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Runs of homozygosity in Italian Holstein bulls: a permutation approach and time-based mapping of the genomic regions potentially under selection

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Abstract

Background Runs of homozygosity (ROH) occurrence in animal genomes may result from a high level of relatedness within the population or from positive selection. ROH investigation enables the assessment of the degree of individual autozygosity at the genome-wide level, providing insights into genetic background and development history of a population. In this context, the main objectives of this study were to: 1) develop a method based on permutation tests for detecting single nucleotide polymorphisms (SNP) significantly included in ROH genomic regions of Italian Holstein bulls; 2) identify candidate genes close to the SNP frequently positioned within ROH; 3) investigate the effects of genetic selection on ROH distribution and ROH islands; and 4) assess potential associations between ROH and economically important traits in Italian Holstein cattle. We used high-density SNP data of 3,009 Italian Holstein bulls for identifying ROH regions. The threshold for declaring a SNP as significantly included in a ROH region was obtained by randomly shuffling SNP within animals for each chromosome. The chromosome-wide threshold was the 99th percentile of the distribution of the number of times a SNP was included in a ROH segment (SNP_{ROH}) for a specific chromosome. To investigate the influence of ROH on relevant traits, a genome-wide association study testing the presence/absence of specific ROH and a mixed model to quantify the effect of ROH-based inbreeding were carried out.

Results The top 24,905 significant SNP were distributed on ten chromosomes, in genomic regions known to harbor genes involved in milk traits. One region on BTA20 exhibited an interesting pattern of SNP_{ROH} occurrence across animals grouped according to the year of birth, underlining an intense selection pressure in this genomic region. The analyses on the relationship between ROH and pseudo-phenotypes for various traits showed a significant influence of inbreeding on milk and protein yields, and a fertility aggregate index, with the presence/absence of 14 ROH regions having a significant effect on different traits (milk, fat, and protein yields; fat percentage; and somatic cell score).

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Conclusions The application of permutation to a high-density (HD) SNP chip data enabled the identification of genomic regions with high levels of homozygosity in Italian Holstein bulls and revealed associations between ROH occurrence and economically important traits in the studied population.

Keywords Selection signatures, Runs of homozygosity, Positive selection, Inbreeding

Background

The comprehensive analysis of population genomes enables the discovery of demographic and geographic effects on gene frequencies in specific populations [1], including signatures of domestication events, population bottlenecks, and footprints of natural or artificial selective breeding [2, 3]. The cosmopolitan dairy cattle breeds, such as the Holstein breed, have been derived through intensive selective breeding [4]. Intense genetic selection coupled with the development of standardized operation systems and improvements in management practices have contributed to substantial increases in performance levels, but resulted in reduced levels of within breed genetic diversity [5, 6].

Extended regions of consecutive homozygous single nucleotide polymorphism (SNP) loci have been observed in the genome of many mammalian species [7–10]. These genomic regions are commonly referred to as runs of homozygosity (ROH) and have been a major research topic in the past few years, as reviewed by Peripolli et al. [11]. Historically, the analysis of homozygosity in case-control studies enabled the genetic mapping of recessive monogenic genetic defects, diseases, or exterior characteristics such as presence or absence of horns in cattle [12], and to obtain more precise estimates of individual and populational genomic inbreeding coefficients [13, 14]. In addition, ROH length enables the dating of the overall genomic inbreeding into more ancient and more recent inbreeding [5, 15]. In humans, long ROH exhibit a higher enrichment of deleterious variants that are less compatible with life than non-ROH [16], and they seem to be involved in complex or multifactorial diseases [17]. ROH analyses have contributed to disentangle the recent selection history of domestic ruminants such as cosmopolitan and local cattle breeds [18–21], to study molecular coancestry and inbreeding depression in sheep (e.g., Purfield et al. [22]) and other species (e.g., Makanjuola et al. [5] and Bem et al. [23]), to infer genetic signatures of climatic adaptation in goat breeds [24, 25], and to associate ROH islands with environmental variables in both buffaloes and sheep populations (e.g., Macciotta et al. [26]).

The analysis of ROH segments attempts to clarify the origin of autozygosity and offers new tools for inbreeding management [14]. In highly selected cattle populations, ROH islands are due to the combined effect of population relatedness, reduced recombination rates, linkage disequilibrium, and oriented selection (targeting specific traits can lead to fixation of beneficial alleles and increased homozygosity in certain genomic regions). The regions under selection and the level of regional autozygosity are generally correlated [4]. Indeed, genomic regions subjected to selection frequently show a reduced nucleotide diversity, and tend to generate ROH islands, which have high levels of homozygosity around a selected locus compared with the rest of the genome. However, there are no standardized methodologies for identifying ROH segments and ROH islands, but only general recommendations based on simulated or real datasets [27, 28]. In general, a commonly accepted approach is based on merging multiple clues from many different genomic analyses. Therefore, the main objectives of this study were to 1) develop a method based on permutation tests for detecting single nucleotide polymorphisms (SNP) significantly included in ROH genomic regions of Italian Holstein bulls; 2) identify candidate genes close to the SNP frequently positioned within ROH; 3) investigate the effects of genetic selection on ROH distribution and ROH islands; and 4) assess potential associations between ROH characteristics and economically important traits in Italian Holstein cattle.

Methods

Genotyped bulls and SNP data editing

A total of 3,009 Italian Holstein bulls were genotyped using two Illumina BeadChip, including 916 animals genotyped using the BovineHD SNP panel with 777,962 SNP and 2,093 individuals assayed with the BovineSNP50 (54 K; Illumina Inc., San Diego, USA) containing 54,001 SNP. Genomic datasets were generated within the SelMol and ProZoo projects [29]. SNP from both SNP panels were mapped to the ARS-UCD1.2 reference genome and subjected to quality control (QC). The QC was performed independently for each dataset using the PLINK v.1.9 software [30] and the following settings: bulls with individual call rate lower than 95% and Mendelian inconsistency in sire-son pairs greater than 2% were removed. We also removed non-autosomal SNP, SNP with unknown or duplicated genomic positions, or with extreme deviation from Hardy-Weinberg equilibrium (p-value $< 10^{-6}$) as an indication of genotyping errors. We did not filter SNP based on MAF for the ROH analyses [31, 32]. After QC, the datasets included 2,024 (with 44,395 SNP) and 900 (with 734,776 SNP) bulls. The 54 K genotypes were imputed to HD using the Beagle software [33]. After genotype imputation and a second round

of QC based on the same criteria defined above, a final dataset containing 2,916 animals and 712,504 SNP were used for subsequent analyses. Although genotype imputation accuracy statistics were not available for this dataset, imputation accuracies greater than 98% from 50 K to HD SNP panel in a very similar number of Italian Holstein bulls was reported elsewhere [34].

The 2,916 bulls retained after the QC, were clustered into five discrete groups of 7 years each according to their birth year (group1: 1979–1985; group2: 1986–1992; group3: 1993–1999; group4: 2000–2006; group5: ≥ 2007). The pedigree file contained 323,421 individuals and it was analyzed using the optiSel R package [35]. The average pedigree-based inbreeding coefficient for genotyped animals (F_{PED}) was 4.89 ± 2.22%, based on 5.83 ± 1.18 number of full known generations and a degree of pedigree completeness index of 1.00 ± 0.03.

De-regressed proofs (DRP) for nine key traits, based on equivalent daughter contributions as described in Degano et al. [36], were provided by the Italian Holstein Association (ANAFI, Cremona, Italy). The traits included in the study were: milk yield (MY), protein yield (PY), protein percentage (PP), fat yield (FY), fat percentage (FP), somatic cell score (SCS), direct cow longevity (LONd; expressed as productive longevity from first calving to culling), combined cow longevity (LONc; a merge of information on bull's daughter longevity with indirect functional traits such as udder and feet and legs), and fertility aggregate index (FAI). Details on Italian Holstein selection index are retrievable from ANAFI [37]. The heritability estimates of the traits and the reliability of DRP are reported in the Table S1 (see Additional File 1).

ROH detection and statistics

ROH were computed using the "consecutiveRuns" function from the R package detectRUNs [38] and the following settings: a) minimum ROH size of 50 SNP; b) minimum ROH length of 1 Mb; c) one heterozygote allowed into a ROH segment to account for genotyping or imputation errors; d) no missing SNP were allowed, since genotype imputation filled the gaps. The following ROH statistics were computed: total and unique ROH counts, average ROH length, number of ROH per animal, and total length of ROH segments per animal. The ROH segments were classified into five different length classes: 1–2, 2–4, 4–8, 8–16, and > 16 Mb, respectively. These thresholds were used to facilitate comparison of our results with literature reports (e.g., [39, 40]). Moreover, we focused on unique ROH, i.e., ROH that covers the same genomic region (starts and ends at a chromosomal location, not considering partial overlaps). Those ROH can be found either in one or in more than one animal; when the same unique ROH was found in more than one animal, it was defined as shared ROH.

ROH-based genomic inbreeding (F_{ROH}) coefficients were estimated from the overall sum of the *j-th* ROH found in the *i-th* individual, divided by the genome length covered by the SNP. F_{ROH} was also computed within each chromosome. Finally, inbreeding coefficients were computed using ROH with different minimum length to date the inbreeding events [13]: F_{ROH} > 2 Mb, F_{ROH} > 4 Mb, F_{ROH} > 8 Mb, and F_{ROH} > 16 Mb.

Permutation approach for ROH islands identification

The number of times that each SNP occurred into a homozygosity region, i.e., SNP_{ROH}, was plotted against the genomic positions. Fixed thresholds are commonly used to identify the outlier SNPs that are frequently included into ROH segments. Here we propose a permutation approach to set 29 chromosome-wide thresholds by using an in-house script based on five steps, as described in Additional File 2. In brief, i) the PLINK format ".map" and ".ped" files (after QC) were used to compute the ROHs in the whole dataset using the *consecutiveRUNS.run* function of the detectRUNS R package; ii) the occurrence of SNP in a ROH was estimated (SNP_{ROH}) using the *snpInsideRuns* function of detectRUNS for the *i-th* SNP; iii) the full dataset was divided in 29 data subsets, and within each chromosomes the animals' genotypes were randomly shuffled; iv) the step ii was repeated 1,000 times to obtain the *i-th* SNP 1,000 permuted values of SNP_{ROH}; and, v) the 99th percentiles of the chromosome-wide permutated SNP_{ROH} values were used as thresholds to select significant SNP to define a ROH island, i.e. those SNP in which SNP_{ROH} was greater than the chromosome-wide permutation threshold.

ROH features by birth-year groups and relationship with pseudo-phenotypes

To assess the effect of both ancient and recent selection on relevant traits, ROH statistics were calculated for the five birth-year groups considered. In particular, the frequency distribution of ROH in the different class of length (< 2, 2–4, 4–8, 8–16, and > 16 Mb) was calculated for all age groups. Local homozygosity (SNP_{ROH}) islands were computed across and within the five considered groups, allowing them to highlight the effect of selection. Within birth-year group, the F_{ROH} values were also compared to pedigree-based inbreeding coefficients by computing their correlation and regression statistics.

To evaluate the effect of ROH-based inbreeding, F_{ROH} was treated as an explanatory variable in a linear model implemented in the R software:

$$y_{jk} = gr_k + \beta_{(k)} \bullet F_{ROH_j} + e_{jk}$$

where y_{jk} is the DRP of the considered traits recorded on *j-th* bull belonging to *k-th* birth-year group, gr_k is the

cross classified fixed effect of birth-year group (accounting for the genetic trend) and $\beta_{(k)}$ are the regression coefficients of F_{ROH} on the DRP (nested within birth-year group). The same linear model was used on 29 chromosome-based F_{ROH}, fitting one chromosome at a time. Since the skewed distribution of chromosome-wide F_{ROH}, those were transformed into the z-score of $log_{10}(F_{ROH})$. To assess the significance of the main effects and to test $\beta_{(k)}$, F and t-test were used.

Genome-wide unique ROH (i.e., ROH that presented the same genomic limits) were analyzed to test the influence of ROH presence/absence (0, 1) on DRP mimicking a genome-wide association study using ROH ($GWAS_{ROH}$). A slightly reduced dataset, including 2,813 bulls with both pseudo-phenotypes and genotypes was used. The following linear model was applied:

$$y_{jk} = gr_k + ROH_x + e_{jk}$$

where y_{jk} is the DRP of the considered traits recorded on *j-th* bull belonging to *k-th* birth-year group, gr_k is the cross classified fixed effect of birth-year group (5 levels as previously defined), and ROH_x is the fixed effect of the tested *x-th* ROH (2 levels, presence or absence). For the ANOVA test, only ROH shared by at least 20 animals (threshold roughly equal to 1% of the considered bulls and already adopted by Cesarani et al. [41] in European Simmental bulls) were held. The significance threshold was fixed as the negative logarithm of the ratio 0.05/n, where n was the number of ROH tested (n = 1,138).

Gene annotation

Annotated genes close to the signals overcoming the threshold of SNP_{ROH} island (± 250 kb; [42]) or identified within the ROH associated to phenotypes by $GWAS_{ROH}$ were searched using the UCSC Genome Browser Gateway [43]. Moreover, quantitative trait loci (QTL) in the ROH from $GWAS_{ROH}$ were investigated using the GALLO R package [44]. During the QTL enrichment, Bonferroni correction was applied to p-values and significance was declared for P corrected < 0.05.

Results

ROH detection and statistics

A total of 186,567 ROH were identified in 2,916 animals. The number of ROH per animal ranged from 26 to 145, with an average of 64 ± 11 ROH segments. The average ROH length was 4.16 ± 5.36 Mb. The longest ROH (106.45 Mb) was found on BTA4 (82,210–106,635,604 bp); the majority of ROH were shorter than 4 Mb.

The number of unique ROH (i.e., those covering the same genomic region) was 86,491 (Table 1): thus, the same unique ROH was repeated 2.16 ± 6.37 times (i.e., found on average on two animals). The relationship between the length of ROH segments and the number of animals sharing those segments approximate a negative exponential function, with few animals sharing long ROH segments and many animals sharing short segments (< 2 Mb) (see Figure S1, Additional File 3). A total of 22 regions were shared by at least 5% of bulls (see Table S2, Additional File 1). These ROH mapped to 14 chromosomes, and most regions (n = 4) were located on BTA6. A ROH located on BTA26 (20,625,613–21,634,327 bp) was shared by 448 bulls (15.4% of the analyzed sample), whereas a ROH on BTA7 at 92,396,022–93,810,221 bp was found to be shared by 383 bulls (13.1%). The third most shared ROH was located on BTA16 from 43,282,296 to 44,575,736 bp and it was found in 9.4% of the animals (274 individuals).

The total length of the genome in homozygosity (considering the length covered by the SNP used for the computation) was on average 266.1 ± 75.9 and 177.9 ± 73.2 Mb for total ROH and unique ROH, respectively. Thus, the average F_{ROH} was 0.11 ± 0.03. As far as the coefficients per chromosomes were concerned, the largest and lowest average values were observed for BTA20 (0.18 ± 0.16) and BTA15 (0.09 ± 0.10), respectively. As expected, the average F_{ROH} values decreased as the minimum length of ROH to compute the coefficients increased: 0.09 ± 0.03 for F_{ROH} > 2 Mb, 0.08 ± 0.03 for F_{ROH} > 4 Mb, 0.06 ± 0.03 for F_{ROH} > 8 Mb, and 0.03 ± 0.02 for F_{ROH} > 16 Mb.

Table 1 Number of total and unique runs of homozygosity (ROH) according to the class of ROH segment length

	Total ROH			Unique ROH[1]		
Class	**Number**	**%[2]**	**Average length**	**Number**	**%**	**Average length**
< 2 Mb	88,281	0.47	1.64 ± 0.05	26,447	0.31	1.42 ± 0.13
2–4 Mb	45,570	0.24	2.77 ± 0.15	21,168	0.24	2.86 ± 0.27
4–8 Mb	28,492	0.15	5.65 ± 0.40	18,855	0.22	5.74 ± 0.54
8–16 Mb	16,817	0.09	11.10 ± 1.07	13,392	0.15	11.20 ± 1.24
> 16 Mb	7407	0.04	24.00 ± 5.80	6629	0.08	24.40 ± 6.39
Total	186,567	1		86,491	1	

[1]Unique ROH cover a specific genomic region (labelled as chromosome, starting, and ending positions) and can be found in one or more animals

[2]Relative proportion of ROH within each class on the total number of segments

A permutation approach for ROH islands identification

The chromosome-wide SNP_{ROH} (i.e., the percentage of bulls that had a SNP falling into a ROH) are reported in Fig. 1. The 99th percentile of SNP_{ROH} distribution of permutated genotypes allowed to tag 24,905 SNP exceeding the chromosome-wide thresholds in 21 out of 29 autosomes (Table 2). The number of outliers SNP_{ROH} ranged from 175 (BTA18) to 5,122 (BTA23). The highest SNP_{ROH} peak was found on BTA16 (64.3%) at 40.14 Mb (3 SNP). In decreasing order of magnitude, SNP_{ROH} of 46% (BTA26) with 6 SNP between 23.46–23.9 Mb, 36% (BTA21) between 1.15–1.16 Mb, and 35% for BTA7 spanning from 92.39 to 93.81 Mb and BTA6 in the range 30.76–33.64 (34%). Thirty-three SNP were found in a ROH island on BTA14 (33% of animals having the SNP within ROH) between 21.33 and 21.39 Mb. Finally, three ROH islands were identified: one located between 49.29 and 49.41 on BTA15 (33%), another one located between 38.64 and 38.69 Mb on BTA29 (30%), and the last one positioned between 49.44 and 49.49 Mb on BTA3 (30%).

ROH features by birth-year groups

The number of ROH per animal increased over time with average values of 51 ± 9, 55 ± 8, 60 ± 8, 68 ± 11, and 74 ± 14 for group1, group2, group3, group4, and group5, respectively. The distribution of ROH in length classes based on birth-year group (Fig. 2) was similar to what observed in the whole dataset (Table 1), with the largest number of ROH in the first two classes of ROH segment length (i.e., ROH shorter than 4 Mb). However, there is a slight difference in the occurrence of longer ROH regions: in the last two ROH length classes (i.e., 8–16 Mb, and > 16 Mb), an increasing trend was found moving from group1 (older animals) to group5 (younger animals).

Local homozygosity (SNP_{ROH}) islands in each birth-year group are reported in Figure S2 (see Additional File 4). On average, the youngest animals showed larger values compared to the previous birth-year groups. Figure 3 shows the occurrence of SNP_{ROH} detected on BTA20 (i.e., the chromosome with the clearest trend across birth years) according to the birth-year group of the animals. Around the center of the chromosome, it is evident the increasing pattern of percentage of SNP into ROH. This increasing trend was evident also when analyzing

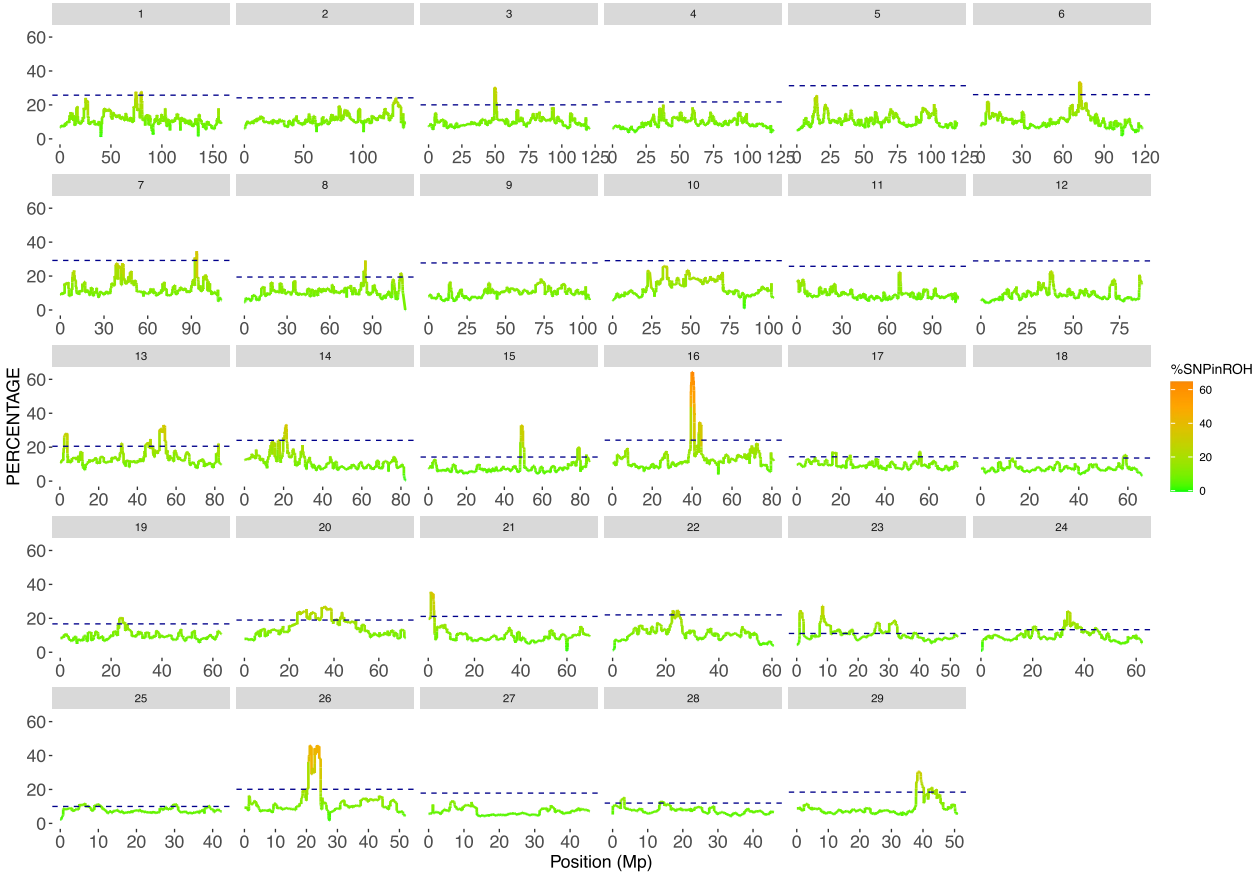

Fig. 1 Results of the permutation approach: number of times (in terms of percentage of animals) each SNP was found within a ROH segment (SNP_{ROH}). The thresholds (i.e., dashed lines) that defined the ROH islands were identified as the 99th percentile of the chromosome-wide permutated SNP_{ROH} values

Table 2 Genomic regions (i.e., ROH islands) with the highest values of the number of times that each SNP fell into a homozygosity region (SNP_{ROH}) identified through the permutation approach

	Threshold		Highest SNP_{ROH}		
Chromosome	**%**	**SNP above**	**%[1]**	**N° SNP[2]**	**Position (bp)**
1	25.75	312	27.88	8	80,024,324–80,039,640
3	20.04	422	30.18	21	49,443,623–49,491,479
6	26.06	349	33.64	1	72,299,004
7	29.21	395	34.67	1	93,647,158
8	19.43	424	29.15	1	84,969,945
13	20.55	2107	33.02	13	53,533,265–53,565,450
14	24.04	581	33.30	33	21,334,532–21,399,339
15	14.19	465	32.72	42	49,292,480–49,419,227
16	24.15	609	64.27	3	40,148,220–40,150,767
17	14.33	949	17.42	13	55,693,967–55,735,039
18	13.61	175	15.43	12	58,822,192–59,010,935
19	16.72	806	20.51	3	24,477,277–24,480,229
20	18.98	4604	26.92	19	35,988,586–36,125,666
21	21.14	193	35.63	3	1,156,168–1,164,298
22	22.02	514	24.97	1	24,285,621
23	11.08	5122	27.30	35	8,394,550–8,570,115
24	13.30	2726	24.31	7	33,394,668–33,419,046
25	9.99	1813	11.87	1	6,559,886
26	20.11	972	46.19	6	23,469,696–23,492,556
28	11.97	477	15.33	2	3,310,204–3,322,748
29	18.43	890	30.45	4	38,641,995–38,693,679

[1]Threshold at which the highest peak of the chromosome-wide permutated SNP_{ROH} values could be highlighted

[2]Number of SNP above the highest peak

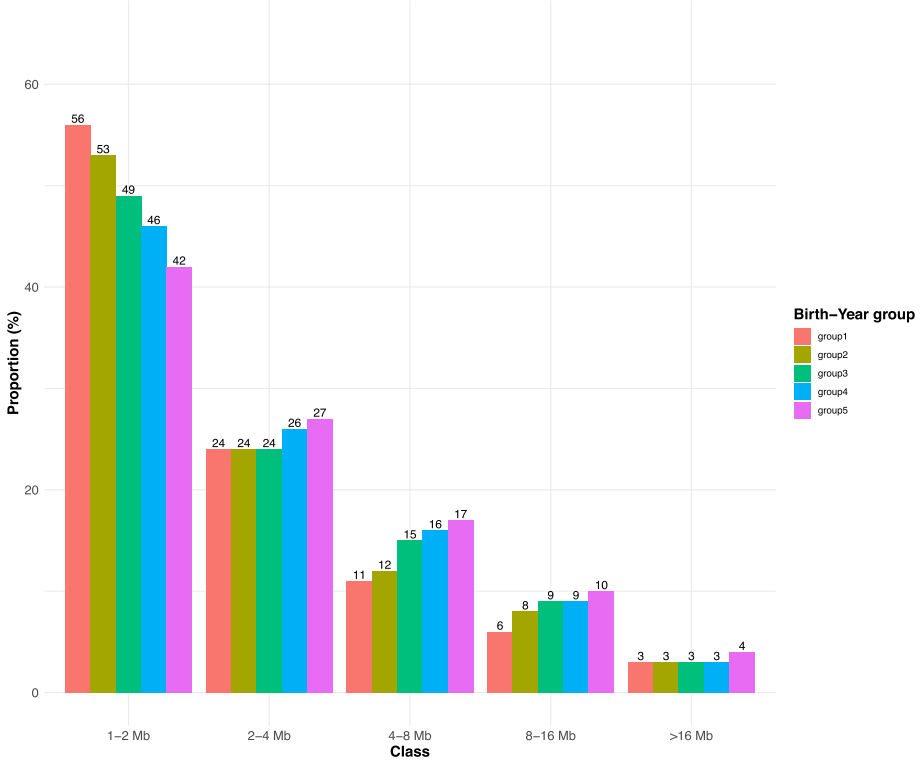

Fig. 2 Proportion (%) of runs of homozygosity (ROH) of different length categories according to the bulls' birth-year group (group1: 1979–1985; group2: 1986–1992; group3: 1993–1999; group4: 2000–2006; group5: ≥ 2007)

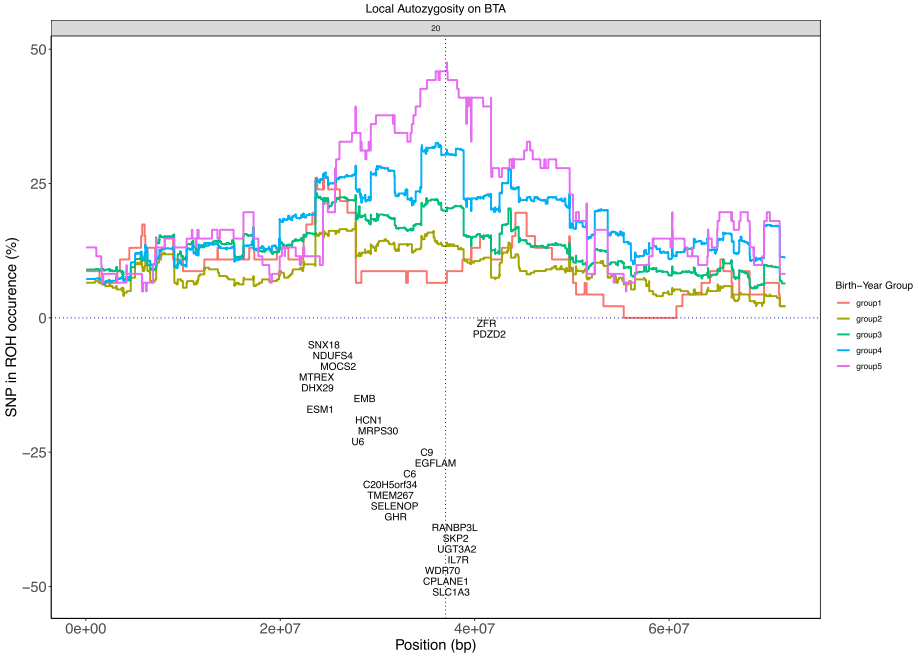

Fig. 3 Percentage occurrence of SNP within runs of homozygosity in chromosome 20 by generation according to the year of birth of the bulls (group1: 1979–1985; group2: 1986–1992; group3: 1993–1999; group4: 2000–2006; group5: ≥ 2007) and genes located on the highlighted region

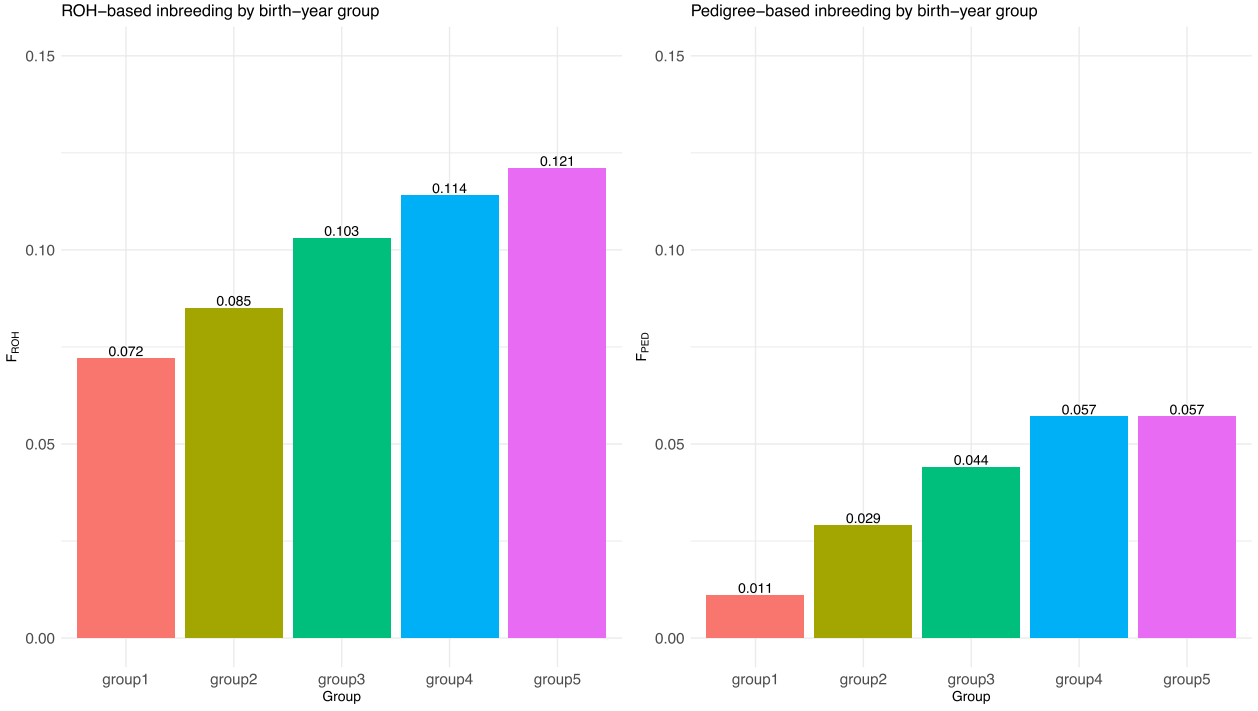

Fig. 4 Trend of inbreeding computed with runs of homozygosity (F_{ROH}) and pedigree in the five considered groups according to the year of birth of the bulls (group1: 1979–1985; group2: 1986–1992; group3: 1993–1999; group4: 2000–2006; group5: ≥ 2007)

inbreeding coefficients by birth-year group (Fig. 4) and by birth-year group at chromosome-level (see Figure S2, Additional File 4). The average ROH-based inbreeding moved from 0.07 ± 0.03 in group1 to 0.12 ± 0.03 in group5; the same increasing trend was observed also for the pedigree-based inbreeding: from 0.01 ± 0.02 to 0.06 ± 0.01 in group1 and group5, respectively (Fig. 4). The overall Pearson correlation between F_{ROH} and F_{PED} is 0.69. This correlation showed a decreasing trend moving from the oldest to youngest animals: 0.74, 0.75. 0.66, 0.60,

Table 3 Results of the linear model testing the effect of ROH-based inbreeding (F_{ROH}) on the considered pseudo-phenotypes at the chromosome and genome-wide levels

Trait	F_{ROH} chromosome[1]	F_{ROH} genome[2]
Milk yield, kg	2, 5, 7, 8, 10, 13, 16, 17, 20, 21, 22, 23	Yes
Protein yield, kg	2, 3, 4, 7, 8, 10, 14, 16, 17, 21, 22	Yes
Protein percentage, %	2, 3, 6, 7, 8, 16, 20, 23, 26	No
Fat yield, kg	1, 2, 4, 9, 11, 17, 26, 28	No
Fat percentage, %	2, 5, 10, 15, 16, 17, 19, 26	No
Somatic cell score	5, 10, 18, 20, 21, 25	No
Longevity combined, index	9, 10, 11, 14, 16, 17	No
Longevity direct, index	3, 9, 11, 14, 16, 17	No
Fertility aggregate index	3, 9, 10, 17, 28	Yes

[1]Chromosomes with a ROH-based inbreeding significant for the pseudo-phenotypes

[2]Significance or not-significance of the ROH-based inbreeding effect on the considered pseudo-phenotypes

Table 4 Shared runs of homozygosity with a significant Bonferroni-corrected effect (presence/absence) on the investigated pseudo-phenotypes

Trait	n[1]	BTA	Position (Mb)	SNP within the ROH
Milk yield, kg	35	16	42.47–43.72	239
	26	26	0.04–1.06	62
Fat yield, kg	64	15	0.04–1.35	226
Protein yield, kg	26	26	0.04–1.06	62
	20	8	96.88–97.92	201
Fat percentage, %	40	1	114.39–119.31	1,160
	24	14	1.26–3.37	312
	35	16	42.47–43.72	239
	22	16	49.92–51.01	286
	28	1	76.82–78.95	444
	43	9	103.36–105.69	589
Somatic cell score	21	16	1.56–3.07	411
	130	20	34.86–39.19	981
	42	2	88.01–89.1	209

[1]Number of animals sharing the ROH segments

and 0.59 for group1, group2, group3, group4, and group5, respectively.

Relationship between ROH features and pseudo-phenotypes

Table 3 shows the results of the linear model testing the effect of ROH-based inbreeding on the considered pseudo-phenotypes.

The genome-wide inbreeding was significant for MY, PY, and FAI. At the chromosomal level, MY showed the largest number of significant associations ($n = 12$) whereas FAI the lowest number ($n = 5$). The inbreeding coefficient computed for BTA17 significantly affected seven different traits (MY, PY, FY, FP, LONc, LONd, and FAI).

The list of ROH exceeding the Bonferroni significance threshold in the association analysis (presence/absence of specific regions) is reported in Table 4. Two ROH regions were found significantly associated with MY: one on BTA16 (42.47–43.72 Mb) shared by 35 bulls, and another one located on BTA26 (0.04–1.06 Mb) shared by 26 bulls. The exact same ROH was significant also for PY; for this trait, another region on BTA8 (96.88–97.92 Mb) was also significant. Only one ROH region shared by 64 bulls was found to be significant for FY (BTA15, 0.04–1.35 Mb). FP was the trait with the largest number of significant regions (n = 6), located on four chromosomes (2 signals on BTA1 and BTA16 each, and 1 signal on BTA9 and BTA14). Lastly, three regions located on BTA2, BTA16, and BTA20 were found to be significantly associated with SCS.

Quantitative trait loci and genes

The ROH significant in the $GWAS_{ROH}$ analysis (Table 4) matched with 7,461 QTL of which 6,239 were significant after Bonferroni correction (see Table S3, Additional File 1). The most interesting result was found on BTA14 from 1.26 to 3.37 Mb, where several QTL were found to be associated with milk fat. Indeed, among the 5,259 significant QTL associations found in this region, 1,376 resulted associated with FP and 968 with milk FY. This result confirms the association between this region and the pseudo-phenotype for FP considered in the $GWAS_{ROH}$ analysis.

Discussion

Over the past few years, various studies have reported ROH segments in Italian Holstein cattle. For instance, Gaspa et al. [45] reported a similar value for the number of ROH per animal (74.2 ± 15.2) using an HD SNP panel on a smaller sample, whereas the average number of SNP per ROH was slightly lower (920.8 ± 171) compared to the value computed in the present study. Szmatola et al. [46] found a higher number of ROH per animal (130.9 ± 94.5), but with a shorter average length (409.8 ± 287.6 Mb). Results reported for Holsteins using lower SNP densities are different from those obtained using HD panels. For example, lower mean ROH length (2.95 and 4.80 Mb) were found using medium density by Liu et al. [47] and Cortes-Hernández et al. [48], respectively. When using the low-density SNP panel (i.e., 50 K), Visser et al. [49] found lower values of ROH per animal (23.70 ± 7.57) but with a larger mean length of 8.15 Mb, whereas Ristanic et al. [50] reported a mean number of 60.42 regions per animal with an average length per ROH of 3.28 Mb. In any case, a common finding is that, especially when analyzing SNP detected at high density, the largest proportion of identified ROH felt in the shortest length class, whereas fewer long ROH were identified. This is probably

due to the better resolution of HD SNP panels that avoids the detection of potentially false long ROH and improves the detection power for the short ROH [51]. Indeed, when using an HD SNP panel, more markers are considered, and those additional SNP may be heterozygotes and break the false long ROH [52] or homozygotes that constitute shorter homozygous regions, both not detectable at lower SNP densities [46]. Regarding the different proportion of total and unique ROH in the five ROH length classes, the lower proportion of unique regions in the shortest class could be due to different recombination events that can occur across generations, breaking the original long ROH [52]. Moreover, when considering unique ROH, larger proportions were found in the medium and long ROH length classes. This suggests that unique long ROH are less prone to be spurious in comparison to short ROH since the recent directional selection tends to generate long haplotype segments around the selection target [53]. In addition, the shortest the ROH segments the more they tend to be shared among animals and vice versa (see Figure S1, Additional File 3). This, instead, could be ascribed to the fact that recombination breaks long ROH more frequently than shorter ones, thus the latter tend to become low-recombination regions [53] and to be inherited as they are.

For ROH islands detection we propose a permutation approach to set a threshold for detecting outlier SNP falling more frequently in a ROH than the other markers. The identification of a ROH island is based on frequency computation, i.e., counting the number of individuals that have a particular SNP within a ROH. The definition of a threshold to be considered as a ROH island is often arbitrary and inconsistent criteria for defining ROH make it difficult to compare results across studies [11]. Permutation-based procedures have been occasionally used in ROH studies, but mainly to assess the significance of ROH abundance at the window level or to compare predefined groups, rather than to define chromosome-specific SNP thresholds. Often the strategies for ROH island analyses are based on indirect evidence from multiple approaches (e.g., ROH analyses and selection signatures [54]). In literature, permutation approaches have been proposed to simulate the null distribution of ROH abundance in genomic windows of certain size in wild animals [55]. Kardos et al. [55] permuted individual genotypes to build a null distribution of ROH counts per genomic window in an isolated wolf population, while Kim et al. [4] compared the number of animals harboring ROH within predefined windows between selected and unselected Holsteins using permutation-derived χ^2 tests. Here, we permuted SNP genotypes within chromosomes to break down ROH integrity and built a reference of distribution to set chromosome-wide thresholds to declare a ROH island. The methodological novelty of this approach allows to overcome the uninformed subjective choice of threshold to define ROH islands, since we proposed the use of the 99th percentile of this permutation distribution as chromosome-specific limits. Compared with fixed, "ad hoc" percentages of animals in ROH [11, 54] or window-based approaches [4, 55], this framework is readily transferable across populations, SNP panels, and species without tuning arbitrary cut-offs.

The average ROH-based inbreeding coefficient was 0.11 ± 0.03, value that agrees with the available literature. In fact, similar F_{ROH} values were reported for Holstein populations worldwide by Lozada-Soto et al. (0.13 ± 0.03 [56]), Ghoreishifar et al. (0.09 ± 0.02 [57]), and Ablondi et al. (0.16 ± 0.03 [58]). Conversely, Ristanic et al. [50] and Visser et al. [49] reported lower values (~ 0.08 and 0.06, respectively), whereas a greater value (0.28 ± 0.07) was computed by Cortes-Hernández et al. [48], all in Holstein populations.

As expected, inbreeding coefficients and ROH features changed across the considered birth-year groups. The youngest animals (group5, 74 ± 14) had 1.45 times more ROH segments than oldest animals (group1, 51 ± 9), and they had a larger proportion of longer ROH (Fig. 2). The larger levels of autozygosity in youngest animals was evident also from the Fig. 3, where an increasing trend from group1 to group5 of SNP falling inside ROH was observed in the central part of BTA20. This suggests the presence of a signal of positive selection for genes associated to milk production, as found also in many studies in the literature. For instance, in this genomic region maps the *GHR* gene, that has been associated with milk yield and protein in Holstein [59, 60]. The larger number of long ROH segments found in youngest animals resulted in increased inbreeding coefficients (Fig. 4). Ablondi et al. [61] analyzed ROH distribution across four birth-year groups (i.e., 2002–2005, 2006–2010, 2011–2015, 2016–2020) of Holstein cattle and reported increasing number of ROH per animal over time, from 109.5 in the first group to 123.2 in the fourth one. As regards the mean length, according to the fact that longer ROH segments are an indicator of more recent inbreeding, the birth-year group with the highest proportion of segments above 16 Mb was the group5, whereas the group1 was the one with the greatest proportion of ROH below 2 Mb. This confirms what was reported by Howrigan et al. [27], who reported that ROH of 10, 5, and 2.5 Mb are correlated with 5, 10, and 20 generations ago, respectively, whereas Cardoso et al. [62] reported that ROH segments longer than 16 Mb originated in the last three generations, whereas those shorter than 8 Mb originated more than six generations ago. The F_{ROH} trend across year-birth group (Fig. 4) also agrees with existing literature. For instance, Ablondi et al. [61] reported that ROH-based inbreeding coefficient value increased as the age

of the considered animals decreased (i.e., from oldest to youngest animals): from 0.15 in the first birth-year group (2002–2005) to 0.19 in the fourth one (2016–2020). The same finding was recently reported in German Brown Swiss cattle [63] and in Nellore cattle [23], when comparing the F_{ROH} of German Brown Swiss animals born from 1990 to 2018 and of Nellore cattle from 1977 to 2020, respectively.

Relationship between ROH features and pseudo-phenotypes

High inbreeding can negatively affect animal health, reproduction, adaptation, resilience, and production traits [64, 65]. This effect has been confirmed in Holstein [5, 56, 57, 66] and other cattle breeds. For example, Bem et al. [23] reported a reduction in birth weight, weight at different ages, and scrotal circumference values in Nellore cattle as the inbreeding coefficient of the animals increased. Pryce et al. [66] estimated a decrease of about 1% in milk, fat, and protein yields for a 1% increase of homozygous SNP in Holstein cattle, confirming previously reported results [67]. Scott et al. [68] reported that, among different production and fertility traits, those most influenced by inbreeding were MY, FY, and PY. Moreover, in the same study it has been reported that with F_{ROH} computed using only ROH > 16 Mb, thus considering the more recent inbreeding, the effects on production and health traits were greater. These findings on the greater power of recent inbreeding were previously reported in various other studies [5, 56–58, 69, 70]. This greater negative power of long ROH in comparison to short ones could be because selection tends to eliminate deleterious mutations, in a process called genetic purging, before inbreeding occurs [66, 71]. Actually, both McParland et al. [69] and Makanjuola et al. [5] reported that inbreeding due to shorter segments (older inbreeding) tended to have a favorable effect on the studied traits, whereas genomic inbreeding based on longer ROH segments tended to have an unfavorable effect on MY and PY. In our study, two ROH on BTA16 and BTA26 were found to influence MY (Table 4). Pryce et al. [66] reported a negative influence of ROH mapped on these two chromosomes on MY, even if at different positions. The negative effect of BTA16 on MY was confirmed also by Scott et al. [68] and Makanjuola et al. [72], who reported genomic regions close to the one highlighted in the present study. The latter found also an effect of ROH presence on BTA8 on PY and on BTA14 on FY, which is similar to our findings. Identifying regions with unfavorable associations with economic traits is fundamental to prevent the effect of inbreeding depression by avoiding matings of individuals that carry them [72]. However, at the same time, inbreeding could also show positive correlation with some traits, because the increased homozygosity at some loci could be due to directional selection in the population, thus being beneficial [67]. For example, Lozada-Soto et al. [56] reported that an increase of inbreeding decreased the probability of displaced abomasum and metabolic diseases.

Gene discovery

In the ROH islands identified through the permutation approach (Fig. 1 and Table 2) 135 genes were mapped. Among them, 24 were from the OR52 family and 20 from OR51 family: the olfactory receptor genes have been previously associated in cattle with several traits linked with sense of smell, such as feed intake and reproduction [73]. As far as the other 91 genes were concerned, in this discussion we focused only on genes associated with traits of interest for the Italian Holstein, such as milk production, fertility, and health traits, whereas more details about all the genes can be found in Table S4 (see Additional File 1). Thus, the whole discussion here mentions only previous reports about Holstein dairy cattle breed. On BTA1, two genes (*RTP4* and *MASP1*) have been previously reported as associated with age at puberty by Stephen et al. [74]. The latter gene, *MBL Associated Serine Protease 1* (*MASP1*), has been also found as associated with milk protein percentage [75] and spontaneous abortion [76]. Another gene mapped on BTA1, the *ST6 Beta-Galactoside Alpha-2,6-Sialyltransferase 1* (*ST6GAL1*) was related to immune response in early lactation [77]. On BTA3, the *Rho GTPase Activating Protein 29* (*ARHGAP29*) has been associated with length of calving interval [78], whereas the *Glutamate-Cysteine Ligase Modifier Subunit* (*GCLM*) resulted involved in energy balance in the early postpartum period [79]. On BTA6, two genes were already reported in Holstein: *RNA Polymerase II Subunit B* (*POLR2B*) was related to Bovine Respiratory Disease in post-weaned heifers [80] and to estrous behavior [81], and *Insulin Like Growth Factor Binding Protein 7* (*IGFBP7*) to fertility traits as well as to milk production [82]. One gene mapped on BTA7, the *Multiple C2 And Transmembrane Domain Containing 1* (*MCTP1*) has been associated with days open [83]. Three genes mapped on BTA13 (*GINS1*, *PCMTD2*, and *MYT1*) have been associated to sire fertility traits [84]. The former was also related to Bovine Respiratory Disease in post-weaned heifers [80], whereas the latter to lactation persistency [85]. Two genes on BTA13 (*NPBWR2* and *OPRL1*) were related to dry matter intake during lactation [86], whereas the *SIRPB1* was reported to be involved in inflammation and immune response [87]. On BTA16, the *Dynamin 3* (*DNM3*) gene has been associated with *Staphylococcus aureus* infection response [88]; the *SUN Domain Containing Ossification Factor* (*SUCO*) is involved in the scurs development [89]; the *Fas Ligand* (*FASLG*) was related to mammary-system related traits

[90] and thermotolerance [91]. On BTA17, a cluster of 4 genes (*CIT*, *PRKAB1*, *TMEM233*, and *CCDC60*) has been associated with eating time and, in general, feeding behavior [92]. On BTA19, two genes (*ASPA* and *TRPV3*) have been reported as related to interval between first and last insemination [93]. The second one, together with the *TRPV1*, was related also to gastrointestinal motility [94]. The *TRPV1* has been previously associated to daughter pregnancy rate [95], too. Moreover, on BTA19, a cluster of 3 genes (*TAX1BP3*, *EMC6*, and *P2RX5*) has been found associated with methane emission per kg of milk [96]. Finally, the *Integrin Subunit Alpha E* (*ITGAE*) was related to resistance to gastrointestinal parasites [97], the *Calcium/Calmodulin Dependent Protein Kinase Kinase 1* (*CAMKK1*) to somatic cell score [98], and the *Purinergic Receptor P2X 1* (*P2RX1*) to heat stress response [99]. On BTA20, two genes (*LIFR* and *EGFLAM*) have been associated with clinical ketosis [100], and the former has also been linked to mastitis susceptibility [101]. On BTA23, five genes (*PACSIN1*, *SNRPC*, *NUDT3*, *RPS10*, and *GRM4*) were related to MY [102, 103]. The *NUDT3* and *RPS10*, together with other four genes (*HMGA1*, *SPDEF*, *ILRUN*, and *SNRPC*), have been also associated with subclinical ketosis [100], whereas the first (*PACSIN1*) has been associated with susceptibility to *Mycobacterium avium* subspecies *Paratuberculosis* infection [104], too. On BTA24, the *RNA Binding Fox-1 Homolog 1* (*RBFOX1*) gene was related with spontaneous abortion [76] and semen quality [105], whereas the *Transmembrane Protein 241* (*TMEM241*) has been associated with success rate at insemination of lactating cows [106]. On BTA26 four genes have been previously related to Holstein traits: the *SUFU Negative Regulator Of Hedgehog Signaling* (*SUFU*) to protein percentage and FY in milk [107], the *ARF Like GTPase 3* (*ARL3*) to Bovine Respiratory Disease in post-weaned heifers [80], and the *BLOC-1 Related Complex Subunit 7* (*BORCS7*), together with the *5′-Nucleotidase, Cytosolic II* (*NT5C2*), to milk fat composition [108]. Finally, on BTA29 three genes (*PAG19*, *PAG20*, and *PAG21*) were found, all related to pregnancy traits, in particular conceptus and placenta [109, 110].

The region on BTA20 that seems to be under selection in all the five considered birth-year groups (Fig. 4), harbored 27 genes. Among them, 21 were previously reported to be associated with important traits in Holstein cattle. Some of them with immune related traits such as *Mycobacterium avium ssp. paratuberculosis* infection status (*SNX18*, *NDUFS4*, *DHX29*, *ESM1*, and *HCN1*; [111]), subclinical (*NDUFS4*, *MOCS2*; [100]) or clinical (*EGFLAM*; [100]) ketosis, clinical mastitis (*IL7R*; [112]), displacement of abomasum (*ZFR*; [113]), and disease resistance (*CPLANE1*; [114]). Many of them were related to milk production traits: milk fat metabolism (*NDUFS4*; [115]), MY (*MTREX*, *MRPS30*, and *SLC1A3*; [107, 116]), milk PP (*HCN1*, *C20H5orf34*, *IL7R*, and *TMEM267*; [107, 117]), lactation persistency (*HCN1* and *MRPS30*; [118]), milk fatty acids (*WDR70*; [119]), and milk yield and composition (*GHR*; [120]). Finally, two genes were previously found associated with heat stress response (*C9* and *C6*; [121]), and two with reproductive traits, such as interval between first and last insemination (*PDZD2*; [93]) and number of services per conception (*MRPS30*; [122]).

Conclusions

The comprehensive investigation on ROH distribution and features carried out in this work has provided interesting insights on the genome structure of the considered sample of Italian Holstein bulls, confirming the usefulness of this metrics for studying the genome structure of livestock species. The importance of the age of inbreeding in affecting production traits has been confirmed. Moreover, the permutation approach proposed provided an objective method for assessing the relevance of the occurrence of a SNP into a ROH, confirmed by the agreement between genomic regions highlighted in this study and previous reports in different Holstein populations.

Supplementary Information

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Supplementary Material 1.

Supplementary Material 2.

Supplementary Material 3.

Supplementary Material 4.

Supplementary Material 5.

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Authors' contributions

AC, NPPM, and GG conceived and designed the manuscript. LF, AC, and GG performed the analyses. LF, AC, LFB, SM, AP, NPPM and GG interpreted the results and edited the manuscript. NPPM and GG contributed resources and funding. LF, AC, and GG wrote the first draft of the paper. All authors revised, read, and approved the submitted manuscript.

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Data availability

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Declarations

Ethics approval and consent to participate
No ethics approval was needed since data came from preexisting databases.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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