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Induction of viable gynogenetic progeny using eggs and UV irradiated sperm from the Chinese tetraploid loach, *Misgurnus anguillicaudatus*

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Abstract When eggs from the Chinese tetraploid loach which had 100 chromosomes were fertilized with UV irradiated sperm, we obtained viable gynogenetic progeny without any additional treatment for the duplication of maternal chromosomes, which survived beyond first-feeding towards adult stage of development. Gynogenetic progeny were determined to be diploid since they possessed 50 chromosomes, along with two chromosomes bearing nucleolar organizing regions (NORs), detected by silver nitrate staining (Ag-NORs), chromomycin-A_3 (CMA_3) positive sites and fluorescence *in situ* hybridization (FISH) signals for rDNA loci. In contrast, when gynogens were induced using eggs from diploid loach fertilized by UV irradiated sperm, but without chromosome doubling, we found that all resultant progeny were non-viable haploid gynogens with 25 chromosomes, along with one NOR-bearing chromosome detected by Ag-NOR, CMA_3 and FISH. These observations demonstrate the true genetic tetraploid nature of the Chinese loach possessing 100 chromosomes and the potential use of this tetraploid as a source of functional diploid gametes for further ploidy manipulation experiments.

Keywords Chromosome・Gametes ・FISH・Polyploid

Introduction

Diploid gametes represent key resources in order to expand the chromosome manipulation for improved breeding techniques. If we are able to use diploid gametes for breeding and subsequent manipulation, then it would allow the mass production of sterile auto- or allo-triploid lines, synthesis of new amphidiploids or allotetraploid lines, recovery of androgenotes from cryopreserved diploid sperm, and other applications for aquaculture (Arai 2000, 2001; Arai et al. 2010). However, successful examples of induced tetraploid fish as a source of diploid gametes have been limited due to the technical difficulties in producing tetraploids by inhibition of early cleavage coupled with very low survival rates of the resultant tetraploid progeny (Chourrout et al. 1986; Nam et al. 2001; Zou et al. 2004; Sakao et al. 2006). An alternative way would be to use the diploid gametes of natural tetraploid lines in certain species or hybrids.

In dojo loach or Oriental weatherfish *Misgurnus anguillicaudatus* (Teleostei: Cobitidae), most individuals of Japanese wild populations are diploid with 50 chromosomes. However, tetraploids possessing 100 chromosomes have often been identified amongst specimens recovered from Japanese markets (Ojima and Takai 1979; Arai et al. 1991a). Using chromosome manipulation techniques, Arai et al. (1991b, 1993) concluded that loaches possessing 100 chromosomes were true tetraploids possessing four sets of homologous chromosomes, because gynogenetic progeny which were artificially induced by fertilizing eggs from these market-sourced specimens with genetically-inert UV irradiated sperm were viable without requiring any treatment to duplicate the maternal chromosome set. In a similar manner, viable androgenetic progeny were successfully induced by fertilizing UV irradiated eggs with the sperm from loach possessing 100 chromosomes (Arai et al. 1995). Gynogenetic and androgenetic progeny produced from the gametes of normal diploid fish are non-viable due to the expression of abnormalities collectively referred as 'haploid syndrome', observed in many species of fish including loach (Suzuki et al. 1985; Arai et al. 1992; Fujimoto et al. 2007), common carp (Nagy et al. 1978) and salmonids (Chourrout et al. 1980; Chourrout 1982; Onozato 1982; Onozato and Yamaha 1983).

In central parts of China, natural tetraploid loach possessing 100 chromosomes co-exist, in the same habitats, with sympatric diploid loach possessing 50 chromosomes (Li et al. 2008). Cytogenetic studies including differential staining with silver nitrate for nucleolar organizing regions (Ag-NOR), fluorochromes such as chromomycin A_3 (CMA_3)/ Distamycin (DA)/ 4',6-diamidino-2-phenylindole (DAPI) and fluorescence *in situ* hybridization (FISH) using human 5.8S+28S rDNA sequences suggested that a loach

possessing 100 chromosomes should be a tetraploid, based upon a karyotype showing quartets of chromosomes and the presence of four FISH signals (Li et al. 2010). However, specific genetic characteristic of these Chinese loaches have not yet been determined using the chromosome manipulation approach reported in Arai et al. (1991b, 1993, 1995). If Chinese loaches possessing 100 chromosomes are genetic tetraploids with four sets of homologous chromosomes, then it should be possible to use these as a source of diploid gametes in order to expand and improve existing breeding techniques. In the present study, we artificially induced gynogenesis using eggs from putative tetraploid females collected from the Chang Jiang River, Hubei Province, China by fertilizing with UV irradiated sperm and then carefully determined survival rates and chromosome numbers in the resultant gynogenetic progeny.

Materials and methods

Fish and gametes

Diploid (4 females, 4 males) and tetraploid (4 females) loach were collected in Chang Jiang River in Hubei Province, China and transported to Dalian Ocean University. Identification of tetraploid and diploid specimens was determined in accordance to Li et al. (2008). Artficial ovulation was induced by the injection of hCG (human Chorionic Gonadotropin, 20 IU per body gram weight, Aska Pharmaceutical Co. Ltd., Tokyo) according to Suzuki and Yamaguchi (1975). Sperm was collected into a capillary hematocrit tube by gently squeezing the male's abdomen (Morishima et al. 2002). Sperm was then diluted 1: 100 with Kurokura solution (Kurokura et al. 1984).

Fertilization and gynogenesis

Eggs taken from diploid (2n) and tetraploid (4n) females were fertilized with sperm from 2n males and then, diploid female x diploid male (control cross, 2n x 2n) and tetraploid female x diploid male (triploid cross, 4n x 2n) crosses were performed. Eggs of 2n and 4n females were then inseminated with UV irradiated sperm from a 2n male (2n x UV and 4n x UV, respectively) to induce gynogenetic development. UV-irradiation of sperm from 2n males was performed according to Suzuki et al. (1985). Fertilized and treated eggs from each cross were reared in a Petri dish (90mm diameter x 15mm depth) containing freshwater (aged tap water) at room temperature.

Fertilization rate was calculated as the proportion of cleaved eggs relative to the initial number of eggs. Hatching rate was calculated as the proportion of hatched larvae relative to the number of

fertilized eggs. The normal rate was calculated as the proportion of normal larvae relative to the number of hatched larvae. Survival rate at 7 days after hatching (dah) was calculated as the proportion of surviving larvae relative to the number of hatched larvae. Water was changed every day and larvae were first fed *Artemia* from 3 days after hatching. Larvae were incubated at a temperature of 20°C.

Chromosome preparation and observation

Embryos at the optic vesicle stage (15 to 20 h after fertilization at 20°C) were used for chromosome preparation. In physiological saline, the chorion was mechanically removed and embryos treated for 45 min by soaking them in 0.0025% demecolcine (Sigma). Hypotonic treatment was performed with 0.8% citric acid for 20 min and samples were fixed with Carnoy's fixative (3 parts of methanol: 1 part of aceteic acid). Chromosome preparations were prepared from developimg embryos as described by Inokuchi et al. (1994). Chromosome slides were stained with Giemsa (Merck) for microscopic observation and karyotyping carried out in accordance with Levan et al. (1964).

Differential staining with CMA_3/DA/ DAPI (Schweizer 1976; Schweizer et al.1978) and the Ag-NOR method (Howell and Black 1980) was applied in accordance with Li et al. (2010). FISH using human 5.8S+28S rDNA sequences as a probe was applied in accordance with Li et al. (2010).

Results

Viability of gynogenetic progeny

All control crosses (2n x 2n) resulted in high rates of fertilization (83-94%), hatching (75-96%), normal fry (84-97%) and survival at 7 dah (72-95%; Table 1). Triploid crosses (4n x 2n) also exhibited high rates of fertilization (77-98%), hatching (51-99%), normal fry (81-99%) and survival at 7 dah (77-90%; Table 1). Most control and triploid larvae exhibited normal appearance (Fig. 1A).

In gynogenetic crosses, differences were observed between 2n x UV and 4n x UV crosses. Gynogenetic progeny from 2n x UV crosses produced lower rates of fertilization (43-84%) and hatching (3.2-56%) than control and triploid crosses and no normal fry were apparent (Table 1). All resultant fry exhibited abnormalities characteristic of haploidy (Fig. 1B). In contrast, 4n x UV crosses produced high rates of fertilization (69-95%) and hatching (81-99%). Whilst 37 to 67% of resultant larvae were normal (Fig. 1C) or malformed (Fig. 1D), 46-60% fry survived to 7 dah (Table 1).

Chromosomal nature of gynogenetic progeny

As shown in Table 2 and Fig. 2AC, the modal chromosome number of embryos from 2n x 2n crosses was diploidy (2n=50), while the mode of embryos from 4n x 2n crosses was triploidy (3n=75). The 2n x UV embryos exhibited a haploid range of chromosomes with a modal number of 1n=25 (Table 2, Fig. 2B). In contrast, 4n x UV embryos exhibited a diploid range of chromosomes with a modal number of 2n=50 (Table 2, Fig. 2D). Consequently, most embryos from crosses 2n x 2n, 4n x 2n, 2n x UV and 4n x UV crosses were considered diploid (2n=50), triploid (3n=75), gynogenetic haploid (1n=25) and gynogenetic diploid (2n =50), respectively. However, hypo- and hyper-chromosome numbers were observed in each ploidy group (Table 2). The 2n x UV embryos possessed one Ag-NOR/ CMA_3 positive region and FISH signal indicating haploidy (Fig 3A-C), while 4n x UV embryos demonstrated two Ag-NOR/ CMA3 positive regions and FISH signals indicating diploidy (Fig. 3 D-F).

Discussion

In the present study, viable gynogenetic progeny were produced without chromosome duplication from the eggs of Chinese tetraploid loach females fertilized with UV-irradiated loach sperm. Progeny possessed 50 chromosomes, equivalent to one half of the 100 chromosomes found in parental tetraploid loach specimens (Li et al. 2010). Gynogenetic fry possessing 25 chromosomes that were induced from the eggs of diploid females could not survive beyond hatching or feeding due to abnormalities. Diploidy of viable gynogenetic progeny was also demonstrated by the presence of two Ag-NORs, CMA_3 positive sites and FISH signals, whereas the haploid nature of non-viable gynogens was indicated by one site and one signal. Consequently, the production of viable gynogenetic progeny suggests that Chinese tetraploid loach are true tetraploids, possessing four sets of homologous chromosomes with the ability of generating diploid gametes. Thus, the tetraploid nature of the Chinese loach possessing 100 chromosomes was conclusively demonstrated by observing the viability of UV-induced gynogenetic progeny. Similar results have already been obtained in gynogenetic and androgenetic progeny induced from the gametes of loach possessing 100 chromosomes obtained from a Japanese market (Arai et al. 1991b, 1993, 1995), and in those induced from the gametes of natural tetraploid population in a different cobitid species *Cobitis biwae* (Kusunoki et al. 1994). Therefore, the overall genetic characteristics of Chinese tetraploid loach are essentially the same as those of tetraploid loach found in Japanese markets (Arai et al. 1991ab, 1993, 1995; Arai 2001, 2003). These results suggest the possibility that the tetraploid loach found in Japanese markets should be transported from China as food or fishing bait.

An important application of genetically tetraploid fish is as a source of diploid gametes for the expansion and improvement of existing ploidy manipulation techniques.

In Chinese loach, it is also possible to expand ploidy manipulation using diploid gametes as reported in our previous studies using natural tetraploid loach found in Japanese markets (Arai 2000, 2001, 2003). The mass production of triploids was first realized by crossing tetraploid and diploid fish (Matsubra et al. 1995; Zhang and Arai 1996) whilst a neo-tetraploid strain was produced by inhibiting second polar body release in the 2n x 4n cross (Zhang and Arai 1996; Fujimoto et al. 2010). Fertile hexaploid lines were also produced by inhibiting second polar body release just after fertilization in 4n x 4n crosses. Second generation hexaploid, pentaploid and tetraploid fish were then produced using fertile triploid gametes from hexaploid loaches (Arai et al. 1999). Diploid sperm from natural tetraploids is particularly useful for producing viable androgenotes by fertilizing UV-irradiated eggs (Arai et al. 1995; Yasui et al. 2010). Consequently, the Chinese tetraploid loach represents a valuable resource for inducing various levels of polyploids, gynogens and androgenotes for aquaculture. Breeding programs using diploid gametes are already implemented in allotetraploid crucian carp x common carp hybrids (Liu et al. 2001).

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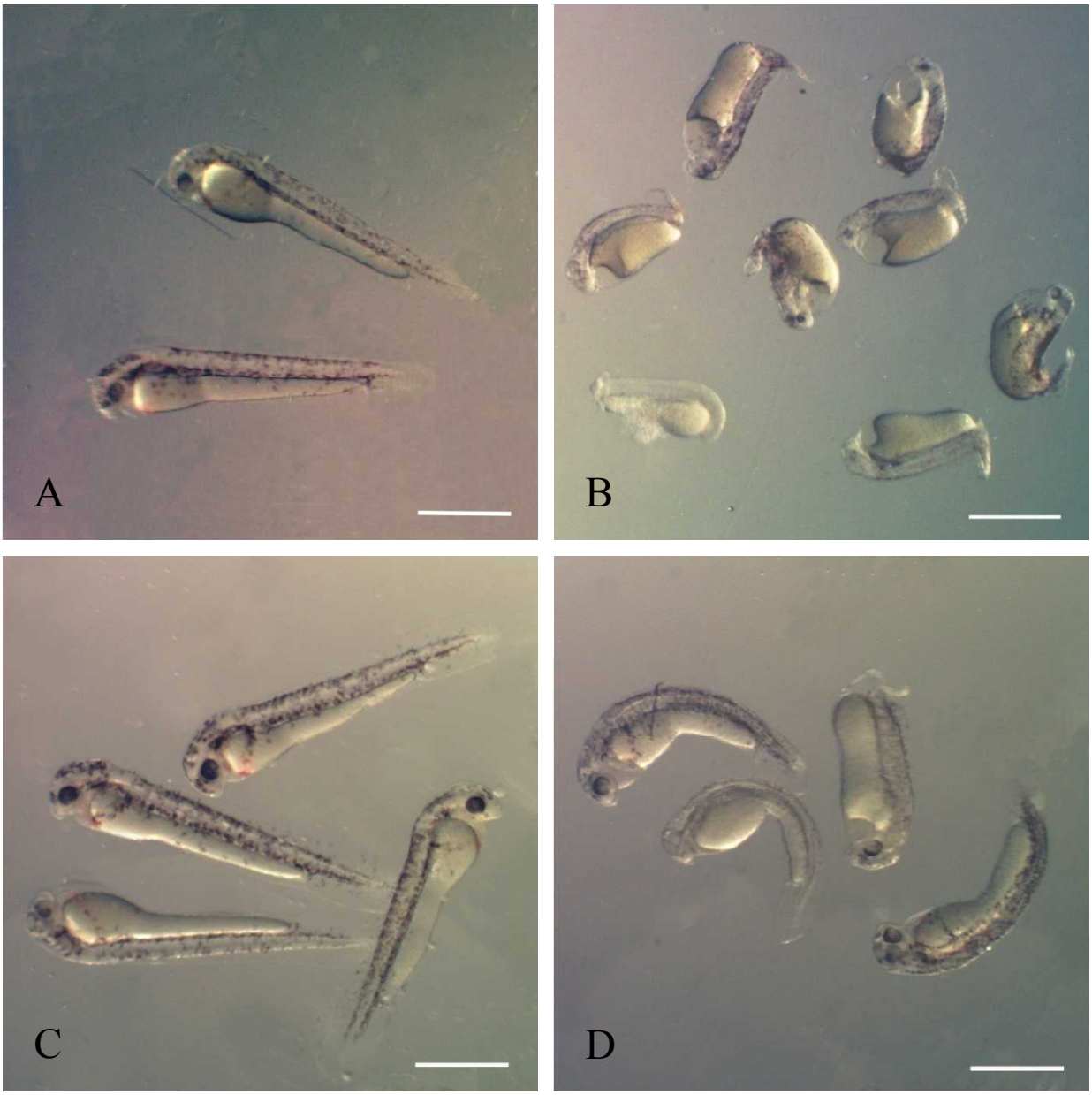
Legends for figures

Figure 1 Normal larvae from 2n x 2n (A), abnormal larvae from 2n x genetically inactivated sperm with UV irradiation (UV) (B), normal larvae from 4n x UV (C) and malformed larvae from 4n x UV (D). Bars indicate 1 mm.

Figure 2 Metaphase spread with (A) 50 chromosomes from a 2n x 2n embryo, (B) 25 chromosomes from a 2n x UV embryo, (C) 75 chromosomes from a 4n x 2n embryo, and (D) 50 chromosomes from a 4n x UV embryo (D). Bars indicate 10 μm.

Figure 3 Metaphase spreads in 2n x UV (A, B, C) and 4n x UV (D, E, F) dojo loach embryos. Ag-NORs (A, D), CMA_3/DA/DAPI staining positive sites (B, E) and FISH signals detected with 5.8S+28S rDNA probe (C, F) are shown. Arrows indicate NORs shown by Ag staining (A, D), CMA3 positive sites (B, E) or FISH signals (C, F). Bars indicate 10 μm.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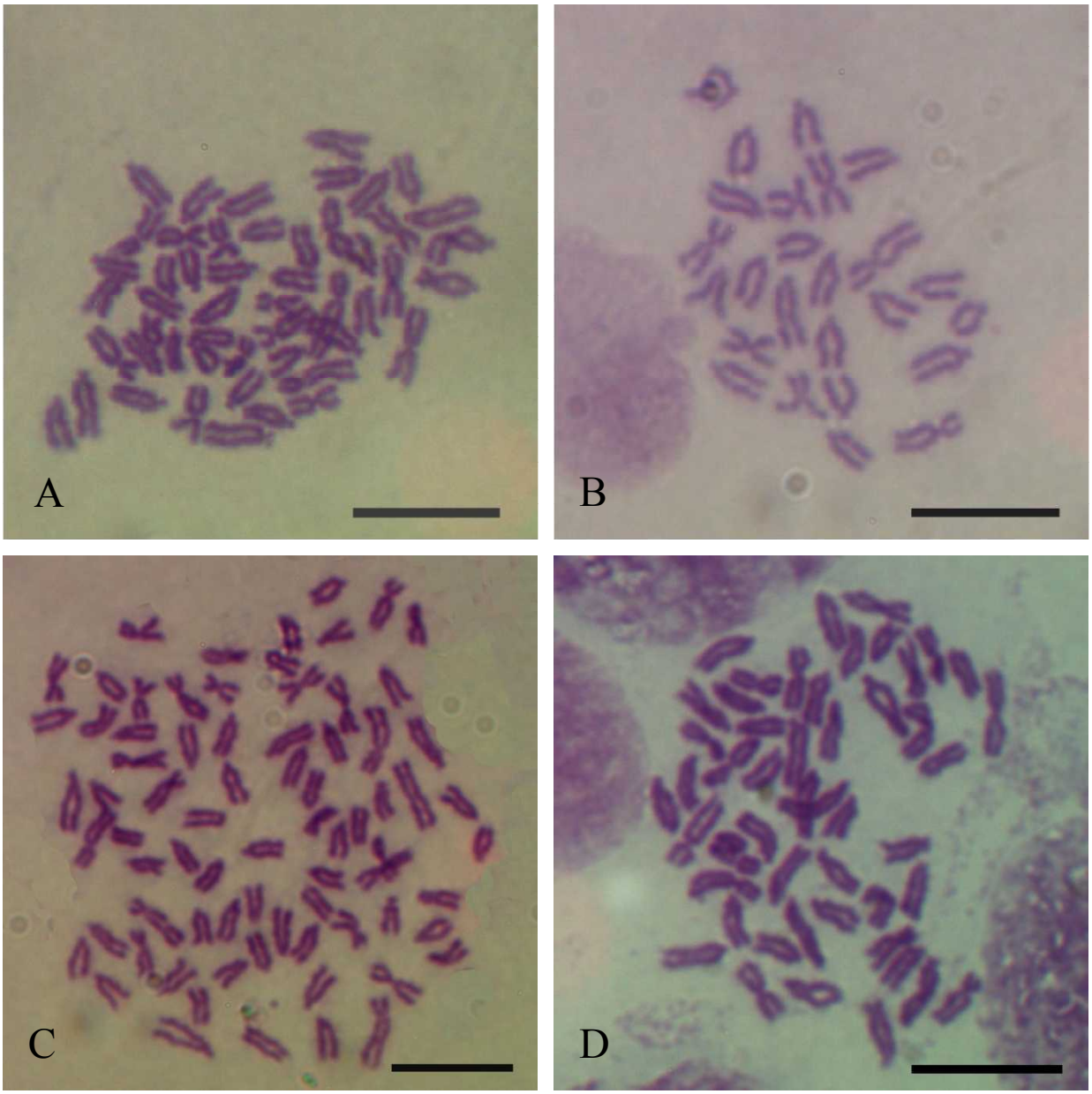
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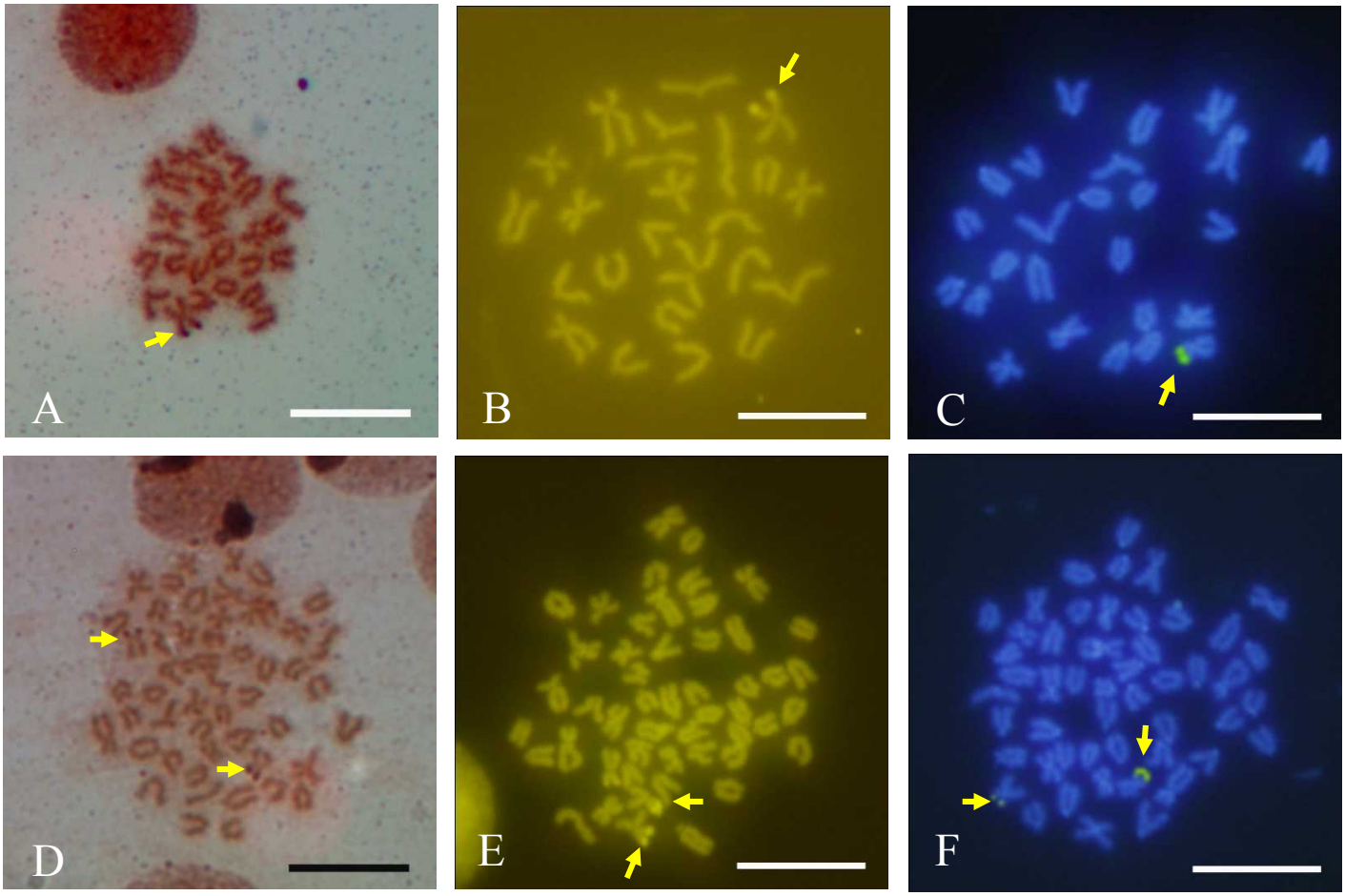
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Table 1. Number of eggs, fertilization rate, hatching rate, normal rate and survival rate at 7 days after hatch in different crosses

Cross	No. of eggs	Fertilization rate (%)	Hatching rate (%)	Normal rate (%)	Survival rate at 7 days after hatch (%)
2nx2n-1	412	92.95	79.36	91.14	75.79
2nx2n-2	716	83.58	74.51	96.7	94.86
2nx2n-3	448	66.07	95.61	91.17	71.73
2nx2n-4	414	93.96	78.41	84.26	73.77
mean±SD		84.14±0.13	81.97±0.09	90.82±0.05	79.04±0.11
2nxUV-1	701	75.89	3.2	0	0
2nxUV-2	766	56.27	9	0	0
2nxUV-3	433	42.96	27.96	0	0
2nx2nUV-	565	83.89	56.33	0	0
mean±SD		64.75±0.19	24.12±0.24	0	0
4nx2n-1	1210	97.69	51.02	88.72	76.78
4nx2n-2	850	77.41	91.79	98.68	89.74
4nx2n-3	778	89.2	99.14	80.52	77.91
4nx2n-4	524	90.65	94.32	84.38	84.38
mean±SD		88.74±0.08	84.06±0.22	88.07±0.08	82.20±0.06
4nxUV-1	480	95.21	81.33	66.67	54.53
4nxUV-2	489	73.62	98.89	44.1	59.55
4nxUV-3	695	68.78	96.23	37.39	49.13
4nxUV-4	744	70.97	92.42	48.57	45.7
mean±SD		77.14±0.12	92.22±0.08	49.18±0.13	52.23±0.06

Table 2. Metaphase chromosome count in Dojo loach embryos originating from different crosses

Cross	Embryo number	Cell number	Chromosome number frequency																	
			23	24	25	26	27	…	40	41	42	43	44	45	46	47	48	49	50	51	52
2n x 2n	17	72	0	0	0	0	0		1	3	2	2	2	1	4	4	5	2	41	2	3
2n x UV	11	39	5	6	26	1	1		0	0	0	0	0	0	0	0	0	0	0	0	0
4n x 2n	16	77	0	0	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0
4n x UV	17	100	0	0	0	0	0		0	0	0	2	1	0	3	10	9	3	44	3	6

Cross	Chromosome number frequency																		
	53	54	56	57	…	66	67	68	69	70	71	72	73	74	75	76	77	78	79
2n x 2n	0	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0
2n x UV	0	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0
4n x 2n	0	0	0	0		1	0	2	4	5	3	8	5	2	31	5	1	4	6
4n x UV	5	11	2	1		0	0	0	0	0	0	0	0	0	0	0	0	0	0

2n x 2n: control cross from fertilization of eggs of diploid female with sperm of diploid male, 2n x UV:gynogenetic cros from fertilization of eggs of diploid female with UV-irradiated sperm, 4n x 2n: cross from fertilization of eggs of tetrap female with sprem of diploid male, 4n xUV: gynogenetic cross from fertilization of eggs of tetraploid female with UV-irradiated sperm