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Copy number variation of *PRAMEY* across breeds and its association with male fertility in Holstein sires

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ABSTRACT

Multi-copy gene families are especially prevalent in the male-specific region (MSY) of the mammalian Y chromosome. Copy number variations (CNV) of these Y-linked gene families have been shown to affect human and animal fertility. The *PRAMEY* (Preferentially expressed antigen in melanoma, Y-linked) gene family is a newly identified, bovid-specific Y-linked gene family, which codes for a cancer/testis antigen that is expressed predominantly in testis and various tumors. The *PRAMEY* gene family is believed to play an important role in spermatogenesis and male fertility in cattle. The objective of this study was to investigate the CNV of *PRAMEY* within and across breeds and to determine whether CNV was associated with reproductive traits in Holstein bulls. A quantitative real-time PCR method was applied to measure the copy number of *PRAMEY* among 460 bulls using a Y-linked single copy gene, *DDX3Y* (DEAD box polypeptide 3, Y-linked), as a reference. The median copy number of *PRAMEY* was 13, ranging from 2 to 31. Significant variations in *PRAMEY* copy number were observed among 15 breeds investigated. Holstein bulls had the lowest median copy number (12), whereas Limousin bulls possessed the highest median copy number (26). Furthermore, bulls in the taurine lineage (13) had a significantly lower median copy number than those bulls in the indicine lineage (20). Association analysis revealed that *PRAMEY* copy number was correlated negatively with scrotal circumference (SC), relative scrotal circumference (RLSC), percentage of normal sperm (PNS), and nonreturn rate (NRR), but had no significant association with postthaw motility (PTM), incubated motility (IM), percentage of intact acrosome (PIA), sire conception rate (SCR), or relative breeding efficiency (RBE). The data from this study indicate that CNV of the *PRAMEY* gene family is associated with male reproductive traits and may serve as a valuable marker for sire fertility selection at an early age in cattle.
Key words: *PRAMEY*, copy number variation, Y chromosome, bull fertility

INTRODUCTION

Genomic selection has been applied in cattle breeding programs to accelerate genetic gain for traits such as milk production (VanRaden et al., 2009) and meat quality (Mannen, 2011). However, limited opportunities exist for genetic improvement through genomic selection of semen quality and male fertility traits, partly because of the lack of molecular studies on male fertility. The Y chromosome is present in males only and carries several testis-specific genes that play fundamental roles in spermatogenesis and male fertility (Lahn and Page, 1997, 1999; Lahn et al., 2001; Cocquet et al., 2009; Reynard et al., 2009; Chang et al., 2011a; Yang et al., 2011). Recent progress in sequencing and structure analysis of the human (Skaletsky et al., 2003), mouse (Alföldi, 2008), and bovine Y chromosomes (Chang et al., 2013) has identified at least 3 types of sequence variations, including SNP, insertion/deletion (indel), and copy number variation (**CNV**), which provide an opportunity to develop genetic markers for male reproduction evaluation. Of particular interest is the deletion or CNV of the Y-linked multi-copy gene families that are all expressed predominantly or solely in testis (Skaletsky et al., 2003; Chang et al., 2013). In mice, deletions of Y-linked multi-copy genes, such as *Sly* (Sycp3-like Y-linked), affect spermatogenesis, with the most extensive deletions resulting in severe sperm-head malformations and infertility (Cocquet et al., 2009; Reynard et al., 2009). In humans, deletions (or microdeletions) in the *AZFa-c* (Azoospermia factor a, b, c) regions on the long arm of the human Y are the most frequent genetic cause of severe oligozoospermia and azoospermia (Krausz et al., 2003; Krausz, 2005), accounting for 10 to 18% of men with spermatogenic

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Table 1. Bull information and the median copy number of *PRAMEY* in 15 cattle breeds

Breed	No. of bulls	Median (range) copy number	Lineage
Angus	14	17 (16–21)	*Bos taurus*
Beefmaster	14	17 (13–24)	Taurine/indicine
Brahman	10	19 (9–31)	*Bos indicus*
Brown Swiss	14	20 (14–27)	*Bos taurus*
Charolais	10	21 (15–27)	*Bos taurus*
Gir	14	20 (19–21)	*Bos indicus*
Hereford	15[1]	17 (11–19)	*Bos taurus*
Holstein	257	12 (2–20)	*Bos taurus*
Jersey	28	14 (10–23)	*Bos taurus*
Limousin	14	26 (3–30)	*Bos taurus*
Nelore	10	25 (24–26)	*Bos indicus*
Norwegian Red	9	14 (8–16)	*Bos taurus*
Qinchuan (*Bos indicus*)	10	14 (7–16)	*Bos indicus*
Qinchuan (*Bos taurus*)	16	16 (11–30)	*Bos taurus*
Red Angus	11	15 (12–20)	*Bos taurus*
Santa Gertrudis	14	24 (20–25)	Taurine/indicine
Total or median	460	13 (2–31)	

[1]The bull used as the calibrator was not used for the median copy number estimation for the Hereford breed.

failure (Foresta et al., 2001; Kleiman et al., 2003; Krausz and Degl'Innocenti, 2006; Poongothai et al., 2009). Copy number variations of Y-linked multi-copy gene families, such as *DAZ* (Deleted in azoospermia) and *TSPY* (Testis-specific protein, Y-encoded), have been found to be associated with semen quality (Vodicka et al., 2007; Giachini et al., 2009) and reproduction in men (Lin et al., 2005).

The gene content of the bovine Y has been recently studied and at least 6 multi-copy, protein-coding gene families, including *TSPY*, *HSFY* (Heat-shock transcription factor, Y-linked), and *PRAMEY*, were identified (Chang et al., 2013). Both *TSPY* and *HSFY* have been analyzed with respect to their CNV in cattle (Hamilton et al., 2009, 2011, 2012). The CNV of *TSPY* is positively correlated with bull fertility and negatively correlated with gene expression level (Hamilton et al., 2012). A similar approach was applied to examine the copy number of *HSFY*, although no variation was observed in the initial report (Hamilton et al., 2011).

The *PRAME* (Preferentially expressed antigen in melanoma) gene is a member of the cancer/testis antigens that are expressed predominantly in normal testis and in a variety of tumors (Chang et al., 2011a); *PRAME* is believed to play important roles in immunity and reproduction (Kastner et al., 1996; Epping et al., 2005; Ortmann et al., 2008; Chang et al., 2011a). It is one of the most amplified gene families in Eutherian mammals, with approximately 90 copies in the mouse, 50 copies in the human, and 30 copies in the bovine genomes (Birtle et al., 2005; Church et al., 2009; Chang et al., 2011a). The *PRAMEY* (PRAME, Y-linked) gene is a bovid-specific Y-chromosome gene originating from transposition of an autosomal *PRAME* paralog on BTA17 and amplified on the Y chromosome during evolution (Chang et al., 2011a; Yang et al., 2011). The *PRAMEY* gene is expressed specifically in testis (Chang et al., 2011a), with the sense RNA of *PRAMEY* expressed specifically in spermatids and the antisense RNA expressed in all cell types in the seminiferous tubules. The highest expression is reported to occur in spermatids, suggesting that it plays an important role in spermatogenesis (Chang et al., 2011a). A recent study on mouse *Pramel1* (Prame-like 1), an ortholog of *PRAME/PRAMEY*, indicated that *Pramel1* is involved in acrosome formation and sperm motility (Mistry et al., 2013). Approximately 10 copies of *PRAMEY* are present in the Y chromosome (based on the draft sequence) of the bull whose DNA was used for the bovine Y sequence project (Chang et al., 2011a, 2013). However, it is unclear whether the copy number of *PRAMEY* varies between individual males and among cattle breeds. The objective of this study was to determine the CNV of *PRAMEY* in different cattle breeds and to test whether the CNV was associated with reproductive traits in Holstein bulls.

MATERIALS AND METHODS

Phenotype and Pedigree of Animals and DNA Preparation

A total of 460 bulls from 15 breeds were used in this study (Table 1). Semen samples were collected from 257 Holstein bulls that had phenotypic records and pedigree information. The remaining animals were either from the bovine HapMap Project (13 breeds; Gibbs et al., 2009) or from a Chinese local cattle breed

(Qinchuan), which did not have any phenotypic records or pedigree information. A Hereford bull L1 Domino 99375 (American Hereford Association registration number 41170496) was sequenced in the bovine Y chromosome sequencing project (https://www.hgsc.bcm.edu/content/y-chromosome-genome-project) and was used as a calibrator. This bull is the sire of the cow L1 Dominette 01449, whose DNA was sequenced in the bovine genome sequence project. Semen DNA was extracted using Qiagen DNeasy blood and tissue kit (Qiagen, Valencia, CA) according to manufacturer's instructions.

Of the 257 Holstein bulls that were used in AI, 140 had records for scrotal circumference (**SC**), age-adjusted relative scrotal circumference (**RLSC**), postthaw motility (**PTM**), incubated motility (**IM**), percentage of normal sperm (**PNS**), and percentage of intact acrosome (**PIA**). In addition, 82 (of these 140) bulls had data on sire conception rate (**SCR**), which is a bull fertility evaluation system recently developed by the US Department of Agriculture (http://aipl.arsusda.gov/reference/arr-scr1.htm), and 102 (of the 140) bulls had data on relative breeding efficiency (**RBE**), an in-house bull fertility evaluation parameter estimated by Select Sires Inc. (Plain City, OH). Relative breeding efficiency uses a similar methodology to SCR but contains only a 1-yr rolling database (Amann and DeJarnette, 2012). The remaining 117 Holstein bulls had data on nonreturn rate (**NRR**), a fertility parameter used by Semex Alliance (Guelph, ON, Canada). Nonreturn rate is a traditional methodology to evaluate bull fertility, indicating that a cow was inseminated and was not called to re-service within a given amount of time (usually 60 d) for the first service (Amann and DeJarnette, 2012). Data for the paternal pedigrees of the Holstein bulls were collected from the public databases, including the US Department of Agriculture-Animal Improvement Programs Laboratory (Beltsville, MD; http://aipl.arsusda.gov/) and the Holstein Association USA (Brattleboro, VT; http://www.holsteinusa.com).

Primer Design

The *PRAMEY* sequence (GenBank accession no. GU144301) was aligned to the bovine Y chromosome draft sequence assembly (GenBank accession no. CM001061, NCBI Project ID: 20275) using Splign (Kapustin et al., 2008). Ten *PRAMEY* loci were predicted and the paralogous sequences were retrieved and aligned by MEGA 5.0 (Tamura et al., 2011). The PCR primers were designed from conserved regions, based on the alignment of *PRAMEY* sequences, using the Primer Premier 5.0 program (http://www.premierbiosoft.com/). A single-copy gene *DDX3Y* (DEAD box polypeptide 3, Y-linked; GenBank accession no. NT182066) was used as reference. The primer sequences were as follows: *PRAMEY*: forward, 5′-GCCCATCCTGTGCCCCTGCT-3′; reverse, 5′-CTCCCTCCCCGCCCACTCTA-3′ and *DDX3Y*: forward, 5′-ATCGTGGGCGGAATGAGTGT-3′; reverse, 5′-CTTGGTGGAAGCGGTTTTGA-3′. To confirm the Y chromosome-specificity of the designed primers, a routine PCR was performed using male and female cattle genomic DNA as templates and water as a negative control. The PCR protocol was as follows: each 20-μL reaction contained 13.76 μL of distilled water, 0.5 μL of each primer (10 pmol/μL), 4 μL of Bioline 5× buffer (Bioline USA Inc., Taunton, MA, including 200 μ*M* deoxyribonucleotide triphosphates), 0.24 μL of Bioline Taq DNA polymerase (Bioline USA Inc.), and 1 μL of either genomic DNA (10 ng/μL) or water. Thermocycling consisted of an initial denaturation at 95°C for 5 min, followed by 35 cycles of at 94°C for 30 s, at 65°C for 30 s, and at 72°C for 30 s, and a final extension at 72°C for 5 min.

Quantitative Real-Time PCR

Quantitative real-time PCR (**qPCR**) was used to measure the copy number of *PRAMEY* in the samples using the 7900HT Fast Real-Time PCR System and Power SYBR Green PCR Master Mix kit (Invitrogen, Carlsbad, CA). Plates with 384 wells were set up to run the qPCR. On each plate, we set up wells for standard curve samples, a calibrator (i.e., bull L1 Domino 99375), and a negative control (distilled water). Standard curves were generated from bovine DNA diluted to 12.5, 5, 2.5, 0.5, and 0.1 ng/μL for both *PRAMEY* and *DDX3Y* primers. For the test bull samples, DNA was concentrated to 5 ng/μL. A PCR with standard curve samples was run in triplicate, and remaining test samples (including calibrator and negative control) were run in duplicate. In this study, we ran a total of 460 bulls on 6 plates (each plate was set up for 1 calibrator plus 87 testing samples). Each reaction contained 5 μL of SYBR Green PCR Master Mix, 0.5 μL of primers (10 pmol/μL), 3 μL of distilled water, and 1 μL of DNA template (5 ng/μL). The qPCR was run with a program of the following steps: predenaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 65°C for 30 s, and extension at 72°C for 30 s; final extension at 72°C for 5 min. A melting curve was then generated by taking fluorescent measurements every 0.1°C from 60°C until 95°C. The resulting reactions had an efficiency of 1.95 and 1.92 for *PRAMEY* and *DDX3Y*, respectively, according to the equation $E = 10^{-1/\text{slope}}$.

Copy Number Estimation

The copy number of *PRAMEY* was estimated for test samples by using the following 3 equations described in Hamilton et al. (2009):

$$\text{Copy number}_{\text{calibrator}} = \frac{(\text{E}_{\text{reference}})^{\text{C}_{\text{T}_{\text{reference}}}}}{(\text{E}_{\text{target}})^{\text{C}_{\text{T}_{\text{target}}}}}, \quad [1]$$

$$\text{Ratio} = \frac{\left(\text{E}_{\text{target}}\right)^{\Delta\text{C}_{\text{T}_{\text{target}}}(\text{calibrator} - \text{sample})}}{\left(\text{E}_{\text{reference}}\right)^{\Delta\text{C}_{\text{T}_{\text{reference}}}(\text{calibrator} - \text{sample})}}, \quad [2]$$

$$\text{Copy number}_{\text{test sample}} = (\text{copy number}_{\text{calibrator}}) \times (\text{ratio}). \quad [3]$$

In the above equations, the DNA sample of the Hereford bull L1 Domino 99375 was used as the calibrator. The cycle threshold ($\mathbf{C_T}$) value of the calibrator for each gene (*PRAMEY* and *DDX3Y*) was determined by the average of 12 C_T values obtained in 6 different plates for this particular sample; E = the PCR efficiency for either *PRAMEY* (1.95) or *DDX3Y* (1.92); and ΔC_T = C_T of the calibrator – C_T of the test sample.

Association and Statistical Analysis

To minimize the technical impact and to have an accurate copy number estimation, raw qPCR data that showed a coefficient of variation >1% between the duplicates were excluded from further analysis (a total of 35 bulls were eliminated in this study). The normality of the *PRAMEY* copy number data was assessed with the Kolmogorov-Smirnov and Shapiro-Wilk normality tests (Shapiro and Wilk, 1965; Justel et al., 1997). The multiple pairwise comparison of the *PRAMEY* median copy number between breeds was analyzed using a nonparametric Mann-Whitney U test (Mann and Whitney, 1947) with a Bonferroni correction (Dunn, 1961). In addition, the Mann-Whitney U test was used to compare the median copy number between groups that were classified based on the origin and formation of the cattle breeds: *Bos taurus* (**BTA**), *Bos indicus* (**BIN**), and composite (**COM**), and those groups that were classified into high fertility (NRR ≥70%), low fertility (55 ≤ NRR ≤ 62%), and subfertility (NRR <55%) based on their NRR records.

Association analyses of the *PRAMEY* copy number with the fertility traits (see above) were performed only in the Holstein bulls by using a Pearson correlation test using SPSS 17.0 software (SPSS Inc., Chicago, IL). A 2-way ANOVA was applied to investigate the effect of the *PRAMEY* copy number and the founder (of a paternal pedigree) on SC, RLSC, PTM, IM, PNS, and PIA using a general linear model in SPSS 17.0. Furthermore, a mixed model in SAS 9.2 (SAS Institute Inc., Cary, NC) was applied to investigate the effect of CNV of *PRAMEY* on RLSC, SC, PTM, IM, PNS, and PIA, in which the sire was included as a random effect. A *P*-value <0.05 was considered statistically significant for each test.

RESULTS

CNV of PRAMEY Across Cattle Breeds

Before the qPCR analysis, we first validated the male specificity of the *PRAMEY* and *DDX3Y* primers by a routine PCR. As shown in Figure 1, both *PRAMEY* and *DDX3Y* primers amplified a male-specific band of the expected size, confirming that the DNA fragments were amplified from the corresponding genes on the bovine Y chromosome, not from the autosomal ortholog (*PRAME*) or the X-linked *DDX3X* (DEAD box polypeptide 3, X-linked).

The copy number of *PRAMEY* in the calibrator was estimated to be 13 using Equation [1] (see Materials and Methods). Subsequently, the gene copy number of testing bulls was calculated using the calibrator as adjustment based on Equations [2] and [3] (see Materials and Methods), and the results are summarized in Table 1. The median copy number of *PRAMEY* was 13, ranging from 2 to 31 among the bulls tested. Statistical analysis indicated significant CNV among these animals ($P < 0.001$). Interestingly, copy number data did not fit the normal distribution ($P < 0.05$) in either the Holstein population (the largest population, with 257 AI bulls) in the study (Figure 2A, Table 1) or all populations in the 15 breeds tested (Figure 2B). The distribution of copy number in the Holstein population

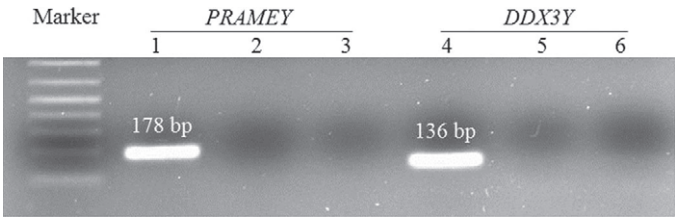

Figure 1. Gel electrophoresis of PCR products of the bovine *PRAMEY* and *DDX3Y* genes. Marker = 1-kb DNA ladder; lane 1: *PRAMEY* PCR product amplified from the Hereford bull L1 Domino 99375 genomic DNA (187 bp); lane 2: *PRAMEY* PCR product amplified from female cattle genomic DNA; lane 3: negative control (distilled water) for *PRAMEY*; lane 4: *DDX3Y* PCR product amplified from the Hereford bull L1 Domino 99375 genomic DNA (136 bp); lane 5: *DDX3Y* PCR product amplified from female cattle genomic DNA; lane 6: negative control (distilled water) for *DDX3Y*.

showed 2 obvious peaks, one at a copy number of 11 and the other at 13 (Figure 2A).

The pairwise comparisons of the median copy number between breeds are listed in Table 2 and indicated a significant difference among breeds ($P < 0.05$). Holstein bulls had the lowest median copy number of *PRAMEY* (12, range: 2–20), whereas Limousin bulls possessed the highest (26, range: 3–30), which was significantly higher than that of the Holstein bulls ($P < 0.001$). Furthermore, bulls in the taurine (BTA) lineage had significantly lower median copy number (13) than bulls in the indicine (BIN) lineage (20), whereas the 3 composite cattle breeds (Beefmaster, Santa Gertrudis, and Qinchuan) had intermediate median copy numbers of 17 (Figure 3).

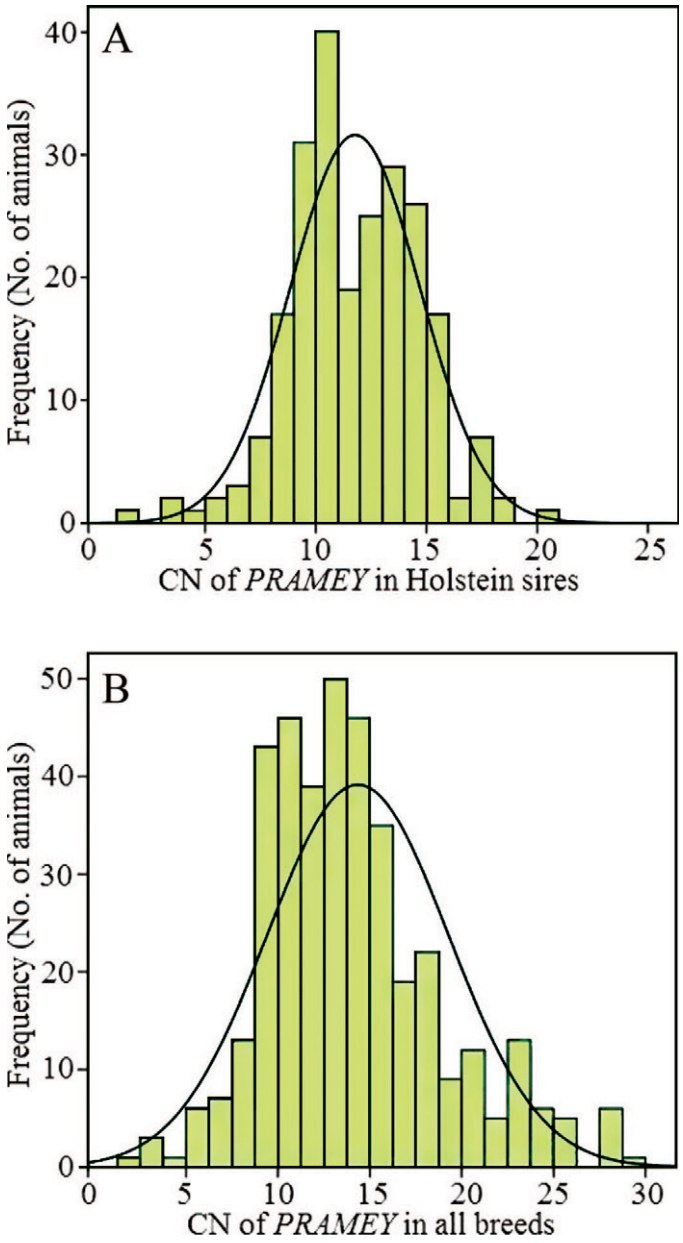

Figure 2. Distribution of the *PRAMEY* copy number (CN) in the bulls. (A) Distribution of *PRAMEY* CN among 257 Holstein bulls; (B) distribution of the *PRAMEY* CN among all 460 bulls analyzed; the CN data do not fit the normal distribution based on the Kolmogorov-Smirnov and Shapiro-Wilk normality tests. Color version available in the online PDF.

In the present study, we analyzed 26 bulls from a population of Qinchuan, a native Chinese composite cattle in central China. Based on a previous Y-chromosome SNP haplotype analysis, 16 of 26 Qinchuan bulls had a BTA Y chromosome, and the remaining 10 had a BIN Y chromosome (Chang et al., 2011b). It was interesting to note that Qinchuan bulls with a BTA-derived Y chromosome had a significantly ($P < 0.01$) higher median copy number of *PRAMEY* (16) than those bulls with a BIN-derived Y chromosome (14; Table 2, Figure 3).

Association of PRAMEY CNV with Male Reproductive Traits in Holstein Bulls

To determine the relationship between *PRAMEY* CNV and male reproductive traits, we analyzed the CNV in 257 Holstein AI bulls by Pearson correlation analyses. We focused our association analysis on 3 types of male reproductive traits: testis size, semen quality, and a general fertility measurement. For testis size, we evaluated 2 traits, SC and RLSC (the latter being an age-adjusted SC). Our results revealed that the CNV of *PRAMEY* was negatively correlated with both SC ($r = -0.26$, $P = 0.003$) and RLSC ($r = -0.27$, $P = 0.002$) (Figure 4A, B), indicating that a lower copy number of *PRAMEY* is associated with a larger testis size.

For the semen quality parameters, we analyzed PTM, IM, PNS, and PIA. Associations of CNV with the semen quality parameters were not significant in PTM ($r = -0.08$, $P = 0.36$), IM ($r = -0.05$, $P = 0.60$), or PIA ($r = 0.03$, $P = 0.73$), but the association between the CNV of *PRAMEY* and PNS tended toward significance ($r = -0.16$, $P = 0.09$; Figure 4C).

For the general fertility measurement, we analyzed 3 traits: SCR, RBE, and NRR. The results demonstrated that the CNV of *PRAMEY* was negatively correlated with NRR ($r = -0.38$, $P < 0.001$), suggesting that a lower copy number of *PRAMEY* was associated with a higher NRR (Figure 4D). In addition, we did pairwise comparisons of the median copy number of *PRAMEY* between the high fertility (NRR $\geq$70%), low fertility (55% $\leq$ NRR $\leq$62%), and subfertility (NRR <55%) groups and found that the high NRR group had a significantly lower median copy number ($P < 0.05$; Figure 3). Association analyses of the *PRAMEY* copy number with RBE ($r = 0$, $P = 0.88$) and SCR ($r = 0.06$, P

Table 2. Comparisons of the copy number of *PRAMEY* among 15 cattle breeds[1,2]

	ANG	BMA	BRM	BSW	CHL	GIR	HFD	HOS	JER	LMS	NEL	NRC	QCI	QCT	RGU	SGT
ANG	—															
BMA	NS	—														
BRM	NS	NS	—													
BSW	NS	*	NS	—												
CHL	NS	*	NS	NS	—											
GIR	NS	NS	NS	NS	NS	—										
HFD	NS	NS	*	**	NS	NS	—									
HOS	***	***	***	***	***	***	***	—								
JER	NS	NS	***	***	*	NS	NS	NS	—							
LMS	***	***	**	**	NS	NS	***	***	***	—						
NEL	*	***	*	*	NS	NS	**	***	***	NS	—					
NRC	NS	***	***	***	***	*	NS	NS	NS	***	***	—				
QCI	NS	**	***	***	***	NS	NS	NS	NS	***	***	NS	—			
QCT	NS	NS	NS	NS	NS	NS	NS	**	NS	**	NS	NS	NS	—		
RGU	NS	NS	*	**	NS	NS	NS	*	NS	***	**	NS	NS	NS	—	
SGT	***	***	*	NS	NS	NS	**	***	***	NS	NS	***	***	NS	***	—

[1]The pairwise comparisons were analyzed by a Mann-Whitney U test with a Bonferroni correction.

[2]ANG = Angus; BMA = Beefmaster; BRM = Brahman; BSW = Brown Swiss; CHL = Charolais; GIR = Gir; HFD = Hereford; HOS = Holstein; JER = Jersey; LMS = Limousin; NEL = Nelore; NRC = Norwegian Red; QCI = Qinchuan (*Bos indicus*); QCT = Qinchuan (*Bos taurus*); RGU = Red Angus; SGT = Santa Gertrudis.

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

= 0.62) were also performed, although no significant associations were found.

As mentioned above, 2 peaks of median copy number were observed in the Holstein population (Figure 2A), which were also reflected in the *PRAMEY* copy number plot analysis in Figure 4A-C. To test whether the genotype (copy number distribution) is associated with the phenotype (bull reproductive traits), we simply divided these 140 Holstein bulls into 2 groups based upon genotype (copy number of *PRAMEY*): Group I (68 bulls) had copy numbers <12, and group II (72 bulls) had copy numbers ≥12 (Figure 4A-C). The reproductive performance of the 2 groups of bulls is listed in Table 3. Statistical analysis (one-way ANOVA) indicated that the difference between the 2 groups was significant for SC ($P = 0.008$), RLSC ($P = 0.01$), and PNS ($P = 0.02$), but not significant in the remaining 3 traits (PTM, IM, and PIA; $P > 0.05$; Table 3). These results, from a different angle, confirmed that the copy number of *PRAMEY* is associated with the bull fertility.

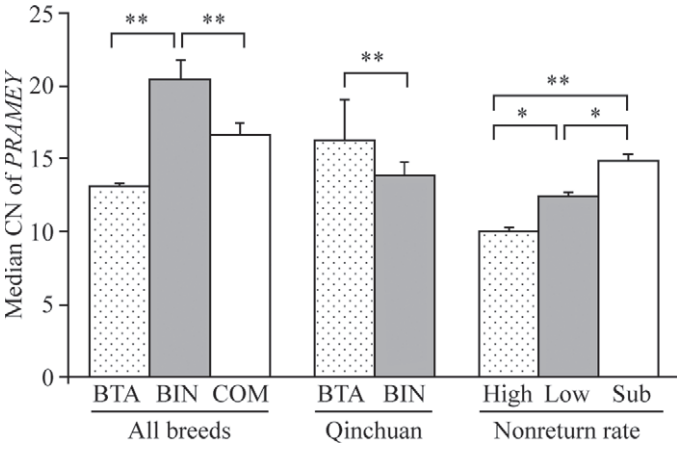

Figure 3. Comparison of the copy number (CN) of *PRAMEY* among *Bos taurus* (BTA), *Bos indicus* (BIN), and composite (COM) lineages and among different groups of nonreturn rate (NRR; high fertility, low fertility, and subfertility). The CN of *PRAMEY* was significantly different between the lineages ($P < 0.01$). In the Qinchuan cattle, the median CN of *PRAMEY* in BTA-derived bulls was significantly higher than that in BIN-derived bulls ($P < 0.01$). In addition, The CN was significantly different among the high NRR, low NRR, and subfertile groups ($P < 0.01$). These comparisons were assessed by a nonparametric Mann-Whitney U test. The error bars represent standard errors; *$P < 0.05$; **$P < 0.01$.

Relationship Between Holstein Bull Pedigrees and Their PRAMEY CNV and Reproductive Traits

We were able to collect and analyze pedigree data for a total of 192 Holstein bulls from public databases (see Materials and Methods). Of these, 94 bulls were half-sib brothers from 27 sires (families), with the largest family having 11 members. To exclude the sire effect from the *PRAMEY* CNV association analyses, we included sire in the mixed model as a random effect. We found that the CNV of *PRAMEY* had a significant effect on SC ($P = 0.04$) and RLSC ($P = 0.05$), and the effect of the *PRAMEY* copy number tended to be significant on PNS ($P = 0.09$), indicating that *PRAMEY* is an important gene involved in male reproduction.

Because AI has been widely used in the dairy industry since the 1960s, we looked further at the Y-pedigree information for all bulls in this study. We found that all the animals were descendants of only 4 patrilineal founders (HOUSA1427381, HOUSA1428104, HOU-

Table 3. Reproductive performance of Holstein bulls grouped by copy number of *PRAMEY*[1]

Reproductive trait[2]	Group I (copy number <12)		Group II (copy number ≥12)		
	No. of bulls	Mean ± SE	No. of bulls	Mean ± SE	*P*-value
SC (cm)	68	40.7 ± 0.4	72	39.0 ± 0.5	0.008
RLSC (%)	68	100.9 ± 0.9	72	97.6 ± 0.8	0.01
PNS (%)	68	80.2 ± 0.9	72	78.0 ± 0.9	0.02
PTM (%)	68	77.8 ± 0.3	72	77.6 ± 0.4	0.30
IM (%)	68	34.2 ± 0.3	72	33.9 ± 0.5	0.55
PIA (%)	68	80.2 ± 0.4	72	80.3 ± 0.4	0.86

[1]The comparison was conducted by a one-way ANOVA.

[2]SC = scrotal circumference; RLSC = relative scrotal circumference; PNS = percentage of normal sperm; PTM = postthaw motility; IM = incubated motility; PIA = percentage of intact acrosome.

SA1441440, and HOUSA1491007) that were born in the 1960s. The median copy number of *PRAMEY* for each founder lineage was recalculated (Supplemental Figure S1; http://dx.doi.org/10.3168/jds.2013-7037). Although the median copy number varied between these lineages, the variations were not significant ($P > 0.05$). The HOUSA1427381 (median copy number = 10.7) and HOUSA1441440 (10.5) founders shared

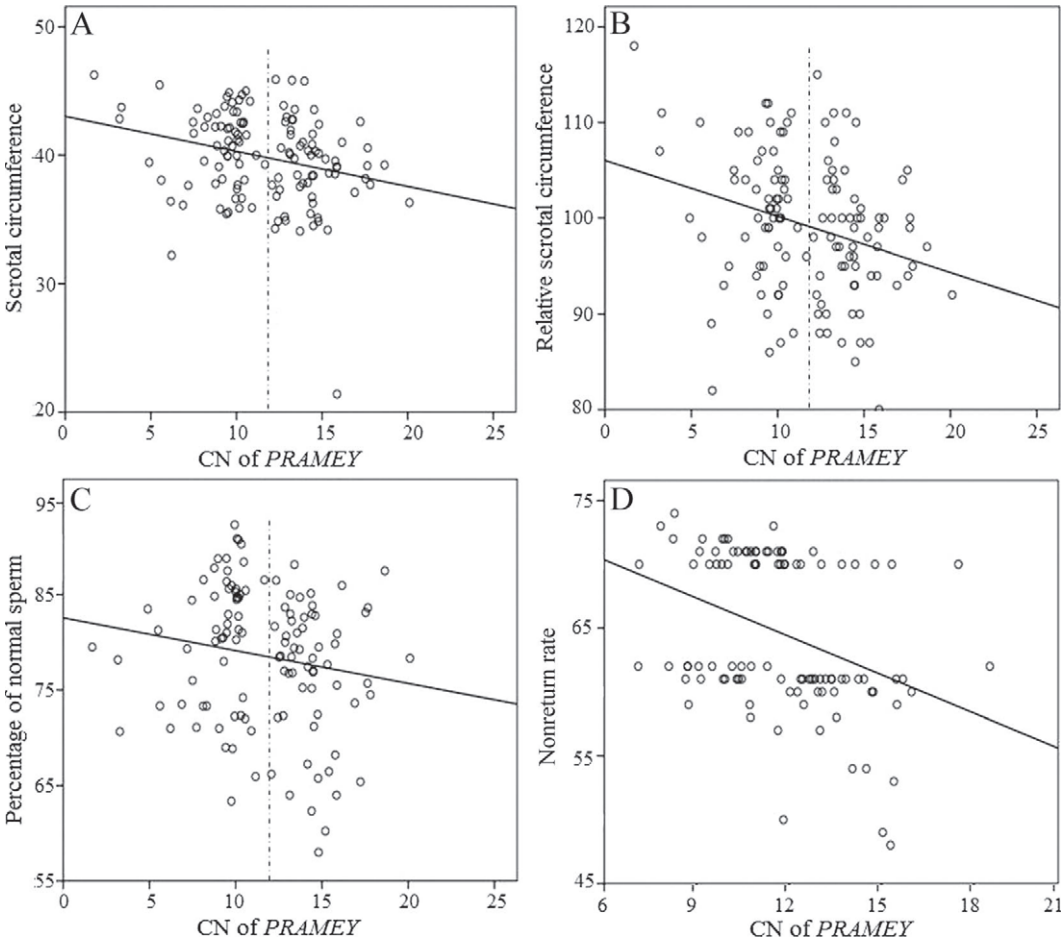

Figure 4. Correlations between copy number (CN) variation of *PRAMEY* and bull reproductive traits. The association of the *PRAMEY* CNV with bull reproductive traits was conducted by the Pearson correlation analysis. The *PRAMEY* CNV was negatively associated with (A) scrotal circumference (r = −0.26, $P = 0.003$), (B) relative scrotal circumference (r = −0.27, $P = 0.002$), (C) percentage of normal sperm (r = −0.16, $P = 0.087$), and (D) nonreturn rate (r = −0.38, $P < 0.001$). Each circle represents one individual. The vertical dashed line in panels A, B, and C marks the cut-off threshold (CN = 12) that was used to divided the tested animals into 2 groups based on their genotypes.

the same ancestor going back to 1860 and their median copy numbers were very similar, whereas founder HOUSA1491007 had a relatively higher median copy number of 12.4, reflecting the evolutionary relics of the paternal Y chromosome lineages.

To test whether the association between the *PRAMEY* CNV with male reproductive traits observed above for the entire Holstein population was still present in the different paternal lineages, we performed 2-way ANOVA with data from 2 founders, HOUSA1427381 and HOUSA1491007, in which a relatively large number (40 and 81) of descendants were included in this study. The results revealed that copy number had a significant effect on SC ($P = 0.01$) and RLSC ($P = 0.02$) in both Y-lineages, whereas pedigree had a significant effect only on PTM ($P = 0.02$; Table 4). In addition, we observed a significant interaction between pedigree and *PRAMEY* copy number on the reproductive traits of PTM ($P = 0.007$), PNS ($P = 0.04$) and PIA ($P = 0.03$).

DISCUSSION

In the present study, we investigated the CNV of the *PRAMEY* gene family by a qPCR method modified from Hamilton et al. (2009). The modified method took the advantage of the bovine Y chromosome draft sequence assembly for PCR primer design to target all *PRAMEY*-related loci on Y. Because genomic DNA from the bull L1 Domino 99375, which was used for the Y chromosome sequence project, was available to us, we decided to use this animal as a calibrator to minimize the technical variations between PCR plates during the estimation of the *PRAMEY* copy number. We used this sequenced animal rather than a randomly selected unsequenced animal as the calibrator to better facilitate comparisons of our results to similar studies in the future. We observed that the *PRAMEY* copy number was 13 for the calibrator in all PCR plates we run, which was different from the 10 copies in the assembled draft sequence of the same Y chromosome (Chang et al., 2011a). This difference is most likely caused by the unfinished Y chromosome sequence assembly in which 2 gaps (~0.5 Mb/gap) are located adjacent to either side of a narrow region where the *PRAMEY* loci are located (Chang et al., 2013).

Previous studies indicated that the bovine Y-linked *TSPY* gene family has been extensively amplified (50–200 copies) across the entire ampliconic region (~30 Mb) during evolution (Hamilton et al., 2009; Chang et al., 2013), whereas the "autosome-to-Y" transposed *PRAMEY* gene family is less amplified in a 5-Mb region located in a so-called transitional region between the pseudoautosomal and ampliconic regions on the bovine Y chromosome (Chang et al., 2013). The data from the current study confirm that the *PRAMEY* gene family is less amplified, with a median *PRAMEY* copy number of 13 across breeds. However, we found that the *PRAMEY* copy number was highly variable among the 460 individuals and 15 cattle breeds investigated. Importantly, *PRAMEY* CNV was also significantly different between the BTA and BIN lineages (Table 1). We found that BTA-derived bulls demonstrated a significantly lower median copy number than bulls in the BIN lineage. Although the reason for this is unclear, Y morphology is different between BTA and BIN at the cytogenetic level. The Y chromosome of taurine bulls is submetacentric, whereas it is acrocentric in indicine bulls (Goldammer et al., 1997). This morphological difference is believed to be caused by a chromosome rearrangement (Meo et al., 2005). However, it is still unknown whether this rearrangement event is associated with the amplification or expansion of *PRAMEY* or any other Y-linked genes. It is worth noting that the range of the *PRAMEY* copy number in BIN (9–31) overlapped with the range observed in the BTA lineage (2–30), although the BIN breeds (Brahman, Gir, and Nelore) showed a higher median copy number (17) than the BTA breeds (13; Table 1). In addition, the Chinese native breed, Qinchuan, whose Y is known to be derived either from BTA or from BIN (Chang et al., 2011b), showed the opposite pattern, with the BTA-derived individuals having a significantly higher median copy number of *PRAMEY* than the BIN-derived individuals; both groups of Qinchuan bulls, however, had relatively low median copy numbers (15 and 13). Furthermore, our pedigree analysis on the Holstein AI bulls identified the difference in the median copy number of *PRAMEY* between the patrilineal (Y chromosome) lineages. Together, these results suggest that the CNV of the *PRAMEY* gene family was influenced by breeding and selection during the formation of a cattle breed, in addition to the evolutionary forces that led to the formation of BTA and BIN lineages. Given

Table 4. The effect of the *PRAMEY* copy number and the founder on reproductive traits as indicated by *P*-values in ANOVA[1]

Reproductive trait[2]	Founder	*PRAMEY* copy number	Founder × copy number
SC	0.18	0.01	0.78
RLSC	0.09	0.02	0.69
PNS	0.96	0.06	0.04
PTM	0.02	0.32	0.007
IM	0.32	0.71	0.18
PIA	0.44	0.36	0.03

[1]A two-way ANOVA was run in a general linear model.

[2]SC = scrotal circumference; RLSC = relative scrotal circumference; PNS = percentage of normal sperm; PTM = postthaw motility; IM = incubated motility; PIA = percentage of intact acrosome.

the fact that the male-specific region (i.e., 95% of the Y chromosome) where *PRAMEY* is located does not recombine during meiosis and any variation (including the CNV) we observed from a patrilineal lineage is an evolutionary relic of that particular Y-chromosome lineage, our data suggest that the ancestor or founder effect will be maintained for many generations.

The relationship between CNV of Y-linked genes and male fertility has been tested in humans and cattle. In humans, an early report found that a significantly higher copy number of *TSPY* was detected in infertile men (Vodicka et al., 2007), whereas another study found a significant positive correlation between the CNV of *TSPY* and sperm count (Giachini et al., 2009), and a subsequent study found no relationship between the CNV of *TSPY* and semen quality (Nickkholgh et al., 2010). These conflicting results were due mainly to biases in study design (Krausz et al., 2010). In cattle, the CNV of *TSPY* was found to be positively correlated with solution index (a modified NRR; Hamilton et al., 2012). In the present study, we found that the CNV of *PRAMEY* was negatively associated with testis size, PNS, and NRR, suggesting that a bull with a lower copy number of *PRAMEY* tends to have larger testes and higher PNS and NRR. It was surprising to observe that the CNV of *PRAMEY* was significantly associated with NRR but not with SCR and RBE. This discrepancy might be explained by the fact that these 3 traits were derived from 3 different fertility evaluation systems (for details, visit http://aipl.arsusda.gov/reference/arr-scr1.htm). Although these evaluation systems have the same objective (to improve the male fertility), they may not measure the genetic effects of the same group of (fertility-related) genes because of the use of the different sets of phenotypic data (http://aipl.arsusda.gov/reference/arr-scr1.htm).

Even though the association analysis performed in the present study was limited to the Holstein breed, we think our general conclusion (that a bull with a lower copy number of *PRAMEY* has higher fertility) may apply to other cattle breeds. This is supported by findings of a previous study to compare male fertility among BTA breeds, which found that Limousin and Charolais were among the worst breeds in terms of fertility (Berry et al., 2011). Indeed, Limousin and Charolais had the highest median copy numbers of *PRAMEY* among all BTA breeds we investigated in this study (Table 1). In terms of reproductive performance between BTA and BIN lineages, a few previous studies have demonstrated that BTA breeds had significantly better sperm quality than BIN breeds (Brito et al., 2002; Beletti et al., 2005), which coincides with our discovery that the BTA lineage had a lower median copy number of *PRAMEY* than the BIN lineage.

Why is a low copy number of *PRAMEY* advantageous for bull fertility? The molecular mechanism behind this is unknown. Our previous study indicated that all copies of *PRAMEY* on the Y chromosome are transcriptionally active (Chang et al., 2011a). However, whether or not a higher or lower copy number of *PRAMEY* affects the transcription and translation of this gene family (as a whole) has not yet been studied. It has been reported that CNV of several genes are negatively associated with their expression level (Stranger et al., 2007; Schuster-Böckler et al., 2010), and the excessive copy number of the human *TSPY* could negatively regulate cell division in the process of spermatogenesis (Vodicka et al., 2007).

It is worth noting that *PRAMEY*, unlike the other Y-linked genes that have no or only a few autosomal paralogs, has approximately 30 copies of autosomal paralogs in the bovine genome (based on the sequenced cow), which may also play an important role in male reproduction (Chang et al., 2011a). A recent study revealed that *Pramel1*, an ortholog of *PRAME*, is involved in acrosome formation and sperm motility in the mouse (Mistry et al., 2013). It is our prediction that, just like the Y-linked *PRAMEY* family, the copy number of autosomal *PRAME* genes likely varies between individuals and among breeds. Because there is low sequence similarity of the *PRAME* family in the autosome (~50%), it is impossible for us to design common primers for the qPCR method, which prevented us from investigating the CNV of autosomal *PRAME* genes in this study. Other methods—for instance, comparative genomic hybridization or next-generation sequencing—could be applied to investigate the CNV of autosomal *PRAME* in the future.

Testicular size is a unique trait for a bull and has been used as a predictive indicator for output of sperm cells for yearling bulls (Parkinson, 2004). It has been estimated that for every 1-cm increase in a sire's SC over the population average, one can expect a 0.25-cm increase in SC in male offspring due to a moderate (0.4) to high (0.7) heritability (Coulter and Foote, 1976; Coulter et al., 1987). However, testicular size, like other reproduction traits, cannot be used for early sire selection (newborn to 8 mo old). To date, knowledge about the molecular basis of SC is very limited. Development of genetic markers, such as the CNV of *PRAMEY* identified in this work, could facilitate earlier prediction of testis size and male fertility (at the newborn stage) and accelerate genetic improvement for male fertility traits.

CONCLUSIONS

The copy number of *PRAMEY* was found to be variable among individuals and breeds in cattle. The copy

number of *PRAMEY* was negatively associated with testis size, percentage of normal sperm, and nonreturn rate in Holsteins; it may serve as a potential marker for sire fertility selection at an early age in cattle.

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