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Study on Molecular OPAY02-Scar Markers Related to Early Body Weight of New Yangzhou Chickens

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Abstract: In order to develop rapid and reliable SCAR markers linked to the QTL, to get better understood of orgin of New Yangzhou chickens, two bands of OPAY02 type marker have been cloned, sequenced and two pairs of primer were designed according to the DNA sequence of two bands of OPAY02 to amplify the SCAR-markers on 86 individuals randomly selected from New Yangzhou chickens. The results of comparing the two bands DNA sequence with red jungle fowl genomic DNA database showed that the large molecular size band is located on the 3rd chromosome, the sequence identities were 98% and 8 SNPs mutations were detected, they were at 195 (T \rightarrow G), 316 (A \rightarrow T), 538 (G \rightarrow A), 731 (T \rightarrow A), 1147 (G \rightarrow A), 1329 (T \rightarrow C), 1927 (C \rightarrow T) and 2081 (C \rightarrow T). On the other hand, the small molecular size band can't be found in red jungle chicken genomic DNA database, speculated that the source of New Yangzhou Chickens may originate from not only red jungle fowl, but also other jungle fowls. SCAR -markers confirmed that the two bands of OPAY02 type marker can be applied to genetic analyses because of its stability and reliability. Genotype equilibrium test showed that New Yangzhou chickens are in equilibrium on the two bands loci, two bands of OPAY02 type marker selection will be in favor of New Yangzhou chickens body weight breeding.

Key words: OPAY02- SCAR genetic markers, DNA sequencing, bioinformatics, genotype equilibrium test

Introduction

Animal breeding is the domestic fowl evolution process manipulated by human being. In order to accelerate breeding process, the breeding experts persevere in searching for genetic markers which can be used for selection, especially those which have close relationship with quantitative traits. The molecular genetic marker which closely related to animal important economic traits assistant selection has become more important theoretic foundation and precondition in animal molecular breeding (Wu, 1998). This research was based on the study results of Dai (2004a, 2004b), Wang (2001); Wang and Chen (2004a); Wang et al. (2004b). They detected that there were significant relationship between the band 2326, band 1660 combination marker of the OPAY02 and the early growth performance of New Yangzhou, Xiaoshan and SR92 A strain chickens respectively. To get better understand of the two polymorphic bands of OPAY02, New Yangzhou chickens were Chosen as experiment materials, the two bands (fragments) of OPAY02 were cloned and sequenced to analyse its bioinformatics, according to the results of sequence of OPAY02 marker, OPAY02 markers were converted to stable SCAR (Sequence characterized amplified region) markers to study their stability and genetic style, establish the foundation of chicken marker-assisted selection.

Materials and Methods

Experiment chickens: 86 healthy individuals were randomly selected from the New Yangzhou chickens reared in Yangzhou University experiment farm, and were runed by the broiler's breeding method and were used as experimental chickens for OPAY02-SCAR molecular genetic marker analyse.

Genome DNA samples isolation: Approximately 1ml of blood was collected from each of the bird into 1.5ml microfuge tubes, transferred to the laboratory and frozen at -20°C before DNA extraction. 300 μl each New Yangzhou chicken blood was added into 3 ml DNA lysate, mixed well until limpidity, and then extract DNA by phenol, chloroform and isoamyl alcohol (Sambrook *et al.*, 1998). DNA purity and concentration were detected by ultraviolet spectrophotometer. The genomic DNA can not be used to PCR amplification except its ratio of OD260 to OD280 were greater than 1.700. The gennome DNA was diluted into 50 ng/μl, stored at 4°C for SCAR genetic marker detect later.

Amplification and Clone of OPAY02 type genetic marker fragments: The optimum PCR condition carried out in OPAY02 type genetic fragments detecting refers to the reference of Dai (2004a, 2004b). The RAPD fragments amplified by the polymerase chain reaction were separated by high-transresolution gel

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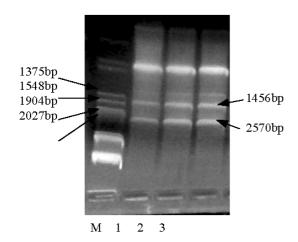


Fig. 1: PCR results of OPAY02 random primer
M: maker (λDNA-EcoR I Hind III);
Lanes 1~3: PCR results of OPAY02 - A type.

electrophoresis using 1.4% agarose gels with a drop (0.5 μ g/ml) of ethidium bromide (EB) used as staining agent. Following electrophoresis, gels were visualized under UV light illuminator and the RAPD profiles were examined with the Kodak Digital Camera (DC120). The two target DNA fragments of OPAY02 were recovered by PCR Fragment Recovery Kit (Produced by Baoshengwu Dalian limited company), and were cloned by PGEM-T easy plasmid vector (Promaga) and then were sequenced (at Baoshengwu Dalian limited company).

SCAR genetic marker detection: After sequencing of two target fragments, two pairs of primers were designed according to the basis of previously randomly 10 base primer sequence. The OPAY02 type genetic markers were converted into SCAR markers (Murayama et al., 1999; Forrest et al., 2002). PCR reaction mixture with the final volume of 25µl included 1µl template DNA. 2.5µl of 10×PCR Buffer, 1µl of 25mM dNTPs, 1µl of each (8pmol/µl) forward and reverse form of the primers and 0.2ul of 5U/ul Tag DNA polymerase (Sangon Company. Shanghai, China) with 2.2µl of 25mmol/l MgCl and 16.1µl sterilized distilled water 2 added. The reaction programme carried out in PCR Hybaid Touchdown Express System (PE 9600) was at 95°C for 8min predenature, the polymerase chain reaction were run for 33 cycles at 94°C denature Tem. for 1min, 55°C annealing Tem. for 1min, 72°C extension Tem. for 2 min, with final extension step at 72°C for 10 min. The methods of separation and examination of the two fragments are described previously.

Results and Discussion

The recovery and purification of two polymorphic OPAY02 bands: The OPAY02 random primer was amplified by using of the optimum PCR program and

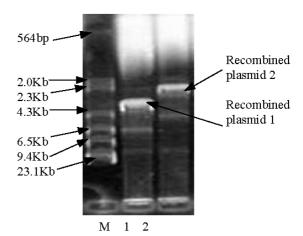


Fig. 2: The results cloned by pGEM-T easy plasmid vector of two polymorphism bands amplified by OPAY02 primer

Lanes 1~2: recombined plasmids; M: marker

conditions. Several tubes of PCR products were put together and loaded into large loading slot in order to get more PCR products of the two targets fragments in favor of cloning. The mixed PCR products were electrophoresed thought new electrophoresis buffer and agarose gel. The electrophoresis results were showed in Fig. 1, λ DNA/Hind III + EcoR I was used as molecular marker to estimate the size of the amplified products. The size of the two target fragments were 2570bp and 1456bp.

The cloning and EcoR digesting characterization of two OPAY02 polymorphic bands: Fig. 2 is the results of two polymorphism bands amplified by OPAY02 RAPD primer cloned by pGEM-T easy plasmid vector, Fig. 3, Fig. 4 were the results of recombined plasmid vectors digested by EcoR for the large size and small size fragments (bands) respectively.

The sequencing results of the two OPAY02 polymorphic bands: By linked by the conting software, the DNA sequence of the two target polymorphic bands was showed as follow, the nucleotide sequence which was italics, overstriking and with line under the characters was the sequence of the OPAY02 random primer, which with the line under the characters only was the sequence of SCAR genetic marker primers.

 GTTGCTTTATTCTAATGTGTGAAGTGGATCTCATTAATAAAGTCCCTTGAAGCTTCATAAAGTATTCCTGTCTCGGCAC GTTTAATGACAAGGAGCAAAATAATTTCCAATCTACATTTCTTAAAAGTAGTTAAAGGTCAAGAATAAATTACTGAGAACTT CCCACAGGAAAATTTCTTTACAATATTTAACATTTTGACACCAAGGGGACCAAGGTTATAATGTCATTACTAAAGATGAC CTTTACTTATCTCTGTGAATAATAAATAAGCAGGTAAAATGCAAAACAGCGCTTGCTGTTTTACATATACTAGGCATTC TGTTTATGTAACTTCCACTTCTTCAAATTCTTTGTGTTCCCACAAATCTGTACAGAAAGCATTTACTAAATATTCTGAAGC TAAACTATCTTCATTAGGAAAGAAATAAAAATATATGTTAAGTAATTACTGTTTTTAAAATCTCCTAAATAACTTTACACTGA CACAATACAAGTTCCTGTTTTCTACCGTTTGGCTCTGCAGATACCAATTCTGGGCTAACTTCAGCAACAAAAAAATTAA GCAGGAAGGCACTGGAATTTGTGAGGCTTTATCTCACAGCTTGGTGACATGTTATATCTTCCTTTCAAAATAATAAT GATAATAATTATAGAGGAAGTAGGAGATACAATTTAAGTAATAACTACATCCTGGTTGTTGAAGACAATATTTTCAAAAAAT TTCTCTTTTGCAACCTATCTTAGTTCTATGAAAGCTTCAAACCATCTGGAGACTGAAGCCTTTTAATTTCAGACAAGTGT GGTGAATGGAGTAGAGGAAAGATTCGATGTGCTACAGCACAAGAACCCATCCTCACTTCTCAACTTTCAACATTGGAG TATTCAATATCTTCATCTAGTTTAAGTGTTACCTAAGTTGAAACCTTTTTTCTAATTCTGAAGCCTTCCTGGATAACCCTA AGAAAGCCAAAAACCTGCCTACCTCTGTTGTGGAGAAGCAAATAATAAGGTGCAAAGGAGAGTATTTTGCTTTTTCATT CAAAGTAAACTGTGCAAACTGTATGCCTATCCACAGGAAATGGTCCAGAATCTCATCCATTCTTACAATTCTTACGATC TTTTTTGTTGCTGTTTTCCTTTAAAAATATGAAGTGATATTTAAATAAGAAACAAGTGATAAAGTCTTGCACCATTCCACAG TAAAATCCTAGTGAAAATAGGATATATTTTCATGCAATATTTTTATGTTCCCTTTGTTCAGTATTATCAAGCATTTTTATTAA GAAGCAAGCGTGTTTACAAAGCCCAGCTCCAGACATGTTCAGGCACAATAAAGGCATATTAAAGGAACACCACATGAT ATCTTTTCCTTCACCAAAATCCCAATATAAAGGAGATCAGAAGTGTTGACACCACCAAAAACCCACACCTTAGGAAACCA CCATGCTATGTTGCTGCTAAAAGCACTGTCTATTGATTGTTTCTTCTAAGAAAAGGGCATGAGAGTAGTAAGAAAAATTT GGGCAACAGAATGCTAAAGAAACGATCTGGCAGAAAAAAGTAATTCACAATGGAACACCAACGAAGATAAAACACAATG TGTGTAATACATGACAAGCCTGGAAATAAACAGTAAACAAATGCCATTTTTTCAAGTTTTCAGGTGATCACAACTAATTGT AAGTAAGAAACTTGGAGTCCTATTCTGTTGATTCTAATGGAAGCTGCTGAGCTCTGAGATCTGGTATAGCCAAGTAATG TGACTACAAATCTGATGGGAATAAAGCACATAAATTGCTGAAGCTTCAC<u>TTTCTAGTCAAACCAGCCTTCGCA</u>AATCAC TAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGGCTCCCAACGCGTTGGATGCATAGCTTGAGT

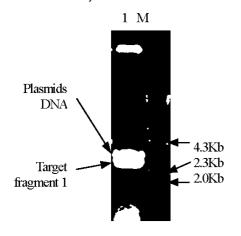
The small size DNA fragment sequencing result:

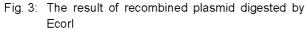
CGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATCGATT CAATAGACTAAAAAATGCTACGGGCACCTTTATTAAGAAAAACGGCGTGCCGCTTACATTCACACAGTTTGAGCGTGC CCTGCAAAAAAGCGATTCTCCTTATTACGATTATACCAATACGGTACAAACCTATAAAGGAACCGCAGTGGATTATTTA AAATCGAAACCAAACACTTTTGATTCGCTGCTTTTGGTGTTAGACCGTTTTCCTTTTTTAAAGGATTCTTTAACCAATCA GAAAGTAACCCTTTTTGCACCGGTAAATGAAAATTTTGCGGCATCGGTTAAATATTTGAACCAAACCAGAAAGCAGAAG GGAAAGCACCTATTTATTTAAGCACTGCAGATCCTCTGGAACTGGCATTTATGCTTTCAAAAATATATTGTAAGGGGAAAC TTAACACCTGCTACTTATGCAAGTACAGTTGATGGCATTTCGGTTACAACCGTAGCCTCCAATTATCCTATGCATGTAA CCATCAGGCTTAACTGGGTAACTACCAATACCAACGCCATTAACATTAAAACCAATAATGCGACCATAAACATTTTAACC CCGCTGCACAATTTTGGGTTTGATGAATTTACATACAGATTAGACCAATAGCCTTATGAAGATGAAAAACTAAAAAACACT TTCTTAGTGTAGCGATAATGCTAACAGGCATGATGCTCGTATTGGTATCGTGTAAGAAAAATTTACCTGATAACAGGTTG TACCTACGGAAATTCGAGCAGGCCTCTGGATTTTAAAATTGTGAATATGCGCACTTTTGGTGGCGAGCCGGCTCCCG AACTCACCAAACTTTATCCTGTTAAAGTATGGAAAACAGCTTATGACGGTACCGAAAAGACGCTGGCCGAAATTGAAGC CAAACGTGTAATCGAAAACCACCCTTTATTTGAAGTACGTCCGCATTCTGGTGAGTTTTTAATGTGGGCAGAAGCCAAT TCGAACATGATCAAAGCACAGCCAGATTCGGGTTACGTTTTTGATGTAGAAATGTCGAACACCGGTGGGAGGAAATAT TTTCAGAATTTCCGTCTTAGCCCACTACG<u>CGAACGTCCTTA</u>CG**AGCCTTCGCA**AATCACTAGTGAATTCGCGGCCG CCTGCAGGTCGACCATATGGGAGAGCTCCCAATCGC

According to the two DNA fragments sequencing results, it is very obvious that there is no simple repetitive

sequence in the two polymorphic bands sequence. Whether it is a gene expression sequence or a part of it,

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Lane 1: recombined plasmid digested by EcoRI; M: marker (λ DNA-EcoRI)

or a molecular genetic marker linked with New Yangzhou chicken early growth QTL, only it can be known after comparing with the cDNA related to a special mRNA or with a certain EST.

The bioinformatics analytical results of two OPAY02 polymorphic bands: The result of comparing of large molecular size band DNA sequence with chickens genome sequence database using Blast MEGBLASTN software program were used to analyse the large molecular size band DNA sequence in chicken genome nucleotide sequence database (Database: chicken_wgs), the BLAST result showed that the large size fragment had 98% (Identities = 2532/2569=98%) [Score = 4782 bits (2487), Expect = 0.0] homologous sequence with the linear chicken contig genome DNA number AADNO1039470.1 (Length = 3629377 bp), which was from the 2872926bp to 2875494bp. The sequence was located on the 3rd chromosome of red jungle fowl and 8 SNP mutations were detected, they were at 195 (T \rightarrow G), 316 (A \rightarrow T), 538 (G \rightarrow A), 731 (T \rightarrow A), 1147 (G \rightarrow A), 1329 (T \rightarrow C), 1927 (C \rightarrow T) and 2081 (C \rightarrow T). By electronic map, features in this part of subject sequence, there was a gene which is similar to T-cell lymphoma breakpoint-associated target 1 (TCBA1). The chicken genome database on the Internet is the genome DNA of the red jungle fowl inbred line UCD001, hen.

The result of comparing of small molecular size band DNA sequence with chickens genome sequence database using Blast MEGBLASTN software program were used to analyse the small molecular size band DNA sequence in chicken genome nucleotide sequence database (Database: chicken_wgs), the BLAST result showed that there was no homogenous sequence in the genome database of chicken.

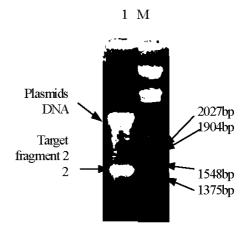


Fig. 4: The result of recombined plasmid digested by EcoRI

Lane 1:recombined plasmid digested by EcoRI; M: marker (λDNA-Ecorl-HmidIII)

The stability and reliability of RAPD detected results compared with SCAR technique: The amplified two RAPD target bands were in accordance with the corresponding SCAR genetic marker analytical result. The two bands detected results using these two techniques are showed in the Table 1 and 2 respectively. The small molecular size band was detected out in 46 chickens while 40 chickens didn't have this band. The large molecular size band was detected out in 63 chickens while 23 chickens without. Regarding the SCAR technique detect result as standard; one false positive case was appeared in the RAPD gel electrophoresis of the band, so its wrong judgment rate is 2.12%. The large molecular size band detected results using the two techniques are the same, so its wrong judgment rate is 0.00%. The results above showed that the RAPD technique detecting results are steady and reliable when the PCR condition is improved or the detect loci is special.

Genotype equilibrium test results of the two loci: SCAR detecting technique was used to test the genotype equilibrium of the two loci when the two fragments were regard as different genes. The presence of small molecular size bands were defined as AA and Aa genotype, whereas the absence was defined as aa genotype; similarly, the presence of large molecular size band were defined as BB and Bb genotype, the absence was defined as bb genotype. The four observed frequency of each genotype were in Table 3. Because 86 New Yangzhou chickens were randomly selected from the random mated population, it might assumed that the two loci were in gene equilibrium (Wang Jinyu et al. 2004a), the four expected frequency of each genotype were in Table 4.

The Chi² test of gene equilibrium of two loci, df = k-c-1= 4-2-1 = 1, = $\text{Chi}^2_{\text{calculated}} = \sum$ [(|observed frequency -

expected frequency| - 0.5)²/ expected frequency| = [(| 37-33.7 |-0.5)²/33.7] + + [(|14-10.7 |-0.5)²/10.7] = 0.2364 + 0.2676 + 0.6374 + 0.7327 = 1.87, $Chi^2_{0.05(1)} = 3.84$, $Chi^2_{calculated} < Chi^2_{0.05(1)} P > 0.05$, there were no significant difference between observed frequency and expected frequency of each genotype, it can be inferred that the two OPAYO2 markers were in gene equilibrium in New Yangzhou chickens.

Discussion

The bioinformatics analysis of OPAY02 molecular genetic markers: The search for similarity DNA sequence in nucleotide database had become one of the most important parts of bioinformatics research. The bioinformatics dummy experiments were quick, convenient, economical when comparing with the reality experiment, and can share the bioinformation resources with each other at the most possible (Ding and Zhang, 2003). The DNA sequencing results of OPAY02 two molecular markers showed that there were no Short tandem repeat sequences in each sequences. Comparing with the red jungle fowl genome sequences , there were 8 SNPs mutations in large molecular size band DNA sequence, which was on the 3rd chromosome, they were at 195 (t \rightarrow g), 316 (a \rightarrow t), 538 (g \rightarrow a), 731 (t \rightarrow a), 1147 (g \rightarrow a),1329 (t \rightarrow c), 1927 (c \rightarrow t) and 2081 (c \rightarrow t). The result also showed that there was no similarlity sequence of OPAY02 small size band in red jungle fowl genome database, it may speculate that the origin of New Yangzhou chickens are not only come from red jungle fowl but also from others. Based on the results of investigation, there are many different indigenous chicken breeds in almost each province of china, even more than ten breeds in some province (Chen et al., 2004). Based on the results of polymorphic molecular genetic markers and Phylogenetic trees analysis, the genetic distance between indigenous china chicken breeds are different, some are great and some are small, so the genetic diversity among indigenous chicken breeds are abundance (Olowofes et al., 2005). Cheng et al. (1996) using protein polymorphic data of china domestic fowls (chahua, taihe shouguan) and jungle fowls (red jungle fowl, Gallus varius green jungle fowl, black jungle fowl and Grey-Streaked jungle fowl) to analyze genetic relationship, the results idecated that the genetic relationship sequence order from close to far were china demestic, red jungle fowl, Grey-Streaked jungle fowl, black jungle fowl and Gallus varius green jungle fowl. Althought red jungle fowl was considered as the origin of modern chicken breeds by Darwin (Wang et al., 2004), based on the DNA sequences alignment search results of OPAY02 two molecular genetic markers, it may be speculated that the source of New Yangzhou Chicken may originate from not only red jungle fowl, but also other jungle fowls, such as Gallus varius green jungle fowl, black jungle fowl and Grey-Streaked jungle fowl.

Table 1: Results detected by RAPD and SCAR technique of small size band

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Results	Results detected	by RAPD	Sum		
detected					
by SCAR	Presence	Absence			
Presence	46	0	46		
Absence	1	39	40		
Sum	47	39	86		

Table 2: Results detected by RAPD and SCAR technique of large size band

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Results	Results detected by RAPD		Sum		
detected					
by SCAR	Presence	Absence			
Presence	63	0	63		
Absence	0	23	23		
Sum	63	23	86		

Table 3: The observed frequency of each genotype

	B-	bb	Sum
A-	37 (0.4302)	26 (0.3023)	63
aa	9 (0.1047)	14 (0.1628)	23
Sum	46	40	86

Note: the actual proportion of each genotype was in bracket The genotype frequency of aa: $q^2 = 0.1047 + 0.1628 = 0.2674$, The genotype frequency of bb: $s^2 = 0.3023 + 0.1628 = 0.4651$

Table 4: The expected frequency of each genotype in gene equilibrium

	B-	bb	Sum
A-	(1-q ²) (1-s ²)	(1-q²)s²	(1-q ²)
	0.3918 (33.70)	0.3407 (29.3)	0.7326
aa	q² (1-s²)	q^2s^2	q^2
	0.1431 (12.3)	0.1244 (10.7)	0.2674
Sum	$(1-s^2)$	s^2	1
	0.5349	0.4651	

Note: the expected frequency of each genotype was in bracket

The stability and population genetic analysis of OPAY02 molecular genetic markers: Comparing the detection results of RAPD molecular genetic markers with SCAR genetic markers of OPAY02, the results showed that the polymorphism of the amplifications of SCAR is identical to the one revealed by the RAPD marker on large molecular size band detection. Supposing SCAR detection technique was the standard methods, the false rate was 0.00%. One false positive case was appeared in 86 chickens RAPD gel electrophoresis of the small molecular size band, so its wrong judgment rate is 2.12%. Based on minor probability principle, the false positive case was impossible or the RAPD result was reliable in doing RAPD detection once. The conclusion of this study was SCAR marker was more suitable for genetic analysis. Dusan Tercic et al. (1998) selected two divergent chicken lines of high and low body weight respectively at 8 weeks of age, 3 kind of RAPD makers related to chickens body weight were detected. Paxton *et al.* (2005) found 19 AFLP makers associated with Round heart syndrome in turkeys, SCAR primers were designed from marker sequences with the hopes of designing a simple MAS strategy. Didion *et al.* (2000) converted RAPD marker DK122 into SCAR marker after sequencing the two ends of the polymorphic DNA fragments, the information about this DNA marker may be useful for building the porcine genetic and physical maps. Bello and Sanchez (1999) used SCAR makers to identify the stability of RAPD makers which was a sex-specific marker. Based on the results of this study, The SCAR marker can be used for marker-assisted selection.

The population of New Yangzhou chickens was in Genotype equilibrium at the two OPAY02 genetic loci. Based on animal breeding principle, it can be changed by selection. By improving the favored OPAY02 markers' frequency and reducing the unfavored, New Yangzhou chickens can grow fast at early growth stage. When the OPAY02 marker was converted into stable SCAR marker and applied to chicken body weight selection, it will be useful, because the detection of SCAR marker are not only simple for operate, quick for analysis, but also may improve the accuracy of selection, reduce the generation interval of breeding and save the breeding money.

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