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Effects of genetic polymorphisms at the growth hormone gene on milk yield in Serra da Estrela sheep

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The five exons and the 5′ and 3′-untranslated regions (5′-UTR and 3′-UTR) of the oGH gene were screened for mutations using PCR-single strand conformation polymorphism (PCR-SSCP) procedures in 523 Serra da Estrela ewes and were found to be highly polymorphic. The region extending across and between the *GH2-N* and *GH2-Z* copies was sequenced allowing the design of primers for the specific PCR amplification of each copy. These were cloned and sequenced in 20 animals representative of all SSCP patterns. The corresponding genotypes were established for each copy following nucleotide sequencing of SSCP alleles. Twenty-four polymorphic sites were found at the *GH2-N* (or *GH1*) and fourteen at the *GH2-Z* copies. Eight amino acid substitutions were predicted at the *GH2*-N and six at the *GH2-Z* copies. Milk yield adjusted to 150 lactation days was analysed for the genotype of each *oGH* gene copy taken separately or together (associated genotypes) by restricted maximum likelihood (REML) through a univariate best linear unbiased prediction (BLUP) animal model with repeated measures. Significant associations between genotypes and milk yield were observed. Within *GH2-N* genotypes there was a milk yield differential of 21·4±0·2 l/150 d between the most (N7) and the least (N5) productive ones. Within *GH2-Z* genotypes there was a differential of 21·6±0·2 l/150 d between the most (Z8) and the least (Z1) productive ones. The effect of associated *GH2-N* and *GH2-Z* genotypes revealed a differential of 39·6±0·3 l/150 d between the most (N1+Z7) and the least (N3+Z2) productive associated genotypes. The results show that *GH2-N* and *GH2-Z* genotypes significantly affect milk yield in Serra da Estrela ewes. Moreover, the apparent joint effect of *GH2-N* and *GH2-Z* genotype could improve milk yield in 25% as compared with the mean milk production of the analysed population.

Keywords: *Ovis aries*, growth hormone gene, polymorphism, PCR-SSCP, genetic markers, milk yield.

Polymorphisms at the growth hormone (GH) gene are associated with variation in milk production traits in dairy cattle (Lucy et al. 1993; Falaki et al. 1996; Lagziel et al. 1996). Small ruminant species have been much less studied. It would be expected that the same association would occur in the dairy sheep because bovine (*bGH*) and ovine GH (*oGH*) genes are 97·5% identical in the coding regions (Orian et al. 1988). Recent studies on dairy goats also show associations between PCR-single strand conformation polymorphisms (PCR-SSCP; Orita et al. 1989) at the GH gene and milk yield (Malveiro et al. 2001; Marques et al. 2003).

In ewes, results from Kann et al. (1999) and from Liebovich et al. (2001) suggest that mammogenesis and/or lactogenesis are partially controlled by somatotrophic hormones such as oGH and to a lesser extent by ovine placental lactogen (oPL) and that insulin-like growth factor-I (IGF-I) could be one of the mediators of these hormones. Therefore, the detection of genetic markers at the *oGH* gene associated with milk yield and quality might contribute to the establishment of early selection criteria. In fact, in the *oGH* gene, restriction fragment length polymorphisms (RFLP) using restriction endonucleases *Taq*I

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and *Pvu*II (Parsons et al. 1992; Gootwine et al. 1993, 1996) and *Eco*RI (Barracosa, 1996; Gootwine et al. 1998) and PCR-SSCP polymorphisms (Bastos et al. 2001; Marques et al. 2001; Santos et al. 2004) have been reported.

The complete *oGH* DNA sequence and its predicted amino acid sequence have been established by Orian et al. (1988) from an ovine pituitary genomic library. In the ovine, two alleles of the GH gene have been described. The Gh1 allele contains a single gene copy (*GH1*), whereas in the Gh2 allele the gene is duplicated (copies *GH2-N* and *GH2-Z*) with the two copies being located 3·5 kb apart (Valinsky et al. 1990). Sequence differences between the *GH2-N* and *GH2-Z* copies have been demonstrated and polymorphisms have been found in oGH coding and non-coding regions (Ofir & Gootwine, 1997).

The autochthonous Serra da Estrela sheep breed is reared under a natural pasture-based production system in the mountainous region of Serra da Estrela located in the centre of Portugal. This breed has two varieties, white and black. It produces on average 148 l of milk adjusted to 150 lactation days (Carolino et al. 2003).

In the present study, the five exons and the 5′-UTR and 3′-UTR regions of the *oGH* gene were analysed by PCR-SSCP in a sample of 523 Serra da Estrela ewes and the nucleotide polymorphisms were characterized. The objectives were to identify and characterize nucleotidic polymorphisms at the *oGH* gene in Serra da Estrela sheep and associate them with milk production potential.

Materials and Methods

Animals and milk records

A total of 523 Serra da Estrela ewes from white (64%) and black (36%) varieties belonging to seven randomly chosen flocks from Associação Nacional de Criadores de Ovinos Serra da Estrela (ANCOSE) associates were studied. Pedigree information and data concerning 1704 valid lactations (A4) from official milk records between 1996 and 2000 were kindly provided by ANCOSE. Milk yield per lactation was estimated using the Fleischmann method and was adjusted for milking length on a reference period of 150 d. Mean milk yield adjusted to 150 lactation days was 159·5±72·2 l.

Copy number genotypes of the oGH *gene*

To determine the copy number genotype of the *oGH* gene, a total of 56 ewes were analysed by Southern blotting. A 2055-bp DNA probe containing the *oGH* gene was amplified by PCR with the primers GHT-Fwd (5′ CCA GAG AAG GAA CGG GAA CAG GAT GAG 3′) and GHT-Rev (5′ATA GAG CCC ACA GCA CCC CTG CTA TTG 3′) designed according to the published *oGH* gene sequence (GenBank accession number: X12546, Orian et al. 1988). The PCR reaction was performed in a final volume of 50 µl according to the following conditions: 500 ng of genomic DNA; 6 pmoles of each primer; 2·0 units of *TaKaRa LA Taq*™ (TAKARA SHUZO CO., Ltd, Japan); 1× of 2× GC Buffer I with 2·5 mM-$MgCl_2$ and 400 µM of each dNTP. Amplification cycles included an initial denaturation at 94 °C for 1 min followed by 30 cycles of denaturation at 98 °C for 20 s, annealing at 62 °C for 12 min, and a final extension at 72 °C for 10 min. The 2055-bp DNA probe was digoxigenin (DIG)-labelled with the PCR DIG Probe Synthesis Kit (Roche Diagnostics GmbH, Indianapolis IN, USA) according to the instruction manual.

Fifteen µg of genomic DNA were digested overnight with *Eco*RI restriction endonuclease (Invitrogen Life Technologies; Carlsbad CA, USA). Digested fragments were separated on a 0·8% agarose gel (2 V/cm) with 0·5× TBE buffer (0·045 M-borate–0·001 M-EDTA) for 13 h, denatured in 0·5 M-NaOH for 30 min, transferred by capillarity to a positively charged nylon membrane (Hybond™-N^+, Amersham Pharmacia Biotech, Ireland), and UV-cross linked to the membrane. The blots were hybridized with the 2055-bp DIG-labelled probe at 45 °C in DIG Easy Hyb solution (Roche Diagnostics GmbH) for 16 h. The probe-target hybrids were immunodetected on the blots with an alkaline phosphatase-conjugated anti-DIG antibody from sheep (Anti-Digoxigenin-AP, Fab fragments; Roche Diagnostics GmbH) and visualized with the chemiluminescent alkaline phosphatase substrate CSPD (Roche Diagnostics GmbH). Then the blots were exposed to X-ray film (Kodak BioMax MS1, Eastman Kodak Company, Rochester NY, USA) for 45 min according to the standard DIG chemiluminescent detection procedure.

The Hardy-Weinberg equilibrium for the *oGH* copy number genotypes was tested by χ^2 analysis (Statistica software, StatSoft, Inc., Tulsa OK, USA).

oGH *gene analysis by PCR-SSCP*

Blood samples (9 ml) from 523 ewes were obtained by jugular venipuncture on potassium-EDTA at a final concentration of 1·6 mg/ml blood). DNA extraction was performed using a phenol/chloroform free method (Puregene DNA Isolation Kit, Gentra Systems, Minneapolis MN, USA).

Seven DNA fragments (I to VII) of the *oGH* gene comprising the five exons (including intron-exon junctions), and the 5′-UTR and 3′-UTR regions, were amplified by PCR with copy-unspecific primer pairs (Invitrogen Life Technologies, Barcelona, Spain) shown in Table 1. Sizes of amplified fragments ranged from 112 to 289 bp. PCR reactions of the fragments II to VI were performed in a final volume of 25 µl using Ready-To-Go PCR Beads (Amersham Biosciences, Buckinghamshire, UK) according to the following conditions: 25–50 ng of genomic DNA; 0·16–0·64 µM of each primer; 1·5 units of *Taq* DNA polymerase; 10 mM Tris–HCl (pH 9); 50 mM-KCl; 1·5 or 2·5 mM $MgCl_2$; 200 µM of each dNTP and stabilizers

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Table 1. Length and localization of PCR-SSCP fragments of the *oGH* gene (E, exon; I, intron; UTR, untranslated region) and primers used for the PCR analysis†

Fragment				Primer		
Name	Type	Length	Localization (bp)	Name	Sequence	Annealing Temp. (°C)
I	5′-UTR, E1	125	205 to 329	GH5′-Fwd: GH5′-Rev:	5′ GGG AAA GGG AGA GAG AAG AAG CCA G 3′ 5′ CAG CCA TCA TAG CTG GTG AGC TGT C 3′	68
II	5′-UTR, E1, I1	112	248 to 359	GH1-Fwd: GH1-Rev:	5′ CAG AGA CCA ATT CCA GGA TC 3′ 5′ TAA TGG AGG GGA TTT TCG TG 3′	57
III	I1, E2, I2	198	569 to 766	GH2-Fwd: GH2-Rev:	5′ CTC TCC CTA GGG CCC CGG AC 3′ 5′ TCT AGG ACA CAT CTC TGG GG 3′	65
IV	I2, E3, I3	154	967 to 1110	GH3-Fwd: GH3-Rev:	5′ CTC CCC CCA GGA GCG CAC CT 3′ 5′ GCT CCT CGG TCC TAG GTG GC 3′	60
V	I3, E4, I4	200	1303 to 1502	GH4-Fwd: GH4-Rev:	5′ CTG CCA GCA GGA CTT GGA GC 3′ 5′ GGA AGG GAC CCA ACA ATG CCA 3′	60
VI	I4, E5, 3′-UTR	289	1740 to 2028	GH5-Fwd: GH5-Rev:	5′ CCC TTG GCA GGA GCT GGA AG 3′ 5′ AAA GGA CAG TGG GCA CTG GA 3′	67
VII	E5, 3′-UTR	150	1943 to 2092	GH3′-Fwd: GH3′-Rev:	5′ CCT TCT AGT TGC CAG CCA TCT GTT G 3′ 5′ CCA CCC CCT AGA ATA GAA TGA CAC CTA C 3′	64·5

† According to the published *oGH* gene sequence GenBank accession number X12546 (Orian et al. 1988)

including BSA. PCR reactions of fragments I and VII were performed in a final volume of 25 µl according to the following conditions: 50 ng of genomic DNA; 12 pmol of each primer; 1·0 unit of *Taq* DNA polymerase (Roche Diagnostics GmbH); 10 mM-Tris–HCl (pH 9); 50 M-KCl; 3·5 mM or 4·5 mM-$MgCl_2$; and 200 µM of each dNTP. Amplification cycles included an initial denaturation at 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C to 68 °C for 30 s (Table 1), extension at 72 °C for 30 s and a final extension at 72 °C for 5 min. Amplification products were analysed by electrophoresis on ethidium bromide stained 2% agarose gels (5 V/cm), with 1× TBE buffer (0·09 M-borate, 0·002 M-EDTA).

For SSCP analysis, 4 µl or 5 µl of each amplification product was added to 12 µl or 15 µl of stop solution (95% formamide, 10 mM-NaOH, 0·05% xylene cyanol and 0·05% bromophenol blue). Samples were heat-denatured at 95 °C for 5 min, immediately chilled on ice, and loaded onto native 8–12% polyacrylamide gels, with 2·5% crosslinking and 0·5 or 1× TBE buffer. Gels were run at constant power (25 or 40 W) and temperature (8–20 °C), for an optimized time (4–9 h) in a DCode™ Universal Mutation Detection System (Bio-Rad, Hercules CA, USA), coupled to a refrigeration system. After the run, gels were silver stained (PlusOne™ DNA Silver Staining Kit, Amersham Biosciences, Uppsala, Sweden). SSCP patterns were identified and assigned a capital letter.

After cloning and sequencing the *oGH* gene copies and the inter-copy region (see following section), further PCR-SSCP analyses were carried out on each *oGH* gene copy separately using the previously described conditions. PCR amplicons of the *oGH* copies as well as cloned *oGH* copies were used as DNA templates. Genotypes were assigned to each amplified fragment (I to VII) produced from each gene copy (Table 2).

Cloning and sequencing of the oGH *gene copies and of the inter-copy region*

To specifically assign the SSCP bands to the *GH2-N* (or *GH1*) or the *GH2-Z* gene copies, cloning and sequencing of the inter-copy region was performed. A DNA fragment 4527 pb long (ranging from the exon 5 of the *GH2-N* copy to the exon 3 of the *GH2-Z* copy) of a Gh2/Gh2 animal was amplified with primers GH5–Fwd and GH3–Rev (Table 1). PCR was performed in a final volume of 50 µl according to the following conditions: 250 ng of genomic DNA; 3 pmol of each primer; 1·0 unit of *TaKaRa LA Taq*™ (TAKARA SHUZO CO., Ltd, Japan); 1× of 2× GC Buffer I with 2·5 mM-$MgCl_2$ and 400 µM of each dNTP. Amplification cycles included an initial denaturation at 94 °C for 1 min followed by 30 cycles of denaturation at 98 °C for 20 s, annealing at 60 °C for 12 min, and a final extension at 72 °C for 10 min. PCR products were column purified, cloned into the pCR®-XL-TOPO® vector according to the instructions of the TOPO XL PCR Cloning Kit (Invitrogen Life Technologies) and transformed into competent *Escherichia coli* One Shot® TOP10 cells. Recombinant plasmids DNA were purified using the QIAGEN® Plasmid Midi Kit (QIAGEN GmbH, Hilden, Germany) following the manufacturer's recommendations. The 4527-pb long DNA fragment was sequenced from both ends (GenBank accession number: DQ238053). Sequencing reactions were performed according to the protocol from the ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems, Warrington, UK) and repeated for confirmation. DNA was purified using

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Table 2. SSCP patterns found at seven *oGH* fragments in Serra da Estrela sheep. The corresponding genotypes are shown in italic figures and pattern frequencies in brackets

SSCP pattern	Fragment genotypes† and SSCP pattern frequencies (%)						
	(I_N)/(I_Z)‡	(II_N)/(II_Z)	(III_N)/(III_Z)	(IV_N)/(IV_Z)	(V_N)/(V_Z)	(VI_N)/(VI_Z)	(VII_N)/(VII_Z)
A	(*1/1*)/(na) (91·88)	(*1/1*)/(*1/1*) (93·12)	(*1/4*)/(*3/3*) (22·41)	(*1/2*)/(*1/1*) (57·36)	(*1/1*)/(*1/1*) or (*1/1*)/(–/–) (84·70)	(*1/1*)/(*1/2*) (48·18)	(*1/1*)/(*1/3*) (36·82)
B	(*2/3*)/(na) (6·77)	(*1/2*)/(*1/1*) or (*2/2*)/(*1/1*) (6·88)	(*1/5*)/(*1/1*) (18·01)	(*1/1*)/(*1/2*) (19·69)	(*1/1*)/(*1/1*) (15·30)	(*1/1*)/(*1/1*) or (*1/1*)/(–/–) (31·74)	(*1/5*)/(*2/3*) (25·19)
C	nd (0·39)		(*1/1*)/(*1/1*) (16·09)	(*1/1*)/(*1/3*) (8·60)		(*2/3*)/(*1/2*) (13·58)	(*1/1*)/(*1/1*) (12·79)
D	nd (0·97)		(*1/1*)/(*2/3*) (11·11)	(*1/2*)/(*1/3*) (5·54)		(*4/5*)/(*1/3*) (5·35)	(*2/2*)/(*2/3*) (9·69)
E			(*1/1*)/(*1/1*) (7·47)	(*1/3*)/(*3/3*) (4·59)		(*6/7*)/(*3/3*) (1·15)	(*3/3*)/(*4/4*) (5·81)
F			(*1/1*)/(*2/2*) (4·98)	(*1/4*)/(*3/4*) (2·10)			(*6/7*)/(*3/3*) (5·43)
G			(*1/1*)/(*2/4*) (4·41)	(*1/1*)/(*5/5*) (1·72)			(*2/2*)/(*2/4*) (4·26)
H			(*1/1*)/(*1/5*) (3·64)	(*1/2*)/(–/–) (0·38)			
I			(*1/3*)/(*2/2*) (3·26)				
J			(*1/2*)/(*1/2*) (2·30)				
K			(*1/1*)/(*1/4*) (1·92)				
L			(*1/6*)/(*4/4*) (1·72)				
M			(*1/1*)/(nd) (1·53)				
O			(*1/1*)/(–/–) (0·38)				
N, P, Q			nd (0·77)				
(*n*)	517	523	522	523	523	523	516

† Genotypes were determined by copy specific fragment sequencing (Table 3); (*n*) – number of animals; (*1/1*)/(*1/1*) – *GH2-N/GH2-Z* homozygous genotype at both *oGH* copies; (*1/2*)/(*1/2*) – *GH2-N/GH2-Z* heterozygous genotype at both *oGH* copies; (*1/2*)/(*1/1*) – *GH2-N/GH2-Z* heterozygous genotype at *GH2-N* copy; (*1/1*)/(*1/2*)– *GH2-N/GH2-Z* heterozygous genotype at *GH2-Z* copy; (*1/1*)/(–/–) – *GH1* homozygous genotype (*GH2-Z* copy absent) etc.; na – not analysed by SSCP; nd – genotype not determined

‡ SSCP analysis performed either at the *GH1* copy or at the *GH2-N* copy of the *oGH* gene

ethanol precipitation and analysed using an Automatic Sequencer 3730xI.

Alignment of the sequences of *GH2-N* and *GH2-Z* gene copies with the inter-copy region sequence using Vector NTI® Suite software (InforMax®, Bethesda MD, USA) revealed nucleotide variations that allowed the design of primers specific for the *GH2-Z* copy and that the GHT–Fwd primer was specific for the *GH1* and *GH2-N* copies. The oGH copies of 20 animals representative of all SSCP patterns were subsequently sequenced. *GH1* and *GH2-N* copies were PCR amplified using the GHT–Fwd and GHT–Rev primers. The *GH2-Z* copy was amplified using primers GHZ–Fwd ($^{5'}$ GAG GAG TAA ATG AAA TGA GGT C $^{3'}$) and GHZ–Rev ($^{5'}$ CCT CTG TGC TAT GTC CTT CAC AAG C $^{3'}$) designed according to GenBank accession numbers: DQ238053 (our results) and M37310 (Byrne et al. 1987), respectively. PCR of the *GH1* and *GH2-N* copies was performed as described previously (see Copy number genotypes of the *oGH* gene). PCR of the *GH2-Z* copy was performed as described above for the inter-copy region using an annealing temperature of 50 °C. PCR products of the *oGH* gene copies were cloned and sequenced as described above and/or directly sequenced after purification using the QIAquick Gel Extraction Kit (QIAGEN GmbH, Hilden, Germany).

After alignment of the sequencing data (Vector NTI® Suite software, InforMax®, Bethesda MD, USA), *GH2-N*, *GH2-Z* and associated *GH2-N* and *GH2-Z* genotypes were established. Haplotypes and their frequencies were inferred using the FBAT software (Horvath et al. 2004).

Statistical analysis

Results were statistically analysed to test possible associations between mean milk yield adjusted to 150 lactation days and copy number genotypes, and *GH2-N* and *GH2-Z* copies genotypes, taken separately or associated. Results were analysed by restricted maximum likelihood (REML) through univariate analyses with repeated measures using the BLUP-Animal Model and the MTDFREML program (Boldman et al. 1993). The following model was used:

$$y = X\beta + Z_a a + Z_p p + e$$

where, y is the vector of milk records; β is the vector of fixed effects which included the effect of year-flock (year 1996 flock 1, year 1997 flock 1, …, year 2000 flock 7), of month of lambing (August, September, …, December), type of lambing (simple or multiple), variety (white or black), the linear and quadratic effect of the ewes' lambing age, and the effect associated with the genotypes studied; a is the vector of random additive genetic effects; p is the vector of random permanent maternal environmental effects; e is the vector of random residual effects. X, Z_a and Z_p are the incidence matrixes which relate the fixed (X) and random (Z_a and Z_p) effects with the vector of milk records, y.

To solve the mixed model equations (1), it was assumed that $\alpha=3$, which corresponds to a heritability of 0·25 (Horstick et al. 2002; heritabilities of 0·20 and 0·30 were also tested) and that $\gamma=5$, which corresponds to a repeatability of 0·40.

$$\begin{bmatrix} X'X & X'Z & X'Z \\ Z'X & Z'Z+A^{-1}\alpha & Z'Z \\ Z'X & Z'Z & Z'Z+I\gamma \end{bmatrix} \begin{bmatrix} b \\ a \\ p \end{bmatrix} = \begin{bmatrix} X'y \\ Z'y \\ Z'y \end{bmatrix} \quad (1)$$

In the mixed model equations, A is the relationship matrix between all animals (genotyped ewes with milk records and their pedigrees: 750 animals); I is the identity matrix; b is the solution for fixed effects; a is the solution for genetic effects and p is the solution for permanent environmental effects, where:

$$\alpha = \frac{\sigma_e^2}{\sigma_a^2} \text{ and } \gamma = \frac{\sigma_e^2}{\sigma_{pe}^2}$$

Solutions for the effects of the analysed genotypes upon milk yield, contrasts between the analysed genotypes and the corresponding significance test were obtained through option 4 of the subroot MTDFRUN (solutions for MME then sampling variances) from the MTDFREML program (Boldman et al. 1993). The number of records used in univariate analyses was 259 lactations from the 56 *oGH* copy number genotyped ewes and 1704 lactations from the 523 SSCP-genotyped ewes. Only genotypes present in more than 2% of the population were considered.

Results

oGH *copy number genotypes*

Sheep can be homozygous for one copy (*GH1*, Gh1 allele), homozygous for the two copies (*GH2-N* and *GH2-Z*, Gh2 allele) or heterozygous for the copy number (Gh1 and Gh2 alleles) (Wallis et al. 1998). Southern blotting analysis of the genomic DNA of the 56 Serra da Estrela ewes digested with *Eco*RI showed the three *oGH* genotypes Gh1/Gh1, Gh1/Gh2 and Gh2/Gh2 previously described (Gootwine et al. 1998). The observed genotypic frequencies were 1·14%, 15·91% and 82·95% for Gh1/Gh1, Gh1/Gh2 and Gh2/Gh2 genotypes, respectively. Allele frequencies were 9·09% for the Gh1 allele and 90·91% for the Gh2 allele. Ewes with Gh2/Gh2 genotype produced more 4·9±0·31 l/150 d than ewes with Gh1/Gh2 genotype ($P<0·001$).

The studied population was in Hard-Weinberg equilibrium ($\chi^2=0·123$; df=2; $P>0·10$).

PCR-SSCP of oGH *gene fragments and sequencing of the inter-copy region*

Seven PCR fragments (I to VII) containing the five *oGH* exons, the 5′-UTR and the 3′-UTR analysed by PCR-SSCP were found to be highly polymorphic (Fig. 1). Except

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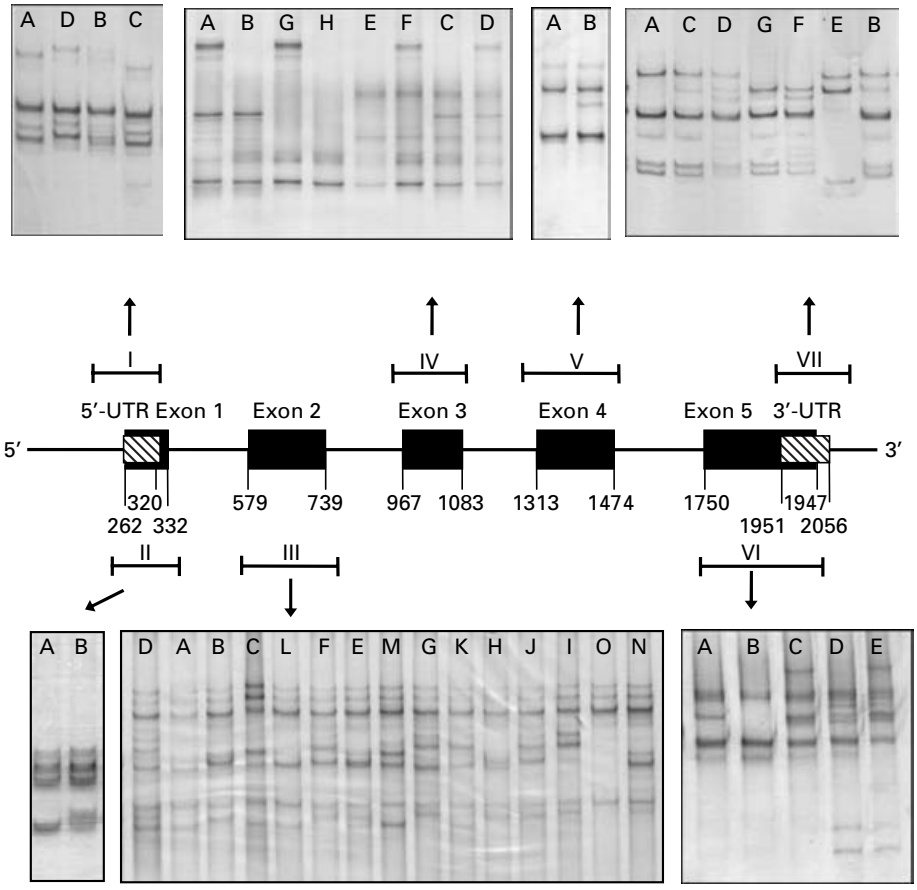

Fig. 1. PCR-SSCP patterns for fragments I (5′-UTR, exon 1), II (5′-UTR, exon 1, intron 1), III (intron 1, exon 2, intron 2), IV (intron 2, exon 3, intron 3), V (intron 3, exon 4, intron 4), VI (intron 4, exon 5, 3′-UTR) and VII (exon 5, 3′-UTR) of Serra da Estrela *oGH* gene separated by native PAGE under non-denaturing conditions. The horizontal line is a schematic representation of the *oGH* gene. Exons are represented by black boxes and UTRs by striped boxes. Boundaries of these fragments are indicated in base pairs according to the published o*GH* gene sequence (exons following Orian et al. 1988; GenBank accession number X12546; 3′-UTR following UTR accession number: 3OAR000234). Different PCR-SSCP patterns within each fragment were randomly identified with a capital letter.

for the 5′-UTR the primers used in the amplification were not copy-specific owing to the high homology of sequences in the 3′-UTR, and in exons and adjacent intronic regions. PCR-SSCP revealed four SSCP patterns in fragment I, two in fragment II, 17 in fragment III, eight in fragment IV, two in fragment V, five in fragment VI, and seven in fragment VII. SSCP pattern frequencies are shown in Table 2. The most frequent SSCP pattern was designated as A for all the fragments. Its frequency was 91·88% in fragment I, 93·12% in fragment II, 22·41% in fragment III, 57·36% in fragment IV, 84·70% in fragment V, 48·18% in fragment VI and 36·82% in fragment VII.

Duplication of the *GH* gene in *Ovis aries* makes its PCR-SSCP analysis complex. A minimum of one and a maximum of four alleles can be differentiated by SSCP analysis depending on the copy number genotype and on the zygotic condition (homo or hetero) of the animal for the analysed DNA fragment. To specifically assign the SSCP bands to each copy the following strategy was followed. The inter-copy region was PCR amplified, cloned and sequenced (GenBank accession number: DQ238053). Sequence analysis disclosed major differences in the 5′ regions of the *oGH* copies allowing the design of primers for the specific amplification of each copy. The products of these specific amplifications were used as templates for separate PCR-SSCP fragment analysis. Superimposition of the SSSP patterns from each copy generated the patterns obtained with the non-specific copy fragment amplification performed on genomic DNA (Fig. 2). For instance, SSCP pattern B of fragment VI has two bands suggesting that animals exhibiting this pattern are homozygous for the copy number and/or for the exon 5 containing fragment (Fig. 1, Table 2). This was confirmed by Southern blotting analysis which showed that one animal presenting this pattern had the Gh1/Gh1 genotype. In addition, sequencing showed that the remaining animals with Gh2/Gh2 genotype were homozygous for fragment VI. The more complex pattern C in fragment IV

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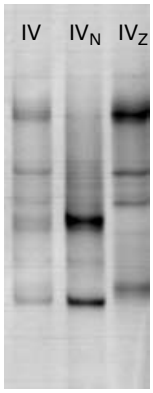

Fig. 2. PCR-SSCP analysis of the fragment IV of the *oGH* gene. IV – PCR-SSCP pattern C of the fragment IV [(*1/1*)/(*1/3*) genotype]: amplification from genomic DNA was not copy specific; IV_N – PCR-SSCP pattern B of the fragment IV_N, a DNA specifically PCR amplified from the *GH2-N* copy was used as a template for fragment IV amplification. IV_Z – PCR-SSCP pattern B of the fragment IV_Z, a DNA specifically PCR amplified from the *GH2-Z* copy was used as a template for fragment IV amplification.

exhibits six bands suggesting that animals presenting this pattern have the Gh2/Gh2 genotype and are heterozygous for fragment IV in one copy and homozygous in the other, or alternatively have the Gh1/Gh2 genotype and are heterozygous for the *GH2-N* copy. Southern blotting analysis and sequencing supported the first hypothesis (Table 2 and Fig. 2).

Molecular characterization of the SSCP patterns

Twenty sheep carrying each of the SSCP patterns shown in Fig. 1 were selected and genotyped for the copy number. One of these animals had the Gh1/Gh1 genotype, two the Gh1/Gh2 genotype and 17 the Gh2/Gh2 genotype. The nucleotide sequence of each *oGH* gene copy fragment was established for the selected animals. SSCP alleles at each fragment were molecularly characterized and designated by an italic figure (Table 3): *e.g.* allele *1* of fragment II_N [at *GH2-N* (*or GH1*) copy] carries bases T_{301} (T at position 301, according to sequence GenBank accession number X12546; Orian et al. 1988) and G_{305}; allele *1* of fragment III_Z (at *GH2-Z* copy) carries bases G_{644}, C_{649}, C_{668}, C_{704} and G_{712}. For each fragment's SSCP patterns, a fragment genotype was thus established (Table 2).

A total of 2055 bp were sequenced at the *GH2-N* (or *GH1*) copy and of 2100 bp at *GH2-Z* copy in each of the selected animals. Twenty-four polymorphic sites were found at the *GH2-N* (or *GH1*) copy and fourteen at the *GH2-Z* copy. Some of these polymorphisms had been reported previously (Table 3). Compared with the *GH1* sequence first reported by Orian et al. (1988) the *GH2-N* (or *GH1*) copy presented a total of sixteen transitions, six transversions and two insertions/deletions of one nucleotide each. Compared with the *GH2-Z* sequence reported by Ofir & Gootwine (1997) the *GH2-Z* copy showed a total of eleven transitions and three transversions. Nucleotide polymorphisms were found in all of the studied fragments of the *GH2-N* (or *GH1*) copy but in fragment V_N and in fragments III_Z, IV_Z, VI_Z and VII_Z of the *GH2-Z* copy. Although two SCCP patterns were found in the fragments V_N and V_Z no polymorphisms were identified.

The polymorphic sites were distributed as follows at the *GH2-N* (or *GH1*) copy: two in fragment I_N, two in fragment II_N, seven in fragment III_N, three in fragment IV_N, four in fragment VI_N and seven in fragment VII_N; at the *GH2-Z* copy: five in fragment III_Z, five in fragment I_Z, two in fragment V_Z and two in fragment VI_Z. The *GH2-N* copy was more polymorphic than the *GH2-Z* copy. The number of alleles ranged from two in fragment I_N to seven in fragment V_N and fragment VI_N.

A total of 86 haplotypes at the *GH2-N* copy and 32 at the *GH2-Z* copy were inferred from SSCP allele composition, and the DNA sequences of the SSCP alleles shown in Table 3. Haplotypes were constructed by joining together, separated by a comma, the alleles of the analysed fragments, *e.g.* a *GH2-N* haplotype composed by allele *1* at fragments I to VII is denominated (*1,1,1,1,1,1,1*). Whenever phase was determined, the haplotypes of a genotype were separated by /, *e.g.* the N1 genotype of the *GH2-N* copy is (*1,1,1,1,1,1,1*)/(*1,1,1,2,1,1,1*). Fragment genotypes were separated by a comma whenever phase could not be determined. For example, an animal with genotype (*1/1*) at fragments I, II and III, genotype (*1/2*) at fragment IV, genotype (*1/1*) at fragments V and VI, and genotype (*1/5*) at fragment VII has a genotype denominated (*1/1,1/1,1/1,1/2,1/1,1/1,1/5*). The three most frequent haplotypes were *Hn1* (*1,1,1,1,1,1,1*; 42·67%), *Hn2* (*1,1,1,1,1,1,5*; 11·60%) and *Hn3* (*1,1,1,2,1,1,1*; 5·04%) for the *GH2-N* copy; and *Hz1* (*1,1,1,1,2,3*; 14·74%), *Hz2* (*1,2,3,1,1,2*; 11·16%), and *Hz3* (*1,1,1,1,1,1*; 7·87%), *Hz4* (*1,1,1,1,1,2*; 7·87%) and *Hz5* (*1,3,1,1,1,1*; 7·87%) for the *GH2-Z* copy.

Synonymous and non-synonymous substitutions were found in the exons 2, 3 and 5 of the *oGH* copies (Table 4). Eight non-synonymous substitutions were predicted in the *GH2-N* copy and six in the *GH2-Z* copy.

Statistical analysis

Statistical analyses were performed considering the *GH2-N* and *GH2-Z* copies genotypes taken separately or associated (Table 5). The contrasts were performed considering the differences of milk yield adjusted to 150 lactation days between the different genotypes and the most frequent homozygous genotype in each fragment or o*GH* gene copy. The different genotypes found in the *GH2-N* and *GH2-Z* copies had significant influence on milk yield in Serra da Estrela ewes ($P<0·05$). Within *GH2-N* genotypes there was a milk yield differential of 21·4±0·2 l/150 d ($P<0·0001$) between the most (N7) and

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Table 3. Nucleotide sequence characterization of SSCP alleles of each amplified fragment at the *GH2-N* (or *GH1*) and *GH2-Z* copies

oGH Fragment (Type)	*GH2-N* (or *GH1*) alleles† Site§	*1*	*2*	*3*	*4*	*5*	*6*	*7*	oGH Fragment (Type)	*GH2-Z* alleles‡ Site§	*1*	*2*	*3*	*4*	*5*
I_N	$5UN_{288}$	C	T	T											
(5′-UTR, E1)	$5UN_{293}$	C	C	A											
II_N	$E1N_{301}$	T	A												
(5′-UTR, E1, I1)	$E1N_{305}$	G	A												
III_N	$E2N_{597}$	T	T	C	T	T	T		III_Z	$E2Z_{644}$	G	G	G	A	G
(I1, E2, I2)	$E2N_{651}$	C	C	C	C	C	T		(I1, E2, I2)	$E2Z_{649}$	C	C	C	C	G
	$E2N_{666}$	C	T	C	C	C	C			$E2Z_{668}$	C	T	C	C	C
	$E2N_{708}$	A	A	A	A	G	A			$E2Z_{704}$	C	C	C	C	G
	$E2N_{717}$	C	C	C	C	C	T			$E2Z_{712}$	G	A	A**	A	G
	$E2N_{733}$	A	A	A	G	A	A								
	$E2N_{738}$	T	T	C	T	T	T								
IV_N	$E3N_{973}$	A	A	T	A				IV_Z	$E3Z_{1035}$	T	C	T	C	C
(I2, E3, I3)	$E3N_{1024}$	T	T	C	C				(I2, E3, I3)	$E3Z_{1047}$	A	A	A	G	G	G
	$E3N_{1047}$	A	G*	A	A					$E3Z_{1057}$	A	G	A	G	G	
										$E3Z_{1062}$	G	G	G	G	C	
										$I3Z_{1100}$	A	G	G	G	G	
VI_N	$E5N_{1872}$	G	A	G	G	G	G	G	VI_Z	$E5Z_{1852}$	G	G	A			
(I4, E5, 3′-UTR)	$E5N_{1938}$	C	C	C	C	C	C	T	(I4, E5, 3′-UTR)	$3UZ_{1965}$	T**	C	C			
	$3UN_{1980}$	—	T	T	C	T	T	T								
	$3UN_{2024}$	—	—	—	—	—	T	T								
VII_N	$3UN_{1980}$	—	T	T	C	—	—	—	VII_Z	$3UZ_{1965}$	T	T	C	C		
(E5, 3′-UTR)	$3UN_{2024}$	—	—	T	—	—	—	—	(E5, 3′-UTR)	$3UZ_{2030}$	T	C	C	T		
	$3UN_{2036}$	A	A	A	A	A	A	G								
	$3UN_{2039}$	C	C	C	C	T	C	C								
	$3UN_{2050}$	C	C	C	C	G	C	C								
	$3UN_{2059}$	G	G	G	G	G	T	T								
	$3UN_{2069}$	G	G	G	G	G	C	C								

† Underlined: variants from the GH1 copy sequence reported by Orian et al. (1988; GenBank accession number X12546); * Australian Merino breed GH sequence reported by Byrne et al. (1987; GenBank accession number M37310)
‡ Underlined: variants from the Awassi breed *GH2-Z* copy sequence reported by Ofir & Gootwine (1997; GenBank accession number AF002124 to AF002129); ** Romanov breed *GH2-Z* copy sequence reported by Ofir & Gootwine (1997; GenBank accession no. AF002118 and AF002120)
§ Site: U – UTR; E – exon; I – intron; *N* – *GH2-N* (or *GH1*) copy; *Z* – *GH2-Z* copy; *e.g.* $5UN_{288}$ – 5′-UTR at *GH2-N* (or *GH1*) copy, position 288 (according to the published *oGH* gene sequence (Orian et al. 1988; GenBank accession no. X12546)

Table 4. Polymorphisms found in the coding regions of the *oGH* gene copies, predicted amino acid changes and protein variants

oGH Fragment	*GH1* or *GH2-N* Site†	nt	aa no.	Aa	Variants a	b	c	d	e	*GH2-Z* Site†	nt	aa no.	Aa	Variants a	b	c
III	$E2N_{597}$	T→C	−16	Leu→Pro	L	L	P	L	L	$E2Z_{644}$	G→A	−1	Gly	G	G	G
(E2)	$E2N_{651}$	C→T	3	Pro→Leu	P	P	P	P	L	$E2Z_{649}$	C→G	2	Phe→Leu	F	F	L
	$E2N_{666}$	C→T	8	Ser→Phe	S	F	S	S	S	$E2Z_{668}$	C→T	9	Arg→Cys	R	C	R
	$E2N_{708}$	A→G	22	His→Arg	H	H	H	R	H	$E2Z_{704}$	C→G	21	Leu→Val	L	L	V
	$E2N_{717}$	C→T	25	Ala→Val	A	A	A	A	V	$E2Z_{712}$	G→A	23	Gln	G	G	G
	$E2N_{733}$	A→G	30	Lys	K	K	K	K	K							
	$E2N_{738}$	T→C	32	Phe→Ser	F	F	S	F	F							
IV	$E3N_{973}$	A→T	35	Thr→Ser	T	S	T			$E3Z_{1035}$	T→C	55	Ser	S	S	S
(E3)	$E3N_{1024}$	T→C	52	Phe→Leu	F	L	L			$E3Z_{1047}$	A→G	59	Pro	P	P	P
	$E3N_{1047}$	A→G	59	Pro	P	P	P			$E3Z_{1057}$	A→G	63	Ser→Gly	S	G	G
										$E3Z_{1062}$	G→C	64	Asn→Lys	K	K	N
VI	$E5N_{1872}$	G→A	166	Arg	R					$E5Z_{1852}$	G→A	160	Gly→Ser	G	S	
(E5)	$E5N_{1938}$	C→T	188	Ser	S											

† According to the published oGH gene sequence (Orian et al. 1988; GenBank accession number X12546). E – exon
nt – nucleotide; aa no. – amino acid number considering N-terminal alanine as amino acid 1 which corresponds to amino acid 27 of the published oGH sequence number CAA31063 (Orian et al. 1988)

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Table 5. *GH2-N* and *GH2-Z* genotypes and phenotypes†, their respective frequencies (%) and milk yield deviation (l/150 d) ± SE from the more frequent homozygous *GH2-N* and *GH2-Z* genotypes and phenotypes

GH2-N				*GH2-Z*				*GH2-N+GH2-Z*		
Genotypes		Freq. (%)	Milk yield (n=1150)	Genotypes		Freq. (%)	Milk yield (n=1007)	Genotypes	Freq. (%)	Milk yield (n=818)
N1	(*1,1,1,1,1,1,1*)/(*1,1,1,2,1,1,1*)	13·67	$4·2 \pm 0·1^{e}$	Z1	(*1,1,1,1,1,2*)/(*1,1,1,1,2,3*)	13·31	$-2·0 \pm 0·2^{a}$	N1+Z7	8·97	0^{k}
N2	(*1,1,1,1,1,1,1*)/(*1,1,1,1,1,1,1*)	9·48	0^{d}	Z2	(*1/1,1/1,1/2,1/1,1/2,2/3*)	10·74	$1·8 \pm 0·2^{d}$	N2+Z1	6·65	$-18·3 \pm 0·3^{f}$
N3	(*1/1,1/1,1/5,1/1,1/1,1/1,1/5*)	8·81	$-3·8 \pm 0·2^{b}$	Z3	(*1,3,1,1,1,1*)/(*1,3,1,1,1,1*)	6·13	0^{c}	N2+Z6	6·27	$-14·9 \pm 0·3^{h}$
N4	(*1/1,1/1,1/4,1/2,1/1,1/1,1/1*)	8·19	$4·3 \pm 0·2^{e}$	Z4	(*1,1,1,1,1,1*)/(*1,1,1,1,2,3*)	5·09	$6·0 \pm 0·2^{g}$	N3+Z2	5·14	$-39·6 \pm 0·3^{a}$
N5	(*1/1,1/1,1/1,1/2,1/1,1/1, 1/5*)	6·53	$-10·9 \pm 0·2^{a}$	Z5	(*1,3,1,1,1,1*)/(*1,3,1,1,1,3*)	5·03	$14·2 \pm 0·2^{j}$	N4+Z2	3·51	$-26·9 \pm 0·3^{b}$
N6	(*1/1,1/1,1/1,1/2,1/1,2/3,2/2*)	5·67	$-3·3 \pm 0·2^{c}$	Z6	(*1,3,1,1,1,2*)/(*1,3,1,1,2,4*)	4·97	$6·7 \pm 0·2^{h}$	N5+Z13	3·39	$-12·3 \pm 0·3^{j}$
N7	(*1/1,1/1,1/4,1/2,1/1,1/1,6/7*)	5·30	$10·5 \pm 0·2^{h}$	Z7	(*1/1,1/1,1/2,1/1,1/2,1/3*)	4·29	$12·8 \pm 0·2^{i}$	N6+Z9	3·26	$-26·2 \pm 0·3^{c}$
N8	(*1,1,1,1,1,1,1*)/(*1,1,5,1,1,1,1*)	3·45	$-4·2 \pm 0·2^{b}$	Z8	(*1/1,2/2,1/3,1/1,1/2,1/3*)	3·19	$19·6 \pm 0·3^{k}$	N7+Z10	3·13	$-13·4 \pm 0·3^{i}$
N9	(*1/1,1/1,1/4,1/2,1/1,2/3,2/2*)	3·33	$8·3 \pm 0·2^{g}$	Z9	(*1/1,3/3,1/1,1/1,1/2,2/3*)	2·45	$-1·5 \pm 0·3^{b}$	N8+Z11	3·01	$-23·9 \pm 0·3^{e}$
N10	(*1,1,1,1,1,1,1*)/(*1,1,2,1,1,1,1*)	2·28	$-0·1 \pm 0·3^{d}$	Z10	(*1/1,1/5,1/1,1/1,1/2,1/3*)	2·33	$4·2 \pm 0·3^{e}$	N9+Z12	2·07	$-16·5 \pm 0·3^{g}$
N11	(*1/1,1/1,1/4,1/2,1/1,2/3,2/2*)	2·09	$4·3 \pm 0·3^{e}$	Z11	(*1,1,1,1,1,2*)/(*1,1,1,1,3,4*)	2·15	$5·2 \pm 0·3^{f}$	N10+Z4	2·01	$-25·1 \pm 0·3^{d}$
N12	(*2/3,1/1,1/1,1/2,1/1,1/1,1/5*)	2·03	$7·2 \pm 0·3^{f}$	Z12	(*1/1,2/3,1/1,1/1,1/2,2/3*)	2·09	$-1·2 \pm 0·3^{b}$	N11+Z5	1·94	$-24·7 \pm 0·2^{d}$
								N12+Z2	1·94	$-12·8 \pm 0·3^{ij}$

† Genotypes and phenotypes with less than 2% of the total lactations are not shown

Genotypes notation: e.g. N1 – phase seven genotype of the *GH2-N* copy composed of haplotypes (*1,1,1,1,1,1,1*) and (*1,1,1,2,1,1,1*) separated by /, fragment (I to VII) individual alleles were represented by an italic figure and were separated with a comma; N3 – (*1/1,1/1,1/5,1/1,1/1,1/1,1/5*) unphased genotype of the *GH2-N* with individual fragment (I to VII) genotypes were comma separated. For individual fragment alleles details see Table 3. Z13 = (*1/1,1/2,1/3,1/1,1/2,1/3*)

n = number of lactations

[a, b, c, d, e, f, g, h, I, j, k] – values in the same column with different letters are significantly different (P<0·05)

the least (N5) productive. Ewes with N7 genotype produced also 6·3±0·2 l/150 d more milk ($P<0·001$) than ewes with N1 genotype which was the most abundant in the studied population. Within *GH2-Z* genotypes there was a differential of 21·6±0·2 l/150 d of milk ($P<0·001$) between the most (Z8) and the least (Z1) productive. The effect of associated *GH2-N* and *GH2-Z* copies genotypes together revealed a differential of 39·6±0·3 l/150 d of milk ($P<0·001$) between the most (N1+Z7) and the least (N3+Z2) productive (Table 5).

Discussion

Duplication of the *oGH* gene has been described in sheep (Valinsky et al. 1990), in goat (Yamano et al. 1991) and in man (Chen et al. 1989), but not in cow. The GH2-N and GH2-Z gene copies are regulated differently. The GH2-N is expressed in anterior pituitary gland somatotrope cells (Warwick et al. 1989). The GH2-Z copy is expressed in placenta trophoblasts and syncitium between days 30 and 60 of pregnancy, a period of maximum placental growth (Lacroix et al. 1996, 1999). It is not expressed in the pituitary (Gootwine et al. 1996). In ovine breeds (our results; Valinsky et al. 1990), animals carrying *GH2-Z* copy (Gh2 allele) represents more than 90% of the population suggesting that it may confer some selective advantage. Moreover, as duplication confers tissue-specific and stage-specific activity to the *GH2-Z* gene, amino acid divergence between the oGH copies was possibly caused by positive Darwinian selection as suggested by Ohta (1993). In the Serra da Estrela breed, an advantage could be the higher milk yield observed at Gh2/Gh2 animals when compared with Gh1/Gh2 ones.

The high level of polymorphism detected at each *oGH* gene copy and between *oGH* gene copies prove that the PCR-SSCP technique is an efficient one to detect mutations (Table 2 and Fig. 1), as has been shown in other studies in sheep (Bastos et al. 2001; Santos et al. 2004), in goats (Malveiro et al. 2001; Marques et al. 2003), in cattle (Lee et al. 1994; Lagziel et al. 1996; Yao et al. 1996; Hines et al. 1998; Lagziel & Soller, 1999) and in pigs (Kaminski & Wachek, 2002). It is also cheaper and faster than techniques like allele-specific PCR or restriction fragment length polymorphism; nevertheless, it has limitations such as the occasional appearance of extra bands that may complicate the analysis. In the *oGH* gene, as *GH1*, *GH2-N* and *GH2-Z* sequences are highly homologous it is not possible to design specific primers for the exons and the 3′-UTR of each copy. However, the approach described in the present study allowed the SSSP patterns to be interpreted and *GH2-N* and *GH2-Z* copies genotypes attributed.

Valinsky et al. (1990), Gootwine et al. (1993; 1996) and Ofir & Gootwine (1997) reported three *Pvu*II-RFLPs related to the *oGH* copy number, located at position 712 in exon 2 (restriction site present only at the *GH2-N* copy), position 932–937 in the intron 2 (restriction site mutated at the *GH2-Z* copy) and position 1935–1939 in the exon 5 (restriction site present in the *GH2-N* and GH2-Z copies). The existence of polymorphisms at *Pvu*II restriction sites in the exon 2 (*GH2-N* copy) and at exon 5 (*GH2-Z* copy) identified in the present work invalidate the use of *Pvu*II-RFLP to discriminate between *oGH* copies (Table 3), but this could be done using PCR-SSCP analysis of exon 2 as described (section *oGH* gene analysis by PCR-SSCP). Results presented in Table 3 revealed copy-specific polymorphisms (24 at the *GH2-N* and 14 at the *GH2-Z* copies) and a common polymorphism at position 1047. Alignment of the GH2-N and GH2-Z predicted mature proteins from all the genotyped sheep exhibiting the two o*GH* copies (17 Gh2/Gh2 and two Gh1/Gh2 animals) revealed that copies always differ at two amino acids (aa): aa(–7) (P→L; site 624) and aa9 (G→R or C; site 668) as reported by Wallis et al. (1998). Amino acids at position 63 differ also between copies (G→S; site 1057) in most animals, however in others G^{63} is present at the *GH2-Z* copy like in *GH2-N* copy.

The present (Fig. 1, Table 2 and Table 3) and previous results in other Portuguese breeds (Bastos et al. 2001; Santos et al. 2004), as well in Assaf breed (Santos et al. unpublished results) and in Indian breeds (GenBank accession numbers: DQ166369-74 and DQ176733-47) show that coding regions were much more polymorphic in ovine than in bovine GH, where polymorphisms were detected by SSCP analysis mainly in non-coding regions and in exon 5 by SSCP analysis (Falaki et al. 1997; Lagziel & Soller, 1999; Ge et al. 2003). The low level of polymorphism observed in the 5′-UTR and in exon 1 (fragments I and II) agrees with previous findings indicating that these regions are highly conserved in GH1 and GH2-N genes in ovine (Ofir & Gootwine, 1997). Exon 2 was the most polymorphic one in Serra da Estrela ewes and also in Merino da Beira Baixa (Santos et al. 2004), but not in caprine breeds (Malveiro et al. 2001; Marques et al. 2003). This suggests that those polymorphisms arose after species differentiation. In exon 4, no polymorphism was revealed in spite of the two observed SSCP patterns, suggesting that DNA modification other than nucleotide mutations could be detected by SSCP analysis. Eight exon 4 variants (GenBank accession numbers: DQ176740-47) were detected in Indian breeds mainly within the exon4-intron4 junction region coincident to the annealing site of the GH4-Rev primer (Table 1) which may partly explain our results. High polymorphism level was also observed in caprine breeds (Malveiro et al. 2001; Marques et al. 2003). Polymorphisms detected in both copies could either involve regulatory regions and affect gene transcription rate (Pesole et al. 2000), or influence the secondary structure of the protein and its post-transcriptional function (Woychik et al. 1984), or be linked to a genotype of interest located elsewhere (Sneyers et al. 1994).

Taking fragment genotypes as a starting point, *oGH* haplotypes were inferred in both copies for some genotypes,

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but it was not possible to determine the phase for all genotypes, so statistical analyses could not be performed concerning haplotypes as it was done in bovine by Lagziel et al. (1996). At the time of blood collection many of the ewes' sires were no longer in the flocks, and therefore could not be genotyped. Nevertheless, considering pedigree information of 750 animals, a large number of *oGH* haplotypes was inferred, thus revealing considerable genetic diversity within the breed at the o*GH* gene. Eighty-six haplotypes were inferred at the *GH2-N* copy, and 32 at the *GH2-Z* copy which is much more than the 14 different haplotypes reported by Lagziel et al. (1996) in bovines.

Predicted protein variants differences resided in amino acids listed at Table 4. At the GH2-N, aa(–16) was in the GH signal peptide, aa3 was near the GH N-terminal amino acid, aa8, 22, 25 and 32 were in helix 1, with aa8 being involved in the second receptor-binding site as defined by Cunningham et al. (1991) for human GH. The remaining amino acids involved in variants differentiation were between helices 1 and 2. At the GH2-Z, aa(–1) was in the GH signal peptide, aa2 was near the GH N-terminal aa, amino acids 9, 21 and 23 were in helix 1, with aa9 being in the second receptor-binding site, amino acids 55, 59, 63 and 64 were between helix 1 and 2, with aa63 being in the first receptor-binding site, and aa160 were between helix 3 and 4. N-terminal residues, involved in GH receptor binding, have substantial galactopoietic activity in cows (Eppard et al. 1992). Hence, polymorphisms found in this region could account for the differences observed in the milk yield.

Homozygous genotypes were not found for all the alleles uncovered by SSCP analysis leading to several unphased genotypes. This limited the statistical analysis (Table 5) to the use of a model where only additive genetic effects were considered. At the GH2-N copy, no protein differences were found between the least (N5) and the most (N7) productive genotypes. The observed effect upon milk yield differences could be associated with the efficiency of mRNA processing or with its stability, not with differences in the protein structure. At the GH2-Z copy, a protein variant with amino acid partial sequence F^{2},R^{9},L^{21},S^{63},K^{64},G^{160} which corresponds to the published oGH sequence number Q95205 (Lacroix et al. 1996) was coded by the Z1 genotype and differed from the one coded by the Z8 genotype in C^{9}. The effect of an arginine at this position was discussed by Wallis et al. (1998), whether the Arg→Cys substitution influences GH binding to its receptor or protein folding remains to be elucidated. In any case, the genotypes that code for C^{9} variant were positively ($P<0{\cdot}05$) associated with milk yield (Table 5).

Associations were previously established between GH gene SSCP patterns and milk yield and composition parameters in bovine (Lagziel et al. 1996) and caprine breeds (Malveiro et al. 2001; Marques et al. 2003). Major effects on milk yield were observed in Serra da Estrela ewes for N1+Z7 associated genotypes: N1 genotype coded for two proteins similar to the published for the GH2-N copy (CAA31063), and Z7 coded for one protein similar to the one published for GH2-Z (Q95205) and to another with G^{63}. Amino acids at 63 belong to the binding site 1 in human GH (de Vos et al. 1992). Results from Wallis et al. (1998) support the notion that the S^{63} substitution together with Arg^{9} at the GH2-Z copy could inhibit GH-GHR binding. The presence of the G^{63}, described for the first time in ovine populations in the present work, and its positive effect on milk yield is in agreement with that assumption. In nature, although acting at different stages, both of the o*GH* gene copies act to control ewes' metabolism. Whether the GH2-Z copy influences directly milk yield, or not, remains controversial. However, the study of associated *GH2-N* and *GH2-Z* genotypes was performed in the present study because the GH2-Z copy influences placental and fetal growth, contributing to successful gestations of twins. It is widely observed that ewes nursing twin lambs produce more milk; this could be explained as an indirect effect of GH2-Z copy.

In conclusion, the *GH2-N* and *GH2-Z* genotypes were shown to significantly affect milk yield in Serra da Estrela ewes. The apparent joint effect of GH2-N and GH2-Z genotypes could on average improve milk yield, adjusted to a 150-d lactation, in 25% as compared with mean milk yield of the analysed population. The higher level of polymorphism observed suggests that genetic variability at the o*GH* gene could be exploited to produce genotypes with increased genetic merit.

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References

Barracosa HMGP 1996 [Study of genetic polymorphism and its association with milk yield potential in Algarvia goat breed and Serra da Estrela sheep breed]. *MSc Thesis,* University of Algarve, 86 pp

Bastos E, Cravador A, Azevedo J & Guedes-Pinto H 2001 Single strand conformation polymorphism (SSCP) detection in six genes in Portuguese indigenous sheep breed Churra da Terra Quente. *Biotechnologie, Agronomie, Société et Environnement* **5** 7–15

Boldman KG, Kriese LA, van Vleck LD & Kachman SD 1993 A set of programs to obtain estimates of variances and covariances. *In*: A manual for use of MTDFREML (draft). USDA ARS, US Meat Animal Research Center, Clay NE, USA

Byrne CR, Wilson BW & Ward KA 1987 The isolation and characterization of the ovine growth hormone gene. *Australian Journal of Biological Science* **40** 459–468

Carolino N, Gama L, Dinis R & de Sá T 2003 [Productive characteristics of Serra da Estrela ewes]. *Archivos de Zootecnia* **52** 3–14

Chen EY, Liao Y-C, Smith DH, Barrera-Saldana HA, Gelinas RE & Seeburg PH 1989 The human growth hormone locus: nucleotide sequence, biology and evolution. *Genomics* **4** 479–497

Cunningham BC, Ultsch M, de Vos AM, Mulkerrin MG, Clauser KR & Wells JA 1991 Dimerization of the extracellular domain of the human growth hormone receptor by a single hormone molecule. *Science* **254** 821–825

de Vos AM, Ultsch M & Kossiakoff AA 1992 Human growth hormone and extracellular domain of its receptor: crystal structure of the complex. *Science* **255** 306–312

Eppard PJ, Bentle LA, Violand BN, Ganguli S, Hintz RL, Kung L, Jr, Krivi GG & Lanza GM 1992 Comparison of the galactopoietic response to pituitary-derived and recombinant-derived variants of bovine growth hormone. *Journal of Endocrinology* **132** 47–56

Falaki M, Sneyers M, Prandi A, Massart S, Corradini C, Formigoni A, Burny A, Portetelle D & Renaville R 1996 TaqI growth hormone gene polymorphism and milk production traits in Holstein-Friesian cattle. *Animal Science* **63** 175–181

Falaki M, Prandi A, Corradini C, Sneyers M, Gengler N, Massart S, Fazzini U, Burny A, Portetelle D & Renaville R 1997 Relationships of growth hormone gene and milk protein polymorphisms to milk production traits in Simmental cattle. *Journal of Dairy Research* **64** 47–56

Ge W, Davis ME, Hines HC, Irvin KM & Simmen RCM 2003 Association of single nucleotide polymorphisms in the growth hormone and growth hormone receptor genes with blood serum insulin-like growth factor I concentration and growth traits in Angus cattle. *Journal of Animal Science* **81** 641–648

Gootwine E, Ofir R & Yossefi S 1996 Characterization of *Pvu*II polymorphisms between the ovine growth hormone GH2-N and GH2-Z gene copies. *Animal Biotechnology* **7** 135–143

Gootwine E, Sise JA, Penty JM & Montgomery GW 1993 The duplicated gene copy of the ovine growth hormone gene contains a *Pvu*II polymorphism in the second intron. *Animal Genetics* **24** 319–321

Gootwine E, Suttie JM, McEwan JC, Veenvliet BA, Littlejohn RP, Fennessy PF & Montgomery GW 1998 The physiological effects of natural variation in growth hormone gene copy number in ram lambs. *Domestic Animal Endocrinology* **14** 381–390

Hines HC, Ge W, Zhao Q & Davis ME 1998 Association of genetic markers in growth hormone and insulin-like growth factor-I loci with lactation traits in Holsteins (Abstract). *Animal Genetics* **29** 63

Horstick A, Hamann H & Distl O 2002 Estimation of genetic parameters for dairy milk performance of East Friesian milk sheep by randon regression models. *7th World Congress on Genetics Applied to Livestock Production* Communication N° 01–53

Horvath S, Xu X, Lake S, Silverman E, Weiss S & Laird N 2004 Tests for associating haplotypes with general phenotype data: application to asthma genetics. *Genetic Epidemiology* **26** 61–69

Kaminski S & Wachek M 2002 Single-strand conformation polymorphism within the porcine growth hormone gene promoter. *Animal Science Papers and Reports* **20** 47–53

Kann G, Delobelle-Deroide A, Belair L, Gertler A & Djiane J 1999 Demonstration of in vivo mammogenic and lactogenic effects of recombinant ovine placental lactogen and mammogenic effect of recombinant ovine GH in ewes during artificial induction of lactation. *Journal of Endocrinology* **160** 365–377

Lacroix MC, Devinoy E, Cassy S, Servely JL, Vidaud M & Kann G 1999 Expression of growth hormone and its receptor in the placental and feto-maternal environment during early pregnancy in sheep. *Endocrinology* **140** 5587–5597

Lacroix MC, Devinoy E, Servely JL, Puissant C & Kann G 1996 Expression of the growth hormone gene in ovine placenta: detection and cellular localization of the protein. *Endocrinology* **137** 4886–4892

Lagziel A & Soller M 1999 DNA sequence of SSCP haplotypes at the bovine growth hormone (bGH) gene. *Animal Genetics* **30** 362–365

Lagziel A, Lipkin E & Soller M 1996 Association between SSCP haplotypes at the bovine growth hormone gene and milk protein percentage. *Genetics* **142** 945–951 (Corrigendum 1997 *Genetics* **146** 442)

Lee BK, Foster DN & Crooker BA 1994 Detection of mutations in the bovine somatotropin (bST) gene by single strand conformation polymorphism (SSCP) analyses. *Journal of Animal Science* **72** 316

Leibovich H, Gertler A, Bazer F & Gootwine E 2001 Effects of recombinant ovine placental lactogen and recombinant ovine growth hormone on growth of lambs and milk production of ewes. *Livestock Production Science* **68** 79–86

Lucy MC, Hauser SD, Eppard PJ, Krivi GG, Clark JH, Bauman DE & Collier RJ 1993 Variants of somatotropin in cattle: gene frequencies in major dairy breeds and associated milk production. *Domestic Animal Endocrinology* **10** 325–333

Malveiro E, Pereira M, Marques PX, Santos IC, Belo C, Renaville R & Cravador A 2001 Polymorphisms at the five exons of the growth hormone gene in the algarvia goat: possible association with milk traits. *Small Ruminant Research* **41** 163–170

Marques MR, Santos IC, Belo CC & Cravador A 2001 Associations between SSCPs in the GH gene and milk traits in Serra da Estrela ewes. *Biotechnologie, Agronomie, Société et Environnement* **5** 57

Marques PX, Pereira M, Marques MR, Santos IC, Belo CC, Renaville R & Cravador A 2003 Association of milk traits with SSCP polymorphisms at the growth hormone gene in the Serrana goat. *Small Ruminant Research* **50** 177–185

Ofir R & Gootwine E 1997 Ovine growth hormone gene duplication—structural and evolutionary implications. *Mammal Genome* **8** 770–772

Ohta T 1993 Pattern of nucleotide substitutions in growth hormone-prolactin gene family: a paradigm for evolution by gene duplication. *Genetics* **134** 1271–1276

Orian JM, O'Mahoney JV & Brandon MR 1988 Cloning and sequencing of the ovine growth hormone gene. *Nucleic Acids Research* **16** 9046

Orita M, Suzuki Y, Sekiya T & Hayashi K 1989 Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. *Genomics* **5** 874–879

Parsons YM, Cooper DW, Piper LR & Adams TE 1992 RFLPs at the ovine locus for growth hormone. *Animal Genetics* **23** 571

Pesole G, Liuni S, Grillo R, Licciulli F, Lariza A, Makaliwski W & Saccone C 2000 UTRdb and UTRsite: specialized databases of sequences and functional elements of 5′ and 3′ untranslated regions of eukatyotic mRNAs. *Nucleic Acids Research* **28** 193–196

Santos IC, Marques MR, Belo CC & Cravador A 2004 Polymorphism analysis at the growth hormone gene in Merino da Beira Baixa ewes. *Biotechnologie, Agronomie, Société et Environnement* **8** 40–41

Sneyers M, Renaville R, Falaki M, Massart S, Devolder A, Boonen F, Marchand E, Prandi A, Burny A & Portetelle D 1994 *Taq*I restriction fragment length polymorphisms for growth hormone in bovine breeds and their association with quantitative traits. *Growth Regulation* **4** 108–112

Valinsky A, Shani M & Gootwine E 1990 Restriction fragment length polymorphism in sheep at the growth hormone locus is the result of variation in gene number. *Animal Biotechnology* **1** 135–144

Wallis M, Lioupis A & Wallis OC 1998 Duplicate growth hormone genes in sheep and goat. *Journal of Molecular Endocrinology* **21** 1–5

Warwick JM, Wallis OC & Wallis M 1989 Cloning, sequence and expression in *Escherichia coli* of cDNA for ovine pregrowth hormone. *Biochim Biophys Acta* **1008** 247–50

Woychik RP, Lyons RH, Post L & Rottman FM 1984 Requirement for the 3′ flanking region of the bovine growth hormone gene for accurate polyadenylylation. *Proceedings of the National Academy of Sciences of the USA* **81** 3944–3948

Yamano Y, Abe M, Mikawa S, Kioka N, Manabe E, Sakai H, Komano T, Utsumi K & Iritani A 1991 Structural analysis of repetitive DNA sequences in the goat growth hormone gene region. *Agricultural and Biological Chemistry* **55** 633–639

Yao J, Aggrey SE, Zadworny D, Hayes JF & Kuhnlein U 1996 Sequence variations in the bovine growth hormone gene characterized by single-strand conformation polymorphism (SSCP) analysis and their association with milk production traits in Holsteins. *Genetics* **144** 1809–1816