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Genome-wide identification and functional characterization of hyperactivated β -recombinase RNA-guided recombinase target sites in the bovine genome

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Abstract

Background: RNA-guided recombinase (RGR) systems have emerged as promising alternatives to nuclease-based genome editing by enabling precise DNA integration without inducing double-strand breaks. Among engineered recombinases, hyperactivated β -recombinase possesses a degenerate recognition motif that offers expanded genome-wide targetability. However, the distribution and biological significance of β -recombinase-compatible target sites in the bovine genome remain largely unexplored.

Methods: A genome-wide computational analysis was performed using the *Bos taurus* reference genome to identify genomic loci compatible with the recognition motif of hyperactivated β -recombinase. Candidate target sites were identified using the EMBOSS dreg program with exact pattern matching and subsequently validated through BLAST alignment against the *Bos taurus* RefSeq genome. Validated loci were annotated according to their genomic location, and associated genes were examined to evaluate their biological relevance and potential applications in livestock genome engineering.

Results: A total of 367 RGR target sites were identified across the bovine genome and distributed throughout all autosomes and the X chromosome. Chromosome 2 contained the highest number of target sites (32), whereas chromosome 29 contained the fewest (5). Among the validated loci, 158 (43.05%) were located within annotated genes and 209 (56.95%) within intergenic regions. Several target sites occurred in genes with important biological functions, including *TG*, *KIF23*, *DOK6*, *PLCL1*, *ATP11B*, *CAPNS1*, *IL6R*, *UCK2*, *NCR1*, and *FRYL*, which are involved in metabolism, cellular signalling, immune regulation, development, and production-related traits. The broad genomic distribution of β -recombinase recognition motifs indicates substantial genome-wide accessibility for programmable recombinase-mediated DNA integration.

Conclusions: This study presents the first comprehensive genome-wide map of hyperactivated β -recombinase RGR target sites in the bovine genome and demonstrates their widespread distribution across coding and non-coding genomic regions. The identification of biologically relevant target loci provides a valuable resource for the development of recombinase-mediated genome engineering strategies in cattle. These findings establish a foundation for future experimental validation and support the potential application of RNA-guided recombinase systems in precision livestock breeding, functional genomics, and sustainable genetic improvement.

Keywords: *Bos taurus*, RNA-guided recombinase, β -recombinase, genome editing, recombinase target sites, computational genomics, precision livestock breeding, functional genomics

1. Introduction

The advent of genome editing technologies has revolutionized genetic improvement in livestock by enabling precise and targeted modification of complex genomes such as that of cattle (Popova *et al.* 2023) [3]. Unlike conventional breeding approaches, which rely on phenotypic selection over multiple generations and are constrained by existing genetic variability, genome editing facilitates direct manipulation of specific genomic loci within a single generation. This significantly accelerates genetic gain and enhances the efficiency of trait introgression (Mueller and Van Eenennaam 2022; Wang *et al.* 2025) [4, 5]. These advancements are particularly important in addressing global challenges in livestock production, including climate change, emerging diseases, and the increasing demand for animal-derived food products (Wood *et al.* 2024) [6]. For instance, improving resistance to heat stress, bovine tuberculosis, and parasitic diseases such as trypanosomiasis is critical for sustainable livestock production, particularly in developing regions. The urgency for improving livestock productivity is further emphasized by the projected global population growth to approximately 11.2 billion by 2100, which is expected to increase

demand for milk and meat by 63% and 76%, respectively (Tait-Burkard *et al.* 2018) [7]. Meeting these demands requires the integration of advanced biotechnological interventions capable of enhancing productivity, resilience, and efficiency in livestock systems. Genome editing technologies therefore represent a key strategy for achieving global food security and economic sustainability in animal agriculture (Popova *et al.* 2023) [3]. However, despite their transformative potential, both conventional breeding and current genome editing approaches face limitations in efficiently addressing complex polygenic traits and rapidly evolving disease challenges (Fischer and Schnieke 2023) [8].

Most widely used genome editing platforms, including CRISPR-Cas9, Zinc Finger Nucleases (ZFNs), and Transcription Activator-Like Effector Nucleases (TALENs), rely on the induction of DNA double-strand breaks (DSBs), which are subsequently repaired by endogenous cellular mechanisms such as non-homologous end joining (NHEJ) or homology-directed repair (HDR) (Sander and Joung 2014; Brookhouser *et al.* 2017) [9, 10]. Although these technologies have significantly improved the efficiency and accessibility of targeted genome modification, their dependence on DSB-mediated repair introduces several limitations, including unpredictable insertions or deletions, off-target effects, genomic instability, and cytotoxicity (Gabriel *et al.* 2011; Fu *et al.* 2013; Ihry *et al.* 2018; Haapaniemi *et al.* 2018) [11-14]. Furthermore, the efficiency of HDR is often low and highly variable across cell types, thereby limiting precise genome editing outcomes.

In contrast, site-specific recombinases have emerged as a promising alternative for genome engineering, enabling precise DNA modification without reliance on DSB-induced repair pathways. These enzymes catalyze DNA strand exchange through coordinated cleavage and religation reactions without requiring high-energy cofactors or inducing extensive DNA damage responses (Grindley *et al.* 2006) [15]. Recombinases are broadly classified into two major families: tyrosine recombinases, such as Cre, FLP, and λ integrase (Grainge and Jayaram 1999) [16], and serine recombinases, including Hin invertase, Sin resolvase, and Gin invertase (Smith and Thorpe 2002) [17]. These systems have been widely applied for site-specific DNA integration and excision in various biological systems (Turan *et al.* 2011; Gaj *et al.* 2013) [1, 18].

Despite their high precision, the broader applicability of recombinases is constrained by their strict sequence specificity, as they typically recognize fixed recombination sites. Consequently, recombination events are limited to endogenous pseudo-sites or artificially introduced recognition sequences, restricting their programmability and genome-wide applicability (Buchholz *et al.* 2001) [19]; Thyagarajan *et al.* 2001; Karpinski *et al.* 2016) [20]. To overcome this limitation, engineered recombinases fused with customizable DNA-binding domains such as zinc finger proteins and transcription activator-like effectors have been developed (Akopian *et al.* 2003; Gersbach *et al.* 2011; Mercer *et al.* 2012) [2, 21, 22]. However, these approaches remain technically complex and often exhibit reduced efficiency due to challenges associated with protein engineering and domain compatibility.

Recent advances in genome engineering have led to the development of RNA-guided recombinase (RGR) systems,

which integrate the targeting specificity of CRISPR-Cas systems with the catalytic activity of recombinases. In these systems, catalytically inactive Cas9 (dCas9) is fused with recombinase domains to enable programmable DNA recombination without inducing double-strand breaks. Notable examples include recombinase Cas9 (recCas9), generated by fusion of dCas9 with hyperactivated Gin invertase Chaikind *et al.* 2016) [23], and integrase Cas9 (iCas9), which incorporates the catalytic domain of Tn3 resolvase (Standage-Beier *et al.* 2019) [24]. Although these systems represent a significant advancement, their broader application is limited by the availability of efficient recombinases and incomplete understanding of genome-wide targeting potential.

Among recombinase proteins, β recombinase of the λ -Red system represents a promising yet underexplored candidate for next-generation genome editing. β recombinase promotes homologous recombination by facilitating the annealing of single-stranded DNA to complementary genomic sequences, operating independently of double-strand break formation (Court *et al.* 2002) [25]. Its ability to function with short homology arms and compatibility with recombinase approaches make it a powerful tool for precise genome modification (Mosberg *et al.* 2010) [26]. Importantly, hyperactivated variants of β recombinase exhibit degenerate recognition sequences, suggesting expanded genome-wide targetability relative to conventional recombinases (Sirk *et al.* 2014) [27]. In RNA-guided recombinase platforms utilizing β recombinase, target site selection is simplified by modification of the central pseudo-recognition sequence. The consensus recognition motif of hyperactivated β recombinase is NNNVNNRGTWWACYNNBNNN, where N=A / T / C / G, V = A/C/G, B=T / C / G, R = A/G, Y = T/C, and W = A/T (Sirk *et al.* 2014; Chaikind *et al.* 2016) [23, 27]. This degenerate motif provides substantial flexibility for genome-wide targeting and highlights the potential of β recombinase for programmable genome engineering applications. Despite these advantages, the application of β recombinase within RNA-guided recombinase systems remains poorly characterized, particularly in complex eukaryotic genomes such as cattle. There is a lack of comprehensive studies investigating the distribution, specificity, and functional relevance of β recombinase target sites across the bovine genome. Furthermore, comparative evaluations with established CRISPR-Cas9 systems in terms of efficiency, precision, and genomic stability are limited. Therefore, the present study aims to systematically investigate the genome-wide targetability of hyperactivated β recombinase within an RNA-guided recombinase framework in cattle. Specifically, a comprehensive map of target loci was constructed across the bovine genome using β recombinase with a spacer length of seven, alongside the identification of genes associated with these loci. It is hypothesized that integration of β recombinase with RNA-guided targeting systems will enable efficient, precise, and double-strand break-free genome editing, with expanded target site availability and improved genomic stability compared to conventional nuclease-based approaches.

2. Materials and Methods

2.1 Genome sequence retrieval

The reference genome sequence of *Bos taurus* was retrieved from the Ensembl database using the assembly *Bos_taurus.UMD3.1.dna.chromosome*. Chromosome-level

FASTA files, including autosomes and sex chromosomes, were downloaded and processed individually. Sequence files were standardized to ensure compatibility with downstream analyses.

2.2 RGR target site architecture

The RNA-guided recombinase (RGR) platform was defined based on a composite recognition site comprising a degenerate pseudo-core recombinase sequence, a spacer region, flanking guide RNA (gRNA) binding sites, and protospacer adjacent motif (PAM) sequences. The central component consists of a 20 bp degenerate recognition motif specific to hyperactivated β recombinase.

The generalized architecture of the RGR target site was defined as: CCN(26–27)-NNNVNNRGTWWACYNNBNNN-N(26–27)-GG, where nucleotide degeneracy follows standard IUPAC notation (N = A/T/C/G; V = A/C/G; B = T/C/G; R = A/G; Y = T/C; W = A/T). The central degenerate motif is flanked by variable-length regions corresponding to spacer and gRNA binding sequences, with PAM sequences (CC and GG) positioned at the termini.

2.3. Search pattern design and genome-wide scanning

A search pattern was constructed to identify RGR target sites across the bovine genome, incorporating the degenerate 20 bp recombinase core, a 7 bp spacer, flanking ~25 bp gRNA binding regions, and PAM sequences at both ends. The explicit search expression used for pattern matching was:

```
CC[ATCG][ATCG][ATCG][ATCG][ATCG][ATCG][ATCG]
[ATCG][ATCG][ATCG][ATCG][ATCG][ATCG][ATCG][A
TCG][ATCG][ATCG][ATCG][ATCG][ATCG][ATCG][ATC
G][ATCG][ATCG][ATCG][ATCG][ATCG][ACG][ATCG][A
TCG][AG]GT[AT][AT]AC[TC][ATCG][ATCG][TCG][ATC
G][ATCG][ATCG][ATCG][ATCG][ATCG][ATCG][ATCG][
ATCG][ATCG][ATCG][ATCG][ATCG][ATCG][ATCG][AT
CG]GG
```

Genome-wide identification of candidate RGR target sites was performed using the *dreg* program implemented in the EMBOSS package (version 6.6.0 or later). Searches were conducted on both forward and reverse strands with exact pattern matching (no mismatches allowed). Each chromosome was analyzed independently, and output included genomic coordinates and matched sequences.

2.4. Sequence extraction and alignment

Identified RGR target sequences were extracted and converted into FASTA format using custom parsing scripts. Redundant and overlapping sequences were removed to retain unique loci.

The resulting sequences were aligned against the *Bos taurus* reference genome from the NCBI RefSeq database (taxid: 9913) using the BLAST (BLASTn, version 2.13.0 or later). Alignments were performed using the megablast algorithm with an E-value cutoff of 10^{-10} and default scoring parameters, with low-complexity filtering disabled.

Only sequences exhibiting complete alignment with 100% identity, full query coverage, and no insertions or deletions were retained for downstream analysis.

2.5 Genomic annotation and characterization

Validated RGR target sites were mapped to genomic features using the corresponding *Bos taurus* annotation dataset. Each locus was classified based on its genomic context, including exonic, intronic, and intergenic regions. Associated genes were identified to assess potential functional relevance of the detected target sites.

2.6 Statistical analysis

Descriptive statistical analyses were performed to evaluate the distribution of RGR target sites across the bovine genome. Metrics included total number of identified loci, chromosome-wise distribution, and density of target sites per megabase. The proportion of loci within genic and intergenic regions was also calculated.

All analyses were conducted using standard computational tools within Python and R environments. Results were summarized using frequencies and percentages, and visual representations were generated to illustrate genome-wide distribution patterns.

3. Results

3.1 Genome-wide identification of hyperactivated β -recombinase target sites

A comprehensive genome-wide computational analysis identified 367 RNA-guided recombinase (RGR) target sites compatible with the recognition motif of hyperactivated β -recombinase containing a seven-nucleotide spacer sequence. Candidate target sites were detected across all bovine autosomes and the X chromosome, demonstrating the widespread occurrence of recombinase-compatible genomic loci within the *Bos taurus* genome.

The identified target sites were unevenly distributed among chromosomes. Chromosome exhibited the highest number of RGR target sites (32 sites), followed by chromosomes 3 and 5 (25 sites each). In contrast, chromosome 29 contained the lowest number of identified target sites (5 sites), while the X chromosome harbored 10 target sites. This variation indicates chromosomespecific differences in the occurrence of hyperactivated β -recombinase recognition motifs across the bovine genome (Fig. 1).

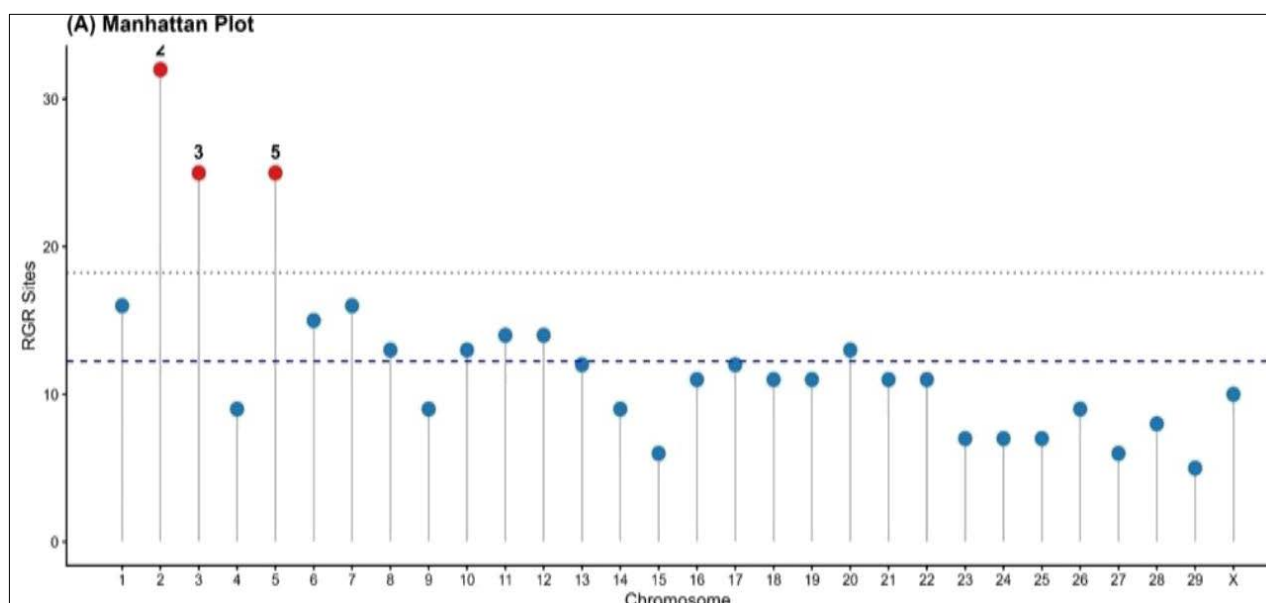


Fig 1: Chromosome-wise distribution of 367 RNA-guided recombinase target sites identified across the bovine genome. The Manhattan plot illustrates the number of target sites detected on each chromosome. Chromosomes 2, 3 and 5 exhibited the highest target-site densities, whereas chromosome 29 showed the lowest density

3.2 Genomic distribution of RGR target sites

Following BLAST validation against the *Bos taurus* RefSeq genome, all identified RGR target sites were classified according to their genomic location. Of the 367 validated target sites, 158 sites (43.05%) were located within annotated genic regions, whereas 209 sites (56.95%) were located in intergenic regions (Fig. 2). The relatively higher proportion of intergenic

target sites suggests the availability of numerous potential genomic loci suitable for targeted DNA integration while minimizing disruption of endogenous coding sequences. Conversely, the substantial number of target sites identified within genes demonstrates that hyperactivated β -recombinase motifs are also distributed among functionally important genomic regions.

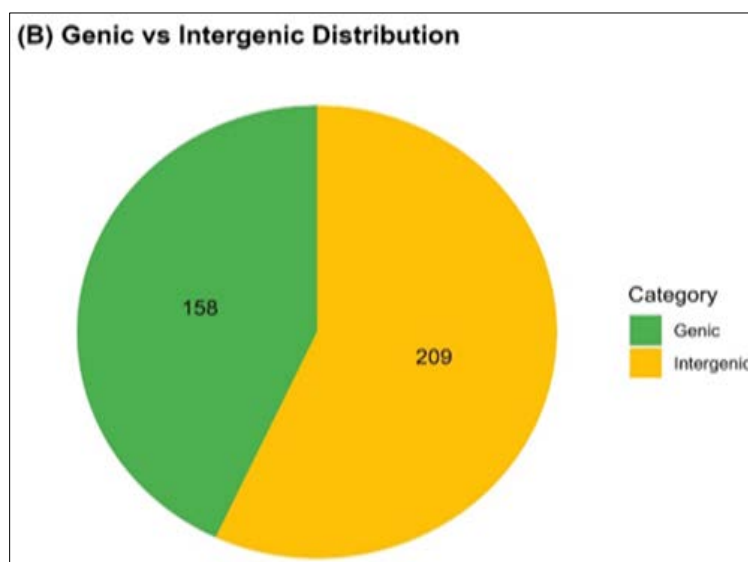


Fig 2: Distribution of validated RNA-guided recombinase target sites across genic and intergenic regions of the bovine genome.

3.3 Chromosome-wise density of RGR target sites

To further evaluate genome-wide distribution patterns, chromosome-specific densities of RGR target sites were visualized using a heatmap (Figure 3). High-density regions were primarily observed on chromosomes 2, and 5, whereas chromosomes 29 and X exhibited comparatively lower densities. The heatmap clearly demonstrated that

hyperactivated β -recombinase recognition motifs are broadly dispersed throughout the bovine genome rather than being restricted to specific chromosomal regions. This widespread distribution substantially expands the number of candidate loci available for future RNA-guided recombinase-mediated genome engineering.

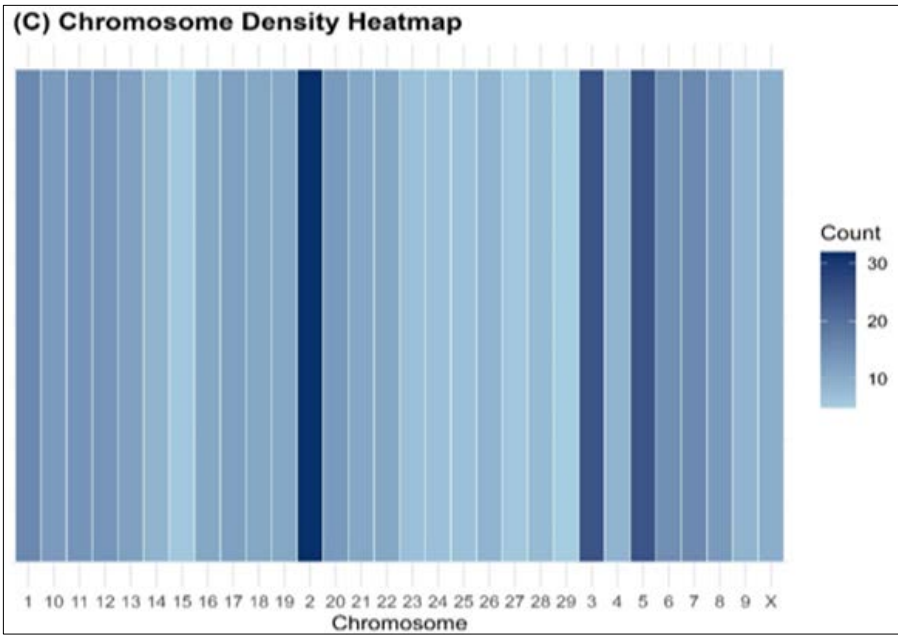
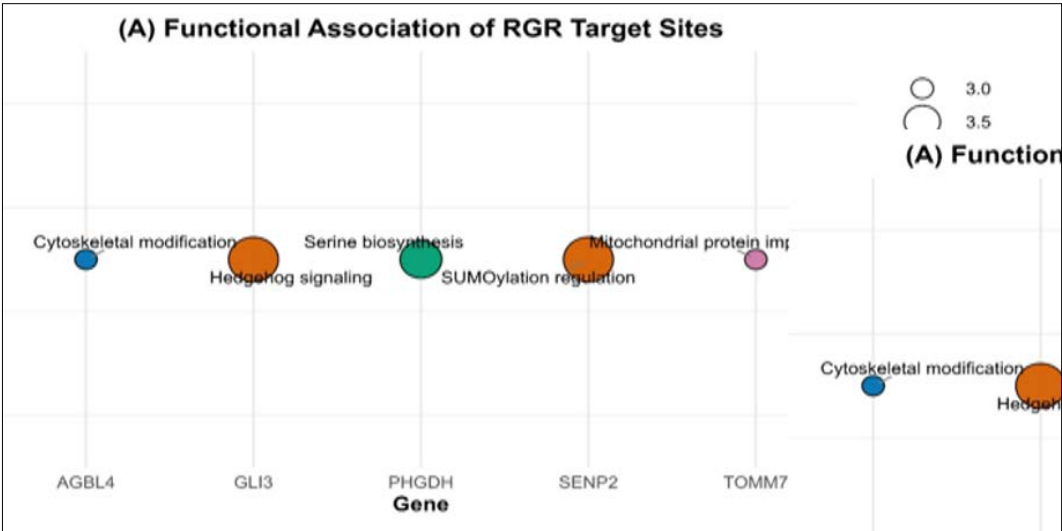


Fig 3: Heatmap illustrating chromosome-wise density of hyperactivated β -recombinase target sites across the bovine genome.

3.4 Functional annotation of genes harboring RGR target sites

Functional characterization of genes containing RGR target sites revealed their association with diverse biological processes, including cellular signaling, metabolism, structural organization, immune regulation, mitochondrial function and cell-cycle regulation (FigureA-B). Representative genes identified during the analysis included *TG* (thyroglobulin), *KIF23*, *PLCL2*, *DOK6*, *UCK2*, *CAPNS2*, *IL6R*, *ATP11B*, *NCR1*, and *FRYL*, each of which performs important physiological functions in cattle. The *TG* gene, identified on chromosome 21, encodes the precursor of thyroid hormones that regulate metabolic activity and influence fat deposition. Previous studies have demonstrated significant associations between polymorphisms within the *TG* gene and economically important meat-quality traits, particularly marbling score.

Similarly, an RGR target site identified within *KIF23* on chromosome 24 is associated with chromosome segregation and spindle organization during mitosis and meiosis, whereas *UCK2* participates in pyrimidine nucleotide metabolism and activation of therapeutic nucleoside analogues. Additional target sites were identified in genes including *DOK6*, involved in neuronal signaling; *PLCL2*, associated with phosphoinositide-mediated intracellular signaling; *ATP11B*, responsible for phospholipid transport and membrane asymmetry; *CAPNS2*, encoding the regulatory subunit of calpain involved in muscle proteolysis and meat tenderness; *IL6R*, an important mediator of cytokine signaling; *NCR1*, involved in natural killer cell-mediated immune responses; and *FRYL*, which contributes to centrosome organization and mitotic spindle formation.



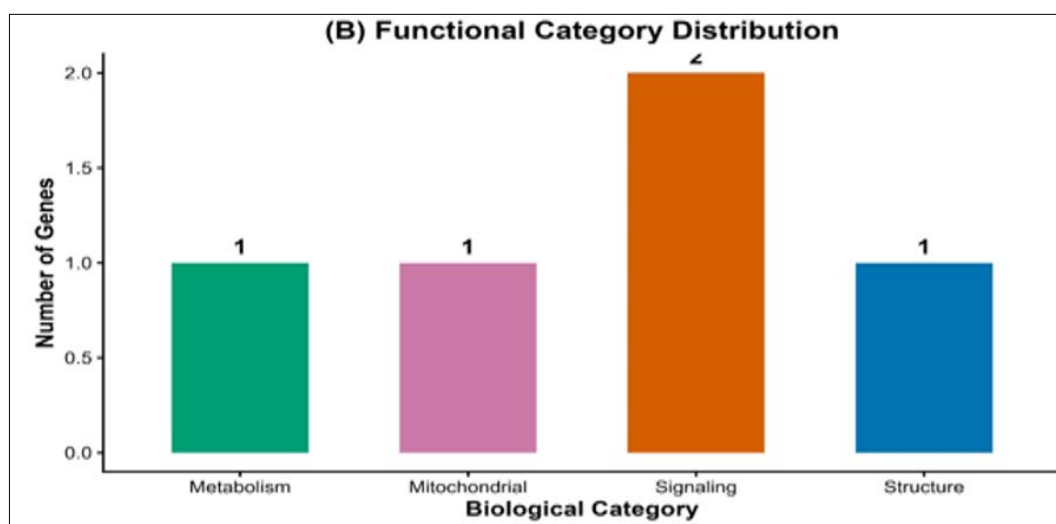


Fig 4: (A-B). Functional classification of representative bovine genes harboring hyperactivated β -recombinase target sites. Bubble size represents relative biological importance, while color intensity indicates functional grouping.

3.5 Genomic targeting capacity of the hyperactivated β -recombinase platform

The genomic targeting capacity of the RNA-guided recombinase platform was estimated based on the observed frequency of target-site occurrence throughout the bovine genome. Using the sevenbase spacer architecture employed in the present study, the platform exhibited an average targeting frequency of approximately one compatible genomic site per 2.7×10^6 nucleotides. Theoretical evaluation further suggested that reducing the spacer length to six or five nucleotides would substantially increase the frequency of target-site occurrence, potentially improving genome-wide targeting capacity by approximately 700-fold and 1000-fold, respectively. These observations indicate that spacer length is an important determinant of genomic target availability and provide valuable guidance for future optimization of RNA-guided recombinase platforms.

4. Discussion

The present study provides the first comprehensive genome-wide map of hyperactivated β -recombinase-compatible RNA-guided recombinase (RGR) target sites in the bovine genome. A total of 367 putative RGR target loci were identified using a degenerate recognition motif specific to hyperactivated β -recombinase and subsequently validated through genomic alignment. These findings demonstrate that recombinase-compatible target sequences are widely distributed throughout the *Bos taurus* genome, thereby supporting the feasibility of programmable recombinase-mediated genome editing in livestock. The broad genomic distribution of these target sites substantially expands the repertoire of potential integration loci that may be exploited for precise DNA insertion without relying on double-strand break (DSB)-mediated repair mechanisms. Current genome editing technologies, including CRISPR/Cas9, Zinc Finger Nucleases (ZFNs), and Transcription Activator-Like Effector Nucleases (TALENs), have transformed animal biotechnology by enabling efficient modification of specific genomic loci. Nevertheless, these systems depend on the induction of DNA double-strand breaks, which are subsequently repaired through non-homologous end joining (NHEJ) or homology-directed repair (HDR). Although highly effective, DSB-dependent editing frequently results in insertion-deletion mutations, chromosomal rearrangements,

off-target cleavage, and variable HDR efficiency, thereby limiting editing precision, particularly in primary cells and post-mitotic tissues (Gabriel *et al.*, 2011; Fu *et al.*, 2013; Haapaniemi *et al.*, 2018; Ihry *et al.*, 2018) [11-14]. Recombinase-based genome engineering represents an attractive alternative because DNA integration occurs through sitespecific strand exchange without extensive DNA damage or dependence on endogenous repair pathways (Grindley *et al.*, 2006; Turan *et al.*, 2013) [15]. The identification of 367 candidate RGR target sites in the bovine genome demonstrates that hyperactivated β -recombinase possesses considerable genome-wide targeting potential. These observations are consistent with the pioneering work of Chaikind *et al.* (2016) [23], who identified approximately 450 compatible target sites for the hyperactivated Gin recombinase in the human genome using the recCas9 platform. Although the total number of target sites differs between the two studies, likely reflecting differences in genome composition, recombinase specificity, recognition motifs, and computational search strategies, both investigations demonstrate that engineered recombinases with degenerate recognition sequences substantially expand the availability of programmable genomic targets compared with conventional site-specific recombinases. Similarly, Sirk *et al.* (2014) [27] reported that hyperactivated recombinase variants exhibit relaxed sequence specificity while maintaining recombination activity, thereby increasing the number of accessible genomic loci for targeted DNA integration.

An important observation of the present study was the distribution of RGR target sites across both coding and non-coding regions of the bovine genome. Of the 367 validated target sites, 158 (43.05%) were located within annotated genes, whereas 209 (56.95%) occurred in intergenic regions. This distribution has important implications for genome engineering. Intergenic target sites may represent suitable genomic locations for targeted insertion of exogenous DNA while minimizing disruption of endogenous coding sequences and gene regulatory elements. Such loci could potentially function as candidate genomic "safe-harbor" sites for stable transgene integration, an important consideration for precision livestock breeding and transgenic animal production. Conversely, the substantial proportion of target sites located within genes indicates that hyperactivated β -recombinase may also enable precise modification of endogenous loci associated with economically

important traits, thereby broadening its potential applications in functional genomics and trait improvement.

The chromosome-wise distribution further demonstrated that RGR target sites were not uniformly represented throughout the bovine genome. Chromosomes 2, 3, and exhibited comparatively higher numbers of target sites, whereas chromosome 29 contained relatively few compatible loci. Although the present study did not investigate the underlying genomic determinants responsible for this variation, chromosome-specific differences in nucleotide composition, local sequence architecture, repetitive DNA content, and distribution of degenerate β -recombinase recognition motifs may collectively contribute to the observed pattern. The widespread occurrence of compatible target sequences across all autosomes and the X chromosome nevertheless suggests that hyperactivated β -recombinase possesses broad genome accessibility, thereby increasing the flexibility of future RNA-guided recombinase-based genome engineering strategies.

Conclusion

The present study provides a comprehensive genome-wide assessment of hyperactivated β -recombinase RNA-guided recombinase (RGR) target sites in the *Bos taurus* genome, establishing a foundational resource for recombinase-mediated genome engineering in cattle. A total of 367 RGR-compatible target sites were identified across the bovine genome, demonstrating the widespread occurrence of degenerate β -recombinase recognition motifs suitable for programmable DNA integration. These target sites were distributed across all autosomes and the X chromosome, with 158 loci located within annotated genes and 209 loci in intergenic regions, indicating substantial opportunities for both targeted gene modification and potential safe-harbor integration.

The identification of RGR target sites within genes associated with metabolism, cellular signaling, immune regulation, development, and production-related traits highlights the potential biological relevance of these loci for precision livestock improvement. The broad genomic accessibility of hyperactivated β -recombinase, together with its ability to facilitate targeted DNA integration without inducing double-strand breaks, supports its potential as a complementary alternative to conventional nuclease-based genome editing systems. In contrast to technologies that rely on DNA cleavage and endogenous repair pathways, the RGR platform offers the prospect of more precise and predictable genome modifications while minimizing the risks associated with off-target mutations and genomic instability.

Collectively, the genome-wide map generated in this study provides a valuable reference for future development of RNA-guided recombinase-based genome editing strategies in cattle. The identified target loci may facilitate the precise introduction of desirable genetic variants associated with economically important traits, disease resistance, reproductive performance, and environmental adaptation. Future experimental validation of these computationally identified target sites will further establish the utility of hyperactivated β -recombinase as a next-generation genome engineering tool for functional genomics, precision breeding, and sustainable livestock improvement.

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Authors contributions

A.C. and A.A.S. conceived the design of this study. S.S.L., A.S., and M.T. participated in data collection. S.S.L., M.R., J.M.M. and A.C. participated in data analysis and manuscript writing. All authors contributed to the revision and review of the manuscript before submission.

Data availability

All the datasets generated in this study are available with the corresponding author with reasonable request.

Declarations

Conflict of interest: The authors declare no competing interests.

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