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GENOTYPING OF FOUR LOCI IN HUNGARIAN YELLOW AND BROILER CHICKENS

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Traditional selection has led to remarkable differences in allele frequencies among various chicken breeds. Indigenous and broiler-type chicken populations were genotyped for polymorphisms in thyroid hormone responsive *Spot14α*, prolactin (*PRL*), IGF-binding protein 2 (*IGFBP2*), and somatostatin (*SST*) genes in order to determine potential utilisation type-associated allele frequencies. Significant ($P < 0.05$) differences were detected between Hungarian Yellow and broiler populations for *Spot14α*, *PRL*, and *IGFBP2* allele frequencies, whereas the same *SST* allele (A) was fixed in both groups. In this study, the most significant associations ($P < 0.05$) were found between the *IGFBP2* genotypes and the measured traits (body weight, carcass weight, breast muscle weight with or without skin, breast muscle weight as a percentage of carcass weight) in the broiler population. The results can be applied for the evaluation of polymorphism effects in the analysed populations; however, contradictory allele effects in different breeds and hybrids indicate the need for cautious marker utilisation in selection programmes.

Key words: Chicken, prolactin, *Spot14α*, *IGFBP2*, somatostatin, polymorphism

In order to identify potential utilisation- and production-related changes in allele frequencies, four polymorphisms in growth-related genes were selected for genotyping, allele frequency and genotype-trait analysis in chicken populations that characteristically differ regarding utilisation and economically important production traits. Allele frequency analyses are often used to identify potential candidate genes and polymorphisms, and are important for the selection of advantageous genotypes.

The intensive selection of broiler chicken has successfully increased growth rate, feed efficiency, carcass yield, and breast muscle percentage. Application of molecular markers associated with meat production is a potential tool to improve production yields.

The thyroid-hormone responsive *Spot14* gene (also known as *THRSP*) was mapped to 1q41-44 in chicken, subsequently a QTL for subcutaneous fatness

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(Ikeobi et al., 2002) and abdominal fatness (Lagarrigue et al., 2003) were detected close to this locus. The *Spot14* gene was identified as duplicated polymorphic paralogs in chicken, namely *Spot14α* and *Spot14β* (Wang et al., 2004), and consists of two exons (D'André et al., 2010). *Spot14α* encodes a small acidic protein, which consists of 132 amino acids, only expressed in lipogenic tissues such as liver, fat and mammary glands in mammals (Cao et al., 2007). *Spot14* is implicated as a transcription factor on lipogenic genes promoters, involved in the control of lipogenic enzymes, although the exact molecular mechanism is not clear (Wang et al., 2004). Furthermore, *Spot14α* is maintaining a connection between thyroid hormone concentrations and growth (Cao et al., 2007; D'André et al., 2010). *Spot14* mRNA levels are increased by carbohydrate feeding or insulin injection and decreased by high plasma glucagon levels or by a diet rich in polyunsaturated fatty acids (Jump et al., 1993). The expression of *Spot14α* mRNA is also regulated by thyroid hormone status (Wang et al., 2004).

Prolactin (*PRL*) is a polypeptide hormone secreted by the anterior pituitary gland, and has a diverse spectrum of functions including growth, development, metabolism, reproduction, behaviour, and immunoregulation in vertebrates, and plays main roles in several reproductive processes in avian species (Angelier and Chastel, 2009). *PRL* takes part in several biological functions; however, only limited genotype–growth associations have been reported in chicken (Bhattacharya et al., 2011).

Insulin-like growth factor 1 (*IGF1*) is a candidate gene for growth, body composition, metabolism, fat deposition and skeletal traits in chicken (Zhou et al., 2005). The actions of IGFs are regulated by many factors. The IGF-binding protein type 2 (IGFBP2), as an important member of IGFBPs family, has comprehensive biological functions *in vivo* via endocrine, autocrine, or paracrine mechanisms, may play an important role in the modification of the growth-supporting effect of circulating IGF1 by producing the IGFBP complex in chicken (Kita et al., 2002), and by regulating IGF transport to tissues and IGF bioavailability to IGF receptors at cell membrane level (Silha and Murphy, 2005). The chicken *IGFBP2* gene spans approximately 38 kb and is located on chromosome 7, consists of four exons, encoding a 275-amino-acid polypeptide hormone, and is regulated by growth hormones (Schoen et al., 1995). The structure and function of the *IGFBP2* gene were analysed in detail; however, its association with body weight has not yet been clarified in chickens (Zhao et al., 2015). The chicken *IGFBP2* gene is highly expressed in most tissues of embryos, such as liver, muscle, kidney, heart, ovary, brain, intestine, and other tissues (Schoen et al., 1995).

Somatostatin (*SST*) regulates the secretion of numerous hormones from the mammalian brain, pituitary and peripheral tissues, affects the inhibition of growth hormone secretion from the anterior pituitary (Geris et al., 2000), inhibits pancreatic insulin and glucagon secretion, lipolysis, thyroid function, and stimu-

lates food intake (Tachibana et al., 2009). The *SST* gene encodes two biologically active cyclic peptide forms (*SST*-14 and *SST*-28). *SST*-14, which consists of 14 amino acids, is almost fully conserved across vertebrates (Gahete et al., 2008). *SST*-28, which consists of 28 amino acids, contains a methionine–leucine substitution in chicken compared to mammals (Hasegawa et al., 1984). Although polymorphisms in the *SST* gene have major effects on growth and body composition in mammals, they are less well characterised in chicken (Nie et al., 2005).

Materials and methods

Experimental birds and sampling

The analysed breed and hybrid were: Hungarian Yellow (indigenous breed; n = 436; female) and Ross 308 end-product chicken (broiler type hybrid; n = 103; male). The individual birds involved in this study were randomly selected. Hungarian Yellow chickens used in the present study originated from the genetic resource farm located in Mosonmagyaróvár, managed by the Széchenyi István University. Blood samples were taken into collection tubes (with EDTA as anticoagulant) from wing veins in the Hungarian Yellow population, whereas feather samples were collected from the broilers for the identification of *Spot14α, PRL, IGFBP2, SST* genotypes, and stored at –20 °C pending processing. The weight of 38-day-old broilers was measured before slaughtering, whereas the weight of individual carcasses (CW) was measured before refrigerating. After refrigerating (2 h at 4 °C), the breast muscle fillets with skin (BMWS) and without skin (BMW), as well as the thighs with skin and bone (TW) were measured, and their percentage related to carcass and live weight were also calculated.

Extraction of DNA from blood and feathers was done with Wizard Genomic DNA Isolation kits (Promega, USA), according to the manufacturer's instructions. DNA concentration and purity were determined using a NanoDrop 2000 spectrophotometer (Thermo Fischer Scientific, USA), and both 260/280 and 260/230 ratios of samples exceeded 1.8.

Genotyping

Polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) techniques with agarose gel electrophoresis were used to identify genotype. With slight amendments the genotyping methods for the A213C locus in *Spot14α* by Cao et al. (2007) and the 24 bp insertion in *PRL* by Jiang et al. (2005) were applied. Primers were designed and RFLP was constructed for non-synonymous SNPs, the G645T SNP in *IGFBP2* and A370G SNP in *SST* (Table 1). These SNPs were described by Nie et al. (2005).

Table 1

Primers and restriction enzymes used

Locus	Sequence (forward/reverse)	Ta	Sequence ID	Length (bp)	RE
A213C exon 1 of *Spot14α*	CAGGAGGGAGCAGAGGGATAG/ GGTCGGTCAGAACCTGCTGC	60	AY568628	419	BsaHI
24 bp insertion promoter of *PRL*	GGTGGGTGAAGAGACAAGGA/ TGCTGAGTATGGCTGGATGT	56	FJ663023 or FJ434669	201 and/or 177	–
G645T exon 2 of *IGFBP2*	AACAGGCATGAAGGAGATGG/ CTCGCCAGCACATCAAAGT	52	U15086	315	BseGI
A370G exon 2 of *SST*	CCTGTTTTCTCTCCCCTCAC/ AGTCTTCGCCTCTCGTGGT	55	X60191	330	BsrBI

Ta – annealing temperature, °C; RE – restriction endonuclease; Length – length of PCR products (bp)

The PCR was performed in 25-µl mixtures containing 1 µl (200 ng) of genomic DNA, 12.5 µl 2xPCR Mastermix (Promega, USA; Thermo Fisher Scientific, USA), 1 µl of appropriate oligonucleotide primer (0.4 µM; Table 1) and nuclease-free water up to 25 µl final volume. PCR was applied with the following conditions: 4 min at 95 °C, followed by 35 cycles of 1 min at 95 °C, 1 min at annealing temperature (Table 1), 1 min at 72 °C, and final extension of 4 min at 72 °C. The PCR products were digested in a total reaction volume of 20 µl containing 10 µl of the PCR products and 10 U of the appropriate restriction enzyme (Table 1) according to the manufacturers' (Promega and Thermo Fischer Scientific) recommendations. Restriction fragments were electrophoresed on 2% agarose gels (Agarose, LE, Analytical Grade; Promega) stained with ethidium bromide (10 mg/ml stock concentration, 0.5 µg/ml final concentration, Promega), and PCR-RFLP fragment size in each sample was determined by FastRuler Low Range DNA Ladder (Thermo Scientific) under UV illumination. The 24-bp insertion of PRL genotyping was done by loading PCR products straight on 3% agarose gel.

Statistical analysis

Hardy–Weinberg Equilibrium (HWE) was tested by Chi-square analysis (SPSS 20.0 for Windows). Differences in allele frequencies between groups were analysed with 2-sided Fisher's exact tests. Heterozygosity (HE) and polymorphism information content (PIC) were calculated according to Nagy et al. (2012). HWE, PIC and HE analyses for *SST* were not adaptable, since only one allele of the examined locus was present in the investigated populations. Genotype–trait

associations were evaluated by variance analysis in SPSS 20.0 (ANOVA) using Least Significant Difference (LSD) tests.

Results

The genotype and allele frequencies of *Spot14α*, *PRL*, *IGFBP2*, and *SST* are shown in Table 2. Chi-square test of HWE was not applicable for *SST* since only allele A was present in the population, whereas other genes were in HWE ($P > 0.05$).

A significant difference ($P < 0.05$) was observed between *Spot14α* allele frequencies in the examined two populations. Allele A was characterised by a 419-bp fragment, whereas allele C was demonstrated by 319- and 100-bp-long fragments. In the two populations all the three genotypes were found in *Spot14α*: AA, AC and CC. In both populations a higher frequency of allele C was represented. The A213C SNP in *Spot14α* results in aspartic (allele C) to glutamic (allele A) acid change. The analysed *Spot14α* SNP had significant ($P < 0.05$) effect on breast muscle weight as a percentage of body weight (Table 3).

The *PRL* allele frequencies in Hungarian Yellow chicken were significantly different ($P < 0.05$) compared to the broiler population (Table 2). In the Hungarian Yellow and the broiler population all the three genotypes were found. Allele I was identified as a 201-bp-long product, whereas allele D was characterised by a 177-bp-long product. In the examined broiler population *PRL* genotype was significantly ($P < 0.05$) associated with thigh weight and thigh weight percentage of body weight (Table 3). Regarding most of the measured traits (BW, CW, BMWS, TW), homozygous (DD) chickens surpassed heterozygotes. However, body and carcass weight did not differ significantly ($P > 0.05$) between heterozygous and homozygous chickens.

The *IGFBP2* allele frequencies significantly ($P < 0.05$) differed between the Hungarian Yellow and the broiler population (Table 2). Two of the three genotypes were found in broilers (GG, GT), while the TT genotype was missing. Allele G was identified by 198- and 117-bp-long fragments, allele T was represented by a 315-bp-long product. Only allele G was found in Hungarian Yellow hens in a previous study (Tempfli et al., 2015).

In the examined broiler population, where two *IGFBP2* genotypes were found, birds with heterozygous GT genotype demonstrated significantly ($P < 0.05$) higher body and carcass weight, breast muscle weight with and without skin and breast muscle weight percentage of carcass weight, than chickens with homozygous (GG) genotype (Table 3).

Allele A at A370G locus in *SST* was fixed both in the Hungarian Yellow and the Ross 308 populations (Table 2). The fixed genotype was characterised by the uncut 330-bp-long PCR product.

Table 2

Genotype and allele frequencies in different populations

Locus	Breed/Hybrid	Allele frequency*		Genotype frequency	χ^2	P value	PIC	HE
A213C in *Spot14α*	Hun. Yellow (n = 436)	A = 0.38 C = 0.62	a	AA = 0.13 AC = 0.51 CC = 0.36	2.213	0.140	0.360	0.470
	Broiler (n = 100)	A = 0.11 C = 0.89	b	AA = 0.01 AC = 0.20 CC = 0.79	1.700	0.190	0.180	0.200
24 bp indel in *PRL*	Hun. Yellow (n = 436)	I = 0.53 D = 0.47	a	II = 0.29 ID = 0.48 DD = 0.23	0.511	0.470	0.370	0.500
	Broiler (n = 117)	I = 0.23 D = 0.77	b	II = 0.03 ID = 0.41 DD = 0.56	0.001	0.970	0.280	0.340
G645T in *IGFBP2*	Hun. Yellow (n = 436)	G = 1.00 T = 0.00	a	GG = 1.00 GT = 0.00 TT = 0.00				
	Broiler (n = 103)	G = 0.92 T = 0.08	b	GG = 0.84 GT = 0.17 TT = 0.00	0.657	0.417	0.120	0.130
A370G in *SST*	Hun. Yellow (n = 436)	A = 1.00 G = 0.00	a	AA = 1.00 AG = 0.00 GG = 0.00				
	Broiler (n = 103)	A = 1.00 G = 0.00	a	AA = 1.00 AG = 0.00 GG = 0.00				

Hun. Yellow – Hungarian Yellow chicken; *different letters (a, b) indicate significantly different ($P < 0.05$) allele frequencies between populations

Table 3

Genotype and carcass trait associations in male broilers

Genotype (n)		BW (g)	CW (g)	BMWS (g)	BMWS percentage of CW (%)	BMWS percentage of BW (%)	BMW (g)	BMW percentage of CW (%)	BMW percentage of BW (%)	TW (g)	TW percentage of CW (%)	TW percentage of BW (%)
Spot14α	AA (n=1)*	2240±0	1733±0	550±0	31.7±0.0	24.6±0.0	488±0	28.2±0.0	21.8±0.0	520±0	30.0±0.0	23.2±0.0
	AC (n=20)	2535±279	1930±212	622±94	36.0±5.3	27.4±3.5	523±110	29.1±1.9	22.3[b]±1.7	579±61	33.7±4.7	25.6±3.2
	CC (n=79)	2509±262	1936±198	631±81	35.2±6.6	28.5±4.2	549±81	28.6±3.1	23.9[a]±3.0	581±72	32.4±5.5	26.2±3.6
PRL	II (n=2)*	2217±117	1699±52	586±24	43.8±0.5	33.6±0.4	417±79	30.5±3.0	23.0±2.8	507±36	37.8±1.5	29.0±0.5
	ID (n=42)	2489±223	1919±181	617±86	34.3±5.7	27.5±3.9	544±93	28.6±2.9	23.4±3.0	563[b]±62	31.4±5.1	25.1[b]±3.1
	DD (n=59)	2534±284	1947±208	637±82	35.6±6.7	28.7±4.2	543±83	28.7±3.0	23.8±2.9	593[a]±71	32.5±5.6	26.8[a]±3.7
IGFBP2	GG (n=86)	2485[b]±263	1911[b]±194	619[b]±81	35.2±6.5	28.3±4.2	528[b]±81	28.4[b]±2.9	23.5±2.9	575±70	32.7±5.5	26.3±3.6
	GT (n=17)	2639[a]±220	2034[a]±195	679[a]±76	35.9±5.7	28.5±3.9	604[a]±91	30.1[a]±2.5	24.3±2.7	603±61	32.0±5.4	25.4±3.1

BW – body weight at day 38; CW – carcass weight; BMWS – breast muscle weight with skin; BMW – breast muscle weight without skin; TW – thigh weight with skin and bone; *This genotype was not included in the statistical analysis due to the small group size; different letters (a, b) indicate significantly different ($P < 0.05$) means between genotypes

Discussion

Tempfli et al. (2015) reported a significant association between *Spot14α* genotypes and body weight of 8- to 14-, 40- and 45-week-old Hungarian Yellow hens, and allele A was associated with higher body weight in this population. There were no significant differences in body weight at younger ages, and genotype effects were stronger ($P < 0.01$) at older ages and at higher body weight in the Hungarian Yellow population. In an F_2 broiler–layer cross (Cao et al., 2007), which had all the three genotypes, this SNP showed significant ($P < 0.05$) effects on body weight of 5- to 12-week-old chickens; however, allele C was associated with increased body weight, since birds with AA genotype had significantly ($P < 0.05$) lower body and carcass weight than those with CC genotype. There was no association between the polymorphism and the measured body composition traits in the experiment of Cao et al. (2007). In the investigated broiler population significant differences ($P < 0.05$) were found between the genotypes and breast muscle weight percentage of body weight. In an F_2 population of White Recessive Rock and Xinghua breeds, SNPs located in the 5' flanking region of this gene were associated with body weight at younger ages (hatch and 28-day weight; D'André et al., 2010). Based on these contradictory results, a non-direct effect of A213C SNP was proposed, suggesting that the actual causative mutation was nearly linked to the investigated SNP, and was in inverse linkage in the different populations. Before using this SNP in marker-assisted selection programmes, attention must be given to survey the actual direction of the allele substitution effect in the particular breed or line.

In the investigated broiler population significant associations ($P < 0.05$) were found between the *PRL* genotypes and the leg weight as well as the leg weight percentage of body weight traits measured. There were no further associations between *PRL* genotypes and other measured traits. Based on the highly polymorphic *PRL* promoter, also significant ($P < 0.05$) *PRL* haplogroup associations were described in White Leghorn lines with body weight of chickens at sexual maturity and at 16 and 64 weeks of age (Bhattacharya et al., 2011).

Association between *IGFBP2* and some growth traits were reported in several chicken populations (Nie et al., 2005; Li et al., 2006). The G645T is non-synonymous, and significant differences ($P < 0.05$) were found between polymorphism and body weight in an F_2 designed population made up of a reciprocal cross between White Recessive Rock and Xinghua chickens (Lei et al., 2005). Additional associations were found between polymorphisms of *IGFBP2* and growth, carcass and body composition traits in several chicken populations (Li et al., 2006; Zhao et al., 2015).

In *SST* only allele A was found in Hungarian Yellow and Ross 308 broiler populations. This SNP was described by Nie et al. (2005), when Leghorn, White Recessive Rock, Taihe silkies and Xinghua breeds were contrasted. Other reports

have not been presented about frequency distribution or genotype–trait association of this polymorphism. Probably the clearly preferred allele rapidly fixed in a continuously selected stock. Therefore, further investigations involving other breeds are necessary to assess the effect and frequency of the polymorphism.

The identified breed-specific genotype frequencies are essential markers of the effects of different alleles on the overall performance of chickens. It was concluded that most of the advantageous alleles are nearly fixed in the highly selected broiler population; however, the identified effects of *Spot14α*, *PRL,* and *IGFBP2* genotypes indicate that there are further possibilities to improve production with specific marker-assisted breeding programmes. Due to contradictions described in this study and the relevant literature in regard to genotype effects on production traits in different breeds and hybrids, it is advised to carefully investigate specific effects in any given population before application of the polymorphisms in marker-assisted selection.

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