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Confirmation of quantitative trait loci using a low-density single nucleotide polymorphism map for twinning and ovulation rate on bovine chromosome 5[1,2]

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ABSTRACT: Traditional genetic selection in cattle for traits with low heritability, such as reproduction, has had very little success. With the addition of DNA technologies to the genetic selection toolbox for livestock, the opportunity may exist to improve reproductive efficiency more rapidly in cattle. The US Meat Animal Research Center Production Efficiency Population has 9,186 twinning and 29,571 ovulation rate records for multiple generations of animals, but a significant number of these animals do not have tissue samples available for DNA genotyping. The objectives of this study were to confirm QTL for twinning and ovulation rate previously found on BTA5 and to evaluate the ability of GenoProb to predict genotypic information in a pedigree containing 16,035 animals when using genotypes for 24 SNP from 3 data sets containing 48, 724, or 2,900 animals. Marker data for 21 microsatellites on BTA5 with 297 to 3,395 animals per marker were used in conjunction with each data set of genotyped animals. Genotypic probabilities for females were used to calculate independent variables for regressions of additive, dominance, and imprinting effects. Genotypic regressions were fitted as fixed effects in a 2-trait mixed model analysis by using multiple-trait derivative-free REML. Each SNP was analyzed individually, followed by backward selection fitting all individually significant SNP simultaneously and then removing the least significant SNP until only significant SNP were left. Five significant SNP associations were detected for twinning rate and 3 were detected for ovulation rate. Two of these SNP, 1 for each trait, were significant for imprinting. Additional modeling of paternal and maternal allelic effects confirmed the initial results of imprinting done by contrasting heterozygotes. These results are supported by comparative mapping of mouse and human imprinted genes to this region of bovine chromosome 5.

Key words: GenoProb, multiple-trait derivative-free restricted maximum likelihood, single nucleotide polymorphism, twinning

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INTRODUCTION

Fine mapping and marker development for marker-assisted selection (**MAS**) in all species has had limited success; however, with technological advances in genomics and the ongoing development of the bovine whole-genome sequence, the rate of success in the discovery of DNA markers for MAS should accelerate. To date, successes in MAS have resulted from phenotypic traits with moderate to high heritability, and commercialization of markers for MAS in beef cattle has been limited to traits related to meat tenderness and fat deposition (Page et al., 2004; Schenkel et al., 2005; Casas et al., 2006).

Reproductive efficiency is a primary component of profitability for the cow-calf producer (Dickerson, 1970; Doyle et al., 2000; Martinez et al., 2005). The theoretical relative economic value of reproductive traits was shown to be 3.24 times greater than for final product consumable traits (Melton, 1995). However, direct improvement for reproductive efficiency using traditional selection methods in the bovine has resulted in limited success because of the long generation interval and low heritability. Discovery of DNA markers for reproduc-

[1]Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the USDA.

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tive traits will give the producer a tool to identify superior or inferior animals and improve selection response, further enhancing economic gains for the cow-calf producer.

The objective of this study was to confirm the region segregating a QTL for ovulation rate on BTA5 by using a low-density SNP map in a multigeneration pedigree. An additional objective was to evaluate the extent to which a previous investment in microsatellite genotyping for linkage analysis could be leveraged to reduce the current cost of SNP genotyping for linkage disequilibrium analysis. To do this, we evaluated the use of GenoProb (Thallman, 2002) with 3 sets of genotyped animals, containing 48, 724, or 2,900 animals, in a large multigenerational pedigree. Comparisons of significance levels between these analyses were evaluated to help understand the predictive power of allelic peeling in GenoProb.

MATERIALS AND METHODS

The US Meat Animal Research Center Animal Care and Use Committee approved these experimental procedures.

Population

A selection experiment was initiated in 1981 at the USDA-ARS US Meat Animal Research Center (**USMARC**) to increase the frequency of dizygotic twin births in cattle (Gregory et al., 1997; Allan et al., 2007). The USMARC Production Efficiency population (formerly USMARC Twinning population) structure is a large multigeneration pedigree representing 12 different breeds of cattle with a broad array of phenotypes for reproduction, growth, and carcass traits (Gregory et al., 1996; Echternkamp and Gregory, 1999a,b). The population was derived from 345 founding gametes. Presently, the population is maintained with a fall- and spring-calving season and continues to undergo selection for twinning rate. Current rates are 48% for multiple ovulations in 12- to 18-mo-old heifers (Cushman et al., 2005) and greater than 60% for twin or multiple births in cows (Echternkamp, 2000; Echternkamp et al., 2007). Additionally, a large repository of DNA samples has been created for mapping production traits that includes most of the founding sires and a portion of the males and females that have undergone selection since 1981. The repository includes essentially all individuals born since 1996 that have produced progeny in the population or that have generated phenotypes for ovulation rate.

Genotyping

Discovery of SNP was done in a selected group of Production Efficiency animals representing sires whose 4 founding alleles constitute 30% of the genetic composition of the population at the peak of the QTL on BTA5. Animals in this group were homozygous for the favorable founding QTL allele or homozygous for the allele not contributing to the effect of the QTL. Additionally, 1 founder sire (Pinzgauer bull 19784403) known to be heterozygous for the QTL (Kappes et al., 2000) was sequenced, bringing the discovery set of animals to 24.

Primers were designed from the bovine genome sequence (3.1) by using Primer 3 (Rozen and Skaletsky, 2000; code available at http://primer3.sourceforge.net; last accessed May 2007), or selected from the USMARC database from earlier bovine sequencing projects. For PCR amplification, 25 ng of genomic bovine DNA extracted from ear notch or semen samples was used in a total 12-μL volume containing 1.5 m*M* $MgCl_2$, 0.25 m*M* each dNTP, 0.5 U of Ampli*Taq* Gold with 1× $MgCl_2$-free supplied buffer (Perkin-Elmer, Branchburg, NJ), and 0.2 μ*M* each primer. Thermal cycling conditions included an initial denaturing step at 95°C for 5 min, followed by 40 cycles of a denaturing step at 95°C for 30 s, annealing at 58°C for 45 s, and extension at 72°C for 1 min, with a final extension period at 72°C for 5 min. A portion of each sample was separated on a 2% agarose gel to verify quality.

A portion of the PCR reaction (3 μL) was incubated with 0.1 U of Exonuclease I (USB, Cleveland, OH) at 37°C for 1 h, followed by heat inactivation at 65°C for 20 min, and the product was purified by using ethanol precipitation. Purified PCR products were sequenced by using ABI BigDye Terminator chemistry (Perkin Elmer, Foster City, CA) and analyzed on an ABI 3730 sequencer.

Sequence information was analyzed from chromatograms stored in the USMARC database; bases were called with Phred and assembled in contigs with Phrap (Ewing and Green 1998; Ewing et al., 1998). The results were visually assessed by using Consed (Gordon et al., 1998). Sequence information was subjected to the BLAST (Basic Local Alignment Search Tool) function of the National Center for Biotechnology Information bovine genome project release 3.1 (http://www.ncbi.nlm.nih.gov/genome/guide/cow/index.html; last accessed December 2007). Scaffolds with significant hits (E-value 0.0) were then queried against the bacterial artificial chromosome (**BAC**) end clone sequence data by using BLAST. The BAC end sequence results were merged with the integrated bovine BAC map (Snelling et al., 2007) to identify chromosome and approximate map position (Table 1). Bovine genome sequence (3.1) order within contigs was used to refine order when the integrated composite map was unresolved.

Characterized SNP were sequenced and genotyped in an additional 24 animals, bringing the total to 48 animals genotyped, representing founding alleles that composed 70% of the genetic composition at the QTL peak for ovulation rate on BTA5 (Kappes et al., 2000). A multiplex assay, including 24 SNP, was designed by using Assay Design 3.1 (Sequenom, San Diego, CA),

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Table 1. Ordered map of BTA5 combining the US Meat Animal Research Center linkage map with bovine bacterial artificial chromosome (BAC) information and bovine genome sequence assembly

Marker name	Marker type[1]	dbSNP and accession no.[2]	BAC contig/ position in BAC[3]	cM[4]	Bt3.1 Mb[5]	Locus ID	No. of genotyped animals
BMS1095	MS	G18613	5005/963.35714	0.00	4.295		855
BMS610	MS	8563170*		11.98	6.995		2,626
BP1	MS	7908653*	5010/117.4	17.29	13.960		1,203
BM6026	MS	G18426	5010/1185.3125	6.06	12.806		297
RM103	MS	U10391	5020/980.5	29.44	18.782		3,404
AGLA293	MS	WO9213102*		32.26	24.857		209
BMS1315	MS	G18653		33.67	25.452		462
BM321	MS	G18515		39.26	29.219		3,719
BMC1009	MS	7908653*		42.08	28.895		2,167
DIK2465	MS	AB165009	5025/1654.0	42.10	29.227		947
2019_224	SNP	ss52084216	5025/1946.6667	45.4	26.249	ITGA5	48 /724/2900
BMS1898	MS	G18705	5025/1947.25	45.59	30.895		3,724
34336_495	SNP	ss38339393	5025/2280.125	45.60	27.050	ATPG52	48 /724/2900
34336_607	SNP	ss38339394	5025/2280.125	45.61	27.050	ATPG52	48 /724/2900
12163_117	SNP	ss28448456	5025/2903	45.7	29.633	LOC534536	48 /724/2900
55069_549	SNP	ss78017549	5025/3194	45.75	29.251	LOC516929	48 /724/2900
55067_558	SNP	ss78017543	5025/3194	45.8	29.253	LOC516929	48 /724/2900
55067_720	SNP	ss78017546	5025/3194	45.85	29.253	LOC516929	48 /724/2900
55057_236	SNP	ss78017538	5025/3224	45.90	29.262	LOC516929	48 /724/2900
55057_461	SNP	ss78017542	5025/3224	45.94	29.262	LOC516929	48 /724/2900
11929_523	SNP	ss28451527	5025/3224	45.98	29.267	LOC51495	48 /724/2900
11929_712	SNP	ss28451543	5025/3224	46.00	29.267	LOC51495	48 /724/2900
55053_598	SNP	ss78017536	5025/3224	46.70	29.268	LOC51495	48 /724/2900
34426_641	SNP	ss38340054	5025/3919.8214	46.88	30.910	LOC540105	48 /724/2900
34444_603	SNP	ss38340978	5025/3919.8214	46.90	30.910	LOC540105	48 /724/2900
DIK4352	MS	AB165612	5025/5150.9167	48.62	39.895		942
BL4	MS	9107677*	5025/9447.5	53.52	43.064		2,679
7189_241	SNP	ss28450253	5025/9496.5	53.60	42.948	PTPRR	48 /724/2900
52337_266	SNP	ss78017532	5025/9812.3333	53.70	40.447	LOC538624	48 /724/2900
52331_182	SNP	ss78017529	5025/9821.7143	53.75	40.435	MGC128852	48 /724/2900
52325_613	SNP	ss78017527	5025/9821.7143	53.80	40.431	MGC128852	48 /724/2900
52321_373	SNP	ss78017521	5025/9821.7143	53.85	40.429	MGC128852	48 /724/2900
52311_539	SNP	ss78017516	5025/9821.7143	53.90	40.426	MGC128852	48 /724/2900
52309_281	SNP	ss78017511	5025/9821.7143	53.95	40.425	MGC128852	48 /724/2900
24370_238	SNP	ss28449534	5025/9821.7143	54.00	40.424	MGC128852	48 /724/2900
36440_654	SNP	ss52084221	5025/9821.7143	54.10	40.422	MGC128852	48 /724/2900
52289_1165	SNP	ss78017507	5025/9821.7143	54.30	40.405	MGC128852	48 /724/2900
CA084	MS	U32916	5025/11682.875	57.74	46.931		1,626
BR2936	MS	G18488		66.29			1,542
BMS1216	MS	G18633	5030/5144.1154	82.94	69.371		2,008
BMS1248	MS	G18644		95.93			875
BM315	MS	G18514		108.20	97.309		3,088
BM2830	MS	G18487	5065/370.22222	122.55	107.135		2,482
BMS597	MS	G18887	5075/39.75	131.81	113.315		172
BM8126	MS	G18854		132.97	114.187		258

[1]Marker type denoted as MS (microsatellite) and SNP.
[2]GenBank accession number available at http://www.ncbi.nlm.nih.gov, with the exception of identification numbers followed by an asterisk (*), which are found in patents or publications (publications listed by PubMed number). Single Nucleotide Polymorphism database (dbSNP) identification numbers are available at http://www.ncbi.nlm.gov/SNP/.
[3]Bovine bacterial artificial chromosome contig and position in BAC contig.
[4]Centimorgan positions for MS markers from the USDA-ARS US Meat Animal Research Center linkage map; cM positions for SNP are arbitrary units to denote order between MS markers.
[5]Bovine genome sequence build 3.1 megabase position.

and genotyping was done by using Sequenom iPLEX MassArray technology (Sequenom). Genotypes were collected for 340 sires and 384 females, for a total of 724 animals in the second data set (this set contained the 48 original genotyped animals). Females were selected to represent as much of the pedigree as possible, using the following criteria. Beginning with 2001 and moving back in time, all females selected were grouped in 2-yr intervals. Additionally, these females needed to have at least 2 parturitions, with no female progeny already included in the data set from more recent years. The final set of genotyped animals included the 724 animals described previously, with additional female DNA from the Production Efficiency Population (n = 2,176), bringing the total number of genotyped animals to 2,900. The multiplex assay was validated by compar-

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ing the genotypes scored for the first 48 animals by the iPLEX MassArray system (Sequenom, San Diego, CA) with their manually scored sequenced genotypes.

Microsatellite genotyping included data for BTA5 generated previously (Kappes et al., 2000) and data generated by using standard PCR and infrared dyes (IRD700, IRD800), followed by electrophoresis and analysis on a LI-COR 4200 IR^2 system (LI-COR, Lincoln, NE). Recently genotyped microsatellite data included an additional 128 progeny-tested sires and all males and females born since 1999. All microsatellite data, including the number of genotyped animals, were used in combination with SNP data in the integrated map described previously (Table 1).

Analysis

Genotype probabilities for all animals in the extended pedigree (n = 16,035) were calculated by using an extended version of the GenoProb software (Thallman, 2002), and the integrated map for BTA5 including 21 microsatellites and 24 SNP (Table 1). GenoProb is an allelic peeling algorithm program that can be used to predict ordered genotypic distributions on pedigreed animals with incomplete genotyping data (Thallman et al., 2001a,b). All GenoProb analyses used the microsatellite data and the whole pedigree. However, varying amounts of SNP genotypes were used. This procedure was repeated on a data set that included either 48 animals genotyped for SNP (sequence population), 724 animals genotyped for SNP (influential animal data set), or 2,900 animals (all animals with DNA sample). When comparing the results of these 3 analyses, the 2,900-animal SNP data set was used as a standard. Only SNP probabilities were used in subsequent association analyses; probabilities were generated and used for all 16,035 animals.

Twinning and ovulation rate were fit together in a 2-trait, repeated records analysis in multiple-trait derivative-free REML (Boldman et al., 1995), using the model described by Van Vleck and Gregory (1996) with additional details listed in Allan et al. (2007). Briefly, the model for twinning rate included the fixed effects of year of parturition, season of parturition, age at parturition, and genotypic regressions of the SNP marker(s). The model for ovulation rate included the fixed effects of birth-year season, age at ovulation, month of ovulation, and genotypic regressions of the SNP marker(s). Both traits included random effects for breeding value, with full relationships accounted for and environmental variance common to the repeated records of the animal in addition to the residual. The number of phenotypic records used in the analyses included 29,571 for ovulation rate and 9,186 for twinning rate records. Independent regression variables representing additive, dominance, and imprinting genetic effects were calculated by using the genotypic probabilities computed by GenoProb (Allan et al., 2007). For each SNP, genotypic regressions for additive, dominance, and imprinting (**ADI**) were fitted as fixed effects in the model and run in a single-marker analysis. When imprinting was not significant, these analyses were repeated for models containing only the regressions of additive and dominance (**AD**). If the additive and dominance effects were confounded because of low minor allele frequencies, models containing only additive and imprinting (**A+I**) regressions or additive (**A**)-only models were fitted. All markers with F-tests that were at least approaching significance were fit simultaneously in one model. A step-down procedure was performed to establish the most significant markers for the region. After each analysis, if nonsignificant SNP associations existed, the SNP with the least significant F was eliminated from the model and the analysis was rerun. This procedure was repeated until only significant SNP remained in the model.

Imprinting in the initial ADI or A+I model was defined as the difference between the effects of the heterozygous genotypes (Allan et al., 2007). To better define imprinting, we developed additional analyses that allowed us to examine whether the pattern of inheritance was maternal (**M**), paternal (**P**), or both (similar to Thomsen et al., 2004) rather than additive or dominant. The values for the independent variables for animal j were obtained from the vector $\mathbf{x}_j$:

$$\mathbf{x}_j^{\mathrm{T}} = \begin{bmatrix} x_M \\ x_P \end{bmatrix}_j = \begin{bmatrix} 0 & 0 & 1 & 1 \\ 0 & 1 & 0 & 1 \end{bmatrix} \begin{bmatrix} p_{aa} \\ p_{aA} \\ p_{Aa} \\ p_{AA} \end{bmatrix}_j,$$

where p_{aa} is the probability that j is homozygous for allele a, p_{AA} is the probability that j is homozygous for allele A, p_{aA} is the probability that j inherited allele a from its dam and allele A from its sire, and p_{Aa} is the probability of the heterozygote with opposite parental origins. The regressors are x_M and x_P for M and P imprinting components of gene action, respectively. Imprinting was replaced by M, P, or both effects in the final 2,900 genotyped animal group model only.

RESULTS

Construction of the map order for BTA5 used a combination of the Shirakawa Institute of Animal Genetics-USMARC linkage map (Snelling et al., 2005), the bovine BAC map, and the 3.1 genome sequence build. The BAC map order agreed with the linkage map order, except for the marker BM6026, which mapped out of order in the BAC map relative to the linkage map and bovine sequence. The linkage map order and BAC map order otherwise were in agreement for the remainder of this region of BTA5. Some discrepancies did exist when comparing the sequence assembly to the BAC and linkage maps. When these disagreements arose, the BAC and linkage map order was used instead of the genome sequence order.

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Table 2. Genotypic distribution of markers for animals with twinning (n = 9,186 records) for each GenoProb run using 48, 724, or 2,900 animals genotyped

		Twin, 48[1]			Twin, 724			Twin, 2,900		
Marker name	cM	aa[2]	Aa	AA	aa[2]	Aa	AA	aa[2]	Aa	AA
2019_224	45.40	0.552	0.299	0.037	0.588	0.343	0.055	0.610	0.337	0.051
34336_495	45.60	0.148	0.485	0.366	0.144	0.496	0.347	0.158	0.505	0.336
34336_607	45.61	0.174	0.515	0.310	0.174	0.502	0.313	0.188	0.509	0.301
12163_117	45.70	0.872	0.124	0.004	0.793	0.195	0.011	0.827	0.162	0.008
55069_549	45.75	0.089	0.386	0.346	0.063	0.392	0.521	0.069	0.398	0.529
55067_558	45.80	0.054	0.393	0.553	0.060	0.378	0.551	0.063	0.388	0.548
55067_720	45.85	0.519	0.245	0.026	0.514	0.393	0.062	0.534	0.397	0.067
55057_236	45.90	0.058	0.372	0.570	0.047	0.342	0.609	0.059	0.348	0.592
55057_461	45.94	0.097	0.382	0.323	0.156	0.475	0.361	0.145	0.480	0.374
11929_523	45.98	0.010	0.164	0.611	0.005	0.142	0.853	0.006	0.142	0.851
11929_712	46.00	0.211	0.388	0.154	0.299	0.512	0.186	0.303	0.518	0.177
55053_598	46.70	0.324	0.405	0.113	0.447	0.450	0.103	0.429	0.464	0.106
34426_641	46.88	0.068	0.375	0.500	0.079	0.398	0.517	0.080	0.410	0.505
34444_603	46.90	0.529	0.387	0.069	0.517	0.402	0.081	0.514	0.402	0.079
7189_241	53.60	0.284	0.499	0.217	0.309	0.482	0.187	0.315	0.505	0.179
52337_266	53.70	0.084	0.426	0.486	0.102	0.457	0.440	0.082	0.439	0.466
52331_182	53.75	0.290	0.225	0.039	0.621	0.333	0.046	0.616	0.342	0.041
52325_613	53.80	0.791	0.199	0.010	0.736	0.238	0.013	0.732	0.252	0.014
52321_373	53.85	0.740	0.244	0.015	0.728	0.258	0.015	0.718	0.264	0.016
52311_539	53.90	0.873	0.122	0.005	0.827	0.167	0.006	0.806	0.185	0.006
52309_281	53.95	0.364	0.483	0.146	0.217	0.516	0.260	0.198	0.515	0.282
24370_238	54.00	0.667	0.305	0.028	0.711	0.254	0.013	0.718	0.263	0.016
36440_654	54.10	0.587	0.210	0.019	0.897	0.100	0.003	0.897	0.099	0.003
52289_1165	54.30	0.736	0.247	0.016	0.686	0.295	0.019	0.683	0.296	0.019

[1]Difference between the sum of the 3 genotype classes and 1.00 equals the percentage of animals containing at least 1 null allele for a marker.
[2]Three genotypic classes for each SNP is represented as 2 homozygous genotypes of aa and AA, with the heterozygote represented as Aa.

Genotypic frequencies for each marker, based on animals with phenotypic records for twinning rate for each GenoProb run, are listed in Table 2. Genotypic frequencies for each marker were similar between the ovulation and twinning rate phenotypes (data not shown). Slight differences can be attributed to the distribution of animals contributing phenotypic observations for each trait. Because of the small number of SNP genotypes used in the 48 animal analyses, GenoProb computed substantial probabilities of null alleles for some of the markers, perhaps because several animals in the sample of 48 were homozygous for these markers. For example, in the 48-animal data set, GenoProb generated genotypes with probabilities greater than 0.95, with null alleles for markers 11929_712 (n = 298), 52331_182 (n = 161), and 55053_598 (n = 33). As sample size increased, the frequencies of the null alleles were almost completely eliminated for the 724- and 2,900-animal data sets (see Table 2 for the previous 3 markers).

Heritability estimates for ovulation and twinning rate were 0.12 and 0.10, with permanent environmental proportions of phenotypic variance of 0.055 and 0.023, and phenotypic variances of 0.172 and 0.216, respectively. Additionally, the genetic correlation between ovulation and twinning rate was high ($r_g = 0.75$). Single-marker analyses for each genotyped data set resulted in a group of markers to begin the step-down analysis by using multiple-trait derivative-free REML. Table 3 lists all single-marker analyses that were included to begin the step-down analyses. Consistent results across analyses were observed for markers 55069_549 and 11929_523 for ovulation rate, and 2019_224 with twinning rate for single-marker AD analyses. Markers 55053_598 and 7819_241 were significant for effects including imprinting for ovulation and twinning rate across all 3 analyses, respectively. Single-marker analyses resulted in significant associations for 52289_1165 for ovulation rate in both the 48 and 724 genotyped data sets. Marker 34336_607 was significant for twinning rate in the 48 and 2,900 genotyped data sets. Only 4 markers were significant in the 724- and 2,900-animal data sets: 2019_224 and 12163_117 for ovulation rate, and 11929_523 and 34444_603 for twinning rate.

Results from the final analysis resulting from the step-down procedure for the ADI, AD, A+I, or A models can be found in Table 4. Markers with at least a trend ($P < 0.1$) in all 3 analyses were 55053_598 for ovulation rate and 2019_224 for twinning rate. For the 724- and 2,900-animal data sets, 2 markers showed association in both analyses: 55069_549 for ovulation rate and 7189_241 for twinning rate. Also included in Table 4 are the marker genetic effects for each trait with SE. For all markers that concurred across the analyses, the direction of effect was the same for the genetic effect that was significant. For marker 55053_598, the size of the effect was similar across all 3 analyses, with SE reducing in size as the number of animals genotyped increased. Standard error was reduced or was the same

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Table 3. Single-marker results using 48, 724, or 2,900 animals genotyped with markers used to start step-down analyses

			48				724				2,900			
SNP	cM	Trait[1]	Model[2]	F[3]	df[4]	*P*-value[5]	Model	F	df	*P*-value	Model	F	df	*P*-value
2019_224	45.40	Ovul					A, D	3.29	2	0.038	A, D	2.38	2	0.093
12163_117	45.70	Ovul					A, D	2.02	2	0.133	A, D	4.19	2	0.015
55069_549	45.75	Ovul	A, D	5.47	2	0.004	A, D	2.34	2	0.097	A, D	5.47	2	0.004
55067_558	45.80	Ovul	A, D, I	2.08	3	0.101					A, D, I	2.86	3	0.036
55067_720	45.85	Ovul	A, D	5.96	2	0.002					A, D	4.45	2	0.012
11929_523	45.98	Ovul	A, D	4.11	2	0.016	A, D	3.54	2	0.029	A, D	3.95	2	0.019
55053_598	46.70	Ovul	A, I	2.71	2	0.038	A, D, I	3.27	3	0.021	A, D, I	4.37	3	0.004
52325_613	53.80	Ovul					A, D	2.09	2	0.124				
52321_373	53.85	Ovul					A, D	2.73	2	0.066				
52309_281	53.95	Ovul									A, D	2.96	2	0.052
24370_238	54.00	Ovul	A, D	2.62	2	0.073								
52289_1165	54.30	Ovul	A, D	2.20	2	0.111	A, D	2.34	2	0.097				
36440_654	54.10	Ovul									A, D	2.76	2	0.063
2019_224	45.40	Twin	A, D	2.90	2	0.055	A, D	3.29	2	0.038	A, D	3.04	2	0.048
34336_607	45.61	Twin	A, D	2.76	2	0.064					A, D, I	2.11	3	0.098
55069_549	45.75	Twin	A, D	2.34	2	0.097								
55057_461	45.94	Twin									A, D	3.80	2	0.023
11929_523	45.98	Twin					A, D	2.53	2	0.080	A, D	2.73	2	0.066
34444_603	46.90	Twin					A, D	3.77	2	0.023	A, D	2.44	2	0.088
7189_241	53.60	Twin	A, D, I,	2.97	3	0.031	A, D, I	2.99	3	0.030	A, D, I	2.20	3	0.087
52331_182	53.75	Twin									A	3.17	1	0.075
52309_281	53.95	Twin	A, D, I	2.07	3	0.103								

[1]Traits used in analysis: ovulation rate (Ovul) and twinning rate (Twin).
[2]Genetic effects used in the model for single-marker analysis: additive (A), dominance (D), and imprinting (I).
[3]Overall *F*-test (F) generated from the single-marker analysis.
[4]Degrees of freedom based on the genetic effects used in the model.
[5]Significance level expressed as a *P*-value calculated from the *F*-test.

for all markers that were significant across data sets, with the smallest SE observed for the 2,900-animal data set.

Results from single-marker 2,900-animal analyses of M and P imprinting, as defined previously, are found in Table 5. Like the ADI analysis of single markers, associations with significant *F*-tests were observed with marker 5053_598 for ovulation rate and with marker 7189_241 for twinning rate. Results including genetic effects of P (M was not significant) in the overall step-down model are shown in Table 6, with individual marker effects and SE. The final list of markers showing association did not change from the previous ADI final analysis. The overall *P*-value did decrease for the 2 markers when fitting P in the model relative to ADI.

DISCUSSION

The USMARC Production Efficiency Population has undergone selection for increased reproductive rate, defined as increased calf production, for nearly 30 yr. The population offers the opportunity to discover and validate markers for low-heritability reproductive traits such as ovulation and twinning rate. To maximize phenotypic information with limited genotypic information, GenoProb software was used to generate information by using all available data from the population (Thallman, 2002). By genotyping ancestral animals with DNA in conjunction with progeny from animals with no DNA, genotypes can be inferred from phase relationships to increase the number of genotypes available for association analyses. Three different GenoProb analyses were run to evaluate SNP for associations by using 48, 724, and 2,900 genotyped animals with the entire multigenerational pedigree (16,035 animals).

Previously, a QTL scan was done to identify regions of the genome affecting ovulation rate in the USMARC Production Efficiency population (Kappes et al., 2000). A significant QTL was detected on bovine chromosome 5. Microsatellite markers for this region are presently being used for MAS in conjunction with 2 regions on BTA7. In addition to the MAS information, predicted breeding values for ovulation rate and twinning rate, and EPD for birth, weaning, and yearling BW are being used to make genetic selection decisions. A previous study found a QTL for twinning rate in a Norwegian cattle population just distal to the QTL peak in this study (Lien et al., 2000). Their peak appeared to be located at 70 cM, with a slight peak nonsignificant, centered at BMC1009 (approximately 42 cM), where the USMARC QTL resides. Additional analyses using combined linkage and linkage disequilibrium confirmed the peak at approximately 65 cM in the Norwegian population (Meuwissen et al., 2002). Additionally, Cruickshank et al. (2004) found evidence for QTL at approximately 70 cM, confirming the QTL found by

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Table 4. Final step-down model results using 48, 700, or 2,900 animals genotyped

No. of animals genotyped	Trait[1]	SNP[2]	Model[3]	F[4]	*P*-value[5]	A effect[6]	SE[7]	*P*-value[8]	D effect[9]	SE	*P*-value	I effect[10]	SE	*P*-value
48	Ovul	11929_523	A, D	4.09	0.017	−0.0015	0.0155	0.921	0.0455	0.0201	0.024			
	Ovul	55053_598	A, I	2.87	0.057	−0.0122	0.0101	0.229	−0.0027	0.0145	0.852	0.0174	0.0083	0.036
	Ovul	24370_238	A, D	2.65	0.071	−0.0583	0.0260	0.025	0.0683	0.0313	0.029			
48	Twin	2019_224	A, D	2.43	0.089	0.0256	0.0206	0.214	0.0596	0.0279	0.027			
	Twin	34336_607	A, D	2.09	0.124	−0.0023	0.0146	0.866	0.0354	0.0178	0.047			
724	Ovul	55069_549	A	3.56	0.059	−0.0161	0.0088	0.059	0.0175	0.0098	0.066			
	Ovul	55053_598	A, D, I	4.12	0.006	−0.0147	0.0093	0.114	−0.0006	0.0092	0.520	−0.0168	0.0062	0.006
	Ovul	52321_373	A, D	2.73	0.066	−0.0305	0.0165	0.065	0.0416	0.0178	0.019			
724	Twin	2019_224	A, D	3.46	0.032	−0.0317	0.0160	0.048	0.0477	0.0188	0.011			
	Twin	34444_603	A, D	3.68	0.025	−0.0142	0.0133	0.289	0.0438	0.0161	0.007			
	Twin	7189_241	A, D, I	3.49	0.015	−0.0281	0.0124	0.023	0.0107	0.0148	0.752	−0.0216	0.0096	0.026
2,900	Ovul	55069_549	A, D	3.30	0.037	−0.0202	0.0076	0.008	0.0023	0.0095	0.808			
	Ovul	55053_598	A, D, I	4.82	0.002	−0.0178	0.0077	0.021	0.0101	0.0083	0.225	−0.0165	0.0056	0.003
	Ovul	52309_281	A, D	3.69	0.025	0.0108	0.0063	0.088	−0.0158	0.0075	0.036			
2,900	Twin	2019_224	A, D	2.41	0.090	−0.0179	0.0151	0.236	0.0362	0.0165	0.028			
	Twin	34336_607	A, D	3.02	0.049	0.0262	0.0105	0.013	−0.0013	0.0123	0.909			
	Twin	55057_461	A, D	4.01	0.018	0.0152	0.0110	0.167	0.0354	0.0128	0.005			
	Twin	7189_241	A, D, I	2.60	0.051	−0.0090	0.0106	0.394	0.0017	0.0124	0.144	−0.0228	0.0085	0.007

[1]Traits used in analysis: ovulation rate (Ovul) and twinning rate (Twin).
[2]Marker ID.
[3]Genetic effects used in model for step-down analysis additive: (A), dominance (D), and imprinting (I).
[4]Overall *F*-test (F) generated from the single-marker analysis.
[5]Significance level expressed as a *P*-value calculated from the *F*-test.
[6]Additive (A) effect for the SNP.
[7]Standard error for the SNP.
[8]Significance level expressed as a *P*-value calculated from the *t*-test.
[9]Dominance (D) effect for the SNP.
[10]Imprinting (I) effect for the SNP.

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Table 5. Results comparing genotypic class effect means for paternal inherited alleles

SNP	Trait[1]	cM	A_D_I single marker[2]	M_P single marker[3]	*P*-value	aa[4]	aA[4]	Aa[4]	AA[4]	Het avg[5]
55053_598	Ovul	46.70	I F	P	0.017	0	−0.0157	0.0234	−0.0106	0.0038
				F	0.001					
7189_241	Twin	53.60	I F	P	0.057	0	−0.0278	0.0110	−0.0249	−0.0083
				F	0.046					

[1]Traits used in analysis: ovulation rate (Ovul) and twinning rate (Twin).
[2]Model used in the single-marker analyses containing effects: additive (A), dominance (D), and imprinting (I); letters represent significant effects with the letter F representing the overall significant *F*-test for the model.
[3]Model used in the single-marker analyses containing effects: paternal (P) and maternal (M); letters represent significant effects, with the letter F representing the overall significant *F*-test for the model.
[4]aa, aA, Aa, AA represent the genotypic mean calculated from the ADI model.
[5]Het avg = average of the heterozygotes.

Meuwissen et al. (2002), but not at the location identified by Kappes et al. (2000).

The practical problem in developing commercial tests based on SNP markers is to identify the minimal set of SNP that account for most of the variation occurring within a region of a chromosome. Using the step-down approach with marker association analyses eliminates markers that may be significant in a single-marker analysis, which may explain a portion of the variation shared with another marker. The linkage of these markers makes it more probable that the markers explain similar sources of variation for the traits of interest. Systematic removal of SNP in each round of the step-down approach resulted in a more concise and meaningful model and perhaps concentrates efforts for the development of additional SNP. As advancements in software and theory in this field enable using large numbers of newly identified SNP, fitting haplotypes and their interactions may become the models of choice (Kuehn et al., 2007).

Allele frequencies can have implications for association results of SNP analysis. Minor alleles that are rare often may result from a single founder or may be breed specific. This classification can result in spurious associations, often referred to as a "stratification" effect, of the single founder or breed. By fitting a polygenic animal model, the chances of finding a spurious association should be greatly reduced. However, significant SNP with low minor allele frequency need to be validated in additional populations to confirm the association (Allan et al., 2007).

Because of the larger number of genotyped animals, the 2,900-animal data set was expected to produce the most accurate set of markers. When only 48 animals were genotyped for SNP, markers were removed from the final model that remained significant in the final analyses of the 724 and 2,900 genotyped animals. Additionally, SNP in the final 48 genotyped animal model failed to remain in the final models for the 724 and 2,900 genotyped animal analyses. Numbers of animals with null alleles were significantly greater in the 48-animal genotyped data set, decreasing the ability to detect associations for markers that were significant in the final model. Additionally, this data set appears to be generating some false positives for associations. The use of 724 genotyped animals did relatively well in predicting marker effect sizes relative to the 2,900 genotyped animal analysis. It should be noted that special emphasis was placed on selection of the 384 females used in the 724 data set. These females had a minimum of 2 parturitions each and represented a broad sampling of the founding genetics over the last 14 yr.

The region examined on BTA5 in the present study is gene dense and provides a strong argument for the limitations of using a candidate gene approach to identify useful genetic markers (quantitative trait nucleotides) within a chromosomal region. In this region of BTA5, there are several strong candidate genes with potential involvement in follicular development, embryonic development, or both that could increase ovulation or twinning rate. There are genes involved in cell proliferation (CDCA3, IGF-I, IGFBP6, KL, MYF5, MYF6, SSR3), apoptosis (Apaf-1, TEGT, SOCS2), and signal transduction (ALK-1, FRS2, PTPRR, PTK9, SKR-3) throughout this region, and by chance, several of the SNP that were used fall within the introns of several these genes (FRS2, PTPPR, TAIP-12). Although it may be tempting to focus in depth on any one of these candidates, the use of a large number of SNP provides the ability to use haplotype analysis and linkage disequilibrium to fine map a region of interest, and could provide informative markers more rapidly and cost effectively than focusing on any single gene or SNP.

Although the genetic correlation between ovulation rate and twinning rate is high, there are SNP within this region on BTA5 that associate with one or the other phenotype, but not both. The association of SNP with one trait, but not the other, may result from the number of measurements for each trait. It should be noted that in single-marker analysis, some of the SNP were significant for both traits, yet none of these remained for both traits in the final models. However, some of these differences may be artifacts of the model selection process. Furthermore, there is some potential biology that would support the possibility of more than one gene under the peak of the QTL with specificity for each trait. The ability to ovulate multiple follicles is a key component of multiple births in cows; however, the

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Table 6. Final results using the 2,900 animal data set including paternal effects in the model for the step-down process

SNP	Model[1]	Trait[2]	F[3]	*P*-value[4]	A effect[5]	SE	*P*-value[6]	D effect[7]	SE	*P*-value	P effect[8]	SE	*P*-value
55069_549	A, D	Ovul	4.68	0.009	−0.0200	0.0082	0.015	0.0025	0.0095	0.788			
55053_598	P	Ovul	12.28	<0.001							−0.0180	0.0051	<0.001
52309_281	A, D	Ovul	3.20	0.041	0.0082	0.0062	0.186	−0.0162	0.0075	0.032			
2019_224	A, D	Twin	2.39	0.092	−0.0186	0.0146	0.204	0.0360	0.0165	0.029			
34336_607	A, D	Twin	2.97	0.052	0.0252	0.0106	0.018	−0.0015	0.0123	0.902			
55057_461	A, D	Twin	3.99	0.019	0.0147	0.0109	0.179	0.0358	0.0130	0.006			
7189_241	P	Twin	7.35	0.007							−0.0212	0.0078	0.007

[1]Genetic effects fit for each SNP: additive (A), dominance (D), and paternal (P).
[2]Traits used in analysis: ovulation rate (Ovul) and twinning rate (Twin).
[3]Overall *F*-test (F) generated from the final step-down analyses. Numerator degrees of freedom are either 1 or 2, depending on the number of effects fitted.
[4]Significance level expressed as a *P*-value calculated from the *F*-test.
[5]Additive (A) effect for the SNP.
[6]Significance level expressed as a *P*-value calculated from the *t*-test.
[7]Dominance (D) effect for the SNP.
[8]Paternal (P) allele effect for the SNP.

twinning rate phenotype represents the ability of a cow to conceive successfully and carry twins to term. Therefore, the components of the 2 phenotypes are different. Ovulation rate requires successfully growing multiple follicles to a stage and functional status at which they can undergo further growth in response to the FSH surge (i.e., be selected). They must synthesize LH receptors and be responsive to the LH surge (i.e., ovulate and form a functional corpus luteum). Therefore, the genes involved in ovulation rate would be those involved with establishment of the ovarian reserve, activation and growth of follicles to the early antral stage, selection of ovulatory follicles, and successful ovulation and luteinization. These would all be components of twinning rate, because multiple ovulations are required as the first step in dizygotic twinning. However, the twinning rate phenotype would also include genes involved in fertilization, early embryonic development, successful maintenance of pregnancy, and uterine capacity.

The value of the twinning rate phenotype to developing genetic markers for fertility in monovulatory cattle seems clear. As stated above, these would represent genetic regions associated with fertilization, early embryonic development, and maintenance of pregnancy, all physiological processes that would need to occur in monovulatory cows as part of successful reproduction. Evaluating these components in the presence of multiple ovulations provides more opportunities for failure, and therefore greater phenotypic variation. The ovulation rate phenotype is less clear. If all the genes associated with ovulation rate are markers of multiple ovulations, then they may have less value to the reproductive efficiency of monovulatory cows. However, successful initiation of ovulation in the postpartum period is one of the greatest reproductive obstacles a beef cow must overcome. It is possible that genes associated with ovulation rate, and therefore associated with successful follicle growth, selection, and ovulation, could be genetic markers for successful initiation of fertile estrous cycles in the postpartum period of monovulatory cows, if they could be used without resulting in multiple ovulations. Additionally, these markers may be useful in decreasing ovulation rate in populations such as dairy cattle, for which twin births are detrimental to the reproductive fitness of the female.

Understanding M and P allele genetic effects, referred to as imprinting, should be included in association analyses. Markers 55053_598 and 7189_241 for ovulation and twinning, respectively, were significant in the final models for both the 724- and 2,900-animal genotyped data sets. Because of these results, we further analyzed a model for P (and M) genetic effects. The *P*-values of both markers decreased relative to the earlier analysis. For the 2,900-animal genotyped data set, we observed paternally influenced inheritance effects for both ovulation and twinning. Comparative mapping of the mouse and human places this region of BTA5 on mouse chromosomes 10 and 15 and human chromosome 12. A maternally expressed gene, Decorin (**Dcn**), in mice

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has been identified on mouse chromosome 10 (Mizuno et al., 2002). This gene maps to 19 Megabases (**Mb**) on BTA5, placing it 10 Mb proximal to the QTL and marker 55053_598. Additionally, Slc38a4, paternally expressed in mice (Smith et al., 2003), maps to 33 Mb, approximately 4 Mb distal from marker 55053_598. These imprinted mouse genes give direct evidence that the paternally inherited effects we observed in the model fit potential biological imprinting evidence for this region of the bovine genome. Khatib (2005) explored the possibility of Dcn being imprinted in cattle by using monoallelic analysis of SNP from the coding region of Dcn for various tissues. Results suggested that bovine Dcn transcripts were not imprinted in bovine tissues. In a similar experiment, Zaitoun and Khatib (2006) also concluded that SLC38A4 transcripts were not imprinted in bovine tissues. Results suggesting that SLC38A4 is not imprinted may decrease the excitement about this gene as a candidate for the QTL on BTA5, but it should be noted that these conclusions were made from 3 cows and fetuses collected from a slaughter facility, which were heterozygous for the polymorphism in SLC38A4. This limited sampling, with no consideration taken of the genetic background of the sampled animals, keeps these conclusions from being definitive. More recently, C12 was found to be methylated in various tissues in humans on human chromosome 12 (Shen et al., 2007). This gene maps to BTA5 at 26 Mb, 3 Mb from marker 55053_598. This additional evidence in humans supports the possibility of a clustering of imprint-regulated genes in this region. This evidence strongly supports the genetic observation found with the maternal-paternal allele analysis and warrants additional investigation of the underlying mechanisms for the reproductive rate QTL in this region.

In conclusion, GenoProb is an extremely valuable tool in generating marker information when DNA may not be available. However, caution should be taken in the number of animals genotyped. In this study, the 48-animal data set worked well for SNP discovery. However, extrapolation of the SNP genotypes from the 48 animals to the rest of the population was not adequate to make conclusions about genetic associations, even with the extremely high number of microsatellite markers. The DNA markers from this study with significant associations with ovulation rate (55069_549, 55053_598, and 52309_281) and twinning rate (2019_224, 34336_607, 55057_461, and 7189_241) may be useful to increase or decrease the incidence of twinning in industry populations for the QTL segregating in this region of BTA5. It should be noted that these markers need to be validated in additional populations before they are used for MAS in an industry setting.

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