Raza asno de los Encartaciones

5. DISCUSIÓN GENERAL

En este apartado pretendemos realizar una discusión global de los capítulos de resultados y discusiones plasmados anteriormente, todo ello enmarcado en la caracterización molecular de las razas asnales españolas.

El objetivo último de esta tesis es poder brindarle a los interesados (criadores, investigadores y estudiantes) en las razas asnales, cierta información que, en conjunción con los demás trabajos de caracterización morfológica, hematológica, bioquímica y citogenética, permita establecer un Plan de Conservación *in situ* de estas poblaciones que actualmente se encuentran en peligro de extinción.

La excelente adecuación y amplificación de los marcadores de caballos en los asnos (pertenecientes ambos al mismo género *Equus*) nos permitió hacer esta primera aproximación del estudio a nivel del ADN en estas razas. Claro está, después de realizar algunas ligeras modificaciones, en especial en las condiciones de PCR, ajustando tanto cantidades de los productos utilizados (Taq, Oligos, $MgCl_2$, etc) como temperaturas de *anneling* y combinaciones de los marcadores (para evitar solapamientos, que en principio no se presentan en los análisis de muestras originarias de caballos), logrando la completa amplificación en 14 de los 15 microsatélites de caballos utilizados en muestras de asnos.

En trabajos previos, Breen *y col.* (1994) ya habían demostrado que ciertos microsatélites de caballos lograban amplificar en individuos del mismo género *Equus,* y que había la posibilidad del uso de estos para estudios poblacionales en especies relacionas y en especial en las razas minoritarias donde la investigación es bastante limitada. Más tarde, en los primeros trabajos de esta Unidad (Unidad de Genética y Mejora Animal de la UAB), se lograban amplificar estos y otros microsatélites equinos en muestras de asnos (Jordana *y col*., 1999).

Es así que, los microsatélites utilizados, salvo la excepción del marcador ASB2 que no logró amplificar y el HMS1 que fue monomórfico (165 pb.), fueron exitosamente polimórficos y con valores de PIC aceptables, lo que los hace altamente informativos para este tipo de estudios.

El principal objetivo de un programa de conservación *"in situ"* es el mantenimiento de animales vivos que retengan la máxima cantidad de variabilidad genética posible (bajo la hipótesis de existencia de correlación con la viabilidad de la población) con el mínimo incremento de consanguinidad por generación. Esto repercute principalmente en nuestro caso en una depresión consanguínea, una reducción de los caracteres reproductivos y la inevitable disminución del número efectivo de la población, llegando inclusive a la extinción de no tomarse medidas. Así que discutiremos cómo la aportación de esta investigación, contribuye a dar respuesta a esta problemática.

En este orden de ideas, podemos indicar que con respecto a la diversidad de las razas asnales españolas, las cinco razas estudiadas (AND, CAT, ENC, MALL y ZAM) mostraron unos niveles similares y aceptables de variabilidad genética, con valores promedios de H_E y H_O de 0.654 y 0.546 respectivamente, y un número promedio de alelos por *locus* de 7.2, sin presentarse diferencias estadísticamente significativas entre ellas.

Así mismo, podemos indicar que tanto para cada una de las razas individuales como a nivel global, la probabilidad de exclusión (PE) fue del 99,99%, lo cuál nos permite contar con una herramienta eficaz a la hora de realizar pruebas de paternidades o descartar una asignación errónea de paternidad. Esto puede ser de gran interés si tenemos en cuenta que en la mayoría de las razas no se dispone de información genealógica. La identificación de alelos y sus respectivas frecuencias,

revelaron pocas o nulas diferencias entre las razas. A pesar de que se presentaron algunos alelos privados, su baja frecuencia no permite utilizarlos como alelos marcadores de raza.

En cuanto al estudio de la estructura genética de las razas, se realizó en primer lugar un análisis global de las mismas y en segundo lugar, y después de detectar un significativo déficit de heterocigotos en la población, se realizó un exhaustivo análisis jerárquico, subdividiendo a cada una de las razas en subpoblaciones (básicamente por su proximidad geográfica) y que nos permitiera de ese modo explicar ese déficit.

A nivel global se presentó una aparente diferenciación racial, del 4.1% ($P< 0.001$), contribuyendo todos los *loci* a ella, por lo que el remanente 95.9% correspondería a diferencias entre los individuos. Como promedio las razas presentaron un déficit de heterocigotos del 17.8%; mientras que en la población total ese déficit fue del 21.1 % ($P < 0.001$).

Este bajo aunque significativo valor de diferenciación genética (4.1%), también nos da indicios de un elevado flujo de genes entre estas razas; siendo mayor entre las razas CAT, MALL y ENC por un lado, y por otro, entre AND y ZAM y mucho menores entre AND y CAT-MALL al igual que entre las razas CAT y ZAM.

El análisis jerárquico mostró, tal y como era de esperar, que las mayores diferencias se presentaran entre razas con respecto al total, más que entre las subpoblaciones dentro de razas y dentro de las subpoblaciones 6.4% vs. 3.5% y 3.0%, respectivamente.

El déficit de heterocigotos en todas las razas osciló entre el 13.0% y el 23.2% para las razas del asno de las Encartaciones y la raza

Andaluza, respectivamente. La principales causas de este déficit podrían variar por raza; atribuyéndose principalmente en las razas AND, CAT y ZAM a la consanguinidad, dado que más del 75% de los *loci* se encontraron en déficit; aunque en estas razas también se presentó un significativo valor de diferenciación genética (F_{ST}) dentro de razas, lo que indica además una cierta subestructuración reproductiva. Mientras que, en las razas ENC y MALL, el déficit podría ser explicable, primordialmente, por la subestructuración reproductiva (efecto Wahlund), más que por consanguinidad, ya que tan solo 6 y 5 de los marcadores, respectivamente, mostraron déficit significativo.

Otro efecto que no podemos olvidar como posible causante de déficit de heterocigotos es el derivado de los alelos nulos, los cuáles, a pesar de no haber podido ser verificados (dada la carencia de información genealógica en la mayoría de las razas), no deberían ser obviados, ya que nos podrían ayudar a explicar parte del déficit presente en todas las razas, en especial la de los marcadores HTG4 y HMS7, estando este último ya reportado en caballos de la raza "Quarter horse" (Bozzini *y col.*, 1997).

La divergencia genética existente entre las razas queda reflejada gráficamente en los árboles (dendrogramas) generados en el estudio de las relaciones filogenéticas. Dependiendo de la distancia utilizada, las mayores divergencias se presentaron entre las razas AND y CAT para todas las distancias evaluadas; mientras que las menores fueron entre AND y ZAM, cuando las distancias fueron la D_A y D_S, y las razas MALL-ENC en la evaluación con la distancia de Reynolds.

Sin embargo, los árboles generados y evaluados por permutaciones bootstrap nos produjeron dos claros patrones de organización: en primer lugar una estrecha relación entre las razas CAT y MALL las cuáles siempre formaron una agrupación más o menos

sólida, y por otro lado, la separación o cierto distanciamiento de la raza AND con el resto de las razas españolas, llegando al caso inclusive, que utilizando la distancia de Reynolds, esta raza clusterizó con el asno de Marruecos, dando indicios de un posible origen común.

Las divergencias genéticas entre las razas, desde un punto de vista general, parecería que por si sola no aportan mucho a un programa de conservación; sin embargo, si tomamos en cuenta que estas poblaciones son de un número efectivo reducido, dichas relaciones podrían servirnos, en casos extremos, a la hora de decidir sobre la utilización de otra raza cercana o próxima a la que está en peligro a la hora de buscar incrementar su *Ne* y así evitar su extinción.

Las representaciones gráficas, tanto de la metodología PCA como D_{AS}, nos dio una distribución más o menos similar a la obtenida previamente con las distancias genéticas, agrupando por un lado de forma más próxima a las razas CAT y MALL y más retirada o distante la raza AND. Con respecto al análisis de Weitzman, el patrón de variación genética o aporte de cada una de las razas al pool genético osciló entre el 19.9% del asno de las Encartaciones y el 29.11% para la raza Catalana; por lo cuál resulta obvio que la pérdida de la raza CAT causaría una mayor pérdida que la extinción de la raza ENC o inclusive la MALL. Este último análisis presenta otra utilidad práctica para la conservación de razas, ya que en situaciones cuando los recursos económicos son reducidos o limitados, nos permite establecer prioridades en base al aporte genético de ciertas razas a la diversidad genética en una población. Pudiendo por lo tanto, elegir para conservar, aquélla (s) raza (s) que nos aporten un mayor nivel de variabilidad genética a la población.

Dado que otro de los puntos a discutir es el mínimo incremento de consanguinidad por generación, obviamente lo ideal sería contar con

los registros genealógicos de las razas y a partir de allí programar aquellos apareamientos que resultasen con una descendencia menos consanguínea; sin embargo, conocida la falta de esta información, podemos, a partir de la información generada por los microsatélites estimar estas relaciones y así poder, mediante las combinaciones haplotípicas de los diferentes marcadores y la proporción de alelos compartidos, programar los apareamientos de mínima consanguinidad.

En este punto podemos citar el trabajo recientemente publicado por Eding y Meuwiseen (2001). En él se realiza el cálculo de un índice de parentesco a partir de la información generada por los microsatélites, denominado "Índice de Similitud", bastante parecido al índice de parentesco calculado a partir de los registros genealógicos (elevada correlación entre ambos). En dicho trabajo se concluye que en casos de poblaciones con falta de pedigrí (como las aquí plasmadas), la información molecular puede sustituir las estimaciones genealógicas de parentesco y así poder tomar decisiones acertadas para los planes de conservación.

Es así que, la información generada en este trabajo, puede ser utilizada para el cálculo del índice de parentesco (índice de similitud) y así poder programar aquel apareamiento más óptimo entre un macho y una hembra que maximice el llamado Índice de Conservación Genética y minimice la Consanguinidad (F) de un hipotético hijo de la pareja, con lo cuál podemos mantener al máximo la variabilidad genética de las poblaciones.

Por otra parte, los resultados obtenidos a partir de los estudios con el mtDNA, además de proveer las primeras evidencias de estudios moleculares a este nivel, nos permitió observar que las razas asnales presentan un bajo nivel de variación genética mitocondrial. Con respecto a las relaciones filogenéticas los resultados parecen indicar

que el origen evolutivo del asno español podría derivarse de un único y común tronco ancestral con las razas africanas estudiadas y que desde entonces han ido evolucionando de acuerdo a las preferencias de los criadores y al ambiente en el cual se han desarrollando; o bien que, procediendo de dos troncos ancestrales, las actuales razas españolas serían producto de una mezcla de diferentes líneas maternas como consecuencia de la gran afluencia africana que ha tenido España, directa o indirectamente, ya sea a través de los movimientos poblacionales e intercambios que han producido un verdadero trasiego de asnos africanos que se han cruzados con asnos españoles y viceversa (Romagosa, 1959).

En segundo lugar, efectos del tipo “cuellos de botella” o “efectos fundadores” explicarían la inesperada separación de la raza ENC del resto de las razas estudiadas, y en tercer lugar, cabe destacar la gran similitud presentada entre las razas AND y MAJ, ya que estas comparten la mayoría de los haplotipos encontrados (tanto del citocromo b como del D-loop) lo que nos sugiere un posible origen ancestral común o bien que ha existido un gran intercambio de reproductores entre ellas.

Y para finalizar podemos corroborar que el tiempo de divergencia del asno y el caballo a partir del fragmento del D-loop, correspondió a cerca de 9 millones de años, coincidiendo con lo referido por Xu *y col.* (1996); aunque más temprano que lo citado por otros autores (Lindsay *y col.*, 1980; George y Ryder, 1986; Kim *y col.*, 1999) de tan solo 1.5 a 3.5 millones. La diferencia entre las aproximaciones de la divergencia, están relacionadas en primer lugar con la región o fragmento utilizado para el cálculo, y en segundo lugar, por las tasas evolutivas de estos.

Raza Majorera

6. CONCLUSIONES

De acuerdo a los resultados obtenidos las principales conclusiones de este trabajo son las siguientes:

1. La utilización de marcadores de caballos resultó exitosa y de gran utilidad para los estudios en poblaciones de asnos. Salvo la excepción del microsatélite ASB2 que no amplificó y el HMS1 que fue monomórfico, los demás fueron altamente polimórficos.

2. Las razas asnales españolas no mostraron diferencias estadísticamente significativas entre ellas en cuanto a sus niveles de variabilidad genética.

3. La probabilidad de exclusión (PE) obtenida con este conjunto de marcadores de 99.99%, resulta de gran utilidad para futuras pruebas de paternidades. El polimorfismo encontrado nos sirve además para la identificación individual de los animales, al igual que la asignación de individuos a raza, la cuál es pieza clave para la gestión del programa de conservación.

4. La diferenciación genética entre las razas asnales fue del 4.1%, siendo como promedio el déficit de heterocigotos por raza del 17.8%. El mayor déficit correspondió a las razas AND, CAT y ZAM, en las cuáles la consanguinidad y la subestructuración reproductiva juegan un papel muy importante. Este último fenómeno parece ser la principal causa de déficit en las razas ENC y MALL.

5. Según el análisis de Weitzman, las razas que aportan mayor diversidad a la población son la CAT con el 29.11% y la AND con el 27.53%, siendo la que menos aporta el asno de las Encartaciones con tan solo un 19.19%. La agrupación AND, CAT y ENC aporta un 78.20% al total de la variabilidad.

6. Las relaciones genéticas mostraron que las razas más próximas fueron siempre la CAT y MALL, estando la AND siempre más alejada de la razas del norte de España y más próxima a la del asno de Marruecos.

7. El análisis del mtDNA permitió detectar tan solo 6 haplotipos a partir del citocromo b y 7 a partir de la región D-loop, mostrando por tanto un bajo nivel de variabilidad en las razas asnales analizadas.

8. Los resultados del mtDNA permiten indicar que el estado actual de las razas asnales españolas parece corresponder al producto de una mezcla de líneas maternas debido posiblemente a un elevado flujo de genes entre ellas, o bien qué, el origen evolutivo del asno español podría derivarse de un único y común tronco ancestral con las razas africanas.

9. El tiempo de divergencia entre el asno y el caballo, correspondió a 9 MYA, similar a otros reportes.

Raza Andaluza

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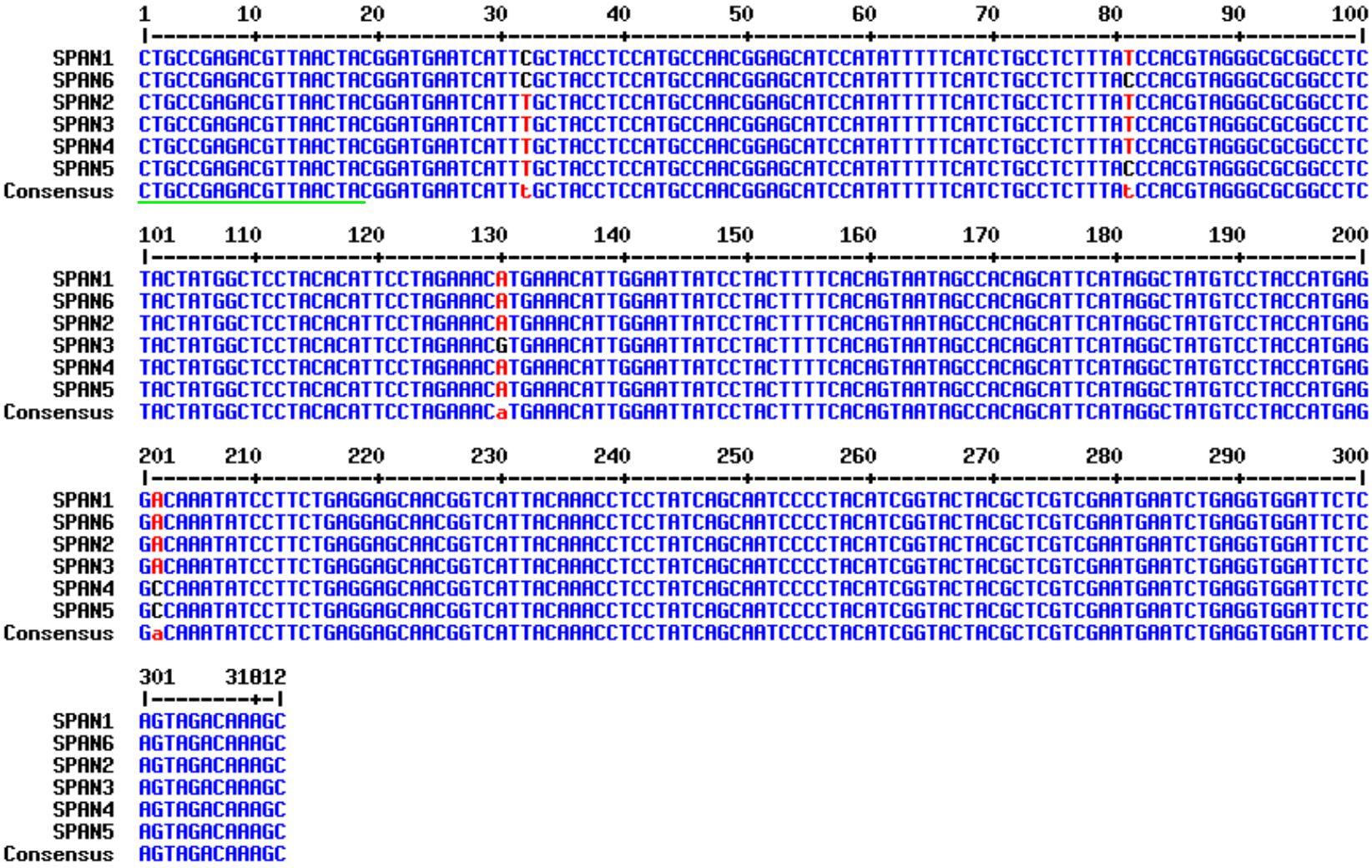
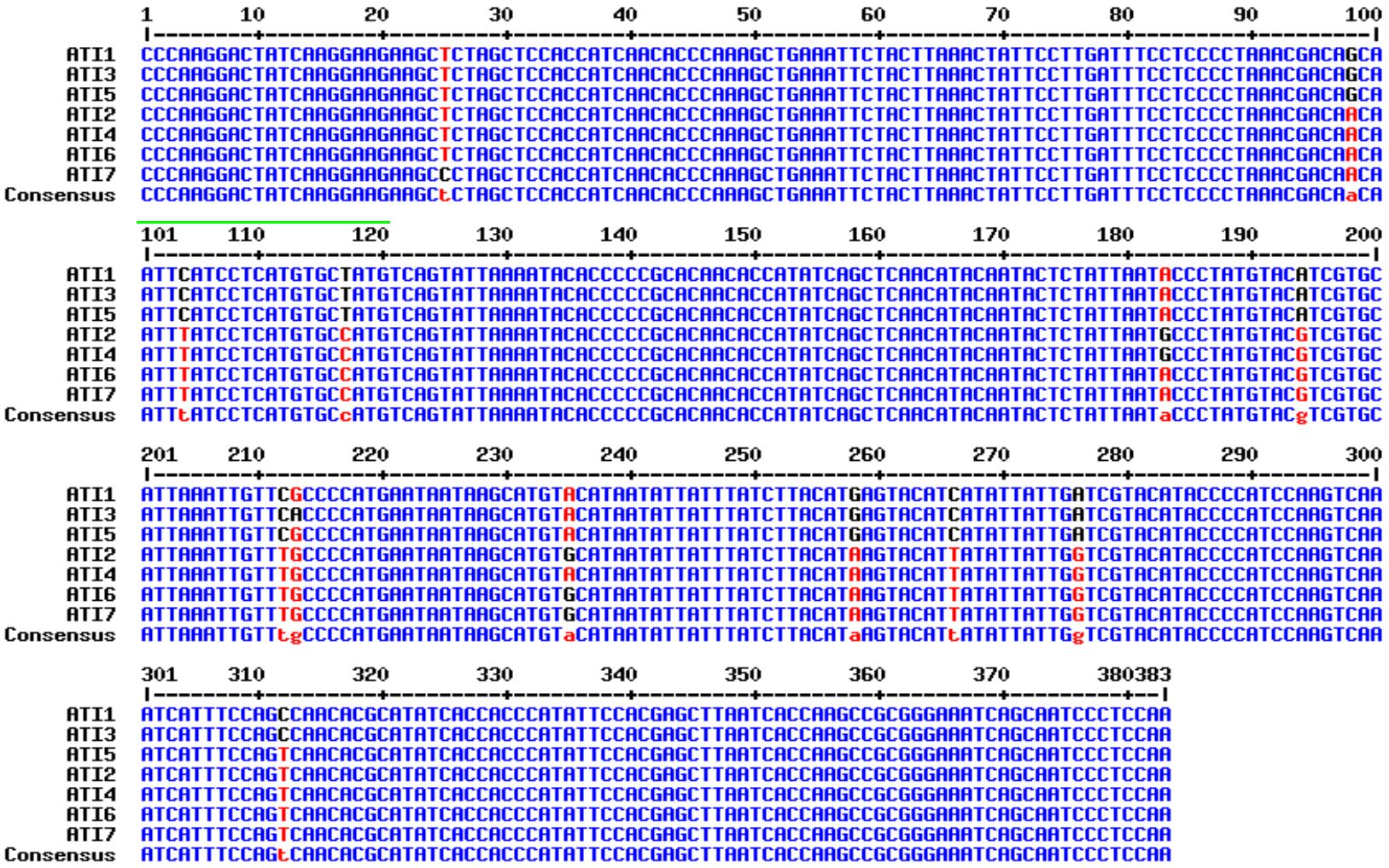
Raza Catalana

8. ANEXOS



Anexo 1. Secuencias haplotípicas del citocromo b en las razas asnales españolas. La región marcada corresponde al *primer* CITB-F.



Anexo 2. Secuencias haplotípicas del D-loop en las razas asnales españolas. La región marcada corresponde al *primer* DONK-A.

Sub Poblaciones	A1	A2	A3	C1	C2	C3	M1	M2	M3	M4	E1	E2	E3	Z1	Z2
AND2	**.072**														
AND3	**.051**	**.049**													
CAT1	.084	.119	.042												
CAT2	.078	.099	.041	**.023**											
CAT3	.118	.188	.084	**.035**	**.057**										
MALL1	.097	.121	.043	.037	.060	.075									
MALL2	.075	.110	.057	.040	.073	.085	**.007**								
MALL3	.081	.124	.057	.040	.073	.085	**.045**	**.037**							
MALL4	.055	.076	.028	.029	.050	.088	**.033**	**.013**	**.014**						
ENC1	.065	.114	.049	.053	.071	.084	.062	.052	.033	.041					
ENC2	.040	.082	.041	.039	.047	.068	.058	.046	.035	.018	**.014**				
ENC3	.053	.078	.029	.036	.036	.071	.043	.045	.036	.022	**.012**	**.012**			
ZAM1	.047	.070	.034	.056	.077	.091	.054	.044	.041	.016	.054	.031	.037		
ZAM2	.046	.070	.034	.059	.060	.106	.065	.064	.071	.037	.070	.040	.026	**.015**	
ZAM3	.076	.118	.043	.053	.074	.092	.059	.072	.090	.006	.084	.067	.058	**.047**	**.052**

Anexo 3. Distancias F_{ST} entre las subpoblaciones de los asnos españoles (en negritas distancias dentro de razas).

ANEXO 4

Genetic diversity in Spanish donkey breeds using microsatellite DNA markers.

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Genetic diversity in Spanish donkey breeds using microsatellite DNA markers

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Abstract – Genetic diversity at 13 equine microsatellite loci was compared in five endangered Spanish donkey breeds: Andaluza, Catalana, Mallorquina, Encartaciones and Zamorano-Leonesa. All of the equine microsatellites used in this study were amplified and were polymorphic in the domestic donkey breeds with the exception of HMS1, which was monomorphic, and ASB2, which failed to amplify. Allele number, frequency distributions and mean heterozygosities were very similar among the Spanish donkey breeds. The unbiased expected heterozygosity (H_E) over all the populations varied between 0.637 and 0.684 in this study. The low G_{ST} value showed that only 3.6% of the diversity was between breeds ($P < 0.01$). Significant deviations from Hardy-Weinberg equilibrium were shown for a number of locus-population combinations, except HMS5 that showed agreement in all analysed populations. The cumulative exclusion probability (PE) was 0.999 in each breed, suggesting that the loci would be suitable for donkey parentage testing. The constructed dendrogram from the D_A distance matrix showed little differentiation between Spanish breeds, but great differentiation between them and the Moroccan ass and also with the horse, used as an outgroup. These results confirm the potential use of equine microsatellite loci as a tool for genetic studies in domestic donkey populations, which could also be useful for conservation plans.

donkey / endangered breed / microsatellite / diversity / genetic variability

1. INTRODUCTION

The local Spanish donkey breeds (*Equus asinus*) Andaluza, Catalana, Encartaciones, Mallorquina and Zamorano-Leonesa have suffered a substantial decrease in population size which might cause high levels of inbreeding

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resulting in inbreeding depression and increasing the risk of breed extinction. The principal cause of the great reduction in population size of up to 80% has been the intense mechanisation of agriculture which took place in Spain during the 60s and the 70s. These Spanish donkey breeds have been officially recognised as breeds for a long time. Currently, the number of animals recorded among these five breeds is very low, and they are included in the FAO (Food and Agriculture Organisation of the United Nations) list of domestic animal breeds to be conserved (FAO, DAD-IS http://fao.org/dad-is). At present, the Spanish donkey breeds comprise approximately 100 to 200 females each (Breed Associations personal communications). These figures fit into the category of an endangered breed as proposed by the FAO Expert Consultation [2]. Without immediate action, the effective population size of these five Spanish breeds will be inadequate to prevent constant genetic loss at each generation [8].

The origin of the modern Spanish donkey breeds remains uncertain. According to several authors [1,12,13,15] current Spanish donkeys seem to derived from two ancestral sources: the Nubian ass (*Equus asinus africanus*), which gave rise to the Andaluza breed [3,16,40], and secondly, the Somalian ass (*Equus asinus somaliensis*) which gave rise to the donkeys of Southwest Asia and probably also to the majority of European breeds, among which the Catalana, Mallorquina, Encartaciones and Zamorano-Leonesa breeds [15].

Notwithstanding this, Dechambre and Sanson, as cited by several authors [3, 24,37,40], support the theory of two different ancestral sources: one which would correspond to the *Equus asinus africanus*, originating from Northeast Africa, and the other one, the *Equus asinus europeus*, whose area of origin is the Mediterranean Basin, in particular the Balearic Isles, which would have given rise to the majority of European donkey breeds, including the four Spanish breeds mentioned in the previous paragraph.

The conservation of genetic variation found in these minor livestock breeds is a growing world-wide concern due to the increasing risk of breed loss. Recently, many studies of breed conservation have used allele frequencies for several DNA markers, such as microsatellites [19,26,36].

Very little literature reporting microsatellite data in domestic donkeys exists; only Breen *et al.* [10], using a set of 13 microsatellite loci isolated from the domestic horse, verified that they were well-amplified in eight individuals. In addition, Bellone and co-workers [7] reported studies in one French donkey breed (Baudet du Poitou) with nine microsatellite loci. Finally, we also performed studies with the Catalonian donkey breed [22,23]. In the present work, 15 equine microsatellite loci were analysed in 5 Spanish donkey breeds, in order to study the genetic variability both within and between these breeds.

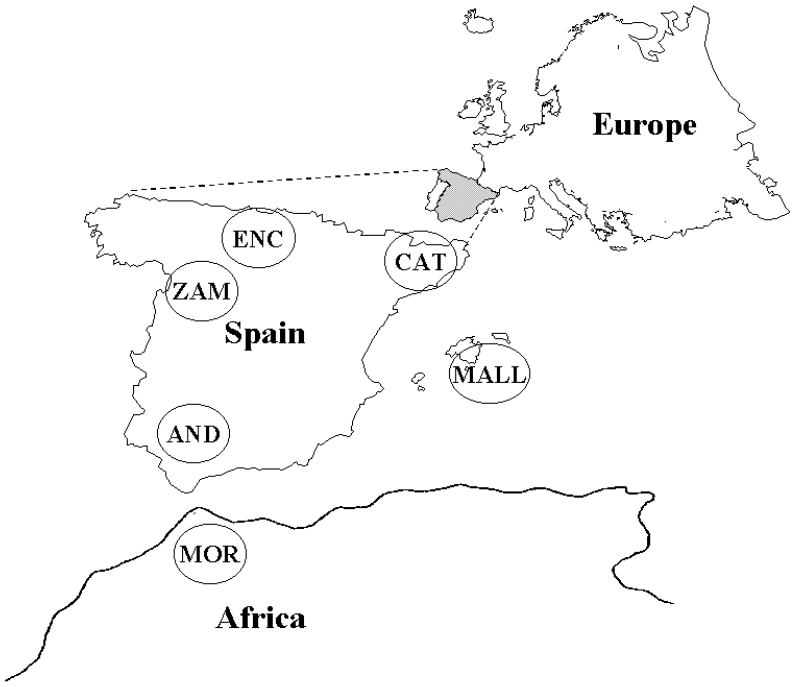

Figure 1. Geographical location of the Spanish donkey breeds.

2. MATERIALS AND METHODS

2.1. Population samples

The number of individuals sampled, of both sexes, was 87 Andaluza (AND), 140 Catalana (CAT), 104 Mallorquina (MALL), 74 Encartaciones (ENC) and 108 Zamorano-Leonesa (ZAM) representing 75 and 95% of the whole population in each case. The area of main distribution of these indigenous Spanish breeds is shown in Figure 1. In addition, 9 Moroccan asses (MOR) were used as genuine members of *E. asinus africanus*, and 24 horses of the Merens breed (*E. caballus*) were used as an outgroup. Donkey DNA was prepared from whole blood according to standard methods involving lysates of the washed white-cells and phenol-chloroform-isoamylalcohol (25:24:1) extraction [4].

2.2. Microsatellite markers

The 15 microsatellite loci studied were AHT4, AHT5 [6], ASB2 [11], HMS1, HMS2, HMS3, HMS5, HMS6, HMS7 [17], HTG4, HTG6 [14], HTG7, HTG10 [25], HTG15 [5] and VHL20 [41].

2.3. Multiplex PCR conditions

The 15 microsatellites were amplified in three multiplexes using fluorescently-labelled primers. The first multiplex included microsatellites ASB2, HMS3, HMS6, HTG6, HTG10, and VHL20. The second was composed of AHT4, AHT5, HMS2, HMS7 and HTG7, while the third contained HMS1, HMS5, HTG15 and HTG4. Multiplex PCRs were carried out in 15 μL reactions containing 30 ng of genomic DNA, 200 μM of dNTP, 0.5 μL of AmpliTaq Gold (5 U $\cdot$ μL^{-1}), 1.5 mM of $MgCl_2$ and 0.5 μL of each primer (AHT4, ASB2, HMS2, HMS3, HTG6, HTG7, HTG10), 0.4 μL of the primer (AHT5, HMS6 and HMS7) while 0.3 μL of primer VHL20 (StockMarks® for Horses, Equine Paternity PCR Typing Kit, PE Applied Biosystems, Foster City, CA), and finally, 0.20 μM of primers HMS1, HMS5, HTG4 and HTG15. PCR was carried out in a 9 700 GeneAmp PCR system (Perkin Elmer) by an initial denaturation at 95 °C for 10 min, followed by 30 cycles at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 60 s. The thermal profile ended with a final extension at 72 °C for 60 min. PCR products were detected by capillary electrophoresis using an Applied Biosystems 310 DNA Sequencer with GENESCAN Analysis software (ABI), using the ROX 350 bp internal-size standard.

2.4. Statistical analyses

Allele frequencies (available from the authors on request) and mean heterozygosity values for each polymorphic locus were obtained using the BIOSYS-2 computer programme [38]. The test of genotype frequencies for deviation from the Hardy-Weinberg Equilibrium (HWE) was calculated using the exact test of the GENEPOP 3.1d computer programme [32], using the Markov-chain method [18]. Polymorphic information content (PIC) was calculated for each microsatellite locus according to Botstein *et al.* [9], and the probability of exclusion (PE) was determined for all informative markers [20].

The average expected heterozygosity for each population (H_S), the gene diversity in the total population (H_T), and the coefficient of gene differentiation G_{ST} [27] were estimated using the computer programme DISPAN [30], and tested by permutation test. Differences in average heterozygosities among breeds were assessed using the ANOVA test of the SAS® package [35].

Genetic distances and phylogenetic trees among populations were obtained with the distance measure D_A [29]. Takezaki and Nei [39] suggested the D_A distance for making phylogenetic trees when the interest of the study mainly focused on the topology rather than evolutionary time. Distance data was analysed with the neighbour-joining (NJ) method of clustering [34]. The NJ method produces only unrooted trees. For this reason we included the data for the Merens breed population as an outgroup to root the tree. The robustness of the dendrogram was evaluated by bootstrap resampling of loci (1 000 replicates). All these calculations were carried out using the DISPAN package [30].

Table I. Total number and range of observed alleles, average heterozygosity H_S and H_T, coefficient of differentiation G_{ST}, PIC and PE, in Spanish donkey breeds.

Microsatellite	No.A.[1]	S. Range[2]	H_T	H_S	G_{ST}	PIC[3]	PE[4]
AHT4	15	126–160	0.773	0.753	0.031***	0.71	0.55
AHT5	14	126–156	0.907	0.852	0.037***	0.85	0.74
ASB2	–	–	–	–	–	–	–
$HMS1_A$	1	165	–	–	–	–	–
HMS2	10	229–247	0.709	0.714	0.016***	0.65	0.47
HMS3	7	152–170	0.618	0.603	0.044***	0.51	0.32
HMS5	3	105–109	0.278	0.336	0.109***	0.20	0.10
HMS6	6	151–167	0.649	0.613	0.041***	0.54	0.33
HMS7	7	165–177	0.626	0.601	0.031***	0.53	0.33
HTG4	5	167–175	0.495	0.439	0.048***	0.40	0.21
HTG6	11	76–102	0.817	0.714	0.053***	0.73	0.55
HTG7	13	134–164	0.843	0.800	0.030***	0.80	0.65
HTG10	12	85–107	0.837	0.790	0.035***	0.78	0.63
HTG15	7	116–136	0.751	0.746	0.014***	0.70	0.51
VHL20	4	75–99	0.579	0.597	0.035***	0.50	0.31
All loci			0.683 (±0.170)	0.658 (±0.147)	0.036*** (±0.023)		0.999

*** $P < 0.001$.

[1] Total number of observed alleles. –: Failed to amplify.

[2] Size range of the observed allele in bp.

[3] Polymorphism information content.

[4] Exclusion probability.

$_A$: Monomorphic.

3. RESULTS

The equine microsatellites were all well-amplified in the donkey, with the exception of locus ASB2, which failed to amplify. All amplified loci were polymorphic except HMS1, which was monomorphic (165 bp) in all breeds. The number of alleles varied between 3 (HMS5) and 15 (AHT4) (Tab. I), with generally little difference between the breeds (data not shown). The average gene diversity H_T [27] over all loci was 0.683 ± 0.170 while, for individual loci, it ranged from 0.278 (HMS5) to 0.907 (AHT5).

The average expected heterozygosity H_S across all loci in the total sample was 0.658 ± 0.147 and ranged from 0.336 (HMS5) to 0.852 (AHT5). The average coefficient of gene differentiation (G_{ST}) over the 13 loci was 0.036 ± 0.023 ($P < 0.01$). The G_{ST} values for single loci ranged from 0.014 for HTG15 to 0.109 for HMS5. The PIC and the exclusion probability (PE) are given in Table I. The combined probability of exclusion was 0.999, across the whole sample as well as for each breed.

Table II. Sample size, number of alleles per locus and heterozygosity (± standard errors) averaged over 13 microsatellites in 5 donkey populations.

Population	Mean sample size per locus	Mean No. of alleles per locus	Mean heterozygosity	
			Observed	Expected*
Andaluza	87	7.0 ± 1.0	0.532 ± 0.052	0.679 ± 0.034
Catalana	140	7.1 ± 1.0	0.528 ± 0.062	0.663 ± 0.055
Mallorquina	104	7.5 ± 0.9	0.570 ± 0.063	0.637 ± 0.054
Encartaciones	74	7.4 ± 1.0	0.564 ± 0.066	0.646 ± 0.059
Zamorano-Leonesa	108	7.3 ± 1.1	0.539 ± 0.058	0.684 ± 0.044
Means		7.2 ± 1.0	0.546 ± 0.060	0.654 ± 0.048

* Unbiased estimate [28].

Among Spanish donkeys the mean number of alleles per locus ranged from 7.0 in the Andaluza breed to 7.5 in the Mallorquina breed (Tab. II). The mean observed heterozygosity (H_O) showed a range of values from 0.528 in the Catalana breed to 0.570 in the Mallorquina breed. Average expected heterozygosities (H_E) ranged from 0.637 in the Mallorquina breed to 0.684 in the Zamorano-Leonesa breed, and were not significantly different. The number of private alleles varied among the five breeds: 1 in Andaluza (HMS6: 151 bp), 4 in Encartaciones (one in HTG4: 175 bp; AHT4: 146 bp; and two in HTG6: 98 and 100 bp), 2 in Mallorquina (HMS3: 160 bp; HTG15: 136 bp) and one in Zamorano-Leonesa (HTG4: 173 bp). Only one private allele showed a frequency $> 5\%$ (HTG6; 100 bp with a frequency of 10.8%, in the Encartaciones breed).

HWE was tested for all breed-locus combinations. Of the 65 contrasts, 48 tests gave significant deviations from HWE showing a significant heterozygote deficit. Only 17 tests showed agreement with HWE, corresponding to the Andaluza, Encartaciones and Mallorquina breeds for four microsatellites (HMS3, HMS5, HTG6 and VHL20; HMS3, HMS5, HTG10 and VHL20; and AHT4, HMS3, HMS5 and HMS6, respectively), the Zamorano-Leonesa breed for three microsatellites (HMS5, HTG10 and VHL20) and the Catalana breed for two microsatellites (HMS5 and HTG15). Only one of the microsatellites (HMS5) showed agreement with H-W proportions in all analysed populations.

The D_A distance, using 13 microsatellites, ranged between 0.057 and 0.093 for the Spanish donkey breeds (Tab. III). A neighbour-joining tree was constructed, and the reliability of the obtained tree was examined by 1 000 bootstrap replicates (Fig. 2). The most robust features of the topology were the Catalana-Mallorquina cluster (70% support) and the cluster (77% support) formed by Andaluza and the four black coated breeds (CAT, ENC, MALL and ZAM) which are all from the North of Spain.

Table III. Matrix of D_A genetic distance among five Spanish donkey breeds (Moroccan ass and horse).

	AND	CAT	MALL	ENC	ZAM	Horse
Moroccan	0.119	0.197	0.154	0.136	0.123	0.685
Andaluza		0.093	0.078	0.063	0.057	0.629
Catalana			0.069	0.071	0.079	0.665
Mallorquina				0.067	0.062	0.649
Encartaciones					0.059	0.640
Zamorano-Leonesa						0.644

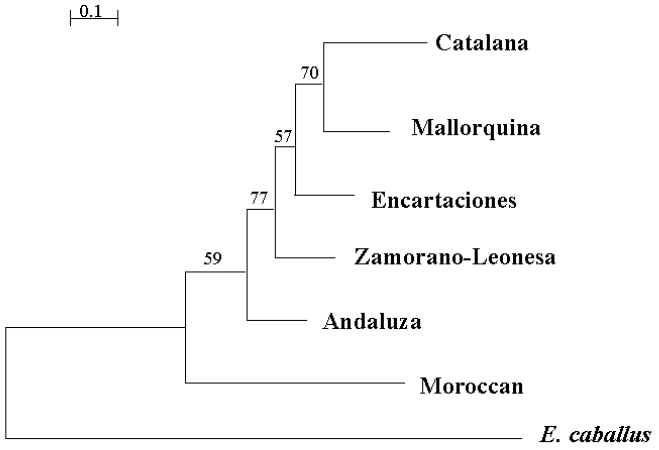

Figure 2. Dendrogram showing the genetic relationships among donkey breeds using the neighbour-joining method and the D_A genetic distance, measured with 13 microsatellite loci. The number at the forks indicate the percentage of group occurrence in a bootstrap resampling of 1 000 trees.

The low genetic distances among Spanish breeds indicated a close relationship among these populations. The phylogenetic tree was constructed based on the matrix of D_A values using the Merens horse breed as an outgroup, and the Moroccan ass breed as the reference population for the Spanish breeds.

4. DISCUSSION

The average number of alleles and the expected heterozygosities (H_E) were similar for all breeds, indicating that there are no appreciable differences in the level of genetic variability among the Spanish breeds. These results are comparable to the previous values reported in Catalonian donkeys [23] and the Baudet du Poitou breed [7].

Average genetic differentiation (G_{ST}) among the breeds was 3.6%, a relatively low but significant ($P < 0.01$) value. All loci were contributing to that differentiation. The global PE value of 0.999 for each breed makes it extremely unlikely that false parentage would not be recognised. These markers are therefore an effective tool in donkey parentage verification. The genetic relationships among the populations correspond with the geographical distribution of the breeds studied. The dendrogram (Fig. 2) groups all of the Spanish donkeys into one cluster (59% support).

Within the Spanish breeds, the four black coated populations (CAT, ENC, MALL and ZAM) form a cluster (77% support), supporting the hypothesis of a common ancestral past from *E. a. europeus*. The Catalana and Mallorquina breeds are the most closely related, supporting both the historic and the archaeological evidence that they show common ancestry [31, 33].

All sources agree that the Andaluza breed descended from the primitive ass of North Africa (*E. a. africanus*) which could have been introduced into the South of the Iberian Peninsula through the Straits of Gibraltar [3, 13, 15, 37]. However, our data fails to clearly position the Andaluza breed within our tree. Further investigations involving more European and African donkey populations, as well as the analysis of $_{mt}$DNA, which could show a possible introgression of African haplotypes into European populations would be useful to clarify this point. Nevertheless, we have concluded that the analyses of genetic markers such as microsatellite sequences are very valuable for the study of genetic variability in donkey populations and to contribute to the establishment of their own conservation plans [21].

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ANEXO 5.

Hierarchical analysis of genetic structure in Spanish donkey breeds using microsatellite markers.

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Hierarchical analysis of genetic structure in Spanish donkey breeds using microsatellite markers.

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Keywords: Donkey, extinction danger, population structure, *F*-statistics, hierarchical analysis, microsatellite.

Short running title**: Hierarchical population structure in Spanish donkey breeds.**

Summary

The hierarchical population structure of five, native-Spanish donkey breeds (Andaluza, Catalana, Mallorquina, Encartaciones and Zamorano-Leonesa) has been studied by using *F*-statistics. In addition, 9 Moroccan asses and 24 Merens breed horses were included. Data came from 15 DNA microsatellites. The analysis showed that Spanish donkeys are substructured at both hierarchical levels studied, among breeds and within breed (subpopulations). In the whole population, the deficit of heterozygotes estimated was about 21%. The proportion of genetic variability attributable to differences between breeds, subpopulations within breeds, and within subpopulations was estimated to be 6.4%, 3.5% and 3.0%, respectively. The dendrogram obtained clearly showed that the Andaluza-Moroccan ass forms a separate cluster from the Northern-Spain breeds (Catalana, Encartaciones, Mallorquina and Zamorana-Leonesa). These racial groupings coincide with the groupings obtained from historical and archaeological data.

Introduction

The ass (*Equus asinus*), is a herbivorous animal of the order *Perissodactyla*, family *Equidae*. Was domesticated about 6,000 years ago, probably in either Egypt or Mesopotamia (Littauer & Crouwel, 1979). In Spain, the development of donkey populations was influenced by their extensive use for riding and as a beast of burden; it is useful as a pack animal. It is capable of carrying over 100 kg a day with little food. Another useful domestic animal, the mule, the hybrid offspring of a male donkey and female horse, is used for vineyard cultivation for which it is suitable (Aparicio, 1960).

The Spanish donkey breeds: Andaluza, Catalana, Mallorquina, Encartaciones and Zamorano-Leonesa have suffered a substantial decrease in population size (Jordana & Folch, 1998) which might create high levels of inbreeding which may result in inbreeding depression, increasing the risk of breed extinction. Currently, the census population size of these five breeds is very low, and they are included in the Food and Agricultural Organization of the United Nation's (FAO) list of domestic animals to be preserved (FAO, DAD-IS http://fao.org/dad-is).

The objective of the present study is to characterize the genetic structure of five Spanish donkey populations in danger of extinction by using *F*-statistics analysis (Wright, 1965; Nei, 1977; Weir & Cockerham, 1984). *F*-statistics have proven to be a very useful tool in elucidating the pattern and extent of genetic variation residing within and among natural populations of animal and plant species.

Genetic characterisation is the first step in breed conservation programs and may have implications for future breeding strategies (Bjørnstad *et al*., 2000). Very little literature reporting microsatellite data in domestic donkeys exists (Breen *et al.,* 1994; Bellone *et al.*, 1998; Jordana *et al.*, 1999, 2001; Aranguren-Méndez *et al.*, 2001); for these reasons is understood the importance of this study.

The results of this analysis are expected to provide us with some insight for making decisions about the conservation of these breeds. These results will allow us to acquire knowledge about the degree of genetic differentiation that exists between breeds and subpopulations and of gene flow between them through the study and analysis of the *F*-statistics.

Materials and methods

Breeds studied and DNA collection

Genomic DNA was prepared from whole blood according to standard methods involving lysates of the washed white cells and phenol-cloroform-isoamylalcohol (25:24:1) extraction. The sample size and breeds involved were: 87 Andaluza (AND), 140 Catalana (CAT), 104 Mallorquina (MALL), 74 Encartaciones (ENC) and 108 Zamorano-Leonesa (ZAM) breeds. The area of main distribution of these indigenous Spanish breeds is shown in Figure 1. In addition, 9 Moroccan asses were used as genuine members of the *E. asinus africanus*, and 24 horses of the Merens breed (*E. caballus*) were used as an outgroup. To perform the intraracial analysis of the populations, the breeds were divided into 16 subpopulations, according to geographical criteria and/or areas of influence of certain breeders (Figure 1).

Microsatellite markers

The 15 equine microsatellite markers chosen for analysis were: AHT4, AHT5, ASB2, HMS1, HMS2, HMS3, HMS5, HMS6, HMS7, HTG4, HTG6, HTG7, HTG10, HTG15 and VHL20. Primer sequences, reaction conditions and data collections (available from the authors on request) have been described previously by Aranguren-Méndez *et al.* (2001).

Statistical analyses

Population structure was analysed by means of *F*-statistics using Weir & Cockerham methods (1984), implemented in the FSTAT computer programme (Goudet, 2000). A hierarchical F_{ST} analysis was performed to determine the amount of variance

attributable to subpopulation substructure (Wrigth, 1978; Johannesen & Loeschcke, 1996). The hierarchical analysis was carried out using analysis of molecular variance (AMOVA) in the ARLEQUIN 2,000 package (Schneider *et al.,* 2000). AMOVA yields estimations of population structure at different levels of the specified hierarchy.

In order to test the significance of the different statistics for the null hypothesis of no differentiation at the corresponding level, permutation and resampling tests (jack-knifing and bootstrapping) were carried out as implemented in the previous programmes.

Additional Reynolds genetic distance (Reynolds *et al*., 1983), a measure based on F_{ST} values [D_R= -ln(1-F_{ST})], with neighbour–joining (NJ) method clustering (Saitou & Nei, 1987), was used to construct a dendrogram of breed relationships, using the PHYLIP package (Felsenstein, 1995). An unrooted consensus tree, evaluated by 1,000 bootstrap replicates, was obtained. The method of Slatkin (1993), implemented in GENEPOP, was used to assess the genetically effective migration rate ($M = N_e m$, the average number of migrants exchanged per generation).

Results

Thirteen of fifteen equine microsatellites investigated amplified well and were polymorphic in the donkey, except for locus ASB2, which failed to amplify, and HMS1, which was monomorphic (165 bp) in all breeds. The F_{ST}, F_{IT} and F_{IS} values, computed over all breeds and loci, were obtained. Levels of apparent breed differentiation were considerable and multilocus F_{ST} values indicate that around 4.1% of the total genetic variation could be explained by breed differences, the remaining 95.9 % corresponding to differences among individuals. Genetic differentiation among

breeds was highly significant (P< 0.001) for all loci. On average, individuals within breeds had a 17.8% (P<0.001) deficit of heterozygotes, whereas the total population had a 21.1% (P<0.001) deficit of heterozygotes.

Values for the *F*-statistics of the Spanish donkey populations at all hierarchical levels are presented in Table 1. The degree of genetic differentiation among subpopulations was highly significant for all breeds, oscillating from 1.3% for the Encartaciones breed to 5.8% for the Andaluza breed (P< 0.001), indicating the existence of a certain reproductive substructure within them all.

The hierarchical analysis of the Spanish donkey populations (Table 2) revealed that, as expected, most of the differentiation occurs among breeds with respect to the total population rather than among subpopulations within breeds, and within subpopulations; 0.064 *vs* 0.035, and 0.030, respectively. The differentiation among breeds in hierarchical analysis was 6.4%, rather than without using hierarchical analysis (4.1%).

Table 3 shows the total inbreeding estimate ($F \cong F_{IT}$) per breed, evaluated by hierarchical analysis. The whole loci showed values significantly different from zero, as well as the estimated average (P<0.001). The F_{IT} average, obtained from jackknifing over loci, ranged between 0.112 ± 0.049 and 0.232 ± 0.058 for the Mallorquina and Andaluza breeds, respectively.

The interbreed genetic distance, or F_{ST} estimates, below the diagonal, and gene flow (N_em) above the diagonal, between pairs of Spanish donkey breeds are shown in Table 4. After 1,000 permutations, performed with FSTAT, all F_{ST} calculated by pairwise breed combinations were significantly different from zero (P<0.001). Least differentiation was detected between Andaluza and Zamorano-Leonesa breeds and

Encartaciones-Mallorquina breeds (F_{ST}=0.031), and the most divergence was observed between Andaluza and Catalana breeds (F_{ST}=0.058). N_em represents the number of effective migrants exchanged per generation. The N_em values for breed pairs varied from 4.16 to 7.88 for the AND-CAT pair and the MALL-ENC pair, respectively.

Figure 2 shows a NJ tree based on Reynolds genetic distance (data not shown) relating the five Spanish donkey breeds studied, the Moroccan ass, and the Merens breed used as outgroup. The numbers at the nodes are bootstrapping values for 1,000 replicates of the 13 loci genotyped.

Discussion

Genetic differentiation among Spanish donkey breeds exists. All loci contributing to this differentiation with values moderately low and similar for all systems studied, but very significant (P<0.001), indicating a relatively high gene flow among the breeds studied. However, it is clear that most of the total genetic variation corresponds to differences among individuals (95.9%) and only less than five percent is due to differences among breeds. These values of total genetic differentiation (F_{ST}) among breeds are close to those found in other domestic species, for example: among river buffalo breeds (F_{ST}=0.038, Barker *et al.,* 1997), among Spanish horse breeds (F_{ST}=0.078, Cañon *et al.,* 2000), though slightly lower than those found in Norwegian horse breeds (F_{ST}=0.12, Bjørnstad *et al.,* 2000), in European cattle breeds (F_{ST}=0.11, MacHugh *et al.,* 1998; Kantanen *et al.,* 2000) and among Spanish dogs (F_{ST}=0.099, Jordana *et al.,* 1992).

The results from the hierarchical analysis further show that populations of Spanish donkeys are substructured at different levels (Table 2). The differentiation

within breeds is only half of that between breeds (3.5% and 6.4%, respectively). This value was similar to that reported for other organisms: Spanish dogs (Jordana *et al.*, 1992), black-tailed dogs (Chesser, 1983) and house mice (Selander & Kaufman, 1975).

The total breed inbreeding estimate ($F \cong F_{IT;}$ Table 3) showed a significant deficit of heterozygotes ($P<0.001$) in all breeds. Consanguinity, which is produced by mating between relatives, can be one cause of loss of heterozygotes, but this deficit affects all or most of the loci in a similar way. AND, CAT and ZAM breeds showed 10 or 11 loci, of the total of 13, with a significant deficit of heterozygotes, therefore possibly being able to attribute this to this phenomenon (Table 3). Although in these breeds high and significant values of F_{ST} (Table 1) (0.058, 0.031 and 0.037, respectively), also suggest that a significant subpopulation structure (Wahlund's effect) exists within these populations. On the other hand, MALL and ENC breeds showed single deficit of heterozygotes in only 5 and 6 loci, respectively, this deficit not being able to essentially be attributed to the consanguinity.

We must remember that there are other factors that can also cause a lack of heterozygotes in the population (Nei, 1987). First, the locus can be under selection, the "genetic hitchhiking" effect, close to some morphological or productive trait of selective interest. Second, "null alleles" (non-amplifying alleles) may be present and lead to a false observation of excess homozygotes. Third, the presence of a population's substructure within the breed may lead to Wahlund's effect.

The reproductive substructure within the breed, in isolated units, would be a very feasible explanation to understand this high deficit of heterozygotes observed in the Spanish donkey populations. Nevertheless, two loci exist, concretely HTG4 and HMS7, which show a very significant deficit of heterozygotes in all breeds ($P <0.001$).

The most coherent interpretation to explain this deficit in those two markers is that these loci can be under selection (genetic hitchhiking effect) with some trait of selective interest, or the possible presence, not detected, of "null alleles" in these populations. Previous reports have indicated the occurrence of a "null allele" present in the HMS7 locus in the Quarter-horse equine breed (Bozzini *et al.*, 1997).

In the context of the conservation and maintenance of animal genetic variability, migration values (N_em) can be interpreted as the upper limit of the number of migrants per generation, which would allow the maintenance of the observed genetic differentiation among the breeds (Cañón *et al*., 2000). For example, an introgression rate of eight individuals per generation between the ENC and MALL breeds would maintain the estimated degree of genetic differentiation between these breeds. Similarly, when we compare the CAT and AND breeds, a gene flow between them greater than only 4 individuals per generation could mean a real threat for both breeds. A gene-flow strategy, which permits a greater introgression rate between genetically close populations rather than in more divergent ones, should be emphasised.

The dendrogram's topology (Figure 2) is very similar to that obtained by Aranguren-Méndez *et al.* (2001) using the D_A genetic distance (Nei, 1987) and the NJ algorithm. In this work, the four breeds of black coat from the North of Spain (CAT, ENC, MALL and ZAM) form a closed cluster (64% support), supporting the hypothesis of a common ancestral past from *E. asinus europeus.*

In this paper, unlike Aranguren-Méndez *et al*. (2001), the African origin of the Andaluza breed is clarified, which forms a cluster with the Moroccan ass, a genuine representative of the ancestral trunk of *E. asinus africanus*. This supports the thesis earlier stated by other authors about its African origin (Aparicio, 1960; Epstein, 1984;

Sotillo and Serrano, 1985). However, the low bootstrap value for this cluster (43% support) reflects the instability of the topology, so additional studies to confirm this hypothesis would be necessary.

Further investigations involving more European and African donkey populations, as well as the analysis of genetic variability of mtDNA, could look for a possible introgression of mtDNA haplotypes of African origin in these populations. This variability is not reflected in the nuclear genome, and it would be useful to clarify this point.

Nevertheless, this study contributes to the knowledge of genetic structure and molecular characterisation of endangered and small populations, such as the Spanish donkey breeds. It also shows how microsatellites can be used to establish the genetic relationships between donkey populations, and help to establish the planning for their conservation (Jordana & Folch, 1998; Jordana *et al*., 2001), as well as in order to integrate this information into the FAO Global Data Bank on Domestic Animal Diversity (DAD-IS).

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Legends to tables and figures

Table 1. Genetic structure of the Spanish donkey breeds through an analysis of their subpopulations.

Table 2. Hierarchical F-statistics and variance components for Spanish donkey breeds with two subdivision levels.

Table 3. Total breed inbreeding estimated ($F \cong F_{IT}$) by hierarchical analysis in the Spanish donkey breeds.

Table 4. F_{ST} estimates (below the diagonal) as a measure of genetic distance between donkey breeds and the number of effective migrants per generation (N_em) (above the diagonal).

Figure 1. Geographical location of the Spanish donkey subpopulations and their reference codes (n is the sample size).

Figure 2. Neighbour-Joining dendrogram showing the genetic relationships among the Spanish donkey breeds, inferred through microsatellite data. This tree is based on Reynolds' genetic distance. The numbers at the nodes are values for 1,000 bootstrap resamplings of the 13 loci genotyped.

Breeds	$F_{IS} \cong f$	$F_{IT} \cong F$	$F_{ST} \cong \theta$
Andaluza (0.009)***	0.184 (0.058)***	0.232 (0.058)***	0.058
	† (0.084-0.306)	(0.132-0.352)	(0.040-0.075)
Catalana (0.007)***	0.189 (0.053)***	0.214 (0.052)***	0.031
	(0.102-0.303)	(0.127-0.324)	(0.018-0.044)
Mallorquina (0.007)***	0.091 (0.045)***	0.112 (0.049)***	0.022
	(0.016-0.193)	(0.032-0.221)	(0.010-0.038)
Encartaciones (0.006)***	0.118 (0.040)***	0.130 (0.041)***	0.013
	(0.053-0.210)	(0.062-0.224)	(0.003-0.026)
Zamorano-Leonesa (0.014)***	0.193 (0.053)***	0.223 (0.061)***	0.037
	(0.099-0.307)	(0.116-0.353)	(0.014-0.068)

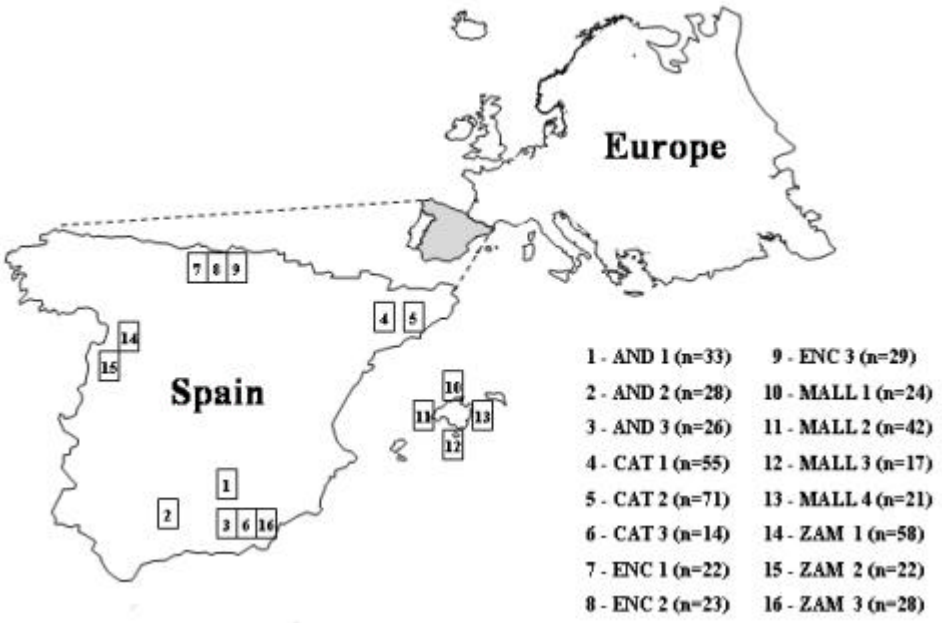
f, within-population inbreeding estimate; F, total inbreeding estimate; θ, measure of population differentiation
Standard deviation in parentheses estimate from jackknife over loci
*** P<0.001, from permutation tests in FSTAT programme
† 95% Confidence Interval

Sources of variation	**Variance** component	Percentage of variation	Fixation Indices (%)
Among breeds	0.133	2.96	6.4
Among subpopulations within breeds	0.153	3.41	3.5
Within subpopulations	4.208	93.63	3.0

Locus	Andaluza	Catalana	Mallorquina	Encartaciones	Zam-Leonesa
AHT4	0.147 ***	0.129**	-0.038	0.146 **	0.151 **
AHT5	0.080 **	0.220 ***	0.138 ***	0.098*	0.253 ***
HMS2	0.250 ***	0.106 *	0.195 ***	0.065	0.223 ***
HMS3	0.144 **	0.117 *	-0.085	0.017	-0.030
HMS5	-0.095	-0.037	-0.105	0.133	-0.015
HMS6	0.479 ***	0.271 ***	0.033	0.141 *	0.315 ***
HMS7	0.661 ***	0.357 ***	0.336 ***	0.478 ***	0.577 ***
HTG4	0.695 ***	0.823 ***	0.782 ***	0.604 ***	0.944 ***
HTG6	0.177 ***	0.364 ***	0.052	0.153 ***	0.206 ***
HTG7	0.224 ***	0.067 **	0.048	0.082	0.246 ***
HTG10	0.144 **	0.125 ***	0.076 **	-0.013	0.139 ***
HTG15	0.138 **	-0.000	0.036	0.093	0.111 *
VHL20	-0.015	0.193 ***	0.031	-0.061	-0.105
Mean	0.232*** (0.058)	0.214*** (0.052)	0.112*** (0.049)	0.130*** (0.041)	0.223*** (0.058)

*P< 0.05, ** P<0.01, ***P<0.001 from permutation tests in FSTAT programme

	Andaluza	Catalana	Mallorquina	Encartaciones	Zam-Leonesa
Andaluza		4.167	4.762	6.532	7.797
Catalana	0.058		6.134	6.585	4.842
Mallorquina	0.051	0.040		7.883	7.118
Encartaciones	0.037	0.037	0.031		7.235
Zamorano-Leonesa	0.031	0.050	0.034	0.034	


Europe
Spain
7
8
9
4
5
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15
16
11
13
12
1
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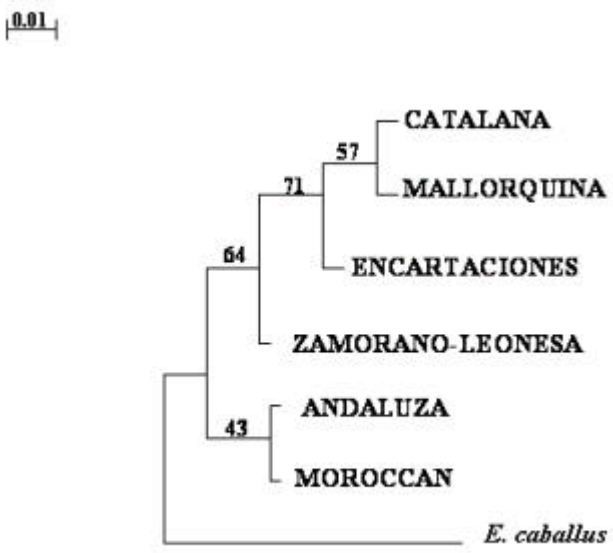
16
1 - AND 1 (n=33)
2 - AND 2 (n=28)
3 - AND 3 (n=26)
4 - CAT 1 (n=55)
5 - CAT 2 (n=71)
6 - CAT 3 (n=14)
7 - ENC 1 (n=22)
8 - ENC 2 (n=23)
9 - ENC 3 (n=29)
10 - MALL 1 (n=24)
11 - MALL 2 (n=42)
12 - MALL 3 (n=17)
13 - MALL 4 (n=21)
14 - ZAM 1 (n=58)
15 - ZAM 2 (n=22)
16 - ZAM 3 (n=28)


0.01
CATALANA
57
MALLORQUINA
71
ENCARTACIONES
64
ZAMORANO-LEONESA
ANDALUZA
43
MOROCCAN
E. caballus

ANEXO 6

Genetic conservation of five endangered Spanish donkey breeds.

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GENETIC CONSERVATION OF FIVE ENDANGERED SPANISH DONKEY BREEDS

By J. ARANGUREN-MENDEZ[1,2] J. JORDANA[1] M. GOMEZ[3]

Summary

The genetic diversity of most livestock species is being reduced, and it is not possible to preserve all of these livestock breeds. In order to preserve as much of the genetic diversity as possible, we must first have a robust method of measuring genetic diversity among breeds. Three different methods of study that graphically represent relationships among breeds are presented; Weitzman's method, principal component analysis (PCA) and a neighbor-joining tree with allele-sharing. Diversity was evaluated on the basis of 15 microsatellite markers typed over a total de 513 DNA samples collected from five Spanish donkey breeds. Breed differentiation was confirmed by the clustering based on the genetic distances between individuals, which essentially grouped all individuals in discrete clusters. The genetic distance among breeds was used to measure the global diversity of the set in breeds considered, and to evaluate the marginal loss of diversity attached to each breed. The Catalana breed appeared to be the most "unique" in the set considered. In addition to this, the usefulness of global evaluations of diversity using molecular markers to choose breeds is worthy of conservation.

Resumen

La diversidad genética en la mayoría de especies domésticas se está reduciendo, no siendo posible poder conservar todas esas razas domésticas. Para intentar preservar la máxima cantidad de diversidad genética, deberíamos disponer, en primer lugar, de un método preciso que nos midiera dicha diversidad entre razas. Presentamos aquí, tres diferentes métodos de estudio que representan gráficamente las relaciones existentes entre las razas; el método de Weitzman, el análisis de componentes principales (PCA) y el árbol "neighbor-joining" con alelos compartidos. La diversidad se evaluó a partir del análisis de 15 marcadores genéticos, de tipo microsatélite, sobre un total de 513 muestras de DNA obtenidas de cinco razas asnales españolas. La diferenciación entre razas se confirmó por el agrupamiento individual obtenido, basado en las distancias genéticas entre los individuos, que agrupó esencialmente a todos los animals de la misma raza en grupos discretos. La distancia genética entre las razas se utilizó para medir la diversidad global de todas ellas, y para evaluar la pérdida marginal de diversidad asociada a cada una. La raza Catalana aparece cómo la más "única" en el conjunto considerado. Las evaluaciones globales de diversidad, utilizando marcadores moleculares, resultan muy útiles para la elección de las posibles razas a conservar.

Introduction

In genetic conservation the main objective consists of preserving variability within populations under the hypothesis of correlation between genetic variation and the population's viability. When economic resources are scarce, it is important to prioritise populations for preservation, decisions of where to allocate resources or to establish preservation plans should be based on information that ensures, to the greatest degree possible, the future viability and success of the preserved populations and of the species (Falk, 1991).

Consequently, one of the first stages in the conservation programme of species consists of the evaluation of their genetic variability, their distribution among their populations, and the possible detection of rare alleles, as an indicator of populations with unique genetic variants (González-Candelas and Montolío, 2000).

The Food and Agriculture Organization of the United Nations (FAO) has been mandated by its member nations to manage global animal genetics resources, and much important progress has been made in the last few years (http://www.fao.org/dad-is/). However, resources are limited, and priorities will have to be set for breed conservation, for breed development programmes and for evaluation studies (Barker, 1999).

This article presents the results of the analysis of microsatellite variability in five endangered Spanish donkey breeds (Andaluza, Catalana, Mallorquina, Encartaciones and Zamorano-Leonesa breeds). Our main goals consist of studying the genetic diversity of the five populations of donkeys, through the analysis of three methods which study genetic variability, based on microsatellite data: Weitzman's diversity, the principal components analysis (PCA) and the allele-shared analysis.

Materials and methods

Population samples

Currently, the 5 Spanish donkey breed figures fit in the category of endangered breeds, proposed by the FAO Expert Consultation (FAO, DAD-IS http://fao.org/dad-is), as well as general information on those breeds, which may be found here. The number of individuals sampled was as follows: 87 Andaluza (AND), 140 Catalana (CAT), 104 Mallorquina (MALL), 74 Encartaciones (ENC) and 108 Zamorano-Leonesa (ZAM). In addition, 9 Moroccan asses (MOR) were included as a reference population. Donkey DNA was prepared from whole blood according to standard methods involving lysates of the washed white-cells and phenol-chloroform-isoamylalcohol (25:24:1) extraction (Ausubel et al. 1987).

Microsatellite markers

Out of the 15 equine microsatellite markers used, 12 (ASB2, AHT4, AHT5, HMS2, HMS3, HMS6, HMS7, HTG4, HTG6, HTG7, HTG10, and VHL20) were included in a kit (Stock-Marks® for horses, Equine Paternity PCR Typing kit, PE Applied Biosystems Division; Foster City, CA), which was used with slight modifications in some PCR conditions. The other 3 markers (HMS1, HMS5 and HTG15) were obtained from the literature of equine markers (Breen et al. 1994). Primer sequences, reaction conditions and data collections have been described previously (Aranguren-Méndez et al. 2001).

Statistical analyses

Genetic distances can also be used to measure diversity, as proposed by Weitzman (1992, 1993). This approach has been implemented here to provide a further upward hierarchical representation of the breeds and to evaluate marginal losses of diversity due to various patterns of breed extinction, as advocated by Thaon d' Arnoldi et al. (1998).

Genetic distances between breeds were calculated based on allelic frequencies in each breed (available from the authors on request). Two measures of distances were used, namely the Reynolds' (Reynolds et al. 1983) and the standard Nei distances (Nei, 1972), taking into account the corrections needed for a small sample size (Nei, 1978).

Principal components analysis (PCA) was performed using the SAS procedure PRINCOMP (SAS, 1990) according to the recommendation of Cavalli-Sforza et al. (1994). A neighbor-joining (NJ) tree (Saitou and Nei, 1987), using individual animals as operational taxonomic units (OTUs), was constructed with a distance matrix derived from the simple allele-sharing statistic (Bowcock et al. 1994). The distance was obtained using the MICROSAT computer programme (Minch et al. 1995). The PHYLIP package (Felsenstein, 1995) was then used to construct the tree from the distance matrix.

Results

Table 1 gives the Reynolds and Nei standard genetic distances. The smallest values were obtained for the AND-ZAM and MALL-ENC pairs by Reynolds and Nei distances, respectively. The largest were between AND-CAT for both distances.

Weitzman's representation, based on Nei's standard distance, is shown in Figure 1, where the branch length for each breed can be read, approximately, measuring its relative contribution to the corresponding diversity function. A clear discrimination is observed between two groups, i.e., a) a first group constituted only of the Andaluza breed (South of Spain), and b) another group involving all breeds of black coat of the North of Spain (Catalana, Encartaciones, Mallorquina, and Zamorano-Leonesa breeds).

The marginal losses of diversity attached to each breed, which may be taken as a measure of their "uniqueness", are shown in Table 2, based on the two distances considered. On average, the highest and lowest losses of diversity are incurred with the extinction of the Catalana and the Encartaciones breeds, respectively. It can also be seen, in Table 2, that the loss of the Andaluza + Catalana + Encartaciones breeds induces a markedly higher loss than the sum of the corresponding individual breeds losses.

The first three principal factors in the principal components (PCs) analysis are plotted in Figure 2. The first PC accounts for 52.0% of the underlying variation, the second PC condenses 15.0% and the third PC accounts for 13.8% of the variation. The first component, which explain more than the half of the existent variation, clearly separates the Spanish donkey breeds from the Moroccan ass population, and within the Spaniards, grouping closely the Catalana and Mallorquina breeds, in a similar way to the obtained in other studies (Aranguren-Méndez et al. 2001).

Figure 3 shows a NJ phylogenetic tree, constructed from the simple allele-sharing distance between 109 individuals, 20 animals taken at random from each population of Spanish donkeys, except for the Moroccan ass population in which all 9 individuals were used. Of the 100 Spanish donkeys represented in the tree, only 11 were

not clustered with animals from the same population, and some of the breeds are very closely clustered. For example, all AND and ENC animals are found in discrete clusters. The other breeds showed a comparable level of clustering, as do the CAT and MALL breeds. The ZAM and Moroccan animals show a more fragmented pattern of clustering, with animals from these two populations split into a number of distinct groups spread over the tree.

Discussion

Three different methods of study and its graphic representation of breed genetic diversity are presented: Weitzman´s method, the principal component analysis (PCA) and the neighbor-joining tree with allele-sharing.

Weitzman's diversity defines the diversity expected after a given period of time based on the extinction probability of each element of the set considered. If *n* elements are endangered, 2^{nd} survival-extinction patterns may occur with given probabilities, and for each pattern the resulting diversity may be calculated (Thaon d' Arnoldi et al. 1998). Knowing the pairwise genetic distances and the risk status of a given set endangered breeds, as expressed through their respective probabilities of extinction, an order of priority for a cryopreservation programme could thus be established.

In this study an opportunity is given for evaluating the global diversity in the set of breeds considered, using Weitzman's approach (1992, 1993). Table 2 clearly shows the wide range of contributions of each breed to overall diversity, ranging from about 19.19% (Encartaciones breed) to 29.11% (Catalana breed). Based on these distances (Reynolds and Nei distances), the AND, CAT and ENC breeds altogether account for

80% of the total diversity, which is an indicator for the potential value to preserve these local endangered breeds in the maintenance of biodiversity. The loss of diversity caused by extinction in a set of breeds can be estimated by the sum of the ordinates in the nodes, which would disappear from the tree if the extinct breeds were to be removed, without any other change. Thus, just by looking at the table, it is obvious that the loss of the Catalana breed would cause the decrease of diversity three or four times greater than the loss of the Mallorquina or Encartaciones breeds. Although the microsatellite-based phylogeny and the Weitzman diversity tree (Fig. 1) showed some identical clusters, it should not be expected that they will give the same information (Barker et al. 2001).

However, the results and the conservation decisions that one could take of the Weitzman's diversity analysis based on genetic distances should be taken with caution, as recent studies seem to indicate that these methods could be inappropriate for within-species breed conservation, because they ignore within-breed variation (Caballero and Toro, 2002).

Multivariate analysis of microsatellite allele frequencies (PCA) has previously been shown to be a powerful tool to reveal the underlying evolutionary history and admixture among distantly related populations (Schmid et al. 1999; Stahlberger-Saitbekova et al. 2001). However, its use as a technique to discern relationships among closely related populations is questionable (MacHugh et al. 1998).

The used markers did not show any breed specific allele allowing simple identification of the breed and allocation of each one of the individuals as to its breed origin. However, the NJ tree of individuals is in very good agreement with its population's structure (Fig. 3). Of the 100 Spanish donkeys examined, 89 (89.0%) form discrete clusters that coincide with the breed of origin of the sample. The position of

eleven individuals (11.0%) is not clearly defined in the tree, and the Moroccan ass shows a more fragmented pattern of clustering. Within-breed, these samples tend to form subclusters and they correspond to their source subpopulations to a very high degree (data not shown). Previous studies, which have used a microsatellite allele-sharing distance among individual organisms, have revealed a similar level of clustering within a population to that observed in Fig. 3 (MacHugh et al. 1998; Laval et al. 2000). More precisely, using breed allelic frequencies to calculate the likelihood that an animal belongs to a given breed and then assigning the animal to the breed showing the largest likelihood (using Bayesian methods as proposed by Rannala and Mountain, 1997) allowed at 90.64% the animals to be correctly assigned (data not shown).

The apparent taxonomic distinctiveness of a breed may not necessarily mean that it carries genes that are adaptively unique, as forces other than selection may have been operating. For example, random drift can affect the genetic distances among populations. When we are selecting breeds for conservation it may be important not just to consider taxonomic distinctiveness or between-population variation, but also to take measures within population diversity (Blott et al. 1998; Caballero and Toro 2002). Such measures could be included into a diversity index and population selected for conservation on the basis of this index. On the other hand, there is a need for measuring the overall diversity of a set breeds, since prospective evaluations of diversity are required for defining appropriate conservation policies, as advocated by Weitzman (1993). In addition to this, the usefulness of global evaluations of diversity using molecular markers for the choice of breeds is worthy of conservation. We hope that this information and that reported previously (Aranguren-Méndez et al. 2001; Jordana et al.

2001) will significantly contribute to the establishment of a sensible preservation strategy for these populations.

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Legends to tables and figures

Table 1. Reynolds' distance estimate (below the diagonal) and standard Nei genetic distance (above the diagonal) among five Spanish donkey breeds.

Table 2. Marginal losses of Weitzman's diversity in Spanish donkey breeds.

Figure 1. Dendrogram of relationships established by Weitzman's method using standard Nei distance among five Spanish donkey breeds.

Figure 2. Principal components analysis of allele frequencies from 13 microsatellite loci typed in seven populations from five Spanish donkey breeds, and one Moroccan ass breed. The first PC accounts for 52.0%, second PC condenses 15.0% and the third PC condenses 13.8% of the variation.

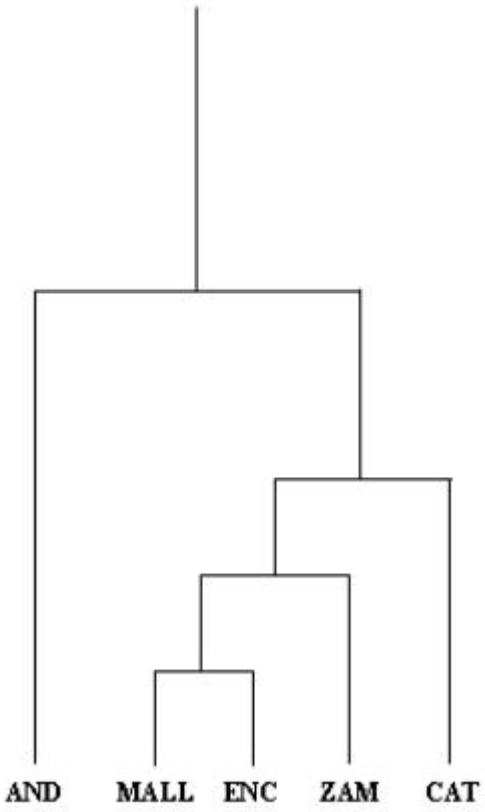
Figure 3. Neighbor-joining dendrogram constructed from allele-sharing distance among 109 individuals from five Spanish donkey breeds, and one Moroccan ass breed. Numbers to the right indicate the fraction of individuals of the breed.

	Andaluza	Catalana	Mallorquina	Encartaciones	Zam-Leonesa
Andaluza	--------	**0.1324**	0.1074	0.0797	0.0737
Catalana	**0.0618**	--------	0.0801	0.0767	0.1140
Mallorquina	0.0561	0.0441	--------	*0.0597*	0.0702
Encartaciones	0.0452	0.0413	0.0379	--------	0.0723
Zamorano-Leonesa	*0.0372*	0.0537	0.0393	0.0407	--------

(greatest distances in bold; smallest distances in italics)

Breed loss (Q)	Reynolds distance: Diversity V(S\Q)	Reynolds distance: Absolute loss ΔV	Reynolds distance: Relative loss ΔV/V (%)	Nei standard distance: Relative loss ΔV/V (%)	Nei standard distance: Diversity V(S\Q)	Nei standard distance: Absolute loss ΔV	Average: Relative loss ΔV/V (%)
None (0)	1810	0	0.00	0.00	3424	0	0.00
Andaluza (1)	1323	487	27.53	26.91	2460	934	28.15
Catalana (2)	1312	498	29.11	27.51	2373	1051	30.70
Mallorquina (3)	1403	407	20.16	22.49	2814	610	17.82
Encartaciones (4)	1431	379	19.19	20.94	2827	597	17.44
Zamorano-Leonesa (5)	1438	372	20.53	20.55	2722	702	20.50
(1) + (2)	786	1024	59.01	56.57	1320	2104	61.45
(2) + (4)	824	986	55.05	54.48	1520	1904	55.61
(1) + (4)	944	866	46.72	47.85	1863	1561	45.59
(1) + (2) + (4)	407	1403	78.20	77.51	723	2701	78.88
(2) + (3) + (5)	452	1358	75.88	75.03	797	2627	76.72

V(S\Q) = diversity after dropping Q from S; ΔV = V(S) - V(S\Q) = absolute value.
ΔV/V = relative value (distance multiplied by 10,000)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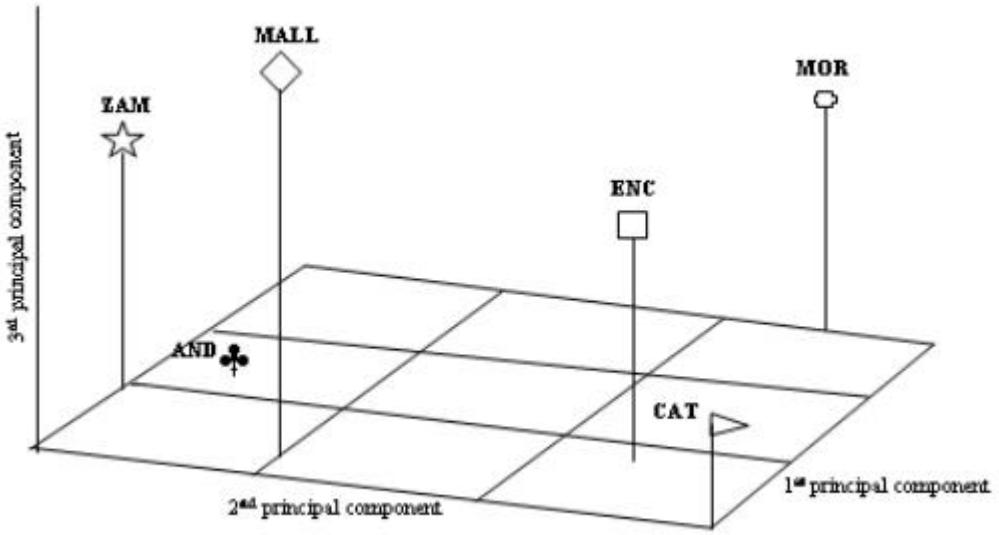
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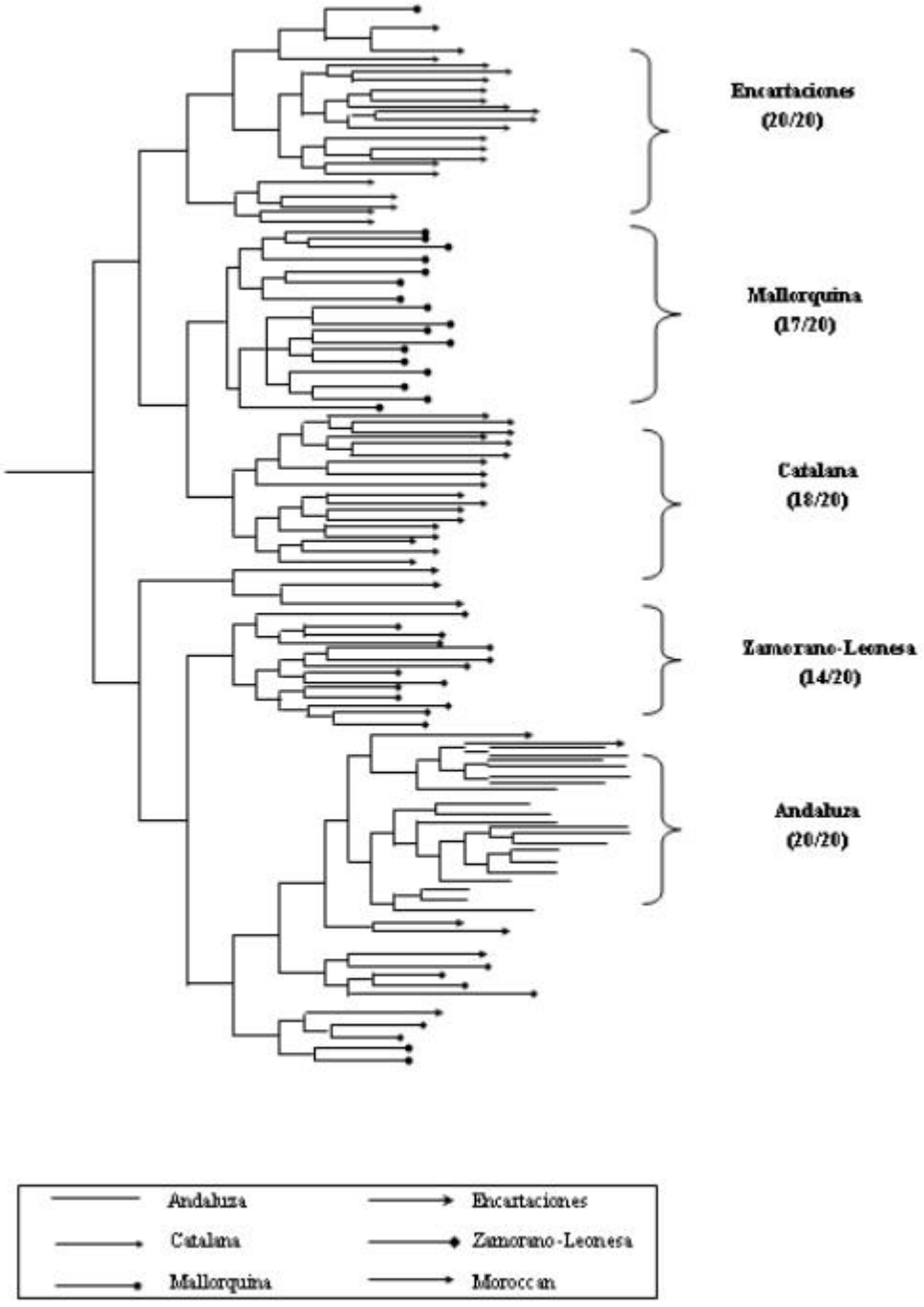
Encartaciones
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