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[Short Communication]

The Complete Sequence of Mitochondrial Genome from a Gynogenetic Triploid "Ginbuna" (*Carassius auratus langsdorfi*)

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ABSTRACT—The complete mitochondrial (mt) genome of the gynogenetic triploid ginbuna (*Carassius auratus langsdorfi*, AZ3 line) has been cloned and sequenced. The genome consisted of 16,578 bp and encoded the same set of genes (13 proteins, 2 rRNAs and 22 tRNAs) in addition to a D-loop region, as described for other vertebrate mtDNAs. Comparison with other teleost mtDNAs demonstrated that the protein/rRNA-coding regions of the ginbuna were highly homologous both in length and nucleotide composition to those of the carp, indicating fairly close relationship between the triploid ginbuna and the carp. Although the size of the ginbuna D-loop was almost the same as that of the carp, the nucleotide sequence showed a moderate variation. More comprehensive sequence data of the D-loop regions will lead to the elucidation of phylogenetic relationships among *Carassius auratus* subspecies.

INTRODUCTION

The so-called ginbuna (*Carassius auratus langsdorfi*, Japanese silver crucian carp) is widely distributed in Japan (Liu *et al*., 1980; Kobayasi, 1982). It is well known that its triploid form reproduces gynogenetically to give clonal female offspring (Kobayasi, 1971; Ojima and Asano, 1977). Such a vertebrate with an exceptional reproductive system is unusual in nature and very attractive as a laboratory animal for clone studies. However, little is known about the evolutionary status of the tripolid ginbuna, such as the origin of its gynogenesis. Even worse, the taxonomic confusion regarding the *C. auratus* makes it difficult to identify the "genuine ginbuna". In an attempt to define the genetic characteristics of the gynogenetic ginbuna, we are focusing on mitochondrial DNA (mtDNA) as well as repetitive DNA sequences in nuclear DNA (Murakami and Fujitani, 1997a,b). The mtDNA has been successfully used as a genetic marker for evolutionary and phylogenetic studies in various vetebrates including fishes in virtue of its small size, maternal inheritance and more rapid evolution than nuclear DNA (Brown *et al*., 1979). As one of the initial steps to elucidate the genetic background of the triploid ginbuna, we cloned and sequenced the entire mtDNA of the gynogenetic triploid ginbuna, and the nucleotide and the deduced amino acid sequences were compared with those of other known fish mtDNAs.

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MATERIALS AND METHODS

Isolation and cloning of mtDNA

Mitochondrial DNA was isolated from mature oocytes of a gynogenetically clonal line of the triploid ginbuna (AZ3 line) which had been maintained in our laboratory, by using ultracentrifugation in CsCl/ethidium bromide equilibrium density gradient. The entire mitochondrial genome was cloned into the *Bam*HI or *Sal*I sites of pUC19 using *E. coli* JM109. The subclones were obtained from these two clones using pUC119 or pUC19, on the basis of the restriction map which had previously been constructed with 15 restriction enzymes; 12 sites for *Ban*II, 9 sites for *Hin*dIII, 8 sites for *Dra*I, 6 sites for *Pvu*II, 5 sites for *Eco*RI, 3 sites for *Sma*I, 2 sites each for *Apa*I, *Cla*I, *Eco*RV and *Pst*I, and a single site each for *Bam*HI, *Sal*I, *Mlu*I, *Sac*I and *Xba*I (unpublished data). A total of 58 overlapping subclones were prepared. Nine out of them, which had large fragments unsuitable for DNA sequencing and lacked appropriate restriction sites, were handled with a Deletion kit (TaKaRa) to obtain a series of mutants deleted by every about 300 bp.

DNA sequencing

Each cloned plasmid was sequenced with an AmpliTaq Dye Terminator Cycle Sequencing FS kit (Perkin-Elmer, Foster, CA, USA) and universal primers using an autosequencer (373A, Applied Biosystems, Foster, CA, USA). Nucleotide sequences of the inserts were determined by analyzing either both strands of subclones with inserts shorter than 350 bp or at least two strands from distinct subclones.

RESULTS AND DISCUSSION

The complete nucleotide sequence of the gynogenetic triploid ginbuna mtDNA of 16,578 bp has been determined and submitted to the DDBJ with the accession number AB006953.

The GC content of the entire genome was 42.6% whereas that of the D (displacement)-loop, which lay between the tRNA-Pro and tRNA-Phe genes and was known to be the most rapidly evolving (Hoelzel *et al*., 1991; Lee *et al*., 1995), was as low as 33.5% (i.e., AT-rich).

The triploid ginbuna mtDNA contained 13 protein genes (12 genes were encoded by the heavy (H)-strand while the ND 6 gene was on the light (L)-strand), 2 rRNA genes (both were on the H-strand) and 22 tRNA genes (14 on the L-strand and 8 on the H-strand) (Table 1). This gene arrangement is identical to those so far obtained in other vertebrates except the chicken.

The lengths of most of the coding genes of the ginbuna were identical to those of the carp (Chang *et al*., 1994). Even the D-loop where the highest size variation was expected, the length difference between the triploid ginbuna and the carp (928 bp) was as few as 7 bp. Altogether, the whole mtDNA of the ginbuna was only 3 bp longer than that of the carp.

As shown in Table 1, all of the protein-coding genes except the CO I gene started with ATG. In case of the CO I gene, GTG was employed as the initiation codon. Such a codon usage is common among fish mtDNAs (Tzeng *et al*., 1992; Chang *et al*., 1994; Zardoya *et al*., 1995). Six of the protein-coding genes terminated with TAA, four terminated with TAG. Of these stop codons, the last nucleotide A of TAA of the ATPase 6 and the CO III genes, and the last two nucleotides AG of TAG of the ND 2 and ND 3 genes were responsible also for the begining of genes for the CO III, tRNA-Gly, tRNA-Trp and the tRNA-Arg, respectively. The genes for the CO II, ND 4 and the Cyt b ended with a single stop nucleotide T where the post-transcriptional polyadenylation could produce a TAA termination codon (Anderson *et al*., 1981; Ojala *et al*., 1981)

All of the protein- and rRNA- coding genes of the triploid ginbuna showed greater similarities to those of the carp than to those of the loach (Tzeng *et al*., 1992) and the rainbow trout (Zardoya *et al*., 1995) at both nucleotide and amino acid sequence levels (Table 2). Moreover, when the sequences of the Cyt b gene were compared, the ginbuna exhibited the higher homology to the carp than to other cyprinid fishes (Table 3). These molecular data confirm the common notion that the triploid ginbuna is more closely related with the carp.

In the D-loop region, which is the major non-coding region involved in regulation of replication of the H-strand and transcription of the both strands, the sequence difference between the triploid ginbuna and the carp was 13.3%. In particular, the first one third of the D-loop (adjacent to the tRNA-Pro gene) showed a higher level of alterations with as much as 15.1%. Such a segment is expected to be variable also within the species *C. auratus*, and may be useful for studying relationships among them. We therefore sequenced the entire D-loop regions from a diploid ginbuna, a gengorobuna (*C. a. cuvieri*) and a goldfish (*C. a. auratus*) by analyzing about 1.2 kbp-fragment of each mtDNA digested with *Eco*RI. They are available for accession numbers, AB012094, AB007838 and AB008802, respectively. Each sequence variation as compared with the triploid ginbuna was 1.2%, 4.6% and 4.3%, respectively, implying the closest relationship of the triploid ginbuna to the diploid ginbuna in terms of the maternal lineage.

The more extensive sequence comparison of the D-loop regions among diploid, triploid and tetraploid forms of the ginbuna, along with that among various subspecies of *Carassius* fishes, should help clarify the origin of the gynogenetic triploids. Studies in this direction are in progress.

Table 1. Localization of the mitochondrial genes of the triploid ginbuna

Gene	Position	Size(bp)	Strand	Codon	
				Start	Stop
D-loop	1– 921	921	H		
tRNA-Phe	922– 990	69	H		
12S rRNA	991– 1944	954	H		
tRNA-Val	1945– 2016	72	H		
16S rRNA	2017– 3697	1681	H		
tRNA-Leu(UUR)	3698– 3775	78	H		
ND 1	3776– 4750	975	H	ATG	TAA
tRNA-Ile	4754– 4827	74	H		
tRNA-Gln	4825– 4895	71	L		
tRNA-Met	4897– 4965	69	H		
ND 2	4966– 6012	1047	H	ATG	T(AG)
tRNA-Trp	6011– 6081	71	H		
tRNA-Ala	6084– 6152	69	L		
tRNA-Asn	6154– 6226	73	L		
tRNA-Cys	6259– 6329	71	L		
tRNA-Tyr	6328– 6398	71	L		
CO I	6400– 7590	1551	H	GTG	TAA
tRNA-Ser(UCN)	7951– 8021	71	L		
tRNA-Asp	8025– 8096	72	H		
CO II	8109– 8799	691	H	ATG	T
tRNA-Lys	8800– 8875	76	H		
ATPase 8	8877– 9041	165	H	ATG	TAG
ATPase 6	9035– 9718	684	H	ATG	TA(A)
CO III	9718–10503	786	H	ATG	TA(A)
tRNA-Gly	10503–10574	72	H		
ND 3	10575–10925	351	H	ATG	T(AG)
tRNA-Arg	10924–10993	70	H		
ND 4L	10994–11290	297	H	ATG	TAA
ND 4	11284–12664	1381	H	ATG	T
tRNA-His	12665–12733	69	H		
tRNA-Ser(AGY)	12734–12802	69	H		
tRNA-Leu(CUN)	12804–12876	73	H		
ND 5	12880–14703	1824	H	ATG	TAA
ND 6	14700–15221	522	L	ATG	TAG
tRNA-Glu	15222–15290	69	L		
Cyt b	15296–16436	1141	H	ATG	T
tRNA-Thr	16437–16508	72	H		
tRNA-Pro	16507–16578	72	L		

Each region was idetified by homology with known vertebrate mitochondrial genomes. The position 1 indicates the first nucleotide of the 5' end of the D-loop.

Table 2. Nucleotide (nt) and amino acid (aa) sequence identities (%) of the mitochondrial genes of the triploid ginbuna to those of the carp, the loach and the rainbow trout

	Carp		Loach		Rainbow trout	
Gene	nt	aa	nt	aa	nt	aa
ND 1	87.9	97.8	79.1	90.4	76.3	90.4
ND 2	84.9	91.7	74.1	79.5	69.5	73.1
CO I	88.3	98.6	82.3	95.3	80.4	95.2
CO II	88.3	97.4	81.4	92.2	80.7	93.9
ATPase 8	95.8	96.3	80.7	74.1	74.4	72.2
ATPase 6	85.4	96.0	78.4	93.0	72.5	85.9
CO III	91.6	99.2	83.7	95.4	80.0	94.3
ND 3	87.2	97.4	78.1	87.9	74.2	84.5
ND 4L	86.9	97.0	81.5	94.9	76.8	91.8
ND 4	86.7	97.0	78.1	91.1	74.1	82.6
ND 5	86.7	93.1	75.3	82.0	72.0	77.4
ND 6	86.4	97.7	77.2	86.7	73.4	85.0
Cyt b	88.2	97.9	79.3	93.9	78.4	91.3
12S rRNA	96.9	—	87.1	—	80.6	—
16S rRNA	94.5	—	83.8	—	78.5	—

Table 3. Homology (%) of the Cyt b gene between the triploid ginbuna and other cyprinid fishes

Fish (accession number)	Nucleotide	Amino acid
Carp (X61010)	88.2	97.9
Tench (Y10451)	82.8	95.0
Bleak (Y10443)	80.3	94.5
Bitterling (Y10454)	79.7	93.9
Gudgeon (Y10452)	80.7	93.4
Pugnose minnow (U17270)	80.8	91.8
Rosefin shiner (U17268)	80.0	94.2

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