

# **Biotechnological interventions in marine finfish breeding and seed production**

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## Introduction

Fish farming is the world's fastest growing sector of agricultural business. Fish and other aquatic products represent an important, underutilized resource for a protein-hungry world. Natural catches are dropping even as demand continues to increase, making aquaculture increasingly attractive. Consumer demand for fish products is increasing. At the same time, wild fish stocks are rapidly declining, mainly because of over-fishing. Biotechnology provides powerful tools for the sustainable development of aquaculture, fisheries, as well as the food industry. Increased public demand for seafood and decreasing natural marine habitats have encouraged scientists to study ways that biotechnology can increase the production of marine food products, and making aquaculture as a growing field of animal research.

In general, biotechnology can be broadly defined as using living organisms or their products for commercial purposes. As such, biotechnology has been practiced by human society since the beginning of recorded history in such activities as baking bread, brewing alcoholic beverages, or breeding food crops or domestic animals. Biotechnology allows scientists to identify and combine traits in fish and shellfish to increase productivity and improve quality. Scientists are investigating genes that will increase production of natural fish growth factors as well as the natural defence compounds marine organisms use to fight microbial infections. Modern biotechnology is already making important contributions and poses significant challenges to aquaculture and fisheries development. It perceives that modern biotechnologies should be used as adjuncts to and not as substitutes for conventional technologies in solv-

ing problems, and that their application should be need-driven rather than technology-driven.

The use of modern biotechnology to enhance production of aquatic species holds great potential not only to meet demand but also to improve aquaculture. Modern biotechnology is concerned with the use and manipulation of the DNA molecules, the genetic code, of living organisms. The emergence of modern biotechnology began with the discovery of a heat stable DNA polymerase enzyme, which produces many copies of small amounts of DNA in the Polymerase Chain Reaction (PCR). With this technique, selected portions of the genome, including genes, can be rapidly amplified to amounts suitable for further analysis. Once the DNA sequence is available, the challenge is to use this new information in the genetic improvement of aquaculture species.

Genetic modification and biotechnology also holds tremendous potential to improve the quality and quantity of fish reared in aquaculture. There is a growing demand for aquaculture; biotechnology can help to meet this demand. Biotech aquaculture also offers environmental benefits. When appropriately integrated with other technologies for the production of food, agricultural products and services, biotechnology can be of significant assistance in meeting the needs of an expanding and increasingly urbanized population in the next millennium. Successful development and application of biotechnology are possible only when a broad research and knowledge base in the biology, variation, breeding, agronomy, physiology, pathology, biochemistry and genetics of the manipulated organism exists. Benefits offered by the new technologies cannot be fulfilled without a continued commitment to basic research. Biotechnological programmes must be fully inte-

grated into a research background and cannot be taken out of context if they are to succeed.

The two areas of modern biotechnology, which will probably have the most significant impact on genetic improvement of aquaculture species, are DNA markers and transgenics. One can safely say that genetic modification is as old as agriculture, at least. As long as man has carried out selective breeding of domestic plants and animals, we have also altered the genetic composition of organisms. Still, it is not too hard to see that there are marked differences between traditional 'old fashioned' breeding techniques and direct transferral of genes from other species. The benefits from producing GMO's are overwhelming in some areas: non-polluting enzyme-based detergents are produced by transgenic microorganisms; genetically engineered yeasts produce human insulin for treatment of diabetics; and bacteria-carrying human genes produce pituitary hormones for improving the lives of people with defective glands.

Aquaculture/ Mariculture area continues to excite particular interest in many forums. Since we know little about how to raise, protect and reproduce most species (except carp, tilapia and a few others), biotechnology techniques could help:

- Clarify the biology and life cycle of new species;
- Promote fertility, growth, meatiness and egg-laying through hormonal treatment;
- Diagnose fish diseases (common in overcrowded ponds) and prevent them with vaccines; and
- Promote the scientific raising of rotifers and other fingerling food.

One important commercial goal could be the economical production of disease-free embryos and fingerlings of food and ornamental fishes.

In global scenario, the fisheries and aquaculture is an important sector of food production, providing nutritional security to the food basket, contributing to the agricultural exports and engaging millions of people in different activities. In this similar line, the marine aquaculture or mariculture using biotechnological interventions has immense potential being a lucrative sector worldwide. The potential area of biotechnology in mariculture include the use of synthetic hormones in induced breeding, transgenic fish, chro-

mosome engineering (uniparental and polyploidy population), cryopreservation and gene banking, marker assisted genetic improvement and health management.

### **Induced breeding of fish**

Gonadotropin releasing hormone (GnRH) is now the best available biotechnological tool for the induced breeding of fish. GnRH is the key regulator and central initiator of reproductive cascade in all vertebrates (Bhattacharya et al.,2002). It is a decapeptide and was first isolated from pig and sheep hypothalamus with the ability to induce pituitary release of luteinising hormone (LH) and follicle stimulating hormone (FSH). Since then only one form of GnRH has been identified in most placental mammals including human beings as the sole neuropeptide causing the release of LH and FSH. However, in non mammalian species (except guinea pig) twelve GnRH variants have now been structurally elucidated, among them seven or eight different forms have been isolated from fish species (Halderet al.,1991). The most recent GnRH purified and characterized was by Carolsfeld et al., (2000). Depending on the structural variant and their biological activities, number of chemical analogues have seen prepared and one of them is salmon GnRH analogue profusely used now in fish breeding and marked commercially under the name of 'Ovaprim' throughout the world. The induced breeding of fish is now successfully achieved by development of GnRH technology.

### **Transgenesis**

Transgenesis or transgenics may be defined as the introduction of exogenous gene/ DNA into host genome resulting in its stable maintenance, transmission and expression. The technology offers an excellent opportunity for modifying or improving the genetic traits of commercially important fishers, molluscs and crustaceans for aquaculture. The idea of producing transgenic animals became popular when the first produced transgenic mouse by introducing metallothionein in human growth hormone fusion gene (mT-hGH) into mouse egg, resulting in dramatic increase in growth. This triggered a series of attempts on gene transfer in economically important animals including fish.

The high fecundity of most fish and external fertilisation and embryonic development make

them especially suitable for transferring specific genes. Successful gene transfer has been demonstrated into a variety of aquatic organisms by applying different techniques, including micro-injection, particle gun bombardment, and electroporation. The transfer of genes into the sperm or directly into the skeletal muscle has become alternatives to the fertilized eggs. The production of transgenic fish is aimed at dramatic improving traits like growth, disease resistance, and environmental tolerance. The nature itself of such quantitative traits, being influenced by multiple genes, makes them difficult to manipulate by gene transfer techniques. It should be noted that the increased growth rate in the transgenic fish is mainly due to the great amounts of growth hormone produced in large tissues like the liver or gonads, and not the result of injecting millions of gene copies into the fertilized eggs.

There are several problems to be overcome before transgenic animals can be produced on a large scale. Indeed, in over 90 % of the microinjected eggs, the transgene is not efficiently integrated in the genome at the one-cell stage. The result is highly mosaic transgenic fish and low frequencies of germ line transmission since only the tissues developing from the transformed cell will carry the transgene. Furthermore, the injected DNA integrates at single or multiple random sites in the genome of the recipient embryo, each developing into a unique hemizygous fish. Hence, the establishment of a stable transgenic broodstock will be a costly endeavour requiring several generations.

The first transgenic fish was produced Zhu et al., (1985) in China, who claimed the transient expression in putative transgenics, although they gave no molecular evidence for the integration of the transgene. The technique has now been successfully applied to a number of fish species. Dramatic growth enhancement has been shown using this technique especially in salmonids. Some studies have revealed enhancement of growth in adult salmon to an average of 3-5 times the size of non-transgenic controls, with some individuals, especially during the first few months of growth, reaching as much as 10-30 times the size of the controls (Hew et al., 1995). The introduction of transgenic technique has simultaneously put more emphasis on the need for production of sterile progeny in order to minimize the risk

of transgenic stocks mixing in the wild populations. The technical development has expanded the possibilities for producing either sterile fish or those whose reproductive activity can be specifically turned on or off using inducible promoters. This would clearly be of considerable value allowing both optimal growth and controlled reproduction of the transgenic stocks while ensuring that any escaped fish would be unable to breed.

An increased resistance of fish to cold temperatures has been another subject of research in fish transgenics for the past several years (Fletcher et al., 2001). Coldwater temperatures pose a considerable stressor to many fish and few are able to survive water temperatures much below 0-1°C. This is often a major problem in aquaculture in cold climates. Interestingly, some marine teleosts have high levels (10-25 mg/ml) of serum antifreeze proteins (AFP) or glycoproteins (AFGP) which effectively reduce the freezing temperature by preventing ice-crystal growth. The isolation, characterization and regulation of these antifreeze proteins particularly of the winter flounder, *Pleuronectes americanus* has been the subject of research for a considerable period in Canada. Consequently, the gene encoding the liver AFP from winter flounder was successfully introduced into the genome of Atlantic salmon where it became integrated into the germ line and then passed onto the off-spring (F3) where it was expressed specifically in the liver (Hew et al., 1995). The introduction of AFPs to goldfish also increased their cold tolerance, to temperatures at which all the control fish died (12 h at 0°C; Wang et al., 1995). Similarly, injection or oral administration of AFP to juvenile milkfish or tilapia led to an increase in resistance to a 26 to 13°C drop in temperature. The development of stocks harbouring this gene would be a major benefit in commercial aquaculture in counties where winter temperatures often border the physiological limits of these species.

The most promising tool for the future of transgenic fish production is undoubtedly in the development of the embryonic stem cell (ESC) technology. These cells are undifferentiated and remain totipotent so they can be manipulated in vitro and subsequently reintroduce into early embryos where they can contribute to the germ line of the host. This would facilitate the genes to be stably introduced or deleted (Melamed et al., 2002). Although significant progress has been made in

several laboratories around the world, there are numerous problems to be resolved before the successful commercialization of the transgenic brood stock for aquaculture. To realize the full potential of the transgenic fish technology in aquaculture, several important scientific breakthroughs are required. There include:

1. More efficient technologies for mass gene transfer
2. Targeted gene transfer technologies such as embryonic stem cell gene transfer
3. Suitable promoters to direct the expression of transgenes at optimal levels during the desired developmental stages
4. Identified genes of desirable traits for aquaculture and other applications
5. Information on the physiological, nutritional, immunological and environmental factors that maximize the performance of the transgenics of the transgenics and
6. Safety and environmental impacts of transgenic fish.

Work on producing aquatic genetically modified organisms (GMO) of relevance for the ornamental trade has, so far, been carried out (in particular) in Asia. In Singapore, for example, transgenic Zebra Fishes (*Danio rerio*) that glow in green or red under UV-light were first produced some years ago. And, in several countries, research continues to find new areas for employing genetic engineering in the production of new fish varieties. Efforts do not only focus on improving colour and shape, but also on developing characteristics such as faster growth, resistance to infection and tolerance of lower temperatures. This last point, in particular, would certainly open up the debate on invasiveness and environmental risks.

### Chromosome Engineering

Chromosome sex manipulation techniques to induce polyploidy (triploidy and tetraploidy) and uniparental chromosome inheritance (gynogenesis and androgenesis) have been applied extensively in cultured fish species (Pandian and Koteeswaran, 1998; Lakra and Das, 1998). These techniques are important in the improvement of fish breeding as they provide a rapid approach for gonadal sterilization, sex control, improvement of hybrid viability and cloning. Most vertebrates are

diploid meaning that they possess two complete chromosome sets in their somatic cells. Polyploidy individuals possess one or more additional chromosome sets, bringing the total to three in triploids, four in tetraploids and so on. Induced triploidy is widely accepted as the most effective method for producing sterile fish for aquaculture and fisheries management. The methods used to induce triploids and other types of chromosome set manipulations in fishes and the applications of these biotechnologies to aquaculture and fisheries management are well described (Purdom, 1983; Chourrout, 1987; Thorgaard, 1983; Pandian and Koteeswan, 1998). Tetraploid breeding lines are of potential benefit to aquaculture, by providing a convenient way to produce large numbers of sterile triploid fish through simple interploidy crosses between tetraploids and diploids (Chourrout et al., 1986; Guo et al., 1996). Although tetraploidy has been induced in many finfish species, the viability of tetraploids was low in most instances (Rothbard et al., 1997).

In teleosts, techniques for inducing sterility include exogenous hormone treatment (Hunter and Donaldson, 1983) and triploidy induction (Thorgaard, 1983). The use of hormone treatments, however could be limited by governmental regulation and a lack of consumer acceptance of hormone treated fish products. Triploidy can be induced by exposing eggs to physical or chemical treatment shortly after fertilization to inhibit extrusion of the second polar body (For reviews see Purdom, 1983; Thorgaard, 1983 and Ihssen et al., 1990) triploid fish are expected to be sterile because of the failure of homologous chromosomes to synapse correctly during the first meiotic division. Methods of triploidy induction include exposing fertilized eggs to temperature shock (hot or cold), hydrostatic pressure shock or chemicals such as ancolchicines, cytochalasin-B or nitrous oxide. Triploid can also be produced by crossing tetraploids and diploids. Tetraploid induction involves fertilizing eggs with normal sperm and exposing the diploid zygote for physical or chemical treatment to suppress the first mitotic division.

Gynogenesis is the process of animal development with exclusive maternal inheritance. The production of gynogenetic individuals is of particular interest to fish breeders because a high level of inbreeding can be induced in single generation. Gynogenesis may also be used to produce

all-female populations in species with female homogamety and to reveal the sex determination mechanisms in fish. It is convenient to use all female gynogenetic progenies (instead of normal bisexual progenies) for sex inversion experiments. Methodologies combining use of induced gynogenesis with hormonal sex inversion have been developed for several aquaculture species (Gomelsky et al., 2000). Androgenesis is the process by which would have commercial application in aquaculture. It can also be used in generating homozygous lines of fish and in the recovery of lost genotypes from the cryopreserved sperms. Androgenetic individuals have been produced in a few species of cyprinids, cichlids and salmonids (Bongers et al., 1994).

### **Cryopreservation of gametes or gene banking**

Cryopreservation is a technique, which involve long-term preservation and storage of biological material at a very low temperature usually at  $-196^{\circ}\text{C}$ , the temperature of liquid nitrogen. It is based on the principle that very low temperature tranquilizes or immobilizes the physiological and biochemical activities of cell, thereby making it possible to keep them viable for very long period. The technology of cryopreservation of fish spermatozoa (milt) has been adopted for animal husbandry. The first success in preserving fish sperm at low temperature was reported by Blaxter (1953) who fertilizes Herring (*Clupea harengus*) eggs with frozen thawed semen. The spermatozoa of almost all cultivable fish species has now been cryopreserved (Lakra, 1993). Cryopreservation overcomes the problem of male maturing before female, allow selective breeding and stock improvement and enables the conservation (Harvey, 1996). Most of the plant varieties that have been produced are based on the gene bank collections. Aquatic gene bank however suffers from the fact that at present it is possible to cryopreserve only the male gametes of finfishes and there is no viable technique for finfish eggs and embryos. However, the report on the freezing of shrimp embryos by Subramoniam and Newton (1993) and Diwan and Kandaswami (1997) look promising. Therefore, it is essential that gene banking of cultivated and cultivable aquatic species be undertaken expeditiously.

### **Marker assisted genetic improvement**

It is difficult to develop a sustainable enhanced production based on wild broodstock. Therefore,

it is of great importance to develop a system independent of wild broodstock by controlled reproduction involving broodstock development, breeding and seed production. The development of selective breeding programme is regarded as an important tool in order to domesticate marine finfishes and improve important economic traits. The information on genetic variation is essential for conservation and stock improvement programmes. In farmed animals and plants, it has been demonstrated that systematic selection is an efficient way of improving production traits and thereby produce fast growing animals with high quality.

Selective breeding programs in aquaculture make use of family information, which requires that families are kept separately until the fish are large enough to be physically tagged. This imposes major economic and practical problems, and can induce environmental effects common to full-sibs. Identification of family groups by use of DNA markers has the potential to overcome these problems. Using this technology, fish from different families could be reared together in the same tank even from the egg stage. As the need to keep each family in a separate tank is circumvented, using DNA markers would allow larger number of families to be tested, without increasing the number of tanks, and thus facilitating the use of higher selection intensities without rapid accumulation of inbreeding.

Due to their high polymorphism, microsatellite markers are useful for genetic tagging. Microsatellites have successfully been used to empirically reconstruct pedigrees in fish populations with families mixed from hatching. In an experiment with Atlantic salmon families, using four highly polymorphic microsatellites was sufficient to assign at least 99% of the offspring to the correct pair with 100 crosses involving 100 males and 100 females. An additional polymorphic microsatellite was required for correctly assigning 99% of the offspring when the 100 crosses were produced with 10 males and 10 females. This study demonstrated that parental assignment is feasible with the DNA markers currently available in several fish species.

Both the efficiency and costs of microsatellite based pedigree analysis should be considered before this method is included in a breeding program. Practically, the parents and the mixed

offspring are genotyped by PCR amplifying the appropriate microsatellite loci from crude DNA extracts from small non-destructively sampled quantities of tissue such as fish scales, mucus, and fin clip. Using this protocol, approximately 2000 fish from a mixture of 500 families can be screened for 10 markers in less than a month allowing 99 % of the fish to be parental assigned. Allocation of offspring to families without parental genotypes is also possible, provided enough fish are sampled to obtain sufficient representation of all families, but a larger number of markers are required than for parentage analysis, with probably more than one hundred microsatellites required.

The steady decreasing costs of genetic tagging may still not compete with traditional physical tagging. As the genotype information is detached from the individual, genetic tagging implies that the fish has to be retyped each time its performance is evaluated or individuals are selected. Alternately, fish can be physically tagged for re-identification following genotyping and parentage assignment. Thus the cost of implementing DNA markers in selection programs could be considerable. However it should soon be possible to apply recent developments in human genetics such as DNA chips, where many SNP markers can be genotyped simultaneously and cheaply, in the aquaculture species, and this could dramatically reduce the cost of genetic tagging. DNA markers have several other applications within fish management as well, including evaluation of inbreeding levels, stock identification, movement of released or escaped fish and their possible genetic interactions with wild stocks.

### **Biotechnology and fish health management**

Disease problem area is the major constraint for development of aquaculture. Biotechnological tools such as molecular diagnostic methods, use of vaccines and immunostimulants are gaining popularity for improving the disease resistance in fish and shellfish species. For viral diseases, avoidance of the pathogen is very important. In this context there is a need to develop rapid method for detection of the pathogen. Biotechnological tools such as gene probes and polymerase chain reaction (PCR) are showing great potential in this area. Gene probes and PCR based diagnostic methods have developed for a number of pathogens affecting fish and shrimp (Karunasagar, 1999). In case of

finfish aquaculture, numbers of vaccines against bacteria and viruses have been developed. Some of these have been conventional vaccines consisting of killed microorganism but new generation of vaccines consisting of protein subunit vaccine, genetically engineered organism and DNA vaccine are currently under development.

In the vertebrate system, immunization against disease is a common strategy. However the immune system of shrimp is rather poorly developed, biotechnological tools are helpful for development of molecule, which can stimulate this immune system of shrimp. Recent studies have shown that the non specific defence system can be stimulated using, microbial product such as lipopolysaccharides (LPS), peptidoglycans or glucans (Itami et al., 1998). Among the immune-stimulants known to be effective in fish, glucan and levamisole enhance phagocytic activities and specific antibody responses (Sakai, 1999).

In the attempts to combat viral and bacterial pathogens threatening commercial stocks, the utilization of DNA vaccines has been promising. This technique is based on the injection of DNA encoding part of the antigen, usually a bacterial outer membrane or viral capsid protein, in the fish muscle. Here the protein will be synthesized, and the production of antibodies against the foreign protein is induced. A significant degree of protection against infectious hematopoietic necrosis virus (IHNV) was found in Atlantic salmon after vaccination with a gene construct containing an IHNV glycoprotein. Similarly, protection against viral haemorrhagic septicaemia virus (VHS) was induced in vaccinated rainbow trout. The main disadvantage of these approaches is they require detailed information about the structure, conformation, and the encoding sequence of the pathogen's protein. An alternative approach to increase the resistance of fish to pathogens is to target the nonspecific immune response through use of antimicrobial peptides, which are found in both vertebrates and invertebrates. Short peptides consisting of 30-50 amino acids with strong antimicrobial activity have been isolated from the skin mucus of several fish species, Recently, manipulation of antimicrobial cecropin genes in the Japanese medaka and channel catfish produced strains of transgenic fish resistant to infection by fish bacterial pathogens.



## Future Prospects

The completion of the Human Genome Project inspired the entire world and triggered the start of a genomics revolution. Accompanying this revolution was a complete change in the way science was conducted in the field of life sciences. Without exception, the waves produced by the genome revolution are now having a tremendous impact on aquaculture genomics and aquaculture genetics in general. This raises new challenges for aquaculture geneticists, breeders, and fisheries managers regarding how to best use the huge amount of genomic information now available, and how to master and apply continuously changing genome technologies to aquaculture and fisheries.

The applications of molecular techniques in aquaculture are promising, but still somewhat uncertain. While high costs seem to be the only hindrance for widespread application of DNA markers for identification purposes and marker assisted selection, the situation regarding commercial use of genetically modified fish is more complex. Although the potential importance of gene transfer technology is large, a major concern relates to the possible impact, which release or accidental escapes of gene-modified individuals may have on natural ecosystems. Other controversial aspects are related to animal welfare, food safety and the public perception of gene manipulation in general. To which extent such issues will constrain the future use transgenic animals in applied aquaculture production remains to be seen.

Other technologies are also rapidly emerging which are either being used or are likely to be used in the future in the aquaculture species. For example micro-array technology, where an array of DNA or protein samples are hybridized with probes to study gene expression of a large number of genes simultaneously, can be used to determine which genes are involved in response to disease, expression of meat colour and other important issues. Already, a microarray containing DNA probes for 3700 DNA sequences or genes is available in salmon, and will be used to compare gene expression in disease challenged and non-challenged fish (Davidson and Koop, 2003). Such technology has the potential to contribute very large amounts of information on the genes and pathways of genes, which affect the economic traits in aquaculture species.

Biotechnological research and development are growing at a very fast rate. The biotechnology has assumed greatest importance in recent years in the development of fisheries, agriculture and human health. The science of biotechnology has endowed us with new tools and tremendous power to create novel genes and genotypes of plants, animals and fish. The application of biotechnology in the fisheries sector is a relatively recent practice. Nevertheless, it is a promising area to enhance fish production. The increased application of biotechnological tools can certainly revolutionise our fish farming besides its role in biodiversity conservation. The paper briefly reports the current progress and thrust areas in the transgenesis, chromosome engineering, use of synthetic hormones in fish breeding, biotechnology in health management and gene banking.

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