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Genetic structure of eighteen local south European beef cattle breeds by comparative *F*-statistics analysis

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Summary

The genetic structure and relationships among 18 local Southwest European beef cattle breeds (10 from Spain, five from Portugal and three from France) have been inferred from 16 DNA microsatellite loci, by using *F*-statistics, for conservation purposes. Level of apparent breed differentiation is considerable and multilocus F_{ST} values indicate that around 6.8% of the total genetic variation could be explained by breed differences and the remaining 93.2% by differences among individuals. For countries of origin, the French breeds were those that showed a higher genetic uniformity. All breeds, except the Portuguese breeds Barrosã and Mirandesa, showed a significant heterozygotes deficit. Several factors that could cause this deficit are discussed, and the within-population inbreeding estimates obtained are compared with those from genealogical data. Gene flow could have played an important role for genetic uniformity in populations of narrow geographical vicinity. Neither isolation by distance and hierarchical structure associated with geography are detected. However, in sight of the obtained results, we suggest the genetic drift as the most important factor of genetic differentiation among the analysed populations. The apparent taxonomic distinctiveness of the breeds could be, in an important way, the result of a random drift, which can affect the genetic distances among populations.

Zusammenfassung

Genetische Struktur von achtzehn lokalen südeuropäischen Fleischrinderrassen anhand vergleichender F-Statistik

Die genetische Struktur von und Beziehungen zwischen 18 lokalen südwesteuropäischen Fleischrinderrassen (10 aus Spanien, 5 aus Portugal und 3 aus Frankreich) wurden zum Zweck der Erhaltung mit Hilfe von F-Statistiken anhand von 16 DNA Mikrosatelliten erschlossen. Der Grad der offensichtlichen Differenzierung der Rassen ist beträchtlich. Die Multilocus F_{ST}-Werte deuten an, dass etwa 6,8% der gesamten genetischen Variation durch Unterschiede zwischen den Rassen erklärt werden können, die verbleibenden 93,2% durch Unterschiede zwischen Individuen. Bezüglich der Herkunftsländer zeigten die französischen Rassen eine höhere genetische Uniformität. Alle Rassen, außer der portugiesischen Rassen Barrosã und Mirandesa, zeigten ein signifikantes Heterozygotie-Defizit. Einige das Defizit verursachende Faktoren werden diskutiert. Die innerhalb der Populationen erzielten Inzuchtschätzungen sind vergleichbar mit denen aus genealogischen Studien. Der Genfluss kann eine wichtige Rolle für die genetische Uniformität in Populationen eingeschränkter geographischer Gebiete gespielt haben. Weder Isolation durch Distanz, noch eine im Zusammenhang mit der Geographie stehende hierarchische Struktur konnten nachgewiesen werden. Jedoch wird vermutet, dass im Hinblick auf die erzielten Resultate genetische Drift der wichtigste Faktor für die genetische Differenzierung zwischen den analysierten Populationen ist. Die ersichtliche taxonomische Beson-

derheit der Rassen könnte maßgeblich durch eine zufällige Drift verursacht sein, welche die genetische Distanz zwischen Populationen beeinflussen kann.

Introduction

Cattle, like other livestock species, are recognized as important components of world biodiversity because the genes and gene combinations they carry may remain useful to agriculture in the future (Hall and Bradley 1995).

Local cattle breeds are considered, for many reasons, precious genetic resources that tend to disappear because of the new market and agricultural demands. Nevertheless, there is a serious risk that most of these breeds will disappear before they have been fully characterized and studied (FAO 2000). According to Hall and Ruane (1993), of the 464 cattle breeds that exist or have existed in Europe during the twentieth century, 33% (154) have become extinct and 22% (101) are considered to be in danger of extinction.

The objective of the present study is to characterize the genetic structure of 18 local Southwest European beef cattle populations by *F*-statistics analysis (Wright 1965; Nei 1977; Weir and Cockerham 1984). These breeds are characterized by their small population size and linked to traditional production systems. Consequently, one of the first stages for breed conservation consists of the evaluation of their genetic variability, its distribution among the populations, and the possible detection of rare alleles, as an indicator of populations with unique genetic variants (González-Candelas and Montolío 2000).

The *F*-statistics have proven to be a very useful tool in elucidating the pattern and extent of genetic variation within and among natural populations of animal and plant species. For a total population that is subdivided into many subpopulations (breeds), Wright (1965) defined the *F*-statistics: F_{IS}, F_{IT} and F_{ST}, as correlations between uniting gametes. F_{IS} and F_{IT} stand for the correlations between two uniting gametes drawn at random from a subpopulation and from the total population, and relate the departure from panmixia in each subpopulation and in the population as a whole, respectively, whereas F_{ST} is the correlation between two gametes randomly drawn from each subpopulation relative to the whole breed. These parameters are related through the following expression:

$$(1 - F_{IT}) = (1 - F_{IS}) \cdot (1 - F_{ST})$$

The study of the *F*-statistics will allow to quantify the existing genetic differentiation among bovine populations. The analysis of deficit or excess of heterozygotes, per locus and population, will allow to discuss the most probable causes that have led to departure from panmixia, and permit an approximate estimate of inbreeding in each breed, comparing it subsequently with those obtained from genealogical data.

Does isolation by distance among the breeds from a country and among countries exist? What are the current levels of gene flow between these breeds? Has gene flow been the main factor of the current genetic similarity between these populations from the Southwest of Europe? Are the genetic relationships derived from estimates of *F* values consistent with those evaluated by other methods? We hope that these answers and the results previously reported for these populations by Cañón et al. (2001) and Beja-Pereira et al. (2003) will contribute to the establishment of a sensible preservation strategy for these local breeds.

Materials and methods

Breeds sampled and microsatellite markers analysed

Blood samples of 889 unrelated individuals (25 males and 25 females per breed) from 18 local European beef cattle breeds were analysed: 10 breeds from Spain: Alistana (ALI), Asturiana de la Montaña (ASM), Asturiana de los Valles (ASV), Avileña (AVI), Bruna dels

Pirineus (BRU), Morucha (MOR), Pirenaica (PIR), Retinta (RET), Sayaguesa (SAY) and Tudanca (TUD); five breeds from Portugal: Alentejana (ALE), Barrosã (BAR), Maronesa (MAR), Mertolenga (MER) and Mirandesa (MIR) and three from France: Aubrac (AUB), Gasconne (GAS) and Salers (SAL). The geographical distribution of these breeds is shown in Fig. 1. Information may be found in Mason (1996) and in the FAO Domestic Animal Diversity Information System (DAD-IS, http://www.fao.org/dad-is/).

A total of 16 microsatellite markers were analysed: CSSM66, ETH 10, ETH 152, ETH 225, ETH 3, HEL 1, HEL 5, HEL 9, ILSTS 5, INRA 23, INRA 32, INRA 35, INRA 37, INRA 5, INRA 63 and TGLA 44. Only the last locus is not included in the European Concerted Action AIRE 2066 list (FAO list). Primer sequences, reaction conditions and data collections have been described previously by Cañón et al. (2001).

Statistical analyses

The *F*-statistics (Wright, 1965, 1978), F_{IT}, F_{ST} and F_{IS} (based on an infinite allele model of mutation) were estimated in the form of F, θ and f, the sample-based, respective estimators of these parameters proposed by Weir and Cockerham (1984). These were computed using fstat program (Goudet 1995). Significance of the *F*-statistics was determined from permutation tests with the sequential Bonferroni procedure (Hochberg 1988) applied over loci.

The F_{ST} values among pairs of breeds were calculated by using the genepop program (Raymond and Rousset 1995), and their statistical significances tested by permutation tests with the genetix program (Belkhir et al. 1998). The Reynolds' distance (Reynolds et al. 1983), a measure based on F_{ST} values $[D_R = -\ln(1 - F_{ST})]$, with the neighbour-joining (NJ) method of clustering (Saitou and Nei, 1987) was used to construct a dendrogram of breed relationships, using the phylip 3.57c program (Felsenstein, 1995). An unrooted consensus tree, evaluated by 1000 bootstrap replicates, was obtained.

genepop program was used to quantify the effects of migration on the genetic structure and gene flow was estimated between each pair of breeds by converting F_{ST} to amount of gene flow (N_em), according to an island model under neutrality and negligible mutation (Slatkin 1993). N_em indicates the average number of effective migrants exchanged per

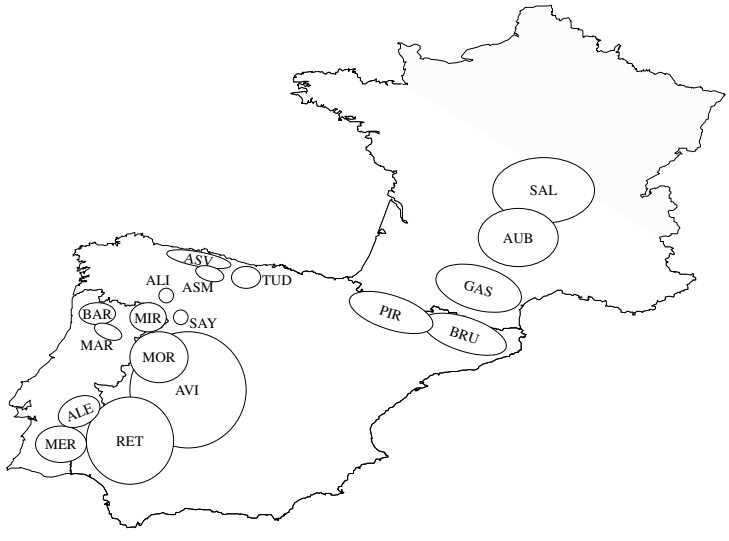

Fig. 1. Geographical location of the 18 South European cattle breeds (see text for acronyms)

generation to produce the observed F_{ST} under the n-island model. The private alleles method (Slatkin 1985) was used to obtain the N_em values.

Slatkin's (1993) and Rousset's (1997) isolation by distance methods were also applied to these cattle populations. In both methods, a linear regression is used to estimate the coefficients:

$$y = \alpha + \beta \log(d) \quad (1)$$

where, in Slatkin's method y is $\log M (M = N_e m)$, as evaluated from population pairwise estimates of Weir and Cockerham's (1984) θ statistic, and in Rousset's method y represents $\theta/(1 - \theta)$. In both methods, d represents the pairwise distance between populations. In order to compare the distance matrices describing genetic and geographical relationships among breeds, the pairwise $\theta/(1 - \theta)$ values were correlated with $\ln(d)$ between each pair of populations and the significance of the association estimated using Mantel's (1967) permutation test (genetix program).

Results

Population differentiation was examined by fixation indices F_{IS}, F_{IT} and F_{ST} for each locus and across all loci. Results of the *F*-statistics for each of the 16 analysed loci in the 18 European bovine breeds are shown in Table 1. We can observe that, as an average, a significant deficit of heterozygotes of 7.6% ($p < 0.001$) exists for each one of the analysed breeds, this deficit being 13.9% ($p < 0.001$) in the whole population. With the exception of ETH 10, ILSTS 5 and INRA 5 (in agreement with Hardy–Weinberg proportions) all markers, but especially INRA 35, contribute to the observed deficit. The significance of INRA 5 ($p < 0.05$ for F_{IT}) is explained, almost exclusively, by the French Gasconne breed.

The average of genetic differentiation among breeds, measured as F_{ST} value, was 6.8% ($p < 0.001$), all loci contributed significantly ($p < 0.001$) to this differentiation. By country of origin (France, Portugal and Spain), we obtain average values of genetic differentiation

Table 1. ***F*-statistics analysis for each of 16 microsatellite markers in 18 European cattle breeds, and mean estimates for these populations**

Locus	$F_{IS} \equiv f$	$F_{IT} \equiv F$	$F_{ST} \equiv \theta$
CSSM 66	0.091***	0.128***	0.042***
ETH 10	−0.028	0.017	0.044***
ETH 152	0.081***	0.152***	0.077***
ETH 225	0.027	0.079***	0.054***
ETH 3	0.086***	0.147***	0.067***
HEL 1	0.058**	0.117***	0.063***
HEL 5	0.051**	0.121***	0.074***
HEL 9	0.088***	0.168***	0.088***
ILSTS 5	−0.055	0.010	0.062***
INRA 23	0.101***	0.198***	0.108***
INRA 32	0.045**	0.115***	0.074***
INRA 35	0.384***	0.444***	0.098***
INRA 37	0.131***	0.210***	0.091***
INRA 5	−0.006	0.044*	0.049***
INRA 63	0.041*	0.076***	0.037***
TGLA 44	0.171***	0.236***	0.079***
Mean estimates†	0.076 (0.019)***	0.139 (0.021)***	0.068 (0.005)***

f, Within-population inbreeding estimate; F, total inbreeding estimate; θ, measure of population differentiation.
†Mean estimates from jack-knife over loci. Standard deviations are given in parentheses.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, from permutation tests in fstat program.

Table 2. ***F*-statistics analysis of the 18 European cattle breeds nested by countries (France, three breeds; Portugal, five breeds; Spain, 10 breeds)**

Country	$F_{IS} \equiv f$	$F_{IT} \equiv F$	$F_{ST} \equiv \theta$
France	0.088 (0.031)***	0.122 (0.031)***	0.038 (0.008)***
Portugal	0.033 (0.021)***	0.101 (0.025)***	0.071 (0.009)***
Spain	0.093 (0.019)***	0.148 (0.021)***	0.060 (0.007)***

f, Within-population inbreeding estimate; F, total inbreeding estimate; θ, measure of population differentiation. Mean estimates from jack-knife over loci. Standard deviations are given in parentheses.
*** $p < 0.001$, from permutation tests in FSTAT program.

between breeds (F_{ST}) of 3.8, 7.1 and 6.0%, respectively (Table 2), highly significant ($p < 0.001$), with the French breeds showing less differentiation.

Table 3 shows the comparison of within-population inbreeding from molecular and genealogical data. All breeds, except Barrosã and Mirandesa, showed a significant heterozygotes deficit, ranging from 4% for the Asturiana de los Valles to 15.1% for the Avileña. Nevertheless, the possible assignment of this deficit to the mating between relatives (inbreeding) has to be evaluated with caution, as inbreeding affects all or most loci in a similar way, and in the present work only the Avileña breed shows 10 loci with significant deficit of heterozygotes. The consanguinity values (F), of some of the breeds, obtained from genealogical information of the studbooks (SB) (Project FAIR1 CT95 702 Final Report) range from 0.2% for Gasconne breed to 7.0% for Alentejana and Sayaguesa breeds. However, the degree of pedigree completeness, measured as the proportion of ancestors known in three preceding generations (great-grandparents), ranged from 4% for Asturiana de los Valles and Bruna dels Pirineus breeds to 64% for Aubrac, Pirenaica and Alentejana breeds.

Table 4 shows F_{ST} estimates and gene flow (N_em), between pairs of populations. After 5000 permutations, performed with GENETIX, all F_{ST} values between pairs of breeds were significantly different from zero ($p < 0.001$). N_em represents the number of effective migrants exchanged per generation. The migrant's number after correction for size, using private alleles method (SLATKIN 1985), averaged 4.38; ranging from 1.4 (Mirandesa–Tudanca pair) to 8.6 (Asturiana de la Montaña–Retinta pair). For countries, the average N_em values were: 4.27 for France, 5.05 for Portugal and 1.02 for Spain, which indicates that a larger reproductive isolation exists among the Spanish breeds.

The application of ROUSSET's (1997) isolation by distance method, as implemented in GENEPOP program, allowed the computation of parameters α and β in equation (1). The values obtained (see Fig. 2) were 0.044 and 0.005 for α and β, respectively, with 2.8% of the total variance explained by the regression model (R^2).

$$F_{ST}/(1 - F_{ST}) = 0.044 + 0.005 \ln(d)$$

However, regression failed to provide enough support for isolation by distance, as indicated by the low R^2 value and by Mantel's test ($G = 1.291$, $r = 0.169$; $p < 0.109$, G being the statistic Z of Mantel normalized; MANLY 1985), that did not support a significant correlation between the genetic and geographical pairwise distances. When the same tests were carried out (data not shown) separately for countries, no significant correlation between both types of distance was obtained.

Lastly, Fig. 3 shows the unrooted consensus tree obtained for the 18 cattle breeds, using the NJ method of clustering with the Reynolds' distance matrix (data not shown). Bootstrap proportions (number of times node was observed in 1000 replicates) are shown at each node. Bootstrap values were generally low indicating that the relationships, inferred from this small number of markers (16 microsatellite loci), are not very robust. Only the

Table 3. **Comparison of within-population inbreeding estimates ($F_{IS} \equiv f$) in 18 European cattle breeds, from molecular and genealogical data**

Locus/breed	Alistana	Alentejana	AsturMont	AsturVall	Aubrac	Avileña	Barrosã	Bruna	Gasconne
(a)									
CSSM 66	−0.025	0.015	0.115**	0.053	0.075*	0.180***	0.032	0.109**	0.091*
ETH 10	0.006	0.018	−0.055	−0.156	−0.058	−0.036	−0.034	0.098	−0.074
ETH 152	0.154**	0.024	0.119*	−0.018	0.298***	0.329***	0.091	0.214***	−0.094
ETH 225	0.145**	−0.053	−0.112	−0.036	0.007	−0.092	−0.050	0.019	0.251***
ETH 3	0.064	0.081	0.114*	0.144**	−0.007	0.157***	0.042	0.138**	0.080
HEL 1	0.028	0.293***	0.101	0.106	0.080	0.279***	−0.002	−0.009	0.105
HEL 5	0.159***	0.090*	0.111**	0.004	−0.063	0.081*	−0.108	0.057	0.003
HEL 9	0.042	−0.080	0.344***	0.053	0.344***	0.113*	0.062	0.023	−0.107
ILSTS 5	0.058	0.013	−0.065	−0.010	0.058	−0.156	−0.397	0.178	0.059
INRA 23	0.068	−0.034	−0.034	−0.006	0.107**	0.262***	0.031	0.196***	0.098*
INRA 32	0.136*	0.062	0.111*	−0.030	0.005	0.021	−0.011	−0.036	0.145**
INRA 35	0.376***	0.137	−0.035	−0.105	0.289***	0.599***	0.098	0.242**	0.691***
INRA 37	0.012	0.285***	0.204***	0.148**	−0.005	0.126*	0.076	0.002	0.157***
INRA 5	0.084	−0.061	0.101	−0.011	0.097	−0.011	−0.067	−0.021	0.228***
INRA 63	−0.022	−0.073	0.118	0.168*	0.095	0.080	−0.005	−0.046	0.061
TGLA 44	0.010	0.147**	0.018	0.288***	−0.075	0.443***	−0.025	0.179***	0.096*
Mean estimates[†]	0.077***	0.050***	0.075***	0.040**	0.068***	0.151***	−0.011	0.079***	0.110***
	(0.023)	(0.029)	(0.031)	(0.029)	(0.030)	(0.044)	(0.023)	(0.023)	(0.044)
Mean F (genealogical data)[‡]	2.5%	7.0%	2.7%	1.5%	0.9%	5.1%	No data	1.6%	0.2%
Pedigree completeness	20%	64%	10%	4%	65%	45%		4%	9%

Table 3. **Continued**

Locus/breed	Maronesa	Mertolenga	Mirandesa	Morucha	Pirenaica	Retinta	Salers	Sayaguesa	Tudanca
(b)									
CSSM 66	0.038	0.208***	0.012	0.112**	0.004	0.103*	0.175***	0.221***	0.079*
ETH 10	−0.002	−0.133	−0.243	0.026	−0.017	0.001	−0.031	0.183**	0.040
ETH 152	0.272***	0.113*	−0.029	0.188**	0.056	0.078	−0.183	−0.015	−0.182
ETH 225	−0.049	−0.163	0.127*	0.170**	0.105	0.154**	0.060	0.072	−0.118
ETH 3	−0.031	0.209***	−0.049	0.017	0.118*	0.102*	0.105	0.035	0.229***
HEL 1	0.015	0.134*	−0.119	0.109	0.081	0.049	0.101	0.004	−0.411
HEL 5	0.103*	0.112*	−0.038	0.006	0.091	0.108**	0.041	0.071	0.013
HEL 9	0.122*	0.100*	−0.093	0.086*	0.200***	0.021	0.029	0.087*	0.227***
ILSTS 5	−0.157	−0.039	−0.095	0.091	0.263**	−0.183	−0.015	−0.162	−0.157
INRA 23	0.289***	0.038	0.096	0.065	0.126*	0.191***	0.065	−0.071	0.342***
INRA 32	0.019	0.104*	0.011	0.233***	−0.061	0.004	−0.121	0.135**	0.049
INRA 35	0.008	0.154	0.453***	0.051	0.597***	0.685***	0.694***	0.322***	0.697***
INRA 37	0.108	0.066	−0.025	0.003	0.165**	0.342***	0.109	0.262***	0.261***
INRA 5	−0.305	−0.124	−0.108	−0.015	0.102	−0.008	0.129*	−0.066	−0.043
INRA 63	−0.065	−0.052	0.215**	0.151**	0.034	−0.014	0.219***	−0.010	−0.118
TGLA 44	0.289***	0.316***	0.115*	0.228***	0.767***	0.167***	0.020	0.080	0.212***
Mean estimates[†]	0.045***	0.068***	0.015	0.100***	0.134	0.116***	0.081***	0.076***	0.086***
	(0.041)	(0.036)	(0.040)	(0.022)	(0.044)***	(0.039)	(0.043)	(0.029)	(0.056)
Mean *F* (genealogical data)[‡]	No data	5.7%	No data	6.7%	2.5%	No data	1.5%	7.0%	No data
Pedigree completeness		46%		15%	63%		48%	27%	

[†]Mean estimates from jack-knife over loci. Standard deviations in parentheses.
[‡]Data obtained from Final Report FAIR1 CT95 702.
*p < 0.05, **p < 0.01, ***p < 0.001, from permutation tests in FSTAT program.

Table 4. **F_{ST} estimates (below the diagonal) and gene flow N_em[†] (above the diagonal) between pairs of European cattle populations**

Breeds	ALI	ALE	ASM	ASV	AUB	AVI	BAR	BRU	GAS	MAR	MER	MIR	MOR	PIR	RET	SAL	SAY	TUD
Alistana		3.5	4.7	4.3	2.4	4.1	3.6	3.8	2.9	3.1	3.5	4.3	3.4	3.1	3.7	2.6	2.6	2.9
Alentejana	0.066		5.1	5.0	2.4	4.2	4.1	3.3	3.0	3.6	6.2	2.3	5.0	3.1	4.9	2.4	2.1	2.5
AsturMont	0.050	0.046		7.5	3.5	4.5	6.1	4.7	5.7	5.7	5.4	2.6	6.7	4.1	8.6	4.0	3.8	4.0
AsturVall	0.055	0.048	0.032		3.2	5.1	4.1	5.7	4.9	3.8	4.7	2.4	6.6	5.1	5.5	4.1	3.2	3.4
Aubrac	0.096	0.093	0.067	0.073		4.6	2.3	3.2	5.7	2.4	3.0	1.8	3.6	3.9	2.9	8.5	2.3	2.5
Avileña	0.058	0.056	0.053	0.047	0.052		3.7	5.1	4.3	3.3	4.1	2.8	5.3	3.9	4.4	4.1	3.1	3.2
Barrosã	0.065	0.057	0.039	0.058	0.098	0.063		3.4	3.5	5.3	5.6	2.2	5.3	2.6	4.6	2.3	3.5	3.8
Bruna	0.062	0.071	0.050	0.042	0.072	0.047	0.069		4.6	3.0	3.8	1.9	5.7	6.0	4.2	4.0	2.6	3.2
Gasconne	0.078	0.078	0.042	0.049	0.042	0.055	0.067	0.051		3.4	3.7	2.1	5.0	6.2	5.0	5.7	2.8	3.2
Maronesa	0.075	0.065	0.042	0.061	0.096	0.070	0.045	0.078	0.068		4.1	2.0	4.0	2.8	4.8	3.0	3.0	2.9
Mertolenga	0.067	0.038	0.044	0.051	0.078	0.058	0.043	0.061	0.063	0.057		2.5	4.9	4.2	6.2	3.2	2.6	2.6
Mirandesa	0.055	0.098	0.086	0.096	0.121	0.083	0.103	0.116	0.106	0.111	0.090		2.1	1.9	2.5	1.8	1.7	1.4
Morucha	0.069	0.048	0.036	0.037	0.066	0.045	0.045	0.042	0.048	0.059	0.049	0.108		3.8	5.0	3.9	3.0	4.3
Pirenaica	0.074	0.074	0.058	0.047	0.060	0.060	0.087	0.040	0.039	0.083	0.056	0.118	0.062		4.0	4.2	2.0	2.3
Retinta	0.064	0.049	0.028	0.043	0.080	0.054	0.052	0.056	0.047	0.049	0.039	0.091	0.048	0.059		3.4	3.8	3.5
Salers	0.087	0.094	0.059	0.057	0.029	0.058	0.099	0.059	0.042	0.077	0.071	0.120	0.061	0.056	0.069		2.3	2.5
Sayaguesa	0.086	0.105	0.062	0.073	0.099	0.076	0.067	0.089	0.082	0.078	0.086	0.131	0.076	0.110	0.062	0.097		3.2
Tudanca	0.080	0.091	0.059	0.068	0.090	0.073	0.061	0.072	0.073	0.079	0.087	0.148	0.055	0.099	0.066	0.091	0.072	

[†]N_em, number of effective migrants exchange per generation.

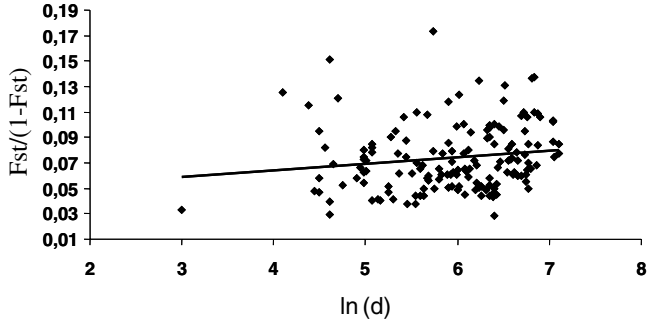

Fig. 2. Plot of relationship between geographical distance, ln(d), and pairwise $F_{ST}/(1 - F_{ST})$ for all pairs of European cattle populations. The fitted line correspond to the equation $F_{ST}/(1 - F_{ST}) = 0.044 + 0.055 \ln(d)$, obtained by least squares linear regression. The fraction of variance explained by this regression, R^2, represents 2.8% of the total variance. The low R^2 value and the Mantel test (see text for details) did not show enough support for a significant correlation between the genetic and geographical pairwise distances

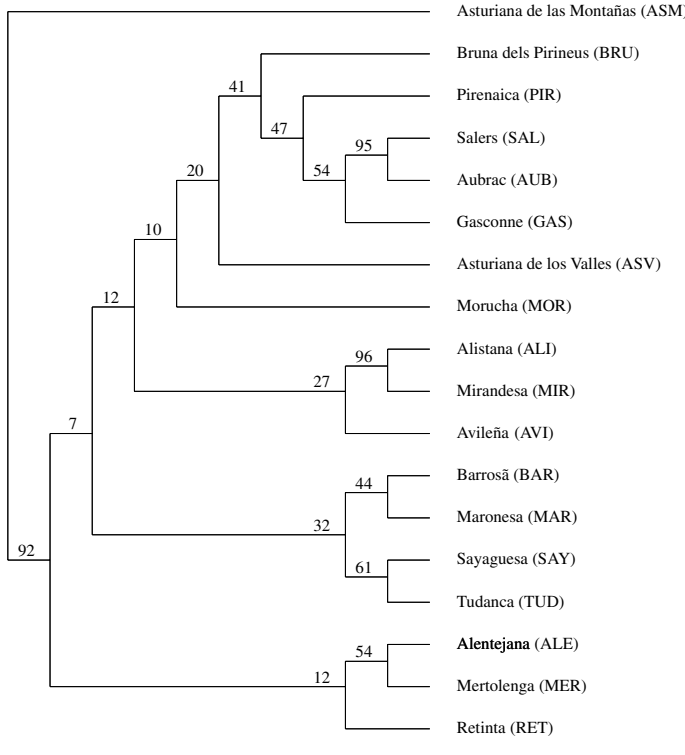

Fig. 3. Unrooted consensus tree showing the genetic relationships among 18 South European cattle breeds, using the neighbour-joining method and the Reynolds' genetic distance. Numbers at the nodes are the values for 1000 bootstrap resamplings of the 16 microsatellites genotyped

clusters formed by the Salers–Aubrac and the Alistana–Mirandesa breeds, overcome the 95% confidence level.

Discussion

On average, the genetic differentiation (F_{ST}) among breeds was 6.8% (Table 1), a relatively low but highly significant ($p < 0.001$) value. All loci contribute to this differentiation. However, it is clear that most of the total genetic variation corresponds to differences among individuals (93.2%) and only <7% is the result of differences among breeds.

This value of genetic differentiation (F_{ST}) among cattle breeds is very similar to the values reported for other species. For example, among the three major human groups, Negroid, Mongoloid and Caucasoid, $F_{ST} = 0.088$ (Nei and Roychoudhury 1982), among Spanish dog breeds, $F_{ST} = 0.099$ (Jordana et al. 1992), $F_{ST} = 0.108$ (Morera et al. 1999), river buffalo breeds, $F_{ST} = 0.038$ (Barker et al. 1997), Spanish horse breeds, $F_{ST} = 0.078$ (Cañón et al. 2000), although lower than in Swiss goat breeds, $F_{ST} = 0.170$ (Saitbekova et al. 1999), in European wild rabbits, $F_{ST} = 0.150$ (Surridge et al. 1999), and among European pig breeds, $F_{ST} = 0.270$ (Laval et al. 2000).

In cattle, values of genetic differentiation have been reported for seven European breeds, $F_{ST} = 0.112$ (MacHugh et al. 1998), Swiss breeds, $F_{ST} = 0.090$ (Schmid et al. 1999), Belgian breeds, $F_{ST} = 0.035$ (Mommens et al. 1999), and 20 North European cattle breeds $F_{ST} = 0.107$ (Kantanen et al. 2000).

For populations mating at random, genes are equally related within or between individuals. In this case $F = \theta$ or $f = 0$. Therefore estimates of F and θ that differ significantly indicate departures from random mating. Any avoidance of mating of relatives will cause θ to exceed F and f to be negative. More commonly, f is positive ($F > \theta$), which could be interpreted as evidence of inbreeding (Weir 1996).

In our study (Table 1) f is positive ($f = 0.076$), and $F = 0.139 > \theta = 0.068$. Neglecting the effects of migration, and assuming a low contribution of mutations to the genetic diversity between these breeds, the differences in allele frequencies may be interpreted as primarily the result of random genetic drift. The genetic differentiation (6.8%) may be seen as the result of an increased mean inbreeding coefficient F over a rather short period of time (Laval et al. 2000). We therefore consider the relatively low mean F_{IS} value (0.076) to be the result of a reduction of heterozygosity within the breeds studied and the relatively high mean F_{IT} value (0.139) as indicative of effective barriers to gene flow between any populations.

The deficit of heterozygotes (measured as F_{IS} or f) for each one of the 18 analysed breeds is given in Table 3. All values were statistically different from zero, with the exception of the Portuguese breeds Barrosã and Mirandesa. The consanguinity, produced by mating between relatives, can be one of the causes for loss of heterozygotes, but this deficit affects all or most of the loci in a similar way. Only the Avileña breed showed 10 of the total of 16 loci, with a significant deficit of heterozygotes. Therefore we might consider consanguinity as the principal cause of loss of heterozygotes, a conclusion that we cannot extrapolate to the remainder of the breeds although differences between them are rarely significant.

Other factors can also cause a lack of heterozygotes in a population (Nei 1987). First, the locus can be under selection, the 'genetic hitchhiking' effect, being close to some morphological or productive trait of selective interest. Secondly, 'null alleles' (non-amplifying alleles) may be present and lead to a false observation of homozygotes. Thirdly, the presence of population substructure within the breed may lead to Wahlund's effect.

A better and more reliable estimate of the consanguinity would be that obtained from genealogical information of the SB of the breeds. Some values of these estimates are shown in Table 3. However these values, of course, are very dependent on the quantity (animals registered in SB) and quality of data (percentage of ancestors known in the whole pedigree

data file). If pedigree information is very incomplete, the obtained F values will therefore be underestimated.

As a general conclusion of the estimates of consanguinity, we can postulate that the true F value would be positioned within the interval between the estimate of the genealogical data (underestimated values, as pedigrees are rather incomplete) and the estimate from the molecular data (overestimated values).

The most reliable estimates of F, from genealogical data (pedigree completeness >60%), would be from Alentejana ($F = 7.0\%$, $f = 5.0\%$), Aubrac ($F = 0.9\%$, $f = 6.8\%$) and Pirenaica ($F = 2.5\%$, $f = 13.4\%$) breeds. In the Aubrac and Pirenaica breeds, these results are in close agreement with the analysis of the F_{IS}-statistic per locus and population (Table 3). Only 5 and 7, of the total of 16 loci, for the Aubrac and Pirenaica breeds, respectively, showed significant deficit of heterozygotes, therefore this deficit cannot be essentially attributed to consanguinity.

The population substructure within the breed, in breeding units, more or less large, and more or less isolated, would be a very feasible explanation to understand the high deficit of heterozygotes observed in some loci (Wahlund's effect).

Nevertheless, two loci (INRA 35 and TGLA 44), show a very significant deficit of heterozygotes in almost all the analysed breeds; 11 breeds for INRA 35 and 12 breeds for TGLA 44. The most likely interpretation to explain this deficit in these two markers is that they can be under selection (genetic hitchhiking effect) with some trait of selective interest.

Possibly the marker CSSM 66 could also be included in this group, although the interpretation is much more doubtful, because, although it shows significant deficit in 11 of the 16 analysed breeds, only in four of them this deficit was highly significant ($p < 0.001$). However, Coppieters et al. (1998) detected a close genetic linkage between CSSM 66 and a quantitative trait loci (QTL) affecting milk yield, milk fat and protein composition in the Holstein–Friesian populations.

In the breeds analysed in this work, the effect of genetic hitchhiking has been described in the Asturiana de los Valles breed for the TGLA 44 locus (Dunner et al. 1997) in relation to the myostatin gene (muscular hypertrophy gene), responsible for double muscling (Ménissier 1982; Charlier et al. 1995; Grobet et al. 1997).

Linkage of this marker with the gene has also been described in other breeds, for example in Charolais, Belgian Blue, Piedmontese and Chianina (Charlier et al. 1995; Casas et al. 1997). Thus, its association is quite distributed among breeds. Nevertheless, it has not been reported in 17 breeds of this study. However, the mating of local cows with sires of specialized meat breeds (i.e. Charolais, Limousin, etc) that manifest the hypertrophy phenomenon clearly, has been a quite frequent practice. Therefore, it is possible that this mutation is present in some breeds of this study (e.g. Bruna dels Pirineus and Pirenaica breeds) and that breeders maintain the mutation through a phenotypic selection for better conformation.

For the marker INRA 35, a similar explanation may also be possible, with some other trait of general selective interest for the breeders, although up to the present day no selective association has been described for this marker. However, the high deficit of heterozygotes observed in the majority of breeds, may be the result of 'null alleles' in this marker, although these should be ancestral and of wide geographical and racial distribution. Kantanen et al. (2000) proposed the same explanation, in order to argue the exceptionally high deficit of heterozygotes observed in a total of 20 North European cattle breeds ($F_{IS} = 0.397$), for the same marker.

However, the situation is reverse in the Alentejana breed. The analysis of the genetic markers tells us that the 5% deficit of heterozygotes could not be attributed to inbreeding.

Two loci (HEL 1 and INRA 37) show very high and significant deficit of heterozygotes ($p < 0.001$), and the reproductive substructure within the breed would be the most coherent explanation for this deficit. However, the pedigree analysis, with a 64% of thoroughness, estimates an F of 7%. The two consanguinity approaches do not agree, and it

is difficult to explain the cause. Possibly a poor quality of the genealogical information in the assignment of paternities is responsible.

The same problem exists for the Sayaguesa and Mertolenga breeds. The F estimates, obtained from genealogical data, are too high compared with the value obtained from molecular data, with the added difficulty of the low pedigree completeness values (27 and 46%, respectively), with the consequence that the genealogical estimates are underestimated. For the remaining breeds, the results obtained from genealogical and molecular data are in good agreement.

Table 4 shows F_{ST} estimates and gene flow ($N_e m$) between pairs of breeds. The populations included in this study are characterized by (a) widespread regional distribution, (b) small population sizes and (c) breeds linked to traditional production systems. As most of the breeds have not been subject to reproductive technology or other breeding tools related to artificial discriminative mating, gene flow between breeds should have been very limited, with individual dispersion only at a local level (FAIR1 CT95 702 Final Report).

However, this empirical observation, with regard to possible gene flow among the breeds analysed in this study, contrasts with our results. On average, the genetic differentiation (F_{ST}) among breeds was 6.8% ($p < 0.001$), similar to other breeds, and an average gene flow of 4.38 effective migrants per generation.

The gene flow ranges from 1.4 to 8.6 between pairs of populations. Trexler (1988) showed that if $N_e m > 1$ (in an infinite island model), gene flow is enough to attenuate the genetic differentiation between populations. However, the $F_{ST} = 0.068$ value, is significantly different from zero ($p < 0.001$), among the 18 analysed breeds, indicating significant subpopulation structure within the south European bovine breeds. Genetic drift could be the factor which contributed most to the observed inter-breed differentiation, because of the low effective population size in the breeds studied.

If we assume that little genetic exchange has existed between these breeds (local beef cattle, small population size, few or no artificial insemination, absence of breed improvement programmes, non-defined objectives of selection, non-evaluated sires and dams, etc), we could not attribute the low F_{ST} values observed between the breeds (see Table 4) to gene flow. The likely explanation for these results can be related to the fact that these breeds separated from their ancestral common trunk only a short time ago (MacHugh et al. 1997).

Genetic migration would have played a relatively important role only between populations of close geographical vicinity (see Fig. 1). This could be the case for the three French breeds (Aubrac, Gasconne and Salers), the two Pyrenean Spanish breeds (Bruna dels Pirineus and Pirenaica) and also for the Portuguese breeds (Alentejana, Barrosã, Maronesa and Mertolenga), and between Mertolenga and the Retinta Spaniard breed. In the Spanish breeds, a possible gene flow exists between the two Asturian breeds (Asturiana de las Montañas and Asturiana de los Valles), between the Avileña and Morucha breeds, and the previously mentioned Bruna and Pirenaica breeds. However, the great gene flow ($N_e m = 8.6$, higher obtained value) between the Asturiana de las Montañas and Retinta breeds, located in opposing ends of the Iberian Peninsula, seems to be unlikely.

The analysis of the Mirandesa breed requires to be mentioned. F_{ST} and gene flow estimates indicate that this population has maintained, and maintains, an important genetic isolation from all other breeds. Only some exchange of breeding animals with the Alistana breed could have existed. A similar situation, although of minor grade, exists in the Spanish Sayaguesa and Tudanca breeds – local and very small breeds – that have maintained reproductive isolation with the other populations.

Although genetic migration between neighbouring populations could have been relatively important in some cases, isolation by distance between the 18 analysed breeds has not been detected. The correlation obtained between genetic and geographical distances was low and non-significant ($r = 0.169$; $p < 0.109$), with only 2.8% of the total variance explained by the regression model (R^2) (see Fig. 2). Therefore genetic drift

appears as the most important factor of genetic differentiation among the analysed populations. Additionally, the inferred conclusions from indirect estimates of gene flow, integral to interpretation of microevolutionary patterns and geographical structure, should be taken with much caution. According to Bossart and Prowell (1998), indirect methods using genetic markers are often ambiguous and open to multiple interpretations. Studies of paternity, kinship and phylogeography generate the most reliable results.

Lastly, Fig. 3 shows the unrooted consensus tree obtained for the 18 cattle breeds, using the NJ method of clustering with the Reynolds distance, considered appropriate for our data because genetic drift is assumed to be the main factor in genetic differentiation among closely related populations or for short-term evolution (Reynolds et al. 1983; Weir 1996; Takezaki and Nei 1996). As the low bootstrap values indicate, the relationships in the dendrogram are not too robust.

The several racial groups do not follow a clear ethnologic pattern (Epstein 1972; Sánchez Belda 1984; Sotillo and Serrano 1985; Alderson 1992). Neither do they show good agreement with the relationships obtained from morphological data (Jordana et al. 1991). And lastly, they neither follow a hierarchical structure associated with geography (significant isolation by distance does not exist, as is shown in the present study). Possibly the racial relationships obtained with microsatellites reflect a combination of the three patterns previously commented.

The apparent distinctiveness of the breeds could be, in an important way, because of a random drift, which can affect the genetic distances among populations. When a population is subject to a bottleneck effect, the genetic distances also increase quickly. This increase of genetic distances might subsequently distort the topology of the evolutive trees (Nei and Roychoudhury 1982; Nei 1987; Takezaki and Nei 1996). Therefore, it should be emphasized that the dendrogram shows the current genetic relationships among breeds, and it cannot show the actual evolutionary history of populations if these are not completely isolated (Nei 1987).

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