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Fish genome manipulation and directional breeding

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Aquaculture is one of the fastest developing agricultural industries worldwide. One of the most important factors for sustainable aquaculture is the development of high performing culture strains. Genome manipulation offers a powerful method to achieve rapid and directional breeding in fish. We review the history of fish breeding methods based on classical genome manipulation, including polyploidy breeding and nuclear transfer. Then, we discuss the advances and applications of fish directional breeding based on transgenic technology and recently developed genome editing technologies. These methods offer increased efficiency, precision and predictability in genetic improvement over traditional methods.

fish directional breeding, polyploidy breeding, nuclear transfer, transgenic fish, genome editing

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Fish are one of the most important sources of protein for humans. As a result of declines in wild fisheries, aquaculture has become one of the fastest developing agricultural industries worldwide [1]. To increase the sustainability of aquaculture, culturists commonly conduct selective breeding to develop strains that perform well in captivity. Unfortunately, the sustainability of many sectors of the industry is negatively affected by inbreeding depression, disease outbreaks, under production, and low meat quality [2]. To address these issues, there is an urgent need for the development of high quality fish strains that have high growth rates, disease-resistance, and/or higher nutritional value.

Traditional crossbreeding methods such as intra-species crossbreeding [3] and inter-species hybridization [4] have been successfully used for several decades. However, crossbreeding requires multiple-generations of hybridization to introduce a desirable trait to a given strain. Additionally, the outcomes of these methods are unpredictable because the underlying mechanisms controlling desirable traits are unknown. Thus, there is a need to develop more efficient, precise and predictable techniques for producing production scale numbers of high-quality fish. We review the history of fish breeding methods based on classical genome manipulation approaches, including polyploidy breeding [5] and nuclear transfer [6]. Then, we discuss directional breeding of fish based on transgenic technology and recently developed genome editing technologies. Advances in breeding methodology based on classical genome manipulation and recently developed genome editing methods will likely play a major role in the future of genetic breeding in fish.

1 Polyploidy breeding

Polyploidy, a way to artificially duplicate the chromosome, is considered to be a classic approach to genome manipulation [5]. In some fish species, growth rates differ between females, males, or infertile individuals. To exploit this, culturists produce monosex or infertile populations to increase productivity. A number of methods have been used to achieve sex- and fertility-control during fish breeding. Of these, polyploidy breeding was one of the earliest and the

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most efficient. This method can also be used to rapidly obtain a homozygous population, thereby decreasing the generational length of the genetic breeding process.

The concept of polyploidy breeding was developed based on research into gynogenesis. In fish, the second meiotic division is completed shortly after ovulation or fertilization. This process can be inhibited by cold-shock treatment, resulting in duplication of chromosomes in the oocyte [7,8]. The combination of physical treatments such as heat-shock and hydrostatic pressure yields the best results [9,10]. Gynogenesis in fish is induced by thermal shock or hydrostatic pressure treatment after fertilization of the egg with inactivated sperm [11,12]. Conversely, androgenesis is induced by thermal shock or hydrostatic pressure treatment of an inactivated egg fertilized with normal sperm. Both gynogenesis and androgenesis have been used on a variety of fish species to produce double haploids (reviewed by [9]).

The production of double haploids can be used to maintain a monosex population. In species with XX-XY sex determination, offspring that are produced by gynogenesis are expected to be all female and the offspring derived by androgenesis have an XX or YY genome. These traits are reversed in species with ZW-ZZ sex determination. The use of gynogenesis and androgenesis for sex-control is only suitable for production of small populations because of the relatively high mortality rate, which is likely caused by irradiation damage, the side-effects from thermal/pressure shock, and inbreeding depression [9]. Notably however, polyploidy breeding methods can be combined with other classical methods, such as induced sex-reversal, to control the sex of offspring and decrease the duration of the breeding process. One successful example of this approach is the production of super, all-male yellow catfish (*Pelteobagrus fulvidraco*), in which males grows faster than females [13]. The YY-super male yellow catfish population is obtained from gynogenesis of induced physiological XY females, and the YY-strain is maintained by incrossing with induced sex-reversed YY females.

As with other animals, sterile fish typically have higher growth rates than fertile individuals. Thus, a number of researchers have evaluated methods to produce sterile triploid fish. The earliest studies used thermal shock treatment after fertilizing the eggs with normal sperm. However, this method has a relatively low success rate and is often accompanied by high mortality. A higher rate of triploidy can be achieved by mating tetraploid fish with diploid fish [14]. Because most fish species are diploid, the most critical step in this process is the production of fertile tetraploids. Researchers successfully obtained fertile allotetraploid fish by hybridizing crucian carp (*Carassius auratus*) with common carp (*Cyprinus carpio*) until the F3 generation [15,16]. The same group also successfully obtained another tetraploid fish by crossing crucian carp with blunt snout bream (*Megalobrama amblycephala*) [17,18]. Although polyploidy breeding has been used for a long time and has yielded several valuable strains, it is not widely applicable because only a few fish species can be used for inter-species crossing to generate tetraploid fish. As this technique is used primarily to breed growth-enhanced strains, other desirable non-growth characters are unlikely to be obtained through this method. Additionally, this method is not of use in species that do not exhibit growth differences between males, females, or infertile individuals.

2 Nuclear transfer

Nuclear transfer (NT), the transfer of one nucleus into another enucleated egg resulting in a re-constructed egg, is a method for whole-genome manipulation. Intra-species NT refers to transfer of donor cells and oocytes (eggs) from the same species. Intra-species NT has been used to study developmental plasticity and nuclear reprogramming of a nucleus and to produce reprogrammed stem cells from differentiated nuclei [19]. Although animal cloning studies have successfully used stem cell nuclei for several decades (reviewed by [6]), this approach was not widely applied until the birth of the sheep, "dolly"—the first mammal cloned from a somatic nucleus [20]. In fish, the "art" of NT was first demonstrated by Tung *et al.* in goldfish (*Carassius auratus auratus*) and bitterling (*Rhodeus amarus*) [21]. The first animal successfully cloned from a short-term cultured somatic nucleus was born in 1984. This study was originally written in Chinese [22], and was not translated and republished in English until 2010 [23]. Intra-species NT has promised significance for generating genetically manipulated fish from genetically modified *in vitro* cultured cells. The first report of successful intra-species nuclear transfer was in zebrafish (*Danio rerio*) using long-term cultured cells [24]. Interestingly, semicloning technology was recently successfully applied by using medaka (*Oryzias latipes*) haploid stem cells to conduct NT and generate haploid cloned fish [25]. However, this technique currently has a low success rate and cloned offspring often exhibit a range of defects.

If the oocytes and donor cells are derived from two different species, the NT will be defined as cross-species NT. Cross-species NT results in the combination of the nuclear genome from one species and the cytoplasmic factors from another species, so offers a range of unique possibilities in breeding programs [26]. Cross-species NT was first described in amphibians, within the genera of *Rana* and *Xenopus* [27,28]. In those studies, all the NT embryos exhibited early developmental arrest, likely due to the incomplete reprogramming of the donor nuclei and/or incompatibility between the nuclei and the egg cytoplasm. In mammals, cross-species NT has been successfully applied to cloning of endangered mammals within a few closely relat-

ed species (reviewed by Sun & Zhu [29]). The cloned animals are totally identical to their nuclear donors, highlighting the importance of the nuclear genome in phenotypic determination. In fish, however, cross-species NT between two distantly related species, which have distinct appearances or phenotypes, has resulted in some interesting and different outcomes.

In fishes, cross-species NT can be successfully conducted between distantly related species. For example, NT was conducted between two genera by combining common carp (*Cyprinus carpio,* genus *Cyprinus*) nuclei with crucian carp (*Carassius auratus,* genus *Carassius*) egg cytoplasm [26] or crucian carp nuclei with common carp egg cytoplasm [30]. In those studies, and in our recent study of cross-genus cloned common carp derived from transgenic common carp nuclei and goldfish enucleated eggs [31], the vertebral number of some cross-genus NT individuals was consistent with that of the egg-donor species, goldfish. This suggests that the fish egg cytoplasm can not only support development driven by the transplanted nuclei from a distantly related species at the genus scale, but can also significantly modulate development of the nuclear transplants. Notably, cross-species NT has also been conducted between members of two different families, such as the goldfish (*Carassius auratus*, family *Cyprinidae*, order *Cypriniformes*) and the loach (*Paramisgurnus dabryanus*, family *Cobitidae*, order *Cypriniformes*), and between two orders, such as the tilapia (*Oreochromis nilotica*, order *Perciformes*) and the goldfish, and the tilapia and the loach [32,33]. However, offspring of these cross-family or cross-order NT experiments were unable to develop to term. Therefore, current evidence suggests that cross-species NT can only be successfully applied to a few species that can be artificially hybridized. However, the success rate of cross-species NT is relatively low, so it is unlikely that cross-species NT can be used to introduce desirable traits from a distantly related species into a target species of commercial importance. Nevertheless, cross-species NT has significant potential for cloning or genetic breeding of endangered fish species.

Because of technical difficulties with the process of nuclear transfer, some studies have focused on alternative methods. Among these, cell fusion is a means of obtaining a large number of re-constructed eggs at a single time. This method has been successfully adapted to create hybrid fish with common carp nuclei and crucian carp egg cytoplasm [34]. The hybrid fish has similar morphology to the nuclear donor, common carp. Additionally, a hybrid fish was generated by fusing grass carp (*Ctenopharyngodon idellus*) hemorrhagic virus (FRV) resistant liver cells with unfertilized eggs [35]. Unfortunately, however, the resistant ability of this strain has not yet been reported. As with nuclear transfer, the success rate of cell fusion-based technologies is extremely low, which limits its application in breeding research.

3 Transgenic breeding

Recently, there has been rapid progress in functional genomic studies in a range of organisms including plants, invertebrates, and vertebrates. As a result, researchers have deduced the function of several thousand genes and evaluated the degree of conservation among different species. Because transgenic methods can be easily applied to incorporate the function of a specific gene, this approach is potentially the most direct and rapid method of obtaining a stable and genetically inherited trait in fish.

A range of gene-delivery methods have been used to conduct transgenesis in fish, including electroporation [36–38], sperm-mediation [39,40], electroporated-sperm-mediation [41,42], retrovirus [43–45], and liposome-mediated methods [46]. However, microinjection is currently the most popular method for generation of transgenic fish [47].

The first transgenic fish was generated by over-expression of humanized growth hormone (hGH) gene driven by a mouse metallothionein-1 (MT) gene promoter in Chinese goldfish [47]. Since then, *growth hormone* (*GH*) transgenic fish have been created using a range of fish species, including loach [48], common carp [49–53], channel catfish (*Silurus asotus*) [37], Atlantic salmon (*Salmo salar*) [54], and tilapia [55,56]. In most cases, the *GH*-transgenic fish grow faster and have higher feed conversion efficiency than their non-transgenic siblings, demonstrating that higher GH levels induce fish growth.

In addition to transferring growth hormone genes to promote growth rate, a number of other genes have also been successfully transferred into fish. For example, the anti-freeze protein gene (AFP) was transferred to promote cold-tolerant traits [54,57] and the lysozyme gene was introduced into Atlantic salmon to confer disease resistance [58,59]. Similarly, the cecropin B gene from *hyalophora cecropia* was inserted into channel catfish genome to increase the survival rate [60]; the human lactoferrin (hLF) gene was transferred into grass carp to promote resistance to grass carp hemorrhagic virus (GCHV) [42]; the *vitreoscilla hemoglobin* (*vhb*) gene was transferred into zebrafish to increase hypoxia tolerance [61].

Because of concerns surrounding transgenic safety and bioethics, researchers have focused on using endogenous fish genes (“all fish” transgenesis) rather than exogenous genes such as human *GH*. For example, an “all-fish” transgenic construct was re-designed by using a common carp *β-actin* promoter to drive expression of the grass carp growth hormone gene (gcGH) [52,53]. Similarly, an AFP promoter from ocean pout (*Zoarces americanus*) was linked to a Chinook salmon (*Oncorhynchus keta*) GH cDNA clone [54]. In both studies, transgenic fish had significantly higher growth rates than non-transgenic controls.

In addition to transgenesis with natural gene, molecular-

ly-designed genes have also been delivered into fish to test their viability. For example, we designed a constitutively activated growth hormone receptor (CA-GHR) gene and transferred it into zebrafish, in which two GHR molecules maintain dimerization by Jun-zippers and constitutively activate downstream signaling. The CA-GHR transgenic fish exhibit higher growth rates than *GH* transgenic fish [62]. Thus, there is considerable scope for future studies of transgenic fish to evaluate the utility of highly-activated transgenes using a "molecular design" approach.

Another application of transgenic technology was derived from synthetic biology approaches used to improve the nutritional value of fish. N3 and n6 polyunsaturated fatty acids such as Omega-3 and Omega-6 are beneficial to human health, and particularly important for brain and retina development [63]. Fish-derived desaturase and elongase were transferred to develop n3 polyunsaturated fatty acid (PUFA) and n6 PUFA rich fish strains [64–66]. However, the production of high levels of n3 or n6 PUFA is dependent on the level of n3 or n6 PUFA in the diet. Thus, feeding costs are increased because of the need to supplement diets with extra n3 or n6 PUFA. Recently, *de novo* LC-PUFA biosynthesis was induced in zebrafish by using fat1 and fat1/fat2 double transgenic fish, resulting in robust n-3 LC-PUFA production even when using low PUFA food [67]. Transgenic fish can also be used as a bioreactor. Several studies have used zebrafish eggs to produce recombinant proteins such as human coagulation factor VII [68], luteinizing hormone [69] and insulin-like growth factors [70].

To obtain multiple traits-related genetic improvement within one fish species, researchers could use of 2A peptides to combine those traits, instead of outcrossing between different transgenic strains. 2A peptides allow for more efficient expression of multiple genes, separated by a 2A sequence, within the same cell. This approach has been used in fish [71], mice [72], and pigs [73]. Introducing 2A peptides into a transgene construct allows for incorporation of multiple desirable characters and significantly shortens the breeding process by combining two or more phenotypes.

Genetically modified animals should be subject to ecological safety assessment because of the risk of escape [74]. Evaluation and analysis should be conducted on a case-by-case basis. In addition to an intensive evaluation of the impact of GM fish on other species [75], the production of sterile triploid transgenic fish can reduce such impacts [76,77]. At least two transgenic fish are close to being market-ready. *Growth hormone* transgenic salmon produced by the AquaBounty Technology Company have been submitted to the Food and Drug Administration (FDA) for approval (http://www.fda.gov/AnimalVeterinary/DevelopmentApprovalProcess/GeneticEngineering/GeneticallyEngineeredAnimals/ucm280853.htm). Similarly, sterile triploid *GH*-transgenic carp are close to satisfying regulatory requirements [77].

4 Genome editing

In recent years, there has been rapid development of targeted nuclease technologies, such as ZFNs (zinc finger nucleases) [78], TALEN (transcription activator-like effector nucleases) [79,80], and CRISPR (clustered regularly interspersed short palindromic repeats)/Cas9 [81–83]. These methods can be used for targeted knock out or targeted genome editing, and they have been applied to quite a few species including zebrafish. Compared to ZFNs and TALENs, the components of the CRISPR/Cas9 system are much simpler but the CRISPR/Cas9 system can achieve similar or even higher efficiencies. Furthermore, the time to prepare constructs for CRISPR/Cas9 is significantly shorter than for the other two. As a result, the CRISPR/Cas9 system has become widely adopted by researchers. The CRISPR/Cas9 system uses a short guide RNA, which contains an ~20-bp target sequence, to bind to its complementary DNA target and direct the Cas9 nuclease to the target site to make double-strand breaks (DSBs). The DSBs are typically repaired by either homology-directed repair (HDR), which results in precise genome editing if a exogenous DNA repair template exists, or non-homologous end-joining (NHEJ), which usually results in indel mutations [84]. To date, there have been limited reports documenting the successful application of this method for directional breeding purposes. For instance, the Celtic POLLED, a non-horned allele, was introduced into the genome of horned dairy cattle breeds, and the endogenous horned POLLED allele was removed by TALEN specific cleavage to obtain the non-horned trait [85]. Several genes were edited in pig, sheep and cattle, in order to obtain virus-resistant or growth-enhanced characteristics in those livestock species [86,87]. In fish, using zebrafish as model and using TALEN technology, we knocked out *socs2* which belongs to the SOCS superfamily—the major negative regulators of the GH signaling pathway. The mutation of *socs2* resulted in increased stimulation of the GH signaling pathway and the zebrafish mutant had higher growth rates during early larval stages [88].

Single stranded oligonucleotides are an effective repair template for HDR via a single strand annealing (SSA) mechanism. Co-injection of targeted nucleases and single stranded DNA was successfully used to introduce single nucleotide alterations in zebrafish and mice, an approach that is comparable to the occurrence of single nucleotide polymorphisms (SNP) in nature [79,89]. This method can also be used to introduce a small DNA fragment such as the HA (Hemagglutinin) tag or loxP sequence at specific sites [79,81,90]. However, off-target effects are common and are caused primarily by NHEJ repair. Instead of constructing DSBs with the general Cas9, a recently improved Cas9 can create single-strand breaks by point mutating the RuvC or HNH nuclease domains on the Cas9 [91–93]. The use of

mutated Cas9$^{HNH+/RuvC-}$ along with pairs of guide RNAs allows efficient indel formation while reducing off-target effects and improves specificity by up to 1500 fold when compared to the general Cas9 [94,95].

TALEN mediated homologous recombination (HR) has been successfully applied in zebrafish [96]. CRISPR/Cas9 mediated HR has been successfully applied in *C. elegans* [97], *Drosophila* [98−100], and even human cells [101,102]. Although, targeted nucleases have largely improved the efficiency of HR, the rate of HR is less than satisfactory. To address this, studies have shown that inhibition of NHEJ by disruption of specific genes involved in the NHEJ process significantly increase the efficiency of HR [103−107]. A number of studies have shown that homology-directed DNA repair occurs primarily in somatic tissue, which makes the screening process longer and more costly. The ability to detect germ cell specific HDR in F0 and subsequent generations would save significant effort screening individuals. We generated a primordial germ cell (PGC) specific manipulation system based on UAS/Gal4 and Cre/LoxP in zebrafish, a tool that may prove useful to fill this gap [108].

The recently developed genome editing techniques allow researchers to modify multiple genes at precise sites with high efficiency and in a comparably short time [109]. These characteristics make the approach suitable for improving aquaculture strains. More importantly, the process is based on homology-directed DNA repair so does not bring in any foreign DNA elements, but instead modifies the endogenous DNA itself. Therefore, when combined with PGC specific manipulation and conventional genome manipulation techniques (such as polyploidy manipulation), this technique should make fish breeding (and other animals) more efficient, more precise and more predictable.

5 Conclusion

The development of scientific technology accelerates scientific research and turns “impossible” into “possible”. Although the commercialization of transgenic fish faces significant non-scientific concerns, there is currently no conclusive evidence of a safety problem associated with commercialized genetically modified organisms (GMO). Nevertheless, there is a need to conduct a careful and long-term evaluation before authorizing GM animals into the commercial market. In addition to transgenesis, recently developed genome editing techniques provide an enormously valuable tool for fish breeding. In the near future, the introduction of genome editing into conventional fish breeding will allow researchers to directly and precisely improve specific traits without affecting other traits. Because this approach no longer uses exogenous gene fragments, but instead modifies the genetic information itself in a minimal manner, it deserves to play a major role in the future of fish genetic breeding and the breeding of other animals.

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