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ORIGINAL PAPER

A SNP in the *HSP90AA1* gene 5′ flanking region is associated with the adaptation to differential thermal conditions in the ovine species

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Abstract Molecular chaperones have long been understood to be preferentially transcribed in response to multiple perturbations of the cellular homeostasis. In this study, several polymorphisms in the gene encoding the inducible form of the cytoplasmic Hsp90 (*HSP90AA1*) were addressed in 24 sheep breeds reared in different climatic regions of Europe, Africa, and Asia. Significant differences in the genotype frequencies for a C/G single nucleotide polymorphism (SNP) located at position −660 in the *HSP90AA1* 5′flanking region were found between the different breeds. Regression analyses reflected significant correlations (from 0.41 to 0.62) between the alternative genotypes of this polymorphism and several climatic and geographic variables characteristic of the regions where these breeds are reared. Real-time analysis revealed that animals bearing the CC_{-660} genotype presented higher expression levels than those presenting the CG_{-660} or GG_{-660} in summer, but not in spring. Mutation at −660 site seems to affect *HSP90AA1* transcription rates which could have important effects on the adaptation to different environmental conditions in sheep. Thus, the variability found in the genotype frequencies for the SNP at −660 in the ovine *HSP90AA1* locus could be the result of the different environmental pressures occurring in the regions where these breed are maintained.

Keywords Heat shock genes · Polymorphisms · Expression · Thermal adaptation · Ovine species

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Introduction

The cellular ability to resist and rapidly adapt to hazardous environmental conditions was one of the main prerequisites for the development of living organisms during evolution. Sudden changes in temperature are among the earliest and most ubiquitous insults that cells had to cope with to preserve their structural and enzymatic integrity (Nadeau and Landry 2006). Temperature can vary on a daily, seasonal, or spatial scale, and this variation can have a major impact on an individual's fitness (Hoffmann et al. 2003; Rohmer et al. 2004) as well as on its daily behavioral activities (Hoffmann and Parsons 1991; Gilchrist and Huey 1999; Gilbert and Huey 2001; David et al. 2003). Thus, the variation in the environmental temperature will generally

impose stress upon the organism, which may result in the evolution of adaptative genetic mechanisms to cope with temperature extremes in nature (Hoffmann and Parsons 1991).

The heat shock response is one of the most ancient and conserved cellular stress responses, which is characterized by the transcriptional activation and accumulation of a set of proteins known as heat shock proteins (Hsp). The common view on the protective function of Hsp relies on their chaperone activity which consists of assisting the non-covalent assembly and/or disassembly of other macromolecular structures (Ellis 2006). The chaperone known as 90-kDa heat shock protein, Hsp90, is one of the most abundant proteins in eukaryotic cells, comprising 1–2% of cellular proteins under non-stress conditions. There are two major cytoplasm isoforms of Hsp90 which have arisen by gene duplication, Hsp90α (inducible form) and Hsp90β (constitutive form). The contribution cytosolic Hsp90 to various cellular processes including signal transduction, protein folding, protein degradation, cell survival, and morphological evolution has been extensively studied. Additionally, a quantitative trait loci study performed on *Drosophila melanogaster* (Morgan and Mackay 2006) revealed a genomic region on chromosome 3 with an important effect on heat stress resistance. That region contained several positional candidate genes, including *HSP83*, which is the *Drosophila* homolog of the mammalian *HSP90* family.

Biodiversity within species concerns the amount, distribution, and adaptive value of variation within and among populations in their natural environment (Ryder 1986). To date, most biodiversity studies within species have focused on random molecular markers, such as microsatellites (Luikart and England 1999). However, some authors (Reed and Frankham 2001) have pointed out that the variation in molecular markers is not necessarily an indication of the adaptive evolutionary potential or differentiation of populations. In many works, association between single nucleotide polymorphisms (SNPs) at certain genes and stress resistance have been found (Li et al. 2009; Reddacliff et al. 2005; Sun et al. 2007). Therefore, although neutral variation is suitable for the reconstruction of historical processes, information about variation in specific genes, such as those affecting resistance to stressful conditions (e.g., temperature, humidity, insulation, etc.) can constitute a valuable tool for ecological genetic studies.

It seems very likely that sheep were among the earliest domesticated species. Archeological bone remains confirm that sheep were domesticated in southwestern Asia about 9000 B.C., 14 millions years after the development of hominids and two millennia before that of other agricultural animals. In 2006, the total number of sheep worldwide was around 1,051 million (FAOSTAT, http://faostat.fao.org/). The hardiness of sheep is well recognized. They are ruminants designed to graze on low-quality pastures and can thrive in very adverse climatic conditions. They can be found all over the world in a wide variety of environments, including highly improved pasture and intensive housed production systems. Belyaev (1979) considered the enormous increase in the rate and range of variability in sheep as a result of the domestication process. Thus, the variation observed nowadays among sheep breeds is the combined effect of artificial selection imposed by humans, natural selection forces specific to the environment in which each breed is developed, and random processes of genetic drift due to finite population size.

The ovine gene encoding the inducible form of the Hsp90α (*HSP90AA1*) has been recently characterized and mapped (Marcos-Carcavilla et al. 2008). In the same study, several SNPs located in the 5′ flanking region and intron 10 were described and found to be associated with the response to scrapie in sheep. Therefore, it was hypothesized that a C/G transversion at −660 position in the 5′ flanking region and six linked SNPs at intron 10 (A40G, C165T, C178G, C205T, C220T, and C239T) could affect *HSP90AA1* expression rate by altering transcription and splicing factors binding sites, respectively.

This work studies the distribution of these polymorphisms in a collection of sheep breeds from different geographic and climatic regions (The Caucasus, Asia, Africa, Spain, and Northern Europe) and determines the possible association between these genotypes and several climatic and geographic variables of the regions where these breeds are reared. Real-time polymerase chain reaction (PCR) studies were also carried out to test the possible effect of the polymorphisms located at −660 on the ovine *HSP90AA1* gene expression.

Materials and methods

HSP90AA1 polymorphisms in sheep from different geographic and climatic regions

Genomic DNA isolation and amplification of DNA fragments Blood samples were collected from 907 sheep pertaining to 24 different breeds reared in diverse geographic and climatic areas. Fifty European mouflons (*Ovis musimon*) were also analyzed (Table 1). Climatic data and geographic coordinates were obtained from the following web sites: http://www.educaplus.org and http://clima.meteored.com.

Genomic DNA was extracted from lymphocytes according to the salting out procedure described by Miller et al. (1988). In the PCR reactions, 60 ng of genomic DNA was amplified in a final volume of 25 μl containing 0.5 μM of

Table 1 Biological material and geographic and climatic parameters studied

Breed	Breed type	Geographical area	Sample size	LAT	LONG	MINaT	MAXaT	MThm	ANT	TW
Assaf	Fat-tailed	Spain (from Israel)	31	31.86	35.21	14.0	27.4	40	20.7	13.4
Awassi	Fat-tailed/coarse wool	Spain (from Israel)	30	31.86	35.21	14.0	27.4	40	20.7	13.4
European Mouflon	Feral	Spain (from Corsica)	50	41.92	8.73	8.2	22.3	32	14.8	14.1
Latxa	Coarse wool	Spain (Alava)	31	42.88	−2.73	4.7	19.1	30	11.4	14.4
Churra Lebrijana	Coarse wool	Spain (Jérez)	22	36.75	−6.06	10.7	25.7	38	17.7	15.0
Churra	Coarse wool	Spain (León)	33	42.58	−5.65	3.1	19.6	37	10.9	16.5
Spanish Merino	Fine wool	Spain (Cáceres)	30	30.46	−6.33	8.2	25.6	39	16.0	17.4
Rasa Aragonesa	Semifine wool	Spain (Zaragoza)	83	41.66	−1.01	6.2	24.3	35	14.6	18.1
Churra Tensina	Coarse wool	Spain (Huesca)	32	42.08	−0.33	4.9	23.4	37	13.6	18.5
Manchega	Semifine wool	Spain (Albacete)	205	38.95	−1.85	5.0	24.1	38	13.5	19.1
D`man	Fine wool	Morocco (Ouarzazate)	32	30.93	−6.90	9.3	29.5	40	18.9	20.2
Sardi	Coarse wool	Morocco (Settat)	32	32.88	−7.55	9.3	29.5	40	18.9	20.2
Boujâad	Fine wool	Morocco (Khouribga)	31	32.86	−6.96	9.3	29.5	39	18.9	20.2
Pramenka	Semifine wool	Serbia	29	41.96	21.65	5.1	25.9	32	15.3	20.8
Arkhar Merino	Merino fine wool	Kazakhstan/Bosnia	18	43.81	18.35	0.2	21.1	35	11.1	20.9
Olkuska	Continental long wool	Poland (Cracovia)	30	50.08	19.80	−2.9	19.3	30	8.6	22.2
Finnsheep	Nordic short-tailed	Finland (Joensuu)	30	62.66	29.63	−5.7	17.0	20	5.3	22.7
Caucasian	Merino finewool	Russia (Stavropol)	30	45.11	42.08	2.2	25.2	36	13.9	23.0
Karabakh	Fat-tailed	Azerbaijan	30	40.45	50.06	−0.4	23.6	38	11.9	24.0
Moldavian Karakul	Double coated/fat-tailed	Moldova	15	47.01	28.86	−3.3	20.9	37	9.6	24.2
Russian Karakul	Double coated/fat-tailed	Uzbekistan	15	41.26	69.26	0.5	27.6	42	14.2	27.1
Bozakh	Fat-tailed	Azerbaijan and Armenia	28	40.13	44.46	−3.5	26.0	41	12.1	29.5
Karachai	Fat-tailed	Russia (North Caucasus)	30	43.11	131.93	−16.0	18.2	37	0.8	34.2
Bajdarakh	Fat-tailed	Russia (Buryatia)	30	51.83	107.43	−18.8	17.5	34	0.0	36.3
Edilbai	Fat-rumped	West Kazakhstan	30	50.28	57.23	−14.1	22.4	39	4.7	36.5

All temperature data are in degree Celsius

LAT latitude, *LON* longitude, *MAXaT* maximum average temperature, *MThm* maximum temperature of the hottest month, *MINaT* minimum average temperature, *ANT* average annual temperature, *TW* (MAXaT − MINaT)=thermal width

each primer, 200 µM of dNTPs, 2 mM $MgCl_2$, 2.5 µl of 10× buffer $MgCl_2$ free (Biotools), and 1 U Taq polymerase (Biotools). The following PCR conditions were used for the amplification of the 5′ flanking region: denaturation at 94°C for 5 min, 35 amplification cycles of denaturation at 94°C for 45 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s followed by a final 10-min extension at 72°C. In the case of intron 10, the PCR conditions consisted of denaturation at 94°C for 5 min, 35 amplification cycles of denaturation at 94°C for 45 s, annealing at 58° for 1 min, and extension at 72°C for 30 s followed by a final 10-min extension at 72°C. The resulting PCR fragments were purified with ExoSAP-IT (USB Corporation) and sequenced with specific primers (see Electronic supplementary material, Table 1).

Statistical analyses ARLEQUIN 3.1 statistical package (Excoffier et al. 2005) was used to perform the exact test for Hardy–Weinberg equilibrium (HWE).

Several analysis of variance (ANOVA) tests were carried out to check the existence of differences for the genotype frequencies of the polymorphisms at the 5′ flanking region and intron 10 within and between breeds. A chi-square test was also developed for the contingency tables of breeds and genotypes. The correlation between the genotypes and some geographic (latitude = LAT and longitude = LON) and climatic parameters (maximum average temperature = MAXaT, maximum temperature of the hottest month = MThm, thermal width = TW, and average annual temperature = ANT) from the different locations of each breed was assessed fitting regression equations, which included the genotype and the environmental data as the dependent and independent variables, respectively. The *F* ratio test was used to determine the statistical significance of the association. As criteria to compare alternative models, the *R*-squared statistic and the lack of fit test were employed. All statistics were calculated using the SAS statistical package (SAS 1990).

Nei's minimum genetic distance (Nei 1972) among breeds was calculated by BIOSYS-1 software (Swoford and Selander 1981). A cluster analysis using unweighted pair group method with arithmetic mean (UPGMA) method based on the Nei's minimum genetic distance estimates between the breeds was also performed to plot groups of breeds in a phenogram. In addition, a principal components analysis (PCA) was also carried out to determine breed grouping, followed by a discriminant analysis (DA) to test the robustness of the grouping by means of SAS procedures (SAS 1990). The variables used in these analyses were frequencies of alternative genotypes of the SNPs located in the 5′ flanking region and intron 10 of the *HSP90AA1* gene and the climatic and geographic parameters mentioned above.

Expression analysis in animals with different genotypes at −660 position in the *HSP90AA1* 5′ flanking region in two different seasons

Total RNA isolation and cDNA synthesis Two sets of blood samples from 22 rams of the Manchega Spanish sheep breed showing the CC_{-660} (n=10), CG_{-660} (n=10), and GG_{-660} (n=2) genotypes for the mutation located at −660 position were collected in two different seasons in a dry region of Central Spain (Ciudad Real).

The first set of samples was collected at midday the 6th of May 2008, with an environmental temperature and a relative humidity at that moment of 28.6°C and 52%, respectively. The second set of samples was collected at midday the 31st of July 2008, with an environmental temperature and a relative humidity in this case of 34.4°C and 35%, respectively (data from Ciudad Real Meteorological Station, 629m-38, 59N-03 55W).

Total RNA was isolated using the LeukoLock kit (Ambion) following the manufacturer's instructions. In order to avoid contamination with genomic DNA, samples were treated with 3 µl of RQ1 RNase-Free DNase (Promega) at 37°C for 30 min. The quality of the RNA was assessed based on the demonstration of distinct intact 28S and 18S ribosomal RNA bands. RNA concentration was determined with the Nano-drop spectrophotometer. cDNA was synthesized from 0.5 µg of each RNA sample using random hexamers (1 µl) and oligodt (1 µl) primers with the ImProm-II reverse transcriptase (Promega).

Quantification of the HSP90AA1 expression Expression of *HSP90AA1* gene was quantified by real-time PCR. The housekeeping genes (HK) *ACTB* and *GAPDH* were used as internal controls. The primer pairs used in the amplification of the HKs were previously described by Lyahyai et al. (2006, 2007). For the analysis of the *HSP90AA1* gene, two primer pairs hybridizing with exons 5 and 6 and 10 and 11 were used to discard the possible effect of transcriptional variants. The identity of all the PCR products was confirmed by sequencing and by BLAST comparison with the GenBank database. Real-time PCR reactions were run in triplicate in an ABI-Prism 7500 (Applied Biosystems). Amplification was carried out in a final volume of 20 µl containing 10 µl of SYBR Premix Ex Taq (Takara), 2 µl of cDNA diluted 1/20, and 200 or 300 nM of each primer. After preheating the mix at 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 60 s were carried out. For the same animal, the PCRs for the two HK and the *HSP90AA1* genes were run at the same time. The same two animals were run in all the plates as plate controls. The primers used in the gene expression analysis are summarized in Electronic supplementary material, Table 2.

Statistical analyses C_t values obtained from the three replicates run for the two HK and *HSP90AA1* genes were averaged in a single value (C_{tmed}). Subsequently, normalization factors were calculated by geNorm v 3.5 software (Vandesompele et al. 2002) and used to normalize the target gene. As two different primers for the *HSP90AA1* gene were used in the real-time PCR reactions, the first analysis was conducted to test expression level differences between them in order to assess the possible existence of different transcript variants. An ANOVA was developed including as main effects the plate and the *HSP90AA1* primer used. Finally, a general linear model analysis, including in the model the plate and the *HSP90AA1* genotype as fixed effects, was developed to assess the association (F ratio test) between the different *HSP90AA1* genotypes and the expression level of the gene. All analyses were carried out by SAS statistical procedures (SAS 1990).

Results

HSP90AA1 polymorphisms in sheep from different geographic and climatic regions

Test for HWE The results of the exact test for HWE for the *HSP90AA1* 5′ flanking region and intron 10 polymorphisms are shown in Table 2. Only Pramenka and Bozakh breeds showed significant departures from HWE. This disequilibrium was due in both cases to a heterozygote deficiency. In the Bozakh breed, CC_{-660} was the most frequent genotype, whereas GG_{-660} was overrepresented in the Pramenka one. For the polymorphisms at intron 10, only the Pramenka and the Karachai breeds showed significant departures from HWE. In this case, disequilibrium was also due to a high heterozygote deficiency in both breeds.

Table 2 Exact tests for HWE for the SNPs at the 5′ flanking region (C/G_{-660}) and intron 10 (A/G_{40}, C/T_{165}, C/G_{178}, C/T_{205}, C/T_{220}, and C/T_{239}) of the *HSP90AA1* gene in the breeds studied

Breed	C/G_{-660} at the 5′ flanking region		A/G_{40}, C/T_{165}, C/G_{178}, C/T_{205}, C/T_{220} and C/T_{239} at intron 10	
	Number of genotypes	p value	Number of genotypes	p value
Assaf	33	0.55778	31	1.00000
Awassi	30	0.50420	30	0.50420
European Mouflon	51	0.78236	NPL	–
Latxa	31	0.23182	31	0.40241
Churra Lebrijana	23	1.00000	15	1.00000
Churra	33	1.00000	32	1.00000
Spanish Merino	30	0.63857	28	0.17663
Rasa Aragonesa	93	0.73316	81	1.00000
Churra Tensina	32	0.70605	29	1.00000
Manchega	210	0.31453	196	1.00000
D'man	32	1.00000	32	0.39211
Sardi	32	1.00000	28	1.00000
Boujâad	31	0.40198	29	1.00000
Pramenka	30	0.03785*	29	0.00010***
Arkhar-Merino	18	1.00000	18	1.00000
Olkuska	30	0.70787	NPL	–
Finnsheep	30	1.00000	30	0.17931
Caucasian	30	1.00000	30	1.0000
Karabakh	30	0.46257	30	0.56049
Moldavian Karakul	15	1.00000	15	1.00000
Russian Karakul	15	0.62848	15	1.00000
Bozakh	28	0.06472**	28	1.00000
Karachai	30	0.29187	30	0.02970*
Bajdarak	30	0.71195	30	1.00000
Edilbai	30	0.14681	30	1.00000

*$p<0.05$; **$p<0.10$; ***$p<0.001$ (significance level of the departure from HW equilibrium)

Distribution of the HSP90AA1 polymorphisms among breeds ANOVA results for the genotype frequencies of the polymorphisms analyzed in the ovine *HSP90AA1* gene 5′ flanking region and intron 10 showed highly significant ($p<0.0001$) differences among breeds. The *F* ratio between breeds/within breeds took values of 7.73 and 3.89 for the 5′ flanking region and intron 10 polymorphisms, respectively. Also, the chi-square test (48 *df*) for the contingency tables of breeds and genotypes yielded highly significant differences among breeds ($p=0.0000$), having chi-square values of 248.52 and 210.35 for the 5′ flanking region and intron 10 polymorphisms, respectively.

Figure 1 shows the phenogram for the *HSP90AA1* gene 5′ flanking region polymorphism obtained by clustering analyses based on Nei minimum distances between breeds. The genotype frequencies in each breed and the average genotype frequency in each group of breeds are also shown. Breeds were grouped into five clusters. The first cluster contains two fat-tailed breeds from Israel (Assaf and Awassi), two Spanish breeds (Churra Lebrijana and Latxa), and one North African breed (D'man). The second cluster included five Spanish breeds (Churra, Spanish Merino, Rasa Aragonesa, Churra Tensina, and Manchega) and the Boujâad African breed. In both groups, a higher proportion of the CC_{-660} genotype (73.6 and 52.5 as average) was observed. Breeds which constituted these clusters are reared in regions located at latitudes ranging from 30° to 43°. The TW of these regions has values from 13°C to 20°C, the ANT being between 10°C and 20°C. Arkhar Merino, Pramenka, Caucasian, and Olkuska breeds were included in cluster 3. Cluster 4 was made up of seven fat-tailed breeds (Bajdarakh, Karachai, Edilbai, Bozakh, Moldavian Karakul, Karabakh, and Russian Karakul) and the European mouflon. Breeds included in groups 3 and 4 come from regions located at high latitudes (40° to 51°) where TW ranged from 21°C to 36°C and ANT from 0°C to 15°C. In cluster 3, the frequency of the GG_{-660} genotype was similar to that of the CG_{-660} (43.8 and 45.8, respectively), and the CC_{-660} frequency was the lowest of all groups (10.5). Finally, cluster 5 was composed of two quite different

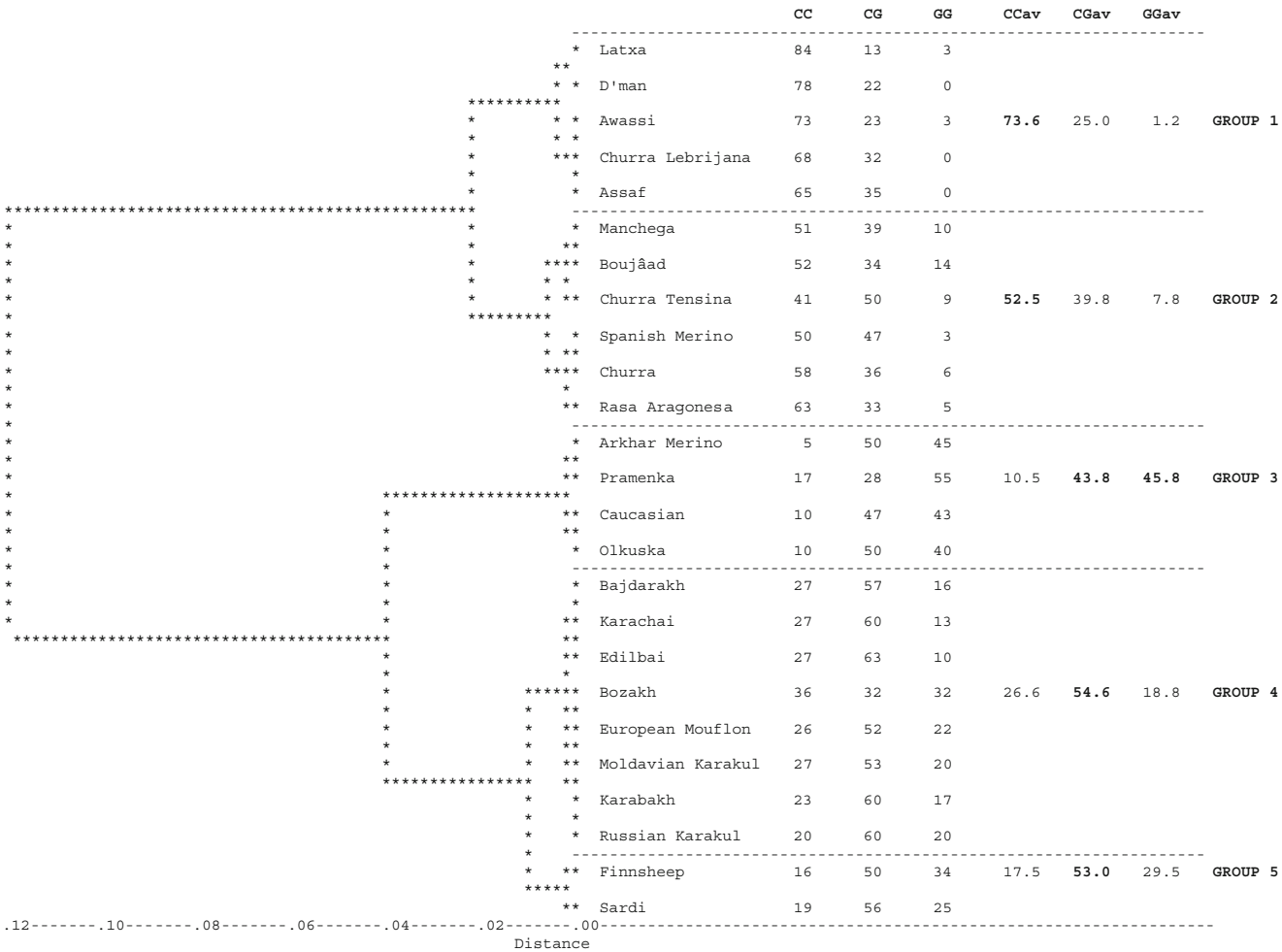


Fig. 1 Phenogram of cluster analysis using the UPGMA algorithm with Nei's minimum distances (D_m) and genotype frequencies of the C/G$_{-660}$ polymorphism at the 5′ flanking region of the *HSP90AA1* gene in 24 European, African, and Asiatic sheep breeds and the European mouflon

breeds, the Finnsheep and the Sardi, which, together with group 3, are the only ones in which the GG$_{-660}$ genotype had a considerable frequency (29.5 and 45.8, respectively).

Figure 2 shows the phenogram for the polymorphism of the *HSP90AA1* intron 10 obtained by clustering analyses based on Nei minimum distances between breeds. The genotype frequencies in each breed and the average genotype frequency in each group of breeds are also shown. In this case, breeds were distributed in five clusters with variable number of breeds per group. From groups 1 to 5, the homozygous $GG_{40}CC_{165}GG_{178}CC_{205}TT_{220}TT_{239}$ frequency decreases from values close to 1 to 0.5. This fact was accompanied by the increase in the heterozygous genotype from very low values to values around 40% and few changes in the $AA_{40}TT_{165}CC_{178}TT_{205}CC_{220}CC_{239}$ homozygous proportion. A mixture of breeds from distant latitudes and very different environmental circumstances was included in each cluster. In this case, the classification of the breeds within the groups did not reflect any relationship with the climatic and geographic variables studied here.

Implication of some environmental parameters in the distribution of the HSP90AA1 polymorphisms among breeds Since a UPGMA tree drawing algorithm is recommended only for cases in which equal rates of evolution for all taxa are assumed, a PCA followed by a DA were performed for breed clustering, taking into account not only genotype frequencies but also environmental variables. Table 3 shows principal components extracted by the PCA analyses which included genetic, climatic, and geographic variables of all breeds studied. For both sets of SNPs (5′ flanking region and intron 10), three principal components with eigenvalues greater than 1 were obtained. They explained an 88.5% and an 86.9% of the variance between breeds for the SNPs at the 5′ flanking region and intron 10, respectively. Component weights for each variable included in the PCA have been summarized in Table 4. In all cases, the heaviest weights of component 1 were those linked to climatic and geographic variables (MAXaT, ANT, and LAT). In the case of the SNP at −660, the greater weights in component 2 were those of MThm

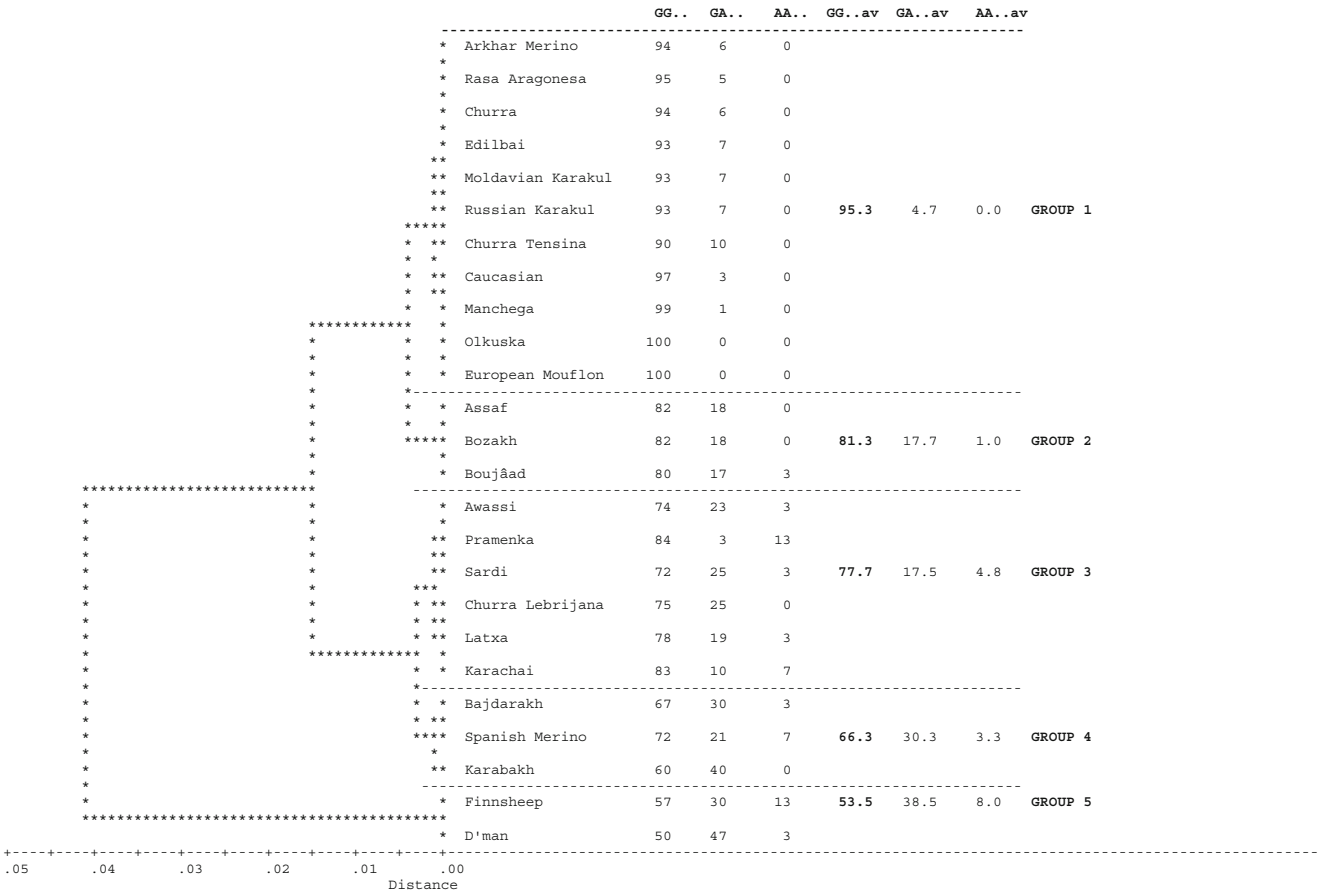


Fig. 2 Phenogram of cluster analysis using the UPGMA algorithm with Nei's minimum distances (D_m) and genotype frequencies of the A/G_{40}C/T_{165}C/G_{178}C/T_{205}C/T_{220}C/T_{239} polymorphism at *HSP90AA1* intron 10 in 24 European, African, and Asiatic sheep breeds and the European mouflon

and TW, while in the case of SNPs at intron 10 were those belonging to the genotype frequencies. Component 3 had the greater weights for the CC_{-660} and GG_{-660} genotype frequencies in the case of the SNP at −660 and for climatic and geographic variables (MThm, TW, and LON) in the case of the SNPs at intron 10. Results from PCA reveal that both genetic and environmental variables have similar and significant responsibility in the observed variability among breeds.

Table 3 Principal component analyses including as variables the genotype frequencies of the SNPs studied in the ovine *HSP90AA1* 5′ flanking region and intron 10 and several climatic and geographic parameters (for complementary information, see "Materials and methods")

Component number	*HSP90AA1* SNP C/G_{-660} 5′ flanking region			*HSP90AA1* SNPs A/G_{40}, C/T_{165}, C/G_{178}, C/T_{205}, C/T_{220} and C/T_{239} at intron 10		
	Eigenvalues	Percent of variance	Cumulative percentage	Eigenvalues	Percent of variance	Cumulative percentage
1	4.76752	52.972	52.972	3.89080	43.231	43.231
2	1.80693	20.077	73.049	2.21546	24.616	67.847
3	1.39065	15.452	88.501	1.71646	19.072	86.919
4	0.53436	5.937	94.438	0.66662	7.407	94.326
5	0.25826	2.870	97.308	0.28318	3.146	97.472
6	0.16743	1.860	99.168	0.16414	1.824	99.296
7	0.07283	0.809	99.978	0.05984	0.665	99.961
8	0.00195	0.022	99.999	0.00217	0.024	99.985
9	0.00006	0.001	100.00	0.00132	0.015	100.00

Table 4 Component weights for the variables included in the principal component analyses of the SNPs studied in the ovine *HSP90AA1* 5′ flanking region and intron 10

HSP90AA1 5′ flanking region				*HSP90AA1* intron 10			
C/G_{-660}				$A/G_{40}C/T_{165}C/G_{178}C/T_{205}C/T_{220}C/T_{239}$			
Variable	Component 1	Component 2	Component 3	Variable	Component 1	Component 2	Component 3
MAXaT	0.36675	0.33917	−0.31674	MAXaT	0.44573	−0.06630	0.18206
MThm	0.23137	0.60668	0.06419	MThm	0.31436	0.11346	0.55662
ANT	0.41731	0.02155	−0.31598	ANT	0.48633	−0.00947	−0.14504
TW	−0.34031	0.39231	0.15344	TW	−0.33694	−0.04783	0.51108
LAT	−0.39826	−0.29110	0.06226	LAT	−0.46316	0.02834	−0.18442
LON	−0.30668	0.37216	0.24536	LON	−0.31957	−0.05985	0.49261
CC_{-660}	0.35003	−0.14059	0.51301	$GG_{40}CC_{165}GG_{178}CC_{205}TT_{220}TT_{239}$	−0.04477	0.66272	−0.03888
CG_{-660}	−0.31892	0.33503	−0.12505	$GA_{40}CT_{165}GC_{178}CT_{205}TC_{220}TC_{239}$	0.09647	−0.60585	0.13634
GG_{-660}	−0.24367	−0.08537	−0.65519	$AA_{40}TT_{165}CC_{178}TT_{205}CC_{220}CC_{239}$	−0.15654	−0.41196	−0.27931

CC_{-660}, CG_{-660}, and GG_{-660} = genotype frequencies of the SNP in the ovine *HSP90AA1* 5′ flanking region

$GG_{40}CC_{165}GG_{178}CC_{205}TT_{220}TT_{239}$, $GA_{40}CT_{165}GC_{178}CT_{205}TC_{220}TC_{239}$ and $AA_{40}TT_{165}CC_{178}TT_{205}CC_{220}CC_{239}$ = genotype frequencies of the SNPs in the ovine *HSP90AA1* intron 10

MAXaT maximum average temperature, *MThm* maximum temperature of the hottest month, *ANT* average annual temperature, *TW* thermal width, *LAT* latitude, *LON* longitude

Table 5 shows Pearson product moment correlations among genetic and environmental variables. As expected, there were many significant correlations among climatic and geographic variables. The CC_{-660} homozygous and the heterozygous (CG_{-660}) frequencies of the SNP at −660 in the *HSP90AA1* gene showed moderate, opposite, and significant correlations with ANT, TW, LAT, and LON. A higher CC_{-660} frequency was found in breeds located in regions with small TW, high ANT, and small values of LAT and LON. No correlation was detected among the genotype frequencies for the SNPs at *HSP90AA1* intron 10 and the environmental factors analyzed. Only the homozygous $AA_{40}TT_{165}CC_{178}TT_{205}CC_{220}CC_{239}$ genotype showed high and negative correlation with MThm. However, this genotype was poorly represented in the breeds analyzed here (detected only in ten out of the 24 breeds), and when apparent, it was always at a low frequency (<13%). This association could thus be spurious.

Furthermore, DA was performed including as the classification variable the breed grouping assessed by UPGMA (Nei's minimum distance) using the data from the SNPs at the *HSP90AA1* gene studied in the present work and climatic (MThm, MAXaT, ANT, and TW) and geographic (LAT and LON) parameters as independent variables. Results from using the derived discriminant functions to classify breeds are shown in Table 6. The percentages of cases correctly classified were 80 and 68 for the SNPs at −660 5′ flanking region and intron 10, respectively. Five and eight breeds were misclassified when comparing UPGMA and discriminant functions groups for both sets of SNPs, respectively.

Results of the significant regression models fitted to genotype frequencies of the *HSP90AA1* polymorphisms and some climatic (MAXaT, MThm, ANT, and TW) and geographical (LAT and LON) variables of the regions of origin of the different breeds are shown in Table 7. The Bonferroni correction was applied to take into account the multiple tests carried out on the same data. Thus, when fitting a reciprocal-X regression model, the CC_{-660} genotype frequency for the polymorphism at the 5′ flanking region showed significant and moderate correlations with TW ($p<0.01$, $r=0.62$) and LAT ($p<0.05$, $r=0.54$). Under linear regression models, no significant correlation between the CC_{-660} frequency and any of the environmental parameters were found. Significant ($p<0.05$) and moderate correlations between the CG_{-660} frequency and ANT ($r=-0.53$) and LONG ($r=0.51$) variables were found by fitting a linear regression model. In this case, TW also showed significant ($p<0.01$) association ($r=-0.61$) with the CG_{-660} frequency under a reciprocal-X regression model. The GG_{-660} genotype frequency only showed a linear ($p<0.05$) moderate negative (−0.40) correlation with the MThm variable. Regarding the polymorphism at *HSP90AA1* intron 10, only the $AA_{40}TT_{165}CC_{178}TT_{205}CC_{220}CC_{239}$ genotype showed a highly significant ($p<0.01$) correlation with MThm ($r=-0.52$). Therefore, a clear relation among the different *HSP90AA1* genotypes and some of the climatic and geographic variables studied here has been detected

Table 5 Pearson product moment correlations between genetic and environmental variables

	CC_{-660}	CG_{-660}	GG_{-660}	GG_{40}…	GA_{40}…	AA_{40}…	MAXaT	MThm	ANT	TW	LAT	LON
CC_{-660}		−0.7621***	−0.8248***	−0.2783	0.3339	−0.1340	0.2901	0.2701	0.4629*	−0.5222**	−0.5393**	−0.4108*
CG_{-660}			0.2627	0.1953	−0.1646	−0.1039	−0.3074	0.0015	−0.5363**	0.6032**	0.4432*	0.5183**
GG_{-660}				0.2480	−0.3574	0.2886	−0.1655	−0.4067*	−0.2234	0.2523	0.4197*	0.1560
GG_{40}…					−0.9571***	−0.4840*	−0.1497	0.0742	−0.0730	−0.0316	0.1221	−0.0375
GA_{40}…						0.2148	0.2066	0.0809	0.1278	0.0175	−0.2172	0.0148
AA_{40}…							−0.1464	−0.5170**	−0.1663	0.0707	0.2721	0.1140
MAXaT								0.6790***	0.8556***	−0.3243	−0.8220***	−0.3738
MThm									0.4337*	0.0619	−0.7386***	0.0359
ANT										−0.7616***	−0.8134***	−0.6670***
TW											0.4776*	0.7805***
LAT												0.3593
LON												

CC_{-660}, CG_{-660} and GG_{-660} = genotype frequencies of the SNP at −660 in the ovine *HSP90AA1* 5′flanking region

GG_{40}…, GA_{40}… and AA_{40}… = genotype frequencies of the SNPs at positions 40, 165, 178, 205, 220 and 239 in the ovine *HSP90AA1* intron 10

MAXaT maximum average temperature, *MThm* maximum temperature of the hottest month, *ANT* average annual temperature, *TW* thermal width, *LAT* latitude, *LON* longitude

*$p<0.05$; **$p<0.01$; ***$p<0.001$

only in the case of the SNP at position −660 in the 5′ flanking region.

Figure 3a–e shows the plots for the equations of the linear and reciprocal-X regressions fitted to the genotype frequencies of the SNP at −660 in the *HSP90AA1* 5′ flanking region and the climatic (MThm, TW, and ANT) and geographic (LAT and LON) parameters. Figure 3a shows in a linear way how, as the ANT increases from 0°C to 20°C, the CC_{-660} frequency increases from 0.13 to 0.52 and the CG_{-660} genotype decreases from 0.60 to 0.30. Therefore, for an increment of 1°C in the ANT, the CG_{-660} frequency diminishes by 1.3%, while the CC_{-660} increases by about 2%. For the LON (Fig. 3b), the opposite situation was observed. In this case, as the LON of the regions to which the different breeds belong increases, the CG_{-660} frequency rises from 0.40 to 0.64, while the CC_{-660} decreases from 0.48 to 0.10. For each LON unit increment, the frequency of CG_{-660} increases by 0.20% and the frequency of the CC_{-660} decreases by 0.28%. Changes in the CC_{-660} and CG_{-660} frequencies as a reciprocal-X function of the TW and LAT are shown in Fig. 3c and 3d, respectively. For increments in 1°C in the TW, a 2% increase in the CG_{-660} frequency and a 1.25% decrease in the CC_{-660} were observed. The same trend was observed for the LAT. Thus, each unit increment was accompanied by a 1.6% decrease and a 0.9% increase in the CC_{-660} and CG_{-660} frequencies, respectively. Finally, Fig. 3e shows the linear change of CC_{-660} and GG_{-660} frequencies as a function of the MThm. Although only the regression coefficient was statistically significant for the GG_{-660} genotype, an opposite trend of both homozygotes can be observed. In this case, as the MThm increases, the frequency of the GG_{-660} genotype decreases and that of the CC_{-660} increases. Thus, for an increase of 1°C in the MThm, the frequencies of CC_{-660} and GG_{-660} genotypes increase and decrease by 1.3%, respectively.

Expression analysis in animals with different genotypes at −660 position in the *HSP90AA1* 5′ flanking region in two different seasons

The first ANOVA conducted to assess the existence of differences between the two primers used in the real-time PCR reactions of the *HSP90AA1* gene, under a model containing plate and primer as main effects, showed a highly significant effect for plate ($p=0.0099$) and null differences between primers ($p=0.9033$).

Results from GLM analyses of the samples collected in spring showed no differences ($p=0.4030$) in the expression level of the *HSP90AA1* gene among alternative genotypes of the SNP located at −660 in the 5′ flaking region. Least square means for the *HSP90AA1* expression levels observed in the different genotypes ranged from 0.40 to 0.45

Table 6 Comparison between breeds grouped by UPGMA algorithm with Nei's minimum genetic distances and by deriving discriminant functions to classify the observations for the SNPs at 5′ flanking region (position −660) and intron 10 (positions 40, 165, 178, 205, 220, and 239) in the ovine *HSP90AA1* gene

Breed	Nei's group SNP −660	Highest probable group by discriminat functions	Value	Nei's group SNPs intron 10	Highest probable group by discriminant functions	Value
Latxa	1	1	517.15	3	3	632.08
D´man	1	1	547.75	5	2[a]	640.11
Awassi	1	1	551.93	3	2[a]	676.99
Churra Lebrijana	1	2[a]	591.84	3	1[a]	711.00
Assaf	1	1	551.93	2	2	676.99
Rasa Aragonesa	2	2	601.45	1	1	715.89
Churra	2	2	611.43	1	1	743.23
Boujâad	2	5[a]	558.10	2	2	654.93
Manchega	2	2	631.77	1	1	726.54
Spanish Merino	2	2	659.47	4	1[a]	771.43
Churra Tensina	2	2	625.45	1	1	749.43
Pramenka	3	5[a]	539.30	3	5[a]	652.49
Caucasian	3	3	605.69	1	1	749.41
Olkuska	3	3	537.76	1	1	684.05
Arkhar Merino	3	3	540.56	1	1	686.00
Bozakh	4	4	561.36	2	2	700.91
Bajdarakh	4	4	556.27	4	4	684.71
Karachai	4	4	552.03	3	3	635.66
Edilbai	4	4	636.14	1	1	779.45
Moldavian Karakul	4	4	581.24	1	1	748.04
European Mouflon	4	1[a]	552.83	1	3[a]	674.57
Karabakh	4	4	556.44	4	4	685.61
Russian Karakul	4	4	649.86	1	4[a]	785.66
Sardi	5	1[a]	547.75	3	2[a]	640.11
Finnsheep	5	5	586.58	5	5	726.73

[a] Incorrectly classified

(arbitrary units). The plate effect was significant (p= 0.0374). Conversely, the analyses of the samples collected in summer revealed that the differences in the expression level of the alternative genotypes for the SNP at 5′ flanking region were significant (p=0.0102). Least square means for CC_{-660}, CG_{-660}, and GG_{-660} genotypes were 0.50, 0.41, and 0.39, respectively. In this case, the plate effect was also significant (p=0.0431). Estimated differences between pairs of means of the CC_{-660}–CG_{-660} and CC_{-660}–GG_{-660} genotypes were 0.09 and 0.10, respectively, which were statistically significant (p<0.05). However, the contrast between the means of the CG_{-660} and GG_{-660} genotypes was not significant. Figure 4 shows *HSP90AA1* relative expression levels in animals having the CC_{-660}, CG_{-660}, and GG_{-660} genotypes in spring and summer. Only when the samples were collected in summer there was a significant overexpression (p<0.02) of the *HSP90AA1* gene in the animals with the CC_{-660} genotype when compared to animals carrying the CG_{-660} and GG_{-660} genotypes.

Discussion

Results from the present work show that the ovine *HSP90AA1* gene could have an important role in the adaptation of the ovine species to different climatic and geographic areas. In this regard, the genotype frequencies for the C/G transversion located at position −660 in the *HSP90AA1* 5′ flanking region were differentially distributed across 24 sheep breeds and the wild European Mouflon according to several climatic and geographic variables characteristic of their regions of origin. Thus, groups 1 and 2, which present the highest CC_{-660} frequency, are constituted by breeds from geographical areas with small TW varying from 13°C to 20°C. These areas correspond to regions located in latitudes between 31° and 42° which undergo elevated ANT (10°C to 20°C) and where the temperature during the hottest months (July and August) can exceed 40°C. Conversely, breeds included in groups 3 and 4, which are reared in higher latitudes (40° to 50°) in

Table 7 Statistics of the regression models fitted to the genotype frequencies of the SNPs at the 5′ flanking region (−660) and intron 10 (40, 165, 178, 205, 220, and 239) in the ovine *HSP90AA1* gene and some climatic and geographical parameters from the regions where the breeds studied are reared

Region	Genotype	Parameter[a]	Models									
			Linear: $y=a+bx$					Reciprocal-X: $y=a+b/x$				
			Rs[b]	*b*	*r*	*F*	*p* value	Rs	*b*	*r*	*F*	*p* value
5′ Flanking region	CC_{-660}	TW						36.42	1052.90	0.62	14.75	0.0032**
		LAT						33.87	3336.66	0.54	11.78	0.0187*
	CG_{-660}	ANT	28.76	−1.31	−0.53	9.29	0.0228*					
		TW						37.45	−605.40	−0.61	13.78	0.0044**
		LON	26.75	0.19	0.51	8.40	0.0325*					
	GG_{-660}	MThm	16.54	−1.36	−0.40	4.56	0.0436*					
Intron 10	$AA_{40}TT_{165}CC_{178}TT_{205}CC_{220}CC_{239}$	MThm	26.72	−0.42	−0.52	8.39	0.0081**					

[a] *ANT* average annual temperature, *TW* thermal width (maximum average temperature − minimum average temperature), *LAT* latitude, *LON* longitude, *MThm* maximum temperature of the hottest month

[b] *Rs R*-squared, *b* regression coefficient, *r* correlation coefficient, *F F* ratio

*$p<0.05$; **$p<0.01$

areas where the TW varies from 20°C to 36°C and the ANT between 0°C and 14°C, showed higher proportions of the CG_{-660} genotype. Interestingly, the GG_{-660} genotype is barely represented in groups 1 and 2, while it has a notorious frequency in groups 3 and 4 (see Fig. 1). Group 5 is constituted of two very different breeds, the Finnsheep and the Sardi, which showed a distribution pattern of polymorphism at −660 similar to that observed in group 3. An unexpected situation was found for the Sardi breed for which a similar situation to that observed for the Spanish and African breeds, with a higher frequency of the homozygous CC_{-660}, would have been expected.

The frequencies of the polymorphisms located in the *HSP90AA1* intron 10 did not show any correlation between the distribution of breeds across groups and the climatic and geographic variables analyzed here. This suggests that there is no advantage associated with the alternative genotypes at this position in response to the selective pressure imposed by the environmental conditions. However, the differential distribution of the $A_{40}T_{165}C_{178}T_{205}C_{220}C_{239}$ allele among the breeds studied might constitute a useful tool, in combination with other molecular markers, for diversity and genetic studies.

Variability in the frequencies of the polymorphism at −660 in these breeds seems to be associated with the animal biological fitness under different climatic conditions. To persist in a particular habitat, organisms must be responsive to the changes in their physical environment. Although these responses are often behavioral or metabolic in nature, one powerful mechanism employed by organisms is the adjustment of gene expression in response to environmental changes (Pigliucci 1996). In this context, a well-described molecular response that provides a degree of physiological plasticity is the stress response, which involves the induced synthesis of chaperones, also known as heat shock proteins. One significant development in the pursuit of the ecological importance of Hsps has been the demonstration that the temperature at which these genes are activated, the threshold induction temperature, is not fixed for a given species but varies within the lifetime of a single organism and is subject to thermal acclimatization.

In a parallel study performed on sheep infected with scrapie (unpublished), we observed that the SNP at −660 in the *HSP90AA1* gene was differentially distributed among animals showing different incubation periods (ip) to this fatal disease. Thus, animals bearing the CC_{-660} genotype presented 170 days longer ip than their CG_{-660} counterparts ($p<0.05$). In this particular case, the source of stress was the exogenous misfolded prion accumulation which stimulated the stress response. No significant differences were found when analyzing the polymorphisms at intron 10. There are numerous works describing the association of SNPs at certain genes with different responses to several

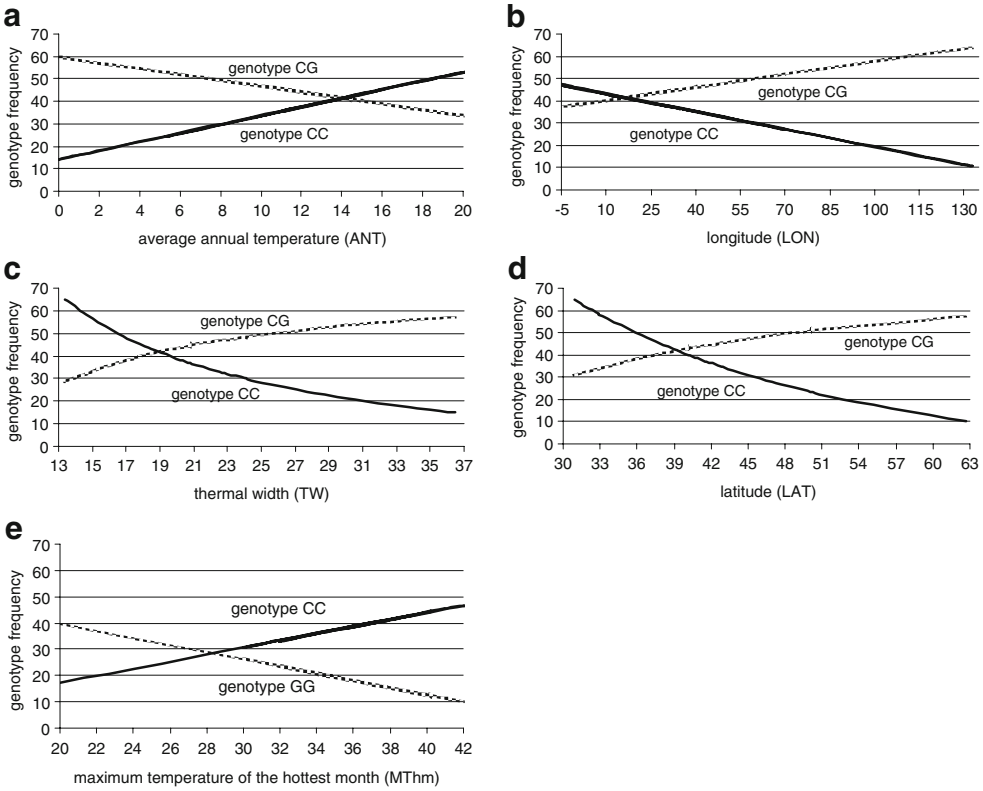
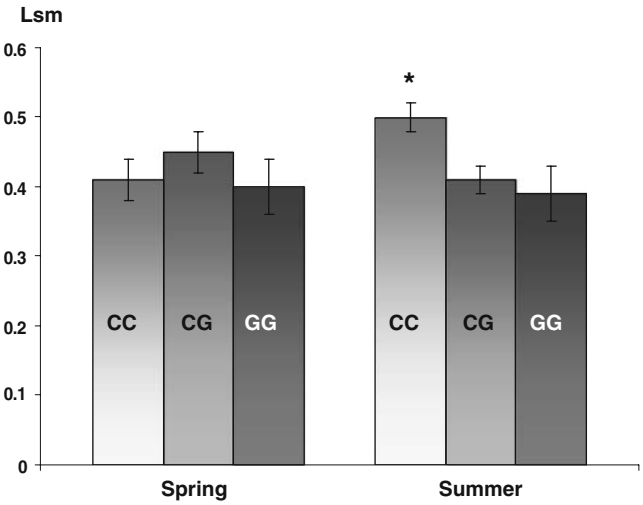


Fig. 3 Plot of the linear (**a**, **b**, **e**) and reciprocal-X (**c**, **d**) single regression models fitted for the genotype frequencies of the SNP located at −660 in the 5′ flanking region of the ovine *HSP90AA1*gene and some of the climatic (ANT, TW, and MThm) and geographic (LON and LAT) parameters studied in the present work

stress conditions. For instance, in Zhikong scallop, a single SNP in the lysozyme gene that showed association with differences in resistance susceptibility to *Listonella anguillarum* has been recently described (Li et al. 2009). Another example is the work of Sun et al. (2007) who found a direct relationship between an insertion–deletion polymorphism in the *CASP8* promoter affecting the expression rate of this gene, which was associated with differential susceptibility to multiple human cancers.

The activation of *HSP* genes is mediated by heat shock transcription factors (HSF) which bind to specific DNA sequences (nGAAn) located in the *HSP* genes promoters, the heat shock elements (HSE). In addition to the HSFs, there are other transcription factors involved in the tight regulation of the HSP gene expression. Concerning the former, the possible relevance of the polymorphism at −660 in the *HSP90AA1* regulation has been previously reported (Marcos-Carcavilla et al. 2008). This and the present studies hypothesize that the mutation at −660 might alter a putative ZBP-89 binding site yielding different rates of *HSP90AA1* transcription due to positive or negative interactions with other transcriptional regulatory elements. ZBP-89 is a Krüppel-like zinc finger protein that is ubiquitously expressed (Merchant et al. 1996). Recent studies have revealed that ZBP-89 possesses multiple functions, including transcriptional regulation (repression or activation) of a variety of genes through G-rich elements (Merchant et al. 1996; Bai et al. 2002; Bai and Merchant 2003), cell growth arrest, and cell death (Bai and Merchant 2001). Therefore, if the SNP at −660 affects *HSP90AA1* transcription, it might have important effects on the



* Significance of test p<0.02. Bars = standard deviation.

Fig. 4 Least square means (arbitrary units) of the expression levels in spring (*light gray*) and summer (*dark gray*) corresponding to animals having alternative genotypes for the SNP located at −660 position in the 5′ flanking region of the ovine *HSP90AA1* gene. **p*<0.02. *Bars* = standard deviation

response to different stress sources, including thermal adaptation.

To test this hypothesis, real-time PCR studies were carried out in 22 Manchega breed sheep bearing the three genotypes for the SNP at −660 position. The first set of samples revealed no differences in the expression of *HSP90AA1* gene. However, the blood was extracted in spring where the MAXaT of the previous days was 27°C. So, taking into account that this gene encodes the inducible form of the cytosolic HSP90 chaperone, a new sample set was collected in the same group of animals in summer, after several days of heat, when the mean temperature of the previous days was 35°C. In this last case, expression analysis revealed that those animals bearing the CC_{-660} genotype presented higher *HSP90AA1* expression (>19%) than animals with both CG_{-660} and GG_{-660} genotypes. Similarly, Podrabsky and Somero (2004) reported the induction of *HSP90AA1* mRNA synthesis by elevated temperatures in *Austrofundulus limnaeus*. On the other hand, we have observed different *HSP90AA1* transcription efficiencies in response to scrapie infection in sheep (unpublished). All these results support the hypothesis that the mutation at −660 in the ovine *HSP90AA1* 5′ flanking region affects its inducible expression under stress conditions.

However, it is necessary to consider that changes in gene expression may arise from a variety of reasons, some of which may not be specifically associated with changes in the activity of gene products. Considering that Dietz and Somero (1992) found that summer-acclimatized fishes had higher levels of Hsp90 protein in brain tissue than winter-acclimatized specimens, these results support the hypothesis that the maintenance of the different distribution for the C/G transversion affecting the −660 position in the ovine *HSP90AA1* gene could be due to an adaptative process to different environmental conditions in order to cope with daily and seasonal temperature changes.

In this sense, our expression results in two different seasons of the year in animals with alternative genotypes for the polymorphism at the 5′ flanking region in the ovine *HSP90AA1* locus confirm this fact. Thus, animals carrying the CC_{-660} genotype might be able to respond to high temperature stimuli (>35°C) through increasing the expression of the *HSP90AA1* inducible heat shock gene. This fact could be interpreted as a response to a source of stress that might be contributing to the denaturation of the cellular protein pool (Parsell and Lindquist 1993).

Groups of breeds here established on the basis of molecular data and environmental variables are very close (80%), revealing a non-random distribution of genetic frequencies of the SNP at −660 position gene across breeds. Thus, from a general point of view, breeds reared in regions with high temperatures in the hot season (Israel, Spain, and Morocco) presented higher significant frequency of the CC_{-660} genotype.

Frequencies of the C and G alleles of the SNP at −660 were 0.53 and 0.49 in the European mouflon. This species was included in a cluster with seven fat-tailed breeds from Eurasian origins in which average heterozygous CG frequency was 0.546. These results agree with those found by Hiendleder et al. (2002) where, on the basis of mitochondrial DNA control region alignments, the European mouflon was also clustered in one domestic sheep group. This work (Hiendleder et al. 2002) revealed that urial and argali species diverged from European mouflon and domestic sheep from 2.83 to 3.21 MyrBP (million years before the present). However, the estimated distance between the European mouflon and actual sheep was 0.38 MyrBP. This fact contributes to the general thought rejecting the European mouflon as an ancestor of domestic sheep breeds and to consider it as the remnant of the first domesticated sheep readapted to feral life.

The transversion C_{-660} G in the ovine *HSP90AA1* gene could have occurred during the evolution process. Domestication of sheep and their spread along regions presenting highly variable environmental conditions, together with genetic drift, would have led to the actual diversity in the genotype frequencies of the SNP at −660 observed here as a product of the adaptation to differential climatic conditions. Thus, breeds reared in regions in which seasonal or daily high temperatures trigger the heat shock response would have taken selective advantage by increasing the frequency of the CC_{-660} genotype. A double dose of the C_{-660} allele could confer a better response to heat stress by, for instance, duplicating the amount of Hsp90α protein to cope with cellular damage or by increasing the number of mRNA molecules contributing to its stabilization and survival rate and therefore to the maintenance of the correct protein levels in the cell.

If this is the case, our results misclassify some breeds. For instance, the Spanish Latxa breed is reared in the North of Spain where climatic conditions would not justify its high proportion of the CC_{-660} genotype. A possible explanation could be that the genetic status of the Latxa breed, regarding the SNP at −660, is linked more to its genetic history than to its environmental adaptation. Recent studies about the genetic relationships among several Spanish sheep breeds revealed that although the Latxa breed shows the phenotypic characteristics of the Churra type group, their ancestral origin is still not well established and is probably different from the rest of the group (Álvarez et al. 2004).

Although results presented here indicate that *HSP90AA1* could be involved in the adaptation to different climatic conditions, additional experiments should be developed. Thus, comparing *HSP90AA1* expression in different breeds

and in different environmental condition would help in clarifying the possible role of the *HSP90AA1* gene in the climatic adaptation and its influence over traits related to animal biological fitness. Additional studies are necessary in order to determine the biological significance of the different expression rates observed among animals with alternative genotypes of the SNP at −660 in the *HSP90AA1* gene. Determining the threshold temperature at which the heat shock response is induced in each genotype and breed will be of great importance due to the new climatic conditions (higher MAXaT and small TW) that would be created by climate change over the world. These new situations could have consequences for both productive and reproductive traits important for the livestock industry within the ovine species.

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