Genomic and transcriptomic analyses reveal selection of genes for puberty in Bama Xiang pigs

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ABSTRACT

The Bama Xiang pig (BMX) is a famous early-maturing Chinese indigenous breed with a two-end black coat. To uncover the genetic basis of the BMX phenotype, we conducted comparative genomic analyses between BMX and East Asian wild boars and Laiwu pigs, respectively. Genes under positive selection were enriched in pathways associated with gonadal hormone and melanin synthesis, consistent with the phenotypic changes observed during development in BMX pigs. We also performed differentially expressed gene analysis based on RNA-seq data from pituitary tissues of BMX and Large White pigs. The CTTNBP2NL, FRS2, KANK4, and KATNAL1 genes were under selection and exhibited expressional changes in the pituitary tissue, which may affect BMX pig puberty. Our study demonstrated the positive selection of early maturity in the development of BMX pigs and advances our knowledge on the role of regulatory elements in puberty evolution in pigs.

Keywords: Puberty; Bama Xiang pig; Pituitary; Differentially expressed genes

INTRODUCTION

Pigs are important protein food source and globally distributed animal domesticated from Eurasian wild boars 9 000 years ago (Larson et al., 2005). During their long domestication history, pigs have exhibited abundant phenotypic variation in coat color, reproduction, and growth. This phenotypic diversity provides valuable animal models for genetic studies (Ciobanu et al., 2001; Rubin et al., 2012).

The Bama Xiang pig (BMX) from Guangxi Zhuang Autonomous Region is a well-known variety noted for its early maturity and two-end black pigmentation. The female BMX pig matures at about three months, in sharp contrast to the five months observed in most other East Asian domestic pigs (China National Commission of Animal Genetic Resources, 2011). Thus, the BMX pig provides an important model to study early maturity and pigmentation evolution during pig domestication.

In this study, we performed genomic screening of signatures of positive selection in the BMX pig in comparison with those of the East Asian wild boar (EAW) and Laiwu (LWU) pigs. The LWU pig has a normal maturity process (at six months) and black coat color (China National Commission of Animal Genetic Resources, 2011), but shares the same domestication origin as that of the BMX pig from East Asian wild boars (Wu et al., 2007). In combination with transcriptomic analysis, we identified genetic variations that were putatively associated with the maturity and pigment phenotype in the BMX pig. The primary goal of this study was to explore the (1) genomic evolutionary pattern shaped by development of the BMX pig, and (2) genetic variations associated with selection for early maturity and coat color in the BMX pig.

MATERIALS AND METHODS

Sample collection and resequencing of genomic DNA

We downloaded the genome resequencing data of six BMX, six LWU, and 13 EAW pigs from the NCBI database (Supplementary Table S1), as reported from earlier studies.
We analyzed selective sweeps in 10-kb non-overlapping sliding windows with an inserted size of 250 bp were prepared using the GATK toolkit (McKenna et al., 2010). Only polymorphic SNPs were included in this analysis. VariantFiltration and SelectVariants were used to filter SNPs by quality score (QUAL <20) and depth of coverage (DP <10). SNPs were annotated with the GTF file included in this analysis. VariantFiltration and SelectVariants were used to filter SNPs by quality score (QUAL <20) and depth of coverage (DP <10). SNPs were annotated with the GTF file downloaded from the NCBI website (https://www.ncbi.nlm.nih.gov/). For annotation, SNPs were classified by their locations in protein-coding (synonymous or non-synonymous), intronic, intergenic, and untranslated region (UTR) sequences.

Single nucleotide polymorphism (SNP) calling and annotation
SNP calling was performed with the DNA sequencing data of the 25 pig genomes using HaplotypeCaller in the GATK program (McKenna et al., 2010). Only polymorphic SNPs were used in this analysis. VariantFiltration and SelectVariants were used to filter SNPs by quality score (QUAL <20) and depth of coverage (DP <10). SNPs were annotated with the GTF file downloaded from the NCBI website (https://www.ncbi.nlm.nih.gov/). For annotation, SNPs were classified by their locations in protein-coding (synonymous or non-synonymous), intronic, intergenic, and untranslated region (UTR) sequences.

Selective sweep detection
Population fixation index (FST) (Weir & Cockerham, 1984), cross population extended haplotype homozygosity (XPEHH) (Sabeti et al., 2007), and nucleotide diversity (π) (Tajima, 1983) were used to search for signals of selection in the BMX pig genome. We analyzed selective sweeps in 10-kb non-overlapping sliding windows. Windows containing <5 SNPs were removed from the analysis.

The FST values between BMX and other pigs (LWU and EAW) were calculated using VCFtools (Danecek et al., 2011). Haplotypes were inferred using Beagle software (Browning & Browning, 2007). Comparing the BMX pigs with LWU and EAW pigs, XPEHH was calculated to examine haplotypes that showed low levels of linkage decay in the BMX populations (Coop et al., 2009; Pickrell et al., 2009; Pritchard et al., 2010). The nucleotide diversities of BMX (πBMX), EAW (πEAW), and LWU (πLWU) were then calculated separately using VCFtools (Danecek et al., 2011). To identify regions that showed low nucleotide diversity in the BMX pigs compared to the LWU and EAW pigs, we calculated πEAW divided by πBMX and πLWU divided by πBMX, respectively.

Genomic windows showing high FST, low nucleotide diversity, and high XPEHH values were identified as having signatures of selection in BMX pigs. Candidate selective sweeps were chosen from genomic windows identified simultaneously by all three statistics as having values above the top 10% threshold in their empirical distributions.

RNA sample collection, sequencing, and identification of differentially expressed genes
Pituitary tissues were collected from three BMX pigs and three Large White pigs for RNA sequencing (RNA-seq). These pigs were females at 85 days after birth. The RNA-seq libraries with an inserted size of 250 bp were prepared using the Illumina standard RNA-seq library preparation pipeline and sequenced on the Illumina HiSeq 2000 platform, with 150-bp paired-end reads generated. In total, the transcriptomes of six pituitaries (GSA number: CRA000876) were used for comparative analysis. The RNA-seq clean reads were mapped onto the Sus scrofa 10.2 genome using TopHat2 (Kim et al., 2013). A new merged GTF annotation file was generated using Cufflinks and Cuffmerge (Trapnell et al., 2010).

Differentially expressed genes (DEGs) were identified by DESeq2 (Love et al., 2014). As DESeq2 required a raw read count table as input, we obtained the raw read count using HTseq (Anders et al., 2014). An FDR of <0.05 was used as the cutoff for DEGs. The ClueGO plugin in Cytoscape (Mlecnik et al., 2018) was used for gene ontology (GO) enrichment analysis. GO terms with a P-value of <0.05 were defined as enriched.

RESULTS
Selective signatures in BMX pig genomes
The whole genomes of six BMX, 13 EAW, and six LWU pigs were used to identify genomic variants underlying the development of BMX pigs. Genomic reads were quality filtered and mapped to the pig reference genome (Sus scrofa 10.2) (see Materials and Methods). A total of 43.5 million SNPs were identified in the 25 individuals. To identify signatures of selection in BMX pig genomes, we analyzed the FST (Weir & Cockerham, 1984), π (Tajima, 1983), and XPEHH (Sabeti et al., 2007) metrics along chromosomes in comparison to those of EAW and LWU pigs (Figure 1). Selective signatures were screened in 10-kb non-overlapping sliding windows and adjacent selective windows were combined. As recent artificial selection produces long stretches of haplotypes in a population (Amaral et al., 2008), a clustering of selective windows with intervals of less than nine windows was chosen as an indicator of a putatively selective sweep. Sweep regions greater than 30 kb in length were included in the following analyses. This procedure resulted in the identification of 257 genes within 339 putative selective sweeps in the BMX pig genomes (Supplementary Table S2).

Functional enrichment analysis of the gene set revealed significant overrepresentation of 59 terms (Table 1). Interestingly, several biological processes related to sexual maturity were included, involving “pituitary gland development” (BMP4, FGF10, and TBX19), “endocrine system development” (BMP4, CRH, FGF10, NEUROD1, and TBX19), and “negative regulation of reproductive process” (BMP4, CSN2, and ZP4). The pituitary is the most important endocrine gland involved in growth and reproduction regulation. In addition, five genes (FGF10, FGF2, MITF, PDGFC, and PDGFDB) were involved in “Melanoma”, which might be associated with the two-end black pigmentation observed in BMX pigs, which differs from most Chinese domestic pigs with black coats (China National Commission of Animal Genetic Resources, 2011).

Although many missense mutations are associated with the domestication of pigs (Fang et al., 2009; Li et al., 2010; Ren et al., 2011; Rubin et al., 2012), whether regulatory variants play a role and their effects on the domestication processes compared to coding variants remain unknown. To address this issue, we compared the evolutionary patterns for SNPs identified from the selective regions in the BMX pig genomes. A
total of 830,949 SNPs were identified from 339 sweeps. Among these, 3,668 SNPs were located in coding sequences and 827,542 SNPs were located in noncoding sequences. We first focused on protein coding region variations located in the 339 sweeps. We examined highly differentiated missense mutations and analyzed their presence in DNA sequences encoding conserved protein sequences. We concentrated on missense SNPs with $F_{ST}$ values up to the chromosomal top 0.1% threshold and compared BMX pigs to LWU and EAW pigs, respectively. We identified a dataset consisting of 11 missense SNPs from 10 genes (Supplementary Table S3). Alignment of the 10 protein sequences showed that the flanking sequences at the residues encoded by the 11 SNPs displayed low level cross-species conservation, implying weak functional constraints on these protein-coding variants. This indicated that regulatory elements rather than missense variants may have greater effects on BMX pig development.

![Figure 1 Whole genome selective signatures of BMX pigs](https://www.zoores.ac.cn)

**Figure 1 Whole genome selective signatures of BMX pigs**

X-axis shows the positions of the windows along the 1–18 autosome chromosomes. Different chromosomes are displayed in different colors. Y-axis shows the $F_{ST}$ (A), XPEHH (B), $\pi_{EAW/BMX}$ (C), and $\pi_{LWU/BMX}$ (D) values. $F_{ST}$ and XPEHH values were calculated between BMX and (LWU+EAW) pigs. $\pi_{EAW/BMX}$ and $\pi_{LWU/BMX}$ were obtained from $\pi_{EAW}$ and $\pi_{LWU}$ divided by $\pi_{BMX}$, respectively.

**Comparative transcriptomic analysis of pituitary tissues**

We conducted transcriptomic profiling to examine gene expression changes due to selection in the BMX genomes as regulatory variants exhibited a profound role during BMX pig development. We chose pituitary tissue as the pituitary gland secretes reproduction-related hormones involved in maturity regulation. In our experimental design, we collected pituitary tissues from three BMX and three Large White pigs at 85 days after birth. At this stage, BMX pigs are close to maturity, whereas Large White pigs do not reach maturity until eight months of age (China National Commission of Animal Genetic Resources, 2011). The transcriptomes from the pituitary tissues were sequenced, from which we obtained 35 million mapped reads, on average, for each individual. We compared the gene expression levels in the pituitary and found 1,600 DEGs between BMX and Large White pigs. Although Large White pigs were domesticated from European wild boars (Larson et al., 2005; Rubin et al., 2012), the DEGs between BMX and Large White pigs provided valuable information on expression changes in BMX pigs if they also exhibited selective signatures in the BMX genome. The DEGs were enriched in the following biological processes: “Estrogen signaling pathway” (ADCY6, ADCY8, CREB3L1, CREB5, FOS, GABBR2, GNAS, GRM1, KCNJ9, MMP9, PIK3CG, PIK3R5, and PRKACA), “Ovarian steroidogenesis” (ADCY6, ADCY8, ALOX5, GNAS, LDLR, LHCGR, and PRKACA), “Oocyte meiosis” (ADCY6, ADCY8, CAMK2B, CAMK2D, CCNE1, CDC20, CPEB2, ESPL1, MAD2L2, MOS, PRKACA, and YWHAZ), “Developmental maturation” (C12H17orf47, CDC20, EDNRB, FAM101B, LTF, PLP1, PRKACA, RBPJ, TAL1, WASH1, and WNT10B), and “Progesterone-mediated oocyte maturation” (ADCY6, ADCY8, CPEB2, MAD2L2, MOS, PIK3CG, PIK3R5, and PRKACA) (Supplementary Table S4). These pathways all participate in sexual maturity.
Table 1 Functional enrichment terms of positively selected genes in BMX pigs.

| GO ID     | GO term                                                                 | P-value   |
|-----------|-------------------------------------------------------------------------|-----------|
| GO:0004080| Neuroactive ligand-receptor interaction                                  | 24.0E-15  |
| GO:0060449| Bud elongation involved in lung branching                                | 100.0E-6  |
| GO:0032740| Positive regulation of interleukin-17 production                         | 140.0E-6  |
| GO:0060393| Regulation of pathway-restricted SMAD protein phosphorylation            | 230.0E-6  |
| GO:0060389| Pathway-restricted SMAD protein phosphorylation                          | 250.0E-6  |
| GO:0032946| Positive regulation of mononuclear cell proliferation                   | 310.0E-6  |
| GO:0050671| Positive regulation of lymphocyte proliferation                          | 310.0E-6  |
| GO:0070665| Positive regulation of leukocyte proliferation                           | 370.0E-6  |
| GO:0048636| Positive regulation of muscle organ development                          | 400.0E-6  |
| GO:0045844| Positive regulation of striated muscle tissue development                | 400.0E-6  |
| GO:1901863| positive regulation of muscle tissue development                         | 450.0E-6  |
| GO:0005412| Arrhythmogenic right ventricular cardiomyopathy (ARVC)                   | 490.0E-6  |
| GO:0005218| Melanoma                                                                | 560.0E-6  |
| GO:0045740| Positive regulation of DNA replication                                   | 600.0E-6  |
| GO:0090798| Axis specification                                                       | 600.0E-6  |
| GO:0090090| Negative regulation of canonical Wnt signaling pathway                   | 600.0E-6  |
| GO:0032660| Regulation of interleukin-17 production                                  | 660.0E-6  |
| GO:0051155| Positive regulation of striated muscle cell differentiation              | 660.0E-6  |
| GO:0032620| Interleukin-17 production                                                | 780.0E-6  |
| GO:0042506| Tyrosine phosphorylation of Stat5 protein                                | 780.0E-6  |
| GO:0051145| Smooth muscle cell differentiation                                       | 800.0E-6  |
| GO:0061037| Negative regulation of cartilage development                            | 920.0E-6  |
| GO:0048546| Digestive tract morphogenesis                                            | 950.0E-6  |
| GO:0005410| Hypertrophic cardiomyopathy (HCM)                                       | 990.0E-6  |
| GO:0051149| Positive regulation of muscle cell differentiation                       | 1.2E-3    |
| GO:0032570| Endocrine system development                                             | 1.3E-3    |
| GO:0055025| Positive regulation of cardiac muscle tissue development                 | 1.3E-3    |
| GO:0042310| Vasoconstriction                                                         | 1.5E-3    |
| GO:0060602| Branch elongation of an epithelium                                      | 1.5E-3    |
| GO:0001709| Cell fate determination                                                  | 2.0E-3    |
| GO:0021536| Diencephalon development                                                 | 1.8E-3    |
| GO:0021983| Pituitary gland development                                              | 2.2E-3    |
| GO:0042307| Positive regulation of protein import into nucleus                       | 2.3E-3    |
| GO:0000561| Glycerolipid metabolism                                                 | 2.4E-3    |
| GO:0004730| Long-term depression                                                     | 2.6E-3    |
| GO:0003338| Metanephrine morphogenesis                                               | 2.4E-3    |
| GO:0030199| Collagen fibril organization                                             | 2.4E-3    |
| GO:0060441| Epithelial tube branching involved in lung morphogenesis                 | 3.0E-3    |
| GO:000219| Megakaryocyte differentiation                                            | 3.3E-3    |
| GO:0042383| Sarcolemma                                                              | 3.3E-3    |
| GO:0009799| Specification of symmetry                                               | 3.3E-3    |
| GO:0051153| Regulation of striated muscle cell differentiation                       | 3.3E-3    |
| GO:0010092| Specification of organ identity                                          | 3.3E-3    |
| GO:1900182| Positive regulation of protein localization to nucleus                   | 3.7E-3    |
| GO:0005033| Nicotine addiction                                                      | 4.0E-3    |
| GO:0004140| Regulation of autophagy                                                 | 5.9E-3    |
| GO:2000242| Negative regulation of reproductive process                             | 5.9E-3    |
| GO:0070664| Negative regulation of leukocyte proliferation                          | 5.1E-3    |
| GO:0003401| Axis elongation                                                          | 5.1E-3    |
| GO:0009948| Anterior/posterior axis specification                                    | 5.5E-3    |
| GO:0055024| Regulation of cardiac muscle tissue development                          | 5.1E-3    |
| GO:0004940| Type I diabetes mellitus                                                | 7.4E-3    |
| GO:0048259| Regulation of receptor-mediated endocytosis                             | 6.9E-3    |
| GO:0070193| Adenylate cyclase-inhibiting G-protein coupled receptor signaling pathway| 11.0E-3   |
| GO:0098802| Embryonic pattern specification                                          | 11.0E-3   |
| GO:0005160| Transforming growth factor beta receptor binding                         | 9.5E-3    |
| GO:0031623| Receptor internalization                                                | 11.0E-3   |
| GO:0048286| Lung alveolus development                                                | 9.5E-3    |
| GO:00010862| Positive regulation of pathway-restricted SMAD protein phosphorylation  | 10.0E-3   |

*: P-value obtained from ClueGO plugin in Cytoscape using default parameters. Only terms with P<0.05 are listed.
As both artificially selected genes and DEGs showed functional enrichment in maturity regulation, it is possible that differential gene expression was favored by the artificial selection of variants from some sweep loci. We therefore identified artificially selected genes with altered gene expression. In our analysis, a total of 22 artificially selected genes exhibited differential gene expression in the pituitary ($P<0.05$; FDR$<0.05$; Table 2). Interestingly, four genes (CTTNBP2NL, FRS2, KANK4, and KATNAL1) were related to reproduction (Figure 2). Compared to that in the Large White pigs, the expression of the CTTNBP2NL gene was increased in BMX pigs. CTTNBP2NL has been reported to affect conception rates in bovines (Sugimoto et al., 2013). FRS2 encodes a docking protein bound to FGF receptors, with a knockout study in mice indicating its involvement in epididymal development and sperm maturation (Xu et al., 2013). In the pituitary tissue, we found that the expression level of FRS2 in BMX pigs was about twice that found in Large White pigs. In addition, KANK4 was found to be differentially expressed in the pituitary tissues of BMX and Large White pigs. Although few studies have reported on the function of KANK4, it is located in a significant pig quantitative trait locus (QTL) for age at puberty (Bidanel et al., 2008). KATNAL1, which encodes Katanin Catalytic subunit A1 like 1, has been implicated in both meiosis and spermiogenesis (Smith et al., 2012).

![Figure 2 Normalized read counts of significantly differentially expressed genes](https://www.zoores.ac.cn)

**Figure 2** Normalized read counts of significantly differentially expressed genes CTTNBP2NL (A), FRS2 (B), KANK4 (C), and KATNAL1 (D) in the pituitary

For each subgraph, red and green boxes represent gene expression in the pituitaries of BMX and Large White pigs, respectively. Upper whisker is maximum value, black horizontal line in box is median, and lower whisker is minimum value.

**DISCUSSION**

We conducted a comparative genomic analysis of BMX pigs with domestic pigs and wild boars from East Asia, and revealed selective signatures potentially associated with selection for early maturity and the two-end black phenotype. During domestication, selection is based on those functions and traits that are favored in pig breeds. As described earlier, selective signatures were detected for the specific phenotype of the BMX pig. This study sheds light on the evolution of puberty in pigs and provides important information and candidate genes for pig breeding.

Puberty is an important trait related to economic output, with early maturity resulting in early slow growth. For instance, Western commercial breeds are characterized with late maturity for enlarged body size (Rubin et al., 2012). The present study revealed four loci (CTTNBP2NL, FRS2, KANK4, and KATNAL1) showing strong signatures of selection and functional association with puberty. In addition, the four genes exhibited differential expression between early and late maturity in pig breeds in the pituitary. This result indicates that regulatory elements may have considerable effect on BMX pig development.

Coat color is a trait that is usually under strong selection in different breeds. In previous studies, several genes have been shown to be associated with coat color variants, including MC1R, KIT, and EDNRB (Kijas et al., 1998; Wiener & Wilkinson, 2011; Wilkinson et al., 2013). However, we found no selective signature of MC1R. The reason for this may be that selection of MC1R occurred during early domestication, and most Chinese indigenous pig breeds share the same haplotype (Li et al., 2010). Earlier research has indicated that mutations in MITF,
**AUTHORS’ CONTRIBUTIONS**

Y.P.Z., H.B.X., and Y.Y. designed the study. H.B.X. supervised the analyses. Y.Y. performed genome assembly and annotation. Y.Y. extracted RNA and wrote the manuscript with the other authors’ input. Y.Y. submitted RNA-seq data to GSA. A.C.A., H.B.X. and Y.P.Z. revised the manuscript. All authors read and approved the final manuscript.

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**Table 2** Overlap between positively selected genes and differentially expressed genes from pituitary tissue in BMX pigs.

| Gene      | Normalized count fold change (BMX/LW) | P-value*
|-----------|--------------------------------------|---------
| MGAT5B    | 0.08                                 | 7.57E-05 |
| BHLHE22   | 2.57                                 | 0.000 538|
| FAM174B   | 0.43                                 | 0.000 589|
| SFT2D2    | 0.28                                 | 0.001 381|
| TRHDE     | 2.13                                 | 0.005 172|
| COL23A1   | 0.12                                 | 0.006 991|
| FRMD4B    | 2.45                                 | 0.007 354|
| CDKN2B    | 0.14                                 | 0.007 441|
| FRS2      | 2.09                                 | 0.008 626|
| ATG4C     | 2.16                                 | 0.010 881|
| PELI2     | 1.90                                 | 0.012 313|
| FEZ2      | 2.12                                 | 0.018 321|
| LGALS12   | 0.13                                 | 0.020 835|
| TYRP1     | 0.13                                 | 0.025 025|
| KCND3     | 1.81                                 | 0.030 48|
| FNDC1     | 0.22                                 | 0.030 808|
| ATL3      | 0.46                                 | 0.033 021|
| KANK4     | 0.34                                 | 0.036 521|
| KATNAL1   | 1.99                                 | 0.037 825|
| CTNNBP2NL | 2.27                                 | 0.039 048|
| ANO3      | 2.50                                 | 0.044 986|
| DYNCL12   | 1.67                                 | 0.047 03 |

*: P-values are corrected by Benjamini-Hochberg FDR<0.05.
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