Aquaculture Research, 2008, **39**, 205–211 doi: 10.1111/j.1365-2109.2007.01886.x

Increasing the genetic uniformity of bighead carp [*Aristichthys nobilis* (Richardson)] by means of spontaneous diploidization of gynogenetically activated eggs

Yuzhen Ye, Zhongwei Wang & Qingjiang Wu

State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, The Chinese Academy of Sciences, Wuhan, China

Correspondence: Wu Qingjiang, Institute of Hydrobiology, the Chinese Academy of Sciences, Wuhan, China. E-mail: qjwu@ihb.ac.cn

Abstract

Three groups of gynogenetic diploid bighead carp were successfully obtained by means of artificial gynogenesis. The activation rates of gynogenesis varied from 75.9% to 98.8%, and the frequency of spontaneous diploidization was around 0.4%. Over 2000 normally gynogenetic diploid fry were obtained in three gynogenetic groups. The haploid karyotype consisted of nine metacentric, 12 submetacentric, three subtelocentric chromosomes and 45 arms. The chromosome number was 48 from gynogenetic diploid. The results showed that the genetic material of offspring was maternal. The aneuploid hybrid embryos of bighead carp and Xingguo red common carp with chromosome numbers ranging from 28 to 73 did not survive post hatch, likely the result of incompatibility between the nucleus and the cytoplasm of two parents. Sixty RAPD primers from three groups were used for total DNA amplification of gynogenetic offspring, maternal and 'paternal' fish. A total of 451 bands were amplified from three kinds of samples above. From maternal bighead carp, 256 bands were amplified; however, there were 251 shared bands between maternal and gynogenetic bighead carp. From artificial gynogenetic offspring, two 'paternal' DNA segments without an expression function were found. An UPGMA tree showed that gynogenetic offspring were closely clustered and the genetic identity among them was very high (0.956).

Keywords: gynogenesis, spontaneous diploidization, chromosome, genetic identity

Introduction

Bighead carp (*Aristichthys nobilis*) is an economically important freshwater fish in China with history of cultivation since the Tang Dynasty (Li & Fang 1990; Liu & He 1992). Although the currently cultivated bighead carp are of wild or of half-wild origin, their genetic structure has altered to a certain extent, because the majority of seeds have originated from long periods of artificial propagation under poor genetic management. In recent years, stocks of bighead carp have suffered from slow growth, size reduction and disease outbreaks, which have hampered their aquaculture development (Xu, Yin, Chen & Wu 1991). During the last decade, numerous studies on genetic resource conservation and breeding of four Chinese major carps have been carried out. These studies mainly focused on biochemical and genetic analysis of fish in the Yangtze River (Li, Lu & Zhou 1995; Wu & Wang 1997; Li & Lu 1998). Development of a new variety of bighead carp is likely to require a substantial investment of time and resources because of its long life cycle when using traditional breeding methods. The approach used by Wu, Chen, Ye and Ke (1981) and by Streisinger, Walker, Dower, Knauber and Singer (1981) in common carp and zebra fish provides a model for the use of gynogenesis for the development of inbred lines. Gynogenesis results in rapid genetic uniformity in offspring and has been investigated extensively, because it can dramatically shorten the time required to produce inbred lines in fish (Wu & Gui 1999). Several reports on the use of gynogenesis in freshwater and marine fish are

currently available (Wu, Ye, Chen & Tong 1991; Guo, Hershberger, Cooper & Chew 1992; Li, Chen & Du 1997; Galbreath, Adama, Wheeler & Thorgaard 1997; Yamamoto 1999; Galbusera, Volckaert & Ollevier 2000; Xia, Wu & Yang 2000). However, there are no reports on the use of gynogenesis in bighead carp. This paper details the production of diploid gynogenetic offspring of bighead carp by means of spontaneous diploidization, and embryological and genetic analysis of induced gynogenetic offspring.

Materials and methods

Collection of eggs and sperm

The gynogenesis trial was carried out at the Guanqiao experimental station of the Chinese Academy of Sciences. Five egg samples were collected from five mature females, of which two (G-3 and 4) were from the Guanqiao experimental station (those derived from wild fries collected from the Yangtze River), and three (G-1, 2 and 5) were from the Donghu fish farm in Wuhan City (offspring of artificial propagation carried out for several generations). The eggs collected from Donghu fish farm were stored in a dry vessel in a thermos at 10 °C, and were transported to the Guanqiao experimental station within 30 min. Sperms of red common carp (*Cyprinus carpio* var. red) were provided by the Guanqiao experimental station.

Production of gynogenetic haploid

Gynogenetic haploids were induced from bighead carp eggs activated by the irradiated red common carp (*C. carpio* var. red) sperm, while normal sperm of red common carp were used in control groups. These were crossed with big head carp eggs or with common carp eggs. The sperms were diluted three times with Hank's solution and transferred to an iced Petri dish (9 cm diameter). The depth of the sperm mixture in the petri dish was not more than 1.5 mm. This was set on a microvibrator and radiated by an ultraviolet lamp (15 W) for 10–20 min. The distance between the lamp and the surface of the mixture was 16–17 cm.

Embryological analysis

For analysis and comparison of karyotypes of haploids, diploids and hybrids, embryo samples at blastula as well as hatching stage were collected from the experimental and control groups. The traditional air-dried chromosome preparation method (Wu, Ye & Chen 1986) was followed.

DNA extraction and RAPD analysis

DNA extraction

Fin samples of one maternal bighead carp and one male red common carp were collected from the experimental station and their 14 gynogenetic offspring (G-03) were randomly sampled. One gram of tissue was digested overnight in 750 μL extracting buffer (10 mmol L^{-1} Tris, 0.1 mol L^{-1} EDTA, 0.5% SDS, pH 8.0) and proteinase K at a final concentration of 200 mg mL^{-1}. The standard method of phenol–chloroform extraction was performed to extract DNA (Sambrook, Fritsch & Maniatis 1989).

Polymerase chain reaction (PCR) amplification and electrophoresis

Sixty random primers (OPJ, OPM and OPO, Operon products) were used. Amplifications were performed in a 25 μL reaction mixture containing 2.5 μL 10 × reaction buffer, 0.5 μL dNTP (2.5 mmol L^{-1}), 0.5 μL primer, 0.5 μL Taq polymerase (2 U μL^{-1}), 1 μL template DNA (100 ng) and 20 μL sterile water. Polymerase chain reaction was operated according to the following programme: an initial denaturation for 5 min at 94 °C followed by 40 cycles of denaturation at 94 °C for 45 s, annealing at 37 °C for 45 s and extendion at 72 °C for 90 s and a final extension at 72 °C for 7 min. Polymerase chain reaction products were separated by 1.4% agarose gel electrophoresis and were visualized under UV light. 1 and 0 denote the presence or absence of the bands respectively. Genetic distance was calculated using the POPGENE32 software, and UPGMA trees were constructed according to the genetic distance using MEGA 2.1 software (Kumar, Tamura, Jakobsen & Nei 2001).

Results

Artificial gynogenesis and hybridization

One million mature eggs produced from five individuals of female bighead carp were used to induce artificial gynogenesis. Four gynogenetic groups were obtained and were marked as G-01, G-03, G-04 and G-05 respectively. No live fry was obtained from the group G-02. Gynogenetic yields were 226, 1100, 750 and 800 in groups G-01, G-03, G-04 and G-05

respectively (Table 1). Activation rates of mature eggs by irradiated sperm ranged from 75.9% to 98.8%. The embryonic development was observed under an optical dissection microscope and no apparent difference was found between diploid and haploid before the tail bud stage. Abnormal embryos occurred after the tail bud stage and majority of them showed the haploid syndrome. Some embryos became blunt, had a short trunk, bent tails, developed retardation of blood cycle and could not develop post hatch. Although some embryos could proceed through the hatching stage, the resulting fry developed abnormalities and died within 2–3 days after hatching. Spontaneously diploidized normal fries occurred in every brood, ranging from 0.01% to 0.4%.

It was demonstrated that the hybrids between bighead carp and red common carp could not survive post hatch. The fertilization rate of hybridization between bighead carp and red common carp was about 90%, and no apparent anomaly was detected in the early stage of embryonic development. However, anomalies could be seen from the gastrula stage and the majority of embryos died when they reached the hatching stage. Only a few embryos could pass through the hatching stage but they died after 3 or 4 days.

Spontaneous diploidization

The majority of the artificial gynogenetic embryos exhibited the haploid syndrome and could not pass through the hatching stage. However, a few spontaneously diploid fry were viable. Altogether, 2080 gynogenetic diploid bighead carp fry in four groups (G-01, 03, 04, 05) were obtained by spontaneous diploidization without application of physical or chemical treatments.

The morphological features of common carp and bighead carp fry after 3 days of hatching were observed and compared. After the eyes were darkly pigmented, the density of the pigment cells increased on the body of common carp, especially on the head, caudal region and along the notochord. However, these features could not be found in gynogenetic haploid bighead carp. The body of bighead carp was transparent and slim, and the dorsal and caudal fin folds were connected while the pectoral and ventral fin was not developed. When bighead carp reached the fingerling stage, the following features were observed: side-depressed body, big head, wide and up-sloping mouth, no barbels, thin scaling and complete lateral line. All these features were the same as those observed in bighead carp. No morphological feature of Xingguo red common carp was found in the gynogenetic offspring.

Embryonic chromosome analysis

Altogether, 100 divisional cells of both gynogenetic haploid and spontaneously diploidized bighead carp were observed and enumerated. The chromosome number of a haploid with a mode of 24 ranged from 21 to 24, while the diploid with a mode of 48 ranged from 45 to 50. This led to the conclusion that the haploid of bighead carp had 24 chromosomes (Figs 1 and 2). The karyotypes of bighead carp and red common carp were in agreement with the report of Yu, Zhou and Li (1989). Chromosomes of haploid bighead carp

Table 1 Artificial activation rate of mature eggs and yield of spontaneously diploidized fries

	G-01	G-02	G-03	G-04	G-05
Mature eggs	150 000	300 000	280 000	300 000	50 000
Eggs from	Donghu	Donghu	Guanqiao	Guanqiao	Donghu
Artificial activation rate (%)	75.9	0	98.8	86.3	91.3
Spontaneously diploidized fries	226	0	1100	750	4
Hatching rate (%)	0.20	0	0.39	0.29	0.01

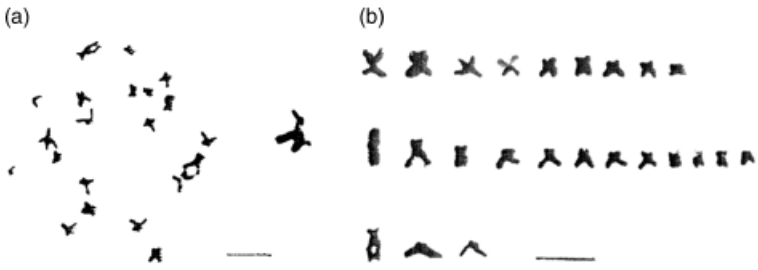

Figure 1 Chromosomes and karyotypes of haploid embryo in bighead carp. (a) Chromosome number (1n): 24, (b) karyotype formula: 9m+12sm+3st, arm number of haploid (NF): 45, rod: 5 μm.

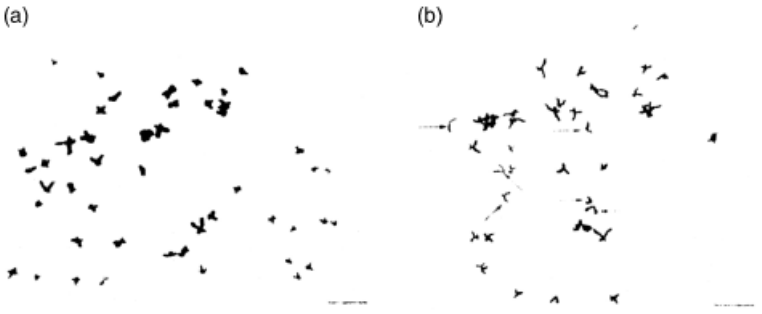

Figure 2 Embryonic chromosomes of hybrid between bighead carp and red common carp. (a) Embryonic chromosomes of diploid bighead carp. (b) Embryonic chromosomes of hybrid between bighead carp and red common carp, the chromosomes pointed by the arrows stand for T chromosomes of red common carp, rod: 5 μm.

Table 2 Karyotype comparisons between bighead carp, Xingguo red common carp and their hybrid

Species	Tissues	Chromosome number	Karyotypes		
			M	SM	ST and T
Haploid bighead carp	Embryonic cell	24	9	12	3
Diploid bighead carp	Kidney cell	48	18	24	6
Haploid Xingguo red common carp	Embryonic cell	50	6	20	24
Hybrid between bighead carp and Xingguo red common carp	Embryonic cell	28–73	10–15	12–31	6–27

included nine metacentrics, 12 submetacentrics and three subtelocentrics. However, no telocentrics could be observed. Fifty chromosomes made up of six metacemtrics, 20 submetacemtrics and 24 telocentrics or subtelocentrics could be observed in the haploid of red common carp (Table 2).

Embryonic cells of hybrid between bighead and red common carp were enumerated and their chromosome numbers ranged from 28 to 73. Therefore, the chromosomes of hybrid could be interpreted as aneuploid, i.e. chromosomes of both bighead and red common carp existed in the embryos of the hybrid. Table 2 shows that the numbers of ST and T chromosome in hybrid embryos range from six to 27, of which three ST chromosomes were contributed by bighead, while the other 3–23 ST and T chromosomes inevitably came from red common carp. However, the numbers of ST and T chromosomes in hybrid decreased gradually during embryonic developmental progress. Telocentric chromosomes unique to the red common carp are shown in Fig. 2 (marked with arrows). In some phases, 27 ST and T chromosomes occurred, but in the other phases only six of these chromosome types could be detected. These phenomena suggest that cell nuclei of the two fish are fused together at the beginning and then chromosomes are lost little by little during the later stage of embryonic development.

RAPD analysis

Genomic DNA samples of 14 gynogenetic offspring fingerlings, their bighead carp mother and 'paternal' red common carp were used for PCR amplification reactions. Sixty primers from three groups (OPJ, OPM, OPO) were used for amplification. Altogether, 451 bands were amplified by 43 primers, but no bands were amplified by the other 17 primers. Among 451 bands, 17 bands were shared between maternal, gynogenetic offspring and red common carp. Another band shared between gynogenetic offspring and red common carp was also detected. Of 256 bands amplified from the maternal side, 251 were shared between maternal and gynogenetic bighead carp. Even though the band numbers amplified from 14 gynogenetic offspring were the same as those from the maternal, amplified bands from individual samples of gynogenetic offspring were lesser than that of the maternal. There were 193 bands peculiar to red common carp. In addition, two fragments peculiar to red common carp were found in two gynogenetic offspring (Fig. 3). The first fragment consists of 1616 bases, and the second consists of 514 bases. No open reading frame was found in these two fragments and no significant similarity was found by a BLAST sequence similarity search.

The genetic distance between red common carp and gynogenetic offspring was 0.8867 while that of

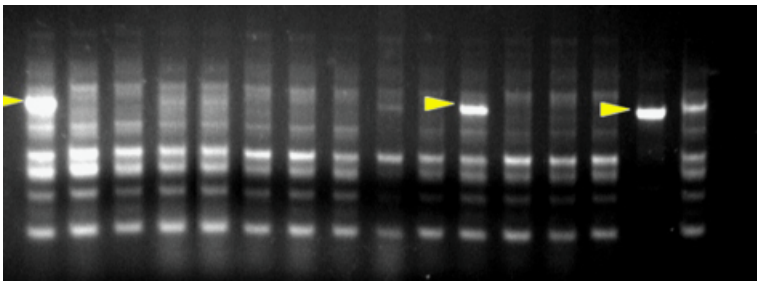

Figure 3 RAPD pattern of gynogenetic bighead, maternal bighead carp and red common carp amplified by primer OPM7. Arrows shows band peculiar to red common carp. Lanes 1–14, gynogenetic bighead carp; 15, red common carp; 16, maternal bighead carp.

Table 3 Nei's genetic distance (below diagonal) and genetic identities (above diagonal) from POPGENE32

	Bighead carp	Gynogenetic offspring	Red common carp
Bighead carp		(0.9802)	(0.0778)
Gynogenetic offspring	0.0198		(0.1133)
Red common carp	0.9222	0.8867	

red common carp and maternal bighead carp was 0.9222. However, the genetic distance between gynogenetic offspring and maternal was 0.0198 (Table 3), and the average genetic identity within gynogenetic offspring was 0.956. Using MEGA 2.1 software, UPGMA trees were constructed (Fig. 4) and it was observed that all gynogenetic offspring, except number 13, were closely clustered.

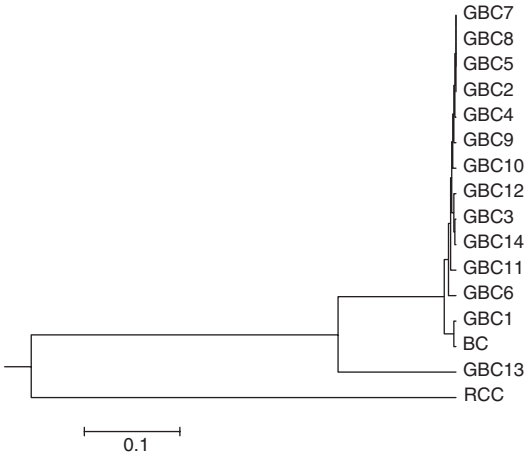

Figure 4 UPGMA tree of gynogenetic bighead carp. GBC, gynogenetic bighead carp; BC, maternal bighead carp; RCC, red common carp.

Discussion

Four gynogenetic groups of bighead carp were obtained successfully by artificial gynogenesis without physical or chemical treatments. As expected, the gonads of all the adult gynogenetic diploids developed normally. Hot or cold shock and chemical drugs may result in changes in the chromosome structure, such as breakage or deletion, which may affect the development of gonad. The development of gynogenetic embroys was controlled by one set of maternal chromosomes. The majority of embryos would show the haploid syndrome and die before hatching if artificial diploidization is not carried out. Physical or chemical diploidization was used widely in the past studies in order to increase the production rate of diploid. It was found that in common carp, 17.4% of haploids were doubled by cold shock (0–3 °C) for 30 min and returned their polar body II. The production rate of diploid embryo was 31.6% when the activated eggs were treated by colchicine to damage the formation of the spindle (Wu *et al.* 1981). Streisinger *et al.* (1981) applied hydrostatic pressure (HP) to convert haploid eggs into diploids and 29% of the HP-treated eggs developed into normal diploid embryos. However, only 20% of the HP-treated diploid embryos developed to maturity. Therefore, the reason for abnormal development of gonads may be due to the loss of genetic information resulting from physical and chemical factors. Although the production rate of diploids was very low, spontaneous diploidization was an efficient method because the offspring were normally gynogenetic diploids with normally developing gonads. In addition, the low production rate of diploid larvae could be compensated by the large quantities of activated eggs.

No offspring was obtained by means of hybridization between bighead carp and red common carp because their genome relationship was distant. Also, chromosomes could not match the cytoplasm because of the asynchronization between the nucleus and cytoplasmic division. The majority of the hybrid embryos were aneuploid according to the karyotyping. The results of this study could be compared with

the hybrid of grass carp and common carp (Ye, Wu & Chen 1989).

Gynogenesis was widely used in the construction of a pure line among fish because of higher homozygosity in gynogenetic offspring. In the present experiment, the maternal was a wild individual containing a certain percentage of heterozygous loci. However, most of the heterozygous loci became homozygous loci within the gynogenetic offspring. The microsatellite MFW1 analysis showed that no heterozygous individual was found in artificial gynogens from heterozygous maternal (Tong, Yu & Liao 2005). Although the band numbers amplified from 14 gynogenetic offsprings, were the same as those from one maternal bighead, amplified bands from samples of gynogenetic individuals were less than that of the maternal. This suggested that some heterozygous loci had become homozygous. The genetic identity of gynogenetic offspring was higher (0.965) than that observed for the natural population (0.896) from the middle reaches of the Yangtze River (Wang, Ye, Zhou & Wu 2004). Among offspring of the first gynogenetic generation, gynogenetic sex-reversed males could match their homozygous sisters in the same brood, and a pure line could be constructed.

A report by Thorgaard, Scheerer and Parsons (1985) indicated that using rainbow trout eggs and irradiated sperm, paternal chromosome fragments can be genetically actived in gynogenetic offspring. Disney, Johnson and Thorgaard (1987) reported that rainbow trout gynogens produced by albino rainbow trout eggs and γ-irradiated brook trout sperm expressed paternal alleles at five isozymes (*Ldh*, *Mdh*, *Aat*, *Gpi* and *Pgd*). These results indicate that fragments with expression gene complete from the paternal genome could be integrated into the genome of gynogens. From our experimental samples, two individuals carrying the 'paternal' DNA segment were found. However, there is no open reading frame in these two segments and no significant similarity could be found in BLAST sequence similarity searching. It appears that these two random segments of male nuclei were integrated into female nuclei but without expression functions. Individuals with segments peculiar to red common carp were fewer in the artificial gynogenetic offspring (2/14). Furthermore, no morphological traits of red common carp or anomalous trait were detected in over 2000 artificial gynogenetic fingerlings. This suggested that segments found in artificial gynogenetic offspring do not appear to be a complete gene, nor disturb the normal expression of the bighead carp genome.

Acknowledgments

This study was supported by the National Tenth Five-Year Key Project (2001BA505B0508) and by the project of the National Natural Science Foundation of China (No. 30471342). We are grateful to Rajeev Raghavan for reading the manuscript and suggesting the necessary changes.

References

Disney J.E., Johnson K.R. & Thorgaard G.H. (1987) Intergeneric gene transfer of six isozyme loci in rainbow trout by sperm chromosome fragmentation and gynogenesis. *Journal of Experimental Zoology* **244**, 151–158.

Galbreath P.F., Adama K.J., Wheeler P.A. & Thorgaard G.H. (1997) Clonal Atlantic salmon X brown trout hybrids produced by gynogenesis. *Journal of Fish Biology* **50**, 1025–1033.

Galbusera P., Volckaert F.A.M.V. & Ollevier F. (2000) Gynogenesis in the African catfish *Clarias gariepinus* (Burchell, 1822): III. Induction of endomitosis and the presence of residual genetic variation. *Aquaculture* **185**, 25–42.

Guo X.M., Hershberger W.K., Cooper K. & Chew K.K. (1992) Genetic consequences of blocking polar body I with cytochalasin B in fertilized eggs of the Pacific oyster, *Crassostrea gigas*: I. Segregation of chromosomes. *Biological Bulletin* **183**, 387–393.

Kumar S., Tamura K., Jakobsen I.B. & Nei M. (2001) MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics* **17**, 1244–1245.

Li S.F. & Lu G.Q. (1998) Diversity of mitochondrial DNA in the populations of silver carp, bighead carp, glass carp, and black carp in the middle and lower reaches of the Yangtze River. *Acta Zoologic Sinica* **44**, 82–93.

Li S.Z. & Fang F. (1990) On the geographical distribution of the four kinds of pond-cultured carps in China. *Acta Zoologica Sinica* **36**, 244–250.

Li S.F., Lu G.Q. & Zhou B.Y. (1995) Feasibility studies on genetic conservation of Chinese carps in swan oxbow of the Changjiang River. *Journal of Fisheries of China* **19**, 194–202.

Li S.Z., Chen L. & Du J.S. (1997) Diploid gynogenesis induced by heat shock in *rainbow trout*. *Journal of Zoologica* **32**, 7–9.

Liu J.K. & He B.W. (1992) *Cultivation of the Chinese Freshwater Fishes*. Sciences Press, Beijing, China.

Sambrook J., Fritsch E.F. & Maniatis T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring harbor Laboratory Press, New York, NY, USA.

Streisinger G., Walker C., Dower N., Knauber D. & Singer F. (1981) Production of clones of homozygous diploid zebrafish (*Brachydanio rerio*). *Nature* **291**, 293–296.

Thorgaard G.H., Scheerer P.D. & Parsons J.E. (1985) Residual paternal inheritance in gynogenetic rainbow trout: implications for gene transfer. *Theoretical and Applied Genetics* **71**, 119–121.

Tong J., Yu X. & Liao X. (2005) Characterization of a highly conserved microsatellite marker with utility potentials in cyprinid fishes. *Journal of Applied Ichthyology* **21**, 232–235.

Wang Z.W., Ye Y.Z., Zhou J.F. & Wu Q.J. (2004) Rapid establishment of pure lines of silver carp (*Hypophthalmichthys molitrix*) and bighead carp (*Aristichthys nobilis*). *Progress in Natural Science* **14**, 60–63.

Wu L.Z. & Wang Z.X. (1997) Biochemical genetic structure and variation in a natural population of silver carp from the middle reaches of the Yangtze River. *Acta Hydrobiologica Sinica* **21**, 157–162.

Wu Q.J. & Gui J.F. (1999) *Fish Genetics and Breeding Engineering*. Shanghai Scientific & Technical Publishers, Shanghai, China.

Wu Q.J., Chen R.D., Ye Y.Z. & Ke H.W. (1981) Investigation on the carp gynogenesis with reference to establishing a pure line. *Acta Genetica Sinica* **8**, 50–55.

Wu C.J., Ye Y.Z. & Chen R.D. (1986) Genome manipulation in carp (*Cyprinus carpio* L.). *Aquaculture* **54**, 57–61.

Wu Q.J., Ye Y.Z., Chen R.D. & Tong J.G. (1991) The production of pure line red carp 8305 and its biological characteristics. *Oceanologia Et Limnologia Sinica* **22**, 295–299.

Xia D.Q., Wu T.T. & Yang H. (2000) Artificial gynogenesis and reversal in *Hypophthalmichthys molitrix*. *Chinese Development and Reproductive Society* **9**, 31–36.

Xu B.H., Yin Z., Chen Y. & Wu Y.S. (1991) A newly infectious of silver carp and bighead carp *Yershinia ruckeri*. *Chinese Science Bulletin* **36**, 620–622.

Ye Y.Z., Wu Q.J. & Chen R.D. (1989) Studies in cytology of crosses between grass carp and carp — asynchronization between nucleus and cytoplasm in distant hybridization of fishes. *Acta Hydrobiologica Sinica* **13**, 234–239.

Yu X.J., Zhou D. & Li Y.C. (1989) *Chromosomes of Chinese Fresh-Water Fishes*. Science Press, Beijing, China, pp. 59–77.

Yamamoto E. (1999) Studies on sex-manipulation and production of cloned populations in hirame, *Paralichthys olivaceus* (Temminck et Schlegel). *Aquaculture* **173**, 235–246.

