Guidelines for research and utilization of genetically modified fish*

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Asian scientists are the first to initiate research in transgenic fish and Asia is the centre of research activity in transgenic fish. Transgenic fish thus far generated display desirable traits, excepting a few, in which reproductive performance seems to have been impaired. Only 12 countries have framed their respective national policy or regulations on genetically modified aquatic organisms. However, transgenic research is growing so fast that these guidelines and regulations will have to be revised from time to time. GH-transgenes are analogous to selected and/or domesticated lines, capable of growing 4–5 times faster. Transgenics, that are anti-freeze protein transformants, may not interfere as much as exotic fish. Regarding biological containment, available methods for inducing sterility of transgenics are briefly described.

A notification by the *Gazette of India* has defined a number of terms like 'cell hybridization', - logy', 'genetic engineering', etc. which are relevant to the term 'Genetically modified organisms (GMO)'[1]. For instance, genetic engineering is defined as the technique by which heritable material, which does not usually and/or naturally occur in the organism or the cell concerned, but is generated outside the organism or cell, is inserted into the said cell or organism and results in its genetic modification. However, a broader version of GMO includes progenies of hybridization, ploidy induction and transgenesis[2,3]. In fish, pre-embryonic events are manipulable and 35 different types of ploidy inductions are possible. Interspecific and interploidy (4*n* vs 2*n*) hybridizations in a few salmonids and cyprinids induce sterile and fertile triploid, respectively[4]. Table 1 shows that almost all ploidy types occur in nature also. As induction of ploidy results in different qualitative and quantitative alterations of the native chromosomes, this aspect is not considered here, although some of its benefits are discussed under biological containment. Transgenics are generated through microinjection or electroporation of solution containing a recombinant DNA construct into the newly fertilized eggs. Thus the recombinant DNA and transfer technology allows the transfer, inheritance and expression of a specific DNA sequence of heterologous or homologous origin. In fish, it is now possible to generate transgenics bearing growth-hormone gene[5] or anti-freeze protein gene[6]. The first one results in the generation of 10–30 times accelerated growth[7] and the second one confers the capacity to tolerate ice-cold waters. This report is therefore confined to the present status of guidelines and regulations imposed on research in transgenic fish and their potential commercial production in farms and natural habitats.

Status of transgenic fish

An overview of technical developments in transgenic fish is a necessary prelude for proper understanding of public policies regarding laboratory production of transgenics and their commercial utilization. Table 2 clearly indicates that Asian scientists were the first to initiate research in transgenic fish and since then Asia is the centre of research activity in transgenic fish. Having on hand, the autotransgenic founder mud loach, *Misgurnus mizolepis*, growing 30 times faster than its siblings[7] and the autotransgenic triploid mud loach with persistent accelerated growth[8] and the possibility of feminization of all transgenic triploid mud loach progenies, or autotransgenic androgenate founders with persistent accelerated growth[9], D. S. Kim and his colleagues are indeed closer to patenting the autotransgenic mud loach as a brood stock and to mass produce all-male triploid transgenic mud loach progenies for sale (see Figure 1; see also ref. 10). In this case, Kim and his colleagues will again become the first Asians to patent a transgenic fish. It must also be stated that of the available 50 and odd growth-promoting genes of piscine origin, most of them are of salmonid-origin and were constructed by the Western scientists. Needless to point out that Asian scientists will have to generate more gene constructs for indigenous fish species of Asia.

Initially, DNA sequences from a variety of organisms, viruses, bacteria, fish, birds, mammals and human were transferred to fish. Although small (heterologous) DNA sequences from these organisms do not impart general characteristics of these organisms in fish, most researchers

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Table 1. Natural occurrence and induced polyploidy in fish (Source: refs 4, 44)

Ploidy	Natural occurrence (no. of species)	Successful induction (no. of species)	Remarks
Haploidy	1	1	Gene regulation on development
Gynogenesis	10	50	Monosex progenies – Homozygous and isogenic strains
Androgenesis	–	5	Monosex progenies – Homozygous and isogenic strains; Restoration of endangered species from cryopreserved sperm
Triploidy	10	40	Useful in introduction of exotics and transgenic studies
Polyploidy	10	5	Useful in induction of androgenates and gene regulation on development

Table 2. Landmark events in transgenic fish research

Scientist, city, country	Event
Zhu *et al.*[45] Wuhan, China	First claim on successful transfer of hGH into goldfish eggs
Ozato *et al.*[46] Kyoto, Japan	First successful nuclear microinjection of transgene into medaka eggs
Inoue *et al.*[47] Kyoto, Japan	First to successfully transfer transgene by electroporation into medaka eggs
Khoo *et al.*[48] Singapore	First to achieve sperm-mediated transfer of transgene into zebrafish
Marian[42] Madurai, India	Generation of transgenic triploid zebrafish
Nam *et al.*[7] Pusan, Korea	First to generate autotransgenic mud loach
Nam *et al.*[9] Pusan, Korea	First to successfully generate transgenic androgenate mud loach

have now chosen to focus on transfer of 'all-fish DNA' (i.e. homologous) construct into fish to increase the likelihood of social acceptance of transgenic fish (see ref. 11). An extreme example of this is that of Nam *et al.*[7], who successfully generated autotransgenic transgenic mud loach, using transgene construct containing mud loach growth hormone fused to mud loach β-actin regulatory region. It is also considered that 'all-fish gene' constructs are more effective; however, Pitkanen *et al.*[12], who fused one of the four different promoters (CMV, OnMT, OnH3 or SsGH2), showed that the ability of CMVGH1 construct to promote growth was greater than that obtained with piscine promoters in *Salvelinus alpinus*, into which the Atlantic GH1 was introduced.

The initial transfer of fusion gene construct into fish embryos usually results in mosaic individuals[13,14]. But when there is a germ-line integration, as in the case of Nam *et al.*[7,9], the introduced DNA is usually inherited in Mendelian ratio and the resulting progenies have the foreign DNA in every cell, allowing accurate evaluation of the expression and biological effects of gene transfer. Most genes transferred to fish have been expressed; the expression of the genes does not always alter performance[15], but many examples of altered performance of transgenic fish are reported[11]. From the point of view of aquaculture, the following traits are desirable in transgenic fish: acceleration of growth, enhanced feed efficiency, tolerance of poor water quality and disease resistance. Devlin *et al.*[16,17] and Nam *et al.*[7] have recorded 11–30-fold growth acceleration in their transgenic salmon and mud loach, respectively. Nam *et al.*[7] also reported increased feed efficiency of the autotransgenic loach. Using rtGH, Chatakondi *et al.*[18] generated transgenic *Cyprinus carpio*, a species of great economic importance to Asia. The transgenic carp is regarded healthier for human consumption, as it contains more protein and less fat. This carcass character is also known to enhance tolerance to low dissolved oxygen[19]. Comparing the CMVOnGH1-transgenic *S. alpinus* and its siblings, Krasnov *et al.*[20] designed a study to examine whether the pattern of utilization of protein and lipids is altered in genetically-modified, growth-accelerated charr. They analysed muscle and plasma composition and plasma metabolites, and estimated rates of gas exchange. There was no difference in the composition of muscle and plasma metabolites. However, the lower ammonia quotient implied reduction in metabolic expenditure of protein; the higher level of total CO_2 in the plasma indicated enhanced oxidation of non-protein nutrients. Decreased plasma triglycerides concentration and lower triglycerides to cholesterol ratio showed faster utilization of lipids. However, this was not accompanied with decrease in lipid content or altered fatty acid composition of muscle triglycerides and phospolipids. Briefly, the observations of Krasnov *et al.*[20] confirm the earlier findings of Chatakondi *et al.*[18]. Regarding disease resistance, the report by Anderson *et al.*[21] of genetically immunizing rainbow trout against infectious haematopoietic necrosis, is promising. Reproductive performance of transgenic carp and catfish bearing growth hormone genes appears to remain unaltered[22]. However, Pandian *et al.*[5] indicated that the transgenic zebrafish bearing pMGH was fast-growing, at the cost of reproductive growth. Records on reduced sperm production in the GH transgenic Nile tilapia, *Oreochromis niloticus*[15] and in the Indian catfish, *Heteropneustes fossilis* (pers. observation) are available. Extremely fast-growing transgenic salmon and loach have low fitness and die early[17,18]. These observations clearly

indicate the need for further research on survival and reproduction of GH transgenic fish.

Status of guidelines for transgenics

In the early days of recombinant DNA research, fears over the safety of such research work led to the development of guidelines for safe laboratory practices[23,24]. That the United States has authorized the sale of transgenic tomatoes is an evidence for the safety of eating some transgenic organisms. These tomatoes are transgenic for a sequence of DNA, which retards the ripening process. On the other hand, since domesticated tomatoes could readily be made transgenic for a toxin gene, it is not safe to eat all transgenic forms of normally edible organisms[25]. Therefore, the United States has imposed certain regulations for undertaking research, and production of transgenics. The National Institute of Health and the US Department of Agriculture have framed different but oft revised guidelines for research and commercial utilization of transgenic organisms[26]. An *ad hoc* group of experts, commissioned by the Organization for Economic Co-operating and Development (OECD), the grouping of 25 industrialized nations, developed recommendations concerning safety in use of recombinant DNA-bearing organisms in industry, agriculture and the environment[27]. Despite a diversity of existing biotechnology regulations among western European nations, a directive on regulations over contained use of genetically modified organisms was adopted by the European Economic Community (EEC)[28]. Responsibility for regulating laboratory production of transgenic animals in Japan is divided among several agencies; for instance, research carried out at the universities is subjected to guidelines, promulgated by the Ministry of Education, other research organizations to guidelines of the Science and Technology Agency, and industry to guidelines of the Ministry of International Trade and Industries[29]. In India, safety guidelines for undertaking such research, are prescribed and revised from time to time by the Department of Biotechnology[30–32]. However, the guidelines and regulations are skewed for pathogenic microorganisms and plants of health and agricultural importance, respectively (see refs 33, 34); very little is mentioned about transgenic fish.

Table 3 lists regulations imposed by selected countries on research and field testing of transgenic fish, in which phenotypic expression of the transgene is confirmed. Briefly, about 12 countries (including India) alone have framed their respective national policy or regulation on genetically modified aquatic organisms. While research on generation of transgenic fish has been permitted/supported in all these 12 countries, it has developed into the field-testing level only in 6 countries, i.e. China, Israel, Hungary, New Zealand, UK and USA, and is limited to putative transgenic carp, salmon, Nile tilapia and channel catfish. To evaluate globally the use, desire and constraints associated with the development of genetically modified organisms in fisheries and aquaculture, Bartley and Hallerman[3] sent a questionnaire to all the 160 member countries of FAO; responses were received from 60 of them. Among Asian countries, China seems not to have replied, but 6 Asian countries consider the GMO positively from the point of moral issues,

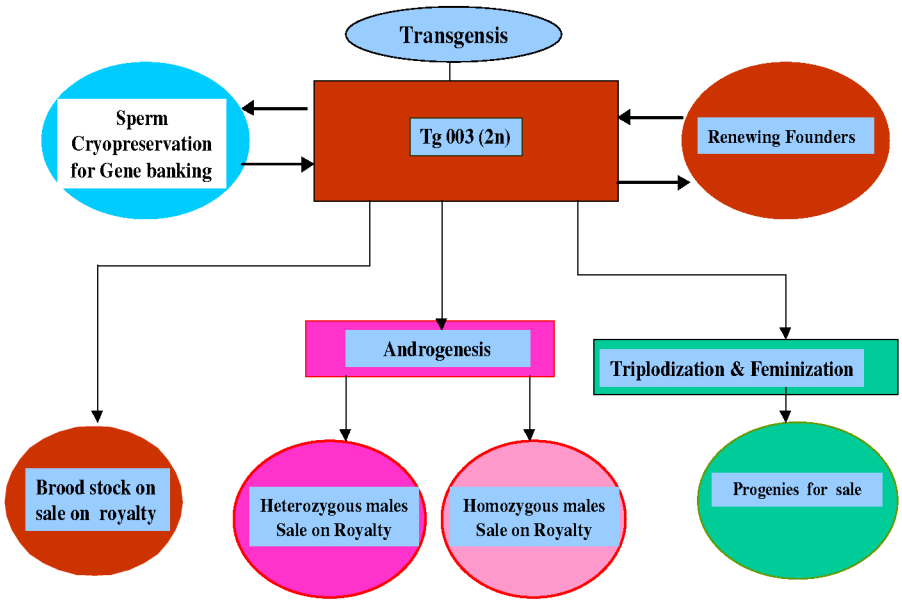

Figure 1. Patenting autotransgenic mud loach. Suggested options for 'containing' founder brood stock and androgenics to be sold on royalty (indicated by red colour) and triploid feminized progenies for free sale (indicated by green colour). Renewing founders and cryopreservation for gene banking must be managed in containment.

economic gain, improved diet and environmental safety. Their interest specifically for transgenic fish is modest, but surprisingly several Latin American countries have evinced keen interest. As much as the transgenic science is young, the preparation of guidelines and regulations is in its infancy; however, two aspects must be indicated: (i) governments in many developing countries do not have an overall knowledge and/or concern for the transgenics and (ii) transgenic research is growing so fast that guidelines and regulations will have to be revised from time to time, even if some governments have framed them.

Definition of GMO

Currently, there is no universally accepted definition for a GMO[1–3,8]. One definition states that it is the one bearing an intergeneric combination of (heterologous) genetic material (Office of Science and Technology, 1986, see ref. 10). The definition thus effectively excludes transgenic fish, that are transformants for DNA constructs bearing non-coding regulatory regions and intrageneric (homologous) protein-encoding sequences. Hence, autotransgenic mud loach generated by Kim and his colleagues as well as those bearing homologous transgene[35] are not considered truly transgenic, and might prove more readily certifiable for deliberate release and distribution. Yet, the genuine concern of other scientists[2] is to insist that the following features of the transgenics must be considered, while defining the GMO: (i) characteristics, (ii) functions of the transferred transgene, (iii) sources of coding DNA, (iv) regulatory DNA, (v) potential for pleiotropic effect, (vi) interaction with remainder genome, (vii) stability of its construction and (viii) ability to transpose within or between genomes. A DNA sequence that does not code for any functional product may do so, when interacting with the remainder of genome through recombination or transposition. Therefore, the distinction between homologous and heterologous protein-encoding genes is largely irrelevant and should not be considered while defining the GMO. There are also other complex issues. For instance, when tilapias, that are anti-freeze transformants, escape into the farms and ponds in temperate countries, a strong possibility for detrimental, environmental impact awaits. But if the same tilapias are GH-transformants, then it does not matter at all, as they may not survive the cold waters. Therefore, framing regulations for the transgenic must have transgene-specific, species-specific and environment-specific considerations. In countries like USA, there is a Recombinant DNA Advisory Committee to consider such cases which do not comply with the guidelines of the respective nations. Keeping this in view, the Ministry of

Table 3. Regulation imposed by countries on field testing of transgenic fish species in which phenotypic expression of the transgene is confirmed (from Dunham[22]; modified and added)

Country/ species	Trait	Phenotypic expression	Field testing	Regulation
China				
Common carp	Growth	+	+	+
Loach	Growth	+		+
India				
Common carp	Growth	+		+
Catfish	Growth	+		+
Israel				
Common carp	Growth	+	+	
Philippines				
Nile tilapia	Growth			+
South Korea				
Loach	Growth	+		
Hungary				
Nile tilapia	Growth		+	
United Kingdom				
Nile tilapia	Growth	+	+	+
Salmon	Growth	+		+
New Zealand				
Salmon	Growth	+	+	+
The United States				
Common carp	Growth	+	+	+
Salmon	Growth	+	+	+
Channel catfish	Growth	+		+
	Disease resistance	+		+
Rainbow trout	Disease resistance	+		+
Canada				
Salmon	Growth	+		+
	Anti-freeze	-		+
Japan				
Medaka	δ crystallin	+		+
Taiwan	Growth	+		

Environment and Forests, Government of India has also framed rules and procedures for handling GMOs, including fishes, as it is known that the longevity of genetically modified fish is shortened[33].

In 1986, the Government of India enacted the Environment (Protection) Act (EPA) to protect and preserve the environment and to minimize the risks from pollutants, and contaminants as well as GMOs. The *Gazette of India* clearly defines the Competent Authorities and their structural composition for handling of all aspects of GMOs and their products. There are six competent authorities, as stated below and among them, the first three were constituted by the Department of Biotechnology, Ministry of Science and Technology, and the fourth by the Ministry of Environment and Forests. (i) The Recombinant DNA Advisory Committee (RDAC) monitors the developments in biotechnology at national and international levels, (ii) The Review Committee on Genetic Manipulation (RCGM) monitors safety aspects of on-going research projects and activities involving genetically engineered organisms, (iii) The Institutional Biosafety Committee (IBSC) keeps track of the identified investigators, and the status and results of their experiments, (iv) The Genetic Engineering Approval Committee (GEAC) is responsible for approval of activities involving large-scale use of GMOs in research, industrial production and applications. The two other committees, namely (v) State Biotechnology Coordination Committee (SBCC), and (vi) District Level Committee (DLC) have powers to inspect, investigate and take suitable action in the case of violations of the statutory provision[34]. However, it must be indicated that most of these committees have framed rules and guidelines, especially for agriculturally important plants and hygienically important pathogens; 'experiments in transgenic animals, including fish are yet at a developmental stage (see Table 3) and India has to go a long way before such products are developed for commercial applications'[33].

Ecological concerns

The primary ecological concerns regarding utilization of transgenic fish are the loss of genetic diversity and loss of biodiversity, and reduction in species richness[26,36]. An evaluation of this is complex, encompassing a wide range of biological processes and field study, including genetics and ecology. Briefly, two aspects related to accelerated growth and thermal tolerance may be considered. GH-transgenics are more analogous to a selected and/or domesticated line, capable of growing 4–5 times faster.

Table 4. Exotic fishes transplanted in India (from Kumar[49], modified)

Species	Home country	Year of introduction	Purpose
Game fish			
Brown trout (*Salmo trutta fario*)	UK	1863–1900	For planting streams, lakes and reservoirs
Loch trout (*Salmo levensis*)	UK	1863	For planting streams, lakes and reservoirs
Rainbow trout (*Salmo gairdneri*)	Sri Lanka and Germany	1907	For planting streams, lakes and reservoirs
Eastern brook trout (*Salvelinus fontinalis*)	UK	1911	For planting streams, lakes and reservoirs
Sockeye salmon (*Oncorhynchus nerka*)	Japan	1968	For planting streams, lakes and reservoirs
Atlantic salmon (*Salmo salar*)	USA	1968	For planting streams, lakes and reservoirs
Food fish			
Golden carp (*Carassius carassius*)	UK	1870	Experimental culture
Tench (*Tinca tinca*)	UK	1870	Experimental culture
Gourami (*Osphronemus goramy*)	Java and Mauritius	1916	Experimental culture
Carp (*Cyprinus carpio*) [German strains]	Sri Lanka	1939	Experimental culture
Tilapia (*Orechromis mossambicus*)	Africa	1952	Experimental culture
Carp (*Cyprinus carpio*) [Bangkok strains]	Thailand	1957	Experimental culture
Grass carp (*Ctenopahryngodon idella*)	Japan	1957	Experimental culture and weed control
Silver carp (*Hypophthalmichthys molitrix*)	Hong Kong	1959	Experimental culture
Tawes (*Puntius javanicus*)	Indonesia	1972	Experimental culture
Larvicidal fish			
Guppy (*Poecillia reticulata*)	South America	1908	Mosquito control
Top minnow (*Gambusia affinis*)	Italy	1928	Mosquito control
Ornamental fish			
Live bearers (27 species)	From various countries		Aquarium keeping
Egg layers (261 species)	From various countries		Aquarium keeping
Unauthorized introduction			
Big-head carp (*Aristichthys nobilis*)			Aquaculture
African catfish (*Clarius gariepinus*)			Aquaculture
Nile tilapia (*Oreochromis niloticus*)			Aquaculture
Red tilapia (*Oreochromis* sp.)			Aquaculture
Red piranha (*Serrasalmus natteren*)			Aquaculture keeping

Thus the altered phenotype is similar to that which could be obtained by strain selection, individual selection, intraspecific cross-breeding and inter-specific hybridization. If a 4–5-fold increase in growth is possible through traditional breeding, ecological impacts would be the same regardless of the mechanism and level of phenotypic alteration, traditional or biotechnological[22]. Carefully designed, long-term researches have been undertaken on more than 20 fish species to assess the competitiveness of triploids with other triploids or diploids of the same species in farms. Unfortunately, no such research work has so far been undertaken to assess the competitiveness of transgenics with their respective siblings. For this kind of investigation, the use of different coloured fluorescence dye (North-west Marine Technology, USA) and other taggings, as has been used by Nam *et al.*[8] may prove beneficial.

Transgenics, that are anti-freeze protein gene transformants, may interfere and compete with the indigenous fish species inhabiting the same geographical area. The number of exotic fish species introduced by man into different countries is more than 300 (Table 4). For instance, there has been repeated and widespread introduction of Indian carps, Chinese carps and common carp throughout Asia[37]. Introduction of exotic species has greater potential to adversely affect biodiversity and genetic diversity[38] than the transgenics, derived from the indigenous aquaculture species[22].

Containment

Lack of data on the potential, ecological and socio-economic impacts of transgenic fish has contributed to a growing debate on commercial utilization of genetically modified fish in the society[22]. That no risk is involved in commercial utilization of at least GH transgenic fish is apparent; yet, there is a need to establish containment facility, both in research laboratories and aquacultural farms, not only to ameliorate social apprehension, but also to facilitate the patenting process, which may require containment for founders (see Figure 1).

Physical containment

The first line of defence against escape of genetically modified fish and their viable gametes into natural aquatic habitats is to establish physical structure and proper management procedures. However, no report is yet available for the kind of physical containment built by Asian countries. Containment of inland-based facility can readily be accompanied by screening in-flow and out-flow water ways, to prevent direct escape of the transgenics and their gametes from ponds and tanks. Sea-pen facilities, which may be required for the Korean hirame *Paralichthys olivaceus*, pose a greater problem for containment. In a given facility, containment is subjected to disruption caused by human error, lack of maintenance and poaching (see also, Devlin and Donaldson[2]).

Biological containment

Clearly, the most secure method for preventing reproductive interaction with wild populations is to induce complete sterility in the stock, for which containment is required. A biological method chosen for containment in aquacultural operations must be completely effective, simple and cheap to implement. Some known biological containing methods of transgenic fish are: (i) surgical removal of gonads, (ii) hormonal induction of sterility, (iii) production of sterile triploid or monosex (gynogen and androgenate) progenies, (iv) hybrid sterility.

Surgical removal of gonad is an effective means to sterilize fish populations. However, the method is difficult to implement, as it involves skilled and intensive labour, and risk of incomplete removal of gonads; secondly, it cannot be practised in small fish species, and thirdly, surgical removal of gonads in cichlids and anabantids results in complete regeneration of gonad of the same or opposite sex[4].

Hormonal induction of sterility, especially by discrete immersion of embryos in super optimum (for masculinization) doses of 17α-methyl testosterone, has been successfully achieved in a number of salmonids and cyprinids[39]. Steroid administration by immersion of embryos and alevins is thousand times cheaper. More than 90–95% of administered steroid is eliminated in less than two weeks after dosing. Estimated residual steroid is too low to cause hazard to human health. Therefore hormonal induction of sterility appears to be an effective, simple and cheaper method[40,41].

Marian[42] was perhaps the first to produce a triploid transgenic fish in an attempt to develop biological containment of transgenics; the transgenic zebrafish expressed the transferred pRSVrtGH. However, survival of the transgenic, which had suffered a double stress of thermal shock (to induce triploidy) and microinjection (to transfer transgene), was less than 2%. Razak *et al.*[43] generated transgenic triploid tilapia, *O. niloticus*, and recorded retarded gonad development. Nam *et al.*[8] produced autotransgenic triploid sterile mud loach, *M. mizolepis*. In all these cases, triploidy conferred complete sterility in female and partial sterility in male. Attempts to fertilize diploid eggs with sperm obtained from conspecific triploid males have not so far succeeded. However, fertile female and male triploids have been generated in a few species of cyprinids and salmonids[4]. Briefly, this method of containment requires skill and ensures only a low yield, but may not completely be effective.

Nam *et al.*[9] were the first to generate homozygous autotransgenic androgenate mud loach, *M. mizolepis*. Their procedure for generation of androgenates yielded

about 30% survival and the males were completely fertile, as evidenced by comparable egg fertilization success. Although a possibility, gynogenetic transgenesis has not been reported so far. Stray occurrence of unexpected male or female has been reported among gynogenate and androgenate progenies of fishes[4].

Hybrid sterility

It is known that hybridization is possible among many closely related species of cyprinids and salmonids; however, progenies of selected hybridization are sterile. Therefore, the technique can be used to induce sterility in transgenics.

Conclusion

About 30 laboratories in about 10 Asian countries are actively engaged in transgenic fish research. 'An establishment of collaborative network to develop protocols for and to conduct sound and safe research on transgenic fish research would assure that the benefits rather than the detriments are the product of aquaculture gene transfer research in developing countries[22].'

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