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Biodiversity studies of ruminant Mediterranean species through DNA molecular markers

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SUMMARY – During the last century the ruminant census has suffered an important decrease and breeds in danger of extinction have increased dramatically. The efforts to preserve these breeds have been essential to maintain their biological and ecological potential. The aim of the present work was to carry out biodiversity studies in some Mediterranean cattle, sheep, and goat breeds, through DNA molecular markers such as microsatellites. In this sense, a DNA data bank has been created for each specie. Moreover, several breeds have been genetically characterised and their differentiation and evolutive relationship have been analysed.

Keywords: Ruminant, breed, microsatellite, diversity.

RESUME – *"Etudes sur la biodiversité des espèces de ruminants méditerranéens à l'aide de marqueurs moléculaires d'ADN". Durant le siècle dernier, le cheptel de ruminants a subi une baisse importante et les races menacées d'extinction ont augmenté de façon spectaculaire. Les efforts visant à conserver ces races ont été cruciaux pour sauvegarder leur potentiel biologique et écologique. La finalité de la présente étude a été d'examiner la biodiversité chez certaines races bovines, ovines et caprines méditerranéennes, à l'aide de marqueurs moléculaires d'ADN tels que les microsatellites. Dans ce sens, une banque de données ADN a été créée pour chaque espèce. En outre, plusieurs races ont été caractérisées génétiquement et leur différentiation et relation évolutive a été analysée.*

Mots-clés : *ruminant, race, microsatellite, diversité.*

Introduction

In recent decades there has been a decrease in population and a marked regression of livestock in the tertiary sector in rural areas. The ruminant census has suffered an important decrease and farm owners are generally the oldest members of the population since young people tend to migrate (Avellanet *et al*., 2005). The FAO alerted that about one third of the world's recognised 5000 livestock and poultry breeds were endangered (FAO, 1999; FAO/UNEP, 1995).

The change from an agricultural landscape to bushy areas of land due to the abandonment of pastures is the immediate consequence of the decrease in the ruminant census and, moreover, the loss of their function as efficient transformers of marginal Mediterranean and Para-Mediterranean vegetation in high quality animal proteins (Zervas *et al.,* 1995). These livestock populations represent a unique resource to respond to the present and future needs of breeding improvement both in developed and developing countries.

Molecular markers, like autosomal microsatellite loci, are being commonly used for the successful implementation and monitoring of *ex-situ* conservation programs. In addition, their analysis is applied to the estimation of population diversity, genetic distances, genetic relationships and population genetic admixture measures.

In the present work, we analyse eleven Spanish populations of three different species including: four cattle breeds, two of them (*Mallorquina*, MAL, and *Menorquina*, MEN) bred in the Balearic Islands and the others in the Peninsula (*Pirenaica*, PIR, and *Serrana de Teruel*, ST), four ovine breeds, two of them bred in the Balearic Islands (*Roja Mallorquina*, RMAL, and *Menorquina*, MEN) and two in the Peninsula (Ansotana, ANSO, and *Rasa Aragonesa*, RASA), and a goat breed bred in the Balearic Islands too (*Mallorquina*, MALL). The aim of this work was to characterize these breeds in terms of genetic variability and to analyse their genetic relationships with other Spanish populations.

Materials and methods

A total of 425 animals were sampled, 174 from the cattle breeds MAL (28), MEN (50), ST (44), and PIR (52), 201 from the ovine breeds RMAL (50), MEN (50), ANSO (43) and RASA (58) and 50 from the goat breed (MALL), avoiding relationship between them whenever was possible. In some cases, the reduced population size made selective sampling impossible.

DNA was extracted from blood using standard protocols and the GFX^{TM} Genomic Blood DNA purification kit (Amersham Biosciences). Thirty microsatellite loci were analysed in the different populations of cattle, and fifteen in the ovine and goat ones, all of them were selected for diversity studies in cattle, sheep or goat by the FAO. Microsatellite allele sizes were differentiated and visualized using $\alpha[^{32}P]$-dCTPs in the PCR and 6% denaturing polyacrylamide gel electrophoresis, and also using the ABI PRISM ® 3130 genetic analyser (Applied Biosystems, Foster City, CA, USA). The internal size standard used was LYS-250 and the software Genemapper 3.7 (Applied Biosystems) were used for sizing alleles. Moreover, some control samples from the ISAG and Resgen Project were also genotyped in order to ensure compatibility of our nomenclature with other research groups.

Allelic frequencies for 21 common microsatellites with a previous study (Martín-Burriel *et al*., 1999) have been used for genetic differentiation and genetic relationship analysis in cattle. The four breeds studied were compared with other seven Spanish populations (*Casta Navarra*, CN, *Betizu*, BET, *Monchina*, MON, *Avileña*, AV, *Asturiana de las Montañas*, AM, *Morenas del Noroeste*, MNO, and *Toro de Lidia*, TL).

Allele frequencies for each locus were calculated by direct count using the program GENEPOP (Raymond and Rousset, 1995). This software was also employed to carry out a probability test to determine possible deviations from Hardy-Weinberg proportions. A Markov chain method was applied to calculate exact P-values, the length of the chain was set to be 100,000 iterations.

The mean number of alleles per locus, unbiased (Nei, 1978) and direct count estimated heterozygosities and the percentage of loci polymorphic, when the frequency of the most common allele was lower than 0.95, were calculated in each population using GENETIX (Belkhir *et al*., 2004).

BOTTLENECK program (Cornuet and Luikart, 1996) was run to evaluate the possibility of a recent reduction of population effective size leading to a temporary excess of heterozygotes. A two-phased model of mutation (TPM) consisting of 90% of one-step mutations (SMM) was applied, as recommended for most microsatellite loci (Di Rienzo *et al*., 1994). To determine whether a population exhibits a significant number of loci with gene diversity excess or deficiency a Wilcoxon sign-rank test was applied (Luikart, 1997).

D_A distance (Nei *et al*., 1983) was also estimated for genetic differentiation. This measurement, based on a model of pure genetic drift, has been successfully applied to the genetic differentiation of breeds using microsatellite loci (Kantanen *et al*., 2000; Kim *et al*., 2001). Phylogenetic trees were constructed by Neighbour-joining (NJ) clustering (Saitou and Nei, 1987) from D_A distances using the PHYLIP package. The robustness of dendogram topologies was evaluated by bootstrap resampling (n=1000). The computer program DISPAN (Ota, 1993) was applied for D_A calculations.

Results and discussion

Genetic characterization of cattle breeds

All microsatellite loci were polymorphic (at 95% level) in every population except SPS115 and

HEL13 in MAL. Predominant alleles differed across populations and the number of Taxon specific alleles (TSA) ranged from 3 to 10, in MEN and ST breeds, respectively. Frequencies for rare alleles were lower or equal to 0.05 in most cases.

Mean expected unbiased heterozygosity estimates, computed across all loci for each cattle population, are shown in Table 1. The heterozygosity values observed ranged from 0.52 (MAL) to 0.69 (ST). All populations showed statistically significant deviation from Hardy-Weinberg equilibrium (HWE) at one or more loci.

Table 1. Genetic variability values for six endangered Spanish cattle populations

Breeds	n†	Pol Loci (0.95)††	MNA†††	TSA††††	Heterozygosity		HWE††††† deviations
					Expected (s.e.)	Observed (s.e.)	
MAL	28	0.900	3.227	2	0.4952 (0.2321)	0.5193 (0.2646)	1
MEN	50	1	5.267	3	0.6156 (0.1288)	0.6151 (0.1479)	4
ST	44	1	6.700	7	0.6997 (0.1323)	0.6924 (0.1504)	4
PIR	52	1	6.000	4	0.6170 (0.033)	0.5880 (0.034)	6

†n = number of individuals per population; ††Pol Loci (0.95) = percentage of polymorphic loci at 95% level; †††MNA = mean number of alleles per locus; ††††TSA=Taxon specific alleles found in 4 Spanish cattle populations using 21 microsatellites markers; †††††HWE deviations = number of loci showing deviations from Hardy-Weinberg Equilibrium.

Microsatellite loci showed a high variability in the four Spanish cattle breeds analysed in the present work. The total number of taxon-specific alleles detected in this study breeds when they were compared with other Spanish populations using 21 common markers was much higher than the one reported by Kantanen *et al*. (2000) Nordic cattle. Animals analysed in the present work were sampled from through-breed populations with a very low gene flow from other related breeds, increasing the probability of detecting taxon specific alleles.

Mallorquina population also displayed the lowest diversity values (mean number of alleles per locus and mean expected heterozygosity) and an excess of heterozygotes that could be due to the existence of a recent bottleneck. By contrast, Minorcan breed showed higher diversity values although its values were lower than those observed in other Spanish populations (Martín-Burriel *et al*., 1999). The low level of intrabreed genetic variation observed these populations corresponds with the expected display of island breeds (MacHugh *et al.,* 1997). Serrana de Teruel, althought it is considered as endangered, has shown high diversity values as well as a very low imbreeding coefficient. Traditionally, this breed has been mainly influenced by Negra Avileña and to a smaller degree by other mountain type breeds of northern Spain (http://www.fao.org/dad-is). The admixture of these breeds might have contributed to the high variability observed now.

Genetic relationship with other Spanish cattle populations

D_A genetic distances ranged from 0.0672 to 0.3408 (data not shown). Considering this distance measurement, the closest breeds were found to be ST and MON, being MAL and MNO the most divergent populations. Distance estimates showed that MAL was genetically the most divergent breed.

High genetic distances were related to the risked status (reduced effective population size) of breeds and reproductive isolation rather than with geographical distances. The phylogenetic tree obtained (Fig. 1) is in agreement with the expected relationship between breeds. On the other hand, although Balearic populations (MAL and MEN) are also grouped, distances between these two breeds were similar to those observed with other Spanish populations. Its risked status and geographical isolation could explain the great divergence.

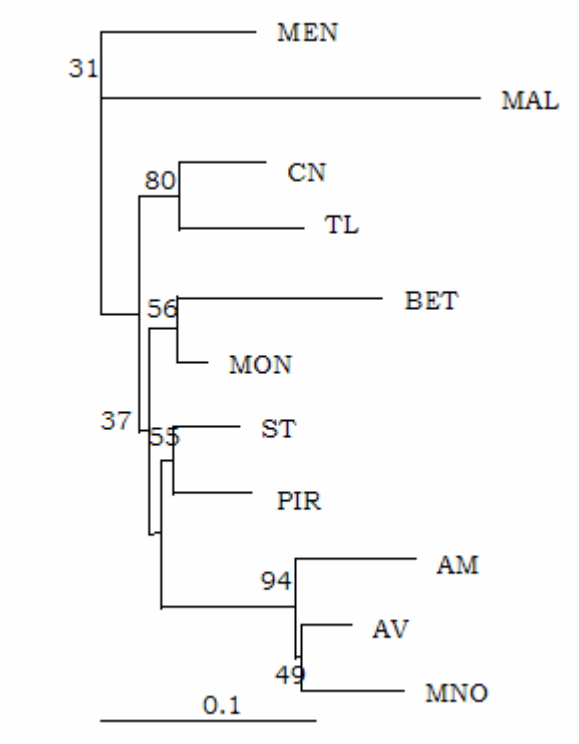

Fig. 1. Unrooted NJ network derived from the D_A genetic distance among 11 Spanish cattle breeds.

Genetic characterization of small ruminants populations

A total of 453 alleles were observed in the four Spanish ovine populations studied, the number of alleles observed at a single locus ranged from 4 to 15 (Menorquina), while a total of 87 alleles were observed in the goat population, and the number of alleles observed at a single locus ranged from 2 to 9. The ovine populations were compared using the allelic frequencies from 15 microsatellites and some Taxon specific alleles were found (Table 2). Normally, allelic frequencies were low, but some alleles showed higher frequencies, so these ones together with the Taxon specific alleles resulted to be ideal for new assignation of individuals to these breeds.

Table 2. Genetic variation in the ovine breeds Roja Mallorquina, Menorquina (20 loci microsatellite), Ansotana and Rasa Aragonesa (27 loci microsatellite), and in the Mallorquina goat breed (15 loci microsatellite)

	Sheep				Goat
	Roja Mallorquina	Menorquina	Ansotana	Rasa Aragonesa	Mallorquina
n†	50	50	43	58	50
Pol Loci (0.95)††	1	1	1	1	1
MNA†††	7.55	7.90	7.48	8.85	5.80
TSA††††	5	2	2	9	-
Mean heterozygosity	0.707	0.680	0.706	0.690	0.507
HWE deviations†††††	1	3	7	2	4

†n = number of individuals per population; ††Pol Loci (0.95) = percentage of polymorphic loci at 95% level; †††MNA = mean number of alleles per locus; ††††TSA = Taxon specific alleles found in 4 Spanish sheep populations using 15 microsatellite markers; †††††HWE deviations = number of loci showing deviations from Hardy-Weinberg Equilibrium.

Observed and expected heterozygosity values were similar in every sheep population. These values were high, around 0.7 (Table 2), displaying also a large mean number of alleles per locus. Neither an excess nor a deficiency of observed heterozygotes with respect to the expected values, was observed.

Most of the microsatellite markers were in Hardy-Weinberg equilibrium in the sheep populations, except for Ansotana breed where the 26% of loci showed deviations from equilibrium. On the other hand, 100% of loci were polymorphic, at 95% and 100% level in both. These results indicate that the sheep populations analysed show a high genetic variability. These breeds will not be in risk if their effective size is maintained.

Nei D_A distances between sheep populations (data not shown) were calculated and used to create a phylogenetic tree grouped (Fig. 2). The obtained dendrogram grouped the two island breeds and the two peninsular populations in different clusters.

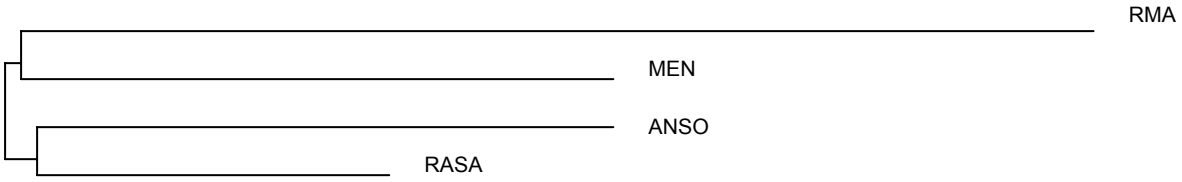

Fig. 2. Phylogenetic tree showing the genetic relations between Roja Mallorquina, Menorquina, Ansotana and Rasa Aragonesa ovine breeds, using the D_A genetic distance and the Neighbour-Joining algorithm.

The goat population studied (Mallorquina) showed diversity values lower than the ones observed for sheep populations, although all microsatellite markers were polymorphic.

Finally, Mallorquina goat population displayed an expected heterozygosity (H_E = 0.597) higher than the observed value (H_O = 0.507). Moreover, 26.67% of loci showed deviations from Hardy-Weinberg equilibrium. The loss of variability would be a consequence of a high imbreeding, as this breed is being considered in risk and it is geographical and reproductively isolated.

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