Prospects and Challenges of Molecular Interventions for Enhancing Aquaculture Production in the Temperate Himalayas

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Abstract

Global harvest of aquatic biota which includes flora and fauna both, through capture fisheries has already reached its maximum potential, or has shown offshoot in some cases. Capture fisheries is now stagnant, and therefore, in order to fulfil the need for growing worldwide population, culture fisheries have come up with a promising way. To further increase the production and sustainability of culture fisheries, various tools and techniques of biotechnology can be used. Aquatic biotechnology, which has both basic and spin-off applications, can help aquaculture producers increase output, efficiency, profitability, and sustainability. Genomic and proteomic research such as whole genome sequence (WGS) and marker-assisted selection (MAS) of economically important cultured fish could have an impact on fish genetic resource development and management as well. In genetically modified (GM) and gene knockout (GKO) fishes, economically important features such as improved growth, enhanced muscle mass, cold tolerance and disease resistance can be further improved. Cryopreservation of gametes (sperms and eggs) and embryos could open up new commercial possibilities for endless seed and fry production, as well as healthier and better-conditioned fish and brood stock management. It could also help with ex situ genome conservation in threatened and endangered species. Biotechnological interventions in intensive aquaculture have shown a considerable potential in using bioremediation and probiotics to regulate effluents, toxicants, and pathogens in the environment. Therefore, molecular tools can be used to minimize the impact of intensive aquaculture in environmental pollution.

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Aquatic biotechnology · Biotechnological interventions · Gene knockout · Brood stock genetic management · Proteome · Marker-assisted selection

14.1 Introduction

Aquaculture is a growing industry globally, as a source of income and easily digestible protein-rich food. But over the last few decades, due to growing global population, aquaculture has shifted from extensive farming to intensive farming. In intensive farming the fishes are stocked at higher density, and therefore, the production enhances tremendously. However, intensive farming also leads to several serious issues such as disease outbreak and effluent generation, which leads to environmental pollution.

Aquaculture in India, Nepal, and Bhutan is practiced based on many fish species such as common carp (*Cyprinus carpio*), brown trout (*Salmo trutta*), rainbow trout (*Oncorhynchus mykiss*), chocolate mahseer (*Neolissochilus hexagonolepis*), and minor carps (*Labeo* spp.). The type of fish, cultured in this region, depends upon the availability of water, topography, availability of seed and the climatic conditions. In India, rainbow trout is the most widely cultured fish species in temperate Himalayan region, followed by common carp and chocolate mahseer. The temperate Himalayan region of India stretches over 2500 km from Arunachal Pradesh to Jammu and Kashmir and 200–400 km from north to south. The principal sources of cold water include upland streams, rivers, high and low altitude lakes, and reservoirs in various hill states. In these regions, rainbow trout and common carp are the most widely cultured food fish species, depending upon the altitude, climatic condition, geography, infrastructure, and resource availability.

Molecular biology provides tremendous tools for developing sustainable, environment-friendly, economically viable, and feasible global aquaculture output. Increased public demand for fish as a protein-rich food and declining capture fisheries have prompted scientists to investigate how biotechnology can boost aquaculture productivity, making aquaculture a topic of great interest. To increase productivity and improve quality, scientists can use biotechnology tools to detect and combine economically important features in fish and shellfish. One example of the economically important trait is enhanced growth rate and disease resistance in fish species, used in aquaculture. Therefore, looking into the genes that promote the synthesis of natural fish growth hormones and natural defensive chemicals is always desirable. In case of aquaculture, enhanced coloration and unique-looking fish are desirable. Therefore, fluorescent fish or glow fish has high market demand than the normal-looking fish. However, it should always be kept in mind that current biotechnology should be utilized in conjunction with traditional technologies rather than as a replacement and that their use should be guided by need rather than the greed.

The need for aquaculture produce is high, and biotechnology can help meet that demand. Aquaculture, like other biotech-based products, will be rigorously vetted before being released to the public. The benefits, promised by developing technologies, would not be realized without a consistent investment to basic research. Synthetic hormones in induced reproduction are one of the biotechnology applications in cold-water aquaculture.

14.2 Biotechnology in Fish Breeding

Several fish species reach maturity but do not breed when kept in confined water bodies such as ponds and tanks. As a result, induced breeding techniques for producing high-quality and abundant cultured fish seed have been developed. It is one of the most reliable methods of producing pure seed of desired fish species. As a result of their efficacy and convenience, synthetic spawning hormones are increasingly being used. Pituitary gonadotropic hormone was purified from murrel and catla for the first time. When administered alone or with carp pituitary, antuitrin-s, leutocyclin, and LH-RH were ineffective. The Central Inland Fisheries Research Institute in Barrackpore, India, conducted extensive research using LH-RH alone or in conjunction with progesterone; breeding success rates ranged from 25 to 49% in carps to 100% in catfishes. The most extensively utilized biotechnological technology for induced breeding in fish is gonadotropin-releasing hormone (GnRH). In all vertebrates, the fundamental regulator and central initiator of the reproductive cascade is GnRH (Battacharya et al. 2002). It is a decapeptide, discovered in the hypothalamus regions of pigs and ships. One type of GnRH has been identified as the sole neuropeptide responsible for LH and FSH synthesis in the most placental mammals, including humans. It should be noted that LH-RH alone is ineffective in inducing fish spawning. The Linpe technique is a highly successful approach that induces ovulation and spawning in farmed fish by combining LH-RH-a (GnRH-a) with a dopamine antagonist. Syndel Laboratories, Inc. of Vancouver, British Columbia, Canada, has marketed this technique, under the trade name Ovaprim. There have been reports of rohu, mrigal, and catla successfully spawning with LH-RH analogues and 100% ovulation with pimozide at 10 mg/kg body weight.

In non-mammalian animals (excluding the guinea pig), 12 GnRH variants have been physically described, with seven or eight distinct forms separated from species of fish (Halder et al. 1991). The same advancement of GnRH technology has now enabled the successfully induced breeding in fish. There are several synthetic hormones, which are currently being widely used in induced breeding. Examples are HCG (human chorionic gonadotropin) hormone; Ovaprim, ovatide, synahorin (CG and mammalian pituitary gland mixture), and ovapel are the most widely used inducing agents. Among all these, Ovaprim is the most widely used synthetic agent in India for induced spawning of fish, including carps. These synthetic hormones are superior to pituitary extract in its performance and are easily available with fish vendors. The dose depends upon the species and sex of the fish. For carps single dose is given for induced spawning. Similarly, indigenous preparations such as Ovatide

(M/s. Hemmopharma Ltd., Mumbai) and WOVA-FH (M/s. WOCKHARDT Ltd., Mumbai) are widely utilized in India for carp and other fish spawning.

The following is the dose of various spawning agents (Ovaprim, Ovatide, WOVA), which can be used in fishes:

Females:

- Catla: 0.4–0.5 mL/kg body weight.
- Rohu: 0.3–0.4 mL/kg body weight.
- Mrigal: 0.25–0.3 mL/kg body weight.
- Fringe-lipped carp: 0.3–0.4 mL/kg body weight.
- Catfishes: 0.6–0.8 mL/kg body weight.
- Males (all carp species): 0.1–0.3 mL/kg body weight.
- Male (catfish): 0.15–0.4 mL/kg body weight.
- Silver carp: 0.4–0.7 mL/kg body weight.
- Grass carp: 0.4–0.8 mL/kg body weight.
- Bighead carp: 0.4–0.5 mL/kg body weight.
- Mahseer: 0.6–0.7 mL/kg body weight.

14.3 Genetically Engineered Fish/Transgenic Fish

Transgenesis or transgenics is the process of introducing a foreign gene or DNA into the host genome, resulting in its stable integration, transmission, and expression. For the first time, transgenic mice were generated by introducing the metallothionein human growth hormone fusion gene (MT-hGH) into mouse eggs, resulting in considerable growth. This generated a flurry of gene transfer research in commercially important fish species.

China created the first transgenic fish (Zhu et al. 1985). The method has now been used to successfully treat a variety of fish species. This method has resulted in dramatic growth enhancement, particularly in salmonids.

Currently, around 35 species of cultured fish species including trout, catfish, salmons, striped bass, tilapia, and flounder are genetically modified. These fish species are being genetically modified to have traits that make them more suitable for large-scale intensive farming, such as better growth, disease resistance, more muscle, and tolerance to extreme temperatures. The genes, used in these genetically modified fish, come from a range of organisms, including other fish, bacteria, coral, mice, and humans. FDA has approved the transgenic salmon, AquAdvantage salmon, produced by AquaBounty Technologies in year 2015. The same company is also developing transgenic tilapia and trout for commercial aquaculture. In other parts of the globe, researchers are also working on transgenic salmon and tilapia. GMO technique can be used to enhance the growth of several slow-growing cold-water fishes such as snow trout and mahseers.

The introduction of transgenic technology has also increased the requirement for sterile offspring production. This would clearly be beneficial, allowing for optimal transgenic stock growth and controlled reproduction while assuring that any

escaping fish would be unable to reproduce. For some years, fish transgenics researchers have been studying how to improve fish resilience to freezing temperatures (Fletcher et al. 2001). Cold-water temperatures stress many fish, and only a few can tolerate below 0 °C. This is a major issue in fisheries especially in cold climates. Some marine teleosts have high levels of serum antifreeze proteins (AFP) or glycoproteins (AFGP), which effectively lower the freezing temperature by preventing the formation of ice crystals. The winter flounder hepatic AFP gene was effectively transplanted into the genome of Atlantic salmon, where it was taken into the germ line and subsequently passed on to the offspring F3, where it was largely expressed in the liver. Developing stocks, expressing this gene, might be tremendously advantageous in commercial aquaculture in places where winter temperatures consistently approach these species' physiological limitations.

These can be used for common carp, allowing them to be farmed in the extreme cold of the Himalayas. Developing embryonic stem cell (ESC) technology is without a doubt the most potential technique for future transgenic fish production. This would make it simple to add and remove genes on a regular basis. Despite tremendous progress in a number of laboratories around the world, a number of challenges remain before transgenic brood stock for aquaculture can be commercialized.

14.4 Production of Sterile Offspring

Transgenic technology has also increased the importance of producing sterilized offspring to reduce the risk of genetically modified stocks combining in wild species. Technology advancements have increased the possibility of producing sterilized fish or fish whose breeding action could be particularly transformed on or off, using transcriptional promoters. It would have also been extremely valuable, allowing for optimal development and limited reproduction of transgenic stocks while ensuring that any escaping fish could not reproduce. For some years, researchers in fish transgenics have been exploring enhanced tolerance to low temperatures (Fletcher et al. 2001).

14.5 Taping Potential Role of Kisspeptin in Sexual Maturity and Reproduction of Reproductive Dysfunctional Fish in Temperate Aquaculture

Reproductive dysfunction in *Tor putitora* is a major issue in temperate aquaculture. Because of its widespread distribution in several South and Southeast Asian countries, as well as its high consumer acceptance, this fish is currently being considered as a candidate species for temperate aquaculture. The primary constraint to golden mahseer seedlings is its fertility disorder and the lack of control throughout its reproductive cycle in captivity (Shahi et al. 2015). The reproduction event in vertebrates, including teleost, is controlled by a series of synchronized actions along the brain-pituitary-gonad (BPG) axis, where kisspeptins, as RF-amide neuropeptide

key upstream signaling molecules, elicits the release of hormone called gonadotropin-releasing hormone (GnRH) from the hypothalamus via GnRH neurons via its receptors (kiss1 and kiss2 receptor). GnRH stimulates the release of gonadotropins (GtHs) in the pituitary gland, as well as follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which regulates the secretion of gonad sex steroids.

Kisspeptins are thought to regulate early development, gonadal sex differentiation, puberty onset, and seasonal reproduction in teleosts (Selvaraj et al. 2010, 2015; Zohar et al. 2009; Taranger et al. 2010; Migaud et al. 2012). Attempts have also been made to clone kisspeptin genes from various fish species in order to better understand their role in sexual maturity and reproduction. Similarly, the transcript level of kiss1 and kiss1r mRNA in the brain, pituitary, and gonad of golden mahseer was investigated during different gonadal development stages (Shahi et al. 2016). Kisspeptins' potential role in temperate aquaculture sexual maturity and reproduction can thus be investigated.

14.6 Fish Health Management

Disease is a significant barrier to aquaculture growth. Biotechnological technologies such as molecular diagnostic methods, vaccinations, and immunostimulants are gaining popularity for enhancing viral disease resistance in fish and shellfish species across the world. In this scenario, infection avoidance is crucial, and a quick pathogen detection approach is necessary. In this arena, biotechnological tools such as polymerase chain reaction (PCR) and gene probes have a tremendous promise. A range of illnesses affecting fish and shrimp have been found using PCR-based diagnostic and gene probe techniques (Karunasagar and Karunasagar 1999). In the case of finfish aquaculture, vaccines against bacteria and viruses have been developed. Traditional vaccinations based on dead microorganisms have been used in some cases, but a new generation of vaccines based on protein subunit vaccines, genetically edited organisms, and DNA vaccines is now being developed.

In the vertebrate system, illness immunity is a typical technique. Despite the fact that the shrimp immune system is still undeveloped, biotechnological technologies can be utilized to create compounds that trigger the shrimp immune response. Recent studies have revealed that microbial compounds such lipopolysaccharides, peptidoglycans, and glucans may be employed to increase the non-specific defensive response in fish (Itami et al. 1998). Glucan and levamisole are two immunostimulants that have been shown to improve phagocytic activity and specific antibody responses in fish (Sakai 1999).

14.7 Transgenes' Pleiotropic Impact on Disease Resistance

The incorporation of a transgene may have pleiotropic effects (Dunham 2011). Pleiotropic effects arise when a transgene impacts many traits, either positively or negatively. Unintentional genotypic or phenotypic alterations may have pleiotropic effects. Overexpression of a transgene can affect other traits, as seen in the case of growth hormone-transgenic salmon, where the hormone's overexpression resulted in a variety of pleiotropic effects other than growth, such as skeletal morphological changes and behavioral changes in feeding and swimming behavior (Devlin et al. 1995). If random transgenic integration occurs into fish functional genes, the insertion might be mutagenic (Gong et al. 2003). The changed phenotype may potentially result in pleiotropic consequences. Lysozyme activity, serum bactericidal activity, leucocratic activity, and the fraction of phagocytic macrophages in the head kidney were all significantly enhanced in transgenic carp. Phagocytic indices and relative spleen weight, on the other hand, did not differ across genotypes (Wang et al. 2006).

14.8 Chromosome Engineering

In farmed fish species, chromosomal sex modification techniques have been widely used to induce uniparental chromosome inheritance (gynogenesis and androgenesis) and polyploidy (triploid and tetraploid) (Lakra and Das 1998). Because they allow for fast gonadal sterilization, sex control, hybrid viability enhancement, and cloning, these treatments are important for enhancing fish breeding. Most vertebrates are diploid, which implies that their somatic cells have two complete sets of chromosomes. Polyploids have one or more extra chromosomal sets, bringing the total number of chromosomal sets to three in triploids, four in tetraploids, and so on. Triploids can be produced by crossing tetraploids with diploids. In fish species with female homogametic, gynogenesis can be used to produce completely female populations and reveal the sex determination processes. For a variety of aquaculture species, methods combining induced gynogenesis and hormonal sex inversion have been developed (Gomelsky et al. 2000). Androgenesis is a method that can be used commercially in aquaculture. It can also be used to create homozygous fish lines and to recover lost genotypes from cryopreserved sperm. A similar approach can be utilized for cold-water fishes such as golden mahseer, which have bigger females than males.

14.9 Gamete Cryopreservation or Gene Banking

Cryopreservation is a technique for preserving and storing biological material for lengthy periods of time at extremely low temperatures, generally −196 °C (liquid nitrogen temperature). Cryogenic preservation of fish spermatozoa (milt) technology has been applied in animal husbandry. Blaxter (1953) claimed to be the first to

maintain fish sperm at low temperatures by fertilizing herring (*Clupea harengus*) eggs, using frozen thawed sperm. Cryopreserved spermatozoa are currently available for almost all cultivable fish species (Lakra 1993). Cryopreservation eliminates the issue of male development occurring before female maturation, allowing for selective breeding, stock augmentation, and conservation. Breeders can develop new strains by utilizing one of the growth criteria. Gene banks are the source of the great majority of plant types. The aquatic gene bank, however, is limited since only male finfish gametes can presently be cryopreserved, with no feasible approach for finfish eggs and embryos. However, new findings on the freezing of shrimp embryos by Subramonium and Newton (1993) and Diwan and Kandasami (1997) sound promising. As a result, gene banking of cultivated and cultivable aquatic species must be completed as soon as feasible.

14.10 Genome Editing in Fish

Genome editing is a relatively new method of genetic engineering in which a sequence of DNA in any organism may be changed, replaced, inserted, deleted, or edited. Unlike prior genetic engineering techniques that randomly insert genetic materials into a host genome, the gene is added to particular regions of the genome in this case. As a result, this is more exact. The genome may be modified via zinc finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), and clustered regularly interspaced short palindromic repeats (CRISPR/Cas9). Because of the multiple biological benefits of fish models, fish species, particularly model species like zebrafish and medaka, have played an important role in testing novel genome editing approaches in recent years. Many more genes have been disrupted or altered in several fish species, including common carp, Atlantic salmon, tilapia, rainbow trout, rohu, and others, for functional studies, notably those linked to reproduction, disease resistance, and growth (Shahi et al. 2022). Gene knockout approach by microinjection (Fig. 14.1) can be utilized in cold-water fishes to increase muscle mass in commercially significant fish species such as common carp (Fig. 14.2).

14.11 Techniques for Genome Editing (GE)

GE-owned techniques include:

- CRISPR/Cas [including all variants under development, the most advanced of which is the CRISPR/Cas9 variant].
- Meganucleases.
- Zinc finger nucleases (ZFN).
- Transcription activator-like effector nucleases (TALEN).
- Oligonucleotide-directed mutagenesis (ODM).

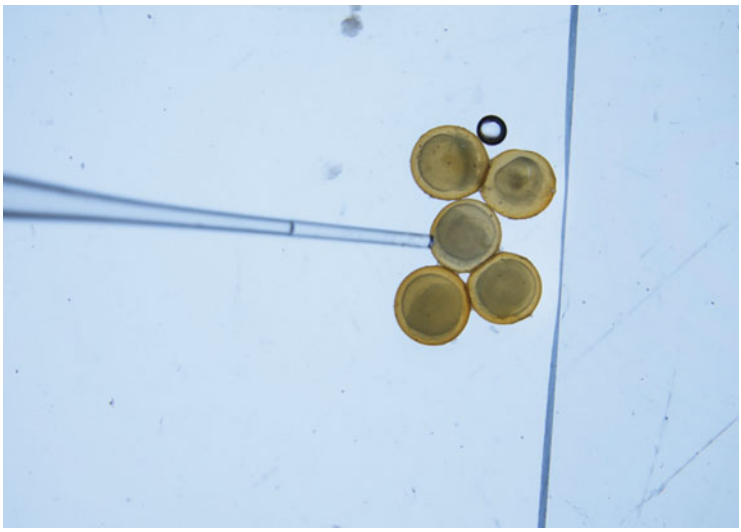

Fig. 14.1 Microinjection in fertilized eggs of common carp

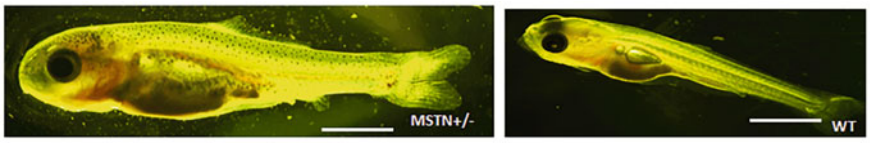

Fig. 14.2 Mstn knockout and wild-type common carp

Site-directed nucleases (SDNs) are used in these GE procedures because, unlike standard genetically modified techniques, SDN techniques are directed to a specific area of the genome and generate precise and targeted alterations (EFSA 2012). According to the EFSA, SDNs are classified into three types. Only this is rapidly transported into SDN-1 with the intention of generating random mutations at the target site, resulting in endogenous nucleotide repair of the damaged DNA of the host cell. SDN-2 uses HDR to produce specific nucleotide sequence alterations by using small non-protein-coding homologous repair DNA (donor DNA). SDN-3 uses HDR to route a lengthy length of protein-coding donor DNA (up to several kilobases) to a specific genomic location for insertion (EFSA 2012).

CRISPR/Cas is the most widely used SDN due to its ease of use, low cost, and high efficiency (Wang et al. 2016). CRISPR/Cas9, the most complex CRISPR/Cas variant, causes double-strand breaks at specific target places on the DNA, causing the cut sites to be repaired by the cell's endogenous DNA repair mechanisms: HDR and non-homologous end joining (NHEJ) (Jiang and Doudna 2017). Because the NHEJ is prone to mistakes, the repair process usually leads in nucleotide deletion, insertion, or substitution modifications to the DNA sequence (Jiang and Doudna 2017). Such modifications can make the target gene dormant or knockout (KO),

which is useful in gene knockout applications. The HDR repair process can employ exogenous DNA sequences as templates to insert donor nucleotide sequences at target locations via substitution and insertions (Jiang and Doudna 2017).

14.11.1 Limitations of CRISPR/Cas Use in Aquaculture

1. Despite the benefits of CRISPR/Cas, attaining the technique's full potential in fish aquaculture is limited by a number of technical hurdles, which are outlined from both a genetic and an application standpoint:
2. *Genetic perspective.*
   (a) The most significant aquatic species have been sequenced, and the aquatic genetic resource is still restricted (Wargelius 2019). A good grasp of genetic background is required for genome editing.
   (b) Trait-related genes need to be identified. Because the genetic dissection of aquatic species lags behind that of humans and plants, trait-related genes must be found.
3. There is fish duplication. In terms of species diversity, fish are the most abundant aquatic creatures. In contrast, teleosts experienced teleost-specific whole genome duplication (TS-WGD) (Glasauer and Neuhauss 2014).
4. *Application perspective.*
   (a) In oviparous fish, the egg membrane decreases the success rate of microinjection. There is no known gene editing platform for ovoviviparous fishes at the moment.
   (b) Off-target effect identification in model organisms focuses on knockout efficiency to optimize CRISPR/Cas design (through the NHEJ/SDN-1 technique). While considered a food resource, off-target effects in aquatic creatures should also take into account the impact of introducing new genes via transgenesis or cisgenesis (i.e., the HDR/SDN-3 strategy).
   (c) Because aquatic creatures have distinct traits that demand species-specific design, such as needle type, injection dose, and so on, there is no standard technique. Because of the rarity of known cell lines and the tiny size of the egg and embryo in crustaceans and mollusks, only *Crepidula fornicata*, *Exopalaemon carinicauda*, and Crassostrea gigas have had successful GE (Gui et al. 2016; Perry and Henry 2015; Yu et al. 2019).
5. *Possible solutions to these issues.*
   (a) As the cost of sequencing decreases (less than $10/sample), more aquatic genomes will be decoded in the future, establishing the genetic groundwork for future GE events.
   (b) As QTL and genetic and molecular biology tools (e.g., QTL mapping, comparative genomics, and pooled CRISPR screens) improve, more trait-related genes will be found (Houston et al. 2020).

(c) For traits involving many genes (quantitative traits), concurrently creating multi-gene knockout mutants using CRISPR/Cas will allow for the production of the desired phenotype.

14.12 Conclusion and Perspective of Genome Editing Tool

Because of the rapid advancement of genome sequencing technology and the completion of whole genome sequencing in multiple fish species, fish breeding has entered the post-genomic age. Not only will genome editing make identifying gene functions at the individual gene level easier, but it will also allow for the insertion of different forms of nucleotide polymorphism or modifications in the fish genome at single-base resolutions. However, before genome editing techniques are widely deployed, it is necessary to address the phenomena of genetic compensation, which can disguise genome-edited phenotypes (Balciunas 2018). As a result, large-scale screening in the zebrafish model with genome editing technologies is necessary, resulting in a comprehensive understanding of the link between sequence changes and gene function dynamics. Furthermore, using genome editing methods, it may be feasible to develop farmed fish with a variety of advantageous qualities, including growth acceleration, high nutrition, disease resistance, stress tolerance, and high fertility, in the future.

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