

# Fine Mapping of a QTL for Fertility on BTA7 and Its Association With a CNV in the Israeli Holsteins

Giora Glick,\* Andrey Shirak,<sup>†</sup> Eyal Seroussi,<sup>†</sup> Yoel Zeron,<sup>\*</sup> Efraim Ezra,<sup>§</sup> Joel I. Weller,<sup>†</sup> and Micha Ron<sup>†,1</sup>

\*The Hebrew University of Jerusalem, The Robert H. Smith Faculty of Agriculture, Rehovot, 76100, Israel, <sup>†</sup>Institute of Animal Sciences, ARO, The Volcani Center, Bet Dagan 50250, Israel, <sup>‡</sup>Sion, AI Institute, Shikmim 79800, Israel, and <sup>§</sup>Israel Cattle Breeders Association, Caesaria Industrial Park, Caesaria 38900, Israel

**ABSTRACT** A quantitative trait locus (QTL) affecting female fertility, scored as the inverse of the number of inseminations to conception, on *Bos taurus* chromosome 7 was detected by a daughter design analysis of the Israeli Holstein population ( $P < 0.0003$ ). Sires of five of the 10 families analyzed were heterozygous for the QTL. The 95% confidence interval of the QTL spans 27 cM from the centromere. Seven hundred and four SNP markers on the Illumina BovineSNP50 BeadChip within the QTL confidence interval were tested for concordance. A single SNP, NGS-58779, was heterozygous for all the five QTL heterozygous patriarchs, and homozygous for the remaining five QTL homozygous sires. A significant effect on fertility was associated with this marker in the sample of 900 sires genotyped ( $P < 10^{-6}$ ). Haplotype phase was the same for four of the five segregating sires. Thus concordance was obtained in nine of the ten families. We identified a common haplotype region associated with the rare and economically favorable allele of the SNP, spanning 270 kbp on BTA7 upstream to 4.72 Mbp. Eleven genes found in the common haplotype region should be considered as positional candidates for the identification of the causative quantitative trait nucleotide. Copy number variation was found in one of these genes, *KIAA1683*. Four gene variants were identified, but only the number of copies of a specific variant ( $V_1$ ) was significantly associated with breeding values of sires for fertility.

## KEYWORDS

quantitative trait locus (QTL)  
copy number variation (CNV)  
*KIAA1683*  
gene-variants  
female-fertility

Poor reproductive performance is one of the most common reasons for premature culling. Low female fertility causes economic losses by increasing the need for additional inseminations, higher veterinary costs, longer than optimal lactations, less calves, and higher replacement costs. Therefore, improving female fertility is important for dairy cattle breeders. Various statistics are used to measure fertility of dairy cattle in different countries. In Israel, fertility is scored as the inversed number of inseminations to conception. Heritability for nearly all measures of fertility is only 1–5% (Gonzalez-Recio *et al.* 2006; Kuhn *et al.* 2006; Weller and Ezra 1997), and has negative genetic correlations with milk production traits (Gonzalez-Recio *et al.* 2006; Veerkamp *et al.* 2001; Weller and Ezra 1997).

Weller *et al.* (2008) conducted a daughter design QTL scan for economic traits on BTA7 in Israeli Holstein population. The scan included genotyping 29 microsatellites spanning the chromosome in 11 sires families. Two significant effects for female fertility QTL were identified; near the centromere with five segregating sire families, and near the end of the chromosome with two segregating sire families. The 95% confidence intervals (CI) of the QTL near the centromere spans 27 cM, which includes hundreds of genes.

The most convincing methodology for identifying the causative polymorphism for segregating QTL, the quantitative trait nucleotide (QTN), is concordance (Ron and Weller 2007). Complete concordance is obtained only if:

1. All individuals known to be homozygous for the QTL are also homozygous for the polymorphism.
2. All individuals heterozygous for the QTL are also heterozygous for the polymorphism.
3. The same QTL allele is associated with the same allele of the putative QTN for all the heterozygous animals.

Testing for concordance requires determination of the QTL genotype of several individuals, which can be determined for family patriarchs by either a daughter or granddaughter design (Ron and Weller 2007). The first two requirements for concordance can be

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<sup>1</sup>Corresponding author: Agricultural Research Organization (ARO), The Volcani Research Center, Institute of Animal Science, Dept. of Quantitative and Molecular Genetics, P.O.B. 6, Bet-Dagan, 50250 Israel. E-mail: [micha@agri.huji.ac.il](mailto:micha@agri.huji.ac.il)

analyzed directly from daughter or granddaughter designs results. The third requirement for concordance requires the identification of the QTL phase of the segregating sires. Concordance can be considered as a proof for QTN detection if the probability of obtaining concordance by chance is sufficiently low for rejection of this hypothesis (Ron and Weller 2007). For a given number of patriarchs with known QTL genotype, probability of obtaining concordance by chance decreases as the fraction of heterozygous sires increases (Weller *et al.* 2008). Therefore, we focused the analysis on the QTL near the centromeric region of BTA7, because five sires were heterozygous for this QTL.

Recently, 900 Israeli Holstein sires with genetic evaluations for milk production traits and female fertility, including the 10 patriarchs of the daughter design, were genotyped for the Illumina BovineSNP50 BeadChip, which includes 54,001 SNPs. The aim of this study was to identify SNPs with significant concordance within the CI of the female fertility QTL in the centromeric region of BTA7, and to use this data to identify likely candidate genes for the QTL.

## MATERIALS AND METHODS

### Illumina BovineSNP50 BeadChip genotyping

DNA samples of 900 Israeli Holstein bulls were genotyped for 54,001 SNPs using the Illumina BovineSNP50 BeadChip. Quality control and genotyping procedure are as shown by Weller *et al.* (2010).

### Concordance testing

A total of 704 SNP markers included on the Illumina BovineSNP50 BeadChip are positioned within the first 30 Mbp of BTA7, which cover the CI of the QTL for female fertility. The genotypes of the 704 SNPs for the 10 sires with inferred genotypes for the QTL from the daughter design were tested for concordance, of which five were heterozygous and five were homozygous for the QTL.

The SNP marker that showed the highest fit to the concordance model is NGS-58779. All five sires, homozygous for the QTL, were homozygous for the marker and all five patriarchs, heterozygous for the QTL were heterozygous for the marker. Nevertheless, the haplotype phase was concordant for only four of the five segregating sires. Thus concordance was obtained in 9 out of the 10 families. The probability to obtain a genetic marker with similar concordance by chance was computed assuming that the QTL allele frequency is 0.5, as half of the families segregated for the QTL. Assuming independence among the sire genotypes, the probability by chance that the five sires homozygous for the QTL will also be homozygous for the marker is  $0.5^5$ , the probability that four sires will be heterozygous for the marker and the QTL with correct allele phase between the QTL and the marker is:  $0.25^4$ . The probability that one out of five heterozygous sires for the QTL will be heterozygous for the marker with incorrect allele phase between the QTL and the marker is:  $0.25 \times 5$ . Thus, the probability to obtain this level of concordance by chance for a single SNP is:  $0.5^5 + 0.25^4 + 0.25 \times 5 = 0.000152$ . As 704 SNPs were analyzed 0.1 SNP is expected to show this level of concordance by chance.

### Haplotype phase analysis

Genotypes of 20 SNP markers from the Illumina BovineSNP50 BeadChip, positioned within the flanking 0.5 Mbp upstream and downstream to NGS-58779, were analyzed for 900 Israeli Holstein sires. Haplotype phase analysis was performed using the Plink software with individuals treated as unrelated (Purcell *et al.* 2007). The resulting haplotypes of the 10 tested sires by the daughter design were verified by LSPH software that is based on known genetic relationships between individuals (Baruch *et al.* 2006).

### Comparative mapping

Comparative mapping between the bovine common haplotype region and the human orthologous region was conducted using Ensembl biomart (<http://www.ensembl.org/biomart/martview>) and NCBI mapviewer (<http://www.ncbi.nlm.nih.gov/projects/mapview/>).

### Positional cloning

A partial screen of the exons of six of the 11 genes within the identified common haplotype region (*KIAA1683*, *PDE4C*, *CIST1*, *JUND*, *LSM4*, and *GDF15*) was performed in order to identify concordant polymorphism for the QTL. Primers were designed by the Primer3plus software (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>) in accordance with the exon-intron borders obtained from the reference sequence of the NCBI Entrez Gene database (<http://www.ncbi.nlm.nih.gov/gene/>). DNA of sires was extracted from semen samples as described previously (Ma *et al.* 1996). The bovine *KIAA1683* genes (*LOC618787* and *LOC788637*) were sequenced simultaneously, due to the very high similarity in their predicted exonic sequences. The primers that were used for the positional cloning are presented in Table 1.

PCR fragments were amplified using Super-Therm Taq DNA polymerase (JMR Holding, London) according to the instructions of the manufacturer and the following conditions: 30 cycles for 30 s at 92°C, 40 s at 63°C, and 1 min at 72°C, using a DNA engine thermocycler (MJ Research Inc., Waltham, MA). PCR products were separated on agarose gels, excised from the gel, purified with DNA Montage Gel Extraction Kit (Millipore, Bedford, MA). Sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit using the 3130 Genetic Analyzer capillary electrophoresis instrument (Applied Biosystems). Sequences were assembled and compared using the GAP4 program (Staden *et al.* 1999).

### Isolation of *KIAA1683* variants

Specific primers (SP) were designed to PCR amplify separately each of the A/G alleles located in *KIAA1683* at position 4856941 /4896528 bp on BTA7, which was heterozygous in all of the sires (Table 1). An “A” to “T” mismatch in the fifth nucleotide from the 5′ end of the SP primers, indicated by lower case letter, was introduced to increase the specificity of the PCR amplification. Standard M-13 phage forward or reverse primer sequences were added to the 3′ core primer sequence to serve as a template for sequencing primers and to decrease primers dimer formation. An additional 26 repeats of T tail was attached to the 3′ end of the SP<sub>1</sub> primer to allow detection of fragment size difference from the SP<sub>2</sub> PCR product on a 2% agarose gel. The *KIAA1683*\_exon3\_R primer was used as a reverse primer (Table 1). PCR products were amplified by high-fidelity BIO-X-ACT Long DNA polymerase (Bioline, London) according to the instructions of the manufacturer and the following conditions: 30 cycles for 30 s at 92°C, 40 s at 64°C, and 1 min at 72°C, using a DNA engine thermocycler (MJ Research Inc., Waltham, MA). SP<sub>1</sub> and SP<sub>2</sub> PCR products were sequenced using the M-13\_F and R primers, respectively.

The sequences of the isolated variants of the *KIAA1683* gene were Blasted against the bovine WGS traces and the HTGS sequence databases (NCBI) for sequence validation, and to identify alternative variants of the gene which were not found among the 10 sires.

### Real-Time qPCR

Determination of the relative copy number of the total *KIAA1683* gene (including all gene variants) was conducted using quantitative real-time PCR (qPCR) analysis. A total of 326 Israeli Holstein bulls with known breeding values for fertility including the 10 sires that were tested by the daughter design were analyzed. Primers were

■ **Table 1 PCR and sequencing primers**

Gene	Forward Primer	Reverse Primer	Product Size
<i>KIAA1683</i>			
Exon 1	GTACCTGCCAAGTGGAGGAG	TGACGCCTGTAGCTGTGAAC	283
Exon 2.1	TTCACAGCTACAGGCGTCAC	CCTTGTGGCTCTGGAAGTT	782
Exon 2.2	ACCCAATGAAGACCAAGCAA	GGCCTTGGTTATTGTGACAG	509
Exon 3	GCCTCGATGATCAAGTCTCC	AGATGTCCGTCCTCGGATGT	700
Exon 4	GAGGACGGACATCTTGAAGG	CACACAGGGAGGCGAGTT	794
Intron 4	GGTCCGTGATCAGTGGTG	ACTGCTGCTCATGGGATCTA	811
Exon 5-6.1	CCCTGACTCCAGCTGACA	CCGCCTTGGTCTGATGAT	795
Exon 6.2	CACCCATCATCAGACCAA	CCCACCAGTAAGCCAAGGT	232
SP <sub>1</sub>	(T) <sub>26</sub> GTAAAACGACGGCCAGTAACTGCTCCTCCCCTGACa		531
SP <sub>2</sub>	CAGGAAACAGCTATGACAACTGCTCCTCCCCTGACg		505
<i>JUND</i>			
Exon 1.1	CCGGGCCGAGGCTATAAG	CTCTGCCTTAATGCGCTCTT	997
Exon 1.2	GTCTACGCGAACCTGAGCA	CCAACGTTTGTCCCGAGTA	911
Exon 1.3	CTCAGACTCGACAAGCTGGA	CCCTGCAGGAGAAGAAGGAG	577
<i>LSM4</i>			
Exon 1	ATTGGTTAGCCTTCCCACGA	ATGACACACTCGCTCTGCAT	236
Exon 2	ATATGCCAGCCCTGCCTAGA	AGCTGCTGCTTCGTGGAC	250
<i>GDF15</i>			
Exon 1	CGGACAAAGTCCAGGGAATA	TGGGGTCTCCGAAATTTTAC	385
Exon 2	AGCTTTCTTTCTCTTTTCCA	CGTCCATGCCATAAATGAAG	700
<i>CIST1</i>			
Exon 1	AACATGCGCCCCCTCACAT	GCCGGATCTTACCTGTTCTT	405
Exon 2	GGGTTGCATATCCTTGTTC	CCCCAATGTCCCAATACTCA	664
Exon 3-4	GGCCCTGGTGTGTCTGTC	GCCTCAGAGGAATGATAGGG	554
NGS-58779	TGAGCTGAGACTGGAAAGAGG	AATCCACACCCAGAAGCAAC	231
M-13	GTA AACGACGGCCAGT	CAGGAAACAGCTATGAC	

designed on a region of the exon 3 which was common and monomorphic to all of the identified gene variants. Relative copy numbers of  $V_1$ ,  $V_2$ , and  $V_3$  were determined using variant specific selective primers.  $V_1$  selective primer was designed to amplify the “T” allele of the 4857096/4896683 polymorphism, which is unique for  $V_1$ .  $V_2$  selective primer was designed to amplify the unique “A” allele of the 4857153/4896740 polymorphism, and the  $V_3$  selective primer was designed to amplify the unique “T” allele of the 4857126/4896713 polymorphism. M-13 phage forward standard primers sequences were attached to the 5' of the core primers to decrease primers dimer formation. The same reverse primer was used for both  $V_1$  and  $V_2$  selective primers. qPCR primers are shown in Table 2.  $V_4$  does not possess a unique polymorphism which can be used for selective qPCR.

Gene copy number was normalized to an autosomal reference gene, Ribonuclease P protein subunit p30 (*RPP30*, GeneID:615098, on BTA26. A fragment of 95 bp at position 12893277), *RPP30*, was used as a reference gene in human CNV studies (Wang *et al.* 2010). No CNV was reported for this gene region in previous studies of CNV in cattle (Bae *et al.* 2010; Fadista *et al.* 2010; Liu *et al.* 2010; Seroussi *et al.*

2010). The qPCR analysis was performed in duplicates using the Absolute Blue SYBER Green ROX mix kit (Thermo Fisher scientific, UK) according to the instructions of the manufacturer in a 17- $\mu$ l reaction volume, which included 2  $\mu$ l of DNA (30 ng/ $\mu$ l), 1  $\mu$ l of each primer (10 pmol/ $\mu$ l), 7.5  $\mu$ l of ultra pure water, and 7.5  $\mu$ l of Absolute Blue SYBER Green ROX Mix. The qPCR reaction was performed in the following conditions: 15 min at 95°C for enzyme activation followed by 40 cycles of 15 s at 95°C, 1 min at 60°C, using an ABI Prism 7000 sequence detection system. Amplification was followed by a dissociation curve analysis to confirm the presence of a single product and the absence of primer dimer. The threshold cycle number ( $C_T$ ) for each tested probe was used to quantify its relative abundance. The qbasePLUS software (Biogazelle, Ghent, Belgium) was used for the calculation of the relative quantities using *RPP30* for normalization (D'haene *et al.* 2010). The qPCR primers that were designed to selectively amplify  $V_3$  failed, thus  $V_3$  was excluded from the analysis.

Correlations between the  $C_T$ s of the two duplicates were 0.8, 0.8, 0.7, and 0.9 for  $V_1$ ,  $V_2$ , total *KIAA1683*, and *RPP30*, respectively. The

■ **Table 2 qPCR primers**

Primer ID	Sequence	Product Size (bp)
<i>RPP30_F</i>	TGCTTCCATTGTTTCTGATGA	96
<i>RPP30_R</i>	TGGGACCAGTTCCATGATC	
Total- <i>KIAA1683_F</i>	CCACCATCCTCAAACCCCTGT	101
Total- <i>KIAA1683_R</i>	GGATGAGGTGTCGGAAATTC	
$V_1_F$	GTAAAACGACGGCCAGTTTTCCGACACCTCATCCGACAT	214/155
$V_2_F$	GTAAAACGACGGCCAGTGAGACAGGCGACCTGGGA	
$V_1/V_2_R$	GGAAACAGCTATGACATTTTCTCGCCTTCCAAAGG	
$V_3_F$	GTAAAACGACGGCCAGTAAATGCTTTCCCGGTCA	121
$V_3_R$	CAGGAAACAGCTATGACCGTGGTTTTCCGTTTTCCCTT	

■ Table 3 Phased haplotypes of the 10 analyzed sires

Sire	QTL <sup>a</sup>	Haplotypes (SNPs Position on BTA7 in Mbp)																			
		4.43	4.50	4.52	4.59	4.63	4.65	4.67	4.72	4.76	4.82	<b>4.92</b>	4.96	5.01	5.03	5.06	5.09	5.11	5.14	5.16	5.20
2278	+	G	A	G	G	A	<b>G</b>	<b>A</b>	<b>A</b>	<b>G</b>	<b>G</b>	<b>A</b>	G	G	A	G	G	G	A	A	G
		G	G	C	A	A	G	A	A	G	G	G	G	G	A	G	G	G	A	A	G
3070	+	G	G	G	G	G	<b>G</b>	<b>A</b>	<b>A</b>	<b>G</b>	<b>G</b>	<b>A</b>	A	G	A	A	G	G	A	G	G
		G	G	G	G	G	G	A	A	<b>G</b>	G	G	A	G	A	G	G	G	G	G	G
3089	+	G	G	G	G	G	<b>G</b>	<b>A</b>	<b>A</b>	<b>G</b>	<b>G</b>	<b>A</b>	A	G	G	G	G	C	G	A	G
		G	G	G	G	G	A	A	A	G	A	G	G	A	G	A	G	G	A	A	G
3208	+	G	G	G	G	G	<b>G</b>	<b>A</b>	<b>A</b>	<b>G</b>	<b>G</b>	<b>A</b>	A	G	A	G	G	C	G	A	G
		G	G	G	G	G	G	A	A	G	G	G	A	G	A	G	A	G	A	G	A
3258	+	G	A	G	G	G	<b>G</b>	<b>A</b>	<b>A</b>	<b>G</b>	<b>G</b>	<b>A</b>	G	G	A	G	G	G	G	G	G
		G	G	G	G	G	G	A	A	G	G	G	G	A	G	G	G	G	A	G	G
3099	-	G	G	G	G	G	<b>G</b>	<b>A</b>	<b>A</b>	<b>G</b>	<b>G</b>	<b>A</b>	A	G	G	G	G	C	G	A	G
		G	A	G	G	G	<b>G</b>	<b>A</b>	<b>A</b>	<b>G</b>	<b>G</b>	<b>A</b>	G	G	A	G	G	G	G	G	G
2357	-	G	G	G	G	G	<b>G</b>	<b>A</b>	<b>A</b>	<b>G</b>	<b>G</b>	<b>A</b>	A	G	G	G	G	C	G	A	G
		G	G	G	G	G	<b>G</b>	<b>A</b>	<b>A</b>	<b>G</b>	<b>G</b>	<b>A</b>	G	G	A	G	G	G	G	G	G
2283	-	G	G	G	G	G	G	A	A	G	A	G	G	G	A	G	G	G	A	G	G
		A	G	G	G	G	G	A	A	G	G	G	G	A	G	G	G	C	G	A	G
3241	-	G	G	G	G	G	G	A	A	G	G	G	G	A	G	A	G	G	A	G	G
		A	G	G	G	G	G	A	A	G	G	G	G	A	G	G	G	C	G	G	A
3274	-	G	G	G	G	G	G	A	A	G	G	G	G	A	G	A	G	G	A	G	A
		G	A	G	G	A	G	G	G	G	G	G	G	A	G	A	G	G	A	G	G

The NGS-58779 SNP in bold is located at 4.92 Mbp. The common sub-haplotype associated with the NGS-58779 “A” allele is in bold.

<sup>a</sup> QTL segregation status of sires (+ for heterozygous and - for homozygous).

standard curves of all qPCR probes were linear in all the tested DNA dilutions with  $R^2 < 0.98$  and with slopes of  $-3.36$ ,  $-3.30$ ,  $-2.99$ , and  $-3.11$ , which indicate efficiency of 101, 100, 93, and 92% for  $V_1$ ,  $V_2$ , total *KIAA1683*, and *RPP30*, respectively. The average  $C_T$  of the lowest detectable DNA concentration point (LOD) in the standard curves were 30.7, 30.6, 26.8, and 25.7, while the average  $C_{Ts}$  of the non-template controls (NTC) were 37.1, 35.1, 32.8, and 31.9 for  $V_1$ ,  $V_2$ , total *KIAA1683*, and *RPP30*, respectively. The differences between the average  $C_T$  of the LOD and NTC were  $>4$  cycles in all tested probes, which indicates nontemplate-specific products of  $<6.25\%$ .

### Association analysis

Association of the NGS58879 SNP marker genotype with the bulls’ breeding values for fertility was analyzed by the PLINK linear/logistic regression option (Purcell *et al.* 2007). The population included 900 sires, including the 10 sires tested by the daughter design. A regression analysis of 326 sires’ breeding values for fertility on the overall CNV or specific variant copy number was performed using the “fit Y by X” option of the JMPIN 5.0.1a statistical software (SAS Institute Inc). In addition, multiple regression was performed for the sires’ breeding values for fertility with NGS58879 and  $V_1$  as independent variables.

## RESULTS

### Concordance and effects associated with NGS-58779

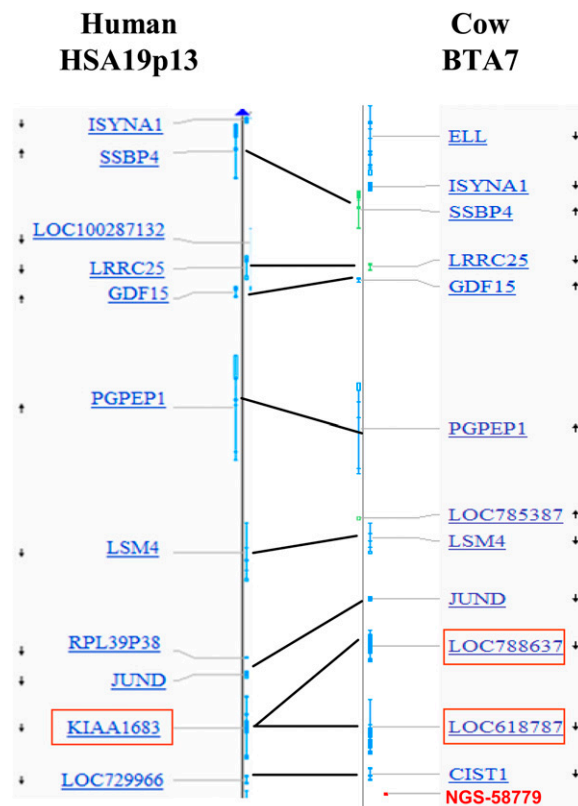
Of the 704 SNPs in the QTL CI on BTA7 that was tested for concordance, only a single intergenic SNP, NGS-58779 positioned at 4922643 bp (Btau 4.0 genome assembly), showed apparent full concordance; all five patriarchs that were heterozygous for the QTL were heterozygous for the marker, and the remaining five sires, homozygous for the QTL, were homozygous for the marker. The “A” allele of the NGS-58779 was associated with the positive haplotype of the fertility QTL identified in the daughter design analysis in four of the five heterozygous sires, and corresponded to the effect associated with the marker in the general sire population. Thus concordance was obtained in 9 out of 10 families.

The NGS-58779 marker showed a significant association to breeding values of sires for fertility in a sample of 900 sires ( $P \leq 1.5 \times 10^{-6}$ ). The frequency of the “A” allele, associated with in-

creased fertility, was 31.5%. The observed allele substitution effect was 0.57 trait units.

### Haplotype phasing analysis

The neighboring genomic region of the concordant SNP was analyzed in order to explore the linkage disequilibrium (LD) boundaries of the



**Figure 1** Bovine/human comparative mapping of the QTL region: lines connect the orthologous genes. The human *KIAA1683* gene and the bovine orthologous duplication (*LOC788637* and *LOC618787*) are marked in red. The SNP marker NGS-58879 is in the bottom of the BTA7 map and appears in red.

■ **Table 4 Partial sequencing of positional genes**

Gene	Number of Exons		Sequencing Size (bp)	Polymorphism Type <sup>a</sup>	Location in Gene	Genomic Location <sup>b</sup>	Concordance <sup>c</sup>
	Total	Sequenced					
<i>GDF15</i>	2	2	1067	A to T non-syn T/S	Exon 2	4,704,137	3/4
<i>LSM4</i>	5	2	486	—			0
<i>JUND</i>	1	1	302	—			0
<i>KIAA1683</i>	6	6	3586	37 SNPs			9/10
<i>CIST1</i>	4	4	1623	T to C non-syn V/A	Exon 1	4,911,890	4/5
				C to T non-syn T/M	Exon 2	4,914,545	9/10
				C to A non-syn L/M	Exon 2	4,914,833	8/10
				A to G 3' UTR	Exon 4	4,916,672	0/3
				T to G	Intron 12	4,920,670	3/5

<sup>a</sup> Non-syn stands for nonsynonymous mutation that is capable of encoding amino acid substitution. UTR stands for untranslated region.

<sup>b</sup> Genomic locations of the identified SNP on BTA7 in bp (Btau4.0).

<sup>c</sup> Concordance of polymorphism with the segregation status of sires for the QTL.

QTL region. Although the Plink haplotype phase analysis was performed without including relationships among bulls, no conflicts were found between the Plink and LSPH results. All chromosomes carrying the “A” allele of NGS-58779 shared a common haplotype extending from 4.65 to 4.92 Mbp (Table 3).

### Comparative mapping and positional cloning

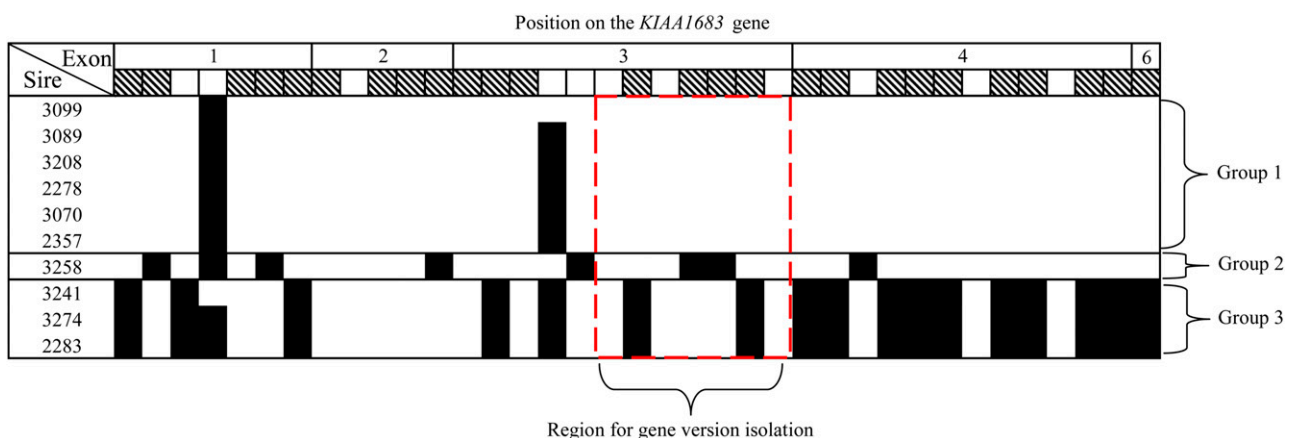
A comparative map of the bovine common haplotype region and its human orthologous region (HSA19p13) is shown in Figure 1. The region contains 11 genes (*CIST1*, *JUND*, *LSM4*, *GDF15*, *KIAA1683*, *LOC785387*, *PGPEP1*, *LRRC25*, *SSBP4*, *ISYNA1*, and *ELL*) in the bovine gene map. Partial sequencing of the exons of four genes (*CIST1*, *JUND*, *LSM4*, and *GDF15*) did not identify a complete concordance between any of the polymorphisms and the QTL status of the patriarchs (Table 4).

The comparative map showed duplication in the bovine *KIAA1683* orthologous genes (*LOC788637* and *LOC6181787*). Alignment analysis demonstrated very high similarity in the sequence of

both genes (~95% identity). Their intron 1 contains a duplication of a section of the exon 1 sequence. *LOC788637* has three copies of the exon 1 sequence, while *LOC6181787* has only two. Two repetitive regions are present in intron 4 of both gene duplicates. Sequencing analysis of the exons regions revealed 37 sites of polymorphism between the gene variants, 26 of which were predicted to be due to the differences between the two paralogues in the reference genome (Btau 4.0). Figure 2 summarizes the polymorphism patterns among the 10 sires for exons 1 to 6 (the full genotyping report is summarized in supporting information, Table S1). These patterns may be clustered into three major groups: 1) “heterozygous” in >95% of the sites (sires 3099, 2357, 3089, 3208, 3070, and 2278); 2) “homozygous” in 8 sites (sire 3258) and 3) “homozygous” in the same 16 sites (sires 2283, 3241, and 3274).

### Isolation of *KIAA1683* variants

The sequencing analysis revealed 11 novel polymorphism sites in the *KIAA1683* gene that were not found in the two reported gene variants.



**Figure 2** Representation of the polymorphic sites found among the 10 sires along exons 1 to 6 of the *KIAA1683* genes: Striped sites indicate the location of differences between the two gene duplicates in the reference genome. Sites in which only a single nucleotide was found for the individual sires are marked in black. The patterns of the 10 sires are clustered into three major groups: 1) “heterozygous” in >95% of the sites (3099, 2357, 3089, 3208, 3070, and 2278); 2) “homozygous” in 8 sites (3258) and 3) “homozygous” in the same 16 sites (2283, 3241, and 3274). The red dashed line indicates the section of exon 3 which was chosen as a target for the gene variants isolation. This region distinguishes between the three cluster groups with at least two unique homozygous sites.



■ **Table 5 Allele-specific amplification and polymorphism of the 10 sires**

Primer	Sire	Cluster Group <sup>b</sup>	Polymorphism on BTA7 (in bp) <sup>a</sup>							Gene Variant
			<b>4856941</b> <b>4896528</b>	4857096 4896683	4857126 4896713	4857153 4896740	4857165 4896752	4857178 4896765	4857214 4896801	
SP <sub>1</sub>	2278	1	A	C/T	C/T	C	C	T/C	G/A	V <sub>3</sub> , V <sub>1</sub>
	3070		A	C/T	C/T	C	C	T/C	G/A	V <sub>3</sub> , V <sub>1</sub>
	3089		A	C/T	C/T	C	C	T/C	G/A	V <sub>3</sub> , V <sub>1</sub>
	3208		A	C/T	C/T	C	C	T/C	G/A	V <sub>3</sub> , V <sub>1</sub>
	2357		A	C/T	C/T	C	C	T/C	G/A	V <sub>3</sub> , V <sub>1</sub>
	3099		A	C/T	C/T	C	C	T/C	G/A	V <sub>3</sub> , V <sub>1</sub>
	3258	2	A	C/T	C/T	C	C	T/C	G/A	V <sub>3</sub> , V <sub>1</sub>
	3241	3	A	C	T	C	C	C	A	V <sub>3</sub>
	3274		A	C	T	C	C	C	A	V <sub>3</sub>
2283	A		C	T	C	C	C	A	V <sub>3</sub>	
SP <sub>2</sub>	2278	1	G	C	C	A	T	C	G	V <sub>2</sub>
	3070		G	C	C	A	T	C	G	V <sub>2</sub>
	3089		G	C	C	A	T	C	G	V <sub>2</sub>
	3208		G	C	C	A	T	C	G	V <sub>2</sub>
	2357		G	C	C	A	T	C	G	V <sub>2</sub>
	3099		G	C	C	C/A	T/C	C	G	V <sub>2</sub> , V <sub>4</sub>
	3258	2	G	C	C	C	C	C	G	V <sub>4</sub>
	3241	3	G	C	C	A	T	C	G	V <sub>2</sub>
	3274		G	C	C	A	T	C	G	V <sub>2</sub>
2283	G		C	C	A	T	C	G	V <sub>2</sub>	

<sup>a</sup> Location of the polymorphic sites on the *KIAA1683* exon 3 in BTA7 on the Btau4.0 genome assembly; positions for each polymorphism are shown for *LOC788637* and *LOC618787* loci, on the upper and lower lines, respectively. The SNP location targeted by the allele-specific primers is in bold.

<sup>b</sup> The polymorphism-pattern cluster of the sires according to their polymorphic pattern (Figure 2).

This indicated that there are additional variants of the gene. In order to isolate these putative variants we focused on exon 3, which showed seven polymorphic sites, including four informative sites that differentiate the three patterns found in the sample of sires (Figure 2).

We used two allele specific primers that selectively amplified the A/G alleles of the 4856941/4896528 polymorphism site (SP<sub>1</sub> and SP<sub>2</sub>, Table 1). All sires amplified both alleles, and the resulting upstream sequence polymorphism is presented in Table 5. The polymorphism of cluster group 3 (sires 2283, 3241, and 3274) was different from that of the other sires, showing only two distinct variants of the *KIAA1683* exon 3 region (V<sub>2</sub> by SP<sub>2</sub> and V<sub>3</sub> by SP<sub>1</sub>). The polymorphism of the other sires upstream to the A/G site was complex, indicating the existence of additional gene variants. The SP<sub>2</sub> primer enabled us to identify two distinct variants of the gene: V<sub>2</sub> (isolated in sires 2278,

3070, 3089, 3208, 2357, 3241, 3274, and 2283), V<sub>4</sub> (isolated in 3258) and both variants in 3099. The SP<sub>1</sub> primer enabled us to isolate V<sub>3</sub> in sires 2283, 3241, and 3274. The SP<sub>1</sub> sequence of the other sires was identical, indicating a common gene version in addition to V<sub>3</sub>. This expected gene version (V<sub>1</sub>) is identical to the *LOC788637* reference genome sequence. Thus, four different variants of the bovine *KIAA1683* exon 3 were identified (Table 6, Figure 3). BLAST analysis of the variants identified perfect matches for the V<sub>1</sub> and V<sub>2</sub> variants in the bovine reference genome. V<sub>3</sub> was found by BLAST analysis against the bovine HTGS sequence database, while V<sub>4</sub> is novel (Table 6, Figure 3).

### Copy number analysis and association with breeding values for fertility

The relative copy number of the 10 sires for *KIAA1683*, and its V<sub>1</sub> and V<sub>2</sub> variants were estimated using qPCR (Figure 4). The results confirm

■ **Table 6 Isolated *KIAA1683* exon 3 variants**

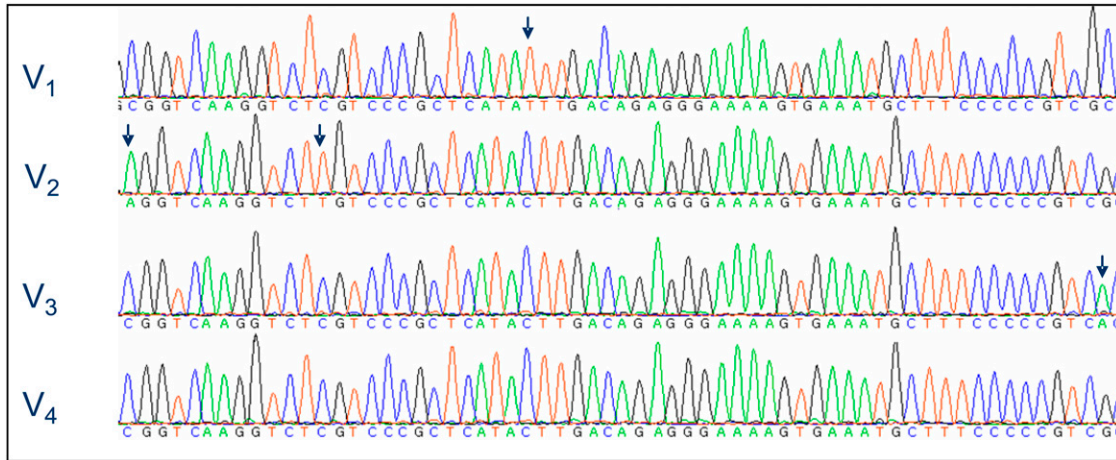
Gene Variant	Reference ID	SNP Location on BTA7 (in bp) <sup>a</sup>						
		4856941 4896528	4857096 4896683	4857126 4896713	4857153 4896740	4857165 4896752	4857178 4896765	4857214 4896801
V <sub>1</sub>	<i>LOC788637</i>	A	T <sup>b</sup>	C	C	C	T	G
V <sub>2</sub>	<i>LOC618787</i>	G	C	C	A <sup>c</sup>	T	C	G
V <sub>3</sub>	ti109805135	A	C	T <sup>d</sup>	C	C	C	A
V <sub>4</sub>	—	G	C	C	C	C	C	G

<sup>a</sup> Location of polymorphic sites on the Btau4.0 genome assembly; positions are shown for the *LOC788637* and the *LOC618787* loci, on the upper and lower lines, respectively.

<sup>b</sup> Polymorphism site used by qPCR to selectively amplify V<sub>1</sub>.

<sup>c</sup> Polymorphism site used by qPCR to selectively amplify V<sub>2</sub>.

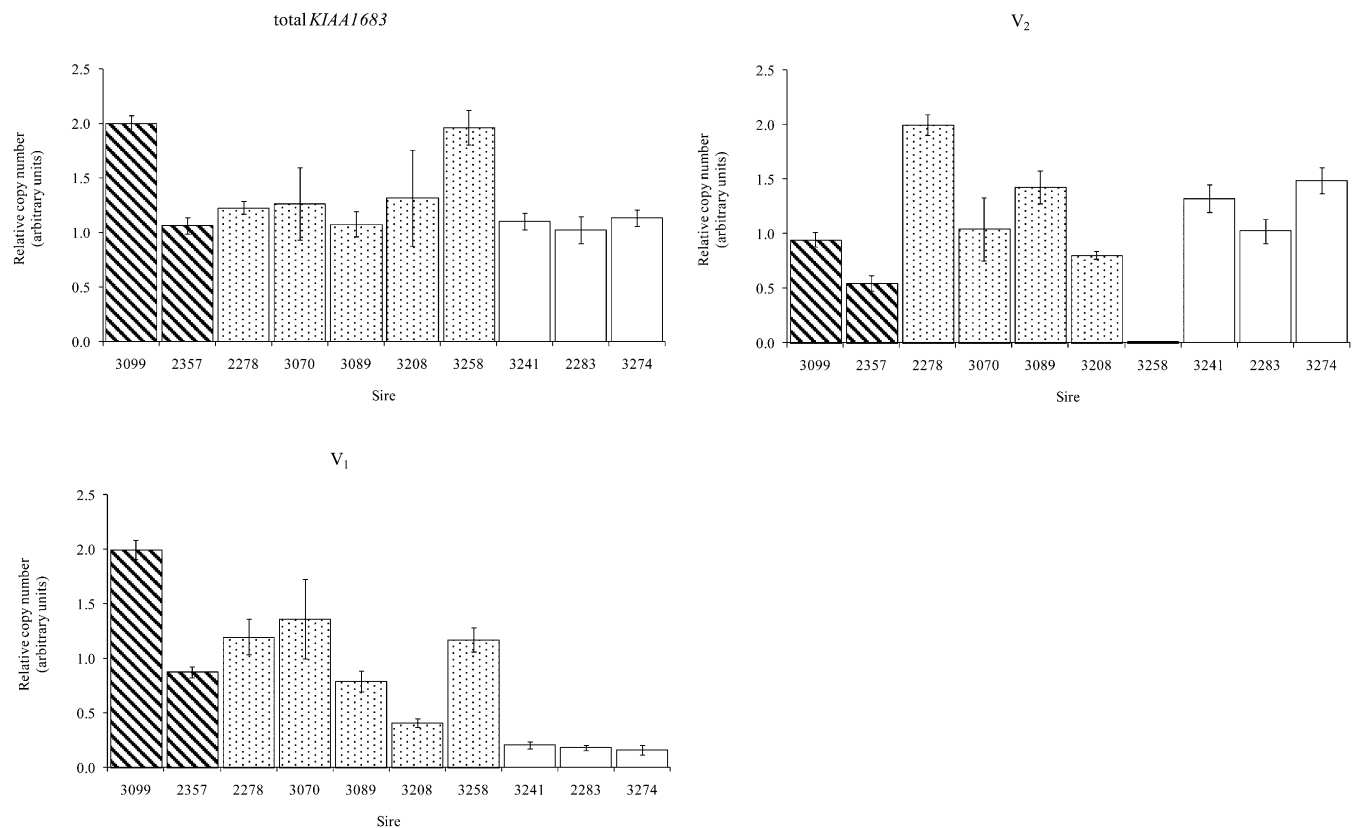
<sup>d</sup> Polymorphism site used by qPCR to selectively amplify V<sub>3</sub>.



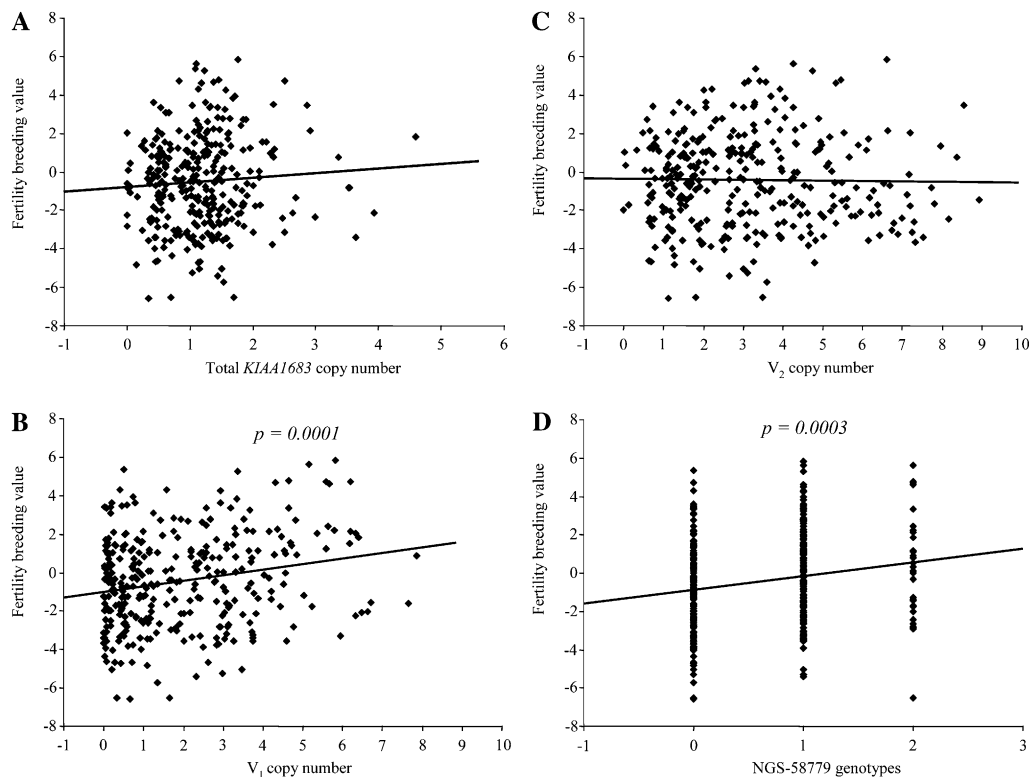
**Figure 3** Sequencing chromatograms of four different *KIAA1683* variants. A 60-bp sequence within the *KIAA1683* third exon is displayed. Arrows mark specific nucleotide changes that differentiate the gene variants.

the genotyping results using the variant specific analysis. Sire 3258 which lacks  $V_2$ , and sires 2283, 3241, and 3274 which lack  $V_1$  showed only residual copy number reflecting the absence of these gene variants. The regressions of breeding values of 326 sires for fertility on their copy number for total *KIAA1683* and  $V_2$  were not significant (Figure 5, A and C). On the other hand, a highly significant regression

was found for  $V_1$  ( $P < 0.0001$ ;  $R^2 = 0.047$ , Figure 5B) which is similar to the association of the NGS-58779 SNP marker using the same population structure ( $P < 0.0003$ ;  $R^2 = 0.037$ , Figure 5D). When both NGS-58779 and  $V_1$  copy number were included in the model, both factors were significantly associated with fertility ( $P < 0.01$  and  $P < 0.03$ , respectively), increasing the  $R^2$  to 0.069.



**Figure 4** Normalized relative copy number (arbitrary units) of total *KIAA1683*,  $V_1$  and  $V_2$  of the 10 sires with known QTL genotypes: QTL genotypes were determined by the daughter design; dotted bars denote the heterozygous sires. Genotypes of the NGS-58779 SNP "AA", "AG" and "GG" are indicated by striped, dotted, and blank bars, respectively. CNV was analyzed by qPCR and quantities were normalized to *RPP30*, an autosomal non-CNV reference gene. Values were normalized to the highest value of sire which was assigned the value of 2 (arbitrary units). Error bars indicates the standard error between the qPCR replicates.



**Figure 5** CNV association test: Linear regressions of sires' breeding values for fertility on copy number of total KIAA1683 (A), V<sub>1</sub> (B), V<sub>2</sub> (C), and genotypes of NGS-58779 (D). NGS-58779 genotypes were scored as 0, 1, and 2 for homozygotes (G), heterozygotes (AG), and homozygotes (A), respectively. Probabilities are given for the significant regressions.

## DISCUSSION

### Concordance testing

In cattle, numerous genome scans were conducted during the past 15 years to identify QTL. These scans, based either on daughter or granddaughter designs (Weller *et al.* 1990), led to the identification of many chromosomal regions with effects on nearly all of the economically important traits (<http://www.genome.iastate.edu/cgi-bin/QTLdb/BT/index>). However, the CI for QTL location is generally >20 cM, thus containing hundreds of genes. Increasing marker density beyond one marker per 10 cM has very limited effect on the length of the CI of the QTL (Ron and Weller 2007). The identification of a putative QTN requires full concordance with the QTL status of individuals. Such information can only be detected by pedigree analysis, such as daughter or granddaughter designs. The probability of obtaining full concordance by chance depends on the number of individuals with known QTL status analyzed, the number of segregating individuals (heterozygous for the QTL), and the number of markers tested (Ron and Weller 2007).

The 27 cM 95% CI interval of the QTL for female fertility in the centromeric region of BTA7 (Weller *et al.* 2008) contains 704 SNPs from the Illumina BovineSNP50 BeadChip. Of these, only a single intergenic SNP, NGS-58779, showed nearly complete concordance (9 out of 10 families). The probability to obtain such concordance by chance is 0.000152 (see *Materials and Methods*). By accounting for multiple testing of 704 SNPs only 0.1 SNP is expected to achieve this concordance level by chance. Furthermore, a highly significant effect on female fertility was associated with this marker in the sample of 900 sires genotyped ( $P < 1.5 \times 10^{-6}$ ).

### Common haplotype identification

Haplotype phase analysis for SNP markers within the flanking 0.5 Mbp upstream and downstream to NGS-58779 interval showed that

all chromosomes carrying the “A” allele of NGS-58779 shared a common haplotype extending from 4.65 to 4.92 Mbp (Table 3). However, because the frequencies of the SNPs alleles included in this haplotype were >0.8, this haplotype was also found in some of the chromosomes carrying the “G” allele of NGS-58779. On the other hand, no common haplotype associated with the “A” allele was discernable on the other side of NGS-58779. We hypothesize that this common haplotype region contains the causative mutation for this QTL. Cohen-Zinder *et al.* (2005) identified a common haplotypes among the two segregating sires for the QTL for milk protein on BTA6. The QTN was eventually identified in *ABCG2*, one of the five genes in this haplotype region.

### Polymorphism in the KIAA1683 gene

Comparative mapping of the common haplotype region to the human orthologous region on HAS19 revealed duplication in the bovine KIAA1683 gene (*LOC788637* and *LOC6181787*). The two gene duplicates shared high sequence similarity (~95%). Among the genes in the region that were sequenced, only KIAA1683 revealed a high number of polymorphic sites that may indicate copy number variation. Twenty six sites were attributed to the differences between the two reference genome gene duplicates. The additional 11 sites indicated the possibility for more unidentified gene variants. The polymorphic patterns identified among the 10 sires, which clustered into three major groups, suggest that there are differences in the gene variants held by the different sires. The gene variants isolation analysis confirmed our assumption. A summary of the gene variants harbored by the 10 sires is presented in Table 7. The three sires homozygous for the NGS-58779 “G” allele—2283, 3241, and 3274—share variants V<sub>2</sub> and V<sub>3</sub>. The two sires homozygous for the NGS-58779 “A” allele—3099 and 3257—also have variant V<sub>1</sub>. Sire 3099 also has variant V<sub>4</sub>. All four sires that are heterozygous for both the fertility QTL and the NGS-58779 marker—2278, 3070, 3089 and 3208—have variants V<sub>1</sub>, V<sub>2</sub>,



■ **Table 7 Summary of the *KIAA1683* gene variants harbored by the 10 sires**

Sire	Gene Variant				NGS-58779 <sup>a</sup>	QTL <sup>b</sup>
	V <sub>1</sub>	V <sub>2</sub>	V <sub>3</sub>	V <sub>4</sub>		
3099	+	+	+	+	A/A	–
2357	+	+	+	–	A/A	–
3089	+	+	+	–	A/G	+
3208	+	+	+	–	A/G	+
2278	+	+	+	–	A/G	+
3070	+	+	+	–	A/G	+
3258	+	–	+	+	A/G	+
3241	–	+	+	–	G/G	–
3274	–	+	+	–	G/G	–
2283	–	+	+	–	G/G	–

<sup>a</sup> Genotypes of the NGS-58779 SNP.

<sup>b</sup> QTL segregation status of the sires (+ for heterozygous and – for homozygous).

and V<sub>3</sub>. Sire 3258, which is heterozygous for the QTL, but shows opposite allelic association between the NGS-58779 and the QTL alleles, has a unique pattern of variants: V<sub>1</sub>, V<sub>3</sub>, and V<sub>4</sub>.

The gene variants' patterns of the 10 sires may indicate the presence of a CNV, as the sires varied not only in the type of gene variants, but also in their number (from two to four). The qPCR analysis revealed a CNV not only in the total *KIAA1683* but also in V<sub>1</sub> and V<sub>2</sub> specific gene variants.

Previous work identified segmental duplication and CNV in this genomic region. Bae *et al.* (2010) used the BovineSNP50 BeadChip signal intensity to identify CNV region in Korean cattle. They identified a CNV region that spans BTA7: 4.65–5.03 Mbp, which overlaps with our common haplotype region (BTA7: 4.65–4.92 Mbp). Liu *et al.* (2009) performed a systematic computational genome-wide analysis of segmental duplications in cattle based on identifying paralogous sequences  $\geq 1$  kb in length with  $\geq 90\%$  sequence identity, and genomic regions that exhibit significant increase in depth of coverage in the whole genome shotgun sequences. The analysis identified significant peaks in both *KIAA1683* genes indicating segmental duplication. In contrast to Bae *et al.* (2010), this analysis indicated that the segmental duplication in this region is limited only to the *KIAA1683* genes.

### ***KIAA1683* Copy number analysis**

Of the four identified *KIAA1683* gene variants identified by the allele specific PCR and sequencing, only V<sub>1</sub> indicated an association with the NGS-58779 marker in the 10 sires analyzed. The sires that were homozygous for the NGS-58779 “G” allele (2283, 3241, and 3274) lack V<sub>1</sub>, while the sires homozygous for the “A” allele (3099 and 3257) and the heterozygous sires (2278, 3070, 3089, 3208, and 3258) all have V<sub>1</sub> (Table 7). In recent years, CNV has been increasingly recognized as a major source of heritable variation that impacts complex traits (Redon *et al.* 2006). Thus, we quantified the copy number of total *KIAA1683* and its variants, V<sub>1</sub> and V<sub>2</sub>. Only V<sub>1</sub> copy number showed a significant association with fertility in the general population ( $P < 0.0001$ ). It appears that copy number variation of different gene variants might be considered as independent genetic markers for association analyses. A similar phenomenon was reported for the human *CYP2D* locus located on chromosome 22 (HSA22q12). *CYP2D* is an enzyme expressed in the human liver. The *CYP2D* locus consists of three tandem repeats of homologous sequences of which *CYP2D6* is the functional gene, while *CYP2D7* and *CYP2D8* are pseudogenes. Apparently only CNV of *CYP2D6* was associated with drug metabolism phenotype (Abraham *et al.* 2010; Ingelman-Sundberg 2004).

Analogously, it is possible that V<sub>2</sub> to V<sub>4</sub> variants of *KIAA1683* are pseudogenes, while V<sub>1</sub> variant is the only active gene. This hypothesis may be tested by gene expression analysis which will identify the gene variants that are actually being transcribed to mRNA in different bovine tissues. Expression data for *LOC788637* and *LOC6181787* genes were searched in the bovine gene atlas but no informative results were found (Harhay *et al.* 2010, <http://bovineatlas.msstate.edu/>).

Inclusion of both NGS-58779 and V<sub>1</sub> copy number in the analysis model yielded statistical significance for both factors and increased the R<sup>2</sup>. It is expected that when a causal mutation is included in the analysis model with additional linked markers, only the causal mutation will be significant (Ron and Weller 2007). Thus both factors contribute to the explained variation, and apparently neither of them is the causal mutation. It should be noted that *CIST1* also showed high concordance between the polymorphism and the segregation status of sires for the QTL.

### **CONCLUSIONS**

We demonstrate a new strategy for fine mapping QTL which combines GWAS data together with daughter design results and concordance testing. We identified a single intergenic SNP, NGS-58779, which showed concordance for 9 out of 10 sires, and a highly significant association with fertility in the population. We further identified a common haplotype associated with the rare favorable allele of the marker, from 4.65 to 4.92 Mbp on BTA7. Thus, the 27 cM CI of the fertility QTL was reduced to a 270 kbp region which contains only 11 genes that should be considered as positional candidates for the identification of the causative quantitative trait nucleotide.

An analysis of the CNV in the *KIAA1683* gene showed that only the number of copies of a specific gene variant (V<sub>1</sub>) was significantly associated with breeding values of sires for fertility. Thus quantifying the copy number of different gene variants is of paramount importance for association analyses.

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