. As in Gutell (1994) and Damberger and Gutell (unpublished), stem 10 is six base-pairs long. At position 10-6, pairing occurs in 72% of the orders/suborders, which is marginally less than the criterion for our core model. However, we are persuaded by numerous instances of positional covariance (e.g., *Vombatus, Caenolestes, Manis*) and have included 10-6 in stem 10. We also note that stem 10 is potentially eight base-pairs long in proboscideans (see *Loxodonta* in Fig. 3).
5. Stem 14 is five base-pairs in the Gutell (1994) models but Damberger and Gutell delete 14-4 and 14-5 from this stem. Potential pairing occurs in 100% of the taxa

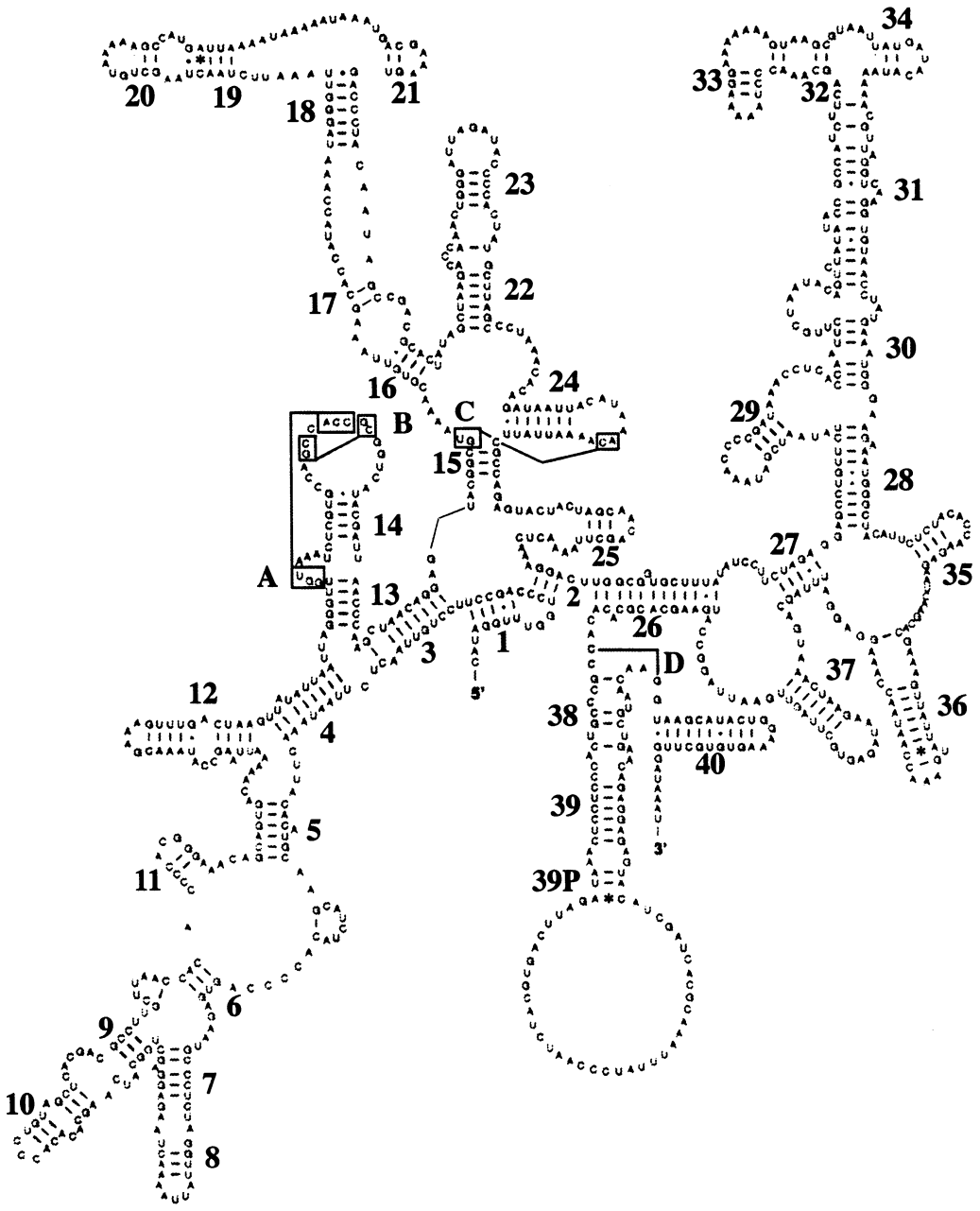

Fig. 2. Core secondary structure model for mammalian 12S rRNA illustrated using *Bos taurus.* Only canonical and U:G base-pairs are indicated. *Asterisks* indicate base-pair interactions that are part of the core model but do not obtain for *Bos taurus. Numbers* refer to stems. *Uppercase letters* refer to tertiary interactions.

at both of these positions. We tentatively retain a five base-pair stem but note that compensatory evidence is minimal (i.e., there are single compensatory mutations in *Dugong* and in hominoids at 14-5).

6. Stem 16 is three base-pairs in the Gutell (1994) *Homo* model and four base-pairs in the Gutell (1994) and Damberger and Gutell (unpublished) *Bos* models. Our analysis agrees with the *Bos* models.
7. Stem 17 was included in the Gutell (1994) *Homo* model but not the Gutell (1994) *Bos* model. However, Damberger and Gutell (unpublished) recognize this stem in their revised *Bos* model. Our analysis indicates 92% and 88% pairing, respectively, at positions 17-1 and 17-2. In addition there are compensatory mutations supporting this stem (see Table 2).
8. Stem 18 is a six-base-pair helix in the Gutell (1994) *Homo* model and in the Damberger and Gutell (unpublished) *Bos* model. Previously, Gutell (1994) did not include this stem in the *Bos* model. Our analysis supports stem 18 as part of the mammalian core model; however, it is seven base-pairs rather than six base-pairs in our model. At the additional position (18-7), pairing occurs in 96% of the orders/suborders and there is positional covariance (e.g., C:G in hyra-

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                          1        2      1'        3            4             5
Loxodonta          CAA AGGTT TGG TCC C GGCCT T CTTATTGGT TA CTAGGAAA CTTA TACaTGC
Bos                CAT AGGTT TGG TCC C AGCCT T CCTGTTAAC TC TTAATAAA CTTA CACaTGC
Equus              CAT AGGCT TGG TCC T AGCCT T TTTATTAGT TA TTAATAGA ATTA CACaTGC
Manis              CAT AGGTT TGG TCC C AGCCT T TTTATTAGT TT ATGATAAA ATTA CACaTGC
Eptesicus          CAT AGGTT TGG TCC T GGCCT T TCTATTAGT TG TTAATAAA TTTA CACaTGC
Phalanger          CAA AGGTT TGG TCC T AGCCT T ACTGTTAAT TA TAATTAGA CCTA CACaTGC
Didelphis          CAT AGGTT TGG TCC T AGCCT T ATTATTAGT TC TAATTAGA CCTA CACaTGC
Ornithorhynchus    TAA AGGTT TGG TCC T AGCCT T ACTGTTAGA TT TGATTAGA TTTA TACaTGC

                                    6          7           8                 8'
Loxodonta          AAGTATCCGCCCGCCA GTG AATAC GCCTT CTAA-- ATC [ATCACC]      GAT
Bos                AAGCATCTACACCCCA GTG AGAAT GCCCT CTAG-- GTT [ATTAA]       AAC
Equus              AAGTATCCGCACCCCA GTG AGAAT GCCCT CTAA-- ATC [ACGTCTCTAC] GAT
Manis              AAGTATCCGCCCTACG GTG AAAAC GCCCT TTAGC- CTT [TACA]        AGT
Eptesicus          AAGAATCCCCGCCCCT GTG AGAAT GCCCT CTAC-- GCC [ATTAATT]     GGC
Phalanger          AAGTTTCCGCTACCCA GTG AGAAT GCCCT CAAA-- GTT [ATCTAA]      AAC
Didelphis          AAGTTTCCGCTACCCA GTG AGAAT GCCCT TTAAGT CTT [ATAAATT]     AAG
Ornithorhynchus    AAGTATCCGCAACCCA GTG AGAAT ACCCT AAAA-- ACT [CTTAACA]     AGT

                          7'      9          10                 10'           9'
Loxodonta          CA-AA GAGaGC T GGC ATCA AGCacACA [CTCTAAG] TGTaGCT CATGAC GTC
Bos                TA-AG AGGaGC T GGC ATCA AGCacACA [CCC]     TGTaGCT CACGAC GCC
Equus              TA-AA AGGaGC A GGT ATCA AGCacACT [AGAA]    AGTaGCT CATAAC ACC
Manis              CA-AA AGGaGC T GGT ATCA AGCacGCC [AATTTAA] GGCaGCT AGTGAC ACC
Eptesicus          AT-AA AGGaGC T GGT ATCA AGCacACT [AAAA]    AGTaGCT CACAAC ACC
Phalanger          AA-TC AGGaGC A GGT ATCA GGTacACT [CTA]     AGTaGCC CATCAC ACC
Didelphis          CA-AA AGGaGC T GGT ATCA GGCacACA [AAA]     TGTaGCC GATAAC ACC
Ornithorhynchus    TA-GA AGGaGT A GAT ATCA GGCacACT [AA]      AGTaGCC CACAAC ATC

                        6'     11      11'       5'                12
Loxodonta          TCGCCT-AGC CAC ACC CC CACG GG AAACA GCAGTA GTAAATA TTtAGcaaTTAAC
Bos                TTGCTT-AAC CAC AC- CC CACG GG AAACA GCAGTG ACAAAAA TTaAGccaTAAAC
Equus              TTGCTC-AGC CAC ACC CC CACG GG ACACA GCAGTG ATAAAAA TTaAGctaTGAAC
Manis              TTGCTTACGC CAC ACC CC CACG GG AGACA GCAGTG ATAAAAA TtAAAccaTTAAC
Eptesicus          TTGCTC-AGC CAC CCC CC CACG GG AGACA GCAGTG ACAAAAA TTaAGcaaTAGAC
Phalanger          TTGCTT-AAC CAC ACC CC CACG GG ACACA GCAGTG ACTAACA TTaAGccaTAAAC
Didelphis          TTGCTT-TAC CAC ACC CC CACG GG AGACA GCAGTG ATTAAAA TTaAGcaaTAAAC
Ornithorhynchus    TTGCCCTAGC CAC ACC CC CACG GG ACACA GCAGTA ATAGAAA TTaGTcaaTAAAC

                       12'          4'             13   A         14
Loxodonta          AAAA GTTAGaCTAA G TTaTCCTAA [TAAA]   GGACT GGT CAATT TCGTG CCA
Bos                GAAA GTTTGaCTAA G TTaTATTAA [TTA]    GGGTT GGT AAATC TCGTG CCA
Equus              GAAA GTTCGaCTAA G TCaTATTAA [ATAA]   GGGTT GGT AAATT TCGTG CCA
Manis              GAAA GTTAGaTTTA G TTaTATCAT [TTAT]   GGGTT GGT AAATC TCGTG CCA
Eptesicus          GAAA GTCTGaCTAA G TTaTATTAT [CCA]    GGACT GGT AAATC TCGTG CCA
Phalanger          GAAA GTTTGaCTAA A TCaCAATTA [TTTAA]  GGGTC GGT AAATC TCGTG CCA
Didelphis          GAAA GTTTGaCTAA G TCaTAATTT [ACATTA] GGGTT GGT CAATT TCGTG CCA
Ornithorhynchus    GCAA GTTTGaACAA G TCaTAATCA [ATAA]   GAGTC GGT AAATT TCGTG CCA

                   B     A' B' ****   14'      13'         3'          15  C
Loxodonta          GC A ACC GC GGCCA TACGA TT AGTCC AA ATTAATAAG CATAC GGC GT
Bos                GC C ACC GC GGTCA TACGA TT AACCC AA GCTAACAGG AGTAC GGC GT
Equus              GC C ACC GC GGTCA TACGA TT AACCC AA ATTAATAAA TCTCC GGC GT
Manis              GC C ACC GC GGTCA TACGA TT AACCC TA ATTAATAAA AAACC GGC GT
Eptesicus          GC C ACC GC GGTCA TACGA TT AGACC AA ACTAATAGA TATTC GGC GT
Phalanger          GC C ACC GC GGTCA TACGA TT GACCC AA ATTAACAGA AAACC GGC GT
Didelphis          GC C ACC GC GGTCA TACGA TT AACCC AA ATTAATAAA -TAAC GGC GT
Ornithorhynchus    GC C ACC GC GGTCA TACGA TT GACTC AA CCTAACAAA -TAAC GGT GT

                        16       17                        18          19
Loxodonta          AAAGC GTAT CAGA AG [AATTAAGAAAA]     TAAAGTT AAATCT TATAC
Bos                AAAAC GTGT TAAA GC [ACCATACCAAA]     TAGGGTT AAATTC TAACT
Equus              AAAGC GTGT CAAA GA [CTAATACCAAAA]    TAAAGTT AAAACC CAGTT
Manis              AAAAA GTGT CAAA GT [GTATTTAAATCAAA]  TAAAATT AAGCCC TAATC
Eptesicus          AAAGG GTGT TTTA GA [AATCAAACAATTAA]  TAAAGTT AAACCC TAACT
Phalanger          AAAGT GTGT TTAA GC [ACTCACCACCAA]    TAAAGCT AAAACC TAACT
Didelphis          AAAGA GTGT TTAA GT [TATATACAAAAA]    TAAAGTT AATAAT TAACT
Ornithorhynchus    AAAAC GTGT TTAA AA [ACTTAAACTAA]     TAAGATT AAAGTA GAACT
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Fig. 3. Multiple alignment for eight representative 12S rRNA gene sequences. Stem *numbers* are given above stems, with base-paired regions indicated as *1* and *1′*, *2* and *2′*, etc. Tertiary interactions are indicated with *uppercase letters* (e.g., *A* and *A′*). Lowercase bases within stems indicate bulges or bases that belong to loops in specific taxa. *Asterisks* indicate bases that participate in the decoding site (Dahlberg 1989). *Brackets* indicate regions of the 12S rRNA gene where alignments are ambiguous. *Equus* is *E. caballus.*

```
                    20    *     20'     19'                    21      21'
Loxodonta         TA GC TGTTTAAA GC TCA AGATA AGACATAAATAACCT AC GAAA GT
Bos               AA GC TGTAAAAA GC CAT GATTA AAATAAAAATAAATG AC GAAA GT
Equus             AA GC CGTAAAAA GC TAC AACCA AAGT-AAAATAGACT AC GAAA GT
Manis             AA GC TGTAAAAA GC CTC GATTA TAGTAAAAATAAACT AT GAAA AT
Eptesicus         AA GC TGTAAAAA GC CCT AGTTA CCGT-AAAACAAATA AC GAAA GT
Phalanger         AA AC TGTAATAC GC TAT AGTTA GAATTAAAATAAACA AC AAAA GT
Didelphis         AA AC TGTAGCAC GT TCT AGTTA ATATTAAAATACATA AT AAAA AT
Ornithorhynchus AA AC TGTGATAA GT CAT AGTTA ATACTAAAGCCATCT AC GAAA GT

                   18'          17'       16'           22           23
Loxodonta         GACTTTA ATAAT- CC TAAAC ATAC GATA GCTAGGgtacA AAC TGAG
Bos               GACCCTA CAATA- GC CGA-C GCAC TATA GCTAAGacccA AAC TGGG
Equus             GACTTTA ATACC- TC TGACT ACAC GATA GCTAAGacccA AAC TGGG
Manis             AATTTTA ATAAA- AC CCA-C ACAC GATA GCTAAGatccA AAC TGGG
Eptesicus         AACTTTA AAAAA- TC TGACT ACAC GATA GCTAAGacccA AAC TGGG
Phalanger         GGCTTTA ACCCC- GC TGAAG ACAC GAAA GCTAAGaaacA AAC TGGG
Didelphis         GACTTTA ATATC- AC CGACT ACAC GAAA ACTAAGacacA AAC TGGG
Ornithorhynchus GATCTTA GACTA- AT TGAAT ACAC GATA GCTAAGgtacA AAC TGGG

                   **  **  23'       22'                  24              C'
Loxodonta         ATTAGATAC CTCA CTA TgCCTAGC CCTAAACTTT GATAGCT [ACCTTT]  AC
Bos               ATTAGATAC CCCA CTA TgCTTAGC CCTAAACACA GATAATT [ACATAA]  AC
Equus             ATTAGATAC CCCA CTA TgCTTAGC CCTAAACTAA AATAGCT [TACCACA] AC
Manis             ATTAGATAC CCCA CTA TgCTTAGC CTTAAACCTA AATAATT [AATTAA]  AC
Eptesicus         ATTAGATAC CCCA CTA TgCTTAGC CATAAACACA GAAGATT [ATAA]    AC
Phalanger         ATTAGATAC CCCA CTA TgCTCAGC CCTAAACCAA GACAATT [AAAATA]  AC
Didelphis         ATTAGATAC CCCA CTA TgCTTAGT AATAAACTAA AATAATT [TAACA]   AC
Ornithorhynchus ATTAGATAC CCCA CTA TgCTTAGC CCTAAACTCA AGTCGTT [TAATA]   AC

                     24'     15'             25       25'     *        2'
Loxodonta         AA AGCTATC C GCC AGAGAACTACT AGC CAGA GCT TAAAACTTAAA GGA CT
Bos               AA AATTATT C GCC AGAGTACTACT AGC AACA GCT TAAAACTCAAA GGA CT
Equus             AA AGCTATT C GCC AGAGTACTACT AGC AACA GCC TAAAACTCAAA GGA CT
Manis             AA AATTATT C GCC AGAGTACTACT AGC AATA GCT TGAAACTCAAA GGA CT
Eptesicus         AA AATTATT C GCC AGAGTACTACC AGC AAGG GCT AGAAACTCAAA GGA CT
Phalanger         AA AATTATC C GCC AGAGAACTACT AGC CAAC GCT TAAAACTCAAA GGA CT
Didelphis         AA AATTATT C GCC AGAGAACTACT AGC AATT GCT TAAAACTCAAA GGA CT
Ornithorhynchus AA AACTACT C ACC AGAGAACTACT AGC AACA GCT TAAAACTCAAA GGA CT

                     * 26              27          28          29    *
Loxodonta         TGGCGgTGCTTTA TATCCAC CTAGG GG AGCCTGTCTC GTA ACCG ATGAACCC
Bos               TGGCGgTGCTTTA TATCCTT CTAGA GG AGCCTGTTCT ATA ATCG ATAAACCC
Equus             TGGCGgTGCTTTA CATCCCT CTAGA GG AGCCTGTTCC ATA ATCG ATAAACCC
Manis             TGGCGgTGCTTCA TATCCCT CTAGA GG AGCCTGTCCT ATA AACG ATAAACCC
Eptesicus         TGGCGgTGCCTTA AATCCAT CTAGA GG AGCCTGTTCT ATA ATCG ATAAACCC
Phalanger         TGGCGgTGTCCTA AACCCAC CTAGA GG AGCCTGTTCT ATA ATCG ATAAACCC
Didelphis         TGGCGgTGCCCTA AACCCAC CTAGA GG AGCCTGTTCT ATA ATCG ATAAACCC
Ornithorhynchus TGGCGgTGCTTCA --CCCCT CTAGA GG AGCCTGTTCT ATA ATCG ATAAACCC

                   29'           30                        31
Loxodonta         CGAT ATACCTTA CCGTCAC TTGCTAATTCA GTcCAtaTACCACCATCTT CA
Bos               CGAT AAACCTCA CCAATTC TTGCTAATACA GTcTAtaTACCGCCATCTT CA
Equus             CGAT AAACCCCA CCATCCC TTGCTAATTCA GCCTATATACCGCCATCTT CA
Manis             CGAT AAACCTCA CCAATCC TAGCTAATGCA GCcTAtaTACCGCCATCCT CA
Eptesicus         CGAT AGACCTCA CCAACCC TTGCCAAATCA GCtTAtaTACCGCCATCTT CA
Phalanger         CGAT AAACCCCA CCTATTC TTGCCAATACA GCcTAtaTACCGCCATCGT CA
Didelphis         CGAT AAACCAGA CCTTATC TTGCCAATACA GCcTAtaTACCGCCATCGT CA
Ornithorhynchus CGAT ACACCTCA CCATCTT TTGCCACTACT GTcTAtaTACCGCCATCGT CA

                  32     33          33'           33'   32'        34           34'
Loxodonta         GC AA ACCCC TAT-- AGG GCACAAAA GT GA GC TTAATC AT [AACCC]  AT
Bos               GC AA ACCCT AAA-A AGG AAAA-AAA GT AA GC GTAATT AT [GATAC]  AT
Equus             GC AA ACCCT AAACA AGG TACC-GAA GT AA GC ACAAAT AT [CCAAC]  AT
Manis             GC AA ACCCT AATAA AGG AACC-ATA GT AA GC AAGATC AT [TGAAAC] AT
Eptesicus         GC AA ACCCT AAA-A AGG AAAA-ACA GT AA GC ATAAAC AT [TAAC]   AT
Phalanger         GC CT ACCCC -AC-A GGG CAAC-AAA GT AA GC AAAATC AT [TAACC]  AT
Didelphis         GC TA ACCTT TAA-A AAG AATT-ACA GT AA GC AAAATC AT [ACAAC]  AT
Ornithorhynchus GC CA ACCCT AAA-A AGG AACA-AAA GT AG GC GTAATC AT [TTTTC]  AT
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Fig. 3. Continued.

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                          31'                  30'           28'
Loxodonta         GAA AAAGTTaGGCcgaGGTGTCGC CTAC GTGACGG TC AAaGATGGGCT ACA
Bos               AAA AAcGTTaGGTcaaGGTGTAAC CTAT GAAATGG GA AGaAATGGGCT ACA
Equus             AAA AACGTTaGGTCAAGGTGTAGC CCAT GGGATGG AG AGaAATGGGCT ACA
Manis             AAA AACGTTaGGTcaaGGTGTAGC TTAT GGATTGG GA AGaGATGGGCT ACA
Eptesicus         AAA AACGTTaGGTcaaGGTGTAGC CTAT GGGTTGG AA AGaAATGGGCT ACA
Phalanger         AAA AACGTTaGGTcaaGGTGTAGC ATAT GAATAGG AA AGaAATGGGCT ACA
Didelphis         AAA AACGTTaGGTcaaGGTGTAGC ATAT GATAAGG AA AGtAATGGGCT ACA
Ornithorhynchus AAA AACGTTaGGTcaaGGTGTAGC CTAT AAGATGG A- AGaAATGGGCT ACA

                     35            35'                              36
Loxodonta         TT TTCT [ATTAT]  AGAA [CAGACAA]            ACGGAT ATCACTCtG
Bos               TT CTCT [ACACCA] AGAG [AATCAAGC]           ACGAAA GTTATTatG
Equus             TT TTCT [ACCCTA] AGAA [CAAGAACTTTAACCCGG] ACGAAA GTCTCCAtG
Manis             TT TTCT [AAAAC]  AGAA [TAAG]               ACGAAT ACCCTTATg
Eptesicus         TT CCTC [GGACC]  GAGG [TAATATTTT]          ACGAAA ACCTATGtG
Phalanger         TT TTCT [AAGCT]  AGAA [AATAA]              ACGAAC TATCTTAtG
Didelphis         TT CTCT [ACTAT]  AGAG [CATA]               ACGAAT CATATTAtG
Ornithorhynchus TT TTCT [ACATT]  AGAA [CAT]                ACGAAA AACTCTAtG

                    36'              **  27'          37
Loxodonta         AAA T-GGGTGGT --TGAAGGCGGA TTTAG TAGTA AACTAAG AATAGAGAG
Bos               AAA C-cAATAAC --CAAAGGAGGA TTTAG CAGTA AACTAAG AATAGAGTG
Equus             AAA C-TGGAGAC --TAAAGGAGGA TTTAG CAGTA AATTAAG AATAGAGAG
Manis             AAA A-TAAGGGT --TAAAGGAGGA TTTAG TAGTA AGACAAG AATAGAGAG
Eptesicus         AAA T-CAACAGT --CAAAGGAGGA TTTAG CAGTA AATTAAG AATAGAGTG
Phalanger         AAA CTcAAGATA -TAGAAGGAGGA TTTAG TAGTA AATTAAG AATAGAGAG
Didelphis         AAA C-TAAAATG CTTGAAGGAGGA TTTAG TAGTA AATTAAG AATAGAGAG
Ornithorhynchus AAA C-TAGAGTA --TTAAGGAGGA TTTAG TAGTA AGCCAAG AATAGAGAG

                                                    *
                  37'           *        26'  *      D * * 38    *     *39
Loxodonta         CTTAATT GAACAAGGCTA TGAAGCGCGTaCA CA C C GccCGtC ACT CTCCTC
Bos               CTTAGTT GAATTAGGCCA TGAAGCACGCaCA CA C C GccCGtC ACC CTCCTC
Equus             CTTAATT GAATCAGGCCA TGAAGCGCGCaCA CA C C GccCGtC ACC CTCCTT
Manis             CTTGACT GAATTAGGCCC TGAAGCACGCaCA CA C C GccCGtC ACC CTCTTC
Eptesicus         CTTAATT GAATAAGGCCA TGAGGCACGCaCA CA C C GccCGtC ACC CTCCTC
Phalanger         CTTAATT GAAATAGGCAA TAGGACGCGCaCA CA C C GccCGtC ACC CTCCTC
Didelphis         CTTAATT GAATTAGGCAA TAGGGCGCGCaCA CA C C GccCGtC ACC CTCCTC
Ornithorhynchus CTTGACT GAACTGGGCAA TGAAGCACGCaCA CA C C GccCGtC ACC CTCCTC

                    39P                                              39P'
Loxodonta         AA GTA [TCCCCACATCAAACAATCATATTACAGATTTAAACAAA]  TAC AA
Bos               AA ATA [GATTCAGTGCATCTAACCCTATTTAAACGCACTAGCTA]  CAT GA
Equus             AA ATA [TCACAAATCATAACATAACATAAAACCGTGACCCAAACA] TAT GA
Manis             AA ATT [TCCAAAAAAACAGTAAATATATTAATGAATAACAAG]    AAT GA
Eptesicus         AA GCG [AAGATATAAAATAATATCTAAATATAAGGTATCAGCC]   CGC AA
Phalanger         AA [    TTATAACCAAATAATAACTAATAAAACTTTAACAAA       ] AA
Didelphis         AA [    CATAATAATCCAACATACCTAATACAATTATTCATTA      ] AA
Ornithorhynchus AA [   TCAAGCAACACAAACATTCCTAAAATCCCAACGCTTTTC     ] AA

                  39'*     **    38'      D'     40              40'
Loxodonta         GAGGAG ACAA GtCGtaaC AA G G TAAGCGTACT GGAA AGTGCGCTTG GATAACTCA
Bos               GAGGAG ACAA GtCGtaaC AA G G TAAGCATACT GGAA AGTGTGCTTG G--ATAAAT
Equus             AAGGAG ACAA GtCGtaaC AA G G TAAGTATACC GGAA GGTGTACTTG G--ATAA-C
Manis             GAAGAG ATAA GtCGtaaC AA G G TAAGCATACT GGAA GGTGTGCTTG G--ATAATC
Eptesicus         GAGGAG ATAA GtCGtaaC AA G G TAAGTGTACT GGAA AGTGCACTTG G--ACAACA
Phalanger         GAGGAG AAAA GtCGtaaC AT G G TAAGTGTACT GGAA AGTGCACTTG G--AATATC
Didelphis         GAGGAG AAAA GtCGtaaC AC G G TAAGTGTACT GGAA AGTGCACTTG G--AATATC
Ornithorhynchus GAGGAG ATAA GtCGtaaC AA G G TAAGCATACC GGAA GGTGTGCTTG G--AATATC
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Fig. 3. Continued.

coids, *Balaenoptera, Tayassu;* U:A in *Felis, Manis, Caenolestes*). Gutell (1994) and Damberger and Gutell (unpublished) suggest alternatives to 18-7 involving stems 19 and 21. These are considered below.

9. Stem 19 is five base-pairs in our model, although one of these positions (19-4) is unpaired in *Bos.* Nevertheless, 19-4 shows pairing in 96% of the orders/suborders with examples of positional covariance in *Atelerix, Manis,* perissodactyls, and hyracoids. In the Damberger and Gutell (unpublished) model, stem 19 also includes an additional three base-pairs that are not present in our model. We find no evidence for

Table 2. Stem number and examples of compensatory evidence provided by mammalian and other vertebrate sequences[a]

Stem number	
2	2-3 (S: *Alligator*)
3	3-1 (D: *Felis*, Cetacea); 3-2 (D: *Equus*, Notoryctes); 3-3 (D: *Rattus* + *Mus*); 3-4 (*Chaetophractus*, Bovidae); 3-5 (D: *Sceloporus*); 3-6 (D: Amniotes); 3-7 (D: *Tayassu;* S: Proboscidea); 3-8 (D: *Phascolarctos, Eptesicus, Amblysomus*); 3-9 (D: *Atelerix, Homo* + *Pongo*)
4	4-1 (D: *Lutreolina, Amblysomus*); 4-2 (D: *Rattus* + *Mus, Caenolestes*); 4-3 (D: *Manis, Macropus*): 4-4 (D: *Odocoileus, Ceratotherium*); 4-5 (D: Proboscidea, *Blarina*); 4-6 (D: Sirenia, *Pongo*); 4-7 (D: *Procavia, Hydrochaeris, Isoodon*); 4-8 (D: *Nyctimene, Rattus*)
8	8-1 (D: *Felis, Didelphis*); 8-2 (D: *Notoryctes, Chaetophractus*); 8-3 (D: *Bos, Manis*);
17	17-1 (D: *Phascolarctos, Isoodon*); 17-2 (D: Didelphidae, *Manis*)
18	18-2 (D: *Atelerix*); 18-3 (D: Bovidae, *Rattus* + *Mus*); 18-4 (D: *Hydrochaeris, Phascolarctos*); 18-5 (D: *Atelerix, Manis*); 18-6 (D: *Phalanger*, Hyracoidea); 18-7 (D: *Balaenoptera,* Hyracoidea; S: *Caenolestes, Eptesicus*)
19	19-1 (D: Hyracoidea, *Phascogale*); 19-2 (D: *Blarina, Notoryctes*); 19-3 (D: *Hydrochaeris, Amblysomus*); 19-4 (D: *Atelerix, Manis,* Perissodactyla)
29	29-3 (D: *Sphenodon, Latimeria*)
34	34-1 (S: *Elephas*); 34-2 (D: Stenella, *Homo* + *Pongo*)
36	36-1 (D: Proboscidea, *Dromiciops*); 36-2 (*Elephas, Eptesicus*); 36-3 (D: Proboscidea, *Capra*); 36-4 (D: *Felis,* Paenungulata); 36-5 (D: *Trichechus, Equus*); 36-6 (Pongo, Vombatus); 36-7 (D: *Loxodonta, Phascogale,* Chiroptera); 36-8 (S: *Dugong, Bos*)

[a] Note. Positions within stems are indicated by numbers after dashes, e.g., 3-1 indicates the first base in stem 3 (and its complement in 3′), 3-2 indicates the second base in stem 3 (and its complement in 3′), etc

pairing or compensation at two of these positions. At the third, which is an alternate to our 18-7, potential-pairing occurs in 92% of the suborders and there is one example of a double-compensatory mutation, i.e., in cetaceans. Mitigating against this are uncompensated mutations in hyracoids and *Tayassu.* Presently, we favor 18-7 because of the more numerous examples of positional covariance.

10. Stem 20 is two base-pairs long in our model and three base-pairs in the Gutell (1994) and Damberger and Gutell (unpublished) models. The additional base-pair shows potential pairing in only 52% of the orders/suborders, including none of the marsupial orders. Also, there is no evidence for compensation at this position.
11. Stem 21 is two base-pairs in our model but three base-pairs in the Damberger and Gutell (unpublished) *Bos* model. The additional base-pair (21-3) is also in apparent conflict with our 18-7. At 21-3, potential-pairing occurs in 84% of the orders/suborders, which is slightly less than for 18.7. However, there are several examples of positional covariance (e.g., C:G in seals, hominoids, *Nyctimene,* U:A in *Rattus* + *Mus, Eptesicus, Caenolestes*). Given the examples of positional covariance that support both 18-7 and 21-3, possibly this is an instance in which two alternate pairings correspond to different conformations of 12S rRNA.
12. Stem 34 is four base-pairs in the Damberger and Gutell (unpublished) model. We tentatively recognize a two-base-pair stem, corresponding to the two internal base-pairs of Damberger and Gutell, with potential pairing in 92% and 96% of the orders/suborders at these two positions, respectively. 34-1 is not supported by any double compensatory mutations among the taxa included in this study but positional covariance does occur in the elephant shrew *Elephantulus rufescens* (Springer, unpublished). At the positions not included in our model, pairing only occurs in 32% and 8% of the orders/suborders, respectively. We note, however, that pairing may occur adjacent to 34-1 in perissodactyls where there is pairing in all three species as well as positional covariance in *Equus.*
13. Stem 36 is two base-pairs longer in our model with positional covariance and/or single-base compensatory mutations supporting each added position (see Table 2). This arrangement is tentative, however, and requires a single-base bulge.
14. The region between 39 and its complement, 39′, is highly variable and difficult to align across divergent mammalian taxa. Moreover, outside Mammalia this region is restricted to only nine nucleotides in *Sphenodon.* Stem 39 in our model is only six base-pairs, which is shorter than the helices in the Gutell (1994) and Damberger and Gutell (unpublished) models. However, three nucleotides downstream from 39 is a three-base-pair region that satisfies our criteria for placental mammals but not for Mammalia as a whole because of lack of pairing in all marsupial orders. Potential base-pairing occurs in the majority of species in 14 of 17 placental orders/suborders at each of these three positions with single-base compensatory substitutions at one of these positions and positional covariance (i.e., in *Eptesicus*) at the other two. This base-paired region is indicated as 39P in Figs. 1–3 to

indicate that it obtains only in placentals. Moreover, the region between 39 and 39′ shows additional base-pairing in the Gutell (1994) *Bos* model (but not *Homo*); while such pairing is supported for ruminant artiodactyls, base-pairing at equivalent positions is not recognizable in most other taxa. Nevertheless, there are apparently helices that are specific to other taxonomic groups in this highly variable region, e.g., a four-base-pair helix in dasyurids (Springer, unpublished). Thus, although this region is indicated as a loop in Fig. 2 because it lacks helices that occur across diverse taxa, we note that there is evidence for helices in select taxa. Thus, this region was omitted from analyses (below) requiring a stem vs loop distinction.

Tertiary Interactions

Gutell (1994) also indicates several tertiary interactions in his *Homo* and *Bos* models that involve canonical and U:G type base-pairing (see Figs. 1 and 2). First, the interaction labeled A and A′ in Fig. 2 involves three bases in an unilateral bulge between stems 13 and 14 and three antiparallel, complementary bases from the terminal loop associated with stem 14. Relatives to *E. coli,* one of the three positions exhibits positional covariance (G:C to A:U transitions). There is also a single-base substitution in the wombat (*Vombatus*) that would maintain pairing at this position. This particular tertiary interaction, referred to as a pseudoknot, is of putative functional importance as it would impose a high degree of constraint on this region if all helices were to pair simultaneously (Noller 1991). A second interaction, two base-pairs in length, involves bases entirely within the terminal loop for stem 14. All of the mammals included in our alignment are invariant at these positions and conserve the same putative base-pairing as in *E. coli* (B and B′ in Figs. 1 and 2). Support for this interaction comes from three independent examples of positional covariance in nonvertebrate mitochondrial small-subunit rRNAs (Gutell et al. 1994). Two additional tertiary interactions (C and C′, D and D′) also show invariant pairing among all mammalian sequences at all of the interacting positions.

Conservation of Decoding Sites

The decoding site comprises the entry site for tRNA (A site) and the peptidyl site (P site). It consists of several regions of the small-subunit rRNA held together in a complex, highly ordered structure (Dahlberg 1989). Moazed and Noller (1986) identified sites of 16S rRNA in *E. coli* that are shielded (or conformationally perturbed) as a consequence of interaction of tRNA with the subunit. Twenty-four bases that participate in formation of the decoding sites are identified in Dahlberg (1989). While these nucleotide sites are widely dispersed in the secondary structure model, three-dimensional folding brings almost all of these sites together to line the cleft of the small subunit with the exception of a few nucleotides that have been identified with maintaining translation accuracy (Dahlberg 1989).

All 24 of these nucleotide sites that contribute to the A and P sites occur in all mammalian 12S rRNAs (see Fig. 3), and 17 are invariant for the same base as in *E. coli,* which suggests conservation of function between *E. coli* 16S and mammalian 12S rRNAs. Included among these invariant sites is a three-base region located between 26′ and 38 that has been implicated in crosslinking to the wobble base of tRNA in *E. coli* (see Dahlberg 1989).

Termination Region

The homolog of stem 31 in *E. coli* 16S rRNA includes tandem 5′ UCA 3′ triplets (complementary to 5′ UGA 3′). Specific interactions between these triplets and stop codons allow for termination. Among mammalian 12S rRNAs, nucleotide sequences are highly conserved in stem 31. There is also an interesting pattern of covariation at the 12th position in stem 31 (and its complement in 31′). Specifically, pairing is U:G in all mammals except elephants (*Elephas* and *Loxodonta*) where instead the complementary bases are C:A. Although C:A pairing is noncanonical, Gutell et al. (1994) discuss a nontypical type of U:G pair that is unusual because it is always U:G except when it is replaced by C:A. Structurally isomorphic U:G and C:A pairs can be formed, for example, by protonating the adenine. Given the pattern of covariation seen among mammals at this location, and that this region of the rRNA is critical in proper termination, C:A in elephants is a likely candidate for noncanonical pairing.

Subunit Association

There is little information about how small and large subunits associate. Herr et al. (1979) suggested that subunit association might require base-pairing between complementary sequences in 16S and 23S rRNAs. Herr et al. (1979), Tapprich and Hill (1986), and Poldermans et al. (1980) have identified two terminal loops that are involved in subunit association in *E. coli.* These loops are homologous to the terminal loops for stems 23 and 40 in our model and are highly conserved between *E. coli* 16S and mammalian 12S sequences. First, the nine-base primary sequence for the terminal loop of stem 23 is, excepting for single-base substitutions in *Hydrochaeris* and *Phascogale,* 100% identical to the *E. coli* sequence. Second, the primary sequence of the terminal loop for stem 40 is 5′ GGAA 3′, identical to the *E. coli* sequence. Thus, there is strong conservation of the primary nucleotide sequences for these two loops (i.e., for stems 23 and 40) that are involved in subunit association.

Base Composition

Table 3 reports nucleotide frequencies for stems and loops in mammalian 12S rRNA genes (variable regions omitted). Consistent with an earlier study that examined fewer taxa and used slightly different demarcations for stems and loops (Springer et al. 1995), base compositions are remarkably uniform across Mammalia.

In loops, adenine is much more abundant than any of the other bases (mean = 48.5%) followed by cytosine (mean = 21.0%), thymine (mean = 19.0%), and guanine (mean = 11.5%). In their study of a phylogenetically more diverse group of metazoans, including other vertebrates as well as invertebrates, Vawter and Brown (1993) also found higher percentages of adenine in unpaired than paired regions. Gutell et al. (1985) suggested this is because adenine is the least polar of the four bases and may foster hydrophobic interactions with proteins.

In stems, in turn, nucleotide frequencies are much more even and the G + C component is appreciably higher (i.e., 50.4% vs 32.5%). The higher G + C composition in stems of mammalian 12S rRNAs is also consistent with findings for other metazoans (Vawter and Brown 1993). Higher G + C composition in paired regions of rRNAs has been predicted based on free energy considerations, i.e., G–C pairs have a lower free energy value than to G–U or A–U pairs (Turner et al. 1988).

In contrast to Vawter and Brown (1993), who found phylogenetic biases in base composition among different metazoans, base composition in both stems and loops is much more uniform among mammals. Mean values for marsupials and placentals, for example, are within 1.6% of each other for all four bases in both stems and loops. Likewise, the values for *Ornithorhynchus*, which is the single monotreme included in our analysis, are well within the range of variation found in both marsupials and placentals. This reduced level of variation is not surprising given that variables such as metabolic rate, which ostensibly influence base composition (e.g., see Martin 1995), exhibit much less variation within Mammalia than across more diverse metazoan groups.

Nucleotide Substitutions in Stems and Loops

The accumulation of substitutions in stems vs loops was investigated using our modified secondary structure model and is illustrated in Fig. 4, which plots the percent substitution against divergence time. The percentage of transitions in loops reaches a plateau at approximately 20 million years (Fig. 4, top panel); beyond this there is little or no increase in the percent transitional difference. In stems there is an apparent increase in transitions beyond 20 million years (Fig. 4, bottom panel). However, a multiple regression showed that the regression weight for stems vs loops is not significant ($P = 0.08$).

Tranversions accumulate at a slower rate than transitions but the increase appears linear as far out as 120 million years in both loops (Fig. 4, top panel) and stems (Fig. 4, bottom panel). However, the slope of the linear regression is approximately threefold higher in loops than stems, which indicates that transversions accumulate more rapidly in unpaired regions. Indeed, comparisons between placentals and marsupials show an average difference (transversions) of only 4.7% in stems compared to 15.6% in loops. A multiple regression examining percent divergence as a function of (1) divergence time and (2) stem vs loop shows that the regression weight associated with stem vs loop is significant at $P = 0.0001$.

It is evident from Fig. 4 that there is a transition/transversion bias in both stems and loops. In loops, this is most evident in pairwise comparisons involving taxa that diverged within the last 20 million years. For taxa that diverged about 80 million years ago, however, saturation obscures this bias and there is an approximate one-to-one ratio of transitions to tranversions. In contrast, the transition/transversion bias in stems is evident in pairwise comparisons involving taxa that diverged 120 million years ago. Vawter and Brown (1993) reported no consistent transition/transversion bias; in that study they used parsimony to trace evolutionary substitutions over a well-substantiated phylogeny. Vawter and Brown (1993) did find higher rates of transition than transversion in stems, but in loops rates for transitions and transversions were approximately equal. Likewise, Springer et al. (1995) used parsimony to trace evolutionary substitutions over a well-substantiated phylogeny that included 18 mammals and obtained a transition/transversion ratio of only 1.16 for loops. However, when this analysis was restricted to a subset of more closely related taxa (i.e., five ruminant artiodactyls) the transition/transversion ratio was higher (3.4). Hixson and Brown (1986) also reported higher transition/transversion ratios in loops for closely related primates. In the context of Fig. 4, it is evident that transition-to-transversion ratios, especially in loops, depend on the level of phylogenetic divergence. Thus, it is not surprising that different values have been estimated in studies that examine closely related vs more distantly related taxa.

Overall, the difference between transition and transversion rates is much more profound in stems than loops. This is apparently a consequence of constraints to maintain pairing in stems (Vawter and Brown 1993; Douzery and Catzeflis 1995; Springer et al. 1995). Importantly, certain transitions allow for the maintenance of pairing through the following pathways: C:G $\leftrightarrow$ U:G $\leftrightarrow$ UA. In contrast, single transversions never maintain base-pairing and a second transversion is always required to restore pairing. Thus, a more pronounced transition: transversion bias in stems is not surprising.

Even though there is apparently a transition:transversion bias among mammalian 12S rRNAs, not all transitions are equally likely, i.e., C $\leftrightarrow$ T substitutions consis-

Table 3. Nucleotide frequencies in stems and loops

	Stems				Loops			
Taxon	G	A	T	C	G	A	T	C
Phoca	25.9	23.8	27.2	23.1	10.7	49.5	17.7	22.1
Halichoerus	26.1	23.5	26.3	24.0	11.0	49.6	17.5	21.9
Felis	26.1	23.5	26.1	24.2	11.7	48.1	17.6	22.6
Procavia	25.9	23.1	26.8	24.2	11.0	50.6	16.0	22.4
Dendrohyrax	24.8	24.4	26.6	24.2	11.2	49.8	16.2	22.9
Trichechus	27.0	22.7	25.9	24.4	12.7	46.0	18.4	22.9
Dugong	27.4	22.0	24.8	25.7	12.4	46.8	18.7	22.1
Loxodonta	27.4	22.2	25.9	24.4	12.6	45.3	21.0	21.0
Elephas	27.2	22.5	25.9	24.4	12.5	45.4	20.3	21.8
B. physalus	26.6	23.1	25.9	24.4	12.6	47.8	17.1	22.5
B. musculus	26.6	23.1	26.6	23.8	11.6	49.1	17.3	22.0
Stenella	26.1	23.8	25.7	24.4	11.6	48.8	19.1	20.5
Bos	26.1	23.8	25.9	24.2	11.0	50.6	18.7	19.7
Tragelaphus	26.1	23.3	26.1	24.4	10.7	50.1	18.5	20.7
Capra	26.3	23.5	24.6	25.5	11.2	49.4	17.6	21.8
Hydropotes	26.6	23.1	26.8	23.5	11.4	49.6	18.9	20.1
Odocoileus	26.3	23.3	25.7	24.6	11.2	49.3	18.9	20.6
Antilocapra	28.1	21.8	25.7	24.4	10.4	50.9	19.0	19.8
Tragulus	29.2	21.2	24.2	25.5	11.6	50.0	16.6	21.8
Sus	25.8	23.4	27.1	23.6	10.8	49.1	18.0	22.1
Tayassu	25.5	24.2	25.3	25.1	9.8	50.5	19.3	20.5
E. caballus	26.1	23.5	25.7	24.6	10.9	49.3	17.7	22.1
E. grevyi	25.9	23.8	26.6	23.8	12.2	48.4	16.6	22.8
Ceratotherium	24.8	25.3	25.9	24.0	11.4	49.5	17.2	21.9
Chaetophractus	25.7	24.0	25.5	24.8	10.6	49.9	17.8	21.7
Manis	25.1	24.8	27.2	22.9	12.1	47.9	19.8	20.2
Rattus	24.7	24.5	28.0	22.8	11.0	49.3	20.3	19.4
Mus	23.5	25.5	28.5	22.5	10.6	49.1	21.7	18.5
Hydrochaeris	25.5	24.4	27.0	23.1	12.1	49.5	18.6	19.8
Cavia	27.2	22.0	26.5	24.3	12.8	47.9	19.5	19.8
Homo	27.2	22.0	25.3	25.5	12.6	45.2	17.6	24.6
Pongo	27.8	21.1	24.1	27.0	13.3	45.0	17.1	24.6
Nyctimene	27.9	21.8	24.2	26.1	11.6	51.0	17.8	19.6
Eptesicus	26.8	23.1	25.7	24.4	11.7	50.0	17.2	21.1
Atelerix	25.3	24.0	28.7	22.0	11.1	49.0	23.0	16.8
Blarina	26.8	23.1	25.5	24.6	12.2	48.5	20.9	18.4
Amblysomus	24.8	24.2	29.2	21.8	10.4	49.5	22.6	17.4
Notoryctes	27.9	21.6	25.1	25.5	14.8	45.4	18.8	21.0
Phalanger	26.1	23.7	25.6	24.6	9.7	51.6	16.6	22.1
Phascolarctos	26.5	23.5	25.6	24.4	10.8	47.5	19.7	21.9
Vombatus	26.9	22.8	26.5	23.7	10.6	47.5	19.7	22.2
Macropus	26.7	23.1	25.6	24.6	12.6	48.1	18.5	20.7
Phascogale	29.6	20.3	25.5	24.6	15.2	44.0	21.4	19.4
Dromiciops	27.0	22.7	25.7	24.6	11.1	47.8	19.5	21.7
Isoodon	25.7	24.2	26.6	23.5	9.9	48.0	21.5	20.5
Caenolestes	26.1	23.5	27.2	23.1	9.8	47.3	23.0	19.9
Lutreolina	25.1	25.1	28.1	21.8	11.5	47.7	22.1	18.7
Didelphis	24.6	25.3	28.1	22.0	10.6	49.8	21.7	18.0
Ornithorhynchus	25.5	25.1	25.7	23.8	12.0	48.2	19.8	20.0
Mean and Standard Deviation (in parentheses) for:								
All 49 taxa	26.3	23.3	26.2	24.1	11.5	48.5	19.0	21.0
	(1.2)	(1.2)	(1.1)	(1.1)	(1.1)	(1.7)	(1.8)	(1.6)
All 11 marsupials	26.6	23.3	26.3	23.9	11.5	47.7	20.2	20.6
	(1.4)	(1.4)	(1.1)	(1.2)	(1.9)	(2.0)	(1.9)	(1.4)
All 37 placentals	26.3	23.3	26.2	24.2	11.5	48.8	18.6	21.1
	(1.1)	(1.1)	(1.2)	(1.1)	(0.8)	(1.6)	(1.7)	(1.7)

tently occur at a high rate in stems and loops but A $\leftrightarrow$ G substitutions in unpaired regions are less likely than certain transversion pathways (i.e., A $\leftrightarrow$ T and A $\leftrightarrow$ C) (Vawter and Brown 1993; Springer et al. 1995). Thus,

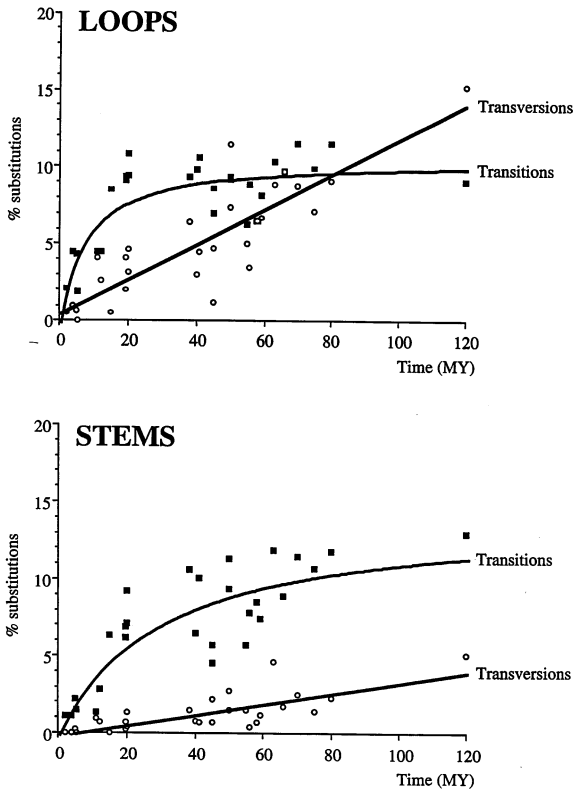

Fig. 4. Percent divergence between taxa (%) plotted against divergence time (t) for transitions (*TS*) and transversions (*TV*) in loops (*upper panel*) and stems (*lower panel*). *Black squares* represent transitions and *open circles* represent transversions. The linear regressions between percent transversions and divergence time are %TV = $0.11t$ + 0.47 (r^2 = 0.74) in loops and %TV = $0.035t - 0.14$ (r^2 = 0.60) in stems. The hyperbolic curves between percent transitions and divergence time are %TS = $10.6t/(t + 7.4)$ (r^2 = 0.64) in loops and %TS = $14.4t/(t + 30.2)$ (r^2 = 0;71) in stems. Estimates of divergence time, which are based primarily on the fossil record and on single-copy DNA hybridization molecular clocks, are as follows: *Halichoerus* to *Phoca* = 2 million years (Arnason et al. 1993); Pinnepedia to Felidae = 58 million years (Garland et al. 1993; and also assuming that pinnepeds are closer to ursids than to fields, see Lento et al. 1995); *B. physalus* to *B. musculus* = 5 million years (Arnason and Gullberg 1993); Balaenopteridae to Delphinidae = 40 million years (Barnes et al. 1985); *Homo* to *Pongo* = 15 million years (Sibley and Ahlquist 1984); *Mus* to *Rattus* = 12 million years (Catzeflis et al. 1992); *Hydrochaeris* to *Cavia* = 20 million years (Carroll 1988); Antilocapridae to Cervidae = 19.5 million years (Garland et al. 1993); *Bos* to *Capra* = 19.5 million years (Garland et al. 1993); [Antilocapridae + Cervidae] to Bovidae = 20 million years; Bovidae to Tragulidae = 38 million years (Garland et al. 1993); Suidae to Tayasuidae = 45 million years (J. Sudre, pers. comm.); Ruminantia to Suiformes = 50 million years (Garland et al. 1993); *E. grevyi* to *E. caballus* = 4 million years (MacFadden 1992); Equidae to Rhinocerotidae = 56 million years (Garland et al. 1993); Artiodactyla to Perissodactyla = 75 million years (mean of Garland et al. 1993; Novacek 1993); *Elephas* to *Loxodonta* = 5 million years (Coppens et al. 1978); *Dugong* to *Trichechus* = 45 million years (Domning 1978); Sirenia to Proboscidea = 70 million years (Novacek 1993); Tethytheria to Hyracoidea = 80 million years (Novacek 1993); Megachiroptera to Microchiroptera = 50 million years (Simmons 1994); Edentata to Pholidota = 63 million years (Novacek 1993); *Phascolarctos* to *Vombatus* = 41 million years (Kirsch et al. submitted); *Phalanger* to *Macropus* = 55 million years (Kirsch et al. submitted); Vombatoidea to Phalangeroidea = 59 million years (Kirsch et al. submitted); Diprotodontia to Dasyuromorphia = 66 million years (Kirsch et al. submitted.); *Lutreolina* to *Didelphis* = 11 million years (Kirsch et al. submitted); Eutheria to Metatheria = 120 million years [mean of 100 million years (Rowe 1993) and 140 million years (Szalay 1994)].

C ↔ T substitutions are primarily responsible for the transition:transversion bias in loops.

Finally, Fig. 4 also illustrates that 12S rRNA sequences will provide maximum resolution in mammalian systematics for divergence events that are within the last 10–20 million years because both transitions and transversions remain unsaturated over this time window. Beyond this, transversions in stems and loops continue to increase linearly beyond 100 million years. Thus, 12S rRNA sequences remain useful over these extended time windows. Indeed, 12S rRNA sequences have proved useful in systematic studies that address interordinal relationships within both Metatheria (Springer et al. 1994) and Eutheria (Springer and Kirsch 1993; Douzery and Catzeflis 1995; Lavergne et al. 1996).

Conclusions

There are several compelling reasons to elucidate the secondary structure of 12S rRNA. First, a firm knowledge of structure is believed to be essential for a rational, molecular amount of the function of ribosomes in protein synthesis (Wool et al. 1990). Comparative sequence analysis, making use of the criteria of potential base-pairing and positional covariance, is one of the most powerful tools available for elucidating secondary structure. In the case of mammalian 12S rRNA, our comparative analysis has confirms much of the Gutell (1994) models but suggests changes as well. In addition, we have identified a number of functional sites (i.e., for decoding and subunit association) in the *E. coli* 16S model that are conserved among all of the mammalian 12S rRNA sequences. This is suggestive of similar function in *E. coli* 16S and mammalian 12S rRNAs for these conserved sites in spite of the much-reduced overall size of the mammalian mitochondrial small-subunit rRNA relative to *E. coli* 16S. Second, secondary structure models for rRNAs provide an essential backbone in certain gene evolution studies. For example, calculations of the strength of selection pressure for compensatory mutations require a model that differentiates between stem and loop bases (e.g., Hillis and Dixon 1991; Dixon and Hillis 1993; Gatesy et al. 1994). Also, this distinction is a fundamental requirement in studies that investigate patterns and rates of nucleotide substitution in stems vs loops. We have shown above, for example, that patterns and rates for transitions and transversions are noticeably different in stems and loops. Finally, secondary structure models are useful in molecular systematics. Specifically, most phylogeny reconstruction algorithms (e.g., maximum parsimony, maximum likelihood) assume that nucleotide changes at different positions are independent. This assumption is clearly violated in stems where there is pressure for compensation. However, it may be possible to develop weighting schemes that address the

problem of substitution dependence in stem regions (Wheeler and Honeycutt 1988; Smith 1989; Dixon and Hillis 1993; Springer et al. 1995). Reliable secondary structure models provide an essential foundation for such studies.

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