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Mertolenga a Well Substructured Population According to their Morphotypes

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Abstract

Genetic structure and diversity of three Mertolenga breed morphotypes were assessed with 30 microsatellites. Allelic richness per locus was relative high, with an overall average of 6.163. The mean number of alleles, corrected for the size of the smaller sample, ranged between 5.6 in the Rosilho variety to 6 alleles in the Malhado de Vermelho and Unicolor varieties. The mean expected and observed heterozygosities ranged between 0.748 in the Unicolor variety, 0.730 in the Rosilho variety, and between 0.735 in the Unicolor variety and 0.685 in Malhado de Vermelho variety, respectively. The Rosilho variety systematically showed the lowest values of genetic diversity excepted for the observed heterozygosity and the number of specific alleles (Private Alleles). The analysis with STRUCTURE has allowed us to get 4 well-defined clusters (one cluster for the Mirandesa breed, an outgroup in the present study, and 3 clusters corresponding to the three morphotypes of the Mertolenga breed), which means that these varieties can be regarded as completely distinct populations in genetic terms. To analyze the substructure among the 58 animals studied, Factorial Correspondence Analysis and a Bayesian approach were carried out using GENETIX and STRUCTURE software. The factorial analysis of correspondences resulted in the formation of 3 well-defined clusters that correspond to one of the three varieties of the Mertolenga breed. The genetic information present in this study demonstrates that the Mertolenga Portuguese cattle breed is genetically well sub-structured in its three morphotypes. Since this has implications with regard to the rational management of animal genetic resource conservation, we believe that the results of this study should be taken into account in future breeding programs and assessments of risk of extinction because each of these populations is a significantly different genetic resource.

Keywords: Diversity; Mertolenga breed; Microsatellites; Portuguese cattle; Substructure

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Introduction

Mertolenga has the largest population (19 142 females and 238 males) (Pais, Personal Communication) of all Portuguese cattle breeds. It consists of a heterogeneous population with origins in the Berrenda en Colorado Andalusian breed, which then was cross-bred with the Alentejana, Brava de Lide and Retinto breeds [1]. With regard to its ethnic characteristics, Mertolenga is part of the Red Convex evolutionary branch originating from the North African path of domesticated cattle [1-4], which is supported by the detection of African-type mtRNA haplotypes [5,6]. Currently, three varieties are recognized in the Mertolenga breed [7], corresponding to three distinct morphotypes with different geographical distribution in the south of Portugal: Unicolor has a uniform reddish coat and is reared mainly along the basins of Sorraia and Sado rivers; Rosilho or Mil Flores has a coat with a mixture of red hair and white hair and is reared in the districts of Portalegre, Beja and Évora; Malhado de Vermelho has a predominantly white coat with large red spots usually on the head and sides and is reared on the left bank of Guadiana river.

The aim of this study was to analyze the genetic diversity and the effect of morphotypes on genetic substructure of the Mertolenga Portuguese cattle breed. For that purpose, we will use the information provided by allele frequencies of 30 microsatellites, 16 of which are part of the list of microsatellites recommended by the ISAG group for genetic diversity studies.

Material and Methods

Animals

A total of 58 unrelated animals registered in respective Herd Books of the Mertolenga breed were included in this study. Mertolenga individuals were sampled according to their morphotypes in different herds and included 12 Malhado de Vermelho, 22 Rosilho or Mil Flores, and 24 Unicolor. A population of 48 animals of the Mirandesa breed were used as reference in the analysis of the substructure of the Mertolenga breed, considered the best genetically distinct population of all Portuguese breeds [4,8,9].

DNA extraction

The DNA was extracted from whole blood samples collected by venipuncture of the jugular and kept in sterile 9 ml vacuette vacuum tubes containing EDTA-K3 as anticoagulant. The DNA was isolated by the saline method proposed by Miller et al. [10].

Microsatellites markers and PCR parameters: Thirty microsatellites (Table 1) distributed across 25 cattle chromosomes were selected

for this study. Sixteen microsatellites included in the analyses are among those recommended for cattle population studies by the ISAG group for Management of Farm Animal Genetic Resources. Microsatellite markers were combined in multiplex-PCR reactions using fluorescently labelled primers and amplified in 12.5µl reaction volume containing 2.5mM $MgCl_2$, 200µM DNTPs, 50-100ng template DNA, 0.5U Taq polymerase and primers at the appropriate concentration (Table 1). Amplification was done with 5 cycles of 1 min at 94°C, 30 sec at specific annealing temperatures (Table 1) and 30 sec at 72°C followed by 25 cycles where the denaturation step at 94°C was reduced to 45 sec [11]. PCR products were separated in denaturing polyacrylamide gels run on ABI 373 DNA Sequencers (Applied Biosystems, Foster City, CA). Fragment size analysis was performed with STRand software [12]. The internal size standard GeneScan™-ROX 350 (PE-Applied Biosystems, Warrington, UK) was used for sizing alleles. In addition, sample #1 from the ISAG 1997/98 comparison test was used as reference to standardise allele sizes [11].

Locus†	BTA	Number of alleles	Allele size range (bp)	Primer (µM)	Ta (°C)
BM1824	1	7	178-190	0.22	58
BM 2113	2	11	121-143	0.11	58
INRA023	3	14	196-222	0.40	58
MGTG4B	4	14	129-155	0.15	60
RM067	4	8	90-106	0.75	58
ETH10	5	11	113-225	0.15	60
ETH152	5	8	193-211	0.12	58
ILSTS035	6	24	210-270	0.80	58
RM006	7	8	110-124	0.25	58
HEL9	8	12	147-169	0.03	52
ETH225	9	10	140-158	0.15	60
SPS113	10	13	133-157	0.15	60
BRRIBO	10	13	238-262	0.30	58
HEL13[c]	11	6	185-195	0.03	52
TGLA345	12	10	112-142	0.05	58
CSSM036	14	10	162-182	0.08	60
SPS115	15	8	246-260	0.40	58
TGLA53	16	18	154-188	0.15	60
ETH185	17	13	221-245	0.03	66
TGLA227	18	12	77-99	0.35	58
ETH3	19	11	103-131	0.20	60
TGLA126	20	8	111-125	0.50	58
TGLA122	21	19	135-181	0.32	58
ETH131	21	29	140-173	0.50	58
BM2613	22	11	159-179	0.16	58
CYP21[e]	23	33	183-222	0.20	58
BM1818	23	7	258-270	0.30	58
ILSTS065	24	11	126-146	0.08	58
HEL11	26	13	184-218	0.10	58
BM203	27	18	115-241	0.04	58

Table 1: Characterization markers and PCR parameters for the 30 microsatellites.

† Microsatellites included in the list of ISAG group are shown in bold face.

Data analysis

With Software Fstat 2.9.3 we obtained allele frequencies for all locus population combinations. Population specific alleles (private alleles, PA) were counted manually. To test whether the populations were in Hardy-Weinberg equilibrium (Ho: random union of gametes), exact tests were performed using software GENEPOP version 4.0 [13]. Non-biased estimates of the exact P were obtained by the Markov Chain Monte Carlo method developed by Guo and Thompson [14]. The excess or deficiency in heterozygosity for each locus in each population was analyzed using a U-test [15]. To test population differentiation, the null hypothesis was Ho: "the alleles were taken from the same distribution in all populations". The method used to reject or accept the null hypothesis was the G-test [16]. The test was repeated for a differentiation of populations, but considered populations pairs. In all the tests, the Markov Chain parameters chosen were 10000 dememorization steps, 2000 batches and 5000 interactions per batch. For each population, the level of significance was adjusted by a strict Bonferoni procedure for multiple comparisons, which allowed us to reduce type II errors [17].

The classical genetic diversity parameters were calculated using GENETIX software version 4.05.2 [18]. The unbiased average expected and observed heterozygosities per population was calculated within the breed. The total and mean number of alleles was corrected for these two parameters, which accounted for all possible combinations of twelve animals (smaller size of an analyzed sample) within each variety of the Mertolenga population. Fstat enabled us to calculate the inbreeding coefficient (F_{IS}) and allelic richness.

Population structure was evaluated using the parameters of hierarchical F-statistics (F_{ST}, F_{IT}, F_{IS}), estimated according to that proposed by Weir and Cockerham [19] and implemented in Fstat, version 2.9.3.2 [20]. The null Hypothesis (Ho) that the estimates are not significantly different from zero was substantiated through testing based on permutations, as proposed by Goudet [20]. To test F_{IS} (*f*), alleles were exchanged between individuals within populations; to test F_{IT} (F), alleles were exchanged between populations; finally, to test F_{ST} (θ), individuals were exchanged between populations. The F_{ST} parameter that measures the proportion of different alleles between all population pairs was also calculated. The distribution of F_{ST} values between pairs of populations, under the assumption that there are no differences between the populations, was obtained through a random sampling of multi-locus genotypes between the two populations. The logarithm of maximum likelihood statistical G test [21] was used to classify the P values (proportion of data in the random sample obtained a value of F_{ST} as great or greater than observed). The significance of the P value, for the comparisons carried out, was corrected by the standard method of Bonferoni, as proposed by Goudet [20].

To have an idea of the degree of genetic separation between the three varieties studied; D_A genetic distances between all pairs of populations were calculated using software populations [22].

Multivariate analysis of correspondences: The analysis of correspondence was carried out using the Factorial Correspondence Analyses module (Analyse Factorielle des Correspondances) implemented by software GENETIX [18]. With this analysis, it was possible to get a graphical representation of the individuals, depending on the variance of their allele frequencies, in the geometric space defined by the three synthetic variables used by this software.

Analyses with STRUCTURE: The genetic structures of the three varieties of the Mertolenga breed were also analysed using STRUCTURE software, version 3.0 [23], to estimate the most likely number of population clusters (*K*) among Mertolenga morphotypes.

For data analysis, we used the Alpha and Lambda parameters defined by the default program of the software. The definition of the groups was based on the admixture model and the assumption that allele frequencies were correlated between the breeds, which are convenient for closely related populations.

To estimate the *K* value for Mertolenga, we used a reference population consisting of 48 animals of the Mirandesa breed, considered the best genetically distinct population of all Portuguese breeds. We varied the value of *K* from 1 to 6 and the software was set to run for 250 000 MCMC repetitions, with a 50 000 burn-in. There were ten runs for each value of *K* and the most likely value of *K* was determined by the highest average of the maximum likelihood of the data (Ln P (D)) with smaller variance.

The STRUCTURE software was also used to allocate individuals to their populations of origin using the strictly Bayesian method implemented by the software. The run was set for 1 000 000 MCMC repetitions, with a 100 000 burn-in, for the most likely value of *K* in order to determine the number of animals classified in each cluster. The percentage of individuals classified in each cluster was determined by considering the estimated proportion of the association of each individual genotype (Q) to each of the clusters. Was also calculated the percentage of subjects not included in their population of origin and misclassified in another cluster. Tests of individual allocation were also performed by STRUCTURE using *a priori* information about the source population of individuals, since the subjects were sampled from different herds and from distinct population with different phenotypes. The run had the same characteristics as before with *K* always equal to 4.

Results

The parameters of genetic diversity (Table 2) found within each varieties of the Mertolenga breed are equivalent to those found in all other Portuguese cattle breeds [9,11], particularly that found within the Mertolenga breed. The expected and observed heterozigoties varied between 0.73 in the Rosilho variety, 0.748 in the Unicolor variety, and between 0.685 in the Malhado de Vermelho variety and 0.735 in the Unicolor variety, respectively. The average number of alleles corrected for the size of the smallest sample ranged from 5.6 alleles in the Rosilho variety and 6.0 alleles in the Malhado de Vermelho and Unicolor varieties. The number of private alleles ranged between 10 alleles and 33 alleles in the Malhado de Vermelho and Unicolor varieties, respectively. The Rosilho variety showed the lowest values of Allelic Richness, while the Unicolor variety showed the highest. F_{IS} values were all very close to zero, indicating that there was not an excess or a deficiency in heterozygotes between the populations studied, which would be confirmed by tests carried out by software Genepop, attesting the excess and deficiency of heterozygotes having all P values as not being significant. All loci and populations combinations are in the Hardy-Weinberg Equilibrium.

The D_A genetic distances (Table 3) are equivalent to those observed among all Portuguese cattle breeds [4,9,11]. The greatest distance was obtained between the Rosilho and Malhado de Vermelho pair and shortest distance between the Rosilho and Unicolor pair. All F_{ST} values are significantly different from zero among all population pairs. They are equivalent to some of the F_{ST} values observed among other Portuguese cattle breeds [4] suggesting that these are entirely genetically distinct populations. The test for genetic differentiation between pairs of populations carried out by Genepop confirmed these results, after obtaining highly significant P values that enabled us to reject the null hypothesis that "The alleles were taken from the same distribution in all populations." Table 4 shows the Wright estimators of genetic differentiation F_{IS} (f) F_{IT} (F) and F_{ST} (θ). None of the estimates of the inbreeding coefficient f was significantly different from zero. The levels of genetic differentiation θ obtained by locus were relatively low and ranged between -0.007 and 0.074 for locus BM1818 and RM006, respectively. For all loci analyzed, estimates of θ were not significantly different from zero except for loci ILSTS035, TGLA122, BM1824, ETH10, HEL11, ranging from highly significant in the first case, very significant in the second, and significant in that of the last three loci. However, when considering all loci, the result was considerably different from zero. The average proportion of genetic variation explained by differences among the varieties was 2.7%, which is quite low when compared with the variation observed among all populations of Portuguese cattle [4,9,11]. Nevertheless, recall that the populations concerned resulted from the subdivision in three phenotypes of a single Portuguese population. We attributed the remaining variation to individual differences existing within each one of the studied varieties.

Figure 1 shows the results of Factorial Correspondence Analysis (FCA) applied to the Mertolenga breed. With regard to the morphotypes, there is a clear substructure among the Mertolenga individuals and the three varieties of the Mertolenga breed that are clearly grouped and separated from each other.

Previous runs with STRUCTURE, without information regarding the source populations of animals, enabled us to define the most probable value of *K* and identify population clusters that best explain the partitioning of all data analysed [24]. For Mertolenga, the Ln P(D) recorded a large increase between $K = 1$ and $K = 2$, then presented a clear tendency to a plateau, reaching its maximum value when K was equal to 4 (Figure 2). The number of analysed populations was exactly four, so this result shows that the three varieties of the Mertolenga breed constitute distinct and well differentiated populations.

Variety	H. Exp.	H. Obs.	MNA	MNAC†	TNA	TNA†	PA	Allelic Richeness	Fis	Dev. HWE
Maver	0.740	0.685	6.0	6.0	179	179	10	5.967	0.078	0
Rosilho	0.730	0.701	6.9	5.6	208	169	25	5.821	0.044	0
Unicolor	0.748	0.735	7.5	6.0	224	180	33	6.112	0.018	0

Table 2: Summary of genetic diversity parameters, including observed and expected heterozygosity, total (TNA) and mean number (MNA) of alleles, mean (MNA_C) and total (TNA_C) number of alleles corrected for the size of the smaller sample, number of Private Alleles (PA), and Allelic Richness, inbreeding coefficient (F_{IS}) and deviations from Hardy-Weinberg equilibrium observed among the three varieties studied.

† Average of all possible combinations of twelve animals within each subpopulation.

Morphotype	MaVer	Rosilho	Unicolor
MaVer	0	0,1488	0,1336
Rosilho	†0,0415	0	0,1029
Unicolor	†0,0221	†0,0235	0

Table 3: DA genetic distances above the diagonal FST values between pairs below .the diagonal

P< 0.01 †

Locus	f	F	θ
BM1818	-0,003	-0,01	-0,007
BRRIBO	0,023	0,064	0,042
SPS115	0,032	0,037	0,004
INRA23	0,098	0,133	0,039
CYP21	0,034	0,051	0,018
ETH152	0,056	0,067	0,012
BM1824	-0,028	0,048	0,073*
ETH131	-0,006	0,011	0,017
TGLA122	-0,053	0,004	0,054‡
BM2113	0,056	0,066	0,011
RM067	-0,082	-0,074	0,008
TGLA227	-0,081	-0,063	0,017
ETH3	0,035	0,067	0,033
ETH225	0,114	0,141	0,03
TGLA53	0,008	0,042	0,035
MGTG4B	0,000	0,025	0,025
SPS113	-0,005	0,034	0,039
ETH10	0,223	0,255‡	0,041*
CSSM36	0,101	0,105	0,004
ILSTS035	0,059	0,127	0,073†
BM2613	0,081	0,103	0,024
ILSTS065	0,053	0,04	-0,014
RM006	-0,134	-0,049	0,074
BM203	0,133	0,154	0,024
HEL11	0,166	0,199*	0,039*
TGLA345	0,019	0,043	0,025
TGLA126	0,201	0,215	0,019
ETH185	-0,015	-0,002	0,012
HEL13	0,008	0,028	0,02
HEL9	0,094	0,112	0,02
All Loci	0,039	0,065†	0,027†

Table 4: Indices of Wright of genetic differentiation for the populations studied.

† P< 0.001

‡ P< 0.01

* P< 0.05

Table 5 shows the results for the longest run with STRUCTURE without knowing the source population of the animals. When the assignment to a cluster was defined as the most likely value of the occurrence of its genotype in this cluster (Qmax), the percentage of correctly classified individuals in their population of origin ranged from 58.3% for the Malhado de Vermelho population to 100% for the Mirandesa breed, as would be expected.

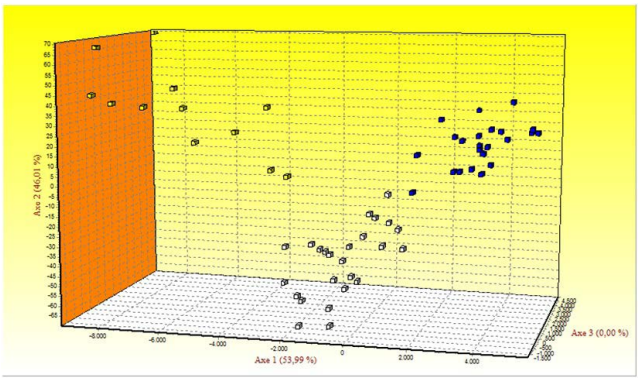

Figure 1: Factorial Correspondence Analysis carried out on the three varieties of the Mertolenga breed.

Malhado de vermelho
Rosilho or Mil Flores
Unicolor

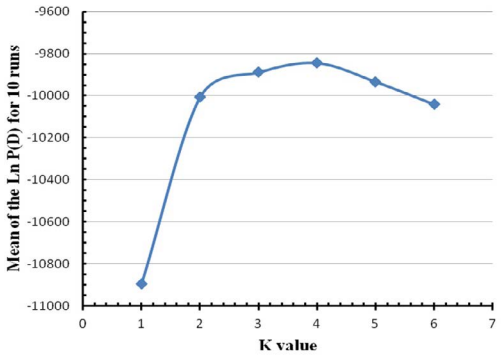

Figure 2: Average value of the Ln P(D) for ten runs without information of the source populations of the animals in the three varieties of the Mertolenga breed and Mirandesa breed.

Varieties	Qmáx	Q> 0,8	Q> 0,9	Mean	Cluster
MaVer	58,3%	45,5%	45,5%	49,8%	1
Rosilho	73%	68%	59%	66,7%	2
Unicolor	75%	71%	58%	68,0%	3
Mirandesa	100%	94%	88%	94,0%	4
Mean	76,6%	69,6%	62,6%	69,6%	

Table 5: Allocation of individuals of the Mertolenga breed for the longest run with STRUCTURE without the information about the source population of the animals and $K = 4$.

Table 6 summarises individuals correctly classified and misclassified in other clusters. As would be expected, for a FST 0.027, all varieties of Mertolenga showed some misclassified animals, mainly of them in favour of the Unicolor morphotype, which is the most representative in terms of total number of animals and geographic distribution. In fact, five animals from Malhado de Vermelho were misclassified as Unicolores, five Rosilho as Unicolor, one Rosilho as Malhado de Vermelho, four Unicolor as Rosilho and two Unicolor as Malhado de Vermelho.

Variety	MalVer	Rosilho	Unicolor	Mirandesa	N	%
MalVer	7	0	5	0	12	58.3%
Rosilho	1	16	5	0	22	73%
Unicolor	2	4	18	0	24	75%
Mirandesa	0	0	0	48	48	100%

Table 6: Individuals correctly classified and misclassified in other clusters for the longest run with STRUCTURE without knowing the source population of the animals and with $K = 4$.

Varieties	Qmáx	Q>0.8	Q>0.9	Mean
MaVer	100%	55%	55%	70.0%
Rosilho	91%	82%	73%	82.0%
Unicolor	96%	87.5%	79%	87.5%
Mirandesa	100%	94%	90%	94.7%
Mean	96.8%	79.6%	74.3%	

Table 7: Allocation of individuals of the Mertolenga breed for the longest run with STRUCTURE with information about the source population of the animals and $K = 4$.

	MalVer	Rosilho	Unicolor	Mirandesa	N	%
MalVer	**12**	0	0	0	12	100%
Rosilho	1	**20**	1	0	22	91%
Unicolor	0	1	**23**	0	24	96%
Mirandesa	0	0	0	**48**	48	100%

Table 8: Individuals correctly classified and misclassified in other clusters for the longest run made with STRUCTURE knowing the source population of the animals and with $K = 4$.

In turn, the allocation of individuals performed by STRUCTURE, with prior knowledge about the animal source population, demonstrated that in 97.0% the assignment to their respective source populations was correct (Table 7). Only three animals were misclassified (Table 8), one Rosilho as Malhado de Vermelho, another Rosilho as Unicolor and one Unicolor as Rosilho.

Discussion and Conclusions

Although this is a subdivision of a Portuguese cattle population, the three varieties of the Mertolenga breed showed indices of genetic diversity within populations comparable to those obtained with all other populations of Portuguese cattle, including the Mertolenga breed [4,9,11]. This outcome indicates this subdivision does not affect the indices of genetic diversity found within each subpopulation of the Mertolenga breed. The genetic differentiation between all pairs of populations all have very significant P-values, which is in agreement with the results of FCA and the results obtained by STRUCTURE where the three varieties of Mertolenga breed are clearly separated from each other, showing that the three phenotypes of the Mertolenga breed can be considered as three completely distinct populations in genetic terms. The DA genetic distances between the varieties of Mertolenga are equivalent to those obtained among the various populations of Portuguese cattle [4,9,11], which again reinforces the idea that these are three genetically distinct populations.

The results of FCA and STRUCTURE analyses demonstrated a clear genetic substructure in three well differentiated populations, each coincident with one of the current three morphotypes (Malhado de Vermelho, Rosilho or Mil Flores and Unicolor), so these may be considered as different populations. These results demonstrate, for the first time, that the three phenotypes of Mertolenga breed are really three distinct populations that can be easily isolated from each other, unlike the results of Ginja et al. [9], that could only isolate one of the three phenotypes. For the first time in Portugal, we have genetic arguments for the subdivision of one population out of three completely distinct populations. Since this fact has implications with regard to the rational management of animal genetic resources for conservation, we believe that the results of this study and others in the same field should be taken into account in future breeding programs. They are relevant to assessments of risk of extinction because each of these varieties is a significantly different genetic resource. At present, the Malhado de Vermelho morphotype population represents a small (less than 20% of the overall population) (Pais, Personal Communication) but effective exemplification of the Mertolenga breed, so their conservation should be a priority.

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