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Genetic characterization of the progeny of a pair of the tetraploid silver crucian carp *Carassius auratus langsdorfii*

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Abstract Silver crucian carp *Carassius auratus langsdorfii* comprises a diploid-polyploid complex in wild Japanese populations. Bisexually reproducing diploids are sympatrically distributed with gynogenetically developing triploids and tetraploids. Triploid and tetraploid males are very rare among Japanese silver crucian carp due to their gynogenetic reproduction. We examined the genetic characteristics of progeny that arose in a tank by natural spawning of a tetraploid silver crucian carp pair. The ploidy status of 120 samples randomly collected from these progeny was determined to be tetraploid by DNA content flow cytometry. DNA fingerprints from a random amplified polymorphic DNA assay indicated that almost all the progeny examined had genotypes identical to the maternal tetraploid female with no paternally derived fragments. Selected specimens' cytogenetic analyses revealed that the progeny examined had tetraploid chromosome numbers, categorized into 40 metacentric, 80 submetacentric, and 80 subtelocentric or telocentric chromosomes, which were arranged into quartets and six supernumerary microchromosomes. Fluorescence in situ hybridization signals were detected in four homologous chromosomes in all analyzed metaphases prepared from diploid goldfish specimens. Contrary, tetraploid silver crucian carp gave eight rDNA signals. These results suggest that gynogenetic development in eggs spawned by tetraploid females should be triggered by tetraploid males' homospecific sperm.

Keywords Silver crucian carp · Tetraploid · Gynogenesis · Flow cytometry · RAPD markers · FISH

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Introduction

Silver crucian carp *C. auratus langsdorfii* comprises a diploid-polyploid complex in the wild populations of Japan. Bisexually reproducing diploids ($2n = 100$) are sympatrically distributed with gynogenetically developing triploids ($3n = 156$) and tetraploids ($4n = 206$) [1–3]. Frequencies of tetraploids are much lower than those of sympatric diploids and triploids [4, 5], and these tetraploids are considered to arise from triploids by accidental incorporation of a haploid sperm nucleus into a triploid egg [6–8]. The occurrence of relatively rare tetraploids from triploids, and the reproductive characteristics of such tetraploid progeny will provide good insights into mechanisms responsible for the origin, diversification and maintenance of such a diploid-polyploid complex in nature. Thus, the wild tetraploid Japanese silver crucian carp *C. auratus langsdorfii* has been considered excellent material to disclose the biological significance of polyploidy in fish reproduction and evolution.

Because unreduced eggs of both triploids and tetraploids develop by gynogenesis (sperm-dependent parthenogenesis) without any genetic contributions from males, the offspring are theoretically an all-female population due to no contribution by male-determinant Y chromosomes.

Although a high male incidence has been observed among Chinese silver crucian carp *C. auratus gibelio*, because these triploid forms undergo a mixture of allogynogenesis (gynogenesis triggered by heterospecific sperm from other species) and bisexual gonochoristic reproduction [9], both triploid and tetraploid males are very rare in the Japanese silver crucian carp *C. auratus langsdorfii* population [10, 11].

In 2003, we happened to discover a tetraploid male among samples from a rearing population in the Gunma Prefecture Fisheries Experiment Station. This male has been reared in the Nanae Fresh-Water Laboratory, Hokkaido University, Nanae, Hokkaido, since 2004. This male is kept in a tank along with a conspecific tetraploid female whose sampling site and year were not recorded. They reproduced in this tank by natural spawning, after which a large number of progeny appeared.

There are three possible genetic results for the progeny of a pair of tetraploids. (1) When some reduced diploid eggs are laid by a tetraploid female and then fertilized with diploid sperm from a tetraploid male, the resulting progeny are likely to be tetraploids with different genotypes. (2) Hexaploid progeny with different genotypes may result when unreduced tetraploid eggs accidentally incorporate a diploid sperm nucleus from a tetraploid male. (3) If unreduced tetraploid eggs of a tetraploid female initiate gynogenetic development due to the intrusion of diploid sperm from a tetraploid male, all of these progeny will grow to a second generation of gynogenetic tetraploids.

In the present study, 120 individuals were randomly selected from the large population of progeny arising from a pair of tetraploids and were assessed for their ploidy status by flow cytometry analysis. Then, using selected samples, the isogenic characteristics of these progeny were verified by random amplified polymorphic (RAPD) DNA-PCR fingerprints. Chromosomes of these progeny were also prepared and assessed by conventional karyotyping and rDNA detection using fluorescence in situ hybridization (FISH).

Materials and methods

Ethics

Research and handling of the animals were performed in line with the Guide for the Care and Use of Laboratory Animals at Hokkaido University.

Source of samples

A tetraploid male *C. a. langsdorfii* was found in a rearing population of the Gunma Prefectural Fisheries Station in 2003, and then transferred and kept at Azabu University. The rearing population of *C. a. langsdorfii* in the Gunma Prefectural Fisheries Station was originated from a wild population of the Jounuma Lake, Gunma Prefecture. The tetraploid male was transferred to the Nanae Freshwater Laboratory, Hokkaido University, Nanae, Hokkaido in 2004. No sampling and rearing record of the female *C. a. langsdorfii* tetraploid individual was available, but it has been reared together with the tetraploid male in the same tank. The tetraploid pair first reproduced in the tank in May, 2009 and a large number of progeny have appeared in the tank since then.

A total of 120 individuals were randomly sampled from these progeny. Due to their small body sizes (body length, 36–54 mm), sex could not be determined for each individual by naked-eye observation. Fin clips were collected for somatic cellular DNA content determinations and stored in 100 % ethanol at −30 °C for genomic DNA extraction. Samples of goldfish *Carassius auratus auratus* which were kept in the aquarium of the Environment Control Experiment Building, Faculty and Graduate School of Fisheries Sciences, Hokkaido University, Hakodate City, Hokkaido, were also used as a source of diploid control samples for these analyses.

Flow cytometry

Ploidy status of 120 individuals was determined by flow cytometry analysis using a previously described procedure [12]. Briefly, fin tissues were minced in DNA-extraction buffer (Cystain DNA 2 step, Partec GmbH, Münster, Germany) and then incubated at room temperature for 20 min. Samples were filtered through a 50-μm mesh filter (Cell TRICS disposable filter units, Partec GmbH), after which 5 volumes of staining solution (Cystain DNA 2 step, Partec GmbH) containing DAPI (4′,6-diamidino-2-phenylindole dihydrochloride) were added. DNA contents were then analyzed using a CyFlow ploidy analyzer (Partec GmbH). Ploidy status was determined by comparing the relative DNA content against standard goldfish diploid DNA content (2C).

DNA Preparation and RAPD-PCR Reactions

Total genomic DNA was isolated from fin-clip samples of 100 individuals by the phenol–chloroform method. RAPD amplification was performed as described previously [13]. From a total of 40 commercially available decamer random primers (Kits A and B, Operon Technologies, Alameda, CA, USA), three primers, OPA-07 (Kit A-7: 5′-GAAACG GGTG-3′), OPB-05 (Kit B-5: 5′-TGCGCCCTTC-3′), and OPB-07 (Kit B-7: 5′-GGTGACGCAG-3′), were selected on the basis of a preliminary screening for stable

amplification, reproducible fragment patterns, and the presence of polymorphisms between parents. The RAPD-PCR mixture contained 1.0 μl of a DNA template (100 ng/μl), 2.0 μl of 10 × PCR buffer (TaKaRa, Japan), 1.6 μl of a dNTP mixture (TaKaRa), 0.2 μl of rTaq polymerase (TaKaRa), 15.2 μl of DDW, and 1.0 μl of a random primer (20 μM). Amplification conditions were 3 min at 95 °C as an initial step, followed by 35 cycles of 95 °C for 30 s, 36 °C for 1 min for primer annealing, and 72 °C for 1 min for elongation. This was followed by a single final step of primer extension at 72 °C for 7 min. Approximately 9 μl of amplification products were separated by 1.5 % agarose gel electrophoresis in TBE buffer (89 mM Tris, 89 mM boric acid, 2.2 mM EDTA). Gels were stained with ethidium bromide and photographed on a UV trans-illuminator using a gel documentation system (Print-graph AE-6915H, Atto, Tokyo).

Chromosome preparation and karyotype analysis

Immature diploid goldfish and tetraploid silver crucian carp (total length: 40–65 mm; body weight: 1.0–3.0 g) were intraperitoneally injected with 50–150 μl of 0.001 % colchicine 3 h prior to sacrifice. Gill tissues were removed and minced finely with forceps and suspended in goldfish Ringer's solution (150 mM NaCl, 3.0 mM KCl, 3.5 mM $MgCl_2$, 5.0 mM $CaCl_2$, 10 mM HEPES, pH 7.5). After centrifugation at $100 \times g$ for 12 min, the cells were resuspended in 0.075 M KCl hypotonic solution for 40–60 min at room temperature. Then, the cells were fixed with chilled Carnoy's solution (methanol:acetic acid = 3:1) and stored at −20 °C. One droplet of a cell suspension was air-dried on a slide, which had been cleaned in advance with chilled 50 % ethanol. After Giemsa staining, karyotypes were analyzed on the basis of metaphase chromosomes, according to Levan et al. [14].

Fluorescence in situ hybridization

FISH was performed using the 5.8S + 28S rDNA probe according to the protocol described by Fujiwara et al. [15] with minor modifications. Briefly, human 5.8S + 28S rDNA was labeled with biotin-16-dUTP using a nick translation kit (Roche, Germany). Chromosome metaphase spread slides (pretreated with RNase) were denatured in 70 % formamide/2 × SSC (pH 7.0) at 70 °C for 2 min, dehydrated in cold 70 and 100 % ethanol for 10 min each, and then air dried. Then, 150–200 ng of labeled rDNA probe was denatured at 75 °C for 10 min and quickly placed on ice before hybridization with each chromosome slide. After washing in 50 % formamide/2 × SSC, 2 × SSC, 1 × SSC, and 4 × SSC, fluorescent signals were generated using an avidin–FITC (N-fluorescein isothiocyanate, Roche) conjugate. Finally, the slides were mounted in 100 μl of DABCO (1,4-Diazabicyclo-octane) antifade solution containing DAPI for counterstaining. The slides were observed under a Nikon ECLIPSE E800 microscope and images were captured with a Pixera Penguin 150CL-CU CCD camera (Pixera, San Jose, CA, USA).

Results

After testing all progenies ($n = 120$), a prominent tetraploid-range DNA content peak (Fig. 1b) was detected when compared with diploid goldfish as a control (Fig. 1a). RAPD-PCR was first performed for 100 (nos. 1–100) out of 120 tetraploid samples using OPA-07. No paternal contribution was detected by this primer, and the fingerprint pattern of progeny was identical to that of the maternal tetraploid female. Furthermore, no variations were detected among these progeny. Similar isogenic genotypes and all-

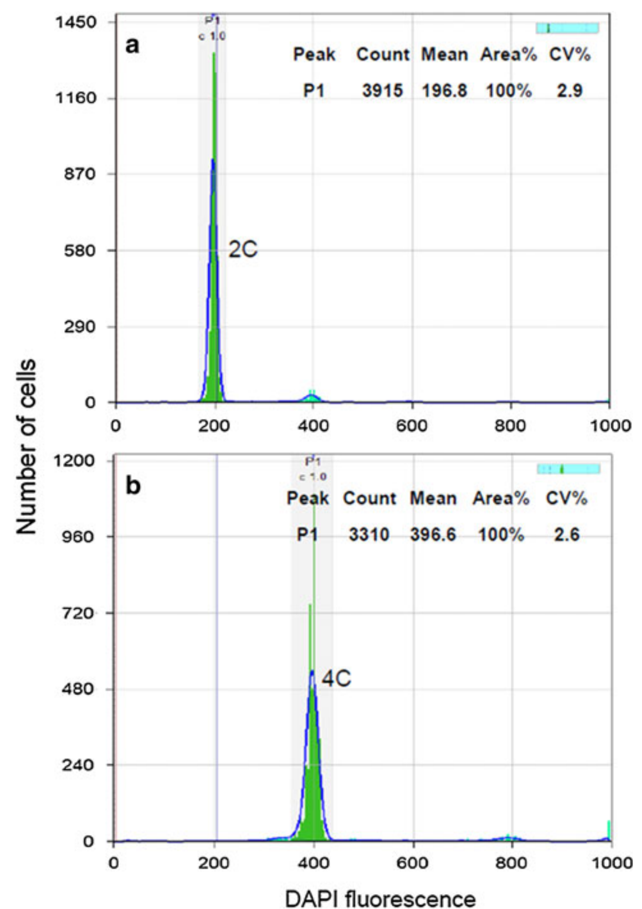

Fig. 1 Flow cytometry results for relative DNA contents in fin cells from control diploid goldfish *Carassius auratus auratus* 2C (**a**) and a tetraploid silver crucian carp *Carassius auratus langsdorfii* progeny 4C (**b**)

female inheritance by progeny were also verified in the same sample set (nos. 1–16) by RAPD-PCR using OPB-05 (Fig. 2).

Fingerprint pattern using OPB-07 primer exhibited no contribution of fragments specific to the father in all the samples examined ($n = 16$). Samples, except for sample #3, demonstrated DNA fingerprints identical to their mother, but a slight variation was found in electropherogram in one out of 16 progeny examined: one maternally derived fragment was faint or lacking when compared with other progeny (Fig. 2). However, this variant individual did not show any polymorphism in fingerprints amplified by other primers. These results indicated that almost all the second generation progeny of the tetraploid female were genetically isogenic and thus likely a gynogenetically generated clone without any contribution of paternal genome, and thus almost all the samples examined were likely genetically identical.

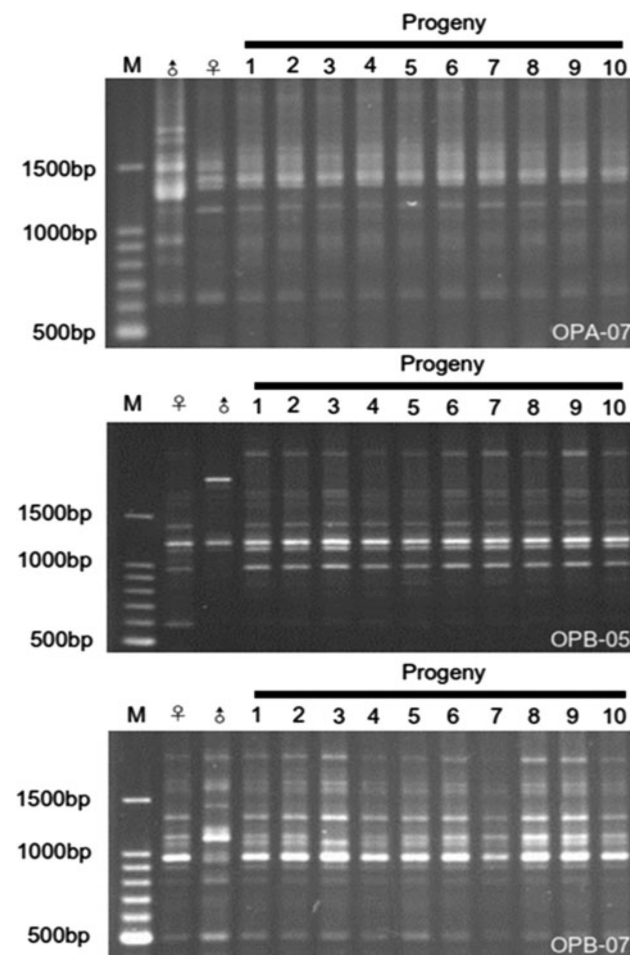

Fig. 2 Random amplified polymorphic DNA (RAPD)-PCR profiles using OPA-07 (*top*), OPB-05 (*middle*), and OPB-07 (*bottom*) primers for silver crucian carp *Carassius auratus langsdorfii*, tetraploid parents (♀ and ♂), and their progeny (nos. 1–10). *M* indicates molecular size markers

Table 1 Chromosome distributions in somatic cells of diploid goldfish *Carassius auratus auratus* and tetraploid silver crucian carp *Carassius auratus langsdorfii*

Fish	Sample no.	No. of cells with chromosomes							
		97	98	99	100	204	205	206	Total
Goldfish	1	0	0	0	12	–	–	–	12
	2	0	0	0	13	–	–	–	13
	3	1	0	0	11	–	–	–	12
Silver crucian carp	1	–	–	–	–	0	1	9	10
	2	–	–	–	–	1	0	10	11
	3	–	–	–	–	1	0	11	12

Table 1 shows the chromosome distributions determined for diploid goldfish and tetraploid silver crucian carp samples. Tetraploid progeny ($n = 3$) had a modal number of chromosomes of $4n = 206$ (Fig. 3c), whereas control goldfish ($n = 3$) had a modal number of $2n = 100$ (Fig. 3a). In goldfish, chromosomes were categorized into 20 metacentric (M), 40 submetacentric (SM), and 40 subtelocentric or telocentric (ST/T) chromosomes that were arranged into 10 M, 20 SM, and 20 ST/T pairs in their karyotypes (Fig. 3b). In tetraploid progeny, the most informative spreads gave karyotypes that were categorized into 40 M, 80 SM, 80 ST/T, and six supernumerary microchromosomes (m). Regular-sized chromosomes were arranged into 10 M, 20 SM, and 20 ST/T quartets in tetraploid karyotypes (Fig. 3d).

FISH signals using the 5.8S + 28S rDNA probe were detected in four chromosomes in all analyzed metaphases prepared from three goldfish samples (Fig. 4a). Tetraploid progeny had rDNA signals in eight chromosomes (Fig. 4b). However, the exact morphologies of rDNA bearing chromosomes and the exact locations of rDNA signals were difficult to identify.

Discussion

Tetraploid-range DNA contents and isogenic RAPD genotypes shown in these progeny clearly indicated the occurrence of gynogenetic development in tetraploid eggs that were spawned by a tetraploid female after fertilization by a tetraploid male sperm. These results indicated that sperm from the tetraploid male had triggered gynogenesis in unreduced tetraploid eggs spawned by the tetraploid female. In the Chinese silver crucian carp *C. auratus gibelio* both allogynogenetic and gonochoristic reproduction are involved [16]. In this species, gynogenesis is initiated only by fertilization with heterospecific sperm from other species (allogynogenesis), whereas

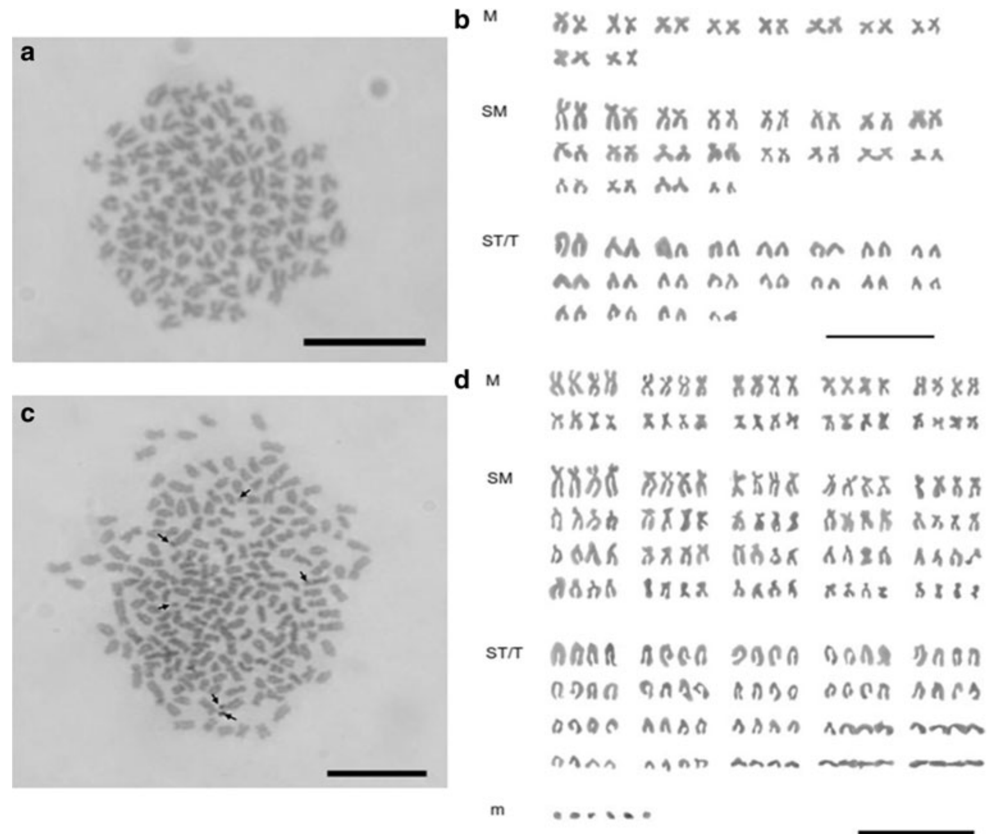

Fig. 3 Mitotic metaphase spread (**a**) and corresponding karyotype of goldfish *Carassius auratus auratus* with 100 chromosomes (**b**). Mitotic metaphase spread (**c**) and corresponding karyotype of silver crucian carp *Carassius auratus langsdorfii* (**d**) with 206 chromosomes. *Arrows* indicate microchromosomes. *Scale bars* 10 μm

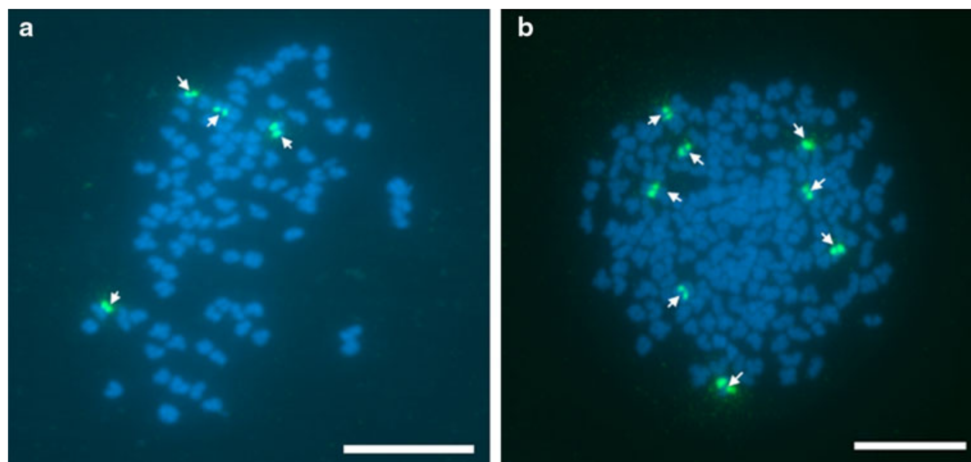

Fig. 4 Mitotic metaphase chromosomes identified after fluorescence in situ hybridization (FISH) probing with 5.8S + 28S rDNA sequences. *Arrows* indicate four fluorescent signals (*green*) in goldfish *Carassius auratus auratus* (**a**) and eight fluorescent signals (*green*) in tetraploid silver crucian carp *Carassius auratus langsdorfii* (**b**). All metaphase chromosomes were counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). *Scale bars* 10 μm

normal bisexual fertilization occurs when eggs are fertilized with homospecific sperm (gonochorism) [16]. As shown in the present study, eggs from a tetraploid female initiated gynogenetic development even after fertilization with homospecific sperm from a tetralploid male. Among 120 individuals examined, no hexaploid individuals arose due to the incorporation of a sperm nucleus from the tetraploid male.

However, an exceptional progeny with a slight difference was detected by RAPD-PCR using OPB-07 in 16 samples examined. This result suggests that the tetraploid female presumably produced large numbers of unreduced clonal tetraploid eggs as well as a very small number of exceptional tetraploid eggs with genetic variation. Such variant eggs can be explained by unusual meiotic recombination during oogenesis of the tetraploid female. Genetic and cytogenetic

results showing the involvement of meiotic recombination by the formation of synapsed bivalents and partially paired trivalents have been reported in gynogenetic triploid silver crucian carp [17, 18]. A few tetraploid eggs may undergo paring and recombination between two homologous and/or homoeologous chromosomes, because they essentially have four sets of chromosomes with relatively higher similarities or affinities. The other possibility may be attributed to unstable reproducibility of RAPD-PCR itself, in which the purity of template DNA often affects amplification of fragments [19]. Therefore, further study is required to confirm the presence or absence of genetic variation among tetraploid eggs using more sensitive and reliable genetic markers such as a microsatellite DNA.

The karyotypes of the goldfish examined here were very similar to those previously reported for diploid *C. auratus* subsp. ($2n = 100$) and diploid Miyazaki race silver crucian carp *C. auratus langsdorfii* ($2n = 100$) [1], which had 20 M + 40 SM + 40 ST/T chromosomes. The karyotype of a tetraploid silver crucian carp *C. auratus langsdorfii* ($4n = 206$), obtained from the Kanto district was reported to be 44 M + 82 SM + 80 T [1], similar to our present results. They had 206 chromosomes, but six supernumerary chromosomes were not distinguished from 200 regular chromosomes. The presence of six microchromosomes found in the present study suggested that a gynogenetic tetraploid may have arisen from a triploid with $3n = 156$ chromosomes [1] by incorporation of haploid chromosomes.

An rDNA locus with 5.8S + 18S + 28S genes and that with 5S genes are located on different chromosomes in most taxa [20]. In triploid silver crucian carp *C. auratus gibelio*, 5S rDNA signals were detected in three STs [21]. In the present study, rDNA signals were detected in four chromosomes of diploid goldfish with $2n = 100$, whereas eight signals were detected in chromosomes of tetraploid silver crucian carp with $4n = 206$. These FISH results indicated that the progeny of silver crucian carp must have a tetraploid karyotype, including four sets of chromosomes. However, the exact morphologies of rDNA-bearing chromosomes have not been determined and additional molecular cytogenetic studies will be required in the near future.

Gynogenesis usually occurs by preventing the decondensation of a sperm nucleus due to a failure in the breakdown of the sperm nuclear envelope [22, 23] in fish species that spawn unreduced diploid or triploid eggs [13, 24–26]. Unreduced diploid eggs are generated by the mechanism of premeiotic endomitosis (chromosome doubling without cytokinesis) in the clonally reproducing diploid loach *Misgurnus anguillicaudatus* [13], whereas clonal triploid eggs are formed by apomixis (i.e., ameiotic division of oocytes by skipping the first meiotic division) in the silver crucian carp *C. auratus gibelio* [24, 25] and *C. auratus langsdorfii* [26], because the formation of a tripolar spindle may physically inhibit the first meiotic division.

The occurrence of a tripolar spindle is presumably related to the presence of three sets of chromosomes in gynogenetic triploid silver crucian carp. If so, then what would occur in unreduced tetraploid eggs that include four sets of chromosomes? This is an enigma that needs to be resolved. In tetraploid eggs with an even number of chromosome sets, normal synapsis and subsequent meiosis can be predicted, although tetraploid silver crucian carp produce unreduced tetraploid eggs that develop by gynogenesis.

In tetraploid silver crucian carp, the cytological mechanisms responsible for the formation of unreduced tetraploid eggs and the initiation of gynogenesis remain to be determined. Our tetraploid population is a reliable set of experimental animals for examining these problems. To identify these mechanisms, the presence or absence of multipolar and other unusual spindles must be investigated in the oocytes of tetraploid silver crucian carp, which can be induced to undergo final maturation by administering 17α-20β-dihydroxy-4-pregnene-3-one during in vitro culture [13, 26]. The configuration of meiotic chromosomes is also informative for determining the reproductive mode [13].

Furthermore, the second generation progeny of tetraploid silver crucian carp are reliable for generating sex-reversed tetraploid males, which are a genetic resource of diploid spermatozoa for further ploidy manipulations. A dimorphism of reproductive mode in the tetraploid silver crucian carp, showing gynogenetic unreduced oogenesis and reduced spermatogenesis, may provide an excellent model for reproductive biology in fishes.

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