Copy number variation of *ZNF280AY* across 21 cattle breeds and its association with the reproductive traits of Holstein and Simmental bulls

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ABSTRACT

The mammalian Y chromosome gene families in the ampliconic region are expressed predominantly or exclusively in the testis, and their copy number variations (CNV) are significantly associated with male reproductive traits, suggesting they have important roles in spermatogenesis and testicular development. *ZNF280AY* (zinc finger protein 280A, Y-linked) is a member of the zinc finger protein family and has been identified as a bovid-specific Y-chromosome gene. The current study applied a reliable quantitative real-time PCR method to estimate the CNV of *ZNF280AY* in 715 bulls across 21 cattle breeds and to further investigate the association of the CNV of *ZNF280AY* with bull reproductive traits and *ZNF280AY* mRNA expression levels in adult testis. The results revealed that the median copy number of *ZNF280AY* was 47, and the copy number varied from 11 to 154, showing significant CNV between and within the investigated cattle breeds. In addition, all 715 bulls were classified into Y1, Y2, and Y3 lineage groups based on a rapid genotyping method described previously. Pairwise comparisons indicated that bulls belonging to the Y1 lineage had a significantly lower median copy number (40) than bulls belonging to the Y2 (52) and Y3 lineages (57). Association analysis revealed that the CNV of *ZNF280AY* was correlated negatively with the percentage of normal sperm and sperm concentration in Holstein bulls, whereas no significant correlation was observed with ejaculation volume, total sperm count, sperm motility, postthaw motility (PTM), and scrotal circumference in Holstein and Simmental bulls. Furthermore, no correlation was observed between *ZNF280AY* copy number and *ZNF280AY* mRNA expression levels in the testis. The

current study suggests that the CNV of the *ZNF280AY* gene family is associated with male reproductive traits and may serve as a valuable marker for early bull fertility selection in Holstein breeding programs.

Key words: *ZNF280AY*, copy number variation, reproductive trait, Y chromosome

INTRODUCTION

Copy number variation (CNV) is an informative class of common genetic variation, revealing larger genetic effects on phenotypic variability, disease susceptibility, and species evolution than SNP (Zhang et al., 2009; Klopocki and Mundlos, 2011). To date, a large number of studies have been conducted to analyze genome-wide CNV in domestic animals, enabling CNV to be used as an important molecular marker for quantitative traits in genome-wide association studies (GWAS; Hou et al., 2012; Duran Aguilar et al., 2017; Gao et al., 2017). Due to its structural complexity and characteristic of harboring highly repetitive sequences, the Y chromosome has only been sequenced in a very limited number of species, which prevents the incorporation of the Y chromosome in genome-wide CNV studies.

Based on the studies of Y chromosome sequencing of human, mouse, and cattle (Skaletsky et al., 2003; Soh et al., 2014; Chang et al., 2013), we found that multicopy gene families are especially prevalent in the male-specific region (MSY) of the mammalian Y chromosome. These multicopy genes show CNV among different individuals. In addition, according to the expression and function analyses, multicopy genes on the mammalian Y chromosome are usually expressed predominantly or solely in the testis and play important roles in spermatogenesis and male fertility (Yue et al., 2014), and CNV of them are significantly associated with male fertility in humans (Giachini et al., 2009; Shen et al., 2013; Yan et al., 2017) and cattle (Hamilton et al., 2009; Yue et al., 2013, 2014).

Received November 27, 2018.

Accepted April 23, 2019.

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On the bovine MSY, 6 multicopy protein-coding genes have been identified (Chang et al., 2013). Of these, *TSPY* and *HSFY* are conserved across different lineages, whereas *PRAMEY*, *ZNF280AY*, and *ZNF280BY* are bovine-specific (Chang et al., 2013). A previous CNV study on bovine Y-linked genes revealed that the copy number (CN) of *PRAMEY* can vary within a range of 2 to 30 copies and is negatively associated with testis size, nonreturn rate, and percentage of normal sperm (PNS) in Holstein sires (Yue et al., 2013). In contrast, *TSPY*, *HSFY*, and *ZNF280BY* are largely amplified on the bovine Y chromosome (up to 350 copies). Association analyses found that the CNV of *HSFY* and *ZNF280BY* correlated negatively with testis size and were positively associated with sire conception rate (Yue et al., 2014), whereas the *TSPY* CN positively correlated with bull fertility and negatively correlated with *TSPY* mRNA expression levels in the testis (Hamilton et al., 2012). Generally, all current reports regarding bovine Y-linked CNV indicate this characteristic may serve as a valuable marker for early bull fertility selection in cattle breeding programs.

ZNF280AY (also known as *SUHW2*, suppressor of hairy wing homolog 2), as well as *ZNF280BY*, originated through the autosome-to-Y transposition of a gene block containing *ZNF280B* and *ZNF280A* on bovine chromosome 17 (Yang et al., 2011) and was subsequently amplified to ~79 copies on the bovine Y chromosome (BTAY) of a Hereford bull whose DNA was used for the bovine Y chromosome sequencing project (Chang et al., 2013). Beyond knowing that its mRNA is expressed predominantly in the spleen and at low levels in the testis and brain of bulls (Yang et al., 2011), the relationship between *ZNF280AY* and bull fertility still remains elusive. A previous study illustrated that zinc finger proteins have extraordinary diversity of structure and function in mammalian genomes (Laity et al., 2001). Several zinc finger genes have been found to play important roles in mammalian spermatogenesis and male fertility (Noce et al., 1992; Pieler and Bellefroid, 1994). For instance, inactivation of a zinc finger gene in human, *ZNF230*, leads to failure in spermatogenesis and azoospermia (Zhang et al., 2001). In addition, the *ZNF280BY* gene family, the paralog of *ZNF280AY*, showed sense RNA expression in different cell types of the seminiferous tubules, and antisense RNA specific in spermatids, suggesting a role in spermatogenesis (Yang et al., 2011); additionally, its CNV is significantly associated with bull fertility (Yue et al., 2014). Taken together, the CNV of *ZNF280AY* may be a valuable marker for the selection of elite sires at an early stage.

To date, no study has been conducted to analyze the CNV of *ZNF280AY* within and among different

cattle breeds and its relationship with bull fertility and mRNA expression levels. The objective of this study is to determine whether bovine *ZNF280AY* CNV differs among individuals and breeds and to unveil the association of *ZNF280AY* CNV with bull reproductive traits and mRNA expression. Here, we provide evidence that *ZNF280AY* varies from 11 to 154 copies among 715 bulls from 21 cattle breeds, and a significant association between CNV and bull fertility is observed ($P < 0.05$), whereas no relationship is present between *ZNF280AY* CNV and its mRNA expression levels in the testis ($r = -0.078$, $P = 0.704$).

MATERIALS AND METHODS

Sample and Reproductive Trait Collection, DNA, and RNA Preparation

A total of 715 bulls from 21 cattle breeds were collected in this study, including 7 introduced cattle breeds (Angus, Simmental, Holstein, Limousin, Charolais, Gir, and Nelore) with semen or DNA samples and 14 indigenous cattle breeds with blood or tissue samples (ear or testis) in China (Table 1). These introduced cattle breeds have a relatively distant genetic relationship with 14 Chinese indigenous cattle breeds. Of these, semen samples were collected from Holstein ($n = 264$) and Simmental ($n = 158$) bulls from the Tianjin Dairy Cattle Center and Gansu Livestock Breeding Center, respectively, who had at least 10 service records of semen quality evaluation, including ejaculation volume (EV), sperm concentration (SPC), total sperm count (TSC), PNS, sperm motility (SM), and postthaw motility (PTM). In addition, scrotal circumference (SC) was measured at 24 mo for 65 Simmental bulls (Supplemental Table S1; <https://doi.org/10.3168/jds.2018-16063>). The DNA samples of Nelore and Gir bulls were collected from the Bovine HapMap Project (Gibbs et al., 2009). Additionally, blood samples of 10 Holstein cows were collected for negative controls. Genomic DNA was extracted from blood, testis, and ear tissues using a standard phenol-chloroform method (Sambrook et al., 2001), and genomic DNA from semen was extracted using a high-concentration salt procedure (Gannes and Matthew, 2014). Total RNA was isolated from testis samples of 26 Xianan adult bulls (at 24 mo) by the TRIzol method (TaKaRa, Otsu, Shiga, Japan) and reverse-transcribed with *TransScript* One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China) using Anchored Oligo (dT)₁₈ Primer (TransGen Biotech) according to the manufacturers' guidelines. The concentrations and quality of DNA or RNA were determined by a

Table 1. Bull information and the median copy number of *ZNF280AY* in 21 cattle breeds

Breed (full name)	Short name	Sample size	Median copy number (range)	Y chromosome lineages
Angus	AGS	15	40 (31–84)	Y1
Anxi	AX	14	29 (16–50)	Y2
Bohai	BH	5	56 (39–62)	Y3
Gir	GIR	13	76 (57–134)	Y3
Hainan	HN	6	43 (30–69)	Y3/Y1
Holstein	HS	264 ¹	42 (11–154)	Y1
Jiaxian	JX	14	51 (28–84)	Y1/Y2/Y3
Limousin	LM	17	55 (32–87)	Y2
Luxi	LX	19	54 (13–77)	Y3
Leizhou	LZ	14	63 (38–100)	Y2/Y3
Mongolian	MG	17	59 (45–78)	Y2
Nelore	NEL	5	59 (46–79)	Y3
Nanyang	NY	29	54 (36–113)	Y2/Y3
Qinchuan	QC	11	49 (35–69)	Y2
Wannan	WN	7	48 (35–69)	Y3
Xuanhan	XH	10	49 (34–90)	Y3
Charolais	XL	34	57 (38–79)	Y2
Simmental	XM	158	53 (13–146)	Y2
Xianan	XN	26	39 (19–57)	Y2
Tibetan cattle	TC	19	30 (15–42)	Y1
Zaosheng	ZS	18	44 (33–89)	Y2
Total		715	47 (11–154)	

¹A Holstein bull was selected randomly as the calibrator to minimize the interassay variation.

NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA) and agarose gel electrophoresis and then stored at -80°C for subsequent analysis.

Primer Design

Based on the annotation of the bovine Y chromosome sequence, a total of 79 loci for *ZNF280AY* were identified on the MSY of a Hereford bull (Chang et al., 2013). To retrieve paralogous sequences of all loci, the *ZNF280AY* sequence (GenBank acc. no. HQ014564) was aligned to the bovine Y chromosome draft sequence assembly (GenBank acc. no. CM001061.2) using Splign (Kapustin et al., 2008). The retrieved sequences were aligned subsequently to obtain conserved regions for primer design using MEGA 5.0 (Tamura et al., 2011). The primers were designed from conserved regions by the Primer Premier 5.0 program ([http://](http://www.premierbiosoft.com/)

www.premierbiosoft.com/). The binding sites of the designed primers for *ZNF280AY* on the draft BTAY assembly (GenBank acc. no. CM001061.2) and the predicted sizes of each amplicon based on In-Silico PCR (<http://genome.ucsc.edu/cgi-bin/hgPcr>) are listed in Supplemental Table S2 (<https://doi.org/10.3168/jds.2018-16063>). A single-copy gene, *SRY* (sex-determining region Y), was used as a reference, and *SRY* primers were described in a previous study (Hamilton et al., 2009). The primer information and primer efficiencies can be found in Table 2.

To validate the male specific amplification of the primers described above, routine PCR was performed to amplify both male and female cattle genomic DNA, and distilled water was used as a blank control. The PCR system contained 12.5 μL of $2 \times$ Easy Taq PCR Super MIX (TransGen Biotech), 0.5 μL of each primer at 10 $\mu\text{mol}/\mu\text{L}$, 1 μL of 50 ng/ μL genomic DNA or

Table 2. Information on the primers used in this study

Gene	Primer sequence ¹ (5'–3')	PCR product size (bp)	Annealing temperature ($^{\circ}\text{C}$)	Primer efficiency
<i>ZNF280AY</i>	F: GGAACAGGTTGGGAAACT R: CTATGGACAGCATACAAGAGAT	203	62.5	2.00
<i>SRY</i>	F: CCAATTAAGCCGGTCACAGT R: GCACAAGAAAGTCCAGGCTC	162	62.5	1.95
<i>GAPDH</i>	F: AATGAAAGGGCCATCACCATC R: GTGGTTCACGCCCATCACA	204	62.5	—
<i>USP9Y</i>	F: AAACCCTTCAAGGTAATAAAACAAAA R: CACAGCTCCTCAAAACCAGA	552	57	—

¹F = forward; R = reverse.

distilled water, and 10.5 μL of distilled water, accounting for a total volume of 25 μL . Amplification reactions consisted of predenaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 62.5°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 5 min. The PCR products of *SRY* and *ZNF280AY* were separated by gel electrophoresis and visualized under UV illumination (GelDoc-It TS Imaging System, Upland, CA).

Determination of Paternal Lineages for Tested Bulls

Previously, 2 *Bos taurus* Y haplogroups, Y1 and Y2, and 1 *Bos indicus* Y haplogroup (Y3) were identified based on Y-SNP and Y-indels on the X-degenerate region of BTAY (Edwards et al., 2011). Afterward, Bonfiglio et al. developed a rapid and unambiguous method to classify the above 3 bovine Y-haplogroups using 2 polymorphisms within *USP9Y* (ubiquitin specific peptidase 9, Y-linked; Bonfiglio et al., 2012). Briefly, an 81-bp insertion can first distinguish the Y1 haplogroup from the Y2 and Y3 haplogroups by PCR and electrophoresis. Subsequently, Y3 can be differentiated from Y2 with an *SspI* endonuclease digestion of the same PCR product containing a Y-SNP (g.76426C > T; Supplemental Figure S1; <https://doi.org/10.3168/jds.2018-16063>). The detailed procedure to determine the 3 Y lineages for all bulls in this study followed the previous description (Bonfiglio et al., 2012).

CNV Estimation and mRNA Expression Levels of *ZNF280AY*

Quantitative real-time PCR (qPCR) was used to measure the CN of *ZNF280AY* for each bull using a CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA) and SYBR Premix Ex TaqII Kit (TaKaRa, Dalian, China). Standard curves were applied to evaluate primer efficiencies from a series of the DNA dilution of 40, 20, 10, 5, and 2.5 ng/ μL for both *ZNF280AY* and *SRY* primers. The resulting reactions had efficiencies of 2.00 and 1.95 for *ZNF280AY* and *SRY*, respectively, according to the equation $E = 10^{-1/\text{slope}}$ (Hamilton et al., 2009; Yue et al., 2013). For both genes, a 10- μL reaction system, containing 1 μL of DNA (5 ng/ μL), 5 μL of SYBR Premix Ex TaqII (TaKaRa), 0.5 μL of each primer at 10 $\mu\text{mol}/\mu\text{L}$, and 3 μL of distilled H_2O was run with the following PCR program: predenaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 62.5°C for 30 s, and extension at 72°C for 30 s, with a final extension at 72°C for 5 min. A melting curve was then generated by taking fluorescent measurements every 0.1°C from 60 to 95°C. Within each 96-well PCR plate, one

fixed bull sample was set as a calibrator to adjust the interassay variation. In addition, one female genomic DNA sample and one distilled water sample were used as a negative and a blank control, respectively. The CN of *ZNF280AY* test samples was estimated with 3 equations described previously (Hamilton et al., 2009; Yue et al., 2013).

The *ZNF280AY* mRNA expression levels in the testes of 26 Xianan bulls were estimated using the $2^{-\Delta\Delta\text{Ct}}$ method (Schmittgen and Livak, 2008) with *GAPDH* as an internal standard. The primers used for mRNA expression were described in a previous study (Yang et al., 2011). The qPCR reaction was run with the same components as those used for CNV estimation, with cDNA (20 ng/ μL) replacing DNA, under the following program: predenaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 62.5°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 5 min. The qPCR for each sample was run in triplicate.

Association and Statistical Analysis

To minimize technical error and to obtain accurate CN estimates, test samples were removed from further analysis if the raw qPCR data showed a coefficient of variation (CV) >1% between triplicates. The normal distribution of the *ZNF280AY* CN data was tested with the Kolmogorov-Smirnov and Shapiro-Wilk normality tests (Shapiro and Wilk, 1965; Justel et al., 1997). Conventional box plot analyses were implemented with the CN data to detect the outliers. Multiple pairwise comparison of the *ZNF280AY* median copy number (MCN) between breeds was performed using a non-parametric Mann-Whitney U test (Mann and Whitney, 1947), with a Bonferroni correction for multiple testing (Dunn, 1961). A 2-factor nested ANOVA model was used to compare the MCN among groups that were classified based on the Y1, Y2, and Y3 lineages of cattle individuals, with the breed nesting within Y lineage group, using the SAS 9.2 software (SAS Institute Inc., Cary, NC).

Association analysis between *ZNF280AY* CNV and reproductive traits was performed for Holstein and Simmental bulls using the Pearson correlation test in SPSS 17.0 (SPSS Inc., Chicago, IL). Furthermore, a general linear model in SAS 9.2 was applied to investigate the effect of CNV of *ZNF280AY* and breed, and their interaction on EV, SPC, and TSC. The Student *t*-test was conducted to compare reproductive traits between 20 Holstein bulls with the highest CN and 20 Holstein bulls with lowest CN of *ZNF280AY*. Additionally, the correlation between *ZNF280AY* CNV and its mRNA expression levels was analyzed using the Spearman test

Figure 1. Gel electrophoresis of PCR products of the bovine *ZNF280AY* and *SRY* genes. The primers of these 2 Y-linked genes amplified male-specific bands; expected fragment size is labeled above each band. M = 2-kb DNA ladder; ♂ = male cattle genomic DNA; ♀ = female cattle genomic DNA; - = negative control (distilled water).

of SPSS 17.0. A P -value of <0.05 was considered statistically significant for all tests except for the multiple pairwise comparison of the *ZNF280AY* MCN between breeds.

RESULTS

CNV of *ZNF280AY* Across Cattle Breeds

Before estimating *ZNF280AY* CN and mRNA expression levels, the male specificity of the *ZNF280AY* and *SRY* primers was validated by routine PCR. The results revealed that primers for both genes showed

amplification only in male genomic DNA with expected fragment sizes, confirming that the designed primers are male specific (Figure 1) and can be used for *ZNF280AY* CNV estimation.

Based on the equations for CNV calculation described previously (Hamilton et al., 2009; Yue et al., 2013), the CN of *ZNF280AY* was 41 for the calibrator sample. Subsequently, significant CNV was observed between and within the investigated cattle breeds. The MCN of 715 bulls from 21 cattle breeds was 47, ranging from 11 to 154 copies (Table 1). According to the Kolmogorov-Smirnov and Shapiro-Wilk normality tests, the CN data for all cattle breeds did not fit a normal distribution ($P < 0.05$). Both Holstein and Simmental had large sample sizes, and the total populations of the 21 cattle breeds showed several outliers based on box plot analyses (Figure 2).

Pairwise comparisons were applied to compare MCN between the tested cattle breeds, which revealed significant differences in MCN between some cattle breeds (Supplemental Table S3; <https://doi.org/10.3168/jds.2018-16063>). Of those, Gir bulls possessed the highest MCN (76 copies, range: 57–134), whereas Anxi bulls showed the lowest MCN (29 copies, range: 16–50; Table 1). It was interesting to note that the CN of *ZNF280AY* in Tibetan bulls (30 copies, range: 15–42) was significantly different ($P < 0.001$) from that of most other breeds (Supplemental Table S3; <https://doi.org/10.3168/jds.2018-16063>).

Figure 2. Box plot analysis of copy number of *ZNF280AY* in cattle breeds. The median copy number of *ZNF280AY* is indicated by a central line in a box, and the black dots represent outliers. The top and bottom of a box and whiskers indicate the first and third quartiles (the 25th and 75th percentiles). The x-axis represents the different cattle breeds. AGS = Angus; AX = Anxi; BH = Bohai; GIR = Gir; HN = Hainan; HS = Holstein; JX = Jiaxian; LM = Limousin; LX = Luxi; LZ = Leizhou; MG = Mongolian; NEL = Nelore; NY = Nanyang; QC = Qinchuan; WN = Wannan; XH = Xuanhan; XL = Charolais; XM = Simmental; XN = Xianan; TC = Tibetan cattle; ZS = Zaosheng.

Comparison of CN Among Cattle Breeds with Different Y Lineages

The 21 cattle breeds in current study can be classified into 4 groups based on genotyping results using 2 polymorphisms in *USP9Y*, including the Y1-lineage group (Angus, Holstein, and Tibetan cattle), Y2-lineage group (Anxi, Limousin, Mongolian, Qinchuan, Charolais, Simmental, Xianan, and Zaosheng), Y3-lineage group (Bohai, Gir, Luxi, Nelore, Wannan, and Xuanhan), and combined Y-lineages group (Hainan, Jiaxian, Leizhou, and Nanyang; Table 1, Supplemental Table S4; <https://doi.org/10.3168/jds.2018-16063>). To explore the potential relationship between the CNV of *ZNF280AY* and paternal lineages, the MCN was compared among the 3 different Y lineages. Bulls with the Y1 lineage showed a significantly lower MCN (40 copies, range of 11 to 154) than bulls with the Y2 lineage (52 copies, range of 13 to 146) and Y3 lineage (57 copies, range of 13 to 134; $P < 0.001$). No significant difference was observed between the Y2 and Y3 lineages ($P > 0.05$), which was further confirmed in the combined Y-lineages group of the Nanyang cattle breed. Among 29 Nanyang cattle, 18 individuals with Y2 possessed a MCN of *ZNF280AY* similar to that of 11 individuals with Y3 (Figure 3). In addition, the low MCN of *ZNF280AY* in the Y1 lineage revealed that *Bos taurus* (BTA) bulls had a significantly lower MCN (46 copies, range of 11 to 154) than that of *Bos indicus* (BIN) bulls (57 copies, range of 13 to 134; $P < 0.001$), potentially because Y1 and Y2 are BTA lineages and Y3 is a BIN lineage (Figure 3).

Association Analysis Between *ZNF280AY* CNV and Bull Reproductive Traits

To obtain robust associations between the CNV of *ZNF280AY* and bull reproductive traits, Holstein and Simmental bulls with at least 10 service records from different seasons were included in association analyses. In 142 Holstein bulls, the CNV of *ZNF280AY* was significantly correlated with PNS ($r = -0.179$, $P = 0.016$; Table 3, Figure 4A), whereas no significant correlation was found between the CNV of *ZNF280AY* and the remaining semen quality parameters, including SPC, EV, TSC, SM, and PTM ($P > 0.05$; Table 3). In 107 Simmental bulls, the CNV of *ZNF280AY* had no significant relationship with either semen quality parameters or SC ($P > 0.05$; Table 3). In addition, 4 outliers were found among the above 142 Holstein bulls based on box plot analyses. To investigate whether the significant association was driven by outliers, these 4 outliers were excluded from a re-association analysis of the remaining 138 Holstein bulls. Notably, stronger correlations were found between the CNV of *ZNF280AY* and SPC ($r =$

-0.148 , $P = 0.042$) and PNS ($r = -0.206$, $P = 0.008$; Table 3, Figure 4B, C), indicating that the low CN of *ZNF280AY* was beneficial for Holstein bull fertility. For Simmental bulls, no significant association with SC measurement was identified after removing 3 outliers from 65 bulls ($P > 0.05$; Table 3).

To further investigate the effect of low and high CN of *ZNF280AY* on the bull productive traits, semen quality of Holstein bulls was compared between 20 bulls with the highest CN (high CN group; $CN \geq 55$) and 20 bulls with the lowest CN (low CN group; $CN \leq 30$) of *ZNF280AY* by a *t*-test. No difference was observed in EV, SPC, TSC, SM, and PTM for Holstein bulls between the high and low CN groups ($P > 0.05$), but PNS for the low CN group was significantly higher than for the high CN group ($P = 0.016$; Table 4), which was in accordance with Pearson correlation results.

Relationship Between *ZNF280AY* CN and mRNA Expression

The mRNA expression levels of *ZNF280AY* were evaluated in the testes of 26 Xianan adult bulls using the $2^{-\Delta\Delta Ct}$ method and then correlated with the CN of *ZNF280AY* in corresponding Xianan bulls. The results revealed no significant relationship between *ZNF280AY* CN and mRNA expression levels ($r = -0.078$, $P = 0.704$), suggesting no dosage effect in the testis.

Figure 3. Comparison of the median copy number (MCN) of *ZNF280AY* between bulls with 3 major Y chromosome lineages (Y1, Y2, and Y3) or 2 paternal lineages [*Bos taurus* (BTA) and *Bos indicus* (BIN)]. The MCN of *ZNF280AY* was significantly different between the Y chromosome lineages or paternal lineages ($P < 0.001$). The MCN of the Y3 lineage was significantly higher than those of the Y1 and Y2 lineages ($P < 0.001$), whereas MCN was not significantly different between the Y2 and Y3 lineages. Similarly, no significant differences were found between the Y1 and Y2 lineages in the Nanyang cattle ($P > 0.05$). The BTA-derived bulls had a significantly lower MCN of *ZNF280AY* than BIN-derived bulls ($P < 0.001$). The error bars represent SE. *** $P < 0.001$.

DISCUSSION

In this study, a robust qPCR method was applied to evaluate the CNV of *ZNF280AY* across 21 cattle breeds. This method has been shown to provide a very effective and accurate measurement of CNV because it takes full advantage of the public bovine Y chromosome assembly (CM001061.2), which enables us to design primers on the conserved regions of different *ZNF280AY* loci (Yue et al., 2014). In previous studies, the CN of *HSFY* was first estimated to be 74 copies in Holstein bulls without CNV (Hamilton et al., 2011), whereas a later study found that the CN of *HSFY* varied from 21 to 308 using the same method as the current study (Yue et al., 2014). In addition, the CN of 3 bovine Y-linked genes, *PRAMEY*, *HSFY*, and *ZNF280BY*, were previously determined to be 10, 192, and 234, respectively, in the bull L1 Domino 99375, which was used for bovine Y chromosome sequencing (Chang et al., 2013), and the current qPCR method estimated the CN for this particular bull to be 13, 204, and 239, respectively (Yue et al., 2013, 2014). Considering some unavoidable experimental errors, these CNV estimations are very reliable. Using this reliable qPCR method, the current study first found that *ZNF280AY* showed a wide range of CNV in 21 cattle breeds, and significant differences were observed in CN between and within cattle breeds (Table 1; Supplemental Table S3, <https://doi.org/10.3168/jds.2018-16063>).

According to the differences in the MCN of *ZNF280AY* among cattle breeds, we found that the MCN of Tibetan bulls was estimated at 30 copies, which was significantly lower than the MCN of other cattle breeds, except Anxi (29 copies; Table 1), and remarkably divergent from those of other cattle breeds (Supplemental Table S3; <https://doi.org/10.3168/jds.2018-16063>).

This finding may be due to differences in evolutionary selection pressure during the domestication process between different cattle breeds. Tibetan bulls have a restricted distribution in the Tibet Autonomous Region of China and have strong adaptive abilities for high-altitude environments (Song et al., 2016), which have led to the accumulation of a large number of domestication signals during long-term natural selection. Copy number variation is a predominant source of genomic structural variation and plays an important role in evolutionary diversification and domestication (Zhang et al., 2009, 2015). We anticipate that our findings in part reflect that the *ZNF280AY* gene evolved under stronger purifying selection in Tibetan bulls (Zhang et al., 2009), which could be interpreted as a signature of adaptation to different local or climatic conditions (Letaief et al., 2017). Simultaneously, our findings also provide new insight into the contribution of Y-linked multicopy genes to evolutionary selection pressure beyond reproductive traits (Hughes and Page, 2015).

Similar to a previous report, CNV were confirmed to be associated with species evolution, and some CNV are lineage specific (Li et al., 2013). In the present study, we found that the CN patterns of *ZNF280AY* vary greatly in different paternal lineages among the 21 breeds tested. The MCN of *ZNF280AY* was significantly reduced ($P < 0.001$) in the Y1-lineage group (40 copies) relative to those in the Y2-lineage (52 copies) and Y3-lineage groups (57 copies), and further analysis showed that the CN of *ZNF280AY* was highly expanded in the BIN lineage and reduced in the BTA lineage (57 copies vs. 46 copies; $P < 0.001$; Figure 3). This finding is congruent with previous reports indicating that the CN of multicopy gene families is significantly different between the BTA and BIN lineages (Chang et al., 2013). For instance, the MCN of *ZNF280BY* and

Table 3. Pearson correlation between the copy number of *ZNF280AY* and reproductive traits in the Holstein and Simmental populations

Breed	Reproductive trait ¹	With outliers		Without outliers ²	
		r	P-value	r	P-value
Holstein	EV	−0.066	0.216	−0.026	0.382
	SPC	−0.081	0.169	−0.148	0.042
	TSC	−0.081	0.169	−0.093	0.139
	SM	−0.007	0.467	−0.040	0.321
	PNS	−0.179	0.016	−0.206	0.008
	PTM	0.008	0.460	0.004	0.482
Simmental	EV	−0.090	0.286	−0.090	0.286
	SPC	−0.101	0.261	−0.101	0.261
	TSC	−0.042	0.397	−0.042	0.397
	SC	0.089	0.239	0.142	0.135

¹EV = ejaculation volume; SPC = sperm concentration; TSC = total sperm count; SM = sperm motility; PNS = percentage of normal sperm; PTM = postthaw motility; SC = scrotal circumference.

²Four and 3 outlier bulls identified in the box plot analysis were excluded from the correlation analysis in the Holstein and Simmental populations, respectively.

Figure 4. Scatter diagram reflecting the correlations between copy number (CN) variation (CNV) of *ZNF280AY* and male reproductive traits. The CNV of *ZNF280AY* was negatively associated with (A) percentage of normal sperm (PNS; $r = -0.179$, $P = 0.016$), (B) PNS ($r = -0.206$, $P = 0.008$; excluding 4 outliers), and (C) sperm concentration (SPC; $r = -0.148$, $P = 0.042$). Each gray triangle represents one individual.

PRAMEY in the BTA lineage were significantly higher than those in the BIN lineage (Yue et al., 2013, 2014). In contrast, the MCN of *HSFY* in the BTA lineage was significantly lower than that in the BIN lineage (Yue et al., 2013). These results lead us to believe that the CN of multicopy genes are associated with paternal

lineages in cattle. Therefore, it is necessary to consider the effects of paternal lineage when investigating the CNV of multicopy gene families among the Y lineages (Yan et al., 2017).

However, the mechanism underlying the differences in CN for gene families in different Y lineages is still unclear. As a consequence of the distinct evolution of the bovine Y chromosome, the morphology of the Y chromosome between *B. taurus* and *B. indicus* is remarkably different, as corroborated by fluorescence in situ hybridization, which revealed that the BAT Y chromosome is submetacentric, whereas the BIN Y chromosome is acrocentric (Goldammer et al., 1997). This remarkable difference is possibly due to a series of cytological events, including centromere transposition, chromosome segment transpositions, gain or loss of constitutive heterochromatin, and pericentric inversions (Iannuzzi et al., 2009). This morphological difference in different lineages likely will lead to diversified gene content. If so, we suggest that the differences in CN among Y chromosome gene families between the BTA and BIN lineages has accelerated the structural remodeling of Y chromosome morphology.

Recently, the mechanism of intrapalindrome gene conversion was theoretically verified, and this mechanism can generate CN changes in the human Y chromosome (Trombetta and Cruciani, 2017; Shi et al., 2018). However, the driving mechanism behind the large expansion and CNV of multicopy genes on the bovine Y chromosome remains unknown. Previous studies have suggested that the unique features identified in the primate MSY are palindromes, based on Y chromosome sequencing, and provide a basis for Y-to-Y gene conversion (Chang et al., 2013). The transcriptional landscape of the bovine MSY has revealed that approximately 80 palindrome-like repeat units (~420 kb per unit) constitute the bovine Y ampliconic region, and each unit resides in 4 multicopy families (*TSPY*, *ZNF280AY*, *ZNF280BY*, and *HSFY*; Chang et al., 2013), which provides an important structural foundation in favor of intra- and interpalindrome gene conversion and potentially regulates the equilibrium between gene loss and acquisition during Y chromosome evolution (Trombetta and Cruciani, 2017). Notably, regardless of the underlying cause of the temporal disparity in multicopy gene families (Oluwole et al., 2017), when combined with the significant differences in CNV of *ZNF280AY* across cattle breeds, we believe that the highly variable CN of this gene is most likely due to frequent gene conversion within the ~80 palindrome-like region.

Currently, the Y chromosome research hotspot mainly focuses on possible associations between male fertility and CNV in multicopy, testis-specific gene families (Hughes and Page, 2015). The majority of studies on

the association between the CNV of Y chromosome testis-specific gene families and male fertility have been proposed to look for some valuable molecular markers for male fertility selection or the diagnosis of male reproductive disorders. The 2 previous studies showed that the CNV of *HSFY*, *ZNF280BY*, and *PRAMEY* are negatively correlated with testis size (SC, age-adjusted relative SC) in Holstein bulls (Yue et al., 2013, 2014). A recent study on the mechanism of cattle-yak hybrid bull infertility indicated that the tremendous expansion of *TSPY* CN in hybrid bulls significantly influenced the success of spermatogenesis, which may be a potential risk factor for hybrid infertility (Zhang et al., 2016).

Our study revealed that the CNV of *ZNF280AY* was negatively associated with SPC and PNS in the Holstein population, suggesting that a bull with a higher CN of *ZNF280AY* tends to have worse semen quality. In contrast, a trend toward a negative correlation between *ZNF280AY* CN and SPC ($r = -0.101$, $P = 0.261$; Table 3) was found in the Simmental population, even though CNV was not significantly associated with semen quality or testicular size ($P > 0.05$). This result might be explained by the fact that the sample size of the Simmental population used for correlation analysis was relatively small and included limited phenotypic data for the assessment of reproductive performance. In addition, differences in genetic background between 2 cattle breeds can also cause bias. Therefore, it would be interesting to study the correlation between CNV and male fertility by performing large-scale phenotyping and assessing diverse types of male reproductive traits.

Gene dosage is the major molecular mechanism that conveys phenotypes associated with CNV (Zhang et al., 2009; Zhou et al., 2011). In most cases, CNV can alter the expression levels of functional genes and affect consequent phenotypes; such genes are described as dosage sensitive. For instance, CNV of spermatogenesis-related genes (*RBM1* and *TSPY*) were closely associated with their levels of gene transcription and translation, and their duplication led to increased or decreased mRNA

and protein levels (Hamilton et al., 2012; Yan et al., 2017). In this study, the CNV of *ZNF280AY* did not show a relationship with its mRNA expression levels in the testis, indicating that *ZNF280AY* may have not a dosage effect in the testis, and in fact, previous research showed that the spleen is the tissue with the highest *ZNF280AY* expression (Yang et al., 2011). Therefore, this correlation should be done in the spleen to check whether a dosage effect exists in a future study. In addition, a previous analysis of *ZNF280AY* genomic sequences predicted a short or no open reading frame. It has been speculated that the majority of the *ZNF280AY* loci on BTA Y chromosomes were pseudogenized over longer time periods (Yang et al., 2011). High sequence similarity (95–100%) between functional copies of *ZNF280AY* and the large number of pseudogenes potentially bias our CN detection (Yan et al., 2017). Until recently, there was no effective way to distinguish functional copies from pseudogenes. Therefore, it is challenging to design primers that capture only functional copies of the *ZNF280AY* gene as well as determine the relationship between *ZNF280AY* CN and gene expression levels. Furthermore, comprehensive understandings of gene function are ultimately required to detect functional copies and determine the correlation between *ZNF280AY* CN and its mRNA/protein levels in testis tissue as well as the subcellular location of the *ZNF280AY* protein.

CONCLUSIONS

The present study is the first to provide evidence that *ZNF280AY* was extensively expanded and showed CNV between and within different cattle breeds. An association study found that the CNV of *ZNF280AY* was negatively associated with sperm quality as measured by PNS and SPC, implying that *ZNF280AY* may play an important role in sperm maturation and providing valuable insight into early selection for bull fertility of Holstein dairy cattle.

Table 4. Comparison of reproductive traits between Holstein bulls with the high and low copy numbers (CN; mean ± SE) of *ZNF280AY*¹

Productive trait ²	Low CN (CN ≤30, n = 20)	High CN (CN ≥55, n = 20)	P-value
EV	6.78 ± 0.54	6.69 ± 0.58	0.904
SPC	14.78 ± 0.82	12.93 ± 1.15	0.197
TSC	102.10 ± 10.75	90.18 ± 13.50	0.494
PNS	85.55 ± 0.32	83.70 ± 0.66	0.016
SM	82.60 ± 1.66	81.00 ± 1.79	0.517
PTM	41.20 ± 0.29	41.50 ± 0.30	0.477

¹The comparison was conducted by Student's *t*-tests.
²EV = ejaculation volume; SPC = sperm concentration; TSC = total sperm count; PNS = percentage of normal sperm; SM = sperm motility; PTM = postthaw motility.

ACKNOWLEDGMENTS

This work was supported by National Natural Science Foundation of China (31501918) and the Program for Changjiang Scholars and Innovative Research Team in University (IRT13019). We are grateful to Wansheng Liu at Pennsylvania State University for sharing DNA samples of Nelore and Gire bulls used in the Bovine HapMap Project.

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