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Production of androgenetic, triploid and tetraploid hybrids from the interspecific hybridization of female Japanese crucian carp and male blunt snout bream

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ABSTRACT

Distant hybridization occurs widely in fishes and is a useful strategy to produce different ploidy offsprings. In this study, we obtained androgenetic blunt snout bream (2n = 48, ADBSB), triploid hybrids (3n = 124, 3nJB) and tetraploid hybrids (4n = 148, 4nJB) from the hybridization of two species from different subfamilies: *Carassius cuvieri* (♀, 2n = 100, JCC) and *Megalobrama amblycephala* (♂, 2n = 48, BSB). The ploidy levels of ADBSB, 3nJB and 4nJB hybrids were confirmed by counting chromosomal numbers, forming chromosomal karyotype, and measuring DNA content. In the phenotypes and reproductive traits, 3nJB and 4nJB exhibited significantly divergences from JCC and BSB, but ADBSB took after BSB. Fluorescence in situ hybridization (FISH) and 5S rDNA analyses revealed that the genetic traits of the offsprings compared with those of their parents. This is the first report on the coexistence in vertebrates of viable androgenetic, triploid and tetraploid hybrids being produced by crossing JCC and BSB. The formation of the different ploidy offspring is great significance in both evolution and fish genetic breeding and it also provides a good model for studying the genomic variation in the first generation of interspecific hybrids.

1. Introduction

Distant hybridization is defined as above-specific or interspecific crossing and is a useful strategy to produce hybrid offspring with altered genotypes and phenotypes or with different ploidy levels (Biradar and Rayburn, 1993; Bullini, 1994; Liu, 2010; Mallet, 2007). Fish distant hybridization is the most frequent in all vertebrate classes, it has been widely conducted between species, genera, subfamilies, families and orders of fish (Schwartz, 1981). Fish chromosomes display plasticity, thus it is easier to produce polyploid and gynogenetic offspring by using distant hybridization between fishes (Liu et al., 2010). For example, F_1 hybrids of grass carp (2n = 48) × blunt snout bream (2n = 48) contained diploid (2n = 48) and triploid (3n = 72) (He et al., 2013); F_1 hybrids of red crucian carp (2n = 100) × blunt snout bream (2n = 48) contained gynogenetic red crucian carp (2n = 100), triploid (3n = 124) and tetraploid hybrids (4n = 148) (Liu et al., 2007; Liu et al., 2010). But, there is rare report on the formation of the bisexual androgenetic fish by hybridization, which is potential to become a new fish population. Stanley (1976) reported that the viable androgenetic grass carp was produced by hybridization of female carp and male grass carp, which was the only reported on the formation of viable androgenetic fish by hybridization. Generally, the very low survival rate of androgenesis in fish is thought to be mainly caused by the expression of homozygous, deleterious alleles on the eggs and the zygotes (Komen and Thorgaard, 2007; Ocalewicz et al., 2013). The exact cause remain unknown.

In vertebrate, the 5S rDNA is composed of a highly conserved 5S rRNA sequence of 120 bp and a variable nontranscribed spacer (NTS) (Bogenhagen and Brown, 1981; Fujiwara et al., 2009; Long and Dawid, 1980), which is typically organized in hundreds to thousands of tandem arrays in one or more chromosome loci (Wasko et al., 2001). The 5S rRNA sequences are highly conserved in length and nucleotide sequence, even among non-related taxa, but the NTSs show high rate of variations in both length and sequence (Pendas et al., 1995; Ferreira

Abbreviations: JCC, Japanese crucian carp; BSB, blunt snout bream; ADBSB, androgenetic blunt snout bream; 3nJB, triploid hybrids; 4nJB, tetraploid hybrids; FISH, Fluorescence in situ hybridization

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et al., 2007; Campo et al., 2009). Although a number of studies have reported the structural and functional organization of the 5S rDNA arrays in teleosts (Pendas et al., 1994; Sajdak et al., 1998; Martins and Galetti, 2001; Gornung et al., 2007; Qin et al., 2010), relatively few studies have done so for polyploid fish hybrids (He et al., 2013; Qin et al., 2015; Qin et al., 2010; Wang et al., 2017).

In the fish catalog, the Japanese crucian carp (*Carassius cuvieri*) (JCC) with 100 chromosomes belongs to the *Cyprininae* subfamily and the blunt snout bream (*Megalobrama amblycephala*) (BSB) with 48 chromosomes belongs to the *Cultrinae* subfamily (Xiao et al., 2014). In this study, we successfully obtained androgenetic blunt snout bream (2n = 48, ADBSB), tetraploid (4n = 148, 4nJB) and triploid (3n = 124, 3nJB) hybrids by crossing JCC (♀) and BSB (♂). The obtainment of these new hybrid offspring has significance in fish genetic breeding and evolutionary study.

2. Materials and methods

2.1. Ethics statement

Administration of Affairs Concerning Animal Experimentation Guidelines states that approval from the Science and Technology Bureau of China and the Department of Wildlife Administration is not necessary when the fish in question are not rare or not near extinction (first-class or second-class state protection level). Therefore, approval is not required for the experiments conducted in this study.

2.2. Animals and crosses

BSB and JCC were obtained from the Protection Station of Polyploidy Fish in Hunan Normal University. During the reproductive seasons (from April to June each year) in 2012–2014, 20 mature females and 20 mature males of both JCC and BSB were chosen as the parents. The crossings were performed by two groups. In the first group, the JCC was used as the maternal, and the BSB was used as the paternal. In the second group, the maternal and paternal were reversed. The mature eggs and sperm of JCC and BSB were fertilized and the embryos developed in the culture dishes at the water temperature of 19 °C–20 °C. In each groups, 5000 embryos were taken at random for the examination of the fertilization rate (number of embryos at the stage of gastrula/number of eggs), the hatching rate (number of hatched fry/number of eggs) and early survival rate (number of survival fry/number of hatched fry). The hatched fry were transferred to the pond for further culture.

2.3. Preparation of chromosome spreads and measurement of DNA content

To determine ploidy, chromosomal preparations were performed from peripheral blood cell cultures of cell cultures of 10 JCC, 10 BSB, 5 ADBSB, 10 3nJB and 10 4nJB at 1-year of age. The chromosomes were prepared in accordance with Xiao et al. (2014). The shape and number of chromosomes were analyzed under microscope. For each type of fish, 200 metaphase spreads (20 metaphase spreads in each sample) of chromosomes were analyzed. Preparations were examined under an oil lens at a magnification of ×330. Good-quality metaphase spreads were photographed and used for analysis of karyotypes. The lengths of the entire chromosome, long and short arms were measured. Chromosomes were classified based on their long-arm to short-arm ratios according to Levan et al. (1964).

The DNA content of erythrocytes of JCC, BSB and their hybrids offsprings were measured using a flow cytometer (cell counter analyzer, Partec) in 2013, 2014 and 2015. About 0.5–1 ml of red blood cells was collected from the caudal vein of the above fish into syringes containing 100–200 units of sodium heparin. The blood samples were treated following the method described in the published paper (Liu et al., 2001). The DNA content of each sample was measured under the same conditions. To calculate the probabilities of the ratios of the DNA content of the polyploidy hybrids to the sum of that of JCC and BSB, the x^2 test with Yate's correction was used for testing deviation from expected ratio values.

2.4. Morphological traits

At 1 year of age, 20 JCC, 20 BSB, 5 ADBSB, 20 3nJB and 20 4nJB were randomly selected for morphological examination following the methods described in previous study (Hu et al., 2012). For both measurable and countable data, we used the software of SPSS to analyze the covariance of the data between hybrid offspring and their parents.

2.5. Gonadal structure and gametes phenotype

At the age of 15 months old, 20 3nJB and 20 4nJB individuals were randomly sampled for examination of gonad development by histological sectioning. The gonads were fixed in Bouin's solution, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Gonadal structures were observed and photographed with a Pixera Pro 600ES digital camera (Nikon, Japan). The gonadal stages were classified in accordance with prior standard series for cyprinid fish (Sun et al., 2003). In addition, at the age of 2-year-old, the mature eggs or white semen were squeezed out from the females and males of the ADBSB, respectively. The mature eggs and semen was collected for the morphology examination.

2.6. Genomic DNA extraction, PCR and sequencing

The genomic DNAs extracted from the blood cells of JCC, BSB, ADBSB, 3nJB and 4nJB hybrids by routine approaches (Sambrook et al., 1989) were used as templates. A set of primers (5S-F, 5′-GCTATGCCC GATCTCGTCTGA-3′: 5S-R, 5′-CAGGTTGGTATGGCCGTAAGC-3′) were designed and synthesized to amplify the 5S rDNA genes directly from genomic DNA by PCR according previous study (He et al., 2012). PCR products were separated on a 1% agarose gel using TBE buffer. The targeted fragments were purified using a gel extraction kit (Sangon, Shanghai, China) and ligated into the pMD18-T vector (TaKaRa, Dalian, China). The plasmids were transformed into *E. coli* DH5a and purified. The inserted targeted fragments in the pMD18-T vector were sequenced by an automated DNA sequencer (ABI PRISM 3730). The sequence homology and variation among the fragments amplified from JCC, BSB, ADBSB, 3nJB and 4nJB were analyzed using BioEdit (Hall, 1999) and Clustal W (Larkin et al., 2007).

2.7. Fluorescence in situ hybridization

The probes for fluorescence in situ hybridization (FISH) for the 5S gene were constructed for JCC and amplified by PCR using the primers 5′-GCTATGCCCGATCTCGTCTGA-3′ and 5′-CAGGTTGGTATG GCCGTA AGC-3′. The FISH probes were produced by Dig-11-dUTP labeling (using a nick translation kit; Roche) of purified PCR products. FISH was performed according to the method described by He et al. (2012). For each type of fish, 100 metaphase spreads (20 metaphase spreads in each sample) of chromosomes were analyzed.

3. Results

3.1. Formation of androgenetic, tetraploid and triploid hybrids

The Japanese crucian carp (JCC, 2n = 100, Fig. 1A) and blunt snout bream (BSB, 2n = 48, Fig. 1B) are widely cultured in Asia. During the reproductive season (from April to June) in 2012–2014, distant hybridization of JCC (♀) × BSB (♂) repeatedly produced three ploidy types (Fig. 1 and Table 1), including the androgenetic blunt snout bream (ADBSB, 2n = 48; 0.03%–0.06%; Fig. 1E), tetraploid hybrids

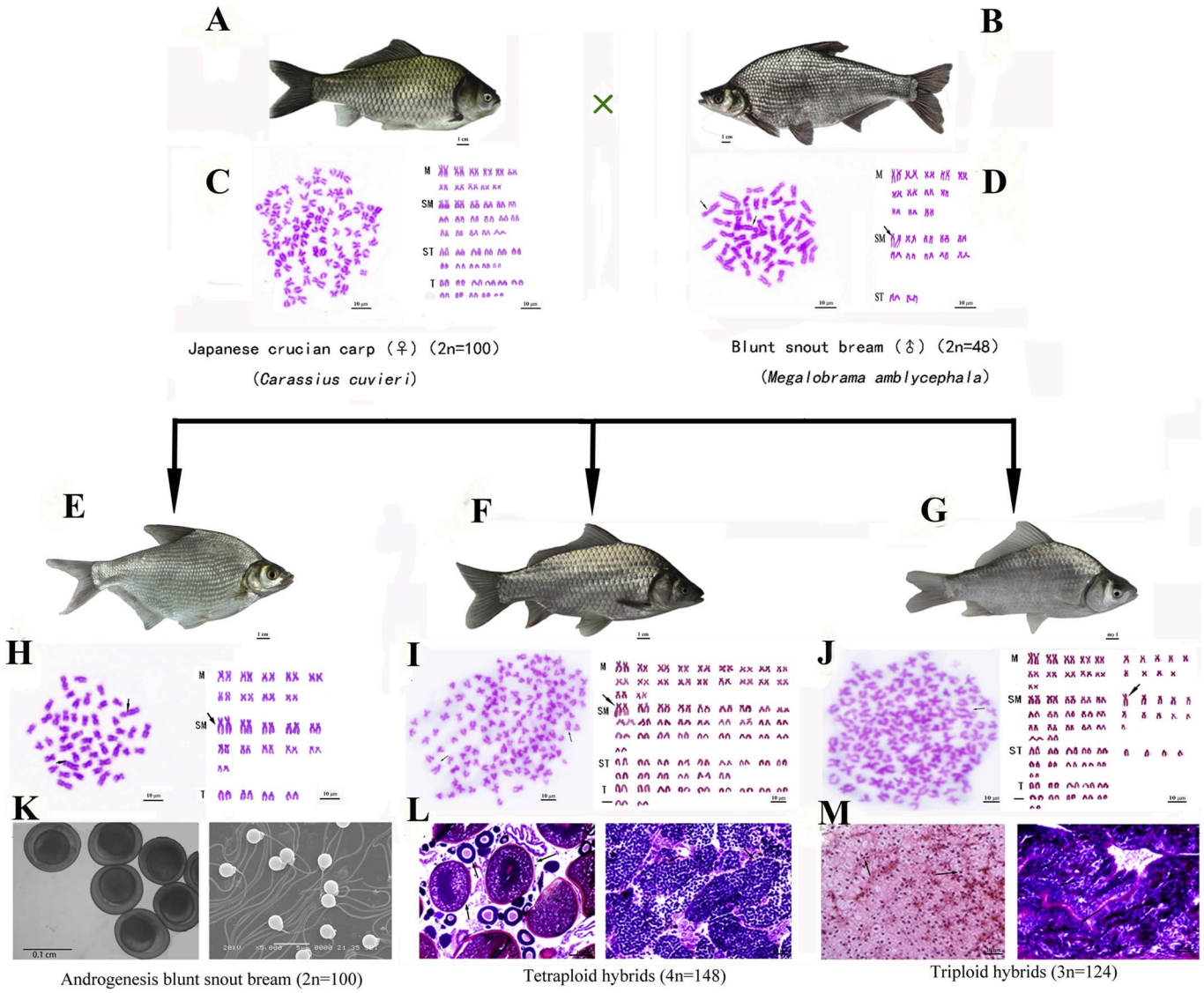

Fig. 1. The chromosomal trait, gonadal development, and appearance of Japanese crucian carp (2n = 100), blunt snout bream (2n = 48), and their hybrid offspring. (A) The appearance of JCC. (B) The appearance of BSB. (C) The chromosomal numbers (Left) and karyotype (Right) of JCC. (D) The chromosomal number (Left) and karyotype (Right) of BSB. (E) The appearance of ADBSB. (F) The appearance of 4nJB. (G) The appearance of 3nJB. (H) The chromosomal number (Left) and karyotype (Right) of ADBSB. (I) The chromosomal number (Left) and karyotype (Right) of 4nJB. (J) The chromosomal number (Left) and karyotype (Right) of 3nJB. (K) Light microscopy of eggs produced by female ADBSB (Left) and Scanning electron micrograph of spermatozoa in semen stripped out from male ADBSB (Right). (L) Ovarian (Left) and testes (Right) microstructure of 4nJB. (M) Ovarian (Left) and testes (Right) microstructure of 3nJB.

Table 1
Percentage of different types of offspring of JCC (♀) × BSB (♂).

Year	Fish type		
	3nJB	4nJB	ADBSB
2014	65.48%	34.47%	0.05%
2015	61.55%	38.39%	0.06%
2016	62.04%	37.93%	0.03%

(4nJB, 4n = 148; 34.47–38.39%; Fig. 1F) and triploid hybrids (3nJB, 3n = 124; 61.55–65.48%; Fig. 1G). However, reverse crosses of BSB (♀) × JCC (♂) did not yield any surviving progeny.

3.2. Measurement of DNA content, examination of chromosome number and formation of karyotype

We used the sum of the DNA content of JCC and BSB as the controls,

Table 2
Mean DNA content of BSB, JCC, ADBSB, 3nJB and 4nJB hybrids.

Fish type	DNA content	Ratio	
		Observed	Expected
BSB	68.8		
JCC	94.72		
ADBSB	74.55	ADBSB/BSB = 1.08[a]	1
3nJB	124.3	3nJB / (JCC + 0.5BSB) = 0.96[a]	1
4nJB	157.43	4nJB / (JCC + BSB) = 0.96[a]	1

[a] The observed ratio was not significantly different ($P > 0.05$) from the expected ratio.

ADBSB, 3nJB and 4nJB DNA content was obtained (Table 2). The mean DNA content of ADBSB was equal ($P > 0.01$) to the BSB, suggesting that ADBSB had two sets of chromosomes completed from BSB. The mean DNA content of 3nJB was equal ($P > 0.01$) to the sum of that of JCC and half of BSB, suggesting that 3nJB hybrids had two sets of

Table 3
Examination of chromosome number in BSB, JCC, 3nJB and 4nJB hybrids.

Fish type	No. in metaphase	Distribution of chromosome number							
		< 48	48	< 100	100	< 124	124	< 148	148
BSB	200	9	191						
JCC	200			15	185				
ADBSB	200	7	193						
3nJB	200					38	162		
4nJB	200							31	179

chromosomes from JCC and one set of chromosomes from BSB. The mean DNA content of 4nJB was equal ($P > 0.01$) to the sum of that of JCC and BSB, indicating that 4nJB had two sets of chromosomes from JCC and two sets of chromosomes from BSB.

Chromosomes were counted in 200 metaphase spreads in each sample of JCC, BSB, NABSB, 3nJB and 4nJB (Table 3 and Fig. 1C, D, H–J). Of the JCC individuals we examined, 92.5% of chromosomal metaphases had 100 chromosomes with the karyotype formula of 22 m + 34 sm + 22 st + 22 t (Table 3, Fig. 1C), which was the same as described in our previous study (Liu et al., 2001). Of the BSB individuals we examined, 95.5% of chromosomal metaphases possessed 48 chromosomes with the karyotype formula of 18 m + 22 sm + 8 st (Table 3, Fig. 1D), which was the same as described in our previous study (Liu et al., 2007). Of the ADBSB individuals we examined, 96.5% of chromosomal metaphases possessed 48 chromosomes with the karyotype formula of 18 m + 22 sm + 8 st (Table 3, Fig. 1H). Of the 4nJB individuals we examined, (Fig. 1G), 89.5% of chromosomal metaphases had 148 chromosomes with the karyotype formula of 40 m + 56 sm + 30 st + 22 t (Table 3, Fig. 2I). Of the 3nJB individuals we examined, 81.0% of chromosomal metaphases had 124 chromosomes with the karyotype formula of 31 m + 45 sm + 26 st + 22 t (Table 3, Fig. 1J). The chromosomal spreads were examined to directly identify the chromosomal number, and the results suggest that ADBSB, 3nJB and 4nJB are diploid, triploid and tetraploid, respectively.

3.3. Morphological traits

The appearance traits of JCC (Fig. 1A), BSB (Fig. 1B), ADBSB (Fig. 1E), 3nJB (Fig. 1F), and 4nJB (Fig. 1G) were shown in Fig. 1. It was easy to distinguish the polyploid hybrids and ADBSB.

The measurable traits and countable traits were examined in each sample of BSB, JCC, ADBSB, 3nJB and 4nJB hybrids (Tables 4 and 5). For ADBSB, all measurable traits were close to those of BSB and significantly different ($P > 0.01$) from those of JCC. For the measurable traits between 3nJB hybrids and BSB, apart from the ratio of TL/TW, which was not significantly different ($P > 0.01$), other ratios were significantly different. Between 3nJB hybrids and JCC, apart from the ratio of BL/HL, which was not significantly different ($P > 0.01$), other ratios were significantly different. Between 4nJB hybrids and BSB, apart from the ratio of BL/BW, which was not significantly different ($P > 0.01$), other ratios were significantly different. Between 4nJB hybrids and JCC, all ratios were significantly different. On the other hand, between 3nJB and 4nJB hybrids, apart from the ratio of BL/HL, which was not significantly different ($P > 0.01$), other ratios were significantly different.

For ADBSB, all countable traits were close to those of BSB and significantly different ($P > 0.01$) from those of JCC. For the countable traits between 3nJB hybrids and BSB, all data were significantly different. Between 3nJB hybrids and JCC, apart from the number of lateral scales, and upper lateral scales, which were not significantly different ($P > 0.01$), other data were significantly different. Between 4nJB hybrids and BSB, all data were significantly different. Between 4nJB hybrids and JCC, apart from the number of upper lateral scales, which were not significantly different ($P > 0.01$), other data were significantly different. On the other hand, between 3nJB and 4nJB hybrids, apart from the number of dorsal fins and anal fins, which were significantly different, other data were not significantly different ($P > 0.01$).

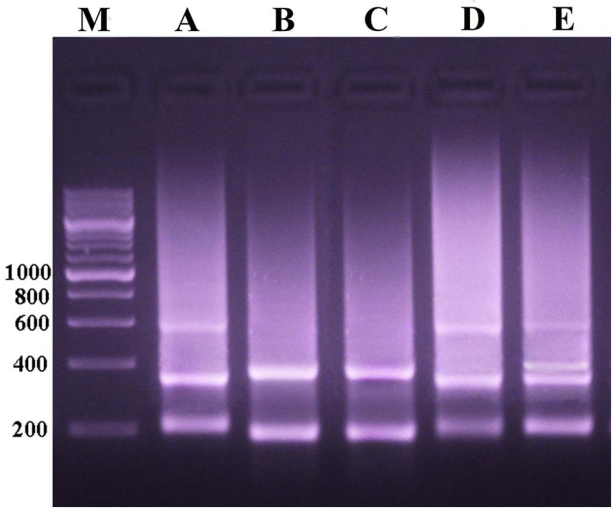

Fig. 2. DNA bands amplified from JCC, BSB, ADBSB, 3nJB and 4nJB. M: DNA 200 bp ladder; 5S amplification resulted in three DNA bands (~200, 350 and 500 bp) from JCC (A); two DNA fragments (approximately 200 and 370 bp) from BSB (B); two DNA fragments (approximately 200 and 370 bp) from ADBSB (C); three DNA fragments (approximately 200, 340, and 500 bp) from the 3nJB (D); four DNA fragments (approximately 200, 340, 400, and 500 bp) from the 4nJB (E).

3.4. Fertility of the hybrids

Our analysis of reproductive traits revealed that ADBSB and 4nJB are fertile and reach sexual maturity at two years of age, but the 3nJB hybrids are sterile. Observations of gonadal development are important for assessing the fertility of the hybrid progenies. Histological sectioning was used to examine gonad development in 4nJB and 3nJB. The testes of the 15-month-old 4nJB were at stage IV, in which a number of secondary spermatocytes were observed in the seminiferous tubules (Fig. 1L, right). The ovaries of the 15-month-old 4nJB were at stage II, were rich in oocytes in synchronized development and were characterized by the location of the yolk nucleus near the cell nucleus (Fig. 1L, left). In addition, during the reproductive season, water-like semen and mature eggs could be stripped out from the two-year-old male and female 4nJB individuals, respectively. The male 4nJB and female 4nJB were used for self-mating and viable autotetraploid (4n = 200) hybrids and diploid hybrids (details not shown) were produced. These results suggest that 4nJB are fertile but at a lower rate than their parents. By contrast, there were three types of gonadal structure in the triploid hybrids. The first type was testis-like gonads that comprised many lobules containing numerous spermatids. Some degenerated spermatids were found, and no mature spermatozoa were observed (Fig. 1M, right). The second type was ovary-like gonads comprising many nests of small, undeveloped cells and a few small

Table 4
Comparison of the measurable traits between the hybrid offspring and JCC and BSB.

Fish type	WL/BL	BL/BW	BL/HL	HL/HW	TL/TW	BW/HW
BSB	1.19 ± 0.03	2.37 ± 0.03	4.75 ± 0.04	1.14 ± 0.03	1.08 ± 0.04	2.09 ± 0.04
JCC	1.24 ± 0.02	2.22 ± 0.15	3.70 ± 0.21	1.17 ± 0.06	0.81 ± 0.01	1.78 ± 0.09
ADBSB	1.18 ± 0.06	2.36 ± 0.06	4.73 ± 0.14	1.15 ± 0.22	1.08 ± 0.25	2.10 ± 0.21
3nJB	1.16 ± 0.03	2.28 ± 0.13	3.80 ± 0.25	1.03 ± 0.07	1.05 ± 0.08	1.72 ± 0.11
4nJB	1.21 ± 0.03	2.35 ± 0.12	3.81 ± 0.14	1.12 ± 0.06	1.02 ± 0.08	1.83 ± 0.10

Table 5
Comparison of the countable traits between the hybrid offspring and JCC and BSB.

Fish type	No. of lateral scales	No. of upper lateral scales	No. of lower lateral scales	No. of dorsal fins	No. of abdominal fins	No. of anal fins
BSB	50.90 ± 0.91 (52–49)	9.65 ± 0.49 (9–10)	10.05 ± 0.69 (9–11)	III +25.85 ± 0.59 (III 25–27)	III +25.70 ± 0.55 (III 25–27)	8.65 ± 0.49 (8–9)
JCC	33.15 ± 0.35 (32–34)	7.5 ± 0.42 (6–8)	7.6 ± 0.31 (5–7)	III +19.35 ± 0.86 (III 18–20)	III +9.05 ± 0.75 (8–10)	6.45 ± 0.31 (6–7)
ADBSB	50.90 ± 0.24 (52–49)	9.47 ± 0.12 (9–10)	9.42 ± 0.69 (9–11)	III +26.22 ± 0.37 (III 25–27)	III +25.50 ± 0.13 (III 25–27)	8.44 ± 0.38 (8–9)
3nJB	33.45 ± 0.74 (32–34)	7.7 ± 0.51 (7–8)	7.45 ± 0.60 (7–9)	III +17.25 ± 1.07 (III 15–20)	III +7.65 ± 0.67 (III 6–9)	7.20 ± 0.61 (6–8)
4nJB	32.05 ± 0.22 (32 − 33)	7.1 ± 0.31 (7–8)	7.95 ± 0.22 (7–8)	III +17.35 ± 0.49 (III 17–18)	III +8.70 ± 0.47 (III 8–9)	7.15 ± 0.37 (7–8)

growing oocytes (Fig. 1M, left). The third type only had fat tissue where the gonads should have been, and neither testes nor ovaries were observed. In the reproductive season, no milt or eggs could be stripped out from the one or two-year old males or females of 3nJB. These results suggest that the 3nJB hybrids are sterile.

The white semen can be stripped out from the male ADBSB individuals at the age of 2-year-old and large numbers of eggs can be stripped out from females ADBSB (Fig. 1K). The sperm scanning electron microscope showed that the heads and tails of the sperm produced by male ADBSB are well-developed. The ADBSB males and females were found to produce mature sperm and eggs, which can fuse to form ADBSB-F_2.

3.5. Molecular organization of 5S rDNA

Using the 5S primer pair, the PCR results show that there were three DNA fragments (approximately 200, 340 and 500 bp) in JCC, two DNA fragments (approximately 200 and 400 bp) in BSB, two DNA fragments (approximately 200 and 400 bp) in ADBSB; three DNA fragments (approximately 200, 340 and 500 bp) in 3nJB and four DNA fragments (approximately 200, 340, 400 and 500 bp) in 4nJB (Fig. 2). To further evaluate differences of 5S rDNA patterns, a total of 230 clones were cloned, including 30 clones from JCC, 20 clones from BSB, 40 clones from ADBSB, 60 clones from 3nJB hybrids and 80 clones from 4nJB hybrids. Sequencing analysis indicated that JCC had three differently sized 5S rDNA fragments (203 bp, 340 and 486 bp); BSB had two differently sized 5S rDNA fragments (188 bp and 376 bp); ADBSB had two differently sized 5S rDNA fragments (188 bp and 376 bp); 3nJB hybrids had five differently sized 5S rDNA fragments (188 bp, 203 bp, 340 bp, 376 bp and 496 bp) and 4nJB hybrids also had three differently sized 5S rDNA fragments (203 bp, 340 bp, 406 bp and 494 bp) (Table 6).

Based on the BLASTn analysis, all fragments of JCC, BSB, ADBSB, 3nJB and 4nJB hybrids were proved to be 5S rDNA sequences. These sequencing results suggest that JCC and BSB are highly conserved in the 5S RNA regions (Fig. 3) but exhibit large variation in the NTS regions (Fig. 4). In JCC, the three monomeric 5S rDNA classes (designated class I: 203 bp; class II: 340 bp; and class III: 486 bp) were characterized by distinct NTS types (designated NTS-I, NTS-II, and NTS-III for the 83, 220, and 366 bp sequences, respectively) (Fig. 4A–C, respectively). In BSB, the only monomeric 5S rDNA (designated class IV: 188 bp) was characterized by one NTS type (designated NTS-IV: 68 bp). Like BSB, the ADBSB also only monomeric 5S rDNA with completely from BSB. The 3nJB hybrids had four monomeric 5S rDNA classes, with three from JCC (class I, class II, and class III) (Fig. 4A–C, respectively) and one from BSB (class IV). The 4nJB had three monomeric 5S rDNA classes, with completely from JCC (class I, class II and class III) (Fig. 4A–C, respectively). The 406 bp DNA fragment from the 4nJB was a dimeric 5S rDNA tandem array comprising two class I sequences.

3.6. Fluorescence in situ hybridization

The results of FISH (Fig. 5) showed that there were two strong and two weak hybridizing signals in JCC (Fig. 5A) and 3nJB (Fig. 5D), two strong and one weak hybridizing signals in 4nJB (Fig. 5E), no hybridizing signal in BSB (Fig. 5B) and ADBSB (Fig. 5C). The FISH analysis also identified the heredity and variation of the chromosomes in the hybrids at the molecular level.

Table 6
The results of 5S rDNA sequences.

Fish type	Number of sequenced clones	DNA fragments			
		~200 bp[a]	~340 bp[a]	~400 bp[a]	~500 bp[a]
JCC	30	Ten sequenced clones of 203	Ten sequenced clones of 340	Absent	Ten sequenced clones of 486
BSB	20	Ten sequenced clones of 188	Ten sequenced clones of 376	Absent	Absent
ADBSB	40	Twenty sequenced clones of 188	Twenty sequenced clones of 376	Absent	Absent
3nJB	60	Fifteen sequenced clones of 205 Five sequenced clones of 188	Eight sequenced clones of 340 Twelve sequenced clones of 376	Absent	Twenty sequenced clones of 496
4nJB	80	Twenty sequenced clones of 203	Twenty sequenced clones of 340	Twenty sequenced clones of 406	Twenty sequenced clones of 494

[a] The approximate size of PCR bands on the agarose gel.

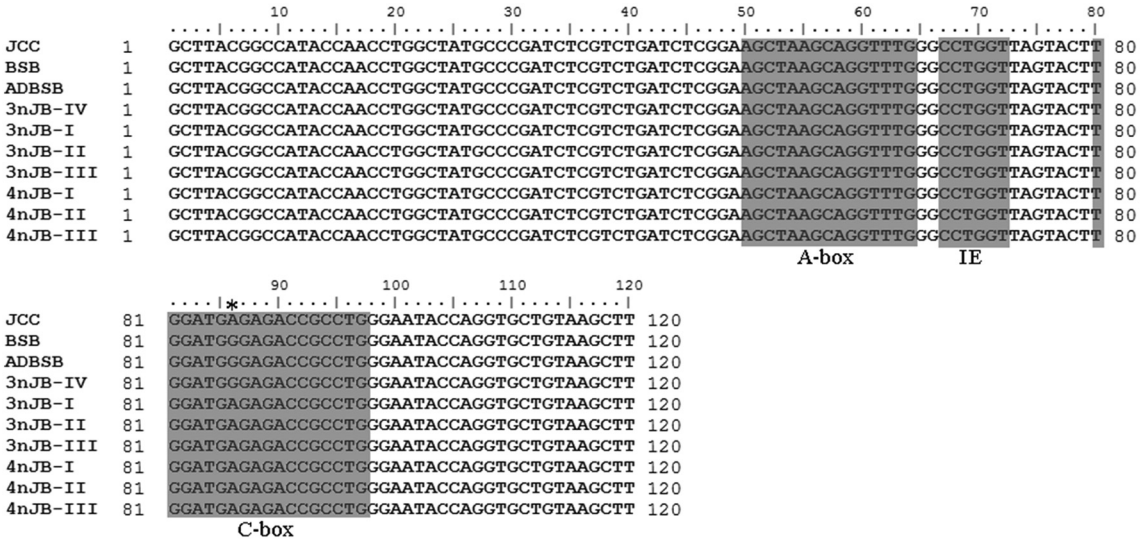

Fig. 3. Complete 5S coding regions from BSB, JCC, ADBSB, 3nJB and 4nJB. Asterisks mark variable sites of the 5S coding regions and internal control regions of the coding region are shaded.

4. Discussion

Distant hybridization offers a unique approach to produce different ploidy offspring in fish species. Prior studies have concluded that gynogenetic, diploid or triploid hybrids are easier to form than androgenetic and tetraploid hybrids in the first generation of the fish distant hybridization (Liu, 2010; Xiao et al., 2014). In this study, the DNA content and chromosomal number were analyzed to confirm the ploidy level of JCC (♀) × BSB (♂) hybrids offspring. All of the above results were in agreement that ADBSB, 3nJB and 4nJB hybrids were diploid androgenetic blunt snout bream, triploid and tetraploid hybrids, respectively.

At the chromosome level, both the chromosome number and karyotype formula of BSB and JCC were consistent with previous reports (Liu et al., 2007; Luo et al., 2011). A pair of the largest submetacentric chromosomes in BSB can be used as marker chromosomes for identifying BSB from JCC (Fig. 1D). Possessing 48 chromosomes and one pair of submetacentric largest chromosome, ADBSB were proved to have obtained two set of chromosomes completed from BSB (Fig. 1H). 4nJB had 148 chromosomes and one pair of submetacentric chromosomes and are suggested to harbor two sets of chromosomes from JCC and two sets of chromosomes from BSB, we concluded that the 4nJB hybrids contain two set of chromosomes from JCC and two set of chromosomes from BSB (Fig. 1I). With 124 chromosomes and the presence of a submetacentric largest chromosome similar to the two in BSB, we confirmed that the 3nJB contain two set of chromosomes from JCC and one set of chromosomes from BSB (Fig. 1J). These results indicate the androgenetic origin of the ADBSB and the hybridization origin of 3nJB and 4nJB of JCC (♀) × BSB (♂).

Distant hybridization not only results in different ploidy levels of hybrid offspring, but also leads to phenotypic changes of the hybrids. The number of countable traits in 3nJB and 4nJB presented the intermediate range compared to that of JCC and BSB (Table 4). The number of measurable traits in 3nJB was beyond the range in HL/HW and BW/HW compared to that of their parents, and 4nJB were closer in BL/BW to that of BSB (Table 5). Interestingly, both 4nJB and 3nJB showed phenotypic differences from their parents. For example, 4nJB have one pair of barbels, but their parents have no barbels; in addition, 3nJB have slightly more prominent eyes than their parents.

In terms of fertility, the three types of offsprings showed different characteristics. Light microscopy and scanning electron micrograph showed that the ADBSB could produce mature sperm and eggs (Fig. 1). Histological observations showed that the 4nJB had normal gonadal development (Fig. 1K) and produced mature sperm and eggs. Further self-mating experiments proved that 4nJB exhibited reduced fertility. However, the gonadal development of 3nJB was retarded and asynchronous and exhibited abundant polymorphisms (Fig. 1M). The 4nJB hybrid exhibited reduced fertility, which might have been caused by aberrant meiotic behavior (Gaeta and Chris Pires, 2010). The 3nJB hybrid was completely infertile, which might have been caused by incompatible genomes and the abnormal expression and regulation of genes related to gonad development (Xu et al., 2015; Zhou et al., 2014).

There were two distinct 5S rDNA classes were characterized by distinct NTSs and base substitutions in the 5S rRNA gene in fish (Moran et al., 1996; Pendas et al., 1994). In this study, three 5S rDNA classes (class I, class II, and class III) were found in the genome of JCC while just one 5S rDNA class was detected in BSB (class IV) (Figs. 3 and 4). For 3nJB, they have four 5S rDNA class with three classes from JCC (class I, class II and class III) and one class from BSB (class IV), indicating that were completely identical to the parental species, and generally preserved the 5S rDNA structural organization characteristic of their parental species. But for 4nJB, they only have three monomeric 5S rDNA classes completely from JCC (class I, class II and class III). Comparative analysis of the NTS sequence was conducted between the hybrids and their parents and the results showed that 3nJB and 4nJB NTS sequences had some nucleotide variations and insertion–deletion (Fig. 4). The results showed that distant hybridization and polyploidization can induce base substitutions and insertion–deletions in the NTS sequence, and that these variations in the NTS are often species-specific.

In the offspring of JCC (♀) × BSB (♂), we observed a very low percentage (0.05–0.08%) of ADBSB. The specific mechanism of the formation androgenetic BSB by distant hybridization is unclear. The survival of androgenetic diploid fish is typically very low, this may be due to the homozygosity of these individuals (Komen and Thorgaard, 2007; Parsons and Thorgaard, 1985). Androgenesis is thus an accidental event that is not likely to be detected unless large numbers of hybrids are examined (Grunina et al., 1990; Stanley, 1976). For 4nJB, we observed about 34.54–37.88% in the offspring of JCC (♀) × BSB (♂). Somatic chromosome doubling after fertilization is the most likely explanation for the formation of 4nJB and in certain organisms, this activity is related to a failure in cell division following mitotic doubling. Chromosome doubling may arise in the zygote, early embryo, or meristem of a plant and will ultimately result in the formation of polyploid tissues and polyploid species (Ramsey and Schemske, 1998; Volff, 2005). For example, the zygote chromosome doubling of Lamarckian

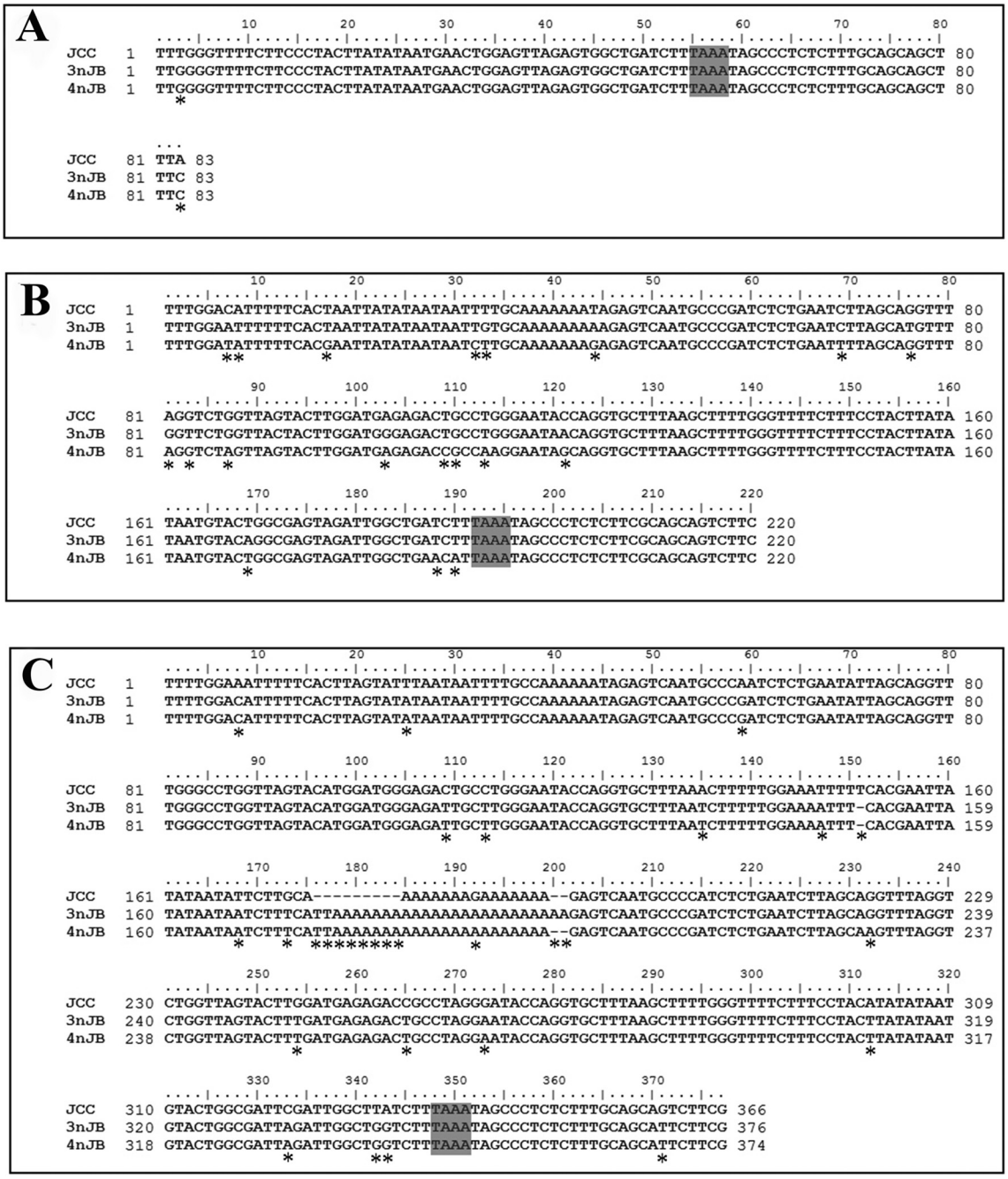

Fig. 4. Comparison of the NTS sequences from 3nJB and 4nJB hybrids, and their parents (JCC and BSB); (A) The 83 bp NTS-I sequences from JCC, 3nJB and 4nJB; (B) NTS-II from JCC, 3nJB and 4nJB hybrids; (C) NTS-III from JCC, 3nJB and 4nJB hybrids. The NTS upstream TATA elements are shaded; asterisks mark variable sites in the NTS.

primrose led to tetraploidization (Gates, 1909). For 3nJB, we observed about 62.04–65.38% in the offspring of JCC (♀) × BSB (♂). In a previous study, triploids *Crepis capillaris* was produced by the fusion of reduced haploid gametes and unreduced diploid gametes (Ramsey and Schemske, 1998). In this situation, the sister chromatids do not separate or the second polar body cannot be released normally in the second meiotic division. Similarly, the 3nJB formation probably occurred because the second polar body extrusion was inhibited during the second division of meiosis.

This study provides genetic evidences at the chromosome, FISH, DNA content, DNA fragment and sequence, and morphological levels to support the successful formation of androgenetic, triploid and tetraploid hybrids of female Japanese crucian carp × male blunt snout bream, which belonged to a different subfamily of fish (*Cyprininae* subfamily and *Cultrinae* subfamily) in the catalog. The formation of these different ploidy offsprings has potential benefit in aquaculture. The 3nJB sterility ensures it are unable to mate with other wild fish and this would play an important role in protecting wild fish resources; the 4nJB has the potential to become a new species and possesses a wider range of genetic material upon which to selectively breed; for ADBSB, due to their high level of homozygosity, they may be useful for genetic mapping and genome sequencing studies as well. Besides, these different ploidy offsprings can be used as a model to test theories about the origin and consequences of polyploidization.

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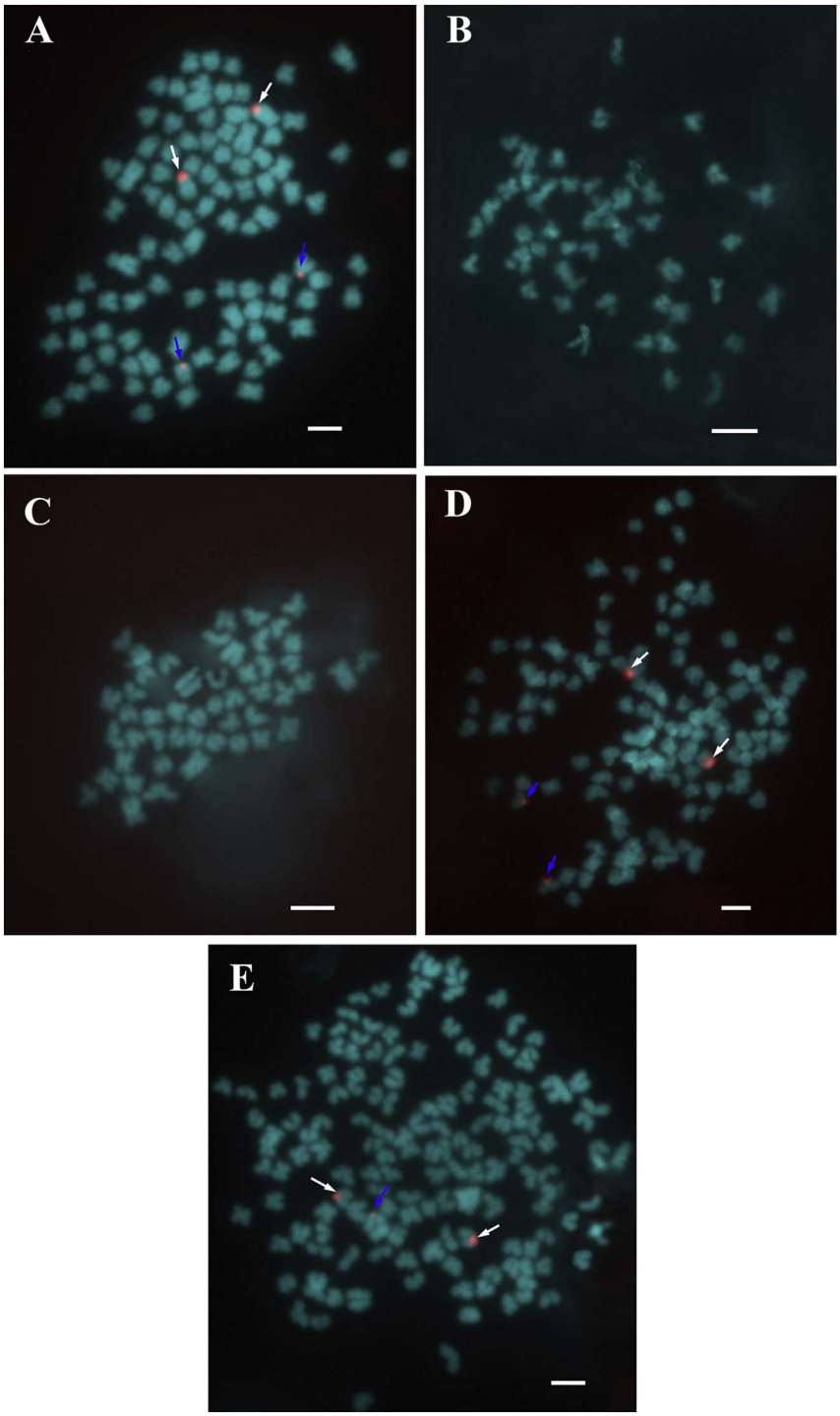

Fig. 5. Examination of FISH signals in JCC, BSB, ADBSB, 3nJB and 4nJB. (A) JCC has two strong fluorescence signals (white arrows) and two weak (blue arrows) fluorescence signals. (B) 2nBSB does not have a 5S gene locus. (C) ADBSB does not have a 5S gene locus. (D) 3nJB has two strong fluorescence signals (white arrows) and two weak (blue arrows) fluorescence signals. (E) 4nJB has two strong (white arrows) and one weak (blue arrows) fluorescence signals. Scale bars in A–E, 3 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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Competing interests

The authors declare that they have no competing interests.

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