SINGLE STRAND CONFORMATIONAL POLYMORPHISM OF *ADRβ3* GENE AND ITS ASSOCIATION WITH LIVE PERFORMANCE TRAITS IN BARKI SHEEP

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Growth rate represents an economically important trait in sheep. Consumer preference for heavier lambs with less body fat has placed considerable pressure on the sheep industry to increase size and weight. Lambs that grow rapidly can reach their market weight at a younger age, which means they need reduced amounts of feedstuffs and have a decreased risk of death (Safari *et al*., 2005).

Non productive traits are either correlated with traits of economic importance or have a direct economic value. In sheep, body conformation traits such as heart girth, body length and height at withers are associated with feed intake, body weight and fat and muscle percentages (Ibiwoye *et al*., 1993; Atta and Elkhidir, 2004; Afolayan *et al*., 2006; Otoikhian *et al*., 2008; Cam *et al*., 2010; Musa *et al*., 2012; Tariq *et al*., 2012; Younas *et al*., 2013). Body size in general has long been considered as a paradigm for quantitative inheritance. It is normally distributed and seems to be controlled by many genes, each with relatively small additive effect on the phenotype (Falconer and Mackay, 1996).

To date, most genetic improvement for these traits in livestock has been made by selection on bases of phenotype or on estimates of breeding values derived from phenotypes, without knowledge of the number of genes affecting the trait. Recent developments in molecular genetics have opened the possibility of using molecular markers for the genetic improvement of livestock. The candidate gene approach has been applied in the past decade for the identification of molecular markers associated with these traits. This approach is motivated by what is known about the trait biologically and can be characterized as a hypothesis testing approach (Barash *et al*., 2013).

Adrenergic Beta 3 receptor (ADRβ3) is a protein belongs to the family of beta-adrenergic receptors, mainly expressed in white and brown adipose tissues and play a crucial role in the regulation of energy balance by increasing the rate of lypolysis and thermogenesis (Takenaka *et al*., 2012). In rodent ADβ3R knock-out model, there was a reduction in lypolysis that stimulated by β3- agonists (Susulic *et al*., 1995) and a decreased body mass (Revelli *et al*., 1997).

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In human, substitution of amino acid, Trp64Arg, in the coding region of ADRβ3 were found to be associated with an impaired ability to produce intracellular cyclic adenosine mono-phosphate (Pietri-Rouxel *et al*., 1997), weight gain (Clement *et al*., 1995), abdominal obesity (Kim-Motoyama, 1997), difficulty losing weight (Yoshida *et al*., 1995) and body mass index in East Asians (Tahara *et al*., 2010; Kurokawa, 2011; Takeuchi *et al*., 2012).

In sheep, few studies concerned the variation in *ADRβ3* gene and its association with economically important traits was done. Five single strand conformational polymorphisms (SSCP) alleles were detected in the intron region of New Zealand breeds of sheep. These alleles were associated with pre-weaning growth rate in Merino-cross and New Zealand Romney sheep (Forrest *et al*., 2003; Horrell *et al*., 2009), carcass compositions in Merino-cross sheep (Forrest *et al*., 2003) , lamb mortality in New Zealand cross-breeds sheep (Forrest *et al*., 2007) and cold survival and wool staple-strength in Merino sheep (Forrest *et al*., 2006; Forrest *et al*., 2009). Another 3 SSCP alleles were detected in New Zealand cross-breed sheep by Byun *et al*. (2008). Sixteen haplotypes were identified by Yang *et al*. (2011) in the 3`untranslated region (3`UTR) and found to be associated with post-weaning growth rate in New Zealand Suffolk sheep (Yang *et al*., 2013). In the Chinese breeds of sheep, 22 single nucleotide polymorphisms (SNPs) were identified in the full length of *ADRβ3* gene; 12 of which in the exon 1 and 10 in the intron region (Wu *et al*., 2012).

According to this background, ovine *ADRβ3* gene which located on chromosome 26 and consists of 2 exons and 1 intron, is a possible candidate gene affecting live performance traits in Barki sheep. The present study was undertaken with the objective of determining the allele and genotype polymorphisms of *ADRβ3* gene and to estimate associations between these polymorphisms and live performance traits in Barki sheep.

MATERIALS AND METHODS

Animals and Phenotypic Data

This study was conducted on 66 males and 70 females of Barki lambs at Maryout Research Station, Desert Research Center. At birth, all lambs were weighted and tagged. The live weights were taken again at weaning and marketing age (9 months). From these weights, pre- and post-weaning daily gains were calculated. Body dimensional measurements were taken at marketing age using a tape. Body length was measured from the point of shoulder to pin bone. Height at withers was measured as the distance from the surface of the platform to the withers of the animal. Height at hips was measured as the distance from the surface of the platform to the hips of the animal. Heart girth was taken by measuring the circumference of the chest. Thigh circumference was taken by measuring the circumference of thigh above knee. From

body dimensional measurements, 4 conformation indices were calculated according to Salako (2006).

- Body mass index (Fleshing index) = (Marketing weight × 100) / Height at withers.
- Skeletal muscle index = (Thigh circumference × 100) / Height at withers.
- Body index = (Body length × 100) / Heart girth. When this measure is greater than 0.90, the animal is longline; between 0.86 and 0.88 is medigline and less than 0.85 is brevigline.
- Relative body index (also called length index) = (Body length × 100) / Height at withers.

Genotyping

Blood samples were collected into 5 ml heparinized tubes from the jugular vein of each sheep and stored at -80°C for several months. Genomic DNA was extracted from whole blood using a commercial kit (Qiagen, Hilden, Germany).

Polymerase chain reaction

According to the reference of Byun *et al*. (2008), a pair of primers (F: 5`-CTAGCTCAGTTCT-TTCTCTGC-3` and R: 5`-CCCAACTCCAACCCGACC-3`) were synthesized to amplify a highly variable fragment (263 bp) within the single intron of the ovine *ADRβ3* gene. PCR was performed in a 20 µl of reaction mixture containing 50 ng of genomic DNA, 0.25 µM of each primer, 150 µM dNTPs (Eppendorf, Hamburg, Germany), 1x polymerase buffer (including 1.5 µM $MgCl_2$) and 0.5 U Taq DNA polymerase (Qiagen, Hilden, Germany). The thermal cycling protocol was 2 min at 94°C followed by 35 cycles (94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec), with a final extension at 72°C for 5 min.

Single strand conformational polymorphism

A volume of 2 µl PCR products was mixed with 8 µl of loading dye (98% formamide, 0.025% bromophenol blue, 0.025% xylene cyanol, 10 mM EDTA). This mixture was denaturated at 105°C for 5 min, then chilled on wet ice for 5 min and loaded on 16 × 18 cm; 14% acrylamide: bisacrylamide (37.5: 1; Bio-Rad) gels. PCR products representative of the first eight known ADRβ3 alleles (A, B, C, D, E, F, G and H) which were detected by Byun *et al*. (2008) were included in the polyacrylamide gel (well numbers 13 and 14 of Fig. 1a), and their banding patterns were used as standards for determining the alleles present in individual Barki sheep. Electrophoresis was performed using protean II xi cells (Bio-Rad) at 200 v and 25°C for 18 h in 0.5 x TBE buffer. Gels were silver stained using the method of Sanguinette *et al*. (1994).

Statistical analysis

The statistical analysis was performed using the general linear model procedures of SPSS, version 19 (SPSS Science Inc., Chicago, IL, USA). The following fixed effect model was employed for assessing the effect of ADRβ3 geno-

types on growth traits, body dimensional measurements and conformation indices:

$$Y_{ijkl} = \mu + S_i + G_j + (SG)_{ij} + R_k + e_{ijkl}$$

Where Y_{ijkl} is phenotypic value of the trait; μ is the overall mean; S_i is the fixed effect of i^{th} sex; G_j is the fixed effect of the j^{th} genotype; $(SG)_{ij}$ is the effect of interaction between sex and genotype; R_k is the random effect of sire k^{th} and e_{ijkl} is random error effect of each observation. In the model assessing genotype effect on weaning weight, weaning age was included as a co-variate.

Only ADRβ3 genotypes with frequency > 0.03 were included in the statistical model. Least squares mean was used for multiple comparisons of these traits among different genotypes. Mean separation procedures were performed using a Duncan test, with a significance level of α = 0.05. The chi squared test was used to assess whether the frequencies of genotypes were in Hardy-Weinberg equilibrium.

RESULTS AND DISCUSSION

Allelic and genotype frequencies

PCR-SSCP analysis for the intron of *ADRβ3* gene (Fig. 1) revealed a total of eighteen SSCP patterns representing only six alleles (A, B, C, D, E and H). Compared to the New Zealand cross-breed sheep in which alleles A, B and C are present at a frequency of 60%, 4.8% and 20.3%, respectively (Byun *et al*., 2008), the genotyped Barki lambs in this study had frequencies of 33%, 18% and 28%, respectively for these alleles. In addition, the genotyped Barki lambs in this study did not have the F and G alleles, unlike the New Zealand cross-breed sheep, which had F and G alleles with frequencies of 3.8% and 5.2%, respectively.

The chi square test showed that the observed genotype frequencies were significantly ($P < 0.05$) different from those predicted, which means that the genotyped sample of Barki lambs is not in Hardy-Weinberg equilibrium. The heterozygote genotypes for this gene are over-represented according to Hardy-Weinberg law. In contrast to the results reported here, the studies carried out on New Zealand breeds of sheep by Forrest *et al*. (2003), Byun *et al*. (2008) and Horrell *et al*. (2009) showed lower level of diversity. This may due to New Zealand sheep have historically been selected for growth.

Association of sex with the studied traits

Sex of animal significantly ($P< 0.05$) affected birth weight and post-weaning daily gain (Table 2).

Association of sex × genotype interaction with the studied traits

The interaction between sex of animal and genotype significantly affected post-weaning daily gain ($P< 0.05$) and marketing weight ($P< 0.01$).

Association of genotypes with the studied traits

The relationships between ADRβ3 genotypes and growth traits are presented

in Table (3). ADRβ3 genotypes were found to be significantly associated with post-weaning daily gain ($P < 0.01$) and marketing weight ($P < 0.05$). Least square means results showed that animals with genotype AC have the highest means and animals with genotype CD have the lowest means for post-weaning daily gain and marketing weight. Multiple comparisons results indicated that the individuals with genotypes AA, AC and CC were higher ($P < 0.05$) than those individuals with genotypes CD, CE and CH in post weaning daily gain and marketing weight. In agreement with our results, genotype AC showed an association with increased post-weaning growth rate in New Zealand Suffolk sheep (Yang *et al*., 2013).

Through association analysis between ADRβ3 genotypes and each body dimensional measurements and conformation indices (Table 4), ADRβ3 genotype was also significantly associated ($P < 0.05$) with thigh circumference, body mass index and skeletal muscle index. Also, there were significant differences in thigh circumference, body mass index and skeletal muscle index between AA, AC and CC individuals and CD and CE individuals after multiple comparisons ($P < 0.05$).

The obtained results indicated that, the major effect of the variation in *ADRβ3* gene is on marketing weight, body mass index and skeletal muscle index. As cited, ADRβ3 is a part of the adrenergic system which plays a crucial role in energy balance regulation. Energy balance is a critical factor that contributes in the regulation of whole body mass and skeletal muscle mass by influencing whole-body and skeletal muscle protein metabolism (Calloway, 1975; Young *et al*., 1991). Decreases in skeletal muscle mass in response to negative energy balance are due to imbalanced rates of muscle protein synthesis and degradation (Carbone *et al*., 2012; Carbone *et al*., 2013). The previous studies approved that the effect of energy deficit resulted in a 19% decrease in skeletal muscle protein synthesis (Pasiakos *et al*., 2010), and 5-10% loss in the initial body mass (Weinheimer *et al*., 2010).

According to our findings, it could be concluded that, SSCPs occurring within *ADRβ3* gene affect growth rate after weaning, marketing weight, whole body mass and skeletal muscle mass in Barki sheep. It may be more advantageous to apply molecular marker selection for AA, CC and AC genotypes to get lambs with higher post-weaning growth rate, heavier marketing weight, increased whole-body mass and increased skeletal muscle mass. Further studies on the *ADRβ3* gene using a lot number of lambs are necessary to evaluate allele submission effects and haplotype association studies in Barki sheep and other local breeds of sheep.

SUMMARY

The adrenergic receptor β3 (ADRβ3) is the major regulator of lipolysis and homeostasis and predominantly expressed in brown and white adipose tissues (Cannon and Nedergaard, 2004). The *ADRβ3* gene which encodes for this receptor was studied as a candidate gene

associated with growth traits, body dimensional measurements and conformation indices of Barki sheep. Single strand conformational polymorphism (SSCP) was used to identify the variation in the intron region of *ADRβ3* gene for 136 male and female Barki lambs. Eighteen SSCP genotypes representing six alleles (A, B, C, D, E and H) were detected. The frequencies of these alleles were 0.331, 0.184, 0.283, 0.044, 0.073 and 0.084, respectively. The results indicated that ADRβ3 genotype significantly ($P < 0.05$) affected marketing weight, thigh circumference, body mass index and skeletal muscle index; and also significantly ($P < 0.01$) affected post-weaning daily gain. Least square means analysis showed that animals with genotypes AA, AC and CC had higher post-weaning daily gain, marketing weight, thigh circumference, body mass index and skeletal muscle index than animals with genotypes CD, CE and CH. In view of the obtained results, molecular marker selection using *ADRβ3* gene is warranted to increase post-weaning growth rate, whole-body mass and skeletal muscle mass in Barki sheep and will be of considerable economic value to sheep producer.

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Table (1): Genotypic and allelic frequencies for *ADRβ3* gene in Barki sheep.

Genotype	N	Frequency	Genotype	N	Frequency	Genotype	N	Frequency	Allele	Frequency
AA	19	0.140	BB	5	0.037	CD	8	0.059	A	0.331
AB	19	0.140	BC	10	0.073	CE	5	0.037	B	0.184
AC	25	0.184	BD	1	0.007	CH	9	0.066	C	0.283
AD	1	0.007	BE	4	0.029	DE	2	0.015	D	0.044
AE	5	0.037	BH	6	0.044	EE	2	0.015	E	0.073
AH	2	0.015	CC	10	0.073	EH	3	0.022	H	0.084

Table (2): Least Square means and standard errors of growth traits, body measurements and conformation indices according to the sex effects in Barki sheep.

Trait	Means ± SE		P-value
	Male	Female	
Birth weight (Kg)	3.64 ± 0.08	3.42 ± 0.07	*
Weaning weight (Kg)	20.24 ± 0.56	19.48 ± 0.40	NS
Pre-weaning daily gain (gm/d)	180.69 ± 5.14	173.98 ± 3.76	NS
Marketing weight (kg)	44.08 ± 0.59	42.59 ± 1.32	NS
Post-weaning daily gain (gm/d)	89.99 ± 1.26	83.24 ± 4.16	*
Height at withers (cm)	72.64 ± 0.56	70.01 ± 0.56	NS
Height at hip (cm)	71.22 ± 0.56	69.55 ± 0.51	NS
Body length (cm)	72.92 ± 0.58	70.73 ± 0.54	NS
Heart girth (cm)	91.80 ± 0.87	89.53 ± 0.67	NS
Thigh circumference (cm)	31.68 ± 0.26	29.55 ± 0.52	NS
Body mass index	62.12 ± 0.74	59.15 ± 1.62	NS
Skeletal muscle index	79.44 ± 0.69	79.35 ± 0.58	NS
Body index	43.29 ± 0.38	41.76 ± 0.61	NS
Relative body index	101.20 ± 0.75	100.50 ± 0.86	NS

NS: no significance; *: refers to significance at ($P < 0.05$); ** refers to significance at ($P < 0.01$).

Table (3): Least Square means and standard errors of growth traits according to the ADRβ3 genotype effects in Barki sheep.

Genotype	N	Birth weight (Kg)	Weaning weight (Kg)	Pre-weaning daily gain (gm/day)	Marketing weight (Kg)	Post-weaning daily gain (gm/day)
AA	19	3.87 ± 0.10	20.87 ± 0.88	183 ± 6.98	46.81 ± 1.54^{ab}	95 ± 4.30^{ab}
AB	19	3.21 ± 0.11	18.58 ± 0.84	163 ± 8.29	39.81 ± 1.38^{bcd}	77 ± 5.44^{bcd}
AC	25	3.49 ± 0.14	20.58 ± 0.83	184 ± 7.78	48.40 ± 1.86^{a}	102 ± 4.70^{a}
AE	6	3.42 ± 0.13	21.25 ± 1.19	192 ± 10.97	44.25 ± 0.66^{abc}	84 ± 4.78^{abc}
BB	5	3.60 ± 0.17	18.20 ± 1.20	156 ± 10.84	42.90 ± 0.82^{abc}	90 ± 3.89^{abc}
BC	10	3.80 ± 0.12	20.60 ± 0.82	186 ± 8.74	44.25 ± 1.28^{abc}	87 ± 6.25^{abc}
BH	6	3.67 ± 0.09	21.17 ± 0.77	183 ± 6.23	44.66 ± 0.81^{abc}	85 ± 4.13^{abc}
CC	10	3.60 ± 0.12	20.50 ± 0.62	185 ± 7.94	46.67 ± 1.63^{ab}	95 ± 9.50^{ab}
CD	8	3.41 ± 0.19	18.50 ± 1.52	161 ± 14.48	34.45 ± 2.59^{d}	68 ± 9.74^{cd}
CE	5	3.25 ± 0.08	15.60 ± 2.02	137 ± 24.92	34.50 ± 3.77^{d}	69 ± 8.95^{cd}
CH	8	3.38 ± 0.08	19.44 ± 1.24	171 ± 9.56	38.80 ± 3.07^{cd}	70 ± 8.93^{cd}
P-value		NS	NS	NS	*	**

NS: no significance; *: refers to significance at ($P < 0.05$); ** refers to significance at ($P < 0.01$).

Table (4): Least Square means and standard errors of body measurements and conformation indices according to the ADRβ3 genotype effects in Barki sheep.

Genotype	N	Height at Withers (cm)	Height at Hip (cm)	Body Length (cm)	Heart Girth (cm)	Thigh circumfe-rence (cm)	Body Mass Index	Skeletal mus-cle index	Body Index	Relative Body index
AA	19	70.9 ± 0.70	69.4 ± 0.74	71.8 ± 1.07	92.6 ± 1.34	31.63 ± 0.55^{a}	64.1 ± 2.23^{a}	44.64 ± 0.72^{a}	77.6 ± 1.02	101 ± 1.42
AB	19	69.2 ± 0.78	67.9 ± 0.68	69.6 ± 0.88	88.0 ± 1.03	28.47 ± 0.64^{bc}	57.5 ± 1.86^{abcd}	41.13 ± 0.80^{abc}	79.2 ± 1.16	100 ± 1.12
AC	25	72.7 ± 1.02	71.1 ± 0.93	73.2 ± 0.94	93.2 ± 1.65	32.00 ± 0.67^{a}	66.4 ± 2.00^{a}	44.71 ± 0.84^{a}	78.8 ± 1.09	101 ± 1.40
AE	6	74.0 ± 1.15	73.2 ± 1.14	72.5 ± 2.31	90.0 ± 2.08	31.00 ± 0.52^{ab}	59.8 ± 0.74^{abc}	41.91 ± 0.73^{abc}	80.6 ± 2.08	97 ± 2.21
BB	5	71.2 ± 2.47	69.8 ± 2.49	71.4 ± 1.02	89.0 ± 3.17	31.00 ± 0.63^{ab}	60.4 ± 1.74^{ab}	43.70 ± 1.39^{ab}	80.5 ± 2.59	100 ± 3.32
BC	10	71.1 ± 1.45	70.9 ± 1.38	72.5 ± 1.00	91.1 ± 1.17	30.80 ± 0.80^{ab}	61.7 ± 2.36^{ab}	42.97 ± 1.67^{ab}	79.6 ± 1.16	100 ± 2.59
BH	6	74.5 ± 1.58	72.0 ± 1.43	73.3 ± 0.95	91.7 ± 1.05	31.00 ± 0.52^{ab}	60.1 ± 1.80^{abc}	41.76 ± 1.49^{abc}	80.0 ± 1.26	98 ± 1.48
CC	10	72.9 ± 1.32	71.4 ± 1.25	72.3 ± 1.27	91.0 ± 1.55	31.72 ± 0.67^{a}	65.9 ± 1.98^{a}	43.93 ± 0.76^{a}	79.6 ± 1.65	99 ± 1.49
CD	8	68.4 ± 0.78	67.1 ± 0.83	70.4 ± 1.41	88.1 ± 1.40	26.50 ± 1.25^{c}	50.3 ± 3.48^{d}	38.74 ± 1.69^{c}	79.9 ± 1.34	102 ± 1.54
CE	5	67.6 ± 1.16	66.2 ± 1.11	68.9 ± 1.76	86.6 ± 1.20	26.80 ± 1.65^{c}	50.3 ± 4.87^{cd}	39.55 ± 1.86^{bc}	79.5 ± 1.77	101 ± 2.66
CH	8	71.0 ± 1.92	69.6 ± 2.04	73.4 ± 1.79	89.4 ± 2.48	29.13 ± 1.09^{abc}	54.2 ± 3.25^{bcd}	40.98 ± 0.82^{abc}	82.4 ± 2.61	103 ± 3.46
P-value		NS	NS	NS	NS	*	*	*	NS	NS

NS: no significance; *: refers to significance at ($P < 0.05$); ** refers to significance at ($P < 0.01$).

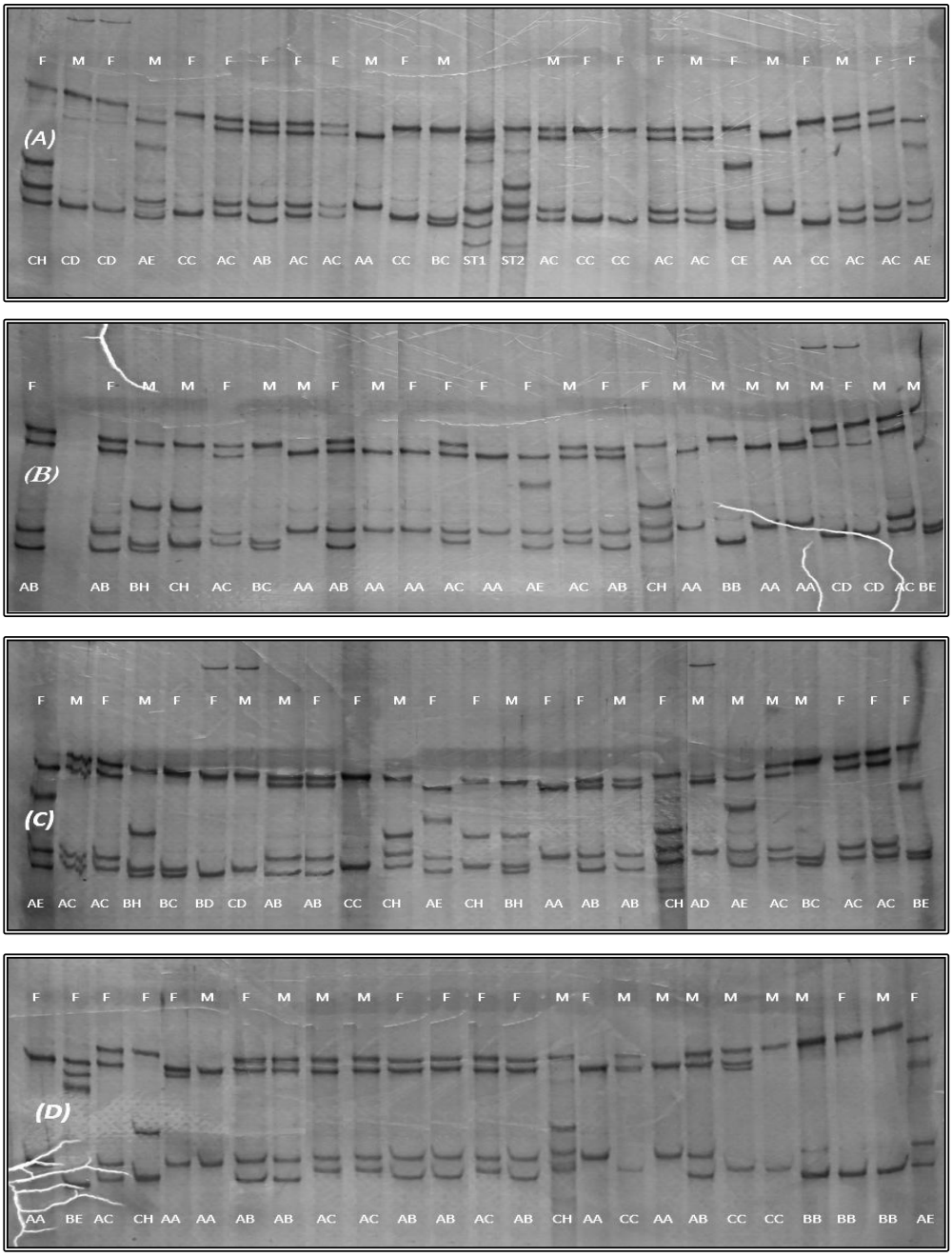

Fig. (1): Polymorphisms in the intron of *ADRβ3* gene in Barki sheep identified using SSCP analysis. M: refers to male; F: refers to female.