ASSOCIATION BETWEEN SSCPs AT ALGARVIA GOAT GH GENE AND MILK TRAITS

ASOCIACIÓN ENTRE LOS SSCPs DEL GEN DE LA GH DE LA CABRA ALGARVIA Y CARACTERÍSTICAS DE LA LECHE

Malveiro, E.[1], P.X. Marques[1], I.C. Santos[1], C. Belo[2] and A. Cravador[1]

[1]Unidade de Ciências e Tecnologias Agrárias. Universidade do Algarve. Campus de Gambelas. 8000-810 Faro. Portugal. E-mail: acravad@ualg.pt

[2]Departamento de Ovinicultura e Caprinicultura. Estação Zootécnica Nacional. Quinta da Fonte Boa. 2000-763 Vale de Santarém. Portugal. E-mail: ezn.inia@mail.telepac.pt.

ADDITIONAL KEYWORDS

Polymorphism. PCR-SSCP. Genetic markers.

PALABRAS CLAVE ADICIONALES

Polimorfismo. PCR-SSCP. Marcadores genéticos.

SUMMARY

The DNA from one hundred eight goats belonging to the indigenous portuguese Algarvia breed was analysed. Single-strand conformation polymorphism's (SSCP) were identified at the five exons of the goat Growth Hormone (gGH) gene. Two conformational patterns were found in each of exons 1 and 2, four in exon 3, seven in exon 4 and five in exon 5. The establishment of an association of these SSCP patterns with milk, fat and protein production, and fat and protein content was attempted. Patterns A, F and D of exons 2, 4 and 5, respectively, are positively associated with milk, fat and protein production ($p<0.05$). Pattern A of exon 2 is also positively associated with fat content ($p<0.05$).

RESUMEN

Se ha analizado el DNA de 108 cabras de la raza autóctona Algarvia. Se determinaron los polimorfismos (SSCPs) en los cinco exones del gen de la GH caprina. Se observaron dos polimorfismos en los exones 1 y 2, cuatro en el exón 3, siete en el exón 4 y cinco en el exón 5. Se ha establecido una asociación de estos SSCPs con la producción de leche, grasa y proteína, así como con el porcentaje de grasa y proteína. Los patrones A, F e D de los exones 2, 4 y 5, respectivamente, están positivamente asociados con la producción de leche y proteína ($p<0,05$). El patrón A del exón 2 está también positivamente asociado com el porcentaje en grasa ($p<0,05$).

INTRODUCTION

Algarvia goat is a Portuguese indigenous breed of unknown origin, whose main potential is milk production that is used for cheese manufacture. Algarvia goats are reared mainly in the Algarve region, where they are very well adapted to dry sylvan areas, and play an important role as an economic resource to the rural populations.

The GH secreted by the pituitary gland, plays an important role on lactation. Some GH secretion parameters and peak frequency are associated with dairy animals of high genetic value (Reinecke *et al.,* 1993). It has been demonstrated that those animals with high milk yield reveal superior GH average levels when compared to those observed in animals with lower production, namely during peak lactation (Bonczeck *et al.,* 1988).

Single-strand conformation polymorphism (SSCP) is a powerful method for identifying sequence variation in amplified DNA. SSCP analysis of DNA have been used for detection of genetic mutations in humans (Orita *et al.,* 1989), rats (Pravenec *et al.,* 1992), cattle (Kirkpatrick, 1992) and in various bacteriological (Morohoshi *et al.,* 1991) and viral (Fujita *et al.,* 1992) systems. Most significant studies using the SSCP approach were accomplished on bovines in linkage analysis (Neibergs *et al.,* 1993) and to define intragenic haplotypes at the growth hormone (Lagziel *et al.,* 1996).

The search for SSCP polymorphism's could lead to the finding of genetic markers useful for improved selection of agricultural populations, namely when applied to candidate genes associated with quantitative genetic variation in traits of economic importance.

As part of a programme of genetic selection aiming at the improvement of production traits, we have identified single-strand conformation polymorphisms at the five exons of the gGH gene, by PCR-SSCP analysis. This work presents preliminary results towards the establishment of an association of those polymorphisms with milk, fat and protein yield, and fat and protein content.

MATERIAL AND METHODS

DNA samples: DNA was obtained from peripheral blood leukocytes of 108 animals of Algarvia goat breed using a DNA Isolation Kit from Puregene.

PCR amplification: The 5 exons from the gGH gene were amplified by PCR using the five primer pairs shown in **table I**. Five amplification fragments were generated ranging in size from 112 to 289 bp. PCR reactions were performed in an thermocycler UNOII from Biometra, using Ready to Go PCR beads, from Amersham Pharmacia Biotech, according to the following conditions: 25 to 50 ng of genomic DNA (**table I**); 1.5 U *Taq* DNA polymerase, 10 mM Tris-HCl, pH 9; 50 mM KCl; 1.5 mM MgCl2; 200 μM of each dNTP; stabilisers, including BSA, for a final volume of 25 μl. The amplification included 30 cycles of denaturation at 95ºC for 30 s, annealing at 57ºC-70ºC (**table I**) during 30 s and extension for 30 s at 72ºC. The product of each amplification was analysed by electrophoresis on 2 p.100 agarose gel (5V/cm), using ethidium bromide staining.

SSCP analysis: For SSCP analysis, 5 μl of each amplification product were added to 15 μl or 20 μl (**table II**) of stop solution (95 p.100 formamide, 10 mM NaOH, 0.05 p.100 xylene cyanol and 0.05 p.100 bromophenol blue). The samples were heat-denatured at 95ºC for 5 min, and chilled at 0ºC, and loaded

Table I. *SSCP fragments at gGH gene and PCR analysis parameters.* (Fragmentos de SSCP en el gen de la gGH y análisis de los parámetros de PCR).

Exon	Length	Primer sequence	$T_{annealing}$ (°C)	Primers (pmol)	DNA (ng)	Fragment localization (bp)
1	112	5'TAA TGG AGG GGA TTT TTG TG3' 5'CAG AGA CCA ATT CCA GGA TC3'	57	16	25	360-471
2	198	5'TCT AGG ACA CAT CTC TGG GG3' 5'CTC TCC CTA GGG CCC CGG AC3'	65	16	50	682-879
3	157	5'GTG TGT TCT CCC CCC AGG AG3' 5'CTC GGT CCT AGG TGG CCA CT3'	60	4	25	1063-1219
4	200	5'GGA AGG GAC CCA ACA ATG CCA3' 5'CTG CCA GCA GGA CTT GGA GC3'	70	8	25	1416-1615
5	289	5'AAA GGA CAG TGG GCA CTG GA3' 5'CCC TTG GCA GGA GCT GGA AG3'	67	16	50	1854-2142

Table II. *SSCP analysis parameters and results for the indicated exons.* (Parámetros de análisis por SSCP y resultados para los exones indicados).

Exon	p.100 T	DNA/ Denat.	Vol loades (µl)	Run temp temp (°C)	Number patterns	Frequencies of patterns (p. 100)
1	12	1/3	20	15	2	A (97.2 p.100); B (2.8 p.100)
2	10	1/3	20	15	2	A (75.9 p.100); B (24.1 p.100)
3	12	1/3	20	15	4	A (18.5 p.100); B (33.3 p.100;); C (39.8 p.100); D (8.3 p.100)
4	10	1/3	20	15	7	A (13.9 p.100); B (27.8 p.100); C (35.2 p.100); D (5.6 p.100); E (11.1); F (2.8 p.100); G (3.7 p.100)
5	8	1/4	25	20	5	A (14.8 p.100); B (27.8 p.100); C (44.4 p.100); D (2.8 p.100); E (10.2 p.100)

(20 µl or 25 µl of each) onto a 8-12 p.100 polyacrylamide/TBE gel. Gels were run at 25 W for 4 to 8 hr at 15-20ºC (**table II**) in a Dcode™ Universal Mutation Detection System, from BIO-RAD, coupled with a refrigerated system. After the run the gel was removed from the apparatus and silver stained.

Table III. *Least Square Means (LSMEAN), and Standard Deviation Error (STD Error) associated, of milk production parameters for SSCP patterns at exon 1, considering 145 days of lactation.* (Media de los mínimos cuadrados (LSMEAN) y respectivo error estándar (STD) de la media de la producción de leche para los patrones de SSCP en el exón 1, considerando 145 días de lactación).

	LSMEAN ± STD Error				
Pattern	Milk (l)	Fat content (p.100)	Protein content (p.100)	Fat (kg)	Protein (kg)
A	115.8 ± 4.68	3.92 ± 0.04	3.94 ± 0.05	4.58 ± 0.21	4.51 ± 0.18
B	150.8 ± 19.35	3.86 ± 0.22	3.84 ± 0.29	5.90 ± 0.86	5.76 ± 0.78
p-value	0.0729	0.7807	0.7191	0.1262	0.1083

Table IV. *Least Square Means (LSMEAN), and Standard Deviation Error (STD Error) associated, of milk production parameters for SSCP patterns at exon 2, considering 145 days of lactation.* (Media de los mínimos cuadrados (LSMEAN) y respectivo error estándar (STD) de la media de la producción de leche para los patrones de SSCP en el exón 2, considerando 145 días de lactación).

	LSMEAN ± STD Error				
Pattern	Milk (l)	Fat content (p.100)	Protein content (p.100)	Fat (kg)	Protein (kg)
A	120.8 ± 4.99[a]	3.97 ± 0.04[a]	3.98 ± 0.06	4.84 ± 0.22[a]	4.72 ± 0.19[a]
B	104.6 ± 7.42[b]	3.76 ± 0.08[b]	3.82 ± 0.10	3.92 ± 0.33[b]	3.96 ± 0.28[b]
p-value	0.0362	0.0174	0.1563	0.0069	0.0115

a, b - Means with different characters differ statistically ($p<0.05$)
a, b - Medias con diferentes caracteres difieren estadísticamente ($p<0,05$)

Statistical analyses: Statistical analysis was performed on the basis of zootechnical parameters (milk, fat and protein production, and fat and protein content) adjusted to 145 days, related to 1996, 1997 and 1998, as well as information about the type of parturition (one, two or more kids) and animal's age. It was adjusted a mixed linear model, by the application of SAS PROC MIXED procedure, to determine associations between SSCP and milk production factors.

With the following mixed linear model, we analysed the five exons separately (because of missing values it was not possible to perform an analysis for the five exons simultaneously).

$$Y_{ijk} = \mu + tp_i + \beta\,(x_{ijk} - \bar{x}) + \\ + \beta\,(x_{ijk} - \bar{x})^2 + Animal_{ijk} + Ex_j + \varepsilon_{ijk}$$

Y_{ijk}: observation of the animal k with parturition type i, pattern level j of exon
μ : overall mean
tp_i: type of parturition

Table V. *Least Square Means (LSMEAN), and Standard Deviation Error (STD Error) associated, of milk production parameters for SSCP patterns at exon 3, considering 145 days of lactation.* (Media de los mínimos cuadrados (LSMEAN) y respectivo error estándar (STD) de la media de la producción de leche para los patrones de SSCP en el exón 3, considerando 145 días de lactación).

	LSMEAN ± STD Error				
Pattern	Milk (l)	Fat content (p.100)	Protein content (p.100)	Fat (kg)	Protein (kg)
A	119.78 ± 7.91	3.92 ± 0.08	4.03 ± 0.10	4.76 ± 0.35	4.73 ± 0.31
B	116.6 ± 6.49	3.98 ± 0.06	4.05 ± 0.08	4.64 ± 0.29	4.64 ± 0.25
C	117.3 ± 6.38	3.93 ± 0.06	3.86 ± 0.08	4.66 ± 0.28	4.48 ± 0.25
D	108.3 ± 11.30	3.67 ± 0.12	3.67 ± 0.16	3.95 ± 0.50	3.91 ± 0.45
p-value	0.8471	0.1931	0.0993	0.5244	0.4176

Table VI. *Least Square Means (LSMEAN), and Standard Deviation Error (STD Error) associated, of milk production parameters for SSCP patterns at exon 4, considering 145 days of lactation.* (Media de los mínimos cuadrados (LSMEAN) y respectivo error estándar (STD) de la media de la producción de leche para los patrones de SSCP en el exón 4, considerando 145 días de lactación).

	LSMEAN ± STD Error				
Pattern	Milk (l)	Fat content (p.100)	Protein content (p.100)	Fat (kg)	Protein (kg)
A	128.9 ± 8.56^{c}	4.03 ± 0.10	4.04 ± 0.13	5.26 ± 0.38^{b}	5.08 ± 0.32^{b}
B	113.0 ± 6.48b^{c}	4.0 ± 0.07	4.03 ± 0.09	4.54 ± 0.29^{b}	4.51 ± 0.24^{b}
C	125.3 ± 6.34^{c}	3.91 ± 0.06	3.91 ± 0.09	4.96 ± 0.28^{b}	4.77 ± 0.24^{b}
D	85.7 ± 11.71^{a}	3.67 ± 0.14	3.83 ± 0.18	3.12 ± 0.52^{a}	3.21 ± 0.44^{a}
E	110.5 ± 10.26^{ac}	3.87 ± 0.12	3.90 ± 0.16	4.26 ± 0.46^{ab}	4.30 ± 0.39^{ab}
F	200.6 ± 21.90^{d}	3.72 ± 0.25	3.52 ± 0.34	7.43 ± 0.98^{c}	6.95 ± 0.83^{c}
G	83.3 ± 15.83^{ab}	3.61 ± 0.18	3.72 ± 0.24	2.98 ± 0.71^{a}	3.01 ± 0.60^{a}
p-value	0.0001	0.1728	0.6093	0.0002	0.0001

a, b - Means with different characters differ statistically (±p<0.05)
a, b - Medias con diferentes caracteres difieren estadísticamente (±p<0,05)

$\beta\,(x_{ijk} - \bar{x})$: linear effect of age covariate
$\beta\,(x_{ijk} - \bar{x})^2$: quadratic effect of age covariate
$Animal_{ijk}$: fixed effect
Ex_j: random effect (exon)
ε_{ijk}: random error

When the effects were significative we made use of multiple comparison tests (t-test) of LSMEAN values,

Table VII. *Least Square Means (LSMEAN), and Standard Deviation Error (STD Error) associated, of milk production parameters for SSCP patterns at exon 5, considering 145 days of lactation.* (Media de los mínimos cuadrados (LSMEAN) y respectivo error estándar (STD) de la media de la producción de leche para los patrones de SSCP en el exón 5, considerando 145 días de lactación).

	LSMEAN ± STD Error				
Pattern	Milk (l)	Fat content (p.100)	Protein content (p.100)	Fat (kg)	Protein (kg)
A	121.2 ± 8.43[a]	3.96 ± 0.10	3.96 ± 0.12	4.84 ± 0.38[a]	4.74 ± 0.34[a]
B	107.2 ± 6.30[a]	3.89 ± 0.07	4.01 ± 0.87	4.21 ± 0.28[a]	4.24 ± 0.25[a]
C	121.2 ± 6.14[a]	3.97 ± 0.06	3.94 ± 0.08	4.86 ± 0.28[a]	4.68 ± 0.24[a]
D	201.0 ± 22.43[b]	3.71 ± 0.25	3.53 ± 0.34	7.45± 1.00[b]	6.98 ± 0.89[b]
E	118.4 ± 10.25[a]	3.77 ± 0.12	3.79 ± 0.15	4.49 ± 0.46[a]	4.43 ± 0.41[a]
P-value	0.0015	0.5109	0.5225	0.0168	0.0390

a, b - Means with different characters differ statistically (±p<0.05)
a, b - Medias con diferentes caracteres difieren estadísticamente (±p<0,05)

in an attempt to find the different level values.

RESULTS AND DISCUSSION

SSCP polymorphisms: We have chosen exons 1, 2, 3, 4 and 5 of the GH gene, for the SSCP analysis. The analysis of the amplified fragments by the PCR method, using the primers described in **table I** is shown in **figure 1**. Their lengths correspond to those expected, according to the position of the primers, deduced from the described nucleotide sequence of the gGH gene from *Capra hircus* (Kioka *et al.,* 1989). Considering the one hundred eight Algarvia goats, we have observed several conformational patterns inside each of those fragments (**figure 2** and **table II**): two conformational patterns (A and B) for each of the exons 1 and 2, four in exon 3 (A, B, C e D), seven in exon 4 (A, B, C, D, E, F e G), and five in exon 5 (A, B, C, D e E). The frequencies found for each pattern are shown in **table II**.

We have made a preliminary attempt towards the search for associations between the SSCP polymorphisms and quantitative variation in the productive traits described below. **Tables III, IV, V, VI** and **VII** summarise the results obtained using the SAS programme applied to the analysis of linear contrasts of SSCPs relating to milk, protein and fat production, and protein and fat content for each exon analysed. Animals with pattern A in exon 2 appear to have a higher milk, fat and protein production ($p<0.05$), and their milk has a higher fat content than those animals having pattern B ($p<0.05$). Animals with patterns F and D for exons 4 and

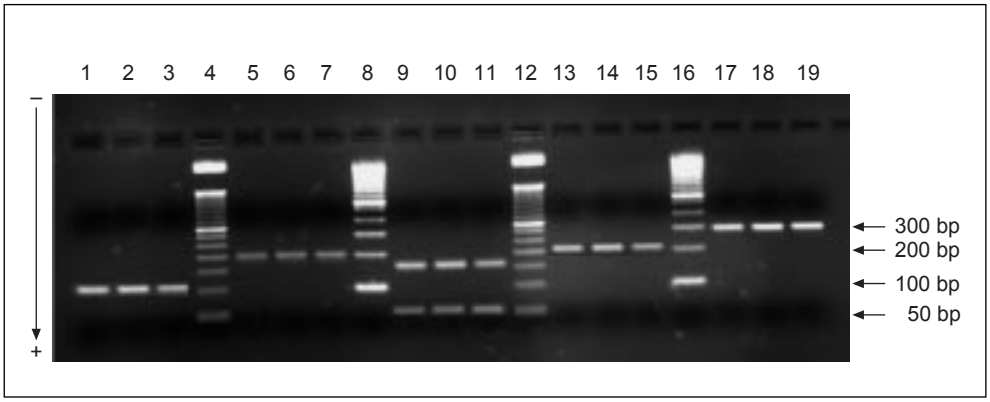

Figure 1. *Electrophoretic separation in agarose gel of PCR products (2 p.100 p/v, 5 V/cm)*
Lanes 1, 2, 3 - exon 1 *Lanes 8, 16 - 100 bp ladder* *Lanes 17, 18, 19 - exon 5*
Lanes 4, 12 - 50 bp ladder *Lanes 9, 10, 11 - exon 3*
Lanes 5, 6, 7 - exon 2 *Lanes 13, 14, 15 - exon 4*
Separación electroforética en gel de agarosa de los productos del PCR (2 p.100 p/v; 5 V/cm)
Bandas 1, 2, 3 - exón 1 Bandas 8, 16 - marcador de 100 pb Bandas 17, 18, 19 - exón 5
Bandas 4, 12 - marcador de 50 pb Bandas 9, 10, 11 - exón 3
Bandas 5, 6, 7 - exón 2 Bandas 13, 14, 15 - exón 4

5 respectively, are superior milk producers ($p<0.05$).

The SSCP analysis of genes, whose product is associated with production traits could be a valuable alternative approach for the establishment of allelic variants useful as markers to aid selection. We have applied this technique to the exons 1, 2, 3, 4 and 5 of GH gene, from the indigenous Portuguese caprine Algarvia breed. The SSCP polymorphisms we have found in the gGH gene coding for a hormone that exerts a positive influence in milk production, hint at the possibility of exploring this approach for the search of genetic markers located in this gene. The SSCP polymorphic variation makes it a potential candidate for the establishment of associations with quantitative traits. Indeed, statistical analyses made clear the existence of associations between the polymorphisms observed in exons 2, 4 and 5 and milk, protein and fat production, and fat content. If specific haplotypes can be defined at this candidate gene that can be associated with milk production, protein and fat content it would be rendered available a valuable genetic resource for improvement of this caprine breed.

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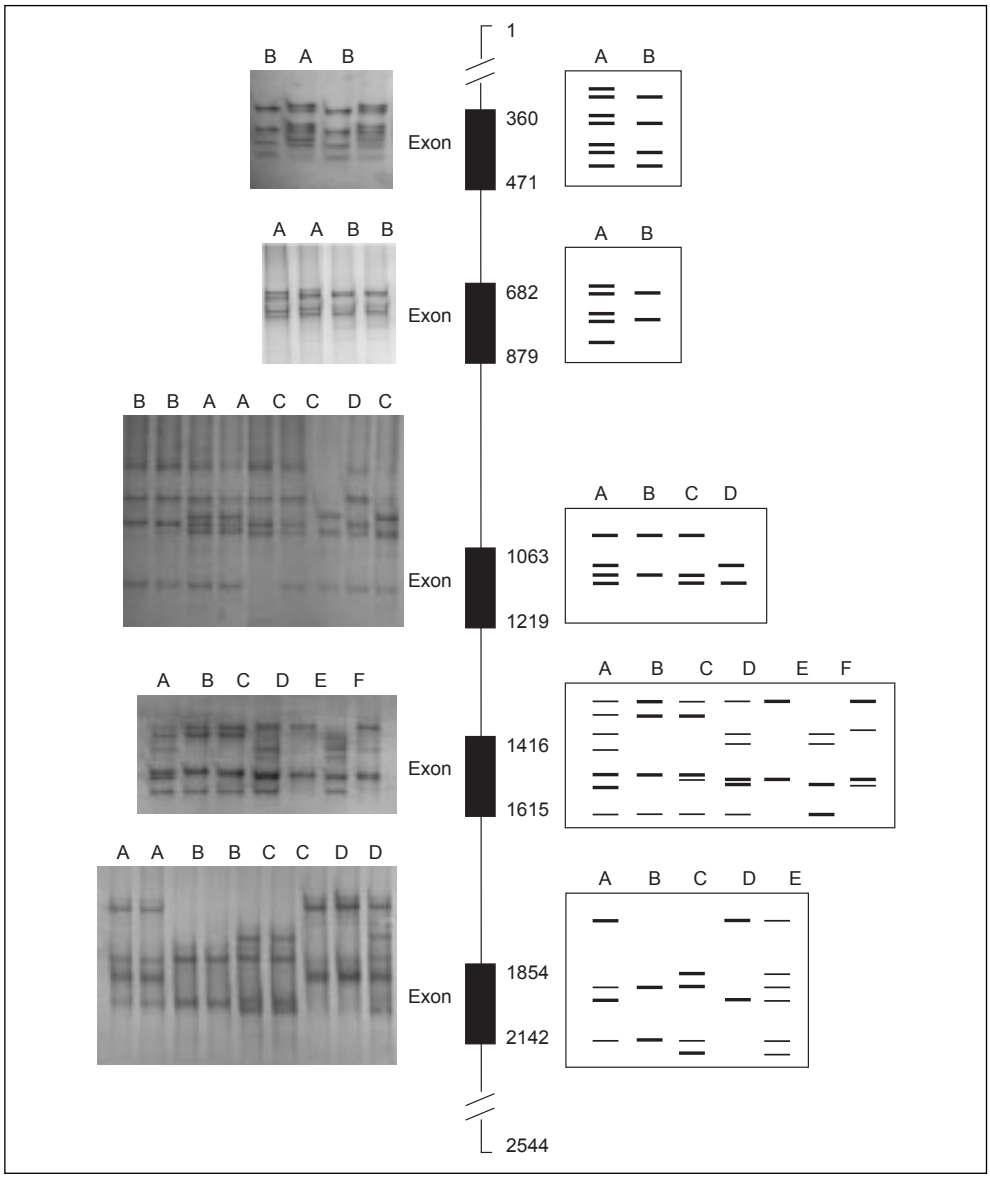

Figure 2. *Left: SSCP's patterns observed in non denaturant PAGE; middle: schematic representation of GH gene with exons represented by black boxes. Numbers represent the localisation of amplified fragments; right: schematic representation of SSCP patterns for each exon.* (Izquierda: patrones SSCP observados en PAGE no desnaturalizante; centro: representación esquemática del gen GH con exones representados por los cuadros negros. Los números representan la localización de los fragmentos amplificados; derecha: representación esquemática de los patrones SSCP para cada exón).

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