Title: Isogenic fish sperm produced by transplanting gynogenetic haploid-derived germ cells

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Running title: Isogenic sperm derive from haploid PGCs

Summary sentence: Artificially induced gynogenetic haploid primordial germ cells, when transplanted into sterile recipients, survived beyond embryogenesis in germline chimeras and differentiated into isogenic sperm via diploidization of their haploid genome in the germ cells.

Keywords: Haploid, Germline chimera, Primordial germ cells, Clonal gamete, Genome doubling, Diploidization

Abstract

Artificially induced haploidy is lethal in vertebrates, although it is useful for genetic screening and genome editing due to its single set of genomes. Haploid embryonic stem (ES) cell lines in mammals contribute to genetic studies and the production of gametes derived from haploid ES cells. In fish breeding, doubled haploids (DHs) induced by artificially induced gynogenesis are used to generate isogenic gametes for cloning purposes. However, gametes have not been directly differentiated from artificially induced haploid cells, even though haploid ES cell lines have been established in medaka. Here, we aimed to demonstrate that fertile haploid sperm with identical genotypes could be differentiated in germline chimeras in zebrafish, wherein primordial germ cells (PGCs) derived from an inviable haploid embryo were transplanted into sterilized recipient embryos. Haploid spermatogonia differentiated from haploid PGCs in germline chimeras, and genome doubling occurred in haploid spermatogonia with one set of chromosomes, which could not pair with counterpart homologs to form bivalents during meiosis. Subsequently, meiosis produced isogenic haploid gametes because identical elements were exchanged between chromosomes doubled from the haploid set during recombination. Consequently, haploid PGCs can survive beyond embryogenesis and potentially differentiate into fertile sperm.

Introduction

Haploid individuals in teleosts are easily induced by artificial gynogenesis and androgenesis using genetically inert sperm and eggs, respectively. However, the induced haploids die before or soon after hatching due to abnormalities known as haploid syndromes. Despite this, haploidy is useful for genetic screening owing to its single genome set [1]. Mutation screening has been performed using haploid zebrafish embryos [2]. Viability can be recovered by genome doubling at the second meiotic stage or the first mitotic division in artificially induced gynogenetic haploid embryos, resulting in heterozygous meiotic and homozygous mitotic gynogens, respectively. Mitotic gynogens, developed as doubled haploids (DHs), are applied to produce isogenic gametes for clonal individuals [3] and are used for whole-genome sequencing due to their complete homozygosity [4, 5]. However, DHs have not been used for practical genetic improvements in fish due to challenges in inducing DHs and their low survival rates. In contrast, haploid plants generated by pollen or anther cultures have dramatically accelerated plant breeding through conversion to DH lines [6, 7].

Haploid embryonic stem (ES) cells have been established in mammals, with cell lines maintained for several generations [8]. Furthermore, transplanted haploid ES cells differentiated into germline cells, and fertile gametes were successfully obtained when haploid ES cells were used to generate chimeras in mice [9-11]. In teleosts, haploid ES cells of medaka are viable *in vitro* and survive *in vivo* after transplantation into diploid individuals [12]. In goldfish, chimerism is established by blastoderm transplantation between unviable haploid cells and viable diploid cells, with haploid cells detected in adult fish [13]. In sterlet *Acipenser ruthenus*, polyspermy is caused by artificial insemination with numerous sperm-induced haploid and diploid mosaic fish, in which haploid cells are distributed in several organs, including the gonads [14]. Thus, fish haploid cells can survive under appropriate conditions when surrounded by normal diploid cells

and tissues (haploid-diploid chimerism). However, germline transmission of haploid cells in chimeras using either ES cells or blastomeres has not been observed in fish.

Germ cell transplantation is widely used to produce germline chimeras in fish, which is considered useful for xenogeneic gamete production in aquaculture and germplasm banking [15, 16]. Germinal stem cells, such as spermatogonia, are commonly used as donor cells in germline chimeras because they can be easily obtained in sufficient numbers from immature testes [16]. Primordial germ cells (PGCs) are also used as donor cells to produce germline chimeras, although distinguishing PGCs from other somatic cells in developing embryos of wild-type fish is challenging [17]. To address this, PGCs can be visualized by injecting exogenous mRNA or using transgenic fluorescent lines to differentiate them in developing embryos [15]. Despite some disadvantages, PGCs offer significant advantages as donor germ cells. In germline chimeras without germ cells derived from recipient individuals, a single transplanted PGC can form one side of a pair of testes or ovaries, which can differentiate into normal functional gametes [18]. Additionally, PGCs from inviable individuals (such as lethal mutants) can be used to generate germline chimeras, with gametes differentiated from the germ cells originating from transplanted PGCs [19, 20]. Therefore, we used PGCs from haploid embryos as donor cells to create germline chimeras.

Furthermore, gametes derived from haploid germinal stem cells are theoretically isogenic because a pair of homologous chromosomes arises from a single chromosome after doubling the haploid genome, preventing variation due to the exchange of identical elements. However, the genetic mechanisms involved in gametogenesis in haploid germinal stem cells remain unclear. The goal of this study is to elucidate the potential and ability of haploid germinal stem cells to differentiate into functional gametes. Therefore, we produced fertile gametes derived from haploid PGCs using the germline chimera technique to confirm the potential of artificially induced haploid germ cells (Fig. 1).

Methods

Ethics

This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals at Hokkaido University. All animal experiments were approved by the Animal Study Ethics Committee of Hokkaido University (Approval number: 19-2).

Fish

Wild-type zebrafish (*Danio rerio*), Tg (*vasa*: DsRed2-*vasa*), and Tg (*bactin*: EGFP) double-transgenic zebrafish [21] and goldfish (*Carassius auratus*) were used to induce haploid donor embryos in germline chimeras. Wild-type zebrafish and pearl danio (*Danio albolineatus*) were used to induce sterile hybrids as recipient embryos in germline chimeras. Golden-strain zebrafish were used for fertilization experiments involving the germline chimeras.

Fish were reared in controlled environments at the Faculty of Fisheries Sciences, Hokkaido University. Zebrafish and pearl danio were reared at 28.5°C with a 14-h light/10-h dark photoperiod. Goldfish were reared at 26°C with natural light cycles.

Gamete collection

Female and male zebrafish were placed in a 10-L aquarium one day before the experiment, separated using a small mesh basket floating in the aquarium. On the morning of the experiment, females and males were mixed, and spawning was induced by lighting. The spawning fish were transferred to another aquarium to obtain ovulated eggs and sperm. The fish were anesthetized using 0.1% ethyl 3-aminobenzoate methanesulfonate salt (Sigma, A5040). Sperm was collected using a micropipette fitted with a 10 -µL pipette tip and diluted with 50 µL of Kurokura's solution (128 mM NaCl, 2.7 mM KCl, 1.4 mM $CaCl_2$, and 2.4 mM $NaHCO_3$) [22]. Zebrafish eggs were collected by gently squeezing the abdomens of the spawned females.

Eggs were collected on a polyvinylidene chloride film (Saran Wrap, Asahikasei Co. Ltd., Tokyo, Japan) stretched over the bottom of a plastic dish and covered with a lid to avoid drying until artificial fertilization.

Sperm from pearl danio was collected in the same manner as zebrafish sperm collection. Spermiation in goldfish was induced by injecting human chorionic gonadotropin (100 U/fish; Aska Pharmaceutical Co. Ltd., Tokyo, Japan) the evening before the experiment. Sperm was collected into glass capillaries and diluted with 500 μL of Kurokura's solution [22].

Inducing a gynogenetic haploid as the donor

Diluted goldfish sperm was genetically inactivated by UV irradiation as previously described [23]. Five hundred μL of diluted goldfish sperm was spread on a hydrophilized plastic dish and irradiated with UV light (1 mW/cm^2) for 50 s (50 mJ) with horizontal agitation. The irradiated sperm was collected in a 1.5 mL test tube and kept on ice until use. Gynogenetic haploids were induced by fertilizing eggs obtained from wild-type or transgenic zebrafish with UV-irradiated goldfish sperm.

Inducing sterile individuals as recipients

Infertile hybrids of *Danio* species [21] and knockdown of the *dead end* (*dnd*) gene [19] were used to sterilize recipient individuals in the germline chimera of zebrafish. The infertile hybrid was produced by artificial fertilization of zebrafish eggs with pearl danio sperm. Eggs were obtained from golden -strain zebrafish and inseminated with sperm collected from pearl danio.

Dechorionated embryos (see the following section for the dechorionation process) were used for the injection of *dnd* morpholino antisense oligonucleotide (*dnd* MO) into the blastodisc of the dechorionated embryos at the one-cell stage.

Incubation and dechorionation of the donor and the recipient embryos

The fertilized eggs were incubated in 90 mm dishes filled with tap water. Some eggs were used as intact controls, while the remaining eggs were dechorionated in another 90 mm dish.

For intact controls, dead and unfertilized eggs were removed, and normally cleaved eggs were transferred to a 96-well plate filled with tap water and incubated at 28.5°C. The hatching rate (hatched embryos/fertilized eggs ×100) and normal/abnormal rates (normal or abnormal embryos/hatched embryos ×100) were calculated at 72 h post-fertilization (hpf).

Dechorionated eggs were prepared by treating fertilized eggs with 0.1% trypsin and 0.4% urea in Ringer's solution (128 mM NaCl, 2.8 mM KCl, 1.9 mM CaCl2, pH 8.0) [24]. The dechorionated eggs were transferred to a 1% agar-coated 90 -mm dish filled with the first culture medium (128 mM NaCl, 2.8 mM KCl, 1.9 mM $CaCl_2$ containing 1.6% albumen) [25] and incubated until the gastrula stage. Embryos at the somite stage were transferred to the second culture medium (0.01% penicillin-streptomycin, 1.8 mM $CaCl_2$, 1.8 mM $MgCl_2$ [25]) and incubated at 28.5°C.

Injection of mRNA to visualize PGCs in donor and recipient embryos

Donor embryos derived from wild-type zebrafish used for blastomere transplantation were injected with GFP-nos3 3'UTR mRNA at the one-cell stage to visualize PGCs via GFP fluorescence. Recipient embryos, hybrids of pearl danio and zebrafish, were injected with DsRed-nos3 3'UTR mRNA at the one-cell stage to visualize their endogenous PGCs via DsRed fluorescence when wild-type zebrafish injected with GFP-nos3 3'UTR served as donors. Conversely, recipient embryos were injected with GFP-nos3 3'UTR at the one-cell stage to visualize their endogenous PGCs via GFP fluorescence when the transgenic zebrafish strain was used as donors.

Primordial germ cell transplantation procedure

Primordial germ cells from donor embryos were transplanted as a blastoderm graft at the blastula stage, as previously described [26]. Briefly, blastomeres containing PGCs were aspirated from the marginal part of the blastoderm of a donor embryo using a glass needle with a 75–80 μm opening. The aspirated blastomeres were then transplanted into the blastoderm of recipient embryos. Post-aspiration, donor embryos were kept individually in a 96-well plate at 28.5°C for subsequent ploidy confirmation at the somite stage by flow cytometry (Cyflow, Partec). Transplanted recipient embryos were observed under a fluorescence microscope to confirm the migration of transplanted donor PGCs and endogenous recipient PGCs at the somite stage. Recipient embryos with endogenous PGCs, indicating failed sterilization, were excluded from the experiment.

Ploidy determination

Ploidy determination of donor embryos was performed using flow cytometry. Fin clips (tissues) were taken from host and donor parents and used as diploid standard samples with 2C DNA content (C refers to the amount of DNA per haploid nucleus). Tissue samples and donor embryos were enucleated with 100 μL and 50 μL of solution A, respectively (Cystain DNA 2 step kit05-5005, Sysmex), and incubated for 20 min at room temperature. This was followed by the addition of 300 μL of solution B containing DAPI (4’,6-diamidino-2-phenylindole) (Cystain DNA 2 step kit05-5005, Sysmex) to 10 μL of tissue and 50 μL of embryo samples in solution A. The sample solutions were filtered using a 45 -μm filter mesh and analyzed by flow cytometry. The ploidy of the sample was determined by the relative value of the fluorescence intensity compared to standard 2C samples.

Observation and histological analysis of gonads

Twelve-month-old 1n-2n chimeras, hybrids, dnd MO-treated hybrids, and 6-month-old male and female wild-type zebrafish were dissected, and their gonads were observed under a

198 microscope. Gonads from the chimeras and wild-type zebrafish were collected for ploidy
199 determination. The remaining fish with gonads were fixed in Bouin's fixative for 12 h. Fixed
200 samples were preserved in 80% ethanol until further use. The samples were embedded in resin
201 (Technovit 8100, Kulzer) and sectioned into 3-μm slices. Sections were stained with
202 hematoxylin and eosin.
203
204 **Chromosome spread of testes**
205 Mitotic and meiotic chromosome spreads were obtained from the testes of wild-type
206 zebrafish and 1n-2n germline chimeras, as described in a previous study [26].
207
208 **Fertilization experiment**
209 Natural and artificial fertilization experiments were conducted using four male chimeras
210 and golden-strain females to confirm the fertilization ability of the sperm. Among these
211 chimeras, one was produced by PGC transplantation using wild-type PGCs, and three were
212 transplanted with PGCs from the Tg strain. For artificial fertilization, sperm from chimeric and
213 golden-strain males were collected, and eggs were obtained from golden females. Fertilized
214 eggs were incubated at 28.5°C in 90 mm dishes filled with tap water. The fertilization rate
215 (normally cleaved eggs/total eggs ×100) was calculated at the 8–16 cell stage. Dead and
216 unfertilized eggs were removed. Hatching and normal hatching rates were calculated at 72 hpf.
217
218 **Genotyping using microsatellite markers**
219 Genotyping using microsatellite markers was conducted in the fertilization experiments
220 with the four chimeras mentioned above. In each cross, 10 progeny, the donor female parent,
221 the parents of the recipient embryos, and the golden female used in the fertilization experiment
222 were genotyped. Forty-two microsatellite loci [28] were tested to identify heterozygous or
223 diagnostic alleles in donor females. Selected loci from different linkage groups were used for the

224 analyses (Table S1). DNA was extracted from the 40 μL of residual solution used for ploidy
225 determination in the progeny, as well as from fin clips of donor and host parents, following a
226 previously described protocol [29]. Extracted DNA was diluted with TE buffer (10 mM Tris-HCl, 1
227 mM EDTA-2Na, pH 8.0) and adjusted to 50 ng/μL. An M13-tailed primer (5'-
228 GCCAGTCACGACGTTGTA-3') labeled with a fluorescent dye (5'-6FAM, VIC, NED, and PET)
229 was used in a polymerase chain reaction (PCR) following a previous study [30]. The PCR was
230 conducted in a reaction mixture (10 μL) containing 50 ng of template DNA, 5 μL of Taq HS
231 Perfect Mix (Takara, R300A), 0.3 pmol M13-tailed forward primer, 3.0 pmol reverse primer, and
232 3.0 pmol M13-tailed primer. The reaction conditions were as follows: one cycle of initial
233 denaturation at 94°C for 3 min, 27 cycles of denaturation at 94°C for 1 min, annealing at 58°C
234 for 30 s, and extension at 72°C for 1 min, followed by a final extension at 72°C for 60 min.
235 Electrophoresis was performed using an automated sequencer (ABI3130xl, Applied
236 Biosystems). Alleles were identified based on molecular size (bp: base pairs) using the
237 GeneScan 500 LIZ size standard (Applied Biosystems, 4322682). Genotyping was conducted
238 using the GeneMapper program ver. 3.7 (Applied Biosystems).
239
240

Results

Producing germline chimera by transplanting haploid PGCs

Germline chimeras were produced by transplanting blastoderm grafts containing PGCs derived from gynogenetic haploid embryos into sterile host embryos. Donor haploid embryos were generated by fertilizing zebrafish eggs with UV-irradiated goldfish sperm. Transgenic zebrafish expressing green fluorescent protein (GFP) throughout the body and DsRed in PGCs [21] or wild-type zebrafish injected with GFP-*nos3* 3'UTR mRNA [31] were used to distinguish PGCs from other cell types in donor haploid embryos. Sterile host embryos were created by hybridizing zebrafish females with pearl danio males [21] and by injecting morpholino oligonucleotide to knock down the *dnd* gene [19]. The haploidy of the donor gynogenetic embryos used for transplantation was confirmed using flow cytometry (Table S2). Chimeric embryos containing haploid donor cells were selected for further analysis. Out of 196 chimeras produced in five trials, 28 germline chimeras were obtained, with transplanted donor PGCs successfully settling at the host genital ridge (Fig. 2, Table S3). Seven adult chimeras survived and exhibited mating behavior. Six chimeras with one to eight transplanted donor PGCs at the hatching stage showed male characteristics and produced sperm, whereas the remaining chimera exhibited female external appearance but did not spawn eggs. Sperm collected from five of the six male chimeras was confirmed to be haploid (Fig. S1, Table S4).

Sperm fertility from the germline chimera

Males copulated and fertilized eggs in four trials when a single female and a single male chimera were placed together in an aquarium to induce mating behavior (Table S5). However, the fertilization rates were lower than those observed in controlled mating between a female and male golden strain zebrafish (Table S5). Artificial fertilization using sperm from two chimeric males resulted in higher fertilization rates than those achieved by natural mating, although

sperm could not be collected from the remaining two males during the fertilization trial (Table S5). Normal larvae hatched from fertilized eggs under both natural mating and artificial fertilization conditions. Among the resultant larvae, 96 out of 101 (95%) progeny were diploid (Table S6). Additionally, embryos from chimeric males derived from transgenic donor embryos exhibited GFP fluorescence (Fig. S2). These results demonstrate that the chimeras successfully produced fertile sperm with the donor genotype transmitted from transplanted PGCs.

Isogenic sperm produced from the chimera

The progeny obtained from natural mating, the donor female for haploid PGCs transplantation, the female fish used for mating, and the recipient parents (female zebrafish and male pearl danio) were genotyped using at least seven microsatellite loci from different linkage groups in each cross (Fig. S3). Representative results are shown in Table 1, with detailed data presented in Table S7. The diploid progeny inherited one allele from the donor female used to induce haploid gynogenesis and another allele from the female fish used for natural mating. Theoretically, sperm differentiated from donor PGCs of single haploid gynogenetic embryos exhibit isogenic genotypes due to the single allele present at all loci in transplanted haploid PGCs. Among the 10 progeny analyzed in each cross, only one allele from the donor female was consistently present at all tested loci.

Spermatogenesis in testis of the chimera

Three male and one female-like germline chimeras (one year old), whose sexes were determined based on external appearance, were dissected to observe gonadal development (Fig. S4). In the control group, testes and ovaries were fully mature and contained several stages of spermatocytes and oocytes (Fig. 3A, 3B). In contrast, hybrids and *dnd* morpholino-treated recipients had immature gonads and lacked germ cells in their gonads, respectively (Fig.

3C, 3D). Although testes were formed in male chimeras, they were not as well -developed as those in wild-type zebrafish (Fig. 3E). No gonads were observed in the female-like chimera (data not shown). Spermatogenesis was observed in the testes of two out of three male chimeras, with all stages of spermatogenesis similar to those seen in wild-type zebrafish (Fig. 3E).

Haploid cells were predominantly detected in the testes of male chimeras when somatic cells from the caudal fin were analyzed as diploids during ploidy analysis of wild-type diploids (Fig. 4A, 4B). The testes of male chimeras mainly consisted of haploid and diploid cells, with some tetraploid cells also observed (Fig. 4C).

Mitotic metaphases with 50 chromosomes and meiotic metaphases with 25 bivalent chromosomes were observed in chromosome spreads prepared from the testes of wild-type diploids (Fig. 5). Chromosome spreads from germline chimeras producing haploid sperm revealed 25 and 50 mitotic chromosomes, corresponding to haploid and diploid numbers, respectively. Occasionally, 100 and 200 mitotic chromosomes were also observed (Fig. 5, Fig. 6A, 6B). In meiotic metaphase, 25 bivalent chromosomes were present, consistent with the bivalent number in wild-type diploids (Fig. 5, Fig. 6C). These results indicate that the testes of germline chimeras contained both original haploid and doubled diploid spermatogonia. Diploid spermatogonia exclusively entered the spermatocyte stage, underwent meiosis, and formed bivalent chromosomes, resembling normal spermatogenesis in wild-type diploids.

Discussion

The production of isogenic gametes has been observed in several unisexual teleosts of hybrid origin [32]. These teleosts generate isogenic, unreduced eggs that produce clonal lineages through sperm-dependent gynogenesis. In some cases, isogenic unreduced sperm has also been reported [33, 34]. Premeiotic endomitosis and apomixis are involved in the formation of unreduced gametes [32]. Germ cells with doubled DNA content are detected during premeiotic endomitosis because chromosome doubling occurs before replication and synapsis in meiosis, followed by two consecutive divisions. In contrast, cells with doubled DNA content in the G1 phase are not observed in apomixis because the first meiotic division is skipped. Thus, the DNA content of germ cells serves as a reliable indicator for predicting the presence or absence of isogenic gamete production.

The ploidy status of germ cells varies depending on the stage of spermatogenesis. In diploid wild-type fish, spermatogonia and spermatocytes are diploid, whereas spermatids and sperm are haploid. Tetraploid DNA content is observed in replicating mitotic cells during the G2/M phase and in meiotic cells during the first meiotic division, where bivalent chromosomes form. In this study, haploid PGCs settled and differentiated into genetically identical sperm in recipient gonads via premeiotic genome doubling, as evidenced by the mitotic metaphase with 50 chromosomes and meiotic metaphase with 25 bivalent chromosomes. Although the exact mechanism of genome doubling in haploid germ cells remains unclear, similar processes have been observed in mammalian haploid ES cells, which spontaneously diploidize their genome through endomitosis [9], mitotic slippage [35], delayed mitosis [36], or transient G2 arrest and abrupt insertion of an extra G1/S phase [37]. Furthermore, haploid parthenogenetic embryos in mammals generate diploid cells during preimplantation development [38, 39]. In human haploid parthenogenetic embryos, self-diploidization due to failed cytokinesis and endomitosis has been observed [40]. These findings suggest that haploid mammalian cells possess an inherent

mechanism for spontaneous diploidization to avoid unstable genomic conditions. However, artificially induced haploid embryos in teleosts develop into abnormal larvae and die before feeding, likely due to the absence of diploidization. Chimeric individuals produced through blastoderm transplantation between haploid and diploid fish retain haploid cells for up to 16 months in goldfish, although whether diploidization of haploid cells occurs in these chimeras is unknown [13]. In medaka, diploidized and polyploidized haploid ES cells are bi- and multinucleated, resulting from endomitosis [12]. In this study, tetraploid and octoploid cells with 100 and 200 mitotic chromosomes, respectively, were detected in the testicular cells of the germline chimeras. Similar ploidy elevations have been observed in the early spermatogonia of sex-reversed clonal dojo loach of hybrid origin, where tetraploid and octoploid cells were identified cytogenetically and through flow cytometry. In clonal loach, unreduced (diploid) isogenic sperm were produced from tetraploid germ cells, whereas somatic cells, such as fins, retained diploidy in adult individuals [27]. Unreduced gametogenesis has been reported in various diploid hybrid fish species [41-43] and fish species of hybrid origin [44]. These findings suggest that genome doubling is intrinsic to sterile hybrids with two non-homologous sets of chromosomes or to haploids with a single chromosome set, as chromosomes may fail to find counterparts to form bivalents. Premeiotic chromosome duplication in hybrid diploid or haploid germ cells resolves this issue, enabling regular meiosis and gametogenesis.

Heterozygous isogenic F1 hybrids exhibiting heterosis could potentially be produced in fish through crossbreeding using two or more DHs. In plants, DH production is primarily achieved through anther or pollen culture, and DHs are widely used in F1 hybrid seed production to enhance crop yields [6]. In teleost fish, DHs can be generated by chromosome manipulation via gynogenesis and androgenesis, but the viability and fertility of DHs are often low, making their application in aquaculture challenging [3]. Low viability is attributed to factors such as genetic inactivation of gametes, thermal or hydro-pressure shock during zygote development, and the expression of deleterious recessive genes due to inbreeding [3]. Our

362 approach of using germline chimeras with haploid PGCs as donors overcomes these limitations
363 by employing robust recipients. The fertility of germline chimeras is unaffected by donor germ
364 cells or recipient gonadal somatic cells, provided there are no mutations or other deleterious
365 effects on gametogenesis. In medaka, maternal zygotic mutant progeny have been produced
366 using germline chimeras [20]. Haploid PGCs used for transplantation are particularly useful for
367 generating gametes with mutations related to embryogenesis, as half of the embryos developed
368 via gynogenesis or androgenesis inherit mutant alleles from heterozygous parents.
369 Furthermore, haploid germ cells used in germline chimeras also contribute to producing mutant
370 progeny for genetic screening, especially when combined with genome editing technologies,
371 such as haploid mouse ES cell biobanks for functional genomics [45].
372 This study demonstrated that haploid germinal stem cells derived from haploid PGCs
373 can differentiate into fertile haploid sperm via genome doubling before meiosis. However, no
374 eggs were obtained from the female germline chimera. It remains unclear whether female
375 germline chimeras with haploid germinal stem cells can produce fertile eggs. In zebrafish
376 germline chimeras, sex differentiation depends on the number of transplanted PGCs [46].
377 Future research should focus on developing techniques to transplant sufficient PGCs or perform
378 male-to-female sex reversal to induce female germline chimeras. Additionally, the potential of
379 this technique to produce isogenic gametes, using germline chimera with haploid PGCs, in other
380 fish species must be explored to facilitate its application in aquaculture breeding programs.

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Conflicts of Interest

The authors declare no competing interest.

Author Contributions

TF, TS, EY, and KA designed the research; TN and TS performed the research; TN and TF analyzed data; TN, TF, and KA wrote the paper.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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Figure Legends

Figure 1. Schematic illustration of the study, showing the production of fertile, genetically identical gametes derived from haploid germ cells of an artificially induced gynogenetic individual using the germline chimera technique.

Figure 2. External appearance of GFP nos3 3'UTR mRNA -injected gynogenetic haploid individual (A), *dead end* morpholino oligonucleotide and DsRed nos3 3'UTR mRNA -injected individual (B), DsRed nos3 3'UTR mRNA -injected individual (C), and germline chimera possessing transplanted primordial germ cells from donor haploid embryos (D). Panels A1, B1, C1, and D1 are bright-field images. Panels A2, B2, C2, D2, and D3 are dark-field images with fluorescent emission wavelengths. Panel D2' is a high-magnification view of the rectangular area shown in D2. Yellow arrowheads and green arrows indicate PGCs at the genital ridge and ectopic positions in recipient larvae, respectively. Scale bars represent 1 mm.

Figure 3. External appearances of gonads and gonadal structures in wild-type male zebrafish (A), wild-type female zebrafish (B), hybrids of zebrafish females and pearl danio males (C), hybrids injected with *dead end* morpholino oligonucleotide as recipients (D), and germline chimeras derived from the transplantation of haploid primordial germ cells (E). Scale bars represent 100 μm.

Figure 4. Ploidy analysis of the caudal fin of wild-type zebrafish (A), testis of wild-type zebrafish (B), and testis of a germline chimera derived from haploid primordial germ cell transplantation (C).

Figure 5. Distribution of mitotic metaphase chromosomes and meiotic bivalent chromosomes at metaphase I in the testes of wild-type zebrafish and germline chimeras derived from haploid primordial germ cell transplantation.

Figure 6. Mitotic metaphases with 25 chromosomes (A) and 50 chromosomes (B) in germline chimeras derived from haploid primordial germ cell transplantation. Meiotic chromosomes at metaphase I with 25 bivalents in the germline chimera (C).

Figure S1. Ploidy of sperm from wild-type zebrafish and germline chimeras induced by the transplantation of haploid primordial germ cells into sterilized recipients. Diploid somatic cells from fins (A) and haploid sperm (B) in wild-type zebrafish, and haploid sperm in germline chimera #2-1 (C), #2-2 (D), #2-3 (E), #3-1 (F), and #4 (G) were detected by flow cytometry.

Figure S2. Resultant progeny from the mating of a golden-strain female zebrafish and a germline chimera male transplanted with haploid donor PGCs derived from transgenic zebrafish. (Left) Embryos under bright-field view. (Right) Embryos under fluorescent view.

Figure S3. Genotyping scheme confirming the genetic contribution of haploid germ cells from donor female zebrafish. If a germline chimera male successfully produces sperm differentiated from transplanted haploid germ cells, the resultant progeny from mating between the germline chimera male and a test-cross female will possess two alleles: one from the female zebrafish donor of the haploid germ cells and the other from the test -cross female. Furthermore, if the

561 donor female is heterozygous at a locus, only one allele will be inherited by the progeny due to
562 the haploidy of donor germ cells.

563

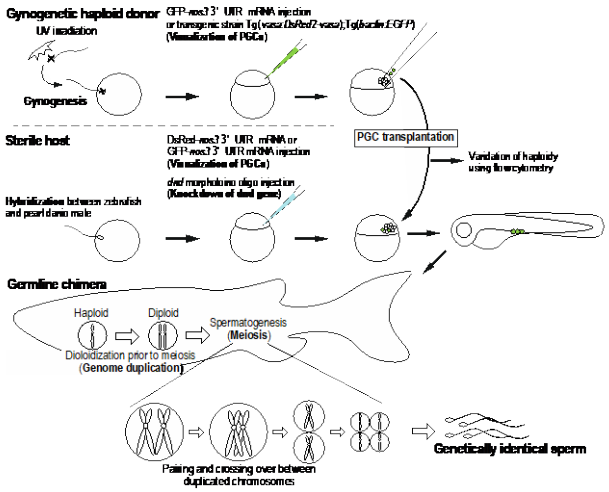
564 Figure S4. External appearance of germline chimeras. The upper fish is the male germline
565 chimera (#3-1), and the lower fish is the germline chimera (#3-2), which exhibited a female
566 external appearance but lacked gonadal structures in the body cavity. Scale bar represents 1
567 cm.

568

Table 1. Representative genotyping results of progeny obtained from the fertilization of eggs from golden strain zebrafish and sperm from germline chimeras.

Crosses	Locus (Linkage group)	Recipient female	Recipient male (pearl danio)	Donor female	Female for test cross	Diploid progeny (the number of individuals)	Triploid progeny (the number of individuals)
Golden x Chimera #1	Z896 (LG5)	205/205	-	210/228	228/228	210/228 (10)	
	Z7576 (LG12)	161/377	-	111/121	160/160	121/160 (10)	
	Z992 (LG16)	139/139	208/216	222/224	222/224	222/224 (5)	
						224/224 (5)	
	Z3123 (LG17)	222/224	122/132	147/175	140/140	140/175 (10)	
	Z1136 (LG18)	138/200	-	108/116	116/116	108/116 (10)	
	Z11113 (LG21)	208/218	-	222/232	208/220	208/232 (6)	
						220/232 (4)	
Golden x Chimera #2-1	Z1402 (LG8)	190/206	-	192/236	192/205	192/205 (7)	
						192/192 (1)	
							192/192/205 (2)
	Z5265(LG23)	184/214	-	195/241	184/214	184/195 (7)	
						195/214 (1)	
							184/195/214 (2)
Golden x Chimera #2-2	Z8495 (LG7)	284/284	-	266/280	284/284	266/284 (10)	
	Z1402 (LG8)	190/206	-	192/236	206/206	192/206 (10)	
	Z5265 (LG23)	184/214	-	195/241	213/213	195/213 (10)	
Golden x Chimera #2-3	Z1402 (LG8)	190/206	-	192/236	190/206	192/206 (5)	
						190/192 (5)	
	Z5265 (LG23)	184/214	-	195/241	184/214	184/195 (5)	
						195/214 (5)	

* Golden strain of zebrafish; -: no amplification



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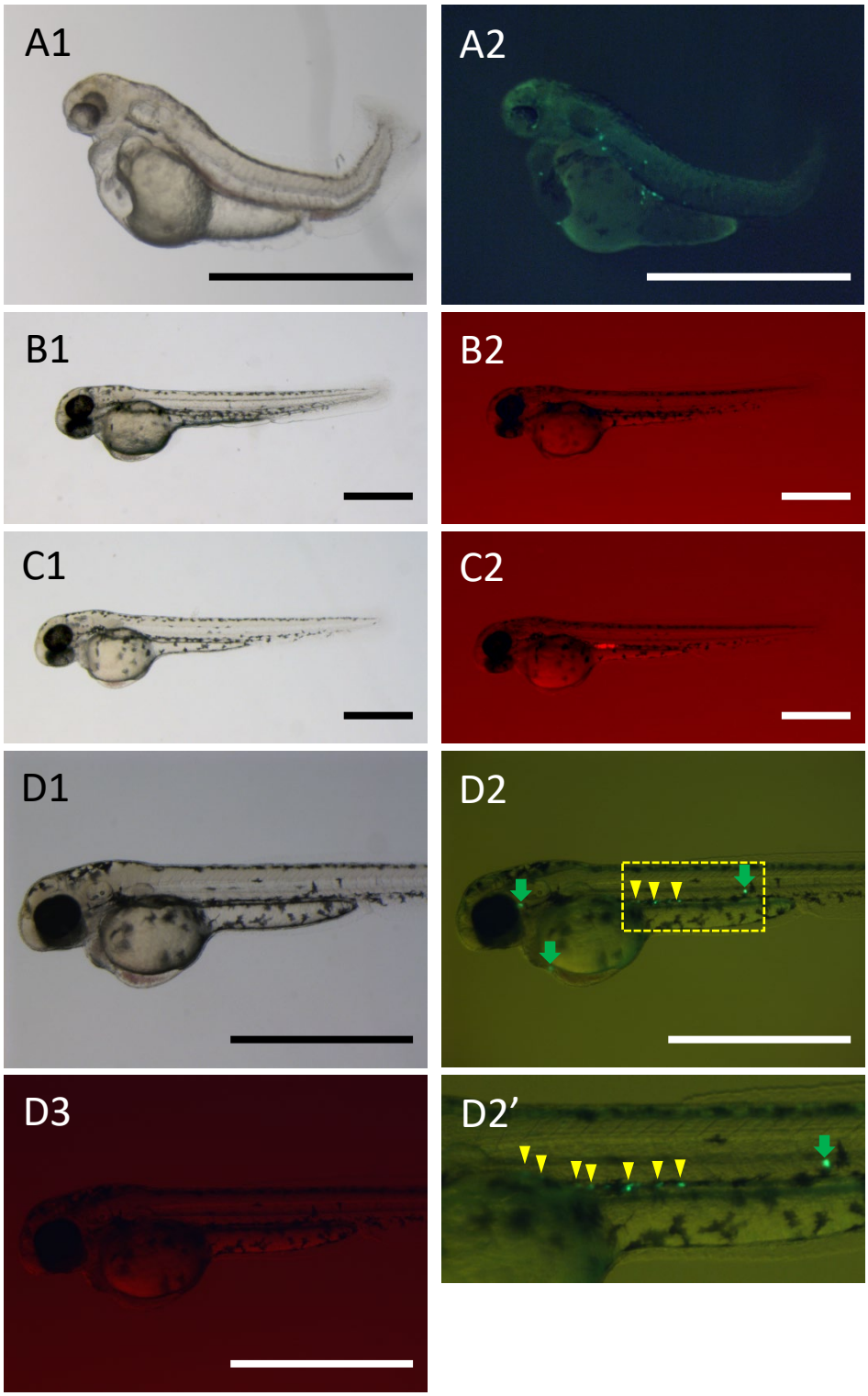

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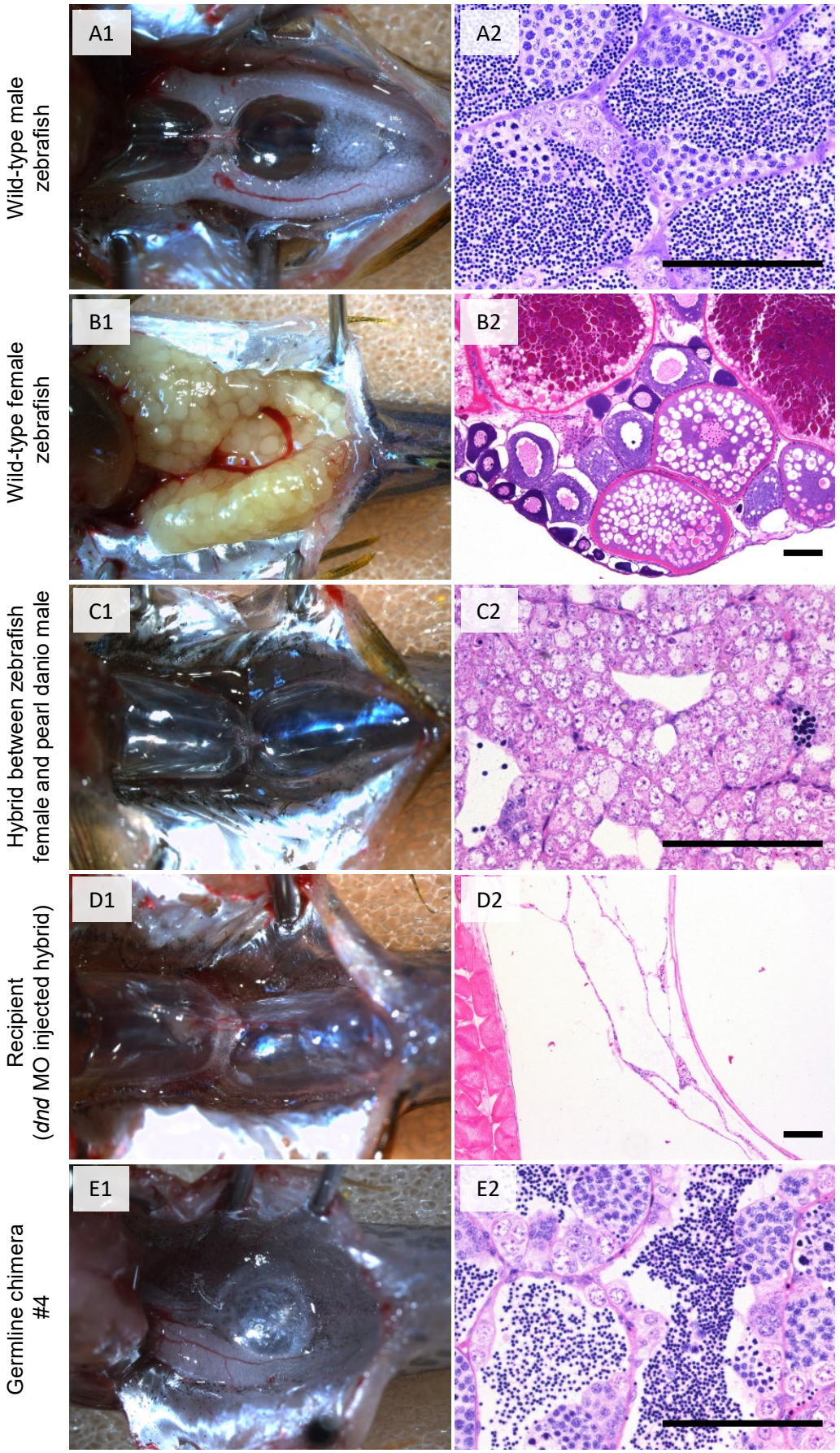

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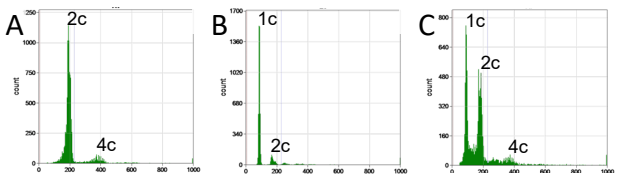

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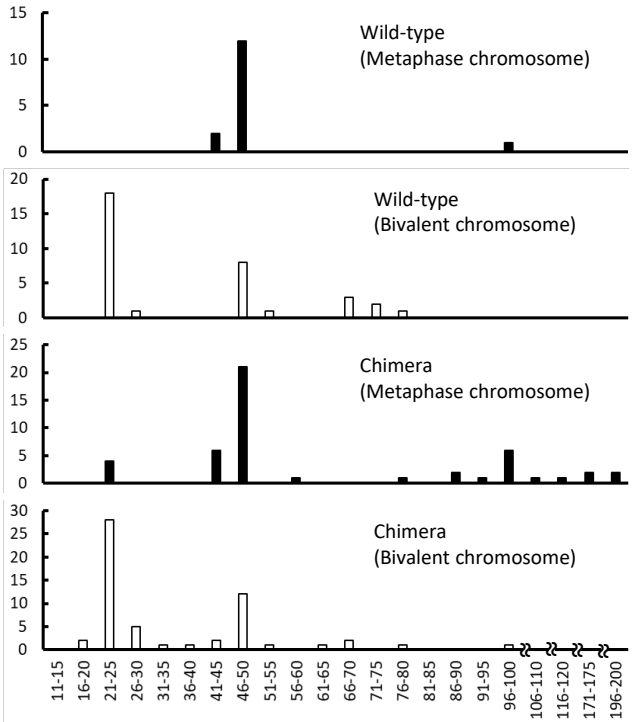


Figure 5. Distribution of mitotic metaphase chromosomes and meiotic bivalent chromosomes at metaphase I in the testes of wild-type zebrafish and germline chimeras derived from haploid primordial germ cell transplantation.

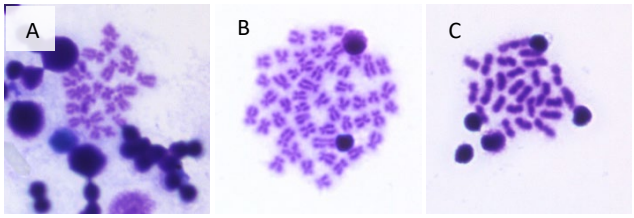
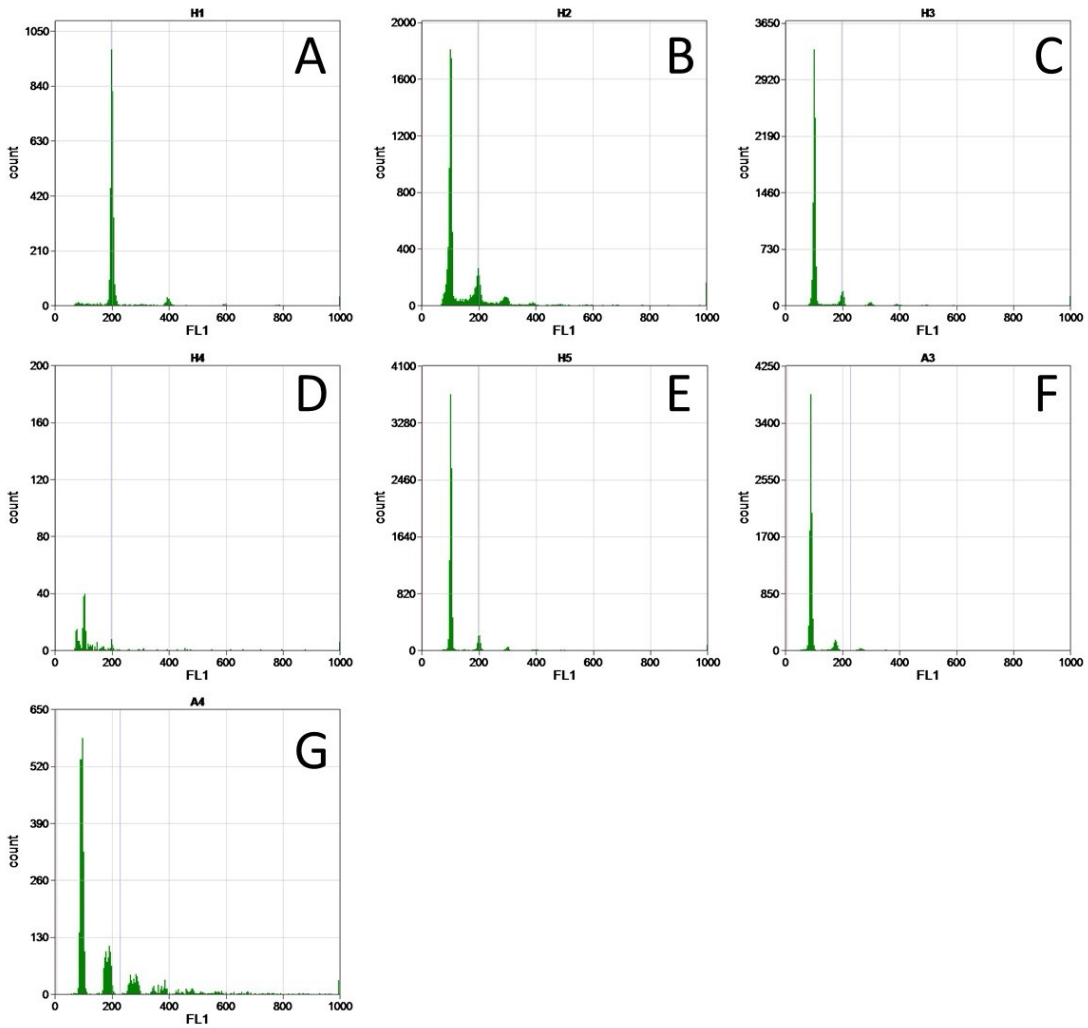


Figure 6. Mitotic metaphases with 25 chromosomes (A) and 50 chromosomes (B) in germline chimeras derived from haploid primordial germ cell transplantation. Meiotic chromosomes at metaphase I with 25 bivalents in the germline chimera (C).



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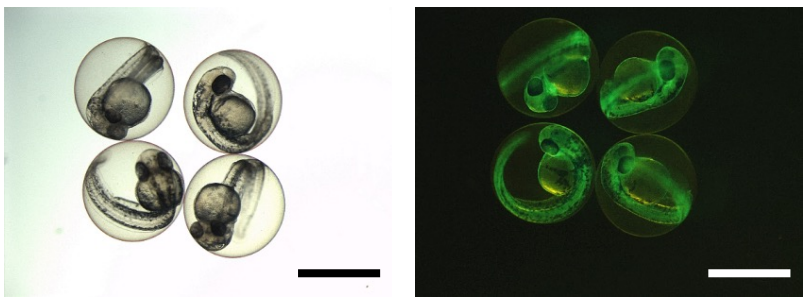

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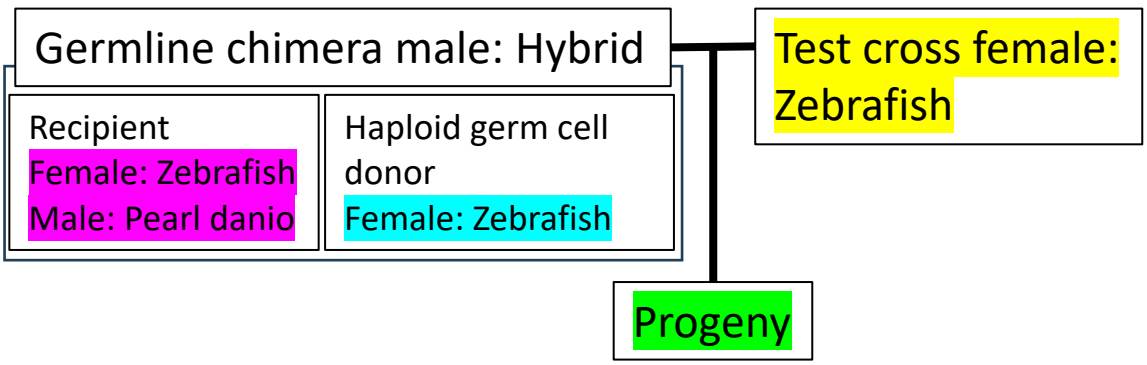

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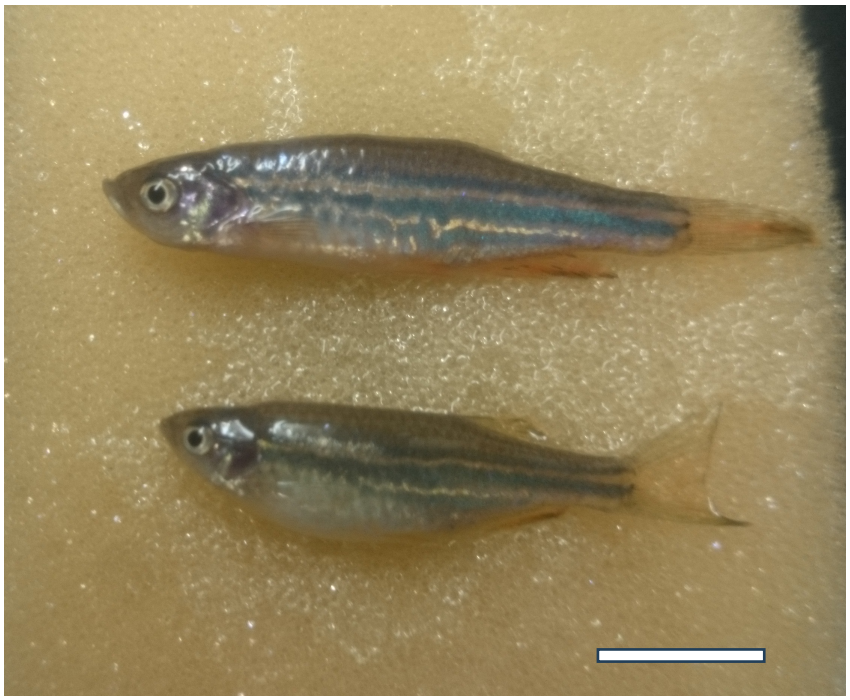

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Table S1. Primer sequences used for microsatellite analysis.

Locus	Linkage group	Forward (5' – 3')	Reverse (5' – 3')
Z8874	1	AGTCACGACGTTGTATGTGTCATCTGCTTTTGTCTGA	ACTGCTGTGAAGTCCACACG
Z1781	1	AGTCACGACGTTGTAGCTCTGGCAGATTCCAAATTCC	AAATTCCAAGGGTCAGCTGCAGGGGT
Z644	2	AGTCACGACGTTGTACACGGACAATCCAGTGTACG	CCCAGCAACAACACTAAGCA
Z7629	4	AGTCACGACGTTGTACGAAACTAACACGAACACCG	CTTTAGCACAACACGACCGA
Z896	5	AGTCACGACGTTGTACCCATCACAGCTGACACAAC	TCAACAGTGGACGAGCTGAC
Z8495	7	AGTCACGACGTTGTATCATAATCATGTGCTCCTCTTGA	TTGTTGTTGACGCTGCTTTC
Z1402	8	AGTCACGACGTTGTAGATCAGGGTGCATGTACTGAA	TCTGCTTTCTGTCAGGGGAT
Z106	9	AGTCACGACGTTGTAGAGACCGACCGTCTGCTAAC	TCCAGAGAACACCCTGCTG
Z7576	12	AGTCACGACGTTGTAAATGATGAAGGTCACCGCTC	GTCCCTTTCCCACAGTCTGA
Z6622	13	AGTCACGACGTTGTACAGTGGAGGCTCTGGCAG	ATCATGCTGGAGAAACCCTG
Z992	16	AGTCACGACGTTGTAACACAGCCGTGTTTGACGTA	TCCCTCACGATTACCCAGAC
Z3123	17	AGTCACGACGTTGTACCTATGGGGAGTCCTGTGAACC	CTTCTGGCCTGTGCGGATTC
Z1136	18	AGTCACGACGTTGTATCATGTGAAGCCTGCAGTTC	AGCTTCAGCTGTTTAAAGGTGC
Z9331	19	AGTCACGACGTTGTAGGATAAATCCTGGCCTTTCC	CAGACACTGCAGAGCTCGAG
Z11113	21	AGTCACGACGTTGTAATGGCTTCGAAAACGGTAAA	GATGCTGCAGACGGACAGA
Z5265	23	AGTCACGACGTTGTACTAACCACAGCTGCAACAACCG	AGTGTCCGTGCAATCAGAGGC

Table S2. Combinations of eggs and sperm to induce haploid donor embryos and the ploidy of donor embryos used for germline chimeras in each experiment.

				Relative DNA content					
Experimental ID	Eggs	Sperm	N	1C	2C	3C	4C	Mosaic	ND[*1]
#1	Wild-type zebrafish	UV irradiated goldfish sperm	8	5	0	0	0	1[*2]	2
#2	Transgenic zebrafish	UV irradiated goldfish sperm	6	6	0	0	0	0	0
#3	Wild-type zebrafish	UV irradiated goldfish sperm	16	12	0	0	0	0	4
#4	Transgenic zebrafish	UV irradiated goldfish sperm	7	7	0	0	0	0	0
#5	Wild-type zebrafish	UV irradiated goldfish sperm	17	12	0	0	1	1[*3]	3

*1: Not detectable

*2: Haploid – diploid mosaic

*3: Haploid – triploid mosaic

Table S3. The number germline chimeras obtained from each experiment, and the external morphology and presence of primordial germ cells of the resultant larvae in each experimental batch.

Experimental ID				The number of embryos used for culture at blastula stage	The number of larvae			Presence of PGCs at the genital ridge of the larvae injected with DsRed/GFP *nos3* 3'UTR			Location of donor PGCs in the recipient with normal external morphology.		
		Type of fish	Experimental batch	N	Hatch	Normal	Deformed	N	Present	Absent	Present at the genital ridge	Present at ectopic position	Absent
#1	Recipient	Hybrid	Intact control	32	32	32	0						
			Dechorionated	32	31	26	5						
			dnd MO injected*	6	6	6	0	6	0	6			
			DsRed-*nos3* 3'UTR	32	28	24	4	10	7	3			
	Donor	Wild-type, Haploid	Intact control	32	20	0	20						
			Dechorionated	32	6	0	6						
			GFP-*nos3* 3'UTR injected	32	7	0	7	7	7	0			
	Chimera			25	25	11	14				3	0	8
#2	Recipient	Hybrid	Intact control	16	16	16	0						
			Dechorionated	4	4	3	1						
			dnd MO injected*	10	10	10	0	10	0	10			
			GFP-*nos3* 3'UTR	16	16	16	0	10	10	0			
	Donor	Transgenic, Haploid	Intact control	21	10	0	10						
			Dechorionated	26	22	0	22						
	Chimera			53	52	48	4				9	3	36
#3	Recipient	Hybrid	Intact control	32	32	30	2						
			Dechorionated	32	31	30	1						
			dnd MO injected*	32	32	30	2	10	5	5			
			DsRed-*nos3* 3'UTR	32	32	30	2	10	10	0			
	Donor	Wild-type, Haploid	Intact control	32	15	0	15						
			Dechorionated	32	16	0	16						
			GFP-*nos3* 3'UTR injected	32	14	0	14	10	10	0			
	Chimera			29	29	25	4				4	4	17
#4	Recipient	Hybrid	Intact control	25	22	21	1						
			Dechorionated	31	28	25	3						
			dnd MO injected*	7	6	5	1	6	0	6			

			GFP-*nos3* 3'UTR	9	8	5	3	8	8	0			
	Donor	Transgenic, Haploid	Intact control	31	24	0	24						
			Dechorionated	8	7	0	7						
	Chimera			62	61	49	12				5	11	33
#5	Recipient	Hybrid	Intact control	19	19	15	4						
			Dechorionated	NA									
			dnd MO injected*	5	4	4	0	4	0	4			
			DsRed-*nos3* 3'UTR	NA									
	Donor	Wild-type, Haploid	Intact control	12	4	0	4						
			Dechorionated	25	8	0	8						
			GFP-*nos3* 3'UTR injected	37	7	0	7	7	7	0			
	Chimera			27	26	19	7				7	3	9

*: In these batches, DsRed-*nos3* 3'UTR mRNA or GFP-*nos3* 3'UTR mRNA was co-injected with morpholino antisense oligo to *dead end* gene.

Table S4. Identification data of each mature germline chimera, the initial number of primordial germ cells at the hatching stage, sex, gamete, and ploidy of the gamete.

ID*	Genotype of donor female	Age	The number of donor PGCs at hatching stage in the recipient	Sex of chimera	Type of gamete	Ploidy of gamete
#1	Wild-type	12 month-old	1	Male	Sperm	ND
#2 – 1	Transgenic	12 month-old	1	Male	Sperm	Haploidy
#2 – 2	Wild-type	12 month-old	3	Male	Sperm	Haploidy
#2 – 3	Transgenic	12 month-old	4	Male	Sperm	Haploidy
#3 – 1	Wild-type	6 month-old	8	Male	Sperm	Haploidy
#3 – 2	Wild-type	6 month-old	3	Female-like**	ND	NA
#4	Transgenic	12 month-old	4	Male	Sperm	Haploidy
Control	-	6 month-old	-	Male	Sperm	Haploidy

*: Sample ID is named Experimental ID – sequential serial number.

**: The germline chimera showed a female-like external shape.

Table S5. Reproductive ability of germline chimeras induced by transplantation of haploid donor PGCs into diploid-sterilized recipients. The reproductive ability was confirmed by the fertilization ability of sperm from the germline chimeras and the developmental ability of the resultant progeny. Fertilization tests were conducted using natural mating and artificial fertilization. In these crosses, the golden strain of zebrafish was used for both females and males. Eggs from a single female golden strain were used for artificial fertilization to avoid variability in fertilization and development due to egg quality.

Males used for test crosses	Fertilization methods	The number of eggs used	Fertilized eggs (%)	Hatching (%)	Normal fry (%)
Golden	Natural mating	186	175 (94.1)	140 (80.0)	135 (96.4)
Golden	Artificial fertilization*	37	32 (86.4)	32 (100)	32 (100)
Chimera #1	Natural mating	243	48 (19.8)	29 (60.4)	26 (89.7)
	Artificial fertilization	-	-	-	-
Chimera #2-1	Natural mating	352	56 (15.9)	39 (69.6)	37 (94.9)
	Artificial fertilization*	98	72 (73.4)	70 (97.2)	70 (100)
Chimera #2-2	Natural mating	332	41 (12.3)	35 (85.4)	35 (100)
	Artificial fertilization	-	-	-	-
Chimera #2-3	Natural mating	656	16 (2.4)	15 (93.8)	15 (100)
	Artificial fertilization*	109	49 (45.0)	47 (95.9)	44 (93.6)

*: Eggs derived from a single female were artificially fertilized.

Table S6. Progeny ploidy arises from fertilization between eggs from golden strain zebrafish and sperm from germline chimeras.

			Ploidy				
Crosses	Fertilization methods	N	1n	2n	3n	4n	Mosaic
Golden x Chimera #1	Natural mating	16	0	15	0	1	0
	Artificial fertilization	ND	-	-	-	-	-
Golden x Chimera #2-1	Natural mating	30	0	28	2	0	0
	Artificial fertilization	16	0	16	0	0	0
Golden x Chimera #2-2	Natural mating	12	0	12	0	0	0
	Artificial fertilization	ND	-	-	-	-	-
Golden x Chimera #2-3	Natural mating	12	0	12	0	0	0
	Artificial fertilization	15	1	13	0	0	1*

* Diploid – triploid mosaic.

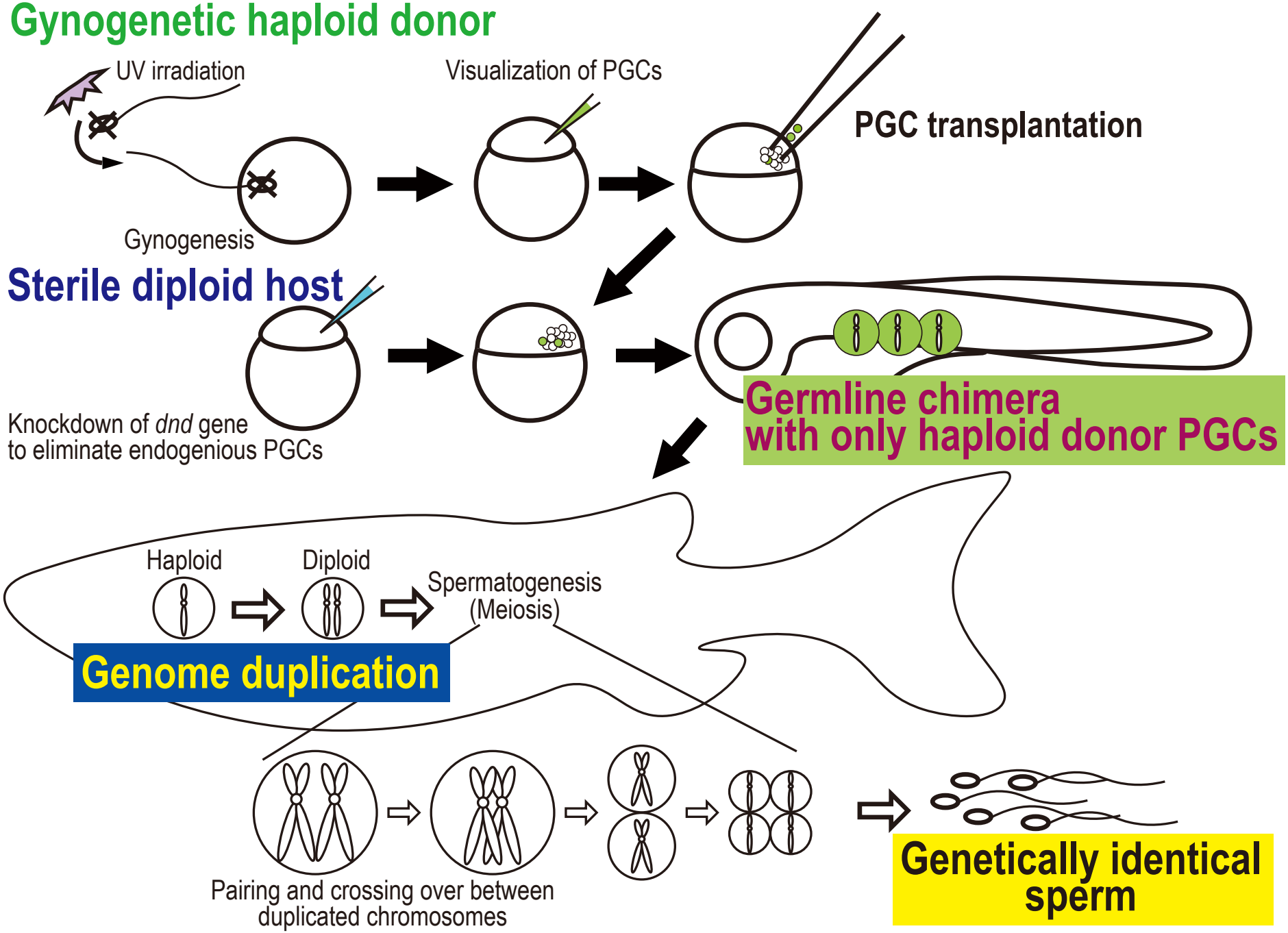
Table S7. Genotyping of progeny obtained from the fertilization of eggs from golden strain zebrafish and sperm from germline chimeras.

Crosses	Locus (Linkage group)	Recipient female	Recipient male (Perl danio)	Donor female	Female for test cross	Diploid progeny (the number of individual)	Triploid progeny (the number of individual)
Golden strain female x Chimera #1 male	Z8874 (LG1)	130/150	114/120	124/130	124/126	126/130 (6)	
						124/130 (4)	
	Z644 (LG2)	223/227	252/254	227/227	223/227	227/227 (5)	
						223/227 (5)	
	Z896 (LG5) *	205/205	-	210/228	228/228	210/228 (10)	
	Z1402 (LG8)	190/206	-	206/206	190/206	190/206 (5)	
						206/206 (5)	
	Z106 (LG9)	186/186	-	186/188	186/186	186/188 (10)	
	Z7576 (LG12) *	161/377	-	111/121	160/160	121/160 (10)	
	Z992 (LG16) *	139/139	208/216	222/224	222/224	222/224 (5)	
						224/224 (5)	
	Z3123 (LG17)**	222/224	122/132	147/175	140/140	140/175 (10)	
	Z1136 (LG18) **	138/200	-	108/116	116/116	108/116 (10)	
	Z11113 (LG21) *	208/218	-	222/232	208/220	208/232 (6)	
						220/232 (4)	
	Z5265 (LG23)	196/216	-	196/208	184/216	184/208 (5)	
						208/216 (5)	
Golden strain female x Chimera #2-1 male	Z8874 (LG1)	126/130	118/122	130/150	124/126	124/150 (3)	
						126/150 (5)	
							124/126/150 (2)
	Z7629 (LG4)	280/280	265/265	256/280	280/280	256/280 (8)	
							256/280/280 (2)

	Z8495 (LG7)	284/284	-	266/284	240/240	240/266 (6)	
						266/284 (2)	
							240/266/284 (2)
	Z1402 (LG8) *	190/206	-	192/236	192/205	192/205 (7)	
						192/192 (1)	
							192/192/205 (2)
	Z6622 (LG13)	273/273	260/260	263/273	273/273	273/273 (8)	
							273/273/273 (2)
	Z9331 (LG19)	288/290	-	288/292	292/292	292/292 (8)	
							292/292/292 (2)
	Z5265(LG23) *	184/214	-	195/241	184/214	184/195 (7)	
						195/214 (1)	
							184/195/214 (2)
Golden strain female x Chimera #2-2 male	Z1781 (LG1)	200/202	230/288	200/214	200/214	200/200 (6)	
						200/214 (4)	
	Z7629 (LG4)	280/280	265/265	256/280	280/280	256/280 (10)	
	Z8495 (LG7) *	284/284	-	266/280	284/284	266/284 (10)	
	Z1402 (LG8) *	190/206	-	192/236	206/206	192/206 (10)	
	Z6622 (LG13)	273/273	260/260	263/273	284/284	273/284 (10)	
	Z9331 (LG19)	288/290	-	288/292	286/290	286/292 (7)	
						290/292 (3)	
	Z5265 (LG23) *	184/214	-	195/241	213/213	195/213 (10)	
Golden of strain female x Chimera #2-3 male	Z8874 (LG1)	126/130	118/122	130/150	124/130	124/150 (6)	
						130/150 (4)	
	Z7629 (LG4)	280/280	265/265	256/280	280/280	256/280 (10)	
	Z8495 (LG7)	284/284	-	266/284	240/284	240/266 (6)	

						266/284 (4)	
	Z1402 (LG8) *	190/206	-	192/236	190/206	192/206 (5)	
						190/192 (5)	
	Z6622 (LG13)	273/273	260/260	263/273	273/283	273/273 (7)	
						273/283 (3)	
	Z9331 (LG19)	288/290	-	288/292	290/292	290/292 (4)	
						292/292 (6)	
	Z5265 (LG23) *	184/214	-	195/241	184/214	184/195 (5)	
						195/214 (5)	

*: Diagnostic alleles to distinguish alleles of donor from alleles of recipient, **: Diagnostic alleles to distinguish alleles of donor from alleles of recipient and female used for test cross, -: No amplification


Gynogenetic haploid donor
UV irradiation
Visualization of PGCs
PGC transplantation
Gynogenesis
Sterile diploid host
Knockdown of *dnd* gene
to eliminate endogenious PGCs
Germline chimera
with only haploid donor PGCs
Haploid
Diploid
Spermatogenesis
(Meiosis)
Genome duplication
Pairing and crossing over between
duplicated chromosomes
Genetically identical
sperm