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A Family of Highly Repetitive DNAs from "Ginbuna" (*Carassius auratus langsdorfi*) Genome Common to *Carassius auratus* Populations

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ABSTRACT—A family of highly repetitive DNAs (Hi-a) in the genome from a population of the crucian carp, tentatively identified as the ginbuna (*Carassius auratus langsdorfi*), was isolated from the *Hin*dIII digests and characterized. The Hi-a monomer (268 or 269 bp) was AT-rich (64.9%) with internal repetitive oligomers. The nucleotide similarity among monomers within the same individual was 88-98%, whose sequence alterations occurred mostly at restricted sites. Hybridization analyses revealed that the Hi-a family was organized into tandem array(s) representing satellite DNAs, and that its variants that share certain restriction sites of the repeat unit were dispersed into the tandem array(s) of Hi-a DNAs at varying periodicities. The lack of sequences homologous to the ginbuna Hi-a in the genomes from cyprinid fishes other than gengorobuna and goldfish suggests that Hi-a DNAs are specifically present in the species *C. auratus*. The genome of goldfish differed somewhat in the quantity or the genomic organization of Hi-a DNAs from that of the ginbuna.

INTRODUCTION

The so-called ginbuna, or silver crucian carp (*Carassius auratus langsdorfi*, also spelled *langsdorfii*), naturally occurs in three types, i.e., diploids, triploids and, rarely, tetraploids, and is distributed throughout Japan. The diploids reproduce bisexually like most other vertebrates, while, interestingly, the triploids reproduce gynogenetically, resulting in clonal female offspring (Kobayasi, 1971; Ojima and Asano, 1977). Such a naturally occurring parthenogenetic vertebrate is unusual and particularly valuable, since it could serve as a precious experimental model not only for the elucidation of the reproductive mechanisms in vertebrates but also for applied biology studies such as those of animal propagation. However, the morphological classification of the species *C. auratus* remains controversial due to the possibility of intersubspecific crossing. The phylogenetic status of the ginbuna is thus currently unclear. The genetic background of the ginbuna, such as the origin of its gynogenesis, has not been studied in detail. Both the establishment of a reliable taxonomy of *C. auratus* and a clarification of the origin of the gynogenetic populations await careful analyses of their genomes. We therefore attempted to define the characteristics of the ginbuna at the DNA level.

It is well known that the heterochromatic regions of the eukaryotic genome consist mainly of highly tandem repeated DNAs, referred to as satellite DNAs (Skinner, 1977; Brutlag, 1980; Singer, 1982). Extensive studies of satellite DNAs have been performed in various organisms. As a consequence, satellite DNAs have been found to be diverse; some vary from one species or population to another in the sequence composition of the repetitive unit, repetition frequency and genomic organization, while others are common over species (Hamilton *et al.*, 1990; Wijers *et al.*, 1993; Capriglione *et al.*, 1994; Sonoda *et al.*, 1995a, b; Mizuno *et al.*, 1995). This reflects both the strict conservation and rapid evolution of satellite DNAs fixed in particular genomes. Investigations of repetitive DNAs will therefore be useful to identify genetic markers for specifying the species or population differentiation, and for determining the evolutionary status among closer species. Such findings could be applied to the elucidation of the genetic relationship of the ginbuna to other members of *C. auratus* as well.

Substantial data on satellite DNAs for a variety of fishes have accumulated to date (Wright, 1989; Denovan and Wright, 1990; Ekker *et al.*, 1992; Goodier and Davidson, 1994; Garrido-Ramos *et al.*, 1994). Highly repetitive DNAs from cyprinid fishes, including carp (*Cyprinus carpio*) (Datta *et al.*, 1988), sheepshead minnow (*Cyprinodon variegatus*) (Turner *et al.*, 1991), *Notropis lutrensis* (Moyer *et al.*, 1988), silver carp (*Hypophthalmichthys molitrix*), bighead carp (*Aristichthys nobilus*) and grass carp (*Ctenopharyngodon idellus*) (Huang *et al.*, 1993) have been examined. However, no reports on the repetitive DNAs from the genus *Carassius* in the family Cyprinidae have been presented. In this paper, we describe for the first time the isolation and characterization of a family of highly repetitive DNAs (Hi-a) from the ginbuna.

In the text below, we refer to the two populations of crucian carp "ginbuna" and "gengorobuna" (deepbodied crucian

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carp, *C. auratus cuvieri*) on the basis of morphology. However, it is with some reservations that we employ this widely used nomenclature (Nakamura, 1982), which seems to be excessively meticulous, for the subspecies of Japanese crucian carp. We adopt it only in reference to the studies from other laboratories that use these designations.

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 the ginbuna was determined by measuring the fluorointensity of erythrocyte DNA stained with DAPI (Hamada and Fujita, 1983), with minor modifications. A gynogenetically clonal line of triploid ginbuna (AZ-3 line) was produced by artificial insemination with the sperm of a diploid ginbuna or a goldfish and had been maintained in this laboratory. Goldfish (wakin and ryukin), rose bitterlings (*Rhodeus ocellatus*) and a commercially cultured form of gengorobuna were obtained from local fish dealers.

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

Isolation and cloning of the repetitive DNAs

Genomic DNA from the ginbuna was digested with one of the following restriction enzymes; *Hin*dIII, *Eco*RI, *Bam*HI, *Pst*I, *Pvu*II, *Hin*cII, *Bgl*II, *Eco*RV, *Sal*I, *Sma*I or *Kpn*I. The digests were electrophoresed on agarose gels in TAE buffer. The separated bands were visualized with ethidium bromide. A *Hin*dIII-digested fragment of about 270 bp in size was cut out and ligated into the plasmid vector pUC19. The ligated DNAs were transformed into competent *E. coli* JM109. Colonies were blue-white selected and the plasmids were purified by Wizard™ Minipreps Resin (Promega, Madison, WI, USA) according to the manufacturer's instructions. The inserts were verified to be about 270 bp by agarose gel electrophoresis.

DNA sequencing

One cloned plasmid from a diploid ginbuna (pCal2nHi-a) and nine cloned plasmids from a triploid AZ line ginbuna (pAZHi-a) were purified with the above resin and used as templates. Each plasmid was sequenced with an Ampli Taq Dye Terminator Cycle Sequencing FS kit (Perkin Elmer, Foster, CA, USA) using an autosequencer (373A, Applied Biosystems, Foster, CA, USA). The complete sequences of the inserts were determined by analyzing both strands.

Southern blot hybridization

Genomic DNAs were separately digested to completion with the following restriction enzymes; *Hin*dIII, *Hae*III, *Dra*I, *Pvu*II, *Afa*I, *Hha*I, *Msp*I, *Mbo*I, and *Sau*3AI. In some experiments, genomic DNAs were partially cleaved with *Hin*dIII. The digested DNAs were fractionated on agarose gels. The gels were depurinated, denatured and then neutralized. They were capillary blotted onto Magnagraph nylon membranes (Micron Separations Inc., Westborough, MA, USA) in 10 × standard saline citrate (SSC) overnight. The transfered DNAs were immobilized by UV crosslinking. The hybridization procedure was essentially according to the membrane manufacturer's instructions. Briefly, hybridization was carried out at 42°C overnight in 45% formamide, 1 × Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS), 5 × SSC, 20 mM sodium phosphate buffer, pH 7.0, and 10% dextran sulfate plus a biotin-labeled probe. The probe was prepared by a polymerase chain reaction (PCR) with a cloned plasmid as the template and the pre-biotinylated universal forward and reverse primers for the pUC vector. The 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. After the product was confirmed to form a single band on an agarose gel, it was used as a probe without further purification. The alkaline-denatured probe was added to the hybridization solution. After hybridization, the membrane was washed with a solution of 0.16 × SSC and 0.1% SDS twice for 3 min each at room temperature and then at 65°C, and was rinsed in 2 × SSC at room temperature. The chemiluminescent detection of hybridized DNAs was performed using a Phototope™ detection kit (New England Biolab, Beverly, MA, USA) according to the manufacturer's protocol.

RESULTS

Isolation of repetitive DNAs from the ginbuna

As shown in Fig. 1, the *Hin*dIII-digested fragments from the ginbuna genome rendered a discrete band of about 270 bp, indicating the existence of repetitive DNAs. Digests with ten other enzymes were spread over a whole size range, appearing as a smear, or stayed in high molecular weight regions. Electrophoresis of the *Hin*dIII-digests on a 3.0% agarose gel revealed the presence of a minor band of about 210 bp, in addition to the prominent 270 bp band (this shorter repetitive DNA is now being characterized and will be described elsewhere) (Fig. 1B). The predominant 270 bp fragment was cloned into pUC19.

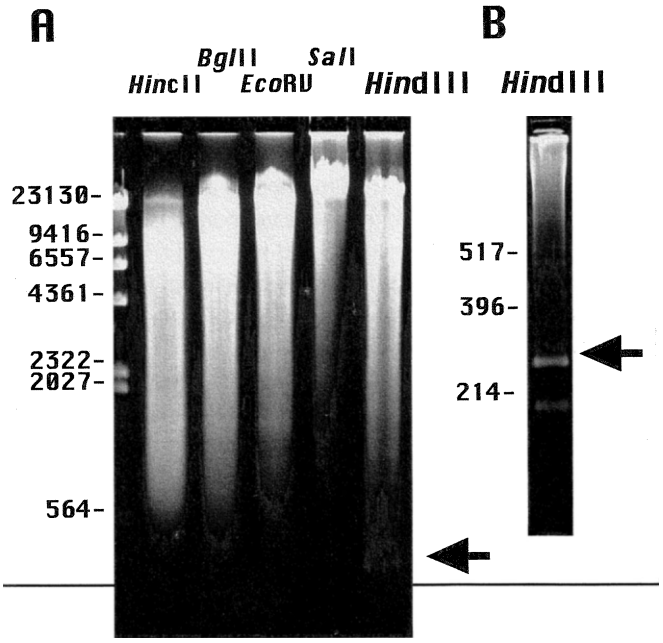

Fig.1. Detection of repetitive DNAs in restriction digests from the ginbuna genome. Five μg of genomic DNA from the ginbuna were digested with *Hin*dIII, *Hin*cII, *Bgl*II, *Eco*RV, *Sal*I or other enzymes (see Materials and Methods; data not shown) and fractionated on a 1% agarose gel (**A**). *Hin*dIII digests were also separated on a 3% agarose gel (**B**). Arrows designate satellite bands of approximately 270 bp in the *Hin*dIII digests. No noticeable discrete band was seen in any other digests. The numbers show the size of marker bands in bp.

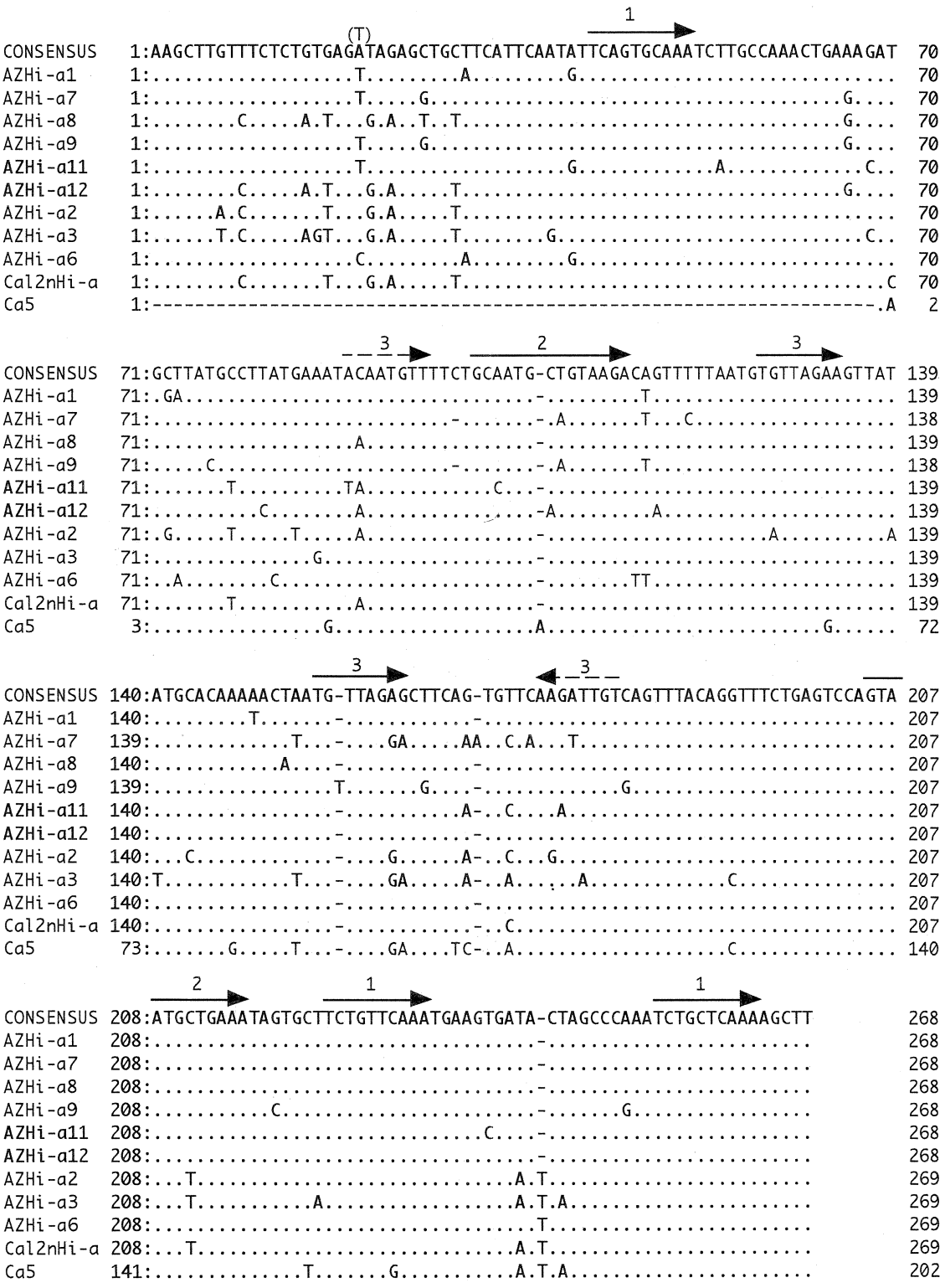

Fig. 2. Nucleotide sequences of the inserts of cloned plasmids (Hi-a family). One clone (pCal2nHi-a) from a diploid ginbuna and nine clones (pAZHi-a) selected randomly from a triploid ginbuna (AZ line) were sequenced and aligned. A consensus sequence was derived from the nine clones of pAZHi-a. The nucleotide with the highest occurrence frequency at each site was assigned as a consensus nucleotide. Dots and dashes indicate identical nucleotides and deletions, respectively. Substitutions relative to the consensus are marked with the appropriate nucleotides. Direct or inverted repeats are indicated with arrows and numbered. Ca5 from goldfish represents the truncated sequence, 202 bp in length. Note that the *Hin*dIII recognition site (AAGCTT) is produced due to the substitution of A for T at position 70 of the consensus sequence. The AZHi-a sequences including the consensus, and the sequences of Cal2nHi-a and Ca5 will appear in the DDBJ/EMBL/GenBank DNA databases with the accession numbers AB001524—1533, D83488 and AB001534, respectively.

Sequencing of repetitive DNAs

The nucleotide sequences of the inserts of one clone (pCal2nHi-a) from a diploid individual and nine clones (pAZHi-a1, 2, 3, 6, 7, 8, 9, 11 and 12) from the triploid line were determined (Fig. 2). These repetitive DNAs, which we named the Hi-a family, were AT-rich (64.9% on the average, ranging from 63.4 to 66.2%) and 268 or 269 bp in length. Clusters of A or T were seen in some regions within the monomers. Three types of direct repeats and one inverted repeat were seen. The extent of sequence identity among AZHi-a monomers and between each monomer and the AZHi-a consensus ranged from 88 to 98% and 91 to 97% (95% on the average), respectively. The similarity between the AZHi-a consensus and the Cal2nHi-a sequence was 96%. Compared with the consensus sequence, the AZHi-a monomers showed variations at 63 positions; substitutions at 59 positions, insertions at 3 positions and a deletion at position 99. Of these 63 positions, sequence variations of the monomers from multiple colonies at the same position were seen for 28 positions, 86% of which were a single nucleotide substitution at each point. Additionally, some streches of 20 to 30 nucleotides with few or no alterations were seen. There was no distinguishable difference in sequence between the AZHi-a monomers from the triploid AZ line and the Cal2nHi-a from a diploid ginbuna, exclusive of the substitution of T for C at position 70. No sequence with significant similarity to this Hi-a family was found in the DDBJ database (release 28).

Organization of Hi-a family in genomes

Complete or partial *Hin*dIII-digests of a triploid ginbuna genome were hybridized to the pCal2nHi-a insert (Fig. 3A). When completely digested, the most remarkable band was a monomer of 270 bp, accounting for the majority of chemiluminescent signals. In addition, small amounts of dimer and faint trimer of the Hi-a repetitive unit were seen, which indicated the presence of mutations at the *Hin*dIII recognition sites between adjacent repetitive units. In the partial digestion experiment, a series of multimers of the repetitive unit was observed as ladders. As many as 15 oligomers of the Hi-a repeat unit were resolved with a moderate digestion. The length of multimers gradually decreased with increasing degrees of digestion until reaching the size of the monomer and dimer. This illustrates tandem arrangement(s) of the Hi-a repetitive units in the ginbuna genome.

The proportion of Hi-a DNAs in the genome was estimated to be approximately 0.15% for both diploid and triploid ginbunas, by comparing the intensity of signals of the monomer and dimer with that of known quantities of the pCal2nH insert (data not shown). As shown in Fig. 3B, in the ginbuna, the hybridization pattern of the digests with *Hae*III, *Dra*I, *Pvu*II, *Afa*I, *Mbo*I or *Sau*3AI showed ladders of multimers (some including monomer, others not) of the Hi-a repetitive unit and also high molecular weight bands. However, most of the hybridization signals in *Msp*I- and *Hha*I-digests were in the region for poorly digested DNAs. There was no significant difference in the hybridization patterns between the diploid and the triploid ginbunas. In the goldfish (wakin and ryukin), the *Mbo*I-digests displayed the presence of prominent Hi-a monomer and dimer bands, and the ladder bands in the *Pvu*II-digests were hardly discernible, contrary to those for the ginbuna.

Specificity of Hi-a family to the species *C. auratus*

To determine whether the Hi-a repeats were conserved within the family Cyprinidae, *Hin*dIII-digests of genomic DNAs from 47 individual cyprinid fishes, i.e., 27 ginbuna (19 triploids, 5 of which were of the AZ line, and 8 diploids), 2 gengorobuna and 4 goldfish (3 wakin and 1 ryukin), all of which belong to the same species *C. auratus*, and 5 carp, 5 rose bitterlings, 2 pale chubs and 2 steed barbels (*Hemibarbus labeo*) were subjected to Southern blot hybridization. In all cyprinid fishes tested except for the species *C. auratus*, no hybridization signal corresponding to Hi-a DNAs was detected (some of these data are shown in Fig. 4A). Hybridization signals for these fishes were not seen even under the conditions of low stringency (the filter was washed with a solution of 1 × SSC and 0.1% SDS at 50°C) in the post-hybridization treatment. The signal intensities of Hi-a monomer for the goldfish and for the gengorobuna were weaker than those for ginbuna, although the signal patterns were essentially identical among the *C. auratus* species, indicating the presence of a lesser amount of typical Hi-a DNAs in the goldfish and gengorobuna genomes. There was no difference between the diploid and triploid ginbuna in the quantity of Hi-a DNAs per haploid genome. Thus, Hi-a repetitive DNAs seem to be present exclusively but in different quantities in the genomes of the subspecies of *C. auratus*.

In the goldfish, a clear band of a smaller size and with a signal weaker than that of the Hi-a monomer was also observed (Fig. 4B). The sequence analysis of the fragment revealed that this was a Hi-a-derived, truncated DNA stretch covering 202 bp from position 69 to the 3' end of the consensus sequence, with 91% similarity (Ca5 in Fig. 2).

DISCUSSION

For a better understanding of the crucian carp, including its gynogenetic populations at the DNA level, we identified satellite DNAs (the Hi-a family) in *Hin*dIII-digests of the ginbuna genome. The repetitive monomers were 268 or 269 bp in length and had an AT-rich nature, which was the most common feature among variable fish satellite DNAs found previously, regardless of the total length of repetitive units (Datta *et al.*, 1988; Wright, 1989; Ekker *et al.*, 1992; Garrido-Ramos *et al.*, 1994). Although the monomers contained several repeats of oligomers as seen in other fish satellite DNAs, no more elaborate internal substructures were seen (Fig. 2). Therefore, the Hi-a sequence might represent a relatively simple evolutionary precursor by itself and might not have evolved from smaller repeating elements (Brutlag, 1980). The slight variation in each Hi-a monomer was in sharp contrast to that of the satellite DNA which shows no intra-individual or intra-population variation in sheepshead minnows (Turner *et al.*, 1991), but was

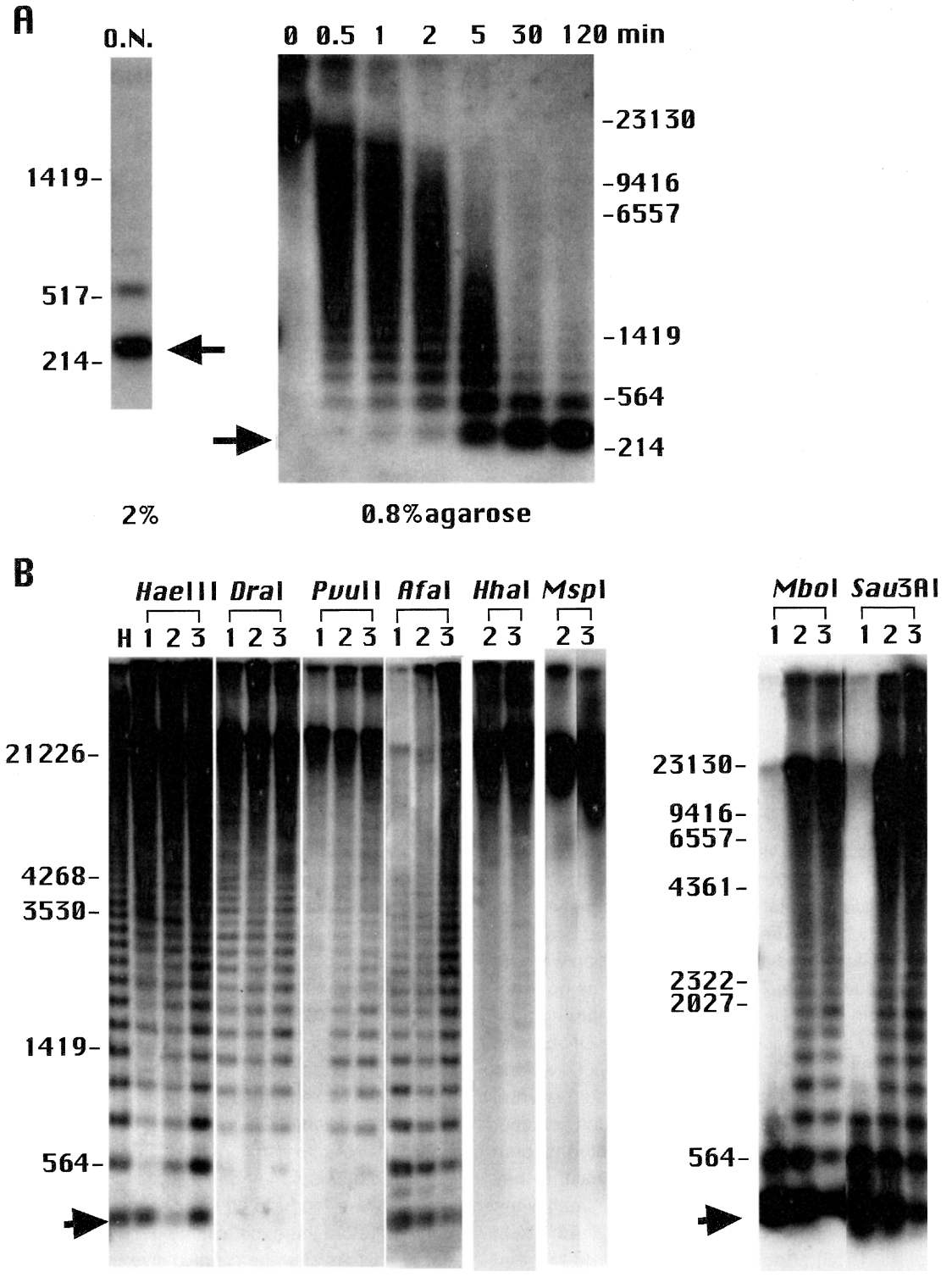

Fig. 3. Genomic organization of Hi-a repetitive DNAs. Genomic DNA from the ginbuna was digested with *Hin*dIII completely (overnight: O.N.) or partially for various incubation periods (0, 0.5, 1, 2, 5, 30, 120 mins) (**A**). Genomic DNAs from the goldfish (lane 1) and ginbuna (triploid, lane 2 and diploid, lane 3) were completely digested with *Hae*III, *Dra*I, *Pvu*II, *Afa*I, *Hha*I, *Msp*I, *Mbo*I or *Sau*3AI (**B**). DNA fragments hybridized to the pCal2nH insert were detected by the chemiluminescent method. The numbers indicate the size of markers in bp. In (**B**), to help estimate the band sizes, partial digests with *Hin*dIII (lane H) were subjected to the hybridization in parallel. The arrows indicate the monomers.

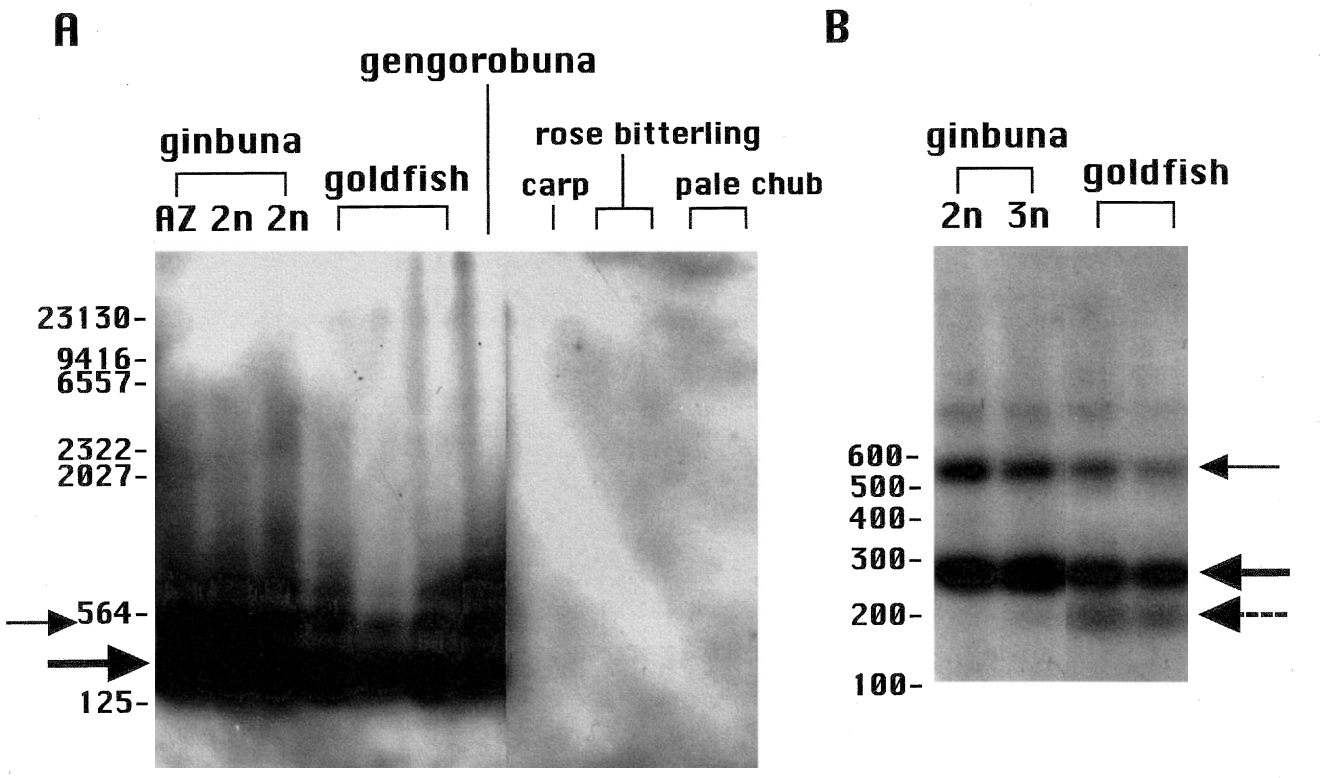

Fig. 4. Distribution of Hi-a family within the genomes in cyprinid fishes. Five μg each of genomic DNAs from various cyprinid fishes were digested with *Hin*dIII. Each digest was fractionated on a 1.5% (**A**) or a 3% (**B**) agarose gel, transferred and subsequently hybridized to the pCal2nH insert under the stringent conditions described in Materials and Methods. 2n and 3n represent diploid and triploid, respectively. The Hi-a monomer and dimer are marked by the solid arrows. The dashed arrow designates the truncated DNA (202 bp). Note that in the goldfish in (**A**), the broad band corresponding to the monomer contains the signals of both the true monomer and the truncated fragment DNA.

similar to those of several other satellite DNAs from tilapia (Wright, 1989), pollock (Denovan and Wright, 1990), zebrafish (Ekker *et al.*, 1992) and *Sparus aurata* (Garrido-ramos *et al.*, 1994). The nucleotide substitutions within Hi-a sequences appeared to occur much more frequently at restricted positions, and most of the substitutions at a particular position were for a single identical nucleotide. This finding indicates non-random alterations and might be involved in a certain homogenizing mechanism as proposed for the satellite DNAs from the mollusc and the beetle (Ruiz-Lara *et al.*, 1992; Davis and Wyatt, 1989). Our hybridization analyses revealed several characteristic structures of Hi-a DNAs in the ginbuna genome. First, Hi-a DNAs were organized as highly repeated tandem array(s) comprising 0.15% of the genome, indicating that they were satellite DNAs. This proportion was rather small when compared with that for other cyprinid satellite DNAs; 8% in carp (Datta *et al.*, 1988) and 5% in silver carp and bighead carp (Huang *et al.*, 1993). Second, most of the hybridized bands of the ginbuna genome digested with *Hae*III, *Dra*I, *Pvu*II, *Afa*I, *Mbo*I or *Sau*3AI corresponded to the integral multimers of the Hi-a repetitive unit. This probably reflects that subsets composed of variants sharing their particular restriction sites at the same positions are interspersed with varying periodicity within the tandem arrangement(s) of Hi-a DNAs. Yet unidentified sequences of Hi-a repetitive units, which should possess a single recognition site for *Hae*III, *Dra*I, *Pvu*II or *Afa*I, would be responsible for these variants. The ladder pattern of *Mbo*I digests may have resulted from the AZHi-a3 sequence with a single *Mbo*I site, which apparently resulted from the substitution of A at position 171 (Fig. 2). These variants may represent transition stages in the course of the homogenization process (Strachan *et al.*, 1985). Both *Mbo*I and *Sau*3AI recognize the identical sequence, GATC, but *Sau*3AI cannot cleave the sequence with 5-methylcytosine. Since the digests with these two enzymes gave similar patterns (Fig. 3B), hardly any methylation of C should have occurred at the GATC sites of Hi-a DNAs. In addition, the CCGG sequence which is recognized by *Msp*I may be lacking in Hi-a DNAs. Thirdly, Hi-a DNAs were found to be common to the *C. auratus* species and therefore there might have been a remarkable expansion of Hi-a DNAs after the cyprinid divergence into the species *C. auratus* or the genus *Carassius*.

In the present study, we could not find a diagnostic difference in the distribution of Hi-a DNAs between the diploid and the triploid ginbuna genomes. However, the identical quantities of the diploid and triploid ginbuna Hi-a DNAs lead us to suggest that Hi-a DNAs are also present in the "extra" genome of the triploid in the same proportion as those in the

diploid. Accordingly, the extra genome with Hi-a DNAs in the triploid may have originated from an ancestral genome common to the *C. auratus*. However, the absolute amounts of typical Hi-a DNAs in the goldfish were lower than that in the ginbuna. Instead, the truncated DNAs of the Hi-a repeat unit were present in abundance in the goldfish, seeming to be more characteristic of this subspecies. The slight differences between the goldfish and ginbuna genomes in the band patterns of their digests with *Mbo*I or *Pvu*II (Fig. 3B) also appear to represent distinct structures of Hi-a DNAs from each fish. Such quantitative or modal differences may provide a clue for the discrimination of the populations in the species *C. auratus*, as demonstrated for satellite DNAs from other organisms (Strachan *et al.*, 1985; Macgregor and Sessions, 1986; Bogenberger *et al.*, 1987; Mizuno *et al.*, 1995). Further sequence analyses of the Hi-a DNAs from various fishes of the species *C. auratus* are in progress.

Future elucidation of the repetitive DNAs including the Hi-a family should greatly accelerate the phylogenetic study of *C. auratus* and contribute not only to the resolution of the taxonomic problems regarding *Carassius* but also to the identification of the genetic background and origin of the gynogenetic triploid crucian carp.

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