

TWO RAPD-SCAR MARKERS IN NEW GIFT NILE TILAPIA (*OREOCHROMIS NILOTICUS*) IMPLICATION FOR SELECTION TRACKING AND STRAINS IDENTIFICATION

SI-FA LI, SHOU-JIE TANG, WAN-QI CAI

Key Laboratory of Aquatic Genetic Resources and Aquacultural Ecosystem, Ministry of Agriculture, Shanghai Ocean University, Shanghai, China

Abstract

The NEW GIFT Nile tilapia is a national certificated new strain produced through 14 years and 9 generations selection from the base strain of GIFT Nile tilapia. From NEW GIFT Nile tilapia two strain-specific RAPD bands namely S₃₀₄^{624bp} and S₃₆^{568bp} were identified from the amplified bands of 50 and 80 10bp-oligo-nucleotide random primers separately after gel extraction. Cloning and sequencing of the strain-specific RAPD bands two pairs of primer (20 base for forward primer and 21 base for reverse primer) was designed according to sequence information, then PCR amplification was carried out in NEW GIFT Nile tilapia and base strains and other 7 farmed strains. For the first primer, one 553bp SCAR was amplified showed a 86.7% appearing frequency in NEW GIFT Nile tilapia but only 17% in base strain. This marker has been used as a specific monitor marker for tracking of selection. For the second primer, one 558bp SCAR was amplified showed a 91.4% appearing frequency in NEW GIFT Nile tilapia and 0-70% in other 7 strains. This marker has been used as a specific molecular marker for identification of Nile tilapia strains. The development of these two markers indicated that the frequency of some alleles related with economically important traits might increase during selection.

Keywords: NEW GIFT Nile tilapia, RAPD-SCAR marker, tracking, identification.

INTRODUCTION

The Genetically Improved Farmed Tilapia (GIFT) Nile tilapia (*Oreochromis niloticus*) (Eknath, Tayamen, Palada-de Vera, Danting, Reyes, Dionisio, Capili, Bolivar, Abella, Circa, Bentsen, Gjerde, Gjedren and Pullin 1993; Gupta and Acosta 2004), bred by the International Center for Living Aquatic Resources Management (ICLARM; now the World Fish Center) and its partners, was introduced to China by Shanghai Fisheries University (now Shanghai Ocean University) in 1994. A series of evaluation studies in China from 1994 to 1996 indicated that the GIFT strain exhibited higher growth performance, salinity tolerance and seinability than previously introduced Nile tilapia strains/lines (Li, Li and Li 1998; Li, Li,

Dey and Dunham 1999). Therefore, the GIFT strain has been certified and promoted by the Agriculture Ministry of China (Li & Li 2001). However, because the introduced GIFT tilapia in 1994 was only the third generation produced by cross-breeding, genetic stability had not yet been achieved, leaving opportunity for further selection. Since 1996, taking the GIFT strain of Nile tilapia as a base population (called F_0 , although it was the 3rd from the Philippines in 1994), a project named "Genetic Selection of Nile Tilapia" was carried out in the 9th (1996-2000), 10th (2001-2005) and 11th (2006-2010) National Five-Year Program. The major purpose is to further improve the aquaculture performance of GIFT Nile tilapia. Compare with the base population, the major improving of 8-9th generations was 30% super in growth, 5~8% higher in fillets ratio, nice stripe pattern on caudal fin, and higher genetic purity (Hu, Li and He 2005; Li, He, Hu, Cai, Deng, and Zhou, 2006; Xie 2006), It had been certified as a super strain by the National Certification Committee of Wild and Bred Varieties in January 2006, renamed as NEW GIFT Nile tilapia, and extended by the Ministry of Agriculture and quickly becomes a principle strain of tilapia cultured in China.

SCAR (sequence characterized amplified regions) is one of the stable markers, generally derived from RFLP, RAPD, AFLP markers. The basic principle is to design a length of around 20 bp specific primers based on the acquired sequence information, and then reveal the polymorphism by ordinary PCR procedure. Because of rapid, simple and low cost in application, particularly very suitable for the analysis of large number of samples, SCAR marker has been applied to the identification of aquatic animal germplasm (Zhou, Wang and Gui 2001; Iturra, Bagley and Vergara 2001; Klinbunga, Amparyup and Leelatanawit 2004; Araneda, Neira and Iturra 2005; Zou, Li and Cai 2005).

During the selection study of NEW GIFT tilapia, besides monitoring of phenotypic characters, monitoring of the genotypic characters was conducted to watch the genetic changes among the new generations and old generations, as well as to identify the new strain from other common used farming strains. Two RAPD SCAR markers have been developed successfully, which provided technical support for selection and criteria for management of genetic improved strains in the tilapia industry in China.

MATERIALS AND METHODS

Materials

For SCAR I: 35 samples of NEW GIFT Nile tilapia F10 (NG, selected 10th generation) and 30 samples of GIFT Nile tilapia F0 (GN, base population,) were collected from Nanhui Fish Breeding Station of Shanghai Ocean University and National Tilapia Seed Farm Qingdao, respectively.

For SCAR II: Besides the samples of NEW GIFT Nile tilapia described above. The other 7 farming strains of Nile tilapia were collected from Hainan Genoma tilapia company(HG), Xiamen Luye tilapia farm(XL), Guangxi Fisheries Research Institute(GF), Guangdong Weiye tilapia farm(WY), Guangdong Zhuhai tilapia farm(ZH) in China, and Egypt strain (EG), and Thailand strain (TL) in Sarvas tilapia farm in Hungary, each were of 20 samples.

A small piece of caudal-fin from each individual was clipped and stored in 95% ethanol. A total of 130 10bp-oligo-nucleotide random primers were synthesized (Sangon, Shanghai).

Genomic DNA extraction

Genomic DNA was extracted using phenol-chloroform procedure (Sambrook and Russell 2001).

RAPD analysis and PCR conditions

PCR mixtures (25 μ L volume) contained 2.5 μ l 10 \times PCR buffer (100mmol/L Trish pH 9.5 \square 500mmol/L KCl 30mmol/L MgCl₂ 0.001% gelatin), 2 μ l dNTP mixtures (0.2 mmol/L each of dATP, dCTP, dGTP, and dTTP), 2 μ L RAPD primer (0.2 μ mol/L), 2 μ l template DNA (50 \square 150ng), 0.5 μ l Taq DNA polymerase (1.25U), and 16 μ l distilled water.

PCR amplification was performed in an Eppendorf Master cycle programmed for initial denaturation at 94 $^{\circ}$ C for 5 min; 45 cycles of denaturation at 94 $^{\circ}$ C for 45 s, annealing at 36 $^{\circ}$ C for 45 s, and extension at 72 $^{\circ}$ C for 1 min30s; and a final extension step at 72 $^{\circ}$ C for 5 min. Reaction tubes were held at 4 $^{\circ}$ C prior to visualization of PCR products in a 1.5% agarose gel stained with ethidium bromide. Each RAPD assay was done three times to ensure reproducibility.

Cloning and sequencing of strain-specific RAPD amplicons

All strain-specific RAPD amplicons was excised from agarose gels and the DNA fragment was recovered using a 3S Spin PCR Product Purification Kit (Biocolor Inc., China) following the manufacturer's protocol. An aliquot of the recovered DNA fragment was reamplified using the corresponding primer to verify that only a single band was excised. The recovered DNA fragment was then ligated into the pGEM^o,R-T Easy Vector (Promega). Then, DH5 α competent cells (TIANGEN) were transformed with ligated DNA following the manufacturer's instructions. In order to detect cloning success, three white colonies were selected from each plate and were screened by PCR using the T7 and Sp6 primers. The cloned fragments were sequenced on an Applied Biosystems ABI 3730 capillary sequencer.

SCAR analysis

The nucleotide sequence of each of the cloned RAPD fragment was used to design pairs of SCAR primers.

PCR mixtures (25 μ L volume) contained 2.5 μ l 10 \times PCR buffer (100mmol/L Tris-HCl \square

pH 9.5, 500mmol/L KCl, 30mmol/L MgCl₂, 0.001% gelatin), 2 µl dNTP mixture (0.2 mmol/L each of dATP, dCTP, dGTP, and dTTP), 1 µL of forward primer (0.2µmol/L), 1 µL of reverse primer (0.2µmol/L), 2 µl template DNA (50-150ng), 0.5 µl Taq DNA polymerase (1.25U), and 16 µl distilled water.

The amplification profile was 5 min initial denaturation at 94°C; 35 cycles of denaturation at 94°C for 30 s, annealing at 57 °C for 45 s, and extension at 72°C for 1 min; and a final extension step at 72°C for 10 min. Reaction tubes were held at 4°C prior to visualization of PCR products in a 1.5% agarose gel stained with ethidium bromide.

RESULTS

SCAR I

RAPD amplification results

RAPD analysis was performed on 10 genomic DNA samples of selected population and foundation population by using fifty 10bp-oligo-nucleotide random primers. In these 50 primers, 23 showed both stable amplification and polymorphism. There was only one specific band between the 2 populations that is S₃₀₄^{624bp}, this fragment served as a specific marker distinguishing the selected population from foundation population. The 624 bp band amplified by S₃₀₄ primer was shown in Figure 1.

Development of SCAR markers

The S₃₀₄^{624bp} fragment was recovered, cloned and sequenced. The sequence of S304624bp fragment was showed in Figure 2. Both the 20bp forward primer and 21bp reverse primer were designed according to the S₃₀₄^{624bp} sequence (Table 1). Due to GC content in the primer, the location of the primer was not at both ends of the fragment, but moved a little closer to the middle part of the fragment.

Both samples of the selected population and foundation population were screened with the specific primer. A 553bp band was produced (Figure 3). The frequency of this 553bp SCAR marker in selected population reached 86.7% the frequency in the foundation population was only 17%. The high frequency of the 553bp marker can be the basis for the identification of NEW GIFT Nile Tilapia.

SCAR II

RAPD amplification results

RAPD analysis was performed on 10 genomic DNA samples of the NEW GIFT groups and the remaining seven groups by using eighty 10bp-oligo-nucleotide random primers. In these 80 primers, 20 showed both stable amplification and polymorphism. There was only one specific band between the 8 groups that is S₃₆^{568bp}, this fragment served as a specific

marker distinguishing the new GIFT group from other groups. The 568 bp band amplified by S₃₆ primer was shown in Figure 4.

Development of SCAR markers

The S₃₆^{568bp} fragment was recovered, cloned and sequenced. The sequence of S₃₆^{568bp} fragment was showed in Figure 5. Both the 20bp forward primer and 21bp reverse primer were designed according to the S₃₆^{568bp} sequence (Table 1). Due to GC content in the primer, the location of the primer was not at both ends of the fragment, but moved a litter closer to the middle part of the fragment.

Both 35 samples of the new GIFT groups and 20 samples of each remaining 7 groups were screened with the specific primer. A 558bp band was produced in those groups. The appearing frequency of this 558bp SCAR marker in NEW GIFT reached 91.4% the frequency in the remaining 7 groups followed by 70 % (ET), 65% (HG), 60 % (TL), 55% (WY), 35% (XL), 30% (ZH), and 0% (GF) (Figure 6). The high frequency of the 558bp marker can be the basis for the identification of NEW GIFT Nile Tilapia.

Bioinformatic analysis of the SCAR markers

In the 558 bp SCAR sequence, AT accounted for 70.43%, showing that the SCAR marker is an AT-rich sequence. The 558bp SCAR sequence was blasted against GenBank database, and the best hit was against a Nile tilapia MHC α gene (accession number: AB270897.1) (Sato, Dongak and Hao 2006). The BLAST result showed that it had 83% homology with the DNA sequence from the first 33878 bp to 34273 bp of the 183264 bp linear genomic DNA. After searching by ORF Finder in NCBI, it could be deduced that the fragment contained an open reading frame; the ORF from the 414 to 557 nucleotide (Figure 5) contained not only a start codon and a stop codon but also 47 codons which can code amino acid. No protein sequence shared a high degree of homology with the sequence, according to the BLAST in the NCBI protein database.

DISCUSSION

Through selective breeding by generation and generation, some economic traits could be improved and stabilized, and as a result, new strains could be created. In the history many good breed were generated by selective breeding (Lou 1999; Hines 1976). The improving of traits are contributed by the natural factors and human factors that may induce mutations, but the selection itself will not create new genes. Nevertheless, the selection may change the allele frequencies, which may cause the changes of traits. Finally, some favorable traits could be accumulated and strengthened.

Breeding requires a long period, how to track the phenotypic variation and genotypic variation of breeding groups is the key towards the success of breeding. The development of modern molecular genetic technology provides an effective measure for selective breeding. RFLP, RAPD, SSR and SCAR markers are commonly used as molecular markers. Among them, RAPD needs less DNA template and is relatively easy to operate, but it is poor in reproducibility and stability, subject to certain restrictions in practical application. However, after converting RAPD markers into SCAR markers, the specificity and stability can be greatly improved, which makes it more convenient and efficient in the testing of different alleles. After designing a pair of 20 bp forward primer and 21 bp reverse primer according to the sequence results of S_{304}^{624bp} , the SCAR marker was slightly shortened to 553 bp. After designing a pair of 20 bp forward primer and 21 bp reverse primer according to the sequence results of S_{36}^{568bp} , the SCAR marker was slightly shortened to 558 bp. In PCR analysis, characterized bands can be clearly distinguished by 1.5% agarose gel electrophoresis for 1.5 h (5V/cm). In addition, based on past experience, the quantity of samples analyzed has a direct impact on resolution of electrophoresis. In this study, a perfect resolution effect could be achieved with only 3 ~ 5 μ L PCR product each hole.

In this study, SCAR-PCR was conducted in the NEW GIFT tilapia and base population as well as other seven farmed Nile tilapias. The frequency of the 553bp marker (SCAR I) reached up to 86.7% in 30 samples of NEW GIFT tilapia, significantly higher than the frequency in the base population(17%), it indicates that the 553bp SCAR band can be used as a marker to distinguish the selected strain from base population. Meanwhile, the frequency of the 558bp marker (SCAR II) reached up to 91.4% in the 35 samples of NEW GIFT tilapia, significantly higher than the frequency(0-70%) in other seven groups, it indicates that the 558bp SCAR band can be used to distinguish NEW GIFT tilapia from other farmed Nile tilapias.

We found (Xie, 2006) that the RAPD analysis of genetic diversity of F_{6-9} generation of selected GIFT strain indicated that there was a clear trend in genetically purification generation by generation, the F_8 and F_9 generation have reached a rather high genetically purification. The microsatellite analysis of genetic diversity of F_0 , F_{6-9} generation of GIFT strain indicated that there were a minimal, but could be monitored genetic differentiation caused by nine generations of selection, the genetic structure of the F_{8-9} was more stable than F_{6-7} . The AFLP analysis of genetic diversity of F_0 , F_{6-9} generation indicated that the genetic distance between F_0 and F_{6-9} increased generation by generation. The genetic diversity of F_0 , F_{6-9} generation by analyzing Mitochondrial DNA sequence indicated that the genetic distances between F_0 and F_6 , F_7 , F_8 , F_9 were increased with selection processing. On

the other side, the growth rate is increased with the accumulation of selected generations, for example, the growth rate increased at an average of 4.85% from F₆ to F₉. It indicates that during the long-term selection (like the 12 years in this study) and high-intensity selection (like the 6% from fry to mature in this study), the frequency of some alleles related with economical traits was changed significantly, e.g., there is a correlation between the phenotypic variation and the genotypic variation over the selection.

Acknowledgement

We are grateful to Dr. C. H. Wang, Dr. X. Y. Xie, Mr. Y. Zhao for help. This research was supported by the 10th (2001-2005) and 11th (2006-2010) National Five-Year Program, named "Genetic Selection of Nile Tilapia".

Table 1. Primer sequence, annealing temperature and size of PCR band of SCAR markers I and II

RAPD primer	RAPD marker (bp)	SCAR Primer sequence	annealing temperature ()	size of PCR band (bp)
SCAR I				
S ₃₀₄	624	5'-GGTGCCTTTTGAATGAGCTA-3' 5'-TGAGATACTGCTACAGCGTGA-3'	57	553
SCAR II				
S ₃₆	568	5'- TGGATGGATGGATTGATGGA -3' 5'- AGCCAGCGAACCAAGATCTAT -3'	57	558

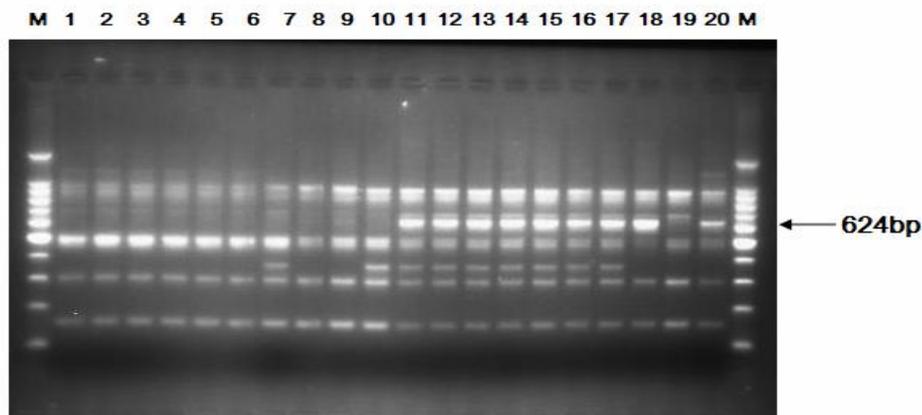


Figure1. The amplification results of primer S_{304} (CCGCTACCGA) in NEW GIFT strain and base strain 1-10 base strain (F0); 11-20 NEW GIFT (F10); M 100bp DNA ladder

GTGTAAAGCCTGGGGTGCCTTTTGAATGAGCTAACTCACATTAATTGCGTTGCGCTCCCTCA
 GCCCGCTTTCTAGCAGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCCAGG
 GGAGAGGCGGTTTTCGTATTGGGCGCTCTCCGCTTCCTCGCTCACTGACTCGCTGCGCACTCG
 GTCGTTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACACAGAA
 TCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAGGCCAGCAAAGGCCAGCAGAACC GTAA
 AAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCTGACGAGCACATCACAAAATCG
 ACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCACAGGCGTTTTCCCCCTGG
 AAGCTCCCTCGTGCCTCTCCTGTTCCGACCCTGCCGCTTACCGGCAATACCTGTCCGCTTTCT
 CCCTTCGGGAAGCGTGGCGCTTTTCTCATAGCTCAGCTGTAGCAGTATCTCAGTTCGGTGTAGGT
 CGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCGTTTCAGCC

Figure 2. Base sequence of the S_{304}^{624bp} band (the underlined bases show SCAR primer sequence)

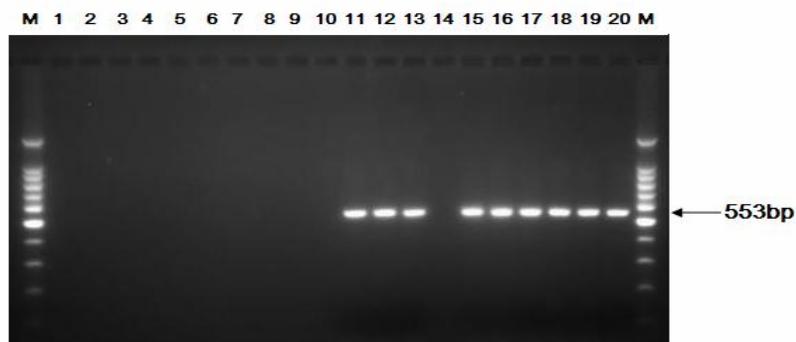


Figure 3. SCAR band from S_{304}^{624bp} marker in NEW GIFT strain and base strain 1-10 base strain (F0) 11-20 NEW GIFT (F10) M 100bp DNA ladder

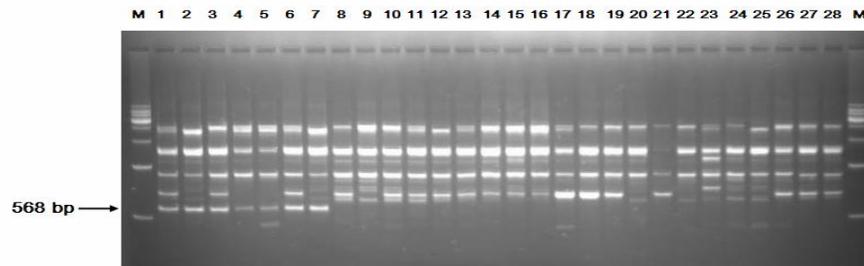


Figure 4. The amplification results of primer S₃₆ [AGCCAGCGAA] in 8 strains of farmed Nile tilapia NEW GIFT: 1-7; Others 8-10 [HG; 11-13 XL; 14-16 GF; 17-19 ET; 20-22 TL; 23-25 WY; 26-28 ZH; M 500bp DNA ladder

AGCCAGCGAATGGATGGATGGATTGATGGATTTTATTTTCGTTTTTCTGCAGTATATAAAAATTG
 GTGTATCTCAAAAATAAACTATGAAGACACTCAAAATAAATTCCTGTTGTTGTAACATTTTT
 TGCAACTTTTCTATATTTAAAGTTTTATATCTTAAATTTTTCTAAGTAGAAATATATGTAAAAAAA
 AAAAAAAAAAGAAAAGATTTTCAATTTTTTGTAGTTTATTGCACTTTTTTGCAATTTATGTAGTTA
 CTATGGACTTAATGCATACATATTATTAACCTTGGGCTATAACAGTTGTATTGATTTATAGTTGA
 AATGCTCCAAAAATGGCACTACAGCATGTAAAAATATAAAGTAAGCTTGGTTCTATGGTAGGTC
 TTAAAGGGTTAAAAAGATAAATGGCTTCT**CAGTAGACCCTCTGCTCAGATGCCTTGATGAAA**
TAAGTGCCTGGATGGCTTTGAACTTTTTACATTTAATGAGCAGAAAACAGAAGTGAT
AGTTTTTGGTGGCGCTTCTGAGACCACTTCTATAGATCTTGGTTCGCTGGCT

Figure 5. Base sequence of the S₃₆^{568bp} band (the underlined bases show SCAR primer sequence [the parts in which the letters are bold show ORF sequence)

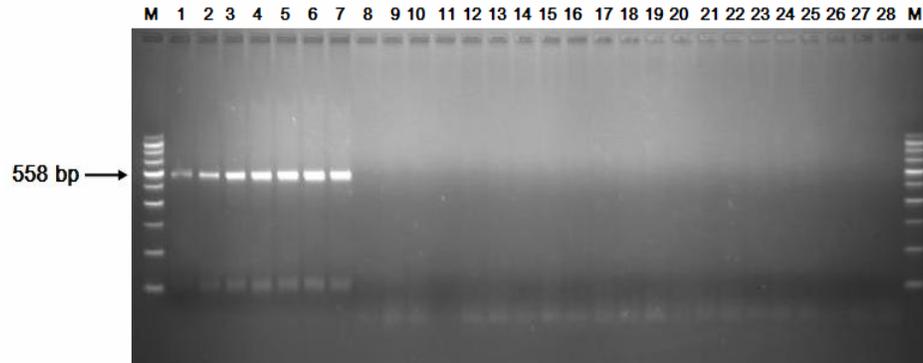


Figure 6. SCAR band from S_{36}^{568bp} marker in eight strains of farmed Nile tilapia NEW GIFT: 1-7; Others 8-10 HG; 11-13 XL; 14-16 GF; 17-19 ET; 20-22 TL; 23-25 WY; 26-28 ZH; M 100bp DNA ladder

REFERENCES

1. Araneda C., R. Neira and P. Iturra. 2005. Identification of a dominant SCAR marker associated with color traits in Coho salmon (*Oncorhynchus kisutch*). *Aquaculture* 247, 67 - 73.
2. Eknath A. E., M. Tayamen, M. S. Palada-de Vera, J. C. Danting, R. A. Reyes, E. E. Dionisio, J. B. Capili, H. L. Bolivar, T. A. Abella, A. V. Circa, H. B. Bentsen, B. Gjerde, T. Gjedren and R. S. V. Pullin. 1993. Genetic improvement of farmed tilapias: the growth performance of eight strains of *Oreochromis niloticus* tested in different farm environments. *Aquaculture* 111,171-188.
3. Gupta M.V. and Acosta B.B. 2004. From drawing board to dining table: the success story of the GIFT project. *NAGA. World fish Center Quarterly* 27, 4-14.
4. Hines N.O. 1976. Fish of rare breeding-salmon and trout of the Donaldson Washington Strains Smithsonian Institution Press 167
5. Hu G. C., S. F. Li and X. J. He. 2005. Selective effects of growth from 6th to 8th generation of GIFT strain *Oreochromis niloticus*. *Journal of Shanghai Fisheries University*, 14,327 - 331.
6. Iturra P., M. Bagley and N. Vergara. 2001. Development and characterization of DNA sequence OmyP9 associated with the sex chromosomes in rainbow trout. *Heredity* 86, 412 - 420.
7. Klinbunga, P. Amparyup and R. Leelatanawit. 2004. Species identification of the tropical abalone (*Haliotis asinina*, *Haliotis ovina* and *Haliotis varia*) in Thailand using RAPD and SCAR markers. *J Biochem Mol Biol* 37, 213 - 220.

8. Li J. L. and S. F. Li. 2001 Introduction and research advances of *Oreochromis niloticus* in Chinese Mainland. *Journal of Fisheries* 25, 90-95 (in Chinese).
9. Li, S. F., C. H. Li, M. Dey and R. Dunham. 1999. Seisability of four strains of Nile tilapia, *Oreochromis niloticus*, in Chinese ponds. *Aquaculture* 174, 223-227.
10. Li, S. F., C. H. Li and J. L. Li. 1998. On-station evaluation of growing performance of five strains of Nile tilapia. *Journal of Fisheries* 22,314-321 (in Chinese).
11. Li, S. F., X. J. He, G. C. Hu, W. Q. Cai, X. W. Deng and P. Y. Zhou. 2006. Improving growth performance and caudal fin stripe pattern in selected F6 -F8 generation of GIFT Nile tilapia (*Oreochromis niloticus* L.). *Aquaculture research*, 37, 1165-1171.
12. Lou Y. D.1999. Fish breeding. Beijing: China Agricultural Publishing House 10-16.
13. Sambrook, J. and D. W. Russell. 2001. *Molecular Cloning* A Laboratory Manual.3rd edition. Cold Spring Harbor Laboratory Press.
14. Sato A., R. Dongak and L. Hao. 2006. Mhc class I genes of the cichlid fish *Oreochromis niloticus*. *Immunogenetics* 58,917-928.
15. Xie X.Y. 2006. A tracking study on the genetic variation during selection of New GIFT strain Nile tilapia (*Oreochromis niloticus*).Doctoral dissertation of Shanghai Fisheries University.
16. Zhou L., Y. Wang and J. F. Gui. 2001. Molecular analysis of silver crucian carp (*Carassius auratus gibelio* Bloch) clones by SCAR markers. *Aquaculture* 201, 219 -228.
17. Zou S. M., S. F. Li and W. Q. Cai. 2005. SCAR transformation of a RAPD marker in blunt snout bream "Pujiang No.1". *Journal of Fisheries of China* 29, 296 – 299.