Polyploid-specific repetitive DNA sequences from triploid ginbuna (Japanese silver crucian carp, *Carassius auratus langsdorfi*)

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(Received 31 March 1997, accepted 16 May 1997)

Repetitive DNA sequences (Cal3nDr) in the genome of a triploid ginbuna (*Carassius auratus langsdorfi*) were isolated from the DraI digests of the genomic DNA. This AT-rich (61%) Cal3nDr monomer was 137 bp in length. The nucleotide similarity among the monomers from the same individual was considerably high (above 97%). Hybridization analyses revealed that the Cal3nDr sequences were organized into tandem arrays. These DNA sequences were present only in triploid and tetraploid ginbunas and were absent from diploid ginbuna, gengorobuna, goldfish, and other cyprinid fishes, and therefore appeared to be specific to polyploid ginbunas. In situ hybridization data showed their localization on one to four out of a total of 150 to 156 chromosomes, depending on the individuals or clonal lines, of the triploid ginbuna. The origin of the Cal3nDr sequences is also discussed on the basis of observation of the artificial triploid ginbuna produced by crossing a diploid female with a tetraploid male.

INTRODUCTION

The Japanese silver crucian carp, so-called ginbuna (*Carassius auratus langsdorfi* or *langsdorfii*) distributed throughout Japan, naturally occurs in three types, namely, diploids, triploids, and rare tetraploids (Kobayasi et al., 1970; Liu et al., 1980; Kobayasi, 1982; Onozato et al., 1983). Although the diploids reproduce bisexually, the triploids and some tetraploids are known to reproduce gynogenetically, resulting in clonal female offspring (Kobayasi, 1971; Kobayasi and Ochi, 1972; Kobayasi et al., 1977; Ojima and Asano, 1977; Dong et al., 1996). A vertebrate with such an exceptional reproductive system is very valuable and should serve as an appropriate experimental model for elucidation of the reproductive mechanisms in vertebrates. Problems are that the diploids and the triploids are morphologically indistinguishable, and even more perplexing, that the morphological classification and evolutionary status of the species *C. auratus* including ginbuna remain obscure.

Defining the characteristics of ginbuna at the molecular level should shed light on this situation. Eukaryotic genomes have been known to contain a variety of highly tandem repeated DNA sequences, referred to as satellite DNAs (Skinner, 1977; Brutlag, 1980; Singer, 1982), which are principally located in the heterochromatic region. Satellite DNAs have been sequenced and analyzed for a wide variety of organisms including various vertebrates. Although the function of satellite DNAs remains yet to be defined, their repetition frequency, sequence composition and genomic organization are useful information for specifying a species or a population and for determining the evolutionary status of the genome in question (Macgregor and Sessions, 1986; Bogenberger et al., 1987; Quinn et al., 1992; Wijers et al., 1993; Mizuno et al., 1995).

Since few studies on satellite DNAs from the species *C. auratus* have been reported, we have been focusing on repetitive DNA sequences such as satellite DNAs in order to foster a better understanding of genomic characteristics of ginbuna. We already found a family of repetitive DNA sequences from ginbuna, which was common to *C. auratus* populations (this will be published elsewhere). In this paper, we will describe another group of intriguing repetitive DNA sequences from triploid ginbuna, which is specific to polyploid ginbuna genomes. The origin of triploid ginbuna will also be discussed.

MATERIALS AND METHODS

Cyprinid fishes and genomic DNA extraction. Ginbuna, common carp and pale chubs (*Zacco platypus*) were collected from the Shibuta River, Kanagawa, Japan. The ploidy of ginbuna was determined by measuring the fluorointensity of erythrocyte DNA stained with DAPI (Hamada and Fujita, 1983), with minor modifications. Goldfish (*C. auratus*), rose bitterlings (*Rhodeus ocellatus*)

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and a commercially cultured form of gengorobuna (*C. a. cuvieri*) were obtained from local fish dealers.

Genomic DNA was extracted from liver tissue or blood according to the standard proteinase K/SDS digestion and phenol extraction procedure (Sambrook et al., 1989).

**Cloning and sequencing of the repetitive DNA sequences.** Genomic DNA from a triploid ginbuna was digested with *Dra*I. The digests were electrophoresed on 3.0% NuSieve GTG agarose (FMC) gel in TAE buffer, and the separated bands were visualized with ethidium bromide. The fragment of about 140 bp in size was excised and ligated into the *Sma*I-linearized plasmid vector pUC19. The ligated DNA was transformed into competent *E. coli* JM109. White colonies with recombinant plasmids were selected on LB agar plates containing ampicillin, X-gal and IPTG, and the plasmids were purified by Wizard™ Minipreps Resin (Promega) according to the manufacturer’s instruction. The inserts were verified to be approximately 140 bp long by agarose gel electrophoresis. Seven cloned plasmids (pCal3nDr’s) purified with the resin were used as templates for sequencing. Both strands of each plasmid were sequenced with AmpliTaq Dye Terminator Cycle Sequencing FS kit (Perkin Elmer) using the Applied Biosystems 373A autosequencer.

**Southern blot hybridization.** Genomic DNA was completely or partially cleaved with *Dra*I. The DNA fragments fractionated on agarose gels were depurinated, denatured and then neutralized. They were transferred to MagnaGraph nylon membranes (MSI) by capillary blotting and were immobilized by UV crosslinking. The membranes were subsequently prewashed and prehybridized under the conditions recommended by the manufacturer. The biotin-labeled probe was prepared by PCR amplification with a cloned plasmid (pCal3nDr11) as a template and pre-biotinylated universal forward and reverse primers for pUC vector. PCR was performed with the first denaturation at 94°C for 5 min, 30 cycles of annealing at 50°C for 1 min, extension at 72°C for 1 min, denaturation at 94°C for 1 min and the last extension at 72°C for 10 min. The product which formed a single band on agarose gel was used as a probe without further purification. Hybridization was carried out at 42°C overnight in 45% formamide, 1 x Denhardt’s solution, 0.1% SDS, 5 x SSC, 20 mM sodium phosphate, pH 7.0, and 10% dextran sulfate plus the alkaline-denatured probe. After the hybridization, the membranes were washed twice in 0.16 x SSC containing 0.1% SDS at room temperature for 3 min and twice in the same solution at 65°C for 3 min, and was rinsed twice in 2 x SSC at room temperature. Chemiluminescent detection of hybridized DNA was performed using Phototope™-Star detection kit (New England Biolab) according to the manufacturer’s protocol. The emitted light was recorded on X-ray film.

**Fluorescence in situ hybridization (FISH).** Metaphase chromosomes were prepared from the culture of whole blood primarily according to Ojima et al. (1970) with slight modifications. Briefly, after blood was cultured in RPMI medium containing 20% fetal calf serum, phytohemagglutinin-M and antibiotics for 3 days at 28°C, it was treated with colchicine for several hours. The KCl-hypotonicization and Carnoy-fixation then followed. Some slides were stained with Giemsa for precise chromosome counting. Hybridization was performed essentially as described previously (Lawrence et al., 1988). Briefly, the chromosome slides were hardened at 65°C for 4 h, denatured in 70% formamide, 2 x SSC at 70°C for 2 min and dehydrated with 70% and 100% ethanol. A hybridization mixture, consisting of 50% formamide, 2 x SSC, 10% dextran sulfate, 2 mg/ml BSA and 400 ng of the formamide-denatured biotinylated probe (see above), was placed on each slide. After overnight incubation at 37°C, the slides were washed with 50% formamide, 2 x SSC at 37°C for 15 min and rinsed sequentially with 2 x SSC, 1 x SSC, and 4 x SSC. Hybridization signals were detected using 5 µg/ml avidine-FITC (Boehringer Mannheim). Chromosomes were counterstained with 1 µg/ml propidium iodide (Sigma) and visualized with fluorescence microscope (Nikon, with 460–485 nm exciter filters, a 505 nm dichroic...
mirror and a 515 nm barrier filter). At least 5 metaphase spreads per specimen were examined.

**RESULTS**

Cloning and sequencing of repetitive DNA sequences. The repetitive DNA sequence of about 140 bp in length was observed as a discrete electrophoretic band in DraI digests from triploid ginbuna, but not from diploid ginbuna (Fig. 1). The predominant fragment from a triploid ginbuna was cloned in the plasmid vector pUC19. The complete nucleotide sequences of seven clones, designated Cal3nDr, were determined (Fig. 2), two of which (Cal3nDr4 and Cal3nDr11) were identical. These repetitive DNA sequences were AT-rich (61% on the average) with a unit length of 137 bp. No elaborate internal structures, except for a perfect 7-bp direct repeat and a perfect 7-bp inverted repeat, were found within the sequence when nucleotide stretches of 7 bp or longer were analyzed. Therefore, the Cal3nDr sequence by itself seems to be a precursor of amplification to form the repetitive sequence. The individual monomers exhibited less than 3% sequence variation between each other with the alterations being restricted to transversions at only 5 positions, where single nucleotide substitutions occurred. Neither insertions nor deletions were seen. Thus the Cal3nDr sequences have apparently been highly conserved and homogeneous, as has been documented for other fish repetitive DNA sequences (Wright, 1989; Ekker et al., 1992; Garrido-Ramos et al., 1994). Process such as unequal exchange or gene conversion might have been involved in the homogenization (Brutlag, 1980; Dover, 1982; Strachan et al., 1985). Homology search with DDBJ using BLAST1.4.9 (Altschul et al., 1990) and FASTA3.0 (Pearson and Lipman, 1988) did not yield any sequence significantly related to the Cal3nDr sequence. The tRNA-related regions such as those contained in most SINEs (Sakamoto and Okada, 1985; Kido et al., 1991) were not detected in the Cal3nDr sequences when compared with twenty different vertebrate tRNA genes. The consensus sequences for polymerase III internal control regions were not seen either.

Southern blot hybridization analyses. In order to examine the organization of the Cal3nDr sequences in the genome of triploid ginbuna, partial digests with DraI were hybridized to the pCal3nDr11 insert as a representative of these repetitive DNA sequences (Fig. 3A). A series of characteristic ladder patterns was generated as digestion progressed: with moderate digestion, as many as 14 bands corresponding to oligomers of the 137-bp repeat unit were distinguishable. These hybridization patterns are indicative of the tandem arrangement of Cal3nDr sequences. Even at complete digestion, a small amount of shorter oligomers (mostly dimer) still remained, most probably resulting from loss of DraI recognition sites within the tandem array. Based on comparison of the intensity of hybridization signals of the monomer and dimer with that of the known quantities of the pCal3nDr11 insert, we estimated that the Cal3nDr sequences accounted for approximately 0.15% of the genome (data not shown). This relative abundance, along with the tandem array, of the repetitive DNA sequences signifies that Cal3nDr sequences are satellite DNA sequences.

Moreover, we investigated whether the Cal3nDr sequences were present in diploid ginbuna in addition to gengorobuna, goldfish, and other cyprinid fishes such as common carp, rose bitterslings and pale chubs. Thirteen individuals of diploid (3 males and 10 females), 11 triploid (all females) and 2 tetraploid ginbunas (both females) were examined. No hybridization signal corresponding to Cal3nDr sequences was observed in their genomic DNA sequences except for all the triploid and tetraploid ginbuna DNA sequences (Fig. 3B). The Cal3nDr se-

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**Fig. 2.** Nucleotide sequences of Cal3nDr monomers. Seven clones from a triploid ginbuna were sequenced and aligned. The sequence from Cal3nDr4 and Cal3nDr11 were identical. All sequences reported here have been submitted to DDBJ and assigned accession Nos. AB001476-AB001481. Dots indicate identical nucleotides. Substitutions relative to the Cal3nDr11 are marked with the appropriate nucleotides. The 7-bp direct and inverted repeats are shown by solid and broken arrows, respectively.
Fig. 3. Southern blot hybridization analysis.  
A. Genomic DNA (5 µg) from a triploid ginbuna was progressively digested with DraI (4 U/µg DNA) for various periods of incubation time (0.5, 1, 2, 5, 20 min, and overnight; O.N.), and fractionated on 1.5% agarose gel.  
B. Genomic DNA (3.5 µg) from various cyprinid fishes was digested to completion with DraI (5 U/µg DNA). In case of a tetraploid ginbuna, 2 µg DNA was used. Each digest was fractionated on 3.0% agarose gel. All digests were blotted and subjected to the hybridization as described in Materials and Methods. Diploid, triploid, and tetraploid show the ploidy of ginbunas. GRB, GF, CC, PC, and RB represent gengorobuna, gold fish, common carp, pale chub, and rose bitterling, respectively. The monomer (repeat unit) is arrowed. The numbers indicate the size of markers in bp.
sequences thus appear to be contained exclusively in polyploid ginbuna genomes.

**In situ hybridization analysis.** While no fluorescent signals were detected in metaphase chromosome spreads from diploid ginbuna (Fig. 4A), clear signals were observed on metaphase chromosomes from all triploid ginbuna tested. However, there was a difference in number of signals between individuals. For instance, some individuals showed two signals whereas others showed three to four signals (Fig. 4B, C and D). The analysis of 11 independent individuals revealed that the number of signals varied from one to four, whereas individuals of clonal triploid ginbuna which were reproduced gynogenetically, exhibited an invariable number of the hybridization signals (data not shown). In accordance with the literature (Kobayasi et al., 1970; Hayashi et al., 1976), Giemsa-staining showed that all the diploid ginbuna tested had 100 chromosomes while the figure for the triploid ginbuna varied from 150 to 156. The number of hybridization signals in the triploid ginbuna was unrelated to the number of chromosomes. We were unable to allocate the Cal3nDr sequences on any particular chromosome due to the considerably large number and small size of the chromosomes (about one third of human chromosomes on the average). The metaphase chromosomes from a tetraploid individual (205 chromosomes) had at least 6 signals (data not shown), indicating that Cal3nDr sequence-carrying chromosomes were distributed more in tetraploid than in triploid ginbuna.

**Artificial triploid ginbuna.** A male tetraploid ginbuna was collected by chance from the Sagami River, Kanagawa, Japan. In 1988, we succeeded in obtaining artificial triploid ginbuna by inseminating eggs from a female diploid ginbuna with the sperm of the male tetraploid ginbuna. The 17 grown-up progeny which are 2 males and 15 females have been kept in our laboratory to date. Examination by Southern blot hybridization revealed that all the 17 progeny possessed Cal3nDr
sequences in their genomes (Fig. 5), as shown for natural triploid ginbuna (Fig. 3). One progeny subjected to FISH analysis displayed only one signal (data not shown). Since their parents had already been dead, it was not possible to retrace the way by which the Cal3nDr sequences of the tetraploid parent were transmitted to the triploid offspring.

**DISCUSSION**

In the present study, we isolated tandemly repetitive DNA sequences from a triploid ginbuna, which turned out to be polyploid-specific. Now we would like to attempt to extend the observation on the polyploid specific DNA sequences into the idea of the origin of triploid ginbuna. Despite the lack of morphological features that distinguish between diploid and polyploid ginbuna, triploid ginbuna has been considered to be created by hybridization. Although experimental evidence has proved that intergeneric cross between carp and gengorobuna or diploid ginbuna, evidence which supported the common belief that the ornamental goldfish was derived from Chinese crucian carp. Shimizu et al. (1993) assumed that an ancestral parent of the Japanese triploid ginbuna was a continental subspecies of *C. auratus* on the basis of electrophoretic polymorphism of amylase isozymes. It thus seems reasonable to suppose that the Japanese polyploid ginbuna has inherited the Cal3nDr sequences from a certain continental crucian carp. However, Chinese crucian carp should be excluded from the candidates because the goldfish genome lacks the Cal3nDr sequences. The most likely origin of Cal3nDr sequences and the origin of triploid ginbuna are attributed to another silver crucian carp, *C. a. gibelio*, which is widely distributed throughout Europe and Russia and resembles ginbuna (*C. a. langsdorfi*) not only in morphology but also in the way of reproduction (Cherfas, 1966, 1972; Kobayasi et al., 1973). Assuming it to be true, the significance of the Eurosiberian crucian carp (*C. carassius*) which have been considered to be the ancestor of *C. a. gibelio* (Raicu et al., 1981) must be born in mind.

The Cal3nDr sequences specific to polyploid ginunas described here should be highly valuable in revealing the genetic background of the Japanese silver crucian carp such as the origin of polyploid and gynogenetic populations.

We are indebted to K. Takayama for his assistance in producing triploid ginbuna by diploid/tetraploid cross. This study was supported in part by a Grant-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan.

**REFERENCES**


