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CRISPR/Cas 9 Genome Editing: Current Trends and Future Prospects in Fisheries

Sukriti M[1], Sourya D[2], Pritam M[1], Yaqoota M[1] and Chandan H[1]*

[1]Department of Fish Genetics and Reproduction, College of Fisheries, Central Agricultural University, India
[2]Aquatic Environment and Health Management Department, ICAR-Central Institute of Fisheries Education, India

***Corresponding author:** Chandan Haldar, College of Fisheries, Central Agricultural University (Imphal), Lembucherra, Tripura-799210, India, Tel: 8779934142; Email: chandanbiotech14@gmail.com

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Abstract

The fisheries sector is a leading player in ensuring food security and contributes to global nutrition. The genome editing technique can enhance aquaculture to meet ever-growing demands and improve fish production's efficiency and sustainability. Older tools, including meganucleases, zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), were used for genetic improvement for decades. CRISPR/Cas9 technology is applied in both diagnosis and therapeutics. The CRISPR/Cas system is popularly known for providing adaptive immunity in bacteria and archaea, is an effective tool for editing genes, and relies on two components: guide RNA (gRNA) and CRISPR-associated protein 9 (Cas-9). The editing mechanism involves three primary steps: recognition, cleavage, and repair. In 2020, Emmanuelle Charpentier and Jennifer Doudna became the first women to be awarded the Nobel Prize for discovering the CRISPR/Cas9 system. CRISPR has broad applications in areas like agriculture, biotechnology and medicine. In agriculture, it can help design new grains with higher nutritional value. In the medical field, it can be used for the detection of HIV and cancers and in gene therapy for sickle cell disease, Duchenne muscular dystrophy and cystic fibrosis. This technology has been utilised in fisheries to develop faster-growing strains of red sea bream, tiger puffer fishes and FLT-01 Nile tilapia. Disease-resistant strains were developed through CRISPR editing in grass carp and farmed rohu. Additionally, this tool has enhanced fish colouration by editing pigment-related genes, as in Nile tilapia, and optimised reproductive traits like fecundity and sex ratios. It prevented the inbreeding and trait reversal issues, with notable successes in sterile Atlantic salmon and all-female common carp populations. This article provides insight into the history of the discovery of CRISPR/ Cas9 technology and focuses on present status and future prospects in fisheries.

Keywords: CRISPR/Cas9; Genome Editing Technologies; Aquaculture; Gene Therapy; Fish Biotechnology; Desirable Traits Enhancement

Introduction

Aquaculture and fisheries have advanced as major sectors in global food production, providing much-desired resources for supplying seafood and quality protein to people worldwide, thus ensuring a higher standard of human nutrition. The total world fisheries and aquaculture production reached 185.4 million tonnes in 2022, with a total aquaculture

production of 94.4 million tonnes. In India, the total aquaculture production in 2022 reached 10,230 thousand tonnes [1]. Despite this growth, the fisheries sector faces challenges regarding disease outbreaks and environmental stressors, which hinder productivity and sustainability. This problem can be eliminated with the application of CRISPR/Cas technology. The advancement of CRISPR/Cas9 genome editing technology has boosted improvement in genetics, surpassing conventional techniques in accuracy and affordability. Many aquaculture species are well-suited for CRISPR/Cas9 applications due to their distinct biological traits, which include high fecundity, external fertilisation, short generation intervals, and well-established breeding procedures. Successful examples include the FLT-01 Nile tilapia created by AquaBounty, categorised as a genetically modified organism under laws, and the red sea bream and tiger puffer fish, which have gained regulatory approval and have become commercially accessible.

In genetic engineering, the targeted nucleases are considered a key tool that helps scientists make precise alterations in an organism's DNA with high accuracy. The four major types of innovative techniques have evolved, each with extraordinary mechanisms and novel applications. These include meganucleases, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and CRISPR/Cas9 (Table 1). Meganucleases, ZFNs and TALENs were early methods of genome editing.

Meganucleases are engineered naturally occurring restriction enzymes that create double-strand breaks by binding to specific large DNA sequences. However, meganucleases could not be reprogrammed for novel DNA targets, making them less versatile and adaptable for diverse applications [2]. This can lead to the insertion or deletion of the DNA segment, resulting in targeted genetic modifications [3]. Zinc Finger Nucleases (ZFNs) consist of zinc finger motifs that recognise and bind to specific DNA triplets with a FokI nuclease domain that cuts the DNA, resulting in double-strand breaks. These breaks are repaired by the cell through non-homologous end joining (NHEJ) or homologous recombination (HR) [4-6]. Regardless of their specificity, ZFNs need significant protein engineering for each target sequence, thus increasing the complexity, costs and risk of off-target effects [7]. TALENs resemble ZFNs and are composed of transcription activator-like (TAL) effectors that recognise DNA, fused to the FokI nuclease domain [9,10]. Even though TALENs provide higher target precision, the large protein size often obstructed their target, making their delivery into the cell difficult and constructing their new target challenging [8]. The spacer region is approximately 4-7 bps in the case of ZNFs, while the spacer region of TALENs is 12-21 bps [11].

Currently, CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) technology is preferred over other methods as CRISPR provides a more straightforward process. In contrast, the different techniques demand complex protein engineering for every distinct DNA target. Unlike ZFNs and TALENs, which utilise protein domains to recognise the target DNA sequences, CRISPR/ Cas9 uses both RNA and protein-based DNA recognition. These technologies have permitted scientists to modify genes precisely, thus revolutionising both agricultural and medical fields. Therefore, these tools improve human health and enhance food security for future generations. CRISPR and its associated protein Cas9 have been recognised as the market's most potent, effective, and accurate tool [12]. Its ability to make precise genetic changes explains its extensive application in many scientific domains.

Criteria	Meganucleases	ZFN	TALEN	CRISPR/Cas9
Origin	Bacteria/ archaea/ eukaryotes	Eukaryotes	Bacteria	Bacteria/ archaea
Length of recognised DNA target	14-40 bp	18-36 bp	24-40 bp	19-22 bp
Off-target effect recognition	Low	High	Low	Variable
Design simplicity	Difficult	Moderate	Slightly complex	Easy
Delivery	Easy	Easy	Difficult	Moderate
Mechanism of recognition of target DNA	Protein-DNA interaction	DNA-Protein interaction	DNA-Protein interaction	DNA-RNA interaction
Mechanism of DNA cleavage and repair	Double-stranded break	Double-stranded break by Fokl nuclease	Double-stranded break created by Fokl nuclease	Single or double-strand break created by Cas9 nuclease

Cost	Low	High	Moderate	Low
Efficiency	High	High	High	High

Table 1: Comparison between different genome editing tools: Meganucleases, ZFN, TALEN and CRISPR/Cas9.

Aquaculture provides food security to the expanding world's population. Aquaculture's capacity to satisfy the need can be substantially boosted by using CRISPR/Cas9 gene editing to increase efficiency and sustainability in fish production. CRISPR technology can make nutrient-rich foods more accessible and inexpensive globally by enhancing characteristics that promote improved growth and disease resistance [13]. Using programmable nucleases as molecular scissors led to the advancement of genetic engineering methods, enabling precise DNA changes in living organisms by adding, removing, or changing specific sequences [14]. Therefore, scientists can precisely alter the genetic material of an organism to produce desirable characteristics.

Recent studies on CRISPR/Cas9 have displayed that this technology has been successfully applied to various fish species, especially freshwater fish in aquaculture, such as Nile tilapia [15], common carp [16], channel catfish [17], southern catfish, rohu [18], grass carp [19], and rainbow trout. It made it possible for the researchers to remove germ cells that are responsible for reproductive cell sex differentiation in Atlantic salmon [20], hastened the growth rate in yellow catfish by improving the feed conversion ratio [21], accomplished effective gene mutations in tilapia, and reduced the off-target effects [22], alter pigmentation pathways, for improving the colour in ornamental fishes such as goldfish and common carp [23,24]. The CRISPR technology has enormous advantages, yet some demerits comprise a nonsupervisory framework and public discernment towards genetically modified organisms. To alleviate these problems, one needs to address the boon and safety of CRISPR-edited fish to ensure proper communication. Overall, incorporating CRISPR/Cas9 technology into fisheries has enhanced food security through sustainable practices.

History

CRISPR (Clustered regularly interspaced short palindromic repeats) was first identified in the *E. coli* genome by Ishino, et al. [25] from Osaka University, Japan, in 1987 while exploring the gene that converts alkaline phosphatase isozyme [25]. The significance and biological function of these sequences were unknown at that time. However, researchers suggested that the information contained within CRISPR loci could be leveraged in medical research, particularly for genotyping various bacterial strains such as *Mycobacterium tuberculosis* [26] and *Streptococcus pyogenes* [27]. This led to the discovery that CRISPR loci exhibit a high degree of polymorphism among different strains of the same bacterial species, facilitating strain identification under various chemical conditions. CRISPR/Cas technology evolved through various historical events (Figure 1).

Another such event involving recognition of the biological role of CRISPR came from the University of Alicante (Spain) by Francisco Mojica, which led to a critical advancement of CRISPR. He discovered a similar structure in archaea's genome, *Haloferax mediterranei,* in 1993 [28] but detailedly described it in 1995. Mojica was the first to report that these unusual loci in the genome of archaea and bacteria represented fragments of foreign DNA that provided an immune mechanism [29]. The CRISPR system and Cas proteins protect prokaryotes from invading viruses and plasmids and perform similarly to eukaryotic RNA interference (RNAi) [30]. These repeated sequences interspersed with non-conserved sequenced were named short regularly spaced repeats (SRSRs) [29], spacers interspersed direct repeats (SPIDRs), and a large cluster of tandem repeats (LCTRs) [31] by different authors. Nevertheless, it was Jansen *et al.* who introduced the term CRISPR in 2002 [32].

In the area of genetic engineering, Mojica laid revolution through his work. In 2012, Jennifer Doudna and Emmanuelle Charpentier showed that CRISPR could be used for targeted DNA cleavage in vitro, thus making it a powerful tool for genome editing [33]. Thereafter, CRISPR technology was incorporated across various fields, such as drug development, diagnostics, cancer research, and agriculture. After comparative genomic studies, several features of CRISPR systems have been outlined, which include:

- Spacers are 17 to 84 bases in length and in the intergenic regions.
- Repetitive sequences that contain numerous short repetitive palindromic sequences, ranging between 23 and 50 bases long [34].
- The interspersed non-conserved sequences are present within non-conserved sequences across different species.
- A typical leader sequence consists of hundreds of base pairs on one side of the repeat cluster [35].

In 2007, French food scientists Rodolphe Barrangou and Philippe Horvath conducted pioneer experiments that elucidated the functioning of the CRISPR system. While working for the Danish company Danisco, they studied *Streptococcus thermophilus*, a bacterium commonly used in yoghurt production. Their research led to the understanding of how CRISPR operates. Both Barrangou

and Horvath revealed that CRISPR sequences conferred immunity to *S. thermophilus* against specific bacteriophages when introducing new spacers to the CRISPR sequences. This discovery provided one of the first patents related to CRISPR technology. Subsequently, in 2005, Danisco started implementing CRISPR-based methods to "vaccinate" bacterial cultures to enhance their resistance to bacteriophage infections [36]. These studies highlighted the adaptive immune capabilities of bacteria and led the foundation for innovative applications of CRISPR technology in biotechnology and food safety.

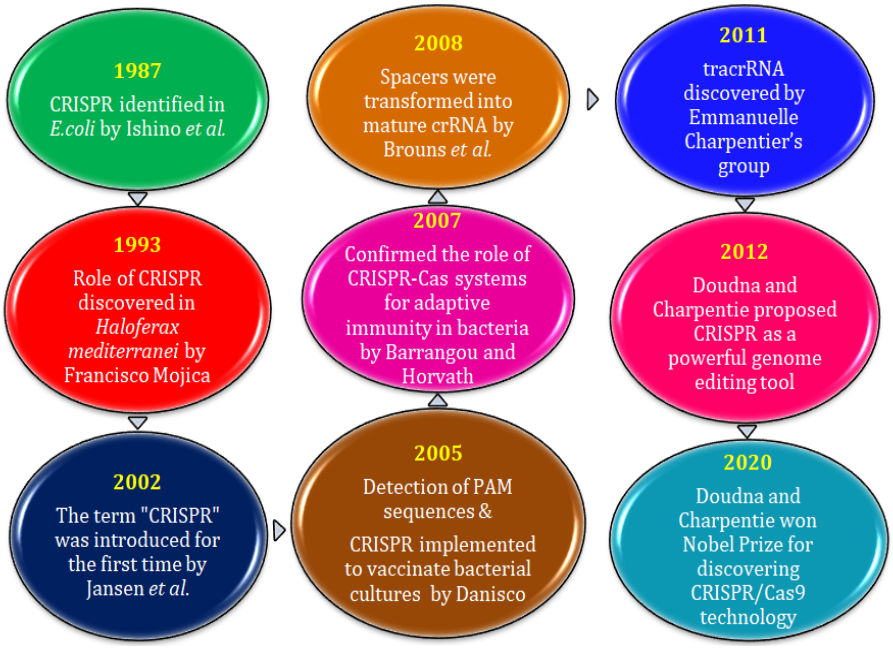

Figure 1: History of CRISPR Technology.

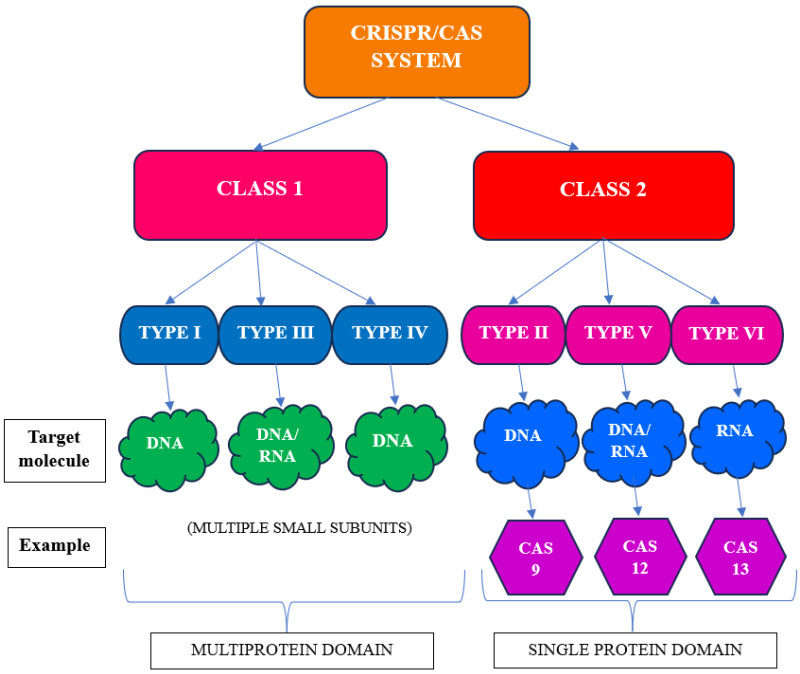

Figure 2: Classification of CRISPR/Cas system.

Components of CRISPR Cas System

Currently, CRISPR/Cas systems are categorised into two main classes and six types, and each type is further divided into numerous subtypes. According to Makarova *et al.,* there are approximately 30 subtypes of CRISPR/Cas system [37]. The key distinction between the two classes lies in their effector modules: Class 1 systems use a complex of multiple proteins, whereas Class 2 systems rely on a single multidomain protein, such as Cas9, Cas12, or Cas13 [38] (Figure 2). CRISPR-associated protein 9 (Cas9) targets double-stranded DNA (dsDNA) and induces a double-strand break using its RuvC and HNH nuclease domains. Even for several hours after the cleavage, the Cas9 protein remains attached to the target DNA [39]. Cas12a, also known as Cpf1, is a class 2, type V CRISPR-Cas system that can cleave both double-stranded (dsDNA) and single-stranded DNA (ssDNA) [40]. Cas13, part of the type VI CRISPR-Cas system, targets single-stranded RNA (ssRNA). It can be programmed with CRISPR RNA (crRNA) to precisely bind and cleave ssRNA sequences [41]. Among the Cas proteins, the best is the one that is involved in the precise cutting of foreign DNA and, in some cases, RNA, with Cas9 being a notable example of these "genetic scissors" (Figure 2).

Short DNA sequences known as Protospacer adjacent motifs (PAMs) [42], which are 2-5 bp long, are located near protospacers. The original spacers of the CRISPR locus lack these protospacers. The protospacers are targeted by the immune system of prokaryotes, which aligns with the corresponding spacers found in the CRISPR locus, except for the PAM sequence. The PAM at the end of a sequence indicates foreign DNA, marking it for destruction. However, DNA stored in the CRISPR locus as spacers lacks PAMs and is recognised as safe and unused by the immune system.

A vital advancement occurred in the CRISPR/Cas9 system with the discovery of a small RNA molecule, CRISPR-associated RNA (crRNA). This molecule is generated by transcribing the CRISPR locus, which helps the immune system proteins target foreign genetic material. This was first described by John van der Oost's team from Wageningen University. They found that the initial transcript from the CRISPR locus forms a longer pre-crRNA molecule containing multiple spacers and repeats, which is then processed into individual crRNA fragments.

When Virginijus Siksnys, from Vilnius University, conducted research, he revealed that the "guide" crRNA, which consists of a sequence of 20 base pairs and is complementary to the target DNA, is crucial as well as sufficient for CRISPR–Cas complex to perform its nuclease activity. Even though the spacer in the CRISPR locus might be longer, the system needs only these 20 base pairs of crRNAs to function. This study was the first to show in vitro how the Cas9 enzyme relies on crRNA to target foreign DNA.

The final essential component discovered was a short RNA molecule, which is required for the functioning of the CRISPR/Cas9 system, identified by Emmanuelle Charpentier's group in 2011. This molecule, called tracrRNA (trans-activating CRISPR RNA), has a vital role in the processing of crRNA and is necessary for nuclease activity. Afterwards, the crRNA and the tracrRNA were fused to form a single chimeric molecule called single-guide RNA (sgRNA). This creation significantly simplified the use of the CRISPR/Cas9 system in subsequent applications.

In June 2012, Emmanuelle Charpentier and Jennifer Doudna made a groundbreaking discovery, demonstrating that the Cas9 protein (SpCas9) derived from the type IIA CRISPR/Cas system of *Streptococcus pyogenes* SF370, is a programmable DNA endonuclease enzyme, directed by crRNA [39]. Just a month later, the Virginijus Siksnys group demonstrated that the Cas9 (StCas9) obtained from the type IIA CRISPR/Cas system in *Streptococcus thermophilus* DGCC7710 functions similarly as a crRNA-guided DNA endonuclease [43]. In both cases, a trans-activating CRISPR RNA (tracrRNA) is required to help activate the nuclease. The two landmark studies revealed the main characteristics of the CRISPR/Cas9 system [39,43]:

- The specificity of Cas9 is determined by the first 20 nucleotides of the crRNA, especially a ~12-nucleotide seed sequence, which is present just before the PAM. SpCas9 recognises the PAM sequence 5'-NGG-3', while StCas9 recognises 5'-NGGNG-3'.
- Cas9 permits up to six mismatches outside the seed region, and single guide RNA (gRNA) can replace crRNA and tracrRNA.
- Cas9 cuts the DNA three nucleotides before the PAM, creating blunt ends, and uses two nuclease domains, RuvC and HNH, to cleave each DNA strand.
- Cas9 can "nick" the DNA even with one inactivated nuclease domain.

CRISPR/Cas9 has been applied to vast areas for genome editing in different types of model organisms. The organisms are the zebrafish *Danio rerio* [44], fruit fly *Drosophila* [45], nematode *Caenorhabditis elegans* [46], rat *Rattus norvegicus* [47], monkey *Macaca fascicularis* [48], and mouse *Mus musculus* [49]. All of these studies demonstrate the versatility and effectiveness of CRISPR/Cas9 in altering genomes across multiple species.

Mechanism of CRISPR/Cas 9 Genome Editing Tool

The CRISPR/Cas9 genome editing approach consists of 3 steps: recognition, cleavage, and repair [50] (Figure 3). A single-guide RNA (sgRNA) is made to be complementary to a specific sequence present in the target DNA. This allows the

Cas9 enzyme to be directed to a particular location within the genome because it has a 5'crRNA complementary base pair component and a tracrRNA sequence needed for Cas9 activation and binding. Without the sgRNA, Cas9 cannot act. The double-stranded breaks (DSBs) are made by Cas 9 protein. The single-guide RNA (sgRNA) attaches to the Cas9 protein, activating it. The recruitment of the Cas9 protein to its target site is permitted by the presence of a short, exact DNA sequence known as the PAM (Protospacer Adjacent Motif), proximal to the target site (Figure 3).

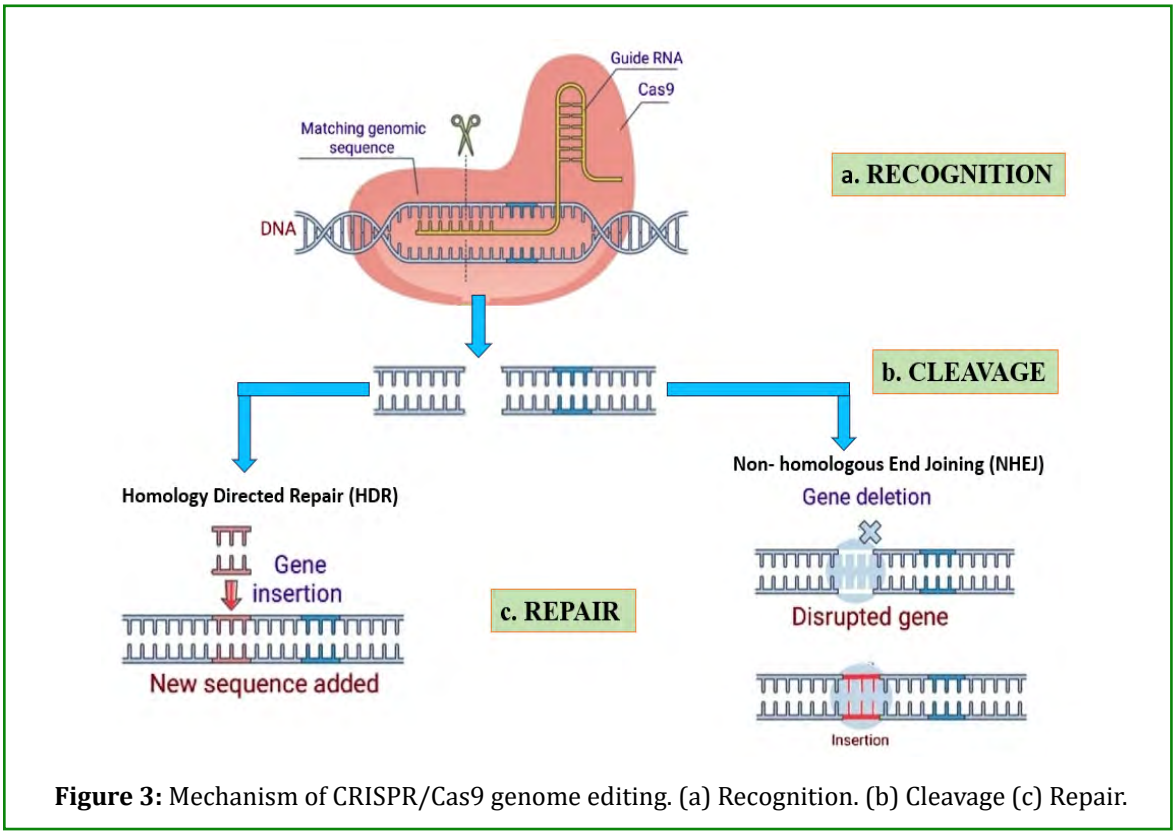

Figure 3: Mechanism of CRISPR/Cas9 genome editing. (a) Recognition. (b) Cleavage (c) Repair.

The PAM sequence is essential for Cas9 to identify where to make its cut. DSB is gently made by Cas 9 at three base pairs [51] upstream of PAM. The "NGG" represents the PAM sequence in the well-recognized CRISPR system, where "N" is any nucleotide. Once Cas9 binds to the PAM sequence and pairs with the target DNA, it causes the DNA to unwind and form a hybrid between the sgRNA and the DNA. Cas9 is then activated to cut the DNA. It has two vital components: (1) the HNH domain, which cleaves one strand of the DNA, and (2) the RuvC domain, which cleaves the other strand. This produces a double-stranded break (DSB) in the DNA. After Cas9 creates the break, the cell's repair mechanism fixes the damaged DNA [52,53]. This repairing process involves inserting new genetic material, correcting errors, or joining the break.

sgRNA Design

Designing a practical single guide RNA (sgRNA) involves the identification of a target site adjacent to a PAM sequence, typically 5'-NGG-3', while minimising off-target effects. Various tools like CHOPCHOP, CRISPOR and CRISPRscan, assess factors like nucleotide composition, GC content and chromatin accessibility to determine sgRNA efficiency and specificity [54,55]. These tools use algorithms, specifically Rule Set 1 and 2, to promote on-target activity while preventing off-target interactions [56].

Combining the sgRNA with Cas9 has revolutionised genome editing, enabling targeted DNA modification across diverse groups of organisms. This technique has enormous potential in research and aquaculture, where traits like reproduction, growth, and disease resistance can be enhanced [57,58].

Mechanism for Repairing the Double-Stranded Break

The double-strand breaks (DSBs) created by the Cas9 in the CRISPR/Cas9 system are repaired in two ways, which include nonhomologous end joining (NHEJ) [59] and homology-directed repair (HDR) [60].

NHEJ is an enzymatic process that can repair the DSBs by directly joining the DNA fragments without using a homologous DNA template. This pathway is active throughout the cell cycle phases, making it a prime cell repair mechanism.

However, NHEJ sometimes leads to small insertions or deletions (indels) at the site of cleavage, thus resulting in errors that lead to frameshift mutation or premature termination codons [12,61]. Due to such variations, the target gene becomes non-functional, popularly known as knocked out (KO) of the target gene, which is beneficial in gene knockout applications.

Compared to NHEJ, HDR is a more accurate repairing mechanism requiring a homologous DNA template. This pathway becomes most active during the late S phase and G2 phases of the cell cycle, where the sister chromatids exist as templates. In CRISPR applications, HDR needs an adequate quantity of donor DNA with a sequence of interest. Due to this, the accurate gene can be inserted or replaced by a donor template with a sequence similar to the DSB site [12,62].

Although HDR offers high fidelity in gene editing, its efficiency is relatively low compared to NHEJ, which often leads to a mixed population of cells containing various alleles post-editing. Therefore, strategies to enhance HDR by suppressing NHEJ are necessary to achieve more controlled and precise genetic modifications [63,64].

Base Editing and Prime Editing

The recent advancement in CRISPR/Cas9 led to the development of base editing and prime editing techniques. Base editing promotes accurate modification of individual bases in DNA without making double-strand breaks (DSBs). These are improved forms of traditional CRISPR gene editing techniques that do not involve NHEJ and HDR.

Base Editing

The base editing technique was first discovered in the laboratory of Drs. David Liu and Akihiko Kondo in 2016 [65] Base editing depends on Cas9 D10A nickase, which breaks a single DNA strand [65]. The nick created directs the deaminase enzyme to convert one base to another. The deaminase domains in base editors are of two types: adenosine deaminase and cytosine deaminase. In 2016, the first base editor, the cytosine base editor (CBE), was developed. The cytosine deaminase changes a C·G base pair to a T·A pair by converting cytosine to uracil, forming a pair with adenine during repair, resulting in a T·A base pair.

In contrast, another base editor known as adenosine deaminase converts an A·T pair to a G·C pair by converting adenosine to inosine, which is recognised as guanine by the cell, thus resulting in a G·C base pair. The cell then uses the modified strand as a template to repair the nick [66]. Therefore, this ensures that the altered base is incorporated into the genome. As this technique avoids DSBs, it eradicates the chances of error due to deletions, insertions, or chromosomal rearrangements, the most prevalent problem with traditional CRISPR techniques [65,66].

Prime Editing

The prime editing technique was described by Anzalone et al. in 2019 [67]. This technique successfully overcame the traditional base editing technologies, which were limited to the induction of transition mutations. Moreover, it avoids double-strand breaks (DSBs). It uses an engineered reverse transcriptase (RT) fused with Cas9 nickase (nCas9) and a prime editing guide RNA (pegRNA), which plays a vital role in the functionality of the system. The pegRNA consists of a target-specific sequence that guides the nCas9 and an extra template for encoding desired sequence change [67].

While editing, the 5’ end of pegRNA binds with the DNA at the primer binding site (PBS), which reveals the non-complementary strand. The nCas9 then creates a break in the strand containing the PAM sequence, which is then extended by the reverse transcriptase using pegRNA as a template, integrating the desired sequence. As a result, two redundant PAM DNA flaps are generated: the edited 3′ flap, synthesised from the pegRNA template, and the unedited 5′ flap. The cellular endonuclease degrades the unedited flap [68] so that the edited flap can be incorporated during DNA replication and repair.

The first-generation prime editor (PE1), consisting of Moloney murine leukaemia virus reverse transcriptase (M-MLV RT) joined to nCas9 and pegRNA, expressed via a second plasmid, achieved the highest editing efficiency of 0.7–5.5% [67]. Subsequently, improvements in PE2 focused on optimising M-MLV RT variants to intensify binding, processivity, and thermostability. The PE3 system incorporated a secondary sgRNA to create a nick in the non-edited strand, guiding the DNA repair machinery to use the edited strand as a template. These enhancements significantly improved editing efficiency, thus generating a next-generation prime editor, achieving an average of 33% (±7.9%) and enabling all 12 possible transition and transversion mutations [67].

The prime editing technique has several benefits over previous CRISPR-based editors, including lowered off-target effects, minimised bystander editing, and decreased PAM requirements, which expands genome accessibility. As a new technology, its specificity, long-term effects, and in vivo applications require further study. Although the base editors are currently more efficient and are better understood, prime editing represents a significant breakthrough with remarkable potential for correcting genetic mutations [67].

CRISPR Delivery Methods in Fishes

The primary reason behind successful CRISPR-based gene editing is the efficient delivery of the CRISPR components into fish cells. The following methods of delivering CRISPR components into fish are as follows:

Physical Delivery Methods- These involve utilizing external forces to facilitate the direct introduction of genetic material into fish cells. These methods are used mainly in the early stages of fish development.

Microinjection: This is the standard method, and it uses a glass micropipette to transfer CRISPR components directly into the cytoplasm or nucleus of fish embryos. This technique allows precise targeting of individual cells. Till now, this technique has been broadly used in zebrafish, medaka, tilapia, and Atlantic salmon [69]. While microinjection provides almost 100% efficiency in delivering CRISPR components, it is labor-intensive and requires skilled personnel [70].

Electroporation: This method uses an electrical field to create pores in the cell membrane, which permits large molecules, such as plasmid DNA and gene constructs, to enter the cells, which otherwise cannot pass through the membrane. The significant drawbacks of this technique are low cell viability and chances of cell damage. This method is highly productive for ex-vivo cultured cells [70]. This technique has been highly successful in medaka [71]. Electroporation can contain multiple embryos simultaneously, making it a flexible method for gene editing in aquaculture.

Induced Transduction by Osmocytosis and Propanebetaine (iTOP): This method was developed by Diego *et al.* in 2015, and it uses a unique buffer solution to deliver proteins into different types of cells. The buffer comprises propanebetaine and sodium chloride [72]. Together, these compounds generate a condition suitable for cells to use external fluids and materials for micropinocytosis. iTOP method generates osmotic shock that causes temporary pores in the membrane, through which CRISPR components enter the cells. It is better than other traditional methods, such as electroporation, because it causes minimum damage to the cell and has higher efficiency for editing genes [73].

Chemical Delivery Methods: Involve using chemical agents to introduce CRISPR components into the fish cell.

Liposome-Mediated Delivery: In this technique, CRISPR components are encapsulated in lipid-based nanoparticles and are transported into the fish cell [74]. The liposome then fuses with the cell membranes and releases their components into the cytoplasm. This technique has been administered to the zebrafish.

Nanoparticle-Mediated Delivery: It is an ideal delivery platform for the CRISPR/Cas9 system, including cationic Lipid-based nanoparticles (LNPs), DNA nanoparticles, lipid complexes, gold-based nanoparticles, and zeolite imidazole frameworks. They have been used for *in vitro* RNP delivery [75]. These nanoparticles can increase the cellular uptake and prevent the CRISPR components from degrading. This method has been applied to various aquaculture species.

Biological Delivery Methods

Viral Vector Delivery: The viral vectors are the engineered viruses that can insert the genetic material within the host genome. This provides a stable expression of CRISPR elements. Usually, two viral vectors are used for delivery: lentiviral and adenoviral [76,77].

Bacterial Delivery: In this method, bacteria such as *Agrobacterium* or *E. coli* are used, and their natural ability is exploited to transfer CRISPR components to the host organism. Till now, it has not been used much in fish research.

Current trend and application of CRISPR/ Cas9 genome editing technology

CRISPR/Cas9 genome editing technology promotes sustainable aquaculture practices by implementing accurate genetic modification in various aquatic species. This technology has enhanced vital traits of various aquatic species necessary for aquaculture. So far, CRISPR research has improved various genetic factors such as disease resistance, growth, coloration patterns, reproduction and sterility in fishes (Table 2). This tool has proved to be highly efficient as it is simple, economical, and can induce targeted changes without incorporating foreign DNA. Incorporating foreign DNA has created ethical and regulatory issues, so this technique can evade all these issues.

Augmentation of Growth Rates in Fishes: The researchers can generate strains that will grow faster by targeting specific genes that regulate growth. Red sea bream and tiger puffer fishes are two CRISPR-edited fishes that were approved by the Ministry of Health, Labour and Welfare (MHLW) and the Ministry of Agriculture, Forestry and Fisheries (MAFF) of Japan after finishing the national application process. These fishes were CRISPR edited to grow bigger and have more edible parts (20 percent more in red sea bream and 90 percent more in tiger puffer fish than the conventional counterpart). Another fish, FLT-01 Nile tilapia, which the AquaBounty Company developed, was also submitted for approval to the regulatory bodies. This fish also contains no foreign DNA; hence, it would not fall into regulations applied for traditional genetically modified organisms [78]. Li, et al. [79] used CRISPR/Cas9 to generate a sterile, all-male population of Nile Tilapia. They displayed that genetically

modified tilapia exhibited higher growth rates. Furthermore, raising farm tilapia, which are sterile, reduces the risk of their escaping, which can otherwise compete and inbreed with the wild fish population, thus increasing aquaculture production.

Target trait	Gene name	Species name	References
Disease resistance	Toll-like receptor TLR22	Labeo rohita (Rohu)	Chakrapani et al., 2016 [18]
	Toll/interleukin 1 receptor domain-containing adapter molecule (TICAM1)	Ictalurus punctatus (Channel catfish)	Elaswad et al., 2018 [80]
	Junctional adhesion molecule-A (JAM-A)	Ctenopharyngodon idella (Grass carp)	Ma et al., 2018 [19]
Growth	Myostatin	Pagrus major (Red Sea bream)	Kishimoto et al., 2018 [81]
		Takifugu rubripes (Tiger puffer)	Kishimoto et al., 2018 [81]
		Oreochromis niloticus (Nile tilapia)	AquaBounty company
Pigmentation	slc45a2 (solute carrier family 45 member 2)	Oreochromis niloticus (Nile tilapia)	Segev-Hadar et al., 2021 [82]
	25 genes involved in pigmentation		Wang et al., 2021 [83]
Reproduction and sterility	Cytochrome P450 17A1 (cyp17a1)	Cyprinus carpio L. (Common carp)	Zhai et al., 2022 [84]
	Dead end (dnd)	Salmo salar (Atlantic salmon)	Wargelius et al., 2016; Kleppe et al., 2017; Güralp et al., 2020 [20,85,86]
	Doublesex- and mab-3-related transcription factor (dmrt6 and dmrt1),	Oreochromis niloticus (Nile tilapia)	Li et al., 2014; Xie et al., 2016; Chen et al., 2017; Jiang et al., 2017 [87-90]

Table 2: Outline of CRISPR/Cas genome editing in various fish species.

Disease resistance in Important Fishes: CRISPR technology has been used for curing fish diseases. It involves two pathways: first, interfering with the pathogen genome, and second, adding mutation in the hosts. In farmed Rohu (*Labeo rohita*), the *Toll-like receptor TLR22* gene has been disrupted. This system assisted in understanding the role of this receptor towards pathogenic bacteria, dsRNA viruses, and parasites like fish lice [18].

Genetic modifications have also improved the immune responses of fish by reducing their susceptibility to common diseases and decreasing the need for antibiotics, thus promoting healthier farming practices [91].

In grass carp, CRISPR/Cas9 technology was used to deal with the hemorrhagic disease caused by grass carp reovirus (GCRV) [19]. Similarly, Wargelius et al. [20]. applied CRISPR/Cas9 gene editing to Atlantic salmon to provide resistance against significant viral pathogens such as infectious pancreatic necrosis virus (IPNV) and salmon alphavirus (SAV). The scientists "knocked down" or modified the host genes, which led to the infection and replication of these viruses, resulting in salmon lines that exhibit enhanced disease resistance. This advancement is crucial for the aquaculture sector, as viral diseases such as IPNV and SAV frequently cause significant economic losses in the farming of Atlantic salmon.

Enhancement of Colour in Ornamental Fishes: Researchers have reported six types of pigment cells in fish [92]. Some of the species may also contain a combination of these pigment cells. Due to the advent of CRISPR/Cas9, it became possible to make advanced studies related to these pigment genes, which resulted in wide varieties of colour in mutant fish [93].

In Nile tilapia (*Oreochromis niloticus*), 25 pigment-related genes were found that were associated with pteridine

metabolism, melanogenesis and pathways for carotenoid absorption and cleavage and all of these were manipulated using CRISPR/Cas9 techniques.

- **Optimizing Reproductive Traits in Aquaculture Species:** Fecundity and sex ratio control can enhance production efficiency, thereby increasing the income for fish farmers. The commonly used methods of optimizing the sex ratio are breeding methods, which take years to develop and may result in inbreeding depression and hamper good production. Sometimes, these traits, accumulated over generations by breeding families or within families, may get reversed once the progenies are reared to be bred, and the next generation has the issues that the first generation was bred to escape [78]. This technology was used in *Salmo salar* (Atlantic salmon) to develop a novel sterile model of this species. This was created by disrupting the germ cell-specific gene known as the *dead-end (dnd)* gene, which resulted in germ cell-free salmon [20]. Recently, an all-female population of Cyprinus carpio (common carp) was produced by knockout of the *cyp17a1* (*Cytochrome P450 17A1*) gene by the use of CRISPR/Cas 9 technology [84].

Besides the applications mentioned above, CRISPR/Cas9 technology can also enhance sustainability in fisheries by decreasing dependence on antibiotics and feeds. This technology can improve disease resistance in fish by more accurate genetic alteration, thus reducing the need for antibiotics. Minimizing the use of antibiotics lowers costs and mitigates the risk of antibiotic resistance and environmental contamination [94].

Additionally, CRISPR/Cas9 enhances growth rates and feed conversion efficiency in fishes by targeting specific genes related to metabolism and growth. This technology can produce fish that require less feed to achieve market size, reducing feed consumption and associated environmental impacts [77]. Implementing CRISPR/Cas9 in aquaculture practices is directly linked with sustainable goals by promoting resource efficiency and reducing chemical inputs. However, it is necessary to address public perception, regulatory frameworks, and potential ecological impacts to ensure the responsible integration of this technology into the fisheries sector [95].

Ethical and Regulatory Challenges of Applying Genome Editing in Aquaculture

The application of CRISPR/Cas genome editing in aquaculture poses significant ethical and regulatory challenges. The primary concern is the ecological risk associated with genetically modified fish, which can escape into the natural surroundings, resulting in gene flow into the wild populations, genetic pollution and damage to biodiversity. CRISPR-modified Atlantic salmon developed for disease resistance against pathogens such as infectious pancreatic necrosis virus (IPNV) has the potential to replace the wild salmon population if escaped, causing alteration in genetic diversity. Additionally, the introduction of increased growth rate traits in genetically modified Nile tilapia can result in unintended competition with the wild species [77].

Public perception towards genome-edited fish is also of significant concern towards its acceptance. Modification of the genome of fish has raised ethical concerns among consumers. Concerns about the safety and ethics of consuming genetically modified salmon such as AquAdvantage salmon marked the importance of labeling, transparency and effective public education for generating trust. Cultural and regional differences also make acceptance of the technology complex. European consumers are more resistant to accepting genetically modified organisms than the United States [77].

The regulatory framework controlling genome editing in aquaculture differs widely, complicating international trade and commercialization. In the case of AquAdvantage salmon, it has been approved for food production in the United States. At the same time, stricter regulatory standards are maintained in other regions, such as the European Union, with comprehensive biosafety assessments and labeling for genetically modified fish. Furthermore, equitable access to CRISPR/Cas9 technology remains challenging as large-scale producers often can use advanced tools like CRISPR/Cas9. Countries like Canada have successfully developed and commercialized genetically modified salmon, but smaller-scale producers in developing areas still face barriers to such innovations, limiting their competitiveness [77].

Concerns regarding animal welfare mark the importance of strict guidelines to reduce suffering and promote the prolonged health of genome-edited fish. For instance, CRISPR-edited fish like Nile tilapia, engineered for sterility to minimize environmental risks, need a careful evaluation to prevent unintended impacts on fish health and ecosystem balance. A balanced approach is required to address these challenges to integrate scientific innovation with societal and environmental factors, ensuring that the advantages of genome editing in aquaculture are achieved responsibly [77].

Future Prospectives of CRISPR/ Cas9 Genome Editing Technology

Since CRISPR is an up-and-coming technology, so much is yet to be discovered. RNAi was used in silencing works. Now, the same thing can be done using CRISPR. The guide RNA, which is compatible with the RNA to be silenced, is injected

along with the remaining parts of the construct. Here, the Cas enzyme cleaves the RNA sequence. Such an experiment was performed to see the effect of cold on *Oryzias sativa and how the OsNCED5* enzyme helps to tolerate cold. Other traits like low gluten and higher protein content in wheat, parthenocarpy, longer shelf life, and desired colour in tomato were also obtained using CRISPR.

Multiplex genome editing lets us simultaneously target multiple genes responsible for a desired trait. Additionally, it considers silencing some of many genes within a pathway that contributes to the targeted product, facilitating more effective silencing of its production [96-98]. Similar to the experiment in rice, the same can be applied to fisheries where abiotic stress can decrease production, and often, genes can be upregulated to ensure better survival. These genetic modifications will make these cultured organisms more tolerant to changing climate.

In an experiment with zebrafish, a comparison was made between single sgRNA genome editing(ssGE) and multiple sgRNA-based multiplex genome editing (msMGE), obtained knocking rates were higher in msMGE without any significant difference in fry survivability and hatch rate [99]. Epigenome editing by modifying epigenome marks is a new way to manipulate gene expression. In an experiment, H3K27me3 of Medaka was used for in vivo modification. In embryos, injection of dCas9-olEzh2 mRNA with single guide RNAs (sgRNA) resulted in downregulating the gene [100]. In another experiment, zebrafish embryos were injected with synthetic gRNA and recombinant Cas9. The researchers found the reagents to be highly efficient in their jobs. During manufacturing, these synthetic reagents were designed and manufactured in a short time frame. Another approach is to use ribonucleoprotein complexes instead of DNA for the Cas sequence and gRNA, which were efficient and degraded over time, resulting in less off-target effects. Moreover, this technique will be adopted to create sterile breeding, disease resistance, targeted therapy, and many more, which could solve the problems of traditional aquaculture practices. Atlantic salmon and tilapia have been selected as model organisms to optimize the aquatic protocol and assess the off-target effects. These are just the things applied in the fisheries sector; many more innovations in other sectors are yet to be adopted here. Once regulatory authorities ensure that CRISPR-based edited fish are safe to consume and receive the much-needed approval, more improvements can be made to the genes of other cultured fishes to improve their productivity and eliminate the constraints.

Conclusion

The CRISPR Cas technology has proven to bring desirable changes to the genome, which will ultimately help improve production. Animals were genetically modified for a long time by various methods like TALEN, ZFN and meganucleases. But they all came with their pitfalls, such as meganucleases not being reprogrammable, ZFN requiring more protein engineering, and TALEN having delivery issues to the cell owing to its large size. CRISPR came as a breakthrough addressing the shortcomings of the predecessor technology.

CRISPR has various applications, including genetic modification for better traits, therapeutics, and disease diagnosis. Some genetically modified species are Nile Tilapia, Common carp, ornamental fishes, etc.

Ornamental fishes were edited for better color. Nile tilapia was edited for better growth, and common carp was modified for sterility. *Labeo rohita* was modified to get disease resistance. These are just a few of the examples of CRISPR-based genome editing.

Collaborative work must be done on CRISPR to ensure the fisheries sector is at the same level as other sectors regarding this technology. We may see more rapid developments soon in this technology, and rapid farm-level deployment can make this technology and everything that goes into research fruitful.

To avoid the taboo surrounding Genetically Modified Organisms, more research is needed to ensure the safety of the modified product; the current literature doesn't mention many shortfalls. One of the significant issues that pester this technology is the promiscuity of the guide RNA since minor changes in sequences don't stop it from binding to non-target sites and cleaving it. These issues are addressed regularly, and new ways are being developed to prevent this.

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