

# Genetic diversity within and among endangered local cattle breeds from Tuscany (Italy)



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## SUMMARY

Italy has a rich diversity in farm animals. In particular, Tuscany hosts a number of autochthonous bovine breeds that are, or have been, relevant for the economic sustainability of the agriculture, the origin and history of which are still poorly documented. Most of these breeds suffered in the past a dramatic loss of economic importance being substituted by cosmopolite and more productive breeds. The aim of the present study was to analyse levels of genetic diversity and relationship among local cattle breeds from Tuscany (Central Italy) using microsatellite markers. DNA from 194 animals (Calvana, 61; Garfagnina, 43; Pontremolese, 23; Chianina, 67) was analyzed at 22 microsatellite loci. Number of alleles (NA) and allelic frequencies were estimated. Locus by locus  $F_{ST}$ , exact tests for deviations from the Hardy-Weinberg equilibrium (HWE) and pair-wise linkage disequilibrium (LD) among microsatellite loci were evaluated. In addition, various among-population genetic distances were calculated. The study evidenced a significant departure from Hardy-Weinberg equilibrium in all the analyzed breeds, probably caused by a rather high level of inbreeding for Calvana, Garfagnina and Pontremolese, whereas likely reflecting the results of recent selective processes in Chianina. The overall fixation index was  $0.115 \pm 0.054$ . Results obtained in this study suggest that the situation of the analyzed breeds is risky as their variability is compromised by a high level of inbreeding, in particular Pontremolese was the most compromised breed. Several distance metrics highlighted a only moderate differentiation between Chianina and Calvana if compared to values found among the other breeds. Also molecular coancestry and kinship distance supported the hypothesis that Calvana originated from Chianina, whereas Garfagnina and Pontremolese should have different ancestral origins. The low genetic variability suggests the need for a careful genetic management of the breeds, in order to maintain long-term population survival and adaptability and to avoid the risk of more excessive increase in the inbreeding level, which would result in significant inbreeding depression and in significant loss of genetic variation.

## KEY WORDS

Genetic variability, cattle breeds, microsatellite markers.

## INTRODUCTION

During the last century, many indigenous domestic breeds became extinct by replacement or crossbreeding with exotic breeds, substitution of draft animals by technology, or unfavourable marketing scenarios. Because of its geographical history, Italy has a rich diversity in farm animals. In particular, Tuscany hosts a number of autochthonous bovine breeds that are, or have been, relevant for the economic sustainability of the agriculture, the origin and history of which are still poorly documented. Most of these breeds suffered in the past a dramatic loss of economic importance being substituted by cosmopolite and more productive breeds. This dramatic size contraction highlights the need for conservation of native genetic resources. In 1985, the Anagraphic Register of local cattle breeds and small-sized ethnic groups was established by the Italian Ministry of Agriculture and Forestry; Calvana, Pontremolese and Garfagnina are among the included breeds. They are all native from Tuscany.

Molecular characterization of animal genetic resources may contribute to a rational approach to conservation<sup>1</sup> by giving a high priority to breeds that are taxonomically most dis-

tinct. For this aim several molecular-based parameters, such as similarity indexes, can be adopted to optimize the management of genetic diversity.

The present study was undertaken with the aim to assess the genetic diversity both within and between breeds and to establishing the relationship among four Tuscany autochthonous cattle breeds, Calvana (CA), Garfagnina (GA), Pontremolese (PO) and Chianina (CH) using a set of 22 bovine-specifying microsatellite markers and evaluating within- and between-breed molecular coancestry, together with other classical genetic parameters.

## MATERIAL AND METHODS

The study was performed on 194 animals belonging to the local cattle breeds Chianina (67), Calvana (61), Garfagnina (43) and Pontremolese (23). Animals from the first three breeds were chosen in order to assure breed representativeness (un-related animals originating from different herds spread all over the rearing area), while all the last living Pontremolese animals were sampled from a single farm.

The Calvana breed is thought to share, together with Maremmana, Romagnola, Marchigiana and Podolica, a *Bos primigenius* x *Bos brachyceros* ancestry and to have originated in the Tuscan Apennine area by recurring introgression of

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ancient-type Chianina blood starting from at least the early Middle Age. The breed counts nowadays 470 heads reared mainly in the provinces of Firenze and Prato, followed by Siena (3 farms) and Pistoia (2 farms). The breed is today recognized by FAO as critical-maintained.

Several hypotheses have been formulated around the origins of the Pontremolese cattle but they still remain uncertain. Despite the begin of a regional conservation program that, only six years later, brought the breed consistencies to almost triplicate, in 2005 the number of animals had again dropped down to 29 (25 of which reared in a single farm). The breed is today recognized by FAO as critical-maintained.

The Garfagnina breed belongs to a wider group, known under different names depending on the place, thought to have originated from the ancient Podolian cattle, that populated, during the 30's, the Provinces of Lucca, Massa Carrara, Reggio Emilia and Modena. Nowadays the breed counts 311 heads reared in 26 farms; they are all crossbred animals derived from Bruna Alpina bulls mated to the few remaining Garfagnina cows. The breed is today recognized by FAO as endangered-maintained.

A quite different scenario happened to the Chianina cattle: widely spread in the hilly zones of Tuscany and Umbria since very old times, the breed underwent a marked number decline starting from the 1950s till 1980s when, due to mutated consumption trends and renewed interest for local breed beef, it turned to assume economic relevance by entering modern meat distribution systems.

It is thought that in the far past podolian animals may have been introgressed into Chianina, thus explaining why calves are born fawn in colour while turning white before reaching the yearling age. The breed counts today over forty-six thousand registered heads.

Genomic DNA was extracted from 5 mL of peripheral blood samples and DNA was isolated using the Genelute blood genomic DNA kit (Sigma-Aldrich, Milano, Italy).

The 22 considered Short Tandem Repeat (STR) markers (Table 1) were located on 13 different chromosomes. Among these, ten (BM1818, BM1824, ETH10, ETH152, ETH3, HEL9, ILST006, INRA63, TGLA126, TGLA227) belonged to the panel of 30 microsatellites recommended by the ISAG-FAO Working Group on domestic animal diversity<sup>2</sup>. STR were amplified in four multiplexes using fluorescently-labelled primers. PCR products were detected by capillary electrophoresis using an Applied Biosystems 310 DNA Sequencer with GENEMAPPER Analysis software (ABI).

Number of alleles (NA) and allelic frequencies were estimated by direct counting. In order to correct for different breed sample-sizes, allelic richness (AR) and private allele richness (PAR) were calculated using the software HP-Rare by rarefying breed samples to 20 individuals each. The presence of null (not amplifying) alleles was tested using the software MICROCHECKER v. 2.2.3.

Locus by locus  $F_{ST}$ , exact tests for deviations from the Hardy-Weinberg equilibrium (HWE) and pair-wise linkage disequilibrium (LD) among microsatellite loci were evaluated using the ARLEQUIN package v. 3.11. The software was also adopted to estimate differentiation among breeds in terms of population pair-wise  $F_{ST}$ . In addition, various among-population genetic distances implemented in the programs PowerMarker v. 3.25 and Genetix v. 4.05.2 were calculated and the relative consensus Neighbour-Joining

(NJ) trees based on 1000 pseudoreplicates from bootstrap analysis were obtained using PHYLIP v. 3.6. At the inter-individual level, the Nei's  $D_A$  genetic distance was calculated by PowerMarker v. 3.25 and used to construct a NJ dendrogram via MEGA v. 4.1.

Breed differentiation was also investigated using the Bayesian clustering algorithm implemented in the STRUCTURE software v. 2.2; we adopted the "admixture model", without providing *a priori* information on population membership. Ten independent runs were performed for each K value from 2 to 10 (where K is the number of cluster to be tested), adopting a burn-in period of 100,000 generations, followed by 100,000 iterations. Moreover, a likelihood test of breed assignment was performed using the ARLEQUIN package v. 3.11.

Molecular coancestry coefficients and kinship distance were computed at the population level using the program Molkin. The molecular coancestry of an individual  $i$  with itself is self-coancestry ( $s_i$ ), which is related to the coefficient of inbreeding of an individual  $i$  ( $F_i$ ) by the formula  $F_i = 2s_i - 1$ . In turn, the kinship distance ( $D_k$ ) between two individuals  $i$  and  $j$  is given by  $D_k = [(s_i + s_j)/2] - f_{ij}$ , where  $f_{ij}$  is the molecular coancestry between the two individuals, i.e. the probability that two randomly sampled alleles from the same locus in the two individuals are identical by state. MolKin computes within- and between-breed molecular coancestry and  $D_k$  by simply averaging the corresponding values for all the within- or between-population pairs of individuals.

## RESULTS

All markers were polymorphic across the four analyzed populations (Table 1), with a mean number of 10.86 ( $\pm 4.2$ ) alleles per locus, ranging from 5 (ETH10, TGLA126) to 20 (BMS1818). Within-population mean number of alleles did not vary significantly among the four population samples, with the exception of the CHI vs. PON comparison ( $P < 0.001$ ); however, no significant differences were observed after applying the rarefaction method implemented in the HP-Rare software in order to correct for variation in sample size (data not shown).

Private alleles were found in all the studied breeds, though only few alleles (Calvana, 3; Garfagnina, 1) scored a frequency higher than 0.2. No significant differences among the four breeds were observed in terms of private allele richness values (0.55, CAL; 0.70, GAR; 0.86, PON; 1.09, CHI; data not shown). Interestingly, all the seven alleles observed at the ETH152 locus in the Calvana breed were absent from the Chianina sample (while being present at a different extent in the other breeds) and all the six ETH152 alleles observed in the Chianina breed were absent from the Calvana and the Garfagnina samples, thus explaining the highest  $F_{ST}$  value observed for the ETH152 locus (Table 1). These results are quite unexpected due to the fact that Calvana has been widely considered as an ecotype of Chianina or, alternatively, as a separate breed still closely related to Chianina.

The average locus-by-locus  $F_{ST}$  value was  $0.115 \pm 0.054$ . The average observed heterozygosity ( $H_{OBS}$ ) in the total sample was  $0.486 \pm 0.117$ ; the within-breed values varied from a minimum of  $0.353 \pm 0.211$  in Pontremolese to a maximum of  $0.603 \pm 0.149$  in Chianina. The average expected heterozygosity

**Table 1** - Microsatellite markers with the corresponding chromosomal location (Chr), number of detected alleles ( $N_A$ ) in the total sample and within each breed, and locus by locus  $F_{ST}$ .

Locus	Chr	$N_A$					$F_{ST}$
		Tot	CAL	CHI	GAR	PON	
BM143	6	12	8	6	5	7	0.157
BMS1678	14	6	5	6	5	5	0.065
ETH131	21	14	7	8	6	11	0.120
ETH152	5	14	7	6	7	7	0.221
INRA53	7	12	4	9	7	6	0.185
TGLA227	18	13	6	7	9	9	0.106
BM1508	14	6	5	4	3	4	0.058
BM1824	1	10	8	7	5	7	0.087
HEL9	8	12	8	9	9	6	0.085
ILSTS006	7	9	6	5	9	7	0.124
INRA11	1	11	8	7	5	5	0.039
INRA63	18	8	6	5	5	5	0.026
TGLA126	20	5	5	5	4	3	0.175
BM1818	23	20	6	17	9	5	0.093
BMS690	6	16	12	11	9	5	0.037
BMS518	6	16	7	15	10	2	0.067
ETH3	19	11	11	6	7	5	0.150
TGLA304	20	17	10	13	8	4	0.141
BMS1747	14	6	5	6	6	3	0.140
BMS1866	2	8	6	6	6	7	0.166
ETH10	5	5	4	4	4	5	0.177
URB011	29	8	7	6	4	6	0.117
Average ( $\pm$ SD)		10.86 ( $\pm$ 4.2)	6.86 ( $\pm$ 2.1)	7.64 ( $\pm$ 3.47)	6.45 ( $\pm$ 2.06)	5.64 ( $\pm$ 2.01)	0.115 ( $\pm$ 0.054)

ity ( $H_{EXP}$ ) was  $0.734 \pm 0.104$  in the total sample, ranging from  $0.603 \pm 0.181$  in Pontremolese to  $0.689 \pm 0.111$  in Garfagnina (Table 2). Within each of the four breeds, several markers were in significant ( $P < 0.01$ ) Hardy-Weinberg disequilibrium (CAL, 95.45%; GAR, 95.45%; PON, 68.18%; CHI, 22.73%) almost exclusively due to heterozygote deficiency.

Heterozygote deficiency may arise also as a consequence of the presence of null (not amplifying) alleles; null alleles have been previously reported for several cattle loci and, among the markers used in our study, for the loci and HEL9. Therefore, we tested the hypothesis of null alleles by using the algorithms implemented in the MICROCHECKER package. The results suggested the possible presence of null alleles at all the 22 loci, which is a quite unlikely scenario.

A significant ( $P < 0.01$ ) pair-wise linkage disequilibrium (or gametic imbalance, for unlinked loci) was observed in the total sample for 81% of all the possible locus-by-locus comparisons ( $N = 231$ ), the within-breeds values being, respectively, 21% in PON, 17% in CHI, 13% in GAR and 9% in CAL (data not shown).

Table 3 shows, for each breed and for the total sample, the average inbreeding coefficients ( $F$ ) as obtained from self-coancestry ( $s_i$ ) using the software Molkin, heterozygote deficiency values ( $F_{IS}$ ), molecular coancestry estimates ( $f_{ij}$ ), kinship distances ( $D_K$ ) and genetic similarities (GS). Average inbreeding coefficient and heterozygote deficiency values were

highly correlated ( $r = 0.99$ ). The highest values of  $F$  and  $F_{IS}$  were observed in Pontremolese (0.648 and 0.406, respectively) while the lowest were observed in Chianina (0.369 and 0.095, respectively).

Molecular coancestry ranged from 0.319 (GAR) to 0.407 (PON) suggesting that Pontremolese had the lowest and Garfagnina the highest ancestral within-breed genetic variability. Kinship distance ranged from 0.366 in CHI to 0.456 in CAL suggesting that Chianina has the lowest and Calvana the highest recent within-breed genetic variability. Genetic similarity values observed within the four breeds were higher than those reported for Podolica, a local cattle breed from Southern Italy<sup>3</sup> (0.281).

Pair-wise  $F_{ST}$  values (Table 4) highlighted a significant ( $P < 0.001$ ) genetic differentiation among the four breeds, the highest values being observed among Pontremolese and the other breeds, while by far the lowest value (0.061) being observed between CAL and CHI. Also genetic similarity values (0.332) suggested CAL and CHI as being the most related breeds.

The branching pattern of the NJ dendrograms based on the pair-wise distances always showed a robust Chianina-Calvana cluster, with an average  $0.96 \pm 0.03$  bootstrap value supporting the node (Figure 1). The NJ dendrogram based on inter-individual Nei's ( $D_A$ ) genetic distance shown in Figure 2 also supports the higher relatedness between CAL and CHI animals.

**Table 2** - Expected ( $H_{EXP}$ ) and observed ( $H_{OBS}$ ) heterozygosity, exact test of Hardy-Weinberg equilibrium (HWE).

	CAL			CHI			GAR			PON		
	$H_{OBS}$	$H_{EXP}$	P									
BM143	0.574	0.731	0.001	0.627	0.675	0.505	0.442	0.689	0.000	0.435	0.615	0.005
BMS1678	0.508	0.631	0.000	0.627	0.549	0.926	0.233	0.752	0.000	0.087	0.584	0.000
ETH131	0.492	0.814	0.000	0.774	0.771	0.302	0.326	0.629	0.000	0.652	0.798	0.029
ETH152	0.443	0.770	0.000	0.656	0.741	0.290	0.465	0.644	0.000	0.435	0.752	0.000
INRA53	0.328	0.433	0.004	0.403	0.614	0.000	0.837	0.770	0.000	0.609	0.657	0.047
TGLA227	0.574	0.719	0.000	0.776	0.807	0.419	0.721	0.780	0.008	0.652	0.836	0.056
BM1508	0.246	0.471	0.000	0.537	0.614	0.525	0.442	0.657	0.000	0.348	0.657	0.000
BM1824	0.508	0.751	0.000	0.567	0.611	0.020	0.581	0.747	0.000	0.522	0.750	0.000
HEL9	0.541	0.807	0.000	0.821	0.819	0.664	0.558	0.711	0.004	0.478	0.741	0.008
ILST006	0.607	0.718	0.038	0.582	0.688	0.089	0.558	0.824	0.000	0.174	0.676	0.000
INRA11	0.197	0.479	0.000	0.484	0.480	0.520	0.116	0.275	0.000	0.087	0.533	0.000
INRA63	0.180	0.680	0.000	0.710	0.682	0.016	0.419	0.721	0.000	0.130	0.698	0.000
TGLA126	0.361	0.647	0.000	0.776	0.768	0.294	0.442	0.643	0.000	0.217	0.428	0.001
BM1818	0.361	0.703	0.000	0.419	0.810	0.000	0.256	0.721	0.000	0.391	0.580	0.023
BMS690	0.180	0.551	0.000	0.403	0.692	0.000	0.628	0.758	0.000	0.348	0.572	0.003
BMS518	0.082	0.445	0.000	0.262	0.530	0.000	0.628	0.759	0.000	0.043	0.043	1.000
ETH3	0.459	0.821	0.000	0.701	0.663	0.246	0.349	0.672	0.000	0.217	0.571	0.000
TGLA304	0.541	0.682	0.000	0.613	0.716	0.000	0.465	0.637	0.000	0.304	0.475	0.016
BMS1747	0.426	0.668	0.000	0.746	0.650	0.628	0.744	0.765	0.000	0.174	0.341	0.001
BMS1866	0.459	0.688	0.000	0.537	0.628	0.148	0.488	0.687	0.000	0.409	0.764	0.000
ETH10	0.328	0.642	0.000	0.493	0.542	0.688	0.395	0.739	0.000	0.217	0.488	0.000
URB011	0.639	0.820	0.000	0.746	0.769	0.372	0.628	0.574	0.673	0.826	0.802	0.044
Average	0.411	0.667		0.603	0.674		0.487	0.689		0.353	0.607	

In our study, quite different patterns of relationship among Tuscan local cattle breeds were depicted by pair-wise molecular coancestry and kinship distance when compared to the classical genetic distances, as highlighted by the low correla-

**Table 3** - Number of individuals (N), inbreeding coefficient (F), heterozygote deficiency ( $F_{IS}$ ), molecular coancestry ( $f_{ij}$ ), kinship distance ( $D_k$ ) and genetic similarity (GS) for each breed and for the total sample.

Breed	N	F	$F_{IS}$	$f_{ij}$	$D_k$	GS
Calvana (CAL)	63	0.589	0.379	0.338	0.456	0.376
Chianina (CHI)	67	0.369	0.095	0.332	0.366	0.404
Garfagnina (GAR)	43	0.512	0.284	0.319	0.437	0.372
Pontremolese (PON)	23	0.648	0.406	0.407	0.417	0.381
Total sample	196	0.512	0.261	0.267	0.416	0.272

**Table 4** - Pair-wise  $F_{ST}$  (below the diagonal) and average genetic similarity among the four breeds (above the diagonal).

Breed	CAL	CHI	GAR	PON
Calvana (CAL)	-	0.332	0.251	0.248
Chianina (CHI)	0.061	-	0.251	0.263
Garfagnina (GAR)	0.131	0.136	-	0.254
Pontremolese (PON)	0.172	0.161	0.166	-

tion coefficients ( $-0.387 \pm 0.180$  and  $0.175 \pm 0.091$  for  $f_{ij}$  and  $D_k$ , respectively). Also  $f_{ij}$  and  $D_k$  resulted to be only moderately correlated ( $-0.157$ ; data not shown). Between-breed molecular coancestry values and kinship distances are given in Table 5. The lowest  $f_{ij}$  values were found between GAR-CHI (0.273) and GAR-CAL (0.278) while Chianina and Calvana displayed the highest  $f_{ij}$  value (0.310).

Breed differentiation was also investigated adopting a likelihood test of population assignment implemented in the ARLEQUIN package v. 3.11. All the animals were correctly allocated to the true breed of origin (data not shown), thus highlighting a clear differentiation among the four Tuscan breeds. This result was supported by the Bayesian clustering analysis carried out using the software STRUCTURE v. 2.2. As shown in Figure 3, each breed was assigned to a separate cluster when testing the hypothesis of  $K = 4$  (where  $K$  is the number of sub-population assumed in the total sample) and almost no admixture was detected among breeds. However, we interestingly found that at lower  $K$  values, Calvana and Chianina clustered together.

## DISCUSSION

The mean number of alleles observed within the four Tuscan breeds was in general agreement with that obtained by Cañón et al.<sup>4</sup> on eighteen local European (Spanish, Por-

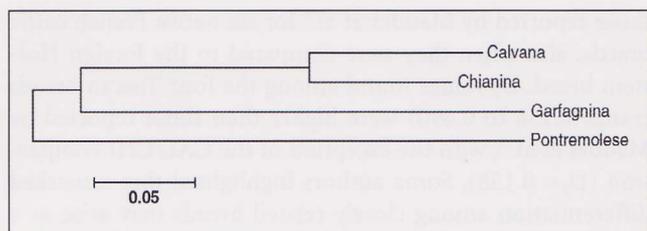


Figure 1 - Neighbor-joining dendrogram.

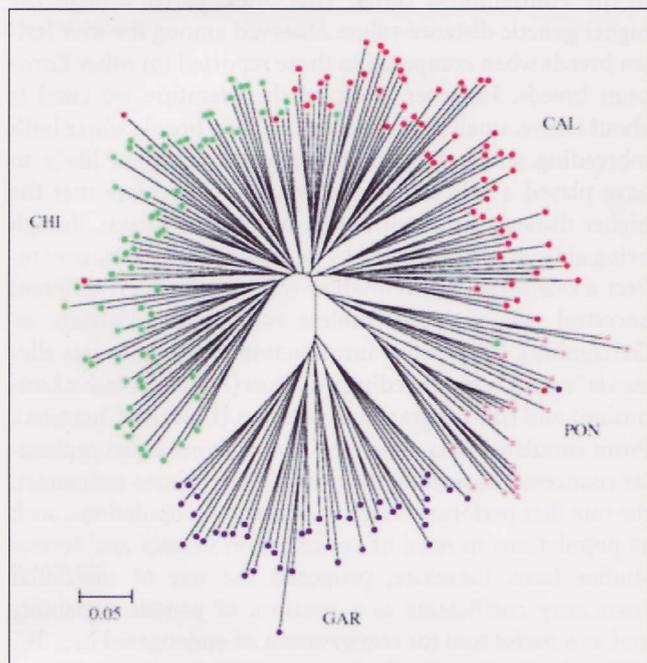


Figure 2 - Neighbor-joining dendrogram based on Nei's ( $D_A$ ) genetic distance (Nei et al., 1983) among individuals belonging to the Calvana (red), Chianina (green), Garfagnina (blue) and Pontremolese (pink) breeds.

tuguese and French) beef cattle breeds using 16 STR loci and by Maudet et al.<sup>5</sup> on seven French cattle breeds using 23 STR loci. Higher values in the mean number of alleles were generally observed by Mateus et al.<sup>6</sup> in native Portuguese cattle breeds by using 30 STR loci and by Moioli et al.<sup>7</sup> in the Podolica cattle breed. A mean number of alleles similar to that observed in the Pontremolese cattle has been reported, using a comparable number of animals and loci, for the Portuguese cattle breed Mirandesa<sup>4,5</sup>, the Portuguese Brava de Lide fighting breed<sup>6</sup>, the Spanish Pirenaica and the Portuguese Alentejana cattle breed<sup>4</sup>. A mean number of alleles lower than that observed in the Pontremolese cattle has been reported by Martin-Burriel et al.<sup>8</sup> for three Spanish native cattle breeds, the Balearic island breeds Mallorquina and Menorquina, and a fighting bull population, where the low values are probably due to small population sizes and reproductive isolation experienced by these breeds.

The average locus by locus  $F_{ST}$  value observed in our study ( $0.115 \pm 0.054$ ) is generally higher than those observed in most of the studies carried out on European cattle breeds. For example, an  $F_{ST}$  value of 0.090 was found by Ciampolini et al.<sup>9</sup> in a comparison among the Chianina, the Italian Friesian and the French Limousine and Charolais beef breeds; an  $F_{ST}$  value of 0.085 was found by Dalvit et al.<sup>10</sup> in a comparison among the local Burlina cattle breed, reared in

Table 5 - Between-breeds molecular coancestry (below diagonal) and kinship distance (above diagonal).

Breed	CAL	GAR	PON	CHI
Calvana (CAL)	–	0.448	0.445	0.409
Garfagnina (GAR)	0.278	–	0.430	0.393
Pontremolese (PON)	0.299	0.285	–	0.379
Chianina (CHI)	0.310	0.273	0.300	–

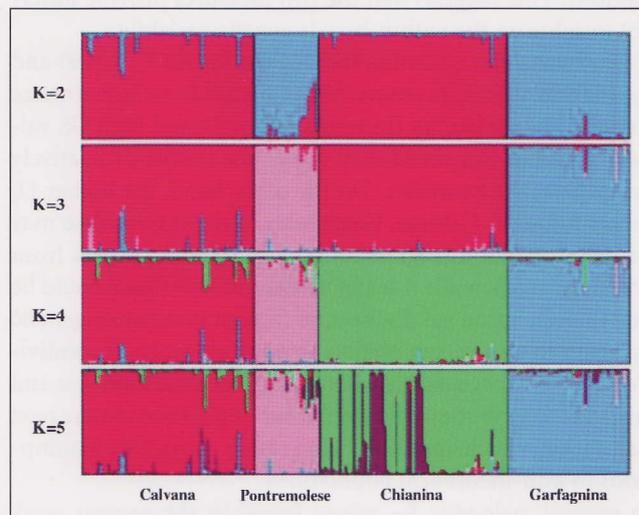


Figure 3 - STRUCTURE estimated membership fractions of sheep breeds for each of the  $k$  inferred clusters assuming  $2 \leq k \leq 5$  subpopulations.

the North-Eastern part of Italy, the Holstein Friesian and the Brown Swiss dairy cattle breeds; lower  $F_{ST}$  values have been observed by Maudet et al.<sup>5</sup> in a comparison among six native French breeds and the French Holstein (0.085), by Canon et al.<sup>4</sup> in local Spanish, French and Portuguese beef cattle breeds (0.070), by Jordana et al.<sup>12</sup> in 18 local South European beef breeds (0.068) and by Moioli et al.<sup>7</sup> in a comparison among the native Italian Piemontese, Maremmana and Podolica cattle (0.060).

Within-breed  $H_{OBS}$  values found in our study were generally much lower than those reported for other European cattle breeds<sup>4,5,6</sup>, while approaching those found by Martin-Burriel et al.<sup>8</sup> for the two Balearic breeds Menorquina and Mallorquina, a Spanish Fighting population and the semiferal Betizu breed. Though we could not exclude that allele drop-out contributed somehow to determining the observed heterozygote deficiency, we discarded the hypothesis that it played a major role and assumed that inbreeding could be the main responsible for the observed disequilibrium; this hypothesis was also suggested by the fact that the lowest percentage of loci in significant disequilibrium was observed in the breed (Chianina) which has the highest population size and the oldest Herd Book (established in 1932); in fact, the genetic management of the other three local breeds has been assigned to the “anagraphic register of small local cattle breeds and populations” only since 1985.

The observed  $F_{IS}$  values (excluding that of the Chianina breed) were much higher than those reported for Italian and French breeds (Italian Friesian, 0.030; Marchigiana 0.052; Chianina 0.056; Charolaise 0.080; Limousine, 0.115, unpub-

lished data) and for French, Spanish and Portuguese beef cattle breeds<sup>11</sup>. The high positive  $F_{IS}$  (and  $F$ ) values therefore support the hypothesis of inbreeding within the four Tuscan cattle breeds and particularly within the Pontremolese cattle that is a very small and closed population.

Surprisingly, no significant correlation was found between molecular coancestry and kinship distance (data not shown), though being the latter influenced by the former ( $D_k$  produces a measure of the recent differentiation corrected for molecular coancestry, i.e. differentiation in the ancestral population). This suggests that the two measures provide different insights on the within-breeds genetic variability.

Considering that Chianina showed the lowest  $F$  (0.369) and  $F_{IS}$  (0.095) values, it seems rather plausible to hypothesize that the low variability (in terms of low  $D_k$  and high  $GS$  values) found in this breed may reflect the results of relatively recent selective processes. On the other hand, the higher  $D_k$  values found in Calvana, Garfagnina and Pontremolese may be due to recent introgression of allochthonous alleles from crossing breeds, while it seems rather unlikely that it could be due to population subdivision; in fact, an increase of genetic diversity in a meta-population as a consequence of subdivision can be expected only when assuming a constant size and an equal contribution of different subpopulations to the next generation, which appear as being rather unrealistic assumptions in non-theoretical populations.

In general, pair-wise  $F_{ST}$  values found in the present work were within the range observed among native Portuguese cattle breeds using 30 STR loci<sup>6</sup>, as well as between Italian Friesian vs. Chianina, Charolaise and Limousine, respectively, using 19 STR loci<sup>9</sup>, while being generally higher than those reported by Jordana et al.<sup>12</sup>, for Spanish, Portuguese and French beef cattle breeds by using 16 STR loci. In particular, the CAL vs. CHI  $F_{ST}$  value (0.061) is similar to those observed by Jordana et al.<sup>11</sup> between most of the south European beef cattle breed pairs, by Ciampolini et al.<sup>9</sup> between Limousine and Charolaise and by Moiola et al.<sup>7</sup> (2004) between Piemontese and Maremmana, thus supporting, together with the genetic similarity values, the hypothesis of Negrini et al.<sup>12</sup> that Calvana could be considered as an original breed, differentiated from, though strictly related to, Chianina. This hypothesis was also supported by the likelihood ratio assignment test results obtained in our study that closely resembled those obtained by Negrini et al.<sup>13</sup> in a study, carried out on 16 Italian cattle breeds using biallelic AFLP markers, where 97% of the CAL animals were assigned to the appropriate breed and only 3% of the CAL individuals were assigned to the Chianina breed.

Breed relationships, as inferred by pair-wise  $F_{ST}$  and genetic similarities, were consistent with the scenarios observed using several genetic distance measures, either for distances based on a simple random drift model and for those assuming a random drift/mutation model (average correlation coefficient  $0.98 \pm 0.01$ ) as well as with the results obtained by Negrini et al.<sup>12</sup> using AFLP markers.

Although accounting for unreliability of the comparative analysis among values obtained in different works due to selection of different loci and different population sample sizes,  $D_A$  values found in the present work (ranging from 0.152 for the CHI/CAL breed pair to 0.308 for the CAL/PON contrast) were within the range observed among native Portuguese cattle breeds<sup>6</sup>, while being generally higher than

those reported by Maudet et al.<sup>5</sup> for six native French cattle breeds, also when they were compared to the foreign Holstein breed.  $D_S$  values found among the four Tuscan breeds (range 0.158 to 0.490) were higher than those reported by Maudet et al.<sup>5</sup>, with the exception of the CAL/CHI comparison ( $D_S = 0.158$ ). Some authors highlighted that a marked differentiation among closely related breeds may arise as a consequence of inbreeding or bottleneck/founder effect. As shown above, marked levels of inbreeding have been observed within the four Tuscan breeds and particularly within the Pontremolese cattle. This could partly explain the higher genetic distance values observed among the four Italian breeds when compared to those reported for other European breeds. However, most of the literature we cited is about native, small population sized cattle breeds where both inbreeding and bottleneck phenomena are quite likely to have played a role. Therefore, we could speculate that the higher distance values observed among our breeds, though being at least partly inflated by demographic factors, may reflect a real breed differentiation resulting from (i) different ancestral origins (Pontremolese vs. Chianina-Calvana vs. Garfagnina), (ii) possible introduction of allochthonous alleles via "recent" crossbreeding practices (Pontremolese x Limousine) and (iii) geographical isolation (Calvana-Chianina). From simulated data, Oliehoek et al.<sup>14</sup> showed that molecular coancestry is, among the possible relatedness estimators, the one that performs better in structured populations, such as populations in need of conservation usually are. Several studies have, therefore, proposed the use of molecular coancestry coefficients as a measure of genetic variability and as a useful tool for conservation of endangered breeds. All  $f_{ij}$  values were rather high (range 0.273-0.310) when compared to data reported on other species such as sheep<sup>1</sup>. The lowest  $f_{ij}$  values found between GAR-CHI (0.273) and GAR-CAL (0.278) suggest for Garfagnina an ancestral origin different from that of Chianina and Calvana. On the contrary, the highest  $f_{ij}$  value found between CHI-CAL (0.310) support the hypothesis that Calvana may have originated from Chianina, probably through introgression of Chianina into local *Bos primigenius* x *Bos brachyceros* cattle, starting at least from early Middle Ages.

## CONCLUSION

The present study contributes to the knowledge and the genetic characterisation of Italian Tuscany autochthonous cattle breeds. The importance and usefulness of genetic diversity studies is greater in cases where the breeds, like Calvana, Garfagnina and Pontremolese, are in a critical or endangered status. Results obtained in this study suggest that the situation of the analyzed breeds is risky as their variability is compromised by a high level of inbreeding, though quite different scenarios were observed for the four breeds. Calvana and Garfagnina had a moderately low genetic variability, as shown by number of alleles, heterozygosity, genetic similarities and molecular coancestry-based parameters. This suggests the need for a careful genetic management of the breeds, in order to maintain long-term population survival and adaptability and to avoid the risk of more excessive increase in the inbreeding level, which would result in significant inbreeding depression and in significant loss of genetic

variation. On a other hand, the low variability found in Chianina may reflect the results of relatively recent selective processes. Pontremolese was the most compromised breed: the very low genetic variability, partly due to strong inbreeding and to the small number of individuals, makes more difficult its genetic management and the only possibility of survival of the breed is to identify the animals with the lowest average molecular coancestry values for the reproduction and to carry out ex situ conservation procedures.

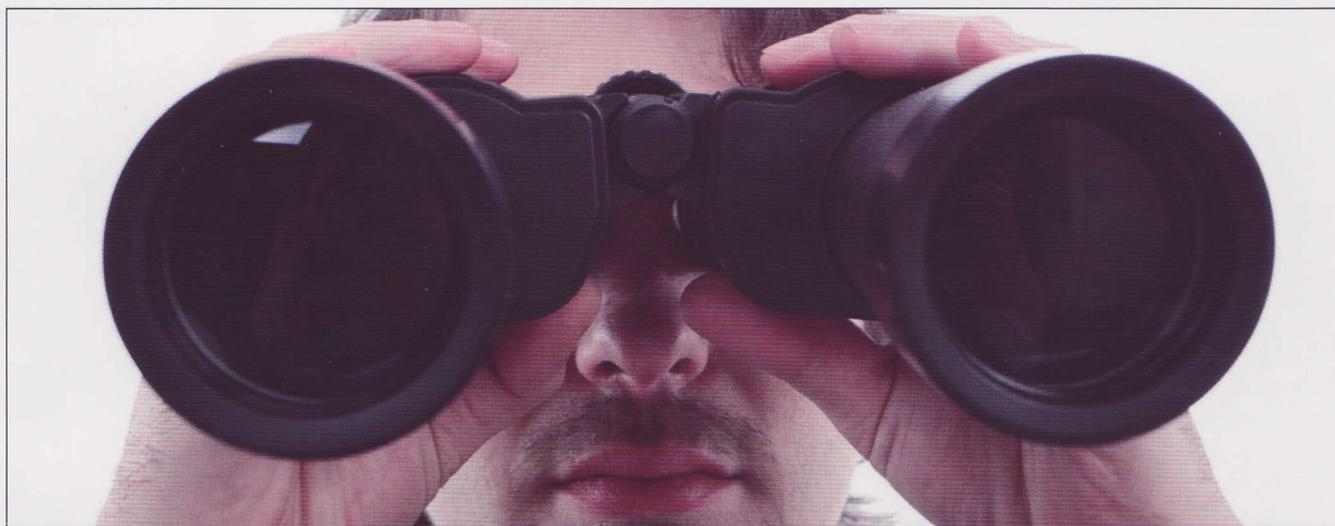
Levels of genetic differentiation observed for the four breeds support their conservation as separate breeds with unique genetic features. This is also true for Calvana, which seems to have ancestrally belonged to the Chianina metapopulation, while having differentiated only relatively recently, as suggested by  $D_k$ .

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