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Chromosome banding and FISH with rDNA in diploid and tetraploid loach, *Misgurnus anguillicaudatus*

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Abstract The chromosomes of diploid and tetraploid loach, *Misgurnus anguillicaudatus*, were analyzed by staining with Ag, Chromomycin A₃ (CMA₃)/Distamycin A (DA), and DA/4',6-diamidino-2-phenylindole (DAPI), and fluorescence *in situ* hybridization (FISH) with 5.8S + 28S rDNA as a probe. Nucleolus organizing regions (NORs) were mapped to the telomeric region of the short arms of the largest (first) metacentric chromosome pair in diploid loach with 2n = 50 and the homologous quartet in tetraploid loach with 4n = 100. The NORs were positive at the same region of the first metacentric chromosome for Ag- and CMA₃/DA-stainings, but negative for DA/DAPI-staining. Four signals at the homologues within the same quartet suggest the duplication of entire genome from diploid to tetraploid status. However, size difference was detected among rDNA signals by FISH and CMA₃-banding.

Keywords Ag-NORs · Cobitidae · Fluorescence *in-situ* hybridization · Polyploidy · Ribosomal genes · Weatherfish

Introduction

A popular freshwater fish, the loach or the oriental weatherfish *Misgurnus anguillicaudatus* is widely distributed in East Asia including Mainland China, the Korean Peninsula, the Japanese Archipelago and other area (Saitoh 1989). In wild populations of Japan, most individuals are bisexually reproducing diploid with $2n = 50$ chromosomes, but a small number of gynogenetically reproducing clonal diploid individuals have been detected in a few localities by experimental reproduction and molecular genetic analyses (Morishima et al. 2002, 2008). The occurrence of natural triploid and diploid-triploid mosaic individuals has also been recorded at low frequencies in a few localities in Japan (Zhang and Arai 1999; Arai 2003; Morishima et al. 2004; Yoshikawa et al. 2007). Although no natural tetraploid individuals have been discovered in wild populations of Japan even after long-term intensive screening with DNA content flow cytometry (Zhang and Arai 1999; Arai 2003; Morishima et al. 2008), bisexually reproducing tetraploid loaches ($4n = 100$) have been found among market samples in Japan but their exact origin has not been identified yet (Ojima and Takai 1979; Zhang and Arai 1999; Arai et al. 1991a). These tetraploid individuals were reported to form diploid gametes and hybrids between tetraploid and diploid were triploid with $3n = 75$ chromosomes (Arai et al. 1991a, b, 1993, 1995; Zhang and Arai 1996). These triploid females produce both fertile haploid and unreduced triploid eggs, but triploid males are sterile (Matsubara et al. 1995; Zhang and Arai 1996; Arai and Mukaino 1997, 1998; Zhang et al. 1998). On the other hand, in China, the occurrence of tetraploid *M. anguillicaudatus* with 100 chromosomes has been reported in samples from Hubei and Guangdong Provinces (Li et al. 1983; Li and Li 1987; Yin et al. 2005). Recently, Li et al. (2008) reported sympatric distribution of diploid and tetraploid individuals in some wild populations in Hubei Province, China, based on chromosome count, nuclear volume of erythrocytes and/or nuclear DNA content flow cytometry.

Molecular cytogenetic approach to reveal structural difference(s) between diploid and

tetraploid *M. anguillicaudatus* is considered important to answer the question whether the loach with 100 chromosomes is a true tetraploid lineage comprising four sets of homologous chromosomes, and whether natural tetraploid form is produced by autotetraploidization or allotetraploidization. Such approaches may give important insights into the origin of natural polyploid and unisexual forms in the species, *M. anguillicaudatus*. In fish cytogenetics, specific chromosomal sites, nuclear organizer regions (NORs) have been used as a useful marker to identify chromosome or chromosomal region, because of no or poor appearance of G- or Q-banding in most fish species due to the lack of genome compartmentalization (Sumner 1990). It has been generally accepted that NORs are the regions comprising multiple copies of the ribosomal RNA genes (rDNAs) and transcriptionally active NORs with certain acidic proteins are preferentially stained with silver nitrates (AgNORs) (Pendas et al. 1993; Fujiwara et al. 1998; Alonso et al. 1999). It is also commonly accepted that NORs or major rDNA clusters can be positive by fluorescent staining with Chromomycin A₃ (CMA₃) that binds to GC-rich segments (Rabova et al. 2001, 2003; Boron et al. 2009), although there are examples to show the lack of correspondence between CMA₃-positive signal and rDNA locus (Almeida-Toledo et al. 2002; Sola et al. 2003; Gromicho et al. 2005).

The species-specific number and location of NORs detected by banding and fluorescence *in situ* hybridization (FISH) with rDNA probe are useful cytogenetic marker to consider evolutionary mechanisms responsible for the formation of polyploid animals. The chromosomal characteristics and/or location of NORs in the species *M. anguillicaudatus* remain unknown. In the present study, we performed molecular cytogenetic analysis on diploid and tetraploid samples of *M. anguillicaudatus* collected in Central China, by Ag-NORs, CMA₃-positive banding and FISH with a 5.8S + 28S rDNA probe.

Materials and methods

All individuals of *Misgurnus anguillicaudatus* ($n = 26$) were collected from Chibi and Honghu, Hubei Province, China, in May 2008 and June 2009 and then transported alive to a laboratory of Dalian Fisheries University, Dalian, Liaoning Province, China and kept in tanks ($22 \pm 1^\circ\text{C}$). Body length of samples ranged between 142 and 180 mm. Body weight ranged between 16.5 and 28.2 g.

Before cytogenetic analyses, all samples were sorted to diploid or tetraploid by analyzing nuclear volume of erythrocytic cells prepared on smear slides (Li et al. 2008) and/or DNA content flow cytometry of blood or fin clip according to the previous papers (Oshima et al. 2005). Chromosome slides were prepared from gill epithelial cells using conventional air-drying method. Samples were killed after two injections of phytohemagglutinin (6 μg per g body weight) followed by one injection of colchicines (6 μg per g body weight), and gill tissues were fixed with Carnoy's solution after hypotonic treatment with 0.0375 M KCl according to previous studies (Lin 1982; Zhang 1993; Li et al. 2008, 2009).

Slides were stained with Giemsa and chromosomes were classified to metacentric (M), submetacentric (SM), subtelocentric (ST) and telocentric (T) chromosomes according to Levan et al. (1964). Active NORs were detected by AgNO₃ staining according to Howell and Black (1980). The fluorochrome CMA₃ (Wako)/DA (Distamycin A, Sigma) staining was performed following to the protocol by Schweizer (1980). DA/DAPI (4', 6-diamidino-2-phenylindole, Sigma) staining was made according to Schweizer et al. (1978). Coverslip was mounted with mixture of equal volume of glycerin and McIlvaine (MI, pH.7.0) buffer for observation under a fluorescence microscope.

FISH with human 5.8S + 28S rDNA sequences as a probe (Fujiwara et al. 1998) after labeling with biotin-16-dUTP by nick translation (Roche) was conducted with slides which were denatured in 70% formamide/2 \times SSC (pH 7.0) at 70°C, followed by hybridization with 150–200 ng of the rDNA probe per slide. After washing, slides were treated for detection with

avidin-FITC (N-fluorescence isothiocyanate, Roche) conjugate and counterstained with DAPI. Signals were detected under an Olympus fluorescence microscope (AH2) using B filter block for single color FISH, and the image was captured with Spot Cooled CCD camera. FISH images were processed by the software program (Adobe Photoshop ver. 7.0). In some parts of prepared slides, hybridization signals were observed and photographed under a Nikon fluorescence microscope (Eclipse E800) using B-2A filter for single colour FISH. The image was captured by CCD camera Pixera Penguin 150CL-CU (Pixera). FISH images were processed using Penguin Mate Ver. 1.0.8 application program (Pixera) on a computer.

Results

Seven (five females and two males) diploid loach samples examined showed 50 chromosomes, classified into 5 M, 2 SM and 18 T chromosome pairs (Fig. 1a, b). While, 19 (12 females and seven males) tetraploid loach samples revealed 100 chromosomes, classified into 5M, 2 SM and 18 T chromosome quartets (Fig. 1c, d). No difference was observed between the female and the male conventional karyotype both in diploid and tetraploid forms.

Telomeric Ag-NORs appeared obviously on the short arm of the largest (first) M chromosome pair or quartet (Fig. 1). Homozygously Ag-NOR active chromosomes occurred in 76% metaphase spreads of diploid loach (Table 1). On the other hand, in tetraploid loach, four Ag signals were detected in 24% metaphase spreads, but three, two and one signal(s) in 34%, 26% and 16% metaphases, respectively (Table 1). Size difference among Ag-NORs was not apparent in the present observation.

In interphases of diploid loach, 67% cells showed two Ag-NORs, while 33% cells gave only one signal (Fig. 2, Table 1). In those of tetraploid loach, only 11% gave maximum four Ag-NORs, whereas 28% cells showed three, 43% two and 18% one signals (Fig. 2, Table 1).

Telomeric position of short arms of the largest M chromosome pair was positive for CMA₃ staining. Two chromosomes with CMA₃-positive site were observed in 16 diploid metaphases prepared from one female, but one was strong and another was weak in all metaphases with two signals (Fig. 3). In six tetraploid loach (three females and three males), four chromosomes with CMA₃-positive site were observed, but in all 50 metaphases examined, three were strong and one was weak (Fig. 3). With DA/DAPI staining, no positive bands were observed in any chromosomes (figure not shown).

The yellowish green rDNA FISH signals were detected in the above telomeric Ag-NOR- and CMA₃-positive sites in the short arms of the largest M chromosome pair or quartet (Fig. 4). Signal intensity was different between two homologous chromosomes in 21 metaphases from two diploid females and two diploid males. FISH signal of one chromosome was stronger than that of its counterpart (Fig. 4). Four FISH signals were detected in the tetraploid chromosome quartet in which signals on three chromosomes were stronger than the other one (Fig. 4). Such a heterogeneity in the signal intensity was found in total 90 metaphase spreads from six samples of tetraploid females and four samples of tetraploid males.

Discussion

The previous and present cytogenetic results are summarized in Table 2. The observed karyotype of Chinese diploid *M. anguillicaudatus* ($2n = 50$; 10 M + 4 SM + 36 T) is concordant with that reported in the previous karyological studies on the Japanese diploid individuals in which 14 bi-arm chromosomes were grouped into only M and SM, while 36 mono-arm chromosomes were classified to A (acrocentric), T to ST or T chromosomes (Hitotsumachi et al. 1969; Ojima and Takai 1979; Arai et al. 1991a; Kusunoki et al. 1994; Zhang and Arai 2003; Itono et al. 2006). Although the present karyotype is also concordant with the results of the Chinese diploid loach

by Li et al. (2009), the slightly different karyotypes (8 M + 6 SM + 36 T, 6 M + 8 SM + 36 T) were reported by Li and Li (1987), Wang et al. (1993), Chang et al. (2000) and Yin et al. (2005). However, the diploid karyotype of *M. anguillicaudatus* is essentially same, because it comprises 14 bi-arm (M,SM) and 36 mono-arm (ST, T) chromosomes and the fundamental number (NF) is constantly 64. The occurrence of hyper-diploid loaches with 51 to 53, or 58 chromosomes plus 0 to 5 supernumerary micro-chromosomes was reported by Zhang and Arai (2003). The other polymorphic diploid karyotype ($2n = 48$, 12 M + 4 SM + 32 T, ST) explained by centric fusion between two T chromosomes was also reported by Ojima and Takai (1979). No such numerical variations were observed in the present study.

Tetraploid karyotype with 100 chromosomes was reported not only in the samples collected from Japanese fish markets and/or farm by Ojima and Takai (1979) and Arai et al. (1991a), but also in the samples from Hubei Province, China by Li et al. (1983), Yin et al.(2005) and Li et al. (2008, 2009). Ojima and Takai (1979) arranged 100 chromosomes to 10 M pairs, 4 SM pairs and 36 ST or T pairs, because they supposed that the entire genomes of tetraploid loach might have been already re-diploidized. On the contrary, Arai et al. (1991a) demonstrated a tetraploid karyotype consisting of 5 quartets of M, 2 quartets of SM and 18 quartets of ST to T chromosomes. They concluded that tetraploid loach should have four sets of homologous chromosomes, because the gynogenetic or androgenetic progeny artificially induced from eggs or sperm of natural tetraploid loach are able to survive beyond feeding and grow to adult stage without any treatment of chromosome doubling (Arai et al. 1991b, 1993, 1995). In most cases, the gynogenetic or androgenetic embryos produced using haploid gametes of normal diploid loach are inviable due to the expression of abnormalities, referred as haploid syndrome (Arai et al. 1991b, 1993, 1995). Li et al. (2009) also arranged 100 chromosomes to 25 quartets including 5 quartets of M, 2 quartets of SM and 18 quartets of T chromosomes based on precise measurements of chromosome arm length. Thus, tetraploid loaches are considered to have just twice the diploid set.

In the present study, rDNA site or major ribosomal gene locus was mapped in the telomeric region of the short arms of the largest M chromosome of both diploid and tetraploid loach. Two rDNA bearing chromosomes were grouped to one pair in diploid loach, while four signals were detected in four M chromosomes within the quartet in tetraploid loach. In other chromosomes, no signal of rDNA was detected. Thus, rDNA site is considered useful marker specific to the first M chromosome in the loach. The observed four rDNA bearing homologues suggest four sets of homologous chromosomes in tetraploid genome. However, size polymorphism is apparently found in major ribosomal gene or NOR of the loach as has been reported in other fish species (Alonso et al. 1999; Boron et al. 2006). In the present FISH study, rDNA site of one chromosome was larger than its counterpart in diploid, while the signal in three rDNA bearing homologues within the quartet was stronger than that of the remaining one. The intensity difference of rDNA signals is commonly observed in vertebrates including teleosts so far examined (e.g., Sola et al. 1992; Rabova et al. 2003) and such heteromorphism is probably due to difference in the copy numbers of repeats as a result of unequal crossing over (Collares-Pereira and Rab 1999; Sola and Gornung 2001). Such structural heterogeneities have been often observed among homologues in some quartet of tetraploid species as well as in some octets of octaploid species in amphibian (Ruiz et al. 1984; Schmid et al. 1985; Wiley et al. 1989; Stoeck et al. 2005).

In both diploid and tetraploid loaches, the position of major ribosomal genes is corresponding to active Ag-NOR and CMA₃-positive site. Since CMA₃-staining suggested GC-rich NORs, two blocks were found in diploid, while four positive sites were observed in tetraploid. On the contrary, different numbers of Ag-NORs were found both in the diploid and tetraploid and the maximum number of two and four was corresponding to the number of genome sets, respectively. Such variations in Ag-NORs are presumably related to transcriptional regulation rather than structural alteration of the major ribosomal loci, as reported in other teleosts (Alonso et al. 1999; Boron et al. 2006, 2009). Lack of correspondence among rDNA locus, Ag-NOR- and CMA₃-positive site reported in other fish (Almeida-Toledo et al. 2002;

Gromicho et al. 2005; Sola et al. 2003) was not found in this species. However, the site of Ag-NORs supported by FISH in the present study is much different from the results reported by Chang et al. (2000), who detected two pairs of Ag-NORs located at telomeric region of the long arm of the third largest SM and the third largest T chromosomes in diploid loach ($2n = 50; 8 M + 6 SM + 36 T$). Chang et al. (2000) also reported that one pair of Ag-NOR-bearing chromosomes was also positive to C-banding, which also stains heterochromatic block.

In the loach, FISH, CMA₃- and Ag-staining clearly detected two and four homologous chromosomes with major rDNA loci in diploid and tetraploid loach, respectively. The cytogenetic results suggest the past duplication event of entire genome from diploid to tetraploid status in the loach. Structural heterogeneities shown by size differences in the rDNA site among chromosomes may be a result of re-diploidization process starting after the duplication event. Nevertheless, it remains to be ascertained how and when the natural tetraploid lineage is formed, and whether tetraploid loach is autotetraploid or allotetraploid. Further molecular cytogenetic studies using FISH and banding on somatic cells as well as meiotic configurations are necessary to elucidate cytogenetic relationship between diploid and tetraploid loach.

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Legends of figures

Fig. 1 Metaphase spreads and karyotypes of diploid (**a, b**) and tetraploid loach (**c, d**), indicating Ag-NORs in telomeric regions of the short arm of the largest (first) metacentric chromosome pair or quartet. Metaphase (**a**) and karyotypes (**b**) of diploid loach. Metaphase (**c**) and karyotype (**d**) of tetraploid loach. *Scales* denote 10 μm . *M* metacentric chromosome, *SM* submetacentric chromosome, *T* telocentric chromosomes

Fig. 2 Maximum two and four Ag-NORs in interphase cell nucleus from diploid (**a**) and tetraploid (**b**) loach. *Scales* denote 10 μm

Fig. 3 CMA₃/DA-staining metaphase spreads of diploid (**a**) and tetraploid (**c**) loach and karyotypes of diploid (**b**) and tetraploid (**d**) loach, indicating CMA₃-positive sites in telomeric regions of the short arm of the largest (first) M chromosome pair and quartet. *Scales* denote 10 μm . *M* metacentric chromosome, *SM* submetacentric chromosome, *T* telocentric chromosomes

Fig. 4 Metaphase spreads of diploid (**a**) and tetraploid (**c**) loach and karyotypes of diploid (**b**) and tetraploid (**d**) loach with FISH signals using 5.8S + 28S rDNA probe in telomeric regions of the short arm of the largest (first) M chromosome pair and quartet, after counterstaining with DAPI. *Scales* denote 10 μm . *M* metacentric chromosome, *SM* submetacentric chromosome, *T* telocentric chromosomes

Table 1 Frequency of Ag-NORs per metaphase and number of nucleolus per interphase nucleus

Ploidy	No. of Ag-NORs	Metaphases	Frequency (%)	No. of Nucleolus	Interphase nuclei	Frequency (%)
Diploid	1	12	24	1	33	33
	2	38	76	2	67	67
Tetraploid	1	8	16	1	18	18
	2	13	26	2	43	43
	3	17	34	3	28	28
	4	12	24	4	11	11

Table 2 Karyological data on diploid, triploid and tetraploid samples of the loach, *Misgurnus anguillicaudatus*

Chromosome no.	Karyotype ^a	NF	Note	Locality	Reference
2n = 50	14M, SM+36T	64	—	Sapporo, Hokkaido, Japan	Hitotsumachi et al. (1969)
2n = 50	10M+4SM+36T, ST	64	—	Chinai River, Shiga + fish market, Japan	Ojima and Takai (1979)
2n = 48	12M+4SM+32T,ST	64	—	*	
3n = 75	15M+6SM+54T, ST	96	—	*	
4n = 100 ^b	20M+8SM+72T, ST	128	—	*	
4n = 100 ^b	16M+12SM+72T	128	—	Wuhan; Shashi, China	Li et al. (1983)
2n = 50	8M+6SM+36T	64	—	Chongqing, Luzhou, China	Li and Li (1987)
2n = 50	10M+4SM+36T, ST	64	—	Fish market, Matsuzaka, Mie, Japan	Arai et al. (1991a)
			—	Fish farm, Tottori, Japan	
			—	Daimon, Toyama, Japan	
			—	Kumayama, Okayama, Japan	
			—	Sera, Hiroshima, Japan	
3n = 75	15M+6SM+54T, ST	96	—	*	
4n = 100	20M+8SM+72T, ST	128	—	*	
2n = 50	6M+8SM+36T	64	—	Hebeiixian, Baoding, China	Wang et al. (1993)
2n = 50	10M+4SM+36A	64	—	Fish Farm, Tottori, Japan	Kusunoki et al. (1994)
				Misasa R, Hiroshima Japan	
				Mihana R, Hiroshima Japan	
2n = 50	8M+6SM+36T	64	Ag-NOR: 3rd SM q, 3rd T q ^c	Guiling, Yashan, China	Chang et al. (2000)
2n = 50	8M+6SM+36T	64	Hyper-diploid, 2n = 51 to 53 or 58 plus 0 to 5 microchromosomes	Progeny of bloodstock from fish market	Zhang and Arai (2003)
4n = 100	16M+12SM+72T	128	—	Wuhan; Shashi, Hubei, China	Yin et al. (2005)
2n = 50	8M+6SM+36T	64	—	Shengnongjia, Yichang, China	
2n = 50	10M+4SM+36ST, T	64	Clonal diploid	Memanbetsu, Hokkaido, Japan	Itono et al. (2006)
2n = 50	10M+4SM+36T	64	—	Honghu, Hubei, China	Li et al. (2009)
3n = 75	15M+6SM+54T	96	—	*	
4n = 100	20M+8SM+72T	128	—	*	
2n = 50	10M+4SM+36T	64	Ag-NOR, CMA ₃ , FISH: 1st M p ^d	Honghu, Hubei, China	Present study
4n = 100	20M+8SM+72T	128		*	

^a M metacentric, SM subtelocentric, ST subtelocentric, A acrocentric, T telocentric chromosome

^b the author described as 2n = 100

^c q means long arm of chromosome

^d p means short arm of chromosome

* Same localities in the above line(s)







