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 largely recognised all over the world. Here, 18 single nucleotide polymorphisms (SNPs) in 12 candidate genes involved on meat traits were investigated on 1055 candidates for selection in order to analyse 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 breeds and the analysis of the relationship between genetic differentiation and heterozygosity indicated significant deviation from a neutral-equilibrium model for LEP-2. 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).

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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; Sharr 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; Ng et al., 2007; Di Stasio et al., 2007; Sherman et al., 2008), the present investigation was carried out in order to analyse the within and between-breed variability in Chianina, Marchigiana, Piemontese and Romagnola breeds with a functional marker approach.

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 specialised breeds for meat production and the quality of their products is widely recognised 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 groupings were obtained by Blott et al. (1998), using blood groups and protein polymorphisms. More recently, molecular markers, such as AFLP (Negreni et al., 2007) and microsatellites (Dalvit et al., 2008), were used to characterise the same breeds in the framework of product traceability.

Materials 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, MO, USA).

According to a preliminary bibliographic survey, 18 single nucleotide polymorphisms (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 assess 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). The inbreeding coefficient within population ($F_{ST}$) per breed across loci was calculated using the software GENETIX version 4.05 (Belkhir et al., 1996-2004), while single-locus fixation index ($F_{ST}$), pairwise $F_{ST}$ and global $F_{ST}$ were estimated using FSTAT software version 2.9.3.2 (Goudet, 2002). The FDIST2 programme (Beaumont and Nichols, 1996) was used to test loci for selective neutrality under an infinite alleles mutational model, setting the confidence limits at 95%. 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 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

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Table 1. Information on the single nucleotide polymorphisms studied.

| Gene | Chromosome | SNP name | Location | Accession No. | SNP |
|------|------------|----------|----------|---------------|-----|
| GH   | BTA19      | GHR      | Promoter | AY45811.g.358 | C>T |
| GHR  | BTA20      | GHR-2    | Promoter | AF12628.g.149 | G>A |
| GHR  | BTA20      | GHR-3    | Intron IV | AY43807.g.300 | A>G |
| GDF8 | BTA2        | GDF8-1   | Promoter | A438578.g.843 | T>A |
| GDF8 | BTA2        | GDF8-3   | Exon I   | AY25215.g.229 | A>C |
| GHRL | BTA22      | GHRL     | Intron III | AY45580.g.446 | A>G |
| LEP  | BTA4       | LEP-1    | Promoter | AB07036.g.752 | C>T |
| LEP  | BTA4       | LEP-2    | Promoter | AB07036.g.1759 | G>C |
| LEP  | BTA4       | LEP-3    | Exon II  | AY13858.g.305 | T>C |
| MNY5 | BTA5       | MNY5     | Intron II | M5684.g.1946 | A>G |
| IGF2 | BTA29      | IGF2     | Exon II  | AY257543.g.150 | C>T |
| LEPR | BTA3       | LEPR     | Exon X   | A558081.g.115 | C>T |
| NPY  | BTA14      | NPY-1    | Intron II | AY49165.g.284 | A>G |
| NPY  | BTA4       | NPY-2    | Intron II | AY49165.g.666 | A>G |
| NPY  | BTA4       | NPY-3    | Intron II | AY49165.g.3032 | C>T |
| POMC | BTA11      | POMC     | Intron II | J00028.g.254 | C>T |
| UCP2 | BTA15      | UCP2     | Intron V  | AY14782.g.380 | G>C |
| UCP3 | BTA15      | UCP3     | Intron III | AY127030.g.1099 | G>A |

SNP, single nucleotide polymorphism.

Table 2. Frequencies of alleles in the single nucleotide polymorphisms studied.

| SNP name | Alleles | Breeds | FST |
|----------|---------|--------|-----|
| GDF8-1   | A       | CHI    | 0.247 | 0.171 | 0.089 | 0.074 |
| GDF8-3   | C       | MAR    | 1.000 | 1.000 | 1.000 | - |
| GH       | C       | PIE    | 1.000 | 1.000 | 1.000 | - |
| GDF8     | A       | ROM    | 0.752 | 0.620 | 0.665 | 0.720 |
| GHR-2    | A       | CHI    | 0.496 | 0.620 | 0.462 | 0.215 |
| GHR-3    | A       | MAR    | 1.000 | 1.000 | 1.000 | - |
| GHRL     | A       | ROM    | 0.857 | 0.932 | 0.797 | 0.952 |
| IGF2     | C       | CHI    | 0.787 | 0.669 | 0.749 | 0.765 |
| LEP-1    | C       | MAR    | 0.937 | 0.833 | 0.597 | 0.633 |
| LEP-2    | C       | PIE    | 0.781 | 0.633 | 0.137 | 0.399 |
| LEP-3    | C       | ROM    | 0.210 | 0.407 | 0.830 | 0.541 |
| LEPR     | C       | CHI    | 0.563 | 0.529 | 0.926 | 0.403 |
| MNY5     | A       | MAR    | 0.416 | 0.560 | 0.426 | 0.242 |
| NPY-1    | A       | PIE    | 0.097 | 0.069 | 0.232 | 0.129 |
| NPY-2    | C       | ROM    | 0.267 | 0.178 | 0.311 | 0.491 |
| NPY-3    | A       | CHI    | 1.000 | 1.000 | 1.000 | - |
| POMC     | C       | MAR    | 0.802 | 0.924 | 0.819 | 0.956 |
| UCP2     | C       | PIE    | 0.930 | 0.917 | 0.810 | 0.853 |
| UCP3     | A       | ROM    | 0.625 | 0.581 | 0.774 | 0.426 |

SNP, single nucleotide polymorphism, CHI, Chianina breed; MAR, Marchigiana breed; PIE, Piemontese breed; ROM, Romagnola breed; FST, fixation index. Only one allele per SNP is reported.

Table 3. Mean observed and expected heterozygosity and inbreeding coefficient within population in the studied breeds.

| Gene | Ho | He | FIS |
|------|----|----|-----|
| CHI  | 0.35 (0.13) | 0.34 (0.13) | -0.027 (0.057-0.001) |
| MAR  | 0.34 (0.15) | 0.34 (0.15) | 0.005 (0.039-0.043) |
| PIE  | 0.34 (0.14) | 0.33 (0.14) | -0.022 (0.061-0.015) |
| ROM  | 0.36 (0.16) | 0.36 (0.15) | -0.008 (0.051-0.029) |

Ho, observed heterozygosity; He, expected heterozygosity; FIS, inbreeding coefficient within population; CHI, Chianina breed; MAR, Marchigiana breed; PIE, Piemontese breed; ROM, Romagnola breed. Values in parentheses represent 95% confidence interval.
allele. The main differences concerned LEP-2, LEP-3 and LEPR loci. For seven SNPs (GHR-2, GHR1, IGFR2, 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 (Narum and Hess, 2011).

The hypothesis of deviation from neutrality was tested using the FDIST2 approach (Beaumont and Nichols, 1996), which analyses the distribution of $F_{ST}$ as a function of heterozygosity, in order to identify markers with outlying values, hence potentially under selection. Of the examined markers, only LEP-2 did not fall within the 95% confidence interval ($\hat{F}_{ST} = 0.985$), which can suggest 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 haplotype 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 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 (Ciampani et al., 2000; Negri et al., 2004; Dalvit et al., 2008), the Piemontese breed had the highest percentage of correct assignment (87.6, with 61% of the values exceeding 95%),

### Table 4. Haplotype frequencies.

| Gene     | Haplotype | 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        | 0.25428 | 0.06200 | 0.22891 | 0.51338 |
|          | GG        | 0.24998 | 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 | 0.53319 | 0.13777 | 0.38868 |
|          | CGC       | 0.11462 | 0.20449 | 0.45569 | 0.24038 |
|          | CGT       | 0.04133 | 0.00000 | 0.00020 | 0.00045 |
|          | TCC       | 0.00000 | 0.00151 | 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 | 0.00343 | 0.07302 |
|          | 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 | 0.70277 | 0.46108 | 0.37193 |

### Table 5. Pairwise and global fixation index.

| Gene | CHI | MAR | PIE | ROM |
|------|-----|-----|-----|-----|
| CHI  |    | MAR |    |    |
|      | MAR |    | PIE |    |
|      | ROM |    |     |    |

| CHI | MAR | PIE | ROM |
|-----|-----|-----|-----|
| CHI | 0.0296 | - | - | - |
| PIE | 0.1877 | 0.1403 | - | - |
| ROM | 0.1029 | 0.0786 | 0.1189 | - |

Global $F_{ST}$ = 0.0848 ($P=0.001$)

### Table 6. Percentage of animals assigned to each breed.

| Assigned to | Mean probability of assignment |
|-------------|-------------------------------|
| CHI | MAR | PIE | ROM |
| CHI | 70.8 | 15.9 | 5.8 | 7.5 |
| MAR | 31.4 | 47.5 | 10.8 | 10.3 |
| PIE | 3.5 | 5.3 | 87.6 | 3.6 |
| ROM | 15.4 | 7.9 | 11.8 | 84.9 |

CHI, Chianina breed; MAR, Marchigiana breed; PIE, Piemontese breed; ROM, Romagnola breed; $F_{ST}$, fixation index.

After Bonferroni’s correction all the values are significant.
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 (FST=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.

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