

# RAPD-SCAR Markers for Genetically Improved NEW GIFT Nile Tilapia (*Oreochromis niloticus niloticus* L.) and Their Application in Strain Identification

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**Abstract:** The NEW GIFT Nile tilapia (*Oreochromis niloticus niloticus* L.) is a nationally certificated new strain selected over 14 years and 9 generations from the base strain of GIFT Nile tilapia, introduced in 1994. This new variety has been extended in most of areas of China. The management of genetically improved strains, including the genetic markers for identification is needed urgently. RAPD analysis was conducted and their conversion to SCAR markers was developed. From NEW GIFT Nile tilapia, two strain-specific RAPD bands, S<sub>304</sub><sup>624 bp</sup> and S<sub>36</sub><sup>568 bp</sup> were identified. The strain-specific RAPD bands were gel-purified, cloned, and sequenced. Locus-specific primers were then designed to amplify the strain-specific bands. PCR amplification was conducted to test the variations in allele frequencies of two converted SCAR markers among the NEW GIFT Nile tilapia and its base strains, as well as 7 additional farmed strains worldwide. The frequency of SCAR marker I (553 bp) was 85.7% in NEW GIFT Nile tilapia, but 16.7% in the base strain. The frequency of SCAR marker II (558 bp) was 91.4% in NEW GIFT Nile tilapia, but 0% – 70% in the 7 other strains. In order to confirm the utility of these two markers, an examination was conducted for a wild population from Egypt, resulted the frequency of SCAR I and II was 10% and 70%, respectively, much lower than that of New GIFT strain. The increase in allele frequency of these two SCAR markers suggests that these markers might be genetically linked to the quantitative trait loci (QTL) underlining the performance traits by long term selection, and indicate the bright potential of SCAR marker technology for tracking generations during selection progress and for distinguishing among genetically improved strain and other strains.

**Key words:** NEW GIFT; Strain; Nile tilapia; RAPD-SCAR marker; Tracking; Identification

## 遗传改良“新吉富”(NEW GIFT)尼罗罗非鱼 RAPD-SCAR 标记开发及其在品系鉴别中的应用

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**摘要:** “新吉富”(NEW GIFT)尼罗罗非鱼是以 1994 年引进的“吉富”(GIFT)品系尼罗罗非鱼为基础群体, 经过 14 年 9 代系统选育后获得的新品种, 该品种已被国家审定良种, 已在全国推广, 迫切需要有效的分子遗传标记来鉴别该新品种并实施科学管理。该研究对良种群体开展了 RAPD 分析, 并进一步将特异性的 RAPD 标记转化成了稳定的 SCAR 标记。通过对“新吉富”群体的 RAPD 分析, 找到了两个品种特异性的 RAPD 条带 (S<sub>304</sub><sup>624 bp</sup> 和 S<sub>36</sub><sup>568 bp</sup>)。对品种特异性的 RAPD 条带进行了胶回收、克隆和序列测定。根据测序结果设计了两对位点特异性引物来扩增这两个品种特异性条带 (简称 SCAR 标记 I 和 II), 检测这两个 SCAR 标记在“新吉富”、“吉富”以及国内外 7 个养殖品系中的出现频率。SCAR 标记 I (553 bp) 在“新吉富”群体中的出现频率为 85.7%, 而在其基础群体 (“吉富”) 中的出现频率仅为 16.7%; SCAR 标记 II (558 bp) 在“新吉富”群体中的出现频率达到 91.4%, 但在其它 7 个养殖品系中的出现频率仅在 0%~70%之间。为验证这两个标记的可靠性, 检测了这两个标记在一个埃及罗非鱼野生群体中的出现频率, 发现 SCAR 标记 I 和 SCAR 标记 II 的出现频率分别为 10%和 70%, 远低于在“新吉富”群体中的出现频率。这两个 SCAR 标记在“新吉富”良种群体中的高出现频率, 预示它们可能与生长性能相关

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QTL 位点间存在连锁关系, 显示 SCAR 标记技术对选育世代间的纵向追溯、以及选育良种群体与其它养殖品系间的横向鉴别有较好应用前景。

关键词: 新吉富; 品系; 尼罗罗非鱼; RAPD-SCAR 标记; 追溯; 鉴别

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The Genetically Improved Farmed Tilapia (GIFT) Nile tilapia (*Oreochromis niloticus*) (Eknath et al, 1993; Gupta & Acosta, 2004), bred by the International Center for Living Aquatic Resources Management (ICLARM; now the WorldFish Center) and its partners, was introduced into 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 and seinability than previously introduced Nile tilapia strains/lines (Li et al, 1998; Li et al, 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 was only the third generation in 1994 produced by cross-breeding, genetic stability had not yet been achieved, leaving opportunity for further selection. Since 1996, the "Genetic Selection of Nile Tilapia" project was carried out in the 9<sup>th</sup> (1996 – 2000), 10<sup>th</sup> (2001 – 2005) and 11<sup>th</sup> (2006 – 2010) National Five-Year Programs, using the GIFT strain of Nile tilapia as a base strain (termed F<sub>0</sub>, although it was the 3rd generation selected from the Philippines in 1994). The purpose was to further improve the aquaculture performance of GIFT Nile tilapia. Compared to the base strain, the major improvements of F<sub>8</sub> and F<sub>9</sub> generations were increased growth by 30%, increased fillet ratio by 5% – 8%, improved stripe pattern on the caudal fin, and higher genetic purity (Hu et al, 2005; Li et al, 2006; Xie, 2006). It was 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 became the principal strain of tilapia cultured in China.

The sequence characterized amplified region (SCAR) marker is one of the stable markers generally derived from random amplified polymorphic DNA (RAPD), or amplified fragment length polymorphism (AFLP) markers (Liu, 2007a; 2007b). The basic principle is to convert the dominant markers into co-dominant markers to reduce the tedious procedures of RAPD or AFLP analysis. With SCAR markers, analysis is reduced to a simple PCR analysis using PCR primers designed from the sequences of the amplicon of RAPD or AFLP.

Because of its simplicity and low cost, SCAR markers have been widely applied to the identification of aquatic animal germplasm (Liu & Cordes, 2004; Zhou et al, 2001; Iturra et al, 2001; Klinbunga et al, 2004; Araneda et al, 2005; Zou et al, 2005). The objective of this study was to develop molecular markers for the NEW GIFT tilapia. Here we report the development of two RAPD-SCAR markers associated with genetically improved NEW GIFT Nile tilapia and their application for identifying various strains of Nile tilapia and tracking of genetic changes over long-term selection.

## 1 Materials and Methods

### 1.1 Fish samples

A total of 65 fish samples including 35 samples of NEW GIFT Nile tilapia F<sub>10</sub> (NG) and 30 samples of GIFT Nile tilapia F<sub>0</sub> (GN; the base strain) were collected from the Nanhui Fish Breeding Station of Shanghai Ocean University and the National Tilapia Seed Farm Qingdao. In addition, 20 samples were collected from each of 7 farmed strains of Nile tilapia from different tilapia farms in China [Hainan Genoma tilapia company (HG), Xiamen Luye tilapia farm (XL), Guangxi Fisheries Research Institute (GF), Guangdong Weiye tilapia farm (WY), and Guangdong Zhuhai tilapia farm (ZH)], and Hungary [Egypt strain (ET) and Thailand strain (TL) in Sjarvas tilapia farm]. Thirty samples of wild Nile tilapia were also collected from the Egyptian Aquaculture Center (Cairo, Egypt) for verification of our developed SCAR markers.

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

### 1.2 Genomic DNA extraction

Genomic DNA was extracted using a phenol-chloroform procedure (Sambrook & Russell, 2001).

### 1.3 RAPD analysis and PCR conditions

PCR mixtures (25  $\mu$ L) contained 2.5  $\mu$ L 10 $\times$ PCR buffer (100 mmol/L Tris-HCl, pH 9.5, 500 mmol/L KCl, 30 mmol/L MgCl<sub>2</sub>, and 0.001% gelatin), 2  $\mu$ L dNTP mixture (0.2 mmol/L each of dATP, dCTP, dGTP, and dTTP), 2  $\mu$ L RAPD primer (0.2  $\mu$ mol/L), 2  $\mu$ L template DNA (50 – 150 ng), 0.5  $\mu$ L Taq DNA polymerase (1.25

U), and 16  $\mu$ L distilled water. PCR amplification was performed in an Eppendorf Mastercycler programmed for initial denaturation at 94°C for 5 min, 45 cycles of denaturation at 94°C for 45 s, annealing at 36°C for 45 s, extension at 72°C for 1 min 30 s, and a final extension at 72°C for 5 min. Reaction tubes were held at 4°C prior to visualization of PCR products in a 1.5% agarose gel stained with ethidium bromide. Each RAPD assay was performed three times to ensure reproducibility.

#### 1.4 Cloning and sequencing of strain-specific RAPD amplicons

All strain-specific RAPD amplicons were 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-T Easy Vector (Promega). Then, DH5 $\alpha$  competent cells (TIANGEN) were transformed with ligated DNA following the manufacturer's protocol. 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 3 730 capillary sequencer.

#### 1.5 SCAR analysis

The nucleotide sequence of each of the cloned RAPD fragments was used to design pairs of SCAR primers (Tab. 1). PCR mixtures (25  $\mu$ L) contained 2.5  $\mu$ L 10 $\times$ PCR buffer (100 mmol/L Tris-HCl, pH 9.5, 500 mmol/L KCl, 30 mmol/L MgCl<sub>2</sub>, and 0.001% gelatin), 2  $\mu$ L dNTP mixture (0.2 mmol/L each of dATP, dCTP, dGTP, and dTTP), 1  $\mu$ L of forward primer (0.2  $\mu$ mol/L), 1  $\mu$ L of reverse primer (0.2  $\mu$ mol/L), 2  $\mu$ L template DNA (50–150 ng), 0.5  $\mu$ L Taq DNA polymerase (1.25 U), and 16  $\mu$ L distilled water. The amplification profile was for 5 min of initial denaturation at 94°C, 35 cycles of

denaturation at 94°C for 30 s, annealing at 57°C for 45 s, extension at 72°C for 1 min, and a final extension 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.

#### 1.6 Verification

Thirty samples of wild Egypt Nile tilapia were tested for verification of the developed SCAR markers.

## 2 Results

### 2.1 SCAR I

2.1.1 RAPD amplification results RAPD analysis was performed on 10 genomic DNA samples of selected strains and base strains by using fifty 10-nucleotide random primers. Of these 50 primers, 23 showed both stable amplification and polymorphism. There was only one specific band between the two groups, S<sub>304</sub><sup>624 bp</sup>; this fragment served as a specific marker distinguishing the selected strain from the base strain. The 624 bp band amplified by S<sub>304</sub> primer is shown in Fig. 1.

2.1.2 Development of SCAR markers The S<sub>304</sub><sup>624 bp</sup> fragment was recovered, cloned and sequenced. The sequence of the S<sub>304</sub><sup>624 bp</sup> fragment is shown in Fig. 2. Both the 20 bp forward primer and 21 bp reverse primer were designed according to the S<sub>304</sub><sup>624 bp</sup> sequence

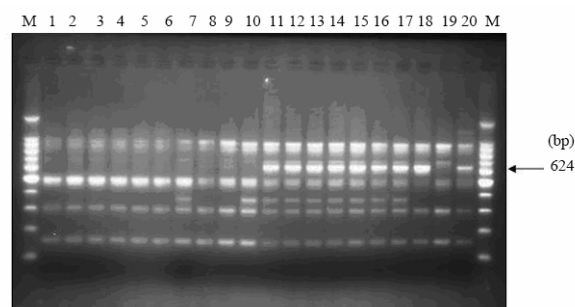


Fig. 1 The amplification results of primer S<sub>304</sub> (CCGCTACCGA) in NEW GIFT strain and base strain 1–10: base strain (F<sub>0</sub>); 11–20: NEW GIFT (F<sub>10</sub>); M: 100 bp DNA ladder.

Tab. 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 (°C)	Size of PCR band (bp)
<b>SCAR I</b>				
S <sub>304</sub>	624	5' -GGTGCCTTTTGAATGAGCTA-3' 5' -TGAGATACTGCTACAGCGTGA-3'	57	553
<b>SCAR II</b>				
S <sub>36</sub>	568	5' -TGGATGGATGGATTGATGGA-3' 5' -AGCCAGCGAACCAAGATCTAT-3'	57	558

GTGTAAAGCCTGGGGTGCCTTTTGAATGAGCTAACTCACATTAATTGCGTT  
 GCGCTCCCTCAGCCCGCTTCTAGCAGGAAACCTGTCTGTCAGCTGCAT  
 TAATGAATCGGCCAACGCGCCAGGGAGAGGCGGTTTGCATATTGGGCGC  
 TCTTCCGCTTCCCTCGCTCACTGACTCGCTGCGCACTCGGTCGTTCCGGCTGC  
 GCGAGCGGTATCAGCTCACTCAAAGCGGTAATACGTTATCCACACAG  
 AATCAGGGGAT AACG CAGG AAAG AACATG TGAGCAAAAGGCCAGCAAAA  
 GGCCAGCAGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCT  
 CCGCCCCCTGACGAGCACATCACAAAATCGACGCTCAAGTCAGAGGTG  
 GCGAAACCCGACAGGACTATAAAGATACCACAGGCGTTCCCCCTGGAAG  
 CTCCCTCGTGCCTCTCCTGTTCCGACCTGCCGCTTACCGGCAATACCTG  
 TCCGCTTTCTCCCTTCGGGAAGCGTGCGCTTTCTCATAGCTCACGCTGT  
AGCAGTATCTCAGTTTCGGTGTAGGTCTGCTCCAAAGCTGGGCTGTGTGC  
ACGAACCCCGTTCAGCC

Fig. 2 Base sequence of the  $S_{304}^{624\text{ bp}}$  band

The underlined bases show SCAR primer sequence.

(Tab. 1). Due to the GC content in the primer, the location of the primer was not at both ends of the fragment, but located slightly closer to the center of the fragment.

Both samples of the selected strain and base strain were screened with the specific primer. A 553 bp band was produced (Fig. 3 and Fig. 4). The frequency of this 553 bp SCAR marker in selected strains reached 85.7%(Fig. 3), while the frequency in the base strain was only 16.7%(Fig. 4). The high frequency of the 553 bp marker can be the criterion for distinguishing NEW GIFT Nile tilapia.

## 2.2 SCAR II

**2.2.1 RAPD amplification results** RAPD analysis was performed on 10 genomic DNA samples of the NEW GIFT strain and the remaining 7 farmed strains by using eighty 10-nucleotide random primers. Of these 80 primers, 20 showed both stable amplification and polymorphism. There was only one specific band between the two groups,  $S_{36}^{568\text{ bp}}$ ; this fragment served as a specific marker distinguishing the NEW GIFT strain from other strains. The 568 bp band amplified by  $S_{36}$  primer is shown in Fig. 5.

**2.2.2 Development of SCAR markers** The  $S_{36}^{568\text{ bp}}$  fragment was recovered, cloned and sequenced. The sequence of the  $S_{36}^{568\text{ bp}}$  fragment is shown in Fig. 6.

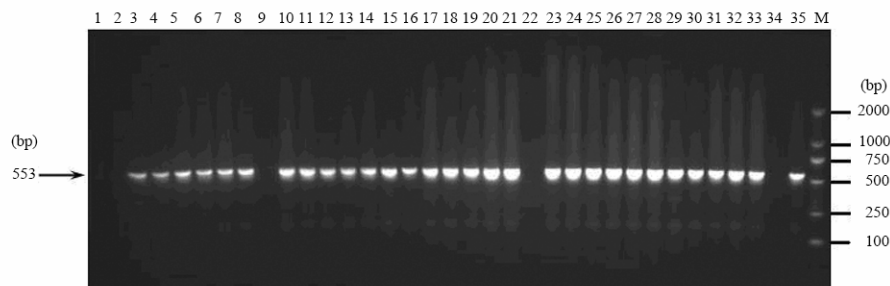


Fig. 3 SCAR band from  $S_{304}^{624\text{ bp}}$  marker in NEW GIFT strain( $F_{10}$ )



Fig. 4 SCAR band from  $S_{304}^{624\text{ bp}}$  marker in base strain ( $F_0$ )

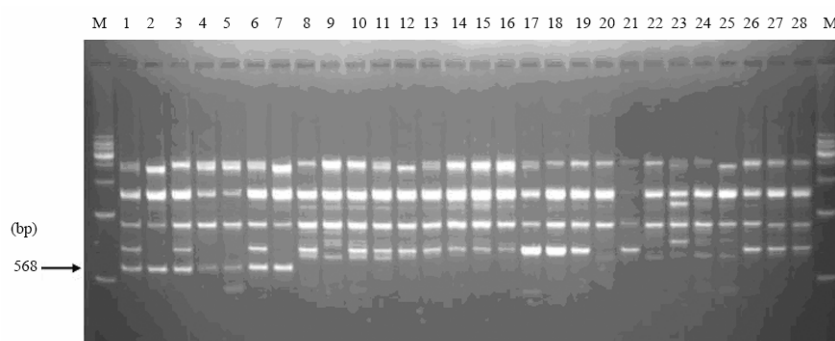


Fig. 5 The amplification results of primer S<sub>36</sub> (AGCCAGCGAA) in NEW GIFT strain and 7 other farmed strains of Nile tilapia

1 – 7: NEW GIFT; 8–10: HG; 11 – 13: XL; 14 –16: GF; 17 – 19: ET; 20–22: TL; 23–25: WY; 26 – 28: ZH; M: 500 bp DNA ladder.

AGCCAGCGAATGGATGGATGGATTGATGGATTTATTTTCGTTTTTCTGCA  
 GTATATAAAAATTGGTGTATCTCAAAAATAAACTATGAAGACACTCAA  
 AATAATTTCTGTTGTTGTAACTATTTTTGCAACTTTCTATATTTAAAG  
 TTTTATATCTTAAATTTTCTAAGTAGAAATATATGTAAAAAAAAAAAA  
 AAAGAAAAGATTTTCAATTTTTTTGTAGTTTATTGCACTTTTTTGCAATTA  
 TGTAGTTACTATGGACTTAATGCATACATATATTAACTTTGGGCTATAA  
 CAGTTGTATTGATTTATAGTTGAAATGCTCCCAAAAATGGCACTACAGCAT  
 GTAAAAATATAAAGTAAGCTTGTTCTATGTTAGGTCTTAAAGGGTTAAA  
 AAGATAAATGGCTTCTCAGTAGACCTCTGCTCAGATGCCTTGATGAAAT  
 AAGTGCCTGATGGCTTTGAACTTTTTACATTTTAATGAGCAGAAAACAG  
 AAGTGATAGTTTTTGTGGCGCTTCTGAGACCACTTCTATAGATCTTGTT  
CGCTGGCT

Fig. 6 Base sequence of the S<sub>36</sub><sup>568 bp</sup> band

The underlined bases are the SCAR primer sequence.

Both the 20 bp forward primer and 21 bp reverse primer were designed according to the S<sub>36</sub><sup>568 bp</sup> sequence (Tab. 1). Due to the GC content in the primer, the location of the primer was not at both ends of the fragment, but located slightly closer to the center of the fragment.

Thirty five samples of the NEW GIFT strain and 20 samples of each of the 7 farmed strains were screened with the specific primer. A 558 bp band was produced in these two groups. The appearing frequency of this 558 bp SCAR marker in NEW GIFT was 91.4% (Fig.7), while the frequency in the 7 farmed strains was 70% (ET), 65% (HG), 60% (TL), 55% (WY), 35% (XL), 30%

(ZH)(Fig. 8), and 0% (GF). The extremely high frequency of the 558 bp marker in NEW GIFT can be used as a criterion for identification of NEW GIFT Nile tilapia.

**2.3 A case of application**

We checked 30 samples of a wild Egyptian population of the Nile tilapia which resulted in a frequency of 10% by SCAR I and 70% by SCAR II, which were very coincident with the 70% in the Egypt strain (ET). This provides evidence for the ability to discern NEW GIFT from other strains.

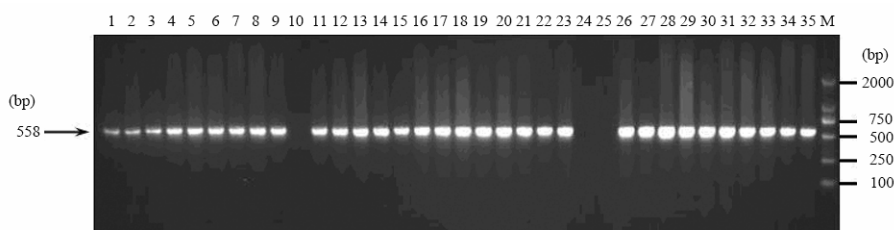


Fig. 7 SCAR band from S<sub>36</sub><sup>568 bp</sup> marker in NEW GIFT strain (F<sub>10</sub>)

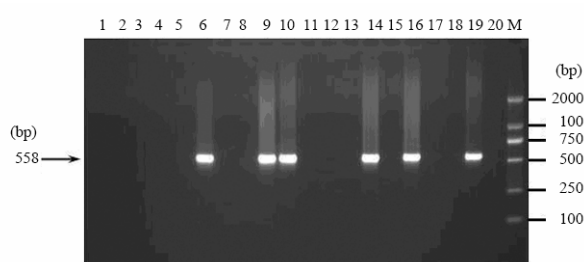


Fig. 8 SCAR band from  $S_{36}^{568 \text{ bp}}$  marker in farmed strain of ZH

### 3 Discussion

Through multi-generations of selection, economic traits could be improved and stabilized, and as a result, new strains could be established (Hines, 1976; Lou, 1999). The improved traits could be attributed to natural factors and selection processes that might induce mutations, but the selection itself would not create new genes. However, selection might significantly change the allele frequencies which might cause the change of traits, then the favorable alleles for the traits could be selected.

Breeding requires a long period of time, and how to track the phenotypic variation and genotypic variation of breeding groups is the key to successful breeding. The development of modern molecular genetic technology provides some effective measures for selective breeding. RFLP, RAPD, SSR and SCAR markers are commonly used molecular markers (Liu & Cordes, 2004). Among them, RAPD needs less DNA template and is relatively easy to operate, but is poor in reproducibility and stability, leading to 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.

In this study, two RAPD-SCARs were developed for the NEW GIFT tilapia: firstly checked in the base

strain and 7 other farmed Nile tilapias, and secondly examined in the wild population. The frequency of the 553 bp marker (SCAR I) was up to 85.7% in NEW GIFT tilapia, significantly higher than the 16.7% in the base strain, suggesting that this marker can be used to distinguish the selected strain from the base strain. Meanwhile, the frequency of the 558 bp marker (SCAR II) was up to 91.4% in NEW GIFT, significantly higher than those in the 7 other farmed strains and the wild population (0% – 70%), suggesting its utility in distinguishing NEW GIFT tilapia from other farmed or wild Nile tilapias. They indicate that there is bright potential of SCAR marker technology for tracking generations during selection process and for distinguishing among genetically improved strain and other strains.

When we analyzed the genetic diversity of  $F_6$ ,  $F_7$ ,  $F_8$  and  $F_9$  of selected GIFT strains, we found that there was a clear trend in genetic purification across generations. The genetic differentiation caused by nine generations of selection was minimal but could be monitored (Xie, 2006). Also, the growth rate increased with selected generations, for example, an average of 4.85% from  $F_6$  to  $F_9$ . This indicates that the frequency of some alleles related to performance traits significantly changed during the long-term selection process, which implies that there is a correlation between the phenotypic variation and the genotypic variation over the selection period. Such genotype-phenotype associations suggest that the identified markers may be genetically linked to the quantitative trait loci (QTL) underlining the performance traits under selection.

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