

Identification of a Short Region on Chromosome 6 Affecting Direct Calving Ease in Piedmontese Cattle Breed

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Abstract

Calving in cattle is affected by calf morphology and by dam characteristics. It is described by two different traits: maternal calving ease, which is the ability to generate dams with good physiological predisposition to calving, and direct calving ease, which is the ability to generate calves that are easily born. The aim of this study was to identify regions of cattle genome harboring genes possibly affecting direct calving ease in the Piedmontese cattle breed. A population of 323 bulls scored for direct calving ease (EBV) was analyzed by a medium-density SNP marker panel (54,001 SNPs) to perform a genome-wide scan. The strongest signal was detected on chromosome 6 between 37.8 and 38.7 Mb where 13 SNPs associated to direct calving ease were found. Three genes are located in this region: *LAP3*, encoding for a leucine aminopeptidase involved in the oxytocin hydrolysis; *NCAPG*, encoding for a non-SMC condensin I complex, which has been associated in cattle with fetal growth and carcass size; and *LCORL*, which has been associated to height in humans and cattle. To further confirm the results of the genome-wide scan we genotyped additional SNPs within these genes and analyzed their association with direct calving ease. The results of this additional analysis fully confirmed the findings of the GWAS and particularly indicated *LAP3* as the most probable gene involved. Linkage Disequilibrium (LD) analysis showed high correlation between SNPs located within *LAP3* and *LCORL* indicating a possible selection signature due either to increased fitness or breeders' selection for the trait.

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Introduction

Reproductive health in cattle is an economically important trait and calving is a key element to improve productivity and animal welfare. Calving difficulty, also known as dystocia, has negative effects on the sustainability and profitability of a herd by adding considerable costs for veterinary care, hampering the next conception, and worsening the animal welfare. Calving is influenced by many environmental and genetic factors: in cattle it is affected by calf morphology [1] and by dam characteristics [1] and can be described by two traits: maternal calving ease and direct calving ease. Both refer to calving without human intervention, the first attributed to the mother and the second to the calf. In addition to birth weight, other important factors as calf shape, the size of cow pelvic area or the gestation length may contribute to calving ease [2]. One of the major causes of dystocia is a disproportion between size of the calf at birth and the cows' birth canal. Thus, the cow physiological predisposition to birth and the calf morphology are the key genetic factors which can be improved by selection [3]. The Estimated Breeding Values (EBVs) for calving ease used in breeding plans are measured from three

sets of records: calving ease score, birth weight and gestation length. Several studies have identified QTLs influencing calving traits [4,5,6,7,8,9]. The estimation of EBV and the identification of genes significantly associated with calving performance is a challenging task since the traits show low heritability and the recording is made on scales which are subject to breeder's interpretation. The recent availability of cattle genome-wide SNP panels could potentially overcome these drawbacks [10]. The use of large-scale single nucleotide polymorphism (SNP) genotyping and genome-wide association (GWA) studies allow to identify genomic regions and hopefully mutations that underlie the desired performance.

The aim of this study was to identify regions of the bovine genome possibly affecting calving performance. We used most of the records registered in the Piedmontese breed, which is known to be at risk of dystocia like all breeds that carry harmful mutations in the myostatin gene [11]. In our work, a medium density SNP marker panel (54,001 SNPs) was used to investigate the genome of 323 Piedmontese bulls for identifying chromosomal regions associated with calving traits. This genome-wide scan let us to discover a 0.9 millions of base pairs (Mbp) region of chromosome 6

(37.8–38.7 Mbp, on the Btau_4.0), associated with direct calving ease and to pinpoint with high probability genes associated to the trait.

Results

Data Editing

Three of the 323 sires were excluded for having a genotype call rate lower than 95% and two for having a too high autosomal heterozygosity. As for markers, 506 out of the 54,001 SNPs of the chip were excluded because the ratio of missing genotypes was higher than 5%; 10870 because showed minor allele frequency below 2%; 1372 because they were out of Hardy-Weinberg Equilibrium (HWE, $P < 0.01$). The final dataset was thus formed by 318 sires and 41333 SNPs. Figure 1 (left panel) shows the distribution of EBV values for direct calving ease partitioned in five equally spaced classes; an acceptable approximation of a normal distribution can be observed for direct calving ease, with the three central intervals accounting for 75% of sample size; a tiny extreme negative tail which is not mirrored by positive values can be observed.

Genome-wide Scan

Distribution of genomic kinship values is shown in Figure 1 (right panel); most sires share a genomic kinship value in the range ± 0.025 and higher values are very uncommon in the sample; thus the chosen statistical approach coupled with genomic control should be able to correctly take into account the population structure while conserving enough statistical power. Results of the genome-wide scan for direct calving ease traits are summarized in the Manhattan plot of Figure 2 (upper panel). Among the 41333 polymorphisms of the Illumina BovineSNP50 retained after quality control, 2030 (roughly 0.5%) were associated to direct calving ease. P-values ranged from 0.05 (chosen as the maximum value to define a SNP as significantly associated to direct calving ease) and 8.17×10^{-15} (see Table 1). A very sharp peak containing several SNPs with very

high $-\log_{10}(P^*)$ values can be observed on the first half of BTA (*Bos taurus* autosome) 6, between 37.8 and 38.7 millions of base pairs (Mbp) (i.e. between 715 and 720 Mbp relatively to total genome length, Btau_4.0). The inflation factor λ obtained for the test was 1.03 showing that, given the number of markers, residual inflation was acceptable; a qq-plot of P-values prior to λ normalization is shown in Figure S1 in the Supplementary Materials. Correction for λ yielded a total of 1876 significant SNPs; of these, 1833 had a λ -corrected P-value in the interval $0.05 > P \geq 0.001$ and 43 had a λ -corrected P-value < 0.001 . Significance levels (unadjusted: P^* , and adjusted with the Benjamini-Hochberg method, Q^* ; see Materials and Methods section) of these SNPs, SNP names and their position on BTA6 according to Illumina BovineSNP50 specifications, and the gene in which they are located within (if any) are shown in Table 1. A clear association of the identified chromosomal region with the target trait can be observed, as statistically confirmed by several markers showing a P-value $< 10^{-7}$ also after the correction for multiple testing. Allelic effects of the minor allele (last column of Table 1) are small and positive except for two markers (Hapmap26308-BTC-057761 and ARS-BFGL-NGS-45457). Figure 2 (lower panel) shows the region on BTA6 spanned by the 13 most significant SNPs showed in the Manhattan plot (Figure 2 upper panel) and listed in Table 1; one marker (Hapmap26308-BTC-057761) is within the sequence of the *LAP3* gene while four (Hapmap 27083-BTC-041166, 23507-BTC-041133, 31285-BTC-041097, 33628-BTC-041023) are located in the *LCORL* gene. Average Linkage Disequilibrium (LD) estimation for all markers in the selected region yielded $r^2 = 0.426$ but higher correlation was observed between SNPs within the *LCORL* gene ($r^2 = 0.637$) and between SNPs located in *LCORL* and *LAP3* ($r^2 = 0.571$).

Association of Additional SNPs Located within LAP3, NCAPG and LCORL Genes

Based on the results of genome wide scan we decided to genotype additional SNPs in the genes located in the region

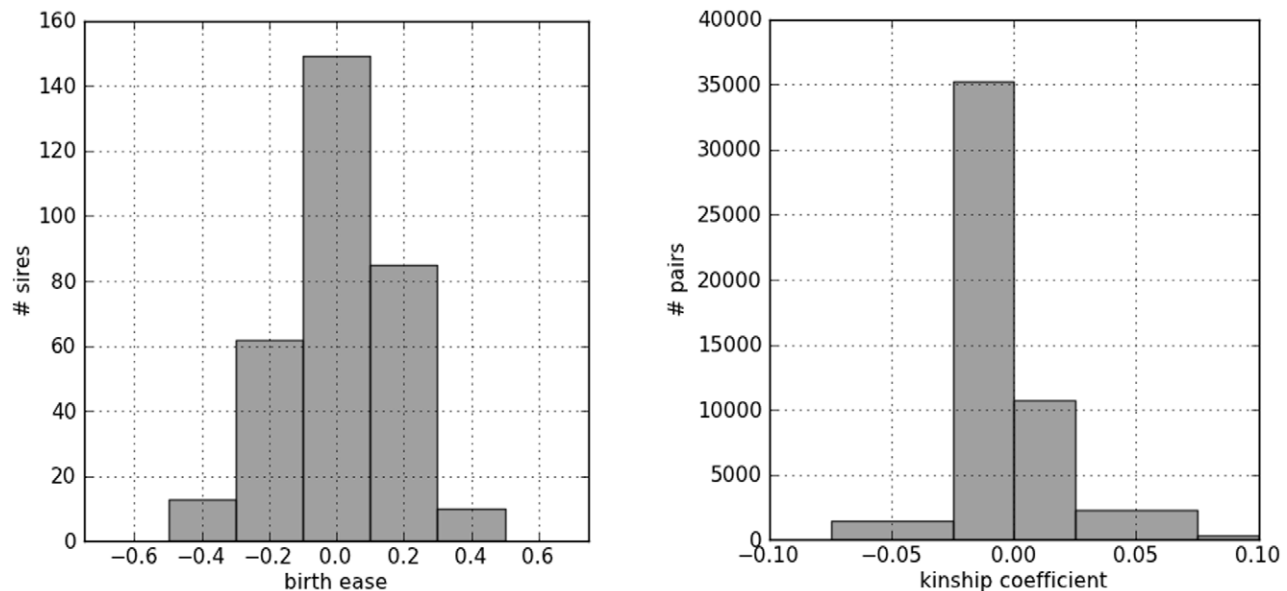


Figure 1. Left Panel: histograms of EBVs for direct calving ease distributed in five equally spaced classes. Right panel: histogram of kinship coefficients between sires in five equally spaced classes. In both panels, the number of sires belonging to each class is plotted as a function of classes.

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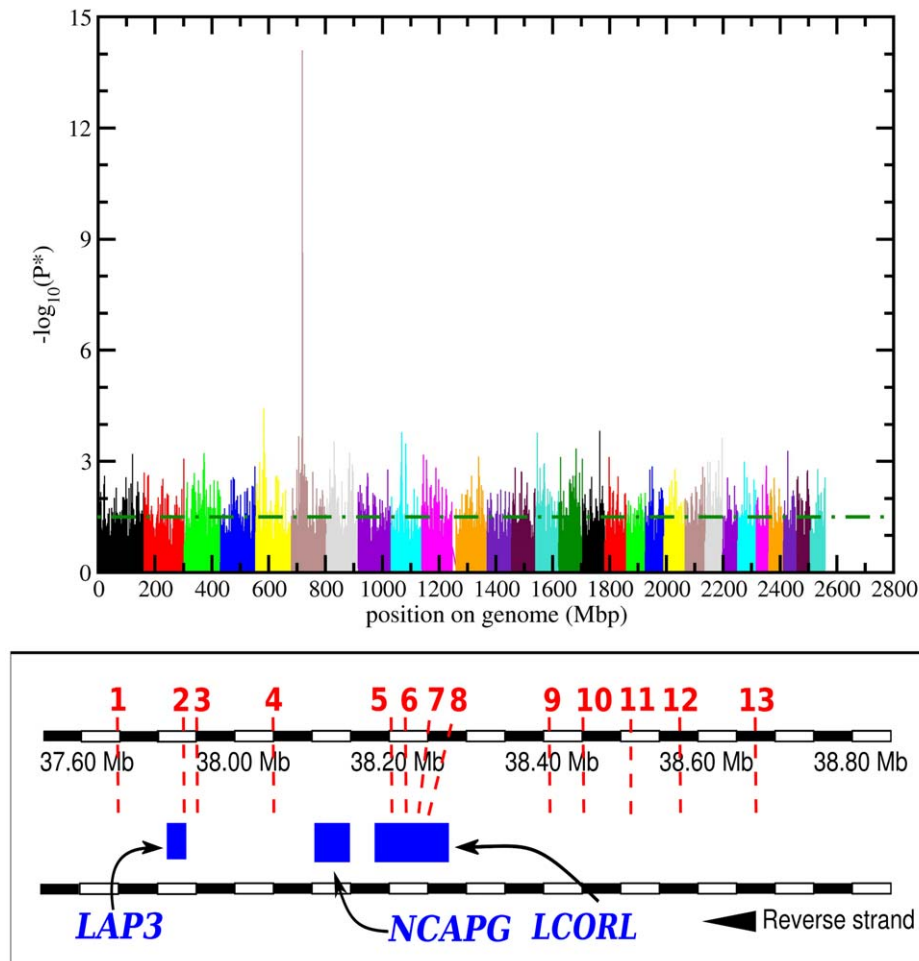


Figure 2. Upper panel: log significance levels of SNPs association with direct calving ease for the entire filtered dataset before the correction for inflation. Each autosome is represented with a different colour and position is calculated along the entire genome length. Values above the dot-dashed green line denote a $P^* < 0.05$. Lower panel: The chromosomal region spanned by the 13 markers listed in Table 1. The approximate position and size of genes located in the region (blue rectangles) together with the position of each SNP (red numbers and red dashed lines) are shown.

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where we found a strong association of the Illumina Bovine SNP50 with direct calving ease. We therefore selected, from NCBI dbSNP (<http://www.ncbi.nlm.nih.gov/snp/>) and from the literature [12,13], 12 SNPs in *LAP3* (only one marker from the Illumina BovineSNP50 panel was located in this gene), 5 in *NCAPG*, and 5 in *LCORL* (see Table S1 for complete information on these additional SNPs). Markers were genotyped on the Piedmontese population, leaving out sires discarded in the GWAS step. However, due to limited availability of biological samples, we were able to obtain further DNA only from 247 subjects of the original population. Data editing of these 22 additional SNPs yielded the following results: of the 247 starting sires, 6 were discarded for having a call rate $< 95\%$; 4 markers were excluded because the call rate was $< 95\%$ and 10 markers were excluded for being monomorphic or for having Minimum Allele Frequency (MAF) below 5%. Checking the EBV distribution of this partially reduced population we verified that it was closely matching the whole one used for GWAS and thus no significant bias should have been introduced by the missing subjects. Figure 3 shows histograms, analogous to those in Figure 1, with the distribution of trait values and kinship coefficients for these 247 sires; in particular, it can be seen that

the greater part of pairs of sires shows a slightly negative kinship (Figure 3, right panel) as seen in the whole population (Figure 1, right panel). The results of the statistical analysis are shown in Table 2. All SNPs resulted associated to calving ease with significance levels ranging from $8.34E-08$ to $7.42E-04$. The strongest association was observed for the SNP *rs110251642* located in the *NCAPG* gene but overall the most important signal was observed for the *LAP3* gene since more than half of the markers within its sequence resulted associated with the studied trait. Moreover, with the exception of *rs4125598* ($P^* = 6.955E-07$, $\beta = 7.335E-02$), all SNPs shared the same P^* and β implying that these markers are in high LD. The average LD estimation among *LAP3* and *LCORL* SNPs was $r^2 = 0.653$ (higher than that obtained analyzing SNPs located within the same genes using the BovineSNP50 v1 panel) but this value dropped to $r^2 = 0.383$ if all significant SNPs located in the peak on BTA6 (i.e. from both GWAS and additional genotyping data) were included. It is also worth to observe that SNPs located in *LAP3* gene showed the highest LD ($r^2 = 0.783$) in agreement with the GWAS results. The significant SNP in the region explained about 22.8% of the EBV variance (Principal Components Regression was used to cope with correlation among SNPs).

Table 1. Single nucleotide polymorphisms associated to direct calving ease and located on BTA6 in the region comprised between 37 and 39 millions of base pairs sorted according to their position on the Illumina BovineSNP50 panel.

SNP name	Position (bp)	gene	P*	Q*	fB	βB
Hapmap30134-BTC-034283	37,852,401	NA	2.38E-09	1.94E-05	0.45	8.43E-02
Hapmap26308-BTC-057761	37,963,148	LAP3	8.17E-15	3.38E-10	0.40	-1.19E-01
ARS-BFGL-NGS-112812	38,014,255	NA	3.87E-06	1.60E-02	0.35	6.85E-02
ARS-BFGL-NGS-45457	38,102,328	NA	7.97E-13	1.65E-08	0.45	-9.97E-02
Hapmap27083-BTC-041166	38,212,942	LCORL	2.58E-07	1.52E-03	0.36	7.50E-02
Hapmap23507-BTC-041133	38,233,089	LCORL	3.89E-07	2.01E-03	0.36	7.39E-02
Hapmap31285-BTC-041097	38,256,890	LCORL	4.11E-11	4.24E-07	0.48	-9.30E-02
Hapmap33628-BTC-041023	38,326,148	LCORL	7.12E-12	9.80E-08	0.46	-9.85E-02
Hapmap25414-BTC-034877	38479644	NA	2.45E-05	7.78E-02	0.27	6.71E-02
Hapmap32207-BTC-034871	38,500,210	NA	2.45E-05	7.78E-02	0.27	6.71E-02
Hapmap28546-BTC-072715	38,558,527	NA	4.84E-07	2.22E-03	0.38	7.38E-02
Hapmap27537-BTC-060891	38,638,963	NA	1.04E-05	3.90E-02	0.39	6.68E-02
Hapmap31044-BTC-071337	38,729,867	NA	2.38E-09	1.94E-05	0.46	8.66E-02

SNP name, position, the gene in which they are located (if any), significance levels both unadjusted (P*) and corrected for multiple testing (Q*), allelic frequencies (fB) and effects of the minor allele (βB).

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Discussion

Today, selection objectives in agriculture pay a great deal of attention to robustness, longevity, welfare and, more generally, sustainability of production. Calving ease is a functional trait having a large impact on animal health and farm profitability, and it also make feasible to breeders the exploitation of sustainable livestock systems [14]. However, antagonistic relationships between calving performance and beef production traits [15] make the classical breeding schemes not efficient and highlights the need for improved selection strategies. The possibility to select individuals based on alleles at genes affecting calving performances may help to overcome this problem. Moreover, Piedmontese is a breed carrying the G1056A mutation in the *myostatin* gene [11,16,17], which affects calving ease, although less severely than in other breeds carrying disruptive mutations, as Belgian Blue [16] and Marchigiana [18]. Furthermore, estimates of heritability of maternal and direct calving related traits for the Italian Piedmontese population are, like many other estimates in several other breeds, between 0.19 and 0.08 depending of the number of parities and the type of effect considered [19] and this proves the difficulty of establishing selection strategies based on traditional breeding programs. Therefore, several attempts have been made to use DNA information for improving this trait. In cattle, QTLs

worth of further study, affecting calving ease and foetus growth-related traits, are described on various chromosomes. A total of 61 and 38 QTLs for direct and maternal calving ease, respectively, are reported in public databases (<http://www.animalgenome.org/cgi-bin/QTLdb/BT/index>). Pausch and collaborators identified by genome-wide association two major loci on BTA 14 and 21 affecting calving ease and growth-related traits, respectively [10]. Other studies point to the existence of QTLs for calving ease and foetal growth on BTA 6 in various cattle populations [20,21,22].

In this study, we performed a genome wide scan of Piedmontese cattle using a medium density chip featuring about 54K SNPs. The chip analysis clearly showed a cluster of 13 SNPs in a 0.9 Mb wide region of BTA6 significantly associated ($P^* < 0.001$) with direct calving ease. Three interesting genes are located in this region: *LAP3*, encoding for a leucine aminopeptidase, which is responsible of the oxytocin hydrolysis [12,13]; *NCAPG*, encoding for a non-SMC condensin I complex, which has been associated in cattle with foetal growth and carcass size [23,24]; *LCORL*, encoding for a ligand dependent nuclear receptor co-repressor-like, which affects height in humans and also controls stature in cattle in high correlation with *NCAPG* [25]. In a crossbred population of beef cattle, Snelling and collaborators identified a region on BTA6 (37.96–38.53 Mb, on the Btau_4.0) associated with feed intake, gain, meat and carcass traits [26,27,28,29]. Four markers typed in *LCORL* were also found associated with carcass weight in Japanese Black steers [24]. We considered *LAP3*, *NCAPG* and *LCORL* as potential positional and functional candidate genes on BTA6 for direct calving ease. Thus, we chose to enrich the SNP set in these genes to be genotyped on the population. Two out of five SNPs in *NCAPG* show a weak association to direct calving ease and a positive effect linked to the homozygote genotypes for the major allele (T/T in *rs109570900* and C/C in *rs110251642*). Recently it has been shown that a polymorphism in the *NCAPG* gene (*rs109570900*, 1326T>G) is significantly associated to an increase in bovine carcass weight at puberty [24,28,33]. Only one of the 5 markers selected in *LCORL* (*rs109572301*) was significantly associated with direct calving ease. *LCORL* is immediately adjacent to the *NCAPG* gene; the *LCORL/NCAPG* locus has been associated with human height [25,30,31,32], with feed intake, gain, meat and carcass traits in beef cattle [28], and very recently with size variation in horses [34,35]. Among the three genes included in the statistically associated region, *LAP3* is the one showing the most suggestive involvement with the trait. It results significant considering both SNPs in the Illumina BovineSNP50 chip and the further genotyped SNPs. Moreover, the SNPs within its sequence show the highest Linkage Disequilibrium, which is probably due to an increased fitness or to breeders selection. It is worth to observe that these results add relevant information to those obtained in the GWAS since the Bovine SNP50 v1 panel contains only one marker in *LAP3* and the strongest signal was obtained for the *LCORL* gene. However, it is not possible to draw definitive conclusions about the relative importance of *LCORL* or *NCAPG* as compared with *LAP3* since most markers genotyped within these genes were not usable as being monomorphic or with $MAF < 0.05$. We cannot rule out that the region was subject to selective sweep that dropped out one allele. *LAP3* is involved in oxytocin metabolism and hence in maternal calving effects, nevertheless we did not find an association with this trait (data not shown). This lack of association is probably due to the scarcity of data since EBV records for maternal calving effects were available for less than one hundred sires and with lower accuracy since calves outnumber dams.

Finally, our genome-wide association study shows that EBVs of traits with low heritability are still able to capture the molecular

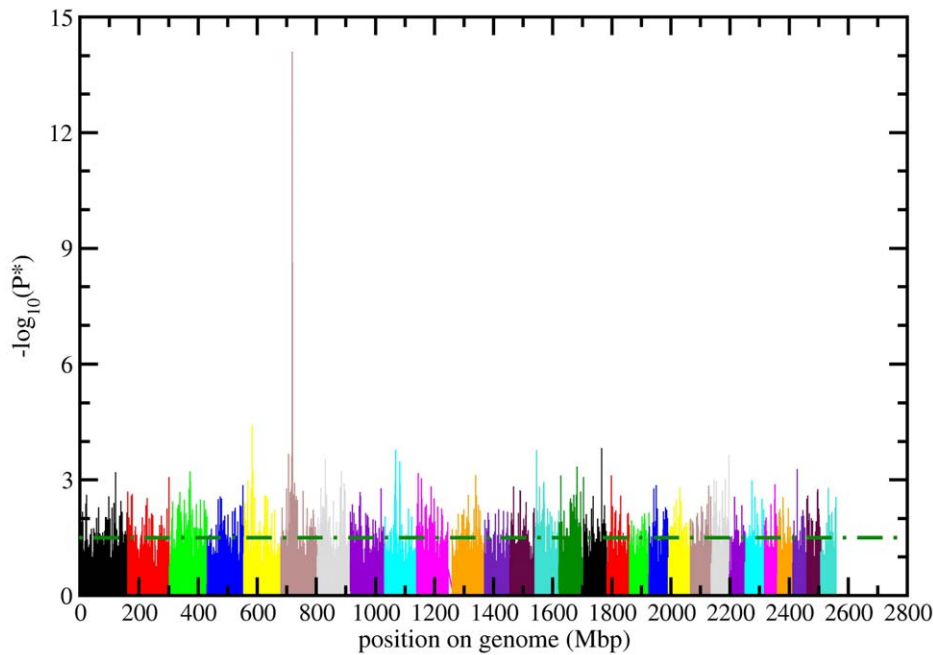


Figure 3. Descriptive histograms for the reduced population of 247 sires used for additional genotyping of SNPs within the *LAP3*, *NCAPG* and *LCORL* genes after the GWAS. Left Panel: histograms of EBVs for direct calving ease distributed in five equally spaced classes. Right panel: histogram of kinship coefficients between sires in five equally spaced classes. In both panels, the number of sires belonging to each class is plotted as a function of classes.

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features of the genome. These can be exploited by a Gene Assisted Selection (GAS), that is comparably much faster and more efficient than the selection based on EBV. Furthermore, traits with low heritability should not be disregarded because their relative objectives of selection can only be reached in a long time, indeed they may be successfully dissected to reveal important insights at molecular level.

Table 2. SNP name, gene name, region within the gene, significance level (P*) and minor allele effect (β) for the SNPs selected in the *LAP3*, *NCAPG* and *LCORL* genes and significantly associated to direct calving ease.

SNP	gene	gene region	P*	β
rs41255598	<i>LAP3</i>	non synonymous codon	7.419E-04	1.658E-02
rs43702364	<i>LAP3</i>	intron 12 (24428-24878)	6.955E-07	7.335E-02
rs43702363	<i>LAP3</i>	intron 12	6.955E-07	7.335E-02
rs43702362	<i>LAP3</i>	intron 12	6.955E-07	7.335E-02
rs110839532	<i>LAP3</i>	3'UTR	6.955E-07	7.335E-02
rs109241256	<i>LAP3</i>	3'UTR	6.955E-07	7.335E-02
rs41255599	<i>LAP3</i>	3'UTR	6.955E-07	7.335E-02
rs110251642	<i>NCAPG</i>	non synonymous codon exon 17	8.341E-08	-8.252E-02
rs110422856	<i>NCAPG</i>	intron variant, splice region variant	4.871E-04	5.394E-02
rs109572301	<i>LCORL</i>	3'UTR variant	4.692E-04	5.414E-02

Where available, rs number links to NCBI dbSNP page for the relative SNP.
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Materials and Methods

Animals and EBV Data

Genomic DNA was extracted from semen of 323 Piedmontese bulls using the NucleoSpin Tissue kit (Macherey & Nagel) according to manufacturer's instruction. DNA was checked for quality on agarose gel and quantified using a DTX microplate reader (Beckman Coulter) after staining with Picogreen (Invitrogen). This Italian breed has been selected for beef production and almost all individuals carry muscular hyperplasia [11]. Estimated Breeding Values (EBVs) were provided by the Italian Piedmontese National Breeders Association (ANABORAPI, <http://www.anaborapi.it/>). The sample includes almost all Piedmontese bulls for which data were available and thus it represents the most complete population available for the association analysis for this breed.

SNP Chip and Genotyping

In total, 323 individuals were genotyped using BovineSNP50 BeadChips (Illumina, San Diego, CA, USA). The Illumina BovineSNP50 array contains 54,001 SNPs distributed across the entire genome, with an average SNP spacing of 51 Kb and a proportion of known chromosome positions of about 97%. Genotyping was outsourced to Geneseek, USA (www.geneseek.com). SNP calls accurate to more than 99.8% were obtained on average. The SNP positions within each chromosome were based on the *Bos taurus* genome assembly Btau_4.0 [36].

Data Editing and Genome-wide Analysis

Sires and markers with a call rate under 95% were discarded, as well as SNPs having a minor allele frequency (MAF) <2.5%. Sires were checked for abnormally high autosomal heterozygosity (FDR 1%) [37,38]. Hardy Weinberg Equilibrium was checked on the

filtered data set [39]; markers were removed from the data set if the P-value was <0.01 . Kinship among sires was estimated directly from genomic data as proposed by Astle and Balding [40]. Association of SNPs with phenotypes was performed using the Family based Test for Association to reduce computing time [41]. Kinship among sires was treated as a random effect, while SNP genotypes were considered as fixed effects. The model used was:

$$y = 1\mu + Zu + u_g g + e$$

where μ is the mean and 1 is a vector of ones, Zu accounts for polygenic effects, u_g is the effect of the marker under investigation and g denotes a genotype, e is a vector of residuals. Polygenic effects were estimated by calculating genomic kinship between sires using the complete set of filtered SNPs [42,43]. Genomic control was applied and the inflation factor λ was calculated and used to correct significance levels [44]. False Discovery Rate control was performed using the Benjamini-Hochberg method [45] using a FDR of 0.1. LD in selected regions was estimated calculating the r^2 coefficient between SNPs using the approach proposed by Hao [46].

Genotyping of Additional SNPs on BTA6

Since the genome wide analysis pointed to a ~ 0.9 Mb region associated to direct calving ease (see Results) we searched for further SNPs not represented in the Illumina BovineSNP50 panel in genes included in this region to validate and enrich the results of the genome wide scan. We selected 12 SNPs in the *LAP3* gene, 5 in the *NCAPG* gene and 5 in *LCORL* (Table S1). For *LAP3* three markers were described by Zheng [12], while nine SNPs were selected by screening *in silico* NCBI dbSNP (<http://www.ncbi.nlm.nih.gov/snp/>). Three SNPs in *NCAPG* gene were described by Setoguchi [13,24], other two were selected in NCBI dbSNP. The five markers in *LCORL* gene were all selected in NCBI dbSNP. The 22 SNPs were genotyped on 247 bulls from the whole population from which we could recover biological samples. SNP

genotyping was outsourced to Kbiosciences (www.kbiosciences.co.uk). Data were filtered using the same criteria illustrated above for the SNP chip data. Association between these SNPs and EBVs for direct calving ease was performed using the same statistical model employed for the genome-wide scan but without correcting for inflation factor or for multiple testing since in this case SNPs were limited; however the full kinship matrix obtained with data from the BovineSNP50 panel and the bulls for which additional genotyping was available was used in the calculations.

The variance explained by the SNPs was evaluated using a Principal Component Analysis regression to cope with correlation (Linkage Disequilibrium) among markers [47].

Supporting Information

Figure S1 QQ-plot of significance levels for the GWAS scan prior to normalization. (PNG)

Table S1 Additional SNPs genotyped in the *LAP3*, *NCAPG* and *LCORL* genes. The r_s number, the gene region and the reference are shown. (PDF)

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Author Contributions

Conceived and designed the experiments: SB LP AV GM. Performed the experiments: SB. Analyzed the data: GM. Wrote the paper: GM SB AV. Collected samples and performed genotyping: SB LP. Designed the software specifically developed for the work and performed statistical analysis: GM. Reviewed the paper: LP GC.

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