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Intersubfamilial Hybridization of Two *Danio* and Six Related Cyprinid Fishes

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Abstract

Production of sterile individuals is the key technique for surrogate propagation in teleosts. Sterile hybrids may be the ideal surrogate host when they do not generate their own germ cells in their gonads. Here, we attempted hybridization experiments between zebrafish (*Danio rerio*) and six closely related species (*Danio albolineatus*, *Aphyocypris chinensis*, *Hemigrammocyppris rasborella*, *Opsariichthys platypus*, *Nipponocypris temminckii*, *N. sieboldii*) and one remotely related species (*Tanichthys albonubes*). Intersubfamilial hybridizations in the family Cyprinidae resulted in the occurrence of inviable abnormal larvae with the two parental genomes, except for the *T. albonubes* x *A. chinensis* hybridization, in which normal larvae survived. Allotetraploidy and spontaneous gynogenetic diploidy were infrequently detected in *T. albonubes* x *A. chinensis* and *D. albolineatus* x *A. chinensis*, respectively

Key words : Chimera, Distant hybrid, DNA content, Flow cytometry, Genome size, Polyploid

Introduction

Efficient production of commercially important fish and gene banking of endangered species can be achieved by the application of reproductive control including germ cell transplantation and sterilization (Yamaha et al., 2007 ; Robles et al., 2017 ; Siqueira-Silva et al., 2018). For successful surrogate production (i.e., allogenes and xenogeneses), germ cells of a donor individual, strain or species are transplanted to a sterile host because fertile or incompletely sterilized hosts induce germ-line chimeras, which produce both host-derived and donor-derived gametes. Thus, production of sterile fish is the key technique. The ideal host for a germ-line chimera should have sterile gonads, which permit proliferation and maturation of transplanted germ cells.

The zebrafish *Danio rerio* is an excellent model animal for basic and applied biological research (Grunwald and Eisen, 2002 ; Sprague et al., 2008). In this model fish, germ-line chimeras have been induced by transplantation of single primordial germ cells (PGCs) to a sterile morphant, in which a *dead end* germline gene was knocked down by the microinjection of morpholino oligonucleotide antisense (Saito et al., 2008). Xenogenetic gametes have been obtained from

germ-line chimeras of the closely related pearl danio *D. albolineatus* (Family Cyprinidae, Subfamily Danioninae) and the remotely related goldfish *Carassius auratus* (Family Cyprinidae, Subfamily Cyprininae) and dojo loach *Misgurnus anguillicaudatus* (Family Cobitidae) (Saito et al., 2008). Another approach to surrogate production was performed by the transplantation of ovarian germ cells to a sterile hybrid host between a zebrafish female and a pearl danio male, and donor-derived gametes were successfully produced by such chimeras (Wong et al., 2011). Although triploid lines have been often used as sterile hosts of germ-line chimeras (Okutsu et al., 2007 ; Yoshikawa et al., 2017) as well as genetically manipulated organisms including transgenics (Devlin et al., 2010), efficacy of hybrids has been recently highlighted as an ideal sterile host (Yoshikawa et al., 2018 ; Xu et al., 2019). Because, hybridization by interspecies cross-fertilization is technically much easier to induce than triploidization, which includes physical (cold, heat, or hydrostatic pressure) or chemical treatments to inhibit the second polar body extrusion just after fertilization (Piferrer et al., 2009 ; Arai and Fujimoto, 2013, 2019). Furthermore, sterility of some fish hybrids is caused not only by meiotic failure of pairing between homologous chromosomes from one species and

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those from other species (Hamaguchi and Sakaizumi, 1992 ; Shimizu et al., 1997 ; Kuroda et al., 2019) as shown in other vertebrate hybrids (Müller, 1977 ; Islam et al., 2013 ; Bhat-tacharyya et al., 2013 ; Torgasheva and Borodin, 2016), but also by mitotic arrest of PGCs, which was recently observed (Yoshikawa et al., 2018 ; Xu et al., 2019 ; Naya et al., 2020).

Besides the above-mentioned research reported by Wong et al. (2011), there have been relatively few hybridization studies in zebrafish. Genetic studies on pigmentation pattern were conducted in several combinations of interspecies cross-fertilization between different *Danio*, *Devatio* and *Tanichthys* species (Parichy and Johnson, 2001 ; Parichy, 2006). However, they did not mention reproductive performance except for the cross between *Danio rerio* and *Danio nigrofasciatus*, which produced a fertile F1 hybrid, but no viable progeny appeared in the backcross of the F1 hybrid. Endoh et al. (2020) observed that female *D. rerio* x male *D. nigrofasciatus* hybrids showed a predominance of males, which produced various aneuploid spermatozoa, which resulted in inviable backcross progeny.

Here, we attempted hybridization experiments between zebrafish and closely or remotely related species. Since heterospecific fertilization sometimes results in unpredicted development, including the occurrence of triploidy, gynogenesis, and rarely androgenesis as reviewed by Arai and Fujimoto (2019), genetic confirmation of hybrid nature, i.e., hybridity, is required to verify the presence of both maternally and paternally derived genomes (chromosome sets) in the resultant progeny. However, no reliable DNA markers have been developed for species identification among zebrafish and other closely or remotely related species in the subfamilies Danioninae, Tanichthyinae and Xenocypridinae. Differences in DNA content among these species could be accessed in an animal genome size database (<http://www.genomesize.com>). However, genome size data fluctuate even in the same species depending on measuring methods, sample sources, standard value, authors and others. Thus, firstly we examined DNA content by the same flow-cytometrical procedure to detect interspecies difference for genetic verification of hybridity among resultant progeny from interspecific cross-fertilization.

As we confirmed a significant genome size difference among fishes of the three subfamilies, we produced intersubfamilial hybrids between *Danio* species from the subfamily Danioninae and *Tanichthys albonubes* from the subfamily Tanichthyinae, and between *Danio* species and *Aphyocypris chinensis* or *Opsariichthys platypus* from the subfamily Xenocypridinae by artificial cross fertilization. We also tried intersubfamilial hybridization between *T. albonubes* and *A. chinensis*. Then, we examined the developmental capacity and nuclear DNA content of resultant progenies from these hybridizations.

Materials and Methods

Fish species

Parental fishes of zebrafish *D. rerio* and pearl danio *D. albolineatus* were taken from the maintained line of each species in the Nanae Fresh-Water Laboratory, Field Science Center of Northern Biosphere, Hokkaido University, Nanae, Hokkaido. Both lines were reared in aquaria at 26°C under 16L (light) and 8D (dark) photoperiod. They were fed *Artemia* nauplii twice a day and TetraMin® once a day.

Other species from Tanichthyinae and Xenocypridinae were obtained from commercial sources. Tang et al. (2013) grouped the genera *Hemigrammocypripis*, *Opsariichthys*, *Nipponocypris* and *Aphyocypris* in the subfamily Oxygastrinae. This subfamily was re-defined as Xenocypridinae based on molecular phylogeny by Yang et al. (2015), so those genera were treated as the subfamily Xenocypridinae in the present study. All fishes in this study were identified according to Nakabo (2013). *Tanichthys albonubes* was reared in aquaria at 26°C water temperature under 16L and 8D photoperiod and fed *Artemia* nauplii twice a day and TetraMin® once a day. *Aphyocypris chinensis*, *Hemigrammocypripis rasborella*, *Opsariichthys platypus*, *Nipponocypris temminckii*, and *N. sieboldii* were reared at 22°C water temperature under 16L and 8D photoperiod. *A. chinensis* and *H. rasborella* were fed TetraMin® three times a day. *O. platypus*, *N. temminckii* and *N. sieboldii* were fed trout pellets (solid No. 4, Nissin-Marubeni) once a day.

Flow-cytometry

DNA content flow cytometry was used to measure the ploidy or the genome size of the caudal fin-clip of each parental fish or the body of each larva or embryo using the Ploidy Analyzer (PA, Partec GmbH, Münster, Germany) with DAPI (4',6-diamidino-2-phenylindole) staining. Sample treatments and flow cytometrical measurements were performed according to Fujimoto et al. (2007). Results were presented as relative values when the DNA content of a somatic cell nucleus of a zebrafish (3.58 pg, Zhou et al., 2011) was 100.

Hybridization and Incubation

For *D. rerio*, *D. albolineatus* and *T. albonubes*, one day before the hybridization experiment, mature female(s) were kept in inside of a sieve set in an aquarium with freshwater at 26°C, while mature conspecific male(s) were kept outside of the sieve in the same aquarium. The next morning, ovulation and spermiation were confirmed by gently pushing the abdomen. For *A. chinensis* and *O. platypus*, sperm was directly taken to a hematocrit capillary by pushing the abdomen from a mature male with external nuptial coloration. Sperm was diluted with artificial seminal plasma for goldfish (Yanagimachi et al., 2017) or common carp (Magyary et al., 1996). Mature eggs were taken on plastic film (Saranwrap®),

Asahi Kasei Co. Ltd.) by pushing the abdomen.

Eggs were inseminated with sperm in a petri dish and ambient water was added to complete the fertilization. The total number and cleaved number of eggs were counted for calculation of fertilization rates. Fertilized eggs were transferred to a petri dish or 96-hole plate for individual rearing of eggs in each hole and incubated at 26°C. At 24 h and 48 h post-fertilization, surviving embryos were counted for calculating survival rates. The external appearance of embryos was observed and photographed.

Results

Nuclear DNA content difference among fishes of three subfamilies

The nuclear DNA content of zebrafish somatic cells (3.58 pg), which was flow-cytometrically determined by Zhou et al. (2011), was used as the standard value in the present study. Our flow cytometric measurements of fin-clip samples revealed $3.58 (\text{mean}) \pm 0.10 (\text{SD})$ pg for *D. rerio* ($n = 30$), 3.38 ± 0.10 pg for *D. albolineatus* ($n = 15$), 2.91 ± 0.05 pg for *T. albonubes* ($n = 20$), 1.98 ± 0.11 pg for *A. chinensis* ($n = 30$), 1.95 ± 0.04 pg for *H. rasborella* ($n = 15$), 1.89 ± 0.06 pg for *O. platypus* ($n = 8$), 1.89 ± 0.11 pg for *N. temminckii* ($n = 11$) and 1.93 ± 0.13 pg for *N. sieboldii* ($n = 6$) in the nuclear DNA content of somatic cells. The DNA content was significantly different among fishes of Danioninae (3.38–3.58 pg), Tanichthyinae (2.91 pg), and Xenocypridinae (1.89–1.98 pg). Thus, intersubfamilial hybrid nature can be detected by DNA content flow cytometry.

Hybridization between *D. rerio* female and *T. albonubes* male

Fertilization and survival rates were always lower or much

lower in hybrids than in the conspecific zebrafish control (Table 1). Early development of *D. rerio* x *T. albonubes* (female x male) progressed in almost the same manner to the control zebrafish staging shown by Kimmel et al. (1995). However, after hatching, malformation of the caudal region and edema were observed in the larvae. After absorption of the yolk sac, all survivors lacked a swim bladder. They did not feed and died within one week after hatching.

Both normal ($n = 10$) and abnormal larvae ($n = 10$) showed flow-cytometrical histograms at about intermediate DNA content (3.24 pg) between *D. rerio* and *T. albonubes* (Fig. 1).

Hybridization between *D. rerio* female and *A. chinensis* male

Fertilization rates of hybrids were similar in two of three experiments, but lower in the third group (Table 2). Survival rates of hybrids in two experiments were higher or similar when compared with the control at 1 to 2 days after fertilization. Another experiment showed a lower survival rate in hybrids than in the control. Early development of *D. rerio* x *A. chinensis* was very similar to normal developmental stages of the control zebrafish by Kimmel et al. (1995). However, embryos of some experimental groups showed abnormalities and abnormal development of the head and tail was frequently observed after hatching. After absorption of the yolk sac, no larvae fed, and all died one week after hatching.

Both normal ($n = 2$) and abnormal larvae ($n = 7$) gave flow-cytometrical histograms at about intermediate DNA content (2.83 pg) between *D. rerio* and *A. chinensis* (Fig. 2).

Hybridization between *D. rerio* female and *O. platypus* male

Fertilization rates of hybrids fluctuated between 31.7% and 93.4%, while those of the control were constant between

Table 1. Fertilization and survival rates in control *Danio rerio* and hybrid between *D. rerio* female and *Tanichthys albonubes* male. hpf : hours post-fertilization

Exp.	Parental Fish		Total	Fertilization		Survival at 24 hpf		Survival at 48 hpf	
	Female	Male	<i>n</i>	<i>n</i>	%	<i>n</i>	(%)	<i>n</i>	(%)
1	<i>Danio rerio</i>	<i>D. rerio</i>	68	67	98.5	67	100.0	65	97.0
		<i>T. albonubes</i>	133	107	80.5	104	97.2	37	34.6
2	<i>Danio rerio</i>	<i>D. rerio</i>	79	75	94.9	70	93.3	70	93.3
		<i>T. albonubes</i>	47	17	36.2	0	0.0	0	0.0
3	<i>Danio rerio</i>	<i>D. rerio</i>	112	109	97.3	105	96.3	97	89.0
		<i>T. albonubes</i>	80	66	82.5	23	34.8	16	24.2
4	<i>Danio rerio</i>	<i>D. rerio</i>	76	74	97.4	65	87.8	50	67.6
		<i>T. albonubes</i>	132	57	43.2	24	42.1	11	19.3
5	<i>Danio rerio</i>	<i>D. rerio</i>	68	62	91.2	23	37.1	19	30.6
		<i>T. albonubes</i>	121	106	87.6	1	0.9	1	0.9
6	<i>Danio rerio</i>	<i>D. rerio</i>	49	44	89.8	5	11.4	5	11.4
		<i>T. albonubes</i>	122	61	50.0	29	47.5	23	37.7

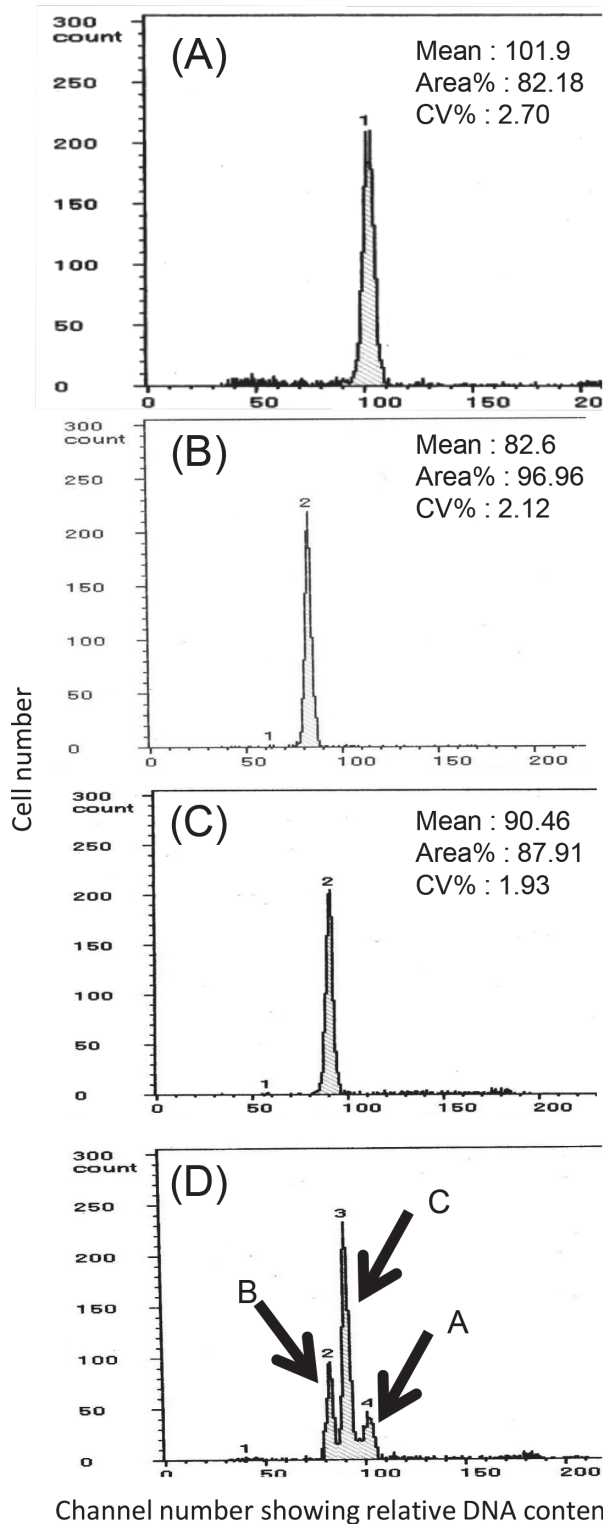


Fig. 1. Flow-cytometrical histograms of a hybrid between *Danio rerio* female and *Tanichthys albonubes* male, and its parental species, A : *D. rerio*, B : *T. albonubes*, C : hybrid, D : mixed sample.

94.9% and 98.5% (Table 3). Survival rates of hybrids were very low, and almost all progeny died within 24 h after fertilization (Table 3). One survivor exhibited lordosis and died

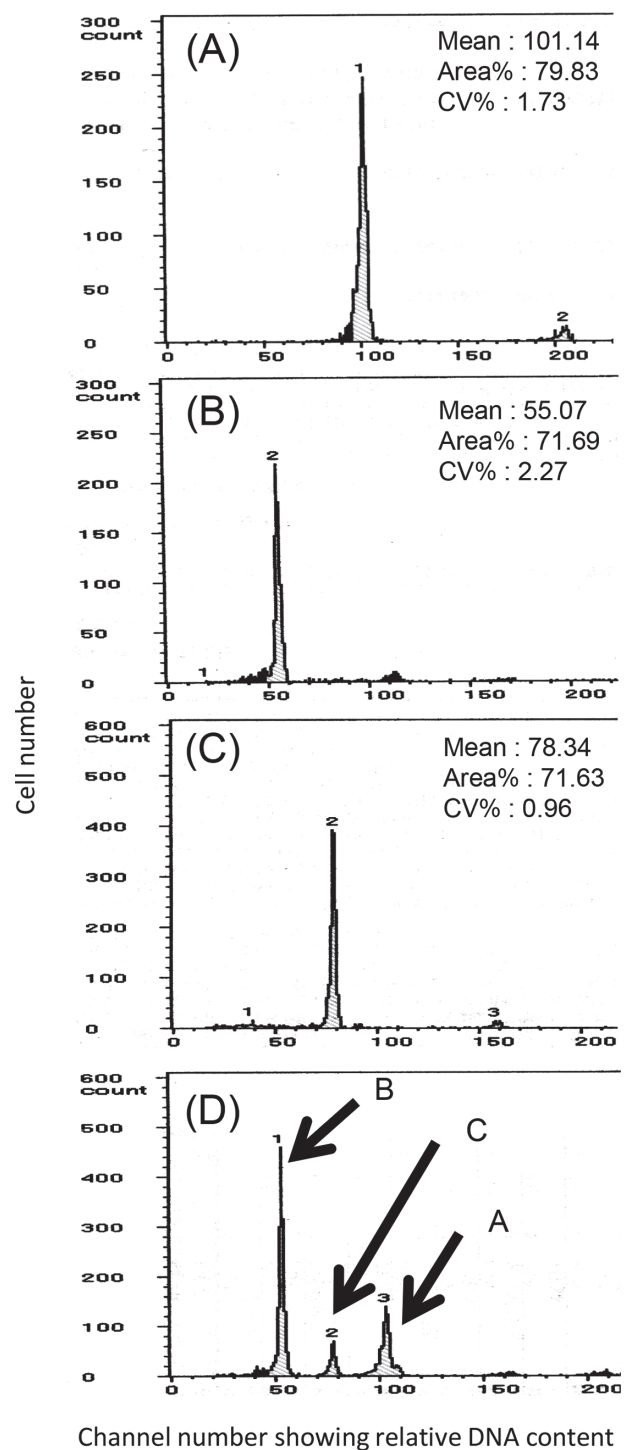


Fig. 2. Flow-cytometrical histograms of a hybrid between *Danio rerio* female and *Aphyocypris chinensis* male, and its parental species, A : *D. rerio*, B : *A. chinensis*, C : hybrid, D : mixed sample.

within 72 h after fertilization. No samples for flow cytometry were obtained due to the abrupt death of the progeny.

Table 2. Fertilization and survival rates in control *Danio rerio* and hybrid between *D. rerio* female and *Aphyocypris chinensis* male. hpf : hours post-fertilization

Exp.	Parental Fish		Total	Fertilization		Survival at 24 hpf		Survival at 48 hpf	
	Female	Male	<i>n</i>	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%
1	<i>Danio rerio</i>	<i>D. rerio</i>	147	103	70.1	99	96.1	99	96.1
		<i>A. chinensis</i>	89	68	76.4	37	54.1	36	52.9
2	<i>Danio rerio</i>	<i>D. rerio</i>	108	81	75.0	67	82.7	38	46.9
		<i>A. chinensis</i>	94	80	85.1	72	90.0	61	76.3
3	<i>Danio rerio</i>	<i>D. rerio</i>	78	28	35.9	25	89.3	25	89.3
		<i>A. chinensis</i>	39	7	17.9	6	85.7	6	85.7

 Table 3. Fertilization and survival rates in control *Danio rerio* and hybrid between *D. rerio* female and *Opsariichthys platypus* male.

Exp.	Parental Fish		Total	Fertilization		Survival at 24 hpf		Survival at 48 hpf	
	Female	Male	<i>n</i>	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%
1	<i>D. rerio</i>	<i>D. rerio</i>	68	67	98.5	67	100.0	65	97.0
		<i>O. platypus</i>	223	185	83.0	1	0.5	0	0.0
2	<i>D. rerio</i>	<i>D. rerio</i>	79	75	94.9	70	93.3	70	93.3
		<i>O. platypus</i>	83	78	93.4	0	0.0	0	0.0
3	<i>D. rerio</i>	<i>D. rerio</i>	112	109	97.3	105	96.3	97	89.0
		<i>O. platypus</i>	103	65	63.1	0	0.0	0	0.0
4	<i>D. rerio</i>	<i>D. rerio</i>	76	74	97.3	65	87.8	50	67.6
		<i>O. platypus</i>	208	66	31.7	0	0.0	0	0.0

Hybridization between *T. albonubes* female and *D. rerio* male

Fertilization and survival rates of hybrids were very low when compared with those of conspecific *T. albonubes* (Table 4). Hybrid larvae showed abnormal external appearance with microphthalmia, lordosis, and edema. All survivors died within 3 days after hatching. No samples were available for flow cytometry.

Hybridization between *T. albonubes* female and *A. chinensis* male

Fertilization and survival rates of hybrid and conspecific *T. albonubes* control are shown in Table 5. Fertilization rates fluctuated among experiments, but were always lower in hybrids (8.5–33.3%) than in the control (41.9–96.3%). Survival rates were similar between hybrid and control progeny

at both 1- and 2-days after fertilization. Both control and hybrid progeny showed normal appearance and growth.

Both normal ($n = 14$) and abnormal larvae ($n = 2$) gave flow-cytometrical histograms at about intermediate DNA content (2.45 pg) between *T. albonubes* and *A. chinensis* (Fig. 3). No difference was detected between normal and abnormal larvae. However, one long-surviving fish with normal appearance gave nuclear DNA content equivalent to amphidiploid or allotetraploid level, comprising both diploid genome of *T. albonubes* and of *A. chinensis* (Fig. 4).

Hybridization between *D. albolineatus* female and *A. chinensis* male

Fertilization and survival rates were lower in hybrids than in the conspecific control *D. albolineatus* (Table 6). Both normal and abnormal larvae appeared at the hatching stage. Normal progeny survived for at least 10 months and were

 Table 4. Fertilization and survival rates in control *Tanichthys albonubes* and hybrid between *T. albonubes* female and *Danio rerio* male. hpf : hours post-fertilization

Exp.	Parental Fish		Total	Fertilization		Survival at 24 hpf		Survival at 48 hpf	
	Female	Male	<i>n</i>	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%
1	<i>T. albonubes</i>	<i>T. albonubes</i>	56	54	96.4	48	88.9	44	81.5
		<i>D. rerio</i>	98	9	9.2	5	55.6	4	44.4

Table 5. Fertilization and survival rates in control *Tanichthys albonubes* and hybrid between *T. albonubes* female and *Aphyocypris chinensis* male. hpf : hours post-fertilization

Exp.	Parent Fish		Total	Fertilization		Survival at 24 hpf		Survival at 48 hpf	
	Female	Male	<i>n</i>	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%
1	<i>T. albonubes</i>	<i>T. albonubes</i>	56	54	96.4	48	88.9	44	81.5
		<i>A. chinensis</i>	105	15	14.3	15	100.0	15	100.0
2	<i>T. albonubes</i>	<i>T. albonubes</i>	93	39	41.9	39	100.0	39	100.0
		<i>A. chinensis</i>	91	27	29.7	27	100.0	27	100.0
3	<i>T. albonubes</i>	<i>T. albonubes</i>	103	15	89.3	90	97.8	85	92.4
		<i>A. chinensis</i>	176	66	8.5	14	93.3	14	93.3
4	<i>T. albonubes</i>	<i>T. albonubes</i>	33	16	66.7	18	81.8	17	77.3
		<i>A. chinensis</i>	102	57	15.7	15	93.8	15	93.8
5	<i>T. albonubes</i>	<i>T. albonubes</i>	45	41	91.1	41	100.0	41	100.0
		<i>A. chinensis</i>	45	15	33.3	15	100.0	15	100.0

Table 6. Fertilization and survival rates in control *Danio albolineatus* and hybrid between *D. albolineatus* female and *Aphyocypris chinensis* male. hpf : hours post-fertilization

Exp.	Parent Fish		Total	Fertilization		Survival at 24 hpf		Survival at 48 hpf	
	Female	Male	<i>n</i>	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%
1	<i>D. albolineatus</i>	<i>D. albolineatus</i>	204	130	63.7	50	38.5	30	23.1
		<i>A. chinensis</i>	181	77	42.5	12	15.6	9	11.7
2	<i>D. albolineatus</i>	<i>D. albolineatus</i>	172	158	91.9	150	94.9	148	93.7
		<i>A. chinensis</i>	127	67	52.8	2	3.0	2	3.0

morphologically quite similar to *D. albolineatus* (Fig. 5).

Both normal ($n = 4$) and abnormal larvae ($n = 4$) gave different flow-cytometrical histograms. Normal-looking larvae showed DNA content (3.43 pg) similar to *D. albolineatus* (Fig. 6), whereas abnormal larvae exhibited intermediate DNA content (2.73 pg) between *D. albolineatus* and *A. chinensis* (Fig. 7). However, one 10-month-old fish with normal appearance gave nuclear DNA content equivalent to diploid genome of *D. albolineatus* (Fig. 8). Thus, abnormal larvae had a hybrid nature between the two parental species, but normal larvae and one long-surviving fish had exclusively two sets of maternally derived chromosomes.

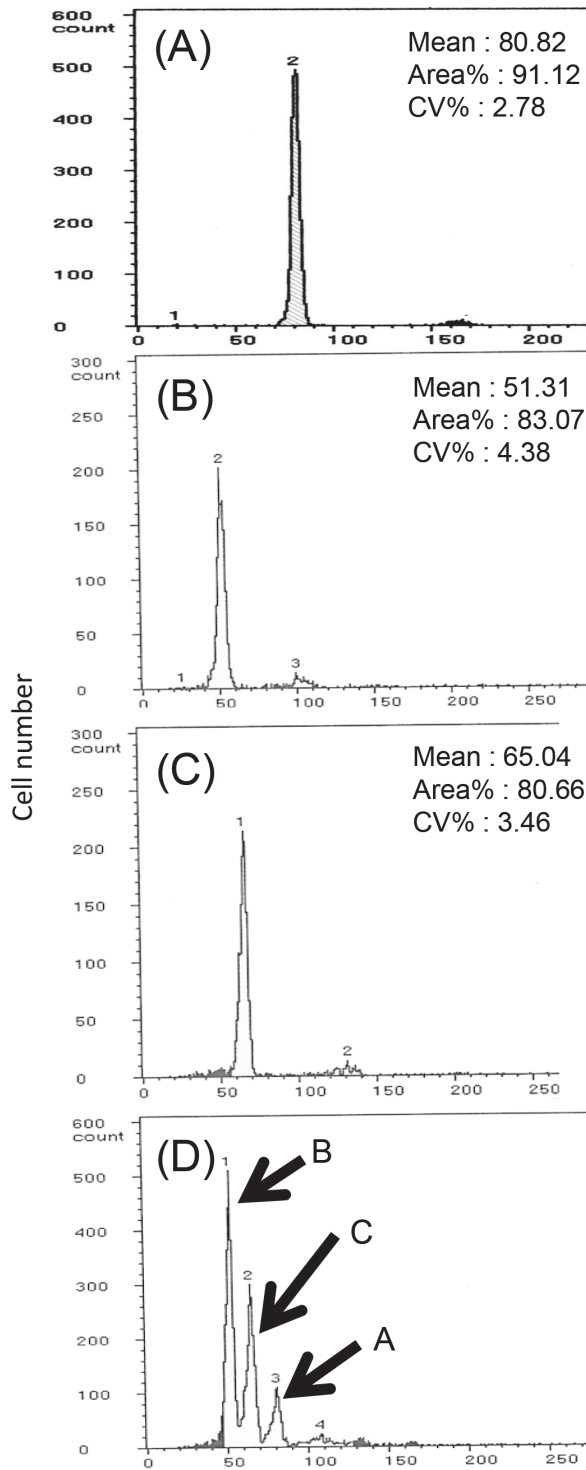
Discussion

Among species of the subfamilies Danioninae, Tanichthyinae, and Xenocypridinae, the nuclear DNA content and genome size differed. Such a difference can be utilized as a useful genomic marker of parental species for hybridization. The tropical *Danio* species, *D. rerio* (3.58 pg) and *D. albolineatus* (3.38 pg) belonging to Danioninae are considered to possess approximately 3.5 pg DNA content per somatic cell nucleus. Similar DNA content of *D. rerio* was previously reported as 3.60 pg by Hinegardner (1968) and Hinegardner and Rosen (1972), 3.56 pg by Ojima and Yamamoto (1990), 3.50 pg by Vinogradov (1991), and 3.36 pg by Ciudad et al.

(2002). All these data listed in an animal genome size database (original data shown by C-value for haploidy in <http://www.genomesize.com>) and Zhou et al. (2011) gave similar values ranging from 3.36 to 3.60 pg, but only Lamatsch et al. (2000) reported much higher 4.56 pg per diploid somatic cells. On the other hand, the DNA content of *D. albolineatus* was reported as 2.84 pg by Suzuki et al. (1995), which is much lower than our result (3.38 pg). Such a difference can be explained by difference in methods. We used flow-cytometry with DAPI staining, but Suzuki et al. (1995) measured DNA content by classic Feulgen densitometry.

The subtropical *T. albonubes* (Tanichthyinae) showed approximately 3 pg DNA content per somatic cell nucleus. This value is much higher than the previously reported 2.3 pg measured by the Feulgen densitometry (Suzuki et al., 1995).

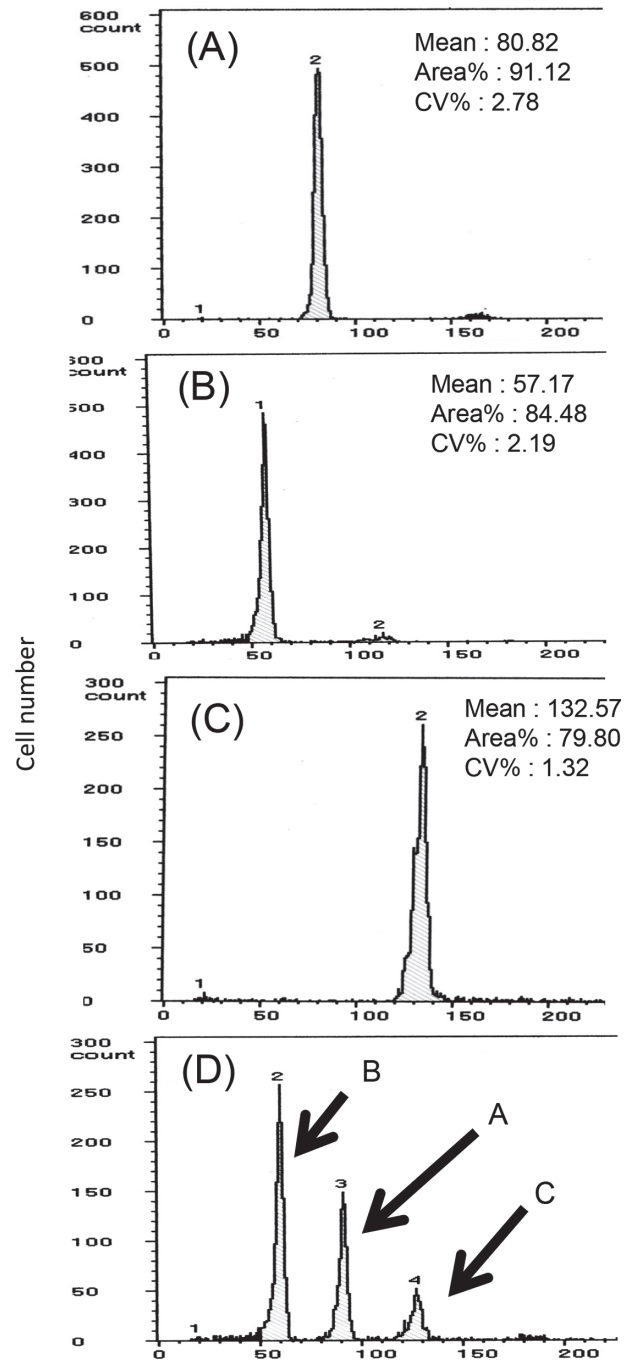
The east Asian and/or Japanese temperate species *A. chinensis*, *H. rasborella*, *O. platypus*, *N. temminckii* and *N. sieboldii*, which are considered members of Xenocypridinae, gave approximately 2 pg (1.89–1.96 pg) DNA content per somatic cell nucleus in the present study. Although the DNA content of *A. chinensis* was reported as 2.32 pg by Ojima and Yamamoto (1990) and 2.30 pg by Suzuki et al. (1995), our flow cytometry showed 1.98 pg. A previous result of *H. rasborella* (1.8 pg, Suzuki et al., 1995) was similar to our result (1.95 pg). The DNA content of our measurement in *O. platypus* (1.89 pg) was very similar to the



Channel number showing relative DNA content

Fig. 3. Flow-cytometrical histograms of a hybrid between *Tanichthys albonubes* female and *Aphyocypris chinensis* male, and its parent species, A : *T. albonubes*, B : *A. chinensis*, C : hybrid, D : mixed sample.

previous result (1.8 pg) by Suzuki et al. (1995), while our DNA content of *N. temminckii* (1.89 pg) was lower than a previously reported value (2.32 pg, Ojima and Yamamoto,



Channel number showing relative DNA content

Fig. 4. Flow-cytometrical histograms of a long surviving hybrid between *Tanichthys albonubes* female and *Aphyocypris chinensis* male, and its parent species, A : *T. albonubes*, B : *A. chinensis*, C : a long surviving hybrid, D : mixed sample. Note allotetraploid or amphidiploid DNA content of the hybrid sample.

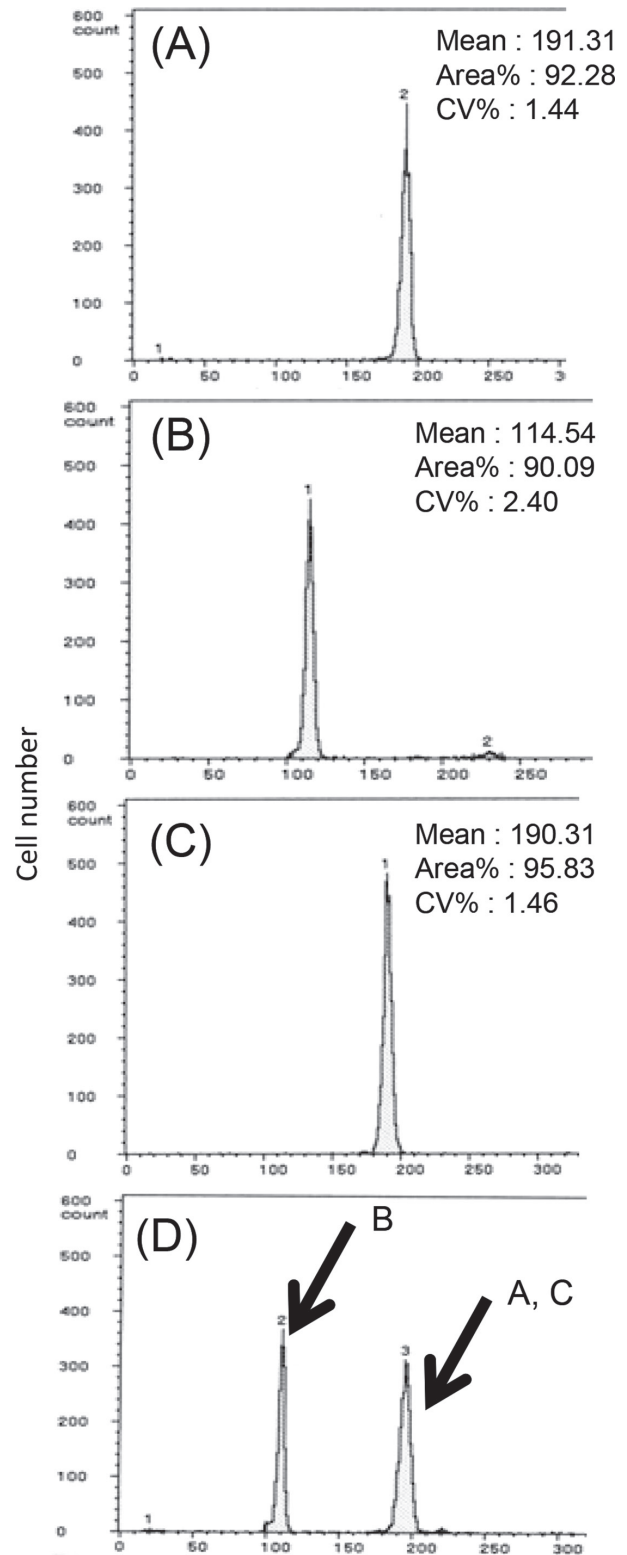
1990). Some differences in DNA content among present and previous results may be explained by the difference of measurement methods (Feulgen densitometry, Feulgen image analysis densitometry, bulk fluorometric assay, and flow cytometry with different machines and protocols), sample



Fig. 5. External appearances of *Danio albolineatus* (A), *Aphyocypris chinensis* (B), and hybrid between *D. albolineatus* female and *Aphyocypris chinensis* male (C) at 10-month after fertilization. Note similarity in external appearance between maternal species and a hybrid progeny. Scale bars show 10 mm.

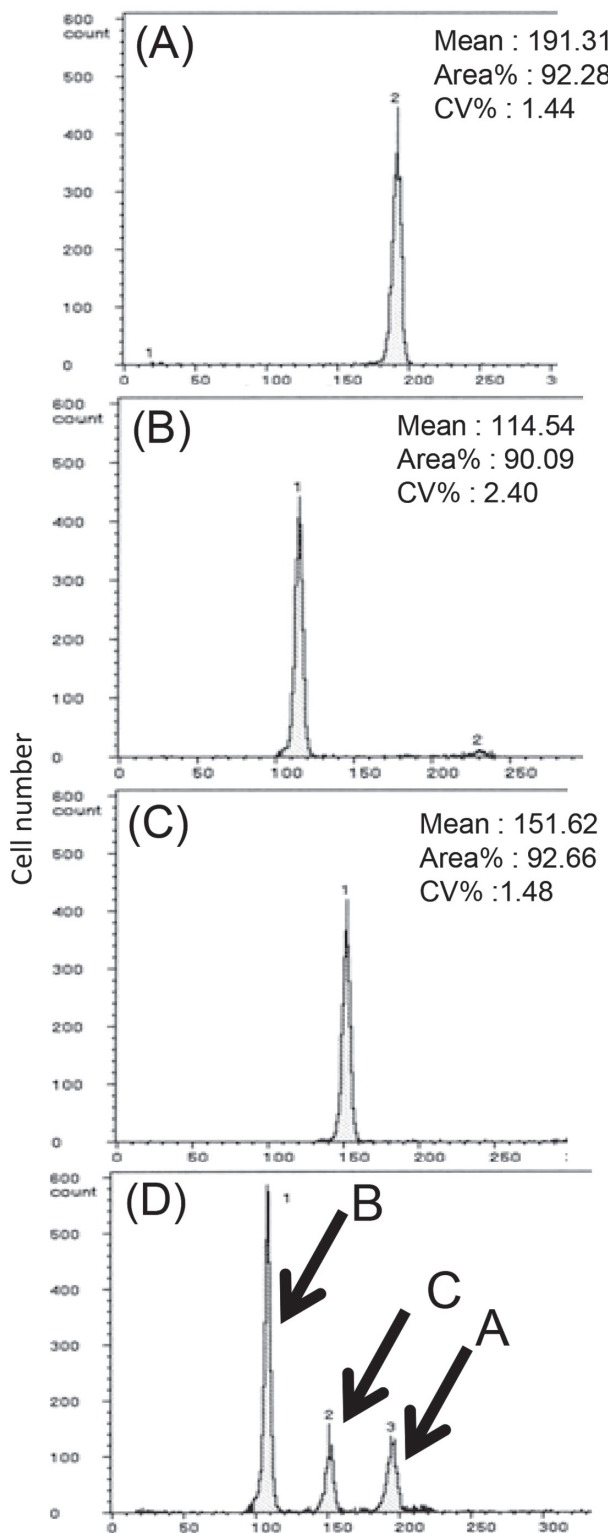
sources (erythrocytes, fin-clip, etc.) and standard sample, but Japanese Tanichthyinae species generally showed approximately 2 pg, giving a useful genomic marker to detect its genome in hybrids when *Danio* or *Tanichthys* species was used as the parental species.

In hybrids *D. rerio* x *T. albonubes* (female x male), *D. rerio* x *A. chinensis*, *D. rerio* x *O. platypus*, *T. albonubes* x *D. rerio*, *D. albolineatus* x *A. chinensis*, various kinds of abnormalities occurred after hatching, and the resultant abnormal progeny soon died. Thus, they are assumed to be inviable hybrids. Among them, the hybrid nature of *D. rerio* x *T. albonubes*, *D. rerio* x *A. chinensis*, and *D. albolineatus* x *A. chinensis* was verified by the approximately intermediate DNA content between the two parental species. These results indicated that even inviable hybrid progeny had both maternally and paternally derived genome. Some inviable hybrids were reported to have aneuploid karyotypes due to sporadic elimination of chromosomes in the course of development after cross-fertilization in salmonids (Arai, 1984; Goodier et al., 1987; Yamazaki et al., 1989; Ueda et al., 1990; Fujiwara et al., 1997) as well as in medaka (Sakai et al., 2007). However, intermediate karyotypes have been reported even in interfamilial hybrids including dojo loach *Misgurnus anguilli-*



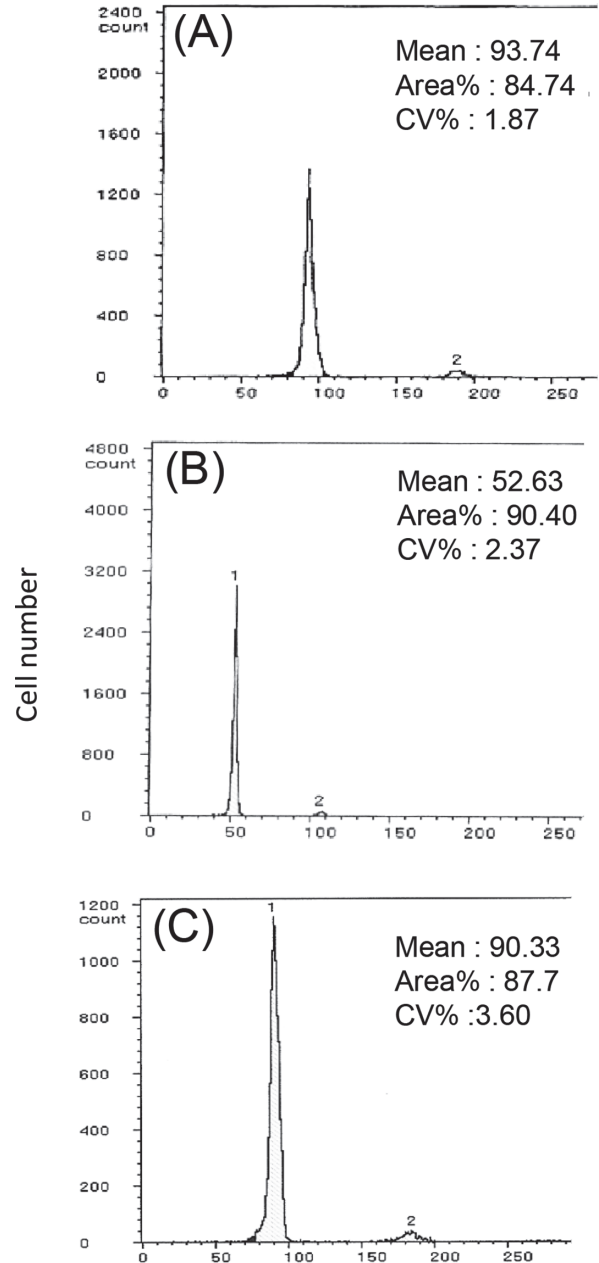
Channel number showing relative DNA content

Fig. 6. Flow-cytometrical histograms of a normal-looking hybrid between *Danio albolineatus* female and *Aphyocypris chinensis* male, and its parent species, A: *D. albolineatus*, B: *A. chinensis*, C: a normal-looking hybrid, D: mixed sample. Note DNA content of the hybrid similar to maternal parent *D. albolineatus*.



Channel number showing relative DNA content

Fig. 7. Flow-cytometrical histograms of an abnormal hybrid between *Danio albolineatus* female and *Aphyocypris chinensis* male, and its parent species, A : *D. albolineatus*, B : *A. chinensis*, C : abnormal hybrid, D : mixed sample.



Channel number showing relative DNA content

Fig. 8. Flow-cytometrical histograms of a long-surviving hybrid between *Danio albolineatus* female and *Aphyocypris chinensis* male, and its parent species, A : *D. albolineatus*, B : *A. chinensis*, C : normal hybrid at 10-month after fertilization. Note DNA content of the hybrid similar to maternal parent *D. albolineatus*.

caudatus female x goldfish *Carassius auratus* male, dojo loach female x minnow *Gnathopogon elongatus elongatus* male, spinous loach *Cobitis biwae* female x ornamental carp (Koi) *Cyprinus carpio* male and dojo loach female x ornamental carp male (Kijima et al., 1996a, 1996b). Thus, in the order Cypriniforms, chromosome elimination did not occur even in interfamilial hybridization, and the occurrence of invi-

able abnormalities in hybrid embryos could not be explained by aneuploidy. Considering these previous results, it is not surprising that inviable progeny with allodiploidy comprising both parental species generally occur in intersubfamilial hybrids in the family Cyprinidae. In *D. rerio* x *O. platypus* and *T. albonubes* x *D. rerio* hybrids, flow cytometry was not performed due to no available samples, but they are expected to be allodiploid hybrids comprising both maternally and paternally derived genome (chromosome set).

In *T. albonubes* x *A. chinensis*, almost all hatching larvae showed normal appearance, and flow cytometry verified their hybrid nature with intermediate DNA content. In addition, some normal larvae gave allotetraploid or amphidiploid DNA content including both two sets of maternally derived chromosomes and two sets of paternally derived chromosomes. Spontaneous occurrence of allotriploidy has been often reported in distant hybridization of fish species, as in common carp *Cyprinus carpio* female x grass carp *Ctenopharyngodon idella* male hybrids (Stanley, 1976), grass carp female x big head carp *Hypophthalmichthys nobilis* male hybrids (Maryan and Krasznai, 1978), and rainbow trout *Oncorhynchus mykiss* female and brook trout *Salvelinus fontinalis* male hybrids (Capanna et al., 1974; Ueda et al., 1984). However, the occurrence of allotetraploidy or amphidiploidy was rarely reported exclusively in bitterling *Rhodeus ocellatus ocellatus* female x *R. atremius atremius* male F1 hybrids (Ueda et al., 1994). Such an unpredictable occurrence of allotetraploidy suggests the presence of a possible mechanism to induce ploidy elevation by the influence of the interspecific fertilization.

In *D. albolineatus* x *A. chinensis*, inviable abnormal larvae were confirmed to be a true hybrid comprising both parental genomes by flow cytometry, but simultaneously appearing viable normal larvae had DNA content equivalent to the sum of the two sets of maternal *D. albolineatus* chromosomes, which strongly suggested the occurrence of spontaneous gynogenesis by intersubfamilial hybridization. Such an unpredictable occurrence of presumable gynogenetic diploidy suggests the presence of possible mechanism to trigger gynogenesis by the heterospecific fertilization, followed by sporadic elevation of ploidy from haploidy to diploidy. There are two possible ways of chromosome duplication: inhibition of the second polar body release or endomitosis of the 1-cell stage embryo. However, it is impossible to identify which mechanism was responsible for chromosome duplication in gynogenetic *D. albolineatus* x *A. chinensis* hybrid embryo. Similar occurrence of gynogenetic diploidy was previously reported in common carp *C. carpio* female x grass carp *Ctenopharyngodon idella* male hybrids (Stanley, 1976) and coho salmon *Oncorhynchus kisutch* female x brook trout *Salvelinus fontinalis* male hybrids (Uyeno, 1972), but cytological and molecular mechanisms responsible for such a phenomenon have not been investigated yet.

In conclusion, intersubfamilial hybridizations in the family Cyprinidae resulted in the occurrence of inviable abnormal larvae with the two parental genomes, except for the *T. albonubes* x *A. chinensis* hybridization, in which normal larvae survived. Allo-tetraploidy and spontaneous gynogenetic diploidy were infrequently detected in *T. albonubes* x *A. chinensis* and *D. albolineatus* x *A. chinensis*, respectively, and further studies are required to identify mechanisms responsible for unpredictable development probably due to distant hybridization.

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