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1 **Genomic constitution and atypical reproduction in polyploid and unisexual lineages of the**
2 ***Misgurnus loach*, a teleost fish**

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8 **Abstract**

9 The loach (*Misgurnus anguillicaudatus*) is an excellent animal model to elucidate biological origin
10 and evolutionary significance of genome duplication and unisexual reproduction because artificially
11 induced and naturally occurring polyploids and parthenogenetic (gynogenetic, androgenetic) animals
12 can be compared. First, we summarize the chromosome manipulation techniques to induce triploids
13 and tetraploids by inhibiting meiotic or mitotic divisions of inseminated eggs, respectively, as well as
14 parthenogenetic animals, obtained after fertilization with genetically inactivated gametes. Then, we
15 review the knowledge on natural polyploid and unisexual lineages found in *Misgurnus* loaches. A
16 natural diploid-tetraploid complex occurs in wild populations in central China, and these diploid and
17 tetraploid loaches reproduce bisexually. Chinese tetraploids are considered autotetraploid, which
18 may have arisen by doubling of the entire genome of an ancestral diploid, based on cytogenetic
19 results from FISH (fluorescence in situ hybridization) karyotypes and meiotic configurations. In
20 contrast, gynogenetically reproducing clonal diploid lineages have been discovered in a few wild
21 populations in Japan, although most wild-type individuals are bisexually reproducing diploids. Such
22 clonal diploid loaches sometimes produce triploid progeny by accidental incorporation of a sperm
23 nucleus into an unreduced diploid egg, and the resulting triploid generates haploid eggs by meiotic
24 hybridogenesis. Unreduced diploid gametes of clonal loaches are generated by a cytological
25 mechanism, premeiotic endomitosis, which likely occurs in the early (gonium stage) germ cells.
26 Initiation of gynogenetic development is related to a failure of decondensation of the male (sperm)
27 pronucleus in unreduced diploid eggs of a clonal loach. Clonal lineages may have arisen from a past
28 hybrid event between genetically divergent groups, but their exact origins are unknown at present.
29 See also sister article focusing on plants by Hegarty et al. in this themed issue.

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33 Artificially induced polyploids and unisexual organisms may exhibit different
34 reproductive traits and other biological characteristics from those found in natural
35 polyploids and unisexual organisms because the origin and the mechanisms of
36 occurrence of these biotypes are presumably different. However, it is difficult to

compare biological traits between induced and natural polyploids and those between bisexual wild-type and unisexual organisms in the same species because not all species have natural polyploids and unisexual organisms. The loach *Misgurnus anguillicaudatus*, or Oriental weatherfish, is a unique model fish suitable for such a comparison because (1) various chromosome set manipulation techniques and genetic backgrounds have been well developed and studied in this species, and (2) certain wild populations include natural polyploid as well as gynogenetically reproducing clonal diploid individuals. In this paper, we briefly describe the status of induction techniques of artificial polyploid and unisexual (gynogenetic and androgenetic) loaches and their performances. Furthermore, we review the probable origin of natural polyploid and clonal loaches based on cytogenetic and experimental genetic results as well as putative mechanisms of atypical reproduction in these natural biotypes and their progeny.

Chromosome Set (genome) Manipulation

Chromosome set or genome manipulation represents a system of techniques to produce artificial polyploid and unisexual progeny by altering the number or combination of the chromosome set of a target species. The biological rationale, technical development and aquaculture applications of genome manipulation have been well reviewed by Purdom [1983], Ihssen et al. [1990], Pandian and Koteeswaran [1998], Arai [2000, 2001], Devlin and Nagahama [2002], Komen and Thorgaard [2007], and Piferrer et al. [2009]. A recent publication by Pandian [2011] also provided a comprehensive review of these topics, especially from the viewpoint of sex determination of fishes.

Like most other vertebrates, teleost males produce haploid spermatozoa by a complete spermatogenetic process, including meiosis and subsequent spermiogenesis, whereas females ovulate mature (physiologically fertile) eggs that have not completed meiosis: ovulated eggs are arrested at the metaphase of the second meiotic division (MII arrest). In teleosts, the resumption of MII occurs in eggs by stimulation of sperm intrusion through the micropyle, and the activated egg extrudes the second polar body with a haploid set of chromosomes to complete meiosis. Next, the egg nucleus becomes the female pronucleus and fuses with the male pronucleus, which is decondensed from the sperm nucleus (karyogamy). The fertilized egg initiates synchronous cell divisions (cleavage) that continue until the mid-blastula transition stage.

Most teleosts are oviparous, and thus, females spawn their eggs to the outer environment; fertilization and subsequent embryogenesis occur outside of the female

body. Therefore, the collected eggs and sperm can be easily inactivated by ultraviolet (UV), X-ray or gamma-ray irradiation. UV has routinely been used to inactivate sperm nuclei for induction of gynogenesis (unisexual development with all-female inheritance) as well as egg nuclei of fish with small egg-sizes for induction of androgenesis (unisexual development with all-male inheritance), without special facilities for safety. When eggs are fertilized with genetically inactivated sperm, gynogenesis commences. When genetically inactivated eggs are fertilized by normal sperm, androgenetic development occurs. Recently, a new androgenesis induction technique, cold-shock androgenesis without any irradiation of the egg nucleus, was described [Morishima et al., 2011].

Gynogenetic and androgenetic haploid embryos can develop until hatching or before first feeding, but these embryos inevitably die due to expression of abnormalities collectively referred to as the 'haploid syndrome'. To obtain viable gynogenetic and androgenetic progeny, a haploid genome should be duplicated just after fertilization or through early cleavage. Mature eggs complete MII after fertilization in teleosts, and thus, chromosomes can be duplicated by inhibiting the release of the second polar body immediately after initiation of haploid development. The release of the second polar body can be suppressed by treating gynogenetically activated eggs with thermal (cold or heat) or hydrostatic pressure shock. When eggs are fertilized by an intact normal haploid sperm and then treated to inhibit the release of the second polar body just after fertilization, the resultant embryos are triploids comprising a haploid set of egg nucleus, a haploid set of sperm nucleus and an additional haploid set of a second polar body nucleus.

Another timing to duplicate the haploid set of chromosomes is during the process of mitosis or cleavage after initiation of embryonic development. The nucleus of a fertilized egg commences replication to enter mitosis, and the nucleus then proceeds to the M phase of the cell cycle, when spindles are formed from the centrosomes located at the poles of the cell and when the chromosomes are aligned along the equator. Subsequently, sister chromatids are separated and distributed into each daughter cell. If cell division can be inhibited at the M phase, more strictly at the pro-metaphase of the first cleavage, just after the replication of the nucleus, by physical shock such as heat, cold, or hydrostatic pressure treatment, the resulting embryos can become doubled haploids (DHs) with 2 sets of homologous chromosomes due to artificially induced endomitosis (endoreduplication). When cell division is inhibited at the optimum timing of the first cleavage of normally fertilized eggs, the resultant progeny are expected to be tetraploid.

As a mechanism of chromosome doubling, the physical treatment at the time of metaphase of the first cleavage (first cell cycle) did not inhibit the first mitosis, but the second mitosis [Zhang and Onozato, 2004]. Because the bipolar spindle was once disrupted by the treatment, it was immediately regenerated, and thus, the first cleavage could proceed. Subsequently, a unipolar spindle was formed in each blastomere of the 2-cell stage embryo. Such a cytological situation must suppress the second cleavage to double chromosomes without cytokinesis (endomitosis). Therefore, treated embryos resulted in considerably longer second mitotic division duration because the cleavage was prevented. DH individuals (completely homozygous diploid gynogenics or androgenics) often demonstrate very low survival rates, but these individuals are indispensable for providing genetically identical gametes for cloning by the second cycle of gynogenesis (polar-body gynogenesis) and androgenesis (inhibition of mitosis).

Artificially Induced Polyploids: General Performance and Reproductive Capacity

Autotriploid Fishes

Autotriploid fish with 3 sets of a conspecific genome or homologous chromosomes can be produced by inhibiting the release of the second polar body just after normal fertilization. Such autotriploid fishes have been artificially induced in many species, and their growth, reproductive and other physiological performances have been studied for aquaculture applications [Piferrer et al., 2009]. In general, most triploid females exhibit sterility because most oocytes cannot enter into the meiotic process due to synaptic abnormalities caused by the presence of an odd chromosome set number. Thus, the triploid ovary never enters into the subsequent vitellogenic stage. In contrast, a different testicular development has been recognized among triploid males. Triploid males can develop their testes until they achieve a similar size of their diploid counterparts. In the testes of triploid males, spermatogonia can proliferate and differentiate into spermatocytes, spermatids and spermatozoa, and subsequently, mature triploid males produce a small quantity of sperm, including aneuploid (approximately $1.5n$) spermatozoa or spermatozoon-like cells, without normal fertility. Induced autotriploid fishes often exhibit better growth than their diploid counterparts, when compared during spawning, and such an outperformance can be explained by energy reallocation from gonadal maturation to somatic growth. Above-mentioned performances are also recognized in artificially induced triploid *Misgurnus loaches*

[Suzuki et al., 1985a; Kim et al., 1994; Arai and Inamori, 1999; Zhang and Arai, 1999b].

Allotriploid Fishes

An allotriploid is defined as a triploid comprising at least 1 set of non-homologous chromosomes from different species and can be artificially induced by inhibition of the release of the second polar body after interspecific hybridization. In several combinations of hybridization among salmonid species, diploid hybrids were inviable and barely hatched due to severe malformation, but artificially induced triploid hybrids exhibited a drastic improvement of survival, and the resultant viable hybrids hatched, survived and grew [Scheerer and Thorgaard, 1983; Arai, 1984, 1986, 1988; Gray et al., 1993]. A similar improvement in viability was also reported for allopolyploid hybridizations between *Misgurnus* or *Cobitis* loaches and goldfish, common carp, and minnow [Kijima et al., 1996a, b]. Although the molecular mechanisms responsible for the recovery of survival by the elevation of ploidy status are still unknown, gene compensation by the presence of additional gene copies supplied by an additional maternal genome is presumably important.

Most allotriploid fishes exhibit sterility, probably due to similar mechanisms acting in autotriploids. However, in the artificial allotriploid *Misgurnus* loach, including 2 chromosome sets of *M. anguillicaudatus* and 1 chromosome set of *M. mizolepis*, fertile males appeared and produced fertile haploid sperm probably by the elimination of the paternal chromosome set of *M. mizolepis*, and subsequent synapsis and meiosis between 2 homologous chromosome sets from conspecific *M. anguillicaudatus* [Fujimoto et al., 2008]. Genome elimination is also found in natural triploid females and is called ‘meiotic hybridogenesis’ (see section *Mechanisms of Unreduced Gametogenesis and Gynogenesis*). Sterility was predicted in female allotriploid hybrids from crosses between *M. anguillicaudatus* and *M. mizolepis* by histological observation [Park et al., 2006].

Tetraploid Fishes

Tetraploids can be induced by inhibition of the first cleavage after normal fertilization, but the survival rates of the resultant tetraploid progeny are extremely low [Piferrer et al., 2009]. Such low viability is likely due to a physiological disturbance caused by an elevation of ploidy status [Sakao et al., 2006]. Once a fertile tetraploid line has been established, it is possible to maintain tetraploid lines by cross-breeding tetraploid females and males because they are expected as a source of diploid gametes

for further expansion of ploidy manipulation [Chourrout et al., 1986; Chourrout and Nakayama, 1987; Nam et al., 2001; Nam and Kim, 2004].

In artificial induction of tetraploidy, mosaics often appeared, and they included both diploid and tetraploid cell populations [Chourrout and Nakayama, 1987; Yamaki et al., 1999; Fujimoto et al., 2012]. Such mosaic individuals simultaneously spawn both haploid and diploid gametes, and some diploid gametes are useful for establishing a tetraploid line [Yamaki et al., 1999; Yamaki and Arai, 2000]. Thus, diploid-tetraploid mosaic individuals are presumably useful sources of diploid gametes, if their gonads include tetraploid germ cells.

Generally, artificially induced tetraploids showed poor survival and growth performance when compared with diploid and induced triploid conspecific counterparts [Chourrout et al., 1986].

Artificially Induced Unisexual Organisms

Meiotic or Polar-Body Gynogenesis

Viable gynogenetic diploid progeny can be induced by fertilization with UV-irradiated sperm, followed by inhibition of the second polar body release as described above in section *Chromosome Set (genome) Manipulation*. Such meiotic or polar-body gynogenetic diploids have been used to induce unisexual (all-female) populations in species such as loach [Suzuki et al., 1985b], with male heterogametic sex determination (XX female, XY male). Sex-reversed gynogenetic diploid males with XX chromosomes have also been utilized to produce all-female progeny in the next generation by cross-breeding with a normal female with XX chromosomes. For a female heterogametic system (ZW female, ZZ male), gynogenesis may produce a WW super-female, which will produce all-female population by cross-breeding between WW females and ZZ males [Cotton and Wedekind, 2007; Omoto et al., 2005].

Meiotic or polar-body gynogenetic diploids are likely homozygous, except in regions of chromosomes where recombination (cross-over) occurs. Such meiotic diploid gynogenics are partially heterozygous due to recombination and cannot generate isogenic gametes. Half-tetrad analysis on frequencies of recombinant heterozygotes among meiotic diploid gynogenics can map gene(s) or DNA marker(s) in relation to the centromere of the chromosome [Thorgaard et al., 1983; Morishima et al., 2001; Komen and Thorgaard, 2007].

DHs, Mitotic Gynogenetic and Androgenetic Diploids

DHs have been produced in both freshwater and marine fish species and used as a source of isogenic gametes because DHs are equivalent to inbred lines [Komen and Thorgaard, 2007]. Cloned lines have been established in a limited number of fish species [Komen and Thorgaard, 2007]. Once homozygous clonal lines have been established, heterozygous clones with better performances, likely due to heterosis, can be produced by cross-breeding between a female from the one clonal line and a male from another clonal line. Heterozygous clone-like lines are produced in F₂ gynogenetic loaches by repeating meiotic or polar-body gynogenesis: proximal chromosome regions become homozygous, i.e. inbred, while distal regions maintain a similar heterozygous genotype [Arai, 2001].

Androgenesis as a Tool for Gene Banking and Restoration of Endangered Genotypes

Sperm is a better material for cryopreservation than eggs because the sperm structure is simple and because a sufficient number of cells are easy to obtain. Therefore, sperm cryopreservation is a powerful tool for gene banking. Theoretically, it is possible to recover an individual from a spermatozoon of an endangered target species by artificial androgenesis, using genetically inactivated eggs from closely related species. However, there are 2 challenges to reduce viabilities of induced androgenesis: (1) genetic inactivation of eggs and (2) genome duplication by inhibition of mitosis; the use of diploid sperm can eliminate the side effect of treatments for chromosome duplication in production of viable androgenetic diploids. Dispermic fertilization using fused sperm has realized the production of viable androgenics [Araki et al., 1995; Nagoya et al., 2010]. Production of viable androgenics was successfully accomplished in loaches using the diploid sperm of natural tetraploid, neo-tetraploid and sex-reversed clonal diploid males (see sections *Experimental Evidence of Genetic Tetraploidy* and *Natural Clone and Clone-Origin Triploid*).

Natural Tetraploid Loaches

Both tetraploid ($4n = 100$) and diploid individuals ($2n = 50$) appear in the loach *M. anguillicaudatus* (Cobitidae) corresponding to wild populations in the Chang Jiang river system, central China [Yin et al., 2005; Li et al., 2008]. These tetraploid loaches reproduce bisexually, similar to their wild-type diploid counterparts [Zhou et al., 2010; Li et al., 2012, 2013]. Therefore, *M. anguillicaudatus* in central China comprises a diploid-tetraploid complex within the same species, and, as a result, this loach can be

used as a model system to elucidate the mechanisms responsible for natural polyploidization. However, no such diploid-tetraploid complex has been identified in other wild populations in China [Li et al., 2008] or Japan [Zhang and Arai, 1999a; Arai, 2003]. Tetraploid loaches have often been found in Japanese market samples, but these loaches are commercially imported from China as foods and live bait for fishing because no tetraploid individuals have yet been discovered in wild populations in Japan, even after intensive screening [Ojima and Takai, 1979; Arai et al., 1991a; Zhang and Arai, 1999a; Arai, 2003].

Karyotype and Meiosis

Cytogenetic research using FISH (fluorescence in situ hybridization) with rDNA probes, AG-NORs (nucleolus organizer regions) and CMA₃ (chromomycin A₃)/DA (distamycin A) staining revealed that diploid loach chromosomes ($2n = 50$) were categorized into 5M (metacentric), 2SM (submetacentric) and 18T (telocentric) chromosome pairs, while tetraploid chromosomes ($4n = 100$) were categorized into 5M, 2SM, and 18T quartets [Li et al., 2010]. Diploid has 2 homologous chromosomes with rDNA sites detected by FISH and differential staining, whereas there are 4 rDNA-bearing homologues in tetraploid [Li et al., 2010]. Thus, tetraploid loaches have twice the number of chromosomes compared with diploid.

Regarding the occurrence of natural tetraploidy in loaches, 2 evolutionary hypotheses have been elucidated: (a) autotetraploidy (doubling of the entire genome) and (b) allotetraploidy (or amphidiploidy, i.e. duplication of each non-homologous genome of a hybrid) (fig. 1). In autotetraploid species, quadrivalent (IV) configurations are expected in meiotic divisions because 4 homologous chromosomes may have a high affinity to form a quartet. For example, tetraploid frog species were reported to exhibit many quadrivalents, suggesting an autotetraploid origin [Beçak et al., 1966; Schmid et al., 1985]. In contrast, allotetraploids (or amphidiploids) presumably exhibit meiotic configurations exclusively with bivalents (II) because 2 sister chromosomes (duplicated from each parent chromosome) can behave as homologous chromosomes forming bivalent pair configurations, similar to polyploid plant species [Jenkins and Jimenez, 1995].

In the diploid-tetraploid complex of the Chinese loach, meiotic chromosome configurations have been examined in spermatocytes by conventional air-dry preparation and in oocytes by in vitro induction of final maturation and subsequent isolation of germinal vesicle during its migration to the animal pole [Li et al., 2011]. Spermatocytes of diploid loaches provided 25II, formed by pairing 50 chromosomes.

Diploid loaches also resulted in 25II in the germinal vesicle of oocytes, which confirmed the diploid chromosome number of 50. Conversely, the tetraploid gave meiotic metaphases, including several quadrivalents in both spermatocytes and oocytes. In tetraploid spermatocytes, metaphase with 4IV and 42II represented the most frequent configurations, but other configurations such as 50II, 2IV + 46II, 3IV + 44II, 5IV + 40II, and 6IV + 38II were also observed [Li et al., 2011]. Various configurations were found in the germinal vesicles of oocytes, but the most frequent configuration was ring-like 3IV and 44II, with 100 chromosomes in total [Li et al., 2011].

Doubling of the entire genome presumably occurred in an ancestral diploid loach, and the resultant 4 homologues would have made quadrivalent configurations during meiosis because of the high affinities of homologues (fig. 1). However, contemporary tetraploid loaches show not only several quadrivalents, but also many bivalents that may have resulted from pairwise differentiation among chromosomes within each quartet, through a chromosome rediploidization process (fig. 1). Because quadrivalents reduce reproductive capacity of zygotes, mainly by irregular meiotic divisions causing the production of unusual (e.g. hypo- and hyper-diploid) gametes, the contemporary tetraploid loach is now genetically and cytogenetically stable, due to the decrease of quadrivalents and the increase of bivalents through immediate rediploidization subsequent to chromosome doubling.

Experimental Evidence of Genetic Tetraploidy

Progeny resulting from cross-breeding of gametes from diploid and tetraploid loaches, followed by chromosome set manipulation, are shown in figure 2. When gynogenetic progeny are artificially induced from eggs of diploid loaches ($2n = 50$) by fertilization of genetically inert (UV-irradiated) sperm (fig. 2a), resultant haploid progeny ($n = 25$) die before hatching or feeding due to the haploid syndrome [Suzuki et al., 1985b; Suwa et al., 1994]. Artificially induced androgenics are also inviable because of the haploid syndrome [Arai et al., 1992; Fujimoto et al., 2007]. Salmonid species and common carp are likely of tetraploid origin, with approximately 100 chromosomes or chromosome arms, but the gynogenetic progeny induced from eggs of these species exhibit the haploid syndrome because they must be genetically diploid at present after completion of rediploidization [Nagy et al., 1978; Chourrout et al., 1980]. The European loach, *M. fossilis*, has 100 chromosomes, but its induced gynogenetic progeny are inviable [Neyfakh, 1964; Romashov and Belyaeva, 1964], suggesting that this species is no longer genetically tetraploid ($4n = 100$), but diploid ($2n = 100$). In contrast, gynogenetic progeny induced from eggs of tetraploid *M. anguillicaudatus* loaches with

100 chromosomes were viable without the expression of the haploid syndrome because these progeny had 2 sets of chromosomes (diploid genomes) [Arai et al., 1991b, 1993; Li et al., 2013]. Androgenics from sperm of natural tetraploid males are also viable [Arai et al., 1995]. These results indicate that loaches with 100 chromosomes are genetically true tetraploid ($4n = 100$) with 4 sets of functional genomes.

Tetraploid lines were established by cross-breeding using diploid gametes of natural tetraploid individuals found in Japanese market samples, as shown in figure 2i [Arai et al., 1991b, 1993; Zhang and Arai, 1996; Arai, 2001, 2003]. Both females and males appeared in the progeny, at a normal sex ratio of 1:1, and these progeny produced fertile diploid gametes [Arai et al., 1999; Arai, 2001, 2003; Li et al., 2012, 2013] (table 1). When the release of the second polar body was artificially inhibited by hydrostatic pressure treatment of the eggs of tetraploid females, just after fertilization with diploid sperm from tetraploid males, hexaploid ($6n$) progeny were successfully induced [Kijima et al., 1996b; Zhang and Arai, 1996] (fig. 2j). These hexaploid loaches produced fertile triploid gametes (eggs and sperm) after growth and maturation [Arai et al., 1999]. These results strongly suggest that contemporary tetraploid loaches are not genetic diploids ($2n = 100$), but genetic tetraploids ($4n = 100$) because the resultant progeny with 150 chromosomes are not sterile triploids ($3n = 150$), but fertile hexaploids ($6n = 150$). In fish, artificially induced triploids with an extra set of chromosomes often exhibit sterility as described in section *Artificially Induced Polyploids: General Performance and Reproductive Capacity*.

Natural tetraploid loaches ($4n = 100$) can be used as a source of diploid gametes (eggs and sperm) for further expansion of chromosome set manipulation studies [Arai, 2001, 2003; Yoshikawa et al., 2008]. Various ploidies and genomic constitutions can be induced by a combination of diploid gametes from tetraploids and using chromosome set manipulation as outlined in part in figure 2b–k [Matsubara et al., 1995; Zhang and Arai, 1996; Arai et al., 1999; Arai, 2001, 2003; Zhang et al., 2002]. Reproductive characteristics of various kinds of progeny of natural tetraploid loaches are summarized in table 1.

Interestingly, tetraploid and hexaploid loaches exhibit retarded growth. When the growth performance was compared among diploid, triploid and tetraploid loaches, under communal rearing conditions in the same tank, 3-year-old tetraploid loaches exhibited significantly slower growth rates than the diploids or triploids [Horie et al., 2004a]. Three-year-old hexaploid loaches exhibited about half the body weight of their tetraploid and pentaploid counterparts under communal rearing conditions [Horie et al., 2004b]. When genetically identical clonal diploid and clonal tetraploid loaches were

communally reared, significantly retarded growth was detected in the tetraploid clone, suggesting that the ploidy level alone influenced the growth-trait [Morishima et al., 2012]. In contrast, higher condition factors were reported in natural tetraploid loaches than in diploids and triploids in wild samples from natural populations of China [Li et al., 2012]. Comparative 15-month studies demonstrated an improved growth of tetraploid loaches compared with diploid loaches [Zhou et al., 2010].

Triploid Loaches from Crosses between Tetraploid and Diploid Loaches

In central China, triploid loaches were detected together with diploid and tetraploid individuals but at a relatively low frequency, and few triploid individuals appeared in other locations [Li et al., 2008]. Considering similar or identical haplotypes (sequences) of mtDNA control regions in diploid, triploid and tetraploid individuals [Morishima et al., 2008a; unpublished data], the differentiation may be recent, and triploid individuals might appear by cross-fertilization between diploid and tetraploid individuals, at least in central China.

In triploid progeny produced by hybridization between tetraploids and diploids under laboratory conditions, males were generally sterile [Matsubara et al., 1995; Arai, 2001, 2003], as reported for artificially induced triploid loaches [Suzuki et al., 1985a; Zhang and Arai, 1999b; Arai and Inamori, 1999] and induced triploids of other fish species [Piferrer et al., 2009] (see section *Artificially Induced Polyploids: General Performance and Reproductive Capacity*). Production of a small quantity of sperm, including aneuploid ($1.2n$ – $2.2n$) spermatozoa, was recently reported for natural triploid males from wild populations in China [Li et al., 2012]. In contrast, triploid (diploid $\times$ tetraploid) females simultaneously generated large triploid eggs, small haploid eggs and sometimes aneuploid ($1.4n$ – $1.5n$) eggs; all of these eggs were fertile [Matsubara et al., 1995; Arai and Mukaino, 1997, 1998; Zhang et al., 1998; Arai, 2001, 2003; Momotani et al., 2002] (table 1). However, the frequencies of triploid, haploid and aneuploid eggs varied between individuals; some females gave a relatively large proportion of triploid eggs, while others exclusively spawned haploid eggs. The simultaneous production of eggs with different sizes is presumably due to the difference in the ploidy status of the early germ cells (oogonia or spermatogonia) from which gametogenesis begins. The mechanisms responsible for the occurrence of large and small eggs will be discussed in section *Mechanisms of Unreduced Gametogenesis and Gynogenesis*.

Pentaploid loaches produced by the tetraploid female $\times$ diploid male cross, followed by the inhibition of the second polar body release (fig. 2h), laid diploid eggs, and triploid ($5n \times 2n$) and tetraploid progeny ($5n \times UV$ and inhibition of the second

polar body release) appeared from the cross using such diploid eggs of pentaploid female [Matsubara et al., 1995] (table 1). Unfortunately, the reproductive traits of pentaploid males were uncertain because there was no access to adult males [Matsubara et al., 1995].

Reproductive traits of triploid (diploid $\times$ tetraploid) female loaches are similar in natural triploid females in China, which mainly generate haploid eggs [Li et al., 2012] (clone-origin triploid females are described in the next section). The reproductive mode of triploid (diploid $\times$ tetraploid) loaches is quite different from that of artificially induced triploid females, which exhibit arrest of oogenesis [Zhang and Arai, 1999a]. The majority of studies on this topic were conducted in the 1990s and early 2000s, and thus, Japanese wild-type diploid loaches were used for the cross-breeding with natural tetraploids found in market samples (probably of Chinese origin). Recent genetic studies indicated the presence of genetically different groups within the species [Morishima et al., 2008a], and therefore, it is difficult to exclude the possibility that the past diploid $\times$ tetraploid crosses were hybridization between Japanese wild-type diploid and Chinese bisexual tetraploid loaches), which presumably have genetic differences. Production and evaluation of triploids and other progeny are required to study crosses between diploid and tetraploid individuals within the same genetic group.

Natural Clonal Loach, an Example in Teleosts

Most Japanese loaches are gonochoristic diploids, and natural triploid variants appear in several locations at relatively low frequencies [Zhang and Arai, 1999a]. In the Hirokami population (Niigata Prefecture, Honsyu Island, Japan), triploid males were essentially sterile, while females laid many haploid and a few triploid eggs [Zhang and Arai, 1999a], similar to the case of triploids resulting from crosses between diploid and tetraploid loaches [Matsubara et al., 1995]. Triploids in this location likely derive from a fertilization of diploid eggs produced by certain diploids with normal haploid sperm of a wild-type diploid [Zhang and Arai, 1999a]. As mentioned above, no natural tetraploid variants producing diploid gametes have been discovered in Japan, except in market samples [Ojima and Takai, 1979; Arai et al., 1991a; Zhang and Arai, 1999a; Arai, 2003]. However, clonal diploid populations propagated by gynogenesis were discovered in the northern area of Hokkaido Island and in Noto Peninsula, Honsyu Island, Japan [Morishima et al., 2002, 2008a]. These populations exhibit different genetic and reproductive characteristics compared with the diploid-tetraploid complex in China and the above-described Hirokami loaches. Natural tetraploid loaches are considered

autotetraploid with 4 functional sets of homologous chromosomes (see section *Natural Tetraploid Loaches*), whereas clonal diploid loaches presumably have a hybrid origin between genetically different groups (or cryptic or extinct species); their origin and ancestors have not been identified. The reproduction and putative progeny of cryptic clonal loaches are summarized in figure 3.

Natural Clone and Clone-Origin Triploid

Clonal lineages of diploid loaches were discovered in the northern area of Hokkaido, Japan, using artificial induction of gynogenetic development with UV-irradiated sperm and heterospecific sperm, and subsequent DNA genotyping; however, the origin of these loaches remains unclear [Morishima et al., 2002]. Although clonal diploid individuals are cryptic due to the lack of a morphological difference from wild-type loaches, the eggs of cryptic clonal females can develop to normal loaches, with identical genotypes, by spontaneous gynogenesis. Clonal diploid loaches spawn genetically identical diploid eggs, most of which develop normally by gynogenesis without any genetic contribution of the sperm donor. Analyses of mtDNA control region sequences revealed that clonal loaches were genetically different from other diploid wild-type loaches in Japan and China [Morishima et al., 2008a]. Multi-locus DNA fingerprinting and random amplified polymorphic DNA analyses have identified at least 4 different clonal lineages with identical or nearly identical mtDNA haplotypes in several locations in Japan [Morishima et al., 2008a].

Clone-origin triploid individuals also appeared by sperm nuclear incorporation into unreduced diploid eggs from clonal diploid females in several wild populations [Morishima et al., 2002, 2008a]. Such triploid males were sterile [Oshima et al., 2005], while the majority of females produced fertile haploid eggs by meiotic hybridogenesis [Morishima et al., 2008b]. However, some females generated diploid, triploid and aneuploid ($1.1n$ – $1.7n$) eggs [Oshima et al., 2005].

Occasionally, diploid-triploid mosaic individuals appear in wild populations [Morishima et al., 2002, 2004]. Such mosaics included both diploid and triploid cell populations; diploid cells had clonal diploid genomes, while triploid cells possessed both, a clonal diploid genome and an additional haploid genome from wild-type diploid males [Morishima et al., 2004]. Diploid-triploid mosaic females laid meiotic haploid, clonal diploid and unreduced triploid eggs simultaneously; clonal diploid eggs likely differentiated from clonal diploid germ cells, while haploid and triploid eggs likely differentiated from clone-derived triploid germ cells [Yoshikawa et al., 2007b]. Interestingly, diploid-triploid mosaic males produced fertile diploid spermatozoa with

genetic identity, i.e. clonal sperm [Morishima et al., 2004]. Clonal diploid spermatozoa differentiate from clonal diploid germ cells in the testis of diploid-triploid mosaics. Clonal diploid lineages are essentially all-female because the sex determination system of the loach is male heterogamety (XX female, XY male) [Suzuki et al., 1985b], where clonal loaches develop by gynogenesis without contribution of a functional Y chromosome. Triploid cells with XXY genotypes may induce testicular development, and the gonadal environment may change the sexual differentiation of genetically female clonal diploid germ cells during the spermatogenesis. Production of clonal males has also been achieved by artificial sex reversal using 17- α methyltestosterone administration, and the resultant sex-reversed clonal diploid males produced genetically identical diploid spermatozoa [Yoshikawa et al., 2007a]. In nature, there is evidence of female-to-male sex reversal by high temperature during sexual differentiation [Nomura et al., 1998]. A putative representation of the cryptic clonal lineages and their derivative progeny is shown in figure 3.

Diploid spermatozoa formed by diploid-triploid mosaics and sex-reversed clonal diploids are useful as genetic materials for ploidy manipulation. However, physiological differences were observed between diploid sperm of clonal diploid males and bisexual tetraploid males. The motility of the diploid spermatozoa of clonal loaches was generally poor due to low content of ATP [Zhao et al., 2012b], but diploid spermatozoa of bisexual tetraploid loaches had a motility and fertility equivalent to haploid sperm of wild-type diploid loaches [Li et al., 2012].

In other unisexual fishes, leakage of genes from a paternal host to a clone via chromosome fragments has been observed [Lamatsch and Stöck, 2009], but no such phenomenon has been detected in loaches. However, chromosome fragments or supernumerary B-chromosomes have been observed in several cases of loach progeny [Zhang and Arai, 1999b, 2003; Zhang et al., 2002; Zhao et al., 2012a].

Mechanisms of Unreduced Gametogenesis and Gynogenesis

In *M. anguillicaudatus*, clonal diploid loaches produce unreduced diploid eggs with genotypes identical to the somatic cells of the mother [Morishima et al., 2002]. Triploid loaches from the cross between diploid and natural tetraploid individuals often lay unreduced triploid eggs that are genetically identical or similar to the somatic cells of the mother [Arai and Mukaino, 1997]. Production of such unreduced isogenic eggs may occur through either (a) apomixis or (b) premeiotic endomitosis [Dawley, 1989]. Alternatively, unreduced egg formation may occur through (c) automixis [Lampert et al., 2007]. In automixis, meiosis is maintained, and diploid gametes are generated after

meiosis by the fusion or duplication of meiotic products. Therefore, resulting diploid products are not genetically identical and are essentially different from (a) and (b) to produce isogenic gametes. Involvement of automixis has not been confirmed in unreduced gametogenesis of *Misgurnus* loaches.

In apomixis, the first meiotic division (MI) is abortive to undergo recombination and segregation of homologous chromosomes. This system is not a mechanism to form unreduced gametes in loaches, but in silver crucian carp, *Carassius auratus gibelio* [Cherfas, 1966] and *C. a. langsdorfii* [Kobayasi, 1976; Yamashita et al., 1993]. These previous studies reported that a tripolar spindle formed at MI, and the first polar body was not released. Thus, MI may be skipped to retain the original ploidy level of eggs after meiosis. Unreduced egg formation in the Amazon molly, *Poecilia formosa* [Monaco et al., 1984; Lamatsch and Stöck, 2009] has also been attributed to apomictic reproduction.

Premeiotic endomitosis is chromosome doubling before entering into meiotic division followed by 2 successive divisions. In clonal diploid loaches, oocytes cultured in vitro in the presence of 17- α -20- β -dihydroxy-4-pregnene-3-one underwent 2 successive meiotic divisions as in normal wild-type diploids: formation of the bipolar spindle, the metaphase of MI, segregation of chromosomes, first polar body release, and the metaphase of MII [Itono et al., 2006]. In addition, the germinal vesicle of clonal oocytes clearly showed the presence of 50 bivalents before MI [Itono et al., 2006]. These results indicate that chromosomes are doubled by mitosis without cytokinesis before meiosis, i.e. premeiotic endomitosis. The same mechanism also occurs in oogenesis for formation of unreduced triploid eggs [Zhang et al., 1998]. Using the testis of sex-reversed clonal males, Yoshikawa et al. [2009] concluded that chromosome doubling occurs in the type A and early type B spermatogonial stage in spermatogenesis for formation of unreduced diploid spermatozoa. This suggests that endomitosis likely occurs to form unreduced eggs during the early oogonial stage, even for oogenesis of clonal females.

Involvement of the premeiotic endomitosis system in unreduced gametogenesis was cytologically confirmed in interspecific hybrids of medaka [Shimizu et al., 2000] and triploid (diploid $\times$ tetraploid) loaches [Zhang et al., 1998]. Formation of unreduced eggs has been reported in several present and past interspecific hybrids of teleosts, including *Poeciliopsis*, *Fundulus*, *Menidia*, *Carassius*, *Phoxinus*, *Squalius*, and *Cobitis* [Lamatsch and Stöck, 2009] and in the interpopulational hybrid *M. anguillicaudatus* between genetic group A (northern area of Hokkaido) and B (central area of Hokkaido) [Arias-Rodriguez et al., 2009, 2010]. These results suggest that a genomic conflict due

to a hybrid event may trigger an atypical mode of reproduction. This process is explained by the ‘balance hypothesis’ [Moritz et al., 1989]: a sufficiently large interspecific genetic divergence causes the production of unreduced eggs, but a moderate divergence decreases the viability and fertility of hybrids.

Formation of haploid eggs in triploid loaches can be explained by meiotic hybridogenesis or the preferential pairing of homologous chromosomes and elimination of unmatched chromosomes [Morishima et al., 2008b]. Clonal diploids are considered products of a past hybridization event between 2 genetic groups (or cryptic species) with putative genomic constitutions of AA and BB [Morishima et al., 2008a]. Hence, the clone may have a heterozygous genomic constitution AB, and clone-origin triploids may have genomic constitutions of AA'B or ABB'. In a clone-origin triploid with AA'B, one genome (A) of the clone is more likely to pair to the genome (A') from present wild-type males in group A, while the unmatched B genome is discarded [Morishima et al., 2008b]. The presence of genetic differences between northern Hokkaido, including clonal lineages, and other locations was also supported by nuclear genetic markers such as allozymes [Khan and Arai, 2002] and microsatellites [Arias-Rodriguez et al., 2007].

The stage corresponding to elimination of the unmatched genome occurs before meiosis because the germinal vesicle of the oocyte exhibits 25 bivalents (50 chromosomes) during MI in triploids [Morishima et al., 2008b]; however, further studies are required in loaches. For meiotic hybridogenetic reproduction in the frog species *Rana esculenta*, Ogielska [1994] concluded that elimination of chromosomes occurs during the interphase in early oogonia and spermatogonia, and the chromosomes or their fragments are rejected from the nucleus as nucleus-like bodies or micronuclei based on histology and electron microscopy. A similar conclusion was reached in the hybridogenetic fish *P. monacha-lucida* using cytology [Cimino, 1972]: the paternal chromosomes could not attach to the mitotic spindle and were thus eliminated to the cytoplasm of dividing oogonia. Ogielska [2009] clarified the timing of genome elimination, but this mechanism is still unclear. Molecular mechanisms of unusual events during gametogenesis such as chromosome doubling and chromosome elimination are unknown, and further molecular biological studies are required to elucidate these mechanisms.

Simultaneous occurrence of eggs with different ploidy status and sizes is frequently observed even in non-mosaic triploid loaches (see section *Triploid Loaches from Crosses between Tetraploid and Diploid Loaches*). This phenomenon may be explained by the difference in the cytological mechanism of gametogenesis: premeiotic

endomitosis will generate much larger triploid eggs in triploid females, whereas meiotic hybridogenesis will generate small (normal) haploid eggs from triploid females. However, the mechanisms of the simultaneous occurrence of 2 reproductive modes in the same individual are unknown at present.

The failure of decondensation of the sperm nucleus is generally accepted as a mechanism of gynogenetic development. In the natural gynogenetic silver crucian carp *C. a. langsdorfii*, insemination by sperm of its diploid bisexual form or by sperm of different species initiated gynogenetic development of eggs, but the incorporated sperm nucleus was not decondensed and did not contribute to zygotic genome [Kobayasi, 1971; Yamashita et al., 1990]. In the clonal lineage of the loach *M. anguillicaudatus*, the sperm nucleus remained condensed in most eggs throughout fertilization. During early embryogenesis, the sperm nucleus was not decondensed to form a male pronucleus and isolated itself from the female pronucleus [Itono et al., 2007]. However, some eggs showed quasinormal syngamy between the female pronucleus and an accidentally formed male nucleus; this individual then developed to a triploid animal. The syngamy between an activated sperm nucleus and a nucleus of a blastomere of gynogenetically developed embryo results in a diploid-triploid mosaic embryo [Itono et al., 2007].

Clonal Diploid versus Clonal Tetraploid

When sex reversal is conducted by androgen treatment or environmental influences (high temperature) during the sex differentiation period, clonal diploid males can be induced (fig. 3). Fertilization of clonal diploid eggs from a clonal female with diploid sperm of a sex-reversed clonal diploid male normally produces gynogenetic diploid progeny; however, tetraploid clones are sometimes generated by accidental incorporation of a diploid nucleus of a clonal sperm [Morishima et al., 2012]. Artificial inhibition of second polar body release after initiation of gynogenetic development in clonal diploid eggs also produced a clonal tetraploid loach comprising a diploid female pronucleus and a diploid polar body nucleus [Morishima et al., 2012]. No genetic differences were observed between the clonal diploid and tetraploid loaches, except for the difference in ploidy level. Interestingly, clonal diploids produced clonal diploid gametes (eggs and sperm) with identical genotypes by unreduced gametogenesis, while clonal tetraploids produced clonal diploid gametes with identical genotypes by regular meiosis and gametogenesis (fig. 3).

Premeiotic endomitosis produces diploid gametes in clonal diploid females and sex-reversed males because their diploid germ cells have non-homologous

chromosomes, presumably derived from different groups (or species) A and B (see section *Mechanisms of Unreduced Gametogenesis and Gynogenesis*). In clonal diploids, diploid germ cells ($2n = 50$) duplicate to tetraploid cells ($4n = 100$), and then sister chromosomes (which double from each non-homologous chromosome in the original diploid germ cell) can pair to form 50 bivalents in meiosis like homologous chromosomes in a normal bisexual diploid. In contrast, in clonal tetraploid females and males, regular meiosis should occur to produce diploid gametes because original tetraploid germ cells have appropriate chromosome pairs that can form 50 bivalents during meiosis [Morishima et al., 2012]. In both cases, however, isogenic diploid eggs were laid by clonal diploid and tetraploid females, which can develop by natural gynogenesis (fig. 3). Atypical gametogenesis (premeiotic endomitosis) occurs in clonal diploids, while regular meiosis occurs in clonal tetraploids. Both clones are genetically identical, except for the ploidy. Therefore, these facts suggest that a difference is controlled by the chromosome (genomic) constitution of germ cells.

Concluding Remarks

The loach *M. anguillicaudatus* is a newcomer to the world of natural unisexual and polyploid fishes (vertebrates). The presence of a diploid-tetraploid complex has been recognized in central China, and these diploid and tetraploid individuals generate fertile haploid and diploid gametes (eggs and sperm) by regular meiosis, respectively, to reproduce bisexually (see section *Karyotype and Meiosis*). Considering the cytogenetic features of mitotic and meiotic chromosomes, tetraploids might have been formed from conspecific diploids by a genome-duplication event, but such autotetraploids are now considered in a process of rediploidization (see section *Experimental Evidence of Genetic Tetraploidy*). In contrast, most loaches are bisexually reproducing diploids in Japan, but gynogenetically reproducing clonal diploids appear in a few populations. Triploids often occur from clonal diploid eggs by accidental incorporation of a sperm nucleus into a diploid egg, and they produce fertile haploid eggs by the atypical reproduction of meiotic hybridogenesis. Clonal loaches likely arise from a past hybridization event between genetically diverse loaches (species or groups, hypothesized genomes, AA and BB in this paper), but their exact origins are unknown due to the shortage of population genetics results (see section *Natural Clone and Clone-Origin Triploid*). To identify the origin of a clone, further genetic studies are required to disclose the relationships among loaches with different ploidy status and reproductive modes, based on different populations and using more sensitive DNA

626 markers and advanced techniques. Regular meiosis and gametogenesis are initiated by
627 checking the presence of 2 homologous chromosome sets to pair in a wild-type diploid,
628 whereas the incidence of atypical reproduction is likely regulated in early germ cells by
629 checking the genome constitution: the presence or absence of homologous
630 chromosomes to pair and form bivalents (see sections *Mechanisms of Unreduced*
631 *Gametogenesis and Gynogenesis* and *Clonal Diploid versus Clonal Tetraploid*). The
632 presence of 2 different non-homologues may cause the duplication of a
633 non-homologous chromosome to produce 2 sister chromosomes, which can pair like
634 regular homologues and then enter meiosis. In contrast, the presence of 2 sets of
635 homologues and 1 set of non-homologues in triploid germ cells may cause elimination
636 of non-homologues and subsequent meiosis with 2 sets of homologues. However,
637 molecular mechanisms regulating chromosome duplication and elimination in early
638 germ cells (by checking chromosome/genome constitutions) are still unknown and
639 require additional studies, including deep sequencing and transcriptome analyses.

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Legends

Fig. 1. Schematic representation of different processes of genomic duplication. **a** autotetraploidization and **b** allotetraploidization.

Fig. 2. Putative progeny of natural diploid and tetraploid loaches *Misgurnus anguillicaudatus* by cross-breeding and chromosome manipulation. UV = ultraviolet ray irradiation of sperm or egg to inactivate nucleus. PTS = hydrostatic pressure or temperature (cold or heat) shock to inhibit the second polar body release after fertilization. Bold arrows indicate the timing to inhibit the second polar body release.

Fig. 3. Putative representation of reproduction of clonal diploid lineages, clone-origin triploid progeny, diploid-triploid mosaic progeny, sex-reversed clonal diploids, and clonal tetraploid progeny of the loach. A and B denote a chromosome set of ancestral species of the clone. CL = Clone; M = meiosis; MH = meiotic hybridogenesis; PE = premeiotic endomitosis.

Table 1. Reproductive capacity of gametes of various biotypes of loaches found in wild populations and formed in the laboratory by artificial cross and chromosome manipulation

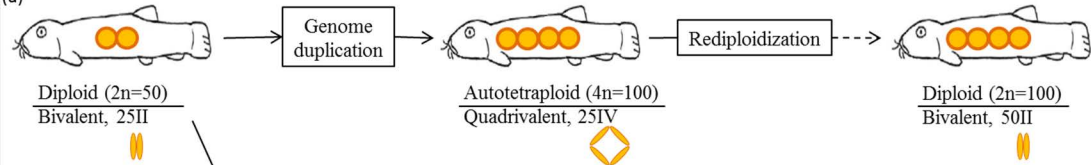
Biotype	Origin/cross/ manipulation	Sex ratio female to male	Egg				Sperm		Reference
			ploidy	diameter (mm)	estimated mecha- nism of oogenesis	gynogenesis	ploidy	estimated mecha- nisms of spermato- genesis	
Diploid wildtype	wild (China)	38F:21M	1n	0.8	M	no	1n	M	Li et al., 2012a
Triploid wildtype	wild (China)	13F:8M	1n	0.8	MH	no	1.2n–2.2n	M	Li et al., 2012a
Tetraploid wildtype	wild (China)	38F:40M	2n	1.0	M	no	2n	M	Li et al., 2012a
Diploid	2n × 2n	16F:14M	1n	1.0–1.2	M	no	1n	M	Arai et al., 1999
Gyno. diploid	2n × UV/CS	56F:1M	n.d.	n.d.	n.d.	n.d.			Suzuki et al., 1985b
Triploid	2n × 2n/CS	63F:27M	sterile						Suzuki et al., 1985a
Triploid	2n × 2n/PS	n.d.	sterile						Zhang and Arai 1999b
Tetraploid	4n × 4n	73F:69M	2n	1.2–1.4	M	no	2n	M	Arai et al., 1999
Gyno. tetraploid	4n × UV/PS	25F:0M	n.d.	n.d.	n.d.	n.d.			Arai et al., 1999
Gyno. diploid	4n × UV	1F:0M	1n	1.1	M	no			Zhang et al., 2002
Triploid	4n × 2n, 2n × 4n	n.d.	1n	1.1	MH	no	sterile		Matsubara et al., 1995 Arai and Mukaino, 1997 Arai and Mukaino, 1998 Zhang et al., 1998
			3n	1.4	PE	no			
Gyno. triploid	3n (2n × 4n) × UV	7F:0M	1n	1.1	MH	no			Momotani et al., 2002
			3n	1.4	PE	no			
			1.4n–1.5n	1.3n–1.4n	M	no			
Pentaploid	4n × 2n/HS	n.d.	2n	1.2	MH	no			Matsubara et al., 1995
Hexaploid	4n × 4n/PS	11F:10M	3n	1.4	M	no	3n	M	Arai et al., 1999
Clonal diploid	CL2n × 2n, UV	n.d.	CL2n	n.d.	PE	yes	CL2n	PE	Morishima et al., 2002 Itono et al., 2006, 2007 Yoshikawa et al., 2007a
	wild (Japan)								
Clone-origin triploid	CL2n × 2n	n.d.	1n	n.d.	MH	no	sterile		Morishima et al., 2008b
			3n	n.d.	PE	no			
Clone-origin triploid	wild (Japan)	n.d.	1n	*	MH	no	sterile		Oshima et al., 2005
			3n	*	PE	no			
			2n	*	?	?			
			1.1n–1.7n	*	M	no			
Clone-origin diploid/ Triploid mosaic	wild (Japan) plus CL2n × 2n	3F:2M	1n	0.8	MH	no	CL2n	M	Yoshikawa et al., 2007b
			CL2n	n.d.	PE	yes			
			3n	1.3	PE	no			
Clonal tetraploid	CL2n × CL2n	23F:0M**	CL2n	n.d.	M	yes	CL2n	M	Morishima et al., 2012
	CL2n × UV/CS	4F:0M	CL2n	n.d.	M	yes	n.d.		Morishima et al., 2012

CL = Clonal individual that normally reproduces gynogenetically; CS = cold shock; PS = hydrostatic pressure shock; HS = heat shock treatment (CS, PS and HS treatment to inhibit second polar body release after fertilization); M = meiosis; MH = meiotic hybridogenesis; n.d. = not determined; PE = premeiotic endomitosis; UV = irradiation to inactivate sperm.

* Eggs which ploidy were not identified gave 3 size groups: 1.0–1.1, 1.2 and 1.3–1.4mm in diameter.

** Spontaneous sex reversal can occur due to high temperature during sexual differentiation. One cross gave 1F:3M.

(a)



(b)

