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Analysis of the polymorphism of expressed sequence tag-Simple sequence repeat in quail

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ABSTRACT

Aiming at accelerating the application of molecular markers in the genetic improvement of quails, six EST-SSR markers were successfully developed using a bioinformatics method. Polymorphisms of three quail populations (Chinese yellow, China black and Korean quail) were detected. The results showed that there were 2-6 alleles in six EST-SSR markers. The mean polymorphism information contents of Chinese yellow , China blackand Korean quail were 0.5451, 0.4962 and 0.4937, respectively. The average heterozygosity values were 0.6134, 0.5759 and 0.5613. Among the six EST-SSR markers, three were highly polymorphicand the others were moderately polymorphic. The newly-developed six EST-SSR markers may be used to determine the genetic diversity of quails. The six EST-SSR markers identified were related to carbohydrate metabolism and melanin synthesis, but the specific mechanisms need to be further analyzed.

Key words: Bioinformatics, Chinese yellow quail, EST-SSR, Genetic diversity.

INTRODUCTION

Expressed Sequence Tag-Simple Sequence Repeat (EST-SSR) is a new type molecular marker developed on the basis of expression sequence by microsatellites. EST-SSR technology avoids the tedious steps of constructed genomic DNA library in the SSR development process. It can provide the absolute marker for gene function and fully reflect the similarity degree of the genomic functional area. Its polymorphism can better explain the phenotypic differences. The universality of SSR derived from traditional genomes is very poor between the species. As a part of genes, EST-SSR flanking sequences are highly conserved between species, and therefore, EST-SSR primers can be commonly used among species. So, EST sequences are valuable resources for SSR markers development. In recent years, with the comprehensive and depth research of different species, a great number of ESTs are accumulated.

The rapid growth of EST data provides a rich source for the development of SSR markers. SSRs developed from ESTs has been reported in many plants, such as grapes (Pen *et al*., 2005), wheat (Eujayi *et al*., 2002) and barley (Thie *et al*., 2003) and also in animals, such as zebra fish (Serapion *et al*., 2004), prawns (Perez *et al*., 2005, Zhang *et al*., 2010), catfish (Ju *et al*., 2005), sheep (Yan *et al*., 2007, Wang *et al*., 2010, Wang *et al*., 2011a, Wang *et al*., 2011b, Zhang *et al*., 2014), goat (Feng *et al*., 2008, Zhao *et al*., 2009)and silkworms (*Bombyx mori*; Mi *et al*., 2011). EST, as a new developed molecular marker, reflects the gene encoding part. EST expression information can be obtained directly through the study on EST, which lays the basis for studies on the functional genome and comparative genomics.

The objective of this study were to develop quail EST-SSR markers by bioinformatics methods, and to detect EST-SSR marker polymorphism in three quail populations, which can provide a new marker and theoretical basis for developing quail genetic diversity research, new strain identificationand evaluation and utilization of genetic resources.

MATERIALS AND METHODS

Materials: Seventy-five Chinese black , 75 Chinese yellow and 75 Korean quails were randomly chosen on the experimental pasture of Henan University of Science and Technology. Two ml of blood were collected from the heart. Acid citrate dextrose solution (ACD) was used as anticoagulation agent (blood:ACD = 6:1). Blood samples were stored in refrigerator at -20°C. Genomic DNA was extracted with the blood tissue genomic DNA extraction kit (Tiangen, Beijing, China).

EST-SSR screening: Firstly, all nucleic acid sequences and EST sequences were retrieved from NCBI public database GenBank at http://www.ncb.inlm.nih.gov/. Then, the microsatellites were searched from these quail sequences using the microsatellite on-line search tool SSRIT at http://www.gramene.org/db/searches/ssrtool. Finally, the homologous sequences of microsatellite markers were compared according to http://blas.tncb.inlm.nih.gov / Blas.tcgi.

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Primers: The following primer design criteria were applied: EST sequence longer than 100bp; the start and terminal ends of SSR sequences with not less than 20 BP from 5' and 3' ends respectively; primer GC content of 40%~60%, and annealing temperature of 55~56°C. These criteria were applied avoid primer dimer hairpin structure, mispairing, and continuous 6-base pairing as much as possible.

Polymorphism detection: Microsatellite primers were synthesized by Shanghai Sangon Biological Engineering Technology Co. The primer sequences are shown in Table 1. The total size of the PCR reaction system was 12.5μL, including 8.65μL of ddH_2O, 1.25μL of 10×buffer, 0.75μL of Mg^{2+}(25 mmol/L), 0.5μL of DNA template, 0.5μL (10 mmol/L) of upstream and downstream primers, 0.25μL of dNTPs, and 0.1μL of Taq enzyme. The PCR amplification process was as follows: denaturation for 3 min at 95°C, denaturation for 45 s at 94°C, annealing for 60s at X°C, extension for 60s at 72°C, and 30 cycles, extension for 12 min at 72°C, and preserving at 4°C. The annealing temperature is shown in Table 1. The PCR products were processed using 10% native polyacrylamide gel electrophoresis for 6~8h under stable voltage of 150~180V, and then fixed for silver nitrate staining, development, and other processes. Finally, imaging was performed with gel imaging system and Excel Microsatellite Toolkit was used to calculate the allele frequency and size range. The polymorphic information content (PIC), heterozygosity (He), and effective amount of alleles (Ne) were calculated using Dispan Software (Ota, 1993).

RESULTS AND DICUSSION

In total, 120 EST-SSR markers were discovered in the published EST quail sequences. The detection rate was 18.9%. The dinucleotide repeat motif, trinucleotide repeat motif, pentanucleotide repeat motif and tetranucleotide repeat motif accounted for 40.0%, 35.8%, 14.2% and 10.0% of the 120 EST-SSR markers respectively. The hexanucleotide repeat motif EST-SSR was not discovered. Ten eligible EST-SSR markers were selected for primer design and primer sequence syntheses in 120 EST-SSRs (Table 1).

The polymorphisms of ten EST-SSR markers (P1, P2, P3, P4, P5, P6, P7, P8, P9 and P10) were detected in the Chinese yellow, Chinese black and Korean quail. The polymorphism detection results of P2 and P3 in quail populations are shown in Figure 1 and Figure 2, respectively. As shown in Figure 1 and Figure 2, EST-SSR markers presented polymorphism in the quail populations. Polymorphisms of six EST-SSR markers (P1, P2, P3, P4, P5 and P6) were detected in the three evaluated quail populations. Polymorphism was not detected in the other markers.

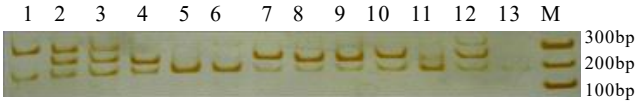

Fig 1: Electrophoresis of EST-SSR markers P2 in Chinese black quail

There were few alleles in which all six EST-SSR markers were detected in Chinese yellow quail, Chinese black quail and Korea quail populations. P2 detected the most alleles (6) while P4 and P6 detected the least (2). The mean polymorphism information contents of the six EST-SSR markers in Chinese yellow quail, China black quail and Korean quails were 0.5451, 0.4962, and 0.4937, respectivelt (Table 2). The mean heterozygosity values were 0.6134, 0.5759 and 0.5613, respectively. The polymorphism

Table 1: Relational information for microsatellite locus

Locus name	Repetitive sequence	Primer sequence(5'→3')	T_A(°C)
P1	(AG)9	CCTCCACACACCATAAAG CCACCATCACATCCATCTCG	55°C
P2	(CCAT)17	AGTGGCAGGTGTTAGTTGACG AGAGGATTGTGGATGGAA	56°C
P3	(AGG)8	CACTTCCCATAACCGTCCG TCACCACCACGTTCCTC	55°C
P4	(CA)13 (CG)4	TGTCTACTCGCAGGTCGG CGCCCTCCTCTATCCGT	56°C
P5	(AT)4 (TA)9	ACTTTGGAGAGGGTAGACAAT CTTCACCTTTGCCTTCA	56°C
P6	(CCA) 8	TCAGAACCACACGAGTCC TCGGAAAGCATAAAGGGA	56 °C
P7	(CAG) 3	TCCCACTTTTGCTGCCC TGGCTGCGCTTTGGAAG	57°C
P8	(GCA) 8	AAAGTGCTGAGTTATCTTCGC TGGCAGTGTTTCGTTCC	55°C
P9	(AGG) 8	GCGAGGAAGTGACTGCG GACGGTGATGGTGCTCA	57°C
P10	(CTTCC) 48	CACTGAGCGCAGGTAGG GCACAGACAGAGGGCAA	55°C

Table 2: Polymorphism information content(PIC) of microsatellite loci

Populations	Locus name	Number of alleles locus (Na)	Effective number of alleles (Ne)	Obs.Hom.	Obs.Het.	Exp.Hom.	Exp.Het.	Polymorphism information content(PIC)	Chi-square
Chinese yellow quails	P1	5	3.7520	0.0500	0.9500	0.2604	0.7396	0.6872	52.1835**
	P2	6	4.7904	0.1333	0.8667	0.2021	0.7979	0.7605	69.3262**
	P3	4	2.0815	0.6833	0.3167	0.4761	0.5239	0.4779	60.2635**
	P4	2	1.9651	0.1333	0.8667	0.5048	0.4952	0.3705	34.3432**
	P5	6	3.1386	0.3167	0.6833	0.3129	0.6871	0.6356	105.9782**
	P6	2	1.7630	1.0000	0.0000	0.5636	0.4364	0.3391	61.3426**
	Mean	4.1667	2.9151	0.3861	0.6139	0.3866	0.6134	0.5451	
Chinese black quails	P1	5	3.4918	0.3636	0.6364	0.2809	0.7191	0.6606	166.9113**
	P2	6	3.4476	0.2576	0.7424	0.2846	0.7154	0.6680	76.2231**
	P3	4	2.2264	0.9848	0.0152	0.4449	0.5551	0.4540	272.4943**
	P4	2	1.9551	0.1515	0.8485	0.5077	0.4923	0.3692	35.1228**
	P5	6	2.2541	0.4545	0.5455	0.4394	0.5606	0.4996	37.6175**
	P6	2	1.6949	1.0000	0.0000	0.5869	0.4131	0.3260	67.4745**
	Mean	4.1667	2.5117	0.5354	0.4646	0.4241	0.5759	0.4962	
Korean quails	P1	4	2.3000	0.2800	0.7200	0.4287	0.5713	0.4982	47.9561**
	P2	6	2.9727	0.7400	0.2600	0.3297	0.6703	0.6098	93.3910**
	P3	5	3.5186	0.8800	0.1200	0.2770	0.7230	0.6669	166.4048**
	P4	2	1.9802	0.1000	0.9000	0.5000	0.5000	0.3725	32.6666**
	P5	5	3.1526	0.3200	0.6800	0.3101	0.6897	0.6260	89.2602**
	P6	2	1.2677	1.0000	0.0000	0.7867	0.2133	0.1889	54.0689**
	Mean	4	2.5323	0.5533	0.4467	0.4387	0.5613	0.4937	

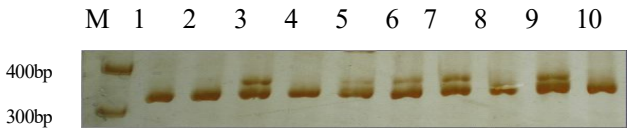

Fig 2: Electrophoresis of EST-SSR markers P3 in Chinese yellow quail

information content and the heterozygosity value of Chinese yellow quails were slightly higher than those of the other two quail populations, suggesting that the genetic diversity of Chinese yellow quails is more abundant. The Hardy-Weinberg equilibrium test showed that the six EST-SSR markers in the three evaluated quail populations deviated significantly from the Hardy-Weinberg law ($P<0.01$).

Table 3 shows that the minimum genetic distance between Chinese black quails and Korean quails is 0.1092, suggesting that Chinese black quails and Korean quails present the closest relationship. The genetic distance between Chinese yellow quails and Korean quails was larger, 0.1359. As shown in Figure 1, firstly Chinese black quail gathered with Korea quail and then gathered with Chinese yellow quail.

The microsatellite polymorphism derived from the variation of the core sequence repetitive number. Generally speaking, the more the repetitive number, the bigger the variation, the higher the number of alleles at this locus and the stronger is the polymorphism. For the microsatellite with the same repeat unit, the repetitive number of mammalian microsatellite is generally higher than that of poultry. For example, the repetitive number of the TG microsatellite is only 4~14 in poultry, but more than 20 in mammals (human and mouse) in general. The polymorphism provided by microsatellite markers in mammals is also very high, which is also the advantage of microsatellite markers. Six EST-SSR markers were firstly developed in three quail populations of Chinese black quails, Chinese yellow quails, and Korean quails in this study. The average number of EST-SSR alleles in the above three quail populations were 4.1667, 4.1667 and 4.000, respectively. The average number of effective alleles were 2.5117, 2.9151 and 2.5323, respectively. The average number of alleles and effective alleles of quail EST-SSR were higher than those of shrimp, developed by Zhang *et al*. (2010), who obtained 2.4000±0.5477 alleles and 2.0600±0.3287 effective alleles, but lower than that of sheep (3.4266), as determined by Wang et al. (2011a). In the present study, except for P6, which was lowly polymorphic, the other five EST-SSR markers were moderately or highly polymorphic in the three quail populations, suggesting that the six newly developed EST-SSR markers may be used as a new molecular marker and are suitable to analyze the genetic diversity of quail populations.

As a part of functional gene, the variation of EST-SSR markers might be correlated with phenotypic traits, which make them valuable for functional markers development. Zhang *et al*. (2014) analyzed the correlation between 6 EST-SSR markers and litter size and birth weight in sheep. The results showed that the locus 114719418 was correlated with the average lamb birth weight and birth weight; 114720463 and 114758122 loci were correlated with total lamb birth weight and litter size, respectively; 114719622 and 114722561 loci were correlated with the total birth weight; and 47952182 locus was correlated with litter size. The EST-SSR marker loci may be closely linked with the control of the sheep birth weight and litter. The study of Wang *et al*. (2011b) showed sheep EST-SSR markers locus 33176918 and locus 33176988 had a significant genetic effect on wool natural length and average fiber diameter, respectively ($P<0.05$). These two loci may be closely linked with the control of wool traits loci.

The comparison between the six EST-SSR markers developed in the present study with the NCBI website showed that EST-SSR marker P1 and quail melanocortin 4 receptor (MC4R) genes are homologous; P2 and transcription factor Fli are homologous; P3 and GAA1 (acid a-glucosidase) genes are homologous; P4 and TOJ3 genes are homologous; P5 and Brn-1 genes were homologous (1, 2, 8-trihydroxynaphthalene reductase gene); and P6 and aspartic acid are homologous. Therefore, it is speculated that these six EST-SSR markers are related to carbohydrate metabolism and melanin synthesis in quails, but the specific mechanisms still needs to be further analyzed.

The current abundance of EST data resources enable development of EST-SSR markers become simple, quick and effective. So this marker has received extensive attention and has been widely applied. At present, the research on quails is not well-developed in China compared with other livestock and poultry; in particular, the research on molecular genetic markers is still at the initial stage. The advantages of the use of EST-SSR in genomics are obvious.

Table 3: genetic distance and similarity coefficient

Populations	Chinese yellow quails	Chinese black quails	Korean quails
Chinese yellow quails		0.8729	0.8355
Chinese black quails	0.1359		0.8966
Korean quails	0.1797	0.1092	

Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

With the implementation of animal, plant, and other model organism genome projects, studies on functional genomics will be the next focus of research. Although the technique of EST-SSR markers has just started to develop, research using this tool is still at the initial stageand its range of application is still relatively limited, it has received widespread attention from the scientific community.

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