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A combination of genome-wide association study and selection signature analysis dissects the genetic architecture underlying bone traits in chickens

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ABSTRACT

The bones of chicken play an important role in supporting and protecting the body. The growth and development of bones have a substantial influence on the health and production performance in chickens. However, genetic architecture underlying chicken bone traits is not well understood. The objectives of this study are to dissect the genetic basis of bone traits in chickens and to identify valuable genes and genetic markers for chicken breeding. We performed a combination of genome-wide association study (**GWAS**) and selection signature analysis (fixation index values and nucleotide diversity ratios) in an F_2 crossbred experimental population with different genetic backgrounds (broiler × layer) to identify candidate genes and significant variants related to femur, shank, keel length, chest width, metatarsal claw weight, metatarsal length, and metatarsal circumference. A total of 545 individuals were genotyped based on the whole genome re-sequencing method (26 F_0 individuals were re-sequenced at 10 × coverage; 519 F_2 individuals were re-sequenced at 3 × coverage). A total of 2 028 112 single-nucleotide polymorphisms (**SNPs**) remained to carry out analysis after quality control and imputation. The integration of GWAS and selection signature analysis indicated that all significant SNPs responsible for bone traits were mainly localized on chicken chromosomes 1, 4, and 27. Finally, we identified 21 positional candidate genes that might regulate chicken bone growth and development, including *LRCH1*, *RB1*, *FNDC3A*, *MLNR*, *CAB39L*, *FOXO1*, *LHFP*, *TRPC4*, *POSTN*, *SMAD9*, *RBPJ*, *PPARGC1A*, *SLIT2*, *NCAPG*, *NKX3-2*, *CPZ*, *SPOP*, *NGFR*, *SOST*, *ZNF652*, and *HOXB3*. Additionally, an array of uncharacterized genes was identified. The findings provide an in-depth understanding of the genetic architecture of chicken bone traits and offer a molecular basis for applying genomics in practical chicken breeding.

Implications

Chicken bone weight and bone length are important parameters of chicken bone health and have an essential impact on actual production. In this study, we investigated the genetic architecture of 17 skeletal traits in an F_2 chicken population. Three genomic regions, including 21 candidate genes, were identified to be significantly associated with bone traits. The findings could enhance our understanding of genetic determinants underlying bone traits in chickens and may aid selective breeding programs in the future.

Introduction

Bones such as femur, tibia, shank, keel, sternum, and metatarsal claw are major parts of skeleton system of the chicken (González-Cerón et al., 2015a and 2015b). Their length and weight are regarded as important parameters for evaluating bone growth and development in chickens (Guo et al., 2020). Due to genetic and nutritional factors, bone development defects such as osteoporosis, rickets, and tibial dyschondroplasia can result in substantial economic losses and compromise bird welfare in the poultry industry (Dinev, 2012; Dale et al., 2015). The reduction in overall fitness is mainly because of tremendously rapid growth in body mass without parallel improvements in the skeleton and internal organs to support the weight of the broiler chickens (Deeb and

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Lamont, 2002; Sharman et al., 2007; Grupioni et al., 2015). The genetic improvement of bone traits has been proposed as a potential solution to these issues.

To date, numerous QTLs and candidate genes that significantly affect chicken bone traits have been identified. Li et al. (2003) found that *TGF-β* family genes might be important in tibia and shank development and growth in two chicken F_2 populations by DNA sequencing and PCR-RFLP methods. Zhou et al. (2007) detected a total of 56 significant QTL for skeletal traits at the 5% chromosome-wise significance in two unique chicken F_2 populations by genome-wide linkage analysis. Gao et al. (2011) found a QTL associated with chest width at 9 weeks of age at 297 cM on Gallus gallus chromosome 3 in an F_2 resource population. Zhang et al. (2020) identified six candidate genes on chromosome 21 by haplotype-based genome-wide association study (**GWAS**), including *TNFRSF1B*, *PLOD1*, *NPPC*, *MTHFR*, *EPHB2*, and *SLC35A3*, which might play essential roles in bone development. De Koning et al. (2020) combined data from the commercial founder White Leghorn population and the F_2 mapping population to locate a QTL on chicken chromosome 1 and identified that the *CBS* gene was linked to osteoporosis in laying hens. Emrani et al. (2020) indicated a unique single-nucleotide polymorphism (**SNP**) (rs16689511) located on chicken Z chromosome within the *LOC101747628* gene that was related to shank length at three different ages of birds (weeks 8, 9 and 11) by GWASs in an F_2 chicken resource population.

In recent years, with the development of SNP array and sequencing technology, a large number of available SNP markers have been obtained, which has made GWAS widely used in the genetic dissection of complex animal traits. However, even with the high resolution of the GWAS, there is relatively limited power to locate actual causal variation because of long-range linkage disequilibrium. In this case, it is essential to combine GWAS with other strategies such as selective signature to improve the accuracy of the functional variant(s) mapping (Zhou et al., 2018).

The domesticated animal species have undergone intensive selection, which has given rise to wide-ranging differences in the phenotype of domestic animals compared to their wild progenitors (Li et al., 2013; Johnsson et al., 2015). In population genetics, reduction in the polymorphism of some chromosome segments caused by selection is called selective sweep and the neutral sites around the selection site show a rapid increase in gene frequency, called the 'Hitch-hiking' effect (Stephan, 2019). These processes will appear in the genome and leave selection characteristics, called selection signatures. The internal mechanism of selection is to select specific genes that are directly related to biological traits. The detection of selection signatures can reveal the genes subject to selection, which is of great significance for understanding the evolution of species and economic traits.

Here, we performed a combination of GWAS and selection signature analysis to dissect the genetic architecture of bone traits in an F_2 chicken population. This study will not only deepen our understanding of the genetic architecture of chicken bone traits but also provide potential genetic markers and candidate genes for chicken molecular breeding.

Material and methods

Experimental populations and phenotypic measurements

The Northeast Agricultural University Resource Population was used in this study. The population is an F_2 population constructed by crossing broiler cocks derived from a fat line with high abdominal fat content (Leng et al., 2009) and Baier layer dams (a Chinese native breed). More details about this population have been reported in previous studies (Zhang et al., 2010; Zhang et al., 2011). A total of 519 F_2 individuals from 12 half-sib families were used in this study. Each individual was the experimental unit. All F_2 birds were provided commercial diets based on corn and soybeans that in line with all NRC (1994) requirements and had free access to feed and water in the process of feeding. The metatarsal length (**MeL**) and metatarsal circumference (**MeC**) of all F_2 individuals were measured every 2 weeks from the age of 4 weeks until 12 weeks. The MeL was measured from the straight-line distance from the superior tibial joint to the third and fourth toes. MeC was measured using a thin piece of string around the middle tibia, and the length of the string was then measured by sliding calipers. We also measured keel length (**KeL**) and chest width (**ChiW**), two indicators of body size and condition of bone growth and development. Keel length and chest width were measured before slaughter at the age of 12 weeks. Femur weight (**FeW**), femur length (**FeL**), shank weight (**ShW**), shank length (**ShL**), and metatarsal claw weight (**MeCW**) were measured after slaughter at the age of 12 weeks. The length of these traits was measured with sliding calipers or a tape measure.

Genome sequencing and quality control

We used the reagent test kit to extract total genomic DNA from the samples. We re-sequenced 26 F_0 and 519 F_2 individuals on the Illumina HiSeq PE150 platform. The F_0 individuals were re-sequenced with an averaged depth of 10×, and F_2 individuals were re-sequenced with an averaged depth of 3×. Library construction and sample indexing were done as described. Single-nucleotide polymorphism calling was performed on a population scale using a Bayesian approach as implemented in the package SAMtools after alignment (Li et al., 2009). Then, we calculated genotype likelihoods from reads for each individual at each genomic location and the allele frequencies in the sample. Only high-quality SNPs (coverage depth ≥ 2, RMS mapping quality ≥ 20, miss ≤ 0.3) were kept for subsequent analysis to exclude SNP calling errors caused by incorrect mapping, and 10 889 955 SNPs were left after filter from 15 868 916 raw SNPs. The missing genotypes were imputed using the F_0 generation 10-fold cross-validation in 519 sequencing individuals of the F_2 generation. Imputation of markers was performed using BEAGLE 4.0 with default parameter settings (Browning and Browning, 2009). A total of 2 028 112 SNPs were left after the imputed 10 889 955 SNPs filtered by MAF ≥ 0.05 and linkage disequilibrium < 0.6 for the 519 individuals.

Single-marker genome-wide association studies

Association analysis was conducted using the Genome-wide Efficient Mixed-Model Association software package (Zhou and Stephens, 2012). For the mixed linear model analysis, the equation is as follows:

$$y = S\beta + X\alpha + K\mu + e$$

In this equation, y represents phenotype; S is the incidence matrix of fixed effects and β is the vector of corresponding coefficients including the intercept; gender, birth weight and top 48 Principal components used for population structure correction were included as covariates to build up the S matrix. X represents the vector of SNP genotype and α is the corresponding effect of the marker; K is incidence matrix for μ and μ is the vector of random additive genetic effects following the multinormal distribution N $(0, G\sigma^2_\mu)$, in which G is the genomic relationship matrix and σ^2_μ is the polygenetic additive variance; e represents random residual with a distribution of N $(0, I\sigma^2_e)$. The genome-wide significance threshold value was set as $P < 10^{-6}$ to control the genome-wide type 1 error rate (Ma et al., 2018).

Heritability, genetic, and phenotypic correlations

SNP-based heritability (h^2_{SNP}) was calculated using the GCTA v1.93.2 beta software (Yang et al., 2011) based on the genetic relationship matrix between pairs of individuals. For pairwise genetic correlation (r_g) analysis of bone traits, bivariate genomic Genome-based Restricted Maximum Likelihood analysis was performed in GCTA v1.93.2. Moreover, Pearson correlation coefficients of pairs of traits were calculated to further check for correlation among the phenotypic characteristics themselves by SPASSAU (https://spssau.com/).

Genome-wide selection signature test

We ranked the phenotypic value of each trait in 519 samples. Selection signature analysis of each trait was carried out between the two groups (15 samples per group) divided according to the highest and lowest phenotypic values. We calculated the genome-wide distribution of fixation index ($\mathbf{F_{ST}}$) values and nucleotide diversity ($\boldsymbol{\theta\pi}$) (pairwise nucleotide differences) ratios for the defined group pairs with VCFtools (40-kb windows sliding in 10-kb steps). The θπ ratios were log2-transformed. After estimating and ranking the empirical percentiles of F_{ST} and log2(θπ ratio) in each window, the windows with the top 5% F_{ST} and log2 (θπ ratio) values simultaneously were considered as candidate outliers under strong selective sweep (Li et al., 2013). Candidate SNPs and genes were grouped into different outlier windows.

Functional annotation and enrichment analysis of the candidate genes

SNP annotation was performed according to the GCF_000002315.6_GRCg6a reference genome using the package ANNOVAR (Wang et al., 2010). Only the high-quality SNPs were annotated. SNPs were categorized in exon regions, intronic regions, splicing sites (within 2 bp of a splicing junction), upstream and downstream regions (within a 1 kb region upstream or downstream), and intergenic regions based on the genome annotation. We identified positional candidate genes according to their physical location on Gallus gallus chromosome and biological functions. Candidate genes were screened in the 40-kb region upstream and downstream of each top SNP according to the analysis results of linkage disequilibrium attenuation distance. Functional enrichment of Gene Ontology and Kyoto Encyclopedia of Genes and Genomes were performed using OmicShare Tools (https://www.omicshare.com/tools/). The data set was further visualized using the R package Gene Ontology plot.

Results

Descriptive statistics

We collected bone trait data from the F_2 population. Descriptive statistics were performed in Table 1. Heritability, genetic, and phenotypic correlations of 17 bone-related traits were summarized in Table 2. In general, there were highly positive phenotypic correlations (0.54–0.96) and genetic correlations (0.53–1.00) between each pair of traits ($P < 0.01$).

Genome-wide association studies for 17 bone-related traits

The statistical results of GWAS were shown by the Manhattan plot (Figs. 1–3). All detected top SNPs of these traits were distributed on chromosomes 1, 4, and 27.

Genome-wide association studies for metatarsus traits

Fig. 1 showed the GWAS of independent SNPs with metatarsus traits, namely MeC at 4–12 weeks, MeL at 4–12 weeks and MeCW. Based on the genome annotation, 295 genome-wide significant SNPs were distributed in exon regions, intronic regions, and intergenic regions. A total of 403 genes were screened according to the physical location of each significant SNP. It could be observed from Fig. 1 that the association signals of both MeC and MeL are increasing with the weeks of age.

For chromosome 1, a top SNP (SNP with the most significant association with phenotype on each chromosome) located in the 170 743 966 bp of the intronic region of *LOC770248* was associated with MeC6, MeC8, MeC10 and MeCW. Another top SNP associated with MeL10 and MeL12 was mapped in the 171 411 019 bp of the intronic region of *SERPINE3* on chromosome 1. For chromosome 4, a top SNP in the 75 121 285 bp of the intronic region of *SLIT2* was significantly related to MeC6, MeC10, MeC12 and MeCW. For chromosome 27, a top SNP located in the 6 070 637 bp of the intronic region of *IGF2BP1* was correlated with MeC8, MeC10, MeC12 and MeCW.

Genome-wide association studies for leg bone traits

Based on the genome annotation, 186 genome-wide significant SNPs for leg bone traits were distributed in exon regions, intronic

Table 1
Numbers of animals (N), mean, SD, minimum (Min) and maximum (Max) values, and CV of 17 bone traits of F_2 chickens.

Classification	Traits	*N*	Mean	SD	Min	Max	CV (%)
Metatarsus	MeL4 (cm)	507	5.68	0.36	4.12	6.97	6.25
	MeL6 (cm)	503	7.12	0.47	5.47	8.39	6.55
	MeL8 (cm)	505	8.52	0.63	6.74	10.17	7.38
	MeL10 (cm)	507	9.34	0.77	7.20	11.45	8.20
	MeL12 (cm)	519	9.90	1.00	7.52	13.70	10.12
	MeC4 (cm)	507	3.07	0.22	2.25	3.70	7.03
	MeC6 (cm)	503	3.84	0.31	0.40	4.65	7.94
	MeC8 (cm)	505	4.00	0.33	3.25	5.05	8.19
	MeC10 (cm)	507	4.17	0.36	3.30	5.30	8.62
	MeC12 (cm)	519	4.32	0.40	3.30	5.80	9.17
	MeCW (g)	518	74.91	21.43	37.00	137.00	28.60
Body size	KeL (cm)	518	13.66	0.93	11.50	16.37	6.84
	ChiW (g)	518	7.69	0.75	5.72	9.44	9.70
Leg bone	ShL (cm)	517	12.71	1.00	10.41	15.81	7.84
	ShW (g)	513	20.27	5.80	10.00	37.00	28.60
	FeL (cm)	510	9.16	0.69	7.60	11.08	7.48
	FeW (g)	507	14.22	3.71	8.00	25.00	26.12

Abbreviations: MeL4-12 = metatarsal length at 4, 6, 8, 10 and 12 weeks of age; MeC4-12 = metatarsal circumference at 4, 6, 8, 10 and 12 weeks of age; KeL = keel length; ChiW = chest width; FeW = femur weight; FeL = femur length; ShW = shank weight; ShL = shank length; MeCW = metatarsal claw weight.

Table 2
Heritability (diagonal), genetic (above diagonal), and phenotypic (below diagonal) correlations of 17 bone traits of F_2 chickens.

Traits	MeL4	MeL6	MeL8	MeL10	MeL12	MeC4	MeC6	MeC8	MeC10	MeC12	MeCW	KeL	ChiW	ShW	ShL	FeL	FeW
MeL4	0.72	1.00	0.99	0.98	0.98	0.93	0.95	0.91	0.93	0.95	0.96	0.94	0.92	0.96	0.95	0.94	0.94
MeL6	0.75	0.76	1.00	0.99	0.99	0.89	0.93	0.90	0.93	0.93	0.96	0.94	0.93	0.97	0.98	0.97	0.96
MeL8	0.72	0.80	0.85	1.00	1.00	0.90	0.95	0.93	0.95	0.96	0.98	0.94	0.90	0.99	0.99	0.98	0.98
MeL10	0.68	0.75	0.81	0.85	0.98	0.91	0.94	0.93	0.95	0.97	0.98	0.94	0.93	0.99	0.99	0.98	0.97
MeL12	0.64	0.72	0.83	0.89	0.85	0.87	0.93	0.93	0.95	0.96	0.98	0.93	0.89	0.98	0.99	0.98	0.97
MeC4	0.63	0.61	0.62	0.60	0.56	0.82	1.00	0.98	0.97	0.97	0.93	0.90	0.93	0.93	0.86	0.85	0.91
MeC6	0.65	0.59	0.70	0.64	0.62	0.78	0.80	0.99	0.99	0.99	0.96	0.92	0.92	0.97	0.92	0.90	0.95
MeC8	0.60	0.63	0.71	0.76	0.77	0.75	0.87	0.93	1.00	0.77	0.97	0.94	0.93	0.97	0.92	0.93	0.96
MeC10	0.62	0.67	0.76	0.79	0.82	0.74	0.73	0.93	0.94	0.89	0.89	0.95	0.95	0.98	0.95	0.95	0.98
MeC12	0.60	0.64	0.74	0.81	0.84	0.71	0.71	0.92	0.93	0.89	0.99	0.95	0.95	0.99	0.95	0.95	0.98
MeCW	0.64	0.71	0.80	0.86	0.90	0.66	0.69	0.89	0.93	0.92	0.93	0.95	0.92	0.63	0.97	0.97	1.00
KeL	0.64	0.68	0.75	0.77	0.77	0.63	0.64	0.80	0.82	0.80	0.84	0.91	0.94	0.95	0.93	0.93	0.96
ChiW	0.54	0.57	0.63	0.63	0.64	0.61	0.58	0.70	0.73	0.70	0.71	0.73	0.82	0.92	0.88	0.89	0.92
ShW	0.60	0.70	0.77	0.83	0.86	0.62	0.67	0.84	0.89	0.88	0.94	0.80	0.67	0.87	0.98	0.98	0.53
ShL	0.64	0.75	0.82	0.89	0.89	0.56	0.63	0.78	0.83	0.81	0.89	0.80	0.64	0.88	0.89	0.99	0.97
FeL	0.63	0.74	0.82	0.87	0.89	0.57	0.63	0.78	0.84	0.82	0.89	0.80	0.65	0.87	0.91	0.91	0.98
FeW	0.62	0.70	0.78	0.84	0.88	0.64	0.68	0.86	0.91	0.89	0.96	0.83	0.69	0.94	0.88	0.91	0.92

Abbreviations: MeL4-12 = metatarsal length at 4, 6, 8, 10 and 12 weeks of age; MeC4-12 = metatarsal circumference at 4, 6, 8, 10 and 12 weeks of age; KeL = keel length; ChiW = chest width; FeW = femur weight; FeL = femur length; ShW = shank weight; ShL = shank length; MeCW = metatarsal claw weight.

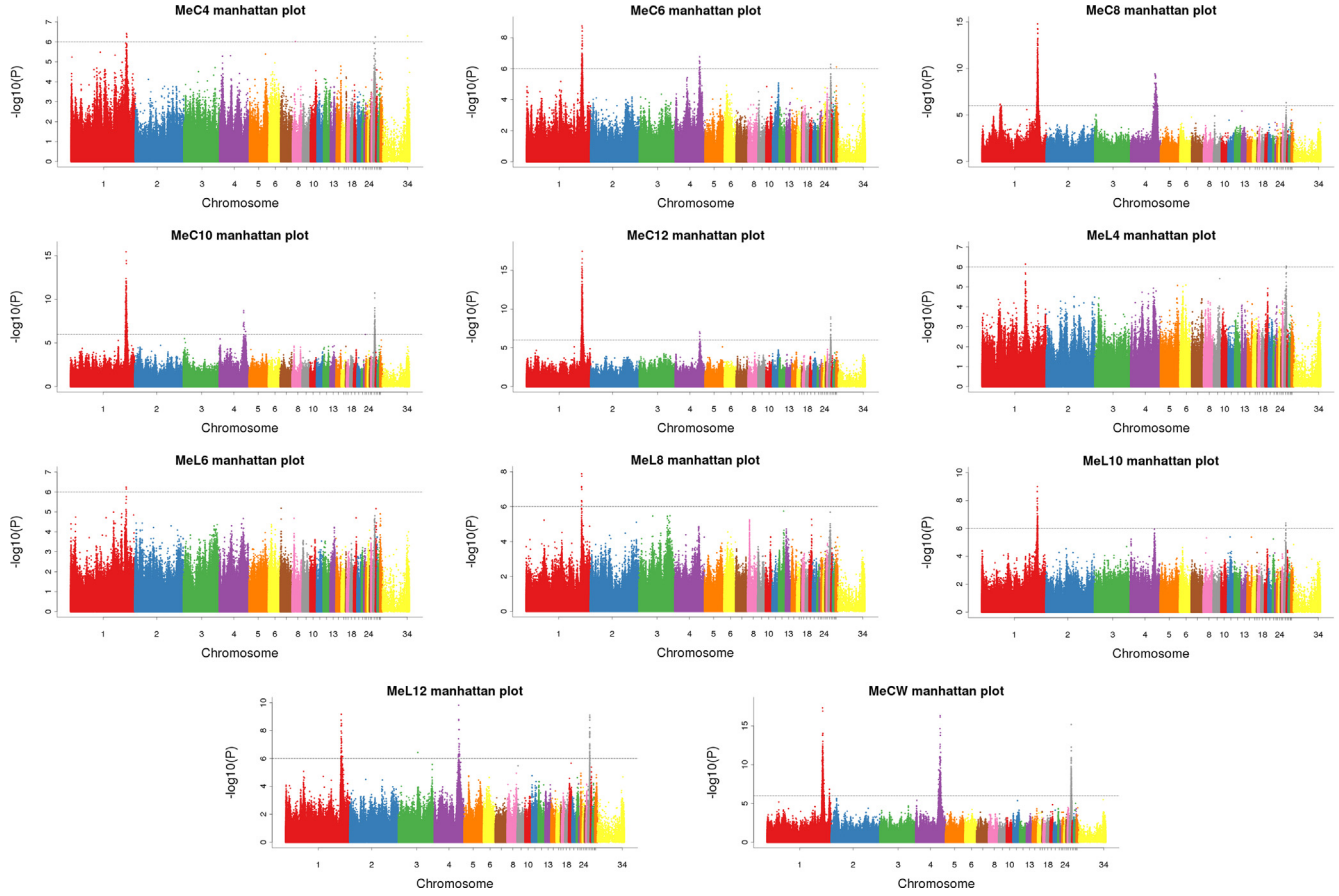

Fig. 1. Manhattan plot for the association analyses of metatarsus traits in an F_2 chicken population. Metatarsus traits include MeC at 4–12 weeks, MeL at 4–12 weeks, and MeCW. In the Manhattan plots, -log10 (*P*-value) of the filtered high-quality SNPs (y-axis) is plotted against their genomic positions (x-axis); SNPs on different chromosomes (1–34) are denoted by different colors. MeC4-12 = metatarsal circumference at 4, 6, 8, 10 and 12 weeks of age, MeL4-12 = metatarsal length at 4, 6, 8, 10 and 12 weeks of age, MeCW = metatarsal claw weight, SNPs = single-nucleotide polymorphisms.

regions, and intergenic regions. A total of 376 genes were screened according to the physical location of each significant SNP. As shown in Fig. 2, for chromosome 1, a top SNP in the 170 743 966 bp of the intronic region of *LOC770248* was correlated with FeL and ShW. Another top SNP in the 171 411 019 bp of the intronic region of *SERPINE3* on chromosome 1 was significantly associated with FeW and ShL. For chromosome 4, there are two top SNPs in the intronic region of the *SLIT2*, one located in the 75 121 285 bp was significantly related to FeL and ShW, and the other located in the 75 112 116 bp was significantly related to FeW. For chromosome 27, a top SNP in the 6 066 378 bp of the intronic region of *IGF2BP1* was associated with FeW, ShL and ShW. Another top

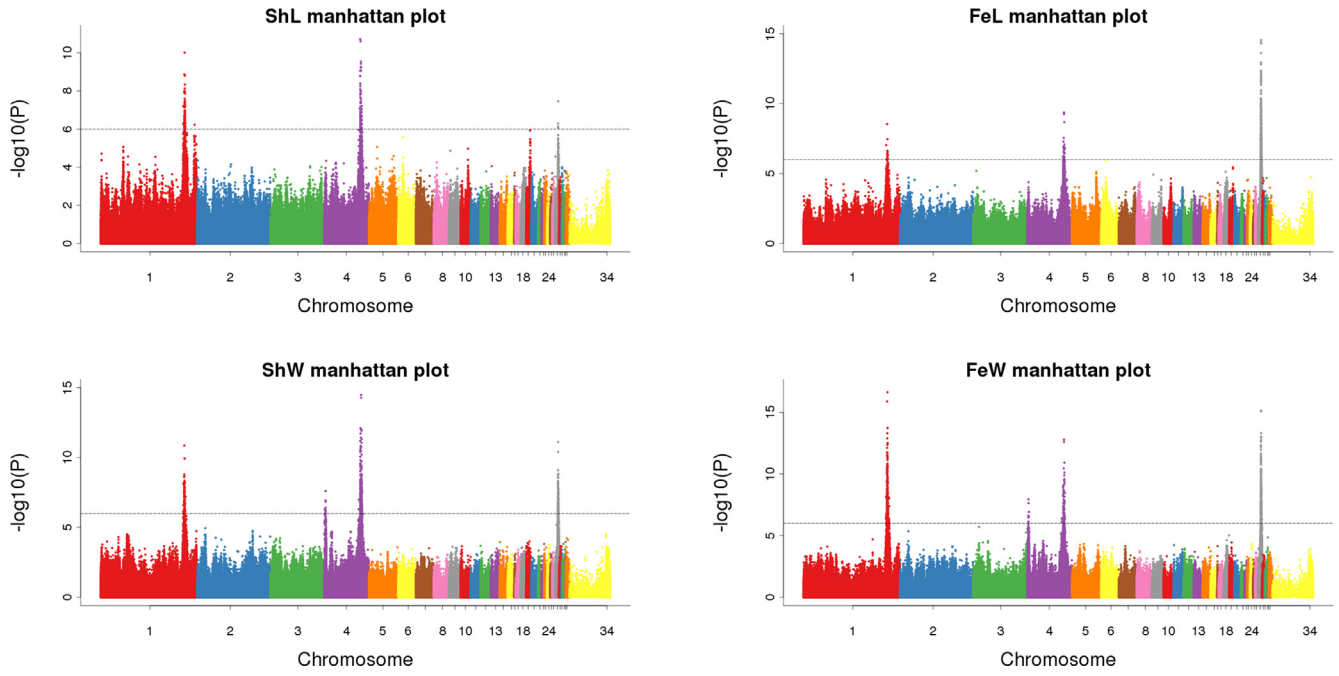

Fig. 2. Manhattan plot for the association analyses of leg bone traits in an F_2 chicken population. Leg bone traits include FeL, FeW, ShL, and ShW. In the Manhattan plots, -log10 (*P*-value) of the filtered high-quality SNPs (y-axis) is plotted against their genomic positions (x-axis); SNPs on different chromosomes (1 to 34) are denoted by different colors. FeL = femur length, FeW = femur weight, ShL = shank length, ShW = shank weight, SNPs = single-nucleotide polymorphisms.

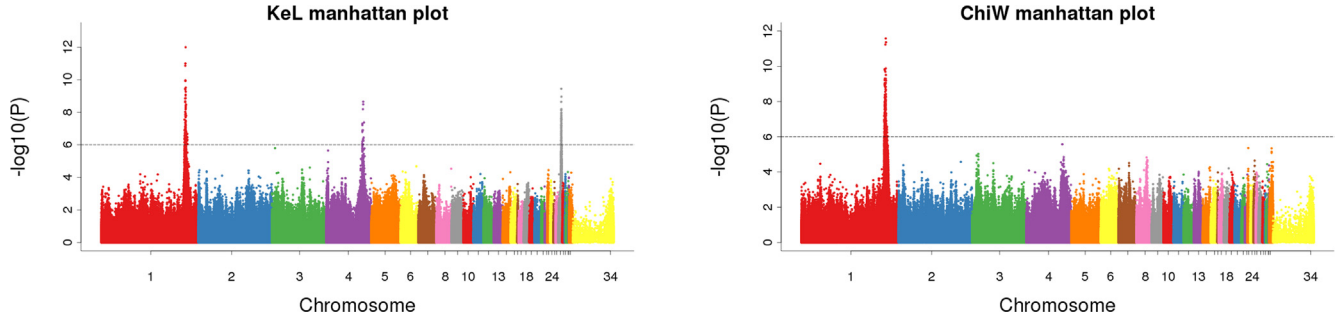

Fig. 3. Manhattan plot for the association analyses of body size traits in an F_2 chicken population. Body size traits include KeL and ChiW. In the Manhattan plots, -log10 (*P*-value) of the filtered high-quality SNPs (y-axis) is plotted against their genomic positions (x-axis); SNPs on different chromosomes (1–34) are denoted by different colors. KeL = keel length, ChiW = chest width, SNPs = single-nucleotide polymorphisms.

SNP ($-\log_{10}(P) = 14.55$) for FeL was resided in the 5 963 295 bp of the intronic region of *ETV4* on chromosome 27.

Genome-wide association studies for body size traits

Based on the genome annotation, 63 genome-wide significant SNPs were distributed in exon regions, intronic regions, and intergenic regions. A total of 179 genes were screened according to the physical location of each significant SNP. As shown in Fig. 3, for chromosome 1, there was a top SNP located in the 171 411 019 bp of the intronic region of *SERPINE3* correlated with KeL and ChiW. For chromosome 4, there was a top SNP($-\log_{10}(P) = 8.66$) in the 75 112 116 bp of the intronic region of the *SLIT2* significantly related to KeL. For chromosome 27, a top SNP in the 5 816 207 bp of intergenic region between two genes *NXPH3* (distance = 14 678 bp) and *NGFR* (distance = 15 455 bp) was associated with KeL.

Selection signature analysis

The distribution of the selection region shared by F_{ST} and log2 ($\theta\pi$ ratio) on the chromosomes was shown in Fig. 4, Supplementary Figs. S1 and S2. We take femur and shank traits as examples to interpret the results in detail (Fig. 4). The selection signature regions of femur and shank on 34 chromosomes during natural selection or artificial domestication in the F_2 population were identified by combining F_{ST} and $\theta\pi$ ratios statistics. It was divided into two distinct selection regions at a 5% empirical distribution in femur and shank traits (High phenotypic values: HFeL, HFeW, HShL, and HShW; Low phenotypic values: LFeL, LFeW, LShL, and LShW). There were 354 candidate genes in the selected region of LShL (log2$\theta\pi$ratio ($\theta\pi$ LShL/$\theta\pi$ HShL) ≤ -0.30, $F_{ST} \geq 0.05$), 361 candidate genes in the selected region of HShL (log2$\theta\pi$ratio ($\theta\pi$ LShL/$\theta\pi$ HShL) ≥ 0.28, $F_{ST} \geq 0.05$). There were 179 candidate genes in the selected region of LShW (log2$\theta\pi$ratio ($\theta\pi$ LShW/$\theta\pi$ HShW) ≤ -0.31, $F_{ST} \geq 0.08$), 456 candidate genes in the selected region of HShW (log2$\theta\pi$ratio ($\theta\pi$ LShW/$\theta\pi$ HShW) ≥ 0.38, $F_{ST} \geq 0.08$). There were 334 candidate genes in the selected region of LFeL (log2$\theta\pi$ratio ($\theta\pi$ LFeL/$\theta\pi$ HFeL) ≤ -0.29, $F_{ST} \geq 0.05$), 438 candidate genes in the selected region of HFeL (log2$\theta\pi$ratio ($\theta\pi$ LFeL/$\theta\pi$ HFeL) ≥ 0.30, $F_{ST} \geq 0.05$). There were 148 candidate genes in the selected region of LFeW (log2$\theta\pi$ratio ($\theta\pi$ LFeW/$\theta\pi$

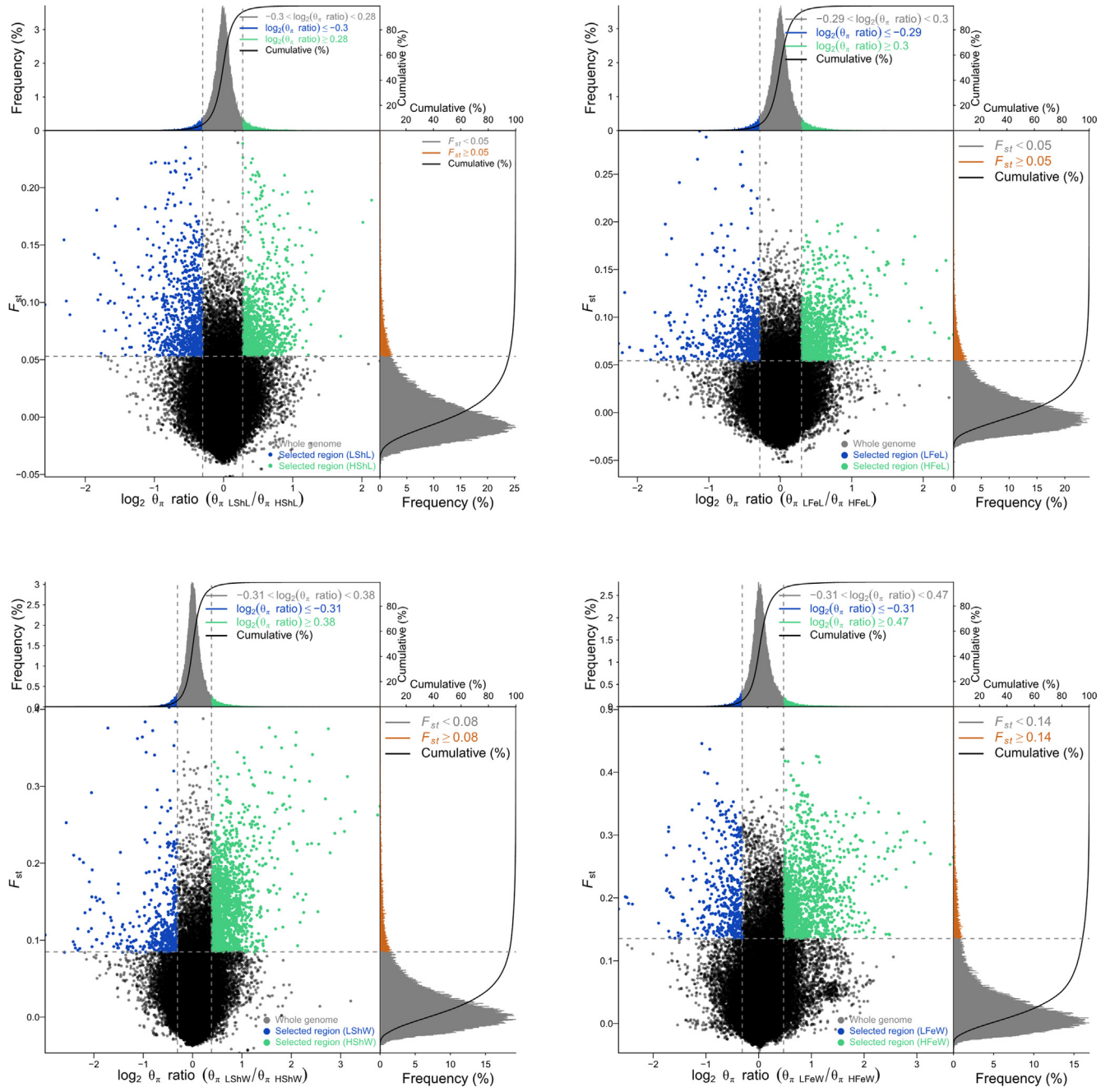

Fig. 4. Identification of high-quality selection regions of femur and shank using fixation index and nucleotide diversity (F_{ST} and θπ) in an F_2 chicken population. Data points (blue and green) are located on both sides of the left and right vertical dashed lines. Different colors represent different intersect regions. The frequency of the two methods' value is distributed in the right and top of the X, Y lines. FeL = femur length, FeW = femur weight, ShL = shank length, ShW = shank weight.

HFeW) ≤ −0.31, F_{ST} ≥ 0.14), 386 candidate genes in the selected region of HFeW (log2θπratio (θπ LFeW/θπ HFeW) ≥ 0.47, F_{ST} ≥ 0.14). It could be concluded that more regions of high phenotypic traits (green data points) were scanned than low phenotypic traits (blue data points) in ShW, FeL, and FeW.

Similar results were also found in KeL, ChiW (Supplementary Fig. S1) and MeCW (Supplementary Fig. S2). The number of detected genes in HKeL (440), HChiW (478), and HMeCW (324) region was higher than LKeL (395), LChiW (295), and LMeCW (195). For MeC and MeL, it can be observed that the genes detected in the HMeC region gradually increase with the weeks of age until it exceeds LMeC. Compared with LMeL, the HMeL region can always detect more genes (Supplementary Figs. S2 and S3).

Identification of candidate genes and functional enrichment analysis

We checked the distribution of selection signature regions and their overlap with GWAS results within the genomic windows to reveal the selected genes of chickens. A total of 166 underlying candidate genes were analyzed for functional enrichment. The bubble chart of the enrichment results of Gene Ontology terms is shown in Fig. 5a. These genes involve 23 biological process categories, 10 molecular functional categories, and participate in the composition of 14 cellular components (Supplementary Fig. S4). These genes are mainly enriched in Gene Ontology terms related to skeletal, such as embryonic skeletal system development and morphogenesis. Kyoto Encyclopedia of Genes and Genomes

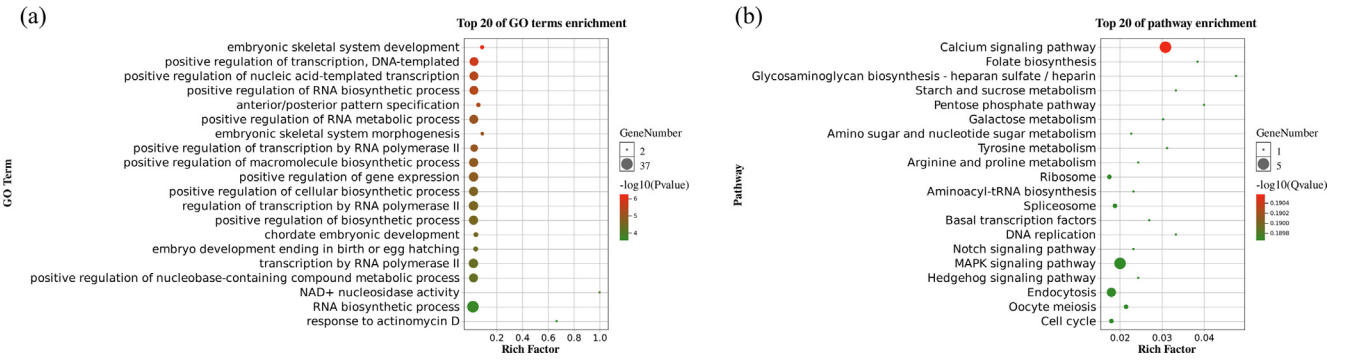

Fig. 5. GO and KEGG enrichment analysis of candidate genes in an F_2 chicken population. Figure (a) showed the top 20 enriched GO terms for bone traits. Figure (b) showed the top 20 pathway enrichments for bone traits. Rich Factor refers to the ratio of the number of genes with the term entry with respect to the total number of genes in the term entry. The larger the Rich Factor, the higher the degree of enrichment. The bubble size indicates the number of genes, and the color of the bubble indicates the level of significance. GO = Gene Ontology, KEGG = Kyoto Encyclopedia of Genes and Genomes.

Table 3
Potential candidate genes for bone traits revealed by integration of genome-wide association study (GWAS) and selection signature analysis in an F_2 chicken population.

Traits	Gene name	Description	Chromosome	Position (bp)
FeW, MeCW, ChiW, MeC8, MeC10, MeC12	*LRCH1*	leucine rich repeats and calponin homology domain containing 1	1	169 520 469–169 644 616
KeL, FeW, MeC8 MeC10, MeC12	*RB1*	retinoblastoma 1	1	170 072 965–170 150 029
ShW, FeW, MeCW, ChiW, MeC6, MeC8, MeC10, MeC12	*FNDC3A*	fibronectin type III domain containing 3A	1	170 318 524–170 431 952
ChiW	*MLNR*	motilin receptor	1	170 432 827–170 436 669
ShW, FeW, MeCW, MeC6, MeC8, MeC10, MeC12	*CAB39L*	calcium binding protein 39 like	1	170 465 092–170 526 727
ChiW, FeL, ShL ShW, MeCW, FeW, MeC8, MeC10, MeC12	*FOXO1*	forkhead box O1	1	171 900 263–171 963 540
FeL, MeL12, ShL, MeL10, ShW, MeC12, MeCW, FeW, MeC8, MeC10	*LHFP*	lipoma HMGIC fusion partner	1	172 287 762–172 427 229
MeC10, MeC12	*TRPC4*	transient receptor potential cation channel subfamily C member 4	1	173 127 352–173 268 522
MeC10	*POSTN*	Periostin	1	173 372 467–173 406 144
MeCW, FeW, ChiW	*SMAD9*	SMAD family member 9	1	173 744 731–173 786 052
KeL, ShL, ShW, FeW, MeCW, MeC8, MeL12	*RBPJ*	recombination signal binding protein for immunoglobulin kappa J region	4	73 221 773–73 366 204
FeL, ShL, ShW, FeW, MeCW, MeC8, MeC10, MeL12	*PPARGC1A*	PPARG coactivator 1 alpha	4	73 757 222–74 103 608
FeL, KeL, ShW, ShL, MeCW, FeW, MeC6, MeC8, MeC10, MeC12, MeL12	*SLIT2*	slit guidance ligand 2	4	74 981 753–75 225 786
ShW, MeCW, MeC8, MeL12	*NCAPG*	non-SMC condensin I complex subunit G	4	75 897 761–75 920 718
MeC8	*NKX3-2*	NK3 homeobox 2	4	77 421 691–77 423 639
MeC8	*CPZ*	carboxypeptidase Z	4	81 163 348–81 197 855
KeL, FeL	*SPOP*	speckle type BTB/POZ protein	27	5 759 689–5 784 701
KeL, FeL	*NGFR*	nerve growth factor receptor	27	5 831 662–5 843 100
FeL, KeL	*SOST*	Sclerostin	27	5 900 500–5 917 997
FeL	*ZNF652*	zinc finger protein 652	27	6 000 724–6 028 986
FeL, ShW	*HOXB3*	homeobox B3	27	6 248 848–6 273 287

Abbreviations: MeL4-12 = metatarsal length at 4, 6, 8, 10 and 12 weeks of age; MeC4-12 = metatarsal circumference at 4, 6, 8, 10 and 12 weeks of age; KeL = keel length; ChiW = chest width; FeW = femur weight; FeL = femur length; ShW = shank weight; ShL = shank length; MeCW = metatarsal claw weight.

analysis also strongly suggests that candidate genes are involved in the calcium signaling pathway (Fig. 5b). To further identify candidate genes related to bone traits, we queried the NCBI database and previous literature to find genes related to animals or human bone growth and development, and bone cell differentiation. Eventually, 21 underlying candidate genes related to bone traits were identified. A list of potential genes was shown in Table 3. In addition to these known genes, we also found 65 uncharacterized genes in this study, including 56 non-coding RNA genes, six protein-coding genes, and three snoRNA genes (Supplementary Table S1).

Discussion

The chicken growth rate has increased significantly in the last 50 years. However, the bone system was not strong enough to support BW in chickens with high growth rates. The bone growth and development of chickens are affected by heredity, nutrition, environment, and diseases, among which heredity is the most critical factor (Guo et al., 2017). Dissection of the genetic architecture of bone traits is conducive to genetic improvement. Due to the genetic hitch-hiking effect and relatively large QTL confidence intervals of F_2 population (Johnsson et al., 2014), we adopted a feasible combining strategy that combines single-marker GWAS methodology and selection signature analysis (F_{ST} & $\theta\pi$) to explore the candidate genes associated with bone traits that exist at the genome-wide level.

In the present study, significant genetic variations for bone traits were identified on chromosomes 1, 4, and 27. A top SNP located on the gene *SERPINE3* was associated with three different types of bone traits (metatarsus, body size, and leg), suggesting

that the same SNP or gene was probably involved in the phenotypic variation of these traits. The same gene or variant influences more than one phenotypic trait, known as pleiotropy, which is pervasive in the genetic architecture of domestication in the chickens (Wright et al., 2010). It is confirmed by our data that there are highly positive genetic correlations among 17 bone traits in this study (Table 2). Also, a large number of previous studies have revealed the positive genetic correlations between the weight and length of the tibia, shank and femur (González-Cerón et al., 2015a and 2015b; Guo et al., 2020).

Selection signature analysis revealed, compared with low phenotypic traits, more genomic regions for high phenotypic traits were scanned in ShW, FeL, and FeW (Fig. 4). It could be attributed to a rapid increase in chicken BW through long-term selection in the past half-century. The leg bones are an indispensable part of chicken body and serve supporting chicken BW. Accompanied with rapid growth of BW, leg bones such as ShW, FeL and FeW must be strong enough to support growing BW. Therefore, there is a close relationship between leg bones and BW. It is evident that long-term selection for BW may also affect leg traits in broilers (González-Cerón et al., 2015a and 2015b). Unlike the above three traits, natural or artificial selection does not seem to be inclined to HShL. It may be due to the fact that shank length affects chicken leg health and longer shanks were considered a source of leg problems in chickens (Deeb and Lamont, 2002).

There were 166 known candidate genes co-mapped by GWAS and selection signature analysis. The annotation strongly revealed a term of skeletal system development and a pathway of calcium signaling (Fig. 5a and b), which were related to bone traits. Importantly, we note that some genes related to bone growth and development have been verified in previous studies. *ZNF652* plays a vital role in the bone growth of chickens (Wang et al., 2020). *LHFP* is identified as a key regulator of osteoblast activity and bone mass in mice by GWAS and system genetics (Mesner et al., 2019). *SAMD9* is a transcriptional regulator of BMP signaling (Tsukamoto et al., 2014) and has a rare mutation associated with high bone mass (Gregson et al., 2020). *SLIT2* plays a role in regulating in vitro osteoblast differentiation (Sun et al., 2009) and can inhibit osteoclastogenesis and bone resorption (Park et al., 2019). *PPARGC1A* plays important roles in skeletal homeostasis (Wei et al., 2010). *NKX3-2* is capable of controlling hypertrophic maturation of cartilage in vivo, and this regulation plays a significant role in endochondral ossification and longitudinal bone growth (Kawato et al., 2011; Jeong et al., 2017). *RBPJ* plays a key role as an upstream negative regulator during osteoclastogenesis (Zhao et al., 2012; Li et al., 2014). *LRCH1* has been proved to be an osteoarthritis susceptibility locus in many studies (Snelling et al., 2007; Zintzaras et al., 2010; Panoutsopoulou et al., 2017). *CPZ* modulates Wnt signaling and regulates the development of skeletal elements in the chicken (Moeller et al., 2003) and rat (Wang et al., 2009). *POSTN* is a candidate gene for bone mineral density variation and vertebral fracture risk (Xiao et al., 2012) and the latest research shows that *POSTN* may be a novel target gene for anti-osteoporosis therapies (Li et al., 2020). In recent years, many pieces of literature supported that *FOXO1* is a major regulator in bone mass homeostasis and osteoblast physiology (Ambrogini et al., 2010; Rached et al., 2010; Zhang et al., 2018). Extensive animal experimentation and clinical studies demonstrated that the deficiency of *SOST* facilitates bone formation (Delgado-Calle et al., 2017). *SOST* inhibition may be advantageous to prevent secondary fracture(s) in adult male rats (Suen et al., 2015). Sclerostin encoded by *SOST* affects bone quality in layer chickens (Guo et al., 2017). A recent GWAS (Guo et al., 2020) showed that *SPOP*, *NGFR*, and *HOXB3* are associated with tibia length and mass, femur length and area, and shank length; *TRPC4* and *CAB39L* are associated with bone size or mass; a promising locus within *NCAPG* on chromosome 4 is associated with tibia length and mass, femur length and area, and shank length. *SPOP* is an important positive regulator of Ihh signaling and skeletal development and homeostasis (Cai and Liu, 2016). It was worth pointing out that the genes *FNDC3A*, *MLNR*, *CAB39L*, and *RB1* related to bone traits we mapped were consistent with the results of our previous fine-mapping of QTL for bone traits in the same F_2 population (Zhang et al., 2011). Compared to our previous study, this study utilized a substantially larger number of SNPs, which resulted in higher resolution and more potent power of detecting QTLs linked to bone traits.

In addition to literature search of the above genes, we also aligned the positions of these genes with bone traits QTL listed in the chicken QTL database. The positions of 19 candidate genes above on chromosomes showed overlapping confidence intervals with or near (distance from the QTL < 1.50 Mb) the chicken QTL database (Supplementary Table S2) except for RBPJ and CPZ. These two genes can be used as new supplements to chicken QTL database.

Conclusion

In this study, the analysis of colocalization of GWAS and selection signatures markedly shortened the list of candidate genes and significant variant(s) in each region. The results exhibited that an array of genes, including *LRCH1*, *RB1*, *FNDC3A*, *MLNR*, *CAB39L*, *FOXO1*, *LHFP*, *TRPC4*, *POSTN*, *SMAD9*, *RBPJ*, *PPARGC1A*, *SLIT2*, *NCAPG*, *NKX3-2*, *CPZ*, *SPOP*, *NGFR*, *SOST*, *ZNF652*, and *HOXB3*, was candidates for kinds of bone traits in chickens. These positional candidate genes will be strong candidates to determine the underlying mechanism of bone growth and development.

Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.animal.2021.100322.

Ethics approval

The Laboratory Animal Management Committee approved all studies involving animals of Northeast Agricultural University. This study was carried out following the Care and Use of Experimental Animals guidelines established by the Ministry of Science and Technology of the People's Republic of China (approval number 2006-398).

Data and model availability statement

None of the data were deposited in an official repository. Data may be available upon request by contacting the corresponding author.

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Declaration of interest

The authors declare to have no conflicts of interest.

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