Research Article

Nucleotide Sequence and Chromosome mapping of 5S Ribosomal DNA from the Dojo Loach, *Misgurnus anguillicaudatus*

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Abstract

There are 2 genetically divergent groups in the dojo loach *Misgurnus anguillicaudatus*: A and B. Although most wild-type diploids reproduce sexually, clonal diploids (clonal loach) reproduce gynogenetically in certain areas. Clonal loaches produce unreduced isogenic eggs by premeiotic endomitosis, and such diploid eggs develop gynogenetically following activation by the sperm of sympatric wild-type diploids. These clonal loaches have presumably arisen from past hybridization events between 2 different ancestors. The genomic differences between these 2 groups have not been completely elucidated. Thus, new genetic and cytogenetic markers are required to distinguish between these 2 groups. Here, we compared the 5S rDNA region to develop markers for the identification of different dojo loach groups. The nontranscribed sequence (NTS) of the 5S rDNA was highly polymorphic and group-specific. NTS sequences were found in clades of 2 different groups in clonal loaches. In contrast, we did not find any group-specific sequences in the coding region of the 5S rRNA gene. Sequences were located near the centromere of the short arm of the largest submetacentric chromosomes in groups A and B and clonal loaches. Thus, the 5S rDNA of the dojo loach is conserved at the chromosomal location. Whereas, the sequences of the NTS regions evolved group-specifically in the dojo loach, with the sequences of both groups being conserved in clonal loaches.

Introduction

Vertebrates reproduce sexually. However, some exceptional animals can reproduce asexually via parthenogenesis, gynogenesis, and hybridogenesis [Lamatsch and Stock, 2009; Kearney et al., 2009]. In gynogenesis, females spawn unreduced eggs with isogenic genotypes, which develop to embryos following triggering by the sperm of a sympatric wildtype species. Thus, the resultant progeny are all female isogenic clones without any genetic contribution from sperm. Molecular genetic studies on clonal animals have revealed that they often exhibit polyploidy and have a hybrid origin between 2 ancestral species [Lamatsch and Stock, 2009; Kearney et al., 2009].

The dojo loach *Misgurnus anguillicaudatus* (Teleostei: Cobitidae) is a freshwater fish found in ponds and rice paddies throughout Japan, Korea, China, Vietnam, and other parts of Asia [Arai and Fujimoto, 2013]. Most dojo loaches are sexually reproducing diploids, but clonal lineages exist in certain areas of Japan [Morishima et al., 2002]. Clonal diploids produce unreduced eggs by premeiotic chromosome doubling [Itono et al., 2006; Kuroda et al., 2018; Kuroda et al., 2021], and such isogenic eggs develop gynogenetically [Itono et al., 2007].

Although dojo loach *M. anguillicaudatus* has been considered a single species, analyses of the mitochondrial DNA control region strongly suggested the presence of 2 genetically diverse A and B groups, with the B group being further divided into B1 and B2 subgroups [Morishima et al., 2008]. Similar results were obtained based on the *cytochrome b* region of mitochondrial DNA [Koizumi et al., 2009]. The presence of 2 distinct A and B groups was not only shown through differences in the sequences of nuclear gene *recombination activating gene 1* (*RAG1*) and *interphotoreceptor retinoid-binding protein 2* (*IRBP2*) [Yamada et al., 2015], but also by the presence of several repetitive sequences [Fujimoto et al., 2017]. As clonal loaches carry 2 different sequences derived from each group in the *RAG1* and *IRBP2* genes, they might have arisen from past hybridization events [Yamada et al., 2015]. In addition, two distinct groups have currently established reproductive isolation [Okada et al., 2017]. The hybrid origin of clones was also cytogenetically supported by the presence of fluorescence in situ hybridization (FISH) signals detected by group A- and B-specific probes [Kuroda et al., 2018; Kuroda et al., 2021].

The sequence and chromosomal location of rDNA regions have been used to characterize species and populations and to clarify phylogenetic relationships and genome structures [Messias et al., 2003; Jansen et al., 2006]. In eukaryotes, there are 2 clusters of rDNA, major rDNA and minor rDNA, each consisting of hundreds to thousands of gene copies [Torres-Machorro et al., 2009]. The minor rDNA, 5S rDNA, comprises a 120 bp gene region flanked by nontranscribed regions (NTS) that form repeating units and tandem repeats. The 5S rRNA gene region is highly conserved among

species and groups, whereas the NTS region is frequently mutated by deletions, insertions, internal subrepeats, and base substitutions [Wasko et al., 2001]. Variations found in the sequence length and nucleotides of the NTS region were used for phylogenetic studies among *Cobitis* and *Sabanejewia* species [Kirtiklis et al., 2011], while the polymorphic 5S rDNA region was used for species identification. In *Misgurnus* loaches, including the dojo loach, the 5S rDNA region has not been investigated; thus, sequences and chromosomal locations are currently unknown. Here, we performed genetic and cytogenetic studies in *Misgurnus* loaches, especially group and clone identification in the dojo loach, and analyzed sequences of the 5S rDNA region, followed by FISH to detect its location on chromosomes.

Materials and Methods

Fish samples

Individuals from each group of dojo loach (*M. anguillicaudatus*) were collected from 3 locations. Both, a A group and clonal loach were collected from Abashiri City, Hokkaido, Japan; a B1 group loach was collected from Nanae Town, Hokkaido, Japan; and a B2 group loach was collected from Fuji City, Shizuoka Prefecture, Japan. In all individuals, species and group identification were carried out using *RAG1* genotyping and ManDra characteristics [Fujimoto et al., 2017].

Sequence analysis of 5S rDNA

Genomic DNA was extracted from the fin clips using a standard phenol/chloroform protocol [Asahida et al., 1996]. The primer pair, 5S-1 (5′ -TAC GCC CGA TCT CGT CCG ATC-3′) and 5S-2 (5′ -CAG GCT GGT ATG GCC GTA AGC-3′) was used to amplify the 5S rDNA fragment [Komiya and Takemura, 1979]. The PCR reaction was performed using 100 ng DNA, 1.2 µL distilled water, 5.0 µL 2× PCR buffer of KOD FX Neo (TOYOBO, Osaka, Japan), 2.0 µL dNTPs (2 mM), 0.2 µL KOD FX Neo DNA polymerase (TOYOBO), and 0.3 µL each of the 5S rDNA primer set (10 µM). PCR cycling conditions were: 2 min at 94 °C; 30 cycles of denaturation at 98 °C for 10 s, annealing at 63 °C for 5 s, and extension at 68 °C for 15 s; and final extension at 68 °C for 7 min. PCR products were electrophoresed on 1.5 % agarose gel at 100 V for 30 min and then stained with ethidium bromide. Subsequently, they were cloned into a plasmid vector by transformation using the Zero Blunt TOPO PCR cloning kit for sequencing (Thermo Fisher Scientific, Waltham, MA, USA) and One Shot TOP10 chemically competent E. coli (Thermo Fisher Scientific) according to the manufacturer's instructions. PCR products from 4 individuals from each group were used for cloning. The inserted fragment size was then checked, and colonies with 500 bp and 760 bp inserts were sequenced. Briefly, 5–8 colonies from each individual with a 500 bp insert and 2–5 colonies from each individual with a 760 bp insert were used for sequence analysis. The obtained sequences were separated into the 5S rRNA gene region and NTS region using BioEdit (version 7.0.5.3). The frequency of polymorphisms in each sequence was then compared among groups, and a phylogenetic tree was created using the CLC genomics workbench (version 9.5.3, CLC bio, Aarhus, Danmark).

Chromosome Preparation

Mitotic chromosomes were prepared from embryos at the optic vesicle stage. Embryos of groups A and B1 were produced by artificial fertilization. Embryos of natural clonal loach were produced by fertilizing their unreduced diploid eggs with UV-irradiated goldfish sperm to ensure gynogenetic development of all eggs by preventing accidental incorporation of sperm nuclei and generation of triploids. Embryonic bodies were incubated in 0.0025 % colchicine (FUJI FILM Wako Pure Chemical Corporation, Osaka, Japan) dissolved in physiological saline (7.5 g/L NaCl, 0.2 g/L KCl, 0.264 g/L $CaCl_2 \cdot 2H_2O$) for 45 min at 20 °C. Embryonic bodies were then placed in a hypotonic solution (0.075 M KCl) for 30 min and fixed with Carnoy's solution (methanol: acetic acid = 3:1). Fixed embryos were stored at -30 °C until the preparation of metaphase spreads. Cell suspensions from embryonic bodies were dropped onto glass slides and air-dried. Slides were incubated at 65 °C for 24 h for hardening.

Two-Color FISH

Plasmids containing the 5S rDNA region of groups A (accession: LC600007.1) and B1 (accession: LC600052.1) were used as 5S rDNA-A and 5S rDNA-B probes, respectively (Fig. S1). The 5S rDNA-A probe was labeled with digoxigenin-11-dUTP using the Dig-Nick translation mix (Roche, Basel, Switzerland). The 5S rDNA-B probe was labeled with biotin-16-dUTP using the Biotin-Nick translation mix (Roche). Two-color FISH was performed according to the method described by Kuroda et al. [2018]. Digoxigenin-labeled 5S rDNA-A and biotin-labeled 5S rDNA-B probes were detected with anti-digoxigenin-rhodamine, Fab fragments (Roche), and streptavidin and Alexa Fluor 488 conjugate (Thermo Fisher Scientific), respectively. Signals were amplified using a biotinylated anti-avidin antibody (Vector Laboratories, Newark, CA, USA). Slides were counterstained with ProLong Gold Antifade Mountant with DAPI (Thermo Fisher Scientific). Metaphases were observed using a fluorescence microscope (DM5500B; Leica, Wetzlar, Germany), while images were captured using a DFC 365FX camera (Leica). Image processing was performed using PhotoShop Elements15 (Adobe, San Jose, CA, USA).

Results

Analysis of 5S rDNA fragments

We observed that the electropherograms of the fragments amplified using the 5S rDNA primers were very similar among all groups (Fig. 1a). In particular, the electropherograms appeared at approximately 240, 500, 760, and 1020 bp in a ladder-like band pattern. We found that in all groups, the 500 bp fragment contained a single 5S rRNA gene and 2 NTS regions between the end and the beginning of the 5S rRNA gene, while the 760 bp fragment contained 2 5S rRNA genes and 3 NTS regions between the end and the beginning of the 5S rRNA gene (Fig. 1b).

Sequence of 5S rRNA gene region

We obtained the sequences of the 5S rRNA gene from 4 individuals in each group: 49, 48, 51, and 49 sequences in A, B1, B2, and clonal loaches, respectively (Table S1). We specifically detected

that among these, certain sequences were frequently observed as major sequences in each group, whereas variations in sequences were also observed with low frequency as minor sequences (Table 1). 34 haplotypes were categorized as belonging to the A group, 28 haplotypes to the B1 group, 37 haplotypes to the B2 group, and 42 haplotypes as clonal loaches. Of these, the type 1 designation was given to the major haplotype identified with the highest frequency, and the type 2 designation was given to the second most frequently identified haplotype (Table 1). The Type 1 haplotype was commonly observed in all groups, whereas the type 2 haplotype was mainly observed in the B1 and B2 groups (Table 1). Interestingly, types 1 and 2, which were the major types in dojo loach, showed substitutions at 2 bases out of 120 bp (Fig.2). When we aligned the sequences of type 1 and 2 with those of *Misgurnus fossilis* and *Cyprinus carpio*, we detected that type 1 and *C. carpio* showed only 1 nucleotide substitution, whereas type 1 and *M. fossilis* showed 7 nucleotide substitutions. In addition, type 2 showed less variation in *C. carpio* than *M. fossilis*. Thus, we concluded that the sequence of the 5S rRNA gene region of loach was more homologous to that of *C. carpio* than *M. fossilis*. We also found that the number of bases in the obtained sequences was approximately 120 bp in all groups (Table S1). Furthermore, when we generated a phylogenetic tree using UPGMA with all obtained haplotypes, we noticed that not each group formed a clade (Fig. 3a).

Sequence of NTS region

In contrast, we obtained 90 sequences in the NTS region in A group, 88 in B1 group, 91 in B2 group, and 87 in clonal loach (Table S2). In the A group, we identified 86 haplotypes. Approximately 89 %, 10 %, and 1 % of haplotypes in the A group had NTS regions of roughly 128 bp (124 to 134 bp), 140 bp (135 to 146 bp), or other sizes (below 123 bp and above 147 bp), respectively (Table 2). We identified 52 haplotypes in the B1 group. Approximately 31 % and 69 % of haplotypes in the B group had NTS regions of roughly 128 bp (124 to 134 bp) and 140 bp (135 to 146 bp), respectively (Table 2). In the B2 group, we identified 76 haplotypes. Approximately 25 %, 69 %, and 6 % of haplotypes in the B2 group had NTS regions of roughly 128 bp (124 to 134 bp), 140 bp (135 to 146 bp), or other sizes (below 123 bp and above 147 bp), respectively (Table 2). Finally, we identified 71 haplotypes in the clonal loach group. Approximately 45 %, 45 %, and 10 % of haplotypes in the clonal loach group had NTS regions of roughly 128 bp (124 to 134 bp), 140 bp (135 to 146 bp), or other sizes (123 bp below and 147 bp above), respectively (Table 2). Of the obtained haplotypes, the major haplotype found at the highest frequency was designated as type N1, and this sequence was identified in the B1, B2, and clonal loach groups (Table 1). When we created a phylogenetic tree using the UPGMA with all haplotypes, 2 clades were formed: 1 clade mostly included haplotypes of group A, whereas the other

clade mostly included haplotypes of B1 and B2 groups. Furthermore, haplotypes detected in the clonal loach were observed in both clades (Fig.3b).

5S rDNA localization

Based on the results of sequence analysis, we prepared probes from plasmids containing different sequences in the NTS region of the A and B1 groups as 5S rDNA-A and 5S rDNA-B probes, respectively (Fig. S1). We found that samples from groups A, B1, and clonal loach contained 50 chromosomes that were classified as 10 metacentric (M), 4 submetacentric (SM), and 36 telocentric (T) chromosomes (Fig. 4). We also detected 2 signals of the 5S rDNA-A and 5S rDNA-B probes near the centromere of the short arm of a pair of the largest SM chromosomes (Fig. 4). The 5S rDNA-A and 5S rDNA-B probe signals were detected at the same position and their strength was similar.

Discussion/Conclusion

Interspecific differences in 5S rDNA tandem repeats are useful genetic markers for identifying populations, species, and their hybrids in fish [Martins and Wasko, 2004]. For instance, PCR amplification of the 5S rDNA region revealed the presence of different fragments among Cobitid species [Kirtiklis et al., 2011]. In *Diplodus sargus* (family: Sparidae), a multiband pattern that differed among individuals was observed in amplified PCR products of the 5S rDNA region, indicating the presence of polymorphism, even within species [Merlo et al., 2013]. However, only a single band with the same fragment size was identified in *Pagrus pagrus* and *P. auriga* of the same Sparidae family; thus, this approach was not useful for identifying hybrids between these 2 species [Merlo et al., 2013]. Many of these 5S rDNA regions, which are identifiable by the fragment size of PCR products, contain highly polymorphic NTS regions [Martins and Wasko, 2004]. The fragment size of PCR products amplified from the 5S rDNA region in the 4 dojo loach groups used in the present study, were almost the same. Therefore, it was not possible to identify groups of dojo loach using electrophoretic images of PCR products of the 5S rDNA region.

The 5S rRNA gene region is highly homologous in closely related species. In fact, in fish species, the average similarity of the 5S rRNA gene region has been reported to be 95 % [Martins and Wasko, 2004]. The 5S rRNA gene is transcribed by RNA polymerase 3, which synthesizes several other types of small RNAs [Nederby-Nielsen et al., 1993]. In addition, 5S rRNA gene sequences contain 3 internal control regions (ICRs) that are active as promoters for transcription: A box, intermediate element (IE), and C box [Martins and Wasko, 2004]. The sequence of ICRs was completely identical in *P. pagrus* and *P. auriga* and was highly conserved among 5S rRNA gene regions [Merlo et al., 2013].

Likewise, ICRs were completely identical in major haplotypes (type 1 and type 2) of the 5S rRNA gene region of dojo loaches and highly conserved compared with other regions. In contrast, the comparison of ICRs of dojo loach with those of *M. fossilis* and *C. carpio* suggested potential differences in the structure of the translated 5S rRNA among different species. In addition, both major and minor haplotypes of the 5S rRNA gene in the dojo loach that were different in sequence due to nucleotide substitutions were identified in all groups. Thus, it was suggested that sequences in the 5S rRNA gene region of the dojo loach were mutated due to base substitutions.

Generally speaking, the sequence of the NTS region of 5S rDNA was more frequently polymorphic than the 5S RNA gene region in teleost [Martins and Wasko, 2004]. There are 2 types of NTS region sequences with different base numbers in several fish species [Atlantic salmon *Salmo salar*: Pendas et al., 1994, rainbow trout *Oncorhynchus mykiss*: Moran et al., 1996, tilapiine cichlid fish *Oreochromis niloticus*: Martins et al., 2000, the genus *Brycon*: Wasko et al., 2001, and the genus *Rhizoprionodon*: Pinhal et al., 2009]. In dojo loach, length polymorphisms were observed in the NTS region. These polymorphisms can be divided into 2 sizes, approximately 128 and 140 bp. However, the difference in length between the 2 types of polymorphisms in dojo loach was 12 bp, and there was no remarkable difference in the number of bases between the types when compared with the 270 bp polymorphism in Atlantic salmon *S. salar* [Pendas et al., 1994], 80 bp in rainbow trout *O.mykiss* [Moran et al., 1996] and–250-550 bp in the genus *Brycon* [Wasko et al., 2001]. However, the high frequency of sequences around 128 bp in group A and 140 bp in group B revealed differences in the frequency type among groups. Multigene families containing the 5S rDNA region evolve according to a homogenization process regulated by molecular drivers and concerted evolution [Dover, 1989]. The cytological mechanisms that lead to such concerted evolution are unequal crossing-over, gene conversion, and replication slippage, but the rate at which mutant repeat types homogenize depends on other aspects, such as the number of repeats in the sequence, the strength of natural selection, and effective population size [Schlötterer and Tautz, 1994]. Thus, sequence homogenization through concerted evolution is expected to be underway within the groups of dojo loach. In addition, approximately half of the sequences around 128 bp and 140 bp of the NTS regions, which were classified into both the A and B clades in the phylogenetic tree, were detected in clonal loaches. Thus, as in previous studies [Yamada et al., 2015], clonal loaches were shown to possess both sequences derived from groups A and B.

In fish, 5S rDNA, located on a single pair of chromosomes, is considered to be of the ancestral type [Martins and Wasko, 2004]. In the dojo loach used in this study, we detected 2 signals on a single homologous chromosome pair in all individuals; thus we considered that 5S rDNA was of the

ancestral type. However, some Cypriniformes species have demonstrated multiple locations of 5S rDNA, for example, *M. fossilis* [Spóz et al., 2017], *Crassius carassius* [Spóz et al., 2014], and *C. taenia* [Boroń et al., 2006]. Of these, *M. fossilis* and *C. carassius* have 2n = 100 chromosomes, which is twice the number of chromosomes compared with dojo loach (2n = 50 chromosomes). Therefore, the multiple signals of 5S rDNA in *M. fossilis* and *C. carassius* might be derived from polyploidization by genome duplication. In contrast, *C. taenia* has 2n = 48 chromosomes, differing from the dojo loach chromosome set by only 1 pair of chromosomes. However, *C. taenia* have an arm number of 76 [Vasilev et al., 1989], which is more than that of the dojo loach (64) [Kim et al., 1995]. In addition, the formation of triploids and tetraploids has been observed in *C. taenia* through hybridization with closely related species, suggesting the occurrence of chromosomal rearrangements [Boroń et al., 2006]. In the dojo loach, the chromosomal position of the 5S rDNA region in groups A and B was the same, indicating that the chromosomal position was conserved within species. Furthermore, although the clonal loach originated from hybridization between groups A and B, there was no change in the chromosomal position of the 5S rDNA region, suggesting no chromosomal rearrangement due to hybridization.

In conclusion, this study revealed the 5S rDNA sequence and its location on the chromosomes of genetically divergent groups of loaches. The sequence of the NTS region of 5S rDNA showed specific features in groups A and B, which were also maintained in clonal loaches. In addition, the chromosomal location of 5S rDNA was conserved near the centromere of the short arm of a pair of the largest SM chromosomes in all groups.

Statements

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Statement of Ethics

This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals of the Hokkaido University. All animal experiments were approved by the Animal Study Ethical Committee of Hokkaido University (approval number 29-3).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Kiko Shibata., Takafumi Fujimoto., Etsuro Yamaha., and Katsutoshi Arai. conceived and designed the study. Kiko Shibata conducted the experiments. Masamichi Kuroda contributed to the development of new analysis tools. Kiko Shibata analyzed data. Kiko Shibata and Takafumi Fujimoto wrote the manuscript. All authors have read and approved the manuscript.

Data Availability Statement

Sequence data for the loach 5S rDNA analyzed in this study are available at NCBI (GenBank accession number LC599981-LC600139). Additional data generated or analyzed during this study are included in this article and its supplementary material files. Further inquiries can be directed to the corresponding authors.

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Figure Legends

Fig. 1. (a) Electrophoregrams of PCR products of the 5S rDNA region in A, B1, and B2 groups, and clonal loaches. In each group, 1–4 indicate different individuals. Bands showed a ladder-like pattern, appearing at approximately 240, 500, 760, and 1020 bp. The 500 and 760 bp bands shown in red rectangles were used for cloning and sequence analysis. (b) Schematic diagram of the sequences obtained from the 500 and 760 bp bands. The 500 bp fragment contained 1 5S rRNA gene region and 2 NTS regions between the end and the beginning of the 5S rRNA gene, whereas the 760 bp fragment contained 2 5S rRNA gene regions and 3 NTS regions between the end and the beginning of the 5S rDNA gene.

Fig. 2. Aligned sequences of 5S rRNA genes from *Misgurnus anguillicaudatus*, *Misgurnus fossilis*, and *Cyprinus carpio*. The start point of transcription is indicated by +1, nucleotide substitutions are indicated in gray shading, and the internal control region (A box, IE, and C box) is indicated in black shading. In *M. anguillicaudatus*, type1 was detected as a major sequence in all groups; typeA_1, 5S rRNA gene typeB1_1, 5S rRNA gene typeB2_1, and 5S rRNA gene typeC_1, whereas type2 was detected as a major sequence in B1 and B2 groups; 5S rRNA gene typeB1_2 and 5S rRNA gene typeB2_2.

Fig. 3. UPGMA phylogenetic tree based on nucleotide sequence divergence of the 5S rRNA gene region (a) and nontranscribed sequence (NTS) region (b). In the 5S rRNA gene region, no clades were detected to cluster with group A (red), B1 (blue), B2 (light blue), and clonal loaches (green). In contrast, in the NTS region, separate clades (clade A and B1-B2) were identified, consisting of group A (red) and B1 and B2 (blue and light blue), respectively. The NTS region of clonal loaches (green) belongs to both A and B1-B2 clades.

Fig. 4. Two-color FISH using 5S rDNA-A and 5S rDNA-B probes in the somatic cells of groups A (a), B (b), and clonal loaches (c). The 5S rDNA-B probe was labeled with biotin-16-dUTP and detected using a streptavidin AlexaFluor 488 conjugate (green). The 5S rDNA-A probe was labeled with digoxigenin-11-dUTP and detected using anti-digoxigenin-rhodamine, Fab fragments (red). All chromosomes were counterstained with DAPI (blue). Scale bars indicate 10 μm.

Table 1. Frequency of polymorphisms in 5S rRNA gene and NTS regions

	5S rRNA gene				NTS region		
	Sequence name	Frequency (%)	Number of types		Sequence name	Frequency (%)	Number of types
A group	type_1	22.4	1	A group		2.2	4
		6.1	1			1.1	82
		4.1	3				
		2.0	29	B1 group	type_N1	17.0	1
						9.1	1
B1 group	type_1	14.6	1			8.0	1
	type_2	12.5	1			4.5	1
		10.4	1			2.3	6
		6.3	1			1.1	42
		4.2	3				
		2.1	21	B2 group	type_N1	7.7	1
						5.5	1
B2 group	type_1	13.7	1			4.4	1
	type_2	11.8	1			2.2	2
		5.9	1			1.1	71
		3.9	1				
		2.0	33	Clonal loach	type_N1	6.9	1
						3.4	2
Clonal loach	type_1	6.1	1			2.3	7
		4.1	5			1.1	61
		2.0	36				

Table 2. Frequency by number of bases in 5S rRNA gene and NTS region

	Frequency (%)		
	124-134 bp	135-146 bp	other size
A group	89	10	1
B1 group	31	69	0
B2 group	25	69	5
Clonal loach	45	45	10

supplemental table

Table S1. The size of the 5S rRNA gene region and number of duplications in each 5S rRNA gene region

A group			B1 group			B2 group			Clonal loach		
Name	Size (bp)	N	Name	Size (bp)	N	Name	Size (bp)	N	Name	Size (bp)	N
TypeA_1	120	11	TypeB1_1	120	7	TypeB2_1	120	7	TypeC_1	120	3
TypeA_2	120	3	TypeB1_2	120	6	TypeB2_2	120	6	minorC_1	120	2
minorA_1	120	2	TypeB1_3	120	5	TypeB2_3	120	3	minorC_2	120	2
minorA_2	120	2	TypeB1_4	120	3	minorB2_1	120	2	minorC_3	120	2
minorA_3	120	2	minorB1_1	120	2	minorB2_2	89	1	minorC_4	120	2
minorA_4	119	1	minorB1_2	120	2	minorB2_3	117	1	minorC_5	120	2
minorA_5	119	1	minorB1_3	120	2	minorB2_4	119	1	minorC_6	119	1
minorA_6	120	1	minorB1_4	55	1	minorB2_5	120	1	minorC_7	120	1
minorA_7	120	1	minorB1_5	120	1	minorB2_6	120	1	minorC_8	120	1
minorA_8	120	1	minorB1_6	120	1	minorB2_7	120	1	minorC_9	120	1
minorA_9	120	1	minorB1_7	120	1	minorB2_8	120	1	minorC_10	120	1
minorA_10	120	1	minorB1_8	120	1	minorB2_9	120	1	minorC_11	120	1
minorA_11	120	1	minorB1_9	120	1	minorB2_10	120	1	minorC_12	120	1
minorA_12	120	1	minorB1_10	120	1	minorB2_11	120	1	minorC_13	120	1
minorA_13	120	1	minorB1_11	120	1	minorB2_12	120	1	minorC_14	120	1
minorA_14	120	1	minorB1_12	120	1	minorB2_13	120	1	minorC_15	120	1
minorA_15	120	1	minorB1_13	120	1	minorB2_14	120	1	minorC_16	120	1
minorA_16	120	1	minorB1_14	120	1	minorB2_15	120	1	minorC_17	120	1
minorA_17	120	1	minorB1_15	120	1	minorB2_16	120	1	minorC_18	120	1
minorA_18	120	1	minorB1_16	120	1	minorB2_17	120	1	minorC_19	120	1
minorA_19	120	1	minorB1_17	120	1	minorB2_18	120	1	minorC_20	120	1
minorA_20	120	1	minorB1_18	120	1	minorB2_19	120	1	minorC_21	120	1
minorA_21	120	1	minorB1_19	120	1	minorB2_20	120	1	minorC_22	120	1
minorA_22	120	1	minorB1_20	120	1	minorB2_21	120	1	minorC_23	120	1
minorA_23	120	1	minorB1_21	120	1	minorB2_22	120	1	minorC_24	120	1

minorA_24	120	1	minorB1_22	120	1	minorB2_23	120	1	minorC_25	120	1
minorA_25	120	1	minorB1_23	120	1	minorB2_24	120	1	minorC_26	120	1
minorA_26	120	1	minorB1_24	121	1	minorB2_25	120	1	minorC_27	120	1
minorA_27	120	1	total		4	minorB2_26	120	1	minorC_28	120	1
					8						
minorA_28	120	1				minorB2_27	120	1	minorC_29	120	1
minorA_29	120	1				minorB2_28	120	1	minorC_30	120	1
minorA_30	120	1				minorB2_29	120	1	minorC_31	120	1
minorA_31	120	1				minorB2_30	120	1	minorC_32	120	1
minorA_32	120	1				minorB2_31	120	1	minorC_33	120	1
total		49				minorB2_32	120	1	minorC_34	120	1
						minorB2_33	120	1	minorC_35	120	1
						minorB2_34	120	1	minorC_36	120	1
						total		5	minorC_37	120	1
								1			
									minorC_38	120	1
									minorC_39	120	1
									minorC_40	120	1
									minorC_41	120	1
									total		49

supplemental table

Table S2. The size of the nontranscribed sequence and number of duplications in each NTS

A group			B1 group			B2 group			Clonal loach		
Name	Size (bp)	N	Name	Size (bp)	N	Name	Size (bp)	N	Name	Size (bp)	N
minorA_N1	124	2	TypeB1_N1	140	15	TypeB2_N1	140	7	TypeC_N1	140	6
minorA_N2	124	2	TypeB1_N2	128	8	TypeB2_N2	140	5	minorC_N1	121	3
minorA_N3	125	2	TypeB1_N3	140	7	minorB2_N1	140	4	minorC_N2	140	3
minorA_N4	129	2	minorB1_N1	128	4	minorB2_N2	128	2	minorC_N3	120	2
minorA_N5	124	1	minorB1_N2	128	2	minorB2_N3	140	2	minorC_N4	128	2
minorA_N6	124	1	minorB1_N3	128	2	minorB2_N4	80	1	minorC_N5	133	2
minorA_N7	124	1	minorB1_N4	139	2	minorB2_N5	119	1	minorC_N6	140	2
minorA_N8	124	1	minorB1_N5	140	2	minorB2_N6	119	1	minorC_N7	140	2
minorA_N9	124	1	minorB1_N6	140	2	minorB2_N7	120	1	minorC_N8	141	2
minorA_N10	124	1	minorB1_N7	140	2	minorB2_N8	123	1	minorC_N9	152	2
minorA_N11	124	1	minorB1_N8	127	1	minorB2_N9	124	1	minorC_N10	117	1
minorA_N12	124	1	minorB1_N9	128	1	minorB2_N10	127	1	minorC_N11	124	1
minorA_N13	125	1	minorB1_N10	128	1	minorB2_N11	127	1	minorC_N12	127	1
minorA_N14	125	1	minorB1_N11	128	1	minorB2_N12	127	1	minorC_N13	127	1
minorA_N15	126	1	minorB1_N12	128	1	minorB2_N13	128	1	minorC_N14	127	1
minorA_N16	127	1	minorB1_N13	132	1	minorB2_N14	128	1	minorC_N15	127	1
minorA_N17	127	1	minorB1_N14	132	1	minorB2_N15	128	1	minorC_N16	128	1
minorA_N18	127	1	minorB1_N15	132	1	minorB2_N16	128	1	minorC_N17	128	1
minorA_N19	127	1	minorB1_N16	132	1	minorB2_N17	128	1	minorC_N18	128	1
minorA_N20	127	1	minorB1_N17	132	1	minorB2_N18	128	1	minorC_N19	128	1
minorA_N21	127	1	minorB1_N18	132	1	minorB2_N19	128	1	minorC_N20	128	1
minorA_N22	127	1	minorB1_N19	138	1	minorB2_N20	128	1	minorC_N21	129	1
minorA_N23	127	1	minorB1_N20	139	1	minorB2_N21	128	1	minorC_N22	129	1
minorA_N24	128	1	minorB1_N21	139	1	minorB2_N22	128	1	minorC_N23	129	1
minorA_N25	128	1	minorB1_N22	139	1	minorB2_N23	128	1	minorC_N24	129	1
minorA_N26	128	1	minorB1_N23	140	1	minorB2_N24	128	1	minorC_N25	129	1

minorA_N27	128	1	minorB1_N24	140	1	minorB2_N25	128	1	minorC_N26	129	1
minorA_N28	128	1	minorB1_N25	140	1	minorB2_N26	128	1	minorC_N27	129	1
minorA_N29	128	1	minorB1_N26	140	1	minorB2_N27	132	1	minorC_N28	129	1
minorA_N30	128	1	minorB1_N27	140	1	minorB2_N28	133	1	minorC_N29	129	1
minorA_N31	128	1	minorB1_N28	140	1	minorB2_N29	137	1	minorC_N30	130	1
minorA_N32	128	1	minorB1_N29	140	1	minorB2_N30	137	1	minorC_N31	130	1
minorA_N33	128	1	minorB1_N30	140	1	minorB2_N31	138	1	minorC_N32	131	1
minorA_N34	128	1	minorB1_N31	140	1	minorB2_N32	139	1	minorC_N33	131	1
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minorA_N36	128	1	minorB1_N33	140	1	minorB2_N34	139	1	minorC_N35	131	1
minorA_N37	128	1	minorB1_N34	140	1	minorB2_N35	140	1	minorC_N36	132	1
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minorA_N41	128	1	minorB1_N38	140	1	minorB2_N39	140	1	minorC_N40	133	1
minorA_N42	128	1	minorB1_N39	140	1	minorB2_N40	140	1	minorC_N41	133	1
minorA_N43	128	1	minorB1_N40	140	1	minorB2_N41	140	1	minorC_N42	133	1
minorA_N44	128	1	minorB1_N41	140	1	minorB2_N42	140	1	minorC_N43	133	1
minorA_N45	129	1	minorB1_N42	140	1	minorB2_N43	140	1	minorC_N44	133	1
minorA_N46	129	1	minorB1_N43	140	1	minorB2_N44	140	1	minorC_N45	133	1
minorA_N47	129	1	minorB1_N44	140	1	minorB2_N45	140	1	minorC_N46	136	1
minorA_N48	129	1	minorB1_N45	140	1	minorB2_N46	140	1	minorC_N47	136	1
minorA_N49	129	1	minorB1_N46	140	1	minorB2_N47	140	1	minorC_N48	137	1
minorA_N50	129	1	minorB1_N47	141	1	minorB2_N48	140	1	minorC_N49	139	1
minorA_N51	129	1	minorB1_N48	141	1	minorB2_N49	140	1	minorC_N50	139	1
minorA_N52	129	1	minorB1_N49	141	1	minorB2_N50	140	1	minorC_N51	139	1
minorA_N53	129	1	total		88	minorB2_N51	140	1	minorC_N52	139	1
minorA_N54	129	1				minorB2_N52	140	1	minorC_N53	140	1
minorA_N55	129	1				minorB2_N53	140	1	minorC_N54	140	1
minorA_N56	129	1				minorB2_N54	140	1	minorC_N55	140	1
minorA_N57	129	1				minorB2_N55	140	1	minorC_N56	140	1
minorA_N58	129	1				minorB2_N56	140	1	minorC_N57	140	1

minorA_N59	129	1
minorA_N60	129	1
minorA_N61	129	1
minorA_N62	129	1
minorA_N63	129	1
minorA_N64	130	1
minorA_N65	131	1
minorA_N66	131	1
minorA_N67	132	1
minorA_N68	132	1
minorA_N69	132	1
minorA_N70	132	1
minorA_N71	132	1
minorA_N72	132	1
minorA_N73	133	1
minorA_N74	133	1
minorA_N75	133	1
minorA_N76	133	1
minorA_N77	135	1
minorA_N78	136	1
minorA_N79	136	1
minorA_N80	139	1
minorA_N81	140	1
minorA_N82	140	1
minorA_N83	140	1
minorA_N84	143	1
minorA_N85	144	1
minorA_N86	167	1
total		90

minorB2_N57	140	1
minorB2_N58	140	1
minorB2_N59	140	1
minorB2_N60	140	1
minorB2_N61	140	1
minorB2_N62	140	1
minorB2_N63	140	1
minorB2_N64	140	1
minorB2_N65	140	1
minorB2_N66	140	1
minorB2_N67	140	1
minorB2_N68	140	1
minorB2_N69	140	1
minorB2_N70	141	1
minorB2_N71	141	1
minorB2_N72	141	1
minorB2_N73	141	1
minorB2_N74	153	1
total		91

minorC_N58	140	1
minorC_N59	140	1
minorC_N60	140	1
minorC_N61	140	1
minorC_N62	140	1
minorC_N63	140	1
minorC_N64	140	1
minorC_N65	140	1
minorC_N66	140	1
minorC_N67	141	1
minorC_N68	144	1
minorC_N69	144	1
minorC_N70	167	1
total		87

figure1

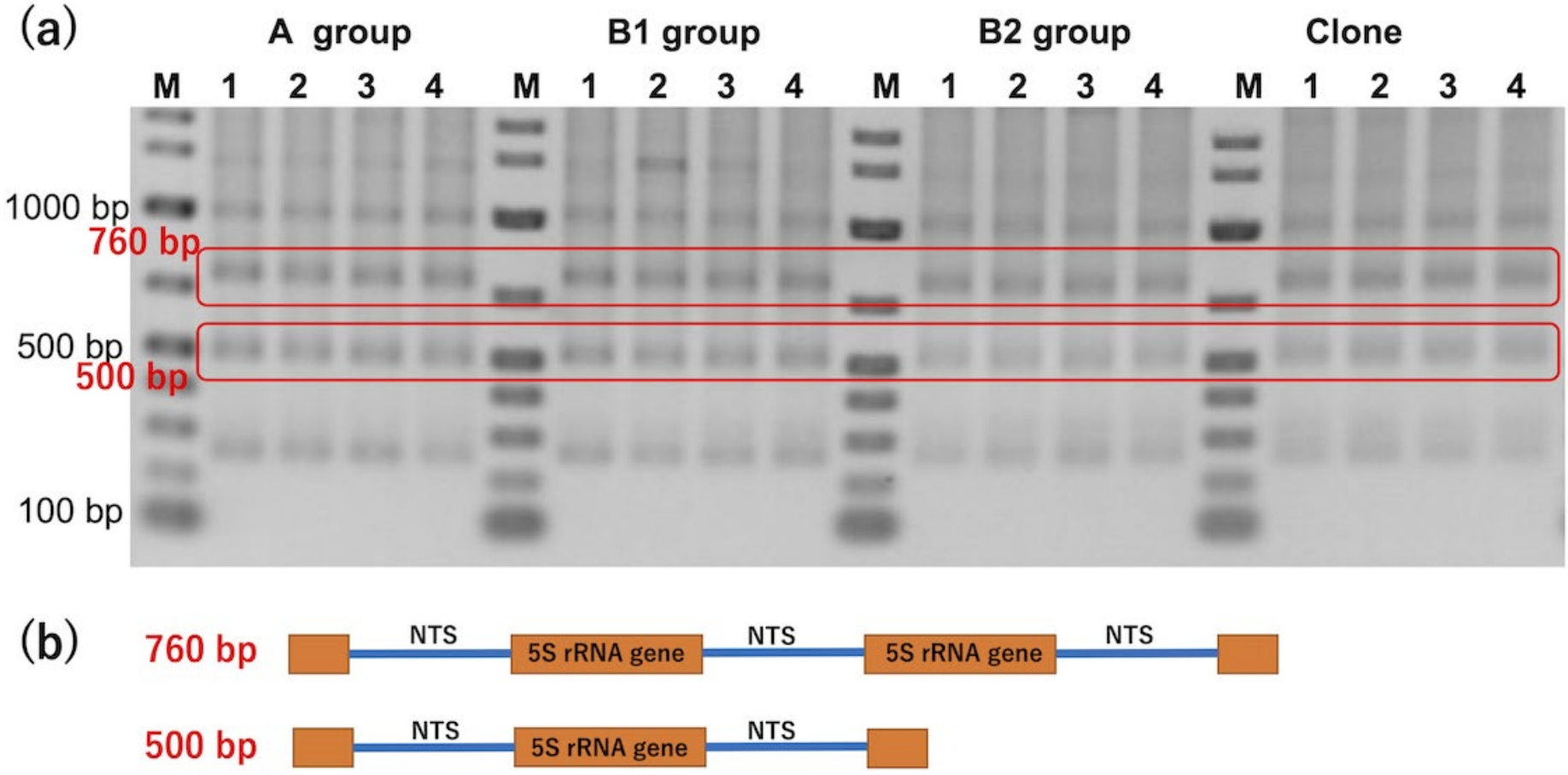

(a)
A group
B1 group
B2 group
Clone
M 1 2 3 4 M 1 2 3 4 M 1 2 3 4 M 1 2 3 4
1000 bp
760 bp
500 bp
500 bp
100 bp
(b)
760 bp
NTS
5S rRNA gene
NTS
5S rRNA gene
NTS
500 bp
NTS
5S rRNA gene
NTS

figure2


+1
A box
+65
M. anguillicaudatus type1 GCTTACGGCC ACACCACCCT GAGCACGCCC GCTCTCGTCT GATCTCGGAA GCCAAGCAGG GTCGG
M. anguillicaudatus type2 GCTTACGGCC ATACCAGCCT GAGCACGCCC GCTCTCGTCT GATCTCGGAA GCCAAGCAGG GTCGG
M. fossilis GCTTACGGCC ACACCAACCT GAGCAAGCCC GATCTCGTCT GATCTCGGAA GCCAAGCAGG GTTGG
Cyprinus carpio GCTTACGGCC ATACCACCCT GAGCACGCCC GATCTCGTCT GATCTCGGAA GCTAAGCAGG GTCGG
IE
C box
+120
M. anguillicaudatus type1 GCCTG GTTAGTACTT GGATGGGAGA CCGCCTGGGA ATACCAGGTG CTGTAAGCAT
M. anguillicaudatus type2 GCCTG GTTAGTACTT GGATGGGAGA CCGCCTGGGA ATACCAGGTG CTGTAAGCAT
M. fossilis GCCTG GTTAGTACTT GGATGGGAGA CTGCCTGGGA ATACCAGGTG TTGTAAGCTT
Cyprinus carpio GCCTG GTTAGTACTT GGATGGGAGA CCGCCTGGGA ATACCAGGTG CTGTAAGCTT

figure3

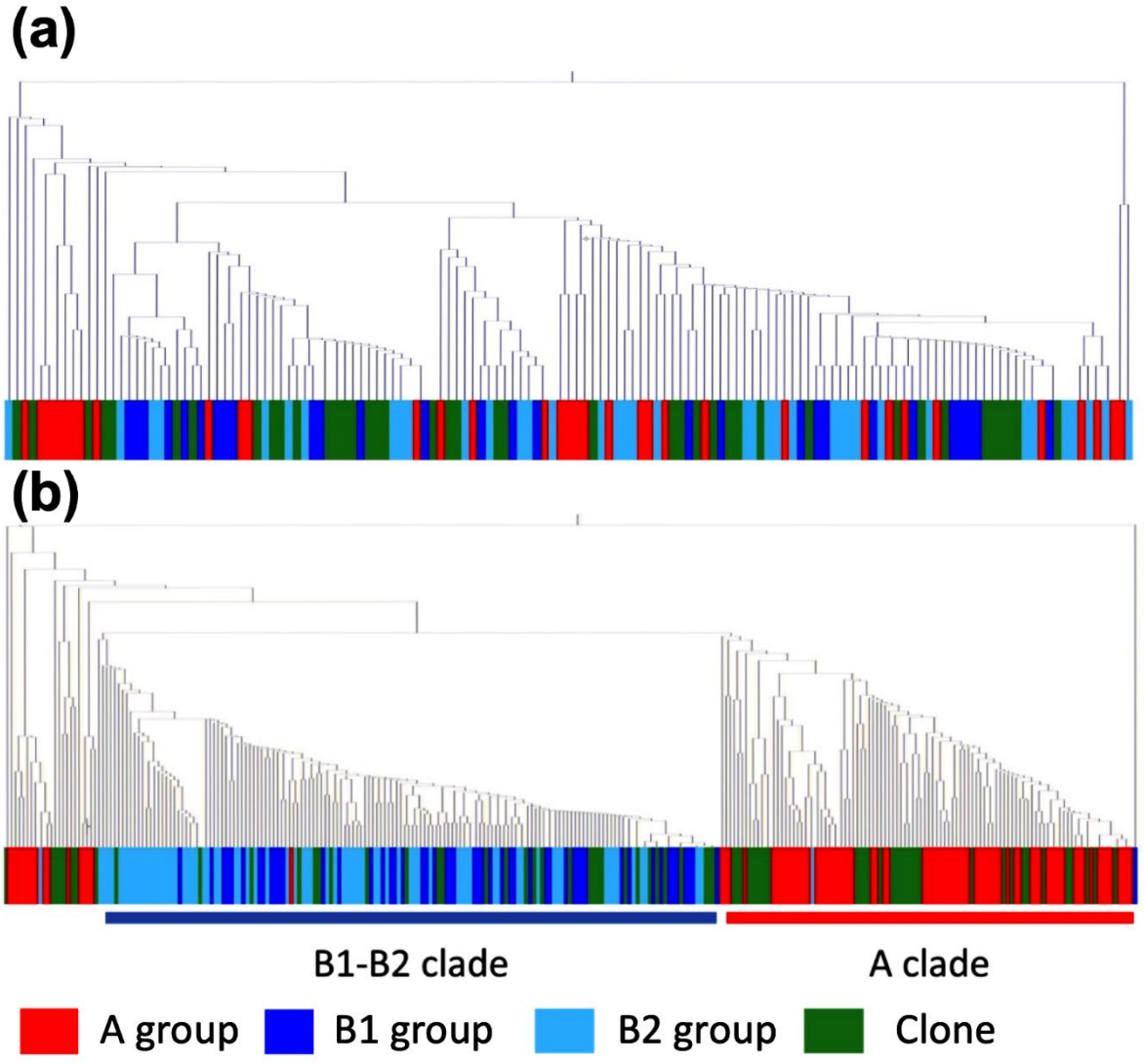
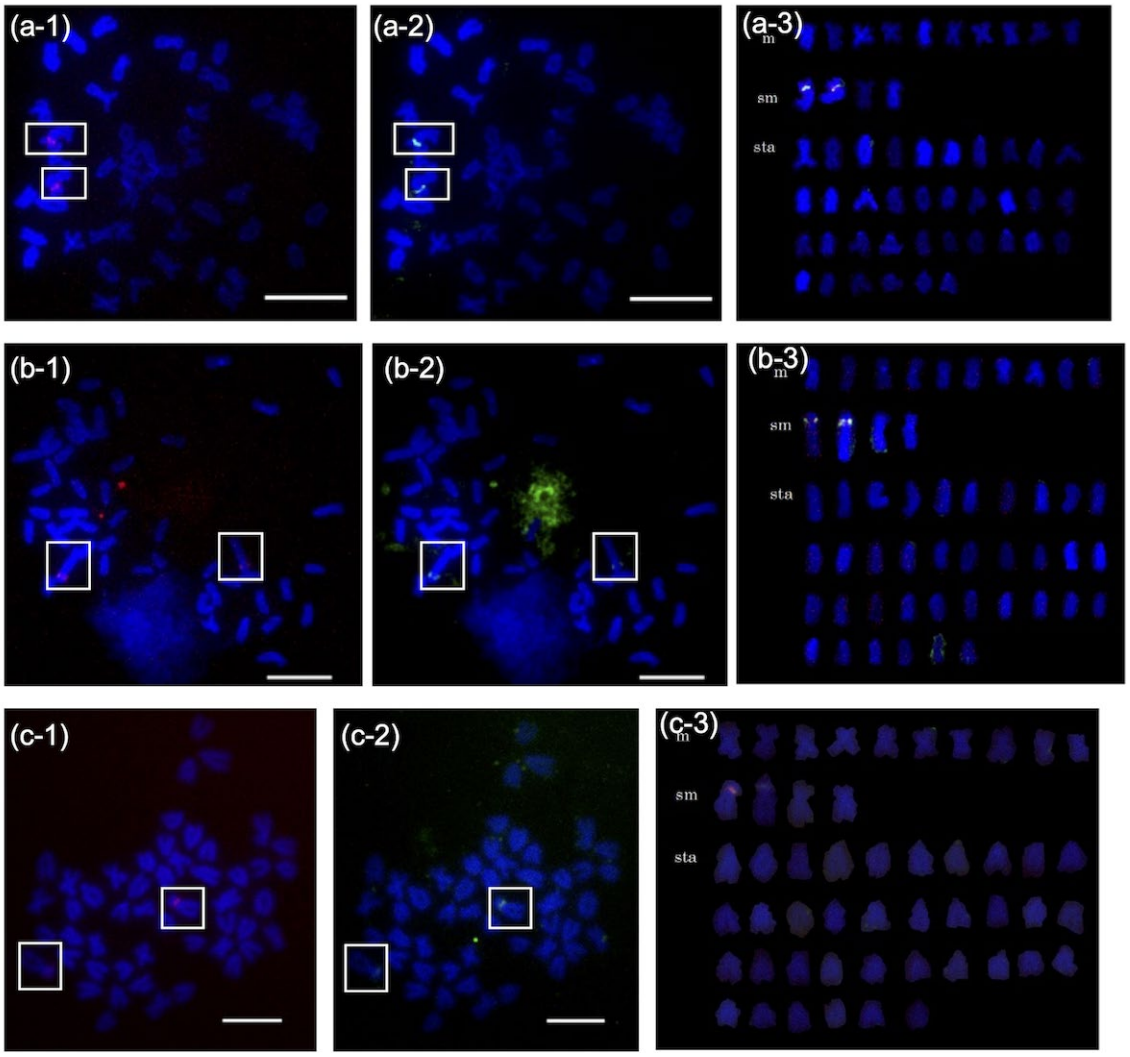


figure4



figureSI

5S rDNA-A probe

```
  1    ataccaggtg ctgtaagcat ttaattattt atttattatt ttttcatgta attttttcc
 61    ataaatgcgt cctttctgta agcgttcgcc cgatggcatg gcgttgccac attagtcctg
121    aagtggctct cgaatcgagt cagcgctcgc ttacggccac accaccctga gcacgcccgc
181    tctcgtctga tctcggaagc caagcagggt cgggcctggt tagtacttgg atgggagacc
241    gcctgggaat accaggtgct gtaagcattt aatttttgtt taattattta ttcatgtaat
301    ttttttccat aaatgcgtcc tttctgtaag cgttcgcgga tggcatggcg ttgccacatt
361    agtcttgaag gggctttcga attgagttaa cgctcgctta cggccatacc agcct
```

5S rDNA-B probe

```
  1    ataccaggtg ctgtaagcat ttaattcttt atttaattaa ttatttaatt atttattcat
 61    ataatttttt tccagaaatg catcctttct gtaagcgttc gccgatggca tggcgttgcc
121    acattagtct tgaagtggct ctcgaatcga gtcagcgctc gcttacggcc acaccaccct
181    gagcacgccc gctctcgtct gatctcggaa gccaagcagg gtcgggcctg gttagtactt
241    ggatgggaga ccgcctggga ataccaggtg ctgtaagcat ttaattcttt atttaattaa
301    ttatttaatt atttattcat ataatttttt tccagaaatg catcctttct gtaagcgttc
361    gccgatggca tggcgttgcc acattaatct tgaagtggct ctcgaatcga gtcagcactc
421    gcttacggcc ataccagcct
```