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Published version:

DOI:10.4081/ijas.2015.3465

Lasagna E.; Ceccobelli S.; Di Lorenzo P.; Albera A.; Filippini F.; Sarti F.M.; Panella F.; Di Stasio L.. Comparison of four Italian beef cattle breeds by means of functional genes.. ITALIAN JOURNAL OF ANIMAL SCIENCE. 14 pp: 86-89.

DOI: 10.4081/ijas.2015.3465

Running title: SNPs for genetic characterization of Italian beef cattle

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

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Abstract

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

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

Keywords: Chianina, Marchigiana, Piemontese, Romagnola, SNPs

Introduction

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

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

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

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

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

Material and methods

Animal sampling and molecular analysis

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

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

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

Statistical analysis

The allele frequencies, observed and expected heterozygosity were calculated by the FSTAT software version 2.9.3.2 (Goudet, 2002). F_{IS} per breed across loci was calculated using the software GENETIX version 4.05 (Belkhir *et al.*, 1996-2004), while single-locus F_{ST}, pairwise F_{ST} and global F_{ST} were estimated using FSTAT software version 2.9.3.2 (Goudet, 2002). The FDIST2 program (Beaumont and Nichols, 1996) was used to test loci for selective neutrality under an infinite alleles mutational model. The linkage disequilibrium between SNPs was tested by the software GENEPOP 4.0 (Raymond and Rousset, 1995), using Bonferroni correction. For the linked SNPs, the haplotype frequencies were estimated by the software PHASE version 2.1 (Stephens and Scheet, 2005). The percentage of correct assignment per breed was calculated by the GeneClass2 software (Piry *et al.*, 2004), using the distance method, which does not require the assumption of independence among loci. Of the different genetic distance option, the Da (Nei *et al*., 1983) was used. The assignment was considered correct when the probability was higher than 50%. For each breed the assignment of 20 individuals not in the reference sample was also tested.

Results and discussion

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

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

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

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

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

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

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

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

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

Conclusions

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

Acknowledgements

The research was financially supported by SelMol and Innovagen projects.

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

Table 1. Information on the studied SNPs.

Gene	Chromosome	SNP name	Location	Accession No and base position	SNP
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

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

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

Table 3. Mean observed heterozygosity (Ho), mean expected heterozygosity (He) and F_{IS} in the studied breeds.

Breeds	Ho	He	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)

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

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

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

Table 5. Pairwise and global F_{ST}.

	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)			

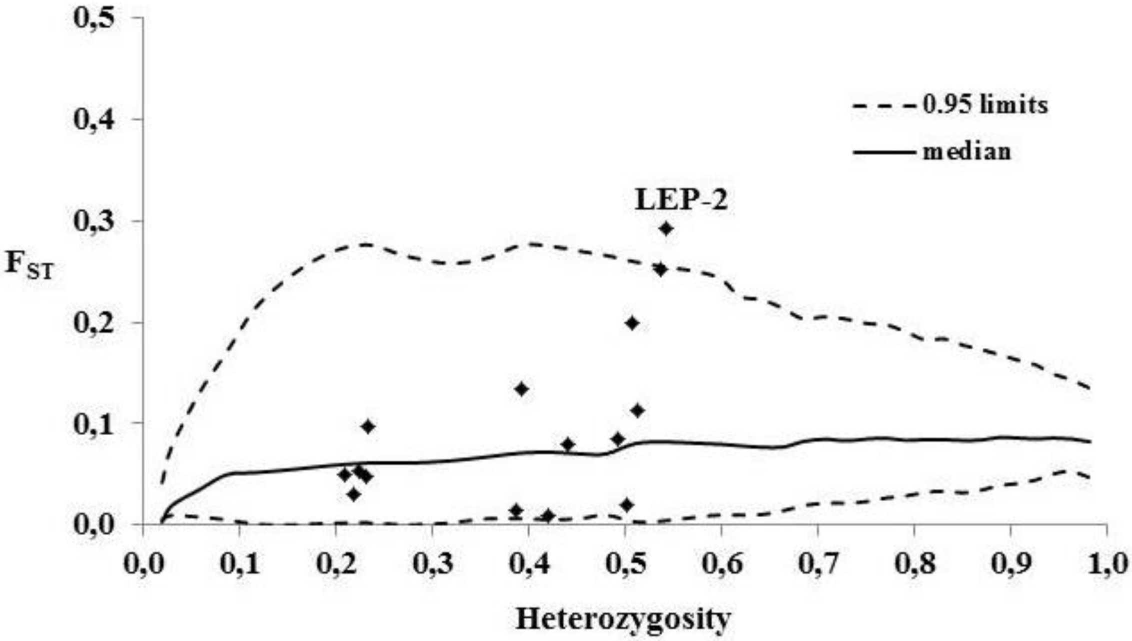
CHI: Chianina, MAR: Marchigiana, PIE: Piemontese, ROM: Romagnola.
After Bonferroni's correction all the values are significant.

Table 6. Percentage of animals assigned to each breed.

	Assigned to				Mean probability of assignment
Breeds	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

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

Figure 1. F_{ST} values estimated for the 15 polymorphic markers, plotted against heterozygosity.



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