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Structure of the mitochondrial control region and flanking tRNA genes of *Mugil cephalus*

Estructura de la región control mitocondrial y genes ARNt adyacentes de *Mugil cephalus*

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ABSTRACT

We cloned and sequenced the mitochondrial (mt) control region (CR) and flanking transfer RNA genes (T, P, and F) of the striped mullet, *Mugil cephalus* and designed species-specific primers to amplify the entire CR in specimens from the Pacific (Hawaii), the Gulf of Mexico, and the Atlantic. We verified the absence of heteroplasmy and nuclear mtDNA duplications of this region in the organisms sampled, finding an extraordinary level of sequence divergence (mean 38-75% Tamura & Nei distance- Γ) between fish from both Oceans, including Japan. The CR of the mullet was variable in length (845-930 bp) and contained structural elements in common with other CRs, including a central conserved segment flanked by hypervariable regions and smaller conserved sequence blocks. Termination associated sequences, however, were not found. The CR of the striped mullet was rich in AT (~67%) and poor in GC.

Key words: Mitochondrial DNA, cloning, molecular divergence, mullet, control region.

RESUMEN

Se clonó y secuenció la región control (RC) mitocondrial (mt) y los genes de ARNt adyacentes (T, P, y F) de la lisa rayada, *Mugil cephalus* y se diseñaron cebadores especie-específicos para amplificar la RC en su totalidad en organismos del Pacífico (Hawai), el Golfo de México y el Atlántico. Se verificó la ausencia de heteroplasmia y de duplicaciones nucleares del ADN mt de esta región en los peces analizados, encontrándose una divergencia genética extraordinaria (38-75% distancia Tamura & Nei- Γ) entre las lisas de ambos océanos, incluyendo a Japón. La longitud de la RC fue variable en la lisa (845-930 pb) y presenta elementos estructurales en común con otras RC, incluyendo un segmento central conservado rodeado por dos regiones hipervariables y además bloques de secuencias conservadas más pequeños. No se encontraron secuencias asociadas a la terminación en la RC de la lisa, que se caracterizó por ser rica en AT (~67%) y pobre en GC.

Palabras clave : ADN mitocondrial, clonación, divergencia molecular, lisa, región de control

INTRODUCTION

The vertebrate mitochondrial (mt) genome shows an extreme structural economy with circa 16,000 base pairs (bp) coding for 37 compactly packed genes: two rRNAs, 13 protein open reading frames, and 22 tRNAs (Boore, 1999). Two important structural and functional features of animal mtDNA are the absence of introns (but see Beagley *et al.*, 1998) and of recombination (but see Smith & Smith, 2002). However, the molecule does contain a non-coding region of varying size known as the "control region" (CR), "Displacement-loop containing region", or just "D-loop" (Brown *et al.*, 1986). This non-coding segment contains conserved motifs, the origin of heavy-strand replication, and both heavy- and light-strand transcription initiation sites (Clayton, 1991). In vertebrates, this region is flanked by the genes coding for tRNA-threonine (tRNA-T), tRNA-proline (tRNA-P) at the 5' end of the light strand and for the tRNA-phenylalanine (tRNA-F) at the 3' end (Meyer, 1993). During the evolution of mitochondria from hypothesized α-proteobacteria several genes were completely translocated to the eukaryotic nucleus (Gray *et al.*, 1999). Data are accumulating showing mitochondrial duplications (known as nuclear mitochondrial or numt DNA) that have also been copied to the nucleus in several taxa, with the potential confounding and misleading effects on the interpretation of the data if they go unrecognized as pseudogenes (Richly & Leister, 2004).

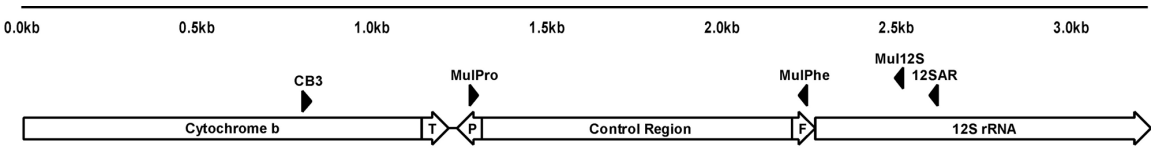
The rapid evolution of mitochondrial protein coding genes compared to nuclear genes has long been established (Brown *et al.*, 1979) and within the mtDNA, the CR has been estimated to evolve 2-5 times faster than protein coding genes (Meyer, 1993). Due to its elevated evolutionary rate, the CR has been the marker of choice to address a variety of intra-specific genetic questions in a wide range of taxa (v.gr., Taberlet, 1996). Comparative studies of the animal CR have revealed a structure consisting of a central conserved region flanked by two hypervariable sections; as well as the existence of conserved sequence blocks (CSB, < 30 bp), which may be found near the heavy strand origin of replication (Walberg & Clayton, 1981) but also several hundred nucleotides downstream from it (Doda *et al.*, 1981). The latter have not been documented in the species of fish examined to date, although some CSBs are conserved from fish to mammals (v. gr., Lee *et al.*, 1995; Liu *et al.*, 2002). Other structural features of the piscine CR are the termination associated sequences (TAS) (Liu *et al.*, 2002). The 5' hypervariable region of the CR adjacent to tRNA-P is characterized by high levels of nucleotide substitution and, due in part to historical circumstances (since universal primers were first designed for it, Kocher *et al.*, 1989), it has been the most widely used in fish micro- and macroevolutionary genetic research (e.g. Rocha-Olivares *et al.*, 1999a; Rocha-Olivares *et al.*, 1999b; Rocha-Olivares & Vetter, 1999; Rocha-Olivares & Sandoval-Castillo, 2003).

Mugil cephalus Linnaeus 1758, known as striped, grey, or black mullet, or "lisa rayada" (Nelson *et al.*, 2004), is a cosmopolitan species inhabiting tropical and subtropical regions of the world between 42ºN and 42ºS (Gilbert, 1993). The species is of commercial importance in most countries including Mexico (v.gr., Ibañez-Aguirre & Gallardo-Cabello, 1996) and the United States of America (Leber *et al.*, 1996). In part because of the economic relevance, but also because its morphological conservatism and challenging systematics, this species has motivated several genetic studies that have brought to light unsuspected levels of genetic differentiation using biochemical markers and mitochondrial DNA (Tsvetnenko, 1991; Crosetti *et al.*, 1993; Crosetti *et al.*, 1994; Rossi *et al.*, 1998; Rocha-Olivares *et al.*, 2000; Garber *et al.*, 2001; see also Miya *et al.*, 2001 for the complete mitochondrial genome). Here, we report the sequence and structure of the complete CR and flanking tRNA genes of *M. cephalus* from the Pacific (Hawaii and Japan) and the Atlantic (Gulf of Mexico and Northwest Atlantic) Oceans to establish base line levels of geographic variation in the structure of this important region of the mitochondrial genome in the context of vertebrate control region evolution.

MATERIALS AND METHODS

Mugil cephalus were collected from the Atlantic coast (North Carolina) and the Gulf of Mexico (Florida, Mississippi, Louisiana, and Texas) (n = 96) and from the Pacific Ocean (Island of Oahu, Hawaii) (n = 19), as reported in Rocha-Olivares et al. (2000). Total genomic DNA was isolated by phenol-chloroform extraction from white muscle tissue and quantified using fluorescence spectrophotometry (Sambrook & Russell, 2001).

A segment containing the 3' end of the cytochrome *b* gene, tRNA-T, tRNA-P, control region, tRNA-F, and the 5' end of the 12S rRNA was amplified by PCR using universal primers CB3 (Palumbi, 1996) and 12SAR (Martin *et al.*, 1992) in replicate 25 µl reactions (100 ng template DNA, 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.3 µM of each primer, 1.75 units of Taq DNA polymerase, 1X PCR buffer Amersham Life Science). Cycling parameters were 3 min at 94ºC, followed by 35 cycles of 45 sec at 94ºC, 1 min at 55ºC, and 2 min at 72ºC, with a final elongation of 7 min at 72ºC. After visualization on a 1% agarose gel, the appropriate PCR product was excised, purified using the QIAquick Gel Extraction Kit (QIAGEN, Inc.), quantified, and direct sequenced. Species specific primers (MulPro and Mul12S) were designed from this product (Fig. 1).



Primer sequences:

CB3:	5´CACATTCAACCAGAATGATATTT3´
12SAR:	5´ATAATAGGGTATCTAATCCTAGTT3´
MulPro:	5´CCAAGGCCAGGATTTTTACGTT3´
Mul12S:	5´CACGAGATTTACCGGCCCTATTAG3´
MulPhe:	5´TCTTGACATCTTCAGCGTCGC3´

Figure 1. Map of the mtDNA control region and adjacent genes constructed from mullet mitochondrial genome (Genebank NC003182) indicating the position and sequence of the primers used in this study relative to the first base of the cytochrome *b* gene. The 3' end of RNA genes and primers is indicated by arrows, tRNA genes are named after IUPAC single letter amino acid codes.

PCR products obtained with species-specific primers were purified and cloned using the pGEM®-T Easy Vector System (Promega, Inc.). Ligated vector DNA was transformed into competent JM109 cells that were cultured on Luria-Bertani (LB)/ampicillin plates with x-gal and IPTG. Colonies containing inserts were identified by blue/white selection and used to inoculate 5 ml minipreps. The cloned plasmid DNA was isolated using the Wizard® *Plus* DNA Purification System (Promega, Inc.). Plasmid DNA was then purified using PEG (Nicoletti & Condorelli, 1993), quantified, and sequenced.

A species-specific primer in the tRNA-F (MulPhe) was subsequently designed and used with the primer MulPro to amplify the entire control region (Fig. 1) in 50 µL reactions (1x PCR buffer, 200 mM dNTPs, 1.5 µM $MgCl_2$, 0.3 µM of each primer, 200 ng template, and 3.5 units *Taq* DNA polymerase) with the above PCR cycling parameters. The appropriate PCR-product was gel-purified, quantified, and direct sequenced. All DNA sequencing was completed with an ABI model 373A Stretch Automated DNA Sequencer at the University of Maine DNA Sequencing Facility.

Because of potential of numt duplications, we corroborated the mitochondrial nature of the source DNA conducting nested PCR with three sets of primers previously used to amplify mtDNA sequences. Following amplification with CB3 and 12SAR PCR products (Fig. 1) were visualized on a 1% agarose gel and the appropriate band excised, gel purified, and quantified. This product was then used as template in subsequent nested PCR reactions with three sets of mtDNA CR primers: L15998-PRO TACCCCAAACTCCCAAAGCTA and H00585-PHE CAGTGTTAAGCTTTAACTAAGCT (Alvarado Bremer *et al.*, 1995); "A" TTCCACCTCTAACTCCCAAAGCTAG and "E" CCTGAAGTAGGAACCAGATG; and "F" CGTCGGATCCAGAGCCTACCACAAGGTGATT and "G" CGTCGGATCCCATCTTCAGTGTTATGCTT (Lee *et al.*, 1995).

DNA sequences were aligned using CLUSTAL-W with the default settings and verified by eye. Sequences from the Pacific and Atlantic were used to construct a majority-rule consensus sequence. We used the tRNAscan SE search server to infer the secondary structure of the mitochondrial tRNA genes (Lowe & Eddy, 1997). The *M. cephalus* sequence used for tRNA analyses was deposited in GenBank accession number AF108270. Intraspecific nucleotide variability in the control region was assessed from all sequence data available that included sequences previously published by us AF108232-352 (Rocha-Olivares *et al.*, 2000), as well as from the complete mitochondrial genome of *M. cephalus* (accession NC003182, Miya *et al.*, 2001). These data were used to produce a new updated multiple alignment different from that used in Rocha-Olivares *et al.* (2000). Sequence divergence was estimated accounting for different rates of transition and transversion, unequal base frequencies and among site rate variation with a gamma corrected (shape parameter $\alpha = 0.5$) Tamura and Nei model (TrN-Γ), conceived to model the evolution of the vertebrate control region (Tamura & Nei, 1993). Phylogenetic relationships among haplotypes were reconstructed using the Neighbor-Joining method (Saitou & Nei, 1987) with the program Mega 2.0 (Kumar *et al.*, 2001).

RESULTS

Mullet DNA from Mississippi and Hawaii amplified with the universal primers CB3 and 12SAR resulted in a PCR product ~2000 bp in length. Sequences obtained from clones of a 1300 bp PCR product from this fragment (using MulPro-Mul12S) were identical for a single amplification, indicating the absence of heteroplasmy in the amplified mtDNA. Except for one pair of primers, nested amplifications within CR primer pairs known to be contained within the fragment produced bands of the expected sizes, suggesting that the priming

Table 1. Mean uncorrected (below diagonal) and TN-Γ corrected (above diagonal) percent sequence divergence among mullet control region haplotypes. Diagonal: within-region mean uncorrected/corrected percent sequence divergence.

	Japan	Hawaii	Atlantic
Japan	-	38.22	70.62
Hawaii	18.61	2.64/2.94	74.73
Atlantic	25.03	23.82	1.45/1.54

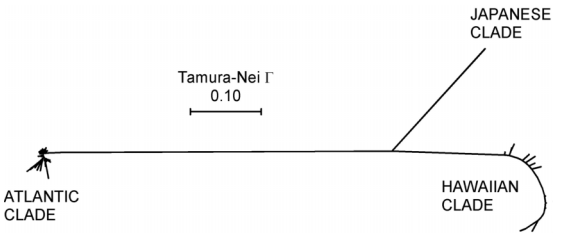


Figure 2. Unrooted Neighbor-Joining phylogenetic reconstruction based on TN-Γ corrected distances of mitochondrial control region sequences of mullets from Japan (n=1), Hawaii (n= 19), and the Atlantic ocean (n= 96).

regions have not experienced run away divergent mutation as expected in a pseudogene. Further corroboration of the authenticity of the mtDNA was obtained from NCBI Blast searches in which the sequences were highly homologous to other teleost CRs and particularly to the CR sequence of *Mugil cephalus* obtained from the entire mitochondrial genome (NC003182).

Species-specific primers (MulPro and MulPhe) designed from the cloned fragment (Genbank accession AF108270) produced a fragment ~880 bp in length. *M. cephalus* control region of the Atlantic specimens (n = 96) ranged in size from 884 - 894 bp (845 bp in one specimen); whereas in the Pacific (Hawaii, n = 19) it ranged from 919-930 bp. All individual sequences were distinct from each other, yielding a total of 115 control region haplotypes. The most relevant finding from the sequencing experiments was the great and unexpected level of DNA divergence found between the Pacific and Atlanic mullets in excess of 23% uncorrected sequence divergence and 70% TrN-Γ corrected divergence (Table 1). This degree of divergence was in sharp contrast with the intra-regional level (Table 1) and resulted in a phylogenetic tree with extremely long branches leading to each cluster of regional haplotypes (Fig. 2). The tree revealed the closer relationship of the Japanese sequence to the Hawaiian haplotypes, despite a considerable level of divergence, and reflected the extreme divergence between mullet from the Pacific and Atlantic oceans (Fig. 2, Table 1).

The sequences of the T, P and F tRNA genes were identified in the cloned DNA sequence and the predicted secondary structure resulted in the expected canonical structure of these molecules (Fig. 3). The multiple alignment of sequences from the two ocean basins (Fig. 4), revealed a conserved central portion flanked by the hypervariable regions, with the 5' end of the control region being more variable. CSB-1, 2 and 3, found in most vertebrate control regions, were present in both the Atlantic and Pacific sequences. In the Pacific we also found a putative CSB-D (Fig. 4). Various insertions and deletions were detected, generally associated to repetitive sequences. Termination associated sequence (TAS) regions were not observed in the CR of *M. cephalus* but the AT-rich 3' end of the control region featured an [AATATTAT] repetitive motif found in several Pacific sequences (from position 984 in Fig. 4). Different

Table 2. Direct (D) and indirect (I) repeats of the *Mugil cephalus* control region from the Atlantic and Pacific Oceans.

D/I	Fragment from base	Repeated from base	Size of repeat[1] (bp)	Repeat sequence (5'→3')
D1 Atl	98	757	8	AAATATAT
D2 Atl	876	900	11	TATAATATTAT
I1 Atl	80	776	8	CTTTTAAA
I2 Atl	775	775	8 (P)	TTTTAAAA
I3 Atl	875	875	14 (P)	TTATAATATTATAA
I4 Atl	879	879	14 (P)	AATATTATAATATT
I5 Atl	901	901	10 (P)	ATAATATTAT
D1 Pac	22	868	8	TGCATATA
D2 Pac	57	884	8	AACATATC
D3 Pac	122	821	8	ACAAGCAG
D4 Pac	896	904, 912 & 928	9	TATAATATT
D5 Pac	383	865	9	ATTTGCATA
D6 Pac	696	743	10	AAACCCCCCC
D7 Pac	698	707 & 745	8	ACCCCCCC
D8 Pac	738	814	9	CCTGAAAAC
I1 Pac	23	23	10 (P)	GCATATATGC
I2 Pac	901	901	18 (P)	TATTATAATATTATAATA

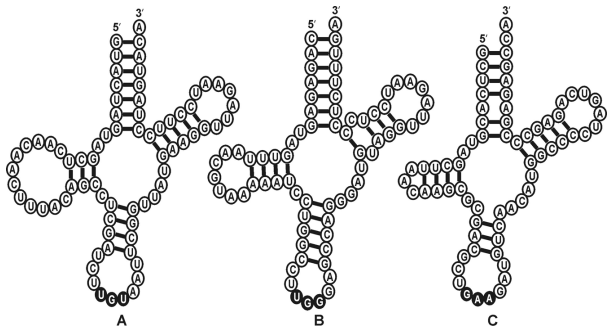


Figure 3. Sequence and structure of mitochondrial tRNAs threonine (A) , proline (B), and phenylalanine (C) of the striped *mullet Mugil* cephalus. Black circles represent the anticodon.

direct and indirect repeated elements of eight or greater nucleotides were identified in all fish. Two direct and five indirect and palindromic repeats were observed in Atlantic specimens; whereas nine direct repeats and two indirect and palindromic sequences were found in the Pacific specimens (Table 2). The nucleotide composition of *M. cephalus* CR was found to be AT rich and poor in G, percent compositions varied from 35.9 -38.1% (T), 34.5 - 38.1% (A), 17.6 - 20.4% (C), and 12.3 - 15.2 % (G), with a ratio AT:CG varying between 2.07 - 2.15.

DISCUSSION

Numt pseudogenes are paralogous mtDNA segments duplicated and transferred to the nuclear genome which can be mistaken for orthologous regions of mtDNA (Lopez *et al*., 1994). The occurrence of nuclear pseudogenes is not uncommon, and has been characterized in many taxa, including marine species (e.g. Zhang & Hewitt, 1996; Schneider-Broussard & Neigel, 1997). Since a PCR reaction may amplify the target mtDNA region (e.g., the CR), a pseudogene, or both, steps must be taken to verify the amplification of the target mtDNA. One method of verification is via "long PCR" (Cheng *et al*., 1994), in which a long piece of DNA is amplified and reamplified with nested primers. The initial amplification product of interest is first gel purified to be used as a template for further amplifications with internal primers. The successful nested amplification of several shorter pieces of DNA of the expected size is a strong indication that the original sequence amplified was of mitochondrial origin, since a pseudogene is more likely to have mutated in several internal priming sites and may not be amplifiable.

Dowling *et al*. (1996) suggested a second verification technique involving enrichment of mtDNA by differential ultra-centrifugation from mitochondria-rich tissues. This decreases the chance of pseudogene amplification but the method is not free of numt contamination. Therefore, the nested PCR approach of long products appears to be more reliable and cost effective. Because two of the three nested primer pairs produced bands of the expected size and because the sequences produced were homologous to a large number of fish CR sequences retrieved from BLAST searches, we conclude that amplified DNA used in this experiment was of mitochondrial origin.

The length of the *M. cephalus* control region in this study (845-930 bp) fell within the range of sizes found for many other fishes (varying from 804 to 1,500 bp and averaging 990 bp) such as the Gadids (e.g. pollock, haddock, and tomcod, with regions of 868, 856, and 853 bp, respectively) (Lee *et al*., 1995); common snook, *Centropomus undecimalis*, 804 bp (Wilson *et al*., 1997); swordfish, *Xiphias gladius*, 842 bp (Alvarado Bremer *et al*., 1995; Rosel & Block, 1996); white sturgeon, *Acipenser transmontanus*, 761-1,007 bp (Buroker *et al*., 1990); Atlantic cod, *Gadus morhus*, 997 bp (Johansen *et al*., 1990); rainbow trout, *Oncorhynchus mykiss*, 1,003 bp (Digby *et al*., 1992); and salmonids, 1,010-1,028 bp (Shedlock *et al*., 1992). Several flatfish species have larger control regions (~1,500 bp) due to longer repetitive sequences at the 3' end (Lee *et al*., 1995).

The structure of the *M. cephalus* control region is similar to that reported for other fish (Buroker *et al*., 1990; Johansen *et al*., 1990; Digby *et al*., 1992), consisting of a conserved central region flanked by two hypervariable segments. Numerous conserved sequence blocks (CSB) have been found in most fish studied (e.g. Lee *et al*., 1995). *Mugil cephalus* from the Atlantic contain CSB-3 and putative CSB-2 and CSB-1, and specimens from the Pacific contain CSB-2, CSB-3, putative CSB-D, and two CSB-1. All CSBs, except CSB-D, are found after nucleotide 572, i.e., toward the 3' end of the control region. The position, order, and sequence of CSB-1, 2, and 3 are similar to those found in swordfish (Rosel & Block,

Table 3. Comparative alignment of CSBs identified in the control region of mammals and piscine mtDNAs (dashes indicate indels). Italicized lower-case letters were not considered CSBs by original authors. In the consensus, upper-case letters denote completely conserved residues and lower-case residues conserved in at least four of the sequences compared (IUPAC codes).

CSB-2

Consensus	yaAAACCCCCcc..taCCCCCcywaa
Mullet (Atlantic)	-AAAACCCCCCC-TACCCCCCCWAA
Mullet (Hawaii)	--AAACCCCCCC-TACCCCCCCAAA
Mullet (Japan)	-AAAACCCCCCC-TACCCCCCCTAA
African clawed frog	t-aaaCCCCC----TACCCCCC-aaa
Mouse	C-AAACCCCCC----ACCCCC-----
Human	C-AAACCCCCCCCCT-CCCCCC----
White sturgeon	c-aaaCCCCC----TACCCCC-----
Atlantic cod	t-AAACCCCCCC--T-CCCCCc-a--
Salmonids	t-aaACCCCCC---TACCCCCC----

Table 3. Continued.

Consensus	yaAAACCCCCcc..taCCCCCcywaa
Mullet (Atlantic)	-AAAACCCCCCC-TACCCCCCCWAA
Mullet (Hawaii)	--AAACCCCCCC-TACCCCCCCAAA
Mullet (Japan)	-AAAACCCCCCC-TACCCCCCCTAA
African clawed frog	t-aaaCCCCC----TACCCCCC-aaa
Mouse	C-AAACCCCCC----ACCCCC-----
Human	C-AAACCCCCCCCCT-CCCCCC----
White sturgeon	c-aaaCCCCC----TACCCCC-----
Atlantic cod	t-AAACCCCCCC--T-CCCCCc-a--
Salmonids	t-aaACCCCCC---TACCCCCC----
Rainbow trout	T-AAACCCCCC---TACCCCCc----
Flounder	-aaaaCCCCCCC-TACCCCCctaaa
Swordfish	tAAAACCCCCC---TACCCCCc-aaa
Common snook	tAAAACCCCCCC--TACCCCCCc---
Freshwater gobiid	TAAAACCCCCC---TACCCCCctaaa

CSB-3

Consensus	tcytgyyAAAaCCCCcc.ggwAAa.sCa.gravaa
Mullet (Atlantic)	TCCTG--AAAACCCCCCCGGAAA---CA-GGAAAA
Mullet (Hawaii)	TCCTG--AAAACCCCCCCGGAAA---CA-GGAAA-
Mullet (Japan)	TCCTG--AAAACCCCCC-GGAAA---CA-GGAAAA
African clawed frog	tcc-GTCAAA-CCCC-----AAAA-CC--gaaaaa
Mouse	---TGCCAAA-CCCC-----AAAAA-CA-------
Human	---TGCCAAA-CCCC-----AAAAA-CA-------
White sturgeon	tcttGTCAAA-CCCC-----AAAA-GCAaggac--
Salmonids	tcctGTTAAA-CCCC-----TAAA-CCa-gga---
Rainbow trout	tccTGTTAAA-CCCC-----TAAA-CCA-gga-a-
Flounder	-cctg--AAAACCCCCC-ggaaa---ca-ggacaa
Swordfish	tccTG--AAAACCCCCc-ggaaa---ca-gga-aa
Common snook	TC-TG--AAAACCCC-----AAAA--CA-GAAGAA
Freshwater gobiid	tccTG--AAAACCCCCC-GGAAA---CA-------

Sources: Mullet, *M. cephalus*– (Atlantic and Hawaii: this report, Japan: Miya *et al.*, 2001); African clawed frog, *Xenopus laevis* (Roe *et al.*, 1985); mouse and human (Chang *et al.*, 1987); white sturgeon (Buroker *et al.*, 1990; Brown *et al.*, 1993); Atlantic cod (Johansen *et al.*, 1990); salmonids (Shedlock *et al.*, 1992); rainbow trout (Digby *et al.*, 1992); plaice and yellowtail flounder (Lee *et al.*, 1995); swordfish (Rosel & Block, 1996); common snook (Wilson *et al.*, 1997); and freshwater gobiid, *Rhinogobius* sp. (Chen *et al.*, 1998).

1996), Atlantic cod (Johansen *et al.*, 1990), common snook (Wilson *et al.*, 1997), and salmonids (Shedlock *et al.*, 1992) (Table 3). CSB-2 and CSB-3 sequences are highly conserved among mammals, an amphibian, and other vertebrates. CSB-1 was identical to, and in the same relative position as, CSB-1 in rainbow trout (Digby *et al.*, 1992) and common snook (Wilson *et al.*, 1997). CSB-1 is much smaller than the 26 bp CSB-1 in mouse (Walberg & Clayton, 1981), but is only one bp smaller than CSB-1 in African clawed frog, *Xenopus laevis* (Roe *et al.*, 1985) and white sturgeon (Buroker *et al.*, 1990).

Fish from Hawaii contain a second CSB-1 close to the 3' end of the control region. This is similar to the arrangement of CSBs found in the white sturgeon (Brown *et al.*, 1993). The CSB-D, found in the swordfish (Rosel & Block, 1996), the freshwater goby *Rhinogobius* sp. (Chen *et al.*, 1998), and several pleuronectids (greysole, plaice, yellowtail, and winter flounder) (Lee *et al.*, 1995), was similar, and in the same relative position to the other CSBs found in fish from Hawaii. A number of direct and indirect repeats were found in *M. cephalus* sequences. Wilson *et al.* (1997) found 18 repeats within the control region of the common snook. In this species a 39 bp tandem repeat spanned nearly half of the control region. Tandem duplications and repeats have been found in many other fish studies producing mtDNA length polymorphisms in the control region (Billington & Hebert, 1991). Bentzen *et al.* (1988) observed two or three copies of a 1,500 bp repeat in the American shad. In a subsequent study, they found in heteroplasmic duplications in the 3' end of the northwest Atlantic redfish CR (Bentzen *et al.*, 1998). However, before these variations can be used as genetic markers, the transmission genetics must be understood, as it is currently uncertain whether these heteroplasmic variations are inherited by offspring (Mulligan & Chapman, 1989). However, in the case of mullet cloning experiments, no evidence of mitochondrial heteroplasmy was revealed.

Indels are common in the non-coding CR of fish (Billington & Hebert, 1991). The 42-bp-long deletion found in a fish from Mississippi occurred within the first 146 bases adjacent to the 5' end. This is the region of highest sequence variability. The deletion may have arisen from folding events, replication slippage, splicing, deletion, and/or duplications (Moritz *et al.*, 1987). In the white sturgeon, Buroker et al. (1990) suggested that length variation might be due to misalignment before replication of a 82 bp repeat region. However it has been hypothesized that these length variations would be of minimal consequence to the organisms due to the non-coding nature of the control region (Brown, 1983; Moritz *et al.*, 1987).

Finally, as is the case with mtDNA in general and with CR in particular, the CR nucleotide composition of mullet was AT rich (~67%). Johansen *et al.* (1990) reported the Atlantic cod control region to be 64% AT. Saccone *et al.* (1987) mentioned that most ATs are found in the hypervariable regions flanking the conserved central domain. This is also true in

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         < tRNA-Pro                                                      60
NC003182 TCGAAGAGGG AGGATTTTAA CCTACATCCC TGGCTCCCAA GGCCAAGATT TTTACATTAA
MulPro                                          CCAA GGCCAGGATT TTTACGTT>

                     CONTROL REGION >>>                                 120
NC003182 ACTACTCTCC GACAAGCTAC ATACACGCAT GTATGTATGT AC-TCCATAT TTGGTTACAC
AF108350                                A....C...C ..-C...... C.A....TGT
AF108352                                A....C.... ..-C...... C.A....TGT
Hawaii                                  ATATGCRTGY AY-CYYATAT CTAGYTATGY
AF108270                                A....C...A G.G.G....C A.A.---...
AF108307                                A....C...A G.G.G....C A.A.---...
Atlantic                                ATATGYAYRR SYGTRCATRC ATAG---YRY

                                                                        180
NC003182 GGACATATTC ATAAACTTTT TTGAAACATT CAACTAACAT TTATACAGTA GCTGCTCTTA
AF108350 AA......CA G.G..... .CA..... A...C..T.. ..-......G AT..A.T.C.
AF108352 AA......CA .......... .CA..... A...C..T.. ..-......C AT..A.T.C.
Hawaii   AAACATATCA RTRAACTTTT TCAAAACATT AAAYCAAYAT TT-TACAGTV ATTGATYTCA
AF108270 .AGT.C..AG .C....C... ..A..... A......TT. ..--....C. AT....T...
AF108307 .AGT.C..A. .C....CC.. ..A..... A......TT. ..--....C. AT....T...
Atlantic RRRYACATRS ACARACCYTT TYAAAACATT AARYTAATTT TT--ACAGCA ATTKCTTTTA

                                                                        240
NC003182 AGAATAGATA AGTAAACCAT ATCAATAAAT TAGCGGTCAT GAGATTAATA AATAATGAAA
AF108350 ..GG.G..C. ..C.G..-.. ..-...G.G. A.TG.CC..C A......... .----.C...
AF108352 ..GG.G..C. ..C.G..-.. ..-...G.G. A.TG.TC..C A......... G----.C...
Hawaii   AGGGTRGACA AGCAGAY-AT AT-ARTRRGY AATGRYYCAC ARGATTAAYA R----YYAAA
AF108270 .-...GA.C. ..C...T-.. ..-...G... A.TAA--... ..A.CC.... ..---.T..G
AF108307 .-...GA.C. ..C...T-.. ..-...G... A.TAA--... ..A.CC.... ..---.T..G
Atlantic A-ARTGARCA AGYAAAT-AT AT-AWKGAAT AATAA--CAY RAAAYCAAYA AA---YYARR

                                                                        300
NC003182 TCAAACGACA TAATTTGTAA AATCAAAAGA TATAACAATT TATTTAAAAT ACTGTAAATG
AF108350 ...T.TA... ....C..... G.C....-.. ...G-T.... .G.....GTA TTAA.G....
AF108352 ...T.TA... ....C....G GGC....-.. ...G-T.... .G.....GTA TTGA......
Hawaii   TCATRTAACA TAATCTGTAR RRYCAAA-GA YATG-YAATT TGTTYAARTR TTRATRAATG
AF108270 .....TA.T. C...CCAC.. GG.T...-.. ....-..... ...C.GG.TA TTA-C.G...
AF108307 .....TA.T. C...CCAC.. GG.T...-.. ....-..... ...C.GG.TA TTA-C.G...
Atlantic YCAAATAAYA CAATCCRCAA GRTTAAA-GA YAYR-CAATW TATSYRRRTA TTA-CARAYR

                                                                        360
NC003182 TAATAAGAGC CTACCATCAG TTGATCTCCT TGTGATGAAA ATTATTGATG TTAAAGACAG
AF108350 ........A. .......... .....T.... CA....A..G .........A ..G.......
AF108352 ........A. .......... .....T.... CA....A..G .......... ..G.......
Hawaii   TAATAAGAAC CTACCATCAR TTGATTTYYT CATGATAAAR RTTATTGATR TTRRAGRCAG
AF108270 ....GGA..T T...-..T.. C..G...... .A....A... G...C....A .C..GA.T..
AF108307 ....GGA..T T...-..T.. C..G...... .A....A..G G........A .C..GA.T..
Atlantic YAATGGAAGT TTAC-ATTAR CTGGTCTCCT TAYRATAAAR RTTAYTRRTA YYAARAATRR

                                                                        420
NC003182 AAATAATAAG GGTTACATAA CTTGA--TCT ATTCCTGGCA TTT-GGTTCC TACTTCAGGG
AF108350 G.....C... ....G....G .....--A.. .......... ...-...... ..T....A..
AF108352 G.....C... .......... .....--A.. .......... ...-...... ..T....A..
Hawaii   GAATARCRAG GGTTAYATAV CTTGA--ACT ATTHCTGRCA TTT-GRTTCC TATTTCAAGG
AF108270 ....G-C..A A...G..CG. TC.C.-GA.. G.CTT..ATG ..CA....TT ..T....A.-
AF108307 ....G-C..A A...G..AG. TC.C.TGA.. G.CTT..ATG ..CA....TT ..T....A.-
Atlantic AARTG-YAAA RRYYGCAHRA TCTCRTGMCT GTYTTTGAYR TYCARRYTYT TRTTTCAAR-
```

Figure 4. Multiple alignment of representative sequences of *Mugil cephalus* mitochondrial control region and adjacent genes from Japan (NC003182), Hawaii (AF108350, 352) and the Atlantic (AF108270, 307). Majority rule consensus sequences (IUPAC codes) of the 19 sequences from Hawaii and 96 from the Atlantic are also aligned. Structural features are indicated (< or >, indicates the 3´ end of RNA genes and primers).

invertebrate mtDNAs, v.gr., in *Drosophila sp*. the control region is known as the A + T-rich region (Avise *et al*., 1987). The extraordinary level of genetic differentiation among these supposedly intra-specific fish has been discussed elsewhere (Rocha-Olivares *et al*., 2000) and will not be repeated here. However, it is worth mentioning that the availability of the Japanese sequence from Miya *et al*. (2001), intermediate between the Hawaiian and Atlantic haplotypes, resulted in a better alignment that, we believe, reflects better the orthology of these divergent sequences and higher estimates of sequence divergence that those previously reported by Rocha-Olivares *et al*. (2000).

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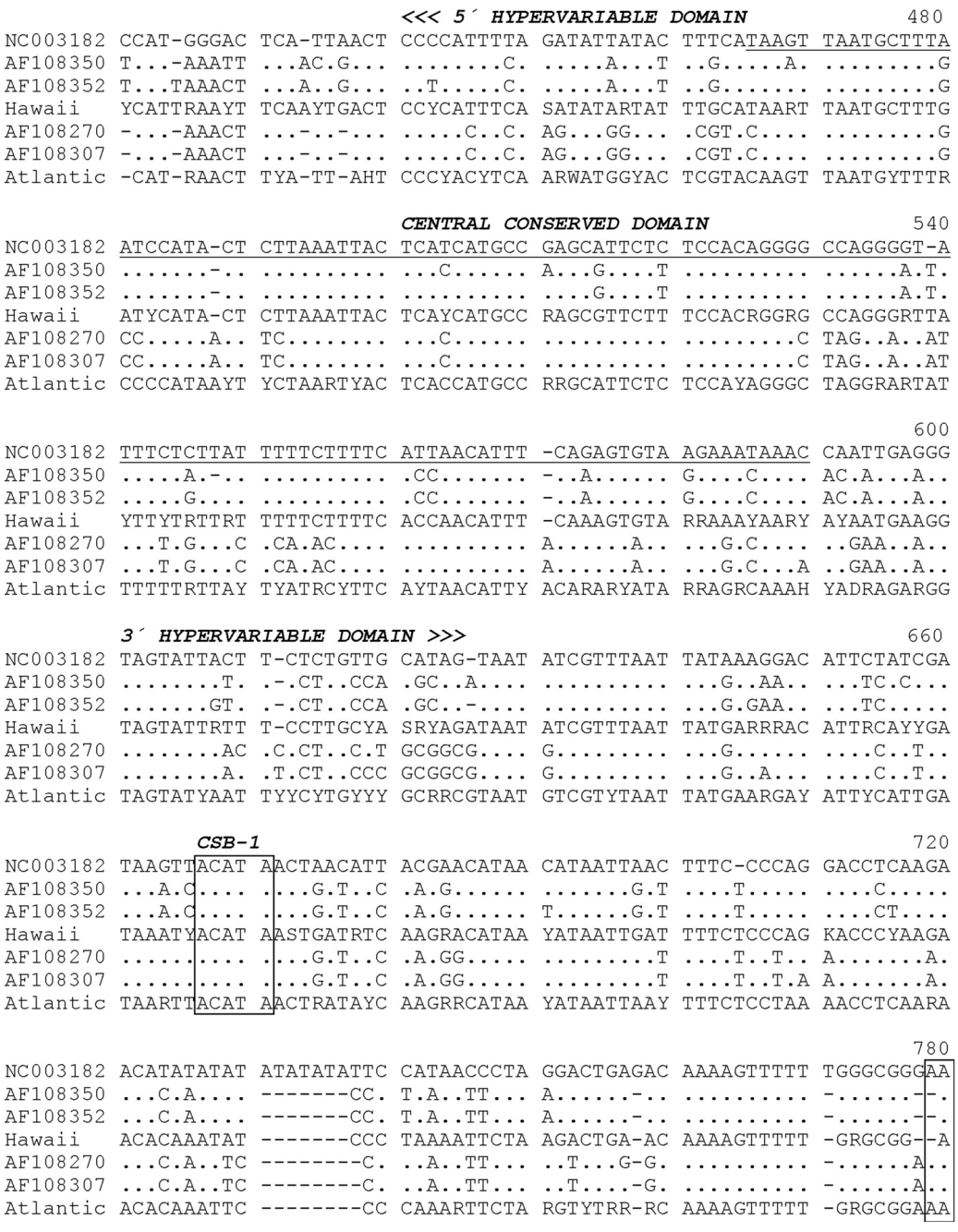

Fig. 4. Continued.

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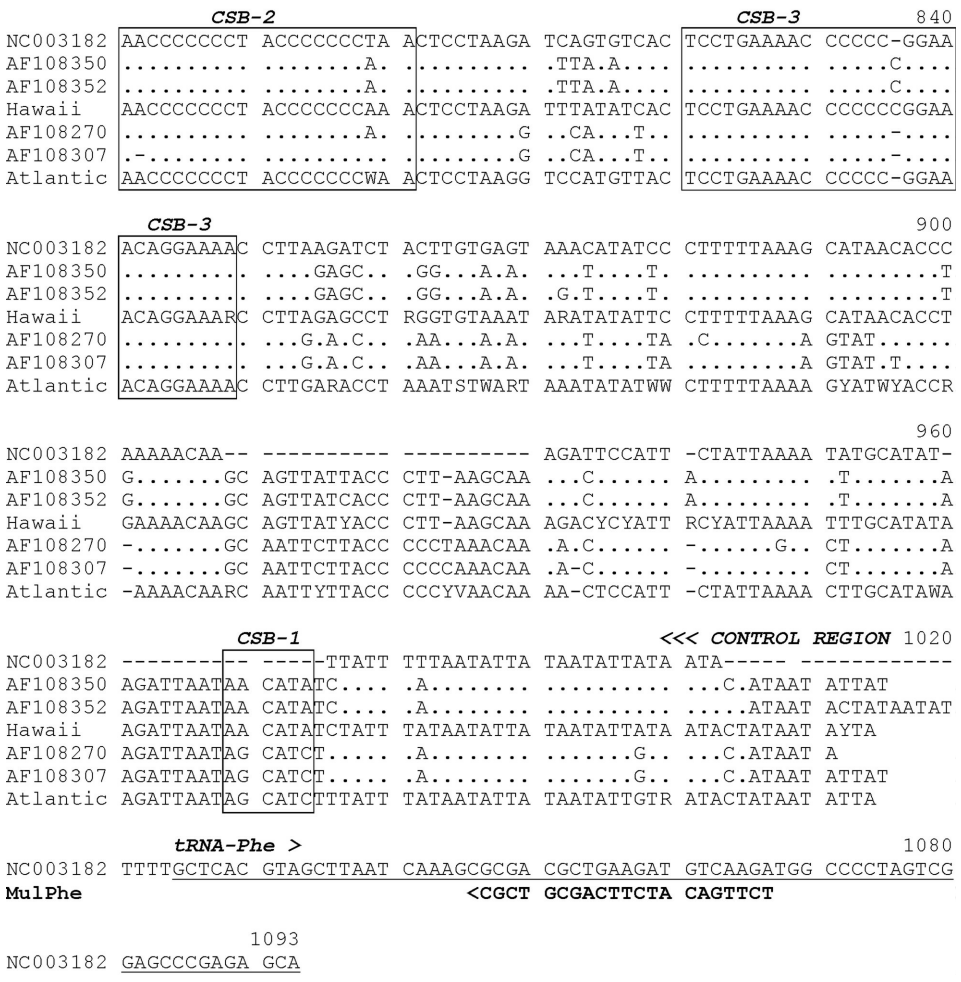

Fig. 4. Continued.

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