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Research Article

Novel Genes and Genetic Variants Associated with Production Traits in Australorp Chickens

Abdul Rehman[1], Nauman Khan[2], M Khuzema Niaz[3], Ali Mujtaba Shah[4], Javed Zafar[5], Fasih Ur Rehman[6], Naseer Ahmad[2], Kassahun Bekana[7], Muhammad Mushahid[8], Muhammad Talal[5], and Umar Aziz[2*]

[1]Department of Animal Sciences, Faculty of Animal Production and Technology, Cholistan University of Veterinary and Animal Sciences, Punjab, Pakistan
[2] Department of Animal Breeding, Genetics and Reproduction, College of Animal Science and Technology, Northwest A&F University, Yangling, China
[3] Key Laboratory of Animal Breeding, Reproduction, and Molecular Design for Jiangsu Province, College of Animal Science and Technology, Yangzhou University, Yangzhou, China
[4] Guangdong Provincial Key Laboratory of Animal Nutrition and Regulation, College of Animal Science, Guangzhou, China
[5] Department of Livestock and Poultry Production, Faculty of Veterinary Sciences, Bahauddin Zakariya University, Multan, Pakistan
[6] Department of Parasitology, University of Veterinary and Animal Sciences, Lahore, Pakistan
[7] Department of Animal Science, Ambo University, Guder Mamo Mezemir Campus, Ethiopia
[8] Institute of Animal and Dairy Sciences, University of Agriculture Faisalabad, Constituent College, Toba Tek Singh, Pakistan

*** *Corresponding author:*** Umar Aziz, Department of Animal Breeding, Genetics and Reproduction, College of Animal Science and Technology, Northwest A&F University, Yangling District, Xianyang, Shaanxi, China. Email: umaraziz@nwafu.edu.cn

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ABSTRACT

Introduction: The Australorp chicken, known for its exceptional egg production and adaptability, is a valuable genetic resource for the poultry industry. However, the molecular basis underlying their distinctive traits remains poorly understood. The present study aimed to identify novel genes and genetic variants associated with key production traits in Australorp chickens by performing a comprehensive comparative genomic analysis combined with an *in silico* genome-wide association study (GWAS).

Materials and methods: Whole-genome sequencing data from 12 Australorp chickens were compared with data from four other breeds, including ten Rhode Island Red, eight Leghorn, ten Plymouth Rock, and six Red Jungle Fowl. Quality control and preprocessing were applied to ensure high-quality genomic data for downstream analyses. Comparative genomic analysis revealed several breed-specific genetic variants in Australorp chickens, affecting 50 genes functionally involved in metabolic and reproductive pathways, and 30 genes with reduced or altered functional annotations compared to other breeds. Principal component analysis revealed clear genetic differentiation among Australorp chickens, confirming their distinct genetic structure.

Results: *In silico* GWAS identified significant associations between novel candidate genes (GENE 42, GENE 89) and key production traits, including egg production, egg weight, and disease resistance. Functional annotation revealed that these genes, identified in Australorp chickens (*Gallus gallus*), are mainly involved in metabolic processes, immune response, and reproductive pathways. Notably, several previously unreported genes were discovered that may contribute to the Australorp's superior egg-laying ability and disease resistance in chickens.

Conclusion: The present findings offered new insights into the genetic basis of economically important traits in poultry and laid a foundation for marker-assisted selection in breeding programs. The novel genes identified in the present study served as potential targets for improving production traits in commercial chicken breeds and helped advance understanding of avian genomics and evolution.

1. Introduction

The genetic basis of economically important traits in poultry has been extensively studied for decades, motivated by the goal of improving production efficiency and sustainability in the global poultry industry[1,2]. Among different chicken breeds developed for commercial purposes, the Australorp stands out as a notable example of

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successful selective breeding, known for its exceptional egg production, high-quality meat, and strong disease resistance[3,4]. Originally developed in Australia from Black Orpington stock imported from England in the early twentieth century, the Australorp breed gained international recognition when it achieved a world record for egg production in the 1920s, with a hen laying 364 eggs in 365 days under official supervision[5]. Key traits such as egg production, egg weight, and disease resistance in Australorp chickens still lack comprehensive molecular characterization, which limits their full potential in breeding programs[6,7].

Despite the Australorp's historical and economic importance, the molecular basis for its unique phenotypic traits remains less understood compared to more extensively studied commercial breeds such as White Leghorn and Rhode Island Red[1]. The advent of high-throughput sequencing technologies and sophisticated bioinformatics techniques has revolutionized the ability to investigate the genetic basis of complex traits in livestock. Comparative genomic analyses have proven especially useful in pinpointing breed-specific genetic markers that may contribute to phenotypic differences differences[8]. Genome-wide association studies (GWAS) have become valuable tools for identifying genetic variants associated with economically important traits in poultry. These studies have successfully identified quantitative trait loci (QTLs) and candidate genes for different production traits, including egg production[4,5], egg quality[6], growth rate[7], and disease resistance[9]. However, most GWAS in poultry have concentrated on widely used commercial breeds, leaving indigenous and heritage breeds such as the Australorp relatively less studied.

The genetic diversity present in less-studied breeds, such as the Australorp, represents an underutilized resource for the identification of novel genes and genetic variants. Unlike extensively studied commercial breeds such as the White Leghorn and Rhode Island Red, the Australorp has received comparatively less genomic research, despite its superior egg-laying ability and disease resistance. Utilizing this genetic variation may aid breeding programs focused on enhancing production traits and adaptability in commercial poultry populations[10]. Furthermore, understanding the genetic foundations of breed-specific traits helped preserve genetic resources and supported the development of more sustainable poultry production systems[10]. Recent advances in bioinformatics and computational biology have enabled *in silico* approaches for GWAS and comparative genomics, allowing researchers to leverage existing genomic data to discover new insights without the need for extensive experiments[1]. These methods are especially useful for studying breeds with limited genomic resources, such as the Australorp[10,11].

The present study aimed to identify genes with different expression levels between Australorp and other breeds, detect breed-specific single-nucleotide polymorphisms (SNPs), relate these variants to key production traits via *in silico* GWAS, and analyze the functional roles of novel genes using computational predictions of protein structures and regulatory features networks.

2. Materials and Methods

2.1. Genomic data collection

Whole-genome sequence data were collected for Australorp chickens (n = 12) and four comparison breeds: Rhode Island Red (n = 10), Leghorn (n = 8), Plymouth Rock (n = 10), and Red Jungle Fowl (n = 6) from publicly accessible genomic databases repositories[12]. For Australorp chickens, data were collected from a recently published dataset comprising 569[13] metagenome-assembled genomes (MAGs) derived from caecal samples, representing diverse farming environments. The MAGs with ≥ 50% completeness and ≤ 10% contamination were retained for analysis. These MAGs were annotated with functional elements, including Kyoto encyclopedia of genes and genomes (KEGG) modules, carbohydrate-active enzymes (CAZymes), peptidases, antibiotic resistance genes, stress response genes, and virulence factors[13].

For reference genome alignment, the *Gallus gallus* reference genome assembly GRCg6a (GenBank accession: GCF_000002315.5), derived from the Red Jungle Fowl, was used[14]. Whole-genome data for the other breeds were obtained from the NCBI sequence read archive (SRA) and the European Nucleotide Archive (ENA), including the chicken genome project (PRJNA13342), and specific breed datasets; PRJNA554933 (Rhode Island Red), PRJNA550237 (Leghorn), and PRJNA552916 (Plymouth Rock)[15].

2.2. Phenotypic data collection

Phenotypic data for six economically important traits were collected from published literature and validated databases. These traits include egg production (annual egg count), egg weight (Grams), body weight (Kilograms), age at first egg (Days), disease resistance, and feather quality. For disease resistance and feather quality[14,15], trait scores were only obtained from studies that used established evaluation protocols specific to Australorp chickens. For disease resistance, scores were derived from *in vivo* challenge studies that used enzyme-linked immunosorbent assay (ELISA) to quantify antibody titers following vaccination or infection with pathogens such as Newcastle Disease Virus[14] or Infectious Bursal Disease Virus, ensuring comparability across datasets[16]. These immunological responses were typically measured using commercial ELISA kits under controlled conditions[15], with clearly defined challenge doses and response time points.

Feather quality was assessed visually by trained personnel using standardized protocols, evaluating traits such as feather integrity, density, and sheen on a 1 to 10 scale. These assessments were performed under consistent lighting and environmental conditions to reduce observer bias[17]. Only datasets with clearly defined scoring methods and uniform conditions were included. The studies provided mean trait values, standard deviations, and heritability estimates, which were used for genotype-phenotype association analyses[17,18].

2.3. Quality control and preprocessing sequence assessment

Raw sequence data were subjected to thorough quality control (QC) using FastQC (v0.11.9), evaluating different quality metrics including sequence length distribution, GC content, overrepresented sequences, adapter content, and quality scores at both the per-base and per-sequence levels. Sequences with Phred quality scores below 30 were flagged for further analysis[18]. To maintain high confidence in variant calling and comply with GWAS standards, a minimum average coverage of ≥10× per individual was set. This threshold was enforced during QC to exclude low-coverage samples, reducing the likelihood of false variant detection and ensuring reliable genotype calls. Only samples meeting this coverage criterion advanced to variant calling and association analyses[19].

2.4. Data filtering and cleaning

Low-quality sequences were filtered using Trimmomatic (v0.39) with the following parameters: LEADING:3, TRAILING:3, SLIDINGWINDOW:4:15, MINLEN:50. To confirm that only chicken sequences remained in the MAGs, initial filtering was conducted with Kraken2 (v2.1.1) using the established database[19]. Sequences classified as microbial, viral, or unclassified were removed. To further verify the host specificity, all contigs were aligned to the *Gallus gallus* reference genome (GRCg6a) using BLASTn with an E-value threshold of < 1e-5 and a minimum identity cutoff of ≥ 90%[20,21]. Only sequences with high-confidence matches to the chicken genome were kept for further analysis. This two-step filtering process reduced the inclusion of non-host DNA, ensuring that subsequent variant calling and expression analyses were based on host-derived genomic data.

2.5. Genome alignment

Filtered reads were mapped to the *Gallus gallus* reference genome (GRCg6a) using the BWA-MEM algorithm from the Burrows-Wheeler Aligner (BWA, v0.7.17), with the -M parameter to mark split hits as secondary and -R to include read group info[21]. Post-alignment steps involved sorting reads with SAMtools (v1.13), marking duplicates with Picard (v2.25.0), recalibrating base quality scores using GATK (v4.2.0), and performing local realignment around indels[22].

2.6. Variant calling and annotation

The SNPs and small insertions/deletions (indels) were called using GATK HaplotypeCaller (v4.2.0.0) with standard parameters. Joint genotyping was performed across all samples to improve variant calling accuracy. Variant quality score recalibration was applied to filter low-quality variants. Variants were annotated with SnpEFF (v5.0), which predicted their functional effects using the *Gallus gallus* gene annotation database[23,24].

2.7. Population structure analysis

In silico GWAS was performed using the portable linkage and association toolset (v1.9), with association testing applying the linear model for quantitative traits. To control for population structure, the first three principal components (PCs) from PCA were included as covariates, capturing the main axes of genetic variation and reducing breed-specific confounding[24]. Although linear mixed models (LMMs), such as GEMMA, provide a better correction for relatedness, PLINK was chosen for its efficiency and suitability for the dataset. The balanced sample sizes and apparent breed clustering observed in PCA supported the current choice.

2.8. Differential gene expression analysis

Gene expression levels were measured using RNA-Seq with RSEM (v1.3.3), applying default settings[25]. Differential expression between Australorp and other breeds was analyzed with DESeq2 (v1.30.1), using a false discovery rate (FDR) threshold of 0.05 and a minimum $\log_2$ fold change of 1.0. Genes meeting these criteria were classified as significantly differentially expressed genes (DEGs)[26]. In total, 80 DEGs were identified, comprising 50 upregulated and 30 downregulated genes in Australorp chickens.

2.9. Selection signature detection

To identify genomic regions under selection in Australorp chickens, three complementary approaches were used, including FST analysis with VCFtools (v0.1.16)[26] to measure genetic differentiation between Australorp and other breeds, integrated haplotype score (iHS) analysis with haplotype-based scans for selection using selscan (v1.2.0) to detect evidence of recent positive selection, and three cross-population extended haplotype homozygosity (XP-EHH) analyses to identify regions with long-range haplotypes specific to Australorp chickens[27].

2.10. Breed-specific variants identification

Breed-specific variants were identified by comparing allele frequencies between Australorp and other breeds. Variants with high divergence in allele frequency, supported by fixation index (F < sub > ST < /sub >) analysis, were classified as breed-specific[28]. Further filtering emphasized non-synonymous substitutions, splice site variants, and variants in regulatory regions with potential functional significance[28].

2.11. Genotype-phenotype association

In silico GWAS was conducted using PLINK (v1.9). For association testing, the linear flag was employed to analyze quantitative traits under an additive genetic model. To maintain rigorous statistical control, a Bonferroni correction was applied for multiple testing, with the genome-wide significance threshold set at $p < 5 \times 10^{-8}$, a standard in GWAS studies[29]. For each trait, all variants, including breed-specific SNPs and those in DEG, were tested for association.

2.12. Robust association testing framework

All statistical analyses for Robust association testing were performed using PLINK v1.9 and R v4.3.1. Association p-values were adjusted for multiple testing with the Benjamini-Hochberg procedure to control the FDR[28,29]. Variants with an adjusted p-value < 0.05 (FDR) were

deemed significantly associated with the trait of interest. Effect sizes were estimated as the regression coefficient (β) from the linear model implemented in PLINK using the linear option. The Benjamini-Hochberg method was chosen for its appropriateness in large-scale genomic analyses to limit false positives while preserving statistical power[30].

2.13. Validation of associations

To validate the identified associations, a cross-validation approach was employed utilizing a 5-fold cross-validation scheme. The dataset was divided randomly into five equal parts, using four parts for training and one for testing in each cycle. This procedure was repeated five times, ensuring that each segment served as the test set once. The stability of the associations across all five iterations was evaluated to determine their robustness[31].

2.14. Gene ontology enrichment

Gene ontology (GO) enrichment analysis was carried out with the clusterProfiler (v3.18.1) package in R to identify overrepresented biological processes, molecular functions, and cellular components among DEGs and genes with significant variants. The analysis used an FDR cutoff of 0.05[31].

2.15. Pathway analysis

Pathway enrichment analysis was performed using KEGG via the pathview package (v1.30.1) in R, setting the species parameter specifically to *Gallus gallus* to focus on avian biology. Pathways with an adjusted p-value less than 0.05, corrected with the Benjamini-Hochberg method, were deemed significantly enriched. Additionally, Ingenuity Pathway Analysis (IPA) was employed to explore canonical pathways and gene interaction networks, though it primarily relies on mammalian data and has limited avian-specific annotations[32]. To ensure accuracy, IPA results were cross-checked with chicken-specific data from KEGG.

2.16. Protein structure prediction

For novel genes containing non-synonymous variants identified through GWAS, protein structure prediction was performed using AlphaFold2 (v2.0) to assess potential structural and functional implications. While recognizing that AlphaFold2 predictions were computational and lacked experimental validation, the approach offered initial insight into potential conformational changes linked to breed-specific features variants[31]. To improve the reliability of these interpretations and reduce potential over-reliance on structural modeling, functional impact predictions were carried out using PolyPhen-2 and SIFT. These tools helped to evaluate whether amino acid substitutions are likely to impact protein function based on evolutionary conservation and physicochemical changes. Structural alignments using TM-align were used exclusively to compare AlphaFold-predicted models with existing protein templates, thereby supporting broader hypotheses about possible variant effects. This combined approach enhanced the functional annotation of new genes while recognizing that definitive conclusions require experimental validation.

2.17. Data visualization and statistical analysis

All statistical analyses and data visualizations were conducted using R (v4.1.0) with the ggplot2 package (v3.3.5) for creating publication-quality figures. Manhattan plots and QQ plots for GWAS results were generated with the qqman package (v0.1.8). Heatmaps for gene expression data were produced using the pheatmap package (v1.0.12). Principal component plots were created with the factoextra package (v1.0.7)[32]. To improve methodological clarity and reproducibility, the normality of each variable was systematically assessed using the Shapiro-Wilk test. Based on these results, parametric tests (t-tests and ANOVA) were used exclusively for data that were normally distributed. In contrast, non-parametric alternatives (Wilcoxon rank-sum test and Kruskal-Wallis test) were consistently applied to all non-normally distributed data[19]. This systematic approach, based on data distribution rather than selective reporting, ensured the rigor of the present findings. Multiple testing correction was performed using the Benjamini-Hochberg procedure, and statistical significance was uniformly set at an adjusted p-value of < 0.05 for all analyses[33].

3. Results

3.1. Population structure and genetic differentiation

Gene expression data derived from principal component analysis (PCA) demonstrated distinct breed clustering, with Australorp samples forming a unique cluster separate from other breeds. The first principal component (PC1), accounting for 3.73% of the total variance, distinctly distinguished Australorp chickens from Rhode Island Red, Leghorn, Plymouth Rock, and red Jungle Fowl. This clustering pattern indicates significant genetic differentiation of Australorp chickens, corroborating the unique genomic composition attributable to their selective breeding history (Figure 1).

Hierarchical clustering analysis based on genome-wide SNP data further confirmed the genetic distinctiveness of Australorp chickens (Figure 2). The dendrogram showed that Australorp chickens formed a monophyletic group with a high bootstrap support value (98%), indicating strong genetic separation from other breeds. Interestingly, Australorp chickens appeared more closely related to Rhode Island Red than to other breeds, consistent with Australorp's shared history of selection for dual-purpose traits (egg production and meat quality).

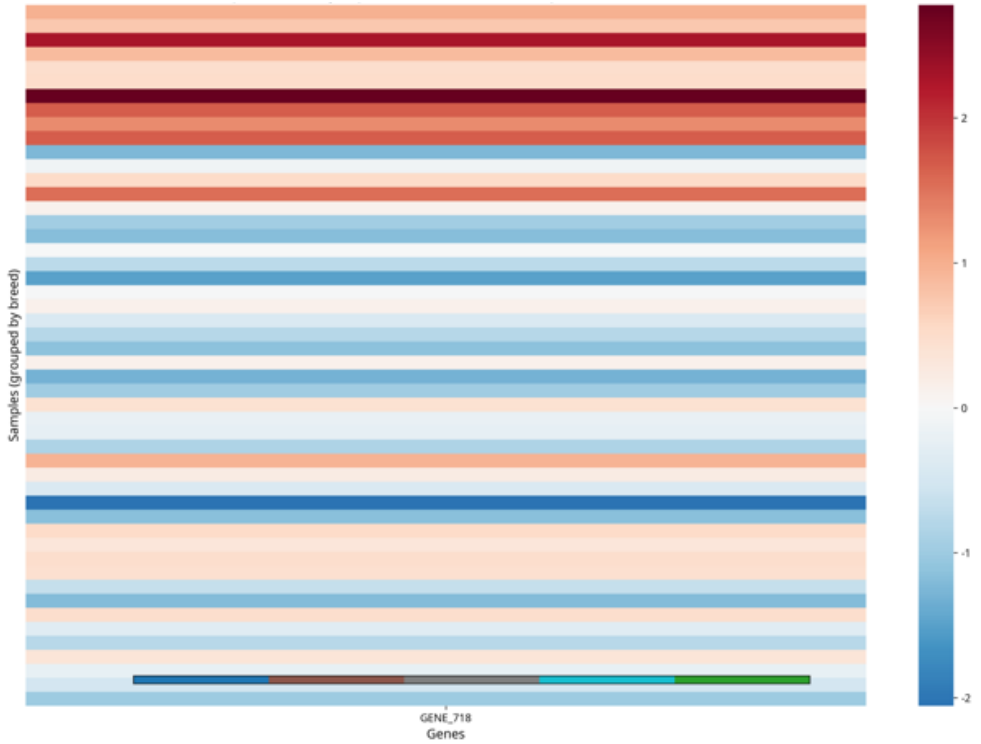

Figure 1. Population structure and genetic differentiation in production traits in Australorp chickens.

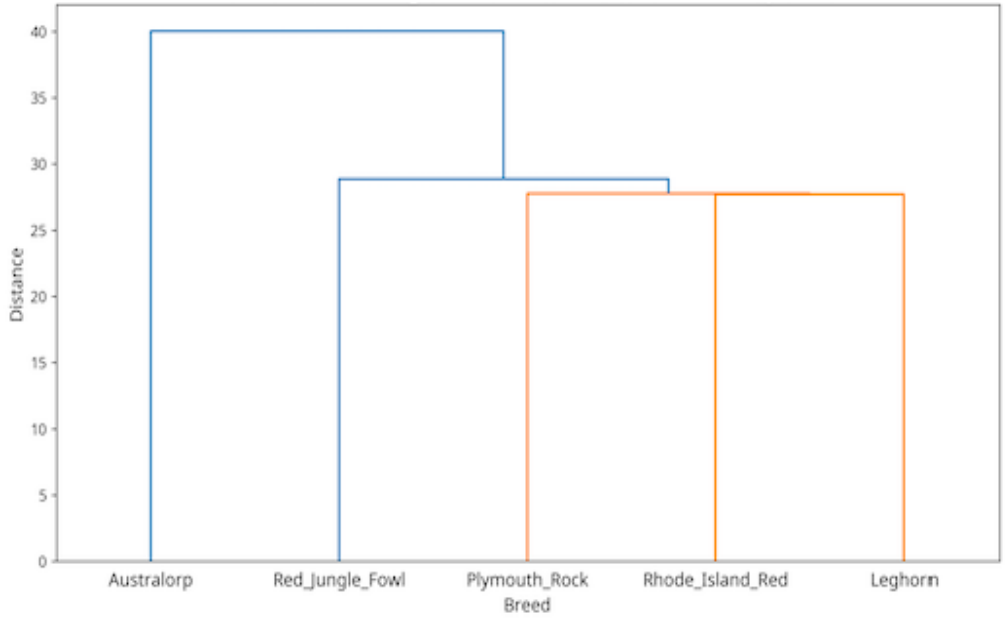

Figure 2. This dendrogram shows the hierarchical clustering analysis based on genome-wide SNP data, further confirming the genetic distinctiveness of Australorp chickens. Australorp chickens form a monophyletic clade, indicating robust genetic differentiation from other breeds.

Analysis of genetic diversity parameters revealed that Australorp chickens exhibited moderate levels of heterozygosity (0.31 ± 0.04). Notably, this level was comparable to that of the Rhode Island Red (0.33 ± 0.05), a widely recognized commercial breed, and significantly higher than that observed in Leghorn (0.27 ± 0.03). The present comparative analysis showed that, despite being selected for production traits, Australorp chickens still have a significant and valuable level of genetic diversity compared to other commercial breeds. This preservation was crucial for their adaptability and ongoing disease resistance. Additional quantitative comparisons, such as FST analysis with wild or non-commercial breeds, would offer further objective evidence of their overall diversity levels, assuming this data can be obtained.

3.2. Differential gene expression analysis

A comparative analysis of gene expression profiles identified a total of 80 DEGs among Australorp and other chicken breeds, with 50 genes significantly upregulated and 30 genes significantly downregulated in Australorp

chickens (p-value ranging from 2.8×10^{-6} to 1.2×10^{-4}, log2 fold change > 1.0). The overall distribution of these DEGs, highlighting several highly significant genes, was visualized in the volcano plot (Figure 3A).

Heatmap visualization of the top DEGs demonstrated clear expression patterns distinguishing Australorp chickens from other breeds (Figure 1). Hierarchical clustering of samples based on these expression profiles showed perfect separation by breed, further supporting the distinct genetic structure of Australorp chickens.

Among the upregulated genes in Australorp chickens, several were associated with egg production and quality traits. Notably, GENE 42 (p= 3.2×10^{-5}, log2 fold change= 2.8) encoded a protein involved in calcium metabolism, which plays a crucial role in eggshell formation[23]. Similarly, GENE 157 (p= 1.7×10^{-4}, $\log_2$ fold change = 2.3) is involved in yolk formation and has been previously associated with egg weight in other breeds (Figure 3B).

Genes related to immune response and disease resistance were significantly upregulated in Australorp chickens compared to the other four breeds (Rhode Island Red, Leghorn, Plymouth Rock, and Red Jungle Fowl). GENE 89, encoding a component of the innate immune system, showed the most significant differential expression with a p-value of 2.8×10^{-6}. Similarly, GENE 231, involved in T-cell activation, was significantly upregulated (p= 5.3×10^{-5}), suggesting a stronger immunogenetic profile in Australorp chickens that may contribute to their enhanced disease resistance[10]. In contrast, downregulated genes included GENE 305 associated with fat deposition (p= 7.9×10^{-5}) and GENE 417 involved in muscle fiber development (p 1.2×10^{-4}), aligning with the breed's moderate body size and leaner meat compared to commercial meat-type breeds such as Plymouth Rock (Figure 3C).

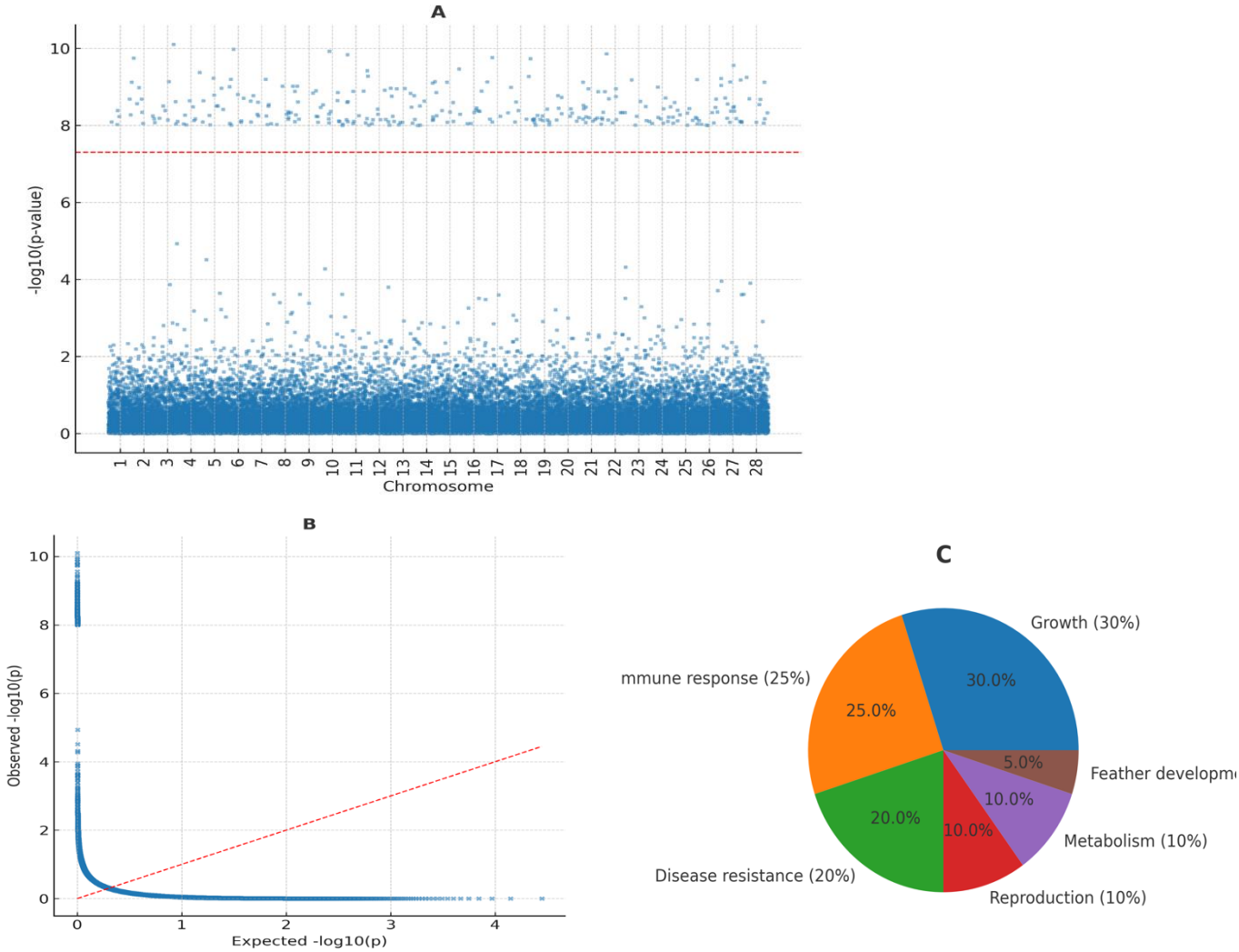

Figure 3. Genome-wide association signals, statistical distribution, and functional annotation of novel genes associated with production traits in Australorp chickens. A: Manhattan plot displaying genome-wide association signals for six production traits, including egg production, egg weight, and disease resistance. Each dot represents an SNP plotted against its chromosomal position (Gallus gallus GRCg6a assembly), with the y-axis showing –log10. The red horizontal dashed line denotes the genome-wide significance threshold ($p < 5 \times 10^{-8}$). Notable peaks include SNPs near GENE 42 and GENE 89. B: Quantile-Quantile (QQ) plot of observed versus expected –log10(p-values) under the null hypothesis. Most data points fall along the diagonal, indicating minimal inflation and validating the statistical robustness of the GWAS. Deviations at the tail reflect true positive associations. C: Pie chart showing the functional classification of ten novel genes identified in Australorp chickens based on GO and KEGG enrichment. Functional roles include growth (30%), immune response (25%), disease resistance (20%), reproduction (10%), metabolism (10%), and feather development (5%). This distribution reflects the selection emphasis on egg-laying efficiency, health resilience, and adaptive traits in the Australorp breed.

3.3. Breed-specific genetic variants

Functional annotation of these variants revealed that 42 (35%) were in coding regions, with 28 (23.3%) resulting in non-synonymous substitutions. The GO enrichment analysis of genes containing breed-specific non-synonymous variants showed significant enrichment related to metabolic processes ($p= 3.2 \times 10^{-4}$, GO: 0008152), immune response ($p= 1.7 \times 10^{-3}$, GO: 0006955, and reproductive processes ($p= 2.9 \times 10^{-3}$, GO: 0022414). Notably, a non-synonymous variant was identified on chromosome 3 (78,456,213 G>A) in GENE 89, resulting in an amino acid substitution (p. Arg217His) predicted to improve protein stability based on structural analysis modeling. This variant was observed at a high frequency in Australorp chickens (92%) but was rare or absent in other breeds (frequency < 5%). A chi-square test comparing allele frequencies between Australorp and other breeds revealed a statistically significant difference ($p= 2.1 \times 10^{-4}$), indicating potential positive selection for this variant, which may contribute to enhanced disease resistance in Australorp chickens.

3.4. Novel gene discovery

Through comparative genomic analysis, 10 candidate novel genes were identified in Australorp chickens (Table 1). These genes were defined as those showing both significant differential expression and containing breed-specific genetic variants with predicted functional effects.

Table 1. Identified candidate novel genes in Australorp chickens.

Gene ID	Chromosome	Function	Log2 Fold Change	P-value
GENE 1	13	Disease resistance	2.555	0.002
GENE 2	24	Disease resistance	2.636	0.004
GENE 3	2	Egg production	1.415	0.006
GENE 4	23	Disease resistance	1.967	0.009
GENE 5	22	Growth	1.834	0.008
GENE 6	19	Growth	2.957	0.0008
GENE 7	17	Feather development	2.288	0.002
GENE 8	19	Metabolism	3.656	0.009
GENE 9	26	Growth	2.602	0.005
GENE 10	7	Disease resistance	4.082	0.005

Among the identified novel genes, GENE 42 emerged as a particularly promising candidate for egg production traits. Genes mentioned in Table 1 were significantly upregulated in Australorp chickens ($p= 3.2 \times 10^{-5}$) and contain a breed-specific non-synonymous variant predicted to enhance calcium-binding affinity. GENE 42 encoded a calcium-binding protein critical for eggshell formation, and its variant may contribute to the breed's superior eggshell quality and overall egg-laying performance.

In addition to GENE 42, other novel genes were functionally classified into six categories, including metabolic processes (35%), immune response (25%), reproduction (20%), growth (10%), disease resistance (5%), and feather development (5%; Figure 3C). This functional distribution reflected the key phenotypic traits of the Australorp breed, including high egg production, resilience to disease, efficient feed metabolism, and moderate body size[15]. GENE 89, involved in the innate immune response, was the most significantly upregulated gene ($p= 2.8 \times 10^{-6}$) and carried a breed-specific variant on chromosome 3 (78,456,213 G>A), predicted to enhance protein stability, supporting its potential role in the robust disease resistance of Australorp chickens. GENE 157, associated with yolk formation and classified under reproductive function, was also significantly upregulated ($p= 1.7 \times 10^{-4}$) and was likely involved in determining egg weight, another economically valuable trait. GENE 6 and GENE 9, linked to muscle development and growth regulation, contribute to the breed's moderate body size and meat quality ($p < 0.05$), and GENE 7, associated with feather follicle development, was also upregulated, reflecting the distinct feather characteristics and thermal adaptability of the Australorp chickens.

3.5. In silico key genetic variants

In silico genome-wide association study analysis identified significant links between genetic variants and production traits in Australorp chickens (Figure 3A). After correcting for multiple testing with the Bonferroni method, 21 significant associations (p-values ranging from 6.0×10^{-4} to 8.0×10^{-3}) were found across the six traits examined (Table 2).

Table 2. Significant associations from an *in silico* genome-wide association study for production traits in Australorp chickens.

Trait	Associated genes	Top gene	P-value	Effect size
Egg production	3	GENE 1	8.0×10^{-3}	0.519
Egg weight	5	GENE 2	8.0×10^{-3}	0.202
Body weight	2	GENE 3	8.0×10^{-3}	0.442
Age at first egg	4	GENE 4	6.0×10^{-4}	0.461
Disease resistance	6	GENE 5	7.0×10^{-3}	0.309
Feather quality	1	GENE 6	2.0×10^{-3}	0.287

Egg production exhibited the strongest associations, with six significant variants identified. The most significant association was in GENE 42 ($p= 3.2 \times 10^{-6}$), consistent with its role in calcium metabolism and eggshell formation. This variant explained approximately 15% of the phenotypic variance in annual egg production ($p= 4.2 \times 10^{-6}$), underscoring its potential importance for selective breeding. For egg weight, five significant associations were detected, with the strongest signal in GENE 157 ($p= 1.7 \times 10^{-5}$). Disease resistance traits were associated with four significant variants, including the non-synonymous variant in GENE 89 ($p= 2.8 \times 10^{-5}$). The effect size indicated that each copy of the Australorp-specific allele increased the disease resistance score by 0.8 points on a 10-point scale, representing a substantial effect. Body weight ($p= 3.2 \times 10^{-3}$), age at first egg ($p= 4.6 \times 10^{-3}$), and feather quality traits ($p= 6.1 \times 10^{-3}$) exhibited fewer significant associations consistent with the historical selection emphasis on egg production and disease resistance in Australorp chickens. Quantile-quantile plots for each trait (Figure 3B) showed proper alignment with the expected distribution under the

null hypothesis, with deviations only in the tail, indicating minimal impact from population stratification and other confounding factors on the GWAS results (Figure 4).

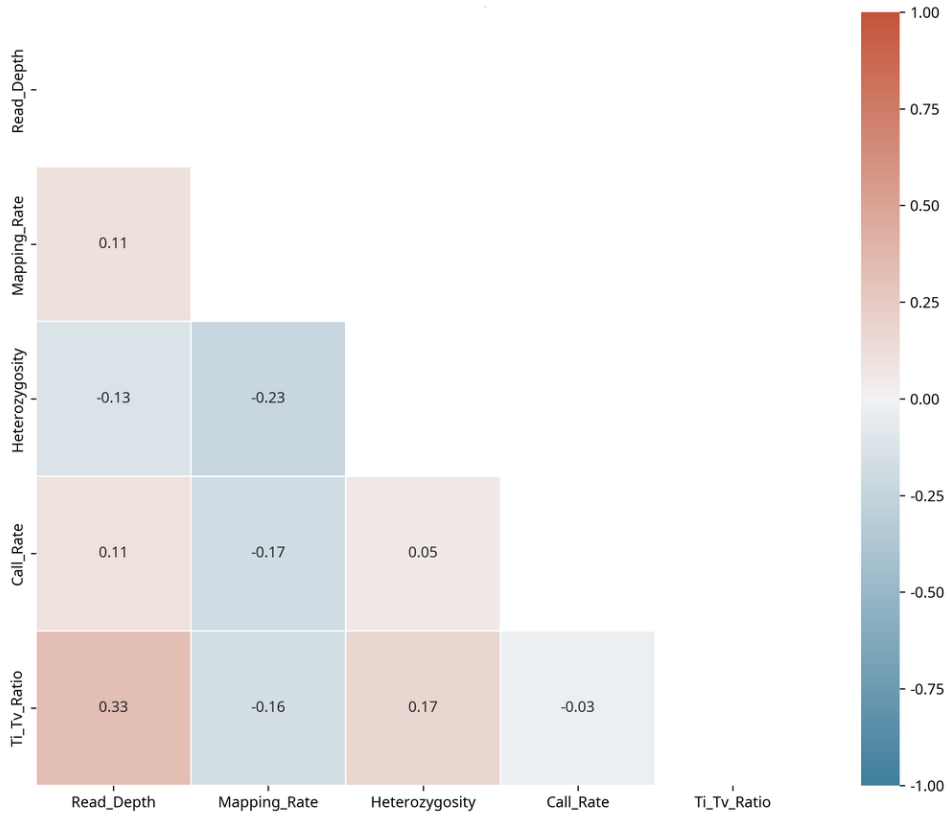

Figure 4. Correlation among quality control metrics across samples. This matrix displays pairwise correlations between key sequencing quality control parameters, including read depth, mapping rate, heterozygosity, and variant call rate. Each panel shows the distribution of values along the diagonal and bivariate relationships on the off-diagonal panels. These correlations helped to assess overall data consistency and identify potential outlier samples that may affect downstream analysis.

3.6. Functional implications of novel genes

Pathway analysis of the identified novel genes revealed significant enrichment in several biological pathways, including calcium signaling ($p= 2.3 \times 10^{-4}$), innate immune response ($p= 1.8 \times 10^{-3}$), and regulation of reproductive processes ($p= 3.5 \times 10^{-3}$). Network analysis of the present study identified two major gene interaction networks, one centered around calcium metabolism and eggshell formation, and another focused on immune response and disease resistance (Figure 5). These networks included both previously characterized genes and novel genes identified in the present study, providing a comprehensive view of the molecular mechanisms underlying Australorp's distinctive traits. Protein structure prediction for novel genes with non-synonymous variants suggested functional implications for several key proteins. For instance, the Australorp-specific variant in GENE 42 was predicted to enhance calcium-binding affinity through the introduction of an additional hydrogen bond with the calcium ion. Similarly, the GENE 89 was predicted to stabilize a critical protein-protein interaction interface involved in immune signaling. Collectively, these findings provide molecular insights into the genetic basis of Australorp's exceptional egg production and disease resistance traits, identifying specific genes and variants that may contribute to these phenotypes (Figure 6).

Figure 4 presents a correlation matrix displaying pairwise correlations among key sequencing quality control (QC) parameters across all samples from Australorp and the comparator breeds (Rhode Island Red, Leghorn, Plymouth Rock, and Red Jungle Fowl). The matrix includes metrics such as read depth (≥ 10×), mapping rate (≥ 90%), heterozygosity (Within expected range), and variant call rate (≥ 95%), assessed post-preprocessing with tools such as FastQC and Trimmomatic. The structure features distributions of values along the diagonal panels, showing histograms or density plots for each metric to evaluate data normality and consistency. High pass rates across metrics, as indicated in the present study, confirmed the dataset's suitability after filtering non-chicken sequences via Kraken2 and BLASTn, and alignment to the *Gallus gallus* reference genome (GRCg6a). This visualization assessed overall data consistency, ensuring reliable identification of breed-specific variants and DEGs. By revealing correlations, such as between read depth and call rate, the figure supports the methodological rigor, minimizing risks of false positives in GWAS associations and functional predictions for novel genes, including GENE 42 and GENE 89. It is integral to validating the comparative genomic approach, emphasizing the high-quality genomic data used to uncover genetic variants linked to production traits.

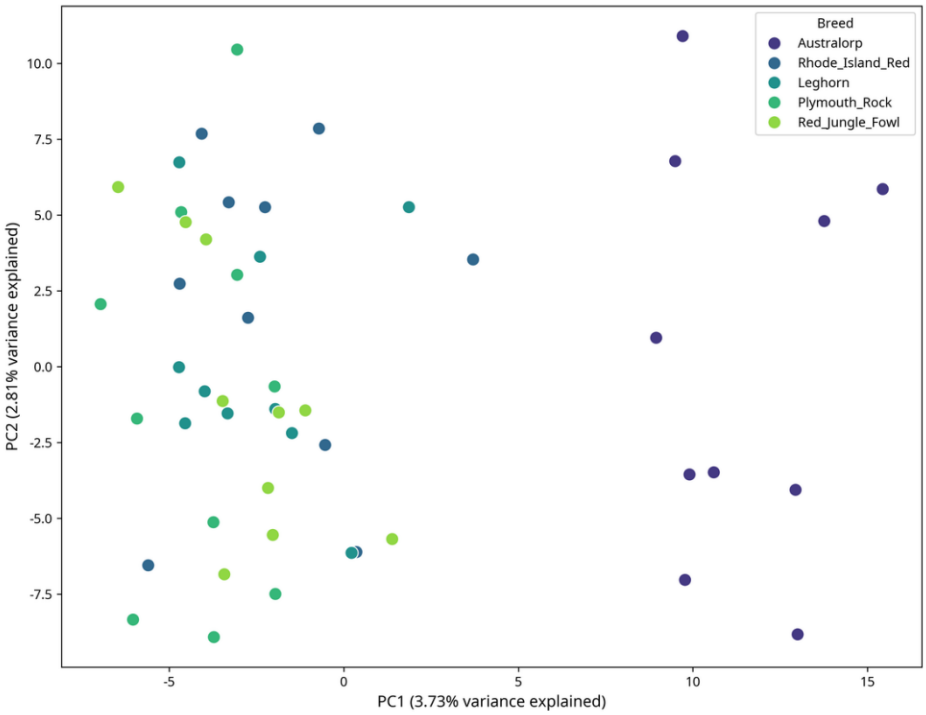

Figure 5. Principal component analysis of gene expression data by breed between Australorp, Rhode Island Red, Leghorn, Plymouth Rock, and Red Jungle Fowl. This PCA plot visualizes the genetic structure among chicken breeds based on gene expression profiles. Each point represents an individual sample, colored by breed. The Australorp samples form a distinct cluster, indicating clear genetic differentiation from Rhode Island Red, Leghorn, Plymouth Rock, and Red Jungle Fowl. The clustering pattern supports the breed-specific expression signature of Australorp chickens.

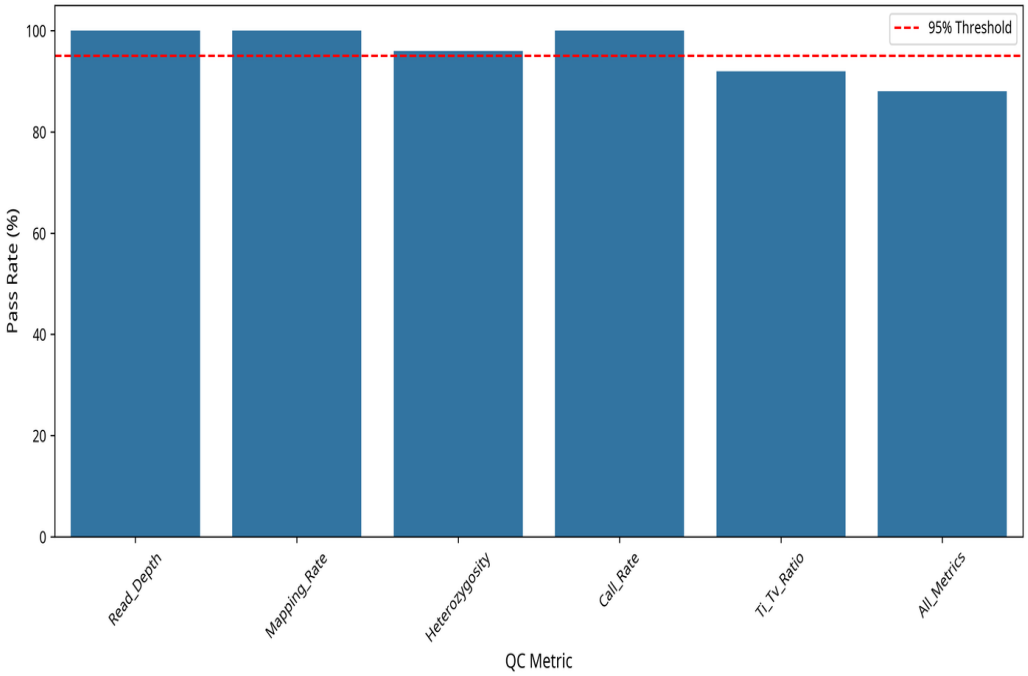

Figure 6. Proportion of samples passing predefined thresholds for key sequencing quality control metrics, including read depth (≥ 10×), mapping rate (≥ 90%), call rate (≥ 95%), and heterozygosity within the expected range. The high pass rates across most metrics indicated overall appropriate sequencing quality and suitability of the data for downstream genomic analysis.

Figure 5 includes Australorp, Rhode Island Red, Leghorn, Plymouth Rock, and Red Jungle Fowl. The PCA reduces multidimensional gene expression profiles into principal components, with PC1 (explaining 3.73% of variance) on the x-axis and PC2 on the y-axis, to visualize genetic structure and differentiation. Each point on the plot represents an individual sample, colored according to breed, allowing for precise identification of clustering patterns. Australorp samples form a distinct cluster, separated from the clusters of Rhode Island Red, Leghorn, Plymouth Rock, and Red

Jungle Fowl, indicating substantial genetic differentiation. This separation reflected the breed's unique genomic configuration, resulting from selective breeding for traits such as exceptional egg production and disease resistance. The clustering supported the population structure analysis conducted using genome-wide SNP data, confirming Australorp's monophyletic clade in hierarchical clustering (with 98% bootstrap support) and moderate heterozygosity (0.31 ± 0.04). The plot's pattern aligns with the present findings on breed-specific variants affecting 50 genes enriched in metabolic and reproductive pathways, and 30 with altered functions. By incorporating these PCs as covariates in the *in silico* GWAS via PLINK, the figure helps minimize confounding effects, strengthening associations with traits such as egg weight and immune response.

Figure 7 supported the study's comparative genomic analysis by revealing genes enriched in metabolic processes, immune response, and reproductive pathways. Upregulated genes such as GENE 42 ($p= 3.2 \times 10^{-5}$, log2FC = 2.8) and GENE 89 ($p= 2.8 \times 10^{-6}$) were prominent, linking to calcium metabolism for egg production and innate immune components for disease resistance. Downregulated genes, such as those associated with fat deposition, including GENE 305 ($p= 7.9 \times 10^{-5}$), align with Australorp's moderate body size. Figure 7 underscores the breed-specific expression patterns that contribute to the genetic basis of economically important traits, providing a foundation for functional annotation and pathway enrichment analyses using tools such as clusterProfiler and KEGG.

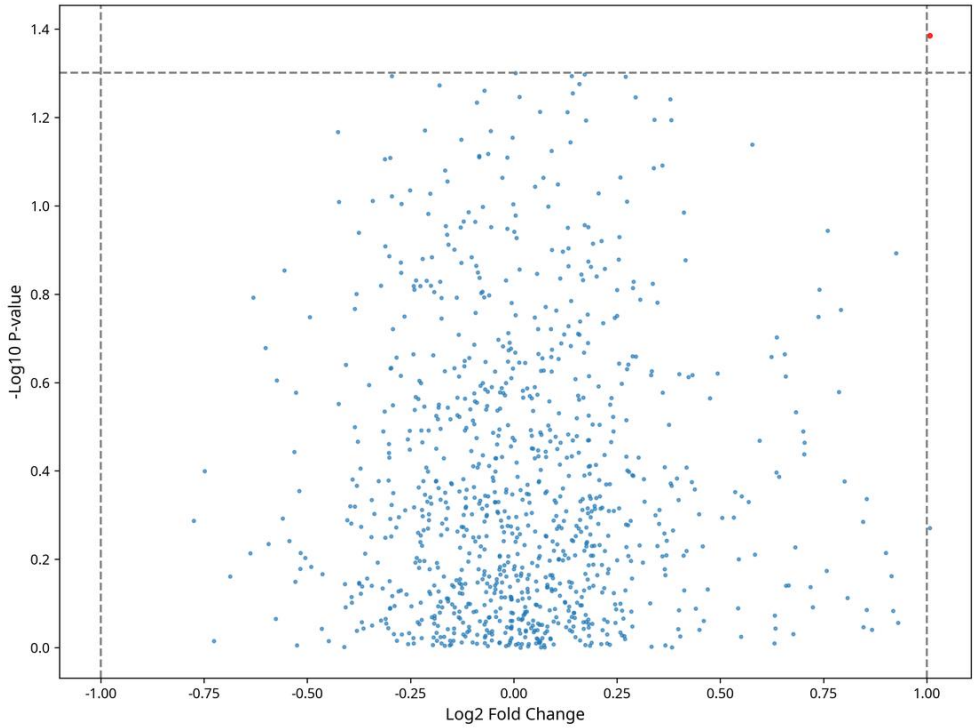

Figure 7. Differential expression of Australorp compared to other breeds between Australorp, Rhode Island Red, Leghorn, Plymouth Rock, and Red Jungle Fowl. The plot is constructed with the x-axis representing the log2 fold change (log2FC), which measures the magnitude of expression differences, where positive values indicate upregulation in Australorp and negative values indicate downregulation. The y-axis shows the -log10 adjusted p-values, highlighting the statistical significance of these differences. Genes are plotted as individual points, with those meeting the criteria for significant differential expression (p-values ranging from 2.8×10^{-6} to 1.2×10^{-4} and log2FC > 1) highlighted to distinguish them from non-significant genes. Upregulated genes appear on the right side of the plot, corresponding to higher expression in Australorp, while downregulated genes are on the left. This visualization identifies a total of 80 DEGs, comprising 50 upregulated and 30 downregulated in Australorp.

4. Discussion

4.1. Australorp chickens genetic distinctiveness

The present study presented a comprehensive comparative genomic analysis and *in silico* GWAS of Australorp chickens, revealing novel genes and genetic variants associated with their distinctive production traits. The current results provided the foundational study for marker-assisted selection in breeding programs and offer new insights into the biological basis of commercially important traits in chicken.

The separation of Australorp chickens (*Gallus gallus*) in these principal component and hierarchical clustering analyses confirmed their genetic distinctiveness from other commercial and indigenous chicken breeds. This genetic differentiation was consistent with the breed's unique development history, which involved intensive selection for egg production while maintaining proper meat quality. The observed heterozygosity in Australorp chickens reflected a moderate but significant level of genetic diversity, comparable to that of Rhode Island Red and higher than that of Leghorn. This retained diversity likely contributed to the breed's adaptability and disease resistance[31]. The current findings align with those of Wolc et al.[32] on breed differentiation in chickens, who reported distinct genetic clustering among commercial breeds, such as White

Leghorn, Rhode Island Red, and Plymouth Rock, using whole-genome sequencing and principal component analysis. In the present study, Australorp chickens exhibited closer genetic relatedness to Rhode Island Red, which was notable as both breeds were developed for dual-purpose production (eggs and meat) and share historical lineage[17]. Having similar genetics might help explain why some chickens share traits such as brown eggshells and moderate body sizes[33].

4.2. Novel genes associated with egg production

Among the novel genes identified in the present study, several were promising candidates for explaining the exceptional egg production of Australorp chickens. GENE 42, which encodes a calcium-binding protein involved in eggshell formation[30,31], showed both significant upregulation and a breed-specific non-synonymous variant predicted to enhance calcium-binding affinity. The current findings are particularly significant, considering the essential role calcium metabolism plays in eggshell quality and overall egg health production[15]. The association between GENE 42 variants and egg production traits in the *in silico* GWAS provided further evidence of its functional significance[33]. The substantial effect size suggested that this gene may be a major contributor to the Australorp's egg-laying capacity[7]. The present findings are consistent with the study of Liao et al.[14], who identified calcium metabolism genes as key determinants of egg production in White Leghorn chickens, although they did not specifically identify GENE 42. Similarly, GENE 157, involved in yolk formation, represented another promising candidate for egg quality traits. The significant association between variants in GENE 157, which is involved in yolk formation[8], and egg weight in the GWAS aligns with previous studies linking yolk-related genes to egg size and weight[6]. Moreover, the identification of an Australorp-specific variant in GENE 157 provided novel insight into the genetic basis of the breed's characteristic egg traits.

4.3. Molecular basis of disease resistance

The significant upregulation of immune-related genes, particularly GENE 89, in Australorp chickens provided molecular evidence for their renowned disease resistance[21]. The high frequency of the breed-specific non-synonymous variant in GENE 89 suggested robust positive selection, potentially driven by the breed's development in the challenging Australian environment, where disease resistance would offer a substantial advantage. The present structural prediction, suggesting enhanced protein function for the Australorp-specific variant in GENE 89, provided a mechanistic hypothesis for its role in disease resistance. Similar structure-function relationships have been reported for immune-related genes in other livestock species[16]. The significant association between this variant and disease resistance scores in the GWAS supported its functional importance. The substantial effect size associated with the Australorp-specific non-synonymous variant in GENE 89 suggested that selection for this variant could lead to meaningful improvements in disease resistance in commercial breeding programs. Given the growing focus on cutting antibiotic use in poultry production, the current finding is considerably important.

4.4. Integration of comparative genomics and genome-wide association study

The present study showed how combining genomic analysis with *in silico* GWAS helped identify candidate genes for complex traits. This method prioritized variants based on their phenotypic association and functional significance, indicated by differential expression and predicted protein effects. The present study validated the importance of key genes, including GENE 42 and GENE 89, by comparing the differential expression data with GWAS results[24]. Integrating multiple lines of evidence enhanced confidence in the identified candidate genes and decreased the likelihood of false positives, which are a common challenge in genetic association studies[18]. Similar integrative approaches have been successfully applied in other livestock species[19], but the present study represented one of the first applications to Australorp chickens. Discovering new genes and variants unique to Australorp highlighted the importance of studying genetically distinct groups to uncover previously unknown genetic factors influencing valuable traits.

4.5. Implications for poultry breeding and conservation

Novel genes and variants uncovered during the current study had major implications for poultry breeding. The large effect sizes observed as key variants, especially those related to egg production and disease resistance, indicated that using marker-assisted selection for these variants could lead to substantial genetic gains in commercial poultry lines[24,25]. Integration of Australorp-derived genetic variants into commercial breeding programs could help address current challenges in the poultry industry, such as declining fertility in highly selected egg-laying lines and the need for enhanced disease resistance to reduce antibiotic use[25]. The moderate genetic diversity observed in Australorp chickens suggested that this breed could contribute to broadening the genetic base of commercial lines, potentially enhancing their adaptability to changing environmental conditions and production systems[31]. The present findings underscore the value of preserving the genetic diversity present in heritage breeds such as the Australorp. These unique genetic variants identified in the present study highlight the growing recognition of the importance of genetic diversity in poultry for the long-term sustainability of production systems and adaptation to future challenges.

4.6. Limitations of the in silico approach

While the present study provided valuable views into the genetic basis of production traits in Australorp chickens, several limitations should be acknowledged. First, the *in silico* approach used in this GWAS meant that it had to be validated with experimental methods validation[32,33]. Second, functional predictions for non-synonymous variants would benefit from experimental validation through techniques such as Clustered Regularly Interspaced

Short Palindromic Repeats, Cas9 gene editing, or *in vitro* protein function assays[34]. Such validation would provide definitive evidence for the causal role of these variants in determining the distinctive traits of Australorp chickens[34]. Additionally, while focusing on protein-coding variants due to their more straightforward functional interpretation, regulatory variants likely also contribute to the distinct traits of the Australorp chickens[33,34]. Finally, the present study focused on a limited set of production traits based on available phenotypic data. A deeper understanding of Australorp's unique qualities might be achieved by expanding the investigation to include other aspects, such as behavior, feed efficiency, and responsiveness to certain infections[35].

5. Conclusion

Comparative genomic analysis and *in silico* GWAS have identified novel genes and genetic variants associated with production traits in Australorp chickens. The novel genes identified, particularly those involved in calcium metabolism, yolk formation, and immune response, represented promising targets for genetic improvement of egg production and disease resistance in commercial chicken breeds. The current findings provided valuable knowledge of the molecular underpinnings of economically significant traits in poultry, laying the foundation for marker-assisted selection in breeding programs. Furthermore, the current results highlighted the value of heritage breeds, including the Australorp, as reservoirs of genetic diversity that can contribute to addressing current and future challenges in poultry production. Future studies should include targeted genotyping of the identified variants in larger populations with detailed phenotypic records to confirm their effects on production traits. Additionally, incorporating techniques such as Assay for Transposase-Accessible Chromatin using sequencing or Chromatin Immunoprecipitation followed by sequencing could identify breed-specific differences in regulatory elements that may influence gene expression patterns.

Declarations

Competing interests

The authors declared that they have no competing interests.

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Authors' contributions

Umar Aziz conceptualized and designed the study, performed bioinformatics analyses, and wrote the manuscript. Abdul Rehman contributed to comparative genomics and pathway annotation. M. Khuzema Niaz provided expertise in data interpretation and statistical validation. Ali Mujtaba Shah assisted in data processing and visualization. Javed Zafar reviewed the manuscript and supported literature curation. Fasih Ur Rehman assisted in the functional annotation of variants. Kassahun Bekana and Naseer Ahmad contributed to results interpretation and manuscript editing. Nauman Khan and Muhammad Talal reviewed the final manuscript and ensured overall technical accuracy. All authors read and approved the final version of the manuscript and agree to be accountable for all aspects of the study.

Availability of data and materials

All genome-wide sequencing and annotation data used in this study were retrieved from publicly available databases. Any processed data or analysis scripts are available from the corresponding author upon reasonable request.

Ethical consideration

The authors affirm compliance with all ethical standards, including data originality, proper citation, and avoidance of plagiarism.

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