A Cryptic Clonal Line of the Loach Misgurnus anguillicaudatus (Teleostei: Cobitidae) Evidenced by Induced Gynogenesis, Interspecific Hybridization, Microsatellite Genotyping and Multilocus DNA Fingerprinting

Authors: Morishima, Kagayaki, Horie, Shin, Yamaha, Etsuro, and Arai, Katsutoshi

Source: Zoological Science, 19(5) : 565-575

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.19.565

ZOOLOGICAL SCIENCE **19**: 565–575 (2002)

A Cryptic Clonal Line of the Loach *Misgurnus anguillicaudatus* (Teleostei: Cobitidae) Evidenced by Induced Gynogenesis, Interspecific Hybridization, Microsatellite Genotyping and Multilocus DNA Fingerprinting

Kagayaki Morishima[1], Shin Horie[1], Etsuro Yamaha[2] and Katsutoshi Arai[1]*

[1]*Graduate School of Fisheries Sciences, Hokkaido University, Hakodate, 041-8611, Japan*
[2]*Nanae Fresh-Water Laboratory, Field Science Center of Northern Biosphere, Hokkaido University, Nanae, 041-1105, Japan*

ABSTRACT—In Memanbetsu town, Hokkaido island, Japan, a high frequency of natural triploid loaches *Misgurnus anguillicaudatus* (7.4% on average) was detected by flow cytometry for relative DNA content. Among sympatric diploid females (n=6) from a single population, we found two unique females that laid unreduced diploid eggs. They gave normal diploid progeny even after induction of gynogenesis with genetically inert UV-irradiated sperm. When fertilized with normal loach sperm, some unreduced eggs developed into triploids, but the rest into diploids. Hybridization using goldfish *Carassius auratus* sperm gave both normal diploid loaches and inviable allotriploid hybrids possessing the diploid loach genome and the haploid goldfish genome. Microsatellite genotyping and DNA fingerprinting demonstrated that the diploid progeny developing from the unreduced eggs were genetically identical to the mother, while the triploids had some of the paternal DNA. These results indicate that the diploid eggs reproduced unisexually as a diploid clone and in other cases developed into triploids after accidental incorporation of sperm nucleus. The presence of at least one clonal line in this area was shown by the identical DNA fingerprint detected in five out of 17 diploid loaches examined.

Key words: unreduced egg, clone, unisexual reproduction, triploid, loach

INTRODUCTION

Unisexual biotypes have been recorded in fishes (Vrijenhoek *et al*., 1989) and they are often associated with interspecific hybridization, all-female sexuality, aberrant gametogenesis and natural polyploidy (Dawley, 1989; Vrijenhoek, 1989). The occurrence of unisexual, clonal and/or polyploid biotypes caused by apparent hybridization has been reported in fish of the genera *Phoxinus* (Goddard and Dawley, 1990), *Fundulus* (Dawley, 1992), *Rutilus* (Alves *et al.,* 1998, 1999), *Cobitis* (Kim and Lee, 2000) and *Oryzias* (Shimizu *et al.,* 2000). Since the unisexual reproduction is generally a rare phenomenon in vertebrates, it is difficult to identify it in wild populations without careful surveillance of unusual occurrences such as aberrant sex ratios or the apparent appearance of hybrids and/or polyploids. However, the cryptic nature of unisexual biotypes suggests that there may be many more examples awaiting discovery (Dawley, 1989).

In the loach *Misgurnus anguillicaudatus* Cantor (Cypriniformes: Cobitidae), bisexual diploid individuals (2n=50) are most common among Japanese populations, but tetraploid and triploid individuals have been found infrequently among specimens obtained from fish dealers in Japan (Ojima and Takai 1979; Arai *et al.,* 1991). The actual origin of these polyploids is still unknown. Recently, Zhang and Arai (1999) examined loaches in 35 populations in Japan using flow cytometry for relative DNA content. They reported no polyploids in 26 places, a few triploids (1.2–3.2%) in 6 places and relatively high frequencies of triploids in Hirokami Village, Niigata Prefecture (average 7.6%) and Ichinomiya town, Aichi Prefecture (7.7%), Honshu island. In the Hirokami population, they also found a unique diploid female that produced unreduced diploid eggs. When eggs of this female were fertilized with genetically inert UV-irradiated sperm, the gynogens from normal haploid eggs died, whereas those from unreduced diploid eggs survived. Fertilization of such diploid eggs with normal haploid sperm could explain the occurrence of natural triploid in this area. How-

* Corresponding author: Tel. +81-138-40-5535;
FAX. +81-138-40-5537.
E-mail: araikt@fish.hokudai.ac.jp

ever, unisexual reproduction was not triggered in any unreduced diploid eggs of the loach from the Hirokami population. No clear genetic evidence of unisexual reproduction has been obtained in diploid individuals of this species.

When we examined the distribution of natural triploid loaches among seven localities in Hokkaido island, Japan, samples collected from the northern area (Memanbetsu and Furen town) included many triploids. We also identified diploid females which were capable of producing unreduced eggs. When these eggs were fertilized with normal loach sperm or with normal sperm of the goldfish *Carassius auratus* (Cypriniformes:Cyprinidae), some progeny were triploid or hybrid but the remainders were diploid and genetically identical to the mother. Therefore, the diploid progeny were actually unisexual clones. In the present paper, we report the presence of a cryptic clonal line in the diploid loach by providing genetic evidence from induced gynogenesis, interspecific hybridization, microsatellite genotyping and multilocus DNA fingerprinting.

MATERIALS AND METHODS

Specimens examined and ploidy determination

To survey the frequency of natural polyploidy in loach, a total of 967 individuals were collected from Memanbetsu town, Furen town, Shibetsu city, Asahikawa city, Akkeshi town, Ebetsu city and Nanae town in Hokkaido island, Japan from 1998 and 2000 (Fig. 1). Due to the small sizes of most samples, sex was not determined in individual specimens except for several loaches used for breeding experiments. In addition, some mature females and males were collected from other two localities, Iwamizawa city and Ohno town, for breeding experiments (Fig. 1). All fishes collected were subjected to flow cytometry for relative DNA content of erythrocytic and caudal fin-epithelium cells in order to determine their ploidy status. When the normal diploid loaches exhibit DNA values of 2C in somatic cells, triploid and tetraploid loaches exhibit values of approximately 3C and 4C, respectively. Aneuploidies exhibit values in the range between two euploid DNA contents. Relative DNA content of fishes collected in 1998 was analyzed by excitation at 488 nm with a laser of the flow cytometer FACS Calibur (Becton Dickinson Biosciences, USA) after sample preparation including PI (propidium iodide) staining according to Zhang and Arai (1996). Samples collected in 1999 and 2000 were measured for DNA content by excitation in the near-ultraviolet (UV) range from a mercury lamp of the flow cytometer PA (Partec GmbH, Germany), after DAPI (4',6-deamidino-2-phenylindole) staining with a preparation kit for animal cells (#06-5-4003) provided by the manufacturer of the flow cytometer (Partec GmbH, Germany).

Induced gynogenesis and interspecific hybridization

In June 2, 2000, six mature diploid females (Nos. 1–6) were selected from the samples (n=136) collected from Memanbetsu town, Hokkaido, and injected with human chorionic gonadotrophic hormone (hCG, Gonatropin ^{R}, Teikoku Zouki, Japan) to induce ovulation according to the procedure described by Suzuki and Yamaguchi (1975). One male (A) from Shibetsu city, one male (B) from Iwamizawa city (Fig. 1) where no triploid has been recorded (Zhang and Arai, 1999) and two males (C, D) from Memanbetsu

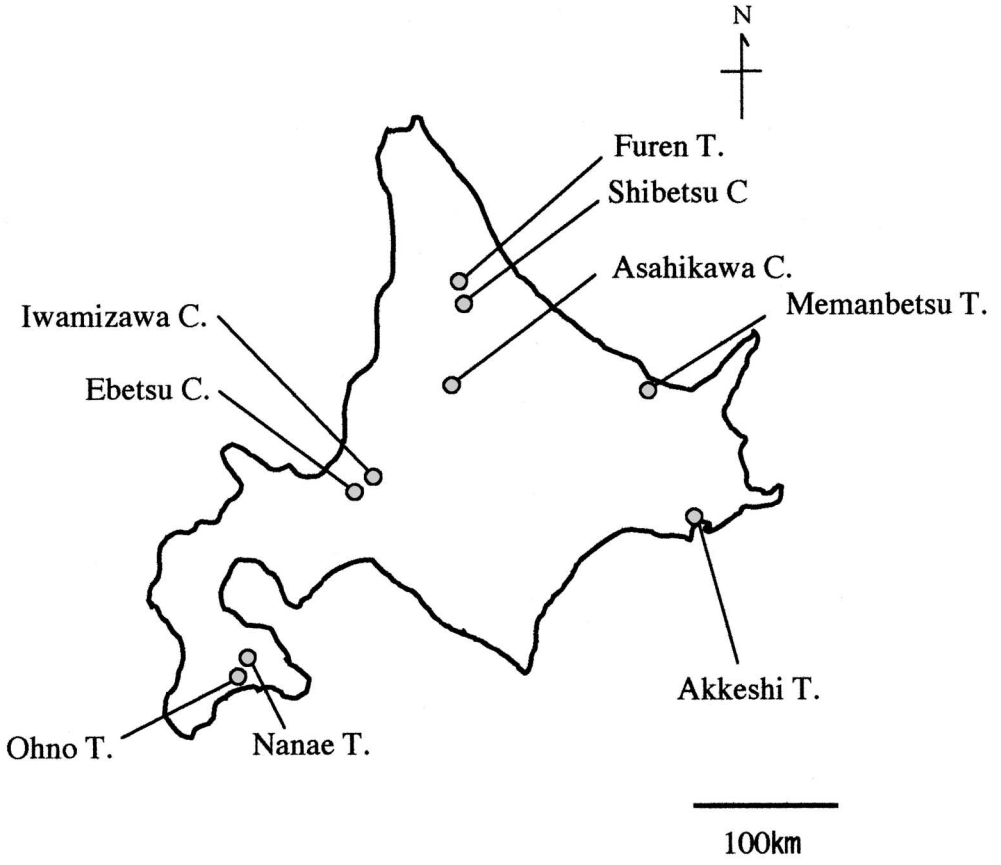


Fig. 1. Sampling localities of the loach *Misgurnus anguillicaudatus* in Hokkaido island, Japan. C and T mean city and town, respectively.

town were used for normal fertilization. Sperm was obtained with a capillary tube or syringe by squeezing the abdomen of each diploid male and then diluted with physiological saline as described in Suzuki *et al*. (1985). Sperm of goldfish (*Carassius auratus,* Cyprinidae) held in the Aquarium center of the Graduate School of Fisheries Sciences, Hokkaido University was also obtained in the same manner. Some eggs from each female were fertilized with sperm from one of the four male loaches. The remainder of each female eggs was fertilized with ultraviolet ray (UV) irradiated sperm of the goldfish in order to induce gynogenetic development, following the procedure described by Suzuki *et al.* (1985). Female No. 5 was not used for normal fertilization due to a shortage of spawned eggs.

Fertilized eggs of each cross were counted and incubated in a plastic pan half-filled with freshwater at room temperature. Hatched fry were counted at one day after fertilization at 28°C. After absorption of the yolk sac, all the resultant progeny were fed brine shrimp *Artemia* sp. Survival rates at 10 days after fertilization were recorded. Ploidy status of the resultant progeny was assayed by measuring relative DNA content of the whole body of hatched fry using DAPI staining and the PA flow cytometer described above.

In August 24, 2000, female No. 3 reached maturation again and a second artificial ovulation was made. Some eggs of this female were fertilized with UV-irradiated goldfish sperm so as to trigger gynogenesis. The remainder was fertilized with functional goldfish sperm. According to Suzuki (1968) and Kijima *et al*. (1996), this latter cross is expected to result in inviable interfamilial hybrids. In addition, diploid female No. 7 collected from Ohno town (Fig. 1) and diploid female No. 8, which was the progeny of the cross between an orange variant and an individual from Saitama Prefecture, Honshu island, Japan, were used for induced gynogenesis using UV-irradiated goldfish sperm as well as interfamilial hybridization using normal goldfish sperm. Normal fertilization of female No. 7 with sperm of diploid loach (male E) collected in Akkeshi town, Hokkaido was performed as a control.

Fertilized eggs were incubated according to the procedure mentioned above. Hatching rate was recorded one day after fertilization and their normal rate of hatched fry was also examined. Flow cytometry with the DAPI staining for DNA content was made to determine the ploidy status as well as the hybrid nature of the resultant progeny.

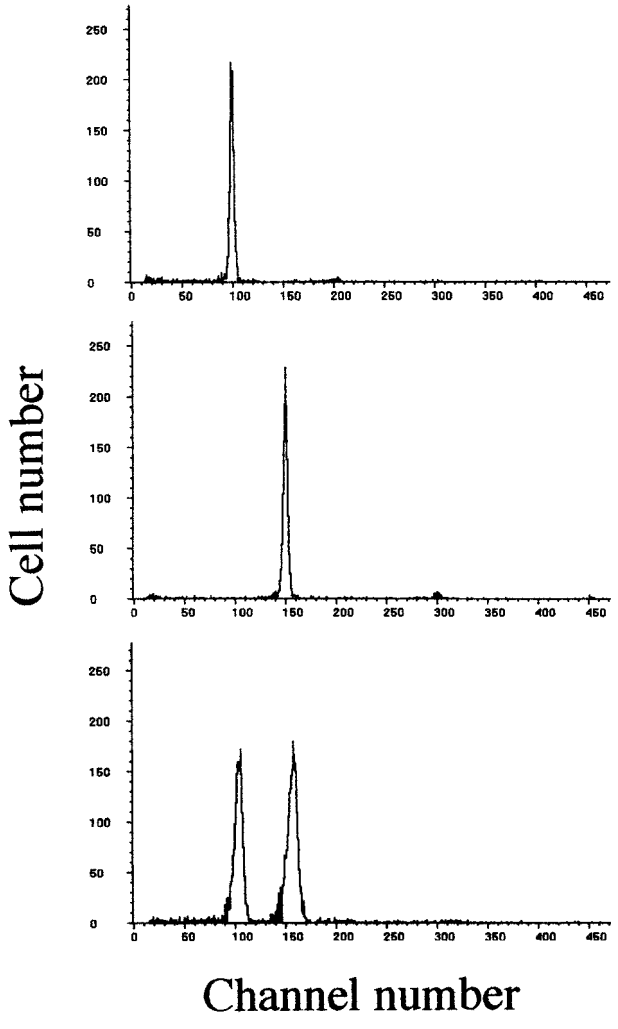


Fig. 2. Flow cytometric histograms for relative DNA content of somatic cells when erythrocytes of normal diploid loach are used as standard of 2C. Top to bottom; diploid, triploid and diplo-triploid mosaic showing 2C, 3C and both 2C and 3C peaks, respectively, in the loach collected in wild populations in Hokkaido, Japan.

Microsatellite analysis

The offspring of female No. 1, No. 3 and No. 6 were genotyped at six microsatellite loci. DNA samples were extracted and purified from the fin or muscle of each parental fish used for the cross and from whole body or muscle of surviving fry and juveniles by the procedure previously reported in Arai and Mukaino (1997). Six primer sets for amplification of *Mac2*, *Mac3*, *Mac24*, *Mac37*, *Mac45* and *Mac49* microsatellite loci developed by Morishima *et al.,* (2001) were used in the present study. Molecular cloning for microsatellite regions and designing of primer sets based on sequence data deposited in DDBJ/EMBL/GENBANK (accession number AB 060172–060186) were already reported in Morishima *et al*. (2001). The PCR was carried out in a reaction mixture (20 μl) containing

Table 1. Frequency of natural triploid loaches in wild populations of Hokkaido island, Japan.

Locality[a]	Year	Fish No.	Ploidy[b] 2n	3n	2n/3n	3n(%)[c]
Memanbetsu T.	1998	76	69	7	0	9.2
	1999	114	109	5	0	4.4
	2000	136	124	10	2	8.8
	Sum	326	302	22	2	7.4
Furen T.	1998	7	6	1	0	14.3
	1999	93	89	4	0	4.3
	2000	108	85	22	1	21.3
	Sum	208	180	27	1	13.5
Shibetsu C.	1999	72	72	0	0	0
	2000	25	24	1	0	4.0
	Sum	97	96	1	0	1.0
Asahikawa C.	1999	54	54	0	0	0.0
	2000	14	14	0	0	0.0
	Sum	68	68	0	0	0.0
Akkeshi T.	1998	29	28	1	0	3.4
Ebetsu C.	1999	14	14	0	0	0.0
Nanae T.	1999	225	225	0	0	0.0
total		967	913	51	3	5.6

[a] T, town ; C, city, [b] Based on relative DNA content by flow cytometry, [c] Including 2n/3n mosaics.

50–100 ng template DNA and 0.1 U *Taq* polymerase (TaKaRa, Japan) in the conditions for each primer set reported by Moroshima *et al.* (2001). PCR product was mixed with loading buffer and electrophoresed in 15% nondenaturing polyacrylamide gel. Amplified fragments were visualized by ethidium bromide staining and UV transillumination. Microsatellite alleles were designated according to their molecular size (base pair).

DNA fingerprinting analysis

For multilocus DNA fingerprinting, purified DNA was digested with *Hae* III (Takara, Japan), underwent electrophoresis at 60V for 37hr or more in 0.8% agarose gel with the size of 200 mm width and 250 mm length, and then transferred to a nylon membrane by capillary action according to the procedure reported in Arai and Mukaino (1997) with some modifications. The membrane was then baked to fix DNA. Tetranucleotide repeats $(GGAT)_4$ were commercially synthesized and then prepared by a labeling kit Light Smith II ™ (Promega, USA). The probe was hybridized and fragments were visualized as described by Spruell *et al.,* (1994). A commercially available NICE™ 33.15 (Jeffreys *et al.,* 1985) probe kit (Cellmark Diagnostic, UK) was also used. Hybridization and visualization were made as described in Arai and Mukaino (1997).

DNA fragments were visually compared and scored in the range between 9 and 6 kilobase pairs to calculate band sharing indices (BSIs) according to the following formula proposed by Wetton *et al.* (1987), $2N_{ab} / (N_a + N_b)$, where N_a and N_b are the total number of DNA fragments scored in individual a and b and N_{ab} is the number of fragments shared by both individuals. BSIs were calculated among 9 diploid and 8 triploid individuals from the Memanbetsu population, analyzed on the same gel and the same membrane.

RESULTS

Frequency of natural triploid loaches

Among 967 loach samples collected from seven localities in three years, the occurrence of normal diploids and spontaneous triploids including diplo-triploid mosaics was confirmed by relative DNA content measured by the flow cytometry (Fig. 2). As shown in Table 1, we detected 913 diploid, 51 triploid and 3 diplo-triploid mosaic individuals. No tetraploid or other polyploidies were observed. Of seven localities, Memanbetsu and Furen town gave remarkably high incidences of triploidy including mosaicism, with averages 7.4% (range 4.4–9.2%) and 13.5% (4.3–21.3%), respectively.

Induced gynogenesis

As shown in Table 2, five diploid females (Nos. 1–4, 6) gave 47.6–94.1% (average 77.9%) hatching rates after fertilization with normal sperm of diploid males. Survival of the 10-day-old feeding fry was 86.6–98.8% (average 96.6%) of the numbers hatched, indicating a good quality of eggs used. When eggs of six diploid females (Nos. 1–6) were inseminated with UV-irradiated goldfish sperm, hatching rates of the resultant gynogenetic progeny were very low (0–7.2%, average 4.5%). Most hatching fry of females No. 1, No. 2, No. 4 and No. 5 exhibited characteristic inviable abnormalities referred to as haploid syndrome (Fig. 3a). Gynogens developing from the other two females No. 3 and No. 6 were externally normal and viable (Fig. 3b). At 10 days after fertilization, a high percentage of these fry (83.7–96.2%) survived and reached the feeding stage. The gynogenetic progeny of females No. 1, No. 2 and No. 5 died before the feeding stage. Only one gynogen was exceptionally survived in the progeny of female No. 4.

Flow cytometry for relative DNA content of surviving fry

Table 2. Developmental potential and relative DNA content of the progenies from normal fertilization and induced gynogenesis of the eggs from six females caught in Memanbetsu, Hokkaido.

Exp.	Cross		Egg	Hatch[a]		Survival[b]		Relative DNA content(C)			
	Female × Male		*n*	*n*	%[c]	*n*	%[d]	*n*	2	3	Others
Normal	1	A	1281	812	63.4	799	98.4	50	50	0	0
fertilization	1	B	592	491	82.9	485	98.8	–	–	–	–
	1	C	747	608	81.4	579	98.2	–	–	–	–
	2	A	812	764	94.1	755	98.8	50	50	–	–
	3	A	629	522	83.0	501	96.0	70	65	4	1[e]
	4	A	955	888	93.0	881	99.2	50	50	–	–
	6	D	485	231	47.6	200	86.6	20	3	12	5[f]
Induced	1	UV[g]	570	32	5.6	0	0	–	–	–	–
gynogenesis	2	UV	951	66	6.9	0	0	–	–	–	–
	3	UV	725	52	7.2	50	96.2	5	5	0	0
	4	UV	826	2	0.2	1	50.0	–	–	–	–
	5	UV	122	0	0	0	0	–	–	–	–
	6	UV	676	49	7.2	41	83.7	5	5	0	0

[a] 1 day after fertilization(incubation temperature 28°C, [b] 10 days after fertilization(incubation temperature 28°C),
[c] Relative to total number of eggs, [d] Relative to total number of hatched fry, [e] 2C/3C(2n/3n) mosaic
[f] 2C/3C(2n/3n) mosaic : 1; 1C/2C/5C(1n/2n/5n) mosaic : 3; 2.5C/3.5C(2.5n/3.5n) mosaic : 1,
[g] Ultraviolet ray irradiated goldfish sperm.

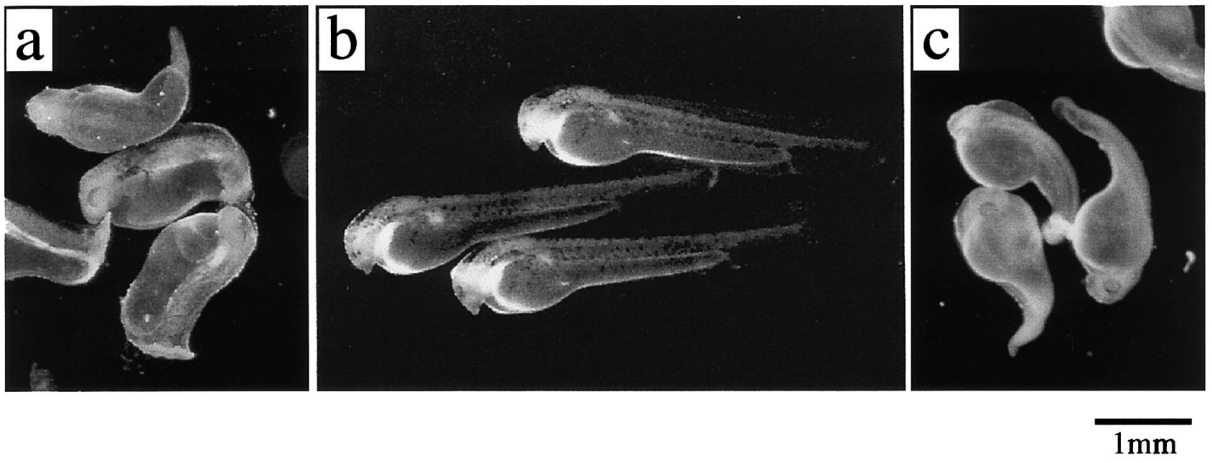


Fig. 3. External appearance of loach progeny developing from induced gynogensis (a, b) and interfamilial hybridization (c). a; abnormal loach larvae, gynogenetically induced from eggs of besexually reproducing females (Nos. 1, 2, 4 and 5) by fertilization with UV-irradiated sperm, b; normal loach larvae, gynogenetically induced from unreduced eggs of putative unisexual females (Nos. 3 and 6), c; abnormal hybrid larvae from the hybridization between loach female and goldfish male.

developing from gynogenetically activated eggs of the two females (No. 3 and No. 6) disclosed that these fry were diploids (Table 2). Thus, the eggs giving rise to viable gynogen even after fertilization with genetically inert sperm are unreduced diploids. In the normal fertilization experiments, female No. 1, No. 2 and No. 4 gave only diploid progeny only with 2C DNA content after normal fertilization. Thus, they generated regular haploid eggs. Females which gave viable diploid gynogens after fertilization with UV-irradiated sperm (No. 3 and No. 6) should give triploids when fertilized with normal sperm. However, female No. 3 gave 93% (65/70) diploid offspring and female No. 6 gave 15% (3/20) diploid offspring in the control crosses (Table 2). Female No. 3 gave 6% (4/70) and female No. 6 gave 60% (12/20) triploid progeny. Both females also produced diplo-triploid, haplo-diplo-pentaploid and 2.5n-3.5n aneuploid mosaic progeny. The occurrence of diploid progeny suggests possible unisexual development in some eggs of the two unique females No. 3 and No. 6.

Interspecific hybridization

To verify unisexual development of the unreduced eggs, ovulation was induced again in female No. 3 and the eggs were fertilized with functional and with UV-irradiated goldfish sperm. At the same time, eggs of the other two females (No. 7 and No. 8) were investigated for comparison.

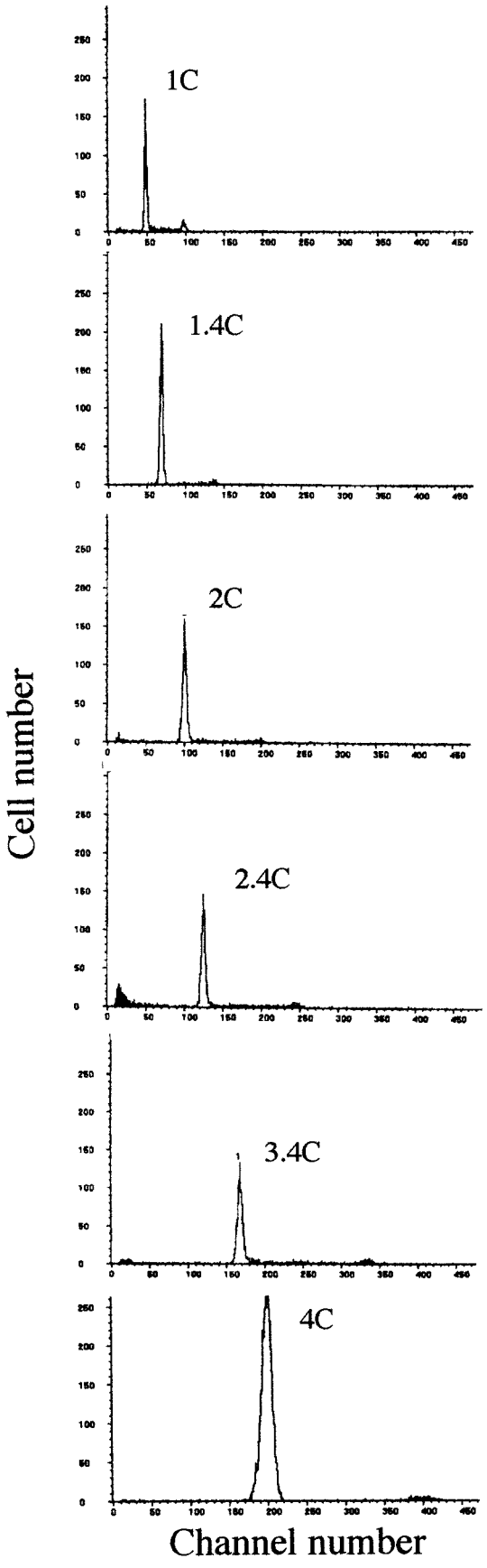
As shown in Table 3, normal hatching fry occurred in the gynogenetic progeny of female No. 3 and 86% (51/59) of them were diploid. However, one triploid, five tetraploids (Fig. 4) and two about 2.5n heteroploids were also observed. In contrast, other two females, No. 7 and No. 8, gave only haploid (1C DNA content) fry after induction of gynogenesis. These results reveal that female No. 3 generated unreduced diploid eggs again, but the other spawned haploid eggs.

In hybridization experiments (Table 3), female No. 3 gave 65.2% (30/46) normal loach fry, whereas only abnormal fry appeared from the eggs of females No. 7 and No. 8 (Table 3, Fig. 3c). Flow-cytometry for relative DNA content

Table 3. Developmental potential and DNA content of the progenies from normal fertilization, induced gynogenesis, and hybridization of eggs from three loach females.

Exp.	Cross		Egg	Hatch[a]		Normal[b]		Relative DNA content(C)							
	Female × Male		*n*	*n*	%	*n*	%	*n*	1	2	2.4	3	3.4	4	Others
Normal fertilization	7	E	452	402	88.9	402	100	–	–	–	–	–	–	–	–
Induced gynogenesis	3	UV[c]	403	114	28.3	112	98.2	59	0	51	0	1	0	5	2[d]
	7	UV	487	167	34.3	1	0.6	20	20	0	0	0	0	0	0
	8	UV	344	71	20.6	0	0	–	–	–	–	–	–	–	–
Hybridization	3	goldfish	140	46	32.9	30	65.2	27	0	13	0	0	12	0	2[e]
	7	goldfish	298	231	77.5	0	0	–	–	–	–	–	–	–	–
	8	goldfish	445	251	56.4	0	0	19	0	0	18	0	0	0	1[f]

[a] 1 day after fertilization, [b] Relative to total number of hatched fry, [c] Ultraviolet ray irradiated goldfish sperm,
[d] 2.5C(2.5n) heteroploid, [e] 3C/3.4C(3n/3.4n) mosaic : 1; 4.4C(4.4n) heteroploid : 1, [f] 1C/2.4C(1n/2.4n) heteroploid mosaic



showed that 48% (13/27) fry from female No. 3 gave just 2C DNA, equivalent to diploid loach, whereas almost all the other fry had 3.4C DNA content (Fig. 4). A small number of aneuploid mosaics were detected. Since the relative DNA content of goldfish sperm was approximately 1.4C in contrast to 1C of the loach sperm (Fig. 4), the fry with 3.4C were allotriploid hybrids possessing two genomes (diploidy, 2C) from the loach and one genome (haploidy, 1.4C) from the goldfish. Abnormal fry developing from the eggs of female No. 8 had 2.4C DNA content in most cases (Fig. 4), thus being diploid hybrids possessing one loach genome (haploidy, 1C) and one goldfish genome (haploidy, 1.4C). Excep-

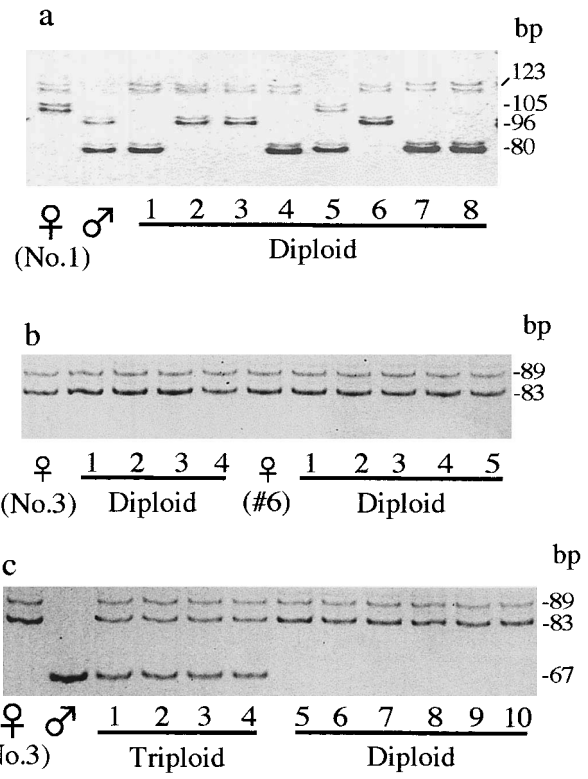


Fig. 5. Microsatellite genotypes in the normally fertilized and gynogenetically induced progeny of the loach. a; *Mac3* genotypes among diploid progeny (Nos. 1–8) developing from normally fertilized eggs of female No. 1, b; *Mac37* genotypes among UV-induced gynogenetic diploid progeny developing from unreduced eggs of female No. 3 (left, Nos. 1–4) and No. 6 (right, Nos. 1–5), c; *Mac37* genotypes among triploid (Nos. 1–4) and diploid (Nos. 5–10) progeny developing from normally fertilized unreduced eggs of female No. 3.

Fig. 4. Flow cytometric histograms for relative DNA content of somatic cells and spermatozoa, when erythrocytes of normal diploid loach are used as standard of 2C. Top to bottom; 1C DNA content of loach spermatozoa, 1.4C DNA content of goldfish spermatozoa, 2C DNA content of diploid loach, 2.4C DNA content of the hybrid between normal eggs of loach female No. 8 and spermatozoa of goldfish male, 3.4C DNA content of the allotriploid hybrid between unreduced eggs of loach female No. 3 and spermatozoa of goldfish male, and 4C DNA content of spontaneous tetraploid fry developing from induced gynogenesis of unreduced eggs of loach female No. 3 after fertilization with UV-irradiated spermatozoa.

tionally, one fry was a mosaic with loach haploid cells and loach-goldfish hybrid cells. These results clearly indicate that some eggs of the female loach No. 3 developed without any genetic contribution from the father fish, but other eggs did incorporate DNA from the sperm nucleus in the process of fertilization.

Microsatellite genotyping

To confirm unisexual development of some unreduced eggs of the females No. 3 and No. 6, genetic transmission of microsatellite markers was analyzed. As shown in Fig. 5a and Table 4, alleles at the microsatellite loci (primer sets) segregated according to Mendelian expectations in the normal diploid progeny of normal loach female No. 1. Mendelian segregation of the six microsatellite loci used in the present study has already been confirmed in some diploid families by Morishima *et al.* (2001). Thus, in that study, diploid progeny of the normal loach females had both maternally and paternally derived alleles in each genotype after normal fertilization, revealing bisexual reproduction.

In UV-induced diploid gynogens developing from putative unreduced eggs of females No. 3 and No. 6, only microsatellite genotypes identical to the mother fish were detected at each locus (Fig. 5b, Table 4), clearly indicating the unisexual transmission of maternal genes. Among the progeny developing from the unreduced eggs after normal fertilization with loach sperm, diploid individuals gave uniparental genotypes identical to the mother, whereas triploid individuals showed genotypes comprising both two alleles from the mother and one allele from the father (Fig. 5c, Table 4). These genetic results indicate that some unreduced eggs of females No. 3 and No. 6 developed unisexually without any DNA contribution from the sperm nucleus, but other eggs incorporated the sperm genome, probably accidentally. Most unreduced eggs are initially diploid as observed in the genotypes of artificially induced gynogenesis.

Interestingly, microsatellite genotypes at the six loci examined were completely identical between the diploid sib-progeny and the mother loaches (No. 3 and No. 6) as shown in Table 4. This indicates that the unreduced diploid eggs of such females develop unisexually as well as clonally. Identical microsatellite genotypes between female No.3 and No.6 also suggest that these two females may be a member of a single cryptic diploid clonal line which reproduces unisexually.

Multilocus DNA fingerprinting

So as to confirm the clonal nature of the progeny, genetic identity was then examined in a wide range of loach genomes by means of the multilocus DNA fingerprinting technique. DNA fingerprints detected with $(GGAT)_4$ probing after *Hae* III digestion depicted that the diploid progeny (n=4) were genetically identical and absolutely clonal to the

Table 4. Microsatellite genotypes in the progenies of normal fertilization and induced gynogenesis using the eggs of loach females No.1, No.3 and No.6.

Female	Microsatellite	Parental genotype		Normal fertilzation		Induced gynogenesis	
No.	locus	Female	Male	*n*	2n genotype : *n*. (3n genotype : *n*.)	*n*	2n genotype : *n*.
1	*Mac 2*	*75/75*	*75/75*	8	*75/75* : 8	–	ND
	Mac 3	*105/123*	*80/96*	8	*80/105* : 1, 96/105 : 4, 80/123 : 3	–	ND
	Mac 24	*90/90*	*90/90*	8	*90/90* : 8	–	ND
	Mac 37	*67/67*	*67/67*	8	*67/67* : 8	–	ND
	Mac 45	*79/79*	*79/79*	8	*79/79* : 8	–	ND
	Mac 49	*92/96*	*94/98*	8	*92/94* : 4, *92/98* : 1, *94/96* : 1, *96/98* : 2	–	ND
3	*Mac 2*	*107/113*	*75/75*	18	*107/113* : 14, (*75/107/113* : 4)	4	*107/113* : 4
	Mac 3	*86/86*	*80/96*	18	*86/86* : 14, (*86/96*[a] : 2, *80/86*[b] : 2)	4	*86/86* : 4
	Mac 24	*92/96*	*90/90*	18	*92/96* : 14, (*90/92/96* : 4)	4	*92/96* : 4
	Mac 37	*83/89*	*67/67*	18	*83/89* : 14, (*67/83/89* : 4)	4	*83/89* : 4
	Mac 45	*77/89*	*79/79*	18	*77/89* : 14, (*77/79/89* : 4)	4	*77/89* : 4
	Mac 49	*92/96*	*94/98*	18	*92/96* : 14, (*92/96/98* : 2, *92/94/96* : 2)	4	*92/96* : 4
6	*Mac 2*	*107/113*	*75/75*	5	*107/113* : 1, (*75/107/113* : 4)	5	*107/113* : 5
	Mac 3	*86/86*	*96/96*	5	*86/86* : 1, (*86/96*[a] : 4)	5	*86/86* : 5
	Mac 24	*92/96*	*90/90*	5	*92/96* : 1, (*90/92/96* : 4)	5	*92/96* : 5
	Mac 37	*83/89*	*67/67*	5	*83/89* : 1, (*67/83/89* : 4)	5	*83/89* : 5
	Mac 45	*77/89*	*79/79*	5	*77/89* : 1, (*77/79/89* : 4)	5	*77/89* : 5
	Mac 49	*92/96*	*86/86*	5	*92/96* : 1, (*86/92/96* : 4)	5	*92/96* : 5

[a] Triploidy was flow-cytometrically confirmed. Probable genotype is *86/86/96*.
[b] Triploidy was flow-cytometrically confirmed. Probable genotype is *80/86/86*.

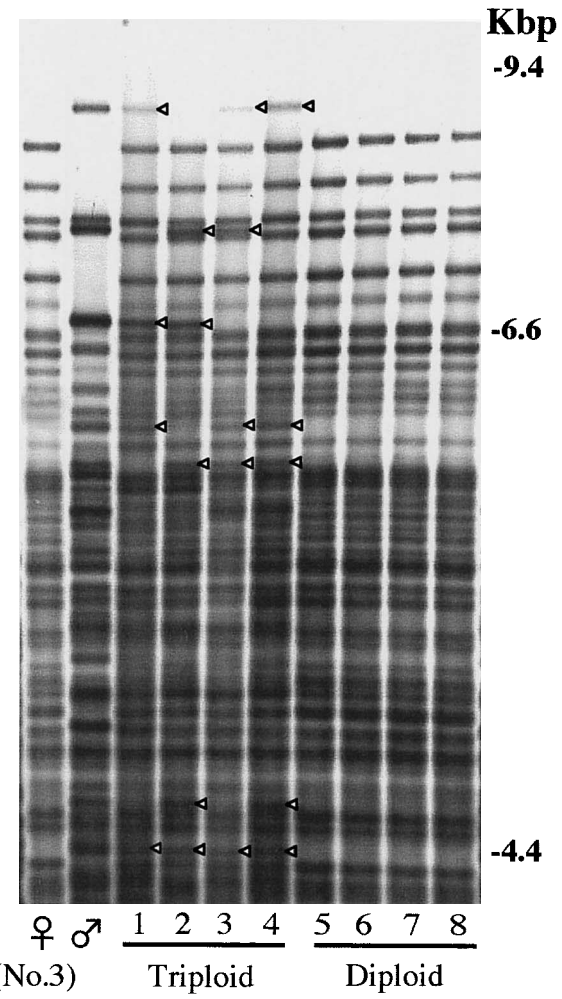


Fig. 6. Multilocus DNA fingerprints hybridized with $(GGAT)_4$ probe in *Hae* III digested samples from diploid (lanes 5–8) and triploid (lanes 1–4) progeny developing from the fertilization of unreduced eggs of female No. 3 with sperm of male A. Triangles denote sperm DNA.

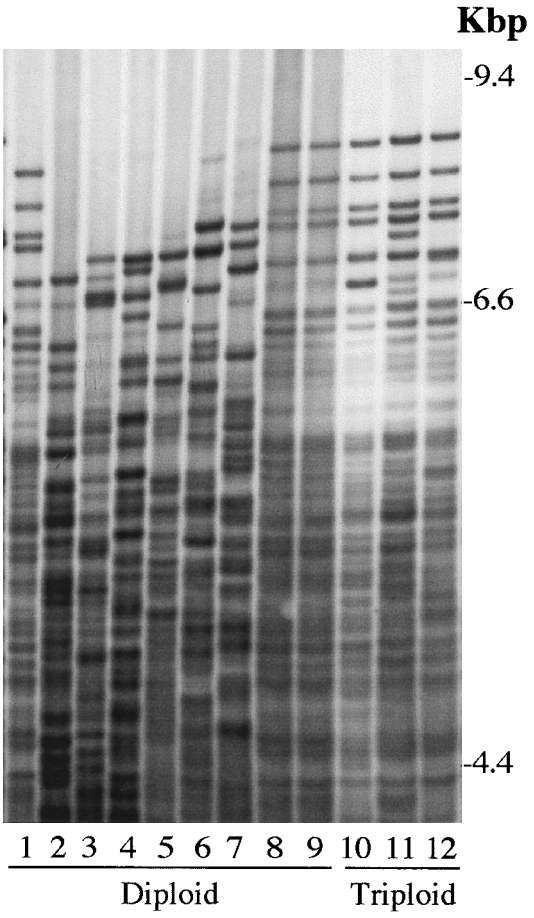


Fig. 7. Multilocus DNA fingerprints hybridized with $(GGAT)_4$ probe in *Hae* III digested diploid (lanes 1–9) and triploid (lanes 10–12) loach samples collected from the Memanbetsu population, Hokkaido, Japan. Note the identical fingerprints among loach in lanes 1, 8 (=female No. 3) and 9 (=female No. 6) and close similarities between clonal diploids (lanes 1, 8 and 9) and natural triploid individuals (lanes 10–12).

mother fish (female No. 3) without any contribution from the alleged father (Fig. 6). DNA fingerprints in the triploid progeny included some of the fragments from the father together with all the fragments possessed by the mother (Fig. 6). DNA fingerprinting with a different probe 33.15 in the same *Hae* III digested samples also gave similar results (figure not shown). Thus, the clonal nature of the diploid eggs is proven and the occurrence of triploid progeny can be explained by genomic incorporation of sperm nuclear DNA. DNA fingerprinting of the diploid and triploid progeny of No. 6 gave the same conclusion.

Identical DNA fingerprints between females No. 3 and No. 6 suggest the existence of a clone in the wild population of Memanbetsu town. Finally, 17 diploid individuals including females No. 3 and No. 6 and 8 triploid individuals from the wild population of the same town were genetically assayed in 2000 to identify clonal individuals by DNA fingerprinting. As shown in Fig. 7, clonal individuals were easily detected by their absolutely identical DNA fingerprints. Five out of 17 diploids examined had an identical pattern and therefore were considered to be a clonal line. At least one clonal line exists in this population.

In the DNA fingerprints probed with $(GGAT)_4$ and probe 33.15, ranging between 6 and 9 kilobase pairs, 6 to 20 (mean 15.3) and 6 to 18 (mean 12.3) fragments were counted, respectively. BSIs analyzed in five clonal diploids, 12 aclonal common diploids and 8 triploids are shown in Table 5. BSIs within clonal line were 1.0 for both probes, while those within common diploid loaches were 0.40 for $(GGAT)_4$ and 0.44 for 33.15 probe. BSIs between clonal and aclonal diploids were 0.28 for $(GGAT)_4$ and 0.25 for 33.15 probe, suggesting a genetically remote relationship between the two.

DNA fingerprinting revealed that all the natural triploid individuals examined (n=8) were not genetically identical, because each individual exhibited a specific pattern as

Table 5. Band sharing indices(BSIs) in DNA fingerprints probed with $(GGAT)_4$ and 33.15 in *Hae* III digested loach samples.

BSIs	$(GGAT)_4$	33.15
Within clonal 2n(*n*=5)	1.00	1.00
Within aclonal 2n(*n*=12)	0.40±0.07	0.44±0.10
Within 2n(*n*=17)	0.39±0.20	0.39±0.20
Within 3n(*n*=8)	0.93±0.03	0.84±0.10
Between clone and aclonal 2n	0.28±0.06	0.25±0.10
Between aclonal 2n and 3n	0.31±0.07	0.32±0.10
Between clonal 2n and 3n	0.96±0.04	0.89±0.10
Between 3n and 2n	0.53±0.31	0.51±0.30

shown in Fig.7. However, all triploids were genetically very similar to each other as indicated by high values of BSIs between triploids, 0.93 for $(GGAT)_4$ and 0.84 for 33.15 probe (Table 5). BSIs between triploids and clonal diploids were high (0.96 for $(GGAT)_4$ and 0.89 for 33.15 probe), but those between triploids and aclonal diploids are very low (0.31 for $(GGAT)_4$ and 0.32 for 33.15 probe). This indicates that natural triploids in this area may be generated by fertilization of clonal diploid eggs with normal haploid sperm.

DISCUSSION

The appearance of viable diploid progeny after UV-induced gynogenesis without any additional treatment to duplicate the chromosome set of the eggs shows that some diploid loaches in the Memanbetsu population, Hokkaido, Japan, generate unreduced diploid eggs. Surprisingly, a considerable number of diploid individuals occurred among the progeny when such unreduced eggs were fertilized with normal haploid loach sperm, although only triploid individuals were predicted. Since viable diploid loach fry were produced in female loach x male goldfish crosses, and only maternal microsatellite genotypes were observed in the diploid progeny of female loach x normal male loach crosses, unisexual development of unreduced eggs is the most likely explanation for the appearance of diploid progeny in these crosses. This is confirmed by DNA fingerprinting which revealed that each diploid fry was genetically identical to its diploid mother. In conclusion, two diploid females (No. 3 and No. 6) and their diploid progeny appear to belong to a clonal line that reproduces unisexually.

All-female inheritance found in these loaches is likely to be gynogenesis because UV-treated sperm always triggered such reproduction in all the unreduced eggs. However, normal sperm was sometimes incorporated into the zygotic genome, probably after its accidental pronucleation as discussed below. In teleosts, unisexual development is exclusively gynogenesis (embryogenesis initiated by sperm stimulation without syngamy) and no parthenogenesis (all-female reproduction in the absence of sperm) has been found (Dawley 1989; Vrijenhoek *et al.* 1989). In the cases of loaches studies here, it is not yet established whether unisexual development is simply gynogenetic or absolute parthenogenetic.

The occurrence of unreduced diploid eggs was previously found in the diploid loach from the Hirokami village, Niigata prefecture, Honshu island, Japan in which a high incidence of natural triploids was recorded (Zhang and Arai, 1999). In that case, the diploid loach laid both normal haploid and unreduced diploid eggs at the same time and both diploid and triploid fry were observed in a normally fertilized batch. Thus, the unreduced eggs of the loach from Niigata are different from those from Hokkaido; the former develop bisexually and the latter reproduce unisexually. All the unreduced eggs examined in the present study were genetically identical to the mother and no genetic variation was found among the samples examined.

The cytological mechanism leading to unreduced oogenesis is unknown in the clonal diploid loach observed in the present study. However, two major systems, apomixis and premeiotic endomitosis, may explain such a phenomenon as reported in other polyploid and unisexual vertebrates (Dawley, 1989). Apomixis is the mechanism in which abortive meiosis I inhibits synapsis of homologous chromosomes that never leads to subsequent recombination and segregation. Thus, mature eggs are formed by somatic division. Apomictic oogenesis has been well documented in the triploid silver crucian carp *Carassius auratus gibelio* (Cherfas, 1966), its related Japanese subspecies *Carassius auratus langsdorfii* (Yamashita *et al.,* 1993) and the amazon molly *Poecilia formosa* and its triploid hybrids (Monaco *et al*., 1984). Premeiotic endomitosis is a process which results in the doubling of chromosomes without cytokinesis before meiosis, followed by two successive divisions in a quasinormal manner. In the loach, unreduced triploid eggs are formed in triploid (diploid female x tetraploid male) individuals by this system (Zhang *et al.,* 1998). A similar mechanism has been found in triploid *Poeciliopsis* (Cimino, 1972) and artificial medaka hybrids between *Oryzias latipes* and *O. curvinotus* (Shimizu *et al*., 2000). In this system synapses and recombination occur essentially between the sister replicate chromosomes produced by the endomitosis. This gives rise to no genetic variation, and the genetic composition of the resultant unreduced eggs is theoretically identical to that of the mother. As the mechanism of the formation of unreduced diploid eggs has not been identified in this study, further cytological studies are required in near future.

When the unreduced diploid eggs laid by the Memanbetsu loaches were fertilized with normal sperm, some eggs unisexually (probably gynogenetically) develop into clonal diploids, while the others develop into triploids incorporating nuclear DNA of the sperm. These observations imply that these loaches are not exclusively gynogenetic. Accidental incorporation of sperm nuclear DNA has also been reported in some other unisexual species. In gynogenetically reproducing triploid crucian carp *C. a. gibelio* in China, a few multiple tetraploid individuals possessing the triploid genome of crucian carp and the haploid genome of common carp

occurred (Gui *et al*., 1993). In Japanese silver crucian carp *C. a. langsdorfii*, artificial tetraploids were produced by the incorporation of a haploid sperm genome into triploid eggs at high or low temperature (Takai and Ojima, 1983; Dong *et al*., 1997; Mada *et al*., 2001). Similar sperm nuclear DNA incorporation at high temperature was also reported in polyploid hybrid salamanders of the genus *Ambyostoma* which produced unreduced eggs and reproduced gynogenetically at low temperature (Bogart *et al*., 1989; Elinson *et al*., 1992). The fact that the two clonal females No. 3 and No. 6 gave different frequencies of triploids after sperm nuclear incorporation suggests that the occurrence of gynogenetic or bisexual reproduction depends on environmental factors at the time of fertilization and subsequent early embryogenesis.

Microsatellite genotyping and DNA fingerprinting reveal the presence of at least one clonal line among the Memanbetsu loaches: a relatively large proportion (29.4%, 5 /17) of diploid loaches appear to be members of the same clone. The distribution and frequency of such clonal lines have not been surveyed in other parts of Hokkaido, but their existence is suggested by the high incidence of triploids. Most unisexual fish species have been shown to comprise several clonal lines as evidenced in natural populations of silver crucian carp (Dong and Taniguchi, 1996; Umino *et al*., 1997; Ohara *et al.,* 1998, 1999, 2000), *Poeciliopsis* (Quattro *et al.* 1992ab), *Rutilus* (Alves *et al.* 1998) and other species reviewed by Dawley(1989). Thus, further genetic analyses are necessary to disclose the number, distribution and diversity of clonal lineage(s) among the loaches in Hokkaido. Apparently low BSIs between clonal and aclonal diploid loach lineage indicate that they are dissimilar, suggesting a remotely distant genetic relationship between them.

Sympatric triploids may be the consequence of sperm nuclear incorporation into the clonal diploid eggs. Thus, although all the triploids are genetically similar, they are not identical due to the genetic variability of each sperm nucleus incorporated, as indicated by the various DNA fingerprints observed. High BSI values within triploid groups and those between clonal diploids and triploids also support this conclusion.

It is believed that unisexuality and polyploidy are closely associated with hybridization between bisexual species and subspecies (Dawley, 1989; Vrijenhoek, 1989). A new unisexual lineage can be established if genetic differences between two hybridizing species are sufficient to disrupt the meiotic mechanism (Vrijjenhoek, 1989). The loach *M. anguillicaudatus* is considered as a single species entity in Japan, but recent population genetic studies using allozymes suggest the presence of at least three genetically different groups with subspeces level differentiation (Khan and Arai, 2000). Two biologically distinct types were also recognized based on genetic and morphological data in the loach specimens from Ibaraki prefecture (Dong *et al.,* 1999). These results suggest the possible existence of some cryptic bisexual species in the loach. An unusually high incidence of triploids was found in Memanbetsu population in the present study. Zhang and Arai (1999) found high frequencies of triploids in Hirokami village, Niigata and Ichinomiya town, Aichi prefecture. These three localities seem to be in a zone between genetically distinct groups shown by Khan and Arai (2000). Possible hybridizations between them may cause ameiotic reproduction in the hybrids with the unbalanced genomes. Further investigations are required before the origin and genetic mechanisms incolved in the occurrence of unisexual reproduction and natural triploidy in the loach can be conclusively determined.

ACKNOWLEDGMENTS

We gratefully acknowledge Prof. N. P. Wilkins, National University of Ireland, galway for his critical reading of our manuscript.

REFERENCES

Alves MJ, Coelho MM, Collares-Pereira MJ (1998) Diversity in the reproductive modes of females of the *Rutilus alburnoides* complex (Teleostei, Cyprinidae): a way to avoid the genetic constraints of uniparentalism. Mol Biol Evol 15: 1233–1242

Alves MJ, Coelho MM, Prospero MI, Collares-Pereira MJ (1999) Production of fertile unreduced sperm by hybrid males of the *Rutilus alburnoides* complex (Teleostei, Cyprinidae): An alternative route to genome tetraploidization in unisexuals. Genetics 151: 277–283

Arai K, Mukaino M (1997) Clonal nature of gynogenetically induced progeny of triploid (diploid x tetraploid) loach *Misgurnus anguillicaudatus* (Pisces: Cobitididae). J Exp Zool 278: 413–421

Arai K, Matsubara K, Suzuki R (1991) Karyotype and erythrocyte size of spontaneous tetraploidy and triploidy in the loach *Misgurnus anguillicaudatus*. Nippon Suisan Gakkaish 57: 2157–2172

Bogart JP, Elinson RP, Licht LE (1989) Temperature and sperm incorporation in polyploid salamanders. Science 246: 1032–1034

Cherfas NB (1966) Natural triploidy in females of the unisexual form of silver crucian carp (*Carassius auratus gibelio* Bloch). Genetika 5: 16–24

Cimino MC (1972) Meiosis in triploid all-female fish (*Poeciliopsis,* Poeciliidae). Science 175: 1484–1486

Dawley RM (1989) An introduction to unisexual vertebrates. In "Evolution and Ecology of Unisexual Vertebrates" Ed by RM Dawley and JP Bogart, New York State Museum, Albany, pp 1–18

Dawley RM (1992) Clonal hybrids of the common laboratory fish *Fundulus heteroclitus*. Proc Natl Acad Sci USA 89: 2485–2488

Dong S, Taniguchi N (1996) Clonal nature of offsprings of ginbuna *Carassius langsdorfii* by RAPD-PCR and isozyme pattern. Nippon Suisan Gakkaish 62: 891–896 (In Japanese with English summary)

Dong S, Taniguchi N, Ishida R (1999) Two types of the loach Misgurnus anguillicaudatus collected from the Torentsu River. Jpn J Ichthyol 46: 83–90 (In Japanese with English summary)

Dong S, Ohara K, Taniguchi N (1997) Introduction of sperm of common carp *Cyprinus carpio* into eggs of ginbuna *Carassius langsdorfii* by heat shock treatment and its confirmation by DNA markers. Nippon Suisan Gakkaishi 63: 201–206 (In Japanese with English summary)

Elinson RP, Bogart JP, Licht LE, Lowcock LA (1992) Gynogenetic mechanisms in polyploid hybrid salamanders. J Exp Zool 264: 93–99

Goddard KA, Dawley RM (1990) Clonal inheritance of a diploid

nuclear genome by a hybrid freshwater minnow (*Phoxinus eos-neogaeus*, Pisces: Cyprinidae). Evolution 44: 1052–1065

Gui JF, Liang SC, Zhu LF, Jiang YG (1993) Discovery of multiple tetraploids in artificially propagated populations of allogynogenetic silver crucian carp and their breeding potentialities. Chin Sci Bull 38: 327–331

Jeffreys AJ, Wilson W, Thein SL (1985) Individual-specific "fingerprint" of human DNA. Nature 316: 76–79

Khan MR, Arai K (2000) Allozyme variation and genetic differentiation in the loach *Misgurnus anguillicaudatus*. Fisheries Sci 66: 211–222

Kijima K, Arai K, Suzuki R (1996) Induced allotriploidy in inviable interfamilial hybrids, female loach x male goldfish and female loach x male minnow. J Fac Appl Biol Sci Hiroshima Univ 35: 1–12 (In Japanese with English summary)

Kim LS, Lee EH (2000) Hybridization experiment of diploid-triploid cobitid fishes, *Cobitis sinensis-longicorpus* complex (Pisces: Cobitidae). Folia Zool 49(Suppl.1): 17–22

Mada Y, Miyagawa M, Hayashi T, Umino T, Arai K (2001) Production of tetraploids by induction of sperm nucleus into the eggs of gynogenetic triploid ginbuna *Carassius langsdorfii*. Suisanzoshoku 49: 103–112 (In Japanese with English summary)

Monaco PJ, Rasch EM, Balsano JS (1984) Apomictic reproduction in the Amazon molly, *Poecilia formosa* and its triploid hybrids. In "Evolutionary Genetics of Fishes" Ed by BJ Turner, Plenum, pp 311–328

Morishima K, Nakayama I, Arai K (2001) Microsatellite-centromere mapping in the loach *Misgurnus anguillicaudatus*. Genetica 111(1–3): 59–69

Ohara K, Dong S, Taniguchi N (1998) Identification and distribution of clonal lines detected by DNA polymorphism in silver crucian carp, *Carassius langsdorfii* collected from the Monobe and Niyodo rivers. Japan J Ichthyol 45: 21–27 (In Japanese with English summary)

Ohara K, Dong S, Taniguchi N (1999) High proportion of heterozygotes in microsatellite DNA loci of wild clonal crucian carp, *Carassius langsdorfii*. Zool Sci 16: 909–913

Ohara K, Ariyoshi T, Sumida E, Sitizyo K, Taniguchi N (2000) Natural hybridization between diploid crucian carp species and genetic independence of triploid crucian carp elucidated by DNA markers. Zool Sci 17: 357–364

Ojima Y, Takai A (1979) The occurrence of spontaneous polyploid in the Japanese common loach *Misgurnus anguillicaudatus*. Proc Japan Acad 55B: 487–491

Quattro JM, Avise JC, Vrijenhoek RC (1992a) An ancient clonal lineage in the fish genus *Poeciliopsis* (Atheriniformes: Poeciliidae). Proc Natl Acad Sci USA 89: 348–352

Quattro JM, Avise JC, Vrijenhoek RC (1992b) Mode of origin and sources of genotypic diversity in triploid gynogenetic fish clones (*Poeciliopsis*: Poeciliidae). Genetics 130: 621–628

Shimizu Y, Shibata N, Sakaizumi M, Yamashita M (2000) Production of diploid eggs through premeiotic endomitosis in the hybrid medaka betweem *Oryzias latipes* and *O. curvinotus*. Zool Sci 17: 951–958

Spruel PS, Cummings SA, Kim Y, Thorgaard G (1994) Comparison of three anadromous rainbow trout (*Oncorhynchus mykiss*) populations using DNA fingerprinting and mixed DNA samples. Can J Fish Aquat Sci 51 (Suppl 1): 252–257

Suzuki R (1968) Hybridization experiments in cyprinid fishes XI. Survival rate of F1 hybrids with special reference to the closeness of taxonomical position of combined fishes. Bull Freshwater Fish Res Lab 18: 112–155

Suzuki R, Yamaguchi M (1975) Influence of water temperature on inducing spawning by hormone injectionin the loach, cyprinid fish. Suisanzoshoku 22: 135–139 (In Japanese)

Suzuki R, Oshiro T, Nakanishi T (1985) Survival, growth and fertility of gynogenetic diploids induced in the cyprinid loach, *Misgurnus anguillicaudatus*. Aquaculture 48: 45–55

Takai A, Ojima Y (1983) Tetraploidy appeared in the offspring of triploid ginbuna, *Carassius auratus langsdorfii* (Cyprinidae, Pisces). Proc Japan Acad 59B: 341–350

Umino T, Arai K, Maeda K, Zhang Q, Sakae K, Niwase I, Nakagawa H (1997) Natural clones detected by multilocus DNA fingerprinting in gynogenetic triploid ginbuna *Carassius langsdorfii* in Kurose river, Hiroshima. Fisheries Sci 63: 147–148

Vrijenhoek RC (1989) Genetic and ecological constraints on the origins and establishment of unisexual vertebrates. In "Evolution and ecology of Unisexual Vertebrates" Ed by RM Dawley and JP Bogart. New York State Museum, Albany, pp 24–31

Vrijenhoek RC, Dawley RM, Cole CJ, Bogart JP (1989) A list of known unisexual vertebrates. In "Evolution and ecology of Unisexual Vertebrates" Ed by RM Dawley and JP Bogart, New York State Museum, Albany, pp 19–23

Wetton JH, Carter RE, Parkin DT, Walters D (1987) Demographic study of a wild house sparrow population by DNA fingerprinting. Nature 327: 147–149

Yamashita M, Jiang J, Onozato H, Nakanishi T, Nagahama Y (1993) A tripolar spindle formed at meiosis I assures the retention of the original ploidy in the gynogenetic triploid crucian carp, ginbuna *Carassius auratus langsdorfii*. Dev Growth Differ 35: 631–633

Zhang Q, Arai K (1996) Flow cytometry for DNA contents of somatic cells and spermatozoa in the progeny of natural tetraploid loach. Fisheries Sci 62: 870–877

Zhang Q, Arai K (1999) Distribution and reproductive capacity of natural triploid individuals and occurrence of unreduced eggs as a cause of polyploidization in the loach, *Misgurnus anguillicaudatus.* Ichthyol Res 46: 153–161

Zhang Q, Arai K, Yamashita M (1998) Cytogenetic mechanisms for triploid and haploid egg formation in the triploid loach *Misgurnus anguillicaudatus*. J Exp Zool 281: 608–619

(Received January 10, 2002 / Accepted February 18, 2002)