Genome-wide Association Study Reveals the *IBSP* Locus Affect Ear Size in Cattle

Jiafei Shen
Northwest A&F University: Northwest Agriculture and Forestry University
Xiaoting Xia
Northwest A&F University: Northwest Agriculture and Forestry University
Qiuming Chen
Northwest A&F University: Northwest Agriculture and Forestry University
Fengwei Zhang
Northwest A&F University: Northwest Agriculture and Forestry University
Bizhi Huang
Yunnan Academy of Grassland and Animal Science
Quratulain Hanif
Pakistan Institute of Engineering and Applied Sciences
Fangyu Li
Northwest A&F University: Northwest Agriculture and Forestry University
Ningbo Chen
Northwest A&F University: Northwest Agriculture and Forestry University
Kaixing Qu
Yunnan Academy of Grassland and Animal Science
Jianyong Liu
Yunnan Academy of Grassland and Animal Science
Jicai Zhang
Yunnan Academy of Grassland and Animal Science
Yongzhen Huang
Northwest A&F University: Northwest Agriculture and Forestry University
Ruihua Dang
Northwest A&F University: Northwest Agriculture and Forestry University
Xianyong Lan
Northwest A&F University: Northwest Agriculture and Forestry University
Hong Chen
Northwest A&F University: Northwest Agriculture and Forestry University
Yu Jiang
Northwest A&F University: Northwest Agriculture and Forestry University
Chuzhao Lei (leichuzhao1118@126.com)

Northwest Agriculture and Forestry University College of Animal Science and Technology
https://orcid.org/0000-0003-1647-1037

Research

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Abstract

Background

Ear size is a classical model for **hot climate** adaptation following evolution, however, the genetic basis of the traits associated with the ear size remains to be elucidated.

Results

In the current study, we performed GWAS on 158 cattle individuals to to explain the genetic mechanism of ear size. The results suggested significant association of *IBSP locus* with ear size. A missense mutation (Threonine-250→Isoleucine) on the seventh exon of *IBSP* was observed, which occurred at a quite conserved site and changed the three-dimensional (3D) structure simulations. In addition to GWAS, 14 cattle breeds were screened for the selection signals associating with the ear size using F_{st} and SweepD. The selective sweep analysis also suggested that *IBSP* was under positive selection amongst 4 breeds with relatively large ear size. The allele distribution of this mutation was validated among 394 samples from 21 worldwide cattle breeds, which strongly implied the origin of the A allele mutation to be from the *Bos taurus*.

Conclusions

These findings not only have important theoretical significance for the exploration of major genes associated with the ear size but also provide important molecular markers for the identification of cattle germplasm resources.

Background

Ear is an auditory organ capable of converting sound waves into nerve impulses (1). The central nervous system in the brain then translates these impulses into a spoken language, birds chirping or the cow's moo, and so on. Besides auditory perception, ear serve other vital functions as well, like heat radiation (2), sense of balance (3–5) and external signals in the mood (6). Ear consist of three sections: the external, middle, and the inner ear (7). The external ear includes an external auditory canal (8), eardrum and a visible pinna that resides outside of the head, and it can collect sound and directs it down the ear canal. Over the course of time, mammals adapted to their respective environmental conditions and evolved an extraordinary variety of ear shapes and sizes. For example, animals bearing high temperature and hot environment, evolved larger ears to combat the heat waves, such as African elephants. African elephants adjust their body temperature through their large ears, providing shade to reduce the loss of water in the body (9). Furthermore, a general observation includes larger ear sizes in indicine cattle harboring in the harsh hot environment, such as Brahman and Burmese. On the contrary, most animals inhabitant in the tropical or colder regions tend to have smaller ear sizes, such as the Arctic fox. The smaller size of ear can help the mammals to reduce heat dissipation and maintain their body temperature. Likewise, the

cattle adapted to the cold environments such as Yanbian, Yakut, and Tibetan cattle, all share smaller ear areas trait.

Size and orthotropism are important conformational characteristics of ears with huge diversity in different species. With the development of whole-genome resequencing technology, genome-wide association study (GWAS) has recently been applied to reveal SNPs associated with complex ear traits in livestock such as pig, dog and sheep. Previous studies have shown that significant quantitative trait loci (QTL) for pig ear size were located on *Sus scrofa* chromosome (SSCs) 5 and 7 (10, 11). A missense mutation (*G32E*) in the *PPARD* gene on SSC7 was considered to be responsible for the differences in porcine ear size (12, 13). QTL fine mapping on SSC5 revealed that *LEMD3* was one of the most important candidates for porcine ear size (14). Moreover, GWAS analysis revealed that *DCC*, *SOX5*, and *PTPRD* were potential candidate genes for ear size in sheep (15). Furthermore, genome fragments containing (*WIF1* and *HMGA2*) also appeared to control ear size in both pigs and dogs (16). However, there is no current report on the ear size of cattle, although the available resources of cattle are very rich worldwide.

Cattle is not only important its meat and milk production but also an important large-animal model for human disease. Among different cattle breeds, ear size is rich in diversity, *Bos indicus*, such as Brahman, with unusually large and lop ears. In contrast, *Bos taurus* have small and erect ears. According to this phenomenon, ear size may be regarded as an important characteristic distinguishing *Bos taurus* and *Bos indicus*. China has a wide geographical latitude span, with taurine and indicine cattle breeds distributed from north to south, and it is a natural cattle breed resource bank for studying ear size. Yunling cattle is a typical hybrid of Brahman bull × Murray Grey bull/Yunnan indigenous cross cow, which is an ideal model for studying the genetic basis of complex traits, such as ear size trait. In the current study, GWAS was performed to screen out potential candidate genes, which were further systematically analyzed to identify the associated loci and the candidate mutation in the particular gene or region. Our findings provide new insights into the genetic basis of ear size of cattle and provide important molecular markers for the identification of cattle germplasm resources.

Methods

Ethics statement

According to the guidelines from the Council of China and the requirements of animal welfare, the relationship between researchers and cattle were handled very kindly. According to the recommendation of the Regulations for the Administration of Affairs Concerning Experimental Animals of China, the Institutional Animal Care and Use Committee of Northwest A&F University approved all animal experiments.

Sample collection and genome sequencing

Individuals used for ear size trait comprised of 158 cattles (39 Brahman and 119 Yunling cattle). Yunling cattle is a typical hybrid cattle breed, bred by Yunnan Academy of Grassland and Animal Science. The ear

tissue and blood samples were collected from adult females. The ear-level photos (with standard tick labels) and body size data of corresponding individuals were also collected in this study. The standard phenol-chloroform protocol was used to extract the genomic DNA from the ear (17). The sequence data used in this paper was obtained from published papers where detailed information about sampling and sequencing was available (18).

Measurement and calculation of ear size

In this study, we used the “living pixel method” to collect the ear shape photo with a standard scale of 475 cattles (39 Brahman, 119 Yunling, 65 Simmental 52 Burmese, 100 Wenshan, and 100 Dabieshan cattle; **Table S1**). The head of the cow was restrained and scale labelled left ear was flattened for the picture. Digital images were taken with a high - pixel camera in a horizontal position. Later the ear size of each animal was measured using Photoshop CS6 (Adobe, American). Firstly, the graph of the ear range and the graph of 1 cm^2 in the scale were obtained through the “magnetic lasso” tool, and the pixel numbers corresponding to the above two were obtained through the “histogram” tool. The size of the ear area could be obtained through the “measuring record” tool and the pixel ratio column.

Reads mapping and SNP calling

Default parameters were used to map clean reads to the cattle reference assembly GCF_002263795.1 using BWA-MEM (19). Duplicate reads were filtered using the “REMOVE_DUPLICATES = true” option of Picard tools. The “HaplotypeCaller”, “GenotypeGVCFs” and “SelectVariants” argument of Genome analysis toolkit 3.8 (GATK) (20) were used for calling the raw SNPs. The average alignment rate and coverage were 99.54% and 5.61×, respectively. The argument “VariantFiltration” of the same software was applied to all raw SNPs with following options: QD < 2, FS > 60, MQRankSum < -12.5, ReadPosRankSum < -8.0 and mean sequence depth (for all individuals) > 1/3 × and < 3x. In addition, the haplotype-phase inference and missing alleles imputation were produced using Beagle (21) to carry out GWAS further. Based on ~ 41 M autosomal SNPs, we estimated the eigenvectors using smartPCA of EIGENSOFT v5.0 package (22) to adjust population structure in GWAS. The principal component 1 (PC1) based on the genotype matrix separated Brahman cattle from Yunling cattle, which is in accordance to the previous studies (18).

GWAS analysis

Based on the 158 sequenced genomes (**Table S2**), a total of 13,057,965 SNPs (MAF > 0.10; missing rate > 0.1) were used in GWAS for ear size trait. The primary association analysis was carried out using a genome-wide efficient mixed-model association (GEMMA) software package (23). The mixed linear model assumed the following model:

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

where, y is a vector of phenotypes; α is a vector of fixed effects representing marker effects; β is a vector of fixed effects representing non-marker effects, and μ is a vector of unknown random effect. X, S, and K represent the incidence matrices relating α, β, and μ, respectively, and ε represents a vector of random

residual effects. The top three PCs and feeding regimes were defined as the S matrix. The kinship matrix calculated from nucleotide polymorphism was defined as the K matrix.

The secondary association analysis used multiple linear regression model using PLINK (24), combining body height, cross high, head length, head width and first PC as covariates to perform genome-wide association analysis on the ear size phenotype. To estimate the correction required for multiple testing, the number of linkage disequilibrium (LD)-pruned SNPs (750367) were defined as the effective number of independent SNPs, was calculated using PLINK (–indep-pairwise 50 5 0.2). Therefore, the significance and suggestive threshold was defined as approximately 5×10^{-8} (0.05/750,367) and 1×10^{-6} (1/750,367), respectively. In fact, the thresholds were widely used by numerous studies.

Identification of candidate genes in the GWAS associated loci

We used the following strategy to narrow down our findings. Firstly, PLINK (24) was used to estimate candidate regions by pairwise linkage disequilibrium correlation ($r^2 > 0.6$) between SNPs related to ear size characteristics and the count of suggestive SNPs < 3 were used as false positives and removed. Secondly, the SNPs (leading SNPs) with the P_{wald} values < 1×10^{-6} were characterized as the candidate SNPs, and functional annotation of suggestive associated SNPs was carried out according to the *Bos taurus* reference genome in package ANNOVAR version (25).

Selective sweep analysis

Based on phenotype relationship of ear size, the genomes of big ear size group and small ear size group was compared to identify signatures of positive selection. Firstly, the genome-wide distribution of F_{ST} values was estimated using VCFtools (26) with the 100 kb window size and 50 kb increment to investigate pairwise genetic differentiation among big and small ear groups. To further indentify the selection signials within each cattle breeds, we used SweepD (Sweep Detector), an open-source tool for the rapid detection of selective sweeps in whole genomes(27). We also calculated the SweepD for 14 cattle breeds (Angus, Brahman, Burmese, Hanwoo, Hereford, Holstein, Kazakh, Ji'an, Longlin, Mishima, Mongolian, Muturu, Simmental and Yanbian cattle) using SweepFinder2 (28) with a sliding window approach (100 kb windows with 50 kb increments). Only the strong regions overlapping both methods (SweepD: top 1%, F_{ST}: top 1%) were defined as candidate regions under positive selection. A custom perl script was used to annotate the regions under selection based on the *Bos taurus* reference genome (ARS-UCD1.2).

Results

Ear size variation traits analyses

The ear size among different cattle individuals and breeds were obviously different. As a general observation, the ear size of *Bos indicus* in Indo-Pak region was rather bigger than the *Bos taurus* in the

world, while hybrid cattle breeds depicted diversified ear size (Fig. 1a**)**. According to this observational phenomenon, the ear size data was collected and calculated from 475 adult female cattle include 6 representative breeds(i.e. Burmese, Brahman, Simmental, Yunling, Wenshan and Dabieshan cattle) using pixel method (29). The overall ear size data from 87.81 to 330.85 cm^2. *Bos indicus* (Brahman and Burmese cattle) have the largest mean values, which were 226.75 cm^2 and 254.06 cm^2, respectively. In contrast, *Bos taurus* (Simmental) has the small mean value, which was 134.62 cm^2. The size of hybrid breed (Yunling) and Chinese indicine (Wenshan and Dabieshan cattle) were relatively scattered, with an average values 164.21, 186.31, and 184.37, respectively (Table 1). The ear size distribution of all individuals showed a unimodal distribution according the histogram and density plot (Fig. 1b). And it can be well distinguished in three distinct intervals: big ear (> 230 cm^2), middle ear (< 230 cm^2 & >148 cm^2) and small ear (< 148 cm^2) with 25% and 75% quantiles of the all individuals' boxplot analysis (Fig. 1c). Boxplot and *F* statistic was used to analyze the variance in the ear size among six cattle breeds. The results showed that *Bos indicus* depicted largest ear size, while *Bos taurus* represented the smallest ear size, while the hybrid (Yunling) and the Chinese indicine (Wenshan and Dabieshan cattle) ranged in the middle (Fig. 1c). Moreover, the *F* statistic results also suggested that the ear size among cattle breeds were significantly different, supporting the above statement.

Table 1
Descriptive statistics of ear size in cattle breeds

Breeds	Origin	Number	Maxmum	Minmum	Mean	CV
Simmental	*Bos Taurus*	65	166.26	92.67	134.62	10.53%
Brahman	*Bos indicus*	39	330.85	140.86	226.75	15.68%
Burmese	*Bos indicus*	52	313.53	212.46	254.06	8.50%
Yunling	*Bos indicus* × *Bos Taurus*	119	268.71	87.81	164.21	18.88%
Wenshan	Chinese indicine	100	315.87	95.47	186.31	22.28%
Dabie mountain	Chinese indicine	100	249.86	120.63	184.37	13.75%

[a]Coefficients of variation

Two genome-wide association studies for ear size traits

Based on 13,057,965 autosomal SNPs derived from the 158 published resequencing cattle data (18), a genome-wide efficient mixed-model association (GEMMA) (23) was used in the primary genome-wide association studies to identify the significant loci. Figure 2a showed the Manhattan plot for the GWAS. The red and green horizontal lines represent the Bonferroni-adjusted genome-wide significant and suggestive threshold. A total of 3 significant SNPs and 40 suggestive SNPs showed genome-wide associations with ear size (**Table S3).** However, most of the significant potential SNPs were located on

BTA 6 (36.79 ~ 38.90 Mb) (Fig. 2b). The significant locus ($P = 1.29 \times 10^{-8}$) was located in *LOC112447053*, and near *MEPE* and *IBSP*. (Fig. 2c). The Quantile-Quantile plot (QQ-plot) in Fig. 2d showed the observed and expected *P*-values of the GWAS for ear size. The dashed line represents the distribution of the SNPs under the null hypothesis that there is no association of SNPs with the trait of interest. The strong deviation of the observed from the expected P-values for QQ-plots indicate that there were more SNPs significantly associated with all of the ear size trait than would be expected by chance.

Since, body size traits and ear size data could have a shared underlying genetic basis, the correlation was measured and analyzed for each trait in **Figure S1**. Pearson correlation coefficient was used to assess the linear relationships of ear size and body size traits. The result showed that the body height, cross high, head length and head width had a weak-to-moderate positive correlation with the ear size trait. The multiple linear regression model was analyzed using these related traits as covariates for secondary GWAS analysis using PLINK (24). The autosomal SNP scan for ear size revealed associated markers as showed in **Figure S2.** A total of 293 SNPs were observed on the potential region and most of them were found on BTA6. The most significant SNP ($P = 5.74 \times 10^{-11}$) was located on the intron of *IBSP*. The two GWAS results strongly suggested that *IBSP* had a strong correlation with ear size and might be a key candidate gene influencing cattle ear size.

Genetic differentiation of the mutation in IBSP among different ear size groups

To clarify whether ear size was selected in different cattle breeds, pairwise fixation index (F_{st})(30) was used to measure the genetic differentiation between big (Brahman, Burmese) and small ear group (Simmental, Yanbian cattle). By annotating the significant regions ($F_{st} > 0.66$, empirical *P*value < 0.005), *IBSP* was found in the most significant region on BTA6 (position: 36,840,001 ~ 36,890,000, $F_{st} = 0.75$) (Fig. 3a). To further investigate the differentiation of these mutation loci across diverse cattle breeds, 14 cattle breeds were used for nucleotide diversity ($\theta\pi$) and SweepD analysis (**Figure S3, S4**) (31, 32). The results showed that the SweepD value on these loci were observed to be near zero in *Bos taurus* (Angus, Kazakh, Hanwoo, Hereford, Holstein, Mishima, Simental, and Yanbian cattle) and over 200 in South Asian indicine (Brahman, Burmese), which means that *IBSP* may be selected in indicine breeds (Fig. 3b). According to the functional annotation, a missense mutation was found on the seventh exon of *IBSP* (Threonine-250→Isoleucine, T250I) (Fig. 3c). To further evaluate the functional impact of the variants, we aligned the mutant *IBSP* protein with its ortholog proteins in bovidae (Fig. 3d) and other diverse vertebrates (**Figure S5**). The comparison reveals that T250I was a quite conserved amino acid mutation, which is invariant among all the other animals we examined except *Bos Taurus*. We calculated the linkage disequilibrium (LD) values of the SNPs, which were shared in the region of *IBSP*. We observed strong linkage in this region (**Figure S6**). At the same time, prediction based 3D structure of protein showed that the mutation site of *IBSP* can change its protein structure (Fig. 3e). All those results imply that T250I is most likely causal mutation for the *IBSP* sweep in cattle ear size.

Allele frequency of IBSP mutation among different cattle breeds indicated the origin

In our study, we genotyped the DNA sequences of *IBSP* with 394 cattle representing *Bos indicus*, *Bos taurus* and hybrid cattle to investigate the genotype frequency of the mutation loci across diverse cattle breeds. Two genotypes (A and G) of the *IBSP* mutation loci were found (Fig. 4a, 4b). The A allele of *IBSP* mutation occurred at high frequency in Burmese (0.06), Brahman (0.30) and Lingnan (0.40), respectively. In Wenshan, Shigatse, Weining, Hainan, Dianzhong, Jinjiang, Ji'an, Wannan and Luxi cattle, the A allele of the two mutation showed a moderate frequency close to 0.5. In contrast, the A allele was almost equal to one in Yanbian, Kazakh, Simmental and other *Bos taurus* (Fig. 4a, **Table S4**). At the same time, we searched the mutation frequency of *IBSP* among different cattle breeds in the world from the Bovine Genome Variation Database and Selective Signatures (BGVD, http://animal.nwsuaf.edu.cn/code/index.php/BosVar) (33). The database contained 24 South Asian indicine cattle, 19 Chinese indicine, 37 East Asian taurine, 38 European taurine, 19 Eurasian taurine and 10 Africa taurine cattle. The results showed that the A allele frequency of *IBSP* was highest in South Asian indicine (0.10), and the frequency was 0.47 in Chinese indicine, respectively. However, the A frequency of *IBSP* was near or equal to one in East Asian taurine, European taurine and Eurasian taurine (Fig. 4b). The allele distribution indicates that the A allele of *IBSP* may have originated in *Bos taurus*. Due to the infiltration of South Asian indicine, the Chinese indicine caused the reduce in the frequency of A allele. We also found the G allele in African taurine at low frequency, which may be due to the large introduction of South Asian indicine after the African rinderpest in 1890 (34).

Discussion

The external ear of cattle is mainly composed of cartilage and thin layers of skin. The function of cartilage in the external ear is a stent which determine the size and the shape of the ear (35). In the current study, *IBSP* as the main candidate gene influencing the ear size of cattle were screened out with two GWAS methods. In addition to the functional analysis, this gene is potentially the biological candidate genes for ear size characteristics. Integrin Binding Sialoprotein (*IBSP*) had been mentioned many times in numerous studies of cartilage and chondrocytes. The main *IBSP*-knockout mice showed a shorter body, reduced chondrocyte proliferation, and impaired cartilage absorption. At the same time, the lack of *IBSP* can alter bone growth, the formation and mineralization of primary bone (36). The protein encoded by *IBSP* is the main structural protein in the bone matrix, and nearly 12% non-collagenous proteins are from *IBSP* in human osteoblasts, hypertrophic chondrocytes and osteoclasts (37–40). *IBSP* is mainly related to tissue development and cell growth, which may relate to its mechanism in promoting the growth and proliferation in bovine ear chondrocytes.

We discovered and validated one missense mutation at *IBSP* in *Bos taurus*, of which T250I occurred in a well-defined domain and quite conserved site (Figure S5). In nearly 70 vertebrate species, this mutation was conserved, except for *Bos Taurus*. We also found this missense mutation in *IBSP* altering the 3D structure of the expressed protein, which may change the function of the protein. At the same time, the statistical analysis of *IBSP* missense genotype among different cattle breeds showed that GG genotype was mainly present in *Bos indicus* with large ear size, AA genotype was mainly present in *Bos taurus* which had small ear size, and three genotypes were present in hybrid cattle which was living in south of

China and with complex ear size. Through the mutation frequency of *IBSP* missense variant among different cattle breeds, we found that the lowest frequency of A allele was in South Asian indicine, the frequency was near or equal to one in East Asian taurine, European taurine and Eurasian taurine. Moreover, the mutations of the A allele was mainly found in cattle with small ear size living in cold environments, while the relatively large ear size of cattle living in tropical or subtropical regions had almost no mutation frequency. We speculated that the missense mutation of *IBSP* may be the main factor that changes the cattle ear size, and speculate that the mutations of the A allele in *IBSP* may originate from *Bos taurus*. The difference in ear size may be due to the variable environmental conditions in which they are located. The larger ear area can help *Bos indicus* to dissipate heat temperature, and the small ear size may be more helpful for *Bos taurus* to keep their temperature in the cold environment. The temperature was not high or low in southern China, so hybrid cattle has the diverse ear size phenotype.

Conclusions

In conclusion, the *IBSP* loci related to cattle ear size was screened out by GWAS, and the missense mutation in *IBSP* (T250I) was speculated to be the candidate mutation affecting ear size among cattle breeds. These findings not only have important theoretical significance for the exploration of major genes of ear area traits but also provide important molecular markers for the identification of cattle germplasm resources.

Abbreviations

None

Declarations

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Availability of data and materials

The raw whole genome sequencing data were reported in our previous study(18) and has been availabled at the NCBI Short Read Archive under the BioProject accession number PRJNA555741.

Author information

Affiliations

Key Laboratory of Animal Genetics, Breeding and Reproduction of Shaanxi Province, College of Animal Science and Technology, Northwest A&F University, Yangling, Shaanxi 712100, China

Jiafei Shen, Xiaoting Xia, Qiuming Chen, Fengwei Zhang, Fangyu Li, Ningbo Chen, Yongzhen Huang, Ruihua Dang, Xianyong Lan, Hong Chen, Yu Jiang & Chuzhao Lei

Yunnan Academy of Grassland and Animal Science, Kunming, Yunnan 650212, China

Bizhi Huang, Kaixing Xu, Jianyong Liu, Jicai Zhang

National Institute for Biotechnology and Genetic Engineering, Pakistan Institute of Engineering and Applied Sciences, Faisalabad, 577, Pakistan

Quratulain Hanif

Contributions

Yu Jiang and Chuzhao Lei designed the study and supported the funding. Jiafei Shen and Qiuming Chen curated and analyzed the data. Jiafei Shen wrote the original manuscript. Quratulain Hanif and Xiaoting Xia reviewed and edited the manuscript. Fengwei Zhang, Kaixing Qu organized sampling and conducted fieldwork. All authors commented on the manuscript and gave final approval for publication.

Corresponding author

Correspondence to Yu Jiang and Chuzhao Lei

Ethics declarations

Ethics approval and consent to participate

This study was approved by Institutional Animal Care and Use Committee of Northwest A&F University following the recommendation of the Regulations for the Administration of Affairs Concerning Experimental Animals of China.

Consent for publication

Not applicable.

Competing interests

The authors declare no potential conflict of interest for this study.

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Figures

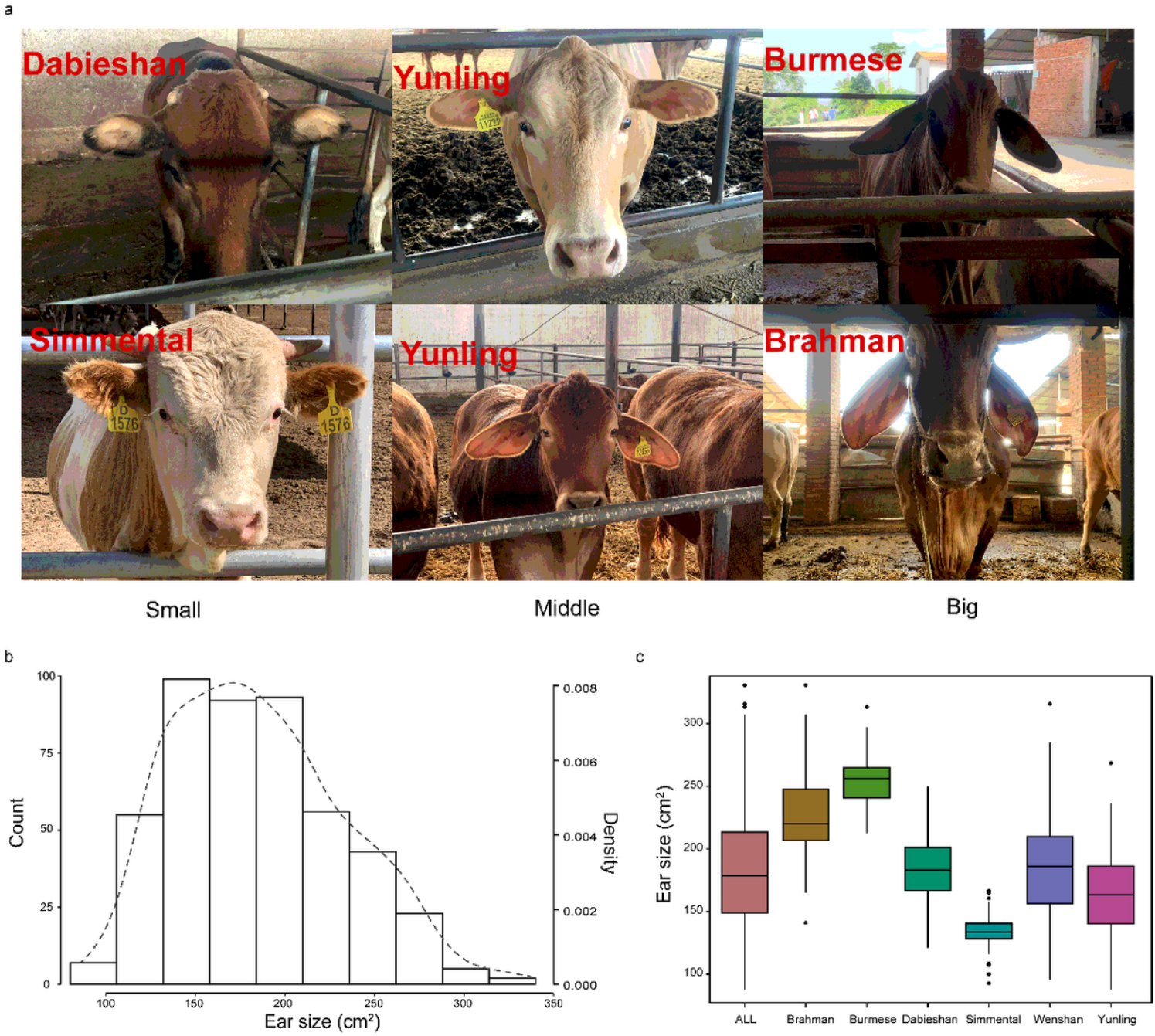
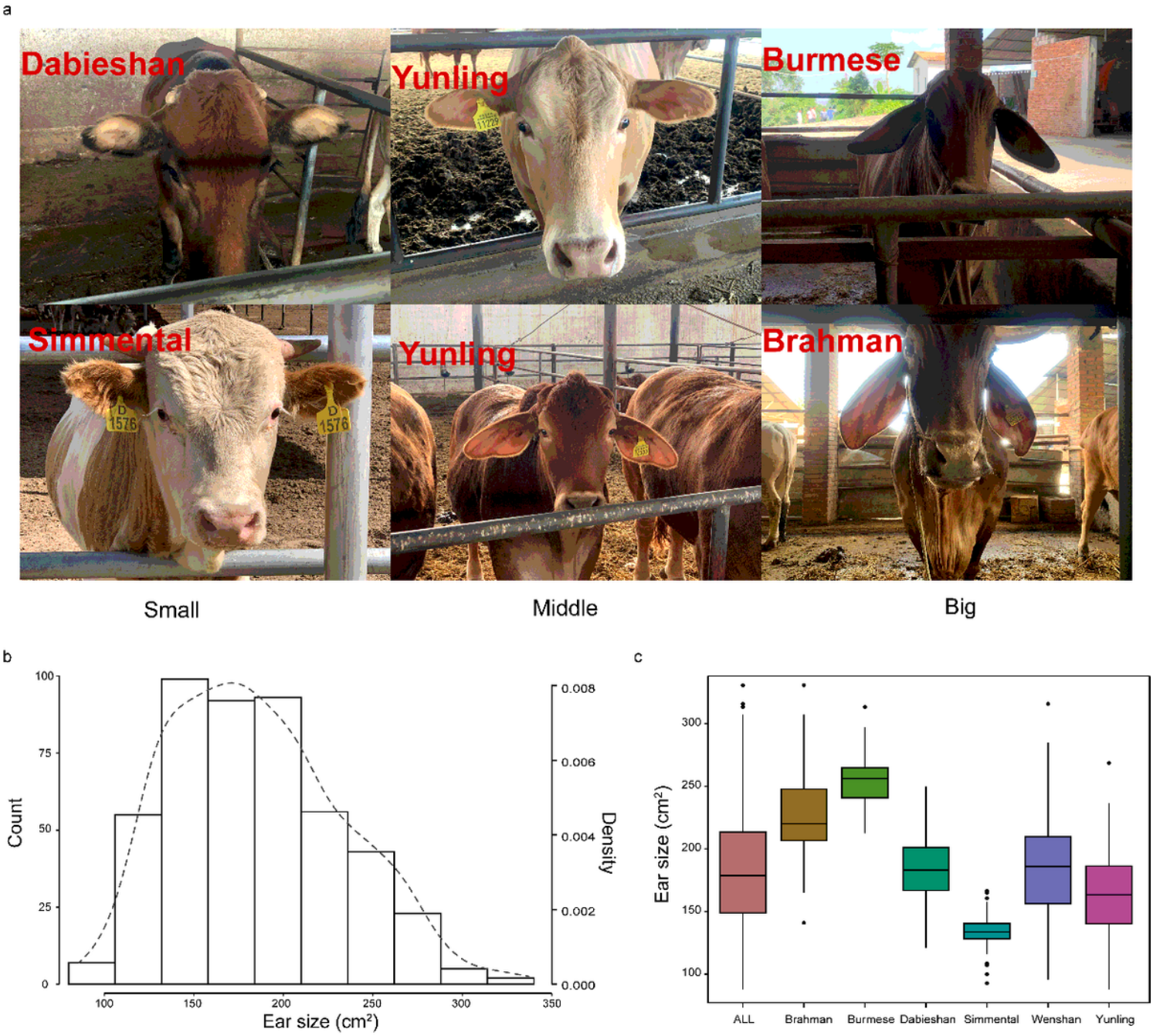


Figure 1

Statistical analysis of ear size. (a) Ear diversity of different cattle breeds, the first column was smaller ear size cattle (Simmental and DBS cattle), and the second column represent hybrid cattle, while the third column shows the large ear size Bos indicus cattle (Brahman and Burmese cattle). (b) Box-plot, histogram and density plot for 475 ear size data, the vertical axis on the left represent number of individuals in different sections, the vertical axis on the right show the percentage of individuals in different sections, and the upper right corner represents the boxplot for 475 individual ear size. (c) Box-plot analysis for ear size among all individuals and six cattle breeds.



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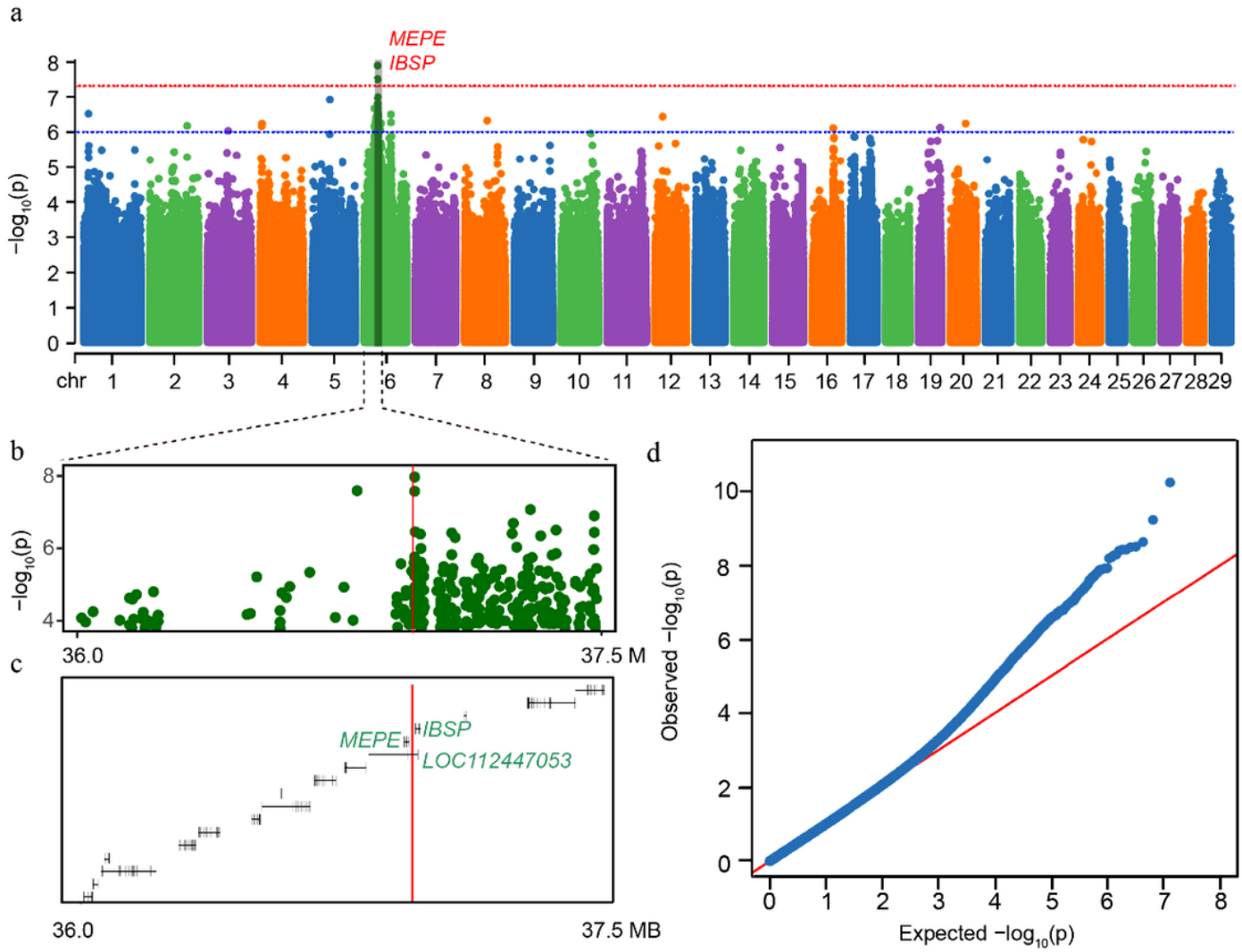
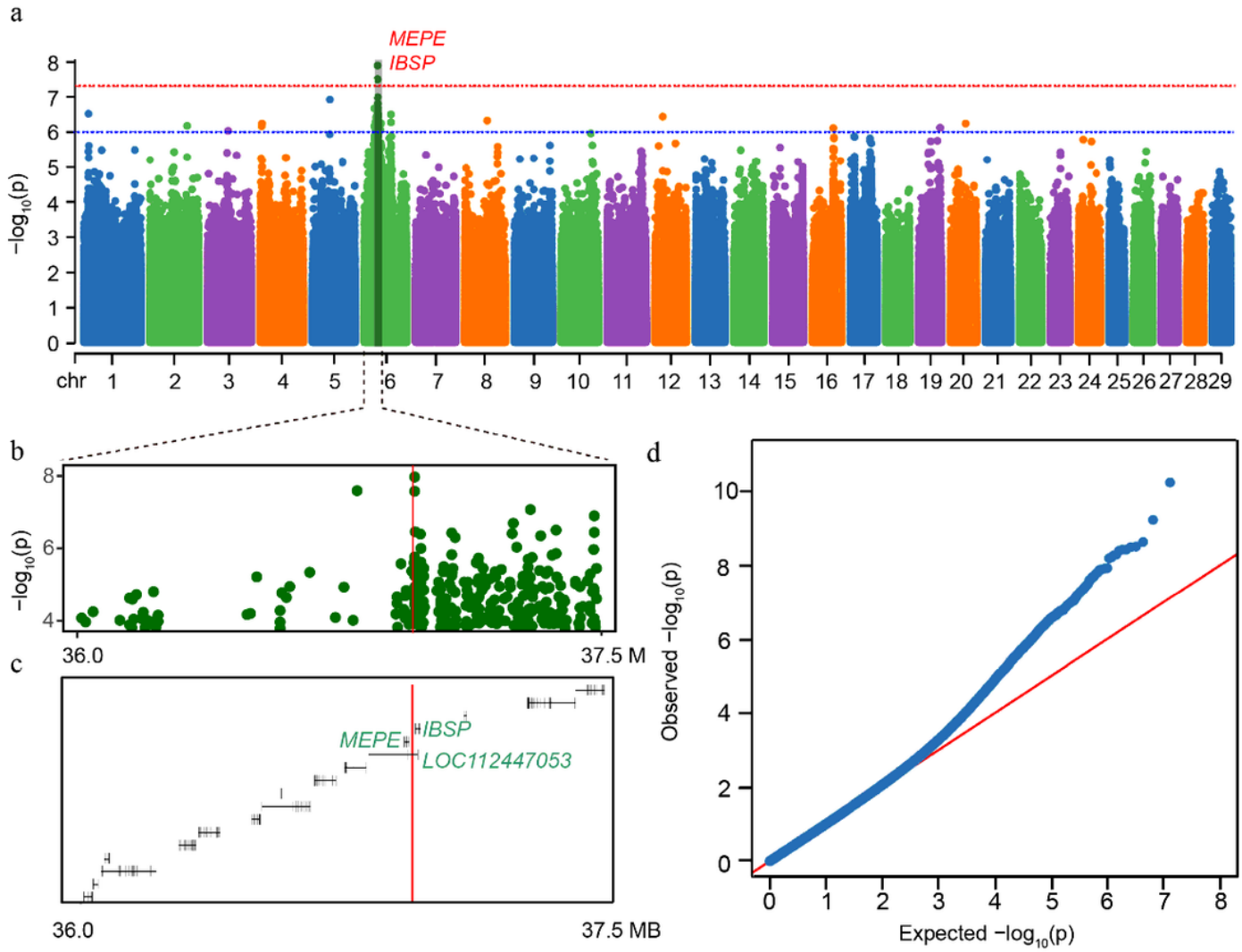


Figure 2

Genome-wide association study of ear size. (a) Manhattan plot for ear size GWAS, (b,c) plot for regional association result for MEPE and IBSP, the functional genes in this region were plotted in the box, (d) quantile–quantile plot observed and expected P-value (expressed as −log10(P)) of the GWAS for ear size.



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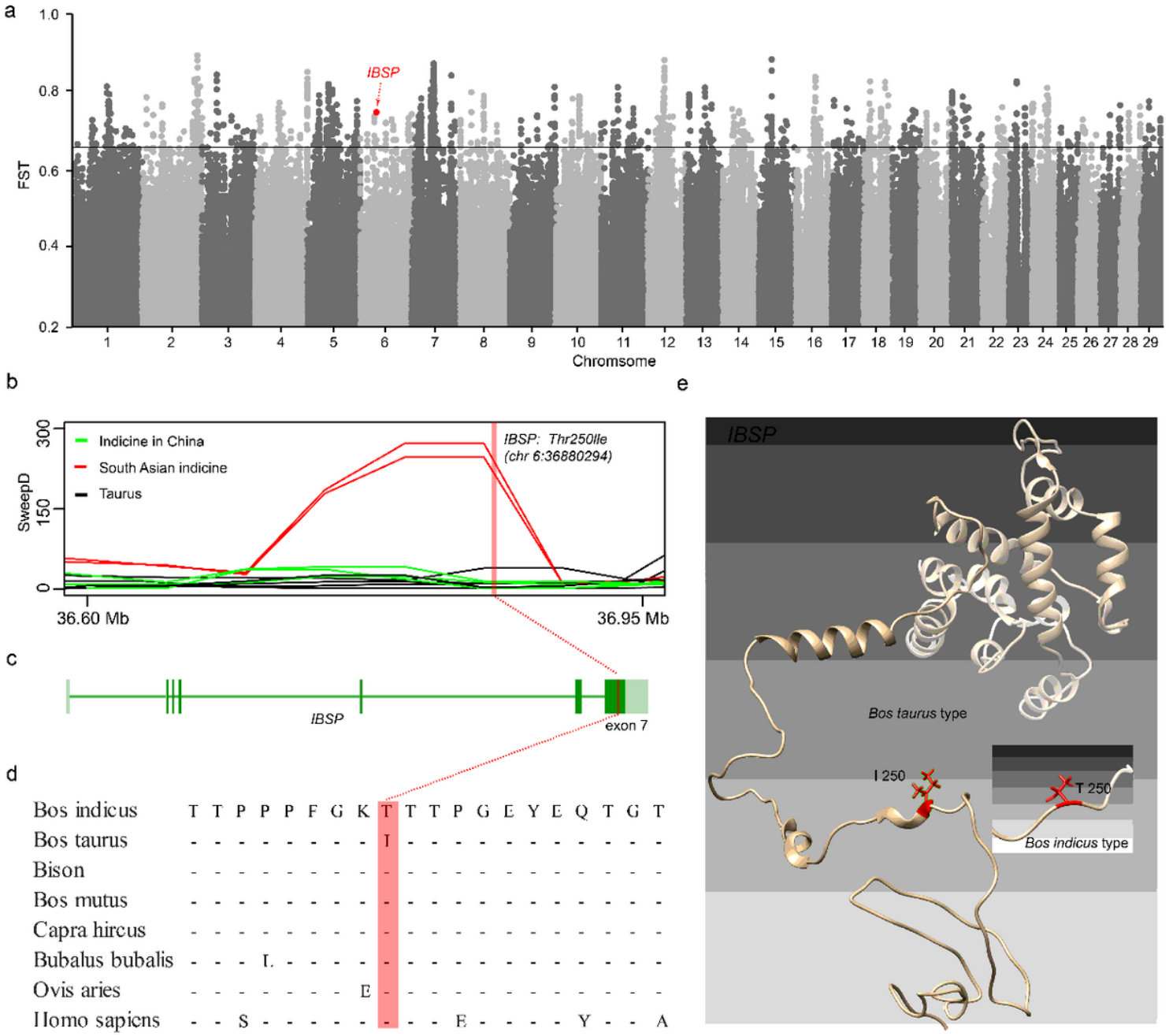
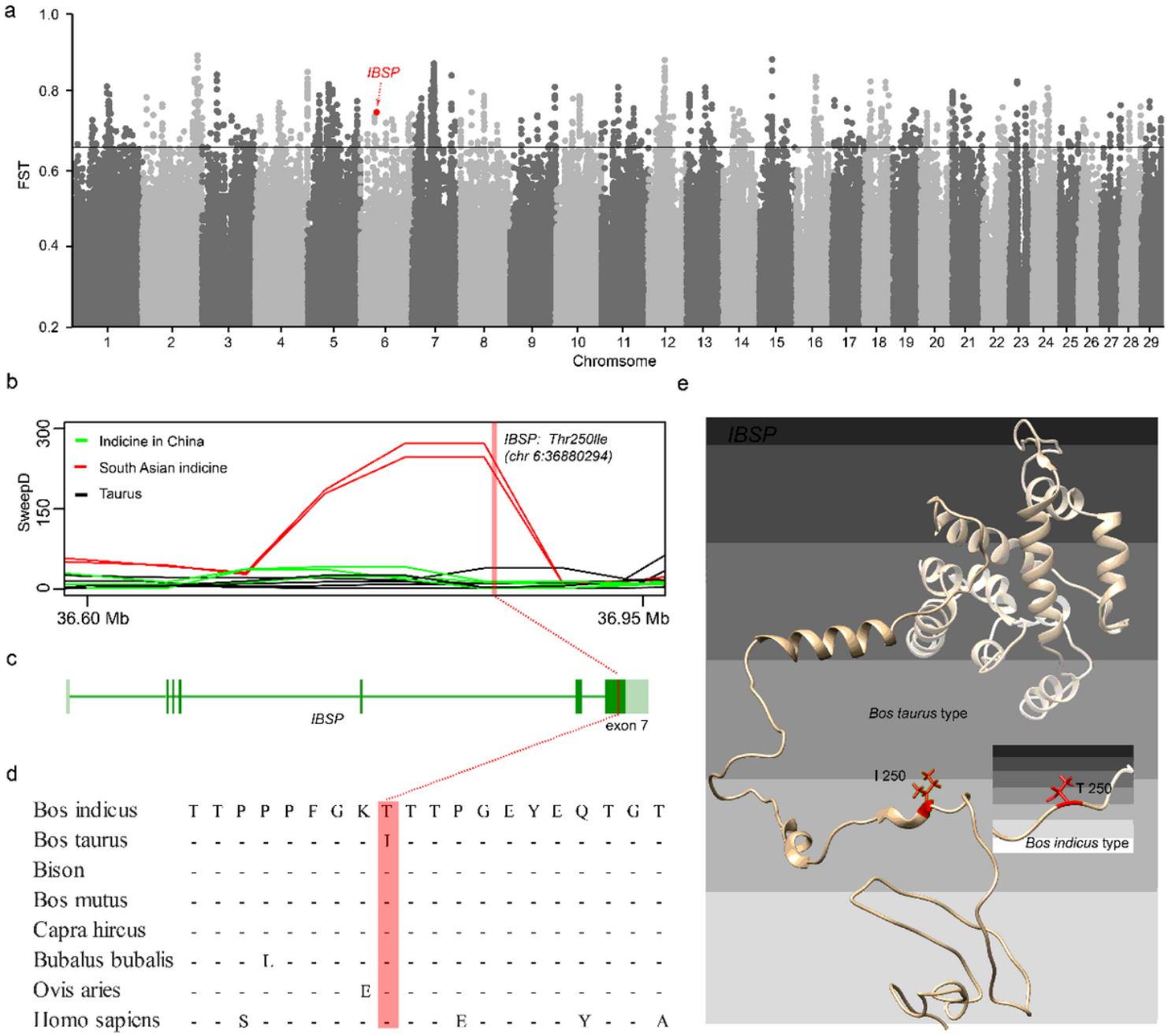


Figure 3

Selection and conservative analysis for IBSP. (a) Manhattan plot for Fst analysis of big and small ear cattle group. The black line in manhattan plots indicate the 0.5% significantly selected regions, while the red box indicated the location of IBSP. (b) Line charts of SweepD in 14 cattle breeds on the gene regions of IBSP. (c) Gene structure of IBSP. (d) Conservative prediction of the mutant IBSP protein with its ortholog proteins in bovidae. (e) 3D protein structure prediction of IBSP.



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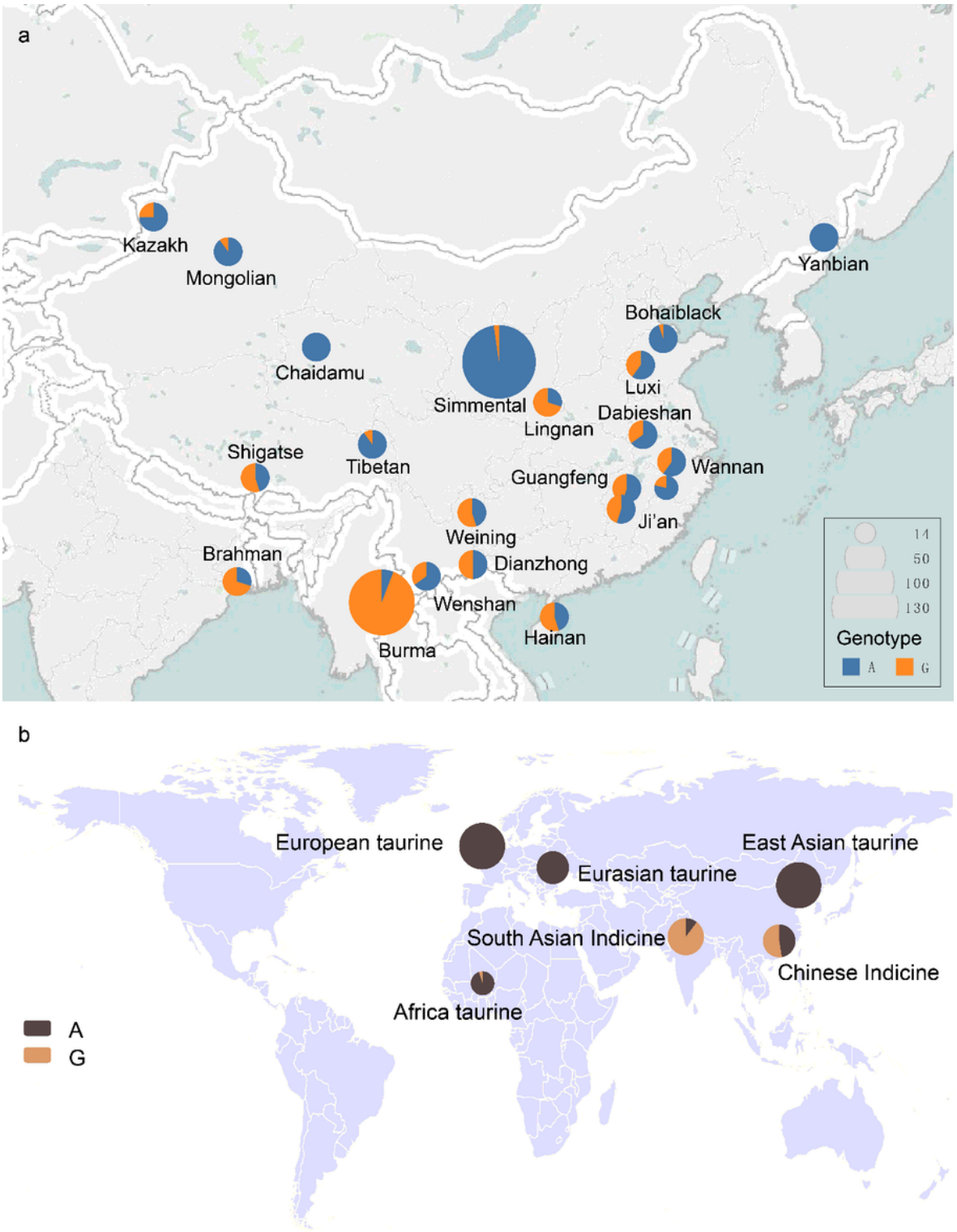

Figure 4

Geographical distribution of the mutation among different cattle breeds. (a) The mutation frequency distribution of IBSP among 21 cattle breeds from China and neighboring countries. The size of the circle represents the count of the samples. The blue and orange color represent G and A allele, respectively. (c) The mutation allele frequency distribution of six ancestral cattle groups. The size of the circle represents the count of the samples. The orange and brown color represent G and A allele, respectively. Note: The

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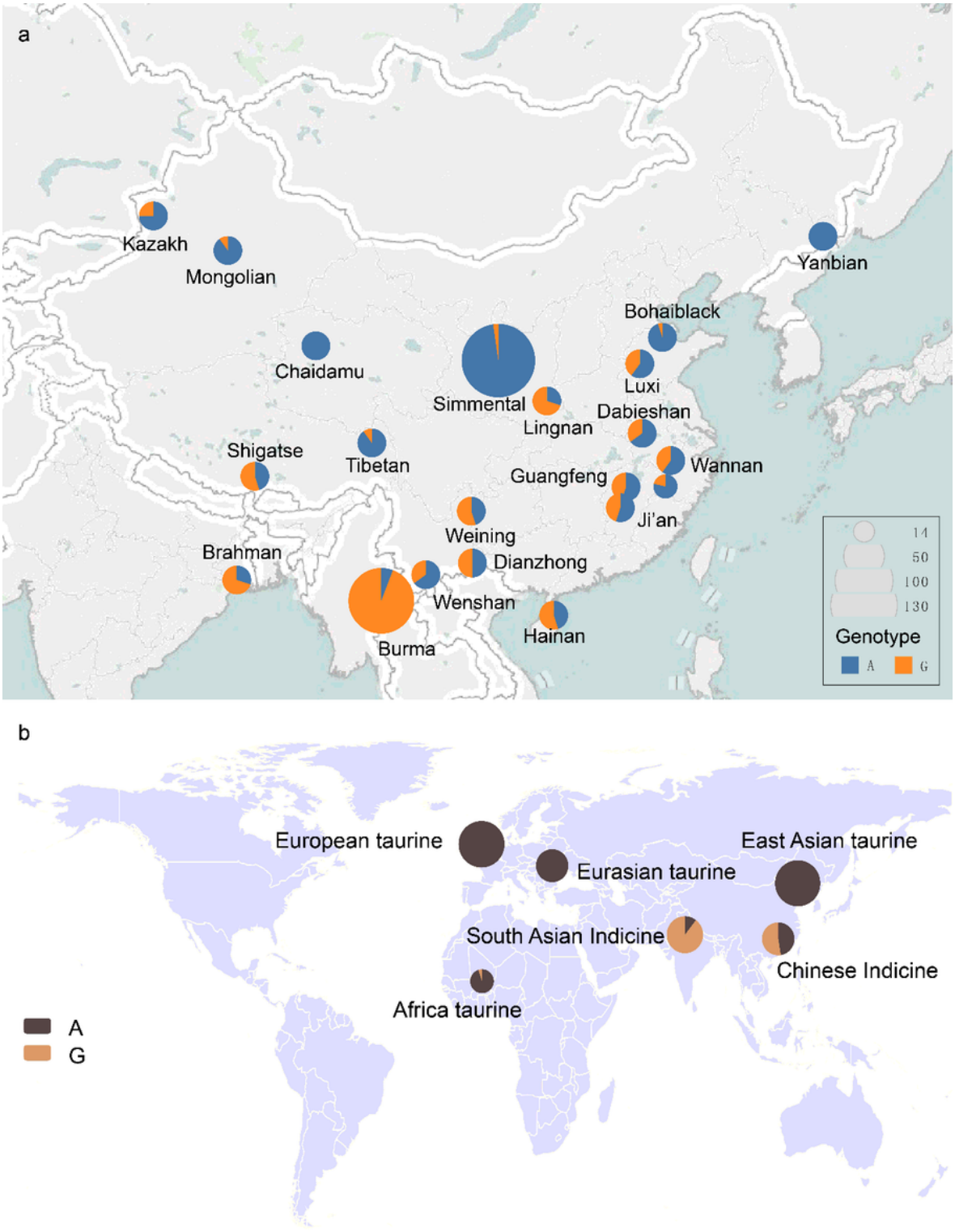

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