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Consistent effects of single and combined SNP(s) within bovine paired box 7 gene (*Pax7*) on growth traits

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Introduction

Paired box 7 (*Pax7*) gene, a member of paired box gene family, is the identification gene of myogenic satellite cells and participates in multistep processes of myogenesis (Le Grand and Rudnicki 2007). Previous studies have identified that there is high expression of Pax7 protein in quiescent, active and proliferating satellite cells (Collins *et al.* 2005; Relaix *et al.* 2006). Consistent with the localization and characteristics, Pax7 plays important regulatory roles in satellite cell regeneration, survival, and anti-apoptosis as well as selfrenewal (Kuang *et al.* 2007; Morrison *et al.* 2010). In addition, as a transcriptional factor, Pax7 also specifies the satellite cells destined to enter the myogenic programme (Le *et al.* 2009), because it triggers the expression of myogenic determination factor and myogenic factor 5, which directly play critical roles in growth and muscle development in animals (Muroya *et al.* 2002). Satellite cells contribute efficiently to postnatal myogenesis and they are localized by the expression of Pax7, leading to the proposal that Pax7 is essential for postnatal skeletal muscle development (Kuang *et al.* 2006).

It has been reported that the Pax7-absent mice are significantly smaller, compared to their wildtype and heterozygous counterparts (Seale *et al.* 2000). In humans, 75 single-nucleotide polymorphisms (SNPs) of *Pax7* gene within the intragenic region have been identified, and they are associated with alveolar rhabdomyosarcoma which seriously inhibits the normal development of skeletal muscle (Syagailo *et al.* 2002; Davicioni *et al.* 2009). Cumulatively, these studies indicate that *Pax7* could be considered as a candidate gene for growth traits due to its fundamental roles in muscle development.

In this study, to assess whether *Pax7* gene could have significant polymorphisms for marker-assisted selection (MAS), we first investigated SNPs of *Pax7* gene in 1226 cattle from five indigenous Chinese breeds by DNA pool, sequencing and PCR-RFLP methods, and then evaluated their effects on growth traits. These will provide some useful information for MAS programmes in cattle breeding industry.

Materials and methods

Experimental animals and DNA preparation

A total of 1226 cattle including five Chinese indigenous breeds (Nanyang cattle (NY, $n = 222$); Jiaxian cattle (JX, $n = 398$); Qinchuan cattle (QC, $n = 210$); Luxi cattle (LX, $n = 163$); Chinese Red Steppe cattle (CRS, $n = 233$)) were used in this study for initial SNP discovery. All these cattle represent the main breeds of P. R. China and are reared in the provinces of Henan, Shaanxi, Shandong and Jilin, respectively. Genomic DNA was isolated from blood samples as per the procedure described by Sambrock and Russell (2001).

Primer designing

Nine primer pairs were designed based on NCBI database (GenBank accession number NC_007300) to amplify all nine exons and partial intron regions of bovine *Pax7* gene. The detailed information about oligonucleotide primers, amplicon sizes and corresponding annealing temperatures are given in table 1.

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Table 1. Primers information of bovine *Pax7* gene.

Locus	Primer sequences (5′ to 3′)	Size (bp)	T_m (°C)	Note (ref. NC_007300)
P1	E1F: GAAGAAGAGGAAAGAAAGAG E1R: AAATAGTGGAATGAGTTGAGA	501	57	Exon 1 and partial intron 1 (1–501)
P2	E2F: CCAGTCCACAGAGCCATCA E2R: CCGCCACCACTATTCCAG	944	65.2	Exon 2 and partial intron 1,2 (2337–3280)
P3	E3F: TCTCCCACCTCCACCTCT E3R: CTCCCTTCTTCCCTGCTC	385	63.2	Exon 3 and partial intron 2,3 (3501–3885)
P4	E4F: ACCGACTGACTGAGGGTATGTG E4R: GATGCTGTGCTTGGCTTTCTT	418	65.2	Exon 4 and partial intron 3 (5066–5483)
P5	E5R: GGTTGCCATTGCCTCCT E5R: TGTGCTATGGTGCGTGCT	632	66	Exon 5 and partial intron 4,5 (56338–56969)
P6	E6F: GAGGAGGACACTGGCTGGAC E6R: CATCAACGCAGACAAACACG	482	58.1	Exon 6 and partial intron 5,6 (64539–65020)
P7	E7F: CAATCACGGAGAACAGGG E7R: AGAACAAAGCCGCAACC	930	63.5	Exon 7 and partial intron 6,7 (65762–66691)
P8	E8F: GCCAGGTAAACGGAGGAT E8R: GGGTCCAGGGCTGTATCA	589	63.5	Exon 8 and partial intron 7,8 (96622–97210)
P9	E9F: CAGAACAGGGATGCGTAG E9R: GATGGAGGGACTGAGGAT	638	62	Exon 9 and partial intron 8 (103395–104032)

SNPs identifying and genotyping

Five DNA pools were constructed with 80–100 individual DNA samples that were randomly chosen from each cattle breed, respectively (Sham *et al.* 2002). Using the DNA pools as PCR templates, amplifications were carried out and then PCR products were sequenced for screening of DNA polymorphisms. *Hin*fI, *Bsp*T104I, *Pst*I, and *Apa*I PCR-RFLP methods were carried out for genotyping SNP loci within bovine *Pax7* gene.

Statistical analysis

Genotypic and allelic frequencies at four polymorphic loci of bovine *Pax7* gene were directly calculated. The frequencies of haplotypes among the polymorphic SNPs in five cattle breeds can be inferred by PHASE software version 2.1 (Stephens and Donnelly 2003). Population genetic parameters, namely, gene homozygosity (H_o) and effective allele numbers (N_e) were calculated using the PopGene software version 3.2 (http://www.exetersoftware.com/cat/Trinity/popgene.html) and the polymorphism information content (PIC) was calculated using the method of Nei and Roychoudhury (1974).

To evaluate the relationships between genotypes of each SNP or diplotypes of several SNPs and growth traits in NY population, marker-trait association analysis was carried out using the least-squares method (LSM) as applied in the general liner models (GLM) procedure of SAS (SAS 2009).

Results

SNP genotyping

Amplification, DNA pool, sequencing and PCR-RFLP methods were applied to identify the polymorphisms of nine fragments (P1 to P9) including nine exons and partial introns of *Pax7* gene among five different cattle breeds. Four mutations were identified: NC_007300: g.3531G>A, g.56626G>A, g.66117C>T and g.66251A>G, which were located at exons 3, 5, 7 and intron 7, respectively. These polymorphic sequences were deposited in the GenBank database (accession numbers: HM804489–HM804496). Among the above mentioned four SNPs, three involving base transitions/transversions revealed synonymous mutations (g.3531G>A (110Ala), g.56626G>A (237Glu) and g.66117C>T (343Ala)).

The four polymorphic loci could be genotyped by the methods of *Hin*fI, *Bsp*T104I, *Pst*I and *Apa*I PCR-RFLP. Here, the four SNPs (g.3531G>A, g.56626G>A, g.66117C>T and g.66251A>G) within P3, P5, P7 and P7 were named *Hin*fI, *Bsp*T104I, *Pst*I and *Apa*I loci, respectively.

Genetic diversity and haplotype analysis

Four SNPs were examined to investigate genotypic and allelic frequencies of *Pax7* gene (table 2). The results showed that all polymorphisms distributed unevenly in the test breeds. G allele was predominant at both *Hin*fI and *Bsp*T104I loci in NY, JX, QC and LX breeds as compared with A allele in CRS cattle, and at *Apa*I locus, the mutant allele G frequency was obviously higher than the A allele in all five breeds. Interestingly, the TC genotype was not found at *Pst*I locus in CRS cattle and the frequency of T allele was close to zero (0.009), which suggested that C allele may be more adapted for the steppe environment.

In addition, the analysis of haplotypes in four SNPs revealed a general consensus that the haplotypes GGTG and GGCG were predominant in NY, JX, QC and LX cattle breeds as compared with the haplotype AACA in CRS breed (data not shown). While taken all breeds together, GGTG, GGCG and AACA were most abundant haplotypes that accounted for 51.7% of total variability.

Table 2. Genetic diversity of four *Pax7* loci in five Chinese indigenous breeds.

Locus	Breed	Genotypic frequency			Allelic frequency		N_e	H_o	PIC
		AA	AG	GG	A	G			
P3 (*Hin*fI)	NY (222)	0.059	0.554	0.387	0.336	0.664	1.805	0.554	0.347
	JX (398)	0.028	0.724	0.248	0.389	0.611	1.907	0.524	0.362
	QC (210)	0.048	0.671	0.281	0.383	0.617	1.897	0.527	0.361
	LX (163)	0.086	0.626	0.288	0.399	0.601	1.921	0.520	0.365
	CRS (233)	0.515	0.137	0.348	0.584	0.416	1.945	0.514	0.368
		AA	AG	GG	A	G			
P5 (*Bsp*T104I)	NY (222)	0.230	0.351	0.419	0.405	0.595	1.931	0.518	0.366
	JX (398)	0.191	0.317	0.492	0.349	0.651	1.833	0.545	0.351
	QC (210)	0.205	0.333	0.462	0.371	0.629	1.876	0.533	0.358
	LX (163)	0.233	0.380	0.387	0.423	0.577	1.954	0.512	0.369
	CRS (233)	0.438	0.442	0.120	0.659	0.341	1.817	0.550	0.349
		TT	TC	CC	T	C			
P7 (*Pst*I)	NY (222)	0.383	0.216	0.401	0.491	0.509	1.999	0.500	0.375
	JX (398)	0.312	0.095	0.593	0.359	0.641	1.853	0.540	0.354
	QC (210)	0.347	0.143	0.510	0.419	0.581	1.949	0.513	0.368
	LX (163)	0.460	0.172	0.368	0.546	0.454	1.983	0.504	0.373
	CRS (233)	0.009	0.000	0.991	0.009	0.991	1.017	0.983	0.017
		AA	AG	GG	A	G			
P7 (*Apa*I)	NY (222)	0.063	0.590	0.347	0.358	0.642	1.851	0.540	0.354
	JX (398)	0.035	0.676	0.289	0.373	0.627	1.879	0.532	0.358
	QC (210)	0.053	0.671	0.276	0.388	0.612	1.905	0.525	0.362
	LX (163)	0.043	0.638	0.319	0.362	0.638	1.858	0.538	0.355
	CRS (233)	0.335	0.180	0.485	0.425	0.575	1.956	0.511	0.369

The values in parentheses indicate the number of animals investigated in five breeds.

The H_e, H_o, N_e and PIC were calculated (table 2), which revealed that all five breeds at *Hin*fI, *Bsp*T104I, *Pst*I and *Apa*I loci possessed middle genetic diversity ($0.250 < \text{PIC} < 0.500$) except for the CRS population (low genetic diversity, $\text{PIC} < 0.250$) at *Pst*I locus.

Association analysis of genotypes in single SNP with growth traits in NY cattle

Comparisons of growth traits between different genotypes in single SNP (*Hin*fI, *Bsp*T104I, *Pst*I and *Apa*I loci) were performed using phenotypic data of 222 NY cattle during the test periods (6, 12, 18 and 24 months of life) and the results are shown in table 3. For the *Hin*fI locus, at the age of six months, cattle with AG genotype appeared superior in body weight and average daily gain as those compared with GG and AA genotypes ($P < 0.05$). At *Apa*I locus, significant differences of body weight and average daily gain were observed among cattle only with A allele (AA genotype) and those with G allele (GG and AG genotypes) ($P < 0.05$). On the other hand, cattle aged 12, 18 and 24 months revealed no significant association with all considered traits at *Hin*fI and *Apa*I loci ($P > 0.05$, data not shown), respectively. Moreover, at the age of 6 and 12 months, chest girth of cattle carrying the TC genotype was higher than in those of CC and TT genotypes for *Pst*I locus ($P < 0.05$), and there was a tendency that TC genotype individuals had better body weight than CC and TT genotypes, although no significant differences appeared ($P > 0.05$), and significance also had not been found at the age of 18 and 24 months ($P > 0.05$, data not shown). The *Bsp*T104I locus had nonsignificant association with all traits measured ($P > 0.05$, data not shown).

Association analysis of diplotypes (haplotype combinations) with growth traits in NY cattle

To further ascertain the association between diplotypes of the significant SNPs (*Hin*fI, *Pst*I, and *Apa*I loci) and growth traits in NY cattle, the haplotypes of three SNPs were constructed at first. Herein, seven haplotypes (GTG, GCG, ACG, GCA, ACA, ATG and GTA) were identified in all individuals and their frequencies were 0.399, 0.198, 0.187, 0.146, 0.043, 0.020 and 0.007, respectively. Because the frequencies of ATG and GTA were small, our association analysis for the effect of diplotype excluded their related diplotypes and the percentages of observations < 5% were also excluded. Therefore, five haplotypes were involved and described as: type B (GTG), type D (GCG), type E (ACG), type F (GCA) and type H (ACA), and eight diplotypes were used for association analysis (table 3). When in combination, three SNPs displayed the consistent significant effects on BW6 and ADG6 with single SNP, which revealed that BH (AGTCAG) was better than all other diplotypes, particularly DD

Table 3. Associations of single and combined SNP(s) within *Pax7* gene with growth traits in NY cattle.

Loci	Genotype	Growth trait			
		BW6 (kg)	ADG6 (kg)	BW12 (kg)	ADG12 (kg)
*Hin*fI (Mean±SE)	AA	161.333±7.452^{ab}	0.728±0.040^{ab}	223.333±9.494	0.344±0.049
	AG	164.604±2.635^{a}	0.747±0.140^{a}	225.417±3.357	0.338±0.017
	GG	153.652±2.691^{b}	0.688±0.140^{b}	219.174±3.429	0.364±0.018
P value		0.017	0.016	0.430	0.568
		BW6 (kg)	CG6 (cm)	BW12 (kg)	CG12 (cm)
*Pst*I (Mean±SE)	TT	160.000±2.832	128.864±1.092^{ab}	223.412±3.980	141.864±1.141^{ab}
	TC	162.088±3.222	130.235±1.242^{a}	224.795±3.498	142.765±1.298^{a}
	CC	153.909±4.005	126.273±1.544^{b}	216.136±4.947	137.364±1.631^{b}
P value		0.275	0.048	0.348	0.028
		BW6 (kg)	ADG6 (kg)	BW12 (kg)	ADG12 (kg)
*Apa*I (Mean±SE)	AA	186.667±10.548^{a}	0.859±0.057^{a}	227.412±7.232	0.465±0.068
	AG	161.417±3.045^{b}	0.728±0.016^{b}	224.694±3.609	0.352±0.020
	GG	156.820±2.339^{b}	0.707±0.013^{b}	218.721±2.772	0.344±0.015
P value		0.018	0.028	0.247	0.231
	Diplotype	BW6 (kg)	ADG6 (kg)	CG6 (cm)	
*Hin*fI-*Pst*I-*Apa*I	EF (0.16)	160.938±4.587^{a}	0.728±0.025^{a}	128.062±1.607^{abc}	
	DF (0.10)	162.800±5.803^{a}	0.741±0.031^{a}	130.688±1.607^{ab}	
	BH (0.06)	164.667±7.491^{a}	0.747±0.041^{a}	130.167±2.625^{ab}	
	BF (0.16)	162.188±4.587^{a}	0.732±0.025^{a}	131.400±2.033^{a}	
	DD (0.08)	140.500±6.487^{b}	0.621±0.035^{b}	122.500±2.273^{c}	
	BE (0.08)	160.000±6.487^{a}	0.723±0.035^{a}	129.750±2.273^{ab}	
	BD (0.06)	162.000±7.491^{a}	0.729±0.041^{a}	129.667±2.625^{ab}	
	BB (0.16)	152.375±4.587^{ab}	0.684±0.025^{ab}	124.312±1.607^{bc}	
P value		0.033	0.021	0.017	

BW6, ADG6 and CG6 represent body weight, average daily gain and chest girth of cattle aged six months; BW12, ADG12 and CG12 represent body weight, average daily gain and chest girth of cattle aged 12 months, respectively. The values in parentheses indicate the frequencies of diplotypes in investigated individuals, and values with different superscripts (a, b, c) within the same column differ significantly at $P < 0.05$. SE, standard error of means.

(GGCCGG). In addition, there was also significant association with CG6 ($P < 0.05$), showing that cattle with the BF (GGTCAG) diplotype had longer chest girth than others. However, significant differences were not found in the rest traits of cattle aged 12, 18 and 24 months ($P > 0.05$, data not shown). All these results showed that the combined effects of the three SNPs were consistent with single SNP.

Discussion

In previous study, *Pax7* gene had been mapped that it was located on the linkage group RT-12 which was the strongest body weight QTL region in rainbow trout (Wringe *et al.* 2010). Additionally, other studies had reported several QTL for growth and production traits were close to *Pax7* gene (Bhuiyan *et al.* 2009; Wringe *et al.* 2010). In the present study, the associations of the variations within *Pax7* gene with growth traits in cattle proved the above prediction, and the effects of genotypes on growth traits were significantly different at *Hin*fI, *Pst*I and *Apa*I loci.

Growth traits are physiological functions under the control of multiple genes. Those genes that have been proved to be significant correlation with related traits can be candidates in the successful application of MAS. When used in practice, the positive effect genes should be chosen simultaneously as molecular markers. Here, *Pax7*, could be used in early selection of beef cattle. *Pax7* gene mainly participates in the early development of animals, and particularly regulates the myogenesis of skeletal muscle in somites and limbs (Morrison *et al.* 2010). Previous studies reported that the Pax7-absent mice were normal at birth, but the body weight was decreased at 1–2 weeks old (Mansouri *et al.* 1996; Seale *et al.* 2000). It was attributable to the absence of myogenic cells and lack of satellite cell fusion during the postnatal growth of muscle (Seale *et al.* 2000). Consistent with that, here we report the SNPs revealed significant association with growth traits in cattle aged 6 and / or 12 months, while no significance was found in cattle aged 18 and 24 months.

The growth traits are complex quantitative traits that are not only affected by single SNPs, but also by SNP–SNP interaction of candidate genes (Orozco *et al.* 2009). So, the associations were analysed between the combination of SNPs (diplotypes) and growth traits. The results showed that the combined SNPs had consistent significant effects on growth traits with single SNP. Cattle with diplotype BH

(AGTCAG) showed better body weight and average daily gain than others. The result was in agreement with Srikanchai *et al.* (2010) who reported that diplotype CAG/CGG had better meat quality traits as with the involved single SNPs.

In conclusion, four novel polymorphisms were identified in the bovine *Pax7* gene in this study. The association analysis of genotypes in the single and combined SNP(s) revealed consistent effects on growth traits in NY cattle. Therefore, they could be used as molecular markers for better growth traits of cattle by early MAS.

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