Using runs of homozygosity to detect genomic regions associated with susceptibility to infectious and metabolic diseases in dairy cows under intensive farming conditions

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Abstract. Runs of homozygosity (ROH) are contiguous stretches of homozygous genome which likely reflect transmission from common ancestors and can be used to track the inheritance of haplotypes of interest. In the present paper, ROH were extracted from 50K SNPs and used to detect regions of the genome associated with susceptibility to diseases in a population of 468 Holstein-Frisian cows. Diagnosed diseases were categorised as infectious diseases, metabolic syndromes, mastitis, reproductive diseases and locomotive disorders. ROH associated with infectious diseases, mastitis and locomotive disorders were found on BTA 12. A long region of homozygosity linked with metabolic syndromes, infectious and reproductive diseases was detected on BTA 15, disclosing complex relationships between immunity, metabolism and functional disorders. ROH associated with infectious and reproductive diseases, mastitis and metabolic syndromes were observed on chromosomes 3, 5, 7, 13 and 18. Previous studies reported QTLs for milk production traits on all of these regions, thus substantiating the known negative relationship between selection for milk production and health in dairy cattle.

Keywords: runs of homozygosity (ROH), disease susceptibility, dairy cattle, genetic associations

Introduction

In dairy cattle intensive farming, diseases are certainly a major concern. They reduce animal welfare, entail considerable direct treatment costs, decrease milk production, may cause mortality and present biosafety risks related to veterinary drugs (e.g. antibiotics) residues in milk and the possible transmission to humans (zoonoses, e.g. tuberculosis). Infectious (e.g. infectious mastitis) and metabolic (e.g. ketosis) diseases comprise most of the diseases observed in dairy cattle.

A strategy to reduce the incidence of diseases and improve the health status of the herd could be to genetically enhance the resistance to diseases. This could address both resistance to specific diseases and a general improvement of the immune system of the animals (~“robustness of dairy cows” [1]).
The identification of genomic regions associated with the susceptibility to diseases is an important preliminary step towards implementing breeding strategies aimed at improving dairy cattle health. This is now made possible by the use of data obtained through next generation genotyping and sequencing technologies (e.g. SNP chips, whole-genome sequences). Genome-wide association studies (GWAS) are commonly used to scan the genome in search of polymorphisms associated with the analyzed phenotype. However, GWAS methods typically analyse one locus at a time, are prone to spurious associations and, but for clear signals, are not always of straightforward interpretation (e.g. [2]).

Runs of homozygosity (ROH, [3]) may be an alternative approach to genome-wide scans for signals of genotype-phenotype association. Runs of homozygosity are contiguous stretches of homozygous genome which likely reflect transmission from a common ancestor, and can therefore be considered as being IBD (identical by descent). ROH have been used to estimate inbreeding both in humans [3] and cattle [4–6]. Hildebrandt et al. [7] applied a similar concept to map recessive disease genes in human populations, and Biscarini et al. [8] used ROH to look for the causal mutations for arthrogryposis and macroglossia in Piedmontese cattle.

Hypothesizing that genetic variants associated with increased risk of disease are more likely to be recessive than dominant (see Hildebrandt et al. for human diseases [7]), looking for associations with homozygous segments of the genome appears to be a reasonable strategy. In this study, ROH were used to detect genomic regions associated with susceptibility to 5 categories of diseases (infectious, metabolic and reproductive diseases, mastitis and locomotive disorders) in dairy cattle.

Material & Methods

Available data

A population of 468 Holstein-Frisian cows between the first and fifth lactation distributed over 4 herds (Table 1 from the Po Valley region in Northern Italy was analysed. All cows were farmed under intensive conditions (use of concentrate feeds, no pasture, indoor housing) in high-yielding dairy farms and were genotyped with the Illumina BovineSNP 50 beadchip version 2 (50k), based on the UMD 3.1 assembly of the *Bos taurus* genome.

Genotypic data were edited for individual and SNP call rate (> 90%). Unmapped SNPs were removed, while those on the sex chromosome were used. This left 458 cows and 53457 SNPs available for the analysis. Phenotype recording was carried out by veterinary practitioners within the framework of the regional project Prozoo [9,11]: diagnosis, onset and treatment for each disease were recorded. Given the limited number of cases for each specific disease, diseases were grouped together in five homogeneous categories in order to increase the statistical power of the analysis: infectious diseases, metabolic syndromes, reproductive diseases, mastitis and locomotive disorders. Categories were partially overlapping (for instance, infectious mastitis was classified both as mastitis...
Table 1: Population size after data editing. On the first row, per herd and total number of cows. Rows 2-6 show n. of cases for each disease category. Disease category may overlap (e.g. infectious mastitis is both infectious disease and mastitis).

|        | herd1 | herd2 | herd3 | herd4 | total |
|--------|-------|-------|-------|-------|-------|
| COWS   | 155   | 88    | 148   | 67    | 458   |
| INFD   | 77    | 29    | 60    | 20    | 189   |
| METD   | 62    | 10    | 68    | 10    | 152   |
| MAST   | 46    | 13    | 44    | 13    | 117   |
| REPRD  | 65    | 16    | 62    | 17    | 163   |
| LOCD   | 21    | 6     | 33    | 13    | 74    |

1INFD: infectious diseases; METD: metabolic diseases; MAST: mastitis; REPRD: reproductive diseases; LOCD: locomotive disorders.

and infectious disease). Table 2 reports the classification of diagnosed diseases into the above mentioned categories. There were 189, 152, 117, 163 and 74 cases respectively for infectious diseases, metabolic syndromes, mastitis, reproductive diseases and locomotive disorders. For each analysis, all animals not in the disease group were used as controls (e.g. for infectious diseases there were 189 cases and 458 - 189 = 269 controls).

Table 2: List of diagnosed diseases falling in each of five (partially overlapping) categories: infectious, metabolic reproductive, locomotive diseases and mastitis.

| Disease category       | Included diseases                                                                 |
|------------------------|----------------------------------------------------------------------------------|
| Infectious diseases    | mastitis, peritonitis, enteritis, traumatic reticuloperitonitis, digital dermatitis, interdigital dermatitis, foot rot, laminitis, pyometra, metritis, endometritis, clostridiosis |
| Metabolic syndromes    | milk fever, ovarian cysts, persistent corpus lumen, ruminal atony, displaced abomasum, indigestion, mesenteric torsion, volvulus, ketosis, steatosis |
| Reproductive diseases  | retained placenta, dystocia, ovarian cysts, hypoplastic ovaries, hypotrophic ovaries, persistent corpus luteum, abortion, embryo resorption, metritis, mummified fetus, stillbirth, endometritis, parauterine abscess, pyometra |
| Mastitis               | mastitis, teat obstruction, teat lesions                                          |
| Locomotive disorders   | digital dermatitis, interdigital dermatitis, sole ulcer, foot rot, white line disease, laminitis, tyloma, carpal arthritis, femoral fracture |
Runs of homozygosity.

Under the hypothesis that complex diseases have a genetic component made up of several recessive variants distributed throughout the genome, each with a small effect [3], runs of homozygosity (ROH) were applied to detect genetic regions associated with susceptibility to diseases. Single-SNP GWAS, which compares allele frequency at each locus, would in fact detect also an excess of dominant alleles. ROH are defined in diploid organisms as contiguous stretches of homozygous genotypes, which reflect transmission of identical haplotypes from common ancestors. Instead of focusing on a single locus, ROH consider also the surrounding regions, thus accounting for the hitch-hiking effect (neighbouring SNPs changing frequency together with a selected locus with which they are in strong linkage disequilibrium; [10]). The observed homozygosity was therefore estimated at each SNP locus and averaged along a sliding window spanning 1000 kbps and progressing SNP by SNP. A maximum of 5 missing genotypes and 1 heterozygous genotypes were permitted for a contiguous stretch of DNA to be considered a ROH.

ROH are basically a model-free statistical technique. Unlike classic GWAS, there is no direct modeling of the phenotype of interest nor a straightforward way to test for the strength of detected associations. However, approaches can be conceived to assess the significance of the detected signals. In this experimental application of ROH to association studies, the significance of phenotype/genotype associations was tested by looking at the difference in homozygosity between cases and controls. The average homozygosity at each SNP locus within the ROH was computed for cases and controls separately, and the significance of the difference tested through a one-tailed t-test:

$$
\begin{align*}
H_0 &: \mu_{\text{cases}} = \mu_{\text{controls}} \\
H_1 &: \mu_{\text{cases}} > \mu_{\text{controls}}
\end{align*}
$$

Additionally, the resulting p-values were compared with those obtained from a standard single-SNP GWAS for the same traits on the same population. Drawing inspiration from the concept of non-inferiority trials [15], the false discovery rate (FDR) was computed for both sets of p-values and tested for equivalence. The null hypothesis was that the FDR was actually larger for ROH than in GWAS:

$$H_0 : FDR_{\text{ROH}} - FDR_{\text{GWAS}} > M$$

where $M$ is a tolerance margin for the difference. This states that ROH are inferior to GWAS. The alternative hypothesis was that the FDR was not different under both methods:

$$H_1 : FDR_{\text{ROH}} - FDR_{\text{GWAS}} < M$$

which implies that ROH are not inferior to GWAS. Non-inferiority was tested for the 5 traits analysed (infectious, metabolic, reproductive, locomotion diseases and mastitis) with a tolerance margin $M = 0.01$. In all cases $H_0$ was rejected,
indicating that the ROH approach was not inferior to standard GWAS (average p-value: \(2.27 \cdot 10^{-6}\)).

**Software**

The software PLINK v1.07 \[18\] was used for the analysis. No restriction on the minimum number of SNPs in a ROH was applied, and the default (1000 kbps) maximum gap between consecutive SNPs was used, in order to account for the lower SNP density in the 50k SNP chip compared to the HD (\(\sim 777k\)) SNP chip. Data preparation, heritability estimates, graphical plots and post-processing analyses were produced within the open source programming environment R \[19\].

**Results & Discussion**

A total of 1273 distinct ROH were detected: this number comprised all ROH found with the chosen parameters, irrespective of whether they were found in all cows or a subset of them. The average ROH length was 285.7 kbps. In principle, stretches of homozygous DNA can appear in all animals, regardless of their health status; those associated with susceptibility to disease are however supposed to be more frequent in cases than in controls. Therefore, the longest ROH (less likely to be due to chance) which were most frequent in cases were retained as results of interest and are reported in Table 3. These include 17 ROH for infectious diseases, 10 for reproductive diseases, 7 for metabolic syndromes and 4 for mastitis and locomotive disorders. For the 42 reported ROH, the frequency in cases relative to controls varied from 51.4% to 100%; their average p-value and FDR were 0.398 and 0.612, respectively. ROH in Table 3 were not always observed in all cases and controls: the number of cows (cases + controls) involved ranged from 124 for infectious and metabolic disease to 56 for reproductive diseases. An important issue in association studies is the expected statistical power of the analysis. This is known to depend on sample size, the heritability of the trait and linkage disequilibrium (LD) between markers and QTLs \[20\]. Heritability was not always estimable for all traits and models; when estimable, it ranged between 0.05 and 0.10 (in line with literature values: e.g. \[14\]). The average LD between adjacent markers in the available Holstein-Frisian population was estimated as \(r^2 = 0.23\). With 458 individuals, the power to detect a QTL explaining 1% of the phenotypic variance would be therefore about 18%.

In the next section a few results of interest are described. The reader is warned that, given the low power of the study and the high FDR for all reported associations, this mostly has only speculative value. It is however indicative of the output obtained from a ROH analysis.

**Regions associated with disease susceptibility**

The longest ROH (117 SNPs, \(\sim 5.3\) Mbps) was found on BTA 11 and was associated with infectious diseases. On BTA 12, three distinct regions (11 414 743 –
13 050 009 bps; 25 878 820 – 30 099 199 bps; 51 834 816 – 53 428 996 bps) were associated with infectious diseases. The first two of these regions were also found in cows with, respectively, locomotive disorders and mastitis. Both conditions often have a microbial etiology (e.g. infectious mastitis, foot rot). Interestingly, the ROH region at ~ 12 Mbps on BTA 12 contains the VWA8 gene (von Willebrand factor A domain), whose mutations might be implied in coagulation abnormalities and may be related with musculoskeletal disorders. Two other regions linked with both infectious diseases and mastitis were found on BTA 24 and BTA 28. On BTA 28 Kolbehdari et al. [17] found polymorphisms (28 149 059 bps and 24 423 785 bps) associated with the conformation of the mammary system and angularity, which might be related to the occurrence of mastitis. A poor conformation of the udder may be a predisposing factor for mastitis, as well as intensive selection for dairy type. The ROH on BTA 28 was found to be associated also with locomotive disorders, pointing at possible detrimental effects of selection for dairy type on locomotion. Homozygous haplotypes linked with locomotive disorders were detected also on BTA 6 between 107 186 270 and 107 835 761 bps; Kolbehdari et al. [17] reported a nearby association with overall rump conformation, which can be related to locomotion anomalies. Upstream along BTA 6 the casein (α-s1, α-s2, β and κ) loci are located which could hint at a link between selection for casein variants and general functionality of the animals.

In some cases, consecutive ROH are quite close together (e.g. BTA 7 and BTA 15) which might reflect the existence of a founder allele broken down by recombination over generations. The parameters for ROH detection (consecutive SNP gap > 1Mbps, more missing or heterozygous SNPs) may also play a role, though. Figure 1 shows the average observed heterozygosity in cases and controls for reproductive diseases along BTA 15: ROH are visible as regions of low heterozygosity (and conversely high homozygosity) in cases (black line) as compared to controls (grey line). ROH associated with impaired reproductive function were found on chromosomes 4, 8, and 18, where other studies detected associations with calving ease [17], calving interval [21] and type traits like body depth, rump width, stature and strength [12].

In this study we identified genetic associations with infectious diseases, mastitis, metabolic syndromes and reproductive diseases on chromosomes 3, 5, 7, 13 and 18 where earlier works reported QTLs for milk production traits [13, 21]: this confirms the known negative relationship between strong selection for milk production and the health of the animals. Negative genetic correlations between milk production and disease resistance in Holstein (e.g. [16]) and dairy cattle in general (Norwegian Red, [23]) were estimated. Though negative, such correlations are larger than -1; this implies that alleles at polymorphic loci with positive effects on both characteristics (or positive effects on production and neutral effect on health) can be found (see for instance Pimentel et al. [22] for fertility and production).
Conclusions

Scanning the genome for runs of homozygosity provides a valid alternative to GWAS studies for the genetic dissection of complex disease traits and for detecting such variants with beneficial effects on both production and the health of the animals. Results could be used in breeding programs aimed at improving milk production while enhancing the resistance to diseases at the same time. Emphasis in selection objectives could be more strongly placed on functional rather than productive traits, thus improving functionality while not completely neglecting production, thanks to residual positive correlations. Alternatively, haplotypes favorably linked to either production, functionality or both could be used to identify carrier animals to breed next generation, in a sort of “haplotype selection”. Several aspects of the use of ROH in association studies still need to be investigated, though. Examples are: the construction of significance tests (e.g. here the difference in homozygosity between cases and controls was tested; however, the differential frequency of ROH could be tested instead); the issue of multiple testing (e.g. permute cases and controls so to create a distribution of ROH under the hypothesis of no association); accounting for systematic effects (e.g. using residuals instead of the original observations, or performing the ROH analysis within class of effects); accounting for selection bias due to culling of animals for health or productive reasons (e.g. ROH analysis within parity/age class, or application of survival analysis). This promises to be an exciting area of research: hopefully, this communication might serve of inspiration.

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Table 3: Most relevant runs of homozygosity (ROH) associated with the five disease categories considered in the analysis. For each ROH, the chromosome (BTA), start and end position and number of SNPs included are reported. In the reported ROH the percentage of cases on total number of haplotypes ranged between 51.4% and 100%.

| Disease category | BTA | start bps | end bps | # SNPs | Production QTLs |<sup>1</sup> |
|------------------|-----|-----------|---------|--------|-----------------|--------|
| Infectious       | 7   | 2991449   | 4655753 | 47     | FY, NM          |<sup>1</sup> |
| Infectious       | 7   | 19163934  | 19461567| 6      | FY, NM          |<sup>1</sup> |
| Infectious       | 7   | 21704630  | 22305419| 13     | FY, NM          |<sup>1</sup> |
| Infectious       | 7   | 23756162  | 24014128| 6      | FY, NM          |<sup>1</sup> |
| Infectious       | 7   | 24236334  | 24330857| 3      | FY, NM, PY      |<sup>1</sup> |
| Infectious       | 7   | 106927241 | 107452906| 12     | NM              |<sup>1</sup> |
| Infectious       | 7   | 108575314 | 108685133| 3      | NM              |<sup>1</sup> |
| Infectious       | 11  | 45688827  | 51041758| 117    |                 |<sup>1</sup> |
| Infectious       | 12  | 11414743  | 13005009| 43     |                 |<sup>1</sup> |
| Infectious       | 12  | 25878820  | 30099199| 84     |                 |<sup>1</sup> |
| Infectious       | 12  | 51834816  | 52573538| 21     |                 |<sup>1</sup> |
| Infectious       | 12  | 53241975  | 53428996| 6      |                 |<sup>1</sup> |
| Infectious       | 15  | 40889434  | 41169953| 4      |                 |<sup>1</sup> |
| Infectious       | 15  | 41780731  | 41971248| 7      |                 |<sup>1</sup> |
| Infectious       | 15  | 42715966  | 42758371| 2      |                 |<sup>1</sup> |
| Infectious       | 24  | 16012254  | 16274083| 8      |                 |<sup>1</sup> |
| Infectious       | 28  | 30070377  | 30158724| 2      |                 |<sup>1</sup> |
| Metabolic        | 3   | 1737421   | 6813316 | 103    | MY, FP          |<sup>1</sup> |
| Metabolic        | 4   | 12868626  | 13148484| 4      |                 |<sup>1</sup> |
| Metabolic        | 8   | 8082781   | 9666303 | 37     |                 |<sup>1</sup> |
| Metabolic        | 15  | 37893014  | 39016114| 22     |                 |<sup>1</sup> |
| Metabolic        | 15  | 40889434  | 41169953| 4      |                 |<sup>1</sup> |
| Metabolic        | 15  | 41780731  | 41971248| 7      |                 |<sup>1</sup> |
| Metabolic        | 15  | 42715966  | 42758371| 2      |                 |<sup>1</sup> |
| Mastitis         | 12  | 25878820  | 30099199| 84     |                 |<sup>1</sup> |
| Mastitis         | 13  | 19816277  | 20687500| 22     | MY, FY          |<sup>1</sup> |
| Mastitis         | 24  | 16012254  | 16274083| 8      |                 |<sup>1</sup> |
| Mastitis         | 28  | 30070377  | 30158724| 2      |                 |<sup>1</sup> |
| Reproduction     | 4   | 8779979   | 10737673| 40     |                 |<sup>1</sup> |
| Reproduction     | 4   | 12868626  | 13148484| 4      |                 |<sup>1</sup> |
| Reproduction     | 5   | 6656617   | 6976839 | 11     | FY, PY          |<sup>1</sup> |
| Reproduction     | 5   | 7832521   | 8063248 | 9      | FY, PY          |<sup>1</sup> |
| Reproduction     | 15  | 40889434  | 41169953| 4      |                 |<sup>1</sup> |
| Reproduction     | 15  | 41780731  | 41971248| 7      |                 |<sup>1</sup> |
| Reproduction     | 15  | 42715966  | 42758371| 2      |                 |<sup>1</sup> |
| Reproduction     | 18  | 23224334  | 23253048| 2      | MY, FP          |<sup>1</sup> |
| Reproduction     | 20  | 19587484  | 19757303| 3      |                 |<sup>1</sup> |
| Reproduction     | 25  | 30824927  | 31525961| 27     |                 |<sup>1</sup> |
| Locomotion       | 6   | 107186270 | 107835731| 11    |                 |<sup>1</sup> |
| Locomotion       | 9   | 78576880  | 79887463| 14     |                 |<sup>1</sup> |
| Locomotion       | 12  | 111414743 | 13005009| 43     |                 |<sup>1</sup> |
| Locomotion       | 28  | 30070377  | 30158724| 2      |                 |<sup>1</sup> |

<sup>1</sup>QTLs for milk production traits from previous GWAS studies: FY=fat yield, NM=net merit, PY=protein yield, MY=milk yield, FP=fat percentage (Cole et al., 2011; Minozzi et al., 2013).