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# Fertility and ploidy of gametes of allodiploid and allotriploid loaches produced by diploid *Misgurnus anguillicaudatus* females and *Paramisgurnus dabryanus* males

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**Abstract** Artificial and natural hybridization of dojo loach *Misgurnus anguillicaudatus* (2N = 50, DD for short) and large-scale loach *Paramisgurnus dabryanus* (2N = 48, PP for short) are well-grown. However, these hybrid loaches have not yet been examined for fertility and ploidy of gametes. Here, histological observations, artificial propagation, observations of embryonic development, larval morphology, and ploidy analyses were conducted to determine the fertility and ploidy of gametes of allodiploid (DP for short) and allotriploid (DDP for short) loaches, produced by DD females × PP males and induced from fertilized eggs of DD females × PP males by cold shock to prevent the second polar body release, respectively. The ovaries of DP and DDP included smaller number of eggs when compared with those of the control DD, while full-grown oocytes were observed. Testes of these two loaches were delayed-developed without spermatids or mature spermatozoa. Results obtained here showed that DP and DDP were fertility-weakened female and sterile male. Moreover, DP females and DDP females could, respectively, produce few viable haploid eggs and few viable haploid and diploid eggs. This study will provide valuable information for fish hybrid researches.

**Keywords** Hybridization · Fertility · Gamete ploidy composition · Diploid *Misgurnus anguillicaudatus* · *Paramisgurnus dabryanus*

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## Introduction

Dojo loach (*Misgurnus anguillicaudatus*) and large-scale loach (*Paramisgurnus dabryanus*) are small-sized freshwater fish species and distributed widely in Asia (Gao et al. 2012). These two loach species, with similar morphological characteristics and nearly overlapping spawning seasons (March to October), are often found sympatric in most areas and a small number of their hybrid progenies were detected in wild environment (You et al. 2009). Viable hybrids between the two loach species were obtained by artificial propagation techniques (Fujimoto et al. 2008; Cui et al. 2013). However, the fertility of hybrids between these two species has not been elucidated. These loach hybrids may give rise to disturbance of freshwater ecosystem of indigenous populations. Are these loach hybrids

biothreats to the natural environment? If the loach hybrids can produce stable offspring, they will cause gene penetration into the two different loach species. Otherwise, if loach hybrids are sterile, they can be used as good hybrid strains for loach farming and risks for natural environment may be much reduced.

In the present study, the fertility and ploidy status were examined in gametes of allodiploid and allotriploid hybrids between dojo loach and large-scale loach. Allodiploid (abbreviated as DP) hybrids were produced by crossbreeding between diploid dojo loach (DD) female and large-scale loach (PP) male. Allotriploid (abbreviated as DDP) hybrids were induced by triploidization of fertilized eggs from DD female × PP male cross by cold shock to inhibit the release of the second polar body.

Firstly, the gonads of DP and DDP hybrids were histologically observed and then ploidy statuses of gonadal cells were determined by flow cytometry. Using gametes of DP and DDP, artificial crossbreeding was done to produce DD (female) × DP (male), DP × DD, DP × DP, DD × DDP, DDP × DDP, and DDP × DDP progenies. Control cross DD × DD was also produced simultaneously. Fertilization, hatching, and survival rates at 7 days after fertilization were measured. Meanwhile, embryonic developments of the seven crosses and external appearances of larvae of DP × DD and DDP × DD were observed. Ploidies of larvae of DP × DD and DDP × DD were last investigated.

## Materials and methods

### Ethics

This study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Huazhong Agricultural University. All efforts were made to minimize suffering of the loaches.

### Parental animal collection

Twenty natural diploid *M. anguillicaudatus* (DD) and *P. dabryanus* (PP) (female/male = 1:1), collected from Liangzi Lake, Ezhou City, Hubei Province, China, were used as parents to produce allodiploid and allotriploid loaches at the College of Fisheries, Huazhong Agricultural University, China, in June 2013. DP loaches were produced by crossing of five DD females and five PP males, while the DDP loaches were induced from fertilized eggs of five DD females and five PP males by cold shock to prevent the second polar body release. The cold shock condition was that after 5 min of fertilization at room temperature, 4 °C ice water was used to do cold shock for 30 min. DP and DDP were reared in tanks with 24–26 °C flowing water and fed with Tubificidae and commercial feeds three times a day.

### Histological observations and ploidy analyses of the gonads of allodiploid and allotriploid loaches

Ten robust (female/male = 1:1) 2-year-old DP were used for gonad histological observations and ploidy analyses. Meanwhile, ten robust (female/male = 1:1) 2-year-old DDP were also used for the same studies and robust wild-type DD were used as controls. After anesthetizing with 100 mg/L MS-222, the ovaries or testes were ws dissected out. Each gonad sample was divided into two parts. One part was fixed at 4 °C for 24 h in Zamboni's fixative (MasterTech, USA) for histological observations (Cao and Wang 2009). And the other part was used for ploidy analyses. The concrete implementing process of the ploidy analyses was firstly cutting the gonad into pieces and then adding 200 μL nuclei extraction buffer and 800 μL staining buffer (Partec, Germany) for DNA staining; the gonad samples were next filtered with a 30-μm strainer and finally tested by Ploidy Analyzer (Partec, Germany). Blood cells of known diploid loach were used as control.

### Artificial propagation crosses

Six DD, four DP, and six DDP (female/male = 1:1) were used as parents to make seven crosses including DD × DD (female before male), DD × DP, DP × DD, DP × DP, DD × DDP, DDP × DD, and DDP × DDP. The DD × DD cross was used as control group. Hormones LRH-$A_2$ (20 mg $kg^{-1}$ body weight) and domperidone (DOM, 6 mg $kg^{-1}$ body weight) were injected to promote ovulation. A half dose of each hormone was injected to each male to promote spermiation. After the injection, females and males were separately kept in different tanks with temperature regulated at 26 °C. Twelve hours later, the females could spawn mature eggs. While, the males were anesthetized with 100 mg/L MS-222, and then the testes were dissected out for semen preparation and preserved in loach's sperm preservation solution

(128.2 mmol NaCl, 4.0 mmol KCl, 2.7 mmol $CaCl_2$, 16.7 mmol glucose) (Yasui et al. 2009). Semen and mature eggs were mixed to activate by adding ambient water for fertilization. Fertilized eggs of each cross were incubated until hatching in plastic tanks (120 × 70 × 30 cm) and then the larvae were reared in cement pools (200 × 200 × 100 cm) at room temperature (approximately 25 °C). The hatching time was between 36 and 48 h after fertilization. The water was changed every day and larvae were firstly fed with *Artemia salina* for 7 days and then fed with commercial feeds. Fertilization rates, hatching rates, and survival rates at 7 days after hatching of each cross were calculated as described by Li et al. (2012). One-way ANOVA was used to compare the differences among different crosses ($P < 0.05$).

### Observations of embryonic development and larval morphologies

The embryonic development was observed in each cross by microscope with a digital camera (Olympus, Japan). The external appearances of the living larvae were also observed.

### Ploidy analyses of viable larvae

There were no hatched larvae surviving in DD × DP, DP × DP, DD × DDP, and DDP × DDP crosses. Viable larvae from DP × DD and DDP × DD crosses were randomly chosen for ploidy analyses. The muscle cells of larvae were separated out and then 500 μL staining buffer (Partec, Germany) was added for DNA staining. After 2 min of staining in the dark, ploidy levels of the larvae were examined by the Ploidy Analyzer. Because of the limited number of muscle cells in one larva, two larvae from the same cross were mixed together for ploidy determination. Blood cells of known diploid loach were used as control.

## Results

### Histological observations and ploidy analyses of the gonads of allodiploid and allotriploid loaches

The ovaries of DP and DDP included relatively smaller numbers of eggs. Immature, perinucleolar, and cortical alveoli stages and oocytes in exogenous vitellogenesis were all found in DP, DDP, and DD ovaries (Fig. 1a, c, e). The testes of DP (Fig. 1b) and DDP (Fig. 1d) were delayed-developed, compared to that of DD (Fig. 1f). Most of the germ cells in the testes of DP and DDP were spermatogonia and primary spermatocytes (Fig. 1b, d), while no spermatids or mature spermatozoa were found. Mature spermatozoa were present in the testis of DD.

Ploidy compositions of DP, DDP, and DD gonads were analyzed here. The ovary and testis of DP were composed of numerous 2N cells and few 4N cells (Fig. 2a, b), which were similar to the ovary of DD (Fig. 2e). The ovary of DDP was composed of numerous 2N and 3N cells and few 4N to 6N cells (Fig. 2c). The testis of DDP was composed of equal numbers of 2.5N and 5N cells (Fig. 2d), which were different from the ploidy analyses of DD testis (Fig. 2f).

### Artificial propagation crosses

Fertilization rates, hatching rates, and survival rates at 7 days after hatching of all crosses are summarized in Table 1. The crosses using DP and DDP as parents had significantly low fertilization rates when compared with the control group ($P < 0.05$), especially the crosses of DD × DP, DP × DP, DD × DDP, and DDP × DDP. Crosses DD × DP, DP × DP, DD × DDP, and DDP × DDP had no viable embryo at hatching, and few viable larvae from DP × DD and DDP × DD crosses could be found.

### Observations of embryonic development and larval morphologies

Embryonic developments of the seven crosses and external appearances of larvae of DP × DD and DDP × DD were observed. The embryos of DD × DP, DP × DP, DD × DDP, and DDP × DDP crosses died before the blastula stage (Fig. 3b, e, f, i), while there were no viable larvae found in these crosses. The larvae of DD × DD showed normal morphology (Fig. 3a). About 35% of DP × DD larvae and 40% of DDP × DD larvae had normal appearances (Fig. 3d, h). Most of the larvae of DP × DD and DDP × DD were malformed, with curved, edematous, and incomplete shape, and eventually dead (Fig. 3c, g).

### Ploidy analyses of viable larvae of DP × DD and DDP × DD

The normal and malformed larvae of DP × DD were all diploid (Fig. 4a), indicating DP females could produce

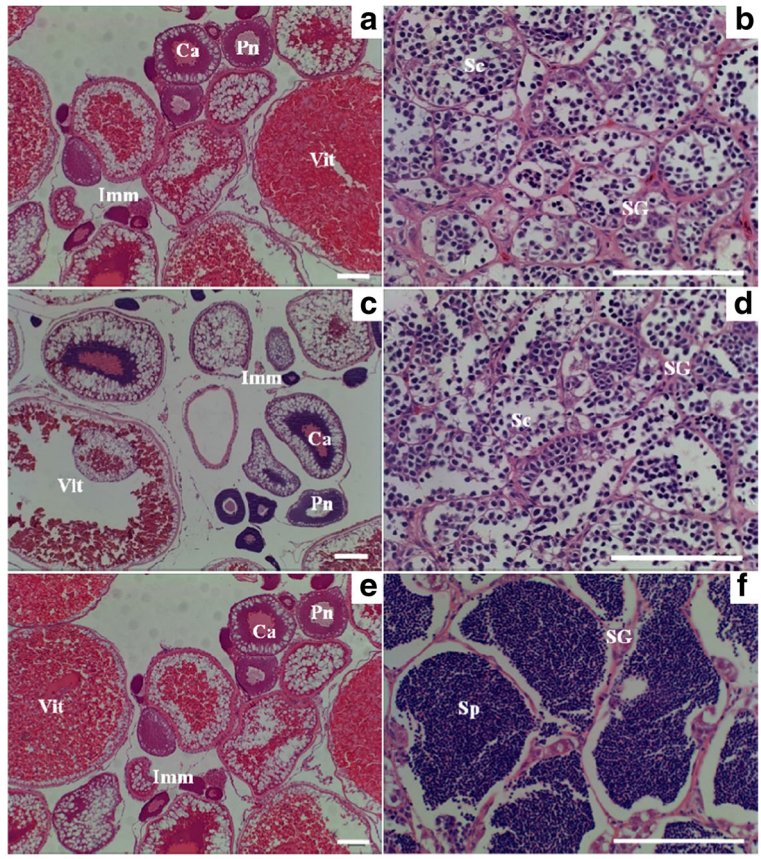


**Fig. 1** Histological observations of the ovary and testis of the allodiploid (DP), allotriploid (DDP), and wild-type diploid (DD) loaches. Immature (*Imm*), perinucleolar (*Pn*), and cortical alveoli (*Ca*) stages and oocytes in exogenous vitellogenesis (*Vit*) in the ovary, and spermatogonium (*SG*), spermatocyte (*Sc*), and spermatozoa (*Sp*) in the testis. *Scale bar* = 200 μm. **a** Ovary of DP. **b** Testis of DP. **c** Ovary of DDP. **d** Testis of DDP. **e** Ovary of wild-type DD. **f** Testis of wild-type DD

viable haploid eggs. At the same time, ploidy levels were determined for normal and malformed larvae of DDP × DD. In malformed DDP × DD larvae, 25% were haploid, while 75% were diploid (Fig. 4c). On the contrary, in normal DDP × DD larvae, 87.5% were diploid, while 12.5% were triploid (Fig. 4b). There were no 2.5N viable larvae found in the DDP × DD cross. The malformed larvae would die within 3 days after hatching and most of the normal larvae could survive to adulthood. In a word, DDP females could produce two kinds of viable eggs: haploid eggs and diploid eggs.

## Discussion

Natural dojo loaches with different ploidy levels coexist with large-scale loaches in China, and their bisexual reproduction and hybridization had been identified by experimental approaches using artificial crosses and chromosome manipulation (You et al. 2009; Li et al. 2012; Cui et al. 2013; Huang et al. 2015). Triploid loach was induced by inhibiting the second polar body release after normal crosses between diploid wild-type loaches and then progeny test between natural tetraploid female and induced triploid males showed that the triploid generated fertile aneuploidy spermatozoa with approximately 1.3N (Zhang and Arai 1999). Artificially induced triploid loaches were sterile, but the reproduction of hybrid triploids between natural tetraploid and wild-type diploid was different. Fertile triploid and haploid eggs were produced in such triploid (diploid × tetraploid) hybrids by premeiotic endomitosis and hybridogenesis-like manner, respectively (Arai and Mukaino 1997, 1998; Zhang et al. 1998). In the present study, no viable progenies were found when using DP or DDP as the male parent, but a small number of progenies survived when using DP or DDP as the female parent. Hence, allodiploid and allotriploid hybrids were fertile in females, but sterile in males. Allodiploid females laid haploid eggs, while allotriploid females spawned both haploid and diploid eggs under laboratory conditions. At present, it is unknown whether they spawn under natural

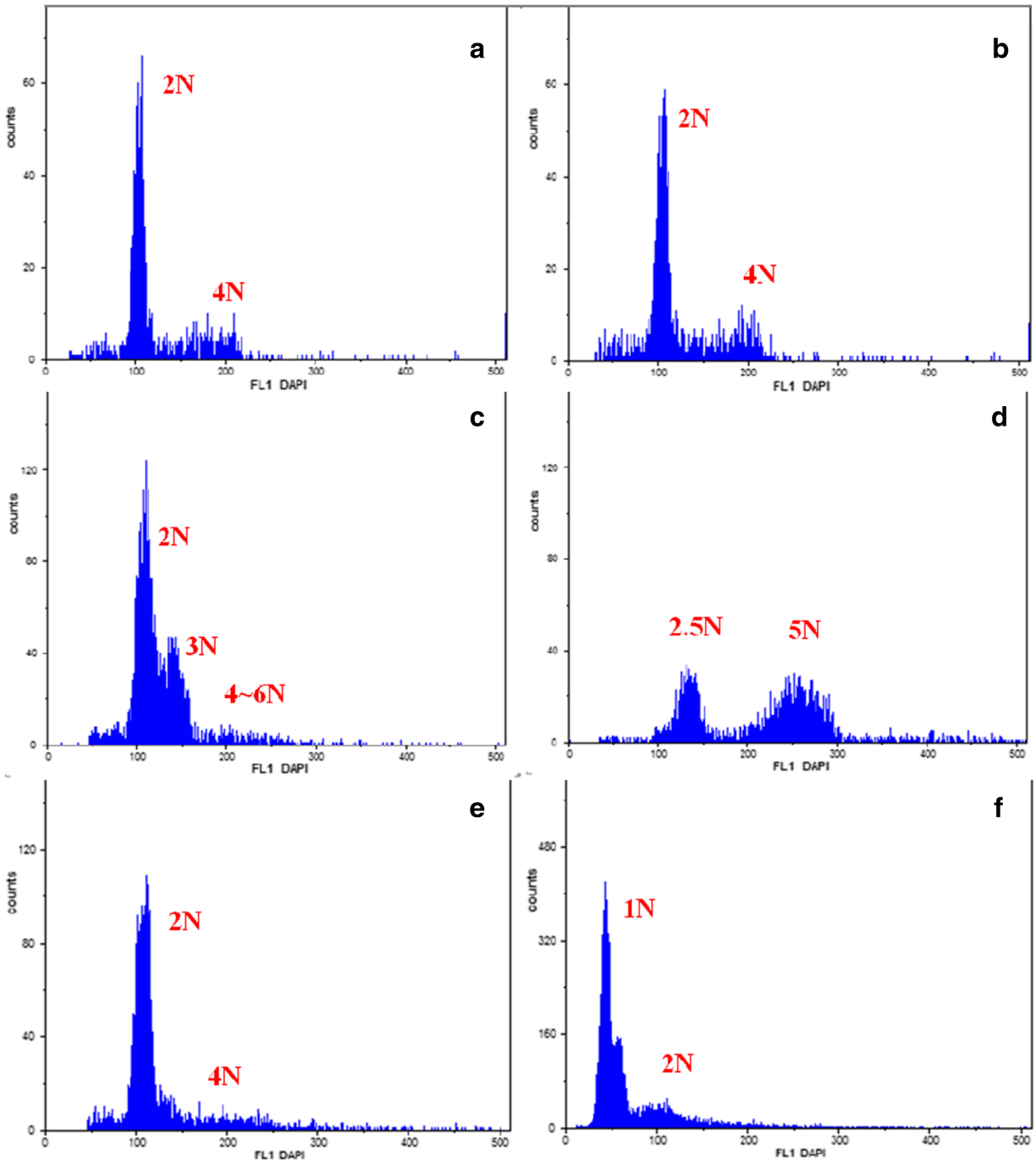


**Fig. 2** Ploidy peaks of the gonads of allodiploid (DP), allotriploid (DDP), and wild-type diploid (DD) loaches. **a** Ovary of DP. **b** Testis of DP. **c** Ovary of DDP. **d** Testis of DDP. **e** Ovary of DD. **f** Testis of DD

environment or not. Sterility or low fertility was already observed in induced triploids of *M. anguillicaudatus* (Suzuki et al. 1985; Zhang and Arai. 1999a) and hybrid diploid and triploid males between *M. anguillicaudatus* and *M. mizoleis* (Fujimoto et al. 2008).

The mechanism of abnormal gametogenesis, especially in formation of unreduced and genome-lost

**Table 1** Number of eggs, fertilization rates, hatching rates, and survival rates at 7 days after fertilization of seven crosses

| Cross | No. of eggs | Fertilization rate (%) | Hatching rate (%) | Survival rate at 7 days after hatching (%) |
|---|---|---|---|---|
| DD × DD-1 | 825 | 82.91 | 87.28 | 89.45 |
| DD × DD-2 | 902 | 84.37 | 89.09 | 90.27 |
| DD × DD-3 | 678 | 80.53 | 90.29 | 91.48 |
| Mean ± SD | | 82.6 ± 1.94[a] | 88.89 ± 1.52[a] | 90.4 ± 1.02[a] |
| DD × DP-1 | 964 | 9.23 | 0 | 0 |
| DD × DP-2 | 859 | 8.61 | 0 | 0 |
| Mean ± SD | | 8.92 ± 0.44[d] | 0[c] | 0[c] |
| DD × DDP-1 | 954 | 8.70 | 0 | 0 |
| DD × DDP-2 | 832 | 8.89 | 0 | 0 |
| DD × DDP-3 | 767 | 8.21 | 0 | 0 |
| Mean ± SD | | 8.6 ± 0.35[d] | 0[c] | 0[c] |
| DP × DD-1 | 502 | 28.29 | 78.87 | 50.00 |
| DP × DD-2 | 576 | 27.95 | 73.91 | 41.18 |
| Mean ± SD | | 28.12 ± 0.24[c] | 76.39 ± 3.51[b] | 45.59 ± 6.24[b] |
| DP × DP-1 | 615 | 2.44 | 0 | 0 |
| DP × DP-2 | 458 | 2.84 | 0 | 0 |
| Mean ± SD | | 2.64 ± 0.28[e] | 0[c] | 0[c] |
| DDP × DD-1 | 555 | 29.73 | 73.33 | 46.28 |
| DDP × DD-2 | 517 | 33.66 | 77.01 | 45.52 |
| DDP × DD-3 | 623 | 30.34 | 74.60 | 50.35 |
| Mean ± SD | | 31.24 ± 2.12[b] | 74.98 ± 1.87[b] | 47.38 ± 2.6[b] |
| DDP × DDP-1 | 544 | 2.94 | 0 | 0 |
| DDP × DDP-2 | 565 | 3.01 | 0 | 0 |
| DDP × DDP-3 | 518 | 2.32 | 0 | 0 |
| Mean ± SD | | 2.76 ± 0.38[e] | 0[c] | 0[c] |

Different lowercase letters within the same columns meant significant differences ($P < 0.05$)

gametes in hybrids, should require more and more attention. In the present study, DP loach produced fertile 1N eggs, and DDP loach produced fertile 1N and 2N eggs. There were abundant 2N secondary oocytes in the allodiploid ovary, and the fertile 1N eggs were presumably produced by normal meiotic process as in a normal diploid organism. While, abundant fertile 1N eggs were also observed in DDP loach, and the ploidy analyses showed numerous 2N secondary oocytes in DDP loach ovary. There were three sets of chromosomes in the spermatogonium as somatic cells in DDP loach: two sets inherited from *M. anguillicaudatus* and the other set inherited from *P. dabryanus*. The homologous chromosomes came from the mother parent paired firstly, and the non-homologous chromosomes generated univalents during the synapsis phase. The 2N secondary oocytes were presumably generated from 3N primary oocytes by eliminating the univalents, which were formed by non-homologous chromosomes in allotriploid loach during meiosis I phase. And the 2N secondary oocytes would produce fertile 1N eggs after meiosis. The small haploid eggs produced by hybrid triploid loach (diploid × tetraploid) were also mainly to exclude haploid genome of one parent in the oogenesis of triploid loach (Arai and Mukaino 1998; Zhang et al. 1998).

Numerous 3N cells were also found in the allotriploid loach's ovary, but no viable larvae were found fertilized by 1.5N mature eggs. We speculated that the 3N cells were possible interstitial cells or secondary oocytes in the gonad, which could not form mature gametes. In a word, the 3N cells in allotriploid loach ovary could not produce 1.5N viable eggs. A high frequency of unexpected triploid

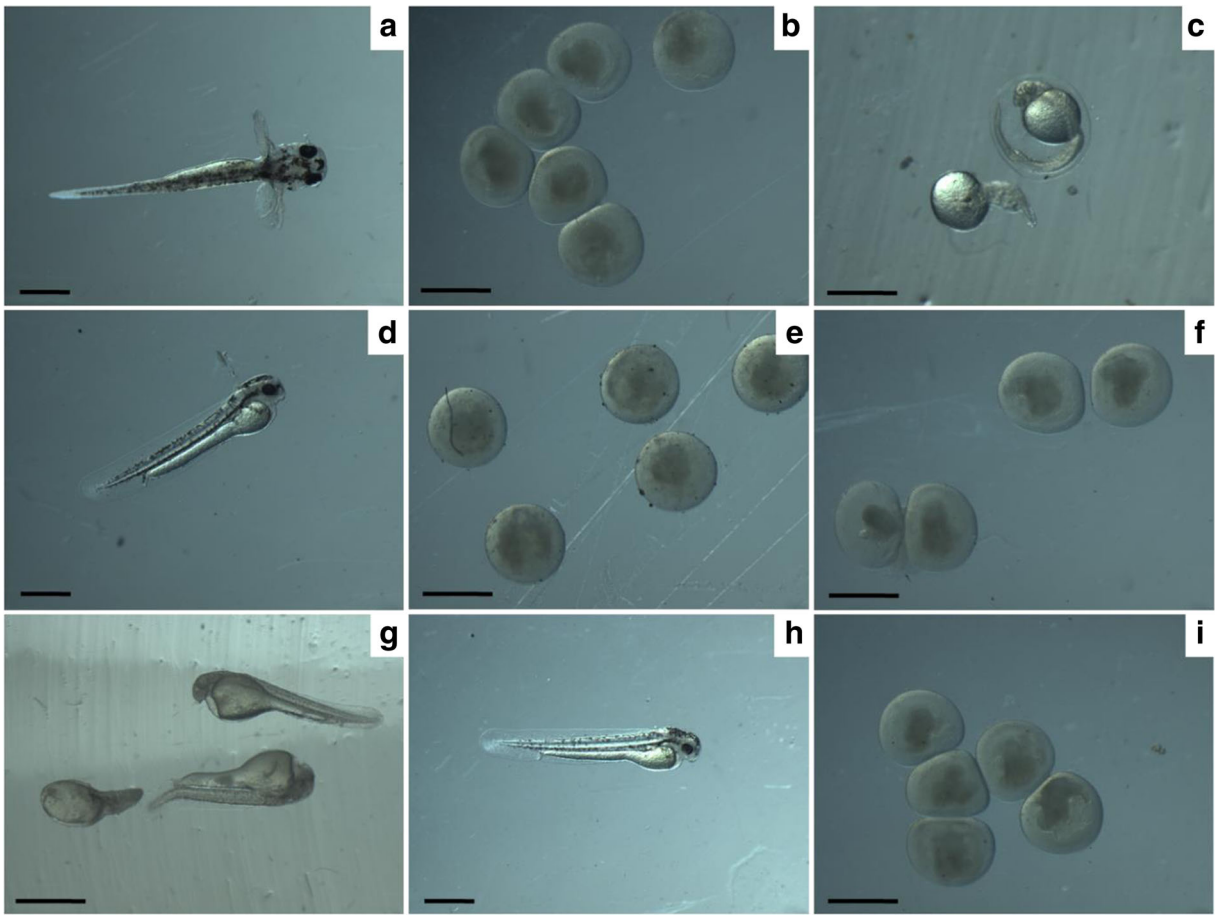


**Fig. 3** External appearances of larvae and embryos of seven crosses. **a** Normal larvae of DD × DD. **b** Dead embryos of DD × DP. **c** Malformed larvae of DP × DD. **d** Normal larvae of DP × DD. **e** Dead embryos of DP × DP. **f** Dead embryos of DD × DDP. **g** Malformed larvae of DDP × DD. **h** Normal larvae of DDP × DD. **i** Dead embryos of DDP × DDP. DD: natural diploid *M. anguillicaudatus*; PP: *P. dabryanus*; DP: allodiploid loach; DDP: allotriploid loach. *Bar* = 1 mm

larvae (12.5%) of the allotriploid loach female × diploid dojo loach male was recorded here. It indicated that the allotriploid loach could produce viable diploid eggs. Such an elevation of egg ploidy status had been explained by spontaneous inhibition of the second polar body released after fertilization, but this was a relatively rare event with a frequency that normally did not exceed 1 to 1.5% (Cherfas 1981).

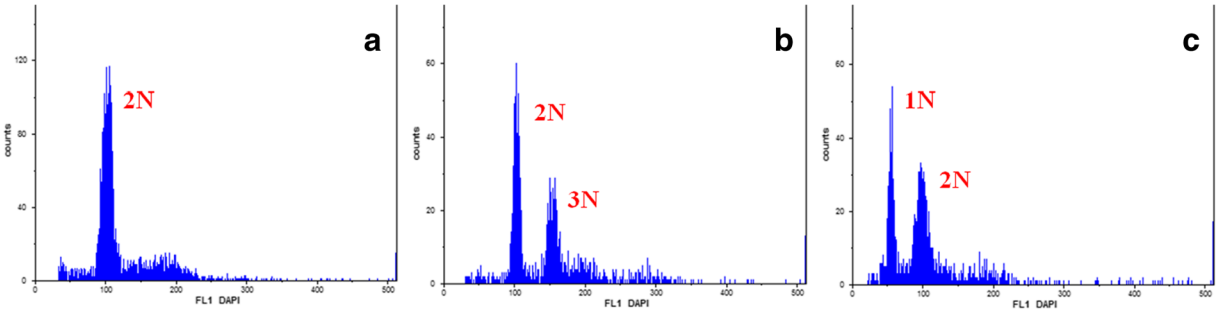


**Fig. 4** Ploidy peaks of hatched larvae of DP × DD and DDP × DD. **a** Larvae of DP × DD. **b** Normal larvae of DDP × DD. **c** Malformed larvae of DDP × DD. DD: natural diploid *M. anguillicaudatus*; DP: allodiploid loach; DDP: allotriploid loach

There were no spermatids or mature spermatozoa in allodiploid and allotriploid loach testes. In combination with the histological observation and ploidy analyses of the gonad, the 2N cells were presumably secondary spermatocytes or somatic cells in allodiploid loach testis. The germ cells stunted at meiosis and could not develop to mature spermatozoa. Allodiploid loach generated 2N secondary spermatocytes regularly with the same ploidy level of somatic cells. While, different ploidy levels of secondary spermatocytes were found in allotriploid loach testis.

The 3N somatic cell generated 5N primary spermatocytes and 2.5N secondary spermatocytes, which was significantly different from normal meiotic process. Until now, there was no such record this happened in fish. Therefore, in the near future, we should examine possible mechanisms for the allotriploid loaches here produced viable diploid eggs and 5N primary spermatocytes.

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**Compliance with ethical standards** This study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Huazhong Agricultural University.

**Conflict of interest** The authors declare that they have no conflict of interest.

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