

Associations of new rSNPs with eggshell thickness in Rhode Island layers*

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Six fragments were sequenced of the regulatory and coding regions of the expressed sequence *ChEST985k21* (accession number CR523443), which had been shown to affect eggshell thickness. Six sites of single nucleotide polymorphism (SNP) were found, five of them located in the regulatory region and one within the ORF. The binding sites of transcriptional factors for all obtained SNPs were revealed to be present in one allelic variant. Based on this fact they were considered as rSNPs. Genotyping of 46 Rhode Island Red birds with thick ($390 \pm 13 \mu\text{m}$) and 45 with thin eggshell ($316 \pm 21 \mu\text{m}$) was done. Significant difference in allelic frequency was shown of rSNPs ST2_1, ST3_1, ST3_2, ST3_3. Genotypic classes of rSNPs ST2_1, ST3_1, ST3_2, ST3_3 and ST6_1 were shown to have significantly different shell thickness. These data could probably provide a tool for marker-assisted selection for optimization of egg shell thickness in Rhode Island layers.

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Identification of genes and alleles affecting economically important traits is one of the most important approaches in farm animal genomics. Usually such traits are characterised by wide variation of gene expression and associated with quantitative trait *loci* (QTLs) – Cheng *et al.* [1995].

Association studies using high resolution genetic maps allowed to dissect complex quantitative traits including growth [Groenen *et al.* 1997; van Kaam *et al.* 1998; Tatsuda *et al.* 2001], feeding efficiency [van Kaam *et al.* 1999a], carcass quality [Vallejo *et al.* 1998], Marek's disease resistance [Vallejo *et al.* 1998; Xu *et al.* 1998; van Kaam *et al.* 1999a], adiposity [Lipkin *et al.* 2002], laying performance [Kawka *et al.* 2012] and egg quality [Ikeobi *et al.* 2002]. Tuiskula-Haavisto *et al.* [2002] carried out the whole genome scan for detection and localization of QTL for layer performance and egg quality traits. These authors detected 14 chromosomal regions affecting egg white thinning, eggshell strength, egg weight, age at first egg, number of eggs, body weight, and feed intake. Six suggestive QTL were located on 2, 3, 4, 5, 8 and Z chromosomes. The most significant results were found for GGA4, where QTL of body weight, egg weight and feeding efficiency were located. Another whole genome scan was done in Green-Legged Partridge (GLP), a native Polish chicken breed maintained as a conservative flock, and a highly productive stock of Rhode Island Red (RIR) – Wardęcka *et al.* [2002]. The significant effect of the genotype (GLP-GLP, RIR-RIR, and GLP-RIR) on egg laying performance and egg quality was found with 23 microsatellite *loci*.

Earlier we have reported data on positional cloning of QTL of shell thickness at week 53 of lay age (ST53) on GGA4 [Sazanov *et al.* 2005, Sazanov *et al.* 2007]. Thirteen BAC-clones were revealed to contain microsatellites *MCW0114* and *ADL0241* which mark boundaries of the QT affecting region. Twenty expressed sequence tags (EST) and were located in the GGA4 region 16,137,294 to 17,826,844 (GGA4q11-12, *MCW0114* - *ADL0241*) in genome sequence of chicken [<http://www.ncbi.nlm.nih.gov/genome/guide/chicken>]. Eight of them were not expressed in chicken oviducts. Expression of 12 positional candidates for QTL shell thickness at week 53 of lay age was investigated by real-time PCR in distal part of chicken oviduct (uterus) with forming eggshell [Sazanov *et al.* 2007]. In the GLP chicken the cDNA *ChEST985k21* (accession number CR523443) was down-regulated with ratio of means 0.49 ($P \leq 0.01$) in the group with low ST53 relative to the group with the highest ST53. Expression of this gene was highly correlated ($r = 0.85$; $P \leq 0.01$) with shell thickness. These data suggested that CR523443 was a candidate gene for QTL ST53 in the chicken [Sazanov *et al.* 2007]. The 2,102 bp sequence CR523443 (*Gallus gallus* finished cDNA, clone *ChEST985k21*) was primarily found in mRNA extracted from adult muscle and then identified in brain, cartilage and female genital tract [<http://www.ncbi.nlm.nih.gov/UniGene>].

Here we report associations of new rSNP located in regulatory and coding regions of the *ChEST985k21* gene with shell thickness in Rhode Island layers.

Material and methods

Animals

Three birds with high ST53 (STH) and 3 birds with low ST53 (STL) from GLP (Green Legged Partridge) were used for DNA-sequencing. The ST53 means with SD values were as follows: $372 \pm 2 \mu\text{m}$ (GLP STH), and $249 \pm 17 \mu\text{m}$ (GLP STL). DNA-samples were prepared from the same animals which were used for expression profiling of candidate genes for ST53 [Sazanov *et al.* 2007]. Six fragments of the regulatory and coding regions of the expressed sequence *ChEST985k21* (accession number CR523443) which was previously shown to affect eggshell thickness, were sequenced.

Ninety one DNA samples used for genotyping were isolated from the two groups of Rhode Island Red breed (RIR) with shell thickness $390 \pm 13 \mu\text{m}$ (46 individuals), and $316 \pm 21 \mu\text{m}$ (45 individuals), kindly provided by the Institute of Farm Animal Genetics and Breeding, Russian Academy of Agricultural Science.

DNA sequencing

The following primers were used for DNA amplification and sequencing of the regulatory and coding regions of the expressed sequence *ChEST985k21* (annealing temperature 60°C):

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ST53Seq_1_FW: AGCAAAGGCAAGCTCGTAAG
ST53Seq_1_RV: TTTTCTGCTTTAGAAGTCCTGCTT
ST53Seq_2_FW: AGTTGCTGAGTACCTCAAACACTACA
ST53Seq_2_RV: TTCCAAGGTAAAGACAGAAGTCG
ST53Seq_3_FW: TGTGAAATGTAAGGGGGTTG
ST53Seq_3_RV: AGCAGAACCTCCAAACTCCA
ST53Seq_4_FW: CTCAGGATGGTGGAGTTTGG
ST53Seq_4_RV: TGCGACAGGAATCCAATACA
ST53Seq_5_FW: TGGATTCCTGTCGCATTAGTC
ST53Seq_5_RV: GCAAACACCTCGATTGCTCT
ST53Seq_6_FW: GTGCTGAGTATGGGGTGCTT
ST53Seq_6_RV: TCAAGGCTTCAACGTCCTC
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The PCR reaction conditions consisted of an initial 5-min hold at 95°C, 30 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 1 min and finally 72°C for 7 min in a iQ5 (BIORAD, USA).

Sequencing reaction was conducted with Big Dye Terminator Sequencing Mix (APPLIED BIOSYSTEM, USA) according to the manufacturer instructions. The electrophoretic separation of the nucleotide fragments and analysis of their nucleotide sequences were made automatically by means of AB3130 (APPLIED BIOSYSTEM, USA).

Search for binding sites of transcriptional factors

The binding sites of transcriptional factors of six SNPs obtained were found using TESS software [<http://www.cbil.upenn.edu>] based on the assumption that targeted sequence should be absolutely complimentary to a sequence represented in TRANSFAC v.6 data base with maximum rigorous selection criterion.

Genotyping

Below are given the allele-specific primers (annealing at 60°C), used in this study.

1. ST2_1_Up_T: CTGCTCAGTGTCTTAGTCTGATCAGT
ST2_1_Up_C: CTGCTCAGTGTCTTAGTCTGATCAGC
ST2_1_Dn: ACAGTCATGATGAGGAAACAGG
2. ST3_1_Up_G: GCGACTCACTCACTCATTTTTG
ST3_1_Up_A: GGCGACTCACTCACTCATTTTA
ST3_1_Dn: AGCAGAACCTCCAAACTCCA
3. ST3_2_Up_T: TTGCTCTCATTAATTAATGAGTTCAGT
ST3_2_Up_C: TTGCTCTCATTAATTAATGAGTTCAGC
ST3_2_Dn: AGAAGGAAGAGAGGTATCAACCAGT
4. ST3_3_Up_T: TGGTCACAGGTGCCTGTAAAT
ST3_3_Up_C: TGGTCACAGGTGCCTGTAAAC
ST3_3_Dn: CATCCTGAGGCACTGCATATT
5. ST3_4_Up_T: ATAGCCCAATCCTGCAGAATT
ST3_4_Up_C: ATAGCCCAATCCTGCAGAATC
ST3_4_Dn: AGCAGAACCTCCAAACTCCA
6. ST6_1_Up_T: TGAGCTTATGAATCTTGCAGTTTTT
ST6_1_Up_C: TGAGCTTATGAATCTTGCAGTTTTT
ST6_1_Dn: TCAACATTGTGGAAAGTAGATACAGA

The PCR conditions consisted of initial 5-min hold at 95°C, 30 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 1 min and finally 72°C for 7 min in a iQ5 (BIORAD, USA).

The statistical estimation of results was carried out through comparison of allelic frequencies in the group with thin and thick eggshell by means of χ^2 criterion and comparison eggshell thickness in different genotypic classes by t-test.

Results and discussion

Six sites of single nucleotide polymorphism (SNP) were found, five of them (S2_1, S3_1, S3_2, S3_3 и S3_4) in regulatory region and one (S6_1) within ORF of the expressed sequence *ChEST985k21* (Tab. 1). Six binding sites covering described SNPs were detected – AP4, F2F, AML1, POUR1F1a, YY1, EFII, MBF-1 – Table 1. The binding sites of transcriptional factors of six SNPs obtained were found based

on the assumption that targeted sequence should be absolutely complimentary to a sequence represented in TRANSFAC v.6 data base with maximum strict options. The binding sites of transcriptional factors for all obtained SNPs were revealed to be present only in one allelic variant.

Table 1. Binding sites of SNP transcriptional factors of regulatory and coding regions of the expressing sequence *ChEST985k21*. Polymorphic nucleotides marked in bold

SNP	Sequence	Distance from the start codon of <i>ChEST985k21</i> (bp)	Transcriptional factors
S2_1	GATCAGYAGTAAA	-958	AP4
S3_1	CATTTTRCTCTCA	-190	F2F
S3_2	GTTCAGYGTGGTC	-288	AML1
S3_3	TGTAAAYGCAGCA	-267	POUR1F1a, YY1
S3_4	CAGAATYGCACAG	-236	EFII
S6_1	AGTTTTYGGGTGC	+1920	MBF-1

Table 2. Numbers of alleles of the investigated rSNPs in the groups with thin and thick eggshell

Layer's groups	rSNP											
	ST2_1*		ST3_1**		ST3_2*		ST3_3		ST3_4		ST6_1	
	C	T	A	G	C	T	C	T	C	T	C	T
Thin eggshell (315.7±21.38 µm)	22	68	30	60	64	26	32	58	43	47	49	41
Thick eggshell (389.9±13.09 µm)	35	65	21	77	60	38	39	63	41	45	42	52

*P<0.05; **P<0.01.

Genotyping was done of 46 Rhode Island Red birds with thick (390±13 µm) and 45 with thin eggshell (316±21 µm). Significant difference in allelic frequency of rSNPs ST2_1, ST3_1, ST3_2 and 3, ST3_3 was shown in between these groups of birds (Tab. 2). Birds from different genotypic classes of rSNPs ST2_1, ST3_1, ST3_2, ST3_3, ST6_1 were shown to have significantly different shell thickness (Tab. 3).

Sequencing of regulatory and coding sequences of EST *ChEST985k21* allowed us to identify six QTN (single nucleotide polymorphism affecting quantitative trait), which could be a tool for establishing of the system of SNPs for marker assisted selection for optimization of egg shell thickness in Rhode Island layers.

The binding sites of transcriptional factors of six SNPs obtained were found using TESS software with most strict options and based on assumption that targeted sequence should be absolutely complimentary to the sequence represented in TRANSFAC v.6 data base. The binding sites of transcriptional factors for all obtained SNPs were revealed to be present in one allelic variant. Based on this fact they were considered as rSNPs [Ponomarenko *et al.* 2002].

Table 3. Eggshell thickness within genotypes of the investigated rSNPs

rSNP	ST2_1		
genotype	CC	CT	TT
eggshell thickness (µm)	389±33	340±43**	351±39**
rSNP	ST3_1		
genotype	AA	AG	GG
eggshell thickness (µm)	336±22	351±38	360±34*
rSNP	ST3_2		
genotype	CC	CT	TT
eggshell thickness (µm)	351±44	353±40	374±34*
rSNP	ST3_3		
genotype	CC	CT	TT
eggshell thickness (µm)	335±13	363±41**	346±43
rSNP	ST3_4		
genotype	CC	CT	TT
eggshell thickness (µm)	343±51	353±39	357±44
rSNP	ST6_1		
genotype	CC	CT	TT
eggshell thickness (µm)	343±41	367±33*	351±46

*P<0.05; **P<0.01.

The use of PCR with allele-specific primers for genotyping allowed to reveal significant difference in allelic frequencies of rSNPs ST2_1, ST3_1, ST3_2 and ST3_3 in groups of layers with thick (390±13 µm) and thin (316±21 µm) egg shell (Tab. 2). It is an evidence of the possible association of genotypes by these polymorphic sites with the trait studied. It was also shown that the genotypic classes of rSNPs ST2_1, ST3_1, ST3_2, ST3_3, ST6_1 were significantly different in shell thickness (Tab. 3). Interestingly, in ST2_1 case both heterozygous and homozygous for T- allele birds had significantly (P<0.01) thinner eggshells than wild type individuals. Significant difference in allelic frequencies was also detected for ST2_1 site that could be interpreted as largest association of rSNP ST2_1 with investigated trait. It is interesting that in ST3_3 and ST6_1 cases heterozygous show essentially thicker eggshell than animals from both homozygous genotypic classes (Tab. 3). In ST3_1 cases ST3_2 genotype affected investigated trait only in homozygotes for mutant allele.

Thus, the data on association of the rSNP with eggshell thickness provide the tool to develop the genotyping system of Rhode Island layers by rSNP alleles affecting this trait. Optimization of shell thickness has economical importance because it can help to reduce transportation losses. It is supposed to adopt this genotyping system to Marker Assisted Selection in chicken. In comparison with present conventional systems of chicken selection, it can reduce the time of selection work by exclusion of the cock breed evaluation and may be useful for the new layer lines and crosses breeding with optimized eggshell. One can't but agree that some molecular markers, such as microsatellite are ineffective in the selection [Boruszewska *et al.* 2009].

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