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Title	Ploidy manipulation using diploid sperm in the loach, <i>Misgurnus anguillicaudatus</i> : a review
Author(s)	Yoshikawa, Hiroyuki; Morishima, Kagayaki; Fujimoto, Takafumi et al.
Citation	Journal of Applied Ichthyology, 24(4), 410-414 https://doi.org/10.1111/j.1439-0426.2008.01129.x
Issue Date	2008-08
Doc URL	https://hdl.handle.net/2115/38891
Rights	The definitive version is available at www.blackwell-synergy.com
Type	journal article
File Information	arai.pdf



PLOIDY MANIPULATION USING DIPLOID SPERM IN THE LOACH,

***Misgurnus anguillicaudatus*: A REVIEW**

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Short title Ploidy manipulation using diploid sperm

Summary

In the loach, *Misgurnus anguillicaudatus* (Teleostei: Cobitidae), diploid sperm can be obtained from natural tetraploid individuals with four sets of homologous chromosomes. Using diploid sperm, various kinds of polyploids and androgenetic diploids have been produced. Cryptic clonal lineages are also recognized in wild populations of the loach. They produce unreduced diploid eggs genetically identical to somatic cells of the mother fish and most diploid eggs develop gynogenetically as a member of the clone. However, some eggs develop to triploid and/or diploid-triploid mosaic individuals by incorporation of sperm nucleus. Diploid-triploid mosaic males exclusively generate fertile diploid sperm with clonal genotypes. Such diploid sperm can also be obtained from artificially sex-reversed clonal individuals. Recent population studies suggested that Japanese *M. anguillicaudatus* might not be a single species, but a complex involving cryptic species, because wild populations exhibited genetic differentiation at interspecific level. This implies possible relationship between atypical reproduction and natural hybridization in the loach.

Key Words: clone, unreduced gametes, polyploid, hybrid, chromosome manipulation

INTRODUCTION

Ploidy manipulation could be further extended to improve economically important aquaculture traits, if fertile diploid spermatozoa were available for mass production of sterile triploid individuals, establishment of tetraploid lines, generation of viable androgenetic diploids without chromosome doubling and so on. However, it is generally difficult to obtain diploid spermatozoa, because artificial tetraploids, which are considered to be a source of diploid sperm, always exhibit very poor survival rates due to the side effect of the treatment to inhibit the 1st cleavage (Pandian and Koteeswaran, 1998; Arai, 2001). Although viable tetraploid individuals have been produced in several fish species (Pandian and Koteeswaran, 1998; Arai, 2001), mature tetraploid fishes have been attained only in rainbow trout (Chourrout et al., 1986; Chourrout and Nakayama, 1987), blunt snout bream (Zou et al., 2004) and mud loach (Nam and Kim, 2004). Using diploid gametes produced by tetraploid rainbow trout, triploid, tetraploid, pentaploid, hexaploid and gynogenetic diploid individuals were generated by combining artificial fertilization and chromosome manipulation (Chourrout et al., 1986; Chourrout and Nakayama, 1987). Tetraploid line was produced by tetraploid female x tetraploid male cross in blunt snout bream (Zou et al., 2004). In the mud loach, however, tetraploid males producing diploid spermatozoa were rather rare, only three out of 48 tetraploid survivors generated diploid spermatozoa and the other were sterile or produced haploid or mosaic sperm (Nam and Kim, 2004). On the other hand, a rare case of fertile diploid sperm was reported in the Iberian minnow, *Squalius (Rutilus) alburnoides* with a natural hybrid origin (Alves et al., 1999). Artificial hybrids between common carp *Cyprinus carpio* and crucian carp *Carassius auratus* were also reported to produce unreduced gametes and diploid spermatozoa have been used in various chromosome manipulations for establishment of aquaculture strains (Cherfas et al., 1994; Liu et al., 2001; Sun et al., 2007). These observations suggest that hybrids may be a source of diploid sperm. The other option to obtain diploid sperm is cell fusion, but successful examples of polyploidy and/or viable androgenetic induction using fused spermatozoa were very rare (Ueda et al., 1986, 1988; Araki et al., 1995).

In the loach *Misgurnus anguillicaudatus*, most individuals are bisexually reproducing diploid with the karyotype $2n=50$ chromosomes, but a small number of natural tetraploid individuals have been recorded mainly in specimens of unknown origin collected from commercial dealers (Arai et al., 1991a; Arai, 2003). These natural tetraploids have been used as a source of fertile diploid gametes (eggs and sperm) to produce various kinds of higher polyploids and gynogenetic and androgenetic diploids (Arai et al., 1991b, 1993, 1995, 1999; Matsubara et al., 1995; Zhang and Arai, 1996). Other source of diploid sperm was found in the derivative lineages of natural clonal diploid loach discovered in the northern area of Hokkaido Island, Japan. These clonal loaches generate genetically uniform diploid eggs, most of which reproduce gynogenetically, while some develop to triploid or diploid-triploid mosaic fish by accidental incorporation of haploid sperm nucleus (Morishima et al., 2002). Since the artificially

sex-reversed clone and diploid-triploid mosaic males were reported to produce fertile diploid spermatozoa (Yoshikawa et al., 2007a; Morishima et al., 2004), they are regarded as the additional source of diploid sperm.

In the present paper, we review ploidy manipulation using diploid sperm of natural tetraploid loache as well as those of derivative lineages of clonal diploid loach.

DIPLOID SPERM OF NATURAL TETRAPLOID LOACH

Natural tetraploid individuals with 100 chromosomes were not found in wild populations so far examined in Japan, but in specimens obtained from commercial dealer (Arai et al., 1991a, Arai, 2003). This suggests that they presumably have an exotic origin because continental *Misgurnus* loaches are being imported to Japan as food and tetraploid individuals with 100 chromosomes were reported in the specimens collected from Hubei Province in China (Li et al., 1983; 2008).

Loaches with 100 chromosomes are not diploid ($2n=100$) with two genome sets, but tetraploid ($4n=100$) with four genome sets, because viable diploid progeny appeared without any treatment to duplicate chromosomes when eggs of tetraploids were gynogenetically activated with UV irradiated sperm, whereas abnormal larvae occurred from eggs of diploid loach after fertilization with UV sperm, because of the expression of haploid syndrome (Arai et al., 1991b, 1993). The same conclusion was also drawn from the results of androgenetic experiments, in which UV irradiated loach eggs were fertilized with sperm of natural tetraploid males and normal androgenetic progeny survived (Arai et al., 1995). These results conclude that natural tetraploid loach is a genetic tetraploid with four sets of homologous chromosomes ($4n=100$). Genetic analysis using allozymes indicated that diploid spermatozoa were generated by meiotic process of tetraploid loach (Arai et al., 1995).

Using diploid sperm of natural tetraploid loaches, various polyploid animals have been created (Matsubara et al., 1995; Zhang and Arai, 1996; Arai, 2001), as summarized in Fig. 1. Triploid loaches were produced by fertilizing haploid eggs with diploid sperm (Fig. 1a). Pure tetraploid lines were established by crossbreeding between diploid eggs and diploid sperm of tetraploid broodstock (Fig.1c). The other type of tetraploid was realized by inhibiting the second meiosis after fertilization of normal eggs with haploid egg nucleus plus haploid polar body nucleus by diploid sperm of tetraploid (Fig. 1b). When the second meiosis was inhibited after the fertilization of eggs with diploid egg nucleus plus diploid polar body nucleus of tetraploid by diploid sperm, hexaploid progeny appeared (Fig. 1d). The second generation was created because founder hexaploids produced fertile triploid eggs and sperm (Kijima et al., 1996; Arai et al., 1999). Growth performances of the above mentioned tetraploid, hexaploid and other polyploids were also evaluated under a laboratory condition: retarded growth was observed in tetraploid and hexaploid lines (Horie et al., 2004ab).

DIPLOID SPERM OF DIPLOID-TRIPLOID MOSAIC LOACHES

Clonal lineages have been found in several localities in Honshu and Hokkaido Island, Japan (Morishima et al., 2002, 2008a). Clonal diploid loaches are genetically identical to the mother and the sib, and they generate unreduced diploid eggs by the mechanism of premeiotic endomitosis: the chromosome duplication without cytokinesis in germ cells before entering to meiosis, followed by quasi-normal two maturation divisions (Itono et al., 2006). Such unreduced diploid eggs normally developed gynogenetically following activation by sperm of bisexual loaches in most cases, but some eggs incorporated sperm nucleus and developed to triploid when sperm nucleus was transformed to male pronucleus-like structure and then fused with female pronucleus before the first cleavage (Itono et al., 2007). When an incorporated sperm nucleus was activated and then fused with static nucleus of a blastomere of cleaved embryo, diploid-triploid mosaic comprising both clonal diploid cell populations and triploid cell populations appeared (Itono et al., 2007).

The diploid-triploid mosaic females laid haploid, unreduced diploid and unreduced triploid eggs, simultaneously (Yoshikawa et al., 2007b). Such a simultaneous formation of eggs varying in ploidy levels was also reported in triploid females derived from the clonal lineage: one triploid produced large number of aneuploid and a few triploid eggs, while the other laid large number of haploid and diploid eggs (Oshima et al., 2005). Similar simultaneous formation of haploid and triploid eggs was already recognized in natural triploid which arose from unreduced diploid eggs of non-clonal diploid (Zhang and Arai, 1999) and in synthetic triploid from the cross between normal diploid and natural tetraploid (Matsubara et al., 1995; Arai and Mukaino, 1997, 1998; Zhang et al., 1998; Momotani et al., 2002). However, in natural triploid males, formation of functional sperm was not reported (Oshima et al., 2005).

On the other hand, diploid-triploid mosaic males were found to generate genetically identical unreduced diploid spermatozoa (Morishima et al., 2004). Flow cytometry for DNA content measurement, microsatellite genotyping and multilocus DNA fingerprinting demonstrated that the mosaic male consisted of diploid cell population with genotypes identical to those of the natural clone and triploid cell populations with diploid chromosome sets (genomes) derived from the clonal lineage plus haploid chromosome set (genome) transmitted from sperm nucleus of a bisexual diploid father. However, various ratios between diploid and triploid cells were detected not only by flow cytometry, but also by the intensity of third microsatellite allele among different tissues taken from the mosaic. Flow cytometry on sperm specimens and testicular tissues taken from the diploid-triploid mosaic indicated that only diploid spermatozoa were generated in the testis of the mosaic. When haploid eggs of normal bisexual diploid loach were fertilized by such diploid sperm, only triploid progeny appeared and they showed microsatellite genotypes comprising two alleles identical to the clonal diploid genotypes and one of two alleles of maternal diploid parent. When UV irradiated, genetically inactivated eggs were fertilized by diploid sperm according to the optimum conditions of androgenetic induction (Arai et al., 1992; Fujimoto et al., 2007), viable androgenetic diploids appeared

(Fig.1e) and they exhibited microsatellite genotypes and DNA fingerprinting profiles, absolutely identical to those of the clone (Morishima et al., 2004). These genetic results clearly concluded that the diploid-triploid mosaic should generate clonal diploid spermatozoa, genetically identical to the clonal diploid loaches.

Clonal diploid spermatozoa must be derived from clonal diploid germ cells in the gonad of diploid-triploid mosaic loach. The diploid members of the clonal lineage must be all-female because sex-determination of the loach is estimated as male heterogamety with the XX female-XY male system (Suzuki et al., 1985) and clonal diploids develop gynogenetically from unreduced eggs without any genetic contribution of Y sperm. However, triploid cells could be XXY genotypes by accidental incorporation of Y sperm. It is supposed that XXY triploid cells might differentiate testicular structure and then such a testicular environment might alter the differentiation of diploid germ cells derived from the clone to the spermatogenesis in the mosaic gonad. Triploid germ cells could not differentiate to functional spermatozoa due to the disruption of normal meiosis by additional chromosome set as in most natural triploid males. Direction of germ cells is easily transformed by gonadal environment. When PGC (primordial germ cell)-containing graft from all-female goldfish (XX) was transplanted to all-male goldfish-carp sterile hybrids (XY) for induction of germ-line chimeras, spermatozoa derived from the all-female goldfish PGC were exclusively produced (Yamaha et al., 2003). Testicular germ cells containing spermatogonial stem cells can produce fully functional eggs in rainbow trout, when those cells were transplanted into peritoneal cavity of newly hatched embryos (Okutsu et al., 2006). Thus, germ cells are capable to differentiate to either sperm or eggs, depending on gonadal environment.

DIPLOID SPERM OF SEX-REVERSED CLONAL LOACHES

If sex reversal of the clonal individual is experimentally realized, sex-reversed clone may generate fertile spermatozoa with diploid genotypes absolutely identical to the clonal member. When clonal loaches were sex-reversed by the treatment with 17-alpha methyltestosterone (MT) for 30 days from one month after hatching, resultant physiological males were verified to produce spermatozoa with a diploid DNA content (Yoshikawa et al., 2007a). These diploid spermatozoa of the sex-reversed clone were essentially fertile, but the resultant fertilization rates were very low, probably due to larger head size and lower motility of diploid spermatozoa. Mean length and width of diploid spermatozoa were 2.51 μ m and 2.39 μ m, respectively, and they were significantly larger than those of haploid spermatozoa (mean length 1.87 μ m, mean width 1.70 μ m) and could be difficult to penetrate into egg through the micro-pyle. Such a sperm head-micro pyle problem in fertilization was already recognized and well discussed in tetraploid rainbow trout (Chourrout et al., 1984). After adding ambient water, more than 80% spermatozoa moved in control, but approximately 10% of all diploid spermatozoa in the view gave movement. Normal haploid spermatozoa were continued to move for 174s, but diploid spermatozoa gave much

shorter periods (27 to 31s).

When normal haploid eggs were fertilized with diploid sperm of sex-reversed clonal males, triploid progeny arose and they comprised two microsatellite alleles transmitted from the clonal father and one of two alleles of the bisexual mother at the three loci examined. These results indicated that diploid spermatozoa were fertile and they were genetically identical clone. Thus, natural clonal loaches are concluded as a source of diploid sperm for further ploidy and genetic manipulation, if they are able to be physiologically sex-reversed. In the loach, higher water temperature in probable period of sex differentiation was reported to induce sex-reversal from genetic females to physiological males (Nomura et al., 1998). This suggests the presence of clonal males which were presumably sex-reversed by temperature in nature. For further observation on sex differentiation in the loach, detailed developmental stages were determined based on morphological features and molecular event of specific gene expression (Fujimoto et al., 2004, 2006)

Unreduced eggs are formed by the system of premeiotic endomitosis in the clonal diploid loach, because 50 bivalents (i.e. 100 chromosomes) were counted in germinal vesicle and the first meiosis was detected in mature eggs (Itono et al., 2006). At present, cytogenetic and cytological mechanisms for diploid spermatogenesis have not been disclosed, but the same system may be involved because diploid spermatozoa have the same clonal genotypes.

DIPLOID SPERM AND HYBRIDIZATION IN LOACH

Atypical reproduction using unreduced eggs and gynogenesis is tightly linked to natural polyploidy and has been considered as a hybrid origin in lower vertebrates (Dawley, 1989; Vrijenhoek, 1989; Vrijenhoek et al., 1989). Since the Japanese loach, *Misgurnus anguillicaudatus* has been identified as a single species entity (Saitoh, 1989), the hybrid origin is not able to explain the natural polyploidy and clonal reproduction observed in Japanese loach. However, the presence of genetically distinct population has been suggested in the loach since the population genetic studies using allozymes as genetic marker (Khan and Arai, 2000). Recently, the sequence analyses on the control region of mtDNA indicated presence of the inter-specific differentiation between the two clades of *M. anguillicaudatu* and then strongly suggested the existence of cryptic loach species in Japan (Morishima et al., 2008a). Presence of genetically divergent populations was also suggested by populational analyses using microsatellite DNA markers (Arias-Rodriguez et al., 2007). All these molecular population genetic results suggest genetic differentiation of Japanese loaches. Highly polymorphic microsatellite markers have been actively developed for population studies to identify paternal origin of the clone as well as for genetic mapping (Morishima et al., 2001, 2008b). If the presence of genetically different loaches is correct, hybrids between two different populations may induce atypical reproduction due to a disruption of normal meiosis between non-homologous chromosomes. In addition to the cases in the Iberian minnow (Alves et al.,

1999) and the common carp x crucian carp hybrids (Cherfas et al., 1994; Liu et al., 2001; Sun et al., 2007) as mentioned earlier, unreduced gametes have been reported in artificial medaka *Oryzias latipes* x *O. curvinotus* hybrids (Sakaizumi et al., 1993; Shimizu et al., 2000), natural *Cobitis* hybrids (Janko et al., 2003, 2005, 2007) and others (Vrijenhoek et al., 1989). These results suggest possible induction of unreduced diploid gametes by artificial hybridization between genetically different populations of the loach.

CONCLUSION

Diploid spermatozoa can be formed in clone and mosaic *Misgurnus* loach by the unreduced spermatogenesis. In contrast, tetraploid loach produce reduced diploid spermatozoa by the meiotic process. These diploid spermatozoa can be applicable for further aquaculture-oriented ploidy manipulations. However, molecular and cellular mechanisms responsible for unreduced spermatogenesis remain to be elucidated.

ACKNOWLEDGMENTS

This study was partly supported by a Grant-in-Aid for the 21st Century COE program from MEXT, Japan and that for the Scientific Research (B) (No.18380108) from JSPS, Japan to K.A., and that for Young Scientists (B) (No. 18780138) from MEXT to T.F.

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Figure legend

Fig. 1. Summary of induction of various polyploid and androgenetic diploid loaches using diploid spermatozoa. **a:** triploid loaches induced from haploid eggs fertilized with diploid spermatozoa; **b:** tetraploid loaches induced by inhibition of the second meiosis after fertilizing haploid eggs with diploid spermatozoa; **c:** tetraploid loaches induced from diploid eggs of tetraploid fertilized with diploid spermatozoa; **d:** hexaploid loaches induced by inhibition of the second meiosis after fertilizing diploid eggs of tetraploid with diploid spermatozoa; **e:** androgenetic diploid loaches induced by fertilizing UV irradiated haploid eggs with diploid spermatozoa.

