# Gene Rearrangements in Snake Mitochondrial Genomes: Highly Concerted Evolution of Control-Region-Like Sequences Duplicated and Inserted into a tRNA Gene Cluster

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Mitochondrial DNA (mtDNA) regions corresponding to two major tRNA gene clusters were amplified and sequenced for the Japanese pit viper, himehabu. In one of these clusters, which in most vertebrates characterized to date contains three tightly connected genes for $tRNA^{Ile}$, $tRNA^{Gln}$, and $tRNA^{Met}$, a sequence of approximately 1.3 kb was found to be inserted between the genes for $tRNA^{Ile}$ and $tRNA^{Gln}$. The insert consists of a control-region-like sequence possessing some conserved sequence blocks, and short flanking sequences which may be folded into $tRNA^{Pro}$, $tRNA^{Phe}$, and $tRNA^{Leu}$ genes. Several other snakes belonging to different families were also found to possess a control-region-like sequence and $tRNA^{Leu}$ gene between the $tRNA^{Ile}$ and $tRNA^{Gln}$ genes. We also sequenced a region surrounded by genes for cytochrome *b* and 12S rRNA, where the control region and genes for $tRNA^{Pro}$ and $tRNA^{Phe}$ are normally located in the mtDNAs of most vertebrates. In this region of three examined snakes, a control-region-like sequence exists that is almost completely identical to the one found between the $tRNA^{Ile}$ and $tRNA^{Gln}$ genes. The mtDNAs of these snakes thus possess two nearly identical control-region-like sequences which are otherwise divergent to a large extent between the species. These results suggest that the duplicate state of the control-region-like sequences has long persisted in snake mtDNAs, possibly since the original insertion of the control-region-like sequence and $tRNA^{Leu}$ gene into the tRNA gene cluster, which occurred in the early stage of the divergence of snakes. It is also suggested that the duplicated control-region-like sequences at two distant locations of mtDNA have evolved concertedly by a mechanism such as frequent gene conversion. The secondary structures of the determined tRNA genes point to the operation of simplification pressure on the T ψ C arm of snake mitochondrial tRNAs.

## Introduction

Complete mitochondrial DNA (mtDNA) sequences have been determined for a number of vertebrates, representing mammals, birds, amphibians, and fishes (Lee and Kocher 1995; Arnason, Xu, and Gullberg 1996; Janke et al. 1996 and references therein). Although many partial mtDNA sequences from a variety of animal groups are known, relatively few have been reported from reptiles. The vertebrate mtDNAs characterized to date are double-stranded, circular DNAs of ~16 kb and encode genes for 13 proteins, 2 rRNAs, and 22 tRNAs, as well as having a major noncoding or control region that contains signals for replicating the heavy strand of mtDNA and for transcription (Anderson et al. 1981; Wolstenholme 1992). Some conserved sequence blocks (CSBs) have been identified by comparing control region sequences from a number of vertebrates (see, e.g., Walberg and Clayton 1981; Lee et al. 1995), although some vertebrates lack certain CSB members (Desjardins and Morais 1990; Quinn and Wilson 1993). CSBs are believed to be important for the replication or transcription of mtDNA (Walberg and Clayton 1981), but their precise functional role(s) has yet to be elucidated at the molecular level.

Vertebrate mtDNAs were once considered to be fairly conservative with respect to gene order. However, recent findings on variations in mtDNA gene organization for an amphibian (*Rana catesbeiana*; Yoneyama 1987), birds (see, e.g., Desjardins and Morais 1990; Quinn and Wilson 1993), opossum (Pääbo et al. 1991; Janke et al. 1994), sea lamprey (Lee and Kocher 1995), crocodilians (Seutin et al. 1994; Kumazawa and Nishida 1995; Quinn and Mindell 1996), and the Texas blind snake (Kumazawa and Nishida 1995) indicate that the gene organization of vertebrate mtDNAs is more variable than previously thought. Transfer RNA genes are involved in most cases of such gene rearrangement.

In our previous work (Kumazawa and Nishida 1993, 1995), we thus focused on the tRNA genes which are encoded in the mitochondrial genome as clusters. We designed a set of polymerase chain reaction (PCR) primers based on the conservative regions in each of the protein genes that surround the three major tRNA gene clusters (IQM, WANCY, and HSL—the tRNA genes are abbreviated by single letters representing the amino acids to be decoded, and the sense strand of the underlined tRNA genes is the heavy strand). These primers proved useful in amplifying the corresponding regions for a wide range of tetrapods (Kumazawa and Nishida 1993, 1995) and enabled us to find tRNA gene rearrangements in crocodilians and the Texas blind snake (Kumazawa and Nishida 1995). The former represents a local rearrangement of tRNA genes within a cluster (HSL to SHL), whereas the latter involves transposition of the $tRNA^{Gln}$ gene from the IQM to the WANCY cluster.

During the course of this work, we noticed that much larger products than expected were amplified from the IQM region of several snakes representing the families Viperidae, Colubridae, and Boidae, which suggest-

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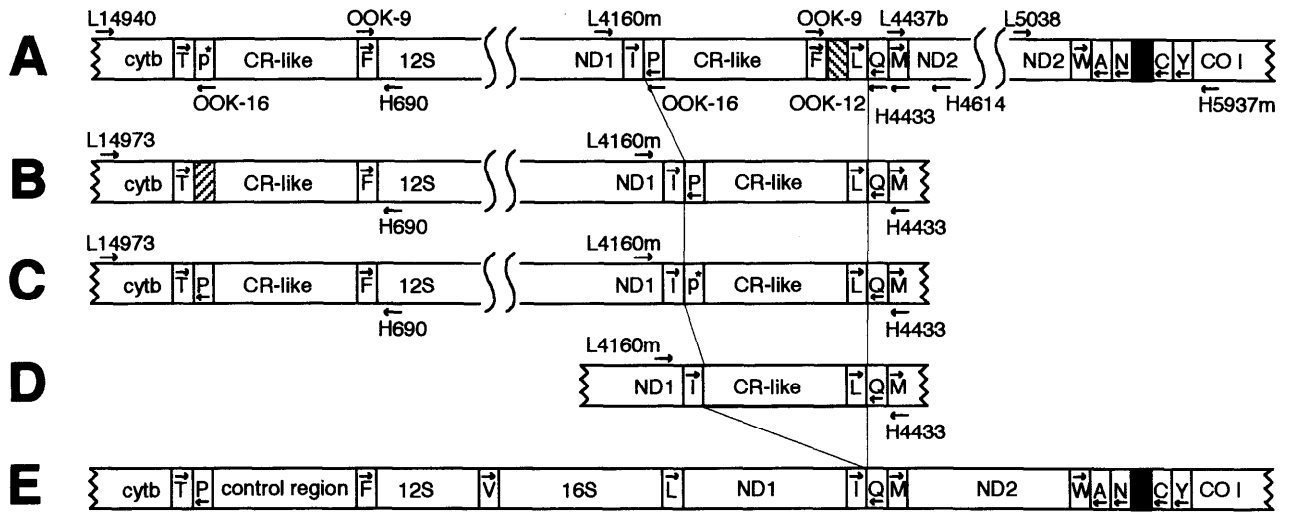



FIG. 1.—Gene organization of snake mtDNAs inferred from determined nucleotide sequences (fig. 4 and data not shown). *A*, Himehabu. *B*, Western rattle snake. *C*, Akamata. *D*, Ball python (possibly boa constrictor). *E*, Typical organization for placental mammals, an amphibian, and bony fishes. Circular mtDNAs are linearly depicted as an open bar divided into individual genes. Only relevant genes are shown, and in a way that does not reflect actual gene lengths. Note that relative positions of individual tRNA gene clusters are presented as deduced from the typical organization, although the organization for himehabu was, in part, confirmed from preliminary PCR amplification experiments (see text). Black and hatched boxes depict the putative light-strand replication origin and the noncoding spacer region, respectively. The corresponding location of each PCR primer is shown by an arrow. Abbreviations: 12S, 16S, and p* stand for 12S rRNA, 16S rRNA, and truncated $tRNA^{Pro}$, respectively.

ed the existence of another novel tRNA gene rearrangement widely present in snakes. This finding prompted us to characterize the large amplified products from these snakes. In this communication, we report on the nucleotide sequences of a long insertion into the IQM cluster of the mtDNAs, together with those of the WANCY and cytochrome *b* (cytb)-12S rRNA regions. An unexpected finding emerged which revealed that snake mtDNAs possess duplicated control-region-like (CR-like) sequences that are nearly identical to each other within the species. We discuss the implications of our findings as to when and how the original duplication of the CR-like sequences occurred in the ancestral lineage of snakes and how the duplicated CR-like sequences have evolved during the evolution of snakes.

## Materials and Methods

Genomic DNAs were extracted from muscle tissues of snakes according to Kocher et al. (1989). The snake species used in this study were the viperids himehabu (*Ovophis okinavensis*, an endemic pit viper of the Ryukyus Islands of Japan) and western rattle snake (*Crotalus viridis*), the boids ball python (*Python regius*) and boa constrictor (*Boa constrictor*), and the colubrids akamata (*Dinodon semicarinatus*) and gopher snake (*Pituophis melanoleucus*). The PCR primers used were mostly derived from previous work: L4160m and H5937m (Kumazawa and Nishida 1993), and H4433, H4614, and L5038 (Kumazawa and Nishida 1995). New PCR primers used for the present study were: L4437b, CAGCTAAAAAAGCTATCGGGCCCATACC (5′ → 3′); L14940, ATTAACTTAGCCTTCTCATC; L14973, CACATCACYCGAGATGTCCCCTACGG; H690, G TTGAGGCTTGCATGTATA; OOK-9, GTCTGTAGC TTAAGCCTAAAGTATAGCACTG; OOK-12, ATTA GAAGTTTTGGGCTTCTAGGTCC; and OOK-16, AG AGAGTAGTTTAAGTAGTAAAATGCTGGC. For the primers L4437b, L14940, L14973, and H690, L or H indicates the primer direction and the accompanying number refers to the 3′ end position according to human mtDNA (Anderson et al. 1981). The matching sites of all the primers to the snake mtDNAs are indicated in figure 1. A number of sequencing primers were also synthesized to determine the CR-like sequences by primer walking (sequences not shown).

PCR-aided direct sequencing of the WANCY cluster was carried out as described previously (Kumazawa and Nishida 1995). Briefly, double-stranded PCR products were amplified from the genomic DNA with pairs of primers and *Taq* polymerase (Takara Shuzo Co.) and subjected to asymmetric PCR. PCR reactions consisted of 30 cycles of denaturation at 92°C for 40 s, annealing at 50–55°C for 1 min, and extension at 72°C for 1–3 min. The single-stranded PCR products were directly sequenced with the amplification primers and appropriate internal primers by the dideoxy chain termination method with [α-$^{35}$S]dATP and SEQUENASE version 2 (Amersham).

Sequences for the other regions were determined in a different manner. Long (>1.5 kb) DNA fragments were amplified from either the total genomic DNA or the nearly complete (~16 kb) mtDNA template obtained by Long and Accurate PCR (LA PCR) (reviewed in Cheng et al. 1994) using an LA PCR kit version 2 (Takara). The condition for LA PCR was 30 cycles of denaturation at 94°C for 30 s and simultaneous annealing and extension at 68°C for 15 min. The long DNA fragments were cloned into the pUC118 vector of *E. coli*,

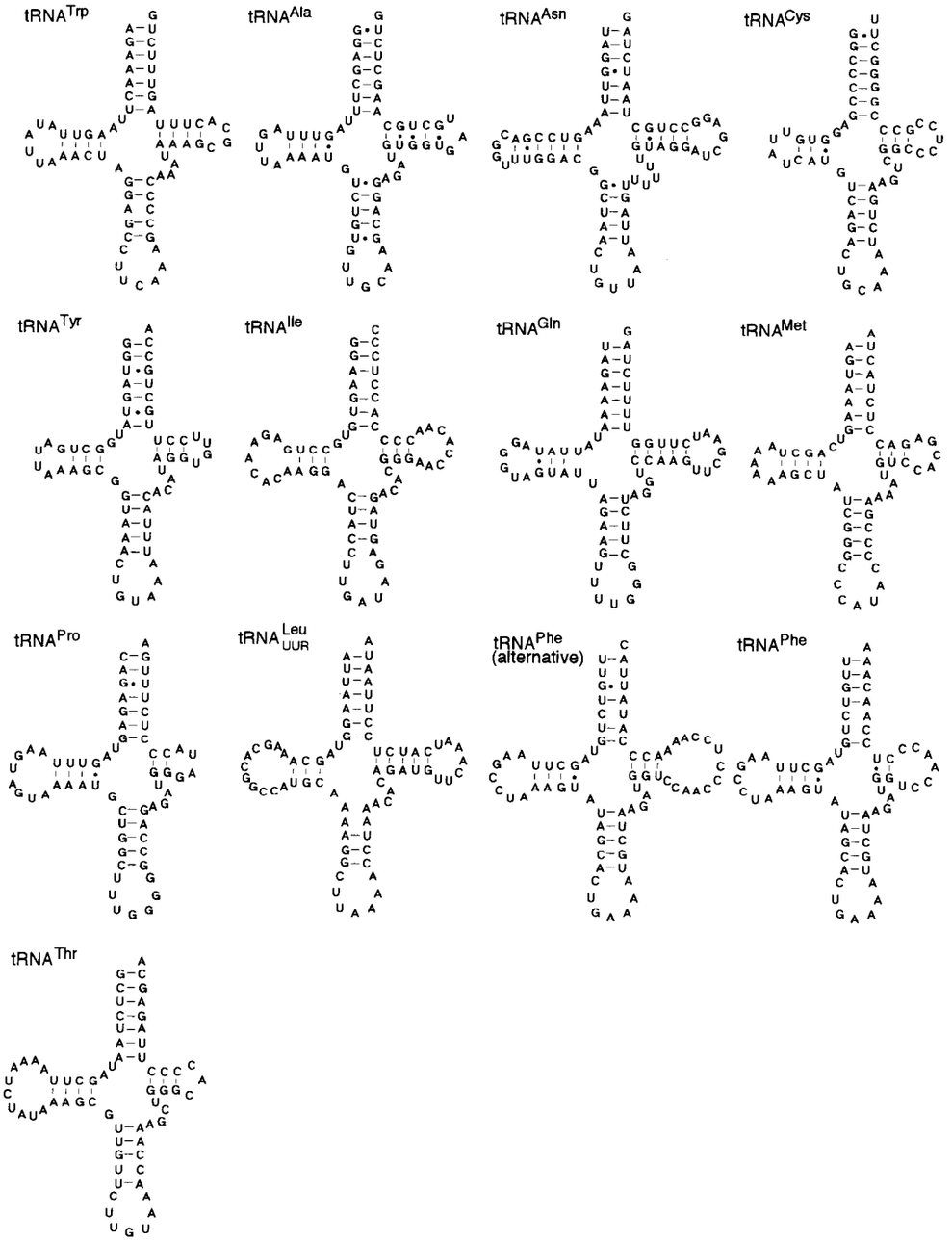



FIG. 2.—Secondary structure representations of tRNA genes found in himehabu mtDNA. Genes for $tRNA^{Trp}$, $tRNA^{Ala}$, $tRNA^{Asn}$, $tRNA^{Cys}$, and $tRNA^{Tyr}$ are from the WANCY cluster, those for $tRNA^{Ile}$, $tRNA^{Gln}$, $tRNA^{Met}$, $tRNA^{Pro}$, $tRNA^{Leu}$(UUR), and alternative $tRNA^{Phe}$ are from the IQM-related region, and those for $tRNA^{Thr}$ and $tRNA^{Phe}$ are from the cytb–12S rRNA region. Bars in stems represent Watson-Crick base pairs and dots stand for wobble G-U pairs. CCA at the 3′ end, which is not encoded in mtDNA but added posttranscriptionally, is omitted.

and relevant sequences of multiple independent clones were determined with an Applied Biosystems 373A DNA sequencer using the primer walking strategy. Nucleotide sequences were determined unambiguously by sequencing both strands.

Transfer RNA genes in the determined sequences were searched in the light of their secondary structure (Kumazawa and Nishida 1993). Protein and rRNA genes were identified by the criterion of sequence similarity with the corresponding genes from other vertebrates. All the nucleotide sequences determined will appear in the GSDB, DDBJ, EMBL, and NCBI databases with accession numbers D84255–D84261 and D86118–D86120.

## Results

### WANCY Cluster

The WANCY region of himehabu was amplified by using primers L5038 and H5937m, whose sequences match the conservative portions of the NADH dehydrogenase subunit 2 (ND2) gene and cytochrome oxidase subunit I (COI) gene, respectively (see fig. 1). PCR amplification gave rise to a discrete product of reasonable size (~900 bp), which was then subjected to asymmetric PCR and sequenced with the help of several of the internal primers used in our previous work (Kumazawa and Nishida 1995). The sequence determined (data not shown) showed that himehabu conserves the usual vertebrate organization of the tRNA genes in the WANCY cluster. The characteristic stem-and-loop structure for the putative light-strand replication origin (Wong and Clayton 1985) located between the $tRNA^{Asn}$ and $tRNA^{Cys}$ genes, shown to be absent in birds, crocodilians, tuatara, and the Texas blind snake (Desjardins and Morais 1990; Quinn and Wilson 1993; Seutin et al. 1994; Kumazawa and Nishida 1995), can be easily identified at the corresponding position in himehabu (fig. 1). Thus, no feature particularly distinctive from the typical vertebrate organization appears to be present in the WANCY region of himehabu. The secondary structures of these tRNA genes are shown in figure 2 and their structural features are discussed below.

### IQM-Related Region

The IQM region was initially amplified by the primers L4160m and H4433, which respectively match conservative portions of the ND1 and $tRNA^{Met}$ genes. Although this amplification should give rise to a product approximately 300 bp in length for the normal ND1-I-Q-M gene organization, a discrete, long product was consistently amplified from several species belonging to different snake families (fig. 3). The length of the product was 1.4–1.6 kb in himehabu and western rattle snake (family Viperidae), akamata and gopher snake (Colubridae), and ball python (Boidae), and longer (~2.2 kb) in boa constrictor (Boidae). These findings suggested the existence of a novel gene rearrangement involving the IQM region of mtDNAs from diverse phylogenetic groups of snakes.

We decided to characterize the large amplified product from himehabu. Because amplification by primers L4160m and H4614, the latter of which matches a part of the ND2 gene, gave rise to a product approximately 1.8 kb in length (data not shown), this product was selected as the sequencing target. The 1.8-kb DNA fragment was cloned into an *E. coli* pUC118 vector and multiple independent clones were sequenced by the primer walking strategy. Figure 4 shows the nucleotide sequence determined and figure 1 depicts the inferred gene organization. Flanked by regions that have an appreciable level of sequence similarity with the ND1 and ND2 genes from the Texas blind snake (Kumazawa and Nishida 1995) and other vertebrates, three tRNA genes specifying isoleucine, glutamine, and methionine can be easily identified.

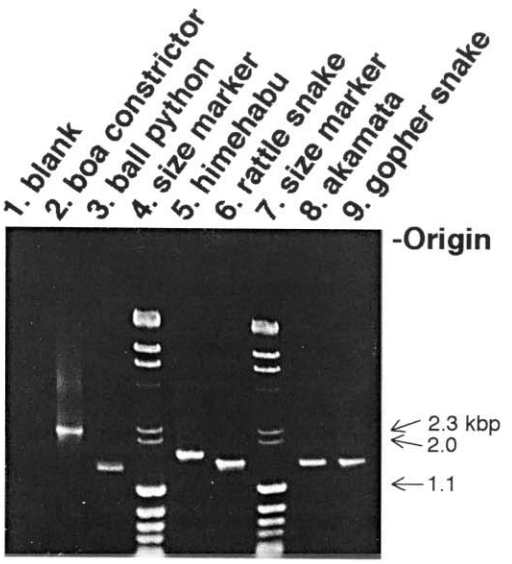



FIG. 3.—PCR amplification of the IQM-related region from various snakes. Conditions for regular PCR are described in *Materials and Methods*. Primers L4160m and H4433 were used and the extension time employed was 3 min. Amplified products were electrophoresed on 0.8% agarose gel and stained with ethidium bromide. The corresponding sizes of some marker bands are shown along the gel. Lanes: 1, no template; 2, boa constrictor; 3, ball python; 4, size marker (λDNA digested with *Hin*dIII plus ϕX174 DNA digested with *Hin*cII); 5, himehabu; 6, western rattle snake; 7, size marker as in lane 4; 8, akamata; and 9, gopher snake.

A long insertion of 1,329 bp is found between the $tRNA^{Ile}$ and $tRNA^{Gln}$ genes (figs. 1 and 4). A sequence which may be folded into a $tRNA^{Pro}$ gene exists immediately 3′ downstream of the $tRNA^{Ile}$ gene. In addition, a possible $tRNA^{Leu}$(UUR) gene occurs immediately 5′ upstream of the $tRNA^{Gln}$ gene. In the middle of the sequenced 1.8-kb DNA there is also a sequence which may be folded into a $tRNA^{Phe}$ gene. No protein or RNA gene could be identified in the regions between the $tRNA^{Pro}$ and $tRNA^{Phe}$ genes, or between the $tRNA^{Phe}$ and $tRNA^{Leu}$ genes. Instead, structural features that are characteristic of a control region occur between the $tRNA^{Pro}$ and $tRNA^{Phe}$ genes (fig. 4). This region includes sequences that have clear sequence similarity to CSB-1 and CSB-3 in the control regions of other vertebrates (fig. 5). Although a structure equivalent to CSB-2 cannot be identified between CSB-1 and CSB-3, a C-rich sequence which has high similarity to the putative CSB-2 sequence for teleosts (Lee et al. 1995) is found near the $tRNA^{Pro}$ gene (fig. 4). Also notable in this noncoding region are hairpin-like structures and repetitive sequences, both of which are often present in the control region. On the basis of these various features, we can regard the noncoding region between the $tRNA^{Pro}$ and $tRNA^{Phe}$ genes to be a CR-like sequence. On the other hand, as far as we can deduce, no functional feature exists in the noncoding region between the $tRNA^{Phe}$ and $tRNA^{Leu}$ genes, where repetition of two kinds of pentamers seems to occur (see fig. 4).

We also cloned and sequenced the 1.4–1.5-kb PCR products amplified from western rattle snake (fig. 3, lane

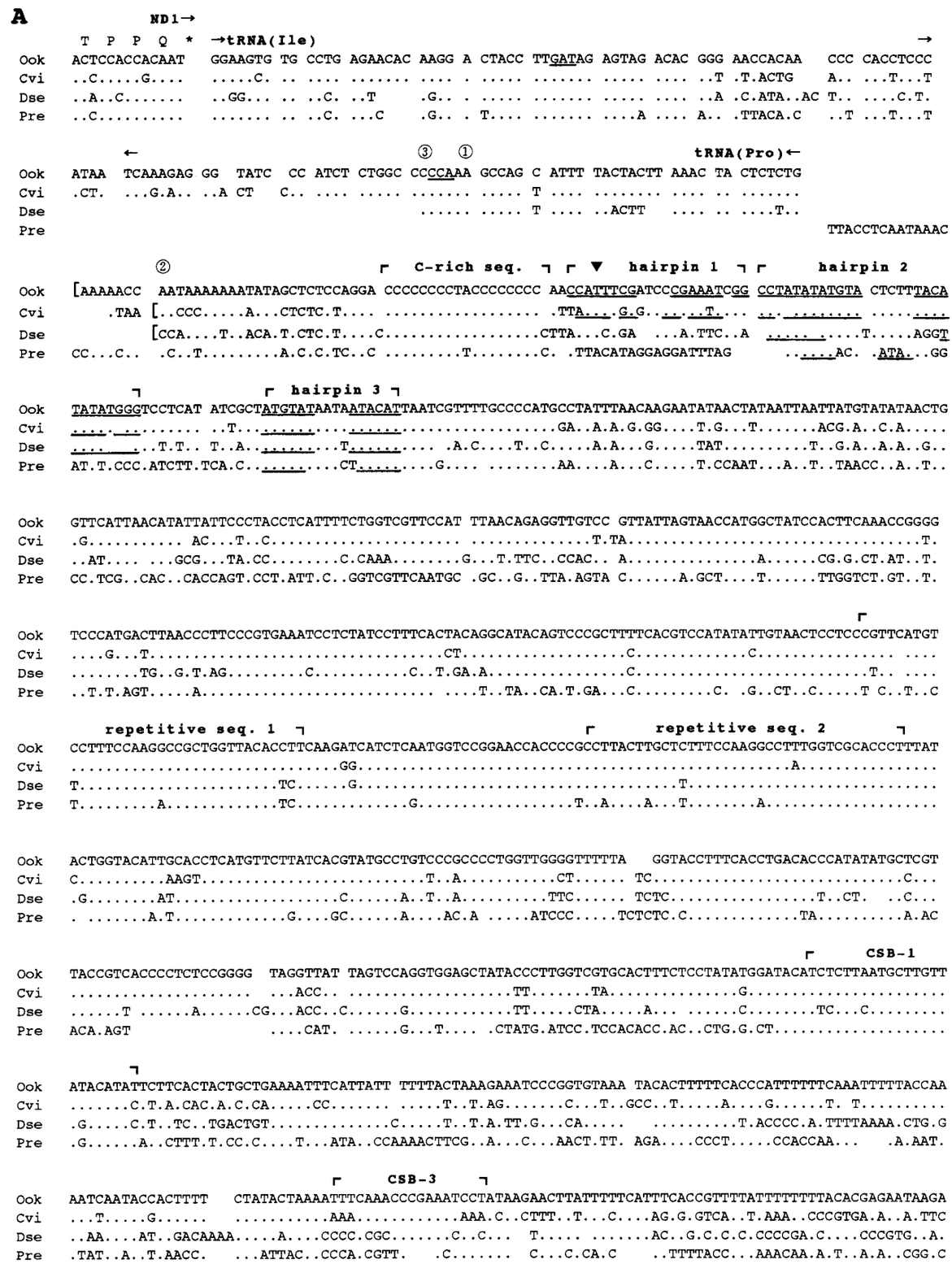
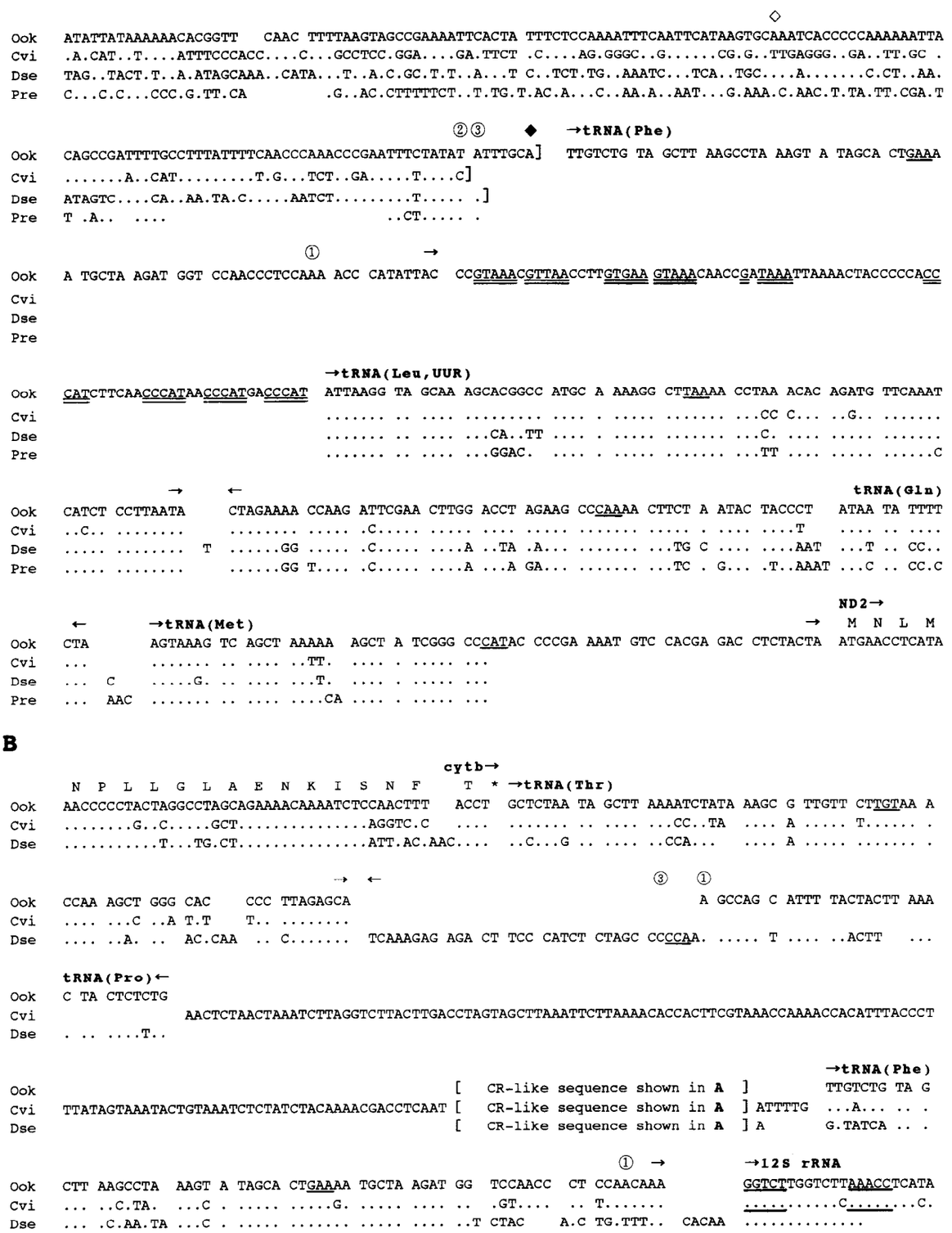



FIG. 4.—Nucleotide sequences of snakes for (*A*) IQM-related and (*B*) cytb-12S rRNA regions. All the nucleotide sequences shown correspond to the light strand. Complete nucleotide and amino acid sequences are presented only for himehabu; dots are used in the nucleotide sequences of the other species where corresponding nucleotides are identical. The CR-like sequences contained within square brackets in *A* are, for each species, identical to the region thus indicated between square brackets in *B* with only a single alteration—in himehabu, the adenosine indicated by a filled




**B**




diamond in *A* is found to be a guanosine in *B*; in western rattle snake, the thymidine indicated by an unfilled diamond in *A* is deleted in *B*; and in akamata, the thymidine indicated by a filled triangle in *A* is a cytidine in *B*. At the left and right junctions of the nearly identical sequence units between the IQM-related and cyth-12S rRNA regions, circled numbers are placed for (1) himehabu, (2) western rattle snake, and (3) akamata. Anticodons of tRNA genes, as well as sequences which may form hairpin structures, are underlined. Repetitive sequence units in noncoding spacer regions are double-underlined. Refer to the nucleotide sequences deposited in the database for more sequence information for parts of ND1, ND2, and cytb genes, as well as for the partially determined IQM-related region sequences of boa constrictor. Species abbreviations: Ook, himehabu (*O. okinavensis*); Cvi, western rattle snake (*Crotalus viridis*); Dse, akamata (*D. semicarinatus*); and Pre, ball python (*P. regius*).

**A** CSB-1

```
himehabu       TCTCTTAATGCTTGTTATACATAT
rattle snake   .......................C
akamata        .TC...C..........G.....C
python         ................G......
boa            -................A......
chicken        .TAG.G.........CGG......
human          .TAA...........AGG.....A
Xenopus        AAATGC....AC..----......
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**B** CSB-3

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himehabu       TTTCAAACCCGAAATCCT
rattle snake   AAA............AAA
akamata        CCC.CGC.......C..C
chicken        .AG.....A.A...C..G
human          .GC.......C...AA.A
Xenopus        CG........C...A..G
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FIG. 5.—Alignment of the (*A*) CSB-1 and (*B*) CSB-3 sequences for snakes together with those for other vertebrates. Complete sequences are presented only for himehabu; for the other species dots are used where corresponding nucleotides are identical. Bars represent gaps in the sequence. The CSB-3 sequence was not clearly identifiable for ball python (see text).

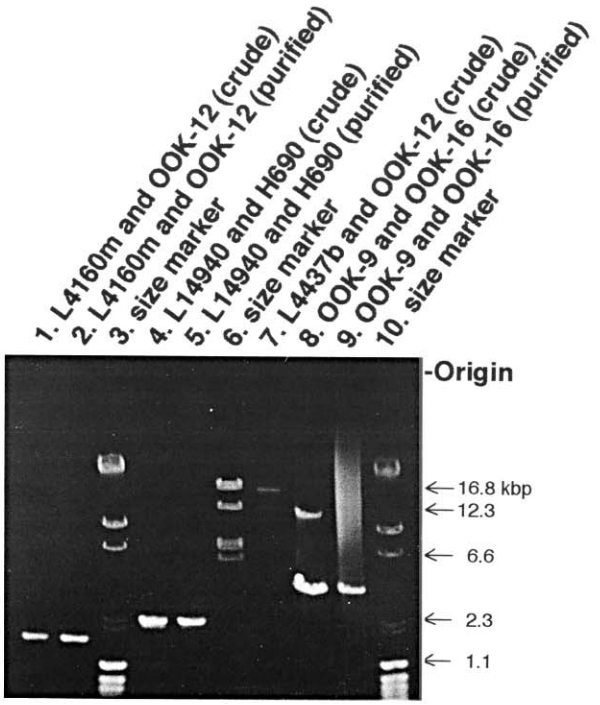


FIG. 6.—PCR amplification for himehabu mtDNA. Conditions for regular PCR (lanes 1–2 and 4–5) and LA PCR (lanes 7–9) are described in *Materials and Methods*. Amplified products were electrophoresed on 0.4% agarose gel and stained with ethidium bromide. The templates used were either the total genomic DNA (lanes 1, 4, 7, and 8) or the purified PCR product of ~16 kb corresponding to a band in lane 7 (lanes 2, 5, and 9). These are respectively indicated by "crude" or "purified" in parentheses following the primer combination used. The size markers were λDNA digested with *Hin*dIII plus ϕX174 DNA digested with *Hin*cII for lanes 3 and 10, and circularized λDNA digested with *Bam*HI for lane 6. LA PCR occasionally produces a smear due to the long extension time or the excessive number of cycles (e.g., lane 9).

6), akamata (fig. 3, lane 8), and ball python (fig. 3, lane 3). As shown in figure 4, these products contain a CR-like sequence and $tRNA^{Leu}$(UUR) gene between the $tRNA^{Ile}$ and $tRNA^{Gln}$ genes. A complete $tRNA^{Pro}$ gene found in himehabu was present only in the western rattle snake, but a partially truncated $tRNA^{Pro}$ gene was found in the corresponding location of akamata (fig. 4). The slightly smaller size of the amplified products from these species as compared with that from himehabu (fig. 3) turned out to be mostly due to the absence of the $tRNA^{Phe}$ gene and its flanking noncoding sequence in this region (figs. 1 and 4). The CR-like sequences for these species conserve CSB-1 and CSB-3 sequences (fig. 5) and CSB-2-like C-rich sequences (fig. 4), although ball python may not have a distinct CSB-3 sequence. These CR-like sequences have clear sequence similarity to that found in himehabu. The pairwise sequence difference in a clearly alignable portion of the CR-like sequences (i.e., from hairpin 3 to CSB-3 of fig. 4) increased in an order consistent with the phylogenetic relationships among the snakes (e.g., Rage 1987; Zug 1993, pp. 443–467; Heise et al. 1995): 13% between himehabu and the western rattle snake, 24% on average between viperids and akamata, and 39% on average between viperids-colubrids and ball python. The PCR product from boa constrictor (~2.2 kb; fig. 3, lane 2) was only partially sequenced to show the existence of genes for $tRNA^{Ile}$, $tRNA^{Leu}$(UUR), and $tRNA^{Gln}$ and the CR-like sequence (data not shown; refer to the sequences deposited in the database with accession numbers D84260 and D84261).

## Cytb-12S rRNA Region

The finding of the long insertion in the IQM cluster region of several snakes consisting of a CR-like sequence and some flanking tRNA genes prompted us to sequence the region surrounded by the cytb and 12S rRNA genes, where the control region and three tRNA genes specifying threonine, proline, and phenylalanine are encoded in the mtDNAs of most vertebrates characterized to date (see fig. 1). For this purpose, we first sequenced a short segment of the cytb gene of each snake species, which can be amplified and sequenced in combination with the well-conserved primers L14841 and H15149 (see Kocher et al. 1989). Primers, named L14940 and L14973, were then synthesized so as to match the determined short cytb sequences (data not shown) for himehabu and for the western rattle snake and akamata, respectively (see fig. 1). Another primer, H690, was also synthesized so as to match a well-conserved region of the vertebrate 12S rRNA gene in proximity to the 5′ end of the gene. Amplifications with L14940 and H690 from himehabu genomic DNA produced a discrete band approximately 2.1 kb long (fig. 6, lane 4). This single band was also seen by LA PCR with the same template and primers (data not shown). The 2.1 kb fragment was cloned into an *E. coli* pUC118 vector and multiple clones were sequenced by primer walking (fig. 4).

The 5′ and 3′ end portions of the 2.1-kb fragment have clear sequence similarity with the corresponding regions of the cytb and 12S rRNA genes of other vertebrates, respectively. In the 3′ end portion, a well-conserved hairpin structure which is characteristic of 12S

rRNA genes is also found (fig. 4). It is therefore unlikely that unrelated regions were amplified by the PCR. Immediately 3′ downstream of the cytb gene, a sequence which may be folded into a $tRNA^{Thr}$ gene is found. Next to the $tRNA^{Thr}$ gene there is a truncated $tRNA^{Pro}$ gene (fig. 4) which has a sequence identical to the 5′ half of the putative $tRNA^{Pro}$ gene found in the IQM-related region (fig. 4). Because the truncation disrupts even the anticodon loop, it is very unlikely that a functional tRNA molecule can be produced from the truncated $tRNA^{Pro}$ gene. Rather, it most likely represents a pseudogene, which may have been generated in association with gene conversion or duplication-and-deletion events, as discussed below. A mitochondrial tRNA pseudogene which may have been generated by the duplication-and-deletion mechanism has been found in a parthenogenetic lizard (Zevering et al. 1991).

Immediately 5′ upstream of the 12S rRNA gene, there is a sequence which may be folded into a $tRNA^{Phe}$ gene (fig. 4). This sequence is nearly identical to an alternative $tRNA^{Phe}$ gene found in the IQM-related region, but lacks the sequence from the T stem to the acceptor stem of the alternative gene (see fig. 2). As shown in figure 2, secondary structures for both of the $tRNA^{Phe}$ genes are similarly unstable in the acceptor- and T-stems, although truncation of the T-stem is common among many snake mitochondrial tRNAs (see *Discussion*). Comparison of the acceptor-stem sequences with $tRNA^{Phe}$ genes from the western rattle snake and akamata (see fig. 4) favors the idea that the gene found in the cytb-12S rRNA region may be the primary $tRNA^{Phe}$ gene for himehabu. However, the possibility that the alternative gene also encodes a functional tRNA cannot be excluded. The question of which (or possibly it is both) of these $tRNA^{Phe}$ genes actually functions in snake mitochondria can only be answered by biochemical characterization of $tRNA^{Phe}$ expressed in the mitochondria.

A long sequence (1,127 bp) between the truncated $tRNA^{Pro}$ and $tRNA^{Phe}$ genes proved to be nearly identical to the sequence between the $tRNA^{Pro}$ and $tRNA^{Phe}$ genes found in the IQM-related region, with the exception of a single A↔G base difference at the immediate 5′ residue of the $tRNA^{Phe}$ gene (refer to the legend of fig. 4). This means that almost the same CR-like sequence appears to be present in both the IQM-related and cytb-12S rRNA regions in himehabu (see fig. 1).

The cytb-12S rRNA regions were also amplified and sequenced with L14973 and H690 from western rattle snake and akamata. As shown in figures 1 and 4, these snakes possess a CR-like sequence and genes for $tRNA^{Thr}$ and $tRNA^{Phe}$ in this region as himehabu does. The western rattle snake, which has a complete $tRNA^{Pro}$ gene in the IQM-related region, lacks another $tRNA^{Pro}$ gene between the $tRNA^{Thr}$ gene and the CR-like sequence of the cytb-12S rRNA region, where an apparently noncoding sequence (130 bp) with no particularly distinctive feature occurs (fig. 4). On the other hand, akamata, which has a truncated $tRNA^{Pro}$ gene in the IQM-related region, possesses a complete $tRNA^{Pro}$ gene in the cytb-12S rRNA region. The CR-like sequence in this region proved to be almost completely identical to the one found in the IQM-related region in both the western rattle snake and akamata. The only differences in the CR-like sequences between the two regions are the deletion or insertion of T in the western rattle snake and a T↔C base substitution in akamata (refer to the legend of fig. 4).

## Two CR-like Sequences in MtDNA

In order to obtain further evidence for the existence of nearly identical CR-like sequences at two different locations of mtDNA, the following experiments were carried out for himehabu. Two primers, named OOK-9 and OOK-16, were synthesized to respectively match the nucleotide sequences determined for the $tRNA^{Phe}$ and $tRNA^{Pro}$ genes of himehabu (see fig. 1). If the CR-like sequence flanked by these tRNA genes were present at only a single location in a circular mtDNA, LA PCR amplification with OOK-9 and OOK-16 should produce a single band covering nearly the entire mtDNA sequence except for the control region. In fact, LA PCR gave rise to two clear bands of approximately 4 and 10–11 kb (fig. 6, lane 8), thus supporting the existence of duplicate sequences. The annealing temperature used for LA PCR (68°C) was high enough to make nonspecific annealing of these primers to unrelated regions unlikely, though not impossible. Although the sequences between the IQM-related and cytb-12S rRNA regions have not been determined, our preliminary PCR amplification experiments using primers designed for the 12S rRNA, 16S rRNA, and ND1 genes suggest that these genes are encoded in this order in the ~4-kb fragment just as in other vertebrates (unpublished data).

Two primers, named L4437b and OOK-12, were then constructed to respectively match the nucleotide sequences determined for the $tRNA^{Met}$ and $tRNA^{Gln}$ genes (see fig. 1) for the purpose of obtaining a nearly entire mtDNA sequence by LA PCR; figure 6 shows a discrete amplified product of ~16 kb (lane 7). This product was highly purified by electrophoresis on 0.4% agarose gel and used as a template for the subsequent amplifications. The amplification from this template with OOK-9 and OOK-16 produced a single band (fig. 6, lane 9) which corresponded to the shorter band (~4 kb) produced from the total genomic DNA with the same primers (fig. 6, lane 8), providing strong evidence for the duplication of the CR-like sequence and flanking tRNAs ~4 kb apart within the mtDNA molecule. The disappearance of the 10–11-kb band may be reasonably accounted for by the fact that a circular mtDNA is linearized at the $tRNA^{Gln}$-$tRNA^{Met}$ junction for the ~16-kb template. With the linearized ~16-kb mtDNA as a template, 1.5- and 2.1-kb products were able to be amplified with L4160m and OOK-12, and with L14940 and H690, respectively (fig. 6, lanes 2 and 5). Partial sequencing of these products from several primer sites revealed no differences from the sequences that were amplified and sequenced from the total genomic DNA (data not shown), thus refuting the possibility that the sequences determined are of nuclear origin.

Frequent polymorphic length mutations are known to occur in and around the control region of lizard mtDNAs (see, e.g., Moritz and Brown 1986, 1987; Zevering et al. 1991). In this respect, it is noteworthy that we have not detected a polymorphic state in himehabu mtDNA; PCR products of the same size were consistently obtained from three individual himehabu with L4160m and H4433, and with L14940 and H690 (data not shown).

## Discussion

### Snake tRNAs with a Truncated T ψ C Arm

Metazoan mitochondrial tRNAs are known to frequently lack invariant and semiinvariant nucleotides, and even an entire arm (see, e.g., Dirheimer and Martin 1990; Wolstenholme 1992; Kumazawa and Nishida 1993). The most extreme examples are the complete lack of the dihydrouridine arm (D arm) for $tRNA^{Ser}$ specifying AGY codons (Anderson et al. 1981; de Bruijn and Klug 1983), the lack of the position 8 residue and the increased number of anticodon stem pairs for $tRNA^{Ser}$ specifying UCN codons (Yokogawa et al. 1991; Steinberg and Cedergren 1994; Watanabe et al. 1994*a*), and the apparent lack of the stable stem-and-loop structure in the T ψ C arm (T arm) for most tRNAs from nematode mitochondria (Wolstenholme et al. 1987; Watanabe et al. 1994*b*). Except for these extreme examples, many mitochondrial tRNAs possess a common cloverleaf secondary structure (Kumazawa and Nishida 1993).

Figure 2 shows secondary structures of possible tRNA genes found in the himehabu mtDNA sequences. It is striking that many of these tRNAs can have only two or three base pairs in the T stem region. For example, $tRNA^{Pro}$ has only two G-C pairs in the T stem. Judging from the excellent base matching in the acceptor and anticodon stem regions, as well as the shortness of the T loop (only four nucleotides), it is hard to envisage alternative secondary structures for the $tRNA^{Pro}$. To the best of our knowledge, this type of tRNA with a severely truncated T arm has not been reported in any of the mitochondria of vertebrates. Table 1 shows the number of nucleotides for the T arm region and the number of possible base pairs in the T stem for 12 tRNA species from various vertebrates. For snakes, both numbers are smaller than or equal to those for other vertebrates in most tRNAs. The smaller numbers for snakes are most clearly seen in $tRNA^{Ala}$, $tRNA^{Pro}$, and $tRNA^{Thr}$. In $tRNA^{Asn}$, $tRNA^{Gln}$, and $tRNA^{Leu(UUR)}$, however, the number of nucleotides in the T arm is constant at 17 for all the animals investigated. As shown in figure 2, these tRNA genes for himehabu conserve GG in the D loop and UCR in the T loop, suggesting the importance of tertiary base pairs between the D and T loops for these particular tRNAs.

These data imply that as snake mitochondrial tRNAs have evolved, their T arm structure has become somewhat simplified to resemble the nematode tRNAs, but that some snake tRNAs, whose D-loop/T-loop interactions are functionally important, have not yet acceded to the simplification pressure. Since it is relatively easy to collect snake tissues in sufficiently large amounts for biochemical characterization, snake mitochondrial tRNAs might provide intriguing subjects for studying the structure–function relationships of tRNA molecules, especially as to how tRNAs with unusual cloverleaf structures are folded into a functional tertiary structure, and how they have evolved the mechanisms by which they interact with recognition enzymes.

**Table 1**
**Number of Nucleotides in the TψC Arm and Number of Base Pairs in the TψC Stem for Mitochondrial tRNAs**

| | Species[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Snakes | | | | | | | |
| tRNA | Ook | Ldu | Bco | Mdo | Bta | Hsa | Gga | Xla |
| Number of Nucleotides in the TψC Arm | | | | | | | | |
| Trp | 12 | 11 | 14 | 14 | 15 | 14 | 17 | 17 |
| Ala | 13 | 15 | 12 | 17 | 17 | 17 | 17 | 17 |
| Asn | 17 | 17 | 17 | 17 | 17 | 17 | 17 | 17 |
| Cys | 11 | 14 | 12 | 16 | 16 | 16 | 16 | 16 |
| Tyr | 10 | 11 | 17 | 17 | 17 | 15 | 17 | 17 |
| Ile | 14 | 16 | 18 | 17 | 17 | 17 | 19 | 17 |
| Gln | 17 | 17 | 17 | 17 | 17 | 17 | 17 | 17 |
| Met | 11 | 13 | — | 17 | 17 | 16 | 17 | 17 |
| Pro | 8 | — | — | 15 | 14 | 15 | 17 | 17 |
| Leu (UUR) | 17 | — | 17 | 17 | 17 | 17 | 17 | 17 |
| Phe | 11[b] | — | — | 14 | 14 | 15 | 15 | 17 |
| Thr | 9 | — | — | 15 | 18 | 13 | 16 | 17 |
| Number of Base Pairs in the TψC Stem | | | | | | | | |
| Trp | 4 | 4 | 4 | 4 | 5 | 4 | 5 | 5 |
| Ala | 5 | 3 | 3 | 7 | 7 | 7 | 5 | 6 |
| Asn | 5 | 3 | 4 | 5 | 5 | 5 | 5 | 5 |
| Cys | 3 | 5 | 5 | 7 | 4 | 6 | 7 | 6 |
| Tyr | 3 | 4 | 4 | 5 | 5 | 6 | 5 | 5 |
| Ile | 3 | 4 | 4 | 5 | 5 | 4 | 5 | 5 |
| Gln | 5 | 4 | 5 | 5 | 5 | 5 | 5 | 5 |
| Met | 3 | 3 | — | 3 | 3 | 3 | 4 | 3 |
| Pro | 2 | — | — | 6 | 6 | 6 | 5 | 5 |
| Leu (UUR) | 5 | — | 5 | 5 | 4 | 5 | 5 | 5 |
| Phe | 2[b] | — | — | 5 | 4 | 3 | 4 | 4 |
| Thr | 3 | — | — | 5 | 4 | 5 | 4 | 4 |

NOTE.—For the assignment of the T arm region (T stem plus T loop), refer to figure 4 of this study and Kumazawa and Nishida (1993, 1995). In order to calculate the number of base pairs in the T stem, only Watson-Crick and G-U wobble pairs are considered under the assumption that at least two nucleotides are required to maintain the T loop. Bars mean that the corresponding genes have not been sequenced.

[a] Species employed: Ook, himehabu (*Ovophis okinavensis*); Ldu, Texas blind snake (*Leptotyphlops dulcis*); Bco, boa constrictor (*Boa constrictor*); Mdo, mouse (*Mus domesticus*); Bta, cow (*Bos taurus*); Hsa, human (*Homo sapiens*); Gga, chicken (*Gallus gallus*); Xla, frog (*Xenopus laevis*).

[b] These numbers are given for the $tRNA^{Phe}$ gene found in the cytb-12S rRNA region. The alternative $tRNA^{Phe}$ structure from the IQM-related region has 19 nucleotides in the T arm and 3 base pairs in the T stem (see fig. 2).

### Hypothetical Gene Rearrangement Mechanism

As Lee and Kocher (1995) have recently noted, two major mechanisms have been proposed to explain gene rearrangements in mtDNAs. One is the duplication of segments during replication, and subsequent internal deletions in a way that results in a different organization. The gene rearrangements in birds may well be explained by such a mechanism (Desjardins and Morais 1990; Quinn and Wilson 1993), and its feasibility is also supported by the frequent polymorphic duplications of

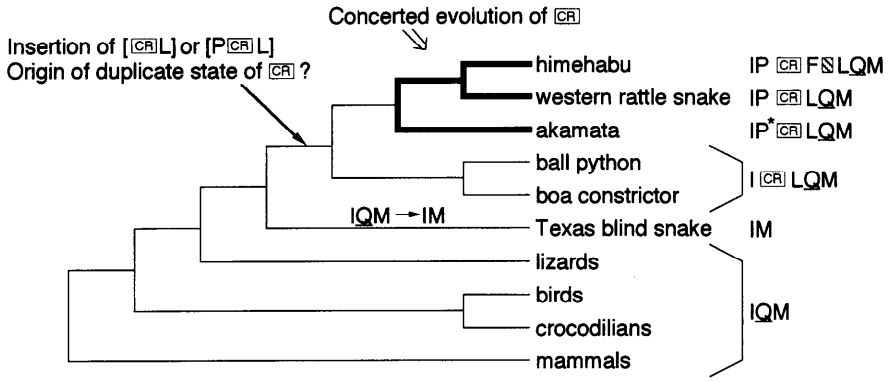


FIG. 7.—Occurrence of gene arrangement features of the IQM-related region in vertebrates. The gene organization of the IQM-related region in amniotes is shown along with a well-established phylogeny (Rage 1987; Benton 1990; Hedges 1994; Heise et al. 1995; Kumazawa and Nishida 1995), on which plausible lineages that account for the occurrence of particular features are highlighted (see text for discussion). Lizards, birds, crocodilians, and mammals are represented by at least three species (Desjardins and Morais 1990; Kumazawa and Nishida 1995; Arnason, Xu, and Gullberg 1996; Janke et al. 1996 and references therein). CR (boxed), the hatched box, and P* stand for the CR-like sequence, the noncoding spacer region, and the truncated $tRNA^{Pro}$ gene, respectively. Lineages on which the concerted evolution of the CR-like sequences may have occurred are highlighted by thick lines. Note that we have not sequenced the cytb-12S rRNA region of boids; the concerted evolution of the CR-like sequences may thus have occurred for boid lineages too (see text).

mtDNA sequences found in lizards (Moritz and Brown 1986, 1987; Stanton et al. 1994). The other suggested mechanism invokes the illicit priming of replication by tRNAs and the resultant integration of tRNA genes around the control region (Cantatore et al. 1987; Jacobs et al. 1989).

We speculate that the original gene rearrangements, which resulted in the insertion of the CR-like sequence and $tRNA^{Leu}$(UUR) gene into the IQM region, may have occurred in the ancestral lineage of the snakes by the first of the mechanisms outlined above. Assuming that a long DNA fragment ranging from the $tRNA^{Pro}$ to $tRNA^{Ile}$ genes of the typical organization (see fig. 1) is duplicated, subsequent deletions of the duplicated portions for $tRNA^{Phe}$-16S rRNA and ND1-$tRNA^{Ile}$ would give rise to the rearranged gene organization either represented by boids with additional deletion of the $tRNA^{Pro}$ gene or by the western rattle snake without such deletion.

## Gene Rearrangement Timing

When did the original gene rearrangements take place? As described above, the insertion of the CR-like sequence and $tRNA^{Leu}$(UUR) gene into the IQM region was commonly found in snakes belonging to the Boidae, Viperidae, and Colubridae families. It is therefore reasonable to infer that the original gene rearrangements that resulted in the insertion of these segments into the IQM cluster took place long ago, i.e., more than 70 Myr as inferred from the minimum divergence time between the Boidae and the Colubridae-Viperidae clade (Rage 1987) (refer to fig. 7). Snakes are phylogenetically derived from a lineage of lizards (see, e.g., Benton 1990; Zug 1993, pp. 438–444), some species of which have been shown to possess the normal IQM cluster like crocodilians, birds, mammals, and fishes (Kumazawa and Nishida 1995). Furthermore, the Texas blind snake, which is considered to belong to the earliest snake lineage (Scolecophidians) (Rage 1987; Zug 1993, pp. 443–467; Heise et al. 1995), has a rearranged organization resulting in an IM cluster (Kumazawa and Nishida 1995). These results suggest that multiple rearrangement events involving the IQM cluster took place early in snake lineages (possibly 70–140 MYA; Rage 1987) (fig. 7).

## Duplicate CR-like Sequences

It was an unexpected finding that himehabu, the western rattle snake, and akamata possess almost completely identical CR-like sequences in two locations of their mtDNAs, there being only a single base alteration between the IQM-related and cytb-12S rRNA regions for each of these snakes, whereas the CR-like sequences otherwise exhibited a large degree of variation among the species (see above). This phenomenon is somewhat reminiscent of the evolutionary mode of the long inverted repeat sequences of chloroplast genomes, the mechanisms of which have yet to be fully understood (reviewed in Sugiura 1992).

How long has the duplicate state of the CR-like sequence persisted in snake mtDNAs? Mitochondrial DNAs are thought to exercise considerable economy with respect to their size and gene number, and, as a consequence, repetitive sequences would be likely to be deleted rapidly. This reasoning predicts that the duplicate state of the CR-like sequence may be only transient. In disagreement with this prevalent notion, our data strongly support the long-term persistence of the duplicate state during the evolution of snakes. As described above, the three snake species all conserve the duplicate state of the CR-like sequence. Although we have not sequenced the cytb-12S rRNA region of boids, preliminary PCR amplification experiments using primers L14940 and H690 produced a band (~3.2 kb, data not shown) from boa constrictor, and the presence of a CR-like sequence within this product was suggested by PCR experiments using this ~3.2-kb template and primers synthesized on the basis of the partially determined CR-like sequence in the IQM-related region of boa constrictor (data not shown). However, discrete amplifica-

tion products were not obtained from ball python with primers L14940, or L14973, and H690 for uncertain reasons (data not shown).

These results suggest that the duplicate state of the CR-like sequence has been maintained in snake mtDNAs for 70 million years (i.e., the minimum divergence time between the Boidae and the Viperidae-Colubridae clade; Rage 1987) and possibly longer—ever since the original duplication-and-insertion event of the CR-like sequence into the IQM region (fig. 7). The reason why one of the duplicated CR-like sequences appears not to have been excised out from the mitochondrial genomes remains unclear. One possible explanation is that the duplication of the control region brings positive functional advantages due to, e.g., efficient initiation of replication or transcription from multiple sites, and that mtDNA molecules with duplicated CR-like sequences become selectively advantageous. Another explanation may be that the duplication and insertion of the CR-like sequence from the cytb-12S rRNA region to the IQM-related region have been driven unceasingly and occurred many times during the evolution of the snakes. In fact, there are many examples of length variation in and around control region sequences that may have been created by frequent duplication and deletion (see, e.g., Moritz and Brown 1986; Solignac, Monnerot, and Mounolou 1986; Buroker et al. 1990).

We consider the latter explanation much less likely, although we do not reject it. A striking difference between the previous examples and ours lies in the fact that the repeated units of snake mtDNAs as indicated by circled numbers in figure 4 are not located closely in tandem but well apart from each other with conserved gene boundaries among the snakes. If the duplication and insertion had been driven by basically the same duplication-and-deletion mechanism outlined earlier, the target sites of the insertion are likely to have been sporadically varied among gene boundaries other than the $tRNA^{Ile}$-$tRNA^{Gln}$ junction. Indeed, Stanton et al. (1994) showed that long duplications with exactly the same gene boundaries can occur independently. However, it seems difficult to find reasons why deletions of excessive parts of duplicated genomes must repeatedly occur with the same gene boundaries among the snakes; none of the snakes permits the insertion of genes for 12S rRNA, 16S rRNA, or ND1 at the CR-like sequence-$tRNA^{Leu}$ junction (fig. 4). In addition, the latter explanation provides no reason why only the region for the excessive CR-like sequence remains undeleted. Finally, duplications and deletions have often been observed as length polymorphism within species and/or heteroplasmy within an individual (see, e.g., Moritz and Brown 1986; Buroker et al. 1990; Zevering et al. 1991). However, no polymorphic or heteroplasmic state could be detected in the length of PCR products containing the CR-like sequences (see above) in spite of the frequent occurrence of the assumed duplication-and-deletion events inferred from the near-identity of the CR-like sequences for each examined species.

## Frequent Gene Conversion?

Why are the duplicated CR-like sequences so similar to each other, whereas they differ to a large extent among the species? If the paralogous CR-like sequences within a mtDNA molecule had evolved independently since the inferred time of the original insertion event (>70 MYA), a much more substantial sequence difference would be expected. Because the origin of the duplicate state at least predates the divergence between the Boidae and the Viperidae-Colubridae clade (see above), the difference between two duplicated CR-like sequences within a mtDNA can be expected to be greater than the difference between the viperids-akamata and ball python (39% on average, see above).

In order to explain this discrepancy, it seems necessary to consider that the duplicated CR-like sequences have evolved in a concerted manner. The control region is thought to contain many nucleotide positions at which base alterations are rapid and selectively neutral (see, e.g., Lee et al. 1995). The concerted evolution between the two CR-like sequences cannot, therefore, simply be explained by directional mutation pressure and/or selection. The most plausible explanation is that frequent gene conversion has persistently homogenized the duplicated CR-like sequences during the evolution of snakes. Gene conversion is a nonreciprocal recombination that results in the complete substitution of a sequence by another relevant sequence. An alternative possibility is that snake mtDNAs have a hitherto unknown replication mechanism that allows duplication of the CR-like sequence from one of two CR-like sequences of a template molecule in each cycle of replication. However, this seems less likely in light of the fact that the duplicated CR-like sequences are not completely identical for each species (fig. 4).

Gene conversion is known to occur commonly in nuclear genomes (see, e.g., Li and Graur 1991, pp. 162–169) but there has been little experimental evidence to show or suggest its occurrence in mitochondrial genomes. On the contrary, any type of recombination has been thought to be absent or very rare in mitochondrial genomes (see, e.g., Wolstenholme 1992). In this respect, the present study may require this prevalent notion to be reevaluated. As shown in figure 4 by circled numbers, the duplicate sequence unit varies from species to species but the left and right junctions are centered around the genes for $tRNA^{Pro}$ and $tRNA^{Phe}$, respectively. Sequencing the IQM-related and cytb-12S rRNA regions from closely related viperids and multiple individuals within the species would provide a key to elucidating the mechanism underlying the concerted evolution of the CR-like sequences in more detail. It would also be important to reveal the entire gene organization and sequence for himehabu mtDNA in order to gain insights into how snake mtDNA replicates with two CR-like sequences. It would be also intriguing to investigate whether any of the gene arrangement features reported herein could serve as a useful molecular marker for snake phylogenetics.

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