Two Novel Gene Orders and the Role of Light-Strand Replication in Rearrangement of the Vertebrate Mitochondrial Genome

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Two novel mitochondrial gene arrangements are identified in an agamid lizard and a ranid frog. Statistical tests incorporating phylogeny indicate a link between novel vertebrate mitochondrial gene orders and movement of the origin of light-strand replication. A mechanism involving errors in light-strand replication and tandem duplication of genes is proposed for rearrangement of vertebrate mitochondrial genes. A second mechanism involving small direct repeats also is identified. These mechanisms implicate gene order as a reliable phylogenetic character. Shifts in gene order define major lineages without evidence of parallelism or reversal. The loss of the origin of light-strand replication from its typical vertebrate position evolves in parallel and, therefore, is a less reliable phylogenetic character. Gene junctions also evolve in parallel. Sequencing across multigenic regions, in particular transfer RNA genes, should be a major focus of future systematic studies to locate novel gene orders and to provide a better understanding of the evolution of the vertebrate mitochondrial genome.

Introduction

Mitochondrial gene order is highly variable among animal phyla and has been considered a useful phylogenetic character (Sankoff et al. 1992; Smith et al. 1993; Boore and Brown 1994; Boore et al. 1995). Vertebrate mitochondrial gene order was initially considered completely conserved. DNA sequences of the entire mitochondrial genome from mammals (Anderson et al. 1981, 1982; Bibb et al. 1981) and the African clawed frog (*Xenopus laevis*) (Roe et al. 1985) suggested that all vertebrates shared a common gene order, and sequences from fish later also revealed this order (Johansen, Guddal, and Johansen 1990; Tzeng et al. 1992; Chang, Huang, and Lo 1994; Zardoya and Meyer 1996). Three types of deviations from the most common gene arrangement subsequently have been identified: duplication of genes (Moritz and Brown 1986, 1987; Wallis 1987), rearrangement of genes, and loss of a recognizable origin for replication of the light strand (O_L) between the genes encoding the asparagine and cysteine tRNAs.

We assessed gene order for one third of the mitochondrial genes spanning from ND1 to COI across squamate reptiles. The agamid lizard, *Uromastix acanthinurus,* has a pair of transfer RNA genes switched in order (fig. 1). Another sequence containing a unique gene order was obtained from the rice frog, *Rana limnocharis,* in a different area of the mitochondrial genome during a search for a region to use in phylogenetic studies.

Gene rearrangements have been reported previously in seven vertebrate lineages: a sea lamprey (*Petromyzon marinus*) (Lee and Kocher 1995), the American bullfrog (*Rana catesbeiana*) (Yoneyama 1987), marsupials (Pääbo et al. 1991; Janke et al. 1994), birds (Desjardins and Morais 1990, 1991; Quinn and Wilson 1993), crocodilians (Kumazawa and Nishida 1995), and the Texas blind snake (*Leptotyphlops dulcis*) (Kumazawa and Nishida 1995). The tuatara (*Sphenodon punctatus*) appears to be heteroplasmic for two nontypical vertebrate gene arrangements (Quinn and Mindell 1996) (fig. 1).

Several mechanisms have been proposed for the formation of tandem duplication of genes and rearrangement. None of the genes involved in vertebrate gene rearrangements have reversed their coding polarity and, thus, an inversion-based mechanism need not be invoked. Transposition previously has been considered unlikely, because most reported duplicated sequences lack the terminal repeats characteristic of most transposable elements (Calos and Miller 1980; Levinson and Gutman 1987). Gene rearrangements have been proposed to occur by tandem duplication of gene regions as a result of slipped-strand mispairing (Moritz and Brown 1986, 1987; Levinson and Gutman 1987), followed by deletion of genes (Moritz and Brown 1986, 1987; Moritz, Dowling and Brown 1987; Pääbo et al. 1991).

A mechanism utilizing stem-and-loop structures during replication may be responsible for tandemly duplicated gene regions. Stem-and-loop structures have been identified at both ends of tandemly duplicated gene regions (Stanton et al. 1994). The stem-and-loop structures of tRNA genes resemble the two replication origins in vertebrates. The $tRNA^{Cys}$ gene has been postulated to have served as a replication origin for light-strand sythesis in human mitochondrial DNA (Clayton 1982), in addition to the O_L stem region. The enzyme that initiates light-strand replication appears to be signaled by a stem-and-loop structure (Wong and Clayton 1985, 1986; Hixson, Wong, and Clayton 1986). This enzyme has been postulated to recognize stem-and-

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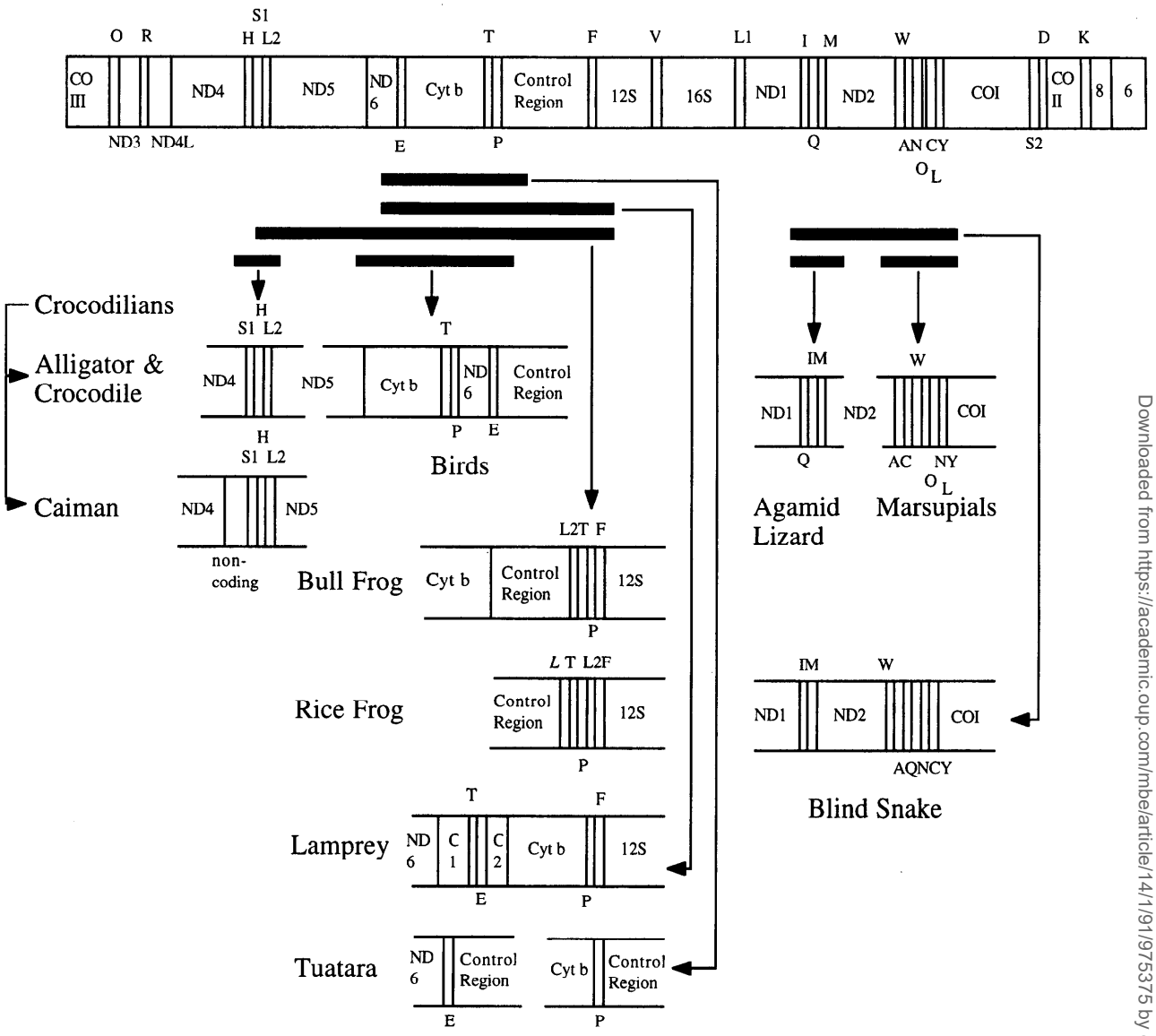

FIG. 1.—Positions of all known vertebrate mitochondrial gene rearrangements. The most common order of genes in the vertebrate mitochondrial genome is depicted as a linear bar at the top. Black bars indicate regions that have undergone rearrangement. Arrows point to the derived gene orders. O_L represents the origin of light-strand replication. Abbreviations for genes are: 12S and 16S for 12S and 16S rRNAs; ND 1–6 and 4L for NADH dehydrogenase subunits 1–6 and 4L; CO I–III for cytochrome *c* oxidase subunits I–III; Cyt b for cytochrome *b*; 6 and 8 for ATPase subunits 6 and 8. All protein-coding genes are transcribed from the heavy strand with the exception of ND6. Transfer RNAs are represented by their standard one-letter amino acid code positioned on the strand encoded, with the top representing the heavy strand and the bottom representing the light strand. S1, S2, L1, and L2 depict $tRNA^{Ser(AGY)}$, $tRNA^{Ser(UCN)}$, $tRNA^{Leu(UUR)}$, and $tRNA^{Leu(CUN)}$, respectively. The italic *L* represents a hypothesized nonfunctional copy of the gene encoding the $tRNA^{Leu(CUN)}$ (see fig. 5). In the lamprey, C1 and C2 illustrate the two portions of the control region. The tuatara has two gene orders. Within the crocodilians, a caiman has an additional noncoding region between the ND4 gene and the gene encoding the $tRNA^{Ser(AGY)}$. Note that all rearrangements involve tRNA genes.

loop structures associated with tandemly duplicated gene regions (Stanton et al. 1994). Rearrangements often may start and end at tRNA genes, which may be involved in the initial production of a tandemly duplicated gene region (Moritz and Brown 1987).

Here a phylogenetic analysis is used to investigate the evolutionary association between rearrangement of the vertebrate mitochondrial genome and movement of the origin of light-strand replication. This analysis examines all known orders of genes in vertebrate mitochondrial genomes. We propose a mechanism by which errors in replication of the light strand of mitochondrial DNA initiate rearrangements of the mitochondrial genome. This mechanism explains most, but not all, of the mitochondrial genomic rearrangements observed in vertebrates. The mechanism predicts that a particular genomic arrangement is very unlikely to evolve in parallel or undergo evolutionary reversal to an ancestral state. Consistent with this prediction, all evolutionarily derived arrangements of vertebrate mitochondrial genes appear to diagnose monophyletic groups. The ordering of genes in the mitochondrial genome may provide important information for assessing phylogenetic relationships in vertebrates.

Table 1
Primers Used in this Study

Human Position[a]	Gene	Sequence[b]	Reference
H692.....	12S rRNA	5′-GGTGTGCTGATACTTGCATGT-3′	This study
H1067.....	12S rRNA	5′-TAGTGGGGTATCTAATCCCAGTTT-3′	This study[c]
L3002.....	16S rRNA	5′-TACGACCTCGATGTTGGATCAGG-3′	This study
L3881.....	ND1	5′-TTTGACCTAACAGAAGGAGA-3′	This study
L3887.....	ND1	5′-GACCTAACAGAAGGAGAATCAGA-3′	This study
L4160.....	ND1	5′-CGATTCCGATATGACCARCT-3′	Kumazawa and Nishida (1993)
L4178a....	ND1	5′-CARCTWATACACYTACTATGAAA-3′	This study
L4178b....	ND1	5′-CAACTAATACACCTACTATGAAA-3′	This study
L4221.....	$tRNA^{Ile}$	5′-AAGGATTACTTTGATAGAGT-3′	This study
H4419a....	$tRNA^{Met}$	5′-GGTATGAGCCCAATTGCTT-3′	This study
H4419b....	$tRNA^{Met}$	5′-GGTATGAGCCCGATAGCTT-3′	This study
L4437.....	$tRNA^{Met}$	5′-AAGCTTTCGGGCCCATACC-3′	This study
L4645.....	ND2	5′-ACAGAAGCCGCAACAAAATA-3′	This study
L4831.....	ND2	5′-TGACTTCCAGAAGTAATACAAGG-3′	This study
L4882.....	ND2	5′-TGACAAAAACTAGCACC-3′	This study
H4980.....	ND2	5′-ATTTTTCGTAGTTGGGTTTGRTT-3′	This study
L5002.....	ND2	5′-AACCAAACCCAACTACGAAAAAT-3′	This study
H5540.....	$tRNA^{Trp}$	5′-TTTAGGGCTTTGAAGGC-3′	This study
L5556a....	$tRNA^{Trp}$	5′-AAGAGCCTTCAAAGCCCTAAG-3′	This study
L5556b....	$tRNA^{Trp}$	5′-GCCTTCAAAGCCCTAAA-3′	This study
H5617a....	$tRNA^{Ala}$	5′-AAAATRTCTGRGTTGCATTCAG-3′	This study
H5617b....	$tRNA^{Ala}$	5′-AAAGTGTCTGAGTTGCATTCAG-3′	This study
L5638a....	$tRNA^{Ala}$	5′-CTGAATGCAACYCAGAYATTTT-3′	This study
L5638b....	$tRNA^{Ala}$	5′-CTGAATGCAACTCAGACACTTT-3′	This study
H5692.....	$tRNA^{Asn}$	5′-TTGGGTGTTTAGCTGTTAA-3′	This study
L5706.....	$tRNA^{Asn}$	5′-TAGTTAACAGCTAAACAC-3′	This study
H5934.....	COI	5′-AGRGTGCCAATGTCTTTGTGRTT-3′	This study
H5937.....	COI	5′-GTGCCAATGTCTTTGTG-3′	This study[d]
H12314....	$tRNA^{Leu(CUN)}$	5′-TTTTACTTGGAGTTGCACCA-3′	This study[e]
L15783....	Cyt *b*	5′-CAACCAGTAGAAGACCC-3′	This study

[a] Primers are designated by their 3′ ends, which correspond to the position in the human mitochondrial genome (Anderson et al. 1981) by convention. H and L designate heavy- and light-strand primers, respectively.

[b] Positions with mixed bases are labeled with their standard one-letter codes: R = G or A; Y = T or C; W = A or T.

[c] Modified from Kocher at al. (1989).

[d] Modified from Kumazawa and Nishida (1993).

[e] Modified from Arévalo, Davis, and Sites (1994).

Materials and Methods

Laboratory Protocols

Genomic DNA was extracted from muscle or liver tissue using the Qiagen QIAamp tissue kit. Genomic DNA was amplified using a denaturation at 94°C for 35 s, annealing at 45–53°C for 35 s, and extension at 70°C for 150 s with 4 s added to the extension per cycle, for 30 cycles. Amplified products were purified on 2.5% Nusieve GTG agarose gels and reamplified under similar conditions. Reamplified double-stranded products were purified on 2.5% acrylamide gels (Maniatis, Fritsch, and Sambrook 1982). Template DNA was eluted from acrylamide passively over 3 days in which Maniatis elution buffer (Maniatis, Fritsch, and Sambrook 1982) was replaced each day. Cycle sequencing reactions were run using the Promega fmol DNA sequencing system with a denaturation at 95°C for 35 s, annealing at 45–53°C for 35 s, and extension at 70°C for 1 min for 30 cycles. Sequencing reactions were run on Long Ranger sequencing gels for 5–12 h at 38–40°C.

Primers are designated by their 3′ ends, which correspond to the position in the human mitochondrial genome (Anderson et al. 1981) by convention, and presented in table 1. Both strands were sequenced except the *Rana limnocharis* control region to 12S rRNA, which was amplified two different ways (see below) from genomic DNA, and the heavy strand was sequenced from each amplification. The two heavy-strand sequences of *Rana limnocharis* were identical. Negative controls were run on all amplifications. The *Rana limnocharis* sequence from ND2 to COI was amplified with L4437 and H5934. Sequencing was done with L5556 and H5934. The *Rana limnocharis* sequence from the control region to 12S rRNA was amplified with L15783 and H692, and L15783 and H1067. Sequencing was done with H692 and H12314. Squamate reptiles from 10 lizard families and Amphibaenia were sequenced completely from ND1 to COI using primers described in table 1. Amplifications used primer H5934 and one of the following: L3881, L3887, L4160, L4178a, or L4178b. DNA samples from some taxa were amplified also with L3002 and either H4419a or H4419b.

Specimen Information

Museum numbers, approximate localities for voucher specimens from which DNA was extracted, and GenBank accession numbers are presented in phyloge-

netic order. Acronyms are AMNH for American Museum of Natural History, New York; CAS for California Academy of Sciences, San Francisco; MVZ for Museum of Vertebrate Zoology, University of California at Berkeley; and ZISP for Zoological Institute, St. Petersburg, Russia. MVZ-RM represents field numbers of the first author for uncataloged specimens being deposited in the Museum of Vertebrate Zoology. *Rana limnocharis* Zhejiang, China (CAS 194255; U71323, U71324); *Uromastix acanthinurus* Morocco (MVZ 162567; U71325); *Teratoscincus przewalskii* Xinjiang, China (CAS 171010; U71326); *Lialis jicari* Manam Island, Madong, New Guinea (AMNH 105099; U71327); *Xantusia vigilis* California, USA (MVZ-RM2299; U71328); *Platysaurus capensis* South Africa (CAS 193465; U71329); *Mabuya aurata* Turkmenistan (CAS 179697; U71330); *Eremias grammica* Turkmenistan (CAS 179206; U71331); *Cnemidophorus tigris* California, USA (MVZ 179799; U71332); *Xenosaurus grandis* Veracruz, Mexico (MVZ 137789; U71333); *Varanus griseus* Turkmenistan (ZISP 19576; U71334); and *Bipes biporus* Baja California Sur, Mexico (MVZ 137543; U71335).

Results

Gene Order in Squamate Reptiles

Sequences obtained from *Teratoscincus przewalskii* (Gekkonidae), *Lialis jicari* (Pygopodidae), *Cnemidophorus tigris* (Teiidae), *Eremias grammica* (Lacertidae), *Platysaurus capensis* (Cordylidae), *Xenosaurus grandis* (Xenosauridae), and *Varanus griseus* (Varanidae) all had a mitochondrial gene order similar to most vertebrates of ND1 (subunit 1 of NADH dehydrogenase), $tRNA^{Ile}$, $tRNA^{Gln}$, $tRNA^{Met}$, ND2, $tRNA^{Trp}$, $tRNA^{Ala}$, $tRNA^{Asn}$, O_L, $tRNA^{Cys}$, $tRNA^{Tyr}$, and COI (subunit I of cytochrome *c* oxidase). Three additional groups, *Bipes biporus* (Amphisbaenia), *Mabuya aurata* (Scincidae), and *Xantusia vigilis* (Xantusidae), had this gene order but do not have a recognizable O_L. While *Xantusia* has only seven bases between $tRNA^{Asn}$ and $tRNA^{Cys}$, *Bipes* and *Mabuya* have strange stem-and-loop structures that may not serve as replication origins because they have little homology with other vertebrate and presumptive squamate lizard replication origins (figs. 2 and 3).

A switch in the order of two transfer RNA genes is observed in the agamid lizard, *Uromastix acanthinurus*. In *Uromastix*, $tRNA^{Gln}$ precedes $tRNA^{Ile}$ and O_L is absent between $tRNA^{Asn}$ and $tRNA^{Cys}$ (figs. 2 and 4). Among vertebrates, this derived gene order is shared only among acrodont lizards (Agamidae and Chameleonidae) (unpublished data). Interestingly, $tRNA^{Gln}$ is also rearranged in the snake, *Leptotyphlops* (Kumazawa and Nishida 1995), but the resulting gene order is different with $tRNA^{Gln}$ located between $tRNA^{Ala}$ and $tRNA^{Asn}$.

In addition, *Uromastix, Lialis, Cnemidophorus, Varanus, Xantusia,* and *Bipes* have an unusual $tRNA^{Cys}$ that lacks a D-stem and, instead, contains a D-arm replacement loop (Macey et al. 1997), similar to that reported in the tuatara, *Sphenodon punctatus* (Seutin et al. 1994) (fig. 2). The lack of a recognizable O_L and the novel secondary structure of the $tRNA^{Cys}$ also characterize acrodont lizards but not most squamate reptiles (Kumazawa and Nishida 1995; Seutin et al. 1994; Macey et al. 1997; unpublished data).

Gene Order in Frogs

Two mitochondrial DNA sequences were obtained from the rice frog, *Rana limnocharis*. The regions examined are from the control region to the 12S rRNA gene, and from the ND2 to COI genes (figs. 2 and 5). Comparison of these sequences with the published sequences from the African clawed frog, *Xenopus* (Roe et al. 1985) and the American bullfrog, *Rana catesbeiana* (Yoneyama 1987), revealed a unique gene order with the same gene content as *Rana catesbeiana* (fig. 5*A*). Whereas *Xenopus* shares the conserved vertebrate gene order containing only the phenylalanine tRNA gene between the control region and the 12S rRNA gene, both species of *Rana* have four tRNA genes in this region. These four tRNA genes are arranged in different orders in the two species: *Rana catesbeiana* has the order control region, $tRNA^{Leu(CUN)}$, $tRNA^{Thr}$, $tRNA^{Pro}$, $tRNA^{Phe}$, 12S rRNA; *Rana limnocharis* has the order control region, $tRNA^{Thr}$, $tRNA^{Pro}$, $tRNA^{Leu(CUN)}$, $tRNA^{Phe}$, 12S rRNA. In *Rana limnocharis,* the $tRNA^{Leu(CUN)}$ gene is flanked by two small noncoding sequences of 15 and 37 bases, respectively. The sequence of the 15-base noncoding region is repeated with a single nucleotide substitution and one deletion in the 37-base noncoding region (fig. 5*A*). In addition, *Rana limnocharis* appears to have a nonfunctional copy of the $tRNA^{Leu(CUN)}$ gene between the control region and $tRNA^{Thr}$. This is especially noteworthy, because *Rana catesbeiana* has a functional $tRNA^{Leu(CUN)}$ gene at this position (fig. 5*B*).

Both *Rana* appear to have a recognizable O_L in the typical vertebrate position (Yoneyama 1987) (fig. 2). These two species share with other vertebrates a region in O_L that forms a stem-and-loop structure. In addition, both species contain the light-strand trinucleotide sequence 5′-CGG-3′, considered to be the initiation site for light-strand elongation in mouse (Brennicke and Clayton 1981) (fig. 2). The only major differences between O_L in the two species of *Rana* is the incorporation of a G_{12} insertion in the loop region and a five-base extension of the stem region in the *Rana limnocharis*. Except for the five-base extension, the O_L stem sequences are identical in the two *Rana* species and *Xenopus*, and these are nearly identical to that in mouse. The O_L-containing region has the typical vertebrate gene order of ND2, $tRNA^{Trp}$, $tRNA^{Ala}$, $tRNA^{Asn}$, O_L, $tRNA^{Cys}$, $tRNA^{Tyr}$, and COI in the *Rana limnocharis*.

Evidence that Amplified Sequences are from Mitochondrial DNA

Several observations support our conclusion that the DNA sequences analyzed here are from the mitochondrial genome and do not represent nuclear integrated copies of mitochondrial genes (see Zhang and Hewitt 1996). All sequences reported here show strong strand bias against guanine on the light strand (reptiles ND1 to COI, G = 11%–14%, T = 21%–28%, A =

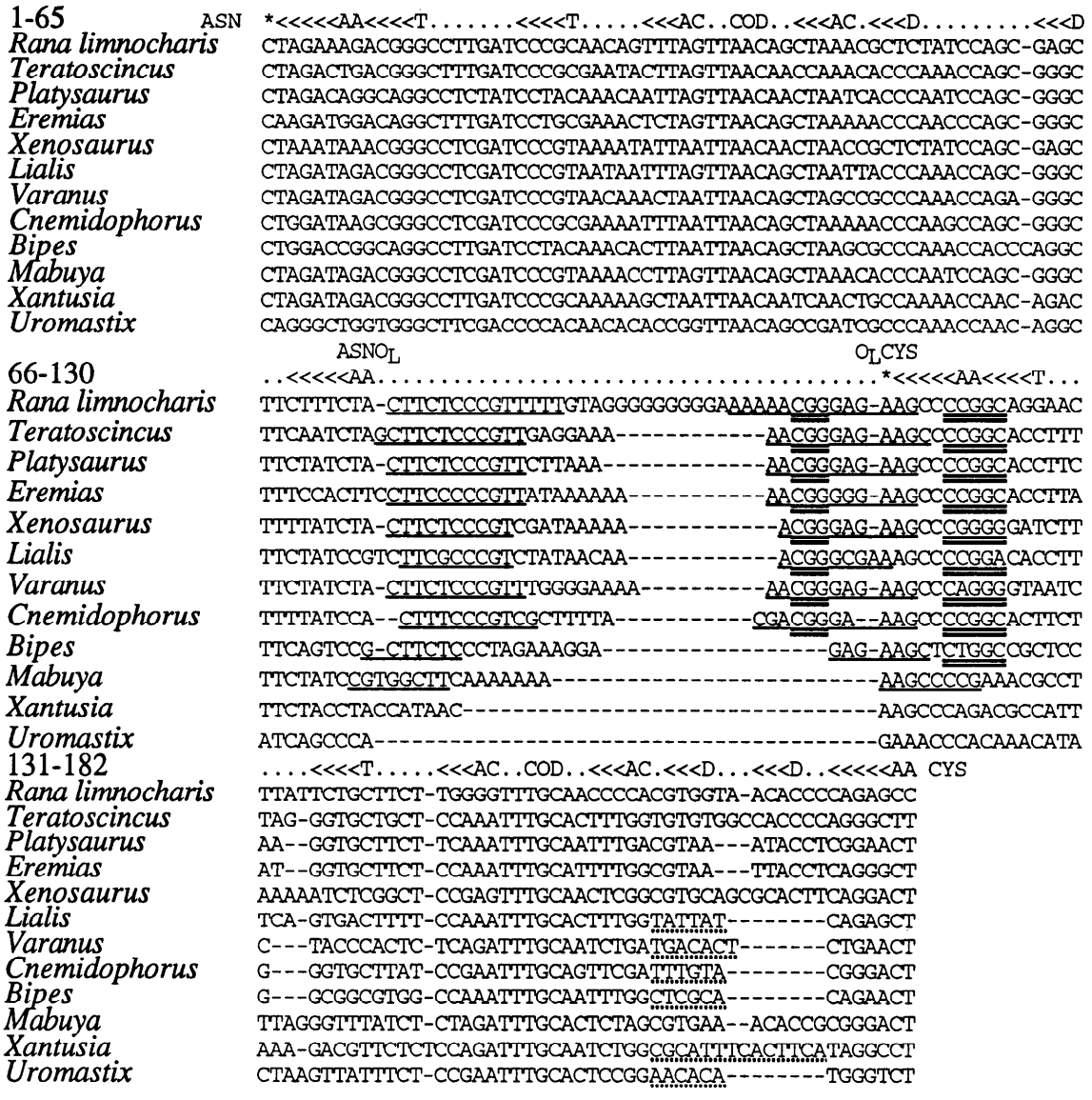

FIG. 2.—Presence of the O_L between the genes encoding the asparagine and cysteine tRNA genes. Stem regions are single underlined (and double underlined 5′-CGG-3′). *Rana limnocharis, Teratoscincus, Platysaurus, Eremias, Xenosaurus, Lialis, Varanus,* and *Cnemidophorus* have sequences presumably representing the O_L. Note in the stem region of the O_L the light-strand trinucleotide sequence 5′-CGG-3′ (double underline) which is considered the initiation site for light-strand replication in mouse (Brennicke and Clayton 1981). Note in these sequences the light-strand sequence 5′-CVGGV-3′ (double underline) complementary to the heavy-strand template sequence 3′-GGCCG-5′ required for *in vitro* replication in humans (Hixson, Wong, and Clayton 1986). No evidence of a related sequence is present in *Xantusia, Mabuya,* or *Uromastix.* Unusual O_L stems are observed in *Mabuya* and *Bipes* (see fig. 3). *Lialis, Varanus, Cnemidophorus, Bipes, Xantusia,* and *Uromastix* have an unusual $tRNA^{Cys}$ which lacks a D-stem and instead contains a D-arm replacement loop (dotted underline). Sequences are presented as light-strand sequence and tRNA secondary structure is designated above the sequence. Stems are indicated by arrows in the direction encoded: AA = amino-acid-acceptor stem, D = dihydrouridine stem, AC = anticodon stem, T = TΨC stem. The positions of anticodons in transfer RNA genes are designated COD. Asterisks indicate the nonpaired 3′ tRNA position 73. Periods represent nonstem bases.

30%–35%, and C = 27%–34%; *Rana limnocharis* control region to 12S rRNA, G = 16%, T = 30%, A = 29%, and C = 25%; *Rana limnocharis* ND2 to COI, G = 20%, T = 28%, A = 26%, and C = 26%), which is characteristic of the mitochondrial genome but not the nuclear genome. All genes sequenced appear functional; transfer RNA sequences form stable secondary structures and protein-coding genes do not have premature stop codons. Therefore we interpret these sequences as authentic mitochondrial DNA.

Transfer RNA Genes

Some common patterns emerge among the gene rearrangements known in vertebrates. All vertebrate rearrangements involve genes for tRNAs, which are stem-and-loop structures. The phenomenon of a high frequency of tRNA genes involved in rearrangement has been noticed both in invertebrates (Brown 1985; Wolstenholme and Clary 1985; Cantatore et al. 1987; Moritz, Dowling, and Brown 1987) and vertebrates (Moritz and Brown 1986, 1987; Stanton et al. 1994). All rear-

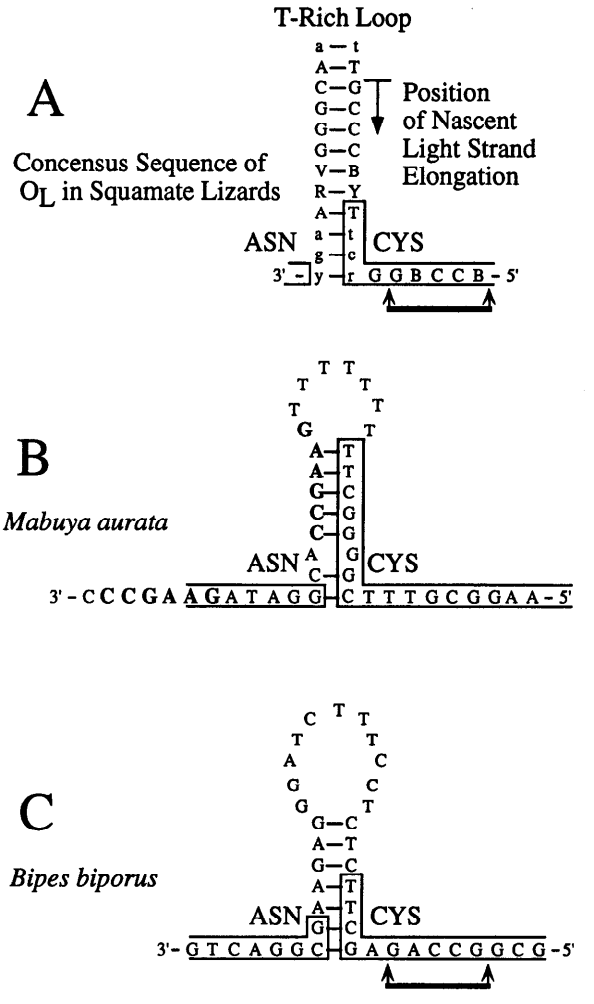

FIG. 3.—Alternative stem-and-loop structures between the tRNA^{Asn} and tRNA^{Cys} genes. *A*, Consensus heavy-strand sequence of putative O_L from squamate lizards. This sequence is based on representatives from the Iguanidae (nine genera, unpublished data), Gekkonidae (*Teratoscincus*), Pygopodidae (*Lialis*), Lacertidae (*Eremias*), Teiidae (*Cnemidophorus*), Cordylidae (*Platysaurus*), Anguidae (*Elgaria*, unpublished data), Xenosauridae (*Xenosaurus*), and Varanidae (*Varanus*). Bases in capitals are conserved pairings and downstream sequence. Bases in lower case are often paired. Variable positions are labeled with their standard one-letter codes: R = G or A; Y = C or T; B = G, C or T; and V = G, C, or A. The 3′-GCC-5′ heavy-strand template sequence identified as the point of light-strand elongation in mouse (Brennicke and Clayton 1981) is indicated (arrow). The heavy-strand sequence 3′-GBCCB-5′ in the tRNA^{Cys} gene related to the 3′-GGCCG-5′ sequence found to be required for *in vitro* replication in humans (Hixson, Wong, and Clayton 1986) is underlined with arrows. *B*, Stem-and-loop structure between the tRNA^{Asn} and tRNA^{Cys} genes observed in the scincid lizard *Mabuya aurata*. Bases in bold indicate a noncontiguous sequence repeated twice. Note the presence of a mispaired position in the stem and a lack of homology with the consensus sequence presented in *A*. *C*, Stem-and-loop structure between the tRNA^{Asn} and tRNA^{Cys} genes observed in the amphisbaenid *Bipes biporus*. Little homology is observed between this stem region and that of the consensus sequence presented in *A*. Note a downstream sequence of 3′-GACCG-5′ is present similar to the 3′-GGCCG-5′ sequence found to be required for *in vitro* replication in humans (Hixson, Wong, and Clayton 1986).

ranged segments begin with a tRNA gene, with respect to the direction of light-strand elongation and typical vertebrate gene order, except in birds and one tuatara gene order. The one tuatara and avian rearrangements begin with the ND6 gene, which is preceded by ND5 and the tRNA$^{Leu(CUN)}$ gene. With the exception of the lamprey, tuatara, and ranid frogs, all rearranged segments end with a tRNA gene. The rearranged segments of the lamprey, tuatara, and ranid frogs end with the control region, which also contains stem-and-loop structures. In addition, endpoints of tandemly duplicated gene regions contain potential stem-and-loop structures, most often in tRNA genes but also in protein-coding regions (Stanton et al. 1994).

Statistics and Phylogeny

All vertebrate lineages that have gene rearrangements, except the two *Rana* species, share the loss of a recognizable O_L between the tRNA^{Asn} and tRNA^{Cys} genes (Desjardins and Morais 1990; Seutin et al. 1994; Kumazawa and Nishida 1995; Lee and Kocher 1995). To determine whether gene rearrangements may be predisposed by movement of O_L, their respective phylogenetic distributions are examined (Donoghue 1989). Phylogenetic distributions are shown in figure 6. In the null model, changes are distributed randomly across the tree. A concentrated change test (Maddison 1990; Maddison and Maddison 1992) shows that rearrangement of genes occurs with displacement of O_L more often than would be expected by chance ($P < 0.001$) (see fig. 6*A* and *C*). Note that expansion of numbers of taxa included in lineages with gene rearrangements (marsupials, crocodilians, birds, and acrodont lizards) and lineages without gene rearrangements (primates, whales, and seals) would make the tests more significant because no variation is observed in these groups.

Discussion

Mechanisms for Rearrangement

We propose the following mechanism to explain all vertebrate gene rearrangements associated with displacement of the O_L:

1. Alternative stem-and-loop structures can initiate replication of the light strand, but O_L is competitively dominant (Wolstenholme, Koike, and Cochran-Fouts 1974; Clayton 1982; Clary and Wolstenholme 1987).
2. A mutational event disrupts O_L and an alternative initiation site is then used for replication of the light strand. A short period of instability follows in which initiation and termination of light-strand replication are situated at various positions having stem-and-loop structures, often tRNA genes. Alternative sites for initiation of light-strand synthesis occur in a region of the genome that remains single-stranded for a lengthy period during the asymmetrical replication process (see Moritz and Brown 1987; Moritz 1991).
3. Light-strand synthesis proceeds to copy the heavy strand.
4. After heavy-strand replication has largely finished, the 5′ end of the nascent light strand becomes de-

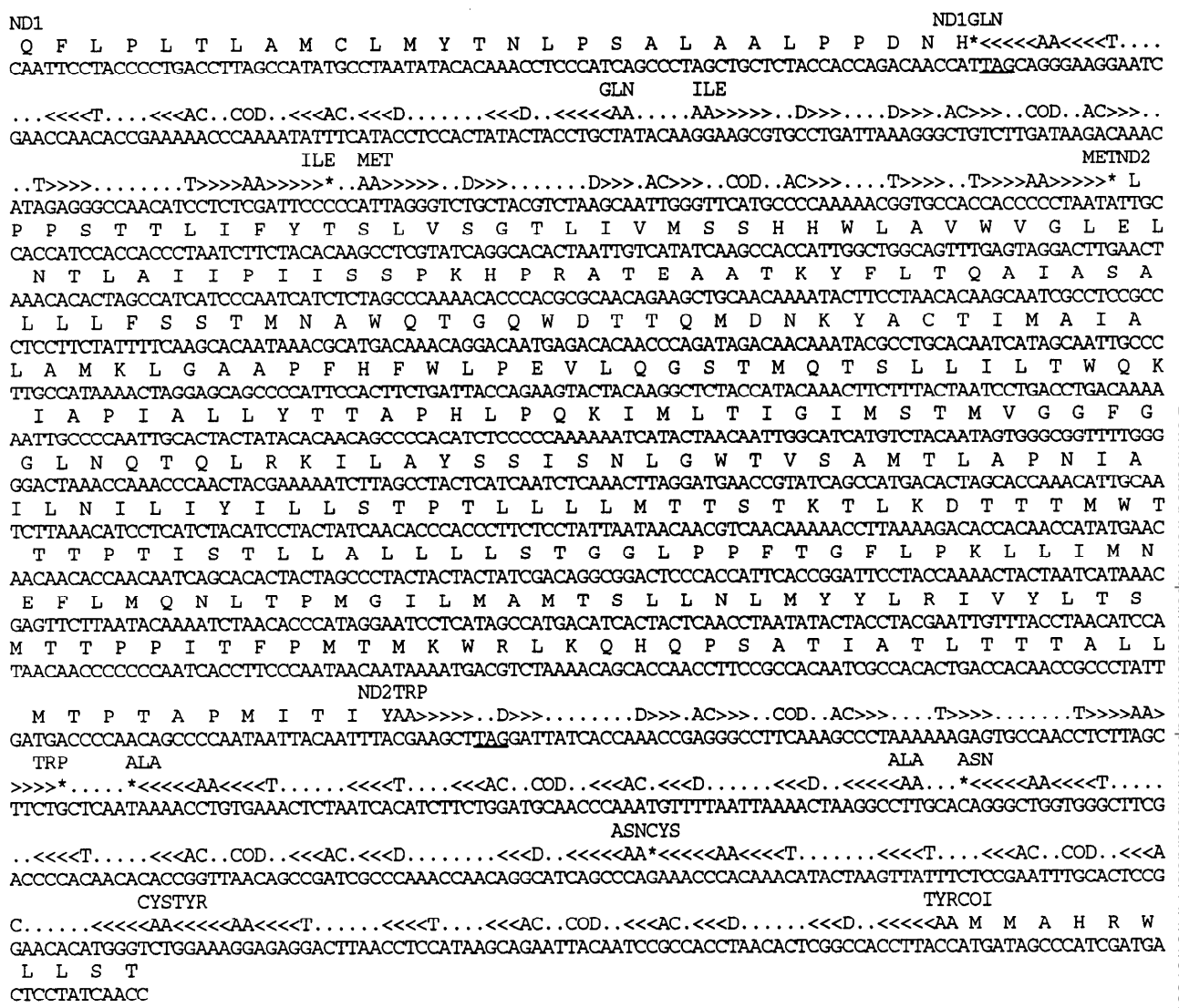

FIG. 4.—Gene arrangement in a segment of mitochondrial DNA from the agamid lizard, *Uromastix acanthinurus*. The most common arrangement for this region in vertebrates consists of the genes encoding ND1, $tRNA^{Ile}$, $tRNA^{Gln}$, $tRNA^{Met}$, ND2, $tRNA^{Trp}$, $tRNA^{Ala}$, $tRNA^{Asn}$, O_L, $tRNA^{Cys}$, $tRNA^{Tyr}$, and COI. In *Uromastix* the $tRNA^{Gln}$ gene precedes the $tRNA^{Ile}$ gene and O_L is missing between the $tRNA^{Asn}$ and $tRNA^{Cys}$ genes. Underlined sequences represent potential stop codons for ND1 and ND2 in adjacent tRNA genes. Sequences are presented as light-strand sequence from 5′ to 3′. Amino acids are positioned over the second codon position in protein-coding genes and tRNA secondary structure is designated above the sequence as in figure 2.

tached and slips ahead to an alternative stem-and-loop structure (slipped-strand mispairing) (Levinson and Gutman 1987; Broughton and Dowling 1994).

5. Light-strand synthesis is completed with termination occuring past the point of initiation. This often appears to be a different tRNA gene.
6. Because initiation and termination are at different sites, a tandem duplication is created.
7. A new site for initiation and termination of the light strand becomes stabilized, preventing recurrence of the events in steps 2–5.
8. Multiple deletions occur, creating a rearrangement of genes (fig. 7).

5′-Nascent Strand Slippage

Slippage of the 5′ end of a nascent strand during elongation (step 4 above) can produce a tandem duplication only in a circular molecule (Broughton and Dowling 1994). Nascent strand slippage involving the 5′ end from either of the two typical replication origins could produce duplications which would be restricted to the vicinity of a particular initiation site (Broughton and Dowling 1994). The control region consistently contains the initiation site for heavy-strand synthesis and slippage of the 5′ end of nascent heavy strands has been postulated to account for relatively large repeats (up to 160 bp, Hoelzel et al. 1994; Fumagalli et al. 1996), which can form stable secondary structures that are upstream from the expected replication origin (Fumagalli et al. 1996). The repeats postulated here cannot be explained by simple replication slippage, slipped-strand mispairing (Levinson and Gutman 1987), or the illegitimate-elongation model (Buroker et al. 1990) involving the displacement strand during initiation of heavy-strand rep-

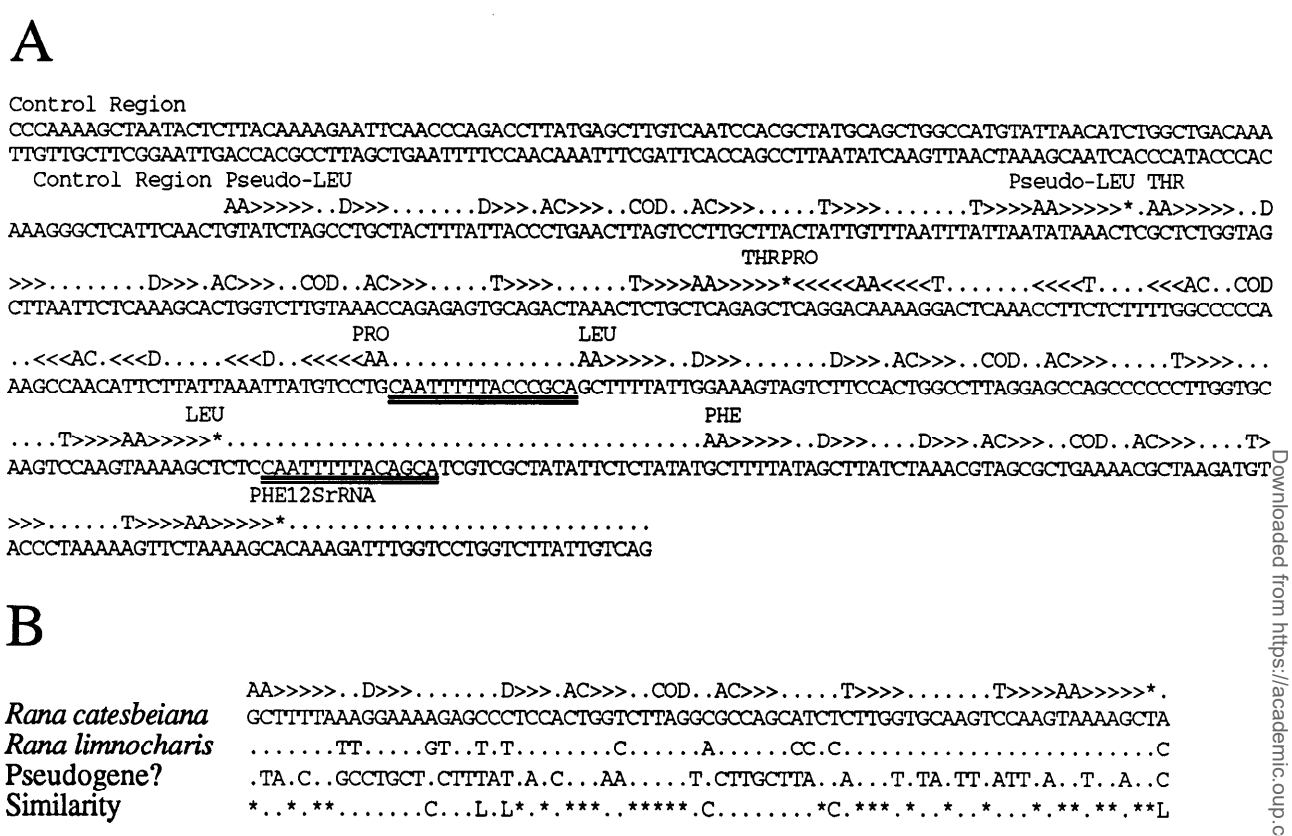

FIG. 5.—*A,* Gene arrangement for the mitochondrial genes between the control region and the 12S rRNA gene in *Rana limnocharis.* Gene regions are designated as in figure 2. The sequences presumed to represent the control region are not alignable to other frog sequences. Note that the same four tRNA genes are present in *Rana limnocharis* as have been reported rearranged in *Rana catesbeiana,* $tRNA^{Leu(CUN)}$, $tRNA^{Pro}$, $tRNA^{Thr}$, and $tRNA^{Phe}$, but the order of these genes is not the same. All other known vertebrate sequences have only the $tRNA^{Phe}$ gene between the control region and the 12S rRNA gene. Double-underlined sequences represent two noncoding direct repeats flanking the rearranged $tRNA^{Leu(CUN)}$ gene of *Rana limnocharis. B,* Alignment of the $tRNA^{Leu(CUN)}$ gene between *Rana catesbeiana* and two regions of sequence from *Rana limnocharis.* Homology was initially inferred by the anticodon "TAG" and no gaps were introduced. A single nucleotide separates the "pseudogene" sequence from the $tRNA^{Thr}$ gene in *Rana limnocharis.* Similarity among the three sequences is shown at the bottom with an asterisk. "L" designates similarity between the *Rana limnocharis* sequences, and a "C" denotes similarity between the "pseudogene" and the *Rana catesbeiana* sequence. The *Rana limnocharis* sequence above is presumed to be functional whereas the *Rana limnocharis* sequence below may be a nonfunctional copy or "pseudogene" because it appears not to form a stable secondary structure common among transfer RNAs. Note that the sequence labeled "pseudogene" is from a gene region homologous to *Rana catesbeiana*; the presumed functional copy of $tRNA^{Leu(CUN)}$ is in a position unique among vertebrates.

lication. The initiation site for light-strand synthesis appears more labile. Slippage of the 5′ end of a nascent light strand that has initiated at an alternative position potentially could produce tandemly duplicated gene regions throughout the mitochondrial genome at appropriate stem-and-loop structures.

Functional Constraints

The use of alternative sites for light-strand replication suggests an exaptation (Gould and Vrba 1982) for stem-and-loop structures in tRNA genes. Transfer RNA stem-and-loop structures standardly used for amino acid transport may be co-opted for the function of light-strand replication, resulting in alternative locations of the O_L. In marsupials the O_L is located in a unique position and includes substantial noncoding sequences on both sides. The total length of the O_L and the noncoding regions is approximately the size of a transfer RNA gene, and this region has been postulated to represent an ancestral $tRNA^{Ala}$ gene (Pääbo et al. 1991). Maizels and Weiner (1995) propose that replication was the original function of tRNA structures and that their standard use in translation is itself an exaptation.

A new location for the O_L may create a new functional burden on some parts of the molecule while releasing functional constraint on others. For example, the $tRNA^{Cys}$ gene normally is situated in a position adjacent to the O_L with some overlap. In the amino-acid-acceptor and TΨC stems of the $tRNA^{Cys}$ gene, the heavy-strand sequence 3′-GGCCG-5′ downstream from the O_L has been found to be required for *in vitro* replication of the human mitochondrial genome (Hixson, Wong, and Clayton 1986). The marsupial O_L is flanked by a noncoding region which contains this sequence or the related heavy-strand sequence 3′-GGCCC-5′ (Pääbo et al. 1991; Janke et al. 1994). Among marsupials, the $tRNA^{Cys}$ gene is in a unique position and only the Australian possum (*Trichosurus vulpecula*) contains the heavy strand sequence 3′-GGCCG-5′ (Pääbo et al. 1991). Other possum sequences in this position are 3′-AACCG-5′ or 3′-AGTCA-5′ (Pääbo et al. 1991; Janke et al. 1994). *Mabuya, Xantusia,* and *Uromastix,* which

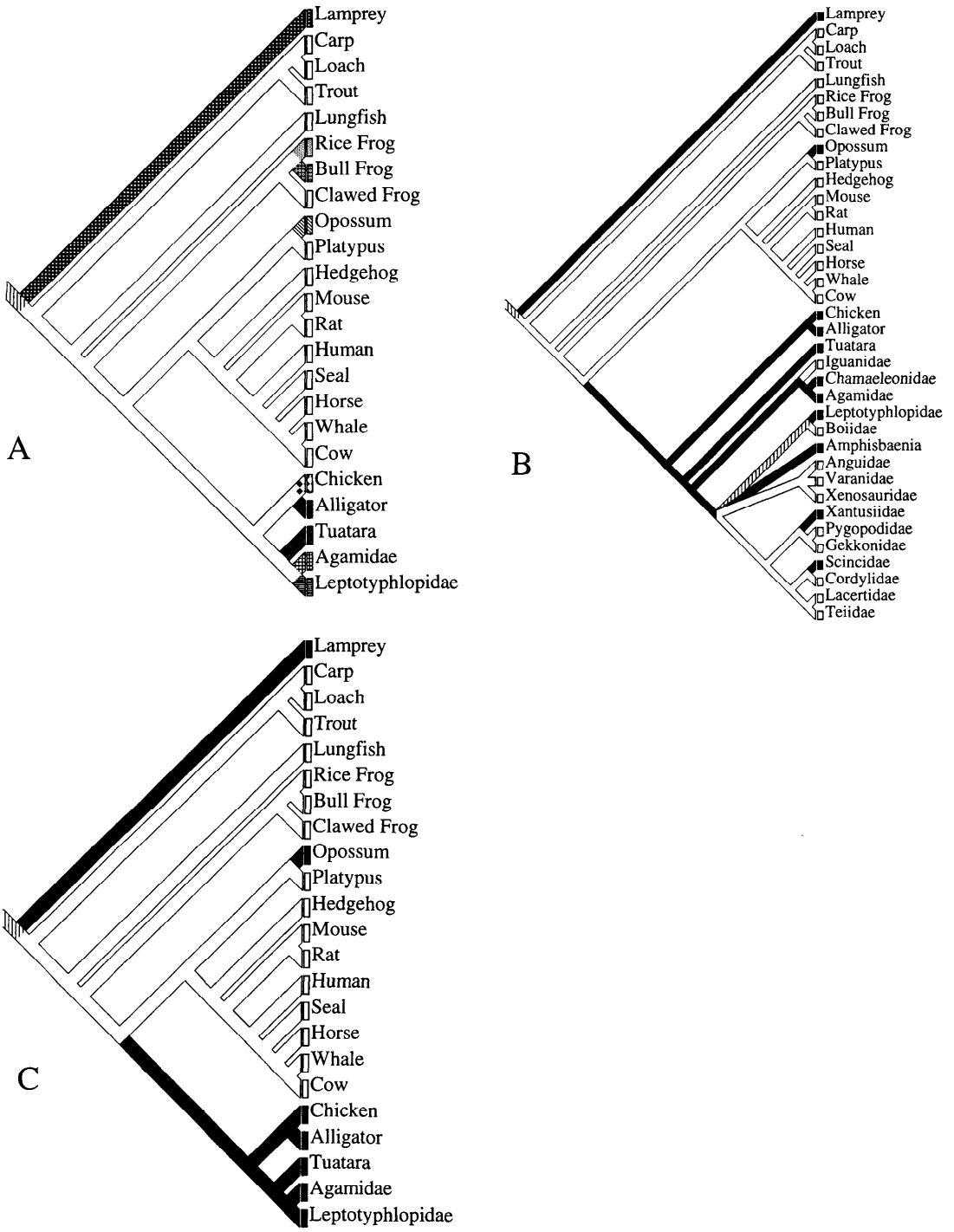

FIG. 6.—Evolution of gene order in the vertebrate mitochondrial genome. The topology for squamate reptiles is based on reanalysis of the morphological data of Estes, de Queiroz, and Gauthier (1988) and Schwenk (1988) (see discussion); the remainder of the topology is from Gauthier, Estes and de Queiroz (1988), Krettek, Gullberg, and Árnason (1995), Janke et al. (1996), and Zardoya and Meyer (1996). *A*, The known vertebrate gene rearrangements (Yoneyama 1987, Desjardins and Morais 1990; Pääbo et al. 1991; Kumazawa and Nishida 1995; Lee and Kocher 1995; Quinn and Mindell 1996). For simplicity, only one marsupial, crocodilian, bird, and acrodont lizard are shown. Taxa illustrated to have the most common vertebrate gene order (Anderson et al. 1981, 1982; Bibb et al. 1981; Roe et al. 1985; Gadaleta et al. 1989; Árnason, Gullberg, and Widegren 1991; Árnason and Johnsson 1992; Tzeng et al. 1992; Chang, Huang, and Lo 1994; Xu and Árnason 1994; Krettek, Gullberg and Árnason 1995; Zardoya, Garrido-Pertierra, and Bautista 1995; Janke et al. 1996; Zardoya and Meyer 1996) have been sequenced for the complete mitochondrial genome. For simplicity only a single primate (for others see Horai et al. 1995), whale (see Árnason and Gullberg 1993) and seal (see Árnason et al. 1993) are shown. The order of the control region, $tRNA^{Glu}$, $tRNA^{Thr}$, $tRNA^{Pro}$, and cytochrome *b* that is ancestral for vertebrates is uncertain and is denoted with hatching (Lee and Kocher 1995). *B* and *C*, Two reconstructions of the absence of a recognizable O_L between the asparagine and cysteine tRNA genes (black). Hatched area is equivocal for presence or absence of a recognizable O_L in the typical vertebrate location. Note that strange stem-and-loop structures are present in Amphisbaenia, Scincidae, and some Acrodonta (Agamidae and Chamaeleonidae) (unpublished data) but lack functional characteristics identified in mamalian light-strand replication (figs. 2 and 3). An atypical stem-and-loop structure found in lungfish (Zardoya and Meyer 1996) has the functional characteristics identified in mammalian light-strand replication (heavy-strand stem sequence of 3′-GCC-5′ and a downstream sequence of 3′-GGCCT-5′ [see fig. 3 for reference]).

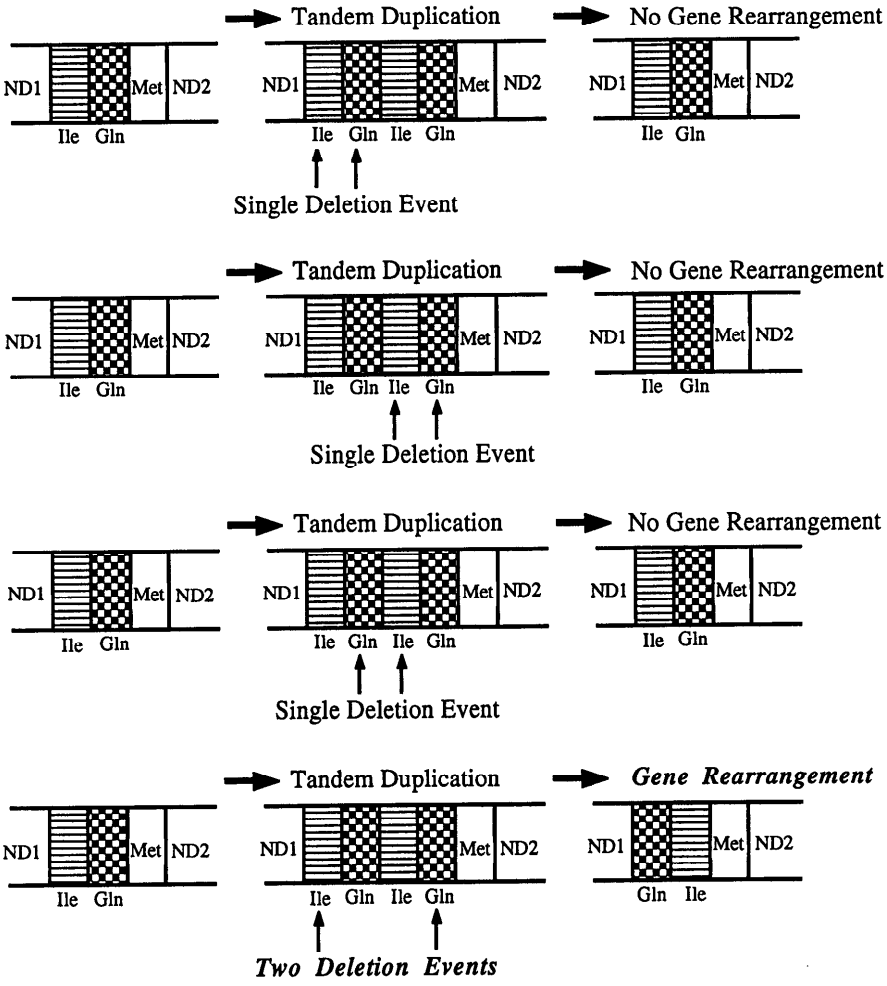

FIG. 7.—Gene reorganization under a model of tandem duplication of genes as illustrated by the rearrangement in the agamid lizard *Uromastix*. After tandem duplication of a gene region is produced, multiple deletions of redundant genes occur (Moritz, Dowling, and Brown 1987). If a single deletion occurs, the gene order will return to the previous state. Only when multiple deletions occur does a rearrangement result. Hence, shifts in gene order result from a nonparsimonious process, suggesting that parallelisms and reversals are unlikely.

lack a recognizable O_L in the normal position, do not have a similar sequence (complement in fig. 2). All other squamate lizards that have a recognizable O_L in the normal position (sampled here and unpublished data) share similar sequences of 3′-GBCCB-5′ (B = G, C or T) (figs. 2 and 3). In addition, note that *Rana limnocharis*, which has a normal O_L position, contains the heavy-strand sequence 3′-GGCCG-5′ (complement in fig. 2).

Transposition

The gene rearrangements observed in ranid frogs may occur by an alternative mechanism that involves small direct repeats. Transposition is associated with terminal inverted or direct repeats (Calos and Miller 1980). The rearranged $tRNA^{Leu(CUN)}$ in *Rana limnocharis* has direct repeats on both flanks (fig. 5*A*). *Rana limnocharis* appears to have a nonfunctional copy of the $tRNA^{Leu(CUN)}$ in the same position as the functional copy observed in *Rana catesbeiana*. If this interpretation is correct, the common ancestor of these two ranid frogs probably had the *Rana catesbeiana* gene order. While slipped-strand mispairing exhibits small direct repeats (Levinson and Gutman 1987), it seems unlikely. Slipped-strand mispairing would have produced a tandem duplicate containing the tRNA genes for leucine, threonine, and proline. It seems improbable that one copy of $tRNA^{Thr}$ and $tRNA^{Pro}$ would be completely excised leaving the $tRNA^{Leu(CUN)}$ gene flanked by the two noncoding repeated sequences. The rearrangements in ranid frogs contain a copy of $tRNA^{Leu(CUN)}$ that is 5 kb from the position observed in other vertebrates. All other known vertebrate gene rearrangements are localized and the bird, crocodilian, blind snake, and agamid lizard rearrangements involve a simple switch of two sequences. However, tandemly duplicated gene regions as large as 9.4 kb have been observed (Moritz and Brown 1987; Wallis 1987; Moritz 1991). If the two ranid rearrangements occurred by an alternative mechanism such as transposition, this would account for their occurrence in the absence of a displacement of O_L.

Phylogenetic Patterns of Genomic Organization

The prospect that gene order within vertebrates is variable opens an opportunity for phylogenetic analysis (Sankoff et al. 1992; Smith et al. 1993; Boore and Brown 1994). Parallelism and reversal are highly un-

likely for gene rearrangements occurring by the proposed mechanisms involving errors in light-strand replication or direct repeats. Under a model of errors in light-strand replication and tandem duplication, multiple deletions are required to create a gene rearrangement (fig. 7). While individual gene regions may not be excised in one piece, multiple regions at least must abandon function and eventually be removed. For a reversal to occur, a gene region must be subjected to tandem duplication twice and a minimum of four deletions. Although tandem duplications have evolved in parallel (Zevering et al. 1991; Stanton et al. 1994), parallelism and reversal are highly improbable for all events required to produce a particular gene rearrangement. Alternatively, a mechanism involving direct repeats may involve transposition. Both parallelism and reversal seem unlikely with transposition, because it would rarely move the same gene region to an identical new site.

Vertebrate mitochondrial gene rearrangement is relatively rare, but when rearrangement occurs it should diagnose monophyletic groups, as shown for birds (Desjardins and Morais 1990, 1991; Quinn and Wilson 1993), crocodilians (Kumazawa and Nishida 1995), and marsupials (Pääbo et al. 1991; Gemmell et al. 1994; Janke et al. 1994) because homoplasy is highly unlikely. Although the gene rearrangements observed in *Uromastix* and *Rana limnocharis* involve genes that are rearranged in other taxa, the derived gene orders in *Uromastix* and *Rana limnocharis* are unique.

Within squamate reptiles, the derived gene order found in *Uromastix* characterizes the Acrodonta (Agamidae, Chameleonidae) (unpublished data). No evidence for reversal is observed across four genera in both subfamilies of the Agamidae and a representative of the Chameleonidae (unpublished data). In figure 8 a reanalysis of morphological data (Estes, de Queiroz, and Gauthier 1988; Schwenk 1988) is presented, showing the phylogenetic distribution of genomic rearrangements from ND1 to COI among squamate reptiles. No evidence for parallelism is observed across all squamate lineages sampled. In addition, among nine genera with representatives of all eight monophyletic groups of the Iguanidae (Etheridge and de Queiroz 1988), the sister taxon to the Aacrodonta, no evidence of parallelism is observed (unpublished data). These data further support the utility of gene order as a phylogenetic character and illustrate how it may be used in phylogenetic reconstruction of vertebrates.

The prospect that gene order is variable within *Rana* suggests that gene order may be useful for reconstructing relationships among more closely related taxa than have been suggested previously (Boore et al. 1995; Kumazawa and Nishida 1995; Stanton et al. 1994).

Recently, gene junctions were found to be useful for phylogenetic inference (Boore et al. 1995). Gene junctions may be much less robust, however, than gene orders as phylogenetic characters. For example, among the known vertebrate gene arrangements, homoplasy is observed for the gene junction between $tRNA^{Pro}$ and $tRNA^{Phe}$. Although lamprey and bullfrog (*Rana catesbeiana*) both possess unique gene arrangements, they share a $tRNA^{Pro}/tRNA^{Phe}$ gene junction (fig. 1) whose evolution requires either parallelism or reversal.

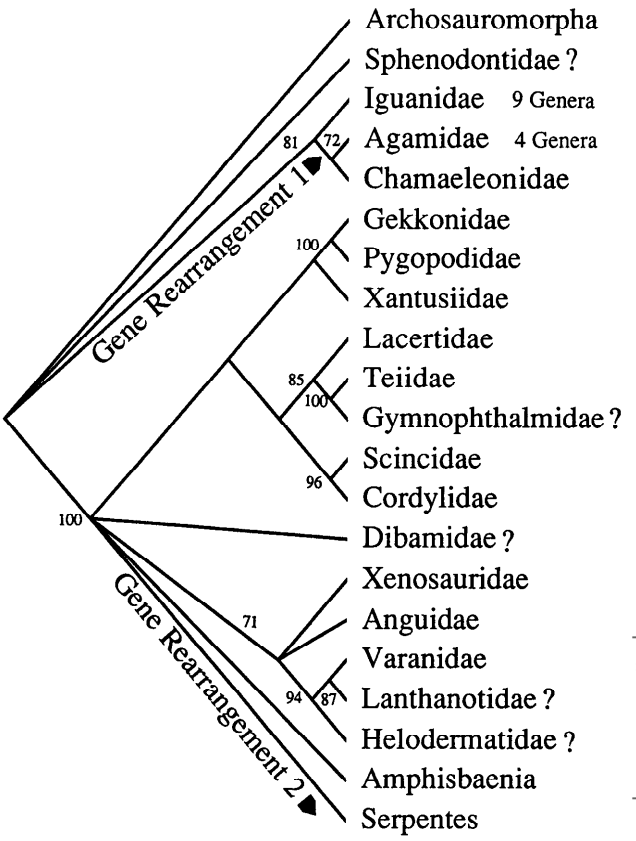

FIG. 8.—Phylogenetic tree of squamate reptiles based on an analysis of 180 morphological characters from the literature (Estes, de Queiroz, and Gauthier 1988; Schwenk 1988) (characters 136–139 from Estes, de Queiroz, and Gauthier [1988] were excluded because of overlap with Schwenk [1988]) showing the position of the gene rearrangement found in *Uromastix* and other acrodont lizards (gene rearrangement 1). The same segment of mitochondrial DNA has undergone a different rearrangement in the blind snake, *Leptotyphlops* (Kumazawa and Nishida 1995) (gene rearrangement 2). This hypothesis is based on 100 randomized heuristic searches using PAUP (Swofford 1993) and produced five equally most parsimonious trees of 367 steps (consistency index of 0.520). Bootstrap values resulting from 1,000 replicates with 10 random additions per replicate are plotted on nodes. All taxa except those with a question mark have been sampled for the mitochondrial DNA region containing the gene rearrangement in *Uromastix* (Kumazawa and Nishida 1995; unpublished data). Note that within squamate reptiles, gene order has shifted twice and no evidence for parallelism or reversal has been observed.

Displacement of the O_L from its typical position between the genes encoding the asparagine and cysteine tRNAs evolves in parallel in association with different evolutionary changes of gene order. Parallel losses of the O_L from the typical vertebrate location (black area in fig. 6*B*) seem more likely than multiple, parallel gains at that site (fig. 6*B*). Most lineages found to lack the O_L in the typical vertebrate position are known to contain gene rearrangements. Previous workers (Seutin et al. 1994; Kumazawa and Nishida 1995) have interpreted the absence of an O_L from the most common vertebrate position as a synapomorphy for birds and crocodilians, although these taxa have different gene rearrangements. If the crocodilian and avian lineages are found to use

different sites for initiation of light-strand replication, relocation of the O_L from the typical vertebrate position probably occurred in two separate events of genomic rearrangement.

The vertebrate mitochondrial genome is much more malleable than originally thought. For historical reasons (Kocher et al. 1989), most DNA sequencing being conducted for phylogenetic inference utilizes sequences of single genes or fragments of genes. Recently, tRNA genes have been found to be useful for phylogenetic inference (Kumazawa and Nishida 1993). All known vertebrate gene rearrangements include tRNA genes. If future phylogenetic studies include clusters of tRNA genes, order of genes also will be assessed. The discovery of additional gene orders will provide phylogenetic characters and test hypotheses of the evolutionary processes that produce the major structural features of the vertebrate mitochondrial genome.

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