Disruption of normal meiosis in artificial inter-populational hybrid females of *Misgurnus* loach

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Abstract Artificial cross between two genetically different populations of Japanese *Misgurnus* loach was made to examine the reproductive capacity of the artificial inter-populational hybrid females. Ploidy status and microsatellite genotypes of the eggs laid by these hybrids were inferred from those determined in progenies developed by normal fertilization with haploid loach sperm, induced gynogenesis with UV-irradiated goldfish sperm and/or hybridization with intact goldfish sperm. Some hybrid females laid unreduced diploid eggs genetically identical to the mother. However, these diploid eggs could not develop by spontaneous gynogenesis, but grow to triploid by incorporation of a sperm nucleus. Other hybrid females laid haploid eggs together with diploid eggs and/or various aneuploid and polyploid eggs. Thus, a disruption of normal meiosis occurred in inter-populational hybrid females. The results suggested that the two populations should be so distant as to give rise to atypical formation of unreduced and other unusual eggs in their hybrids.

Key words: clone, gynogenesis, microsatellite, polyploid, unreduced eggs

Introduction

In Japanese loach *Misgurnus anguillicaudatus* (Teleostei:Cobitidae), most individuals are bisexually reproducing diploids, but rare natural triploids and clonal diploids have been recognized in several localities in Japan (Morishima et al., 2002; 2008a). Gynogenetically reproducing clonal diploid loaches were first discovered in the wild population at the northern area of Hokkaido Island, Japan, because they gave viable diploid progeny even after induction of artificial gynogenesis with UV-irradiated sperm and hybridization with goldfish sperm (Morishima et al. 2002). Other clonal lineages with minor genetic variations were also found in several other localities in Japan by screening absolutely identical DNA fingerprints in two or more candidates (Morishima et al. 2008a). Clonal diploid loaches produce genetically identical diploid eggs, most of which develop to clonal diploids by gynogenetic activation with sperm of bisexually reproducing diploid males, but some eggs develop to triploids and diploid-triploid mosaics due to the infrequent incorporation of a sperm nucleus before and after cleavage, respectively (Morishima et al., 2002; Itono et al., 2006, 2007). A majority of such clone-derived triploid females were reported to produce fertile haploid eggs (Oshima et al., 2005; Morishima et al., 2008b), but males are sterile (Oshima et al.,

2005). Diploid-triploid mosaic individuals revealed more complicated reproductive modes; diploid-triploid mosaic males generate fertile diploid sperm with genetically identical clonal genotypes (Morishima et al., 2004), whereas mosaic females produce haploid, clonal diploid and triploid eggs, simultaneously (Yoshikawa et al., 2007). Clonal diploid and its derivative triploid and mosaic individuals are absolutely cryptic in nature, because they are impossible to distinguish from normal bisexual diploid individuals based on external appearances and other morphological characteristics, but both bisexual and clonal diploids inhabit at the same locality, sympatrically in the northern part of Hokkaido Island and a few other localities in Honshu Island, Japan (Morishima et al., 2002, 2008a).

There is a consensus that the occurrence of asexual vertebrates is tightly linked with the natural hybridization of different species, because inter-specific hybridization often causes a disruption of normal meiosis leading to an atypical mode of reproduction such as unreduced gametogenesis, parthenogenesis, gynogenesis, or hybridogenesis (reviewed in Dawley 1989; Vrijenhoek 1989; Beukeboom & Vrijenhoek, 1998). However, it has been very difficult to explain the occurrence of clonal diploid lineage within Japanese loach species by a hybrid-origin, because *M.anguillicaudatus* has been identified as a single species entity (Saitoh 1989). Recently, genetic studies analyzing

sequences of the control region of mtDNA suggested presence of two genetically distinct groups, probably corresponding to independent cryptic species in Japanese *Misgurnus* loach (Morishima et al., 2008a). The same conclusion was also supported by previous studies using allozymes (Khan and Arai, 2000) and microsatellite DNA markers (Arias-Rodriguez et al., 2007). It was also reported that triploid loaches which were derived from a clonal lineage and comprised intact diploid genomes of the clone and haploid genome of the sperm donor, produce haploid gametes by meiotic hybridogenesis: preferential pairing between more matched homologous chromosomes, one set of the clone and counterpart one set of a sperm donor, as well as the elimination of unmatched set of the clone, suggesting presence of two distinct genomes in the clone (Morishima et al., 2008b).

Although the above-mentioned studies suggest the presence of genetically different loaches in Japan, the relationship between the occurrence of atypical mode of reproduction and the hybridization between these loaches has not been examined yet. The present study examined the reproductive capacity of artificial inter-populational hybrids between loaches collected from geographically different populations, probably belonging to different genetic groups. We produced artificial inter-populational hybrids between females taken from the northern area and males from the southern area of

Hokkaido by artificial ovulation and fertilization, and then reared their inter-populational hybrids until maturation. Here, we produced progenies from eggs of these inter-populational hybrid females by normal fertilization with haploid loach sperm, induced gynogenesis with UV-irradiated goldfish sperm and/or interspecific hybridization with intact goldfish sperm. And then, ploidy status and microsatellite genotypes in these progenies were examined to identify modes of gametogenesis and reproduction in these inter-populational hybrids in *Misgurnus* loach.

Materials and methods

Fish specimens and inter-populational hybridization

Bisexual diploid females and males were collected from the wild population in Memanbetsu (abbreviated as M) town, Abashiri county, located in the northern area of Hokkaido and Kita (K) village, Sorachi county in the southern area of Hokkaido, Japan. The name of the locality was designated by the conventional name before the consolidation of municipalities in March 2006 and Memanbetsu and Kita now belonging to Ozora town and Iwamizawa city, respectively. In mtDNA control region, the RFLP (restriction fragment length polymorphism)-haplotype of females taken from

the Memanbetsu population was haplotype I belonging to clade A, while males taken from the Kita population had haplotype V belonging to clade B (Morishima et al., 2008a).

Mature eggs were obtained by induction of ovulation with an injection with human chorionic gonadotropic hormone (hCG: Gonatropin, Aska (formerly, Teikoku Hormone) Pharmaceutical Co. Ltd., Kawasaki, Japan) according to Suzuki and Yamaguchi (1979) or two injections of homogenized carp pituitary gland (1.0 mg/Kg body weight) every 12 hours at 26.0±1.0℃. Sperm was collected with a glass capillary by squeezing the abdomen gently. Cross breeding was performed in 2004 by a routine fertilization method by mixing eggs of a Memanbetsu (M) female and sperm of a Kita (K) male, followed by activation with ambient fresh water. Hybridization of opposite direction was not conducted at this time due to poor egg quality of mature females from Kita.

From 2125 eggs of M females after fertilization by sperm of K male, 1001 larvae hatched (47.1%). Resultant MK-F1 hybrid larvae were fed daily with *Artemia* for 3 months and then a combination of commercial carp pellets, dried zooplankton, and goldfish flakes until maturation in 2006. Microsatellite genotyping was conducted to indicate hybrid nature of inter-populational MK-F1 larvae by genetic analyses described

bellow.

Breeding of hybrid females

Eggs from six mature MK-F1 hybrid females (MK1-6) were obtained by the above mentioned procedure in 2006. Eggs from one mature wild female from Kita (K) were also obtained in the same manner. Sperm from Kita (K1), Memanbetsu (M1), and goldfish (GF) males were obtained by the above mentioned method. For gynogenetic induction, goldfish sperm was UV irradiated according to Suzuki et al. (1985) and Suwa et al. (1994).

Eggs from each hybrid female were divided into batches for fertilization with normal loach sperm (K1 or M1), UV irradiated goldfish sperm (UV), and normal goldfish sperm (GF). Induced gynogenesis using UV irradiated goldfish sperm was performed to examine ploidy status and genotype of eggs by eliminating genetic contribution of paternal parents. After induced gynogenesis, resultant progeny from normal eggs will die due to the expression of haploid syndrome (Arai et al., 1991, 1993), but those from unreduced diploid eggs are viable due to the presence of two sets of chromosomes (Zhang and Arai, 1999; Morishima et al., 2002). Intact goldfish sperm was used to check the possibility of natural gynogenesis with heterospecific sperm,

because natural clonal diploids are able to reproduce by gynogenesis with sperm from different species (Morishima et al., 2002). In fish with gynogenetic reproduction, sperm cannot contribute to zygotic genome, but trigger the initiation of embryonic development. Rates of hatched larvae were calculated based on the total number of fertilized eggs in each batch. Rates of normal larvae were calculated on the total number of hatched larvae. Survivors from each cross were reared as described above.

Genetic analyses

Relative DNA content (C value) of sperm and/or fin tissue of parental fish and progeny of hybrids was measured to determine ploidy status by flow-cytometry as described in Morishima et al. (2002).

Genomic DNA was extracted from fin-clip samples of parental fish and partial body samples of larvae after ploidy determination according to Asahida et al. (1996) and Arai and Mukaino (1997). Microsatellite loci were amplified and genotyped according to Arias-Rodriguez et al. (2007). RFLP (restriction fragment length polymorphism)-haplotype was determined according to Morishima et al. (2008a).

Results

Survival and ploidy status of progeny of hybrid females

Progeny of the first generation of inter-populational hybrids (MK-F1) were genotyped at six different microsatellite loci and all the hybrids between Mamanbetsu female (M) and Kita male (K) had heterozygous genotypes including both maternally and paternally derived alleles at microsatellite loci examined (Table 1). Thus, MK-F1 cross was a true hybridization between parents from the two different populations.

Mature eggs of six inter-populational hybrid females (MK1 to 6) were used to produce progenies by fertilization with normal loach (K1 or M1), UV irradiated goldfish (UV), or intact goldfish (GF) sperm. The number of eggs used, rate of hatching, percentage of normal larvae, and relative DNA content of larvae are shown in Table 2.

When gynogenetic development was induced for eggs from MK1 female by activation with UV-irradiated goldfish sperm, normal larvae with diploidy (DNA content 2C) appeared. Normal fertilization of MK1 eggs by haploid sperm of K1 diploid male produced triploid (DNA content 3C) larvae with normal appearance, but one exceptional diploid appeared. Thus, most eggs laid by MK1 were diploid.

Eggs of MK2 were fertilized by goldfish sperm with 1.4C DNA content when the loach sperm was 1.0C (Morishima et al., 2002). All the inter-specific loach-goldfish

hybrid progeny of MK2 were abnormal and inviable. As a high rate of 2.4n (DNA content 2.4C) and a low rate of 3.4n (DNA content 3.4C) progeny appeared, MK2 hybrid laid both haploid and diploid eggs, simultaneously. But, haploid eggs were dominantly formed.

When eggs of MK3 hybrid were fertilized by sperm of M1 diploid male, 81.9% hatched and 72.3% exhibited normal appearance. Flow-cytometry showed that most larvae (146/152) were diploid (DNA content 2C), but a few (6/152) were triploid (DNA content 3C). Thus, MK3 hybrid laid large number of haploid eggs and a small number of diploid eggs, simultaneously.

MK4 hybrid female gave 66.3 % hatched larvae, in which 73.7% were normal larvae after fertilization by sperm of M1 loach male. Both diploid (DNA content 2C) and triploid (3C) larvae appeared in the progeny. Thus, MK4 female laid both haploid and diploid eggs, simultaneously. However, rate of haploid eggs was higher than that of diploid eggs.

In the gynogenetic progeny of MK5, both haploid and diploid larvae with abnormal appearances were present. In the progeny from normal fertilization by sperm of M1 male, hatching (37.1%) and normal rates (48.0%) were poorer than those recorded in progenies of other hybrid females. In this cross, relatively large number of

diploid and very few triploid larvae appeared together with large number of unexpected hypotriploid or hyperdiploid (DNA content 2.5-2.8C) and hyperpentaploid (5.5C) larvae with severely abnormal appearances. Similar tendency was recorded in the inviable progeny from interspecific cross between MK5 female and goldfish male. Judging from DNA contents (2.4C, 3.4C) of loach-goldfish hybrid larvae, both haploid and diploid eggs were spawned by this hybrid female. However, in this cross, triploid eggs were also detected because larvae with DNA content of 4.4C occurred.

In gynogenetic progeny of MK6, a few haploid (DNA content 1C) and many diploid (2C) larvae appeared together with unexpected aneuploid larvae with hyperdiploidy or hypotriploidy (DNA content 2.5-2.8C) and polyploid larvae with hypertetraploidy (4.4C) and hypoheptaploidy (6.8C). All these gynogenetic larvae except for diploid larvae exhibited abnormal appearances. In normal fertilization by sperm of M1 male, a few diploid (DNA content 2C) and a large number of triploid (3C) larvae appeared. In addition to these larvae, tetraploid (4C), hexaploid (6C) and hypoheptaploid (6.8C) larvae with abnormalities appeared. In abnormal progeny of loach-goldfish interspecific cross, the occurrence of haploid, diploid and triploid eggs were verified by the presence of 2.4C, 3.4C and 4.4C larvae, respectively. A larva with DNA content of 6.4C indicated the production of very few pentaploid eggs by this

hybrid female. A relatively high rate of abnormal tetraploid larvae was also detected.

In inter-populational hybrid females, gametogenetic modes could be generally categorized to following two cases: (1) females mainly producing unreduced diploid eggs, (2) females producing both haploid and diploid eggs, but rate of haploid eggs being higher. MK1 and MK6 were the first case, but MK2, 3, 4, and 5 were the second one.

Genetic analysis in the progeny of hybrid females

The gynogenetic diploid progeny of hybrid females MK1 (MK1 x UV) gave microsatellite genotypes absolutely identical to somatic cells of the mother at the six selected loci (Table 3). Triploid progenies of MK1xK1 cross gave microsatellite genotypes comprising two alleles derived from the hybrid female (MK1) and one allele from the normal male (K1). Thus, most diploid eggs of MK1 hybrid were genetically identical to the mother. One exceptional diploid progeny which appeared in the cross MK1 x K1 showed a genotype including paternal allele at *Mado15* locus (Table 3). Thus, this individual is not a product of gynogenesis, but of syngamy.

Microsatellite genotypes were also analyzed in the diploid and triploid progeny that developed from MK3 x M1 cross (Table 3). In all the diploid progeny examined,

one allele was transmitted from MK3 female and the other counterpart allele was from M1 male. Thus, haploid eggs were formed by normal meiosis and diploid progeny appeared by normal syngamy with haploid sperm nucleus. Triploid progeny of hybrid female MK3 exhibited genotypes comprising intact two alleles or a double dose of one allele from MK3 female and one allele from M1 male.

In MK4 X M1 cross (Table 3), diploid progeny had genotypes with both paternal and maternal alleles. Triploid progeny showed genotypes comprising intact diploid genotypes with two different alleles of MK4 female and one allele from M1 male. No triploid genotypes with a double dose of one of the two maternal alleles appeared in the progeny of MK4. Thus, diploid eggs of MK4 were genetically identical to the soma of MK4.

In MK5 X M1 cross (Table 3), diploid genotypes including maternal and paternal alleles were detected. Triploid progeny exhibited genotypes comprising intact two maternal alleles or a double dose of one of the two maternal alleles from MK5 female and one allele from M1 male.

Progeny of MK6 x UV gave diploid genotypes completely identical to those of MK6 female (Table 3). Thus, all the triploid progeny of MK6 X M1 cross gave genotypes including intact diploid genotypes with two alleles of MK6 and one of the

two alleles of M1 female (Table 3).

Thus, unreduced diploid eggs of hybrid females MK1, MK4 and MK6 were genetically identical to the somatic cells of the mother. Diploid eggs of MK3 and MK5 females are not genetically identical to somatic cells of each mother fish. On the other hand, MK3 and MK5 females laid haploid eggs with genetic variations.

Discussion

Viable diploid larvae appeared exclusively when gynogenesis was artificially induced in eggs of two hybrid females (MK1 and MK6). Triploid larvae occurred in high percentages when eggs of these hybrid females were fertilized normally. Therefore, the eggs of these two females were apparently unreduced diploids. Genetic analyses indicated that unreduced eggs of each hybrid female were genetically identical to the somatic cells of each mother hybrid. Thus, genetically identical diploid eggs were formed in inter-populational hybrids, but they always incorporated a sperm nucleus. The unreduced eggs of the above two females are quite similar to those reported in a natural clonal lineage (Morishima et al., 2002), because these eggs are genetically identical to the mother. However, unreduced diploid eggs of inter-populational hybrids did not

develop gynogenetically, but incorporated a haploid sperm nucleus by regular syngamy.

The simultaneous occurrence of haploid and diploid eggs was observed in other hybrid females, but the proportion of haploid eggs differed: approximately 90% in MK2, 96% in MK3, and 63% in MK4. The reproductive traits observed in these inter-populational hybrids are similar to those reported in a diploid loach found in Niigata prefecture, Honshu, which laid both haploid and diploid eggs, simultaneously (Zhang & Arai, 1999). Thus, some hybrids mainly produce haploid eggs together with diploid, aneuploid, and other unusual eggs. The simultaneous appearance of various ploidy-types of eggs has been reported in natural triploids and diploid-triploid mosaics, accidentally derived from a natural clonal lineage of the loach (Oshima et al., 2005; Yoshikawa et al., 2007). Similar observations of simultaneous formation of different types of eggs were reported in triploid (diploid x tetraploid) loach (Matsubara et al. 1995; Arai and Mukaino, 1997, 1998; Momotani et al., 2002), spontaneous triploid in central area of Honshu Island, Japan (Zhang and Arai, 1999) and other triploid teleosts including minnow in the genus *Squalius* (Alves et al., 2004) and *Phoxinus* (Dawley and Goddard, 1988; Goddard and Dawley, 1990).

Genetic analyses in diploid progeny of females MK3, MK4, and MK5 demonstrated that haploid eggs should include one of the two alleles from a hybrid

female. These results indicate that normal or near-normal meiotic divisions could occur in these hybrid females. In triploid progenies of these females, although intact female genotypes were transmitted to diploid eggs in MK4, double doses of one maternally derived allele of the hybrid mother were observed in MK3 and MK5. Thus, unreduced diploid eggs could be formed in MK4 as in MK1 and MK6. However, diploid eggs generated by MK3 and MK5 females were not genetically identical to the mother and the mechanism of diploid egg formation might be different from that occurring in the unreduced diploid eggs of MK1, MK4, and MK6 females. The system involved in such diploid ova formation could not be identified in the present study due to the shortage of triploid samples from MK3 and MK5. Occurrence of slight variation was reported in unreduced triploid eggs of triploid (diploid female x natural tetraploid) loach and might have been explained by pairing not between sister chromosomes duplicated from an original chromosome by premeiotic endomitosis, but between homologous chromosomes in oocytes (Zhang et al., 1998; Momotani et al., 2002). In production of unreduced diploid eggs in the inter-populational hybrids MK3 and MK5, similar mechanisms might have acted to generate minor variations. The other possibility is spontaneous inhibition of the second polar body release, in which recombinant heterozygous and non-recombinant homozygous genotypes should occur in diploid eggs

(Arai, 2001).

The mechanisms responsible for differences in egg formation among hybrid females from the same parental combination, i.e. mostly unreduced diploid eggs in MK1 and MK6, but a relatively high percentage of reduced haploid eggs in other four females, are difficult to understand at present and this question remains to be resolved in the future. The cytological mechanisms for the occurrence of various aneuploidies and unusually high polyploidies in MK5 and MK6 are also difficult to explain at present.

The present results indicate that the hybridization between the populations is a primary cause for the production of unreduced and other unusual gametogenetic events observed in hybrid females. As unreduced diploid eggs of some inter-populatinal hybrids were genetically identical to the mother, they are similar to diploid eggs laid by natural clonal lineage. But they are different from the natural clone, because they did not develop by gynogenesis. This result is not surprising because the sequence of mtDNA control region of the maternal fish (M) of the present inter-populational hybrids was categorized to RFLP(restriction fragment length polymorphism)-I belonging to the same clade A, but absolutely different from that of the natural clonal lineages (III-1 or III-2) (Morishima et al., 2008a). Therefore, the present inter-populational hybrids are not a direct origin of the natural clone. But they may act as a supplier of unreduced

diploid eggs, which give rise to triploid development after fertilization with sperm of bisexual diploids in nature. The production of such unreduced eggs by interspecific hybridization has been reported in teleosts including *Salmo* species (Johnson and Wright, 1986), *Phoxinus* species (Dawley et al., 1987; Dawley and Goddard, 1988; Goddard and Dawley, 1990), sunfish (Dawley et al., 1985; Dawley, 1987), medaka species (Sakaizumi et al., 1993; Kurita et al., 1995; Shimizu et al., 2000), and *Fundulus* species (Dawley, 1992). Similar results were also reported in *Cnemidophorus* in Reptiles (Dessauer and Cole, 1989; Moritz et al., 1989). Judging from these results, the present results obtained by inter-populational hybridization in the loach are very similar to those reported in interspecific hybridization in other vertebrate species. Therefore, the two populations used for the hybridization in *Misgurnus* loaches are considered to be so genetically distant as to give rise to atypical oogenesis for the formation of unreduced diploid and other unusual eggs.

According to the generally accepted “Balance Hypothesis” (Moritz et al., 1989), the origin of asexual lineage via hybridization is a restricted event and the transition from bisexual to asexual, gynogenetic development is very difficult in nature due to potent genetic constraints. The experimental results obtained in the present study support this hypothesis, because artificial inter-populational loach hybrids produced

unreduced eggs and other kinds of disruption of normal meiosis, but no eggs developed by gynogenesis. On the other hand, actual occurrence of such inter-populational loach hybrids and its impact on genetic structure of wild populations which are mainly composed of bisexual diploids still remain unknown. Further studies on hybridizations with opposite direction as well as those with bisexual individuals with sequence of mtDNA identical to the clonal lineages are required to elucidate the origin and evolutionary mechanisms of asexual and/or polyploid lineages in *Misgurnus* loaches.

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References

Alves MJ, Gromicho M, Collares-Pereira MJ, Crespo-López E, Coelho MM (2004) **S**imultaneous production of triploid and haploid eggs by triploid *Squalius alburnoides* (Teleostei: Cyprinidae)**.** J Exp Zool 301: 552-558

Arai K (2001) Genetic improvement of aquculture finfish species by chromosome namipulation techniques in Japan. Aquaculture 197:205-228.

Arai K, Matsubara K, Suzuki R (1991) Chromosomes and developmental potential of progeny of spontaneous tetraploid loach, *Misgurnus anguillicaudatus*. Nippon Suisan Gakkaishi 51:2173-2178.

Arai K, Matsubara K, Suzuki R (1993) Production of polyploids and viable gynogens using spontaneously occurring tetraploid loach, *Misgurnus anguillicaudatus*. Aquaculture 117:227-235.

Arai K, Mukaino M (1997) Clonal nature of gynogenetically induced progeny of triploid (diploid x tetraploid) loach, *Misgurnus anguillicaudatus* (Pisces:Cobitididae). J Exp Zool 278:412–421.

Arai K, Mukaino, M (1998) Electrophoretic analysis of the diploid progenies from triploid x diploid crosses in the loach *Misgurnus anguillicaudatus* (Pisces: Cobitidae). J Exp Zool 280: 368-374(1998)

Arias-Rodriguez L, Morishima K, Arai K (2007) Genetically diversified populations in the loach *Misgurnus anguillicaudatus* inferred from newly developed microsatellite markers. Mol Ecol Notes 7:82-85.

Asahida T, Kobayashi T, Saitoh K, Nakayama I (1996) Tissue preservation and total DNA extraction from fish stored at ambient temperature using buffers containing high concentration of urea. Fish Sci 62:727-730.

Beukeboom LW, Vrijenhoek RC (1998) Evolutionary genetics and ecology of sperm dependent parthenogenesis. J Evol Biol 11:755-782.

Dawley RM (1987) Hybridization and polyploidy in a community of three sunfish species. Copeia 2:326-335.

Dawley RM (1989) An introduction to unisexual vertebrates. In: Dawley R, Bogart JP (eds) Evolution and ecology of unisexual vertebrates. Bull 466, New York State

Museum, Albany, New York, pp.1-23

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

Dawley, RM, Goddard KA (1988) Diploid-triploid mosaics in unisexual hybrids of *Phoxinus eos* and *Phoxinus neogaeus*. Evolution 62:649-659.

Dawley RM, Graham JH, Schultz RJ (1985) The triploid progeny of pumpkinseed x green sunfish hybrids. J Heredity 76:251-257.

Dawley RM, Schultz RJ, Goddard KA (1987) Clonal reproduction and polyploidy in hybrids of *Phoxinus eos* and *P. neogaeu*s. Copeia 2:275-283.

Dessauer HC, Cole CJ (1989) Diversity between and within nominal forms of unisexual teiid lizards. In: Dawley RM, Bogart JP (eds) Evolution and ecology of unisexual vertebrate. New York State Museum, Bull 466, new York, pp49-71.

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.

Itono M, Morishima K, Fujimoto T, Yamaha E, Arai K (2006) Premeiotic endomitosis produces diploid eggs in the natural clone loach, *Misgurnus anguillicaudatus* (Teleostei:Cobitidae). J Exp Zool 305A:513-523.

Itono M, Okabayashi N, Morishima K, Fujimoto T, Yoshikawa H, Yamaha E, Arai K (2007) Cytological mechanisms of gynogenesis and sperm incorporation in unreduced diploid eggs of the clonal loach, *Misgurnus anguillicaudatus* (Teleostei: Cobitidae). J.Exp.Zool. 307A:35-50.

Johnson KR, Wright JE Jr (1986) Female brown trout x Atlantic salmon hybrids produce triploids and gynogens when backcrossed to male Atlantic salmon.

Aquaculture 57:345-358.

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

Kurita, J, Oshiro, T, Takashima F, Sakaizumi M **(**1995**)** Cytogenetic studies on diploid and triploid oogenesis in interspecific hybrid fish between *Oryzias latipes* and *O. curvinotus*. J Exp Zool 273:234-241.

Matsubara K, Arai K, Suzuki R (1995) Survival potential and chromosomes of progeny of triploid and pentaploid females in the loach *Misgurnus anguillicaudatus*. Aquaculture 131:37–48.

Momotani S, Morishima K, Zhang Q, Arai K (2002) Genetic analyses of the progeny of triploid gynogens induced from unreduced eggs of triploid (diploid female tetraploid male) loach. Aquaculture 204:311-322.

Morishima K, Horie S, Yamaha E, Arai K (2002) A cryptic clonal line of the loach *Misgurnus anguillicaudatus* (Teleostie:Cobitidae) evidenced by induced gynogenesis, interspecific hybridization, microsatellite genotyping and multilocus DNA fingerprinting. Zool Sci 19:565-575.

Morishima K, Nakamura-Shiokawa Y, Bando E, Li YJ, Boron A, Khan MMR, Arai K (2008a) Cryptic clonal lineages and genetic diversity in the loach *Misgurnus anguillicaudatus* (Teleostei:Cobitidae) inferred from nuclear and mitochondrial DNA analyses. Genetica 132:159-171.

Morishima K, Oshima K, Horie S, Fujimoto T, Yamaha E, Arai K (2004) Clonal diploid sperm of the diploid-triploid mosaic loach, *Misgurnus anguillicaudatus* (Teleostei:Cobitidae). J Exp Zool 301A:502-511. doi:10.1002/jez.a.49

Morishima K, Yoshikawa H, Arai K (2008b) Meiotic hybridogenesis in triploid

Misgurnus loach derived from a clonal lineage. Heredity 100:581-586. doi:10.1038/hdy.2008.17

Moritz C, Brown WM, Densmore LD, Wright JW, Vyas D, Donellan S, Adams M, Baverstock P (1989) Genetic diversity and the dynamics of hybrid parthenogenesis in *Cnemidophorus* (Teiidae) and *Heteronotia* (Gekkonidae). In: Dawley RM, Bogart JP (eds) Evolution and ecology of unisexual vertebrate. New York State Museum, Bull 466, new York, pp 268-280.

Oshima K, Morishima K, Yamaha E, Arai K (2005) Reproductive capacity of triploid loaches obtained from Hokkaido Island, Japan. Ichtyol Res 52:1-8.

Saitoh, K. 1989. Asian pond loach. In :Kawanabe H, Mizuno N (eds) Freshwater fishes of Japan. Yamakei Publ, Tokyo. pp 382-385.

Sakaizumi M, Shimizu Y, Matsuzaki T, Hamaguchi, S. (1993) Unreduced diploid eggs produced by interspecific hybrids between *Oryzias latipes* and *O. curvinotus*. J Exp Zool 266:312-318.

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

Suwa M, Arai K, Suzuki R (1994) Suppression of the first cleavage and cytogenetic studies on the gynogenetic loach. Fish Sci. 60:673–681

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.

Suzuki R, Yamaguchi M (1975) Influence of water temperature on inducing spawning by hormone injection in the loach, cyprinid fish. Suisanzoshoku 22:135-139

Vrijenhoek RC (1989) Genetic and evolutionary constraints on the origin and establishment of unisexual vertebrates. In: Dawley RM, Bogart JP (eds) Evolution and ecology of unisexual vertebrate. New York State Museum, Bull 466, new York, pp24-31

Yoshikawa H, Morishima K, Fujimoto T, Yamaha E, Arai K (2007) Spontaneous formation of haploid, diploid and triploid eggs in diploid-triploid mosaic loaches. J Fish Biol 71(Supplement B):250-263.

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.

Table 1. Microsatellite genotypes in artificial inter-populational hybrids MK-F1 between a female of Memanbetsu population and a male of Kita population

	Genotype of parents		Genotype of progeny						
Locus	Female	Male	observed (expected)				Total	df	X2
Mado1	169/169	110/110	110/169 20(20.0)				20		
Mado3	72/74	77/80	72/80 10(5.0)	74/77 4(5.0)	72/77 4(5.0)	74/80 2(5.0)	20	3	7.20
Mado7	129/140	129/140	129/129 2(5.0)	129/140 12(10.0)	140/140 6(5.0)		20	2	2.40
Mado15	82/82	78/78	78/82 20(20.0)				20		
Mado16	61/61	63/63	61/63 20(20.0)				20		
Mado21	73/76	100/116	76/100 3(5.0)	76/116 4(5.0)	73/100 10(5.0)	73/116 3(5.0)	20	3	6.80

Table 2. Viability and relative DNA content of larvae developed from eggs of inter-populational hybrid females fertilized with UV-irradiated sperm (UV), normal loach sperm (K1 and M1) and goldfish sperm (GF)

Parents		Egg	Hatch	Norma	Larvae no	Relative DNA content (abnormal larvae)											
F	M	no.	(%)	(%)	(abnormal	1C	2C	2.4C	2.5-2.8C	3C	3.4C	4C	4.4C	5.5C	6C	6.4C	6.8C
MK1	UV	65	38.4	100	23(0)	0	23(0)	0	0	0	0	0	0	0	0	0	0
MK1	K1	36	61.1	100	22(0)	0	1(0)	0	0	21(0)	0	0	0	0	0	0	0
MK2	GF	584	33	0	193(193)	0	0	173(173)	0	0	20(20)	0	0	0	0	0	0
MK3	M1	1638	81.9	72.3	152(41)	0	146(39)	0	0	6(2)	0	0	0	0	0	0	0
MK4	M1	299	66.3	78.7	155(33)	0	97(7)	0	0	58(26)	0	0	0	0	0	0	0
MK5	UV	885	1.6	0	14(14)	5(5)	9(9)	0	0	0	0	0	0	0	0	0	0
MK5	M1	1004	37.1	48	175(91)	0	87(5)	0	82(82)	5(3)	0	0	0	1(1)	0	0	0
MK5	GF	770	41.1	0	67(67)	0	0	29(29)	0	0	21(21)	0	16(16)	0	0	0	0
MK6	UV	727	22.1	77	148(34)	8(8)	117(3)	0	17(17)	0	0	0	4(4)	0	0	0	2(2)
MK6	M1	1094	16.6	77.4	182(41)	0	4(4)	0	0	151(10)	0	22(22)	0	0	3(3)	0	2(2)
MK6	GF	701	32.7	0	160(160)	0	0	18(18)	0	0	19(19)	40(40)	82(82)	0	0	1(1)	0

Table 3. Microsatellite genotypes at six different loci in gynogenetic diploid, diploid and triploid progenies of inter-populational hybrid females

Cross	*Mado 1* genotype				*Mado3* genotype				*Mado7* genotype			
Female x Male	F	M	Ploidy	Progeny: *n*	F	M	Ploidy	Progeny: *n*	F	M	Ploidy	Progeny: *n*
M1 x UV	110/169	UV	2n	110/169: 23	72/80	UV	2n	72/80: 23	129/140	UV	2n	129/140: 23
M1 x K1	110/169	110/110	2n	110/169: 1	72/80	77/80	2n	72/80: 1	129/140	129/140	2n	129/140: 1
			3n*	110/110/169: 21			3n	72/77/80: 11			3n*	129/129/140 or
							3n*	72/80/80: 10				129/140/140: 22
MK3 x M1	110/169	133/155	2n	110/133: 4	72/80	72/72	2n	72/72:7	129/140	126/129	2n	126/129: 7
			2n	110/155: 6			2n	72/80: 11			2n	129/140: 11
			2n	133/169: 6			3n*	72/72/80: 1			3n	126/129/140: 1
			2n	155/169: 2			3n*	72/72/72: 1			3n*	129/129/140: 1
			3n*	110/110/133: 1								
			3n*	155/169/169: 1								
MK4 x M1	110/169	133/155	2n	110/133: 4	72/80	72/72	2n	72/72: 7	129/140	126/129	2n	126/129: 5
			2n	110/155: 3			2n	72/80: 3			2n	129/140: 5
			2n	133/169: 3			3n*	72/72/80:10			3n	126/129/140: 6
			3n	110/155/169: 8							3n*	129/129/140: 4
			3n	110/133/168: 2								
MK5 x M1	110/169	133/155	2n	133/169: 7	72/80	72/72	2n	72/72: 8	140/140	126/129	2n	126/140: 11
			2n	110/133: 6			2n	72/80: 10			2n	129/140: 7
			2n	155/169: 1			3n*	72/72/80: 10			3n*	129/140/140: 2
			2n	110/155: 4								
			3n*	133/169/169: 1								
			3n	110/155/169: 1								
MK6 x UV	110/169	UV	2n	110/169: 20	72/80	UV	2n	72/80: 20	140/140	UV	2n	140/140: 20
MK6 x M1	110/169	133/155	3n	110/133/169: 6	72/80	72/72	3n*	72/72/80: 20	140/140	126/129	3n*	126/140/140: 12
			3n	110/155/169:14							3n*	129/140/140: 8

Cross	*Mado15* genotype				*Mado16* genotype				*Mado21* genotype			
Female x Male	F	M	Ploidy	Progeny: *n*	F	M	Ploidy	Progeny: *n*	F	M	Ploidy	Progeny: *n*
MK1 x UV	78/82	UV	2n	78/82: 23	61/63	UV	2n	61/63: 23	73/116	UV	2n	73/116: 23
MK1 x K1	78/82	78/78	2n	78/78: 1	61/63	63/63	2n	61/63: 1	73/116	100/116	2n	73/116: 1
			3n*	78/78/82: 22			3n*	61/63/63: 22			3n	73/100/116: 11
											3n*	73/116/116:11
MK3 x M1	78/82	82/82	2n	78/82: 18	61/63	61/61	2n	61/63: 18	73/116	73/73	2n	73/116: 18
			3n*	78/82/82: 2			3n*	61/61/63: 2			3n*	73/73/116: 2
MK4 x M1	78/82	82/82	2n	78/82: 10	61/63	61/61	2n	61/63: 10	73/116	73/73	2n	73/116: 10
			3n*	78/82/82: 10			3n*	61/61/63: 10			3n*	73/73/116: 10
MK5 x M1	78/82	82/82	2n	78/82: 18	61/63	61/61	2n	61/63: 18	76/116	73/73	2n	73/116: 9
			3n*	78/82/82:2			3n*	61/61/63: 2			2n	73/76: 9
											3n	73/76/116: 2
MK6 x UV	78/82	UV	2n	78/82: 20	61/63	UV	2n	61/63: 20	76/116	UV	2n	76/116: 20
MK6 x M1	78/82	82/82	3n*	78/82/82: 20	61/63	61/61	3n*	61/61/63: 20	76/116	73/73	3n	73/76/116: 20

* Triploid genotypes estimated because of only two alleles detected in electrophoresis