

Association of *RUNX2* and *TNFSF11* genes with production traits in a paternal broiler line

N.V. Grupioni¹, N.B. Stafuzza¹, A.B. Carvajal¹, A.M.G. Ibelli², J.O. Peixoto², M.C. Ledur² and D.P. Munari¹

¹Departamento de Ciências Exatas, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista, Jaboticabal, SP, Brasil

²Embrapa Suínos e Aves, Concórdia, SC, Brasil

Corresponding author: D.P. Munari

E-mail: danisio@fcav.unesp.br

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ABSTRACT. Intense selection for production traits has improved the genetic gain of important economic traits. However, selection for performance and carcass traits has led to the onset of locomotor problems and decreasing bone strength in broilers. Thus, genes associated with bone integrity traits have become candidates for genetic studies in order to reduce the impact of bone disorders in broilers. This study investigated the association of the *RUNX2* and *TNFSF11* genes with 79 traits related to performance, carcass composition, organs, and bone integrity in a paternal broiler line. Analyses of genetic association between single-nucleotide polymorphisms (SNPs) and traits were carried out using the maximum likelihood procedures for mixed models. Genetic associations ($P < 0.05$) were found between SNP *g.124,883A>G* in the *RUNX2* gene and chilled femur weight (additive plus dominance deviation effects within sex) and with performance traits (additive within sex and additive effects). The SNP *g.14,862T>C* in the *TNFSF11* gene presented genetic associations ($P < 0.05$) with additive plus dominance deviation effects within sex for performance

traits. Suggestive genetic associations ($P < 0.10$) were found with abdominal fat and its yield. Selection based on SNPs *g.14,862T>C* in *TNFSF11* and *g.124,883A>G* in *RUNX2* could be used to improve performance and carcass quality traits in the population studied, although SNP *g.14,862T>C* was not in Hardy-Weinberg equilibrium because it was not undergoing a selection process. Furthermore, it is important to validate these markers in an unrelated population for use in the selection process.

Key words: Body weight; Bone integrity; Femur; PCR-RFLP; SNP

INTRODUCTION

Intense selection for phenotypic traits in broilers has resulted in genetic improvement for rapid growth and meat yield (Dransfield and Sosnicki, 1999; Li et al., 2003). However, it has had a negative impact on the skeletal structure in broilers, because animals with high growth rates do not present a skeletal structure strong enough to support their body weight (Ragognetti et al., 2015). Animals with a fast growth rate exhibit decreased locomotor activity and spend more time sitting or lying down (Bessei, 2006). Thus, the incidence of bone disorder-related problems has also increased, because bone development requires more time than the rapid body growth allows. According to Julian (1998), high nutrient intake induces rapid growth and results in bone disorders and severe lameness. Furthermore, the same author pointed out that musculoskeletal disorders may be caused by rapid growth and that defects could be related to high body weight, immature bone tissues, insufficient time to produce strong bone tissues, high specific nutrient requirements, and increase of metabolic products. Fractures such as osteoporosis can result in serious animal welfare concerns because they cause acute and chronic pain as well as mortality (Danbury et al., 2000). It is necessary to better understand problems related to bone integrity because they contribute to increased mortality and carcass condemnations and reduce productivity (Rath et al., 2000).

Animal skeletal development can be influenced by genes involved in growth rates (Zhou et al., 2005; Locatelli and Bianchi, 2014), which can be observed through genetic correlations between weight, weight gain, and bone integrity traits and can be manipulated through selection (Lamont et al., 2004; González-Cerón et al., 2015). Grupioni et al. (2015) found that the breaking strength resistance of the femur is highly genetically correlated with mineral content in a TT Reference Population of broilers. Da Cruz et al. (2015) observed significant associations ($P < 0.05$) of single-nucleotide polymorphism (SNP) *g.729C>T* of the *ADIPOR1* (adiponectin receptor 1) gene with bone integrity traits and breast skin yield in the same population of broilers, suggesting that this gene can act in metabolism, fat deposition, and bone integrity. Fornari et al. (2014) found that the SNP *g.9144C>G* in the *TNFRSF11B* (osteoprotegerin) gene was associated with skeletal structure, performance, and carcass traits in the TT Reference Population of broilers. Genetic associations were also found between the *ACTA1* (actin, alpha1, skeletal muscle) gene and performance and carcass traits in the same population (Venturini et al., 2015).

Association studies of candidate genes functionally related to bone integrity may help to identify markers that can be used in genetic selection for traits related to bone strength, in order to reduce bone disorders resulting from the intense selection process for broiler growth

(Zhou et al., 2007). The runt-related transcription factor 2 gene (*RUNX2*) is a potential candidate for the study of bone integrity-related traits since it is involved in bone tissue development and maintenance, acting as a transcription factor in osteoblast formation (Kammerer et al., 2013). Mutations in *RUNX2* can cause diseases such as cleidocranial dysplasia (Otto et al., 2002), which primarily affects the skeletal system, resulting in membranous bones and endochondral ossification, which may be categorized as a generalized disorder of the skeletal structure (Neville et al., 2002).

The tumor necrosis factor (ligand) superfamily member 11 gene (*TNFSF11*), also known as receptor activator of nuclear factor- κ B ligand (*RANKL*) (Boyle et al., 2003), plays an important role in the regulation of the bone remodeling process (Genetics and Home Reference, 2013), acting on osteoclast differentiation and expression, the function of which is to reabsorb the mineralized bone matrix (Usui et al., 2008).

Association studies of these two genes with meat production and bone integrity traits in broiler lines are important, especially for skeletal structure, because these traits are difficult to measure and have low genetic gains, since they have low to moderate heritability estimates (Grupioni et al., 2015) and require the slaughter of animals for data collection. Hence, the aim of this study was to investigate the association of polymorphisms in the *RUNX2* and *TNFSF11* genes with performance, carcass composition, organs, and bone integrity-related traits in a paternal broiler line.

MATERIAL AND METHODS

Ethics statement

This study was approved by the Embrapa Swine and Poultry Ethical Committee for Animal Use (CEUA) under protocol number 011/2011, following international guidelines for animal welfare.

Experimental population and phenotypic traits

The TT Reference Population database used in this study belongs to the Embrapa Swine and Poultry National Research Center, Concordia, SC, Brazil. The TT Reference Population is a paternal broiler line originating from the crossing of Cornish, Hampshire, and White Plymouth Rock breeds, which was formed from the expansion of the pure line of broilers by mating 20 males with 92 females (1:5) in 5 hatches, resulting in about 1,500 progeny. Selection of this broiler line has been performed since 1992 in order to increase body weight and carcass traits, decrease abdominal fat percentage, and improve reproductive traits and feed conversion rates (da Cruz et al., 2015).

The chicks were tagged with metal rings for pedigree control at birth. The broilers were kept in collective boxes until 35 days of age, and from 35 to 41 days they were housed in individual cages to allow the evaluation of feed conversion. Diets consisted of 3150 kcal/kg metabolizable energy (ME) and 21% crude protein (CP) from 1 to 21 days, 3200 kcal/kg ME and 20% CP from 22 to 34 days, and 3200 kcal/kg ME and 18.5% CP from 35 to 41 days of age.

Broilers were slaughtered at 42 days of age and 2 mL blood was collected during bleeding from the jugular and stored in 2 mL microtubes containing 100 μ L (10% v/v) 0.5 M EDTA anticoagulant for DNA extraction. A total of 79 traits from the TT Reference Population

database were evaluated, including traits related to broiler performance, carcass composition, organs, and bone integrity (see **Table S1**). The descriptive statistics of those traits were described by Fornari et al. (2014).

The methodology used to determine bone dry matter percentage, ashes, and mineral content was previously described by Grupioni et al. (2015). These analyses were performed at the Physical-Chemical Laboratory at Embrapa Swine and Poultry, Concordia, SC, Brazil.

Extraction and quantification of DNA samples

Genomic DNA was extracted using the DNA extraction protocol with DNAzol® reagent (Invitrogen). A total of 500 µL DNAzol® was added to 15 µL whole blood stored in 0.5 M EDTA and homogenized. About 500 µL of absolute ethanol was added to the tubes and mixed gently several times. The precipitate was removed and placed in another tube using a micropipette and washed in 1 mL 95% ethanol. Samples were centrifuged at 3500 g for 10 min, washed in 1 mL 75% ethanol, and centrifuged under the same conditions. Next, the supernatant was discarded and the pellet dried at 22°C for 30 min. The DNA was re-suspended in 150 µL ultra-pure water, incubated at 37°C for 1 h in a water bath and stored at -20°C until use. DNA integrity was verified using 1% agarose gel stained with ethidium bromide and quantified in a BioPhotometer™ (Eppendorf, Hamburg, Germany) spectrophotometer. The concentrations of the samples were adjusted to 25 ng/µL.

Amplification and genotyping by PCR-RFLP

Sequence information regarding the *RUNX2* and *TNFSF11* genes was retrieved from the NCBI database (<http://www.ncbi.nlm.nih.gov>) using the genome annotation Reference Gallus_gallus-5.0 Primary Assembly. *RUNX2* presents a total of 214,603 bp, located on chromosome 3 between 109,768,999 and 109,983,601 bp (gene ID: 373919, updated on 5 Jan 2016). *TNFSF11* contains a total of 22,682 bp, located on chromosome 1 between 166,505,163 and 166,527,844 bp (gene ID: 428067, updated on 5 Jan 2016). The PCR primers were designed using Primer3Plus software (Untergasser et al., 2007) (see **Table S2**).

To amplify the target DNA of *RUNX2* and *TNFSF11*, PCR reactions were standardized as follows: 50 ng genomic DNA was added to a reaction mix composed of 1X buffer (10X High Fidelity buffer [600 mM Tris-SO₄ (pH 8.9), 180 mM (NH₄)₂SO₄], 1 µL MgSO₄ (50 mM), 0.4 mM dNTP, 0.2 mM each primer, and 1 U Platinum Taq DNA Polymerase High Fidelity (Invitrogen), for a total final volume of 25 µL.

The amplifications were carried out under the following conditions: initial denaturation for 5 min at 95°C, followed by 35 cycles of denaturation for 1 min at 95°C, 60°C for 1 min (annealing), extension at 72°C for 1 min, and a final extension at 72°C for 10 min. The restriction enzymes that recognize specific SNP sites were selected using NEBcutter software (Vincze et al., 2003). The *HinfI* (5' G↓ANTC 3') and *BtsCI* (5' GGATGNN↓ 3') restriction enzymes were used to genotype the SNPs of *TNFSF11* and *RUNX2*, respectively. The SNPs evaluated were submitted to dbSNP (<http://www.ncbi.nlm.nih.gov/snp>), under accession number ss1966531575 for the SNP *g.14,862T>C* located in the *TNFSF11* gene and ss1966531593 for the SNP *g.124,883A>G* in the *RUNX2* gene. For RFLP analysis, 10 µL of the PCR reaction was performed using 5 µL PCR products, 1U restriction enzyme, and 1X enzyme buffer. The digestion occurred overnight at 37°C and 50°C for *HinfI* and *BtsCI* restriction enzymes, respectively.

The PCR-RFLP products were visualized on 2% agarose gels stained with ethidium bromide, submitted to electric current at 80 V for 120 min in 1X TBE buffer, and analyzed in a transilluminator under UV light. To identify the size of the amplified fragments, a 100 bp molecular marker (Invitrogen) was used.

Statistical analysis

Genotypic frequency analyses were performed using the SAS FREQ procedure (Statistical Analysis System, v.9.3, Cary, NC). Hardy-Weinberg equilibrium was tested for each SNP using the chi-square (χ^2) test with 1 degree of freedom at the 5% significance level ($P < 0.05$).

The association analyses between SNPs and phenotypic traits were performed using QxPak v. 4.1 software (Pérez-Enciso and Misztal, 2004), which uses maximum likelihood procedures for mixed models through the following general linear model:

$$y_{ijklm} = \mu + H_i + S_k + SNP_l + a_{ijklm} + e_{ijklm} \quad (\text{Equation 1})$$

where y_{ijklm} is the record of the phenotypic trait; μ is the trait's overall mean; H_i is the fixed effect of the i^{th} hatch (with $i = 1 \dots 5$); S_k is the fixed effect of the k^{th} sex ($k = 1, 2$); SNP_l is the fixed effect of the l^{th} genotype ($l = 11, 12, 22$); a_{ijklm} is the random effect of the j^{th} ($j = 1 \dots 510$) animal from i hatch and k sex; and e_{ijklm} is the random error.

Analyses based on the likelihood ratio test ($P < 0.05$) (Dobson, 1990) were carried out using additive (a), additive within sex (a(sex)), additive plus dominance deviations (a+d) and additive plus dominance deviations within sex (a+d(sex)) effects. The following procedures were used in Qxpak software to check the possible effects of SNPs on the studied traits. For SNP additive effects, individuals with genotype markers '11', '12' and '22' assumed the breeding values of K_{AA} , 0, and K_{GG} , respectively. The genotypes '12' and '21' were treated identically. For SNP additive and dominance effects, individuals with genotype markers '11', '12' and '22' assumed the breeding values of K_{AA} , K_{AG} , and K_{GG} , respectively. Subsequently, the models were fitted within sex and the best model was chosen for each significant ($P < 0.05$) trait.

RESULTS AND DISCUSSION

Genotyping and SNP detection

SNP *g.14,862T>C* (ss1966531575), which is located on exon 5 of *TNFSF11*, and SNP *g.124,883A>G* (ss1966531593), which is located on exon 6 of *RUNX2*, were first described in this study. SNP *g.14,862T>C* alters the codon TCA to CCA so the amino acid proline (nonpolar) is produced instead of serine (polar). The SNP *g.124,883A>G* alters the codon TCA to TCG, without changing the amino acid produced (serine).

The PCR-RFLP analysis of the *RUNX2* gene (Figure 1) identified three genotypes: GG (566, 244, 198, 35, and 1 bp), AG (566, 320, 246-244, 198, 35, and 1 bp), and AA (320, 246-244, 198, 35, and 1 bp). For the *TNFSF11* gene (Figure 2), the PCR-RFLP analysis showed three genotypes: GG (863 and 98 bp), AG (863, 544, 319, and 98 bp), and AA (544, 319, and 98 bp).

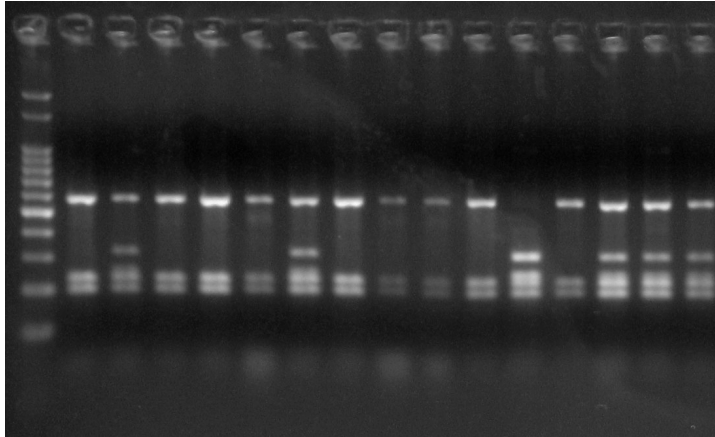


Figure 1. Genotyping by PCR-RFLP for *RUNX2*.

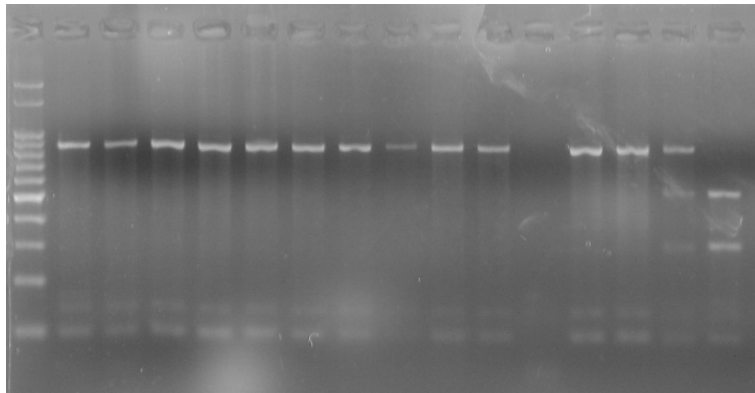


Figure 2. Genotyping by PCR-RFLP for *TNFSF11*.

Statistical analysis

The SNPs on *TNFSF11* (*g.14,862T>C*) and *RUNX2* (*g.124,883A>G*) presented three genotypic classes in the studied population (Table 1). The G allele had higher frequency than the A allele for *RUNX2* and the C allele had higher frequency than T for *TNFSF11*, indicating that selection in this population is close to fixation of the C allele for the SNP on *TNFSF11*. According to the chi-square test (χ^2), the population was in Hardy-Weinberg equilibrium for the SNP in *RUNX2*, but was not in Hardy-Weinberg equilibrium ($\chi^2 = 50.51$; $P < 0.05$) for the SNP in *TNFSF11*.

Table 1. Genes and their SNPs, genotypes, and allele frequencies in a paternal broiler line. Number of genotyped animals (N); number of animals per genotype, and their genotype frequencies (in parentheses) and allele frequencies.

SNP/Gene	N	Genotype			Allele	
		AA	AG	GG	A	G
SNP <i>g.124,883A>G</i> (<i>RUNX2</i>)	1154	165 (14.3)	536 (46.4)	453 (39.3)	0.3750	0.6250
SNP <i>g.14,862T>C</i> (<i>TNFSF11</i>)	1264	30 (2.4)	162 (12.8)	1,072 (84.8)	0.0878	0.9122

The fact that the population studied is an experimental population and was not in Hardy-Weinberg equilibrium for the SNP *g.14,862T>C* indicates that these allelic frequencies were modified by some factor that this population is susceptible, resulting in excessive heterozygosity. This may be because this population has been selected for performance traits, mainly for body weight, and not selected in favor of this SNP, which may be considered a limitation of this study. da Cruz et al. (2015) studied the SNPs *g.102A>T* (apolipoprotein B) and *g.729C>T* (adiponectin receptor 1), which are related to regulation of feed intake, fat metabolism, and protein deposition in the same experimental population of this study, and also observed that the population was not in Hardy-Weinberg equilibrium, as related in this study.

The SNPs in *RUNX2* had a high frequency of heterozygotes in the studied population, reflecting high genetic variability for this particular locus. The fact that this population presented Hardy-Weinberg equilibrium for this SNP can be attributed to indirect selection that this marker is experiencing.

Genetic association analysis

Global results of the association analysis considering the best model fitted and the descriptive analyses of associated traits in this population are shown in Table 2.

Table 2. Significant associations of the SNPs in *RUNX2* and *TNFSF11* under different models and descriptive analyses of the associated traits.

SNP/Gene	Trait	Best model fitted	N	Mean (SD)	CV (%)	Min	Max	
<i>g.124,883A>G</i> (<i>RUNX2</i>)	FI35-41	a	1147	1092.08 (156.21)	14.30	544.00	1590.00	
	W21	a	1135	657.79 (135.07)	20.53	256.00	1034.00	
	FBY	a	1146	7.62 (1.03)	13.52	3.88	11.31	
	BSY	a	1139	1.41 (0.25)	17.73	0.61	2.42	
	WW	a+d	1134	166.94 (19.69)	11.80	72.00	236.40	
	TWW	a+d	1141	85.45 (10.99)	12.86	35.80	119.60	
	BSW	a(sex)	1140	31.35 (6.93)	22.10	8.40	61.70	
	FBW	a(sex)	1146	168.84 (26.65)	15.78	82.00	293.00	
	WG35-41	a(sex)	1144	482.79 (105.92)	21.94	128.00	790.00	
	LUNW	a(sex)	1139	15.25 (3.02)	19.80	7.40	24.60	
	CFW	a+d(sex)	1143	32.49 (5.58)	17.17	18.00	56.60	
	<i>g.14,862T>C</i> (<i>TNFSF11</i>)	HTW	a	1234	12.42 (2.16)	17.40	6.30	19.70
		BW	a+d(sex)	1258	47.66 (3.72)	7.80	37.40	61.80
WG35-41		a+d(sex)	1251	490.14 (106.40)	21.71	158.00	802.00	
FC35-41		a+d(sex)	1251	2.31 (0.45)	19.48	1.42	4.95	

$P < 0.05$. N = number of observations, means and respective standard deviation (SD), CV (%) = coefficient of variation, Min = minimum values observed, Max = maximum values observed. Traits: feed intake from 3 to 41 days (FI35-41), feathers and blood yield (FBY), breast skin yield (BSY), wing weight (WW), thigh wing weight (TWW), breast skin weight (BSW), feathers and blood weight (FBW), weight gain from 35 to 41 days (WG35-41), lung weight (LUNW), chilled femur weight (CFW), body weight at 21 days (W21), body weight at birth (BW), heart yield (HTY), heart weight (HTW), and feed conversion 35-41 days (FC35-41). Weights were measured in grams and yield in percentages (%). The letters indicate the SNP effects: a, additive effects; a(sex), additive effects within sex; a+d, additive plus dominance effects; a+d(sex), additive plus dominance effects within sex.

The effects of the SNPs in *RUNX2* and *TNFSF11* that were significantly associated ($P < 0.05$) with several production traits in the TT Reference Population are shown in Tables 3, 4, 5, and 6. The additive plus dominance effects of *RUNX2* within sex were significant for chilled femur weight (CFW) and birth weight (BW), while two performance traits (weight gain from 35 to 41 days and feed conversion from 35 to 41 days) were significant for *TNFSF11* (Table 3).

Table 3. Additive plus dominance deviation effects within sex from the association analysis of *RUNX2* and *TNFSF11* and production traits.

SNP/Gene	Trait	a (SE) Male	a (SE) Female	d (SE) Male	d (SE) Female	P value	LR
<i>g.124,883A>G (RUNX2)</i>	CFW	0.48 (0.31)	0.19 (0.27)	1.31 (0.39)	-0.13 (0.36)	0.014	12.39
	BW	0.71 (0.31)	0.68 (0.31)	-0.68 (0.41)	-0.18 (0.42)	0.016	12.13
<i>g.14,862T>C (TNFSF11)</i>	WG35-41	23.64 (10.92)	-4.32 (10.92)	-42.89 (14.22)	-3.91 (14.68)	0.044	9.78
	FC35-41	-0.11 (0.04)	-0.01 (0.04)	0.23 (0.06)	-0.03 (0.06)	0.003	15.62

Additive effects: a, dominance deviations effects; d, standard errors in parentheses. LR: Likelihood-ratio test ($P < 0.05$). Traits: chilled femur weight (CFW), body weight at birth (BW), weight gain from 35 to 41 days (WG35-41), and feed conversion 35-41 days (FC35-41). Weights were measured in grams and yields in percentages (%).

For the *RUNX2* gene, genetic association with additive plus dominance deviation effects within sex was found only for chilled femur weight in males (Table 3). Although this trait is related to bone integrity, we observed genetic correlation of this trait only with femur length in a previous study (Grupioni et al., 2015).

Traits related to animal performance with significant ($P < 0.05$) additive plus dominance deviation effects within sex for the allelic substitution of T>C in *TNFSF11* were favorable in males for all traits shown in Table 3. Males presented favorable additive effects for performance traits (BW, WG35-41, and FC35-41). However, unfavorable dominance effects were significant for WG35-41 and FC35-41 in males. Depending on the intralocus relationship, these SNPs can have an important influence on those performance traits. However, it is the genic combination intralocus effect that shows the dominance relationship. The increase in G allele frequency for *RUNX2* and C allele frequency for *TNFSF11* contributes to improving the performance of these phenotypic traits, and their expression also depends on the genetic combination of these alleles.

Results from the association fitting the additive plus dominance effects (a+d) are shown in Table 4. Feathers and blood weight (FBW), thigh wing weight (TWW), and wing weight (WW) were associated with the *RUNX2* SNP only when this model was fitted. The observed dominance degree was classified as overdominance for WW and partial for FBW and TWW.

Table 4. Additive plus dominance deviation effects and dominance degree from the association analysis between *RUNX2* and production traits.

Trait	a (SE)	d (SE)	P value	LR	Dominance degree (d/a)
FBW	3.31 (1.18)	1.73 (1.48)	0.020	7.80	Partial
TWW	1.07 (0.46)	0.98 (0.57)	0.039	6.49	Partial
WW	1.60 (0.73)	1.64 (0.91)	0.043	6.29	Overdominance

Additive effects, a; dominance deviations effects, d; standard errors in parentheses; dominance degree, d/a. LR: Likelihood-ratio test ($P < 0.05$). Traits: feathers and blood weight (FBW), thigh wing weight (TWW), wing weight (WW). Weights measured in grams.

Regarding the additive plus dominance deviation effects for the *g.124,883A>G* allelic substitution in the *RUNX2* gene, significant ($P < 0.05$) effects were observed for FBW, TWW, and WW traits (Table 4). The amount of blood and feathers may influence the increase in waste and consequently decrease the final weight of meat produced. Regarding the TW and WW wing traits, direct selection for the G allele may not be efficient in phenotypic expression of these traits because part of the dominance deviation relationship presented similar magnitude values to the additive effects (Table 4). Dominance deviation effects are non-linear associations

with non-additive effects owing to the interaction between alleles at the same locus. According to Crnokrak and Rpoiff (1995), the study of non-additive gene action of dominance is important because it is part of the phenotypic expression and may affect the heritability estimates of traits.

The additive effects within sex with significant associations between production traits are shown in Table 5. The *RUNX2* SNP had a positive additive effect only in males for the weight gain from 35 to 41 days (WG35-41), FBW, lung weight (LUNW), and breast skin weight (BSW) traits. The SNP in the *TNFSF11* gene was not influenced by the sex of the broilers.

Table 5. Additive effects within sex from the association analysis between *RUNX2* and production traits.

SNP/Gene	Trait	a (SE) Male	a (SE) Female	P value	LR
<i>g.124,883A>G (RUNX2)</i>	WG35-41	14.48 (5.71)	4.72 (5.28)	0.034	6.72
	LUNW	0.41 (0.17)	0.12 (0.15)	0.049	6.04
	BSW	0.82 (0.42)	0.74 (0.39)	0.040	6.21

Additive effects, a; standard errors in parentheses. LR: Likelihood-ratio test ($P < 0.05$). Traits: weight gain from 35 to 41 days (WG35-41), feathers and blood weight (FBW), lung weight (LUNW), breast skin weight (BSW). Weights were measured in grams and yields as percentages (%).

The association analyses using the additive model effects for the SNPs in *RUNX2* and *TNFSF11* are shown in Table 6. The allelic substitution of the SNP *g.124,883A>G* in the *RUNX2* gene significantly influenced ($P < 0.05$) the following performance traits: body weight at 21 days (W21), feed intake from 3 to 41 days (FI35-41), feathers and blood yield (FBY) and breast skin yield (BSY). The allelic substitution of SNP *g.14,862 T>C* in the *TNFSF11* gene had a negative effect on HTW (-0.21 ± 0.10). This SNP also presented suggestive associations ($P < 0.10$; not shown) with abdominal fat weight (AFW) and its yield (AFY), where T to C allelic substitution could be favorable to reduce fat deposition.

Considering the additive effects within sex (Table 5), it can be stated that the selection for allelic substitution in *RUNX2* (A > G) for the WG35-41, FBW, LUNW, and BSW traits will be effective only in males. The selection of the *RUNX2* SNP favoring the G allele could result in broilers with higher phenotypic performance for those traits. In addition, the G allele also increased BSW (Table 5) and breast skin yield, which are traits related to fat deposition. Jennen et al. (2005) identified a QTL (Quantitative Trait Locus) region where the *RUNX2* gene is mapped, associated with body weight at 49 days of age, abdominal fat, and abdominal fat percentage.

Table 6. Additive effects (a) from the association analysis of *RUNX2* and *TNFSF11* and production traits.

SNP/Gene	Trait	a (SE)	P value	LR
<i>g.124,883A>G (RUNX2)</i>	W21	7.84 (3.59)	0.032	1.24
	FI35-41	13.16 (6.48)	0.042	4.10
	FBY	0.09 (0.04)	0.043	4.08
	BSY	0.02 (0.01)	0.055	3.66
<i>g.14,862T>C (TNFSF11)</i>	HTW	-0.21 (0.10)	0.028	4.86

Additive effects, a; standard errors in parentheses. LR: Likelihood-ratio test ($P < 0.05$). Traits: weight at 21 days (W21), feed intake from 35 to 41 days (FI35-41), feathers and blood yield (FBY), breast skin yield (BSY), heart weight (HTW). Weights were measured in grams and yields as percentages (%).

Moderate associations ($P < 0.10$; not shown) found with AFW and AFY traits suggested that the C allele of the *TNFSF11* SNP could reduce carcass fat deposition. These results indicate that this marker could be used in selection to reduce abdominal fat in broilers, after appropriate

validation. Park et al. (2006) identified a QTL region associated with abdominal fat in broilers, where the *TNFSF11* gene is mapped. Moreover, Fornari et al. (2014) found significant ($P < 0.05$) associations between the osteoprotegerin gene (*TNFRSF11B*) and AFW and AFY traits in the same population used in this study. The *TNFRSF11B* gene acts as a decoy receptor of the *TNFSF11* gene (Boyle et al., 2003). Therefore, *TNFRSF11B/TNFRSF11* balance signaling may be directly involved in fat deposition. Most likely, this mechanism is mediated by adiponectin hormone (*ADIPOQ*). According to Luo et al. (2006), *ADIPOQ* hormone inhibits *TNFRSF11B* gene expression in osteoblasts and stimulates *TNFRSF11* gene expression in humans.

da Cruz et al. (2015), studying a SNP in the adiponectin receptor 1 gene (*ADIPOR1*) in the same population studied here, found an additive effect ($P = 0.001$; -0.0016 ± 0.0005) with the breast skin yield trait and dominance effect ($P = 0.014$; 20.47 ± 8.25) with the FI35-41 trait. In the present study, additive effects were also found for *RUNX2* for FI35-41 and BSY traits (Table 6).

Further studies are required to elucidate the possible causes of the associations found in this study since the direct effect of the mutations, interactions with other genes, and/or the linkage disequilibrium between the SNPs and causative mutations resulting from an indirect effect (Fornari et al., 2014) may be responsible for the associations of the two SNPs studied here with several traits in this broiler population.

CONCLUSIONS

Despite the functional importance of the *RUNX2* and *TNFSF11* genes in bone metabolism, associations with bone integrity-related traits were found only for chilled femur weight and the *RUNX2* gene. However, the chilled femur weight is not a good parameter for use as a selection criterion in this population. Furthermore, associations of *RUNX2* and *TNFSF11* were identified with performance, carcass, and organ traits and could be used in the selection process to improve performance and carcass composition traits of broilers, after appropriate validation.

Although the limiting factor that *TNFSF11* (*g.14,862T>C*) is not in Hardy-Weinberg equilibrium and the negative pleiotropic effects involving important traits compromise their potential use in marker-assisted selection, it is important to emphasize the relevance of candidate gene studies to improve our understanding of the genetic architecture control of traits involved in broiler production.

Conflicts of interest

The authors declare no conflict of interest.

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Supplementary material

Table S1. Production traits evaluated from the TT Reference Population database.

Table S2. PCR primer sequences, GenBank accession No. of *RUNX2* and *TNFSF11* gene sequences, and amplicon size.