Chapter 11

Principles of genome editing and its applications in fisheries

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1. Introduction

Aquaculture is one of the fastest-growing food-production sectors and is speedily establishing itself as the chief source of seafood for human and animal nutrition. Selective breeding techniques allow for genetic improvement and production attributes like resistance to disease, but improvement is limited due to the trait heritability of genes and the species' generation gap. Clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9)−mediated genome editing (GE) technology offers one of the most effective ways to speed up genetic gain in aquaculture. GE can make advantageous alterations to the genome at a quicker rate, producing new alleles, integrating alleles from diverse species or strains, or correcting alleles at trait governing loci. Most aquaculture species have great fertility, and external fertilization can facilitate GE for study and application conceivable at a scale not feasible in cultured land mammals.

Food security, along with an increasing need for high-quality animal protein, is a major and growing concern. Crop and animal production will be severely limited due to competition for land utilization [1]. As a result, aquaculture is projected to play a major part in satisfying this expanding nutrition and food requirement. Upscaling and increasing production dependability will necessitate disruptive technologies in engineering, health, nutrition, and genetic enhancement.

Better use of existing genetic resources is required for the development of more effective aquaculture. This additionally includes substantial genetic information in selective breeding methods like marker-assisted breeding. Genome modification like insertion, deletion, or single base alteration is all possible with GE tools [2]. GE technologies opened up several potential possibilities for improving aquaculture stocks, with implications for long-term sustainability and efficiency. Zinc finger nucleases (ZFNs) and transcription activator−like endonucleases (TALENs) were among the earliest GE technologies used. CRISPR-Cas technology, the most recent GE technique, now reigns supreme. This option enables modifications to be made by engineering alleles and trait loci. The CRISPR technology is now recognized as a powerful, most precise, easy-to-use, and cost-effective GE technology [3]. In this chapter, we briefly discuss the CRISPR-Cas−mediated technologies available for performing diverse kinds of GE, review their current status of applications in aquaculture, and highlight the challenges to use those technologies.

2. Genome editing technologies

Researchers are tirelessly working toward gaining the capability to change genomic information since the discovery of DNA structure and function. Restriction endonucleases, ligases, and polymerases are enzymes that allow breaking, ligating, and synthesizing DNA, respectively. Endogenous mechanisms in cells are available to fix DNA strand breaks. The core for GE is the ability to generate breaks at a preferred sequence [4]. Several approaches for changing DNA have evolved in the last 20 years, including nuclease-mediated site-specific mutagenesis and oligonucleotide-directed mutagenesis procedures. The four types of site-directed nucleases are meganucleases, TALEN, ZFN, and CRISPR-Cas [5] (Fig. 11.1). ZFN is constituted of modular DNA recognition and binding proteins combined with FokI nuclease. The complex can be designed to detect certain genomic regions of 9−18 nucleotides, and the FokI enzyme can generate double-strand breaks (DSBs) during dimerization [6]. The use of ZFN was impeded by difficulties in designing,

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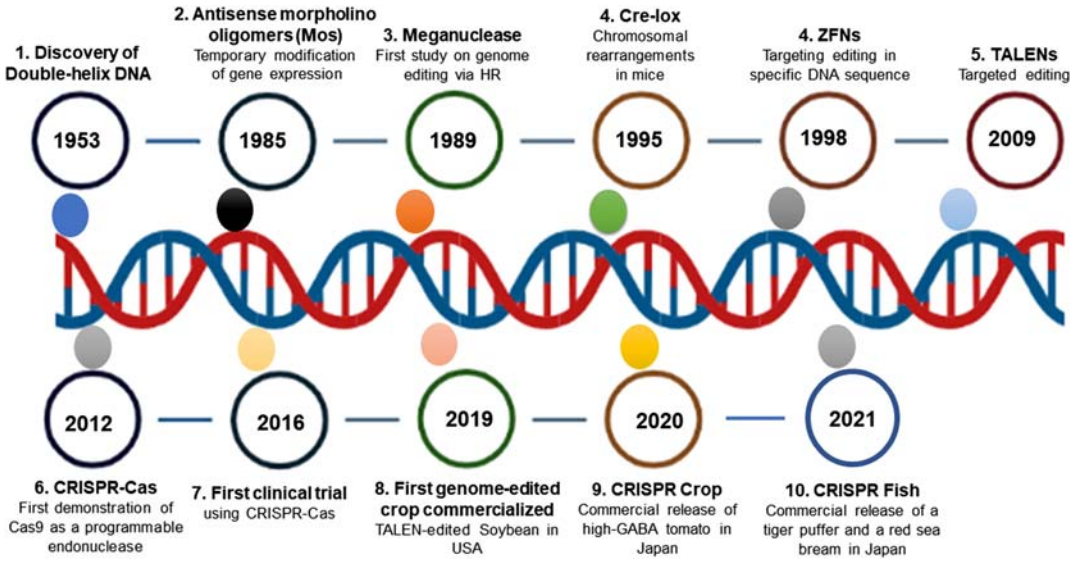


FIGURE 11.1 History and advances of genome editing technologies. CRISPR, clustered regularly interspaced short palindromic repeat; Cas, CRISPR-associated protein; HR, homologous recombination; TALENs, transcription activator–like effector nucleases; ZFNs, zinc finger nucleases.

constructing, and validating. Due to these difficulties, TALEN, a novel tool, was developed in the year 2010–11 [7]. TALEN is though easier to validate and design than ZFN and recognizes fewer nucleotides, making it better than ZFN. However, validation, design, and synthesis of proteins are still cumbersome, limiting the use of this technology.

CRISPR-Cas GE technology, the most recent breakthrough, has been developed by repurposing a prokaryotic immune system [8]. This RNA-mediated mechanism detects and silences alien nucleic acids based on their sequence. Bacterial cells harbor CRISPR arrays consisting of genome-targeting sequences (spacers) interspaced with similar repetitions and Cas enzymes coded in operons. The repurposed CRISPR-Cas9 is a two-component system for GE. The first one is a small guide RNA (gRNA) and the second one is an RNA-directed DNA nuclease, Cas9. The gRNA directs the Cas9 to the specific target DNA sequence to bind and create DSB.

ZFN, TALEN, and CRISPR-Cas systems all can cause targeted DSB at a specific site in DNA. The formation of DSB activates the cell's own repairing systems, viz. nonhomologous end joining (NHEJ) or homology-directed repair (HDR) (Fig. 11.2) [9]. NHEJ is an error-prone repairing process that often leads to alteration of the DNA sequence in the form of deletion, insertion, or substitution of nucleotides [10].

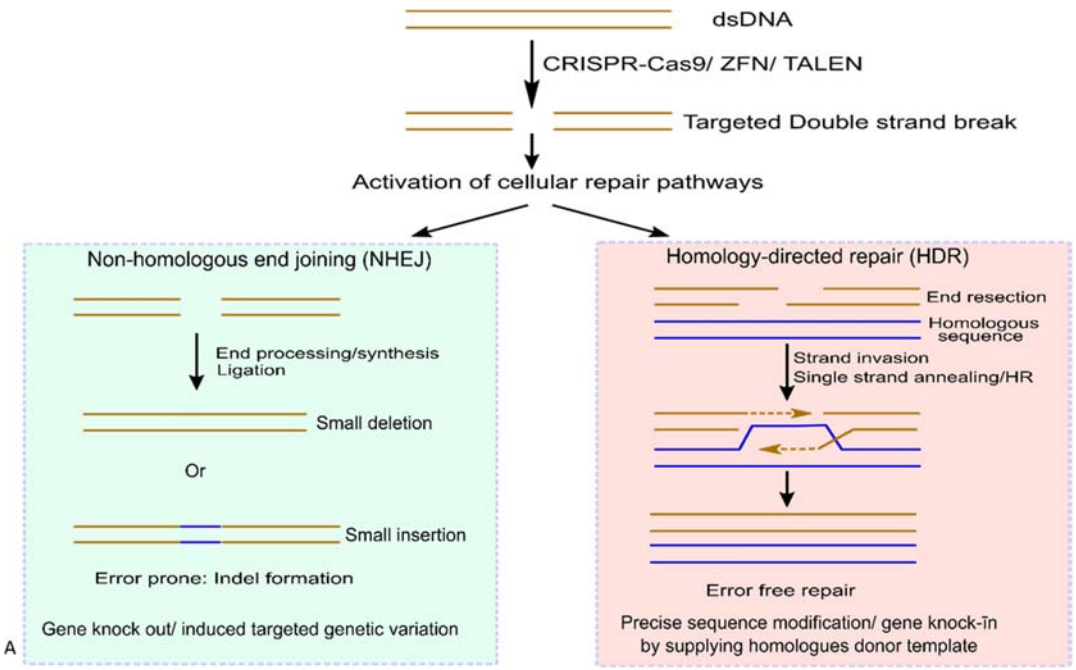
HDR is activated when a donor DNA strand homologous to the flanking sequences of DBS is supplied (Fig. 11.2). The type of insertion, substitution, or restoration of a sequence within DBS is defined by the type of donor. These mutations result in either knock-in (KI) or knockout (KO) of DNA sequence or gene.

To fill the vital gap in precision editing, base editing and prime editing technologies have recently been developed and demonstrated in numerous organisms. These technologies, which are mainly based on Cas9 nickases, can introduce precise changes into the target genome at a single-base resolution [11]. Three types of DNA base editing tools are currently available, viz., cytosine base editor (CBE), adenine base editor (ABE), and C-to-G base editor (CGBE). CBEs were developed to enable C-G to T-A base transition, while ABEs were developed for performing A-T to G-C transition [12–15]. Recently, transversion base editors in the form of CGBE have been developed by several groups to perform C to G base editing [16]. Altogether, these three kinds of base editors present great resources for the precise installation of point mutations in the genome.

For precise insertion and deletion in the genome, a recent GE technology, prime editing, has been developed [17]. Prime editing requires a modified gRNA called prime editing guide RNA and can perform all 12 types of single nucleotide changes and small insertions and small deletions in a precise and targeted manner (Fig. 11.2). However, prime editing needs further improvement to increase the editing efficiency [11,18].

3. Genome editing in aquaculture: an up-to-date status

Currently, GE technology offers plenty of opportunities in aquaculture (Fig. 11.3). Several aquaculture species of Salmonidae, Siluridae, Cyprinidae, as well as gilthead sea bream, Nile tilapia, and Pacific oyster have recently been subjected



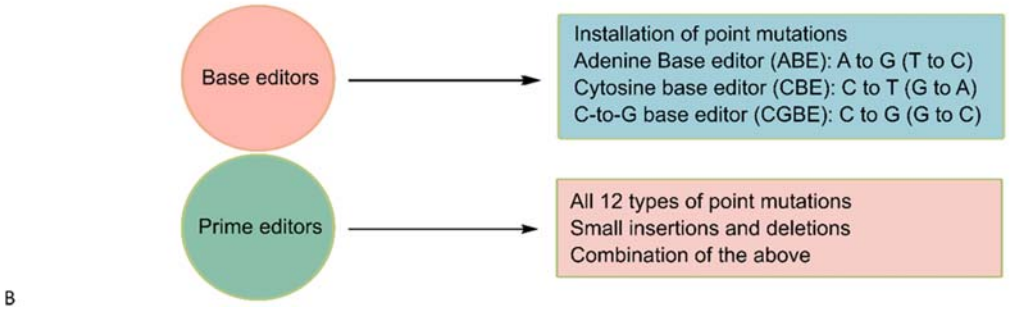


FIGURE 11.2 A, DNA double-strand break (DSB) by CRISPR-Cas or ZFN, or TALEN leads to the activation of cellular repair pathways. NHEJ leads to indel (insertion or deletion) formation causing frameshift mutation and gene knockout. With an additional supply of donor templates, precise modification could be incorporated at or a nearby location of DSB by utilizing the HDR pathway. B, The spectrum of mutations that can be installed with base editors and prime editors. CRISPR, clustered regularly interspaced short palindromic repeat; Cas, CRISPR-associated protein; TALEN, transcription activator–like nuclease; ZFN, zinc finger nuclease.

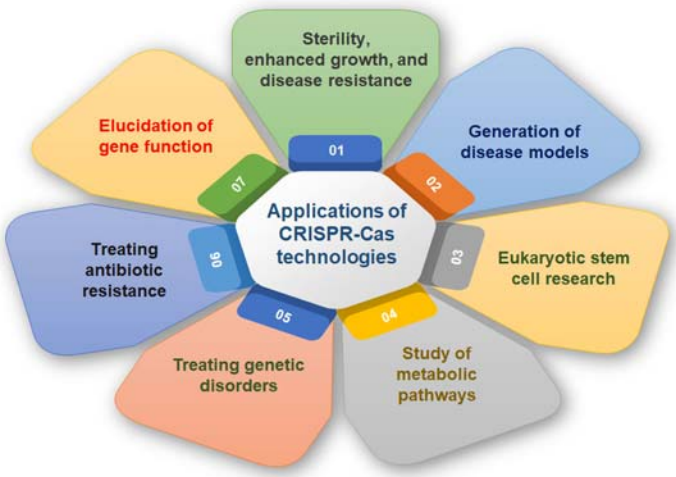


FIGURE 11.3 Application of CRISPR-Cas technologies in basic research and aquaculture. CRISPR, clustered regularly interspaced short palindromic repeat; Cas, CRISPR-associated protein.

to CRISPR-Cas9–based GE in cell lines and/or in vivo (Table 11.1). Successful CRISPR-Cas9–based genome engineering in shrimp (*Penaeus* sp.), one of the primary groups of aquatic organisms, is yet to be documented, which could be due to practical constraints, as addressed briefly below. The majority of the studies were prototypes and applied CRISPR-Cas9 in model organisms like *Danio rerio* (zebrafish) [19]. Most of the studies usually targeted genes with obvious phenotypes to evaluate the effectiveness of editing (e.g., pigmentation). Recently, the CRISPR-Cas9 tool has been used to generate stable and heritable red tilapia phenotype by targeting the *slc45a2* gene [20]. In aquaculture species, the conventional strategy for carrying out in vivo mutagenesis is to inject CRISPR-Cas9 reagents into freshly fertilized eggs at one-cell growth or a near possible stage. In general, mRNA encrypting Cas9 is inserted simultaneously along with gRNA, resulting in high editing efficiency that has been confirmed in various species (Table 11.1); using Cas9 protein in place of mRNA has been also effective [21]. Although, in most of the experiments, NHEJ has been used to make mutations, HDR method has been effectively employed in rohu carp by inserting a template DNA [38]. In addition, GE of germline transmission has been described in various studies so far (Table 11.1). Because of widespread mosaicism in edited animals, it has been suggested that Cas9-induced cutting and GE extend beyond the one-cell stage; it is considered as an important issue and needs to be looked after in future research [40].

In aquaculture species, GE has previously been used to target traits like infertility, growth, and resistance to diseases. CRISPR/Cas9-based GE has also been worked out to generate infertility in catfish and Atlantic salmon in order to prevent gene flow with wild stock and to prevent negative production impacts of early maturation [40]. The myostatin gene (known for its main role in double-muscled cattle) has been edited by several groups, resulting in better fish with improved growth-related traits [40]. Immunity responses in Grass carp and Rohu have already been examined via GE, and this area of research is expected to expand as a way of enhancing and comprehending disease resistance. For example, the purposeful mutation of the *TLR22* gene in carp demonstrates how GE might be used [38]. Such simulations can aid in a better understanding of the fish's host reaction to disease, potentially leading to more successful treatment regimens. In a similar manner, GE technologies can be used to improve fish cell lines by wiping off important

TABLE 11.1 Demonstrated cases of genome editing in aquaculture species.

Species	Trait of interest	Target gene	References
Channel catfish, *Ictalurus punctatus*	Growth	*mstn*	[21]
	Sterility	*LH*	[22] and [23]
	Immunity	*ticam1/rbl*	
Tilapia, *Oreochromis niloticus*	Reproduction	*dmrt1/nanos2-3/foxl2*	[24]
	Reproduction	*sf-1*	[25]
	Reproduction	*wt1a/wt1b*	[26]
	Reproduction	*gsdf*	[27]
	Reproduction	*aldh1a2/cyp26a1*	[28]
Atlantic salmon, *Salmo salar*	Omega-3 metabolism	*elov2*	[29]
	Sterility	*dnd*	[30]
	Pigmentation	*tyr/slc45a2*	[31]
Red sea bream, *Pagrus major*	Growth	*mstn*	[32]
Common carp, *Cyprinus carpio*	Muscle development	*sp7a/sp7b/mstn(ba)*	[33]
Pacific oyster, *Crassostrea gigas*	Growth	*mstn*	[34]
Rainbow trout, *Oncorhynchus mykiss*	Growth	*2b1/2b2*	[35]
Grass carp, *Ctenopharyngodon idella*	Disease resistance	*gcJAM-A*	[36]
Southern catfish, *Silurus meridionalis*	Germ cell development	*cyp26a1*	[37]
Rohu carp, *Labeo rohita*	Immunity	*TLR22*	[38]
Northern Chinese lamprey, *Lethenteron morii*	Pigmentation/development	*slc24a5/kctd10/wee1/soxe2/wnt7b*	[39]

constituents of interferon pathway mechanisms, for instance, to enable efficient virus output for subsequent vaccine manufacturing. Two pragmatic reasons why genetic modification has huge potential for research and applications in farmed fish are the large size of their embryos that permits microinjection by hand and the availability of access to many thousands of externally fertiliz. The ability to use enormous nuclear families provides for some control over background genetic influences as well as relatively large sizes for assessments of successfully transformed individuals with their full-sibling counterparts later on. It is also typical to be able to perform extensive phenotyping during the early stages of life, such as using well-built illness experiment prototypes so to evaluate defense mechanisms for a number of bacterial and viral infections. Finally, if promising alleles for a specific feature (e.g., resistance against disease) are generated or identified, the enhanced germplasm may be widely disseminated for speedy impact through the above mentioned selective breeding programs. Well-interpreted, high-quality reference genomes are also known for most of the significant species. Given the prevalence of recent whole-genome duplication events in diverse finfish lineages, including salmonids, for successful target gRNA design with discernment and little off-target editing, a superior species-specific reference genome is a prerequisite [41]. Among many constraints, infectious diseases are of the greatest dangers to aquaculture's long-term existence, with an assessed of 40% of total potential productivity loss each year. Due to preliminary stages of taming of many aquaculture species, new option and disease constrains may upsurge the probability that standup genetic variability in populations has to include loci of key effect, which may signify potential "low-hanging fruit' for GE to surge the rate of the advantageous allele. GE unlocks new exciting possibilities for aquaculture sustainability and productivity. The three major categories in which GE technologies might be used to improve genetics, each one necessitates a unique approach for the study that leads to the finding of operational alleles, are as follows: (1) deleting, detecting, boosting, or solving specified functional sets of genes at multiple or single quantitative trait locus (QTL) segregating within a selective breeding program's current brood stock populations; (2) directed introgression via editing favorable variations from diverse species, strains, or populations to improve or implement novel features in a population; and (3) energy production using de novo useful alleles that have not been seen previously and are used to initiate or improve new features.

4. Harnessing causative variants of quantitative trait locus

The majority of animal breeding and genetics research tries to uncover and apply causal variants for QTL that impact production characteristics; however, there have been few successes. Simulations suggest that employing editing for advantageous causal alleles at many QTLs as part of program can accelerate genetic gain when compared to genomic selection or pedigree alone. The effective identification of functional dissimilarity beneath QTLs, especially those of modest importance, is nevertheless a major stumbling block to the strategy's effective implementation [40]. To do so, researchers can employ a number of functional genomic technologies to limit down a bunch of candidate variants discovered in vast genome-wide association studies. GE could play a major role as functional genomics tools in this regard. Large effect deviations and polygenic deleterious burdens, which are invariably found in populations, could be eliminated using a similar method. Moreover, to have a significant effect with this strategy, many alleles must be edited at once in the same broodstock animal(s), necessitating the improvement and development of multiplex GE strategies.

5. Mimicking beneficial genes/alleles from unlike species or strains by genome editing

One of the most incredibly interesting applications of GE is the ability to acquire genetic variation outside of restricted breeding populations devoid of any need for time-consuming and expensive introgression efforts, or in situations where introgression is inconceivable [40]. CRISPR technology could be used to edit the negative or lethal genes in the target species and/or strain to complement the sequence of the advantageous or nonlethal gene in a related species and/or strain. GE opens up new ways to avoid conventional means of introgression while also preventing the disadvantages of linkage drag (e.g., the deleterious effects of introgression genes from wild strains on growth rate) and permitting utilization of genetic variability in other species and strains that would not be conceivable by means of traditional methods. Parasitic copepod sea lice (*Caligus rogercresseyi* in the Southern Hemisphere and *Lepeophtheirus salmonis* in Northern Hemisphere) cripple Atlantic salmon aquaculture, causing a loss of USD 880 million per year. Aquaculture is distinguished by the close vicinity of farmed grown species to existing wild populations that may have advantageous features. Coho salmon (*Oncorhynchus kisutch*) and pink salmon (*Oncorhynchus gorbuscha*) are tolerant to sea lice attacks and proved to have an efficient immune reaction to the parasite [42]. This tends to raise the exciting prospect of trying to transfer host defenses to

Atlantic salmon, and an extensive effort has been made by scientific community to figure out the reasons governing dissimilarities of resistance mechanisms. It is likely that the major regulatory genes in the pathways that promote species-specific resistance may be altered by GE in Atlantic salmon to mimic the Coho salmon's reaction to sea lice [40]. Targeted GE of coding area and/or alteration of the regulatory sequence may be employed.

6. Creating new variants from scratch based on trait knowledge

Since GE is centered on the known genetic variation and has already made a contribution to possible outcome solutions regarding quality, production, and welfare issues of animals, generating completely novel favorable alleles (distinctive from any naturally existing alleles) is another exciting avenue [40]. Newer interesting alleles can be developed by employing CRISPR/Cas9 system based on pre-existing knowledge of the genetics of character governing genes of interest, or candidate genes can be discovered using genome-wide genetic interruption approaches. Alteration of the *dnd* allele to cause sterility in Atlantic salmon and aiming the *mstn1* gene in various fish species to increase growth have both been utilized in aquaculture (Table 11.1).

CRISPR screens for resistance to disease through genome-wide CRISPR KO strategy is one of the most potent strategies to identify variants responsible for disease-causing or disease-resistant mutations. However, to apply CRISPR screens, well-established cell lines need to be available.

A key barrier in aquaculture research lies in the lack of viable, well-tested and characterized cell lines for several concerned species. Cell lines from chinook salmon, medaka, and carp have been recently transformed with CRISPR/Cas9 reagents with positive outcomes. Virus-related infections are high-priority target features for in vitro research using CRISPR/Cas9. This is because of innate mechanisms of host response that are generally cell intrinsic and therefore feasible to examination. By means of such technologies, it would be simpler to combine large-scale genetic screens to learn more about the genetics of resistance to diseases and generate a pipeline of candidate genes/alleles identification for profitable and commercial aquaculture breeding.

7. Challenges of applying genome editing

To optimize the prospect for using GE systems in aquaculture species, a number of significant technological challenges must be overcome. Firstly, in species where CRISPR/Cas9 has been used, technique enhancement is desired to increase editing efficiency and minimize off-target effects and mosaicism in the F_0 generation. Off-target editing could cause unwanted and nonspecific genomic modifications and could have unanticipated consequences for the organism. Along with targeted sequencing of predicted off-target genomic sites, the cost-effective whole-genome resequencing can allow regular screening for off-target editing. Improved information on sequences of aquaculture species will help to develop unique gRNAs to a precise targeted region. To combat mosaicism, Cas9 proteins with limited half-life have been developed to cause DSBs exclusively during fertilized embryo's one-cell stage of development [43]. It is also plausible to screen for most often generated mutations in F_1 crossings between mosaic F_0 mutants to recognize animals with a homozygous single mutated gene/allele (reasonably than mosaic for more than one edited gene/allele). Base editing, prime editing, and HDR would enable precision editing in F_0 generation, for example, SNP exchange, to generate a premature stop codon or change the amino acid, or install small insertions and deletions of interest. Cleavage efficiency of gRNAs can be evaluated in vitro or in cell culture before in vivo editing to choose the best among in silico—designed gRNAs [44]. Alternative methods of CRISPR/Cas9 delivery such as editing primordial germ cells, microinjection of unfertilized ova, and sperm-mediated transfer could be tested, especially for species where access to newly fertilized embryos is exaggerating, for example, in some shrimp species [40]. Germ cell replacement in animals with a selective edit into numerous sterile surrogates is an alternative way to improve edited alleles of concern [45].

8. Commercialized CRISPR-edited fish

Recently, the sale of two CRISPR-edited fish has been approved in Japan [46]. Genomes of a tiger puffer and a red sea bream were edited by the Kyoto-based company, Regional Fish Institute, in collaboration with Kyoto University and Kindai University. The genome-edited fish grow larger than wild-type counterparts. Deleting the leptin receptor gene, which controls appetite, leads the tiger puffer to consume more and gain weight faster. On the other hand, the *myostatin* gene was disabled in red sea bream to increase muscle growth. The study reported that the tiger puffer grows 1.9 times and red sea bream grows 1.2 times larger than their natural counterpart on the same amount of food.

9. Concluding remarks

Aquaculture species have high fecundity and external fertilization provides excellent possibilities for high-resolution genetic studies for better understanding and improving complex features. CRISPR/Cas9 and other GE technologies have the ability to gear up genetic gain for desired production traits. Infectious disease is one of the important constraints to aquaculture production and productivity, making it a prime goal for GE and selective breeding techniques [40]. Several types of GE applications are possible, which include the following: (1) discovery of contributing variations underlying one or many QTLs affecting attributes of importance, and installing favorable alleles through editing; (2) introgression of beneficial genes from neighboring populations, strains, or species into closed breeding systems through editing; and (3) creation of new alleles with beneficial properties on the trait of interest and subsequent use [40]. Along with the canonical CRISPR-Cas9, advanced tools like base editing and prime editing would greatly facilitate precise genetic modifications for unraveling gene functions and developing the desired trait in fish. Nevertheless, the availability of those tools would unfold new research opportunities that in turn would benefit the sector in long term.

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