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Analysis of Anhui Indigenous Pig Germplasm Characteristics Based on Copy Number Variation

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Abstract

Background: As one of the most widely covered variations in the genome, copy number variation (CNV) is an important form of expression, which play an important role in phenotypic variations, diseases, and biodiversity of animals. In order to understand the impact of CNV on economic traits of Anhui indigenous pig breeds (AHIPs), in this study, we evaluated CNV differences in five AHIPs and revealed germplasm characteristics of AHIPs based on data from the Porcine 80K SNP BeadChip.

Result: After strict quality control, we identified a total of 3863 CNVs on 18 autosomes of five AHIPs. We obtained 316 loss events and 33 gain events in AHIPs by defining and overlapping copy number variation regions (CNVRs), accounting for an average of 3.61% of the porcine autosomal genome. And then, we obtained a total of 179 genes by annotation of CNVR and conducted GO terms and KEGG pathway analysis, identifying some candidate genes related to fat deposition (*ELOVL4*, *ACSF3*, *ACLY*) and reproductive (*TET1*, *SNCA*) traits.

Conclusion: This studying provides insights and new molecular markers for further genetic analysis, and provide a theoretical basis for improvement and assisted breeding selection of Chinese indigenous pigs.

Keywords: Copy Number Variation; Anhui Indigenous Pig Breeds; Fat Deposition, Reproductive Traits

Introduction

The domestication of wild boars into domestic pigs took a long time, and then many varieties were formed as a result of selection and evolution during this process [1]. In China, these domestic pigs are widely distributed and generally retain the characteristics of wild boars, and they have strong adaptability, forming many populations with different characteristics, resulting in a large number of variations in their genomes, which are important driving forces for biological evolution. The CNV is a relatively large structural variation, which refers to variations that occurred within 1 kb to several Mb of the genome in comparison to the reference sequence and that mostly include multilocus variations like duplications, deletions, inversions, and translocations, were another important genetic variation complementary to SNPs [2]. It is prevalent in domestic animal and human genomes and is crucial for biological evolution and genetic diversity [3]. The CNV was the first identified in the human genome [4,5], Zhang which describing the potential effects of CNV on individual genetic characteristics and disease susceptibility and reveals the complexity and diversity of the human genome. There are many research reports on the factors that contribute to the generation of CNV, some may be inherited from their parents, while others may be newly formed mutations. In the review by Zhang et al. [6], there are four main mechanisms for the formation of CNVs, including non-allelic homologous recombination, non-homologous end-joining, fork stalling and template switching model and long interspersed nucleotide element-1. At present, the technical means for detecting CNVs are also very mature. We can use array comparative genomic hybridization (aCGH), single nucleotide polymorphism (SNP) genotyping platforms, and next-generation sequencing to detect CNVs and determine their functions and effects [7,8].

As one of the important genetic variation types in the biological genome, CNV has been widely used, and genetic maps of CNVs have been constructed in many species. And the effects of CNVs are reflected throughout the whole life activities, from adaptive characteristics to embryonic lethality. In early studies, CNV was associated with human diseases, such as Down syndrome (MIM 190685) [9], color blindness [10], and neurological diseases [11].We generally consider that the factors of diseases are caused by the dose effect caused by CNV disrupting the protein coding sequence, but some study found that CNV were occurred in some noncoding regions and cause congenital disorders, such as long non-coding RNA [12].With further research show that there were also a large number of CNVs in the genetic ancestors of livestock, which affected the phenotype and important economic traits of animals through dosage effects and position effects [13]. Such as Wright et al. [14] found that pea-comb was triggered in chicken species mainly because of the presence of a large duplication on intron 1 of the gene for the SOX5 transcription factor, which interferes with the regulation of SRY-box transcription factor 5 expression due to this dosage effect during cell differentiation. In the study of domestic goats, it was found that thirteen copy number variation genes related to coat color, with agouti signaling protein gene duplication being the major cause of lighter coat color [15]. There has been some progress in CNV research related to pigs, such as variation in coat color in pigs (450 kb duplication of the KIT gene with particularities in different breeds of pigs) [16,17]. Zheng et al. [18] found that the type of AHR genes in Meishan Pigs has a positive effect on litter sizes and birth weight by next--generation sequencing. Wang et al. [19] revealed that differences in CNVs among six indigenous pig breeds by PorcineSNP60K and found that these CNVRs were involved in a variety of molecular functions that may play an important role in the phenotypes and production traits of these breeds.

Although the Chinese indigenous pig breeds were rich in resources and have excellent germplasm characteristics with the CNV map construction of pig breeds was increasingly well, but there have not been reports in AHIPs, which included Anqing Six-end-white pig (ASP) breeds, Huai pig (HP) breeds, Wannan Black pig (WBP) breeds, Wannan Spotted pig (WSP) breeds, and Wei pig (WP) breeds, and these pig breeds have large differences for each other, such as disease resistance [20], coat color, fecundity, etc. Thus, in this study, we performed genome-wide detection of CNVs in five indigenous pig breeds in Anhui based on data from the Porcine 80K SNP Bead Chip to identify possible CNVRs and potential candidate genes particular to these breeds in order to recognize these variety germplasm variations.

Materials and Methods

Ethics Statement

All experiments in this study were carried out in accordance

with the recommendations of the Animal Care Committee of the Anhui Academy of Agricultural Sciences (Hefei, China). The program was approved by the Animal Protection Committee of Anhui Provincial Agricultural Science (Hefei, China; No. AAAS2020-04).

Samples Collection

A total of 150 blood samples were collected from five Anhui indigenous pig breeds (ASP = 30, HP 92 = 30, WBP = 30, WSP = 30, WP = 30), and these individuals were sampled from the nucleus population of local protected farms in Anhui Province, China. Genomic DNA from all the samples was extracted according to standard protocols [21]. The concentration and the purity of genomic DNA were assessed using a NanoDrop™ 2000 (Thermo Fisher).

Genotyping and Statistical Analyses

Individual genotyping was conducted using the Illumina porcine 80K SNP BeadChip (Illumina, San Diego, CA, USA) which contained 68,528 SNPs with an average gap length of 38 kb on each chromosome. Single-nucleotide polymorphism clustering and genotype calling were performed using Genome Studio version 2011 (Illumina, version 1.9.4), and strict quality control was used for SNP filtering to increase the accuracy of the CNV detection. Single-nucleotide polymorphisms with a call rate < 90%, minor allele frequencies < 0.03 and Hardy–Weinberg equilibrium p-value < 1e−6 were removed. In addition, SNPs located on sex chromosomes were also excluded. Finally, we integrated multiple sources of information, include LRR and BAF at each SNP marker as well as the population frequency of B allele (PFB) of SNP sinto a Hidden Markov Model to identify CNVs based on Penn CNV software [22]. In order to decrease potential false-positive CNVs, we conduct quality control on each sample before analysis, include samples with LRR < 0.3, BAF drift < 0.01 and GC wave factor of LRR < 0.05. In addition, inferred raw CNVs were further filtered using two criteria to reduce false positives: a. each CNVs was detected in at least two samples; b. the number of SNPs in each CNVs was more than 3.

The software CNV Ruler was used to integrate the overlapping CNVs to create CNVRs [23]. We chose the CNV method by region approach for our investigation. Subsequently, we applied a recurrence value of 0.3, as advised by the CNV Ruler manual and previous studies [24], to avoid overestimating the size and frequency of CNVRs. Finally, control raw data filtered for occurrence in more than three individuals were used as the last of our results.

Gene Contents and Functional Annotation in Copy Number Variation Regions

We used BioMart (http://asia.ensembl.org/biomart/martview/, accessed on 2 March 2023) to identify genes overlapping with the CNVRs, including gene ID, gene symbol, gene type, from the Ensembl gene 80 databases, which is based on the Sscrofa 10.2 genome assembly, the homology was updated to the latest version (Ensembl Genes 109, Pig genes (Sscrofa11.1)). In order to evaluate the function of these genes, we performed Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis by David [24] (https://david.ncifcrf.gov/, accessed on 8 March 2023). The statistical method used in the enrichment analysis is Fisher's exact test. In addition, we downloaded Sus scrofa 10.2 version quantitative trait 92392502143125 00locus (QTL) information from the pig QTL database and find QTL located in the identified CNVRs or QTL partially overlapping with the CNVRs (all inside the CNVR) for further analysis (https://www.animalgenome.org/cgi-bin/QTLdb/SS/index, accessed on 3 March 2023). Finally, the visualization of data is achieved through the online website tools (https://www.bioinformatics.com.cn, last accessed on 20 March 2023) to conduct the display.

Results

Copy Number Variation Identification in Anhui indigenous pig breeds

After the strict calling pipeline of PennCNV software, we identified a total of 3863 CNVs on 18 autosomes of five AHIPs (Table S1). The statistics of the CNV numbers and length categories (0-10kb, 10-50kb, 50-100kb, 100-500 kb, 500-1000 kb, >1000 kb) in each AHIPs is in Fig 1. A. And on average, approximately 54.16% of the five indigenous pig breeds were located within the 100-500 kb categories. Finally, the overlapping segments were examined by CNVRuler software, which identified 73 CNVRs in the ASP breed, 66 CNVRs in the HP breed, 88 CNVRs in the WBP breed, 69 CN-

VRs in the WSP breed, and 53 CNVRs in the WP breed, respectively.

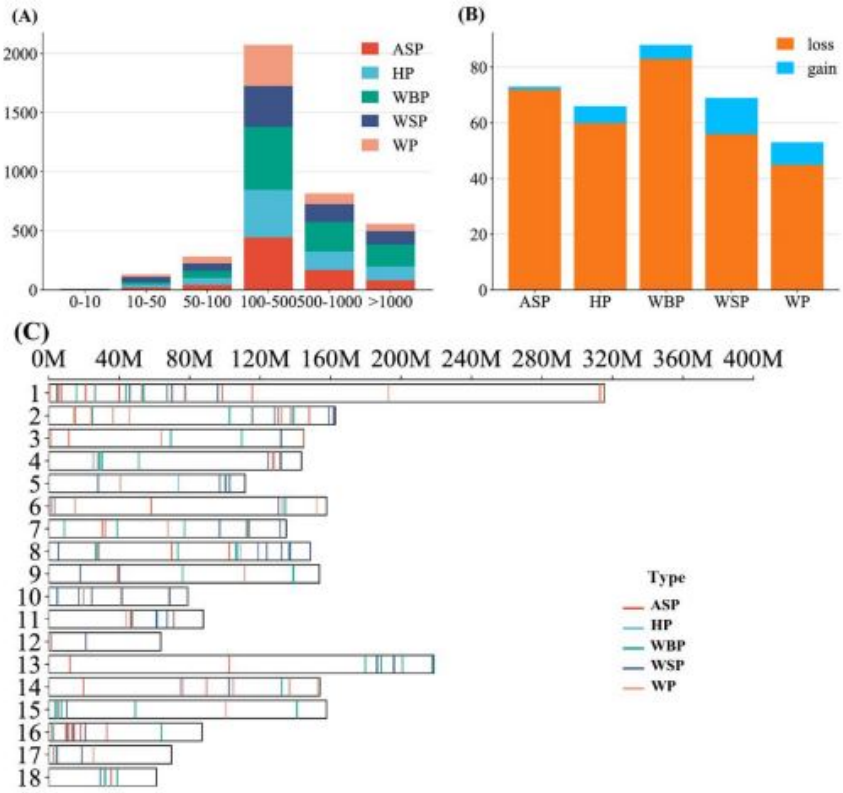

Figure 1: Distribution and statistics of CNV in pig breeds. (A) Size distribution of CNVs in AHIPs. (B) Statistical of CNVR in AHIPs. (C) The distribution of CNVR in pig autosomes

There were 349 CNVR events across the five breeds, 316 loss events, and 33 gain events. (Figure 1. B; Table S1). A descriptive summary of the CNVR numbers and length categories in the five AHIPs on the autosome is listed in Table 1. and distributed in Figure 1. C. We found that the CNVR of five AHIPs accounts for 2.27% to 4.84% of the pig genome, with the largest number of CNVR in the WBP breed, accounting for 4.84% of the pig genome. And the WP breed had the smallest number of CNVRs, average length of CNVRs, and proportion of the porcine genome. The number of CNVR events on chromosome 1 is the largest among all breeds, with a total of 30 CNVR events occurring in more than 50% of the population.

Table 1: The distribution of CNVRs in the pig autosomes

Breed	Number	CNVs	CNVR			Average length	Percentage (%)
			loss	gain	total		
ASP	30	761	72	1	73	1164922	0.0347
HP	30	758	60	6	66	1332014	0.0359
WBP	30	1055	83	5	88	1348604	0.0484
WSP	30	716	56	13	69	1380182	0.0389
WP	30	573	45	8	53	1049275	0.0227
Total	150	3863	316	33	349	6274997	0.1805

Annotation of the Merged Cnvs

The distribution of CNVR length was shown in the figure 2. A. Approximately 53.58% of CNVR segments were in between 100 and 1000 kb in size, as shown in figure 2. A. We annotated CNVR genes through the Ensemble database. And finally, we annotated a total of 179 genes, which were 68 genes in the ASP breed, 51 genes in the HP breed, 67 genes in the WBP breed, 79 genes in the WSP breed, and 53 genes in the WP breed. And then, for CNVRs and annotated genes in each population, we performed a Venn diagram display, as shown in figure 2. B, only one CNVR was common among the five AHIPs. The results of gene annotation showed that there were 34 unique genes in the ASP breed, 8 unique genes in the HP breed, 18 unique genes in the WBP breed, 33 unique genes in the WSP breed, 11 unique genes in the WP breed, and 13 common genes among the five Anhui indigenous pig breeds.

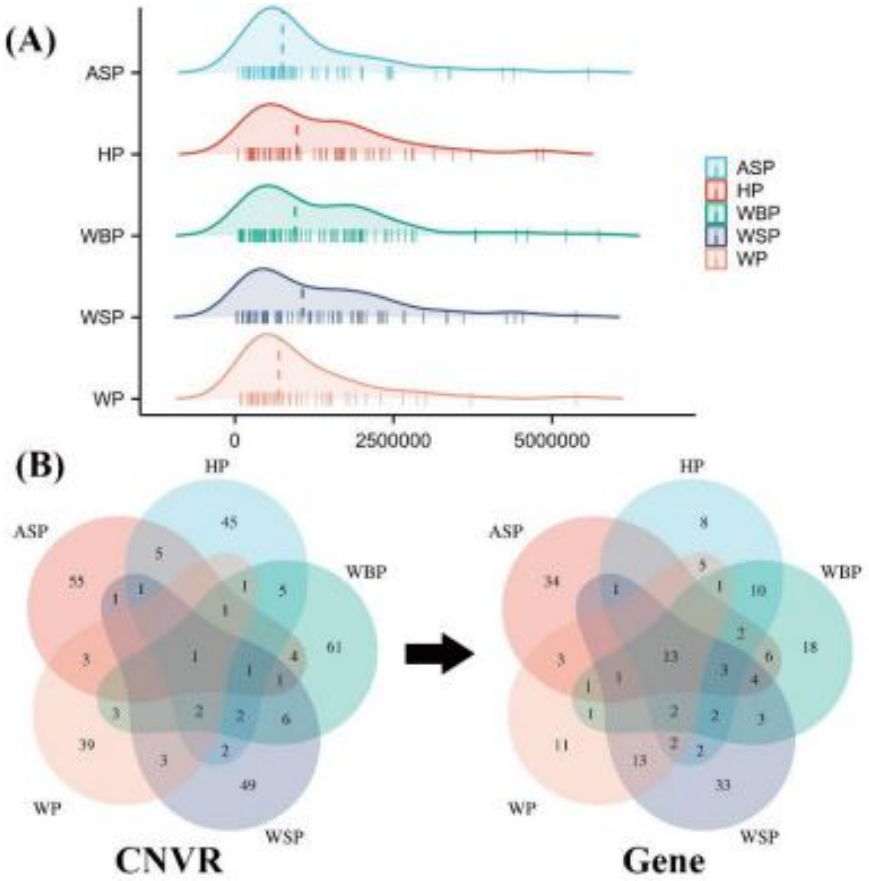

Figure 2: Distribution and annotated of CNVR in AHIPs. (A) Distribution of CNVR length in AHIPs. (B) CNVR and annotated total gene Venn diagram in AHIPs

Functional Enrichment Analysis of Genes in CNVR

In our previous study, we found that we found that ASP, HP, WBP, WSP and WP were clustered together through principal component analysis, indicating that the five breeds had high genetic similarity. Thus, in order to evaluate the function of these genes, we annotated and updated CNVR to Ensemble Genes 109 version, and ultimately obtained 172 genes. We performed GO terms and KEGG pathway enrichment analysis, as shown in Figure 3. The results of GO enrichment analysis showed that these genes enriched in fatty acid biosynthetic process (GO: 0006633, p = 4.99×10-2, 3 genes), protein destabilization (GO:0031648, p = 4.55×10-2, 2 genes), protein-ribulosamine 3-kinase activity (GO:0102193, p = 1.59×10-2, 2 genes). In the KEGG pathway analysis, there are five pathways that were significantly enriched, including the taste transduction (ssc04742, p = 1.56×10-2, 4 genes), gastric acid secretion (ssc04971, p = 1.68×10-2, 4 genes), carbohydrate digestion and absorption (ssc04973, p = 4.62×10-2, 3 genes) and glutamatergic synapse (ssc04724, p = 4.96×10-2, 4 genes).

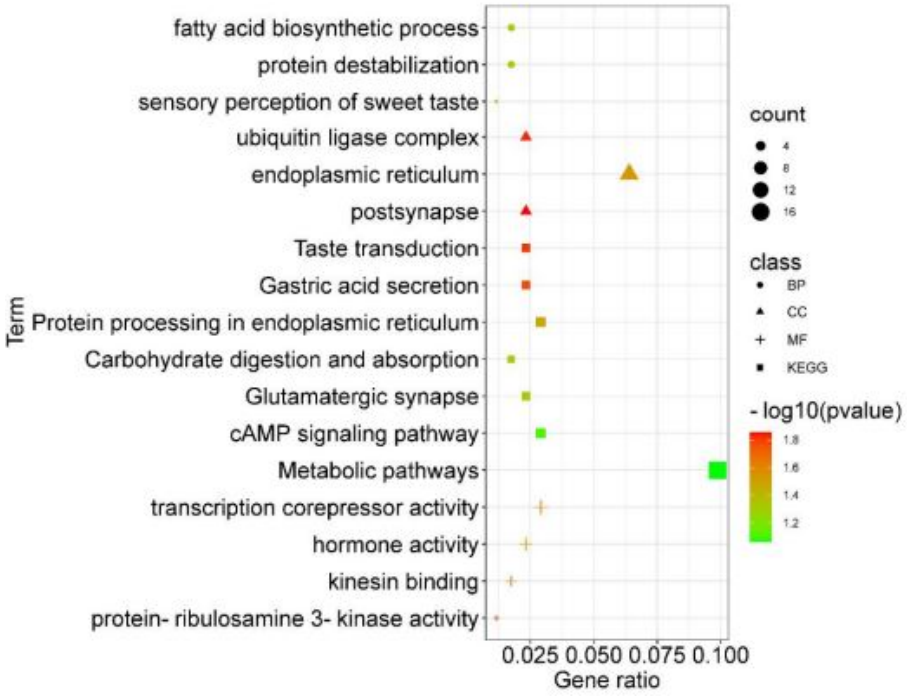

Figure 3: GO term and KEGG pathways analysis of the annotated genes in CNVR in AHIPs, circular referring to biological process, triangle referring to cellular component and cross referring to molecular function and square referring to KEGG pathways analysis, the size of dot refers to the count genes related to pathway, and the red to green indicate the significant p-value change

Discussion

The number of Chinese indigenous pig breeds is one of the richest in the world, and these pig breeds which have some independent characteristics are widely distributed in China, and provide us with a large amount of material for studying genetic characteristics and population diversity. Previously, we have researched runs of homozygosity and selection signals among five Anhui indigenous pig breeds, and found the characteristics of selection among these breeds during domestication and long-term breeding [25,26]. Whereas CNVs are large structural variations that can affect gene activity by disrupting the active portion of the encoded protein of the gene, altering the amount of expression of the gene, or disrupting regulatory regions of the genome that control gene activity. Not only that, some lethal CNV or CNV characteristics that are not conducive to individual adaptation to the environment have gradually disappeared from the genome or population in the long-term domestication and breeding process of animals [27]. And those benign CNVs, which coexist with animal individuals, may already be adaptive [7]. However, many studies have shown that the impact of CNV on important economic traits in pigs exists [28,19].

In our study, we compared with the study of Wang et al. [29] in Large White (LW) pig breeds, which used the same detection tool with our study, and found that the number, average length, and the percentage of CNVs in the pig genome were lower than those of the Anhui indigenous pig breeds. We considered that the reason for this phenomenon might be because the Porcine 80K SNP Bead chip was designed according to the mutation site of imported pig breeds and resulting in imported pig breeds with higher alignments. And then, we found that the CNVR identified in this study has also been reported in other indigenous pig breeds in China [15,30]. Finally, we also found that a total of 30 CNVR occurring within more than half of the breeds, with the largest number in the WBP breed, with 15 CNVRs. We considered that breeding objectives and selection intensity maybe influence CNV variation across the genome because of previous studies reporting that CNVs can be inherited [13]. We compared them with the QTL database and identified QTLs related to important economic traits in pigs, such as meat quality traits, growth traits and reproductive traits.

We performed enrichment analysis on CNVR annotated genes. We found that several candidate genes associated important economic traits in pigs. Fatty acids are involved in various biological processes in the organism, such as immune regulatory functions, metabolism. They are important

8334375326707500nutrients and metabolites in living organisms, and play an important role in the metabolic homeostasis and determining meat quality [31]. The elongase of very long chain fatty acids (ELOVL) family is a family of genes encoding very long chain fatty acids (VLCFA) in mammals and is the rate limiting enzyme catalyzing a cycle reaction of VLCFA synthesis. It plays an important role in the regulation of lipid biosynthesis, fatty acid metabolism, and the development of several metabolic diseases [32].Fan et al. [33] found that copy number variations in one of the Elovl family members in ASP breeds. One of these, the *ELOVL4* gene was first studied in fish [34], which main expressed in retina [35], brain [36], and nests [37] in mammals and involved in the synthesis of polyunsaturated fatty acids [38]. In our study, we found that the *ELOVL4* gene was loss type in ASP breeds. In studies in mice found that mice lacking a functional Elovl4 protein died perinatally, and it was guessed that dehydration from faulty permeability barrier formation in the skin [39]. These results implicate the importance of these long chain fatty acids in the life activities of animals. We also found the acyl-CoA synthetase family member 3 (*ACSF3*) gene, which encodes malonyl COA produced in mammals and is a key regulator of metabolism that can coordinate fatty acid production and oxidation and promote adipogenesis [40,41]. Not only that, *ACSF3* plays a key role in the regulation of cellular triacylglycerol and long-chain polyunsaturated fatty acid levels, and the polymorphism of *ACSF3* may serve as useful molecular markers in the beef breeding of intramuscular fat deposition [42]. Zhang et al. [43][45] compared that the expression patterns of mammary glands from goats, sheep, and cows during the non-lactation and lactation phases, indicating that the *ACSF3* gene may be involved in the formation mechanism of goat flavor in goat milk. Similarly, the expression of fatty acid metabolism genes has been shown to be an important factor associated with the intramuscular fat content (IMF) [44]. Cai et al. [45] compared the difference of transcriptome of longissimus thoracis muscle between Mashen pigs and LW pigs at different age stages based on RNA Seq technology, and found that the dorsal longest muscle IMF began to accumulate at around 90 days of age and the expression of *ACSF3* gene was up regulated, which might explain that the more IMF phenotype accumulated in MS breed than in LW breed. In our study, we found that the *ACSF3* gene was in loss type in all breeds except for the WSP breeds. And also, only in the WSP breeds, we found that the ATP citrate lyase (*ACLY*) gene was gain type. The *ACLY* gene is a cytosolic enzyme that is a key factor linking glucose metabolism and lipids. Albuquerque et al. [46] demonstrated that the *ACLY* gene plays an important role in fatty acid synthesis by real-time qPCR, as *ACLY* is responsible for catalyzing reactions that produce specific non lipid precursors. Similarly, variation in *ACLY* affects multiple traits associated with animal production [47], as SNP polymorphism in the *ACLY* gene was found to be associated with growth traits in beef cattle research. And the expression of *ACLY* is associated with intramuscular fat percentage in sheep [48].

In this study, we also found genes associated with reproductive traits. A series of methylation 8124825181927500events were occurred following a sperm egg binding event in female mammals [49], including tet methylcytosine dioxygenase 1 (TET1) catalyzes the conversion of 5-methylcytosine to 5-hydroxymethylcytosine, starting the process of DNA methylation [50]. Therefore, the research of *TET1* gene in germ cell development has attracted more and more attention. It is involved in regulating multiple physiological processes such as germ cell genesis [51], early embryo formation [52], etc. The lack of *TET1* gene will lead to DNA methylation defects, which will reduce the expression of meiosis gene subsets in oocytes [49]. Research reports have found that the synuclein alpha (*SNCA*) gene was found to be differential expressed in the genital tract of female animals and the testicular maturation process of male animals, indicating that *SNCA* may play an important role in the morphogenesis of the ovaries and testes [53]. The *SNCA* belongs to a family of small and highly conserved proteins in vertebrates [54]. And Li et al.[55,57] found that the SNCA gene was selected by comparing the high-fecundity and low-fecundity sows, which is beneficial for improving the reproductive traits of sows. In our study, we mapped to the CNVR by the location where the *SNCA* gene was located and found that this CNVR segment was present as a loss type in the WSP and WBP populations, respectively, and that their frequencies in the populations were 40.00% and 66.67%, respectively. We contrasted the QTL database and identified 20 and 15 QTLs related to growth (ID =17783, 139167) and meat quality (ID = 7636, 21653) traits, etc.

Although we have found some interesting information in the AHIPs, but there have some limitations in our study. Due to the virus invasion of African pig fever, it has had an impact

on the AHIPs. Therefore, the sample size in our study was small, and we did not collect phenotypic data for these breeds and did not search for CNVs associated with traits. Second, we did not conduct some molecular experiments for verification because of the impact of the epidemic and the fact that these breeds are currently in a state of protection and reproduction. Our follow-up study was hoped to expand the sample size, collect phenotypic data, and then perform validation of our results in the Anhui indigenous pig breeds.

Conclusion

In this study, we comprehensively detected CNV in five AHIPs based on the Porcine 80K SNP BeadChip, and then analyzed the distribution and size difference of CNV segments between each breed. Finally, we conducted GO terms and KEGG pathway analysis and find some candidate genes related to fat deposition and reproduction traits. These results reveal the possible role of these CNV in contributing to functional evolution and help us expanded our understanding of the impact of CNV on important economic traits in indigenous pig breeds. It will provide a theoretical basis for our future work on variety conservation and genetic improvement.

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Author Contributions

XLZ and CLW conceived the study. MZ and WZ collected the samples and recorded the phenotypes. HW and SGS supervised the study and proposed revisions to the manuscript. CLX and LQL performed analysis. CLX and CLW wrote and revised the manuscript. All authors read and approved the manuscript.

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Data Availability Statement

The 150 individuals of total signal intensity (LRR) and B Allele frequency (BAF) data in the present study are available from the FigShare Repository: https://figshare.com/s/bea6b416df538aee3809.

Declarations

Ethics approval and consent to participate

The experiments were performed according to the Regulations for the Administration of Affairs Concerning Experimental Animals and approved by the Animal Research Committee of the Anhui Academy of Agriculture Sciences.

Consent for Publication

Not applicable.

Competing Interests

The authors declare that they have no competing interests.

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