INTRODUCTION

Microsatellites, also known as simple sequence repeats (SSRs) or short tandem repeats (STR), are repeat sequences of 2–6 nt length that play important roles in genomes (Liu et al., 2017). They have become powerful DNA markers in animal breeding using marker-assisted selection (MAS; Beuzen et al., 2000). They can be used in the identification of economically important traits and diseases, individuals and infer parent–child relationships. In humans, SSRs are associated with a variety of diseases, such as cancer and Kennedy’s disease (Breza & Koutsis, 2019; Fujimoto et al., 2020; Vieira et al., 2016). In cattle and goats, they significantly affect growth traits, milk quality, and virus resistance (Avondo et al., 2019; Dux et al., 2018; Karim et al., 2011). However, few studies have

A novel (ATC)_n microsatellite locus is associated with litter size in an indigenous Chinese pig

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Abstract

Simple sequence repeats (SSRs) are an important part of the genome and have become powerful auxiliary DNA markers in animal breeding using marker-assisted selection (MAS). Based on previous sequencing data of Qinghai Bamei pigs, a total of three novel candidate SSR loci were analysed in this study. Time-of-flight mass spectrometry (TOF-MS) was used for SSR genotyping, and association analyses between SSRs and the litter size of Qinghai Bamei sows was also performed. The results of genotyping showed that the (ATC)_n-P1, (AC)_n-P2 and (AC)_n-P3 loci had 2, 3 and 18 genotypes, respectively; 2, 3 and 8 alleles were also identified at these loci. Except for the (AC)_n-P2 locus, the polymorphism information content (PIC) values of other loci were greater than 0.25. Association analyses indicated that only the (ATC)_n-P1 locus was significantly associated with the litter size of Qinghai Bamei sows (p = .047). Compared to 189-/189- genotype, individuals with the 189-/195-genotype had the senior litter size, which was 9.04 ± 0.21. Our results enrich the data on SSRs in Qinghai Bamei pigs and indicate that (ATC)_n-P1 is a candidate locus for MAS in the pig industry.

KEYWORDS
litter size, pig, simple sequence repeat (SSR), time-of-flight mass spectrometry (TOF-MS)
considered the associations between SSRs and economic traits in indigenous Chinese pigs.

There are two traditional methods for detecting SSRs, namely, sequencing and gel-based approaches. However, these two methods are time-consuming and provide very little information about quantitative differences (Vogel et al., 2009). Fortunately, in recent years, SSR detection technology has been reformed. Time-of-flight mass spectrometry (TOF-MS), a powerful tool for qualitative research and relative quantitative determination of variation (Seichter et al., 2004), can greatly improve the accuracy and efficiency of SSR detection (Bonk et al., 2003). It measures the molecular mass of the target and essentially minimizes inaccuracies (Ahlstrom et al., 2014). In a single experiment, it can reveal variability in several repeat lengths (Vogel et al., 2009). Therefore, screening of SSRs with TOF-MS can provide a theoretical basis for MAS of pigs and technical support for the breeding of superior traits.

The litter size of females has an important influence on the reproductive success of livestock. Litter size can be affected by genetics, nutrition, the environment and disease. Genetic factors have far-reaching effects. For example, the bone morphogenetic protein receptor type 1B (BMPR-1B, also known as FecB) gene in sheep may have major effect on their reproduction (Qi et al., 2020). When A746G mutation occurred in FecB gene sequence, BB genotype would show more litter size (Chen et al., 2015). The equivalent gene in pigs is follicle stimulating hormone subunit beta (FSHR; Bernard & Tran, 2013). However, like most economically important traits, litter size is a complex quantitative trait that might be controlled by a major gene along with several minor ones. Apart from the FSHR gene, mutations in Cytochrome B, prolactin (PRL), swine leukocyte antigen 11 (SLA-11) and other genes can also significantly affect the litter size of sows (Korwin-Kossakowska et al., 2009; Pradhan et al., 2018; Zhang et al., 2019). Therefore, to improve the litter size and thus production efficiency of females, it is important to select dominant stable genotypes using an advanced technology such as MAS.

Qinghai Bamei pigs are strongly adaptive and resistant to stress, have high fat deposition, resist of crude feed and feature stable genetics (Zhang et al., 2018), which are one of the important economic breeds in Qinghai. However, its female fertility is lower than that of commercial pigs in China. On the one hand, Qinghai Bamei sows reach sexual maturity at 120 days and can be bred at 300 days after birth. On the other hand, Qinghai Bamei sows have fewer litter size with only about eight live litter size (China National Commission of Animal Genetic Resources, 2011). Enhancing its fecundity, increasing the number of litter size is a complex quantitative trait that might be controlled by a major gene (Hui et al., 2020; Wang, et al., 2020). The Nanodrop 2000 was used to detect the purity (A260/A280 ratio) and quality of DNA.

### 2.1 | Samples and phenotypic data collection

Ear samples of 256 adult Qinghai Bamei sows were randomly selected from the breeding farms (Qinghai, China). Some of these sows had records of litter size. All tissues were immediately frozen in liquid nitrogen, brought back to our lab and stored at −80°C.

### 2.2 | DNA extraction

DNA was extracted using a high salt-extraction method (Aljanabi & Martinez, 1997), and then diluted to a standard concentration (10 ng/μl) and stored at −20°C for the detection of genetic variation (Hui et al., 2020; Wang, et al., 2020). The Nanodrop 2000 was used to detect the purity (A260/A280 ratio) and quality of DNA.

### 2.3 | Primer design

Based on preliminary sequencing results (data not shown), three SSR loci were selected for further analysis. (ATC)n−P1, (AC)n−P2 and (AC)n−P3 represented (ATC)n, (AC)n and (AC)n, respectively. Primer 5.0 and the NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) primer designing tool were used to design the primers (Zhang et al., 2020). Primer information is shown in Table 1.

### 2.4 | Detection of SSR variants using TOF-MS

The specific primers and genome DNA of Qinghai Bamei sows were used to perform polymerase chain reaction (PCR; Smofuch et al., 2019). The products were sent to Saisike Biotechnology Co., Ltd. for TOF-MS analyses. The specific experimental methods followed Seichter et al. (2004) and Rau et al. (2019).

### 2.5 | Statistical analyses

The methods of Nei and Roychoudhury (1974) and Botstein et al. (1980) were used to detect homozygosity (Ho), heterozygosity (He), effective allele numbers (Ne) and polymorphism information content (PIC) using PopGene version 1.3.1 (Molecular Biology and Biotechnology Centre, University of Alberta, Edmonton, AB, Canada). The SHEsis program (http://analysis.bio-x.cn) was used to calculate Hardy–Weinberg equilibrium (HWE; Wang, et al., 2020).
A Student’s t test (T-test) with a paired two-tailed distribution and ANOVA were used to analyse the associations between SSR variation and litter size.

3 | RESULTS

3.1 | SSR Identification

As shown in Table 2, TOF-MS identified two alleles (189- and 195-) and two genotypes (189-/189- and 189-/195-) at the (ATC)n-P1 locus (Figure 1). The frequency of the 189-/195- (0.930) genotype was higher than that of 189-/189- (0.070). Three alleles (126-, 128- and 136-) and three genotypes (126-/136-, 128-/136- and 136-/136-) were found at the (AC)n-P2 locus (Figure 2). In addition, the 136-/136- genotype showed the highest frequency (0.929). Similarly, there were various alleles (8 types) and genotypes (18 types) at the (AC)n-P3 locus (Figure 3).

3.2 | Genetic parameters

Next, the genotypes, allele frequencies and genetic parameters, including Ho, He, Ne and PIC, were calculated and analysed (Table 2). The values of Ho, He, Ne and PIC were 0.502, 0.498, 1.992 and 0.374 at the (ATC)n-P1 locus; 0.932, 0.068, 1.073 and 0.067 at the (AC)n-P2 locus; and 0.253, 0.747, 3.958 and 0.709 at the (AC)n-P3 locus.

### Table 1

| Loci        | Primer names | Primer sequences (5′-3′) | Sizes (bp) | Repeat motif | Location                        |
|-------------|--------------|--------------------------|------------|--------------|---------------------------------|
| (ATC)n-P1   | F: TTCTCCTTCCCCCATATGCTT R: GTGTCAGATGAGTTCGA | 190         | (ATC)n     | Chr17:13600112-13600301 (LOC100155289 gene) |
| (AC)n-P2    | F: AACTTCAGGTGCAACAGCAA R: ATAGATGGGTAACGGGGACC | 139         | (AC)n     | Chr13:145368763-145368901 (ZBTB20 gene) |
| (AC)n-P3    | F: GTGATCCCTTTCAAGTAGTGAGGAA R: GCATTTTGCAATCTGGGAGT | 134         | (AC)n     | Chr15:2003259-203396 (intergenic region) |

### Table 2

| Loci        | Sample size | Genotypes | Genotypic frequencies | Allelic frequencies | Population parameters | P (HWE) |
|-------------|-------------|-----------|-----------------------|---------------------|-----------------------|---------|
| (ATC)n-P1   | n = 256     | 189-/189- | 0.070                 | 0.535               | Ho: 0.502 He: 0.498  Ne: 1.992  PIC: 0.374  | p < .05 |
|             |             | 189-/195- | 0.930                 | 0.465               |                       |         |
| (AC)n-P2    | n = 255     | 126-/136- | 0.055                 | 0.027               | Ho: 0.932 He: 0.068  Ne: 1.073  PIC: 0.067  | p > .05 |
|             |             | 128-/136- | 0.016                 | 0.008               |                       |         |
|             |             | 136-/136- | 0.929                 | 0.965               |                       |         |
| (AC)n-P3    | n = 242     | 128-/128- | 0.116                 | 0.353               | Ho: 0.253 He: 0.747  Ne: 3.958  PIC: 0.709  | p < .05 |
|             |             | 128-/130- | 0.198                 | 0.308               |                       |         |
|             |             | 128-/134- | 0.041                 | 0.081               |                       |         |
|             |             | 128-/136- | 0.161                 | 0.151               |                       |         |
|             |             | 128-/140- | 0.012                 | 0.002               |                       |         |
|             |             | 128-/142- | 0.041                 | 0.027               |                       |         |
|             |             | 128-/146- | 0.021                 | 0.041               |                       |         |
|             |             | 130-/130- | 0.112                 | 0.037               |                       |         |
|             |             | 130-/134- | 0.029                 | -                   |                       |         |
|             |             | 130-/136- | 0.058                 | -                   |                       |         |
|             |             | 130-/138- | 0.004                 | -                   |                       |         |
|             |             | 130-/140- | 0.041                 | -                   |                       |         |
|             |             | 130-/142- | 0.025                 | -                   |                       |         |
|             |             | 130-/146- | 0.037                 | -                   |                       |         |
|             |             | 134-/134- | 0.045                 | -                   |                       |         |
|             |             | 136-/136- | 0.025                 | -                   |                       |         |
|             |             | 136-/142- | 0.017                 | -                   |                       |         |
|             |             | 136-/146- | 0.017                 | -                   |                       |         |
locus, respectively. According to their respective $p$ values, only the (AC)$_n$-P2 locus was at HWE.

### 3.3 Association analysis of mutations

The T-test and ANOVA results indicated that among the three SSR loci, only (ATC)$_n$-P1 was associated with litter size (Table 3). In particular, 189-195- individuals had larger litters ($9.04 \pm 0.21$), followed by 189-189- sows ($p = .047$). There were no significant differences in litter size at the other two loci, with $p$ values of .328 and .220, respectively.

### 4 DISCUSSION

As DNA markers, SSRs were highly emphasized two or three decades ago, but have received less attention in the last decade. The main reasons for this are their low accuracy of detection and the low degree of automation in the techniques used to identify them. However, it is worth noting that SSRs are widespread in the animal genome (Zhao et al., 2018). Moreover, compared to the more commonly used single nucleotide polymorphisms (SNPs) and insertions/deletions (indels), the mutation rates of SSRs are higher (Fotsing et al., 2019). In addition, some SSRs have important physiological functions in animals. Karim and his colleagues found that SSRs in PLAG1-CHCHD7 intergenic region can affect promoter activity and influence the binding of nuclear factor (Karim et al., 2011). Not only that, the SSRs of IGF2R and LEP genes in cattle may influence the milk traits, feed intake, milk fatty acid composition and metabolic state (Avondo et al., 2019; Dux et al., 2018). Therefore, they are important targets to inform breeding practices. Fortunately, the emergence of TOF-MS and other methods makes the screening of SSRs more accurate and convenient than what can be attained using the traditional methods (Beuzen et al., 2000).

In previous work, we identified some potential microsatellites in the genome of Qinghai Bamei pigs (data not shown). The (ATC)$_n$-P1 locus was located on Chr17: 13600112–13600301, which belonged to an uncharacterized LOC100155289 gene (Table 1). Similarly, the (AC)$_n$-P2 locus was located in the zinc finger and BTB domain containing 20 (ZBTB20) gene belonging to chromosome 13. The (AC)$_n$-P3 locus was found between the uncharacterized LOC106506168 gene...
and the uncharacterized LOC102159607 gene in the intergenic region on chromosome 15.

After genotyping the three novel SSR loci using TOF-MS, we analysed the allelic frequency, genotypic frequency and genetic diversity of each locus following the method of Botstein et al. (1980). In genetic breeding, markers with $PIC$ values greater than 0.5 are considered very informative and values between 0.25 and 0.5 are considered somewhat informative (Serrote et al., 2020). Our results indicated that the (ATC)$_n$-P1 and (AC)$_n$-P3 loci could be used as potential markers for selecting sows that would produce large litters ($PIC$ values of 0.374 and 0.709, respectively).

Genetic parameter analyses indicated that these loci were not at HWE except for (AC)$_n$-P2 locus. The direct reason for this result was that there are more heterozygotes in the population of Qinghai Bamei sows (Graffelman et al., 2017). In addition, purification selection, inbreeding and population substructure in the breeding process were all potential causes for HWE-departure (Chen et al., 2017; Lee et al., 2008; Wang & Shete, 2012). These results were consistent with the genetic background of the Qinghai Bamei pigs.

Finally, association analyses indicated that only the (ATC)$_n$-P1 locus was significantly associated with litter size ($p = .047$). Individuals with the 189-/195- genotype had larger litter size compared to the other genotypes. Although the (ATC)$_n$-P1 locus is located on the intron of the LOC100155289 gene, it may affect litter size in various ways. For instance, SSR mutations could directly regulate ontology gene expression (Meloni et al., 1998) or could
co-regulate gene expression with SSRs in the 5′-UTR region (Akai et al., 1999). In addition, the locus could increase abnormal splicing of gene (Sirand-Pugnet et al., 1995) and even lead to gene silencing (Saveliev et al., 2003). However, the specific mechanism of (ATC)n-P1 affecting litter size of Qinghai Bamei sows needs to be studied further. In addition, the LOC100155289 gene is uncharacterized. According to our experimental results, the LOC100155289 gene and the (ATC)n-P1 locus have the potential to be important candidates for MAS to improve the litter size of Qinghai Bamei pigs.

In the future, further experiments will be carried out to explore how mutations in the introns of LOC100155289 affect the litter size of Qinghai Bamei sows.

### 5 | CONCLUSIONS

Three SSRs of Qinghai Bamei sows were identified through TOF-MS. Among them, the (ATC)n-P1 locus was significantly associated with the litter size of Qinghai Bamei sows. Meanwhile, 189/-195- was the dominant genotype. These findings provide a scientific basis and theoretical guidance for the subsequent improvement of litter size in Qinghai Bamei pigs.

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### CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

### AUTHOR CONTRIBUTION

Guofang Wu: Conceptualization; Formal analysis; Funding acquisition; Project administration; Resources; Visualization; Writing-original draft; Writing-review & editing. Wenjuan Shen: Conceptualization; Investigation; Software; Visualization; Writing-original draft; Writing-review & editing. Xingxing Xue: Investigation; Methodology; Software. Lei Wang: Conceptualization; Formal analysis; Funding acquisition; Investigation; Project administration; Resources; Supervision; Validation; Writing-review & editing. Yuhong Ma: Investigation; Methodology; Resources. Jiping Zhou: Methodology; Resources.

### PEER REVIEW

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**TABLE 3** The association between different genotypes of different loci and the multiparity litter size in pigs

| Loci       | Genotypes  | Mean ± SEM (number) | p values |
|------------|------------|---------------------|----------|
| (ATC)n-P1  | 189/-195-  | 9.04 ± 0.21 (n = 79) | p = .047 |
|            | 189/-189-  | 7.85 ± 0.43 (n = 11) |          |
| (AC)n-P2   | 126/-136-  | 8.00 ± 0.00 (n = 2)  | p = .328 |
|            | 128/-136-  | 10.84 ± 0.84 (n = 2) |          |
|            | 136/-136-  | 8.98 ± 0.21 (n = 88) |          |
| (AC)n-P3   | 128/-128-  | 8.00 ± 0.50 (n = 9)  | p = .220 |
|            | 128/-130-  | 8.47 ± 0.35 (n = 25) |          |
|            | 128/-136-  | 9.40 ± 0.56 (n = 15) |          |
|            | 130/-130-  | 9.20 ± 0.43 (n = 15) |          |
|            | 130/-136-  | 9.43 ± 1.09 (n = 7)  |          |
|            | 130/-140-  | 10.31 ± 1.07 (n = 4) |          |
|            | 130/-146-  | 10.22 ± 1.13 (n = 3) |          |

Note: Values with different letters (a, b) differ significantly at p < .05.
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