VARIATION IN THE FORKHEAD BOX CLASS O3 (FOXO3) GENE AND ITS ASSOCIATION WITH LIFESPAN TRAITS IN BARKI EWES

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In sheep industry, the most important costs are related to feeding, replacement and housing. Replacement contributes about 21% of the total costs of sheep production (Ktbl, 2009). Extension of sheep lifespan could increase average age of the flock and female lamb number that will be sold for slaughtering and reduce the costs associated with raising or/and purchasing replacement females (Vukasinovic, 1999). However, selection for lifespan is not commonly the focus of breeding programs as direct selection for long-lived breeding stock is virtually impossible until late in the reproductive life of the animal (Byun *et al*., 2011).

Reproductive traits, generally, have not been selected for in sheep improvement programs because they have low heritabilities, discrete phenotypic expressions and are expressed on in a sexually mature ewe leading to low selection intensities and long generation intervals (Bradford, 1985). Among these reproductive traits, lambing number, total number of lambs born per ewe (TNLB), total weight of lambs born per ewe (TWLB), total number of lambs weaned per ewe (TNLW), total weight of lambs weaned per ewe (TWLW), twining rate and ewe rearing ability, are the most important and thus most studied as increases in these traits offer an opportunity for increasing the efficiency of any kinds of sheep production systems.

Understanding the genetic control of ewes' age and reproductive traits would offer the opportunity to utilize natural variation and improve selective breeding programs through genetic markers. Information from genetic markers could be used to select rams that carry desirable alleles for ewes' age and reproductive traits and to select ewes without waiting for them to reach sexual maturity and have their first litter.

Forkhead Box Class O3 (FOXO3) is a member of the forkhead family of transcription factors, encoded by FOXO3 gene, expressed in the cell nucleus of various tissues and participates in post-transcriptional regulations of some genes that have biological functions controlling the insulin/insulin like growth factor-1 pathway and glucose metabolism (Pawlikowska *et al*., 2009), cell differentiation and proliferation (Seoane *et al*., 2004), muscle mass and atrophy (Mammucari *et al*., 2007; Hudson *et al*., 2014), antiviral responses (Litvak *et al*., 2012; Lee *et al*., 2013), DNA damage

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reparation (Chung *et al*., 2012), oxidative stress protection (Marinkovic *et al*. 2007; Salcher *et al*., 2014) and tumor suppression (Renault *et al*., 2011; Savkovic, 2013).

It is assumed that the previous cellular functions have potential roles affecting age of most lived organisms. Evidence-based on several studies approved that the variation in FOXO3 gene or in the expression of FOXO3 factor was associated with both aging and age of human (Willcox *et al*., 2008; Anselmi *et al*., 2009; Flachsbart *et al*., 2009; Gravina *et al*., 2009; Li *et al*., 2009; Soerensen *et al*., 2010; He *et al*., 2014).

During female reproductive life of mammals, primordial follicles are critically important for fertility and dictate the onset of menopause. FOXO3 factor serves an essential role to control the balance between the conservation and activation of primordial follicles preserving them until later in life (John *et al*., 2008). Overexpression of FOXO3 increased ovarian reproductive capacity and follicle number and decreased gonadotropin levels which enhanced the fertility in mice and pig by 31-49% (Moniruzzaman *et al*., 2010; Pelosi *et al*., 2013). A mutation in FOXO3 gene increased abnormal oocyte apoptosis and primordial follicle activation which caused premature ovarian failure in Chinese women (Wang *et al*., 2010).

In agriculture animal species, there are only two studies carried out by Byun *et al*. (2011 & 2013) to identify the variation in ovine FOXO3 gene and its association with age and fertility in New Zealand sheep. They amplified and analyzed 2 regions in exon 1 and 4 regions in exon 2 by polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP) tool, and identified 10 single nucleotide polymorphisms (SNP) defining 7 haplotypes in the first two regions in exon 2. They suggested that the haplotypes derived from those two regions are associated with the lifespan of sheep.

According to this sort of information about FOXO3 factor, the FOXO3 gene which encodes for this factor could be considered as a candidate gene associated with age and reproductive traits of Barki ewes. The purposes of this study were to identify the variation in a highly variable region in exon 2 of the ovine FOXO3 gene and examine association of this variation with age and reproductive traits in Barki ewes.

MATERIAL AND METHODS

Animal resources and rearing procedures

A total number of 96 ewes from 8 sires (their ages were 4-10 years), grew up at Maryout Research Station, Desert Research Center, were used to carry out this study.

Usually, ewes are allowed to breed for the first time at about 18-19 months of age. The mating season is usually starts in September and lasts for a period of 35 days (3 estrus cycles). All rams and ewes in the flock are weighted and then selected

for mating according to their visual appraisal of general health and conditions as well as their individual performance and parent offspring. The selected rams and ewes are divided into mating groups depending on their pedigree to avoid inbreeding. Ewes are joined in pens with single rams in groups of 20-25 ewes. After the mating period, ewes are separated from rams and kept as one group until lambing. The lambing season usually starts in March. At birth, each lamb is tagged and weighted. Lambs suckle until weaning at about 90 days.

The composite traits of ewe's lifespan were calculated from the ewes' records as: age of ewe, the total number of lambs born per ewe (TNLB), the total weight of lambs born per ewe (TWLB), the total number of lambs weaned per ewe (TNLW), the total weight of lambs weaned per ewe (TWLW), the rearing ability of ewe (TNLW/TNLB), the lambing rate (lambing number / joining number) and the twining rate of ewe (TNLB/ lambing number).

Blood samples were collected from the phenotyped ewes using 5 ml heparinized tubes and stored at -80°C whereupon the extraction of genomic DNA using DNA extraction kit (Promega).

Polymerase chain reaction

Previously primers described by Byun *et al*. (2011), (F: 5`-AACGCCAGCACAGTCAGC-3`; R: 5`-CTTGTTCTCTTGGATGGTCT -3`) were used to amplify a 420 bp fragment in exon 2 of the ovine FOXO3 gene. All PCR reactions were carried out in a total volume of 25 µl containing 0.30 µM of each primer, 1X of high fidelity reaction buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.3), 2 mM of $MgCl_2$, 200 µM of dNTP and 0.7 U of *Taq* DNA polymerase. Reaction parameters were: denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 64°C for 30 sec and 72°C for 30 sec. The final extension was at 72°C for 10 min.

Single strand conformational polymorphism

PCR products were prepared for electrophoresis as follows: 2 µl of PCR product was mixed with 8 µl of denaturing loading buffer (95% formamide, 0.025% bromophenol blue, 0.025% xylene cyanol and 20 mM EDTA; all reagent from Sigma-Aldrich, St. Louis, Missouri). The mixture was heated to 105°C for 5 min, rapidly cooled on wet ice and then was loaded on 16×18 cm; 12% acrylamide: bisacrylamide (37.5:1; Bio-Rad) gels. Electrophoresis was run, using Protein II xi cells (Bio-Rad), for 16 h at 200 v and 25°C in 0.5 x TBE buffer. Gels were silver stained according to the method of Sanguinette *et al*. (1994).

Statistical analysis

All statistical analyses were performed using SPSS software, version 19 (SPSS Science Inc., Chicago, IL, USA). Statistical analyses exploring the effects of variation in FOXO3 gene on age and reproductive traits were undertaken using

one-way analysis of variance. Three different sets of modeling approaches were used to test these effects.

The first set of general linear mixed model (GLMMs) was used to assess the effect of FOXO3 genotypes on age and reproductive traits, the second set of GLMMs was used to explore the effect of the presence/absence of each FOXO3 alleles on age and reproductive traits and the third set of GLMMs was performed to test the effect of the number of FOXO3 allele copies present on age and reproductive traits. FOXO3 genotype was fitted as a fixed factor while sire was fitted as a random factor in each model. Age of ewe at first mating and weight of ewe at first mating were included in the model as covariates. Where significant, these were further explored using pairwise comparison (Duncin test; $P \leq 0.05$).

The generalized statistical model that was used: $Y_{ijk} = \mu + G_i + S_j + \varepsilon_{ijK}$

Where Y_{ijK} = trait value, μ = general mean, G_i = *the* fixed effect of FOXO3 genotype in the first set of GLMMs, the presence/ absence of each FOXO3 alleles in the second set of GLMMs or the number of FOXO3 allele copies present in the third set of GLMMs, S_j is the random effect of sire and ε_{ijK} = the random error associated with each observation, assumed to be normally and independently distributed with zero mean and variance σ^2.

RESULTS AND DISCUSSION

Genotyping at the exon 2 of ovine FOXO3 gene revealed the presence of three alleles, A, B, and C with frequencies of 0.57, 0.28 and 0.15, respectively and five genotypes, AA, AB, AC, BB and CC with frequencies of 0.26, 0.44, 0.18, 0.06 and 0.06, respectively (Fig. 1). The detected alleles and genotypes in Barki ewes are similar to the findings of Byun *et al*. (2011) in New Zealand sheep with the exception of disappearing BC genotype in Barki ewes. However the observed frequency of A allele was lower and the frequencies of B and C alleles were higher than their corresponding alleles observed in New Zealand sheep.

Sire significantly ($P < 0.05$) affected TWLW and twining rate. No associations were found between all the studied traits and the interaction between sire and genotype.

Age of ewe at first mating had no effect on the studied traits, however weight of ewe at first mating significantly ($P < 0.05$) affected rearing ability, TWLB and TWLW.

The obtained results (Table 1) demonstrated that the FOXO3 genotype had significant ($P < 0.05$) effect on age of ewe, twining rate, TNLB and TWLB. Comparisons of ewe age and reproductive traits of the FOXO3 genotypes showed that ewes with the CC genotype had longer age and produced more TNLB and TWLB than ewes with the AA and BB genotypes. However the ewes with BB genotype had more twining rate than the other genotypes. The significantly longer age of ewes with the CC genotype was in

agreement with the result of Byun *et al*. (2011).

The effects of the presence/absence of the detected alleles on the studied traits are shown in Table (2). The presence of C allele was associated with longer age, lambing number and TNLB, whereas the presence of allele B was associated with increased twining rate, shorter age and decreased ewe rearing ability and TNLW. No associations were found between the presence/absence of allele A and the studied traits.

The associations between the number of FOXO3 allele copies present and age and reproductive traits of ewes are presented in Table (3). The presence of two copies of allele C was associated with longer age and increased TNLB. Having two copies of B allele also was associated with shorter age and increased twinning rate, rearing ability and TNLW. There were no associations between the number of A allele copies present and all traits.

A surprising of our findings is the presence of C allele, which was found to be associated with long age and high rearing ability, in a high frequency of 0.15 in Barki ewes in compare to a low frequency of 0.01 in New Zealand ewes. This means that the short age of Barki ewes in compare to the New Zealand sheep may due to the environmental factors.

Our results proved that the variation in FOXO3 genotypes affected the age of Barki ewes, whereas ewes with CC genotype were found to have longer age than ewes with other genotypes. Numerous studies demonstrated that the FOXO transcription factors promote age from worms to mammals. FOXO3 protein plays a critical role affecting the antioxidant system through controlling the transcription activation of genes encode for the antioxidant enzymes such as superoxide dismutase (SD), catalase (CAT) and gluthioneperoxidase (GPX) that have been shown to have higher level of activity in longer lived strains of species. In *Drosophila*, overexpressing in SD, GPX and CAT reduced protein oxidative damage, increased metabolism rate and delayed losing motor ability, and these phenomena extended the age by~ 30% (Sun *et al*., 2002; Mockett *et al*., 1999). An extension in the age of Murine had been achieved by overexpression of CAT enzyme (Schriner *et al*., 2005). Also, FOXO3 controls some biological functions like detoxification of reactive oxygen species (Kops *et al*., 2002) and repair of damaged DNA (Tran *et al*., 2002). Detoxifying reactive oxygen species and repairing damaged DNA was associated with increased organismal lifespan in human and another 35 species (Kirkwood and Austad, 2000) and these additional functions of FOXO3 may be relevant to FOXO3's ability to control lifespan.

This study demonstrated that, ewes with genotype BB have higher twining rate in compare to other genotypes. This means that the variation in FOXO3 plays a role in sheep reproduction. An increasing number of studies have provided evidence that FOXO3 controls crucial steps in em-

bryogenesis and is important for the development of ovarian follicles and reproductive organs (John *et al*., 2008; Pelosi *et al*., 2013). A study confirmed a function for FOXO3 in the control of follicular development through its effect on glucose metabolism (Hosaka *et al*., 2004). Another work on FOXO3-null mice showed abnormal ovary development, and are sterile (Castrillon *et al*., 2003). In spite of having higher twining rate, the ewes with genotype BB had shorter age and decreased rearing ability and TNLW. This result may due to the decreased milk production in Barki ewes which caused a negative correlation between twining rate and each lambing rate and pre-weaning survival rate.

CONCLUSION

Based on the results of this study, we could conclude FOXO3 gene appears to be obvious candidate gene for Barki ewe selection aiming at improving age and reproductive traits. CC genotype is favored in the farm to get ewes with longer age and high rearing ability. Further investigations are needed to confirm the effect of variation in FOXO3 gene on lifespan traits in Barki sheep and other local breeds using more number of animals.

SUMMARY

In this study the polymorphisms in exon 2 of ovine Forkhead Box Class O3 (FOXO3) gene were detected in 96 Barki ewes by the polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP) analysis. The association of FOXO3 gene polymorphisms with lifespan traits was tested using general linear mixed effect models. Three alleles (A, B and C) were detected with frequencies of 0.57, 0.28 and 0.15, respectively. Also five genotypes (AA, AB, AC, BB and CC) were identified with frequencies of 0.26, 0.44, 0.18, 0.06 and 0.06, respectively. Genotypes of the FOXO3 gene were shown to be associated with age, twining rate, total number of lambs born per ewe (TNLB) and total weight of lambs born per ewe (TWLB). The presence of allele C was associated with longer age and increased TNLB, whereas the presence of allele B was associated with shorter age, increased twining rate and decreased rearing ability and total number of lams weaned per ewe (TNLW). The effect of the number of allele copies present was tested and it was found that the presence of two copies of allele C was associated with longer age and increased TNLB and the presence of two copies of allele B was associated with shorter age, increased twining rate and decreased rearing ability and TNLW. These results suggest that variation in ovine FOXO3 gene is associated with lifespan traits of Barki ewes.

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Table (1): Least Square means and standard errors of lifespan traits according to the FOXO3 genotypes effects in Barki ewes.

Trait	Mean ± SE					P-value
	AA (25)	AB (42)	AC (17)	BB (6)	CC (6)	
Age	6.20 ± 0.28^{b}	5.79 ± 0.21^{b}	6.71 ± 0.32^{b}	5.67 ± 0.42 ^{b}	7.50 ± 0.42 ^{a}	*
Lambing Number	4.24 ± 0.26	3.71 ± 0.22	4.53 ± 0.24	3.83 ± 0.60	5.00 ± 0.68	NS
Lambing Rate	0.82 ± 0.03	0.78 ± 0.03	0.81 ± 0.03	0.83 ± 0.11	0.77 ± 0.08	NS
Twining Rate	1.00 ± 0.03^{b}	1.03 ± 0.01^{ab}	1.02 ± 0.02^{ab}	1.10 ± 0.01^{a}	1.03 ± 0.03^{ab}	*
Rearing Ability	0.84 ± 0.03	0.87 ± 0.02	0.83 ± 0.03	0.71 ± 0.01	0.81 ± 0.05	NS
TNLB	4.76 ± 0.35^{ab}	3.71 ± 0.23^{b}	4.71 ± 0.24^{ab}	4.13 ± 0.60^{ab}	5.17 ± 0.75^{a}	*
TWLB	16.86 ± 1.33^{ab}	13.07 ± 0.84^{b}	16.62 ± 0.92^{ab}	14.25 ± 2.26^{b}	18.22 ± 2.83^{a}	*
TNLW	3.96 ± 0.30	3.49 ± 0.20	3.88 ± 0.20	3.19 ± 0.20	4.17 ± 0.60	NS
TWLW	81.86 ± 6.14	68.39 ± 4.17	81.97 ± 3.99	81.92 ± 12.68	84.75 ± 10.66	NS

TNLB: total number of lambs born per ewe; TWLB: total weight of lambs born per ewe; TNLW: total number of lambs weaned per ewe; TWLW: total weight of lambs weaned per ewe; NS: no significance; *: refers to significance at ($P < 0.05$); ** refers to significance at ($P < 0.01$).

Table (2): Association of FOXO3 alleles with various lifespan traits of Barki ewes.

Trait	Allele being assessed	Mean ± SE				P-value
		Allele absent	N	Allele present	N	
Age	A	6.58 ± 0.39	12	6.10 ± 0.16	84	NS
	B	6.54 ± 0.20	48	5.77 ± 0.19	48	**
	C	5.92 ± 0.16	73	6.91 ± 0.27	23	**
Lambing Number	A	4.42 ± 0.46	12	4.04 ± 0.15	84	NS
	B	4.14 ± 0.18	48	3.93 ± 0.21	48	NS
	C	3.90 ± 0.17	73	4.65 ± 0.25	23	*
Lambing Rate	A	0.80 ± 0.07	12	0.80 ± 0.02	84	NS
	B	0.81 ± 0.02	48	0.79 ± 0.03	48	NS
	C	0.80 ± 0.02	73	0.80 ± 0.31	23	NS
Twining Rate	A	1.01 ± 0.01	12	1.04 ± 0.01	84	NS
	B	1.01 ± 0.02	48	1.07 ± 0.01	48	*
	C	1.04 ± 0.01	73	1.03 ± 0.02	23	NS
Rearing Ability	A	0.91 ± 0.04	12	0.85 ± 0.01	84	NS
	B	0.87 ± 0.02	48	0.75 ± 0.02	48	**
	C	0.87 ± 0.02	73	0.82 ± 0.02	23	NS
TNLB	A	4.50 ± 0.50	12	4.23 ± 0.17	84	NS
	B	4.29 ± 0.22	48	3.93 ± 0.22	48	NS
	C	4.08 ± 0.19	73	4.83 ± 0.26	23	*
TWLB	A	16.23 ± 1.83	12	4.92 ± 0.63	84	NS
	B	15.95 ± 0.83	48	14.22 ± 0.79	48	NS
	C	14.47 ± 0.71	73	17.04 ± 0.98	23	NS
TNLW	A	4.00 ± 0.41	12	3.56 ± 0.15	84	NS
	B	3.96 ± 0.20	48	3.27 ± 0.20	48	*
	C	3.51 ± 0.17	73	3.96 ± 0.21	23	NS
TWLW	A	83.33 ± 7.91	12	75.15 ± 2.95	84	NS
	B	82.26 ± 3.67	48	75.08 ± 3.98	48	NS
	C	74.12 ± 3.40	73	82.70 ± 3.92	23	NS

TNLB: total number of lambs born per ewe; TWLB: total weight of lambs born per ewe; TNLW: total number of lambs weaned per ewe; TWLW: total weight of lambs weaned per ewe; NS: no significance; *: refers to significance at ($P < 0.05$); ** refers to significance at ($P < 0.01$).

Table (3): Association of FOXO3 allele copy number with lifespan traits of Barki ewes.

Trait	Allele being assessed	Mean ± SE						P-value
		Allele absent	N	Allele 1 copy	N	Allele 2 copies	N	
Age	A	6.58 ± 0.39	12	6.05 ± 0.18	59	6.20 ± 0.20	25	NS
	B	6.54 ± 0.20^{a}	48	5.79 ± 0.21^{ab}	42	5.67 ± 0.42^{ab}	6	*
	C	5.92 ± 0.16^{b}	73	6.71 ± 0.33^{ab}	17	7.50 ± 0.43^{a}	6	**
Lambing Number	A	4.42 ± 0.46	12	3.95 ± 0.18	59	4.24 ± 0.26	25	NS
	B	4.14 ± 0.18^{a}	48	3.71 ± 0.22^{b}	42	3.83 ± 0.60^{ab}	6	*
	C	3.90 ± 0.17	73	4.53 ± 0.24	17	5.00 ± 0.68	6	NS
Lambing Rate	A	0.80 ± 0.07	12	0.79 ± 0.02	59	0.82 ± 0.03	25	NS
	B	0.81 ± 0.02	48	0.78 ± 0.03	42	0.83 ± 0.11	6	NS
	C	0.80 ± 0.02	73	0.81 ± 0.03	17	0.77 ± 0.08	6	NS
Twining Rate	A	1.01 ± 0.01	12	1.02 ± 0.01	59	1.00 ± 0.16	25	NS
	B	1.01 ± 0.02^{b}	48	1.02 ± 0.01^{b}	42	1.10 ± 0.01^{a}	6	*
	C	1.04 ± 0.01	73	1.03 ± 0.02	17	1.03 ± 0.03	6	NS
Rearing Ability	A	0.91 ± 0.04	12	0.86 ± 0.02	59	0.84 ± 0.03	25	NS
	B	0.87 ± 0.02^{a}	48	0.81 ± 0.02^{ab}	42	0.71 ± 0.01^{b}	6	*
	C	0.87 ± 0.02	73	0.83 ± 0.03	17	0.81 ± 0.05	6	NS
TNLB	A	4.50 ± 0.50	12	4.00 ± 0.19	59	4.76 ± 0.35	25	NS
	B	4.29 ± 0.22	48	4.11 ± 0.23	42	4.13 ± 0.60	6	NS
	C	4.08 ± 0.19^{b}	73	4.71 ± 0.24^{ab}	17	5.17 ± 0.75^{a}	6	*
TWLB	A	16.23 ± 1.83	12	14.09 ± 0.68	59	16.86 ± 1.33	25	NS
	B	15.95 ± 0.83^{a}	48	13.07 ± 0.85^{b}	42	14.25 ± 2.26^{ab}	6	**
	C	14.47 ± 0.71	73	16.62 ± 0.92	17	18.22 ± 2.84	6	NS
TNLW	A	4.00 ± 0.41	12	3.39 ± 0.16	59	3.96 ± 0.31	25	NS
	B	3.96 ± 0.20^{a}	48	3.83 ± 0.60^{ab}	42	3.19 ± 0.20^{b}	6	*
	C	3.51 ± 0.17	73	3.88 ± 0.21	17	4.17 ± 0.60	6	NS
TWLW	A	83.33 ± 7.91	12	72.31 ± 3.27	59	81.86 ± 6.14	25	NS
	B	82.26 ± 3.67	48	75.39 ± 4.17	42	81.92 ± 2.76	6	NS
	C	74.12 ± 3.40	73	81.97 ± 3.99	17	84.75 ± 10.66	6	NS

TNLB: total number of lambs born per ewe; TWLB: total weight of lambs born per ewe; TNLW: total number of lambs weaned per ewe; TWLW: total weight of lambs weaned per ewe; NS: no significance; *: refers to significance at ($P < 0.05$); ** refers to significance at ($P < 0.01$).

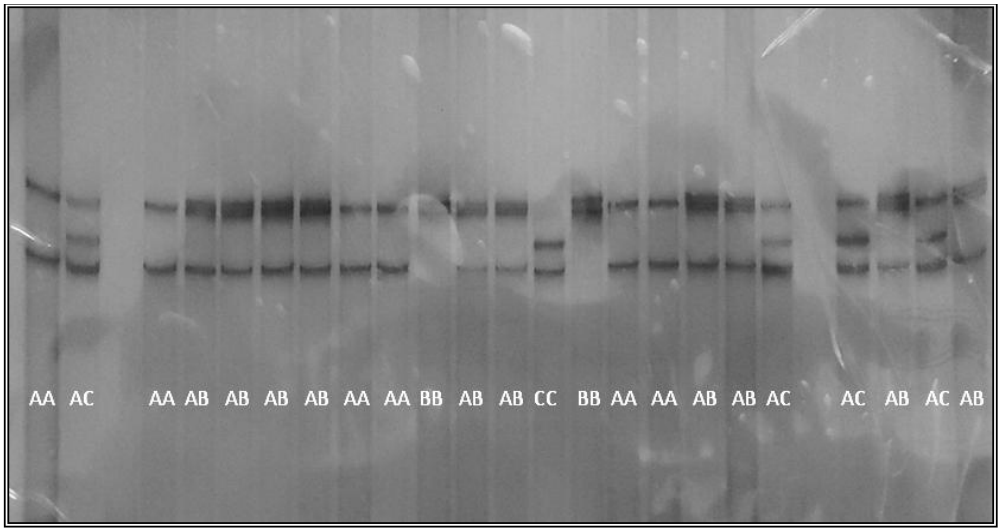

Fig. (1): Polymorphisms in exon 2 of ovine FOXO3 identified using single strand conformational polymorphism analysis.