Chapter

Applied Molecular Cloning: Present and Future for Aquaculture

Tapas Chakraborty, Sipra Mohapatra, Chimwar Wanglar and Dipak Pandey

Abstract

With the grim picture of millions of people living in poverty and hunger, there is also an international alarm over future world food supply. This global concern of food scarcity has established the need to not only increase the production of traditional staples but also fisheries and aquaculture. Genetically, physiologically and phenotypically, fish are the most diverse group of livings. Similar to mammals, molecular biology is being extensively used in aquaculture, be it in disease management, or growth and reproduction enhancement. In this chapter we aim to discuss the molecular methodologies applied to uplift and attain sustainability in aqua farming.

Keywords: aquaculture, fish, disease diagnosis, genome editing, genomic marker

1. Introduction

Agriculture, livestock (bird, cattle, pig, etc.) farming and fish rearing are traditionally used to cater the nutritional requirement since ages. Evidence of agriculture, including meat farming, can be found as far back as the end of the Pleistocene Era, roughly around 12,000 years ago. In contrast, fish have only been farmed in aquaculture setting for over 2000 years [1]. Our world roughly comprises of 70% water and majority of them are unutilized due to inadequate knowledge and resources. Additionally, the availability of terrestrial space of agriculture and livestock farming are now on a decline. The lower FCR values of various aquaculturable species (e.g., cobia 0.96–1.50:1) than various terrestrial animals (e.g., cattle 5.15–6.95:1; poultry 2.13–2.61:1) [2, 3] are not only important for reducing the production cost but also have less environmental burden to bear. However, aquaculture, the farming of fish and aquatic plants, is the fastest growing food sector in the world, recently (since 1970s) growing exponentially to meet the increasing population and declining wild fish stock availability. The aquaculture industry's contribution to the total food supply has increased dramatically since 2000–2012 by 6.2% [4], and it is expected that by 2030, 60% of the total fish supply intended for direct human consumption will be produced by the aquaculture industry [5]. With the development of new and environment friendly Silvofisheries (fish integrated with mangroves), Aquaponics and IMTA

(integrated multitrophic aquaculture), etc., the probability of sustainable growth of aquaculture has been raised several folds. But, unlike agriculture and livestock farming, aquaculture has lot of geographical restriction, such as in North America and Europe, clams, oysters, and other shellfish are the main species being farmed, while in Japan, edible seaweed, marine shrimp, and yellowtail are the desired species for culture. Likewise, carps in India, freshwater prawns in Hawaii, and eels in Taiwan are the preferred culturable species [1]. Although numerous species (>694) have made their way into aquaculture and have international consumer market, only the Norwegian salmon has gained commercially popularity in recent years. If we analyze deeply, it is clear that it is neither the geographical restriction nor the consumer demand, but rather the huge industrial success of this specific salmon is related to meticulous research, better strain availability through years of selective breeding, authenticated and steady high quality seed availability and one stop consultancy [6].

Molecular biology and cloning set sailed its journey with the DNA molecule in 1950s and encountered several breakthrough including RNA and restriction endonucleases, however in reality, the recombinant DNA technology has made a revolution in modern molecular biology. Through this technique, large quantities of proteins present in trace amount, as well as other biologically active substances, could be generated through biotechnology and these genetically engineered macromolecules have very little side effects. Emerging technologies promise even greater possibilities, such as enabling researchers to seamlessly stitch together multiple DNA fragments and transform the resulting plasmids into bacteria in under 2 h, or the use of swappable gene cassettes, which can be easily moved between different constructs, to maximize speed and flexibility. During the past 2–3 decades, fish molecular biology has been intensively investigated in all aspects of fisheries, including diseases, genetics, nutrition, and ecology. Molecular tools are used to investigate changes in the DNA, RNA or proteins to detect certain genetic or biochemical changes that are associated with certain disease-causing pathogens [7–9]. Another advantage of molecular tools is that the analysis can be done on stored specimens and abundance of genetic information in the database. In recent years, great advances have been made to simplify the techniques and reduce the cost without compromising on the sensitivity. In this chapter we will discuss about the issues of aquaculture, and the potential of molecular cloning/biology in fish.

2. Aquatic animal and molecular cloning

2.1 Major hurdles of aquaculture

Fish live in a complex 3D environment, so whether it is the density of the fish, or extra feed given by farmer, or local environment and water quality, everything impacts the aquaculture output. Although new concepts like precision fish farming are emerging, the following categories still are a cause of major concern.

2.1.1 Adequate disease diagnosis and health management

Diseases are the major constricting factor for expansion of aquaculture industry, and they potentially cost the sector nearly $6 billion in yield loss each year [10]. Aquatic environments impose a constant risk of exposure to disease-causing pathogens and poor knowledge of background microbial "diversity" in aquatic farm systems often leads to frequent emergence of previously unknown diseases. Healthy

looking fish can carry pathogens without a clinical sign and disease become evident only under stressful conditions. Therefore, disease management and assessment of cultured fish is a major concern to commercial aquaculturists. The ability to identify the presence or absence and concentration of a pathogenic organism in fish would have significant economic benefits. Statistically, relevant disease surveillance and monitoring requires testing large numbers of fish as it increases the probability of detecting pathogen from clinically normal fish. Reliable detection of fish pathogens in a fish population is difficult if fish with disease are not available or only a low percentage of the fish is infected. To detect pathogen carrying fish, a cost effective, sensitive, and specific system is required for surveillance and monitoring of fish population. Traditionally, the diagnosis and management of diseases is carried out by culture dependent methods which are slow, require skill, and only selective organism can be detected [11]. Potentially faster, more sensitive diagnostic techniques for identification and characterization of pathogens, even from asymptomatic carrier fish, are of utmost necessary.

2.1.2 Maintenance of the environment and biodiversity

Since farmed fish are selected and bred for certain genetic criteria like size, quick growth and hardiness, escaped species can become invasive and pose a massive threat to global biodiversity. The ever-growing aquaculture industries also have to bear the public concern in regard to pollution and other environmental effects and thus maintaining and sustaining the environment is of paramount importance. Attention to genetic variability and biodiversity in aquaculture development, proper stock maintenance and aquatic resource management are therefore crucial elements for sustainable environment. In this sense, traceability tools are essential to assess the impact of aquaculture escapees in natural populations or distinguish the farmed and wild specimens.

2.1.3 Reproductive medley

Reproduction is crucial for steady and quality seed supply and hence of utmost importance for aquaculture sustainability. Fish gonadal development is influenced by intrinsic (genetics, growth, behavior, etc.), and extrinsic (temperature, hormone, environmental pollution, etc.) factors. Though, large diversity of aqua animals has its own advantages, each species has distinct reproductive and embryonic development biology that hinders the timely breeding and smooth progression of commercial aquaculture. For instance, some gonochoristic fish harbors sex chromosome while others do not, and several commercially lucrative fish sequentially changes their sex. Moreover, some hybrids tend to grow bigger with the expense of reproductive unfitness (e.g., hybrids of Atlantic and pacific salmon).

2.1.4 Improper growth

Fish growth largely depends on feeding, environment and genetic background. For example, farmed Atlantic salmon tend to grow faster than wild ones, and genetically modified (GM) farmed salmons are even better. Though FDA recently approved GM salmon, till date it is not ethically preferable to use GM fish for commercial aquaculture. There are few more success stories of using myostatin knockout to improve growth of tilapia, red sea bream and common carp; however, yellow catfish [12] did not display similar results, suggesting functional variation among species.

2.2 Application of molecular cloning in aquaculture

2.2.1 Restriction enzyme/endonuclease digestion

Restriction enzymes (or restriction endonucleases, RE) are enzymes or better known as "molecular scissors" that recognize and cleave the DNA into fragments at or near specific "recognition" sites. The DNA fragments are observed by gel electrophoresis and the pattern of bands are used to generate the "fingerprint" of a particular DNA molecule. The cut DNA can be observed by gel electrophoresis and the pattern of bands compiled to create a restriction enzyme map [13]. This map is useful to identify and characterize a particular DNA region and analyze genetic variation. Restriction enzymes are used to manipulate DNA and are vital tools in molecular cloning. They form the basis for several diagnostic tools like RFLP, AFLP, Southern blotting, etc. For instance, RFLP recognizes size variations, and in combination with PCR can be used to reduce the labor-intensive DNA isolation for RFLP analysis [14]. SNPs (single-nucleotide polymorphism) or INDELs change the restriction endonuclease recognition sites that cause differences in restriction fragment lengths. AFLP technique is based on cutting with two Res (one average (e.g., EcoRI), and another rare (e.g., MseI) cutter), ligation of adapters to these restriction fragments and followed by a PCR-based selective amplification with adapter-specific radioactive or fluorescent-labeled primers.

2.2.2 Random amplified polymorphic DNA (RAPD)

RAPDs are DNA fragments that are amplified using short random primers (~10 bp) and are used to detect polymorphisms. RAPDs are randomly distributed throughout the genome and have high abundance. This technique is quick and easy and requires low quantity of DNA. Fish pathogens have been studied using RAPD, but problems with reproducibility and risks of contamination render the method unsuitable as a stand-alone method of diagnosis. However, RAPD can be a useful technique as a first step in the development of specific primers or probes and has been used in such a way in the study of bacteria.

2.2.3 Polymerase chain reaction (PCR)

The polymerase chain reaction is a robust technique used to produce large copies of the target DNA sequence by amplifying the specific region of interest. The reaction includes template DNA, primers, polymerase enzyme to catalyze creation of new copies of DNA, and nucleotides to form the new copies. The template DNA can be collected from sample tissue, blood, serum, fluid, mucus or can be a purified DNA. The principle of PCR is based on the repetitive cycling of denaturation, annealing and extension. Each copy of the DNA then serves as another template for further amplification and copy number of PCR products then doubles in each cycle. After "n" rounds of replication, 2^n copies of the target sequence are theoretically produced. After thirty cycles, PCR can produce 2^{30} or more than ten billion copies of a single target DNA sequence. The PCR product can be detected by gel electrophoresis. The whole process just needs 2–5 h depending on the number and types of nucleotide. PCR has distinct advantages over conventional microbiological diagnostic methods as it can detect slow growing and unculturable pathogens. PCR is faster, extremely efficient and sensitive and can be used to amplify sequences from wide variety of samples even if they only have a small amount of DNA. Some of the shortcomings of PCR are the false positive results from DNA contamination, limited detection platform for simultaneous identification of multiple samples, etc.

In most cases, the target DNA sequence is the rRNA operon and in bacteria, the most frequently used is the variable region of the evolutionary conserved 16S rRNA gene. Nevertheless, other types of genes or sequences of unknown sequences can also be used.

To overcome the shortcoming and to increase the diagnostic capacity of conventional PCR, multiplex PCR was developed to simultaneously amplify several target sequences by using more than one pair of primers. It can detect multiple pathogens, which save time and cost without compromising test utility, but might require further analysis such as DNA sequencing to confirm the identity of the species.

Nested PCR, which uses two pairs of primers and two successive PCR run, was developed to increase specificity and sensitivity of conventional PCR. The first set of primers is used to amplify target sequence in first run and the PCR products are used as template for the second run and amplification is conducted with the second set of primers. Though, it is popular for unknown/homologous gene identification, due to the lengthy process and complexity, this type of PCR is limited to cases where single PCR is not sufficient to identify pathogen.

Though DNA is reliable, RNA is often a more accurate indicator of viable microorganism. Therefore, Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was developed to first synthesize cDNA from RNA by reverse transcription (RT) and later amplify the cDNA by PCR. However, for effective detection, sufficient amount of detectable RNA concentrations is required, and the RNA sample should be free of genomic DNA to avoid false positive results.

Most recently, real time PCR is used to detect, confirm and quantify PCR products at "real time" during the amplification process using Fluorescent dyes. Two types of dyes are generally used; one is the use of non-sequence specific dyes like SYBR green I or ethidium bromide and the second is the use of fluorescently labeled internal probe like TaqMan, FRET (fluorescence resonance energy transfer), etc. The real time PCR has three novel features—temperature cycling occurs considerable faster than in standard PCR assays, hybridization of specific DNA probes occurs continuously during the amplification and the dye fluoresces only when hybridization takes place. This technique is quick and convenient, and with the recent introduction of multiplex real time PCR, detection of multiple targets in a single reaction can be achieved at cheaper cost, shorter time and faster diagnosis.

2.2.4 Loop-mediated isothermal amplification (LAMP)

It is a novel nucleic acid amplification method that amplifies DNA with high specificity, efficiency and rapidity under isothermal conditions. This method employs a DNA polymerase and a set of four specially designed primers to recognize six distinct regions of the target DNA. Unlike PCR, LAMP is carried out in constant temperature (60–65°C) using an auto-cycling strand displacement DNA synthesis and does not require thermal cycler. The amplified product can be detected as white precipitate or yellow green color solution after addition of SYBR Green. It is cost effective and when combined with reverse transcription, this method can also amplify RNA sequences with high efficiency. It can be used to detect the identification of genus and species-specific parasites. However, this technique is not effective for detection of different pathogens simultaneously.

2.2.5 Fluorescence in situ hybridization (FISH)

In situ hybridization refers to detection of DNA or RNA on actual tissues, cells, or any biological sample in their natural positions within a chromosome, by using a complementary probe. *ISH* correlates DNA localization and mRNA expression

with morphological findings [15]. Most current *in situ* hybridization methods use *FISH* [16, 17] in which fluorescent labeled pieces of DNA or RNA (probe) hybridize to target nucleic acid in cells under appropriate conditions. These labeled cells can then be visualized by flow cytometry or fluorescence microscopy. *FISH* can be used on formalin fixed paraffin embedded tissues, frozen tissues, etc. The technique has also been used to detect bacterial and viral DNA in an infected cell. Since the probe has to reach the target inside the cells, only probes that are small (~300 bases) can be used for tissue penetration, hence sensitivity is limited to the accessibility of the target in the cell.

2.2.6 Molecular padlock probes (MPP)

Padlock probes (PLPs) are single stranded long oligonucleotides whose 5′ and 3′ ends are complementary to two immediately adjacent target sequences. Upon hybridization to the target, the two ends are brought into contact, effectively circularizing the probe with a nick. DNA ligase is added to convert this linear PLP into a covalently closed circular molecule. Single strand specific DNA exonucleases can be used to "chew up" the linear strands and only make available the intact circular molecules. PLPs provide extremely specific target recognition, which is followed by universal amplification and microarray. However, synthesis of long probes can be little expensive as compared to short primers for PCR. At present, the most common application for PLPs is the detection of single nucleotide polymorphisms (SNPs) and multiplex pathogen detection assays.

2.2.7 Rolling circle amplification (RCA)

RCA is an isothermal enzymatic process where short DNA/RNA primer amplified to form a long single stranded DNA/RNA using a circular DNA template and special DNA/RNA polymerases. The product is a concatemer containing tens to hundreds of tandem repeats that are complementary to the circular template. By manipulating the circular template, RCA can be employed to generate complex DNA nanostructures such as DNA origami, nanotubes, nanoribbons and DNA based metamaterials which can be used for bio-detection, drug delivery, etc. Millard et al. [18] combined RCA, MPP and hyperbranching (Hbr) to develop a multiplex detection assay for IHNV and ISAV.

2.2.8 Microarray

This technology is used to assess expression rate of thousands of genes and identify wide range of pathogens from complex samples in one single reaction. This technique usually involves hybridization of DNA with large number of probes and can overcome the shortcomings of multiplex PCR, which can detect only a maximum of six pathogens at a time. There are two types of DNA microarrays that are widely used—cDNA microarrays and oligonucleotide/DNA chips. There are a number of ways of using DNA microarrays. One method is the use of fluorescent labeled DNA sequences that are hybridized to the microarray slide. The data is detected by fluorescent array detection and analyzed by computer programs. The second and more practical method is the use of fluorescent labeled competitor oligonucleotide. When target DNA does not hybridize to the tethered oligonucleotide in the microarray, fluorescent labeled competitor oligonucleotide will bind to the tethered oligonucleotide on the chip and displace the test DNA. Then the fluorescent microarray detector and computer program will analyze the fluorescent array for the presence or absence of the species/strain specific DNA sequence. Microarray does not require

clear length differences between PCR products and therefore, PCR assays can be designed to generate smaller sized amplicons that can improve efficiency and probability of template recovery from degraded DNA and reduces PCR template biasedness. Compared to traditional nucleic acid hybridization with membranes, microarrays offer the additional advantages of high density, high sensitivity, rapid detection, lower cost, automation, and low background levels. Since most of the pathogens genetic sequences are known, oligonucleotide probes complementary to all pathogens can be used for microarray. Although the set-up cost for the use of DNA microarrays is high, once the equipment is available and microarrays are prepared, cost per unit of sample analyzed becomes low. In the post-genome sequencing era, microarrays have been developed from model and non-model fish and have the possibility of heterologous application. Though majority of them are publicly available, however, they vary in type, size, complexity, methodological development and motivation and degree of annotation, so it is advisable to carefully select the array beforehand [19].

2.2.9 DNA sequencing

DNA sequencing is used to determine the four chemical blocks—adenine, guanine, thymine and cytosine, that make up the DNA molecule. The sequence information can help determine changes in the gene that may cause disease. First generation sequencing techniques include the Sanger method and the Maxam-Gilbert techniques. Maxam-Gilbert are based on chemical modification of DNA and subsequent cleavage at specific bases while Sanger method requires that each read start be cloned for production of single-stranded DNA. Maxam–Gilbert sequencing is less popular due to its technical complexity. The chain-terminator method or Frederick Sanger method, which uses dideoxynucleotide triphosphates (ddNTPs) as DNA chain terminators, became a popular method of DNA sequencing due to its greater efficiency, use of fewer toxic chemicals and lower amounts of radioactivity than Maxam-Gilbert method. Second generation sequencing includes technologies such as Illumina and Ion Torrent that produce massive parallel sequencing of short read length of reads of DNA (150–400 bp), which require extensive assembly. Third generation sequencing method includes PacBio and ONT and involves sequencing through extended repetitive regions in the genome to produce much longer reads (6–20 kb) but far fewer reads per run (typically hundreds of thousands). The second and third generation sequencing methods, collectively known as the next generation sequencing (NGS) or high throughput sequencing allows the sequencing of DNA and RNA more quickly and cheaply. The goal of NGS is to investigate functional genome, epigenome and transcriptome elements in cells and tissues, and their temporal expression, which permits the definition of variation in gene expression among the different types of tissue, organs or life stages of the target organism. Over the past decade, the cost of NGS has decreased significantly, making it possible to use non-model fish species to investigate emerging environmental issues, understand the cell-cell interactions, and whole organismal physiology. To cope with it, bioinformatics is also rapidly evolving and new algorithms are being published. It is expected that NGS with bioinformatics is the way to revolutionize the field of fisheries and might also help clarify the previous findings and dogmas prevalent in aquaculture and biology.

2.2.10 RAD sequencing

Restriction-site associated DNA sequencing (RAD sequencing or RAD-Seq) combine the use of genome complexity reduction with REs and the high sequencing

output of NGS technologies. Original RAD-Seq was first described by Baird et al. [20] and several variants of this methodology have been described since then [21]. But, only the original RAD-Seq [20], 2b-RAD and ddRAD are extensively used in aquaculture research. In aquaculture, RAD-Seq has been used in genetic mapping [22], reference genome assembly sex determination loci mapping [23–26], etc. Some of the main reasons for its instant success is that RAD-Seq does not require any prior genomic knowledge, it allows generation of population-specific genotype data (i.e., no ascertainment bias) and it offers flexibility in terms of desired marker density across the genome. The use of different REs or innovative modifications to the base technique allows a high level of control over the number of markers obtained for a specific study. RAD-Seq and similar techniques are also amenable tools for aquaculture breeding, where genetic markers have typically been used in family assignment and pedigree reconstruction [27]. Mass spawning species are common in aquaculture, where mixed rearing and unknown parental contribution necessitate the use of genotyping for family-based breeding. RAD-Seq potentially facilitates a single experiment whereby pedigrees are reconstructed, genetic diversity is quantified, QTL are mapped, and genomic breeding values calculated [28].

2.2.11 Genomic marker development

Most of the genetic improvement in fish and shellfish species to date has been made through the use of traditional selective breeding of Atlantic salmon, Rainbow trout, tilapia and many other fish [29]. Notably, spontaneous mutations in the genome create genetic variability (or polymorphism) and this variability can be an effective means to analyze fish trait and geological pedigree. Boom in whole genome sequencing technology, though still costly, encourage fish researchers to investigate genomic marker's potential in selective breeding and aquaculture production. There are several available markers for fish research: AFLP, RAPD, etc., but most prevalent ones are microsatellite and SNPs. Microsatellite markers, identified using microsatellite sequence enriched genomic library or Expressed tagged sequence library, are simple tandem sequence repeats scattered across the genome and used increasingly in aquaculture species [29]. SNPs are generally identified using in depth genome sequencing and require huge financial and bioinformatical investment. MAS (marker assisted selection) is useful for traits that are difficult to measure on breeding candidates, particularly when they are largely linked to QTL (quantitative trait loci). With the help of MAS and GS (genomic selection), several studies have demonstrated increased accuracy of breeding value predictions in growth and disease resistance in yellowtail and Atlantic salmon [30–33]. Nevertheless, this approach requires a great amount of detailed information in order to choose which gene explains the greatest effect and to have sufficient power to detect the association.

2.2.12 Metagenomics

There are two main methods for studying the microbiome using high-throughput sequencing: marker-gene studies and whole-genome-shotgun (WGS) metagenomics. While marker-gene studies, amplify a particular gene (16S rRNA for bacteria/archaea, 18S for fungi), metagenomics refer to the sequencing of DNA from the entire genome of samples obtained directly from the environment (water, soil) or tissues. Advances in metagenomics have themselves been driven by advances in second- and third-generation sequencing technologies, which are now capable of producing hundreds of gigabases of DNA sequenced data at a very low cost [34]. Unlike bacteria that use the 16S ribosomal RNA as a common gene

for their identification, viruses lack a single common gene for their identification which makes it difficult to monitor their population dynamics in different aquatic environments [35]. Metagenomics also holds the promise of revealing the genomes of the majority of microorganisms that cannot be readily obtained in pure culture [36]. Breitbart et al. [37] have shown that it is possible to sequence entire genomes of uncultured marine viruses using metagenomics. For metagenomic sequences linked to novel diseases, there is need to isolate the virus involved followed by verification using conventional diagnostic approaches such as cell culture to exhibit the cytopathic effect (CPE), morphological characterization using electron microscopy, and molecular characterization using PCR ([38], **Table 1**).

2.2.13 DNA vaccines

DNA vaccines are composed of bacterial plasmids which has two units-antigen expressing unit that comprises of promoter/enhancer sequences, antigen coding and polyadenylation sequences; and the production unit comprising of sequences necessary for plasmid amplification and selection [39]. The vaccine inserts are constructed by molecular cloning and transformed into bacterial cells, and the purified plasmid DNA is injected into fish. Hansen et al. [40] first introduced vaccination in fish by injecting plasmid constructs encoding viral glycoprotein directly into skeletal muscle of common carp that resulted in efficient protection of the fish against rhabdoviruses. More than 20 different virus DNA vaccines have been developed experimentally for prophylactic use in fish targeting viruses such as rhabdoviridae, orthomyxoviridae, togaviridae and nodaviridae [41, 42]. However, despite this huge prospect, DNA vaccines for farmed animals remain at the moment experimental. DNA vaccines seem to be more harmless and more stable than ordinary vaccines [42]. Plasmids are non-viable and do not multiply, and therefore have a low risk of developing secondary disease and infection. The main concern about the potential DNA vaccines is that they might integrate into the host genome and generate immune responses. However, extensive surveys have found little evidence of integration, and the merger risk appears to be less than normal mutation. Significant advantages of these vaccines include cheapness, simplicity of

Pathogens	**Detection method**
V. vulnificus, *L. anguillarum*, *P. damselae*, *V. parahaemolytocus*	Multiplex PCR, DNA microarray
Y. ruckeri, *A. salmonicida*, *F. psychrophilum*	Multiplex PCR
Infectious salmon anemia virus (ISAV)	RT PCR
Myxobolus cerebralis	Real time PCR
Edwardsiella tarda	LAMP
Infectious hematopoietic necrosis virus (IHNV) & ISAV	Molecular padlock
R. salmoninarum, *A. salmonicida*, *E. ictaluri*, *F. columnare*, *F. psychrophilum*, *Y. ruckeri*, *P. salmonis*, *T. maritimum*	DNA microarray
A. salmonicida, *E. ictaluri* and *F. psychrophilum*	PCR and DNA microarrays
Aeromonas (*A. hydrophila*, *A. sobria*, *A. caviae* and *A. veronii*)	Multiplex PCR
P. salmonis (Salmonid Rickettsial Septicaemia)	PCR-RFLP

Table 1.
Prevalent examples of established disease diagnostics in aquaculture.

production and consumption, transport and higher resistance. The other important feature of these vaccines is the ability to put several antigens in the plasmid, resulting in immunization against all agents [43]. In 2005, APEX-IHN (Novartis/Elanco) became the first DNA vaccine licensed for commercial use in aquaculture for protection of Atlantic salmon against Infectious Hematopoietic Necrosis Virus (IHNV) in British Colombia. In 2017, the European Commission through the European Medicines Agency (EMA) granted marketing authorization of CLYNAV (Elanco), a polyprotein-encoding DNA vaccine against Salmon Pancreas Disease Virus (SPDV) infection in Atlantic salmon (*Salmo salar*) for use within the EU. However, administration of vaccines typically requires individual handling and treatment of all production fish, which can be expensive and impractical in a large-scale production environment.

2.2.14 Transgenesis

Transgenics are those genetically engineered organisms which have heterologous DNA (transgene) integrated stably into their genome through artificial means like microinjection, electroporation, sperm mediated transfer, lipofection, retrovirus, etc. The transgene construct carries a target gene, encoding product of interest and regulatory elements that regulate the expression of the gene in a spatial, temporal and developmental manner [44]. Since the development of the first transgenic fish in 1984, a wide number of transgenic fish species have been produced (**Table 2**) to improve growth, disease resistance, cold resistance, etc. [45].

2.2.15 Gene therapy

In the mid-twentieth century, researcher demonstrated that the rate of mutagenesis could be enhanced with radiation or chemical treatment [46, 47]. Later with the help of transposons, targeted genomic changes were made in various model organism including medaka and zebrafish [48–50]. But due to prevalence of transposon machinery in these fish, longer time requirement for generating particular line and

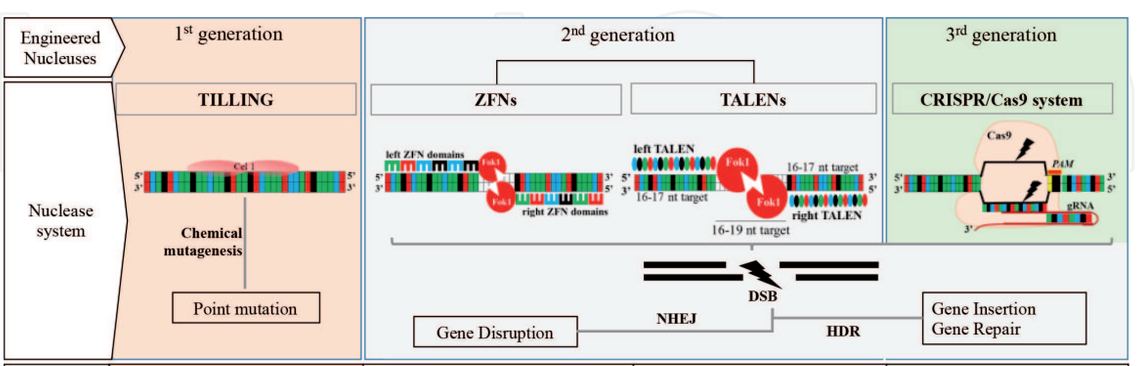
Species	Foreign gene	Desired effect
Striped bass (*Morone saxatilis)*	Insect genes	Disease resistance
Common carp (*Cyprinus carpio)*	Salmon and human GH; rainbow trout GH	Improved disease resistance
Grass carp (*Ctenopharyngodon idellus)*	hLF hLF + common carp β-actin promoter	Increased disease resistance to bacterial pathogen Increased disease resistance to grass carp hemorrhage virus
Channel catfish (*Ictalurus punctatus)*	Silk moth (*Hyalophora cecropia)* cecropin genes	Enhance bactericidal activity
Japanese Medaka (*Oryzias latipes)*	Insect cecropin or pig cecropin-like peptide genes + CMV	Enhanced bactericidal activity against common fish pathogens
Atlantic Salmon (*Salmo salar)*	Mx genes	Potential resistance to pathogens following treatment with poly I:C
Nile Tilapia (*O. niloticus)* and Redbelly Tilapia (*Tilapia zillii)*	Shark (*Squalus acanthias* L.) IgM genes	Enhanced immune response

Table 2.
Transgenesis in aquaculture.

concerns about transgenics associated wild genepool contamination and biodiversity degradation has led aquaculture researchers to focus on other knockdown and knockout technologies.

In fish, antisense morpholinos, small interfering RNA (siRNA) and PNAs (peptide nucleic acid) are widely used to transiently interfere with gene function. Morpholinos are typically 25 bp long oligos that specifically interfere with gene function based on their complementarity to the target sequence either by blocking translation initiation or by interfering with splicing. The non-ribose-based backbone renders morpholinos insensitive to enzymatic degradation. PNAs have a higher affinity for RNA, yet they are less soluble and therefore the *in vivo* use is limited. Dorn et al. [51] changed the chemical composition of the PNA backbone to increase solubility and showed efficient knockdown of the *six3* gene in medaka. In most cases, the chemical/RNA is micro-injected or electroporated into fertilized eggs at early cleavage stages to ensure a ubiquitous distribution to all cells of the developing embryo. If thus applied, they interfere with gene function during early development. To study gene function during later stages, morpholinos can be activated conditionally by light-induced uncaging. However, recent results in zebrafish indicate that morpholino-based gene knockdown often results in unspecific off-target effects [52].

To overcome abovementioned complications advanced genome editing techniques were developed, in which, no genetic material from another species is introduced and thus the genome remains untainted. Although tilling (target induced local lesion in genome) was first of this kind, it mostly creates single point mutation and requires large screening. Some of the next generation gene editing tools used in fish are zinc finger nucleases (ZFNs), transcription activator like effector nucleases (TALENs) and CRISPR/Cas system. Mutations can be achieved by introducing double strand breaks into the target gene and non-homologous end joining (NHEJ) repair mechanism is used to produce insertions or deletions in a site-specific manner resulting in permanent disruption of the function of the target gene. On the other hand, exogenous gene sequence can be introduced into the genome by co-delivering the targeted nucleases along with a target vector containing the DNA homologous to the break site for gene correction (**Figure 1**).



	1st generation	2nd generation		3rd generation
Recognition	None	3 bp/Finger Protein-DNA	1 bp/Module Protein-DNA	1 bp/Base RNA-DNA
Advantage	• Suitable for any organism • Independent of genome size and reproductive system • No transgenic manipulation required	• Target any genomic sequences • Low immunity • Small size: efficient delivery into cells	• Target any genomic sequences • Limited off-target effects • High efficiency	• Target any genomic sequences • Multiple sites target at one time • High efficiency • Cost and time effective
Disadvantage	• Poor efficiency • High cost, labor-intensive and time consuming • Based on random mutation, so the desired mutation might never be found	• Low efficiency, high off-target effects • Single site target at once • Designing construct is time consuming, laborious and expensive, • Risk of an immunological response	• Sensitive to target DNA methylation • Single site target at once • Time consuming and costly (compared to CRISPR/Cas9).	• Low to High off-targets • Target selection limited on PAM sequence adjacent • Big size might complicate delivery

Figure 1.
Comparative evaluation of various knockout technologies used in fish manipulation.

Fishes	Genes (method)	Fishes	Genes (method)
Atlantic salmon	*dnd*, *tyr*, *slc24a5* (C) [60, 61]	Sturgeon	*dnd* (C) [62]
Atlantic killifish	*ahr2* (C) [63]	Tilapia	*fox12a*, *cyp19a1a*, *dmrt1*, *nanos*, *gsdf*, *sf-1/nr5a*, *mstn* (C); *rspo1*, *fox12a*, *cyp19a1a* (T) [64–69]
Cavefish	*oca2* (T) [70]		
Channel catfish	*lh* (Z) [71]		
Chinese lamprey	*slc24a5* (C) [72]	Yellow catfish	*mstn* (Z) [12]
Common carp	*sp7a/b*, *runx2*, *bmp2a*, *opg & mstn* (T & C) [73]	Zebrafish	*dnd* (M); *ntl*, *slc24a5*, *kdr1*, *prl*, (Z); *gria3a*, *hey2*, *cyp19a1a*, *ryr3*, *ryr1a*, *tbx6*, *slc24a*, *slc45a2*, *fsh*, *lh*, *fshr*, *ihcgr*, *pgr*, *rb1*, *bmp15*, *mesp*, *gnrh3*, *zap70*, *nrld1*, *leg1a*, *mstn*, *rnf213a*, *mpl*, *dmrt1*, *cyp17a1*, *stat3*, *kiss1/2 & kissr1/2* (T); *mitfa*, *ddx19*, *slc24a5*, *slc45a2*, *seta/b*, *nrg1-I*, *stxbp1*, *nERs*, *gspt11*, *fus*, *akt2*, *atp6v1h*, *cyp19a1a* (C) [62, 74–108]
Japanese anchovy	*mstn* (T & C) [109]		
Medaka	*dnd* (M); *fox13*, *dmy*, *dmc1*, *fshb*, *gnrh1* (T); *gsdf* (Z) [110–114]		
Red sea bream	*mstn* (C) [115]		
Rice field eel	*dmrt1*, *foxl2*, *cyp19ala* (T) [116]		
Rohu	*tlr22* (C) [117]		
Starlet and sturgeon	*Ntl*, *dnd* (T & C) [118, 119]		

M, morpholino; Z, zinc finger nuclease (ZFN); T, transcription activator-like effector nucleases (TALEN), C, clustered regularly interspaced short palindromic repeats (CRISPR).

Table 3.
Genome editing using ZFN, TALEN and CRISPR system in varies model and non-model fish species.

Theoretically, ZFN is an ideal tool for inducing mutations at target DNA sites in any organisms [53]. However, its application has been constrained by limitations in zinc finger domain design and construction as well as low efficiency [54]. Compared with ZFN, the recently emerged TALEN provides us a more advanced approach for genome editing; it is much easier to construct plasmids for expressing TALE proteins, making this technology easily available to most molecular biology laboratories. Because of this and its high specificity and efficiency, TALEN has quickly replaced ZFN as a dominant platform for genome editing since its establishment in 2011 [55]. Unlike ZFN and TALEN, the nuclease Cas9 is guided towards the target DNA site by a small guide RNA followed by random cleavage of the DNA. Particularly, the rapid emergence of CRISPR/Cas9 caused a paradigm shift in the research community [56]. There is complementary usage of these two technologies in recent years, as CRISPR/Cas9 works as monomer, it consists of protein and RNA and produces blunt end, while TALEN works as dimer, it consists of protein only and produces cohesive ends [57]. Although each one has its associated pros and cons [58], TALENs and CRISPR technologies have comparatively high specificity and efficiency with low off target effect [59]. Not only the methodology, but selection of delivery methodology (microinjection, electroporation, etc.), target tissue, and host is critical for ensured success in

aquaculturable strain production. Numerous genes are being knocked out using various techniques and some of them are already adapted for commercial aquaculture (**Table 3**).

3. Conclusions

With the continual growth of global aquaculture, fish production continues to grow globally and till date only a small proportion of the aquatic animals come from managed breeding especially through applied molecular cloning and genomics (**Table 4**). The molecular biology of aquatic organisms offers many opportunities for rapid genetic gains as new genetic techniques make the improvement feasible in a wider range of model and non-model species. The future of molecular biology in aquaculture is bright with the technologies mentioned above being cheaper than ever, widely available and easily applicable in laboratories. However, the results obtained from these methods should not be conclusive without additional information, such as clinical diagnosis, as the mere detection of a certain pathogen does not

Category	Type of approach	Popular methods
Genomics	High throughput analysis	Microarray, NGS, whole genome bisulfate sequencing
	Marker based analysis	Rad sequencing, microsatellite, SNP, AFLP, RAPD, RFLP
Forward genetics	Chemical mutagenesis	ENU mutagenesis
	Transposon mutagenesis	Sleeping beauty, AcDs, Tol2, EnSpm-N6
Reverse genetics	Antisense and small RNA	Morpholino, PNA, SiRNA, shRNA
	Micro RNA	miRNA sponges, miRNA knockdown, miRNA mimics
	Conditional knockdown	Tet on/off
	Tilling	ENU mutagenesis
	Genome editing	ZFN, CRISPR/Cas9, TALEN
Transgenesis	Meganuclease	ISecI
	Transposon	Sleeping beauty, AcDs, Tol2, EnSpm-N6
	Recombinases (site specific)	PhiC3, Cre-loxP, BAC, Fosmid, YAC
Molecular genetics	Reporter cell line	Promoter analysis,
	Cell lineage	Gaudi toolbox
	Transactivation	LexPR, Gal4, tet on/off, heat shock protein
Transcriptomics	RNA detection	In situ hybridization, expressed sequence tagging, CDNA library, RNA-seq, microarray, QPCR, PCR
Proteomics	Protein detection	Antibody based analysis, chromatography and spectrophotometry

Table 4.
Summary of molecular biology application in fish.

imply necessarily that it is responsible for or even involved in a disease. Effective use of these techniques will reduce economic losses as well as risk of infection among wild fish species. Taking advantage of the numerous tissue specific sequence information available in the database, predictions of gene function by bioinformatics tools such as *in silico* and *in vitro* can be employed to identify candidate genes responsible for diseases or disease resistance that will reduce labor and cost of diagnosis and treatment. *In silico* approaches use computational tools to analyze raw DNA sequence data to simulate and predict the function and structural features of protein. In addition, the use of *in vitro* organoid models that refer to growing stem cells in 3D to generate cellular units that mimic an organ in both structure and function, is advancing rapidly. This method can also be applied in fish to study organ development, reproductive enhancement, fast tract selective breeding, disease and drug interactions as well. The new diagnostic techniques like, droplet digital PCR, Hybrid fusion FISH might improve the credibility and cost effectiveness of disease diagnosis.

Genome editing though has the advantage over traditional selective breeding and a trait can be introduced in a single generation without disrupting a favorable genetic background. Many traits of great significance in aquaculture could be targets for improvement by genome editing, including growth and reproductive performance, disease resistance, feed conversion efficiency, and tolerance to environmental stressors (temperature, salinity and oxygen). Keeping the animal welfare issues of "genetically modified organisms" in mind, fish that carry more muscle mass have also been produced by the disruption of a single gene (Myostatin, an inhibitor of skeletal muscle growth) in Common carp, Tilapia, Red sea bream and Japanese anchovy [74, 75, 82, 91]. But still the key question is whether the precise natural genome modifications will find greater public acceptance and make a way to commercial aquaculture. The long-term impacts of these non-transgenic GMOs on wild biodiversity and environment are an uncharted area too. So, in the coming era, we must rethink to what extent we can and should use these molecular advancements for aquaculture betterment.

Acknowledgements

This work was in part supported by Grants from Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japanese Society for the Promotion of Science (JSPS) Kakenhi, Grant Nos. 16H04981, 18 K14520 and 19H03049; Sumitomo Grant No. 180959.

DOI: http://dx.doi.org/10.5772/intechopen.88197

Author details

Tapas Chakraborty[1*], Sipra Mohapatra[1], Chimwar Wanglar[2] and Dipak Pandey[1]

1 South Ehime Fisheries Research Center, Ehime University, Ainan, Japan

2 College of Fisheries, Central Agricultural University, Imphal, India

*Address all correspondence to: tchakraborty83@gmail.com

References

[1] Nash CE. The History of Aquaculture. Ames Iowa: Wiley-Blackwell; 2011. 227 pp. ISBN: 978-0-8138-2163-4

[2] Smith P, Christofilogiannis P. Application of normalised resistance interpretation to the detection of multiple low-level resistances in strains of vibrio anguillarum obtained from Greek fish farms. Aquaculture. 2007;**272**(1):223-230

[3] Grace S. Lessons for aquaculture from agriculture: Selected comparisons of fish and animal farming. Masters thesis. University of Miami; 2014

[4] FAO Statistical Yearbook. Asia and the Pacific Food and Agriculture. 2014. Available from: http://www.fao.org/3/a-i3590e.pdf

[5] The World Bank annual report. End extreme poverty, promote shared prosperity. 2013. Available from: http://documents.worldbank.org/curated/en/947341468338396810/Main-report

[6] Houston RD, Macqueen DJ. Atlantic salmon (*Salmo salar* L.) genetics in the 21st century: Taking leaps forward in aquaculture and biological understanding. Animal Genetics. 2019;**50**(1):3-14

[7] Mialhe E, Bachère E, Boulo V, Cadoret JP. Strategy for research and international cooperation in marine invertebrate pathology immunology and genetics. Aquaculture. 1995;**132**:33-41

[8] Abollo E, Ramilo A, Casas SM, Comesaña P, Cao A, Carballal MJ, et al. First detection of the protozoan parasite Bonamia exitiosa (Haplosporidia) infecting flat oyster Ostrea edulis grown in European waters. Aquaculture. 2008;**274**:201-207

[9] Grijalva-Chon JM, Castro-Longoria R, Enriquez-Espinoza TL, Maeda-Martinez AN, Mendoza-Cano F. Molecular evidence of the protozoan parasite Marteilia refringens in Crassostrea gigas and Crassostrea corteziensis del Golfo de California. Latin American Journal of Aquatic Research. 2015;**43**:776-780

[10] Stentiford GD, Sritunyalucksana K, Flegel TW, Williams BAP, Withyachumnarnkul B, Itsathitphaisarn O, et al. New paradigms to help solve the global aquaculture disease crisis. PLoS Pathogens. 2017;**13**(2):e1006160

[11] Proxton IR. Molecular techniques in the diagnosis and management of infectious diseases: Do they have a role in bacteriology? Medical Principles and Practice. 2005;**14**(1):20-26

[12] Dong Z, Ge J, Li K, Xu Z, Liang D, et al. Heritable targeted inactivation of myostatin gene in yellow catfish (*Pelteobagrus fulvidraco*) using engineered zinc finger nucleases. PLoS One. 2011;**6**:e28897

[13] Grizzle JM, Altinok I, Fraser WA, Francis-Floyd R. First isolation of largemouth bass virus. Diseases of Aquatic Organisms. 2002;**50**:233-235

[14] Heppell J, Berthiaume L, Tarrab E, Lecomte J, Arella M. Evidence of genomic variations between infectious pancreatic necrosis virus strains determined by restriction fragment profiles. Journal of General Virology. 1992;**73**:2863-2870

[15] Radinsky R, Bucana CD, Ellis LM, Sanchez R, Cleary KR, Brigati DJ, et al. A rapid colorimetrie in situ messenger RNA hybridization technique for analysis of epidermal growth factor receptor in paraffin-embedded surgical specimens of human colon carcinomas. Cancer Research. 1993;**5**:937-943

[16] Trask BJ. Human cytogenetics: 46 chromosomes 46 years and counting. Nature Reviews Genetics. 2002;**3**:769-778

[17] Speicher MR, Carter NP. The new cytogenetics: Blurring the boundaries with molecular biology. Nature Reviews Genetics. 2005;**6**:782-792

[18] Millard PJ, Bickerstaff LE, LaPatra SE, Kim CH. Detection of infectious haematopoietic necrosis virus and infectious salmon anemia virus by molecular padlock amplification. Journal of Fish Diseases. 2006;**29**:201-213

[19] Miller KM, Maclean N. Teleost microarrays: Development in a broad phylogenetic range refleting diverse application. Journal of Fish Biology. 2008;**72**:2039-2050

[20] Baird NA, Etter PD, Atwood TS, Currey MC, Shiver AL, Lewis ZA, et al. Rapid SNP discovery and genetic mapping using sequenced RAD markers. PLoS One. 2008;**3**:e3376

[21] Andrews KR, Good JM, Miller MR, Luikart G, Hohenlohe PA. Harnessing the power of RADseq for ecological and evolutionary genomics. Nature Reviews Genetics. 2016;**17**:81-92

[22] Gonen S, Bishop SC, Houston RD. Exploring the utility of cross-laboratory RAD-sequencing datasets for phylogenetic analysis. BMC Research Notes. 2015;**8**:299

[23] Palaiokostas C, Bekaert M, Davie A, Cowan ME, Oral M, Taggart JB, et al. Mapping the sex determination locus in Atlantic halibut (*Hippoglossus hippoglossus*) using RAD sequencing. BMC Genomics. 2013;**14**:566

[24] Palaiokostas C, Bekaert M, Khan MG, Taggart JB, Gharbi K, McAndrew BJ, et al. A novel sex-determining QTL in Nile tilapia (*Oreochromis niloticus*). BMC Genomics. 2015;**16**:171

[25] Palaiokostas C, Bekaert M, Taggart JB, Gharbi K, McAndrew BJ, Chatain B, et al. A new SNP-based vision of the genetics of sex determination in European sea bass (*Dicentrarchus labrax*). Genetics Selection Evolution. 2015;**47**:68

[26] Wang W, Hu Y, Ma Y, Xu L, Guan J, Kong J. High-density genetic linkage mapping in turbot (*Scophthalmus maximus* L.) based on SNP markers and major sex- and growth-related regions detection. PLoS One. 2015;**13**:e0120410

[27] Vandeputte M, Haffray P. Parentage assignment with genomic markers: A major advance for understanding and exploiting genetic variation of quantitative traits in farmed aquatic animals. Frontiers in Genetics. 2014;**5**:432

[28] Palaiokostas C, Ferraresso S, Franch R, Houston RD, Bargelloni L. Genomic prediction of resistance to pasteurellosis in gilthead sea bream (*Sparus aurata*) using 2b-RAD sequencing. G3 (Bethesda). 2016;**6**:3693-3700

[29] Martnez V. Marker assisted selection in fish and shellfish breeding schemes. In: Guimarães E, Ruane J, Scherf B, Sonnino A, Dargie J, editors. Marker-Assisted Selection: Current Status and Future Perspectives in Crops Livestock Forestry and Fish. Rome, Italy: Electronic Publishing Policy and Support Branch Communication Division FAO; 2007. pp. 329-362

[30] Tsai HY, Hamilton A, Tinch AE, Guy DR, Gharbi K, Stear MJ, et al. Genome wide association and genomic prediction for growth traits in juvenile farmed Atlantic salmon using a high density SNP array. BMC Genomics. 2015;**16**:969

[31] Nguyen HN, Rastas PMA, Premachandra HKA, Knibb W. First high-density linkage map and single

nucleotide polymorphisms significantly associated with traits of economic importance in Yellowtail Kingfish *Seriola lalandi*. Frontiers in Genetics. 2018;**2018**:9-127

[32] Bangera R, Correa K, Lhorente JP, Figueroa R, Yanez JM. Genomic predictions can accelerate selection for resistance against Piscirickettsia salmonis in Atlantic salmon (*Salmo salar*). BMC Genomics. 2017;**18**:121

[33] Robledo D, Matika O, Hamilton A, Houston RD. Genome-wide association and genomic selection for resistance to amoebic gill disease in Atlantic salmon. G3. 2018;**8**:1195-1203

[34] Watson M. Illuminating the future of DNA sequencing. Genome Biology. 2014;**15**:108

[35] Munang'andu HM. Environmental viral metagenomics analyses in aquaculture: Applications in epidemiology and disease control. Frontiers in Microbiology. 2016;**7**:1986

[36] Hugenhotlz P. Exploring prokaryotic diversity in the genomic era. Genome Biology. 2002;**3**:reviews0003.1

[37] Breitbart M, Salamon P, Andresen B, Mahaffy JM, Segall AM, Mead D, et al. Genomic analysis of uncultured marine viral communities. Proceedings of the National Academy of Sciences of the United States of America. 2002;**99**:14250-14255

[38] Bibby K. Metagenomic identification of viral pathogens. Trends in Biotechnology. 2013;**31**:275-279

[39] Schirmbeck R, Reimann J. Revealing the potential of DNA-based vaccination lessons learned from the hepatitits B virus surface antigen. Biological Chemistry. 2001;**382**:543-552

[40] Hansen E, Fernandes K, Goldspink G, Butterworth P, Umeda PK, Chang KC. Strong expression of foreign genes following direct injection into fish muscle. FEBS Letters. 1991;**290**:307-312

[41] Dalmo RA. DNA vaccines for fish: Review and perspectives on correlates of protection. Journal of Fish Diseases. 2018;**41**:1-9

[42] Yoshikawa T, Kawamura Y, Ohashi M. Universal varicella vaccine immunization in Japan. Vaccine. 2016;**34**(19):1965-1970

[43] Soltani S, Farahani A, Dastranj M, Momenifar N, Mohajeri P, Emamie DA. DNA vaccine: Methods and mechanisms. Advances in Human Biology. 2018;**8**:132-139

[44] Chen TT, Lu JK, Fahs II R. Transgenic fish technology and its application in fish production. In: Altman A, editor. Agricultural Biotechnology. Marcel Dekker, Inc.; 1998. pp. 527-547

[45] Beardmore JA, Porter JS. Genetically modified organisms and aquaculture. FAO Fisheries Circular NO 989. Rome FAO 38. 2003

[46] Muller HJ. Artificial transmutation of the gene. Science. 1927;**66**:84-87

[47] Auerbach C, Robson JM, Carr JG. Chemical production of mutations. Science. 1947;**105**:243-247

[48] Rothstein RJ. One-step gene disruption in yeast. Methods in Enzymology. 1983;**101**:202-211

[49] Scherer S, Davis RW. Replacement of chromosome segments with altered DNA sequences constructed in vitro. Proceedings of the National Academy of Sciences of the United States of America. 1979;**76**:4951-4955

[50] Thomas KR, Folger KR, Capecchi MR. High frequency targeting of genes to specific sites in the mammalian genome. Cell. 1986;**44**:419-428

[51] Dorn S, Aghaallaei N, Jung G, Bajoghli B, Werner B, et al. Side chain modified peptide nucleic acids (PNA) for knock-down of six3 in medaka embryos. BMC Biotechnology. 2012;**12**:50

[52] Kok FO, Shin M, Ni CW, Gupta A, Grosse AS, et al. Reverse genetic screening reveals poor correlation between morpholino-induced and mutant phenotypes in zebrafish. Developmental Cell. 2014;**32**(1):97-108

[53] Gupta RM, Musunuru K. Expanding the genetic editing tool kit: ZFNs TALENs and CRISPR-Cas9. The Journal of Clinical Investigation. 2014;**124**:4154-4161

[54] Urnov FD, Rebar EJ, Holmes MC, Zhang HS, Gregory PD. Genome editing with engineered zinc finger nucleases. Nature Reviews. Genetics. 2010;**11**:636-646

[55] Miller JC, Tan S, Qiao G, Barlow KA, Wang J, Xia DF, et al. A TALE nuclease architecture for efficient genome editing. Nature Biotechnology. 2011;**29**:143-148

[56] Sakuma T, Yamamoto T. CRISPR/Cas9: The leading edge of genome editing technology. In: Yamamoto T, editor. Targeted Genome Editing Using Site-Specific Nucleases. Tokyo: Springer; 2015. pp. 25-41

[57] Sakuma T, Yamamoto T. Acceleration of cancer science with genome editing and related technologies. Cancer Science. 2018;**109**:3679-3685

[58] Zhu B, Wei G. Genome editing in fishes and their application. General and Comparative Endocrinology. 2018;**257**:3-12

[59] Wright DA, Li T, Yang B, Spalding MH. TALEN-mediated genome editing: Prospects and perspectives. The Biochemical Journal. 2014;**482**:15-24

[60] Wargelius A, Leininger S, Skaftnesmo KO, Kleppe L, Andersson E, Taranger GL, et al. Dnd knockout ablates germ cells and demonstrates germ cell independent sex differentiation in Atlantic salmon. Scientific Reports. 2016;**6**:21284

[61] Edvardsen RB, Leininger S, Kleppe L, Skaftnesmo KO, Wargelius A. Targeted mutagenesis in Atlantic salmon (*Salmo salar* L.) using the CRISPR/Cas9 system induces complete knockout individuals in the F0 generation. PLoS One. 2014;**9**:e108622

[62] Solin SL, Shive HR, Woolard KD, Essner JJ, McGrail M. Rapid tumor induction in zebrafish by TALEN-mediated somatic inactivation of the retinoblastoma1 tumor suppressor rb1. Scientific Reports. 2015;**5**:13745

[63] Aluru N, Karchner SI, Franks DG, Nacci D, Champlin D, Hahn ME. Targeted mutagenesis of aryl hydrocarbon receptor 2a and 2b genes in Atlantic killifish (*Fundulus heteroclitus*). Aquatic Toxicology. 2015;**158**:192-201

[64] Baloch AR, Franek R, Tichopad T, Fucikova M, Rodina M, Psenicka M. Dnd1 knockout in sturgeons by CRISPR/Cas9 generates germ cell free host for surrogate production. Animals. 2019;**9**(4):174

[65] Li MH, Yang HH, Li MR, Sun YL, Jiang XL, Xie QP, et al. Antagonistic roles of Dmrt1 and Foxl2 in sex differentiation via estrogen production in tilapia as demonstrated by TALENs. Endocrinology. 2013;**154**:4814-4825

[66] Li M, Yang H, Zhao J, Fang L, Shi H, Li M, et al. Efficient and heritable gene targeting in tilapia by CRISPR/Cas9. Genetics. 2014;**197**:591-599

[67] Jiang DN, Yang H, Li MH, Shi HJ, Zhang XB, Wang DS. Gsdf is a downstream gene of dmrt1 that functions in the male sex determination pathway of the Nile tilapia. Molecular Reproduction and Development. 2016;**83**:497-508

[68] Wu L, Yang P, Luo F, Wang D, Zhou L. R-spondin1 signaling pathway is required for both the ovarian and testicular development in a teleosts Nile tilapia (*Oreochromis niloticus*). General and Comparative Endocrinology. 2016;**230-231**:177-185

[69] Xie QP, He X, Sui YN, Chen LL, Sun LN, Wang DS. Haploinsufficiency of SF-1 causes female to male sex reversal in Nile tilapia *Oreochromis niloticus*. Endocrinology. 2016;**157**:2500-2514

[70] Ma L, Jeffery WR, Essner JJ, Kowalko JE. Genome editing using TALENs in blind Mexican cavefish Astyanax mexicanus. PLoS One. 2015;**10**:e0119370

[71] Qin Z, Li Y, Su B, Cheng Q, Ye Z, Perera DA, et al. Editing of the luteinizing hormone gene to sterilize channel catfish *Ictalurus punctatus* using a modified zinc finger nuclease technology with electroporation. Marine Biotechnology (New York, N.Y.). 2016;**18**:255-263

[72] Zu Y, Zhang XS, Ren JF, Dong XH, Zhu Z, Jia L, et al. Biallelic editing of a lamprey genome using the CRISPR/Cas9 system. Scientific Reports. 2016;**6**:23496

[73] Zhong Z, Niu P, Wang M, Huang G, Xu S, Sun Y, et al. Targeted disruption of sp7 and myostatin with CRISPR-Cas9 results in severe bone defects and more muscular cells in common carp. Scientific Reports. 2016;**6**:22953

[74] Yeh YC, Kinoshita M, Ng TH, Chang YH, Maekawa S, Chiang YA, et al. Using CRISPR/Cas9-mediated gene editing to further explore growth and trade-off effects in myostatin mutated F4 medaka (*Oryzias latipes*). Scientific Reports. 2017;**7**:11435

[75] Doyon Y, McCammon JM, Miller JC, Faraji F, Ngo C, Katibah GE, et al. Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases. Nature Biotechnology. 2008;**26**:702-708

[76] Siegfried KR, Nusslein-Volhard C. Germ line control of female sex determination in zebrafish. Developmental Biology. 2008;**324**:277-287

[77] Sander JD, Cade L, Khayter C, Reyon D, Peterson RT, Joung JK, et al. Targeted gene disruption in somatic zebrafish cells using engineered TALENs. Nature Biotechnology. 2011;**29**:697-698

[78] Dahlem TJ, Hoshijima K, Jurynec MJ, Gunther D, Starker CG, Locke AS, et al. Simple methods for generating and detecting locus-specific mutations induced with TALENs in the zebrafish genome. PLoS Genetics. 2012;**8**:e1002861

[79] Jao LE, Wente SR, Chen W. Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system. Proceedings of the National Academy of Sciences of the United States of America. 2013;**110**:13904-13909

[80] Irion U, Krauss J, Nusslein-Volhard C. Precise and efficient genome editing in zebrafish using the CRISPR/Cas9 system. Development. 2014;**141**:4827-4830

[81] Chu L, Li J, Liu Y, Hu W, Cheng CH. Targeted gene disruption in zebrafish reveals non canonical functions of LH signaling in reproduction. Molecular Endocrinology. 2014;**28**:1785-1795

[82] Chu L, Li J, Liu Y, Cheng CH. Gonadotropin signaling in zebrafish ovary and testis development: Insights from gene knockout study. Molecular Endocrinology. 2015;**29**:1743-1758

[83] Zhang Z, Lau SW, Zhang L, Ge W. Disruption of zebrafish follicle-stimulating hormone receptor (fshr) but not luteinizing hormone receptor (lhcgr) gene by TALEN leads to failed follicle activation in females followed by sexual reversal to males. Endocrinology. 2015;**156**:3747-3762

[84] Zhang Z, Zhu B, Ge W. Genetic analysis of zebrafish gonadotropin (FSH and LH) functions by TALEN-mediated gene disruption. Molecular Endocrinology. 2015;**29**:76-98

[85] Tang HP, Liu Y, Luo DJ, Ogawa S, Yin YK, Li SS, et al. The kiss/kissr systems are dispensable for zebrafish reproduction: Evidence from gene knockout studies. Endocrinology. 2015;**156**:589-599

[86] Shu Y, Lou Q, Dai Z, Dai X, He J, Hu W, et al. The basal function of teleost prolactin as a key regulator on ion uptake identified with zebrafish knockout models. Scientific Reports. 2016;**6**:18597

[87] Dranow DB, Hu K, Bird AM, Lawry ST, Adams MT, Sanchez A, et al. Bmp15 is an oocyte-produced signal required for maintenance of the adult female sexual phenotype in zebrafish. PLoS Genetics. 2016;**12**:e1006323

[88] Yabe T, Hoshijima K, Yamamoto T, Takada S. Quadruple zebrafish mutant reveals different roles of Mesp genes in somite segmentation between mouse and zebrafish. Development. 2016;**143**:2842-2852

[89] Spicer OS, Wong TT, Zmora N, Zohar Y. Targeted mutagenesis of the hypophysiotropic gnrh3 in zebrafish (*Danio rerio*) reveals no effects on reproductive performance. PLoS One. 2016;**11**(6):e0158141

[90] Moore JC, Mulligan TS, Torres Yordan N, Castranova D, Pham VN, Tang Q, et al. T cell immune deficiency in zap70 mutant zebrafish. Molecular and Cellular Biology. 2016;**23**:2868-2876

[91] Huang G, Zhang F, Ye Q, Wang H. The circadian clock regulates autophagy directly through the nuclear hormone receptor Nr1d1/Rev-erb and indirectly via Cebpb/(C/ebp.) in zebrafish. Autophagy. 2016;**12**:1292-1309

[92] Hu M, Bai Y, Zhang C, Liu F, Cui Z, Chen J, et al. Liver-enriched gene 1 a glycosylated secretory protein binds to FGFR and mediates an anti-stress pathway to protect liver development in zebrafish. PLoS Genetics. 2016;**12**:e1005881

[93] Gao Y, Dai Z, Shi C, Zhai G, Jin X, He J, et al. Depletion of Myostatin b promotes somatic growth and lipid metabolism in zebrafish. Frontiers in Endocrinology. 2016;**7**:88

[94] Wen J, Sun X, Chen H, Liu H, Lai R, Li J, et al. Mutation of rnf213a by TALEN causes abnormal angiogenesis and circulation defects in zebrafish. Brain Research. 1644;**2016**:70-78

[95] Serifi I, Tzima E, Soupsana K, Karetsou Z, Beis D, Papamarcaki T. The zebrafish homologs of SET/I2PP2A oncoprotein: Expression patterns and insights into their physiological roles during development. The Biochemical Journal. 2016;**473**:4609-4627

[96] Samsa LA, Ito CE, Brown DR, Qian L, Liu J. IgG-containing isoforms of neuregulin-1 are dispensable for cardiac trabeculation in zebrafish. PLoS One. 2016;**11**:e0166734

[97] Grone BP, Marchese M, Hamling KR, Kumar MG, Krasniak CS, Sicca F, et al. Epilepsy behavioral abnormalities and physiological comorbidities in syntaxin-binding protein 1 (STXBP1) mutant zebrafish. PLoS One. 2016;**11**:e0151148

[98] Lau ES, Zhang Z, Qin M, Ge W. Knockout of zebrafish ovarian aromatase gene (cyp19a1a) by TALEN and CRISPR/Cas9 leads to all-male offspring due to failed ovarian differentiation. Scientific Reports. 2016;**6**:37357

[99] Lin Q, Zhang Y, Zhou R, Zheng Y, Zhao L, Huang M, et al. Establishment of a congenital amegakaryocytic thrombocytopenia model and a thrombocyte-specific reporter line in zebrafish. Leukemia. 2017;**31**:1206-1216

[100] Webster KA, Schach U, Ordaz A, Steinfeld JS, Draper BW, Siegfried KR. Dmrt1 is necessary for male sexual development in zebrafish. Developmental Biology. 2017;**422**:33-46

[101] Zhai G, Shu TT, Xia YG, Jin X, He JY, Yin Z. Androgen signaling regulates the transcription of anti-Mullerian hormone via synergy with SRY-related protein SOX9A. Scientific Bulletin. 2017;**62**:197-203

[102] Lu H, Cui Y, Jiang L, Ge W. Functional analysis of nuclear estrogen receptors (nERs) in zebrafish reproduction by genome editing approach. Endocrinology. 2017;**158**:2292-2308

[103] Wang H, Luo L, Yang D. Loss of Gspt1l disturbs the patterning of the brain central arteries in zebrafish. Biochemical and Biophysical Research Communications. 2017;**486**:156-162

[104] Xiong ST, Wu JJ, Jing J, Huang PP, Li Z, Mei J, et al. Loss of stat3 function leads to spine malformation and immune disorder in zebrafish. Scientific Bulletin. 2017;**62**:185-196

[105] Lebedeva S, de Jesus Domingues AM, Butter F, Ketting RF. Characterization of genetic loss-of-function of Fus in zebrafish. RNA Biology. 2017;**14**:29-35

[106] Zhang D, Wang J, Zhou C, Xiao W. Zebrafish akt2 is essential for survival growth bone development and glucose homeostasis. Mechanisms of Development. 2017;**143**:42-52

[107] Liu Y, Tang HP, Xie R, Li SS, Liu XC, Lin HR, et al. Genetic evidence for multifactorial control of the reproductive axis in zebrafish. Endocrinology. 2017;**158**:604-611

[108] Zhang Y, Huang H, Zhao G, Yokoyama T, Vega H, Huang Y, et al. ATP6V1H deficiency impairs bone development through activation of MMP9 and MMP13. PLoS Genetics. 2017;**13**:e1006481

[109] Sakaguchi K, Yoneda M, Sakai N, Nakashima K, Kitano H, Matsuyama M. Comprehensive experimental system for a promising model organism candidate for marine teleosts. Scientific Reports. 2019;**9**:4948

[110] Kurokawa H, Saito D, Nakamura S, Katoh-Fukui Y, Ohta K, Baba T, et al. Germ cells are essential for sexual dimorphism in the medaka gonad. Proceedings of the National Academy of Sciences of the United States of America. 2007;**104**:16958-16963

[111] Nishimura T, Sato T, Yamamoto Y, Watakabe I, Ohkawa Y, Suyama M, et al. foxl3 is a germ cell-intrinsic factor involved in sperm-egg fate decision in medaka. Science. 2015;**349**:328-331

[112] Luo D, Liu Y, Chen J, Xia X, Cao M, Cheng B, et al. Direct production of XYDMY− sex reversal female medaka (*Oryzias latipes*) by embryo

microinjection of TALENs. Scientific Reports. 2015;**5**:14057

[113] Chen J, Cui XJ, Jia ST, Luo DJ, Cao MX, Zhang YS, et al. Disruption of dmc1 produces abnormal sperm in medaka (*Oryzias latipes*). Scientific Reports. 2016;**6**:30912

[114] Zhang X, Guan G, Li M, Zhu F, Liu Q, Naruse K, et al. Autosomal gsdf acts as a male sex initiator in the fish medaka. Scientific Reports. 2016;**6**:19738

[115] Takahashi A, Kanda S, Abe T, Oka Y. Evolution of the hypothalamic-pituitary-gonadal axis regulation in vertebrates revealed by knockout medaka. Endocrinology. 2016;**157**:3994-4002

[116] Kishimoto K, Washio Y, Yoshiura Y, Toyoda A, Ueno T, Fukuyama H, et al. Production of a breed of red sea bream *Pagrus major* with an increase of skeletal muscle mass and reduced body length by genome editing with CRISPR/Cas9. Aquaculture. 2018;**495**:415-427

[117] Feng K, Luo H, Li Y, Chen J, Wang Y, Sun Y, et al. High efficient gene targeting in rice field eel *Monopterus albus* by transcription activator-like effector nucleases. Science Bulletin. 2017;**62**:162-164

[118] Chakrapani V, Patra SK, Panda RP, Rasal KD, Jayasankar P, Barman HK. Establishing targeted carp TLR22 gene disruption via homologous recombination using CRISPR/Cas9. Developmental and Comparative Immunology. 2016;**61**:242-247

[119] Chen J, Wang W, Tian Z, Dong Y, Zhu H, Zhu HH, et al. Efficient gene transfer and gene editing in Sterlet (*Acipenser ruthenus*). Frontiers in Genetics. 2018;**9**:117