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## Comparison of four Italian beef cattle breeds by means of functional genes.

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1 Running title: SNPs for genetic characterization of Italian beef cattle

2 **Comparison of four Italian beef cattle breeds by means of functional genes**

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20 **Abstract**

21 Piemontese, Chianina, Marchigiana and Romagnola are the main Italian beef breeds, and the quality  
22 of their products is largest recognized all over the world. Here, 18 SNPs in 12 candidate genes  
23 involved on meat traits were investigated on 1055 candidates for selection in order to analyze the  
24 within and between breed variability with a functional marker approach.

25 Three SNPs (GDF8-3, GH and NPY-3) were monomorphic and most of the polymorphic SNPs  
26 showed an allele distribution quite similar in the four breeds. High variability at LEP-2, LEP-3 and  
27 LEPR markers was detected across breed and the analysis of the relationship between  $F_{ST}$  and  
28 heterozygosity suggested a different selection intensity by breeds for LEP-2. The highest pairwise  
29  $F_{ST}$  values (0.1189 to 0.1877) were obtained for the comparisons of Piemontese with the other  
30 breeds, while the lowest value (0.0296) was observed between Chianina and Marchigiana. The  
31 Piemontese differentiation from the other breeds could be due to its geographical isolation and  
32 selection targets. The results for breed assignment follows the genetic differentiation, in fact,  
33 Piemontese had the highest percentage of correct assignment (87.6), while Marchigiana had the  
34 lowest one (47.5). These findings suggest that the functional markers can be more suitable than  
35 neutral markers in discriminating breeds in similar morphology if selection played some role in  
36 their differentiation.

37

38 **Keywords:** Chianina, Marchigiana, Piemontese, Romagnola, SNPs

39

40 **Introduction**

41 The Italian beef cattle breeds have always been connected with rural and ethnic traditions, therefore  
42 they represent a historical and cultural heritage which exceeds their economic value. Among them,  
43 Piemontese, Chianina, Marchigiana and Romagnola are the main specialized breeds for meat  
44 production and the quality of their products is widely recognized all over the world.

45 Several studies focused on the genetic description of these breeds and their relationships. For  
46 example, on the basis of biochemical markers, Baker and Manwell (1980) included Chianina,  
47 Marchigiana and Romagnola in the Italian podolic group belonging to the *Primigenius* taxon, while  
48 Piemontese was included in the *Primigenius-brachyceros* Mixed taxon. Concordant results on the  
49 four studied breed grouping were obtained by Blott *et al.* (1998), using blood groups and protein  
50 polymorphisms. More recently, molecular markers, such as AFLP (Negrini *et al.*, 2007) and  
51 microsatellites (Dalvit *et al.*, 2008), were used to characterize the same breeds in the framework of  
52 product traceability.

53 The latter two studies were based on neutral markers, which are routinely used to analyse the  
54 genetic structuring of populations, being the most effective in detecting the relationships among  
55 breeds determined by processes such as migration and genetic drift. However, there is a growing  
56 evidence that variation in functional sequences can be more efficient in highlighting differences  
57 among breeds induced by selection (van Tienderen *et al.*, 2002; Kirk and Freeland, 2011,  
58 Pampoulie *et al.*, 2011).

59 The breeds here considered are all beef breeds, but the selection programmes implemented by the  
60 respective National Breeders' Associations in the course of time are quite different (Albera *et al.*,  
61 2001; Sbarra *et al.*, 2009). At present the emphasis of the selection in the Piemontese breed is on  
62 reducing calving problems, while improving growth rate and meat conformation (ANABORAPI,  
63 2013). For Chianina, Marchigiana and Romagnola the selection has always been focused on  
64 improving daily gain and muscle conformation (ANABIC, 2013).

65 As many candidate genes have been suggested for their potential effects on meat traits (Li *et al.*,  
66 2004; Buchanan *et al.*, 2005; Nkrumah *et al.*, 2005; Di Stasio *et al.*, 2007; Sherman *et al.*, 2008),  
67 the present investigation was carried out in order to analyze the within and between breed  
68 variability in Chianina, Marchigiana, Piemontese and Romagnola breeds with a functional marker  
69 approach.

70

71 **Material and methods**

72 *Animal sampling and molecular analysis*

73 Blood samples were collected from a total of 1055 candidates evaluated using a performance  
74 testing: 359 Chianina (CHI), 242 Marchigiana (MAR), 226 Piemontese (PIE) and 228 Romagnola  
75 (ROM). Genomic DNA was extracted from blood using the GenElute Blood Genomic DNA kit  
76 (Sigma Aldrich, St. Louis, MO, USA).

77 According to a preliminary bibliographic survey, 18 SNPs in the following 12 genes were selected  
78 on the basis of the reported correlations with beef traits: *growth hormone* (GH), *growth hormone*  
79 *receptor* (GHR), *growth differentiation factor 8* (GDF8), *ghrelin* (GHRL), *leptin* (LEP), *myogenic*  
80 *factor 5* (MYF5), *insulin-like growth factor 2* (IGF2), *leptin receptor* (LEPR), *neuropeptide Y*  
81 (NPY), *proopiomelanocortin* (POMC), *uncoupling protein 2* (UCP2) and *uncoupling protein 3*  
82 (UCP3). The list of the studied SNPs is reported in Table 1.

83 The genotyping of the investigated SNPs was performed by LGC Genomics (Hoddesdon, Herts,  
84 UK) using KASPar technology. To assess the genotyping accuracy, 10% of the samples were  
85 genotyped in duplicates.

86

87 *Statistical analysis*

88 The allele frequencies, observed and expected heterozygosity were calculated by the FSTAT  
89 software version 2.9.3.2 (Goudet, 2002).  $F_{IS}$  per breed across loci was calculated using the software  
90 GENETIX version 4.05 (Belkhir *et al.*, 1996-2004), while single-locus  $F_{ST}$ , pairwise  $F_{ST}$  and global  
91  $F_{ST}$  were estimated using FSTAT software version 2.9.3.2 (Goudet, 2002). The FDIST2 program  
92 (Beaumont and Nichols, 1996) was used to test loci for selective neutrality under an infinite alleles  
93 mutational model. The linkage disequilibrium between SNPs was tested by the software GENEPOP  
94 4.0 (Raymond and Rousset, 1995), using Bonferroni correction. For the linked SNPs, the haplotype  
95 frequencies were estimated by the software PHASE version 2.1 (Stephens and Scheet, 2005). The  
96 percentage of correct assignment per breed was calculated by the GeneClass2 software (Piry *et al.*,

97 2004), using the distance method, which does not require the assumption of independence among  
98 loci. Of the different genetic distance option, the  $D_a$  (Nei *et al.*, 1983) was used. The assignment  
99 was considered correct when the probability was higher than 50%. For each breed the assignment of  
100 20 individuals not in the reference sample was also tested.

101

## 102 **Results and discussion**

103 Three SNPs (GDF8-3, GH and NPY-3) were monomorphic in all the breeds (Table 2). The finding  
104 is not surprising for GH and NPY-3, which were reported to be polymorphic only in one or few  
105 breeds (Kim *et al.*, 2004; Sherman *et al.*, 2008), while it was unexpected for GDF8-3, for which  
106 polymorphism had been described in the Piemontese breed, though in a more limited sample  
107 (Vankan *et al.*, 2010). It is also interesting to note that in the Piemontese GDF8-1 was  
108 monomorphic too, while variability was reported by Crisà *et al.* (2003) in the same breed.

109 For most of the polymorphic SNPs, the allele distribution was quite similar in the four breeds, with  
110 the predominance of the same allele. The main differences concerned LEP-2, LEP-3 and LEPR loci.  
111 For seven SNPs (GHR-2, GHRL, IGF2, NPY-1, NPY-2, UCP-2 and UCP-3) the observed  
112 frequencies are in the range reported by Sherman *et al.* (2008) for European beef cattle breeds.

113 The variability of the single loci across breed, estimated by  $F_{ST}$ , showed a wide range, between  
114 0.005 (GHR-3) and 0.238 (LEP-2). High levels of genetic divergence were also observed for LEP-3  
115 (0.204) and, to a lesser extent, LEPR (0.159). It has been shown that  $F_{ST}$  values can help in  
116 detecting markers under directional selection or experiencing different strength of selection,  
117 because they are expected to show higher differentiation across breeds than neutral loci (Beaumont  
118 and Nichols, 1996; Narum and Hess, 2011). The distribution of  $F_{ST}$  as a function of heterozygosity  
119 indicated that all the markers, except for LEP-2, fall within the 0.95 limits (Figure 1). This finding  
120 suggests for LEP-2 deviations from a neutral-equilibrium model, possibly due to selection acting  
121 with different intensity in different breeds.

122 The heterozygosity values at single loci (data not shown) differed between breeds according to the  
123 allele frequencies, but the overall values were very similar. The  $F_{IS}$  values were not significant,  
124 indicating a low level of inbreeding in the four breeds (Table 3).

125 A significant ( $P = 0.0005$ ) linkage disequilibrium was observed only for the SNPs located in the  
126 same gene: GHR-1 - GHR-2, LEP-1 - LEP-2 - LEP-3, NPY-1 - NPY-2.

127 The haplotypes frequencies (Table 4) showed a quite different situation across breeds. For example,  
128 Romagnola differed from the other breeds for the most frequent haplotype at GHR and NPY loci.  
129 For LEP gene, a total of 8 haplotypes were observed, with CCT more frequent, except for  
130 Piemontese. Some of the rarest haplotypes were absent in a given breed: TCC in Chianina, CGT  
131 and TGT in Marchigiana, TCT in Piemontese.

132 The genetic differentiation ( $F_{ST}$ ) in the overall sample (Table 5) was high (0.085;  $P=0.001$ ) with  
133 respect to the value of 0.049 obtained in a comparable study on the same breeds using microsatellite  
134 markers (Dalvit *et al.*, 2008). The pairwise  $F_{ST}$  also detected a higher degree of between breed  
135 variability, so that the functional markers seemed to be even more valuable than neutral markers in  
136 detecting variability among these breeds. The picture of the relationships among breeds was also  
137 different from the one shown by neutral markers. In fact, the highest pairwise  $F_{ST}$  values (0.1189 to  
138 0.1877) were obtained in the comparisons of Piemontese with the other breeds, while the lowest  
139 value (0.0296) was observed between Chianina and Marchigiana. The differentiation of Piemontese  
140 from the others three breeds, already observed with different markers (Ciampolini *et al.*, 1995; Blott  
141 *et al.*, 1998), supports the phylogenetic origin described by Baker and Manwell (1980). Moreover,  
142 the geographical isolation of the Piemontese and, more recently, the difference in selection indexes  
143 could have contributed to its differentiation. The higher similarity among the breeds of the Central  
144 Italy is consistent with both their known history and common selection programmes. In particular,  
145 the closeness of Marchigiana with Romagnola and especially Chianina is expected on the basis of  
146 its documented origin from crossing of local Marche cattle with the two breeds (Bonadonna, 1976).



147 The results for breed assignment reflected the genetic differentiation of the breeds (Table 6). In  
148 agreement with data reported in different studies with different breeds and markers (Ciampolini *et*  
149 *al.*, 2000; Negrini *et al.*, 2007; Dalvit *et al.*, 2008), the Piemontese breed had the highest percentage  
150 of correct assignment (87.6, with 61% of the values exceeding 95%), while Marchigiana had the  
151 lowest one (47.5, with only 4% of the values exceeding 95%). Moreover, the wrongly assigned  
152 Marchigiana animals were mainly classified as Chianina because of their low genetic differentiation  
153 ( $F_{ST} = 0.03$ ).

154 The assignment test of independent samples confirmed the best results for the Piemontese breed,  
155 with 19 out of 20 animals correctly assigned. For the other breeds, in the same test, the percentage  
156 of correct assignment ranged from 55% for Romagnola to 70% for Chianina.

157

## 158 **Conclusions**

159 The results showed that for the breeds here considered functional markers allowed to detect a  
160 greater level of genetic differentiation compared to that observed for the same breeds with neutral  
161 markers. The two classes of markers reflect between-breed differences due to different sources of  
162 variation, mainly genetic drift for neutral markers and selection for functional markers. Therefore,  
163 in a more general view, the combined study of neutral markers and SNPs in functional regions can  
164 provide complementary information about the genetic dynamics of the breeds within a species.

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168 The authors want to thank the two anonymous referees for their valuable comments and  
169 constructive suggestions.

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**Table 1.** Information on the studied SNPs.

<b>Gene</b>	<b>Chromosome</b>	<b>SNP name</b>	<b>Location</b>	<b>Accession No and base position</b>	<b>SNP</b>
GH	BTA19	GH	Promoter	AY445811:g.358	C>T
GHR	BTA20	GHR-2	Promoter	AF126288:g.149	G>A
GHR	BTA20	GHR-3	Intron IV	AY643807:g.300	A>G
GDF8	BTA2	GDF8-1	Promoter	AJ438578:g.843	T>A
GDF8	BTA2	GDF8-3	Exon I	AY725215:g.229	A>C
GHRL	BTA22	GHRL	Intron III	AY455980:g.446	A>G
LEP	BTA4	LEP-1	Promoter	AB070368:g.528	C>T
LEP	BTA4	LEP-2	Promoter	AB070368:g.1759	G>C
LEP	BTA4	LEP-3	Exon II	AY138588:g.305	T>C
MYF5	BTA5	MYF5	Intron II	M95684:g.1948	A>G
IGF2	BTA29	IGF2	Exon II	AY237543:g.150	C>T
LEPR	BTA3	LEPR	Exon XX	AJ580801:g.115	C>T
NPY	BTA14	NPY-1	Intron II	AY491054:g.284	A>G
NPY	BTA4	NPY-2	Intron II	AY491054:g.666	A>G
NPY	BTA4	NPY-3	Intron II	AY491054:g.3032	C>T
POMC	BTA11	POMC	Intron II	J00021:g.254	C>T
UCP2	BTA15	UCP2	Intron V	AY14782:g.380	G>C
UCP3	BTA15	UCP3	Intron III	AF127030:g.1099	G>A

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**Table 2.** Alleles frequencies in the studied SNPs (only one allele per SNP is reported).

SNP name	Alleles	Breeds				$F_{ST}$
		CHI	MAR	PIE	ROM	
GDF8-1	A	0.247	0.171	0.000	0.099	0.074
GDF8-3	C	1.000	1.000	1.000	1.000	-
GH	C	1.000	1.000	1.000	1.000	-
GHR-2	A	0.496	0.620	0.462	0.215	0.087
GHR-3	A	0.752	0.682	0.665	0.720	0.005
GHRL	A	0.857	0.932	0.797	0.952	0.037
IGF2	C	0.787	0.669	0.749	0.765	0.010
LEP-1	C	0.937	0.833	0.597	0.633	0.105
LEP-2	C	0.781	0.633	0.137	0.399	0.238
LEP-3	C	0.210	0.407	0.830	0.541	0.204
LEPR	C	0.563	0.529	0.926	0.403	0.159
MYF5	A	0.416	0.560	0.426	0.424	0.014
NPY-1	A	0.097	0.060	0.232	0.129	0.036
NPY-2	C	0.267	0.178	0.311	0.491	0.061
NPY-3	A	1.000	1.000	1.000	1.000	-
POMC	C	0.802	0.924	0.819	0.956	0.039
UCP2	C	0.930	0.917	0.810	0.853	0.022
UCP3	A	0.625	0.581	0.774	0.426	0.064

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CHI: Chianina, MAR: Marchigiana, PIE: Piemontese, ROM: Romagnola.

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**Table 3.** Mean observed heterozygosity ( $H_o$ ), mean expected heterozygosity ( $H_e$ ) and  $F_{IS}$  in the studied breeds.

Breeds	$H_o$	$H_e$	$F_{IS}$
CHI	0.35 (0.13)	0.34 (0.13)	-0.027 (-0.057 – 0.001)
MAR	0.34 (0.159)	0.34 (0.15)	0.005 (-0.039 - 0.043)
PIE	0.34 (0.14)	0.33 (0.14)	-0.022 (-0.061 - 0.013)
ROM	0.36 (0.16)	0.36 (0.15)	-0.008 (-0.051 - 0.029)

187 CHI: Chianina, MAR: Marchigiana, PIE: Piemontese, ROM: Romagnola.

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**Table 4.** Haplotype frequencies.

Gene	Haplotype	Breeds			
		CHI	MAR	PIE	ROM
GHR	[GHR-2, GHR-3]				
	AA	0.49574	0.61981	0.43393	0.20685
	AG	0.00004	0.00002	0.02708	0.01064
	GA	0.25426	0.06200	0.22891	0.51288
	GG	0.24996	0.31816	0.31008	0.26963
LEP	[LEP-1, LEP-2, LEP-3]				
	CCC	0.03602	0.03427	0.00485	0.00493
	CCT	0.74430	0.59319	0.13779	0.38868
	CGC	0.11462	0.20449	0.45569	0.24038
	CGT	0.04133	0.00000	0.00020	0.00045
	TCC	0.00000	0.00515	0.00022	0.00014
	TCT	0.00014	0.00018	0.00000	0.00012
	TGC	0.06183	0.16273	0.36683	0.29229
	TGT	0.00178	0.00000	0.03443	0.07302
NPY	[NPY-1, NPY-2]				
	AC	0.00034	0.00037	0.00025	0.00020
	AT	0.09715	0.05955	0.23017	0.12760
	GC	0.26707	0.17731	0.30851	0.49307
	GT	0.63544	0.76277	0.46108	0.37913

193 CHI: Chianina, MAR: Marchigiana, PIE: Piemontese, ROM: Romagnola.

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197 **Table 5.** Pairwise and global  $F_{ST}$ .  
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	CHI	MAR	PIE	ROM
CHI	-			
MAR	0.0296	-		
PIE	0.1877	0.1403	-	
ROM	0.1029	0.0786	0.1189	-
Global $F_{ST}$	0.0848 ( $P = 0.001$ )			

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 200 CHI: Chianina, MAR: Marchigiana, PIE: Piemontese, ROM: Romagnola.  
 201 After Bonferroni's correction all the values are significant.  
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**Table 6.** Percentage of animals assigned to each breed.

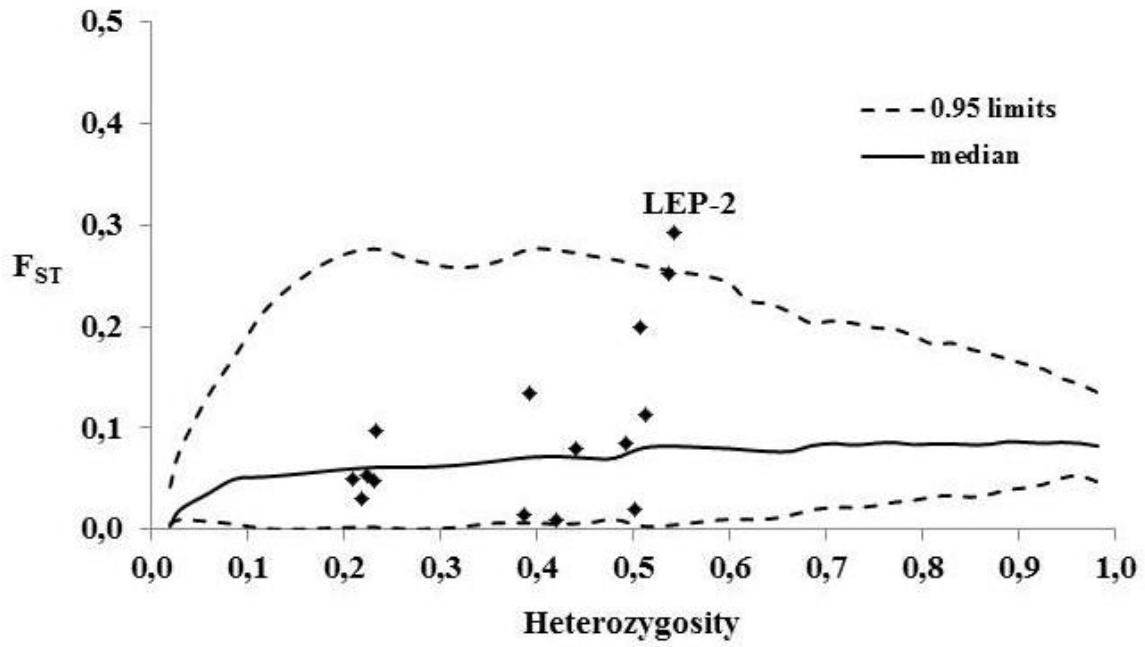
Breeds	Assigned to				Mean probability of assignment
	CHI	MAR	PIE	ROM	
CHI	70.8	15.9	5.8	7.5	79.3
MAR	31.4	47.5	10.8	10.3	69.5
PIE	3.5	5.3	87.6	3.6	91.0
ROM	15.4	7.9	11.8	64.9	82.9

207 CHI: Chianina, MAR: Marchigiana, PIE: Piemontese ROM: Romagnola.  
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210 Figure 1.  $F_{ST}$  values estimated for the 15 polymorphic markers, plotted against heterozygosity.  
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