Genetics and Molecular Research

Molecular markers located on the *DGAT1*, *CAST*, and *LEPR* genes and their associations with milk production and fertility traits in Holstein cattle

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Genet. Mol. Res. 15 (1): gmr.15017794
Received October 6, 2015
Accepted January 6, 2016
Published March 31, 2016
DOI http://dx.doi.org/10.4238/gmr.15017794

ABSTRACT. The objective of the present study was to investigate single nucleotide polymorphisms (SNPs) located in three candidate genes previously reported to have effects on fertility and milk production traits in a population of 123 Holstein cows. The milk production traits evaluated included lifetime averages of milk yield, protein concentration, and fat concentration. Fertility traits evaluated included lifetime averages of services per conception and days-open. Candidate genes included those encoding diacylglycerol acyltransferase (*DGAT1*), leptin receptor (*LEPR*), and calpastatin (*CAST*). A total of 60 SNPs were selected (20 per gene) at equidistant locations on each candidate gene to identify potential linkage with causative mutations. Four SNPs were identified as being significantly associated with the evaluated fertility traits. Specifically, SNPs rs109663724 and rs137673193 were significantly associated with lifetime average days-open, while rs109663724 and rs135560721 were significantly associated with lifetime average number of services per conception. Five SNP (rs109663724, rs132699547, rs135423283,

rs135576599, and rs13675432) were significantly associated with lifetime averages of milk protein concentration and milk fat concentration, with only one SNP (rs109663724) being significantly associated with the average lifetime milk yield. Although multiple SNPs were identified in the current study as being significantly associated with milk production and fertility traits, it is essential that these SNPs are validated in larger populations, under more diverse environments, and that additional SNPs and candidate genes are evaluated prior to their implementation into selection strategies.

Key words: SNP; Milk; Candidate gene; Cattle; Holstein; Fertility

INTRODUCTION

Dramatic improvements in milk yield have been observed over the past five decades (Butler and Smith, 1989; Washburn et al., 2002), and modern Holstein cattle are now producing significantly more milk than in previous decades. The lactation cycle is initiated and renewed by parturition; therefore, an animal must have the ability to conceive, maintain a pregnancy, and rebreed in a timely manner (Lucy, 2001). However, over recent decades, the major focus of selection in Holstein cattle has continued to center on increasing milk production. Consequently, over the same time period, a dramatic decline in reproductive efficiency has been reported in female Holstein. Washburn et al. (2002) reported that days-open and services per conception in Holstein cattle had increased from 124 to 168 days and 1.91 to 2.94, respectively, between 1976 and 1999. However, milk yield increased from 4753 to 6375 kg and fat yield increased from 228 to 282 kg during the same period.

Identification of single nucleotide polymorphisms (SNPs) located in candidate genes or regions of the genome reported to be associated with milk production and fertility traits may provide researchers and producers with a method that can be used to increase the accuracy of selection for lowly heritable traits like fertility while maintaining or improving milking ability. Three candidate genes of known physiological function were selected for investigation in the current study. The leptin receptor (*LEPR*), calpastatin (*CAST*), and diacylglycerol acyltransferase genes (*DGAT1*) were selected based on previously reported associations with fertility and milk production traits in Holstein cattle (Liefers et al., 2002; Ashwell et al, 2004; Garcia et al., 2006). The three candidate genes utilized in the current study have been associated with triglyceride synthesis (Thaller et al., 2003), expression in the mammary glands (Bartha et al., 2005), first service per conception (Clempson et al., 2011), and polymorphisms associated with daughter pregnancy rate (DPR) in dairy cattle (Garcia et al., 2006).

The objective of the current study was to evaluate potential SNP associations in three candidate genes with fertility and milk production traits in a population of Holstein cows located in the southeastern United States.

MATERIAL AND METHODS

Experimental procedures were approved and performed in accordance with the International Animal Care and Use Committee guidelines for the use and care of animals in agricultural research (Approval No. AE-2009-21). A total of 123 female Holsteins born between 2004 and 2010 at the Louisiana State University Agricultural Center Research and Teaching Dairy Farm located in Baton

Rouge, Louisiana, were utilized for the current study. All female Holstein were maintained on a dry mixed diet and were managed and evaluated at the above location. Lactating females were milked twice daily during their lactation cycle and milk production traits were collected and recorded for each individual for future analyses. Average lifetime production was calculated for milk yield, protein yield, fat yield, services per conception, and days-open, owing to animals of different ages varying in the number of lactation cycles.

For breeding purposes, a controlled internal drug release (CIDR) Synch protocol (Accelerated Genetics, Baraboo, WI, USA) was utilized prior to artificial insemination with frozen/thawed semen to synchronize female Holstein. Females that did not respond to the CIDR Synch protocol were identified by visual heat detection and artificial insemination was repeated during the next observed estrus. Pregnancy status was determined utilizing the rectal palpation technique and subsequent management decisions regarding rebreeding or culling were made.

Blood samples were collected from all female Holsteins via tail vein vena puncture. Blood was transferred to 20-mL tubes and centrifuged at 4000 rpm at 4°C for 20 min. White blood cell buffy coats were extracted and transferred to 250-μL micro-centrifuge tubes. Genomic DNA was isolated and purified from buffy coats using a previously described saturated salt procedure (Miller et al., 1988). DNA working solutions (200 μL) were prepared by diluting 25 ng/μL stock DNA with Tris EDTA buffer.

Sequences of SNPs located in the candidate genes (*CAST*, *DGAT1*, and *LEPR*) were identified from dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/). A total of 20 SNPs per candidate gene were selected by identifying SNPs that were evenly distributed over the entire length of each gene. This selection method was selected to account for possible linkage associations with potential causative mutations located on the candidate genes. Flanking sequences of SNPs and allele substitutions for *LEPR*, *CAST*, and *DGAT1* are reported in Tables 1, 2, and 3. IPLEX reactions for all SNPs were generated by NeoGen LLC (Lincoln, NE, USA) and genotyping assays were conducted by NeoGen LLC utilizing Sequenom genotyping technology (San Diego, CA, USA). The Sequenom technology is a MassArray platform that utilizes MALDI-TOF technology to detect the specific mass of uniquely amplified SNP alleles.

Table 1. Single nucleotide polymorphism (SNP) ID numbers, allele substitution, and flanking sequences used to amplify and visualize *LEPR* genotypes.

SNP ID	Allele substitution	Forward primer	Reverse primer
rs135977111	A/G	CTTCTGTTCTCTTCCTTGCAAAACATGTAA	CAAGCTCCCTGGCAGTGGGATTTCCAGACA
rs133145962	A/G	TATCTTTGGCAGGAATGCAATCAAATGTGT	TTAATCAGTCATGTCTGACTCTTTGTGACC
rs43347905	A/G	TTTTCTCTGTGTCTTTTAAATGTCCTAACA	AATTTATTTATGTAATAACTGCATTTAACT
rs133109480	A/G	GGTTTACAGTCCATAGAGTCGCAAAGAGTC	GACATAACTGAGCTGCTAAGCTCAAGCACG
rs43347912	G/T	CTGGACGGCCAGGGGGTTCCCTGAACTAAT	TTTAAAGTCACCCTAGGAGTAGAACAGATA
rs43347914	A/G	AAGCTCTTCCCTGCCTTCCCTTTGATTTTT	CTCAGAAGCCATTTCATAGTTCTAACATTG
rs43347917	A/T	TTTAACCAATCCATTGATTTTTAATGTATG	AGTGTAACATTTTCAAATATCAAGTGAAAA
rs136901371	C/T	GAGACAAGAGAGAAGAGTTCAGAATAAAAT	GGGCTTGATTAATGGAGCAGAATACTCAAA
rs43348634	A/G	CTAAGCTGCTAAGTCACTTCAGTCATGTCC	ACTCTGTGCGAACCCATAGATGGCCTCCCA
rs134577752	A/G	CTGAGCACACTTGTTTACTTTACAAATAAC	CATGTTTCTTCTCTCAAAATTTTAGTTGGT
rs135915491	C/G	AGCAGCAAAGTGGTTTGAAAAATTGAAGTA	ATAGTGATCCTCAAGATGTTTTGTGTGCAT
rs43348652	A/G	TCTCTGCCCAGTATTGTCTACCCCTGCTCT	TGAGGCAGGAACTTTGTCTCACTCACCATT
rs134375381	G/T	CAAAGACAAGAGCCTTTTGCTTGGAGTAAT	AAGGTAGGAGAACATTCAGAGATGTGGTTA
rs135560721	C/T	TTTTGAGGAGATTCAGTCATACTTCAATAT	GTACATTCAAGCTTTCATTCAAGATCAGCA
rs137541136	A/G	GCTATTTCAAATCCTAAAAGATGATGCTGT	AAAGTGTGGCACTCAATATGCCGGCAAATT
rs43348655	C/T	ACAGTCCATGGGGTCACAAAGAGTTGGACA	GACTGAGCAAAATCACTTGGTGCTGCATAA
rs43348659	A/C	AAGAATAATATTTTAGAGAAATATTGATTC	CCTTGTCCTCGCCACACGACACTGGCACTG
rs137111668	C/G	CTCTCCTTATTAGAAAATTGTCATTTACTT	AATTGCATACCCACTTACTGTCAAGCAAAA
rs137842817	G/T	AAAGTTTAATTGGATGTTCTGATGGTTTTT	AAATCTGAGTAGTCATAACTCAAAGCTTAG
rs135263435	A/G	TACTAGAAGACACTGTGAAAATTCAACTTT	GGAATGACAGCTCCTCATTTTACTAGCTTT

Table 2. SNP ID numbers, allele substitution, flanking sequences utilized to amplify and visualize *CAST* genotypes.

SNP ID	Allele substitution	Forward primer	Reverse primer
rs43529864	G/T	GTGGGAGCCAGCTCGGACGTACACGTGCTA	TCGGCGTGAGTTCAGGCTCACAAGTTGAAT
rs133108534	C/T	TTGTCCTATTTTTGATTGCAATGATTCTTT	TTCAGCCTCCTCAAGTCTGCCTTTGAATCC
rs134804900	A/G	TCTGAGTGAAATGTCTCCTACTTTAGGACC	GCATCCTGCACTTCCTGTCTTTGCTCCCGT
rs109727429	C/T	AGCTGGCTGACAGAGAGGAGAGCCAGGCTT	GCCCTGCTCCCGTGACATAAATCACTGCAG
rs133978255	G/T	CACAGAGTCGGACACGACTGAAGCGACTTA	CAGCAGCAGCATACTCTTAACTAGTATCCA
rs135802918	G/T	AATTGGTCATTATATCACCACTGCCTAGAG	AGGACCAGGCTTCTAGCCAGGGTTCAGTAA
rs134187714	C/T	AATCCCATGGACAGAGGAGCCGCAAAGAGT	GGACAGGAATGAGCCACTTCACTTTCACTT
rs135598419	A/C	AGAGCGGTGCTTTGTATCTGTCTTTCAAGA	TGCAAAGTGTTTTCGTGGAGATTTGACAGT
rs133440731	A/G	GGGTCACAAAGAGTCAGACATGTCTCAGCA	TCAGACAAACAGCAAGGGTGTTAATGCTTG
rs135336850	C/T	ATTCAGTGTTGGCTGAAATTCTACCGGTCT	GAGTCCAGAGTCCGCTCTCGCTCTCTTAGC
rs137673193	C/T	CAATTGCACCTGTGGAAGGACAGTCATTAA	ATATAGATAGTGAAAGTGAAACTGTTAGTT
rs110972443	A/C	CATCTGTTGATAGACTTATAGGTTGCTTCC	TGTGTTGGCTATTGTAAACAGTGCTTCAAT
rs134668965	G/T	TTATTGTTTTCAGACTGTTGCTAGGATTAT	ATCAACCAGACACCAACAGCCATTTCTCTC
rs133997237	C/T	AATGAATAAAAGAGCACAGGGCAATCCGTT	ATGAGATGCATTTTATTTGGAAGAGGTGGA
rs133149410	A/G	TAATGTCTCTGCTTTTTAATACCAGGGAAT	TGTTAAATTTCCTCTAGAAAGCTAGCAAAC
rs110647227	A/G	TCCTTAGGCATTCAAGAAAATCATGCTCAC	GCGGGTAGGGTAGCAGACYGTATTGTTGGT
rs109491082	G/T	TACAGAGATCGGGCTTCTGAGTCTCATGTT	TCCACCCGGTTTCCATTGCCAAGGACCAAG
rs111010631	C/T	ACACACTGAAGGAGCTTAATATATTGTTGC	TTATTAGAATTGAAGTGCAATAATGCATAT
rs133820366	A/C	AAGGCCTGCTGTCTCTCTTTCTTCCCCAAC	CCACCACCACCGGTGCTGTTGAGAACGAAG
rs136073124	C/T	GCCCTGTGTTTGATTCTACTTTACAGTAAC	GAAGAGCTGGTTTGGATGAGGGAGACTCTG

Table 3. SNP ID numbers, allele substitution, and flanking sequences used to amplify and visualize *DGAT1* genotypes.

SNP ID	Allele substitution	Forward primer	Reverse primer
rs134049142	A/G	GGCACCCTGTATGATGAGGGGCATGTGCCA	AGGGTGCCTGTGGCGAGCTCCCCACCTTGC
rs135576599	A/G	CCCCAGGGGATTCATGCAGGGAGGCCGTAG	AGCAGGCAGGGCCAGATGCCCAGCAAGACC
rs109711965	G/T	TGCCTGCCCTTTGGTGTGGCAGCCCCTTCA	GCCTCACCTCAGCCTTGGCGCCGGCAGCCT
rs134455341	A/G	GGAAAGGGAGTGGAGATGACCTTGAACACC	TGTCCTTTGCTTTTCTCGGGTCTCTGACCC
rs134374261	A/C	GCACAGCCGGGCCGCAGCAGCTGTCAGCCC	CCTGCCGCCCCTGCAAGTCCTGTCTCCCCA
rs137617619	A/G	TGCCCGACTCCTGTGACCCCATGGATTGTA	CCCACCAAGCTCCTCTATCCATGGGATTTT
rs135048973	C/T	ATTGCCACCTAGGAAGCCCCCCCCCCCCACC	CCTTTGAATATTCTTGTCTCTTTTCCTTGT
rs136875432	A/G	TGCCCCCCTCCTCTTCGGGAGACCATGCAC	TTCTACGCAGCCTGGCACATCTGGCAGACA
rs132679620	A/G	TCCTGGGGCCTCGGGGGCAGAGTGTGTGTT	TGCAAAGACAAGGCCATCTGCCAGCAACCC
rs132778108	C/G	AGGAGCTGCAGCTTCGGCACCCCCCAACCC	CCCCCCGCCACTCACCCTCGGGTAGGTTCT
rs109701809	A/G	CTGTCTGCCCGCGGGGGTATGTGTATCCTG	TGTCGTGTCCCGGGTTTGCTTGGCCCCTCC
rs134718967	C/G	GTGCTCCCTCAACCTCAGGGGCACTCGGGT	ACACCGGGCACAGTCAGGTTAGCAACCCCC
rs109663724	A/T	GTGCTGAACCACGCGCGTGGCGTGTACCAT	TCTCCATCCAGGGCCGCACCGTGTGTCAGG
rs135423283	G/T	GCTGCTGTGGGAGCAGAGAAGTCACTTCGG	TTCCTGTCAGGGTTTTTCCTCAGGGCCATG
rs132669273	C/T	CACGAATGTAAGTAGCCCACCACAGTCCAC	ATCTGGCTCCTCCCAAGACCTCCAGCATCT
rs109169510	A/T	GGCTAAGGGGATGTTCCTGCCCAAAAAGGA	GCAGGCAGGGTCTGGTGGGACTTCCTAGTA
rs137584522	C/G	AGATGAACCGCTCGGCCGAGGGGGATCCCT	CCCCCACCCCCACTGCGGTCCCGCCGGCTG
rs132699547	C/G	GGCCGCCCACCTATCGGGGCAGAGGCAGTA	CAGTGCCCCCATCCCTGGAGCAGGGTCAGG
rs134110051	A/G	ACGGCCGCTGGGCAGCAGGTTTCTTCTGCC	CGGTGGCACAGGCACCTGGGGTTGTGGTTG
rs135143198	C/T	GGGGCTCAGCTCACTGTCCGCTTGCTTCCT	CCCCAGCTGTTCCTCACCCAGCTCCAGGTG

Animal data were analyzed using the mixed model procedure of SAS version 9.3 (SAS Institute Inc., Cary, NC, USA). The model included independent variables for birth year and individual candidate gene SNPs. Dependent variables fit to the model included average lifetime services per conception, average lifetime days-open, average lifetime milk yield, average lifetime fat yield, and average lifetime protein yield, which were evaluated to test for potential associations

between SNPs and the previously described traits. Sire was fitted in the model as a random effect. The pdiff function of LSMEANS was utilized to evaluate significant differences in the performance of genotypes for SNPs that were identified as significant. All statistical analyses were conducted using previously described methodologies (White et al., 2005). Any SNP with only one genotype was excluded from the evaluation owing to a lack of genotypic effects. Statistical significance was set at $P < 0.05$ and all SNPs reaching this level of significance were reported as being significantly associated with the trait of interest.

RESULTS

A total of four unique SNPs located on the three candidate genes were found to be significantly associated with the fertility traits of average lifetime days-open, and average lifetime services per conception (Table 4). When evaluating days-open, two unique SNPs were reported, one located on each of the *DGAT1* and *CAST* candidate genes. Animals inheriting the heterozygous genotype for the *DGAT1* SNP had significantly longer periods of days-open than animals inheriting the major allele genotype. However, the opposite effect was observed for the significant SNP located in the *CAST* gene, where animals inheriting the major allele genotype had significantly longer periods of days-open than animals inheriting the heterozygous genotype. A similar effect was observed when evaluating average lifetime services per conception, for which two unique SNPs located on the *DGAT1* and *LEPR* genes were identified as significant ($P < 0.05$). Animals inheriting the heterozygous genotype for the SNP located on the *DGAT1* gene had significantly ($P < 0.05$) more average services per conception than animals inheriting the major allele genotype. However, animals inheriting the major allele genotype for the SNP located on the *LEPR* gene had significantly ($P < 0.05$) more services per conception than animals inheriting the heterozygous genotype of the same gene. No animals in the current study inherited the minor allele genotype for the evaluated SNPs.

Table 4. Least significant means of SNP genotypes located in three candidate genes significantly associated with fertility traits in dairy cattle.

SNP ID	Trait	Gene	Allele[a]	Major genotype	Heterozygous genotype	P value
rs109663724[b]	Average lifetime DO	*DGAT1*	T/A	114.08 ± 15.5	251.75 ± 61.12	0.03
rs137673193[b]	Average lifetime DO	*CAST*	C/T	182.17 ± 34.42	113.06 ± 15.64	0.05
rs109663724[b]	Average lifetime services to conception	*DGAT1*	T/A	2.07 ± 0.11	4.40 ± 1.12	0.05
rs135560721[b]	Average lifetime services to conception	*LEPR*	C/T	2.30 ± 0.14	1.14 ± 0.33	0.03

[a]Major allele is shown to the left and the minor allele is shown on the left. [b]All means differ at $P < 0.05$ within a row.

Five unique SNPs were significantly associated ($P < 0.05$) or exhibited a statistical trend for association ($P < 0.1$) with the milk production traits of average lifetime milk yield, average lifetime protein yield, and average lifetime fat yield (Table 5). Furthermore, all significant SNPs were located in the *DGAT1* gene, with no animals inheriting the minor allele genotype and no SNP from the other candidate genes being significantly associated with milk production traits. Animals inheriting the major allele genotype for all SNPs had lower levels of average milk yield, average protein yield, and average fat yield as compared with animals inheriting the heterozygous genotype. Furthermore, SNP rs109663724 was significantly associated with both fertility traits evaluated in the current study as well as all evaluated milk production traits.

Table 5. Performance LSMEANS of SNP located in three candidate genes significantly associated with average lifetime protein yield, average lifetime average milk yield, and average lifetime fat yield in dairy cattle.

SNP ID	Gene	Trait	Allele[a]	Major allele genotype	Heterozygous genotype	P value
rs109663724[b]	*DGAT1*		T/A			
		Protein yield		503.21 ± 20.61	1,172.21 ± 215.5	0.03
		Milk yield		16,770 ± 712.84	38,581 ± 7,452.11	0.005
		Fat yield		659.32 ± 24.40	1,479.32 ± 253.42	0.002
rs132699547[b]	*DGAT1*		C/G			
		Protein yield		487.17 ± 22.55	567.67 ± 4.40	0.05
		Fat yield		597.53 ± 26.46	766.68 ± 47.91	0.002
rs135423283[b]	*DGAT1*		G/T			
		Protein yield		486.13 ± 24.20	576.31 ± 41.46	0.05
		Fat yield		622.37 ± 27.11	805.22 ± 45.79	0.0005
rs135576599[b]	*DGAT1*		A/G			
		Protein yield		482.52 ± 24.03	572.80 ± 40.13	0.05
		Fat yield		623.15 ± 26.97	789.49 ± 44.40	0.0006
rs13675432[b]	*DGAT1*		A/G			
		Protein yield		486.52 ± 24.03	572.80 ± 40.13	0.05
		Fat yield		623.15 ± 26.97	789.49 ± 44.40	0.0006

[a]Major allele is shown on the left and the minor allele is shown on the left. [b]All means differ at $P < 0.05$ within a row.

DISCUSSION

The results of the present study support the hypothesis that SNPs located within three candidate genes may be associated with both fertility and milk production traits in the dairy industry. Specifically, two SNPs located in the *DGAT1* gene were found to be associated with both average lifetime services per conception and average lifetime days-open. SNPs associated with fertility traits have also been reported in dairy cattle for both the *CAST* (Liefers et al., 2002) and *LEPR* genes (Almeida et al., 2008). However, these previous studies identified associations with DPR, longevity, and fertility during early lactation. Furthermore, previous research (de Vries and Veerkamp, 2000) has identified associations between SNPs on the *DGAT1* gene with fat/protein ratios during early lactation that had negative effects on dairy cattle fertility. However, to our knowledge, this is the first study that has reported SNPs located on the *DGAT1* gene to have direct effects on fertility traits.

It is surprising that no significant associations were found between SNPs located in the *LEPR* and *CAST* genes and milk production traits, since both genes have been previously reported to affect multiple production traits in both the beef and dairy industry (Casas et al., 2006; Schenkel et al., 2006; Juszczuk-Kubiak et al., 2008; Komisarek, 2010; Clempson et al., 2011). However, previous studies have also reported candidate genes that are significantly associated with diverse traits such as milk production traits and disease resistance (Wang et al., 2015). The results described herein indicate that five SNPs located in the *DGAT1* gene are significantly associated ($P < 0.05$), or show a statistical trend for association ($P < 0.1$), with milk yield, milk fat, and milk protein. These findings are not surprising, since they are in agreement with those of previous studies that have associated the *DGAT1* gene with milk yield, milk protein, and fat yield (Winter et al., 2002; Schenkel et al., 2006).

The current study shows that a single SNP could be associated with multiple complex traits such as fertility and milk production. However, prior to implementing these SNPs into a marker assisted selection strategy, further experimentation is required. SNPs identified in the current study must be evaluated in larger populations and in diverse production environments. Furthermore, a greater number of SNPs and candidate genes must be evaluated in order to identify significant marker associations and SNPs that account for the largest degree of variability for the

trait of interest. Multiple trait interactions must also be evaluated so that detrimental effects on other economically important traits can be avoided. The identification of numerous SNPs associated with fertility and milk production traits could potentially increase the accuracy of selection for dairy producers trying to incorporate increased performance, profit, and sustainability into their herds.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

The current study was approved and funded by the Louisiana Agricultural Experiment Station through the use of State and Federal Hatch funds.

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