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Molecular cytogenetic characterization of 5S ribosomal DNA families in giant salamanders (Amphibia, Urodela)

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Abstract. Entire 5S ribosomal DNA (rDNA), including coding and non-transcribed spacer (NTS) regions from three giant salamander species, *Andrias japonicus*, *A. davidianus*, and *Cryptobranchus alleganiensis*, were sequenced to know the organization of the 5S rDNA multigene family in the giant salamander genome. Owing to the similarity of the sequences in the gene region, the 5S rDNA in these species were clearly divided into four types. Types A and D were detected as species-specific for *A. japonicus* and *A. davidianus*, respectively, while types B and C were isolated from all three species. Comparative analysis suggests the presence of two or three types of the 5S rDNA in the genome of giant salamanders such as the somatic and oocyte types observed in other amphibian species. Further analysis using fluorescence *in-situ* hybridization (FISH) to the embryonic chromosomes from *A. japonicus* localized 5S rDNA to the subterminal region of a single medium-sized acrocentric chromosome pair.

Keywords: Cryptobranchid salamanders, amphibian, 5S rDNA, fluorescence *in-situ* hybridization (FISH)

Introduction

Eukaryotic genomes contain numerous families of repeated sequences, including some multicopy genes, selfish elements, noncoding structural elements, and nongenic repeats. A rough positive correlation between genome size and the number of ribosomal RNA genes has been revealed in ribosomal RNA gene (rDNA) families, which are one of the multicopy gene families (Graur and Li, 2000). In addition, giant salamanders of urodela species have the largest amounts of genomic DNA among vertebrates (Olmo, 1983), and thus should have numerous rRNA genes as well as repetitive DNAs. In higher eukaryotes, rRNA gene families are generally organized into two distinct gene clusters, major (18S, 5.8S and 28S) and minor (5S) rDNA clusters, and these are usually found in loci that are separated from each other. Unlike major rDNA, 5S rDNA is not involved in nucleolus formation, but both rDNA classes are arranged in long, tandemly repeating units. In the 5S rDNA cluster, the repeating unit is comprised of highly conserved 120-nucleotide coding sequences and a non-transcribed spacer, NTS (Little and Braaten, 1989).

Some amphibians have several types of 5S rDNA, which are expressed preferentially as either oocytes or somatic cells (Peterson *et al.*, 1980 ; Van Den Eynde *et al.*, 1989). The most famous example is the two different sets of 5S rDNA, termed the somatic type and oocyte type, which were found in *Xenopus laevis* (Fedoroff and Brown, 1978 ; Miller *et al.*, 1978). In this species, the large oocyte type 5SrDNA loci are dispersed to the telomeric regions of most chromosomes, whereas the somatic type is mostly clustered on a single pair of chromosomes (Harper *et al.*, 1983). In contrast to *X. laevis*, the 5S rDNA data, including the entire nucleotide sequence and chromosomal location, is still lacking in urodela species. Moreover, there are no comparative studies of the 5S rDNA between closely related species in urodela. Family Cryptobranchidae, known as the giant salamanders, is thought to be one of

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the most primitive families in amphibia, and these salamanders are called living fossils since their morphology seems to have remained unchanged for at least 24 million years (Naylor, 1981). This family contains only three living species in two genera, *Andrias* (*A. japonicus* in Japan and *A. davidianus* in China), and *Cryptobranchus* (*C. alleganiensis* in North America), and the two Asian species belonging to *Andrias* demonstrate an indistinguishable phenotype and karyotype. In this study, to get an insight into the organization of the 5S rDNA multigene family in the giant salamander genome, we isolated and determined the 5S rDNA sequences from all three giant salamander species, and compared these sequences within and between species, including other amphibians. We also mapped the isolated 5S rDNA sequence to the chromosomes of *A. japonicus* using fluorescence *in-situ* hybridization (FISH).

Materials and Methods

DNA samples

Four individuals of the Japanese giant salamander, *Andrias japonicus*, were provided from Asa Zoological Park, Hiroshima (Aj1), Kitazato University School of Fisheries Science, Hyogo (Aj2), Nihondaira Zoo, Shizuoka (Aj3), and Nagoya Higashiyama Zoo, Aichi (Aj4). Two individuals of the Chinese giant salamander, *A. davidianus*, were provided from Kyoto University, Kyoto (Ad1), and the Laboratory of the Giant Salamander, Okayama (Ad2). The hellbender (North American giant salamander), *Cryptobranchus alleganiensis* (Ca), was supplied by a specialized dealer in Tokyo, Japan. The tissues of animals were stored at −80°C after dissection. Extraction and purification of genomic DNA from the stored tissue samples were carried out using the procedures described by Tone *et al.* (1982) with minor modifications (Kubota *et al.*, 1993).

PCR amplification and sequencing analyses

Amplification of the 5S rDNA sequences from each genomic DNA was accomplished by polymerase chain reaction (PCR). The primers used for this analysis, 5Sr-1a (5′-GAGACTGCCTGGGAATACC-3′) and 5Sr-1b (5′-GTCTCCCATCAAGTACTAACC-3′), were designed from the most conserved sequences in the gene region of vertebrate 5S rDNA reported previously (Roy and Enns, 1976; Brown *et al.*, 1977; Komiya and Takemura, 1979; Mashkova *et al.*, 1981; Komiya *et al.*, 1986; Pendas *et al.*, 1994; Inafuku *et al.*, 2000). To avoid amplification of the pseudogenes, we selected the sequences in the internal control region. Primers 5SS-1 (5′-CCAAGGAGCATTCGTTTCACCTCC-3′) and 5SS-2 (5′-CACACGTCCACCTTGACTGTGTGCC-3′) were additionally designed from the most conserved part of the NTS sequences obtained in the present study. The reaction mixture (100 μl) for PCR contained 0.5 μg of genomic template DNA, 10 μl of 10 × reaction buffer, 0.4 mM of dNTP mixture, 2.5 units of TaKaRa Ex *Taq* polymerase (TaKaRa), and 1 μM of each primer (constructed by Sawady Technology) and overlaid with mineral oil (Sigma). Amplification was done in a programmable thermal controller (Astec). The reaction conditions were 30 cycles of 30 s at 94°C, 2 min at 57°C (60°C for 5SS-1 and 5SS-2), and 30 s at 72°C (73°C for 5SS-1 and 5SS-2) with a 5 min final extension. The amplified DNA was subjected to electrophoresis on 1.0% agarose slab gels.

The amplified products were purified and ligated into a plasmid pT7Blue Vector (Novagen) or *Hinc*II site of pUC118 (TaKaRa) using a TaKaRa BKL kit (TaKaRa), and *Escherichia coli* strain JM 109 was transformed with the resultant plasmids. Recombinant clones were screened and sequenced as described previously (Nabeyama *et al.*, 2000). All nucleotide sequences were aligned by CLUSTAL W (Thompson *et al.*, 1994). After visual inspection of the resulting alignment, minor modifications were made as necessary. Sequence diversity among the clones was examined with DnaSP software (Rozas and Rozas, 1995). Cloned sequences were grouped after multiple alignment. Phylogenetic lineage of cloned sequences, with a 5S rDNA gene sequence of tiger salamander, *Ambystoma salmoides* (DDBJ/EMBL/GenBank accession number, L49407; Ogilvie and Hanas, 1995) as an outgroup, was estimated by the neighbor-joining method (Saitou and Nei, 1987) using pairwise genetic distance matrices obtained according to Kimura's two-parameter method (1980). A consensus tree of sequence cluster or typing was obtained with bootstrap support for the estimated topology using 1000 replications. These phylogenetic analyses were done using the PHYLIP software package Version 3.66 (Felsenstein, 2006) on a Macintosh computer. The intra-typic nucleotide diversity was calculated as the average number of differences per region between sequences, and the inter-typic nucleotide diversity was calculated as the average number of substitutions per region between types (Nei, 1987).

The copy numbers of the 5S rDNA sequences in the diploid genomes were estimated by slot-blot hybridization, as described previously (Nabeyama *et al.*, 2000). The nuclear DNA amount of each giant salamander was obtained from Morescalchi *et al.* (1977).

Chromosome preparation and fluorescence in-situ hybridization (FISH)

Embryos in egg sacs of *A. japonicus* were provided from Asa Zoological Park in Hiroshima, Japan. Metaphases were obtained from the embryos at stages 31-33, using the method described previously (Kohno *et*

al., 1987). The preparations, after treatment with RNase A, were allowed to hybridize with DNA probes that had been labeled by the enzymatic incorporation of biotin-7-dUTP (Gibco BRL) with a nick translation kit as the protocol from the supplier (Gibco BRL). The denaturation of the chromosome DNA and probe DNA, hybridization, and washing were accomplished as described previously (Kubota *et al.*, 1993). The slides were mounted in Vectashield mounting medium with PI (Vector Laboratories). The hybridization signals were photographed under a microscope (Zeiss). Photographs were taken on Ektachrome ISO 400 color-slide film (Kodak).

Results

PCR amplification with the primers 5Sr-1a and 5Sr-1b generated approximately 470 bp fragments, using genomic DNA isolated from four individuals of Japanese giant salamander, *A. japonicus*, two individuals of Chinese giant salamander, *A. davidianus*, and a hellbender, *C. alleganiensis* (data not shown). After cloning and sequencing of these PCR products, we designated new primers, 5SS-1 and 5SS-2 based on the most conserved region of their NTS. PCR amplification with the new primers generated one or two bands of DNA fragments; a single band of approximately 470 bp was observed in both individuals of *A. davidianus* and *C. alleganiensis*, and two close bands of approximately 470 bp and 480 bp were observed in all individuals of *A. japonicus* (data not shown). After cloning and sequencing of the PCR products, a complete nucleotide sequence of 483 bp of 5S rDNA from *A. japonicus* was determined (Fig. 1). The 5S rDNA sequence of this species included 120 bp of the 5S rRNA gene and NTS with varying length between 345 bp to 364 bp. Since deletions were observed in 4 of 36 cloned sequences, Aj1-1, Aj1-6, Aj2-7 and Aj3-9 in their gene region (Fig. 2), these clones were possibly regarded as nonfunctional genes of 5S rDNA, and were removed from subsequent analysis. After multiple alignment, a total of 32 clones could be divided into three types based on their specific nucleotide substitutions; one type, termed type A, had a characteristic 11-bp insertion in its NTS nearest to the 3′ end of the gene region (Figs. 1 and 3). In the other types, termed types B and C, 10 and 15 nucleotides were substituted from both the gene and NTS regions of type A, respectively (Fig. 3).

After sequencing of the cloned PCR products amplified from the other two giant salamanders, *A.*

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-162
CCAAGGAGCA TTCGTTTCAC CTCCTAAGCA CCTGACTGCA GCTGACAGAC

AGAAAATGCT GCTGGAGTCG CACTCAACTG CAAATTCCTC TGCAAAATGT

GAGCGAAATG GCAGGTGATA GCAGACGGCA AGGACAAGAG GCCTAAAGAC
              +1
AGCTTTCTTG ACGCCTATGG CCACACCACC CTGAATGTGC CCGATCTTGT

CTGCTCTCGG AAGCTAAGCA GTGTTGGGCC TGGTTAGTAC TTGGATGGGA
                                        +120
GACCACCTGG GAATACCAGG TGCTGTAGGC TTTTGCTACT CTCTGGGCAC

TAGTTGGCAC TCTGCTCATT GTCTTTTACC CATTCGTTTT CAGACACTCC

CCCAATCGCA TTGTCTTTTT TGGAAAAGAG AAGGTAGGCA AGCACCAAGC

ATGGCATGGG CCAGCAACTA TCACATCAGG GTCATAGTCG AGCAATAAAA
                                          +321
CAGTTTCAGG CACACAGTCA AGGTGGACGT GTG
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Figure 1. A nucleotide sequence of a cloned 5S rDNA (Aj1-289) from *A. japonicus*, amplified with primers, 5SS-1 and 5SS-2. The number above the sequence indicates the position from the starting nucleotide of the 5S rRNA gene (+1). Primers 5SS-1 and 5SS-2 are double underlined. The sequences corresponding to primers 5Sr-1a and 5Sr-1b are underlined. The sequence of 5S rRNA gene is indicated in bold. The characteristic 11-bp insertion in NTS observed in type A is shaded.

davidianus and *C. alleganiensis*, we also found deletions or insertions in 2 (Ad1-5 and Ad2-225) of 22 cloned sequences, and 7 (Ca37, Ca48, Ca54, Ca58, Ca203, Ca205 and Ca220) of 18 cloned sequences in their gene regions, respectively (Fig. 2). Owing to the observed indels, we regarded these clones as nonfunctional genes, and removed them from subsequent analysis. After alignment, the 5S rDNA sequences from both giant salamanders were also grouped to two and three types, respectively (Fig. 3). In *A. davidianus*, all 20 clones were grouped to three types by their sequence similarity. Eight of these 20 cloned sequences were quite similar to the sequence of type B in *A. japonicus*, except for position 52, while the other two cloned sequences showed close similarities to the sequence of type C in *A. japonicus*. Similarly, all 11 sequences from *C. alleganiensis* could be grouped to two types. Eight of 11 clones showed similar sequences to the type C, except for position 106, whereas the remaining three clones showed close sequence similarities to the type B. However, the remaining 10 clones of *A. davidianus* had similar sequence to the type C, but clearly showed fixed differences at positions 26 and 36 in the gene region, thereby being termed type D (Fig. 3).

A neighbor-joining consensus tree of the 63 cloned gene sequences, with tiger salamander as outgroup, showed two major clusters, one with the sequences of the types A and B, and the other with those of the types C and D. Each of the major clusters was subdivided into minor clusters of types A and B, or types C and D (Fig. 4), in agreement with the results of multiple alignment (Fig. 3).

To understand the molecular evolution and sequence differentiation of the 5S rDNA of giant salamanders, we calculated the sequence divergences between and within the types, in addition to inter- and intraspecific estimations. In each species, the intra-typic nucleotide diversity in both the gene and NTS regions was lower than the inter-typic diversity. Irrespective of the region, there was a slight tendency for the interspecific diversity within a type to be lower than the inter-typic diversity within a species (Table 1).

To quantify the copy number of the 5S rDNA in the genomes of giant salamanders, a slot-blot hybridization was performed using a cloned 5S rDNA of *A. japonicus*, Aj4-208, as a probe. By densitometric scanning and comparison of the autoradiographic intensities, we calculated that the unit of the 5S rDNA sequence was repeated approximately 3.7×10^5 times in the diploid genome of *A. japonicus*. The number of 5S rDNA copies was also calculated as approximately 1.0×10^5 and 4.0×10^4 in the diploid genome of *A. davidianus* and *C. alleganiensis*, respectively (Table 2).

To determine the chromosomal distribution of the 5S rDNA locus on mitotic chromosomes in *A. japonicus*,

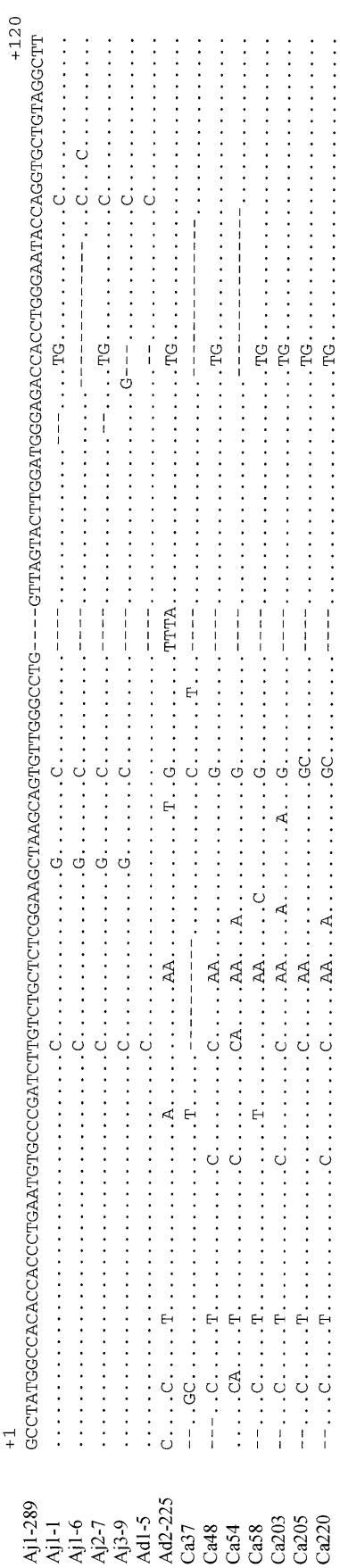

Figure 2. 5S rRNA gene sequence of Aj1-289 (top) and presumptive nonfunctional 5S rRNA gene sequences excluded from comparative analyses. Dots indicate the same state as the corresponding nucleotide of Aj1-289. Dashes indicate gaps.

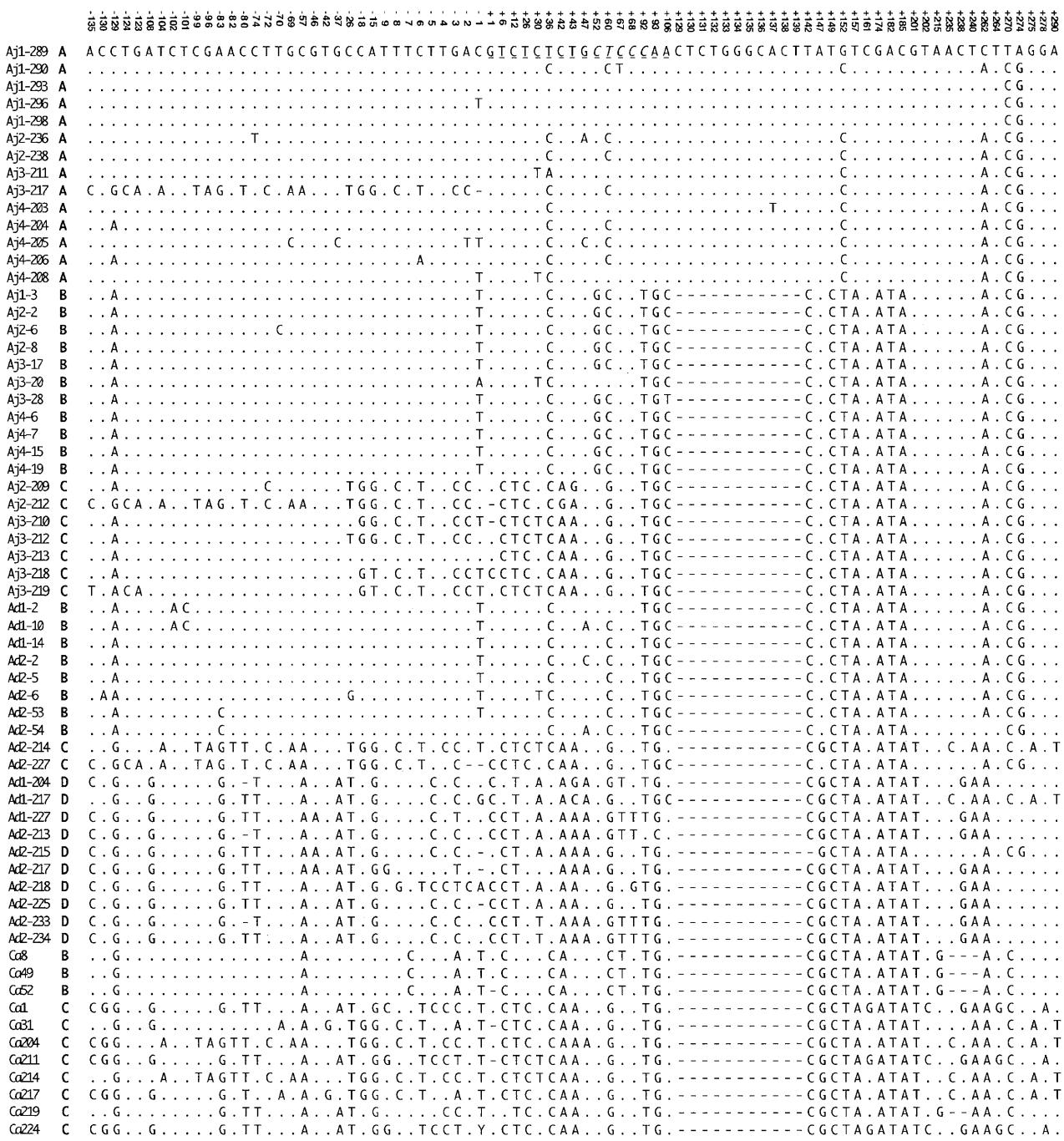

Figure 3. Variable sites of 5S rDNA sequences in all sequences from *A. japonicus*, *A. davidianus*, and *C. alleganiensis*, compared with a sequence of Aj1-289 (top) from *A. japonicus* are shown in this figure. Following the name of the cloned 5S rDNA sequences, A, B, C, and D in bold denote the types of the 5S rDNA. Dots indicate the same state as the corresponding nucleotide of Aj1-289. Dashes indicate gaps. Nucleotides including the gene region (1 to 120) are underlined. The nucleotides corresponding to ICR are indicated in Italic type. Accession numbers are listed in the DDBJ/EMBL/GenBank as follows: Aj1-293, AB066106; Aj1-1, AB066107; Aj3-213, AB066108; Ad1-14, AB066109; Ad1-227, AB066110; Ca1, AB066111; Ca8, AB066112; Ca204, AB066113.

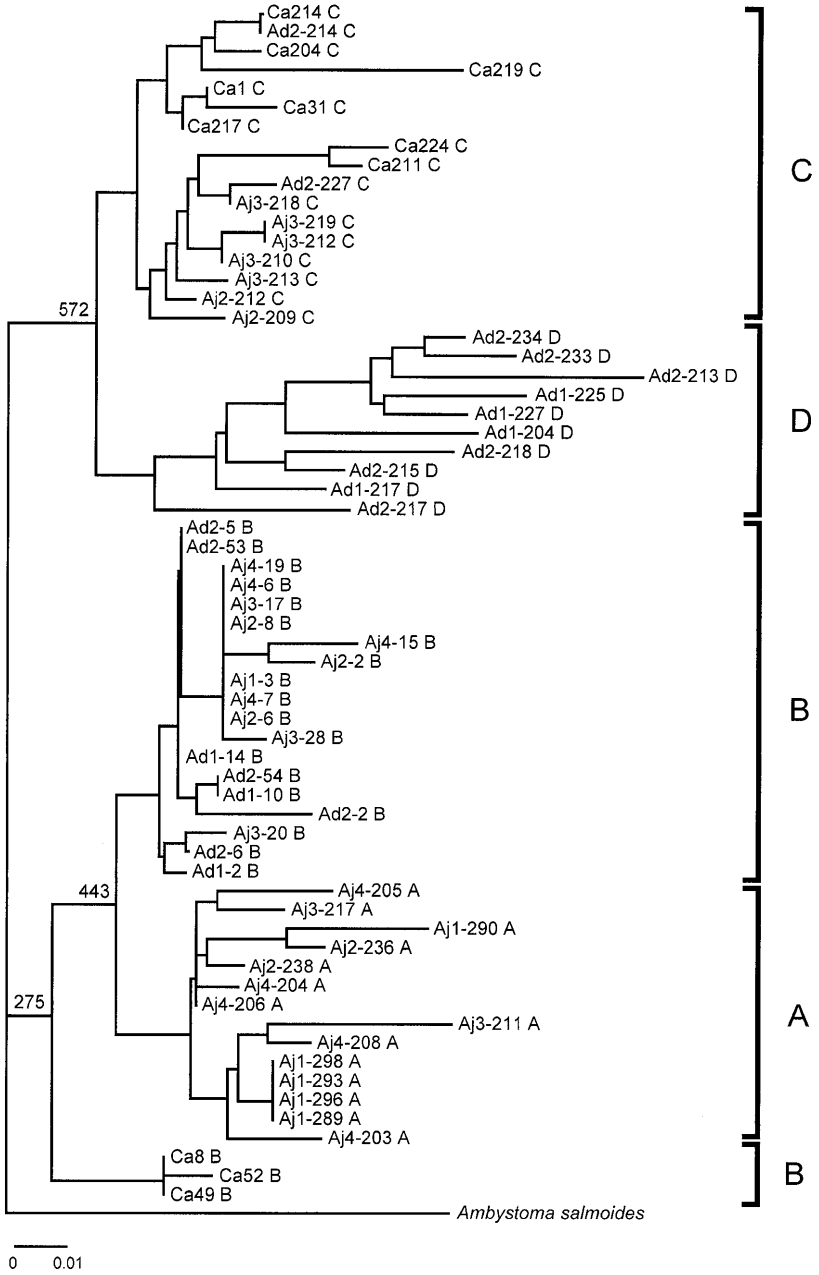

Figure 4. A neighbor-joining dendrogram based on genetic distances by Kimura's two-parameter method using 63 sequences of 5S rDNA gene from giant salamanders. This tree is rooted from a 5S rDNA gene sequence of tiger salamander, *Ambystoma salmoides* (DDBJ/EMBL/GenBank accession number, L49407; Ogilvie and Hanas, 1995) as a outgroup. The values beside the internal branches indicate bootstrap probabilities based on 1000 replicates. The branch length is drawn to the scale of the genetic distances shown at the bottom in this Figure.

we performed FISH analysis using cloned 5S rDNA as the probe. As shown in Figure 5, all fluorescent signals specific for 5S rDNA were located on the subterminal region of a single medium-sized acrocentric chromosome pair.

Discussion

It is known that some amphibians have several types of 5S rDNA (Peterson *et al.*, 1980; Van Den Eynde *et al.*, 1989; Nietfeld *et al.*, 1988). In this study, we demonstrated that giant salamanders also had at least two or three types of 5S rDNA. On the basis of the sequence homologies, we divided the 63 sequences of 5S

Table 1. Nucleotide diversity among gene (left[a] or above[b] diagonal) and NTS (right[a] or below[b] diagonal) regions.

	Aj-A[c]	Aj-B	Aj-C	Ad-B	Ad-C	Ad-D	Ca-B	Ca-C
Aj-A	0.035/0.031	0.058	0.102	0.051	0.104	0.127	0.073	0.104
Aj-B	0.043	0.013/0.004	0.072	0.021	0.083	0.123	0.058	0.091
Aj-C	0.073	0.043	0.019/0.006	0.065	0.024	0.084	0.072	0.039
Ad-B	0.046	0.008	0.046	0.013/0.011	0.076	0.111	0.052	0.084
Ad-C	0.105	0.081	0.061	0.082	0.042/0.049	0.085	0.070	0.031
Ad-D	0.101	0.074	0.085	0.076	0.083	0.071/0.031	0.094	0.085
Ca-B	0.079	0.044	0.075	0.047	0.086	0.067	0.006/0.009	0.073
Ca-C	0.123	0.092	0.099	0.094	0.074	0.068	0.078	0.038/0.070

[a]Average number of differences per region between sequences (Nei 1987, Eq. 10.5).
[b]Average number of substitutions per region between types (Nei 1987, Eq. 10.20).
[c]Symbols indicate the types.
Key to abbreviations: Aj=*A. japonicus*, Ad=*A. davidianus*, Ca=*C. alleganiensis*.

Table 2. Numbers of 5S rRNA genes per haploid genome in amphibians.

Genome source	5S rDNA copy number	DNA pg/N
Andrias japonicus[a]	3.7×10^5	92.9
A. davidianus[a]	1.0×10^5	100.1
Cryptobranchus alleganiensis[a]	4.0×10^4	112.5
Ambystoma mexycanum[b]	6.1×10^4	76.0
Triturus cristatus carnifex[b]	3.2×10^4	46.0
Xenopus laevis[b]	2.4×10^4	6.0

[a]DNA amounts were referred from Morescalchi *et al.* (1977).
[b]DNA amounts and 5S rDNA copy numbers were referred from Hilder *et al.* (1983).

rDNAs from three giant salamanders into four types, A, B, C and D (Fig. 3). Although types A and D were isolated as species-specific, types B and C were commonly detected in all three species. Accordingly, we assume that types B and C are paralogous genes that originated from a common ancestral gene cluster, and that the differentiation of these two types must have occurred in ancestral species before the speciation between genera *Andrias* and *Cryptobranchus*. The result of neighbor-joining analysis clearly supports this evolutional history of 5S rDNA in giant salamanders (Fig. 4). Moreover, the dendrogram may imply that the differentiation of 5S rDNA sequences ascend to the ancestral species before the speciation between families Cryptobranchidae and Ambystomatidae.

The typical example of paralogous 5S rDNA in amphibians was observed in *Xenopus* species, having two types of 5S rDNA, somatic and oocyte types (Peter-

Figure 5. A metaphase of *A. japonicus* hybridized with a cloned entire 5S rDNA sequence (Aj2-238) from *A. japonicus*, as a probe. The bar represents 20 μm.

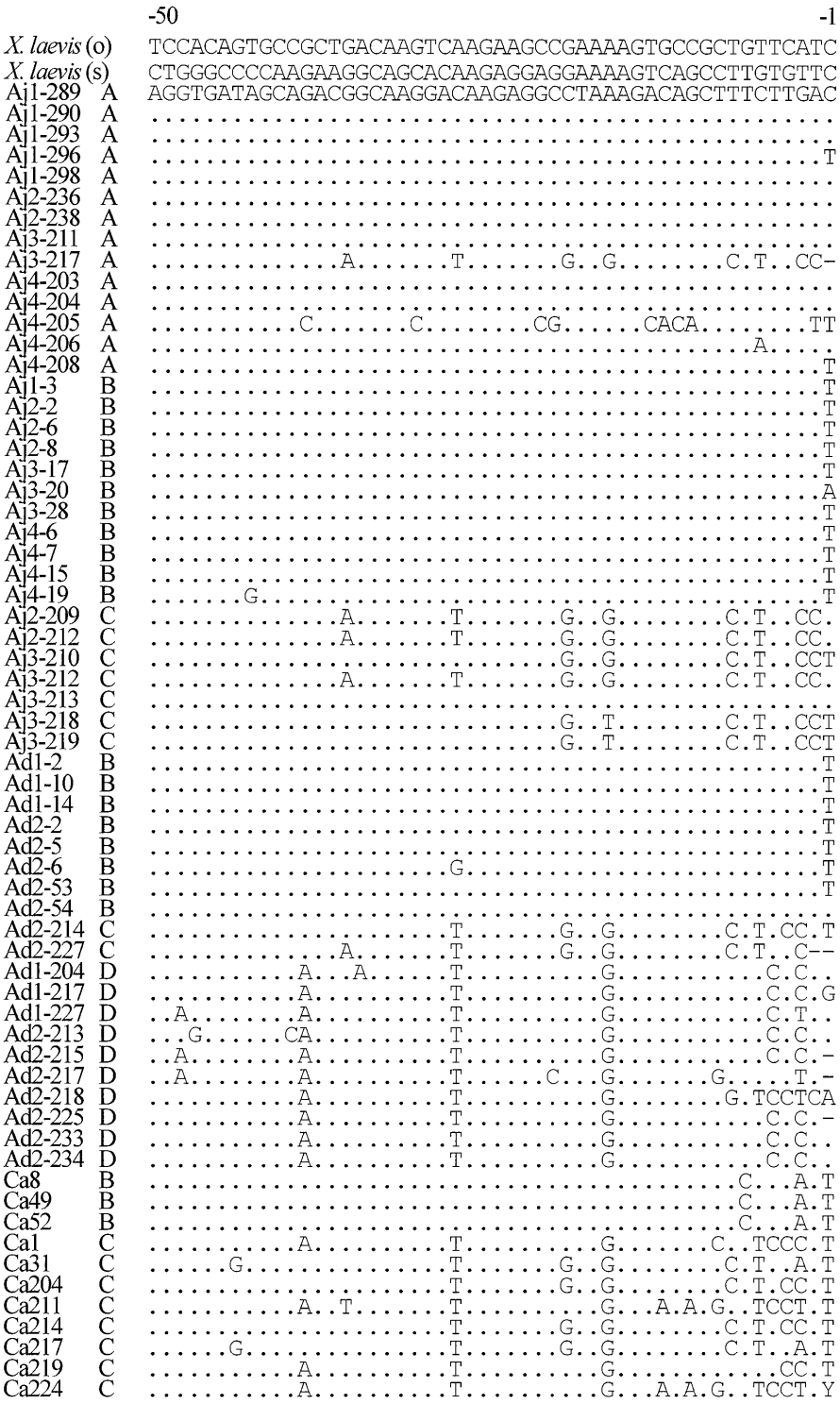

Figure 6. Alignment of upstream NTS sequences of the 5S rDNA gene from giant salamanders. *X. laevis* (o) and (s) indicate the oocyte type and somatic type 5S rDNA of *X. laevis*, respectively (Reynolds and Azer, 1988). The other notations in this figure correspond to those of Figure 3. Y stands for T or C, and S stands for G or C.

son *et al.*, 1980). The 5S rRNA gene includes the minimal promoter element, a 50 bp internal control region (ICR), required for accurate initiation by RNA pol III (Wolffe, 1994). The ICR is the binding site for transcription factor IIIA (TFIIIA), a 38 kDa protein composed of nine tandemly repeated zinc fingers and an additional C-terminal domain. Upon binding, TFIIIA makes strong contacts, mainly with the NTS (Hayes and Tullius, 1992). After formation of the TFIIIA-5S rDNA unit, TFIIIC and TFIIIB sequentially bind, and this multiprotein complex directs the initiation of transcription. Consequently, this complex bound to the 5S rDNA is in the direct path of the transcribing RNA pol III. In *Xenopus* species, the differentiation of the nucleotide sequence in ICR between types has influenced the transcriptional activity (Reynolds and Azer, 1988; Wolffe, 1994). As shown in Figure 3, one to three type-specific substitutions in ICR were also observed in giant salamanders. Therefore, these substitutions might affect the transcriptional advantages as *Xenopus* species. In fact, a substituted site between types B and C, at position 60 in ICR, was identical with those between 5S rRNAs isolated from oocyte and somatic cells in a newt, *Pleurodeles waltl* (Van Den Eynde *et al.*, 1989). In this species, this position is fixed as G in somatic transcripts but substituted as C in several kinds of oocyte-transcripts.

Since the 5′-upstream NTS region is known as another important region for transcription in various vertebrate species (Reynolds and Azer, 1988; Felgenhauer *et al.*, 1990; Nielsen *et al.*, 1993), we analyzed the corresponding region in giant salamanders. Consequently, most of the nucleotides in the 5′-upstream region have been conserved among all types, whereas several type-specific substitutions were observed at positions -1 to -7, -17, -20, and -28 (Fig. 6). This implies that the important sites for transcription are located at these conserved regions. Actually, presumptive transcriptional important nucleotide sequences for RNA pol III at positions -10 to -13 (CAGC) in *X. laevis* (Reynolds and Azer, 1988) are identical with the corresponding region which is conserved among all types from giant salamanders (Fig. 6). This observation strongly suggested that all of these types are transcriptional active genes. However, the nucleotides at -17 and -28 of types A and B were substituted in types C and D. Since a type-specific transcriptional advantage also depends on the nucleotide differences in the 5′-sides of the gene region and 5′-upstream NTS region in *X. laevis*, (Reynolds and Azer, 1988), similar transcriptional regulation of 5S rDNA may be occurring between the former two types (A and B) and latter two types (C and D) in giant salamanders.

Komiya *et al.* (1986) postulated that the oocyte-type of 5S rDNA originated from the somatic type, and has evolved rapidly under lower selective pressure than the somatic type. In giant salamanders, nucleotide diversities in the gene region revealed that the rate of intraspecific sequence homogenization of type C is lower than that of type B (Table 1). Additionally, sequences of type C clearly exhibited more frequent substitutions than those of type B through three salamander species, and sequences of type B showed an interspecifically high similarity. These observations might indicate that the sequences of type B are predominantly transcribed in their somatic cells. The lower selective pressure against the type C than the type B was also supported by the neighbor-joining tree of the 5S gene sequences, in which longer length branching mostly occurred within a type suggesting higher intra-typic sequence variability (Fig. 4).

The 5S rDNA in Cryptobranchoid salamanders was highly repeated in their genomes, as observed in other urodela species (Brown and Weber, 1968; Pukkila, 1975), and our data supports the previous postulation that the 5S copy number tends to increase as the C-value increases in amphibians (Hilder *et al.*, 1983) (Table 2). However, we cannot exclude the possibility that the 5S rDNA sequences isolated in this study were only a part of all sequences among the numerous 5S rDNA copies. In fact, the lack of type C in Aj1, Aj4, and Ad1 indicated that the sequencing of 63 clones was not sufficient to elucidate all of the variations (Fig. 3).

In *X. laevis*, the oocyte-type of 5S rDNA genes were located at the telomere region of the long arm of most chromosomes, and most of the somatic type genes were clustered on a single pair of chromosomes (Pardue *et al.*, 1973; Harper *et al.*, 1983). The uniformity of the oocyte-type of 5S rDNA sequences on non-homologous chromosomes might be maintained by crossing-over between the chromosomes, because these 5S rDNA form a single cluster during meiosis. However, three types of 5S rDNA were included in three separated clusters, respectively, in *Drosophira virilis* (Kress *et al.*, 2001). Interestingly, FISH analyses of two salmonid fishes, *Salmo salar* and *Coregonus atredi*, having two types of 5S rDNA, demonstrated that these 5S rDNA localized to a single locus (Pendas *et al.*, 1994; Sajdak *et al.*, 1998). In the previous karyological study of *Andrias* and *Cryptobranchus*, several distinctive differences were observed between these two genera, whereas a significant difference was absent between two *Andrias* species (Morescalchi *et al.*, 1977). The present study demonstrated that the 5S rDNA of *A. japonicus* has been localized to the subtelomeric region of a medium-sized acrocentric chromosome pair (Fig. 5), and this result supports the previous observation in *A. davidianus* (Sessions *et al.*, 1982). In this study, we cannot exclude the possibility that the minor cluster might be located at a different chromosomal locus in *A. japonicus*. How-

ever, we detected a chimeric sequence (Aj3-213) between the two types A and C in *A. japonicus*; the gene and downstream sequence of the gene is typical of type C, but the upstream sequence of the gene is similar to type A. Additionally, the gene and downstream sequence of the gene of Aj2-212 and Aj3-217 clearly belong to type C and type A, respectively, while their upstream sequence is identical and unique that is quite similar to that of type C in *A. davidianus* (Fig. 3). These observations might mean that the differentiation of the types is not completed in the giant salamander genome, implying that the locus of each type of 5S rDNA sequences exists adjacently.

In the present study, we demonstrated that the features of 5S rDNA from giant salamanders, such as the variation of types and the large copy numbers, are similar to those of other urodela species. Since several types of 5S rDNA were found in this study, this multigene family may contribute to classification of the species, in addition to phenotypic discrimination. Further detailed molecular and cytogenetic studies in other urodela species are needed to understand the molecular evolution and differentiation of the 5S rDNA sequence in amphibians.

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