Review Article

FISH BREEDING AND BIOTECHNOLOGY

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SUMMARY : In this review four different methods which have been used for cultivating new breeds of fish of agricultural importance are discussed. They are; 1. Natural and artificial selection, 2. Artificial sexual hybridization, 3. Nuclear transplantation and 4. Gene transfer. Among them, natural and artificial selections and sexual hybridization are traditional, sexual and non-biotechnical methods. They are popularly and successfully used in fish farming practice in the history. But their further application is limited because of the difficulties of composition of sexual combinations between distantly related species. Furthermore identification and composition of the characteristics which are to appear in the hybrid fish presents many problems. Nuclear transplantation and gene transfer are two newly developed methods for attempting to cultivate new breeds of fish. Biotechnology thus offers the chance of transferring genetic materials or molecules between different fish species. It is expected that these two methods will ultimately replace the sexual methods for obtaining more stable, predictable hybrid fishes. Because no species-specific limitation will appear in those genetic transfer combinations and the target genes which were the newly developed fish in unique ways. Some important approaches have already been made in those research areas, but some special problems, theoretically or technically, have to be solved before those biotechnical methods can successfully be applied to fish culture on commercial scales.

Key Words : Fish breeding, hybridization.

In the literature several kinds of methods which have either already been applied or are intended to be used for cultivating new breeds in animals of agricultural importance have been recorded. These include :

I. Natural and artificial selection

To artificially select of better animals from naturally existing animal species is one of the traditional methods for this purpose. Those selected animals represent mutants accumulated in long-term natural environmental situation. Their characteristics were successfully modified. Through sexual breeding among those individuals, they produced offsprings generation by generation and finally some new breeds, varieties or species of those animals were obtained. This represents 'domestication'.

Since the frequency of natural mutation is very low and to obtain new breeds or varieties will usually take a long time of evolution history. For example, when the same species of animal distributed in different areas were influenced by the different local environmental factors for a long time, firstly some of them and their

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offsprings may change their phenotypes but without modifications to their genotype. This phenomenon is called as the results of 'domestic adoption'. In some cases, when long environmental influences were accumulated strong enough, some of them may also change their genotypes at a very low frequency, i.e., a sort of 'mutagenesis'.

Since those new breeds or varieties, either with different phenotypes or genotypes, appeared in the same animal species in different locations were believed to be caused by long-term influence of geological, climate, food and other unknown factors in various ambient conditions. The way they were formed is thus based on the long-term of 'natural' and 'artificial selection'.

However, the environmental factors which may induce 'domestic adoption' or 'mutagenesis' of animals are very complicated. It will be most difficult to clarify those factors in detail or to try simulating them in artificial conditions for producing new breeds or varieties of animals on reproducible basis.

Therefore, human societies had to search for other possibilities for cultivating new breeds of animal of agricultural importance in order to meet more and more demands for producing better food and other living supplies. Among those possibilities, sexual hybridization has become a most useful method.

II. Artificial sexual hybridization

Sexual hybridization is a traditional method for cultivating new animal breeds or varieties. On the basis of natural and artificial selection, some animal individuals with various ideal characteristics can be selected and obtained from some taxonomally different but closely related species. To make 'sexual hybridization' between them can produce new hybrids with some 'hybrid vigor'. That is, with better dominant characters will be appeared in their hybrid offsprings which may improve the value of their original parent animals.

Sexual hybridization might occasionally happen in nature. But most were conducted artificially. The principle of artificial sexual hybridization is to impose the male and female gametes of different animals to fuse together as a zygote by artificial methods that does not happen in natural conditions. Then those 'hybrid zygotes', in some cases, will develop into hybrids with some improved characteristics. According to modern scientific terminology, this method can be recognized as the recombination of different groups of gene which came from two diploid genomes of different male and

Figure 1: Pictures of a common carp (*Cyprinus carpio*, genus Cyprinus Linnaeus, 2N=100) (A), a crucian carp (Carassius auratus, genus Carassius Jarocki, 2N=100) (B), a one-year old sexual hybrid fish (C) obtained in the combination of common carp ♀ x crucian carp ♂ and a one-year old sexual hybrid fish (D) obtained in the combination of crucian carp ♀ x common carp ♂.

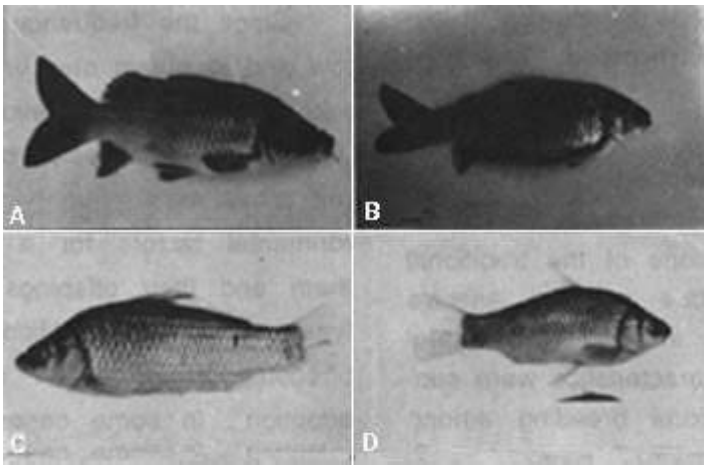
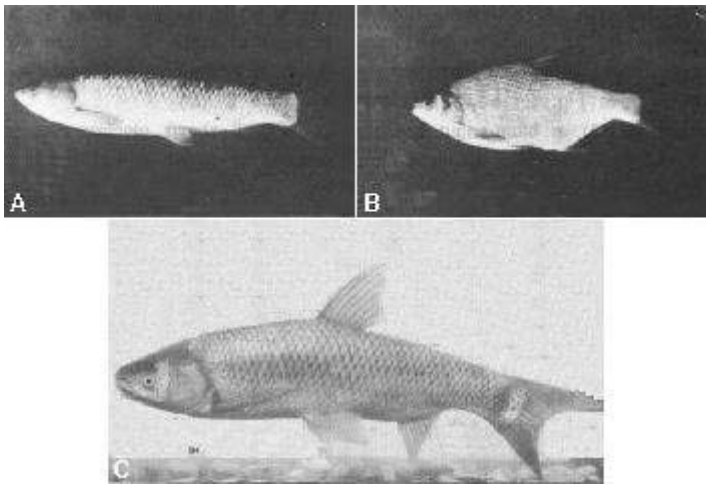


Figure 2: Pictures of a grass carp (*Ctenopharyngoden idellus,* subfamily Leucine, family Cyprinidae, 2N=48) (A), a blunt-snout bream (*Megalobrama amblycephala*, subfamily Abramidinae, family Cyprinidae, 2N=48) (B) and a two-year old sexual hybrid fish (C) obtained in the combination of grass carp ♀x blunt-snout bream ♂.



female animals with obviously different genetic backgrounds. The modified phenotypes appeared in those hybrids were explained as the results of 'hybridity expression' of the newly reconstructed genomes of the animal individuals.

General speaking, when taxonomy closely related varieties or species of animals were used in sexual hybridization, the F1 hybrids with 'hybrid vigor' can be obtained. Their next generations of progeny will, however, be produced following Mendel's law of inheritance. That is, the characters with original differences form the male and female parent animals will segregate and eventually might reappear in the offsprings after the second generation (F2) when those F1 hybrids are mated among themselves.

Therefore, in many kinds of animals, sexual hybridization is very useful for producing better F1 hybrid animals. It will, however, not be performed as an efficient method to cultivate really 'stable new breeds or varieties' of animals.

Moreover, due to natural, biological incompatibilities between the different species of most animals (the exact mechanisms of those incompatibilities are still unknown), such kind of sexual hybridization only can be done between taxonomally closely related species. When the male and female gametes which are from distantly related species were used in hybridization, some of them may not be able to fuse together. Only in rare cases they can be fused as hybrid zygotes but those hybrid eggs are lethal and could not develop into the adults, or they may develop into sterile adults due to poor gonad development. This principle has proved to be very true in almost all higher animals from classes amphibian to mammal. A good example of this kind of sexual hybridization is that of a female horse and a male donkey can be sexual hybridized to produce the hybrid 'mule'. It obtained some better characters from both its parents, but it is unfertile.

However, some exceptions exist in cases of lower vertebrate species. In fish, sexual hybridization not only can be successfully made among closely related species for obtaining better hybrids in fish farming practice, but also can apply to those fish species which belong to rather distantly related species. For example, we have obtained several kinds of sexual hybrid adults, larval fish, or embryos between different genera, subfamilies, families and orders. Following are some examples :

1. Inter-genus sexual hybridization

a. Common carp (Cyprinus carpio, genus Cyprinus Linnaeus, 2N=100) ♀x crucian carp

(Carassius auratus, genus Carassius Jarocki, 2N=100) ♂. Adult hybrid fish were obtained. They look like common carp. The male hybrid is unfertile. Figure 1 shows pictures of a common carp (A), a crucian carp (B) and a one-year old sexual hybrid fish (C) obtained in this combination.

b. Crucian carp ♀ x common carp ♂. Adult hybrid fish were obtained. They look like crucian carp. The male hybrid is unfertile. Figure 1(d) shows the picture of a one-year old sexual hybrid fish obtained in this combination.

2. Inter-subfamily sexual hybridization

a) Grass carp (Ctenophryngoden idellus, subfamily Leucinae, family Cyprinidae, 2N=48) ♀ x Blunt-snout bream (Megalobrama amblycephala, subfamily Abramidinae, family Cyprinidae, 2N=48) ♂. Adult hybrid fish were obtained. Both male and female hybrids are unfertile. Figure 2 shows pictures of a grass carp (A), a blunt-snout bream (B) and a two-year old hybrid fish (C) obtained from this combination.

b. Dace (Lobeo rohita, subfamily Labeoninae, family Cyprinidae, 2N=50) ♀x common carp (Cyprinus carpio, subfamily Cyprininae, family Cyprinidae, 2N=100) ♂. Adult hybrid fish were obtained. Both male and female hybrids are unfertile. Figure 3 shows pictures of a dace (a), a common carp (b) and a one-year old hybrid fish (c) obtained from this combination.

Other sexual larval fish or embryos were also obtained. For example: In inter-order combination

(1) Goldfish (Carassius auratus, order Cypriniformes, 2N=100) ♀x tilapia (Oreochromis nilotica, order Perciformes, 2N=44) ♂. Sexual hybrid young fish were obtained. Figure 4 shows pictures of a tilapia (A), goldfish (B) and a 15-day sexual hybrid fish (C) obtained from this combination.

(2) Loach (Paramisgurnus dabryanus, order Cypriniformes, 2N=48) ♀x tilapia (Oreochromis nilotica, order Perciformes, 2N=44) ♂, and its back cross combination. Sexual hybrid gastrula were obtained. Figure 5 shows pictures of a tilapia (A), a loach (B), a 60 hr sexual hybrid fish obtained in the combination of loach ♀ x tilapia ♂ (C), and a sexual hybrid gastrula obtained in the combination of tilapia ♀ x loach ♂ (D).

Figure 6 shows electron-microscopic pictures of the process of tilapia sperm penetrating into loach egg. Figure 7 shows pronuclei of tilapia sperm and loach egg are going to fuse together (A). A metaphase pic-

Figure 3: Pictures of a Dace (*Labeo rohita*, subfamily Labeoninae, family Cyprinidae, 2N=50) (A), a common carp (Cyprinus carpio, subfamily Cyprininae, family Cyprinidae, 2N=100) (B) and a one-year old sexual hybrid fish (C) obtained in the combination of Dace ♀x common carp ♂.

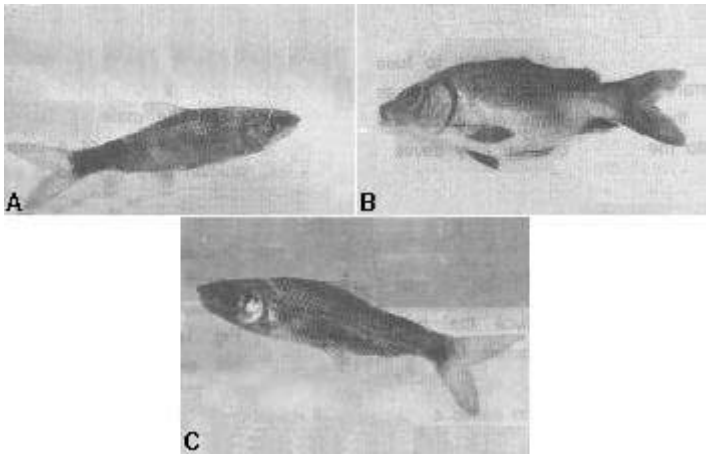
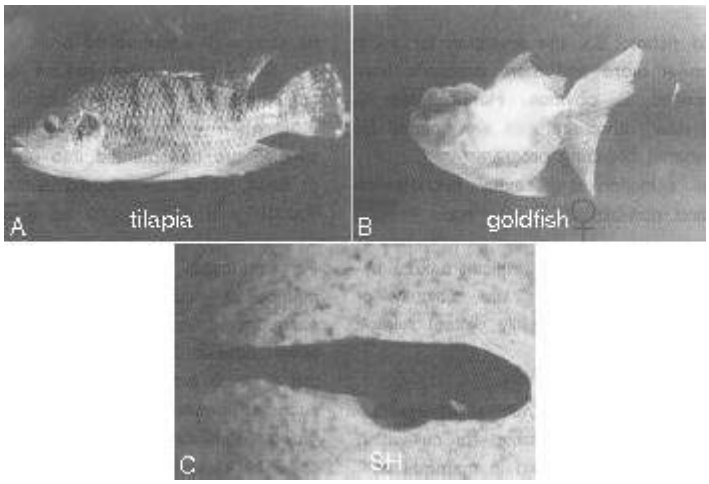


Figure 4: Pictures of a tilapia (Oreochromis nilotica, order Perciformes, 2N=44) (A), a goldfish (Carassius auratus, order Cypriniformes, 2N=100) (B) and a 15-day old sexual hybrid young fish (C) obtained in the combination of goldfish ♀ x tilapia ♂.



ture of hybrid embryo foom the combination of loach egg and tilapia sperm (B). A 432 hrs hybrid larval fish obtained from the some combination.

According to the above examples, there is some good evidence which demonstrate that, it is possible to make some successful artificial sexual hybridizations among distantly related fish species. If we can improve those traditional sexual hybridization methods, they may make some further contribution to fish farming practice in the future.

On the other hand, many existing fish species of agricultural importance are degenerating due to a long history of inbreeding among the individuals of their own species but different strains in fish farming practice. Thus, to try making more successful combinations by sexual hybridization among fishes of different varieties, species, and even more distantly related species seems both necessary and possible.

However, it is still very difficult to overcome natural barrier between the distantly related fish species only by using traditional artificial sexual hybridization methods. That is, "just to mix their sperms and eggs together in an in vitro system" for producing hybrids is not sufficient.

Detailed mechanisms which prevent distantly related animal species from successful sexual hybridization are not clear. It was observed that there are several biological steps which are involved in sexual hybridization. Disruption of any of those steps may make sexual hybridization between distantly related animal species unsuccessful :

a. In most cases, the first most difficult step in sexual hybridization among distantly related animal species is the failure of foreign sperm to penetrate the egg. For example, in some cases, they cannot pass through the micro Pyle of the egg chorion in fish and the zona pellucida of the egg in mammals.
b. In other cases, foreign sperm can enter the eggs but will be degenerated and disappear in the egg cytoplasm without performing any vital function.
c. In some cases, foreign sperm can enter the egg and enlarge as a male pronucleus but it cannot fuse with the egg pronucleus to form a zygote nucleus.
d. In yet other cases, foreign sperm can enter the egg, enlarge as a male pronucleus, fused with the egg pronucleus as a zygote nucleus and divide in coordinating with egg cytoplasmic division. In this case, the fertilization process of sexual hybridization between the sperm and

egg was completed and the fertilized hybrid egg started to develop into an embryo. However, due to the unknown natural incompatibilities which existed between the sperm and egg which were obtained from distantly related animal species, especially in the case when to fuse a male and a female pronucleus with different numbers or pairs of chromosomes, the hybrid zygote nucleus, will become heteroploid. The hybrid embryo always develops abnormally.

Up to now, no practical methods have been available for overcoming the aforesaid natural barriers in sexual hybridization for fish. However, some new biotechnical methods such as microinjection, cell fusion or electroporation might be used to help foreign sperms to enter the eggs. In addition, to select sperms and eggs of different fish with the same or similar chromosome numbers may also be helpful to reach this goal. All those possibilities need further investigation.

The most obvious disadvantage of sexual hybridization for obtaining hybrid animals is that we cannot predict which kinds of new modified characteristic will appear in hybrid fishes. So, the limitation for using this method to meet more and more demands from aqua-cultural purposes is obvious. Hence, from a practical point of view, other methods are needed for developing new animal breeding programs.

Both artificial selection and sexual hybridization are traditional and non-biotechnical methods for culturing animal breeds. However, some new biotechnical techniques could be applied to artificial sexual hybridization in order to overcome the difficulty of performing this method in taxonomally distant related animal species.

III. Nuclear transplantation

During the last two decades, nuclear transplantation has become one possible method for cultivating new breeds not only in fish but also in mammals.

The concept of nuclear transplantation (to transfer a diploid nucleus into an enucleated egg for investigating the roles of nucleus in initiating embryonic development) was first proposed by a German embryologist, Hans Spemann, early in 1938.

In the 1950's, a number of biologists working in fields of fundamental genetic research made the first discovery that by using nuclear transplantation produce unicellular organism strains with some new characters. For example, to transfer the nucleus of one kind of amoeba into an enucleated amoeba of another kind (8), some reconstructed amoeba systems with the

Figure 5: Pictures of a tilapia (Oreochromis nilotica, order Perciformes, 2N=44) (A), a loach (Paramisgurnus dabyranus, order Cypriniformes, 2N=48) (B) and a 60-hour old sexual hybrid larval fish (C) obtained in the combination of loach ♀ x tilapia ♂ and a sexual hybrid gastrula (D) obtained in the reverse combination, combination of tilapia ♀ x loach ♂.

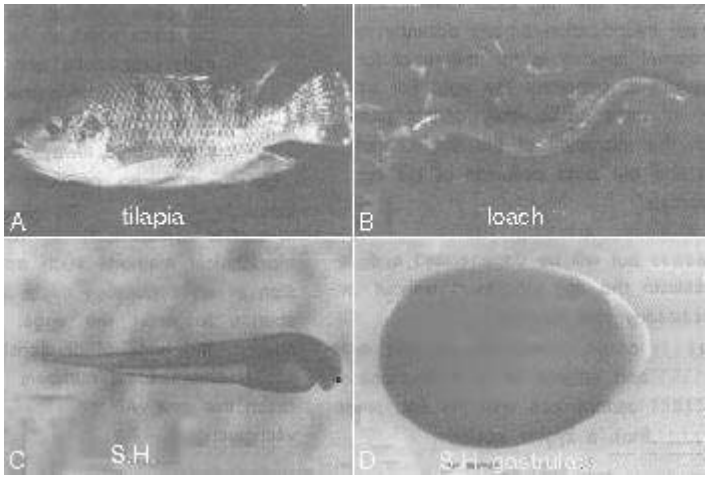
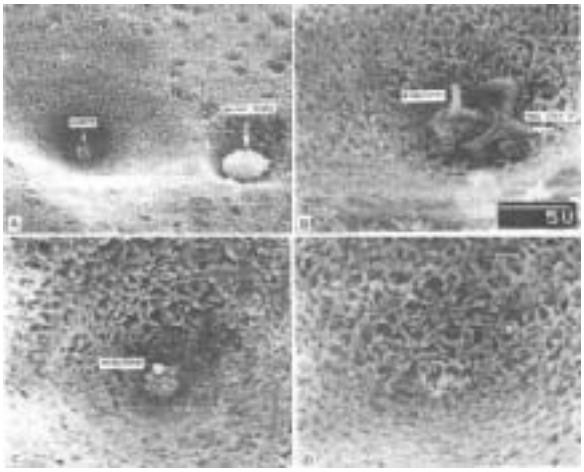


Figure 6: Pictures show the process of tilapia sperm penetrating into loach egg observed by scanning electron microscope. SEM of a de-chorionated loach egg at 5 seconds after insemination to show the head of a tilapia sperm was surrounded by microvilli of the egg surface at the sperm entry site of the animal pole (small arrow) near by the location of the first polar body of the egg (big arrow) (A), SEM of a dechorionated loach egg at 20 seconds after insemination to show the head of tilapia sperm has embedded in the cytoplasm of loach egg. A portion of midpiece of the sperm still remained in outside of the egg. The thick are which surrounded sperm head showed by arrow is the unremoved part of egg chorion (B), SEM of dechorionated loach egg at 30 seconds after insemination to show the head of tilapia sperm completely embedded in loach egg cytoplasm. Only a little part of its midpiece remained in outside of the egg surface (C) and SEM of a de-chorionated loach egg at 40 seconds after insemination to show the tilapia sperm fully incorporated into the egg cytoplasm. The site of sperm penetration is still visible as a patch of microvilli (D).



characteristics from both parent amoeba strains or intermediate ones were obtained.

Since then, other groups of embryologists also confirmed that, either in amphibians (19) or in fish (40), when embryonic cell nuclei of one variety or species are transplanted into enucleated eggs of other ones, some nucleo-cytoplasmic hybrid (NCH) individuals with intermediate and modified characteristics were obtained.

Fortunately, like sexual hybridization in fish, the method of nuclear transplantation can be used to make non-sexual hybridization in fish not only between different varieties or species, but also between those which belong to more distantly related species such as between different subfamilies, families and orders. Previously, nuclear transplantation was only done between a few different species of amphibians and different strains in mammals.

Since the nuclei used in nuclear transplantation experiments are diploid, this method can be recognized as the transfer of an 'intact genome' (49).

This method was introduced into fish in the early 1960's. So far several kinds of inter-species combinations of the nucleus and cytoplasm, to produce nucleo-cytoplasmic hybrids in fish, have been successfully made. The identification of different genetic roles of nucleus and cytoplasm in a nuclear transplantation system of fish became easier and more feasible than those observed in amphibians because the nucleus and cytoplasm could be combined from different kinds of fish with obviously different characteristics.

Following are some examples of nuclear transplantation in fish which were obtained from the combinations of blastula cell nuclei and enucleated eggs of different species :

I. Inter-genus combination

Nucleus from common carp (Cyprinus carpio,

genus Cyprinus Linnaeus, 2N=100) + cytoplasm from crucian carp (Carassius auratus, genus Carassius Jarocki, 2N=100). Adult NCH fish with essentials of common carp phenotype plus some intermediate and cytoplasmic influenced character at morphological, physiological and biochemical levels were obtained (40). Both male and female hybrids were obtained and they are fertile. Figure 8 shows pictures of a common carp (A), a crucin carp (B), and a NCH fish (C) obtained in this way.

2. Inter-subfamily combination

Nucleus from grass carp (*Ctenopharyngodon idellus,* subfamily Leucinae, 2N=48) + cytoplasm from blunt-snout bream (*Megallobrama amblycephala*, subfamily Abramidinae, 2N=48). Adult NCH fish with essentials of the grass carp phenotype and some biochemical, immuno-electrophoretic changes of its blood serum were obtained (45). The male hybrids were fertile but the female hybrids couldn't be identified before they were lost or died due to the careless culture in fish ponds (the female grass carp usually need one more year than the male to become mature. That is, they need about four years of culture. Figure 9 shows pictures of a grass carp (A), a blunt-snout bream (B), and a NCH fish (C) obtained in this way.

Other NCH adult, larval fish and embryos have also been obtained (47-49). For example, in an Inter-order combination : Nucleus from tilapia (order Perciformes, 2N=44) + cytoplasm from loach (order Cypriniformes, 2N=48). Late NCH larval fish unlike tilapia or loach types in morphology were obtained (49).

It is very interesting to point out that in the combination of the nucleus of common carp and the egg cytoplasm of crucian carp (Inter-genus combination), both male and female hybrid fish were obtained and they are fertile. In addition to morphological and biochemical changes which appear in this kind of hybrid fish, their economic value is also to be improved. For example, at least 4 generations of their offsprings were obtained in fish farming firms in different provinces in China in the middle scale of field production. Their modified characteristics are stable. The average enhancement of the body weight growth of such kinds of NCH fish is 22% higher than that of common carp cultured in the same conditions. Figure 10 shows pictures of a six month cultured NCH fish (A) and a sibling control, common carp (B). The nutritional value of this kind of NCH fish ia also improved. That is, the protein

Figure 7: Pictures show the pronuclei of tilapia sperm and loach egg are going to fuse together (A), A metaphase picture of hybrid embryo obtained in the combination of tilapia sperm and loach egg (2N=44) (B) and a 432 hr hybrid fish in this combination (C).

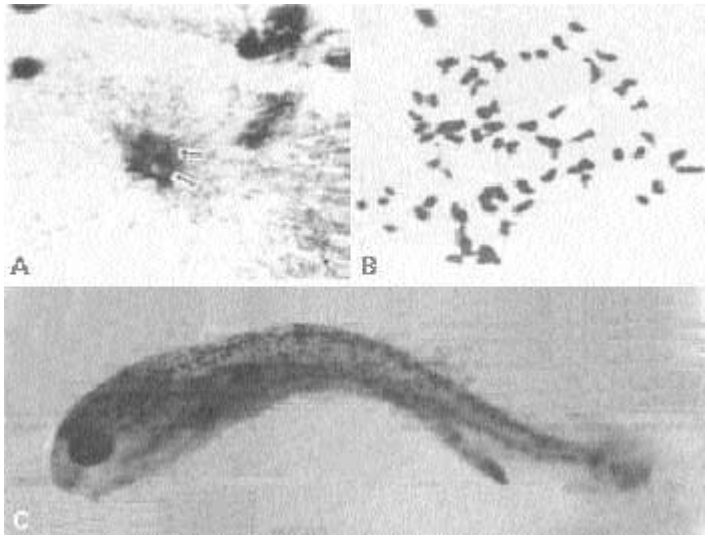
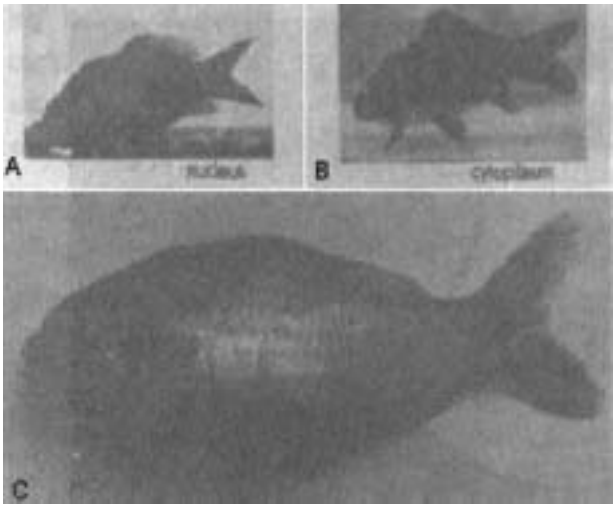


Figure 8: Pictures of a common carp (Cyprinus carpio, genus Cyprinus Linnaeux, 2N=100) (A), a crucian carp (Carassius autratus, genus Carassius Jarocki, 2N=100) (B) and a two- year old nucleo-cytoplasmic hybrid fish (C) obtained in the combination of the nucleus of common carp and the cytoplasm of crucian carp.

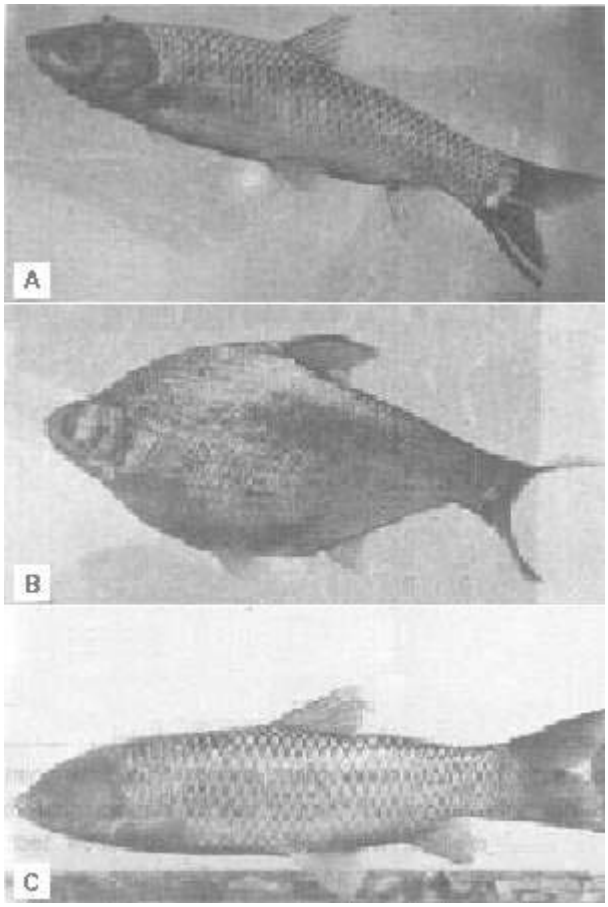


content of fish muscle is 3.78% higher and fat content 5.58% lower, than those of the nucleus-donor fish, the common carp (45). This kind of nucleo-cytoplasmic hybrid fish has been welcomed in various fish farming stations in a number of Provinces in China and has a good market.

Moreover, by using male fish of this kind of NCH fish to fertilize the female mirror carp (a mutant of common carp), the Chang Jiang Fisheries Institute, the Chinese Academy of Fisheries in Shashi, China, had successfully cultured another kind of sexual hybrid fish, 'Ying carp'. This fish has even much better economic quality than its male nucleocytoplasmic hybrid parent fish (personal communication).

The results obtained by nuclear transplantation in mammals are even more interesting. A recent report stated that "When groups of British and American scientists tried to use non-sexual reproductive methods to produce cows and sheep of better qualities, some unexpected 'super animals' were obtained which gave a big shock to scientific circles. The normal body weight of a newborn calf is about 36 kg., but among

Figure 9: Pictures of a grass carp (Ctenopharyngo idellus, subfamily Leucinae, 2N=48) (A), a blunt-snout bream (Megalobrama amblycephala, subfamily Abramidinae, 2N=48) (B), and a two-year old nucleo-cytoplasmic hybrid fish (C) obtained in the combination of the nucleus of grass carp and the cytoplasm of blunt-snout bream.



1000 pregnant cows which were manipulated with the non-sexual reproductive method, one fifth of their fetuses had a body weight of more than 68 kg. Those cows have to use Cesarean Section for helping them to give birth of their baby calf. Scientists pointed out that this unexpected 'achievement' is the most important discovery during the last decade. As to the reasons why those giant animals could be produced, it needs further investigation. This event caused the expansion of their bovine embryo transfer program. In addition, by using the non-sexual reproductive method, scientists

Figure 10: Pictures of a six-month old nucleo-cytoplasmic hybrid fish (A) obtained in the combination of the nucleus of common carp and the cytoplasm of crucian carp, a sibling control (common carp) (B) of same age which was cultured in the same conditions.

also helped female sheep to produce many giant sheep. Scientists pointed out that so called 'non - sexual reproduction' is a sort of method which was used micro-operation technique to transplant a somatic nucleus into an enucleated egg. Then the egg was reimplanted into the uterus of a foster female animal and it can continue to develop without fertilization. This kind of technique can make stable animal clones with better genetic quality as well as produce a great deal of artificially made living stocks (News article from World Journal, Chinese edition published in New York, USA, August 12, 1992).

Another advantage for using the nuclear transplantation method to produce non-sexual hybrid fish with modified characters existed. At least in some cases, the more distantly related species could be used as parent fish whereas in sexual hybridization only closely related species could be used; the NCH fish obtained by this method among distantly related species are fertile and could be cultured as new clones, whereas those obtained by sexual hybridization are infertile.

In addition, when the cell nuclei of the same embryo were transplanted into the same kind of enucleated eggs, NCH hybrid fish with completely identical genetic background could be obtained. These kinds of genetically identical fish clones will be difficult to find in natural conditions or even in long-term inbreeding systems. They will be very useful for use as experimental material for gene transfer and gene targeting research.

What are the mechanisms or factors which induce NCH animals to change their characteristics, from those of their original nucleus donor fish. It is still an unknown area and needs further investigations.

However, the results obtained from the nuclear transplantation experiments in fish or other animals are possible to be tentatively interpreted. According to our speculation that interactions between the cell nucleus (genome) and cytoplasm (control or regulating factors of genomic activities and other genetic components in cytoplasm) of heterogeneous origins within a reconstructed cell or egg may induce different gene expression activities of the genomes during the cell differentiation or developmental process of animals (47).

According to the aforesaid facts, why we do not consider this method as one of the useful technique in biotechnology to produce new animal breeds. Of course, to perform nuclear transplantation experiments is much more difficult in fish rather than those to be done in amphibians or mammals. This is because in amphibians, enucleated eggs can be obtained easily by using UV or laser beams to damage the nuclei of many of their eggs. At the same time after the unfertilized eggs were artificially activated and their animal poles became automatically rotated to the up side. To

remove the nucleus of fish egg require a more complicated technique (47). In mammals, even their eggs are much smaller than those of amphibians and fish, but their male and female pronuclei or zygote nucleus are easier seen under the microscope for micromanipulation, whereas the metaphase stage of the nucleus of unfertilized egg, the male and female pronuclei as well as the zygote nucleus of fertilized eggs of amphibian and fish are impossible to see under the microscope for micro-manipulation purpose.

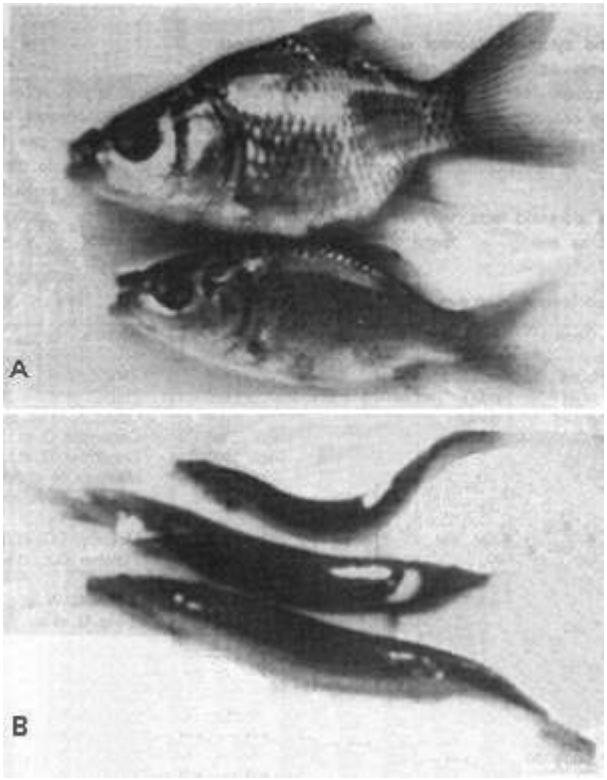
However, the practical techniques for doing nuclear transplantation in fish is already available now. Probably a great challenge for developing more and more research activities by using this method in differ-

Table 1: Brief introduction of transgenic fish studies.+

Fish	Gene	Promotor	I*	E*	T*	Year	Authors
Goldfish	hGH	mMT	+			1985	Zhu, Z. et al.
Loach	hGH	mMT	+			1986	Zhu, Z. et al.
Medaka	cdCR	SV40	+	+		1986	Ozato, K. et al.
Trout	hGHcDNA	mMT	+	+		1986	Chourrout, D. et al.
						1988	Chourrout, D. et al.
Trout	rGH	mMT	+			1987	Maclean, N. et al.
Catfish	hGH	mMT	+			1987	Dunham, R.A. et al.
Salmon	E. Coli						
	B-gal	mMT	+	+		1988	McEvoy, T.G. et al.
Salmon	fAFP	fAFP	+	+	+	1988	Fletcher, G.L. et al.
Zebrafish	E. Coli						
	hygro	mMT	+		+	1988	Stuart, G. W. et al.
Tilapia	fGH	mMT	+			1988	Brem, G. et al.
Common carp	rtGH-						
	cDNA	RSV	+	+	+	1989	Chen, T. T. et al.
Chinese carp	hGH	mMT	+			1989	Chen, T. T. et al.
Common carp	hGH				+	1989	Shen, X. Z.
							(sperm carrier)
Catfish	rtGH-						
	cDNA	RSV	+			1989	Powers, D. A. et al.
Medaka	cdCR	SV40	+	+		1989	Inoue, K. et al.
Salmon	hGH	mMT	+	+		1989	Rokkones, G. L. et al.
Trout	cG	cG	+			1989	Oshiro, T. et al.
Medaka	CAT					1989	Chong, S. S. et al.
Loach	hGH	mMT	+	+		1989	Xie, Y.F.
Zebrafish	CAT	RSV					
		SV-40	+	+	+	1990	Stuart, G. W. et al.
Medaka	lucif	RSV		+		1990	Tamiya, E. et al.
Goldfish	fAFP		+			1991	Wang, R. et al.
Zebrafish	SVCA 7						
	GH5					1991	Khao. H.W. et al.
Cichlid							(sperm carrier)
fish	hGH	mMT	+			1991	Ken, C. F. et al.
Salmon	fAFP	AFP	+	+	+	1991	Shears, M. A. et al.
Medaka	CAT	SV40	+	+		1991	Winkier, C. et al.
Salmon	GHcDNA	AFP	+	+	+	1992	Du, S. J. et al.
Goldfish	AFP		+			1992	Yu Jiankang et al.
							(sperm carrier)

+ Table modified from Powers' (1991). * Abbreviations: I, integration; E. expression; T, transmission.

Figure 11: Pictures of transgenic crucian carp and the sibling control (A), transgenic loach and the sibling control (B) (From Zhou, Z. 1992).



ent animal materials will also happen in the near future.

More recently, another new method for producing novel breeds of animal is being developed in a number of animals, i.e. 'gene transfer'.

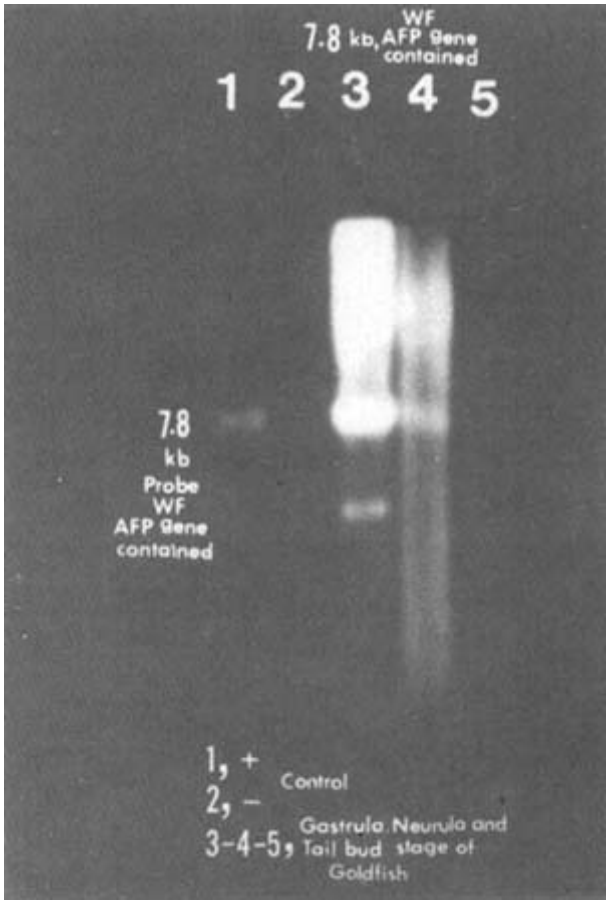
IV. Gene transfer

In the 1980s, due to the fast development of molecular biology and the establishment of DNA recombination techniques, it has become possible for molecular biologists to isolate single gene sequences, assemble them into plasmid vectors, amplify them in vitro and introduce them into micro-organisms for investigating their expression activity and regulation mechanisms.

By using this new technology, molecular biologists also speculated that when a foreign gene was introduced into an animal cell or fertilized egg, it would be integrated into the cell or egg genome and express its activity during cell differentiation or developmental process. It was also thought that, in some cases, the integrated genes could be keep in the germ line of the developed animal individuals and finally be transmitted into further generations. They also believe that since the unique function of genes that have been transferred into the fertilized animal eggs are well known, so the transferred genes must be a sort of 'target genes'. Therefore, changes of phenotypes of animals that are caused by expression of those integrated foreign genes in recipient eggs or embryos can be predicted to perform their function in unique ways in developing animals. The animal thus obtained is so called 'transgenic animals'. Theoretically, this method seems to be a very attractive and ideal method for cultivating new animal breeds.

Palmiter *et. al.* (28) first reported that they obtained 'super mouse' (transgenic mouse) by introducing a growth hormone (GH) gene into mouse eggs. Later on, other authors reported that some transgenic

Figure 12: A picture of Southern blot hybridizations. It shows the injected 7.8 kb AFP gene of winter flounder were presented in gastrula and neural stages and disappeared in tail bud stage of goldfish embryos.



chickens, cows, pigs, rabbits and sheep were also obtained (30).

As to the studies on transgenic fish, the first report was published in 1985 (52). Up to 1990, there were more than 20 articles, abstracts and reviews published by about 14 laboratories in different countries. The summary of the publications of various laboratories between 1985 to 1992 are summarized in Table 1.

According to the above publications, it was shown that so far research topics in transgenic fish can be divided into following categories :

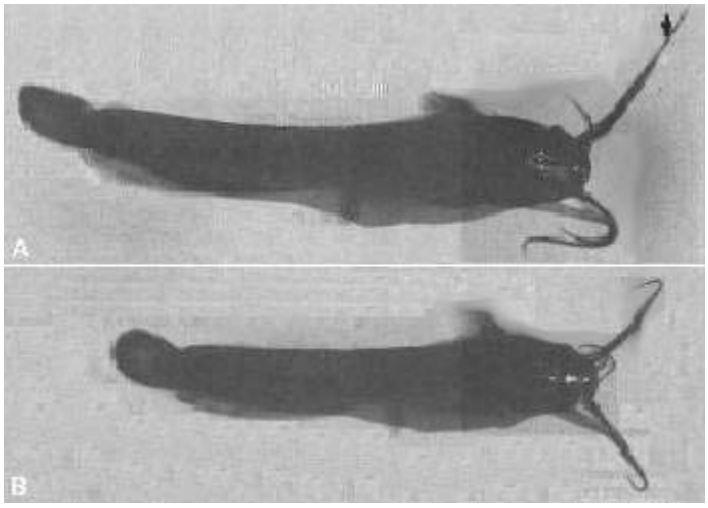
1. Applied attempts : Growth promotion; Low temperature resistance.
2. Fundamental research : Insertional mutagenesis; Developmental gene expression.
3. Technology : Micro-injection; Electroporation; Sperm carrier.
4. Model fish : Zebrafish (Brachydanio rerio) Medaka (Oryzias latipes) Cichlid fish (Cichlasoma nigrofasoiutum) Goldfish (Carassius auratus)
5. Environmental problems : Ecological uncertainties. Methods of containment.

Applied attempts

Since the improvement of fish quality and other aquatic animals of aqua-cultural importance is one of the main demands in fish farming practice, new research projects on 'transgenic fish and other aquatical animals' have become more and more attractive to scientists working in the field of biotechnology. In order to make those kind of research activities more efficient and direct, several kinds of edible fishes have already been used as experimental materials for this purpose by many authors. So far, two main genes or their cDNAs were used in those studies : 1) Growth hormone (GH) genes or their cDNAs obtained from human, mouse, rat, cow, and fish; 2) Anti-freeze protein (AFP) genes or their cDNAs obtained from fish. Most of the promoters linked to those genes were metallothionein (MT) promotor of mammals or viral sequences (RSV, SV40), except in a few cases the fish AFP promotor sequences were linked to fish AFP genes (11-13,34) in Salmon.

Zhu *et. al.* (52, 53) first reported that successful production of transgenic crucian carp and loach were achieved by using a mouse MT promotor - human growth gene construct. The transgenic loach obtained by them at 135 days were 3.1 to 3.8 times larger than controls. The transgenic crucian carp obtained by them at 208 days were 78% larger than controls. They also found that some of the transgenic loach changed their morphological shapes. Figure 11 shows pictures of transgenic crucian carp and the sibling control (A) and

Figure 13: Pictures of a 36-day transgenic young catfish (Clarias leager) obtained from the fertilized eggs of catfish which were injected with AFP gene of ocean pout (Macrozoarces americanus) 10^{7-8} copies/egg) (A) and its sibling control at same age (B). The average total length of transgenic fish: The average total length of sibling control=1.23:1.00.



transgenic loach and the sibling control (B) which were obtained by Zhu *et. al.* (54). Zhang *et. al.* (55) reported that when the RSV-GH-cDNA of trout were injected into the early embryos of common carp, about 10% of the survivors were found to possess that kind of foreign sequences. The size of those P1 transgenic fish exhibit considerable variation. The average size is 20% larger than their sibling controls. Du *et. al.* (11) succeeded in obtaining transgenic Atlantic salmon when the ocean pout AFP promotor sequences linked to the GH-cDNA of Chinook salmon were used in the experiments. Some of the transgenic individuals are 4 to 6 fold larger than the controls. Obviously, the results reported by different authors are quite different from each other. It is very difficult to compare those results on a common standard basis because the species of fish, the kinds of promotor linked to GH genes, the forms of DNA (GH genes or cDNAs, linear or circular) used by various authors as well as their cultural conditions were different. Besides, the copies of genes which were used in different authors' experiments were quite different too. Moreover, in some of those brief reports, none of detailed experimental parameters were available to make accurate comparison possible.

In several other reports, including the experiments, with Chinese carp (2), trout (3-4,25), catfish (12,29), salmon (32), tilapia (1) and Medaka (16), mouse MT or viral promoters linked to a mammalian GH gene or cDNA sequence were used. However, the analyses in those experiments were only carried out with experimental material at the early embryo stages or at hatching stage. In those cases, no enhancement of growth was measured.

So far, more and more scientists convinced that the MT or viral promoters are not suitable for making edible transgenic fish or other aquatic animals. That is because the former one requires heavy metal iron for initiating its activity and the latter one is obviously a bio-hazard. Therefore, even though there is some good evidence demonstrating that the GH gene is non-species-specific in constructing transgenic animals, Hew *et. al.* (15) pointed out that to use all-fish gene constructs (including the promotor and the gene sequences) will be more safe and effective in making transgenic fishes.

It is possible that some negative or unexpected results obtained in experiments of transgenic animals were not reported in the published articles. For exam-

ple, there is a research group in the Institute of Developmental Biology, the Chinese Academy of Sciences, Beijing, China, that introduced 7.8 kb AFP gene of winter flounder (*Pseudopleuronectes americanus*) into fertilized eggs of goldfish (Carassius auratus) and found out that the injected AFP gene sequences gradually disappeared when those eggs developed to the tail bud stage (Figure 12) (Yu Jiankong, personal communication). Another example : When they introduced AFP gene of ocean pout (10^{7-8} copies/egg) into fertilized eggs of catfish (Clarias leager), they found that young fish grew faster than the non-injected controls (Figure 13) (Yu Jiankong, personal communication). Why did the AFP genes disappear during fish embryogenesis so quickly and why they can induce the growth enhancement of fish like the function of GH genes? Those results are quite different.

Therefore, at least, some key problems remain to be solved until any genuine GH gene-transgenic fish are available for aqua-cultural application at commercial level :

1) To select hazard-free promoters with high efficiency expression linked to the GH gene sequence, and especially to design all-fish constructs seems necessary. Otherwise, the transgenic fish which carry potentially harmful MT or viral promoters will not be welcomed in the fish markets. Those two kinds of promotor will also be a dangerous source of environmental pollution in fish farming regions. Hew *et. al.* (15) suggested that the promotor regions of B actin, AFP, albumin and vitellogenin genes of fish be considered as potential candidates to be linked to GH gene sequences. This kind of selection seems necessary.

2) The method for identification of DNA integration, transcription and expression in transgenic fish should be improved. For example, how to accurately identify whether the foreign gene sequences are completely integrated into transgenic fish genomes is still hard work because in various animals, for example, fishes, the homologous frequency of GH gene sequences is very high (between 47-100%, Ito, 1988). The dot and Southern blot hybridization seemed not sensitive enough to analyze the results very accurately. The polymerase chain reaction (PCR) seems to be a better method for improving the sensitivity and simplifying the screening protocols used to detect transgenes (15), but much better methods are still needed.

3) To establish an accurate method for examining the growth enhancement of transgenic fish, and to compare with their sibling controls in field testing levels, a statistical analysis is required. There is a big difference in ways to feed water-living and land animals. For example, if compared with land animals, the feeding of fish in water is not easy to quantitatively control individually and calculate the amount of feed. Even in carefully managed culture conditions, the difference of growth size among individual fish of the same stock (they were even hatched from the same batch of fertilized eggs and cultivated in the same pond) is always obvious. Some of them can catch food actively and some not. The more food individual fish catch the bigger the individuals grow. The feeding behavior for fish is related to various unknown genetic factors in physiology of fish. So, in the same natural conditions, it was always found that some fish individuals are as several times bigger than others. It is reasonable to believe that the different size of fish individuals in a cultured population always occur naturally without GH gene treatment. In other words, the enhancement of growth in fish individual does not merely reflect the role of the introduced GH gene. It may be also influenced by their own breeding behavior which was determined by the inheritance imprinted by their parents. Therefore, if the results were only obtained from few samples in the laboratory, any kind of conclusion should be made carefully.

It is very interesting to note that in some reports no complete identification of integration, transcription and expression of the introduced GH gene were shown for the same experimental materials. So, how can one make a definite conclusion about the enhancement of growth on such an incomplete data basis? For example, Shen (35) reported that they used sperm as carrier to introduce the hGH gene into common carp eggs. Bigger transgenic common carp adults were obtained, but neither integration nor expression were identified in

the transgenic eggs or embryos.

4) Seamark *et. al.* (33) and Rexroad *et. al.* (31) reported that some physiologically abnormal phenomenon appeared in transgenic pigs owing to the over-production of GH (most likely induced by the transferred GH gene) in those animals. Those abnormal transgenic animals will not have any market value. Will those kinds of phenomena happen in transgenic fish? So far, only positive results have been reported and no attempt has yet been made on this aspect of research. However, we must first investigate it in order to prevent it from obtaining unhealthy or harmful transgenic fish.

It is well known that the anti-freeze protein (AFP) in blood serum of some fish species living in the polar regions functions to prevent serum from forming ice crystals. Those fishes survive in severely cold marine conditions. Therefore, to isolate the AFP gene and make AFP gene transgenic fish is also a very attractive target for fish farming research. According to personal communications with several research groups of the Fisheries Institutes in China, great efforts have been made by researchers in using AFP gene for making low-temperature resistant transgenic fish during the past few years. No good results were yet obtained. Fletcher *et. al.* (13) and Shears (34) reported that the AFP gene of winter flounder were successfully integrated into the Atlantic salmon genome and a small number (around 3%) of salmon with low levels of expression of foreign AFP genes developed from micro-injected eggs. Approximately 50% of the progeny produced by crosses between transgenic F1 and wild-type individuals contained the foreign AFP genes. Unfortunately, those AFP gene transgenic salmon are not yet freeze-resistant. That may be due to the low level of AFP expression observed (9). In addition, there is no evidence to show that the mechanisms which control the anti-freezing and anti-chilling behavior in fish physiology are the same ones. So, any attempt for using the AFP gene to make low temperature resistant transgenic fish might not be a good idea. However, since the AFP genes were uniquely discovered in few ice-resistant polar marine fish, when those genes are transferred into the eggs of other fish species which do not possess the sequence homolog to them, the results (either positive or negative) will be easier to be analyzed if compared with the results in GH genes transfer experiments.

The conclusion may therefore be drawn that according to results obtained either in the case of GH or AFP gene transfer, although to construct transgenic fish of aqua-cultural importance has an attractive future, it is still in the beginning stages.

Attempts were also made for producing transgenic fish with disease-resistance or improved nutritional value, but no reports have been published yet.

Foundamental research

Some authors are interested in using gene transfer in fish eggs to study insertional mutagenesis and gene expression control during development. In these cases, to use fish species fish with well-known genetic background, short life cycle, suitable eggs for operation and cultivation in experimental conditions have been selected for this purpose. For example, Stuart *et. al.* (38) reported that they used a linearized form of a 5.2 kb bacterial plasmid, SV40-hygro, containing the hygromycin- resistant gene which was linked to the SV40 promotor to transfer into zebra fish (*Brachydanio rerio*). The plasmid was expected to produced hygromycin phosphotransferase in the host and thereby enable the antibiotic selection of transgenic fish. About 5% of the 4-month adult host zebra fish were detected to carry the foreign gene. When 20 of them were crossed with uninjected fish, each transgenic fish transmitted the foreign gene to approximately 20% of the F1 offspring. In the second out-crossed generation, using one of the F1 fish which was carrying the foreign gene, nine out of eighteen (50%) F2 progeny expressed the foreign gene. These results indicated that the foreign gene introduced into zebra fish embryos could be transmitted to subsequent generations in a Mendelian fashion. However, no expression of the introduced gene could be detected in the transgenic zebra fish. In other words, the integrated foreign gene in this case is of no real function. Do those integrated genes become 'silent genes'?

It is interesting to note that the same group of scientists (38) extend their observations by demonstrating that, following the cytoplasmic injection of recombinant chloramphenicol acetyltransferase (CAT) genes into early zebra fish embryos, as many as one in 20 survivors are germ-line transformants (a success rate) approaching 5%). Those injected CAT genes are expressed, both transiently in developing zebra fish embryos and fry, and continuously in stable transformants. Unfortunately, no similar data were provided by many other authors who are working on other transgenic fishes.

Ozato *et. al.* (26) have reviewed their significant experiments regarding gene transfer in another small fish without aqua-cultural importance. They introduced the chicken d-crystalline gene (the injected plasmid pd-C-1B consisted of a 11.4 kb chicken d-crystalline sequence and a 3.0 kb pAT153 sequence) into Medaka (Oryzias latipes) oocytes. About 50% of the injected oocytes reached maturation in vitro, fertilized and developed into 7-day old embryos. The δ-crystalline sequence could be detected in about 50% of the examined embryos and the δ-crystalline protein was detected in 30% of the examined embryos. The tissue-specific expression of the foreign gene was also examined by using DNA-DNA in situ hybridization and immuno-histological staining technologies. It was shown that only 50% or less of the nuclei carried the foreign sequence. Thus, d-crystalline gene-carrying and non-carrying cells were distributed mosaically in every tissue. In addition, it also showed that δ-crystalline gene was expressed in the retina, brain, spinal cord, muscle, gill and gut, but was rarely expressed in the lens, where the gene should be primarily expressed except that the expression of the δ-crystalline gene in the lens was just detected in 3-day old embryo as the lens are not yet completely formed. They explained that the tissue-specific expression of chicken δ-crystalline gene will depend on developmental stage : It was temporally regulated in Medaka embryos.

From a technical stand-point, it is rather difficult to do manipulation for tissue-specificity of gene expression during development in transgenic fish, even though their embryos are usually transparent and their embryo-genesis could be monitored in in vitro. Tamiya *et. al.* (39) reported that the luciferase gene contained in pRSVDNA was micro-injected into the nuclei of Medaka oocytes. The luciferase activity was detected in injected embryos at 6 days after fertilization by using the photon counting acquisition system and the luminescence patterns in those embryos were demonstrated. So, the luciferase gene is considered to be a potential source in non-invasive and continuous monitoring of gene expression during embryo-genesis of animals including fish.

Technology

Since fish eggs are much larger (about 1 mm in diameter) than those of mammals, to make gene injection in fish eggs seems not very difficult. In many kinds of fish eggs, their chorion is not hard and can be removed either by forceps or enzyme treatment before injection. The micro needle can then penetrate into the egg directly even without removing the chorion. Fish eggs are usually either transparent or semi-transparent and their blastodisc is in the animal pole. It is easy to identify where the dividing nucleus is located. The injection should be made there. So, in most experiments, manipulation for transferring exogenous genes for constructing transgenic fish use the micro-injection method. This method also has advantages for accurate finding the location in the egg cytoplasm which is nearby the dividing nucleus. The dosage of injected genes can be accurately controlled or adjusted. However, in order to expect that the injected gene could be integrated into nuclear genome more efficiently, like in mice, the genes need to be injected into the nucleus of the oocyte. That is because there is no way to accurately identify the site of the nucleus in a fertilized fish egg. When this method is used for fish oocytes, they need to be transparent enough for identifying the location of their nuclei (germinal viscle). In addition, to establish technology for successful culture of those injected oocytes to maturation, as well as to accomplish artificial fertilization in vitro. So far, this method has been well developed in Medaka (27) and just

started in goldfish (43) and zebra fish (Li *et. al.* to be published).

In some special cases, like in Salmonids, the egg chorion is too hard for micro-injection with glass needles. However, micro-injection can be well done through the micro Pyle (13).

The cytoplasm and yolk in some kinds of fish eggs, such as Medaka, Fundulus, Tilappia, Chinese bittering and others, are more or less liquefied or very fragile for operation due to their compact structure between the chorion and egg membrane. When the micro-glass needles penetrates into or is withdraw from those eggs, the positive and negative pressure produced by injection action on the egg surface during operating will make the egg cytoplasm and yolk leak out through the injection hole of the egg surface and finally produce damage to the egg itself. In these cases the electroporation method seems to be a better alternative than micro-injection.

For example, Inoue *et. al.* (17) used the electroporation method to introduce DNA sequences into Medaka eggs. The mortality rate of recipient embryos is about 70% and the integration frequency of the examined embryo is 5%. The simplicity of this method is the advantage of the operation can be controlled and modified to fit individual fish species. This method is also well suited for mass gene transfer in large quantities of fish eggs. The disadvantages of this method is that even in the same batch of fish eggs, every egg may have some physiological differences which will cause different reactions against electric pulses. It is also difficult to make sure, after electroporation, where is the site of the genes to be incorporated into the egg is, which may not closely attach to the egg nucleus. Accordingly, the integration frequency of foreign genes into the genome will decrease. In addition, when eggs are treated in the electroporation medium, many more gene copies should be used for making a proper concentration. Whereas in the micro-injection only a small number of gene copies is used. So, it seems that this method is not an economic way when using limited gene resources.

In 1989, Lavitrona *et. al.* (23) reported that mouse sperm incubated with DNA can carry DNA molecules and introduce them into mouse eggs during fertilization. Transgenic mice were obtained. Unfortunately, this result needs to be re-confirmed. There are also few brief reports which declare that fish sperm can be used as DNA carrier for gene transfer. For example, Shen (35) reported in a review that when hGH gene were carried by sperm and introduced into common carp, the expression of hGH gene was as high as 50% in the transgenic fish embryos and the growth rate of the transgenic carp in some experimental groups was about 2-fold higher than controls. Khao *et. al.* (21) also reported that zebra fish sperm can carry plasmid DNA PUSVCAT and pxGH5 into fertilized eggs but the introduced plasmid DNA was not integrated into the genome. It persisted in the cytoplasm, however. Unfortunately, no detailed experimental data or descriptions were provided in those two brief reports, so it is difficult to make proper comments about those results.

Recently, our group (51) reported that goldfish sperm incubated with antifreeze protein (AFP) gene can fertilize eggs and produce transgenic goldfish. A sperm suspension of $4x10^{(7-8)}$ cells/ml was prepared by using Niu-Twitty solution, containing 4% sucrose, 3% glycerol and 1% DMSO. Linear AFP gene from ocean pout was added to the sperm suspension to yield a final concentration of 3 ug/ml and incubated together with sperm suspension at 4°C for 30 minutes. Polymerase Chain Reaction (PCR) and Southern blot hybridization are used for identifying DNA extract of blood cells of transgenic fish. Three of the 45 adult fish samples were found positive. The positive rate is about 7%. However, no proper methods are available to identify how can sperm carry the AFP gene into the eggs.

It is well known that the physiological behavior of mammalian and fish sperm is quite different. When the mammalian sperm are ejected into the female genetic tract, they can maintain there for several hours to wait for fertilization with the eggs; while in fish, when the sperm are ejected into the water and don't have the chance to fertilize eggs, they lose their activity or die within a few minutes. Sperm may carry DNA molecules either by absorption on its surface or by penetration

into its head portion. According to aforesaid reason, to realize the absorption of DNA molecules by fish sperm within a rather short period of time in a medium may be very difficult. On the other hand, if the DNA molecules can only be absorbed on the sperm surface, then they will remain in the outside of the fertilized egg because the sperm membrane will be fused with the egg plasma membrane as soon as they attach with each other during the fertilization process. The only possibility for making sperm-carrying DNA molecules penetrate the egg is to let the DNA molecules enter the space inside of the sperm membrane within a very short period of time. Probably, electroporation will be helpful to reach this goal. However, from a technical point of view, it seems unnecessary to do so if the transfer of DNA molecules through electroporation into egg will be successfully done. The only difference for using electroporation method between the eggs and sperm is that the foreign DNA molecules can be integrated into sperm DNA, then it can be directly fused into the genome of the zygote, whereas the DNA molecules can only be incorporated into the egg cytoplasm by electroporation.

Recently, Vielkind (42) reviewed methodology for making transgenic fish in Medaka and Zebra fish. A series of techniques were summarized. They indicated that DNA should be cytoplasmically injected into fertilized eggs preferably prior to cleavage of the egg. Injected DNA in linear and super coiled form or transferred in phage particles is replicated, degraded but also retained in a fraction of young fish. Injected DNA is transiently expressed, but after a mosaic integration into the germ line of 5% of injected fish, is stably integrated into the chromosomes, expressed in the progeny and inherited in a Mendelian fashion. It also appears that the reporter genes, like CAT gene, are transiently expressed as well as in the progeny in a tissue specific manner (38).

In summary, although various methods for gene transfer were used in transgenic fish studies, none is perfect or suitable to every case. Generally speaking, the micro-injection method has more advantages, such as to make the accurate injection site, dosage and time as well as saving the gene source. After fertilization, the egg nucleus will continuously divide at a rather short interval. So, if the site of DNA injection is close to the dividing nucleus, more rapid division of the nucleus will provide more possibilities for injected DNA to be integrated into the reorganized chromosomes in division cycles of the nucleus. As to the difference of DNA integration frequency between the oocyte nucleus and egg cytoplasm injections, there is no report about this kind of comparison. It needs further investigations.

A significant problem is that Coffin (7) reported that usually introduced DNA molecules in eukaryote cells will be degraded after a few cell divisions and the integration frequency in cell genome is only 10^{-7}. Chong and Vielkind (5) reported that in Medaka the injected DNA began to show signs of degradation as early as the blastula/gastrula stage. In goldfish, the injected winter flounder AFP gene disappeared at the tail bud stage (Yu, personal communication). However, in most transgenic fish experiments, it was reported that the introduced DNA sequences or genes could be maintained in eggs or embryos for a longer period of time and the integration frequency of DNA sequence into the egg genome is in the range of as high as 3% to 70%. How to explain this kind of difference of DNA integration in cells and eggs will require further investigation.

Model fish

Gene transfer is not only expected to be an important tool to improve fish stocks for aquaculture but also a powerful technique to study gene regulation in living cells or organisms (15). As it was mentioned in above paragraphs, so far, the results obtained from the studies on transgenic fish are more or less confused and incomparable. So, it is necessary to establish studies and will be helpful to examine the experimental results according to some generally accepted standard criteria. Those model fish should have following characteristics :

1) With well-studied genetic background and any change of its phenotype at different levels could be identified.

2) Short life cycle.

3) Easy to be cultured in laboratory.

4) Artificial spawning and fertilization can be easily conducted.

5) Easy to obtain their oocytes for maturation in vitro.

6) Their oocytes and eggs should be technically tolerant to micro-operation or other kinds of gene transfer treatments and easy to be managed for various kinds of analysis.

7) Their oocytes or eggs should be transparent enough in judding the locations of their nuclei or egg blastodisc for accurate gene transfer.

8) The embryo-genesis of their transgenic eggs can be easily monitored in in vitro conditions.

So far, a few fish have been considered by a number of authors to be the candidates of model fish in transgenic research :

1) Zebra fish (*Brachydanio rerio*). It is a kind of tropic decorative fish, small in size, easy to culture in water tanks in the laboratory, its sexual maturation period is 2 to 3 months. It can produce up to few hundreds of eggs daily, its hatching period is 3-4 days, its transparent eggs has a diameter of about 0.7 mm, its soft egg chorion is easily removed mechanically or by trypsin treatment, its egg has strong tolerance against micro-injection and other kinds of manipulation. It is easy to obtain oocytes at various stages and matured eggs by light and temperature control (42, to be published).

2) Medaka (Oryzias latipes). It is also a small kind of fresh-water fish. Several kinds of mutants with different known phenotypes are available. It is easy to culture in laboratory water tanks; its sexual maturation period is about 3 months; its spawning cycle is 24 hours; its hatching period is about 10 days. Its transparent egg has a diameter of 1 mm but the chorion of its fertilized egg is difficult to remove; its egg is delicate for micro-injection due to its tough chorion and liquefied cytoplasm; its oocytes and matured eggs can be obtained at any time by light and temperature adjustment. The method of injection of gene into its oocytes; to culture its oocytes into mature eggs and fertilize them in vitro has been well-established by Japanese scientists. For example, the chicken d-crystalline gene was injected into oocytes, after culture them to maturation, and fertilized in normal condition. 50% of the injected oocytes developed into 7-day old fry. Among them, 30% transgenic larval fish express the gene (27,42).

3) Cichlid fish (Cichlasoma nigrofasciutum). It is also a kind of tropic decorative fish, small in size, easy to culture in laboratory water tanks; spawning several times per year; produces eggs every two weeks; hatching period of 3 days; transparent eggs with sticky chorion. Ken *et. al.* (20) reported that when the mMT-hGH gene was micro-injected into its eggs at 1-32 cell stages, the gene sequences can be integrated into the genome of the embryos.

4) Goldfish (*Carassius auratus*). It is a common decorative fish originally produced in China. Now it is distributed in many places in the world. It has many varieties or strains with very different shapes which are genetically stable. Either from genetic or embryological point of views, goldfish is a very useful experimental material like Drosophila, frogs or Xenopus, etc. Generally speaking, it can mature and produce eggs after once year of culture in suitable cultural conditions. Its natural spawning season is from spring to early summer, when the water temperature is not lower than 20°C and higher than 30°C. In some warm places it can spawn earlier when the water temperature reaches that range. Generally, every one female fish can produce eggs once per year. Sometime, some fish can produce eggs twice a year, once in the spring and other in autumn. In the spawning season if there are many goldfish as a stock available, the male and female fish are cultured separately and if their cultural temperature is controlled below 20°C, their spawning time can be postponed. Then we can select some male and female from this stock, put them together, increase the water temperature up to 20° to 24°C for obtaining their sperms and eggs daily. We can also control them to produce sperms and eggs earlier by increasing the water temperature and combine with pituitary gland or reproductive hormone treatment. Its semi-transparent eggs have a diameter of 1 mm and the chorion of unfertilized or fertilized eggs can easily be removed either by forceps or by enzyme treatment. Its fertilized eggs are easily cultured and their complete embryo-genesis can be observed in in vitro conditions. The in vitro maturation of goldfish oocytes can be accomplished and

Table 2: The comparison of methods for obtaining altered fish*.

	Non-biotechnical		Biotechnical	
	Artificial selection	Sexual hybridization	Nuclear transplantation	Gene transfer
Via natural procedure or chemical treatment	Natural	Natural	Natural	Chemical treatment
Characters modified	+	+	+	+
Efficacy of modification	+	+	+	+
Modification predicated	-	-	-	+
Potential bio-hazard	-	-	-	+ or -
Fertile or infertile	-	+ or -	+	+
Pure genetic clone formed	-	-	+	+ or -
Inter-species restriction	+	+	+	- ?
Hamful ecological influence	-	-	-	+ ?
Necessity of ontainment	-	-	-	+

* This comparison was made on the basis of the methods which are presently used. Further improvement of those methods are expected.

AFP genes were transferred into their germinal vesicle successfully (43).

More suitable candidate fish models for transgenic fish studies could be found if other investigators will pay more attention to this important problem.

In conclusion, it is obvious that so far all the results obtained in transgenic fish research are preliminary and more efforts should be made to focus on the research topics mentioned in the previous paragraphs of this review.

Environmental problems

Even though some positive results were obtained in transgenic research, but other two severe problems dealing with transgenic fish are still worth mentioning :

(1) The 'ecological and regulatory uncertainties' which may be caused by improper management of the transgenic fish in fish farming programs.

(2) The 'methods of containment required to allow safe implementation of them' because those genetically altered transgenic fish have the potential for long-term, sustainable changes and failure to effectively contain them could result in transient effects on the ecosystem resulting from direct competition of escaped individuals with other members of the community, or more long-term effects resulting from genetic introduction of the modifications into the natural gene pools (10).

Hallerman and Kapuscinski (14) pointed out that rapid development and commercial use of transgenic fish will not, however, depend on technical capabilities alone; development, field testing and commercial use of transgenic fish introduce a range of ecological and public policy uncertainties which must be solved before transgenic fish can reach practical utilization. In some developed countries, such as in USA, France, Ger-

many, Japan, United Kingdom, the regulations for controlling the research and application of transgenic fish were made for protecting their natural wild resources and human health from the potential harmful effects of transgenic fish. Unfortunately, no regulations were made in all of the developing countries even though research activities in transgenic fish or other animals are much more encouraged to be done due to urgent demands for improving the economic situation in those countries.

Devlin and Donaldson (10) have given a very detailed analysis about the containment problem and several kinds of methods were suggested for solving this issue such as physical containment, biological containment (chromosome set manipulation and hormonal ablation of maturation) and some other techniques (surgery, radiation treatment, autoimmune sterility, chemo-sterilization, behavioral control, hybrid sterility, monosex populations and other transgenic methods for inducing sterility).

It means that scientists are making their great efforts to use modern gene transfer method to culture new fish breeds on one hand and on the other hand, they also have to use some other existed traditional methods to eliminate the potential harmful effects which might be introduced into human society and nature by transgenic fish.

Table 2 shows the comparison of aforesaid four kinds of methods.

As was shown in Table 2, any one of aforesaid methods has its own advantages and disadvantages. It seems still difficult to say which one is the best. As to those biotechnical methods, including the improved artificial sexual hybridization, nuclear transplantation and gene transfer, some new approaches were already made, but each of them has some special problems, theoretically or technically, to be solved before they can be successfully applied to fish culture on commercial scales. Therefore, at the present time, to only rely on biotechnology to improve fish culture seems not practical.

The scientists who are working in those important areas should not be encouraged to make unnecessary competition among themselves by giving some unreliable promises to the public in order to get more research support from national or international financial sources. Scientists should reveal the true situation of those kinds of biotechnical research, to let them understand how many difficulties have to be overcome before we can reach our final goals and make a real 'revolution' in fish farming practice by using biotechnology. Scientists should explain to them that this is the exact reason why more and more financial supports will be needed in such kind of biotechnical research.

Biological phenomenon are always very complicated, so to make great collective efforts by uniting scientists from different disciplines to work together for solving the key common problems in those research areas seems necessary, urgent and most important, especially in the developing countries.

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