Title page

Title (120 characters):

Developmental potential of somatic and germ cells of hybrids between *Carassius auratus* females and *Hemigrammocypris rasborella* males.

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Running headline (35 characters): Goldfish × golden venus chub hybrids

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Summary

The cause of hybrid sterility and inviability has not been analyzed in fin-fish hybrid, although a large number of hybridizations have been carried out. In this study, we produced allo-diploid hybrids by cross-fertilization between female goldfish (*Carassius auratus*) and male golden venus chub (*Hemigrammocypris rasborella*). Inviability of these hybrids was due to breakage of the enveloping layer during epiboly or due to malformation with serious cardiac edema around the hatching stage. Spontaneous allo-triploid hybrids with two sets of the goldfish genome and one set of the golden venus chub genome developed normally and survived beyond the feeding stage. This improved survival was confirmed by generating heat-shock induced allo-triploid hybrids that possessed an extra goldfish genome. When inviable allo-diploid hybrid cells were transplanted into goldfish host embryos at the blastula stage, these embryos hatched normally, incorporating the allo-diploid cells. These allo-diploid hybrid cells persisted, and were genetically detected in a 6-month-old fish. In contrast, primordial germ cells taken from allo-diploid hybrids and transplanted into goldfish hosts at the blastula stage had disappeared by 10 days post-fertilization, even in chimeric conditions. In allo-triploid hybrid embryos, germ cells proliferated in the gonad, but had disappeared by 10 weeks post-fertilization. These results show that while hybrid germ cells are inviable even in chimeric conditions, hybrid somatic cells remain viable.

Key words (5): chimera, fertility, hybrid, polyploid, sterility.

Introduction

A relatively large number of hybridizations have been carried out in fin-fish species for basic research and aquaculture-oriented studies (Swartz 1981). Especially in salmonid species, various inter-specific and inter-generic hybrids, i.e. allo-diploids, have been produced. While some of these hybrids die in early developmental stages, others are viable and can grow to adult stages (Suzuki and Fukuda, 1971, Blanc and Chevassus, 1979; Chevassus *et al.,* 1983: Hulata, 2001). Inviability of some hybrids has been partially explained by aneuploidies due to uniparental chromosome elimination during early embryonic stages (Arai 1984; Fujiwara *et al.* 1997). Even in viable hybrids, sterility can arise during gonadal development or gamete production, and inviable zygotes have often appeared after back-crossing and/or inter-crossing of hybrids (Suzuki and Fukuda, 1973; Suzuki, 1974; Chevassus *et al.,* 1983). The induction of triploid hybrids (allo-triploids) has given rise to drastic recovery or increased survival potential in several inviable hybrids (Chevassus *et al.,* 1983; Scheerer and Thorgaard, 1983; Arai, 1984, 1986, 1988; Ueda *et al.,* 1984; Parsons *et al.,* 1986; Yamano *et al*., 1988; Seeb *et al.,* 1988; Gray *et al.,* 1993). Such kinds of viable allo-triploid hybrid strains exhibit sterility and have been commercially farmed to support the vitalization of local economies in Japan (Arai, 2000, 2001; Arai and Fujimoto, 2019).

In teleost fish, it has been thought that natural hybridization between different species occurs frequently and contributes genetic introgression in the process of speciation (Scribner *et al*., 2001). However, almost all artificial hybrid cannot reproduce their filial generations, because of above mentioned phenomena, such as inviablity and sterility. Therefore, the analysis of hybrid inviability and sterility between species is of great importance in genetic breeding and biological evolution.

In cyprinid species, many hybrids have been produced via inter-species, inter-genus, and even inter-family combinations (Suzuki, 1956, 1961, 1962, 1968; Ojima, 1973). In case of inter-subfamilial hybrids which have been produced using species from the subfamilies Acheilognathinae, Gobioninae or Cyprininae, most of these progeny exhibited inviability at hatching stage (Suzuki, 1956, 1961, 1962, 1968), while those between species in Oxygastrinae exceptionally developed normally and produced mature eggs (Li *et al.*, 2019). Although inviable cyprinid hybrids have not been cytogenetically studied, Kijima *et al.* (1996 a, b) reported that inviable inter-familial hybrids between dojo loach females (family Cobitidae) and goldfish, minnow or common carp males (family Cyprinidae), had allo-diploid karyotypes intermediate between the two parental species. Thus, inviability of these inter-familial hybrids cannot be explained by aneuploidies due to chromosome elimination during embryogenesis. These authors also reported that induction of allo-triploidization did not enable the recovery of survival potential and produce viable triploid hybrids, though it did improve the external appearance of the resultant hybrid larvae (Kijima *et al.,* 1996 a, b).

As seen in the inviable hybrids from certain combinations of fish species, artificially-induced

haploid embryos exhibit severe abnormalities collectively referred to as haploid syndrome. These abnormalities include dwarfism, microcephaly, microphthalmia and edema, thus almost all gynogenetic or androgenetic haploids die before hatching or first feeding (Arai, 2000, 2001; Arai and Fujimoto, 2019). In goldfish, gynogenetically-induced haploid embryos die before or around hatching; however, haploid cells were able to survive when they were transplanted into diploid blastula embryos, and consequently, viable haploid-diploid chimeras can be produced (Tanaka *et al.,* 2004). The appearance of spontaneous haploid-diploid mosaic charr also supports the survival of haploid cells within diploid cell populations under chimeric or mosaic conditions (Yamaki *et al.,* 1999). Were this also to be the case for inviable hybrids, cells transplanted from inviable hybrids may survive and function in conspecific hosts under chimeric conditions.

In viable hybrids, disturbance can also frequently occur during meiosis and the subsequent differentiation of gonads or gametes because of the presence of non-homologous genomes from genetically distant species. The loss of germ cells has also been reported in viable marine red dram hybrids (Yoshikawa *et al*., 2018). Hybrid fishes are thus often sexually abnormal and can exhibit reproductive performances that range widely, from near-normal fertility to complete sterility or infertility in either or both of the sexes. However, as sterility is a useful characteristic for aquaculture—because sterile fish diminish the risk of genetic contamination of indigenous populations by escaped farmed fishes—sterile hybrids are considered highly useful hosts for transgenesis, genome editing and surrogate reproduction for biological containment (Arai and Fujimoto, 2019). For example, hybrids between female crucian carp (the origin of goldfish) *Carassius auratus* and male common carp *Cyprinus carpio* show male sterility due to the death of spermatocytes, which presumably results from a germ-cell-autonomous abnormality (Makino *et al.,* 1958; Ojima, 1973). When exotic primordial germ cells (PGCs) were transplanted from goldfish, one parental fish, the germline of the resultant chimera would produce sperm that is exclusively derived from the transplanted PGCs, because the host PGCs are abnormal (Yamaha *et al.,* 2003).

In inviable hybrids, the process of PGC differentiation into gametes has not been investigated. In normal teleost development, PGCs differentiate from blastomeres that are inherited along with maternal germplasm (Saito *et al.,* 2014) and in various species, PGCs have been visualized by injecting GFP-*nos*3 3′UTR mRNA to mimic maternal mRNA in the germplasm (Saito *et al.,* 2006; Nagasawa *et al.,* 2013; Goto *et al*., 2015). PGCs visualized in this way maintain their migratory potential under *in vivo* conditions, even after transplantation (Saito *et al.,* 2008). This technique enables the analysis of PGC differentiation and potential (i.e., cellular viability/inviability) in germline chimeras.

Goldfish (*Carassius auratus*) is a popular ornamental fish in Japan that has long been a useful experimental model for reproductive biology, developmental biology, endocrinology and other disciplines (Devlin and Nagahama, 2002; Habibi *et al.,* 2012; Tsai *et al.,* 2013; Urushibata *et al*.,

2019). In particular, as a model for micro-manipulation, embryos of this species have revealed specific insights of several biological processes. For example, graft transplantations have revealed that blastoderm cells are highly pluripotent at the blastula stage (Yamaha *et al.,* 1997; Kazama-Wakabayashi *et al.,* 1999). Germline chimeras are also easily induced by graft transplantation (Yamaha *et al.,* 2001, 2003), as well as by PGC transplantation (Goto-Kazeto *et al.,* 2012). These data suggest that hybrids between goldfish females and males of other species can be highly advantageous for analyses of hybrid cells, including germline cells. Specifically, developmental potential, such as the viability and fertility of hybrid somatic and germline cells themselves, can be confirmed *in vivo* by transplantation of hybrid cells into normal goldfish. Because intrinsic survival and differentiating potentials of hybrid cells, which gave rise to lethal effect to an individual, could be examined under the chimeric condition with viable host individual.

Golden venus chub (*Hemigrammocypris rasborella*) is a small freshwater cyprinid fish that lives in the swamps and rivers of Japan. Recently, this species has been designated as an endangered species 1B, because its population is in decline (https://www.env.go.jp/press/files/jp/109165.pdf). Previously induced artificial hybrids between Gobioninae female fish—including *Biwia zezera, Squalidus japonicus,* and *Gnathopogon elongatus*—and *Hemigrammocypris rasborella* males were inviable (Suzuki, 1968). However, hybridization between goldfish and golden venus chub has not yet been reported.

Here, we induced hybrids between female goldfish, *Carassius auratus,* and male golden venus chub, *Hemigrammocypris rasborella* (hereafter HR), and analyzed their ploidy and development potential. The resultant hybrid progeny with allo-diploidy were inviable, but spontaneously occurring allo-triploid hybrids were viable. Thereafter, we confirmed whether artificial allo-triploidization increased viability in this hybrid combination, and we examined the cause of inviability by transplanting diploid hybrid cells into a goldfish host. Finally, we observed PGCs' specification and gonadal development.

Materials and Methods

Ethics

This study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals in Hokkaido University and Field Science Center for Northern Biosphere, Hokkaido University (approval ID: 22-1).

Fish

Parent goldfish and golden venus chub were kept at the Nanae Fresh-Water Laboratory, Hokkaido University. Parent goldfish were maintained at 10–14 °C. Parent HR originated from Hyogo Prefecture and were purchased from a commercial pet shop. Parent HR were kept in laboratory

aquaria at 22–25 °C and with a 16 hours light, 8 hours dark photoperiod. Collection of goldfish sperm and mature eggs was performed according to Yamaha *et al.* (2001). HR sperm was collected with a 10 μl crystal tip from anesthetized male fish, and diluted in artificial seminal plasma for goldfish (NaCl 5.61g, KCl 5.23g, $CaCl_2 \cdot 2H_2O$ 0.33g, $MgCl_2 \cdot 6H_2O$ 0.22g, $NaHCO_3$ 0.2g/L in distilled water) (Yanagimachi *et al.,* 2017).

Artificial fertilization of goldfish eggs with HR sperm was performed as follows: goldfish eggs were inseminated with diluted HR sperm on a polyvinylidene film, fertilized by mixing with a small amount of freshwater, and then scattered into plastic petri-dishes containing urea water (0.2% urea and 0.24% NaCl in tap water) to reduce viscosity of eggs. In control goldfish fertilizations, eggs inseminated with diluted sperm were directly scattered into urea water. Eggs were fertilized in several different dishes in each cross. We used a representative dish for calculation of fertilization and survival rates by counting cleaved and surviving (and dead) eggs. Other dishes were used for samples of flow cytometry (FCM), histology and/or micromanipulation.

Dechorionation and incubation conditions

Dechorionation of fertilized eggs was performed using the method described by Yamaha and Yamazaki (1993). Prior to experimental manipulations, dechorionated embryos were incubated in 1% agar-coated petri-dishes filled with Ringer's culture solution (128 mM NaCl, 2.8 mM KCl, 1.8 mM $CaCl_2$) with 1.6% albumen.

Thermal treatment of fertilized eggs

To induce allo-triploid progeny, goldfish eggs fertilized with either HR or goldfish sperm were heat-shocked at 40 °C for 50–75 s, 5 min after fertilization (Nagoya *et al.,* 1990). Immediately after treatment, petri-dishes with fertilized eggs were washed and filled with urea water at 20 °C, and then cultured in an incubator at 10 or 20 °C. Hatched fish with a normal appearance were reared by feeding with brine shrimp nauplii and artificial crumble fish feed.

Microinjection

Fertilized and dechorionated eggs were injected with several solutions in order to label the egg cytoplasm, germplasm or PGCs. PGCs were visualized by injecting artificial mRNA for GFP and a 3′UTR region of *nos*3, a germplasm specific RNA of zebrafish (Saito *et al.,* 2006). In order to label donor cells for the blastoderm and blastomere transplantations, 5% FITC (Sigma) and 5% biotin-dextran-lysine (Sigma) in 0.2M KCl solution were injected for fluorescent and histological detection, respectively. To detect germline cells before or after PGC differentiation, a mixture of GFP-Bucky ball mRNA and GFP-*nos*3 3′UTR mRNA in 0.2M KCl solution was injected or co-injected into egg cytoplasm at the 1- to 2-cell stage (Saito *et al.,* 2006, 2014). The labeled

germplasm or cells were observed under a fluorescent microscope (Leica MZ16F) and photographed with an attached digital camera (Leica DFC7000T). For histological detection of labeled cells, serial paraffin sections were made from embryos in which labeled cells were transplanted, as described below.

Cell transplantation

In this study, we performed two types of transplantation. In the first type, various grafts of hybrid blastoderm labeled with FITC and biotin-dextran were transplanted to goldfish blastulae according to Yamaha *et al.* (1997) and Kazama-Wakabayashi *et al*. (1999). Specifically: (i) the entire blastoderm was dissected away from the yolk cells of a hybrid blastula and transplanted onto the animal part of a goldfish blastula that had had the upper part of its blastoderm removed—resultant embryos are referred to as goldfish-hybrid chimeras (Fig. SD1-A); (ii) the lower part of the blastoderm was dissected from a hybrid blastula and transplanted into the middle of a goldfish blastula in which the blastoderm had been horizontally bisected—resultant embryos are referred to as sandwich chimeras (Fig. SD1-B); and (iii) the upper part of the blastoderm from hybrid and goldfish blastulae was removed by glass needle and exchanged—resultant embryos are referred to as goldfish-base chimeras and hybrid-base chimeras, respectively (Fig. SD1-C1, C2). Controls for these micro-surgeries were intact embryos with chorion, and intact dechorionated embryos. After transplantation, controls and chimeric embryos were cultured separately for 1 day in 96-well culture plates filled with Ringer's culture solution. They were then individually moved to a 96-well culture plate filled with 1.8 mM $CaCl_2$ and 1.8 mM $MgCl_2$ solution and cultured until the intact controls hatched.

In the second type of transplantation, small numbers of blastomeres were transplanted into goldfish blastulae according to Saito *et al.* (2010). At the blastula stage, 20 to 30 marginal blastomeres were extracted with a glass needle from hybrid embryos co-injected with GFP-*nos*3 3′UTR mRNA and GFP-Bucky ball mRNA, and transplanted into the marginal part of a goldfish blastula. At the time of operation, donor cells were checked as to whether or not they included a blastomere expressing GFP-Bucky ball using a fluorescent microscope. Transplanted embryos with a GFP-Bucky ball signal are referred to as blastomere transplanted chimeras (BT chimeras). Post-transplantation, embryos were cultured as described above. Chimeric embryos were observed and photographed using a Leica MZ16F fluorescence stereomicroscope equipped with a digital camera (Leica DFC300FX). Images of the embryos were obtained using filters appropriate for GFP.

Ploidy analysis

The relative DNA content of control goldfish, hybrids, several types of chimeric embryos and larvae, and cells from several organs was measured by FCM. According to the animal genome size

database, goldfish have 3.58 pg of DNA per cell, while HR cells have 2.73 pg (http://www.genomesize.com). This analysis was performed according to Tanaka *et al*. (2004).

Genetic analysis

To detect hybrid cells, recombination activating gene 1 (*rag*1) was used as a genetic marker. Species-specific primers were designed for the *rag*1 genes of goldfish (DQ196520) and golden venus chub (HM224045) (Table SD1).

DNA samples were extracted from cell suspensions after FCM analysis, embryos, and tissue samples, using the alkaline lysis method. Briefly, embryos or small pieces of tissue were immersed in 180 µl of 0.1N NaOH solution at 95 °C for 10 min and then mixed with 20 µl of Tris-HCl solution (pH 8.0). After gentle mixing and centrifugation at 12,000 x g for 10 min, supernatants were used as template DNA for PCR analysis.

PCR reactions contained 5 µl of Quick Taq HS DyeMix (TOYOBO), 0.2 µl of each primer, 1 µl of template DNA (50–100 ng/µl) and 3.2 µl of water, for a total volume of 10 µl. PCR was conducted in a Thermal Cycler (Takara Bio Inc.) and consisted of an initial denaturation step of 120 s at 94 °C, followed by 40 cycles of 30 s at 94 °C, 30 s at the primer-specific annealing temperature, and 60 s at 68 °C. Amplified products were separated by 1.5% agarose gel electrophoresis and stained with ethidium bromide.

Histology

Chimeric fish induced by transplantation of biotin-labeled hybrid blastoderm to goldfish were fixed at hatching stage with Bouin's fixative for over three hours. Fixed embryos were embedded in paraffin and 8 µm sections were taken. Deparaffinized sections were treated with peroxidase-conjugated streptavidin and colored with DAB, according to the manufacturer's protocol (Histo-fine, SAB-PO(M), Nichirei).

Allo-triploid individuals and control goldfish were fixed at 1, 3, 7 and 10 weeks after fertilization to analyze gonadal differentiation. Ploidy was analyzed by sampling small fin pieces before fixation. After fixation, gonadal regions were trimmed from the whole body, embedded in paraffin, and then sectioned at 8 µm thickness. Deparaffinized sections were stained with hematoxylin and eosin.

Statistics

The data for developmental potential of the control goldfish and hybrid between female *C. auratus* and male *H. rasborella* were shown as mean ± standard deviation, and were analyzed by Student's t test or Welch's t test ($p < 0.05$).

Results

Developmental potential and genomic constitution of hybrids

Hybrid eggs cleaved normally into blastulae and started epiboly. The speed of epiboly in hybrid embryos was slightly slower than in goldfish (Fig. 1). Hybrid embryos began to die during epiboly, because of breakage of the enveloping layer (Table 1). Surviving embryos developed until around hatching stage (day 4), then most died because of malformation of the body, including edema (Table 1, Fig. 2). A few fish developed normally and survived until after yolk sac absorption (Fig. 2D). Survival rate of hybrid was significantly lower than that of control. At the hatching stage, the ploidy status of surviving embryos was analyzed by FCM (Table 1, Fig. SD2). The DNA content of abnormal progeny was intermediate between that of goldfish and HR, indicating allo-diploidy of the hybrid—i.e., one haploid set of goldfish chromosomes and one haploid set of HR chromosomes. In contrast, the DNA content of normally developing progeny was the sum of the diploid goldfish and haploid HR genomes, indicating allo-triploidy of this type of hybrid. Normal progeny with a DNA content equivalent to the diploid goldfish genome were presumed to be gynogenetically-developing auto-diploid goldfish.

When genetic analysis was performed using species-specific PCR primers for the *rag*1 gene, abnormal progeny from the cross-fertilization between goldfish and HR were found to have both the 742 bp HR fragment and the 332 bp goldfish fragment (Fig. 3). These results genetically confirm that the abnormal progeny were hybrid, as they had both the goldfish and HR genome.

To induce allo-triploids, heat shock was performed 5 min after fertilization (Tables 2 and SD2). These experiments produced externally normal progeny (data not shown). Allo-diploidy and -triploidy were identified in individuals sampled at 7 dpf by FCM (Tables 2 and SD2). After ploidy determination of sampled individuals, remaining progeny were fed. They survived for over 4 months after hatching (Fig. 2D). In surviving progeny that appeared normal, ploidy status was determined by FCM at 1, 3, 7, and 10 weeks after hatching, at the same time as samples were taken for histological observations of the gonad. Almost all progeny were found to be allo-triploid hybrids, except for one auto-triploid (i.e., triploid goldfish), and no allo-diploid hybrids occurred.

Survival of allo-diploid cells under chimeric conditions

To confirm the viability of hybrid cells, blastomere transplantations were performed into goldfish (Fig. SD1). These transplantations occurred at blastula stage and generated: a sandwich chimera, in which the lower part of the hybrid blastoderm was transplanted into the middle of a goldfish blastula; a hybrid-base chimera, in which goldfish blastoderm was transplanted onto the animal part of a hybrid blastula; and a goldfish-base chimera (Fig. 4A), in which hybrid blastoderm was transplanted onto the animal part of a goldfish blastula. The survival rates of these chimeras were

drastically increased when compared to allo-diploid hybrids between goldfish and HR, and to both intact and dechorionated controls (Table 3). Moreover, at hatching stage, some normal progeny appeared within these chimeric experiments. When observed under a fluorescent microscope, hybrid cells were mainly distributed around the anterior part of goldfish-base chimera (Fig. 4C1), but were found in all parts of the hybrid-base (Fig. 4D1) and sandwich chimeras (Fig. 4E1). Histological analysis revealed that hybrid cells were distributed through all three germ layers and the organs containing more hybrid cells than host goldfish cells showed a tendency to be malformed (Fig. 4F, G). In particular, muscle fibers were less differentiated in myomeres that contained many hybrid cells (Fig. 4G2).

When the entire blastoderm of a hybrid was transplanted onto a goldfish host blastula, five of these goldfish-base chimeric fish survived for over 6 months and one such individual had a tumor on its head (Fig. 5). In these chimeras, the persistence of hybrid cells was confirmed by FCM and by genetic analysis, as described above. When we analyzed the tissue samples from fin, muscle, skin, brain, eye and tumor, hybrid peak was not detected. When PCR analysis was conducted in the tissue samples used for FCM, HR-specific fragment was detected exclusively in the brain and both eyes of the single chimera possessing a tumor (Fig. 6).

Germ cell differentiation

When an artificial mRNA comprising GFP and the 3′UTR region of zebrafish *nos*3 (a germplasm specific RNA) was injected into hybrid zygotes immediately post-fertilization, cells with bright GFP fluorescence were observed after epiboly (Fig. 7). These bright GFP cells were detected even in embryos that were seemingly dead during epiboly (Fig. SD3). Histologically, PGCs were detected in the genital anlage of newly hatched hybrids.

As viable allo-triploid progeny were obtained by heat-shock treatment, germ cell differentiation could be histologically analyzed (Fig. 8). Similar to control goldfish (Fig. 8A), single PGCs were detected in the genital anlage of allo-triploid hybrids at 1 week post-fertilization (wpf) (Fig. 8B). Allo-triploid PGCs were encased in somatic cells and proliferated in the gonad at 7 wpf (Fig. 8D–E)—as determined by the similar histological appearance of controls (Fig. 8C). At 10 wpf, a small allo-triploid fish (12 mm in body length) had a similar appearance to that of control fish at 7 wpf (Fig. 8F–G), while allo-triploid progeny over 24 mm long had abnormal gonads with large vacuolar spaces (Fig. 8H).These spaces were similar in size to germ cells in control gonads.

When blastomeres including PGCs were transplanted from allo-diploid hybrids into goldfish blastulae (n=53), about half of these chimeric embryos survived until 10 dpf (Table SD3). GFP-positive cells were detected on the usual migration route of PGCs and in ectopic regions in the chimeric embryos (11.3%) at 1 dpf (Fig. SD4, Table 4). The frequencies of embryos with GFP-positive cells decreased over time, and all GFP-positive cells had disappeared by 10 dpf (Table

4). This indicates that PGCs from allo-diploid hybrids were not able to persist, even in chimeric conditions.

Discussion

Inviable allo-diploid and viable allo-triploid hybrids

In the present study, allo-diploid hybrid between goldfish eggs fertilized with HR sperm died during embryonic and pre-larva. Survivors were also verified to be either allo-triploid hybrids with two sets of goldfish chromosomes and one set of HR chromosomes, or gynogenetically-developed diploid goldfish. Thus, allo-diploid hybrids with a maternally derived haploid set of goldfish chromosomes and a paternally derived haploid set of HR chromosomes were inviable and died soon after hatching, while allo-triploid hybrids (which sporadically occurred) survived. The occurrence of spontaneous viable allo-triploid hybrids has often been reported in crosses of *Ctenopharyngodon idella* × *Hypophthalmichthys molitrix* (Marian and Krasznai, 1978), and *Oncorynchus mykiss* × *Salvelinus fontinalis* (Capanna *et al*., 1974; Ueda *et al*., 1984). It is rare that hetero-specific fertilization gives rise to the occurrence of spontaneous gynogenesis; however, the occurrence of goldfish after hybridization indicates presumable ploidy elevation. This may be occurring by inhibition of the second polar body release or endomitosis in cleavage, followed by the initiation of spontaneous gynogenesis triggered by hetero-specific sperm. Mechanisms underlying such events are presently unknown, but similar cases of spontaneous gynogenesis have been reported in hybrid *Oncorhynchus kisutch* × *S. fontinalis* (Uyeno, 1972), *Pleuronectes platessa* × *Platichtys flesus* (Purdom and Lincoln, 1974) and *Ctenopharyngodon idella* × *Cyprinus carpio* (Stanley, 1976).

In certain salmonid hybrids, inviable development has been explained by the occurrence of aneuploidies due to the elimination of chromosomes (Arai, 1984; Fujiwara *et al*., 1997) or by no, weak or delayed expression of paternal genes in inviable hybrid between *Oncorhynchus keta* female and *O. masou* male (Arai, 1984). In the present study, goldfish × HR hybrids were allo-diploid, and comprised a haploid chromosome set from each parental species and thus inviability of this hybrid cannot be explained by aneuploidy. This result indicates there were no harmful cytological effects, like chromosome elimination, on the HR genome in the development of resultant hybrids; however, maternal cytoplasm and/or genome may suppress the expression of certain paternal HR genes during early development. As most allo-diploid hybrid dies in burst during epiboly, hybrid condition may affect cytoskeleton of enveloping layer cells in blastoderm. In order to elucidate probable nucleo-cytoplasmic effects, the analysis of an inter-specific androgenetic doubled haploid is required.

In our cross-fertilization experiment, allo-diploid hybrids died in the early stages of embryogenesis, while sporadically occurring allo-triploid hybrids showed better survival and were viable. We were also able to easily induce allo-triploidy by heat shock—which inhibits the release

of the second polar body—soon after the fertilization of eggs with hetero-specific sperm. Increased survival of allo-triploids has been well-studied in salmonid hybrids, including *O. mykiss* (female) × *Salmo trutta* (male), *O. mykiss* × *S. fontinalis* (Chevassus *et al*., 1983; Scheerer and Thorgaard, 1983), *Oncorhynchus keta* × *S. fontinalis*, *O. keta* × *S. leucomaenis* (Arai, 1984, 1986) and *Oncorhynchus gorbuscha* × *S. leucomaenis* (Yamano *et al*. 1988). This increased viability is presumably caused by the doubling of the female genome. Thorgaard (1983) suggested that a decrease in the ratio of paternally derived genes might relate to the increased viability of allo-triploids. However, if the paternal genome positively inhibit the normal development under allo-diploid genomic condition, female genome doubling is not sufficient to recover the developmental potential of hybrids (Yamano *et al*., 1988). Here, we conclude that the HR genome is not harmful to development under allo-diploid genomic condition, but partially suppressed in their expression, because allo-triploid hybrids developed beyond hatching and feeding stages.

Survival of goldfish-hybrid chimera

Doubling of the maternally derived genome gives rise to increased survival in certain combinations of hybrids (Chevassus *et al*., 1983; Scheerer and Thorgaard, 1983; Arai, 1984, 1986; Yamano *et al*., 1988). In addition to analyzing the effect of allo-triploidization on the survival of goldfish × HR hybrids, we examined the survival potential of somatic cells from inviable allo-diploid hybrids by experimental induction of interspecific chimera. When allo-diploid hybrid cells were transplanted into host goldfish embryos, hybrid cells could be tracked in 6-month-old individuals under chimeric conditions. Some of the chimeras that included hybrid cells had better survival than intact allo-diploid hybrids. Therefore, we propose that insufficiencies in developmental potential of the hybrid cells are mitigated under the chimeric condition. To our knowledge, this is the first report to show survival recovery of inviable allo-diploid hybrid cells in the chimeric condition.

A similar phenomenon has been reported in haploid-diploid goldfish chimera (Tanaka *et al*., 2004), in which haploid cells were able to survive under chimeric conditions. When haploid blastoderm was transplanted to the animal region of diploid goldfish, the resultant haploid-diploid chimeric goldfish survived to adult size (Tanaka *et al*., 2004). Thus, the poor viability of haploid blastoderm was recovered by transplantation onto diploid blastoderm and yolk cells. In our study, although a large number of chimeras in which hybrid blastomeres were contained in the lower, middle or upper part of host blastulae had an abnormal appearance, a few developed normally. This suggests that the poor viability of hybrid was recovered under mixed condition with diploid cells. However, histological observation revealed that an increase of hybrid cells often induced higher abnormality in tissue structures. Furthermore, in allo-diploid hybrid-goldfish chimeras, hybrid cell-rich myomeres were generally undifferentiated, compared to those with normal diploid goldfish

cells. These results suggest the presence of a community effect, because cell populations comprising more hybrid cells were more abnormal.

Here, in PCR analysis, hybrid cells were not detected in any tissues of 6-month-old chimeric individuals, except for brain and both eyes of a single chimeric fish. Cell lineage analysis by fluorescent label tracking showed that donor hybrid cells were mainly distributed around the head region of hatched larvae. Somatic cells are constantly replaced by new cells over time. If hybrid cells have weaker and/or lower proliferative potential than host cells, then they are destined to be lost during development. Alternatively, as the nerve cells of the central nervous system terminally differentiate at an early stage of embryonic development, hybrid cells may persist in such tissues.

PGC differentiation and the reproductive potential of allo-triploid hybrids

PGCs were identified histologically in the gonadal anlage of surviving hybrid larvae at around hatching, and visualized by GFP-*nos*3 mRNA in hybrids that died at epiboly. In many teleosts, PGCs are destined by maternally supplied cytoplasm that clustered at the ends of early cleavage furrows (zebrafish, Yoon *et al*., 1997; goldfish, Otani *et al*., 2002; dojo loach, Fujimoto *et al*., 2006; Atlantic salmon Nagasawa *et al*., 2013; sturgeon, Saito *et al*., 2014; turbot, Lin *et al.,* 2012; barfin flounder, Goto *et al*., 2015). It is thus reasonable to expect that blastomeres with maternal germplasm would be able to differentiate into PGCs, even in inviable hybrids. However, we observed that hybrid PGCs transplanted into goldfish blastulae disappeared in host embryos by 10 dpf, while somatic cells survived. Usually visualized PGCs with GFP-nos3 mRNA are able to trace at least one month in goldfish. Notably, even in xeno-transplantation, donor PGCs from different species do not always survive and differentiate into gametes in the host species (Saito *et al*., 2008, 2014). Therefore, whether hybrid PGCs are autonomously inviable remains unclear.

We were able to observe germ cells of allo-triploid hybrids until 7 wpf, after which they disappeared from the gonads. As gonads were abundant with vacuoles at 10 wpf, it is likely that the PGCs had undergone apoptosis. This result suggests that allo-triploid PGCs, with two goldfish genomes and one HR genome, are as inviable as allo-diploid PGCs; however, allo-triploid PGCs survived longer than allo-diploid PGCs, and proliferated in the gonad. Therefore, an increase in maternal genome in hybrid PGCs improved their proliferative potential.

Conclusion

In the present study, allo-diploid hybrids between goldfish females and HR males were inviable; however, the cells from these inviable hybrid embryos could survive in chimeric environment together with auto-diploid cells of maternal species. The elevation of the maternal genome by allo-triploidization could also recover subsequent differentiation. Many factors and their interaction, such as that between two non-homologous genomes and that between maternal

cytoplasm and paternal genome, should play roles in embryonic development of allo-diploid, allo-triploid and interspecific chimeras. Even in the case of auto-diploid development, it is obscure whether maternal and paternal genome have different epigenetic program (Labbe et al., 2017). It is very difficult to analyze precisely these interactions. To analyze possible incompatibility between maternal and paternal genomes, homogeneous gametes should be required in both species to produce isogenic hybrids. While to analyze interactions between maternal cytoplasm and paternal genetic materials, interspecific androgenetic diploid (cybrids) should be analyzed. Neither allo-diploid nor allo-triploid PGCs could differentiate into functional gametes, suggesting that additional HR genomes did not bring any harmonious interaction to maternal genome for further differentiation. To reveal this point, the allo-tetraploid (amphidiploid) condition should be analyzed in near future. Chromosome set manipulation and chimeric induction are powerful techniques to answer such questions. Genomic combination involved in gamete differentiation of hybrid should give insights into genetic breeding and biological evolution.

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Figure Legends

Figure SD1. Schematic illustration of blastoderm transplantation. Transplantation was performed between goldfish *C. auratus* and hybrid female goldfish × male golden venus chub *H. rasborella* (HR). (A) The entire blastoderm was cut off from yolk cells of a hybrid blastula and transplanted onto the animal part of a goldfish blastula. (B) The lower part of the blastoderm was cut off a hybrid blastula and transplanted into the middle of a goldfish blastula. (C) The upper blastoderm from both hybrid and goldfish blastulae was cut off by glass needle and reciprocally exchanged.

Figure 1. Development during epiboly of hybrid and goldfish control. (A) Hybrid between female goldfish *C. auratus* × male golden venus chub *H. rasborella*. (B) Goldfish control. Scale bars show 1 mm.

Figure 2. External appearance of goldfish control and hybrid. (A, B) Goldfish control. (C, D) Hybrid between female goldfish *C. auratus* and male golden venus chub *H. rasborella* at 4 days and 4 months post-fertilization, respectively. Hybrid fish (D) were induced by heat-shock treatment at 5 min after fertilization and allo-triploidy was confirmed by flow cytometry.

Figure SD2. Relative DNA contents of hybrid between female goldfish *C. auratus* and male golden venus chub *H. rasborella* and parent species. (A) *H. rasborella*. (B) *C. auratus*. (C, D) Hybrids.

Figure 3. Genetic analysis of *rag*1 gene in hybrid progeny of female goldfish *C. auratus* and male golden venus chub *H. rasborella*. Lanes: 1) goldfish female, 2-3) control goldfish progeny, 4-6) allo-diploid hybrids, 7-9) allo-triploid hybrids, 10) golden venus chub male, 11) negative control. MM indicates molecular size marker.

Figure 4. Development of chimeric fish. Chimeric fish in which blastoderm grafts from hybrid female goldfish *C. auratus* × male golden venus chub *H. rasborella* were transplanted to goldfish blastula. (A) Immediately after transplantation of the entire hybrid blastoderm onto the upper part of a host goldfish blastula. (B) Control goldfish progeny at 3 dpf. (C) Goldfish-base chimera at 3 dpf. (D) Hybrid-base chimera at 3 dpf. (E) 3-day-old sandwich chimera. (C1, D1, E1) Fluorescent images. (B, C2, D2, E2) Brightfield images. (F, G) Histo-chemical analysis of 3-day-old goldfish-base chimeric embryos; head and trunk region, respectively. Brown cells originated from hybrid blastomeres.

Figure 5. External appearance of control goldfish and progeny from chimeric embryos 3 months after operations. (A) Control goldfish. (B) Experimental fish that developed from a chimeric embryo in which hybrid blastoderm was transplanted to a goldfish blastula. (B2) Higher magnification of white rectangles in B1).

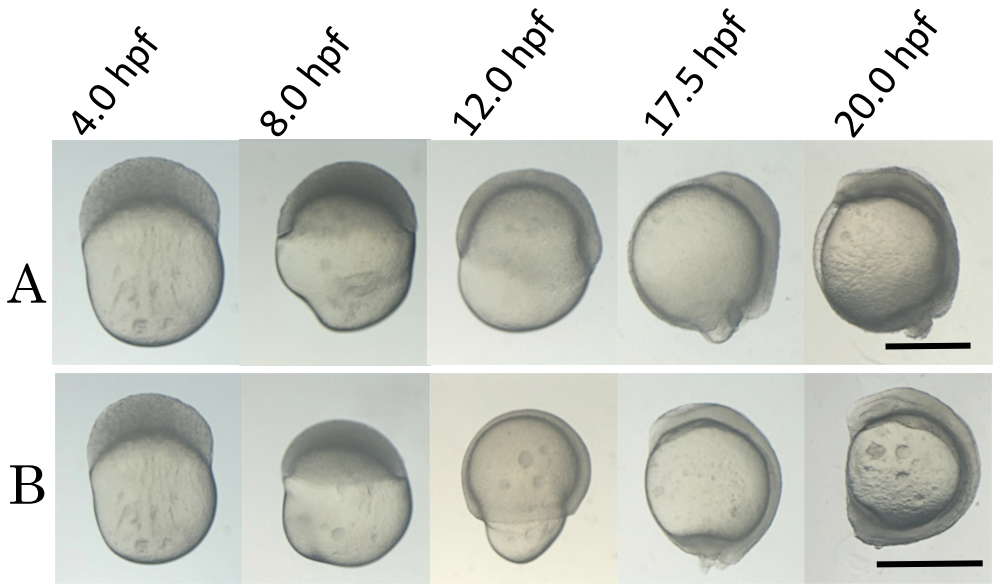
Figure 6. Genetic analysis of *rag*1 gene in fish B2 (Fig. 5). 1) Fin of goldfish control. 2) Fin, 3) left eye, 4) right eye, and 5) brain of chimera. 6) Fin of golden venus chub *H. rasborella.* Both goldfish and *H. rasborella* specific bands are detected in the samples from the chimeric progeny.

Figure 7. GFP fluorescence of PGCs in hybrid between female goldfish *C. auratus* × male golden venus chub *H. rasborella.* (A) 24h, (B) 48 h and (C) 72 h after fertilization. GFP-positive cells are located around the presumptive gonadal region, suggesting that hybrid PGCs have the ability to migrate to the genital ridge. Scale bars indicate 1 mm. Yellow squares indicate lower columns of each picture.

Figure 8. Histological sections of gonads from goldfish and hybrids. (A, C, F) Control auto-diploid goldfish. (B, D, E, G, H) Allo-triploid hybrid between *C. auratus* and *H. rasborella*. (A–B) 1 week post-fertilization. (C–E) 7 weeks post-fertilization. (F–H) 10 weeks post-fertilization. Yellow arrowheads indicate germ cells; black arrowheads indicate intercellular spaces of a similar size to germ cells. A black arrow indicates the ovarian cavity.

Figure SD3. Backlit (A) and GFP fluorescent (B) view of hybrid between female goldfish *C. auratus* × male golden venus chub *H. rasborella* at 1 day after fertilization. The embryo is seemingly dead, but the GFP-positive cells are alive, suggesting the PGC differentiation occurs in seemingly dead embryos. Yellow arrows indicate PGCs.

Figure SD4. GFP fluorescence of a goldfish embryo transplanted with hybrid PGCs. (A) 1 dpf, (B) 2 dpf, and (C) 5 dpf post-transplantation with blastomeres from hybrid female goldfish *C. auratus* × male golden venus chub *H. rasborella*. GFP-positive cells from the hybrid are located around the host genital ridge, suggesting that hybrid PGCs have migratory ability. Scale bars indicate 1 mm. A yellow arrow indicates a PGC from the hybrid near the host genital ridge.



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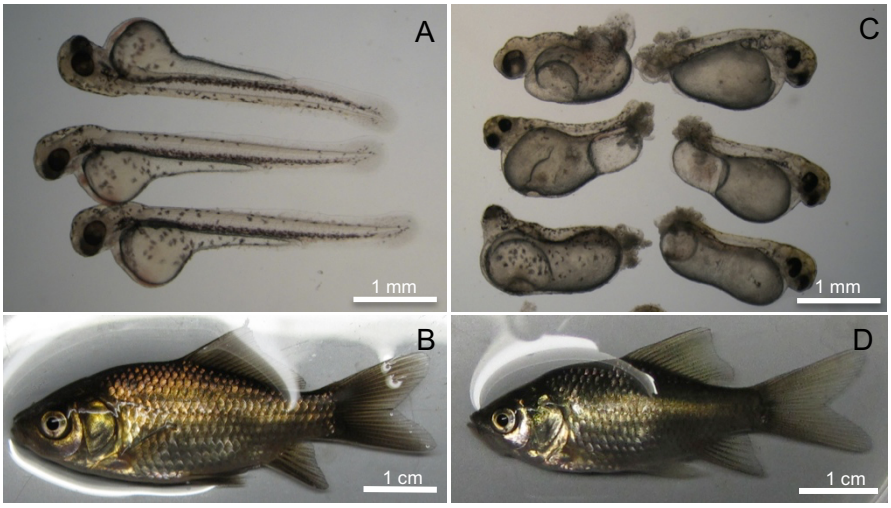

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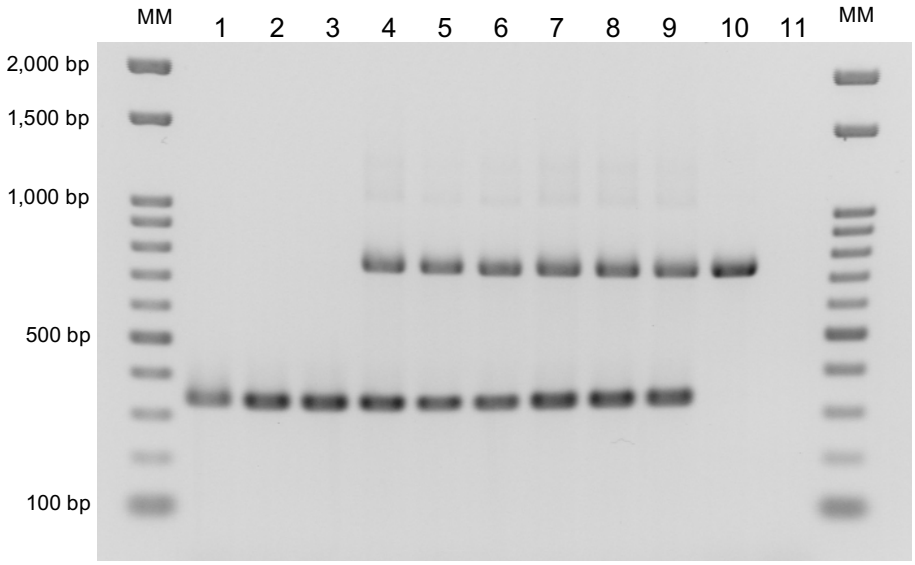

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673

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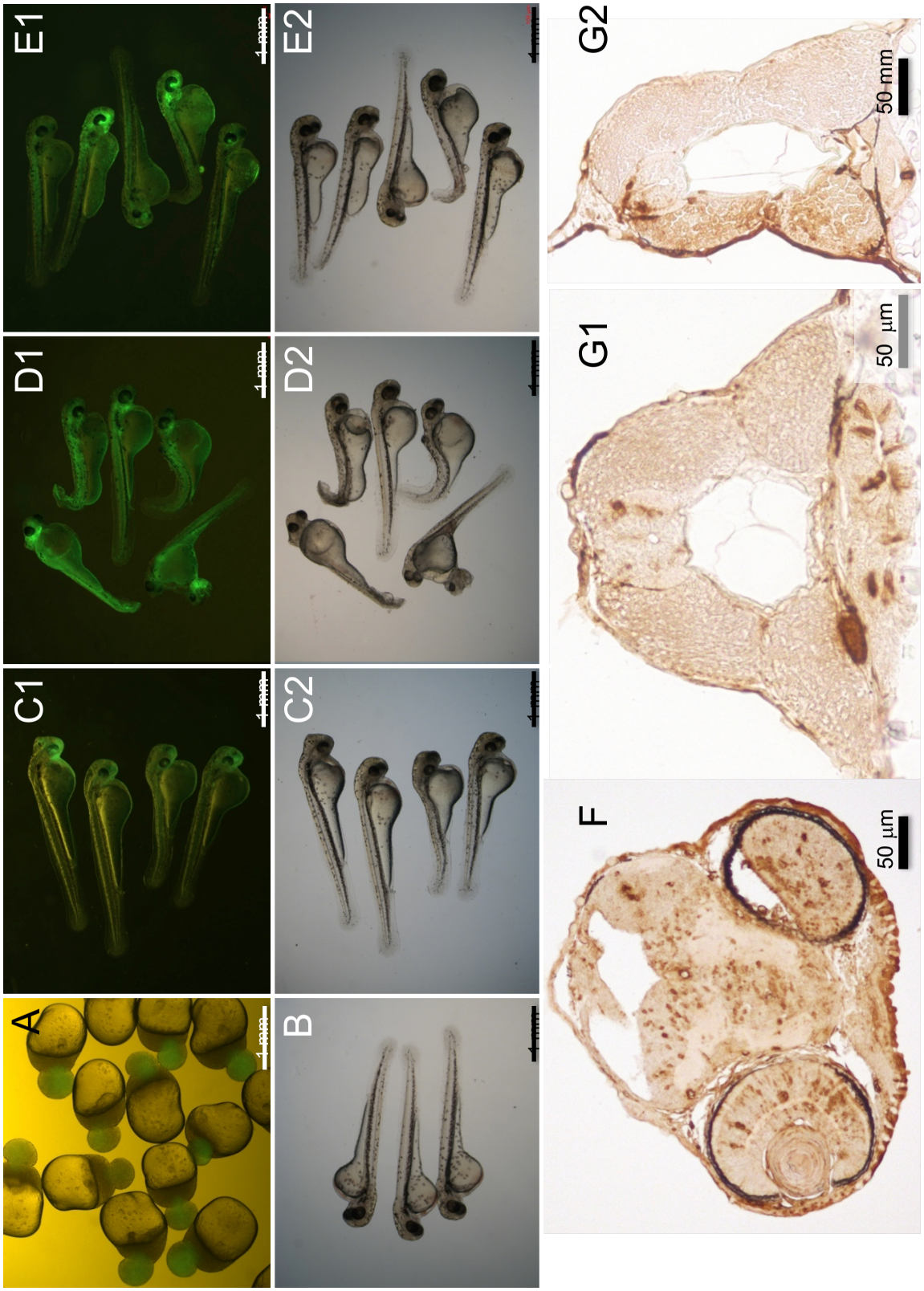

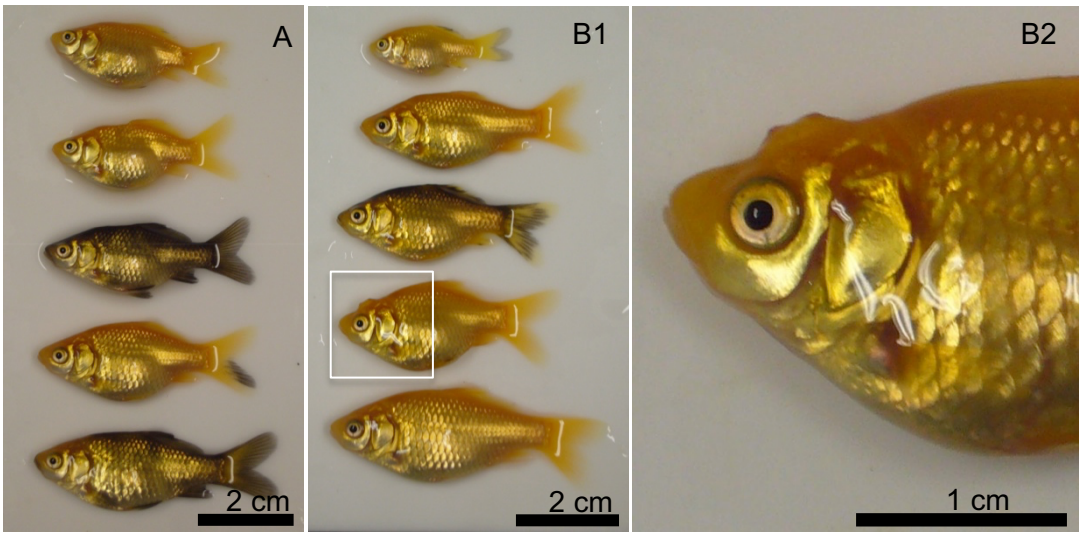

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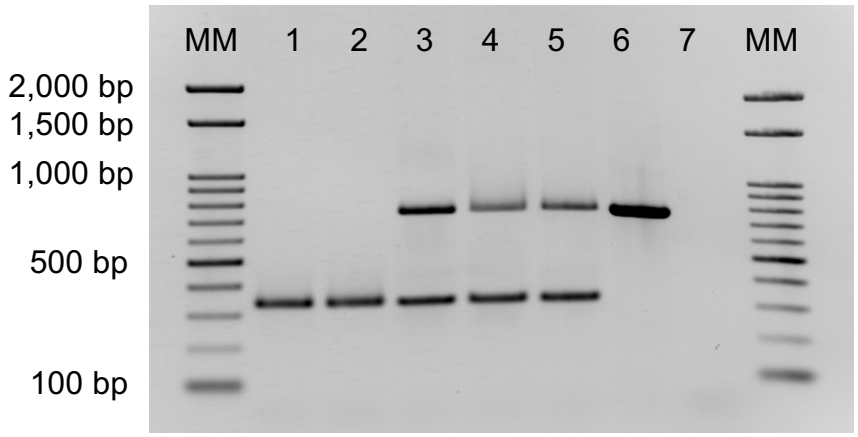

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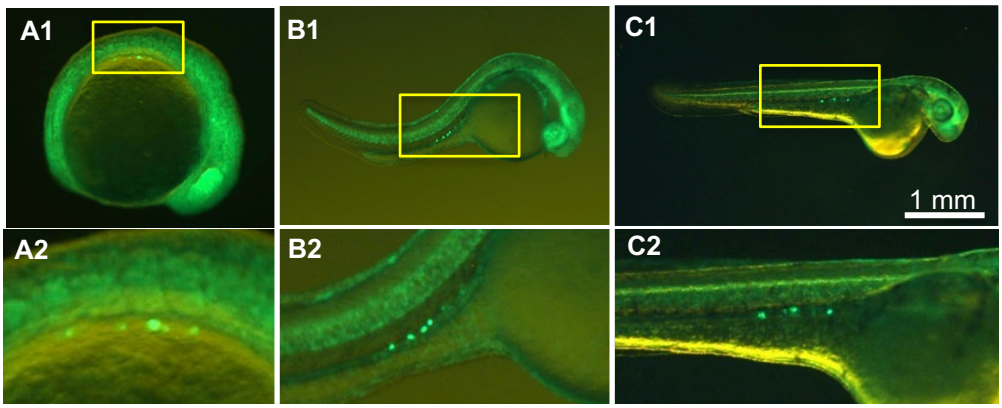

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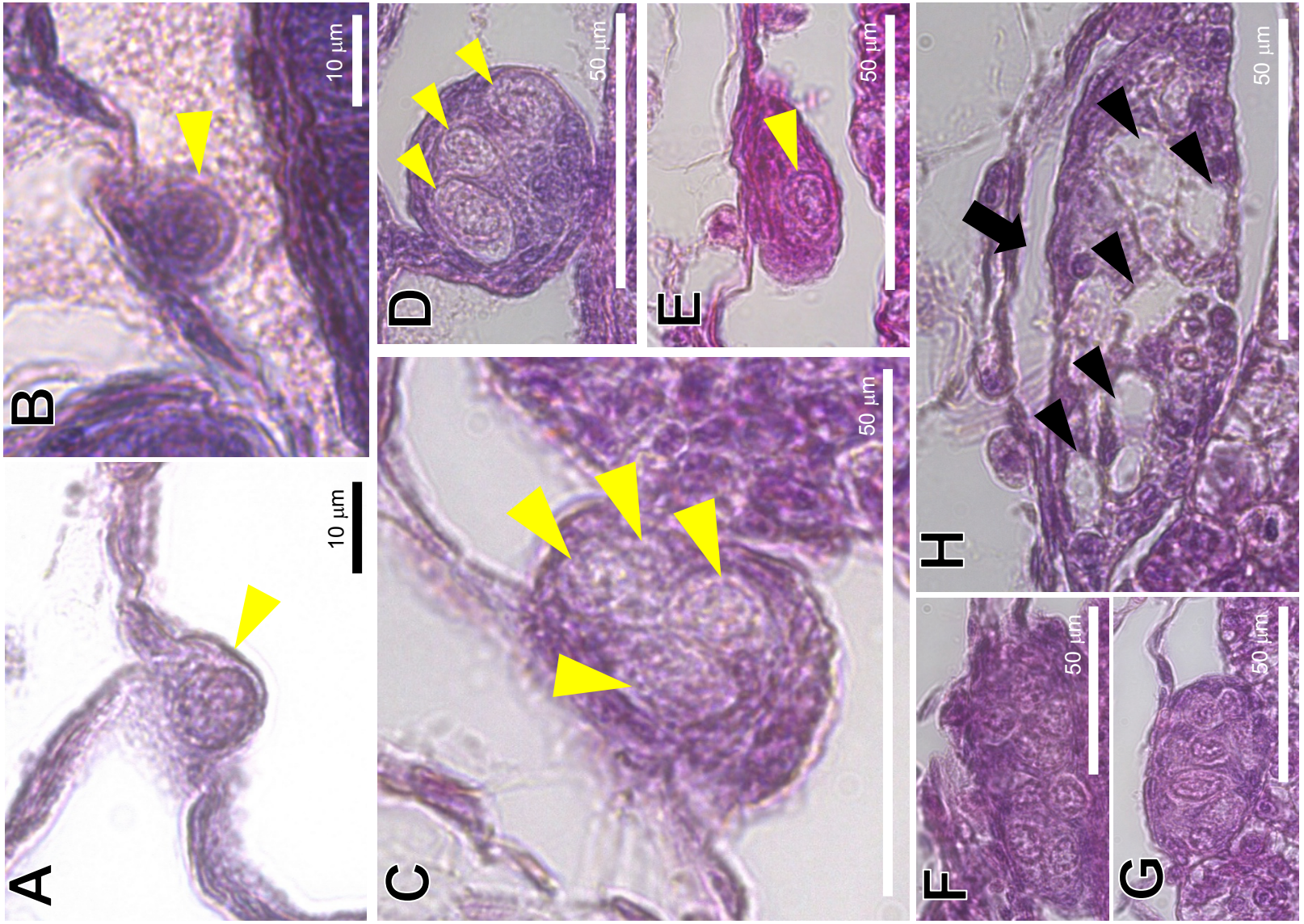

Table 1. Fertilization, survival and ploidy status of the control goldfish and hybrid between female *C. auratus* and male *H. rasborella*.

No. of experiment	Experiment	Total egg No.	Fertilized egg		No. of survival eggs（%）								Ploidy status *2			
			no.	(%)	1 dpf *1		3 dpf		5 dpf		7 dpf		*n*	gyno-1n	auto 2n	allo 2n
1	Control	63	46	(73.0)	37	(80.4)	32	(69.6)	32	(69.6)	32	(69.6)	10	-	10	-
	Hybrid	67	47	(70.1)	0	0.0	-		-		-		17	4	-	13
2	Control	143	124	(83.1)	103	(83.1)	102	(82.3)	97	(78.2)	88	(71.0)	17	-	17	-
	Hybrid	160	155	(74.2)	115	(74.2)	113	(72.9)	110	(71.1)	88	(56.8)	85	-	1	79
3	Control	206	200	(94.0)	188	(94.0)	187	(93.5)	187	(93.5)	114	(57.0)	-	-	-	-
	Hybrid	326	305	(51.5)	157	(51.5)	155	(50.8)	128	(42.0)	128	(42.0)	-	-	-	-
4	Control	104	84	(92.9)	78	(92.9)	72	(85.7)	72	(85.7)	72	(85.7)	-	-	-	-
	Hybrid	214	123	(86.2)	106	(86.2)	101	(82.1)	101	(82.1)	28	(22.8)	4	-	1	-
Mean±SD	Control	129±61	(84.4±10.2^{a})		(87.6±6.8^{a})		(82.8±10.0^{a})		(81.8±10.2^{a})		(70.8±11.8^{a})					
	Hybrid	192±108	(78.3±10.6^{a})		(53.0±38.1^{a})		(68.6±16.1^{a})		(65.1±20.7^{a})		(40.5±17.0^{b})					

*1 dpf: days post-fertilization.

*2 gyno-1n: gynogenetic haploid goldfish, auto-2n: diploid goldfish, allo-2n: allo-diploid hybrid, allo-3n: allo-triploid, ND: Not detected.

Different superscript letters in each column except for the survival rate at 1 dpf indicate significant differences as determined by Student's t test ($P < 0.05$). There was no significant dif

survival rate at 1 dpf as determined by Welch's t test ($P < 0.05$).

Table 2. Fertilization, survival, and ploidy status of control goldfish *C. auratus* and hybrid between female goldfish and male golden venus chub *H. rasborella* after heat shoc
post-fertilization.

Exp No.	Experiment	Duration of HS (s)	Total egg no	Fertilized egg		No. of survival eggs (%)									
				no.	(%)	1 dpf *1		3 dpf		5 dpf		7 dpf		*n*	auto 2n
1	*Control*	-	171	152	(88.9)	142	(93.4)	141	(92.8)	139	(91.4)	137	(90.1)	10	10
	Control	50	190	129	(67.9)	105	(81.4)	72	(55,8)	44	(34.1)	32	(24.8)	10	3
	Control	60	121	65	(53.7)	43	(66.2)	28	(43.1)	24	(36.9)	23	(35.4)	10	3
	Control	75	125	69	(55.2)	42	(60.9)	24	(34.8)	12	(17.4)	9	(13.0)	10	1
	Hybrid	-	155	70	(45.2)	36	(51.4)	20	(28.6)	13	(18.6)	12	(17.1)	10	0
	Hybrid	50	142	75	(52.8)	49	(65.3)	37	(49.3)	22	(29.3)	22	(29.3)	10	0
	Hybrid	60	174	45	(25.9)	27	(60.0)	24	(53.3)	14	(31.1)	13	(28.9)	10	0
	Hybrid	75	163	34	(20.9)	22	(64.7)	13	(38.2)	11	(32.4)	6	(17.6)	10	0
2	*Control*	-	191	189	(99.0)	189	(100)	166	(87.8)	132	(69.8)	108	(57.1)	_	_
	Control	50	133	129	(97.0)	124	(96.1)	16	(12.4)	12	(9.3)	9	(7.0)	_	_
	Control	60	145	144	(99.3)	132	(91.7)	14	(9.7)	11	(7.6)	3	(2.1)	_	_
	Control	75	82	81	(98.8)	75	(92.6)	4	(4.9)	4	(4.9)	4	(4.9)	_	_
	Hybrid	-	125	117	(93.6)	75	(64.1)	42	(35.9)	33	(28.2)	26	(22.2)	_	_
	Hybrid	50	219	211	(96.3)	191	(90.5)	41	(19.4)	29	(13.7)	19	(9.0)	_	_
	Hybrid	60	219	211	(96.3)	177	(83.9)	39	(18.5)	25	(11.8)	16	(7.6)	_	_
	Hybrid	75	228	220	(96.5)	182	(82.7)	19	(8.6)	10	(4.5)	6	(2.7)	_	_

*1 dpf: days post-fertilization.

*2 gyno-1n: gynogenetic hploid goldfish, auto-2n: diploid goldfish, allo-2n: allo-diploid hybrid, allo-3n: allo-triploid, ND: Not detected.

Table 3. Incresed survival and occurrence of normal progeny under chimeric conditions in hybrid between female goldfish *C. auratus* and male golden venus chub *H. rasborella.*

Experiments	Total No.	Survivied progeny		Normal progeny	
		no.	(%)	no.	(%)
Sandwich chimera	16	14	(87.5)	5	(35.7)
Hybrid-base chimera	26	22	(84.6)	1	(4.5)
Goldfish-base chimera	48	40	(83.3)	6	(15.0)
Hybrid dechorionated-control	60	31	(51.7)	0	(0)
Hybrid intact control	671	211	(31.4)	-	-
Goldfish dechorionated-control	36	35	(97.2)	22	(62.9)
Goldfish intact control	561	453	(80.7)	-	-

Table 4. Number of PGCs from goldfish x HR embryo in host goldfish x goldfish during development.

Position of PGC at gonadal chimera	No. of chimera	The rate of chimera has PGC derived donor hybrid(%)			
		1 dpf	3 dpf	7 dpf	10 dpf
Gonadal ridge	53	6 (11.3)	3 (5.8)	2 (4.0)	0 (0.0)
Ectopic position		30 (54.7)	18 (34.6)	1 (2.0)	0 (0.0)

Table SD1. Species specific PCR primers for goldfish *C. auratus* and golden venus chub *rag1* gene.

Primer name	Primer sequence	Amplified length
KinRAG1_1024F	5′-CTG GAC AAA CAG CTG AGA AAG AAG-3′	332 bp
Cyp_RAG1_1355R	5′-ACA TGG GCC AGA GTC TTG TG-3′	
KawaRAG1_330F	5′-AGA TGT CAG TGA GAA GCA TGG AAC-3′	742 bp
KawaRAG1_1071R	5′-CCT CAT CAC AGG CTT GAG TTT CAT T-3′	

Table SD2. Fertilization, survivial rates of control goldfish C. auratus and hybrid between female goldfish and male golden venus chub *H. rasborella* after

Exp. No.	Experiment	Duration of HS (s)	Total eggs	Fertilized egg		No. of surviva					
				no.	(%)	1 dpf *1		3 dpf		5 dpf	
1	Control	-	191	189	99.0%	189	(100)	166	(87.8)	132	(69.8)
	Control	50	133	129	97.0%	124	(96.1)	16	(12.4)	12	(9.3)
	Control	60	145	144	99.3%	132	(91.7)	14	(9.7)	11	(7.6)
	Control	75	82	81	98.8%	75	(92.6)	4	(4.9)	4	(4.9)
	Hybrid	-	125	117	93.6%	75	(64.1)	42	(35.9)	33	(28.2)
	Hybrid	50	219	211	96.3%	191	(90.5)	41	(19.4)	29	(13.7)
	Hybrid	60	219	211	96.3%	177	(83.9)	39	(18.5)	25	(11.8)
	Hybrid	75	228	220	96.5%	182	(82.7)	19	(8.6)	10	(4.5)
2	Control	-	399	-	-	277	(69.4)	248	(62.2)	242	(60.7)
	Control	50	136	-	-	68	(50.0)	58	(42.6)	56	(41.2)
	Control	60	124	-	-	74	(59.7)	64	(51.6)	64	(51.6)
	Control	75	125	-	-	68	(54.4)	51	(40.8)	49	(39.2)
	Hybrid	-	259	-	-	134	(51.7)	114	(44.0)	106	(40.9)
	Hybrid	50	394	-	-	180	(45.7)	126	(32.0)	117	(29.7)
	Hybrid	60	359	-	-	157	(43.7)	107	(29.8)	96	(26.7)
	Hybrid	75	280	-	-	175	(62.5)	119	(42.5)	108	(38.6)
3	Control	-	84	-	-	79	(94.0)	79	(94.0)	79	(94.0)
	Control	50	96	-	-	88	(91.7)	84	(87.5)	83	(86.5)
	Hybrid	-	197	-	-	154	(78.2)	149	(75.6)	135	(68.5)
	Hybrid	50	185	-	-	139	(75.1)	118	(63.8)	108	(58.4)

*1 dpf: days post-fertilization.

Table SD3. Survival of chimeric embryos transplanted with goldfish x HR blastomeres (donor) into goldfish x goldfish (host) at blastula stage.

Eggs	Egg no.	Fertilized egg		Survival rate (%)					
		no.	(%)	1 dpf		3 dpf		7 dpf	
Host (goldfish x goldfish)	63	46	(73.0)	37	(80.4)	32	(69.6)	32	(69.6)
Donor (goldfish x HR)	67	47	(70.1)	0	(0)	0	(0)	0	(0)
Chimera	53	-		53	(100)	52	(98.1)	50	(94.3)

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