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ASSOCIATION STUDY FOR GENETIC VARIANTS LINKED WITH REPRODUCTIVE TRAITS IN ROMANIAN SPOTTED CATTLE

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Abstract

In this work, we performed a targeted candidate-gene association study to identify genetic variants linked with reproductive traits in cattle. We investigated the relationship between 348 SNPs on Axiom BovMDv3 SNP array and the following reproductive traits in cattle: calving interval (CI), gestation length (GL), age at first calving (AFC), artificial inseminations to conception (A2C), presence of twins (TW) and history of abortions (AB). The dataset included reproductive information of 587 Romanian Spotted and Romanian Brown cattle. Poisson regression was applied to test associations for CI, GL, A2C, and AFC phenotypes, whereas TW and AB traits were examined using logistic regression. The results indicate significant associations between certain SNPs and fertility-related traits. The genes SPRY1, ILDR1, SLC24A4, TANC2, LOXL2, and SLC24A4 showed the strongest associations with CI, with extremely small p-values even after correction for multiple testing. In addition, SNPs on the ACOXL, SLC4A4, ABCC9, DDR2 and TANC2 genes were significantly associated with AFC. Our findings suggest that these genes are promising candidates for future studies aimed at elucidating the mechanisms influencing bovine fertility.

Key words*: association study, candidate gene, cattle, reproductive traits.*

INTRODUCTION

Reproductive efficiency represents a foundation of sustainable and profitable dairy cattle production systems, with direct implications for herd productivity, animal welfare, and economic performance (Schwarz et al., 2024). Reproductive traits such as calving interval, gestation length, age at first calving, and conception success influence the efficiency of dairy cows, the overall management, and the replacement strategies within herds. Suboptimal reproductive performance is associated with prolonged calving intervals, increased insemination costs, and higher culling rates, ultimately reducing lifetime productivity. Moreover, it has been widely documented that reproductive performance is often negatively correlated with high milk yield (Oltenacu & Broom, 2010; Pryce et al., 2004), emphasizing the need for balanced breeding strategies that integrate both production and functional traits. Despite their importance, research consistently identifies bovine reproductive traits as having low heritability and complex genetic architecture, being influenced by numerous genes and strong environmental interactions (Berry et al., 2014; Cammack et al., 2009; Panigrahi et al., 2024). This polygenic nature makes the identification of causative genetic factors particularly challenging. Advances in bovine genomic technologies, especially the development of high-density single nucleotide polymorphism (SNP) arrays and whole genome sequencing approaches, have significantly improved the ability to detect genomic regions associated with reproductive traits. Genome-wide association studies (GWAS) have become a powerful tool

for identifying genomic variants associated with economically important traits in cattle, enabling the discovery of candidate genes involved in key biological pathways of reproductive performance (Ortega, 2018). Numerous studies have reported significant associations between SNPs and fertility related phenotypes (Chen et al., 2022; Sbardella et al., 2021; Gonzalez Berrios et al., 2024), highlighting the complex architecture of reproductive traits and validating the idea that they are controlled by a large number of loci with small individual effects (Tahir et al., 2021). Furthermore, expanding genomic research to autochthonous and dual-purpose breeds, which represent unexploited sources of genetic diversity, is essential for identifying breed specific markers and previously undetected genetic architectures that may be absent in specialized cattle breeds.

In recent years, GWAS analyses in dairy and beef cattle populations have uncovered the complex genetic architecture that governs reproductive success and have identified multiple genomic regions and candidate genes associated with both male and female fertility traits. For example, studies using SNP chip data and imputed sequence level genotypes for genetic characterization of reproduction traits in German Holstein cattle have revealed more than 2700 significant SNPs representing about 90 different genes affecting fertility in dairy cattle, with the majority of association signals on BTA6 and BTA18 and others candidate genes on BTAX (Schwarz et al., 2024). Similarly, association studies for beef bull semen attributes have identified genes and quantitative trait loci (QTL) associated with fertility traits in bulls, widely distributed across the bovine genome (Butler et al., 2022), further highlighting the complexity of fertility traits. These findings underscore the potential of incorporating genomic information into breeding programs to enhance reproductive performance through marker-assisted or genomic selection.

However, most genomic studies on cattle fertility have been conducted in specialized breeds, while locally adapted breeds remain significantly underrepresented. Romanian Spotted and Romanian Brown cattle are important dairy breeds within regional production systems, valued for their longevity, adaptability, resilience, and balanced productive traits. Nevertheless, the genetic basis of their reproductive performance has not been extensively investigated, creating a significant knowledge gap. Given that breed-specific genetic backgrounds and selection histories may influence the architecture of reproductive traits, it is essential to conduct targeted association studies in these populations. Therefore, using targeted candidate-gene association study, we aim to identify genetic variants associated with key reproductive traits in Romanian Spotted and Romanian Brown cattle. We investigate the relationship between 348 pre-selected SNPs from the Axiom BovMDv3 array and six phenotypes: calving interval (CI), gestation length (GL), age at first calving (AFC), artificial inseminations to conception (A2C), presence of twins (TW), and history of abortions (AB) in a total of 587 animals. By identifying high-priority candidate genes, this research contributes to the understanding of fertility genetics in autochthonous breeds and provides valuable insights for future genomic selection strategies.

MATERIALS AND METHODS

The study involved a total number of 587 Romanian Spotted and Romanian Brown multiparous cattle from the Research and Development Station for Bovine in Arad. All animals were maintained under uniform nutritional and veterinary protocols and were included in the Romanian Official Performance and Recording Scheme. A total of six reproduction traits were analysed: calving interval (CI), gestation length (GL), age at first calving (AFC), artificial inseminations to conception (A2C), presence of twins (TW) and history of abortions (AB).

Genotyping was performed on blood samples collected from the coccygeal vein using K3EDTA tubes. All samples were subsequently processed by IFN Schönow GmbH in Germany using the Axiom Bovine v3 microarray (Thermo Fisher Scientific), mapped against the Bos_taurus_UMD_3.1.1 reference genome. We selected a panel of 348 SNPs on the Axiom BovMDv3 SNP array, located on 156 genes that have previously been associated with reproductive traits in cattle (Table 1) and investigated the relationship between selected SNPs and the six reproductive traits.

Furthermore, we set up a quality control pipeline, which excluded markers with call rates below 0.95, minor allele frequencies (MAF) less than 0.05, and significant deviations from Hardy-Weinberg Equilibrium.

Genotype and allele frequencies were calculated using a custom R script. Departure from Hardy-Weinberg equilibrium (HWE) was assessed with the chi-square test (χ^2), and a p-value below 0.05 was considered statistically significant, indicating a potential deviation from HWE in the population. Reproductive data were analysed statistically and are presented as mean ± SD. Associations between SNP markers and the first four phenotypes (CI, GL, A2C, and AFC) were evaluated using Poisson regression, while logistic regression was applied to the last two traits (TW and AB). In all analyses, the glm function in R was used. To control the error rate, we applied the Bonferroni correction on the resulting p-values, calculating the adjusted significance threshold.

The study design, sample collection, and all experimental procedures were reviewed and approved by the Ethics Committee of the Research and Development Station for Bovine. All research activities conducted in this study complied with the European Union Directive 2010/63/EU regarding the protection of animals used for scientific purposes.

Table 1. Genes associated with reproductive traits

Gene ID	Trait	Bibliography
GC, COX17, ILDR1, KALRN, SIPA1L3, IGF2BP2, UBXN7	pregnancy rate and cow conception rate	Jiang et al., 2019
CRAMP1L, PTX4, TELO2	calving ease	Frischknecht et al., 2017
AFF1, TSPAN4, NPAS1, SLC25A48, IL9, SPOCK1, B9D1	heifer conception rate	Jiang et al., 2019
ACOXL, RAB11FIP5, SFXN5, BMP6, RREB1, CREB1, FZD5, PLEKHM3, IDH1, PIKFYVE, MAP2, FOXO6	age at first calving	Sbardella et al., 2021
EPRS	age at first service	Zhou et al., 2019
ZBTB20, BTBD11, SLC4A4, PPFIBP1	pregnancy rate	Jiang et al., 2019
GAS2L1, ASCC2	interval from first to last insemination in heifers	Frischknecht et al., 2017
ACAP2	cow conception rate	Jiang et al., 2019
FAM181A, ASB2, SLC24A4, NKX2-1, FAM188A / MINDY3, MRC1, ABCC9,	calving to first service interval	Jiang et al., 2019
CABIN1, IGLL1	non-return rate in cows	Frischknecht et al., 2017
PYGM, PLCB3	days to first service	Frischknecht et al., 2017
ATP10A, MAGEL2	gestation length and calving ease	Frischknecht et al., 2017
ADRA1B, FABP6, TANC2	Calving-difficulty	Keogh et al., 2021
TUBD1	mortality rates	Frischknecht et al., 2017
SPRY1	gestation length	Zhou et al., 2019
CPNE1, SPAG4, CTNNBL1	gestation length	Frischknecht et al., 2017
CREB1, FZD5, PLEKHM3, IDH1, PIKFYVE, MAP2, LOXL2, STC1, PELI2, TMEM260, OTX2, RNF144A, CMPK2	reproductive tract scores	Stegemiller et al., 2021
MSTN	calving interval	Keogh et al., 2021
ACAT2, APBB1, AP3B1, BCAS1, BSP3, CAST, CD14, CD40, CSNK1E, FCER1G, FSHR, FUT1, FYB, GCNT3, HSD17B12, HSD17B7, IBSP, LDB3, OCLN, PCCB, PMM2, RABEP2, TBC1D24	fertility traits	Ortega, 2018
NUF2, RGS5, RGS4, HSD17B7, DDR2, UAP1, UHMK1, FYN, TRAF3IP2, REV3L, SLC16A10, CDK19, AMD1, SLC4A1AP, SUPT7L, GPN1, ZNF512, GCKR, FNDC4, IFT172, KRTCAP3, NRBP1, PPM1G, ZNF513, SNX17, EIF2B4, GTF3C2, MPV17, UCN, TRIM54, SLC30A3, ATRAID, SLC5A6, C16H1orf174, DFFB, CEP104, LRRC47, SMIM1, CCDC27, TP73, WRAP73, TPRG1L, MEGF6, ARHGEF16, TTC28, CHEK2, HSCB, CCDC117, XBP1, ZNRF3, LOC615587, KREMEN1, EMID1, RHBDD3, EWSR1, GAS2L1, RASL10A, AP1B1, NEFH, THOC5, NIPSNAP1	number of calving	Melo et al., 2017

RESULTS AND DISCUSSIONS

In this work, we performed a targeted candidate-gene association study to identify genetic variants linked with reproductive traits in cattle. The statistical analysis employed a two-model approach to account for the specific distributional properties of the reproductive phenotypes under investigation. Traits including CI, GL, A2C and AFC were analysed using Poisson regression. Conversely, the binary outcomes of TW and AB were examined through logistic regression. Following the quality control pipeline for genotypic data, a final set of 232 SNPs was utilized for statistical analysis. Furthermore, a number of 446 animals remained after quality control, of which 415 were Romanian Spotted and 31 Romanian Brown. Due to the small number of Romanian Brown individuals left, we decided to eliminate

them from the analysis due to concerns regarding low statistical power. In conclusion, we kept a total number of 415 Romanian Spotted individuals with phenotypic and genotypic records.

For the binary traits, TW and AB, we report the numbers of individuals in the two respective classes. For TW, there were 391 individuals that never had a twin and 23 individuals that had at least one twinning event until the study was performed. Similarly, for AB, 336 individuals had zero abortions, and 79 animals had at least one occurrence of abortion. The descriptive statistics for CI, GL, AFC and A2C are reported in Table 2. On average, results for reproductive traits in 415 cows showed a calving interval of 397.27 ± 52.58 days, gestation length 284.69 ± 9.04 days, age at first calving 875.48 ± 68.80 days, and the number of artificial inseminations per conception 1.65 ± 0.88.

Table 2. Descriptive statistics for reproductive traits in Romanian Spotted cattle

Trait	Mean ± SEM	Min.	Max.
CI (days)	397.27 ± 52.58	361	514
GL (days)	284.69 ± 9.04	272	306
AFC (days)	875.48 ± 68.80	752	1037
A2C (no)	1.65 ± 0.88	1	5

Calving interval (CI), Gestation length (GL), Age at first calving (AFC), Artificial inseminations to conception (A2C).

Those results reveal a reproductive profile that aligns closely with reproductive performance for Romanian dual-purpose cattle. The mean calving interval of 397.27 ± 52.58 days is consistent with recent findings for Romanian Brown cattle, which reported an average CI of 411±38.8 days (Neamt, 2021). While these results exceed the ideal biological threshold of 365 days recommended for optimal farm efficiency, they fall within the common range for Romanian Spotted (Simmental type), which typically fluctuates up to 429 days depending on the lactation stage (Cighi et al., 2021). In contrast to the variability observed in calving intervals, the mean GL of 284.69 ± 9.04 days demonstrates remarkable physiological stability. This value is nearly identical to the average recorded in Romanian Spotted populations, where GL showed values of 283.61 and 283.24 days, respectively (Potra et al., 2020). The mean AFC of 875.48 ± 68.80 days (approximately 29 months) characterizes the studied population as semi-precocious. This finding is closely aligned with results obtained for Romanian Spotted cows, which noted an average AFC of 29 months and 14 days (Mihuta et al., 2022). The observed mean of 1.65 ± 0.88 artificial inseminations per conception (A2C) suggests good reproductive efficiency for the Romanian Spotted cattle in this study. These results demonstrate improved fertility compared to the findings of Toledo-Alvarado et al. (2017), who reported a higher mean A2C of 1.82±1.10 for Simmental cattle (Toledo-Alvarado et al., 2017). Collectively, these descriptive statistics underscore the dual-purpose nature of the Romanian Spotted breed, which prioritizes environmental resilience over the reproductive speed of specialized dairy breeds. These findings emphasize that genomic selection focusing on markers for earlier maturation and shorter post-partum intervals could significantly bridge the gap between current performance and optimal economic thresholds for the Romanian cattle sector.

The results of the Poisson regression analysis provided valuable information about how different genotypes influence the reproductive traits. Significant genomic associations were only detected for CI and AFC, suggesting that these traits possess a more discernible genetic architecture within the studied population. In total, 41 SNPs showed significant association with CI (Table 3) and 13 SNPs with AFC (Table 4). These SNPs map to various chromosomes and genes.

In Table 3 we include the most significant associations for each SNP identified for CI analysis. Each row represents the genotype with the lowest p-value and its associated coefficient, indicating the effect on the expected interval duration.

Table 3. Significant SNPs associated with Calving Interval (CI) in Romanian Spotted cattle breed

SNP ID	dbSNP ID	Gene ID	Chr	A1	A2	Genotype	P-value	Coefficient
AX_106758358	rs110865913	*SPRY1*	17	A	G	GG	1.11×10^{-15}	0.1132
AX_124377040	---	*ILDR1*	1	G	A	GG	1.28×10^{-14}	-0.1350
AX_117080166	rs109148864	*SLC24A4*	21	A	G	GG	1.87×10^{-13}	-0.0691
AX_124379423	---	*TANC2*	19	A	G	GG	1.18×10^{-13}	-0.0514
AX_106734258	rs109942542	*LOXL2*	8	T	C	TT	3.01×10^{-12}	0.0794
AX_117085492	rs110034825	*SLC24A4*	21	G	T	TT	2.03×10^{-12}	-0.0449
AX_117081263	rs132661844	*SLC24A4*	21	T	C	CC	4.58×10^{-11}	0.0423
AX_117083489	rs109820230	*SLC24A4*	21	G	A	AA	5.58×10^{-11}	0.0423
AX_117090447	rs133058937	*LOC615587*	17	G	A	AA	7.79×10^{-11}	0.0568
AX_106724830	rs41627459	*BCAS1*	13	A	G	GG	3.73×10^{-11}	-0.0420
AX_106727425	---	*ACOXL*	11	T	C	TT	3.51×10^{-11}	0.0736
AX_106740902	---	*KALRN*	1	T	C	TT	1.43×10^{-10}	0.0878
AX_117083000	rs135194517	*SLC24A4*	21	C	A	AA	3.89×10^{-10}	0.0401
AX_106752630	rs110125976	*UBXN7*	1	C	G	GG	5.06×10^{-10}	-0.0997
AX_106730474	rs29019798	*KALRN*	1	T	C	TT	2.59×10^{-10}	-0.0509
AX_115107195	---	*MAP2*	2	A	G	GG	1.02×10^{-9}	0.0396
AX_115111653	rs42889182	*IGF2BP2*	1	C	A	AA	2.96×10^{-9}	-0.0417
AX_117079758	rs133863748	*SLC24A4*	21	C	T	TT	2.18×10^{-9}	-0.0392
AX_117090789	rs110763504	*SLC24A4*	21	C	T	TT	1.97×10^{-9}	-0.0392
AX_117090924	rs109893119	*SLC24A4*	21	T	C	CC	1.71×10^{-9}	0.0392
AX_117085161	rs135732284	*SLC24A4*	21	T	G	GG	1.35×10^{-9}	0.0392
AX_168294373	rs132677621	*SLC24A4*	21	C	T	TT	1.26×10^{-9}	0.0526
AX_169407036	rs109249576	*SLC24A4*	21	A	G	GG	1.92×10^{-9}	-0.0392
AX_169408636	rs135589268	*SLC24A4*	21	A	G	GG	1.07×10^{-9}	0.0683
AX_106731399	rs108944935	*SLC24A4*	21	A	G	GG	1.97×10^{-9}	-0.0392
AX_106734241	---	*SLC4A4*	6	T	C	TT	2.29×10^{-8}	0.0977
AX_106739150	rs110404266	*DDR2*	3	C	T	TT	7.85×10^{-8}	-0.0349
AX_106743037	rs110704402	*ASB2*	21	A	G	GG	8.05×10^{-8}	-0.0552
AX_169504949	rs109703871	*SLC24A4*	21	C	T	TT	1.72×10^{-8}	-0.0370
AX_117085844	rs137491953	*SLC24A4*	21	A	G	GG	7.93×10^{-8}	0.0467
AX_117116602	rs134798153	*SLC24A4*	21	A	G	GG	1.13×10^{-8}	-0.0376
AX_117081043	rs109292204	*SLC24A4*	21	A	G	GG	1.14×10^{-8}	-0.0376
AX_117083128	rs110293188	*SLC24A4*	21	G	A	GG	1.51×10^{-8}	0.0531
AX_117086508	rs43657798	*ACOXL*	11	A	G	GG	1.61×10^{-8}	-0.0468
AX_117084106	rs136185842	*SLC24A4*	21	G	A	GG	1.79×10^{-7}	-0.0466
AX_117086824	rs29010907	*CEP104*	16	A	G	GG	1.83×10^{-7}	-0.0373
AX_106749545	rs42287687	*TTC28*	17	G	A	GG	2.19×10^{-7}	0.0497
AX_115099183	rs109516473	*BTBD11*	5	A	G	GG	1.76×10^{-7}	-0.0337
AX_185114987	rs109154186	*ZBTB20*	1	G	A	AA	1.33×10^{-7}	0.0338
AX_106743349	rs43244044	*ILDR1*	1	C	T	CC	3.99×10^{-6}	0.1540

Each row shows the SNP ID (Axiom BovMD and dbSNP ID), annotated gene (Gene ID), chromosome (Chr), alleles (A1/A2), the genotype with the most significant p-value, the p-value (P), and the Poisson regression coefficient.

The analysis of CI data revealed a significant background of genetic variants distributed across several bovine chromosomes (BTA). In total, 41 SNPs were significant at the chromosome-wide level for CI, distributed on BTA 1, 2, 3, 5, 6, 8, 11, 13, 16, 17, 19 and 21. The most significant and densely populated cluster of associations for CI was found on chromosome 21, specifically within the *SLC24A4* gene. This gene encodes a potassium-dependent sodium/calcium exchanger, which is involved in calcium homeostasis (Nayeri et al., 2016). In our study, 18 SNPs from the *SLC24A4* gene showed robust associations with CI. In different genomic studies, several variants of the *SLC24A4* gene were associated with reproduction, production, body conformation and milk urea concentration traits in cattle (Velayudhan et al., 2023; Nayeri et al., 2016; Maiorano et al., 2018; Yan et al., 2020; Ariyarathne et al., 2021). The presence of numerous significant markers in this region across different studies suggests that ion homeostasis in the reproductive tract may be an important determinant of fertility in cattle.

The most significant association ($p = 1.11 \times 10^{-15}$) with a positive coefficient of 0.1132 was found for AX_106758358 SNP (rs110865913) in the *SPRY1* gene. This gene plays a significant role in male fertility and testicular health,

particularly in protecting the integrity of the blood-testis barrier and preventing excessive inflammatory responses in the testis (Zhang et al., 2023). A previous genetic study conducted to dissect the genetic architecture related to reproductive traits in dual-purpose cattle has identified significant SNPs near the *SPRY1* gene on BTA 17 associated with gestation length (Zhou et al., 2019). By extension, variants of *SPRY1* may influence the inflammatory environment of the bovine uterus postpartum. The positive regression coefficient for the GG genotype of rs110865913 in the *SPRY1* gene suggests that this specific variant is associated with a slower return to reproductive function, potentially through suboptimal modulation of the inflammatory cascade. Integrating these findings with previous research, our results suggest that while the SPRY1 gene may not be the primary driver of the calving interval trait, it is part of the broader genomic landscape that influences calving intervals and overall reproductive efficiency.

For BTA 1, the most significant associations were identified within the *ILDR1* and *KALRN* genes. SNP AX_106740902, located on the *KALRN* gene, showed a highly significant association ($p = 1.43 \times 10^{-10}$) with a positive coefficient of 0.0878, indicating that certain genotypes at this position are linked to a prolongation of the calving interval. *KALRN* is known for its role in embryo development and has been consistently associated with pregnancy rates and conception status in U.S. Holstein populations (Yang et al., 2025). The identification of *KALRN* in the Romanian Spotted population may suggest that genetic defects in early cytoskeletal organization of the embryo may contribute to early embryonic loss, which clinically manifests as an extended calving interval.

On chromosome 13, the *BCAS1* gene was identified as a candidate locus through SNP AX_106724830. The GG genotype at this locus showed a significant reduction in the expected calving interval ($p = 3.73 \times 10^{-11}$, coefficient = -0.0420), suggesting its potential as a marker for improved reproductive efficiency. *BCAS1* has been implicated in genomic studies of bovine reproduction and was associated with fertility across different cattle populations (Ortega et al., 2017). The CI analysis identified other significant associations, involving *ZBTB20, IGF2BP2, UBXN7, MAP2, DDR2, BTBD11, SLC4A4, LOXL2, ACOXL, CEP104, TTC28, LOC615587, TANC2*, and *ASB2* genes, suggesting that the genetic architecture of CI in Romanian cattle is influenced by large-effect variants.

The results of significant genomic associations for AFC are presented in Table 4. The genetic architecture of AFC in Romanian dual-purpose breeds also exhibited significant SNP markers, where thirteen SNPs reached significance. Each entry details the genotype most significantly associated with the trait and the corresponding coefficient derived from the Poisson regression.

Table 4. Significant SNPs associated with age at first calving (AFC) in Romanian Spotted cattle breed

SNP ID	dbSNP ID	Gene ID	Chr	A1	A2	Genotype	P-value	Coefficient
AX_26649835	rs110405210	*SLC4A4*	6	G	A	AG	1.39×10^{-11}	0.0324
AX_106753905	---	*ACOXL*	11	G	T	TT	3.12×10^{-11}	0.0299
AX_106735416	---	*ABCC9*	5	T	C	TT	6.52×10^{-10}	-0.0494
AX_106739150	rs110404266	*DDR2*	3	C	T	CC	6.01×10^{-9}	-0.0387
AX_106746160	---	*TANC2*	19	T	C	TT	1.23×10^{-9}	0.0313
AX_106752999	rs41578409	*ABCC9*	5	A	G	AA	1.47×10^{-9}	0.0469
AX_106733878	---	*IBSP*	6	T	C	CT	7.98×10^{-8}	0.0316
AX_106757386	rs43480825	*AFF1*	6	T	C	TT	2.10×10^{-8}	0.1094
AX_117082950	rs109635380	*PELI2*	10	A	C	CC	2.67×10^{-8}	-0.0344
AX_124381692	rs42286695	*TTC28*	17	T	C	TT	2.24×10^{-8}	-0.0328
AX_169413127	rs133717146	*APBB1*	15	A	G	GG	3.37×10^{-8}	-0.0330
AX_106727919	rs41619788	*SLC4A4*	6	G	T	TT	3.03×10^{-7}	-0.0219
AX_115114961	rs109665442	*IGLL1*	17	T	C	CC	1.99×10^{-7}	0.0585

Each row shows the SNP ID (Axiom BovMD and dbSNP ID), annotated gene (Gene ID), chromosome (Chr), alleles (A1/A2), the genotype with the most significant p-value, the p-value (P), and the logistic regression coefficient.

The top AFC SNP was AX_26649835 in *SLC4A4* (chr6, P = 1.394×10^{-11}). Other notable associations include AX_106753905 (*ACOXL*) and two SNPs in ABCC9 (chr5): AX_106735416 and AX_106752999.

The most significant SNPs highlighted in our study was observed on BTA 6 in *SLC4A4* gene. SNP AX_26649835 (rs110405210), associated with *SLC4A4*, showed high significance (p = 1.39×10^{-11}) with a positive coefficient, reflecting an increased age at first calving for the AG genotype. The SLC4A4 locus is a well-known QTL for pregnancy rates in Holstein cows (Liang et al., 2023). The *ACOXL* gene on BTA 11, represented by SNP AX_106753905, also showed a significant association (p = 3.12×10^{-11}) with a positive coefficient on AFC (0.0299) for the TT genotype. This gene was previously found in the regions associated with AFC (Sbardella et al., 2021) and has also been identified in association with pregnancy outcome in Brahman heifers (Porto-Neto et al., 2015).

The *ABCC9* gene on BTA 5 emerged as a candidate locus for AFC. SNP AX_106735416 within this gene demonstrated a significant association (p = 6.52×10^{-10}) where the TT genotype indicating association with shorter AFC (negative coefficient = –0.0494). The *ABCC9* gene is involved in ATP-sensitive potassium channels and has been linked in previously study with fat yield in Holstein cattle (Jiang et al., 2019).

Additional significant SNPs were identified in genes with diverse biological functions, including *DDR2, TANC2, ABCC9, IBSP, AFF1, PELI2, TTC28, APBB1, SLC4A4* and *IGLL1*, highlighting the complex genetic basis of AFC.

The genomic associations identified in this study point toward several biological pathways that likely coordinate reproductive efficiency in dual-purpose cattle. A consistent pattern observed in the results is the prominent involvement of the Solute Carrier (SLC) gene family, particularly members of the SLC24 and SLC4 families, in modulating bovine reproductive efficiency. The *SLC24A4* gene, which exhibited multiple significant markers on BTA21 for CI, encodes a member of potassium-dependent sodium/calcium exchanger protein family (Nayeri et al., 2016), which is fundamental to cellular ion homeostasis. In mammalian reproduction, calcium is an important signalling molecule, and intracellular Ca^{2+} oscillations are a key signalling mechanism during oocyte maturation, activation, and fertilization (Chen et al., 2024). Given this central role of Ca^{2+} signalling, genetic variation in *SLC24A4* may influence the success of fertilization or the timing of the return to cyclicity postpartum. Similarly, the *SLC4A4* gene on BTA6, associated with both CI and AFC, encodes a bicarbonate cotransporter that plays a key role in regulating pH in the reproductive tract and oviductal fluid. Proper bicarbonate balance is essential for maintaining the microenvironment that supports sperm viability and development of the embryo in cattle (Lane & Gardner, 2000). Therefore, variation in *SLC4A4* could influence fertility traits by altering pH homeostasis during these critical reproductive stages. In this study, the genetic architecture of AFC was dominated by a signal on BTA6 involving the *SLC4A4* gene. For Romanian Spotted heifers, the AG genotype of SNP AX_26649835 was associated with a positive coefficient, indicating a delayed age at first calving. This implies that genomic differences in the homeostatic control of the oviductal environment might influence the success of the heifer's first breeding attempts, thereby determining the age at which it officially enters the productive milking herd.

CONCLUSIONS

The current targeted candidate-gene association study identified a series of genomic markers significantly linked to calving interval and age at first calving in Romanian Spotted cattle. Traits such as twinning, abortions, artificial inseminations to conception and gestation length did not yield significant signals. The recurrent prominence of genes including *SLC24A4*, *SPRY1*, and *SLC4A4* highlights that reproductive traits such as calving interval and age at first calving are controlled by fundamental biochemical regulators governing oocyte activation and uterine microenvironmental dynamics. However, the scientific utility of these results is currently constrained by technical limitations in sample size. Future research will be required, prioritizing the use of whole-genome sequence

WGS imputation to ensure that the identified SNPs are in high linkage disequilibrium with the true causal variants.

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