Original Article

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ASSOCIATION BETWEEN SNP MARKERS AND MILK PRODUCTION TRAIT OF SWAMP BUFFALO

Kalaya Boonyanuwat[1,*], Attapong Kongraksa[2] and Changyeon Cho[3]

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ABSTRACT

The research of association between SNP markers and milk production trait of swamp buffalo was conducted to identify polymorphisms of SNP markers associated with milk/day trait of swamp buffalo. Milk/day data and hair bud samples were collected from 28 second parity milking swamp buffalo at 2 to 3 months at farmer farm in Prachinburi province for DNA extraction. DNA samples were used for 40 SNP genotyping by Mass Array method. The results showed 10 polymorphism SNP and associated with milk/day trait of swamp buffalo ($P<0.01$). They were 3 groups: 1) associated hormones (2 Leptin), 2) energy balance (2 GHRL, 1 ARHGAP39 and 3 MC4R) and 3) immune system (1 BOLADQA1, 1 TFAP2D). The 2 SNPs of Leptin genes (from exon 2 and 3, AY495587.1), There were 2 genotypes of Leptin_2 SNP at G1072A mutation point (GG and GA). The average milk/day of GG and GA were 4.03+0.05 and 5.89±0.26 kg respectively ($P<0.01$). The mutation point of Leptin_5 was G3441A with 3 genotypes GG, GA and AA. The average milk/day of GG, GA and AA genotypes were 5.01±0.07, 4.83±0.10 and 4.95±0.19 kg respectively ($P<0.01$). The GHRL genes (EF583468.1) had 2 SNPs (GHRL_1 and GHRL_2). The GHRL_1 (A122G) had 3 genotypes. There were AA, AG and GG, with average milk/day 5.82±0.09, 3.94±0.13 and 5.02±0.07 kg respectively ($P<0.01$). There were 3 genotypes of GHRL_2 (T380C), TT, TC and CC. The average milk/day were 5.01±0.08, 4.38±0.13 and 5.45±0.08 kg, respectively ($P<0.01$). The BOLADQA1 (rs110525467, C-T) had 2 genotypes CC and TT. The average milk/day was 4.28±0.06 and 5.60±0.09 kg respectively ($P<0.01$). In consideration with the allele frequency, the Leptin_5 SNP (G3441A) was suitable to develop as MAS for milk/day trait. The SNP of GHRL_2(T380C) and BOLADQA1 (rs110525467) were moderate for MAS. However, 3 SNPs can be used as MAS for selection and breeding and introducing new genetics from outside herd for milk/day trait development of swamp buffalo in this farm.

Keywords: *Bubalus bubalis*, buffaloes, snp marker, milk, swamp buffalo, genotype

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[1]Biodiversity Research and Development Section, Bureau of Animal Husbandry and Genetic Improvement, Department of Livestock Development, Thailand, *E-mail: kalayabo@gmail.com

[2]Prachinburi Livestock Research and Breeding Center, Bureau of Animal Husbandry and Genetic Improvement, Department of Livestock Development, Thailand

[3]Animal Genetic Resources Research Center, National Institute of Animal Science, Rda, Korea

INTRODUCTION

There is very little raising of dairy buffalo in Thailand. There are demonstrations and research dairy buffalo farms in government research centers. Thai people rarely drink buffalo milk. Dairy buffalo raised in Thailand integrated with paddy rice production. They are swamp buffalo. Buffalo milk is the second part of consumption in foreign countries compared with cattle milk (Ahmad *et al*., 2008).

In Thailand, the dairy buffalo raising is still in starting stage. Farmers raise Mourah and Murah crossbred to produce mozzarella cheese for value added. Mozzarella cheese are used for making pizza that are popular in Thailand. This is partly reducing the amount of imports from abroad. They can be a source of income for farmers in the future. Since 20 years, dairy buffalo had gained more and more attention from farmers, researchers, consumers and other agencies. Some farmers brought the swamp buffalo for milking by selecting the female that could produce about 2 kg milk per day. They bring milk to be processed as ice cream, yogurt, pasteurized milk for sale, which can make more money. In addition, they are raised at the Livestock Research and Breeding Center of the Department of Livestock Development and the Royal Project.

The study of association between SNP markers and milk production trait of swamp buffalo such as Leptin, can be developed as a marker assisted selection (MAS) for milk production traits. Leptin is a 16 kDa protein (Gong *et al*., 1996). Leptin also plays a role in regulating the energy balance, immune system, and reproductive function (Block *et al*., 2001; Buchanan *et al*., 2003). Leptin and Leptin receptors induce an energy balance and improved efficiency of production, fertility and immune system (Magni *et al*., 2000). Leptin genes at 2 and 3 exon regions in chromosome 4 of cattle (Stone *et al*., 1996) and chromosome 8 of of buffalo (Vallinoto *et al*., 2004). The TT genotype plays a key role in increasing milk production in Holstein dairy cows (Buchanan *et al*., 2003; Chebel *et al*., 2008; Matteis *et al*., 2012).

The other groups of energy balance function genes, such as GHRL, ARHGAP39, and MC4R involved in energy balance traits. These genes also affect other economic traits. According to a study by Braz *et al*. (2015), the use of molecular genetic markers involved in these traits. This allowed for more accurate animal selection, thereby reducing intergenerational intervals and increasing genetic progress. It was found that 6 SNP are located of the GHRL gene, which were in intron 3, intron 4, and exon 5 regions. The positions were g.2184A>G, g.2347T>C, g.4469T>C, g.4548A>G, g.4663T>C and g.4729T>C (GenBank accession No. JX565585). These SNP effected on the body weight at the age of 1 year of the male and female cattle, feed intake, loin eye area and fat thickness significantly ($P<0.05$). Therefore, GHRL was an important gene that could be used for genetic selection. Gil *et al*. (2013) studied 240 riverine buffalo, using 11 SNPs, 8 SNPs were found on GHRL genes such as g.960G>A and g.778C>T. They were correlated with fat yield. The g.905T>C SNP was related to fat percentage. The Rho GTPase activating protein 39 (ARHGAP39) gene was involved in the development of the central nervous system (Ma and Nowak, 2011), related to milk production and milk fat composition (Buitenhuis *et al*., 2014; Nayeri and Stothard, 2016). Melanocortin-4 receptor gene (MC4R) was associated with appetite regulation and energy balance (Barb *et al*., 2004; Seeley *et. al*., 2004; Razquin *et al*., 2011). In humans, there were more than 150 MC4R gene mutations, some of which were associated with obesity (Huang *et al*., 2010). The mutations in MC4R were associated with feed intake, growth, obesity and carcass traits in pigs (Van den Maagdenberg *et al*., 2007; Piorkowska *et al*., 2010). Similarly, SNP polymorphisms were identified with carcass weight, back fat thickness in cattle (Liu *et al*., 2010), sheep (Song *et al*., 2012) and chickens (Yan *et al*., 2013).

The BoLA gene in cattle was extensively studied as a marker for disease resistance traits and immune function (Takeshima *et al*., 2007).

However, there were a small number of studies on the genotype polymorphism of these genes and associated to milk production traits. Especially in Thailand, it is in the beginning phase to develop and promote the raising of dairy buffalo, which includes both riverine and swamp buffalo. Therefore, research studies of genetic selection and breeding are very important. To make progress in breeding together in production, fertility and feed efficiency. Therefore, a study of the association between SNP markers and milk yield in swamp buffalo were conducted.

MATERIALS AND METHODS

Animal

The 28 milking swamp buffalo that were in the period of the 2nd to 3rd month of the lactation in the farmer's farm in Prachinburi Province. These buffaloes were milked once a day (morning). All buffaloes were fed according to the nutrient requirements based on the NRC guidelines (2001) with 18% protein concentrate and the main roughage consisted of corn silage, fresh grass and rice straw.

Data

1. Milk/day at the 2 to 3 month of lactation period by weighing the milk every day.

2. 10 SNP (from 40 SNP) from 3 groups of genes:

2.1 Related hormones (2 SNPs of Leptin Genes)

2.2 Energy balance (2 SNPs of GHRL, 1 SNP of ARHGAP39, 3 SNPs of MC4R)

2.3 Immune system (1 SNP of BOLADQA1, 1 SNP of TFAP2D)

Supporting data

1. Feed intake per day

2. Lactation no. (All of them were the 2nd lactation in this study)

3. Month and year of recording

4. Temperature and humidity

The 28 hair follicles were collected from each buffalo for DNA extraction. They were kept in each bag, labeled, and stored at 4°C. The DNA was extracted at the Molecular Genetics Laboratory, Swine Research and Development Center, Pakchong district, Nakhonratchasima province.

DNA was extracted using silica gel method as described by Boom *et al*. (1990). DNA samples were genotyped by Polymerase Chain Reaction (PCR) using MassARRAY technique.

Data analysis

1. Allele and genotype frequency were analyzed by Popgen32 version 1.32

2. The association between genotype and milk/day trait, since the data were collected from the 2 to 3 months of the 2 lactation milking, there was only one independent variable left (genotype).

$$Yij = u + Gi + eij$$

Yij = Milk/day at j and genotype i

u = Over all mean
Gi = Genotype i (i=1,2,3)
eij = Random error

RESULTS AND DISCUSSIONS

Genotype and allele frequency

The genotypes of the swamp buffalo used in this study consisted of 3 groups of genes (10 SNPs from 40 SNPs): 1) related hormones, 2) energy balance, and 3) immune system. Related hormone group, Leptin gene (AY495587.1) was found to be significantly associated with the milk/day trait of the swamp buffalo (P<0.01). The SNP markers of the Leptin genes were polymorphism of Leptin_2 (G1072A) and Leptin_5 (G3441A) (Table 1), which was located on the exon2 of Leptin gene. Corresponded to Buchanan *et al.* (2002); Lagonigro *et al.* (2003), the MC4R or Melanocortin-4 receptor gene correlated with feed efficacy, growth and carcass quality traits in many species. In this study, MC4R (KJ139982.1) had SNP associated with milk/day trait of swamp buffalo. There were MC4R_2 (C226T), MC4R_3 (A345G) and MC4R_4 (G429A). Major histocompatibility complex (BoLA) class II DQA1 gene was found 1 SNP, BOLADQA1 (rs110525467).

Leptin gene (AY495587.1), G1072A SNP was showed a greater frequency of G alleles than A, 98.00 and 2.00%, respectively (P<0.01) according to Orru *et al.* (2007) studied the Leptin gene (AY495587.1) in riverine buffaloes (Italian and Egyptian rivers) found G1072A SNP with a greater frequency of G than A allele, 84.30 and 15.60%, respectively. But G3441A SNP had a greater frequency of G than A allele, 78.00 and 22.00% respectively (P<0.01), different from Orru *et al.* (2007) where only the G allele was found, it was possible that A allele was found only in swamp buffalo.

The MC4R gene (KJ139982.1), the C226T SNP showed a greater frequency of C than T allele, 96.00 and 4.00%, respectively (P<0.01). According to Deng *et al.* (2015) studied the MC4R gene (KJ139982.1) in buffalo. The C226T SNP had a greater frequency of all C than T allele, 93.00 and 7.00%, respectively. The A345G SNP had a less frequency of A than G allele, 2.00 and 98.00%, respectively (P<0.01). This was different from the Deng *et al.* (2015), found that the A was higher than G allele, 54 and 46%, respectively. Because these milking buffaloes were swamp buffalo.

For the BOLA-DQA1 or MHC classII gene that is associated to disease resistance traits. Based on the mutation point of 25,426,985 bp of chromosome 23 in dairy cows (Carignano *et al.*, 2018) in swamp buffalo, the mutation was found from C to T with a greater frequency of C than T alleles, 64 and 36%, respectively (P<0.01).

Association between SNP and milk/day trait

Related hormone genes

The swamp buffalo from this study had an average milk/day 4.95 kg. The Leptin_2 and Leptin_5 SNP were significantly associated with the milk/day trait. Leptin_2 genotypes were GG and GA with average milk/day 4.03 and 5.89 kg respectively (P<0.01). Leptin_5 genotypes were GG, GA and AA with average milk/day 5.01, 4.83 and 4.95 kg respectively (P<0.01) (Table 2). Due to leptin hormone secreted by adipose tissue, it played an important role in Body weight, diet, immune function and reproduction (Santos-Alvarez *et al.*, 1999; Kadokawa *et al.*, 2000; Block *et al.*, 2001). Based on a study in dairy cows, the exon2 SNP position of Leptin gene, C to T conversion was associated with fat deposition in cattle (Buchanan *et al*., 2002). It influenced the function of Leptin gene that regulated the secretion of leptin in adipose tissue and affected milk production.

Considered with allele and genotypic frequencies, Leptin_5 (G3441A) was an interesting SNP for developing as genetic marker for selection of milk/day trait of swamp buffalo. Since the G allele had a frequency of 78% and the GG genotype had the highest (5.01 kg / day). This would make progress in the breeding system.

Energy balance genes

The MC4R gene (KJ139982.1) or Melanocortin-4 Receptor is primarily responsible for the regulation of energy balance. It was associated to feed intake trait. It was important to many economic animals such as beef cattle, dairy cattle, pigs and chickens. This study found SNP polymorphic and correlated with milk yield of swamp buffalo at 3 positions: MC4R_2 (C226T), MC4R_3. (A345G) and MC4R_4 (G429A). The MC4R_2 SNP had two genotypes: CC and TT, with average milk/day of 4.58 and 5.30 kg, respectively (P<0.01). It was consistent with the Deng *et al.* (2016) studied in buffalo. The riverine buffalo showed that SNP of the MC4R gene (C226T) had 3 genotypes, with TT genotypes having the highest 305d milk followed by CC and TC, average 305d milk yield of 1616.84, 1514.81 and 1458.67 kg per lactation. The MC4R_3 SNP had 2 genotypes, AG and GG, had average milk/day of 5.43 and 4.52 kg respectively (P<0.01) and similar to MC4R_4 SNP.

Considered with allele and genotype frequency, the SNP markers of the MC4R gene were not suitable for the determination of the milk/day trait of swamp buffalo. All 3 SNPs had high alleles and genotypes frequency for low milk/day trait. It will be difficult for genetic selection.

The GHRL gene (EF583468.1) controls the secretion of ghrelin, a peptide hormone made from the stomach. This study showed polymorphic SNP and associated milk/day trait of swamp buffalo. There were 2 SNPs, GHRL_1 (A122G) and GHRL_2 (T380C) SNP. The GHRL_1 position found 3 genotypes, AA, AG and GG. The average milk/day was 5.82, 3.94 and 5.02 kg respectively (P<0.01). The GHRL_2 SNP showed 3 genotypes TT, TC and CC. The average milk/day was 5.01, 4.38 and 5.45 kg respectively (P<0.01). This was different from Gil *et al*. (2013) studied in riverine buffaloes of the same SNP group as this study. It was found that each genotype was not significantly differences. However, according to a study conducted by Prajapati *et al*. (2018) in Murah buffaloes, it was clear that ghrelin supported feed intake, which increased blood sugar levels. But it had a negative effect on fertility.

Considering the alleles and genotypes frequency, the GHRL_2 (T380C) SNP was suitable for MAS development. Because high alleles and genotypes frequencies had high average milk/day trait (5.45 kg per day)

The ARHGAP39 gene produced the Rho GTPase Activating Protein 39, which was in the RHO-related protein group, was important in various cellular processes, movement, contraction, growth (Uniprot, 2021). From this study, there was 1 SNP, rs134892687, with 2 genotypes, CC and TT. The average milk/day was 5.91 and 3.93 kg respectively (P<0.01). Allele frequency and SNP genotypes, rs134892687, were not suitable to develop as a genetic marker for milk/day trait.

Immune system genes

BOLADQA1 or MHC Class 2 genes, from this study showed one SNP marker, rs110525467,

Table 1. Genotype and allele frequencies (%) in the 6 gene loci.

Gene (Accession no)	Mutation	SNP	Allele frequency (%)		Genotype frequency (%)		
1) associated hormones**							
Leptin (AY495587.1)	G1072A	Leptin_2	G 98.00	A 2.00	GG 96.00	GA 4.00	AA -
Leptin (AY495587.1)	G3441A	Leptin_5	G 78.00	A 22.00	GG 64.00	GA 28.00	AA 8.00
2) energy balance**							
GHRL(EF583468.1)	A122G	GHRL_1	A 40.00	G 60.00	AA 32.00	AG 6.00	GG 52.00
GHRL(EF583468.1)	T380C	GHRL_2	T 48.00	C 52.00	TT 40.00	TC 16.00	CC 44.00
ARHGAP39 (BTA27)	C-T	ARHGAP39 (rs134892687)	C 4.00	T 96.00	CC 4.00	CT -	TT 96.00
MC4R (KJ139982.1)	C226T	MC4R_2	C 96.00	T 4.00	CC 96.00	CT -	TT 4.00
MC4R (KJ139982.1)	A345G	MC4R_3	A 2.00	G 98.00	AA -	AG 4.00	GG 96.00
MC4R (KJ139982.1)	G429A	MC4R_4	G 2.00	A 98.00	GG -	GA 4.00	AA 96.00
3) immune system**							
BOLADQA1(BTA23)	C-T 25,426,985	BOLADQA1 (rs110525467)	C 64.00	T 36.00	CC 64.00	CT -	TT 36.00
TFAP2D (BTA23)	G-C 22,997,898	TFAP2D (rs41566363)	G 96.00	C 4.00	GG 96.00	GC -	CC 4.00

**Highly significantly associated with milk/day trait ($P<0.01$).

Table 2. Associations of SNP genotype and milk yield.

Gene (Accession no)	**SNP**	**Genotype MY* (kg/day)**		
1) associated hormones				
Leptin (AY495587.1)	Leptin_2**	GG 4.03±0.05	GA 5.89±0.26	AA -
Leptin (AY495587.1)	Leptin_5**	GG 5.01±0.07	GA 4.83±0.10	AA 4.95±0.19
2) energy balance				
GHRL(EF583468.1)	GHRL_1**	AA 5.82±0.09	AG 3.94±0.13	GG 5.02±0.07
GHRL(EF583468.1)	GHRL_2**	TT 5.01±0.08	TC 4.38±0.13	CC 5.45±0.08
ARHGAP39 (BTA27)	ARHGAP39** (rs134892687)	CC 5.91±0.26	CT -	TT 3.93±0.05
MC4R (KJ139982.1)	MC4R_2**	CC 4.58±0.06	CT -	TT 5.30±0.27
MC4R (KJ139982.1)	MC4R_3**	AA -	AG 5.43±0.27	GG 4.52±0.06
MC4R (KJ139982.1)	MC4R_4**	GG -	GA 5.70±0.27	AA 4.15±0.05
3) immune system				
BOLADQA1(BTA23)	BOLADQA1** (rs110525467)	CC 4.28±0.06	CT -	TT 5.60±0.09
TFAP2D (BTA23)	TFAP2D** (rs41566363)	GG 3.93±0.05	GC -	CC 5.91±0.26

*MY = milk yield (kg/day) ** highly significantly associated with milk/day trait ($P<0.01$).

with 2 genotypes, CC and TT, with average milk/day of 4.28 and 5.60 kg respectively (P<0.01). It corresponded to Al-Waith *et al.* (2018), which was studied in Holstein Friesian cattle. It was found that total milk yield was significantly associated with BOLADQA1 (P<0.01). Genotype C had the highest average milk yield (1699.65±65.78 kg), followed by genotype A (1586.19±63.83 kg) and genotype B had the lowest average milk yield, 1506.62±82.05 kg. The BOLADQA1 associated with disease resistance traits. Therefore, alleles with good immune function made animals healthy and provide high milk yield as well.

Considering allele and genotype frequency, the rs110525467 SNP was suitable to develop as a genetic marker for milk/day of swamp buffalo.

The TFAP2D (Transcription factor AP-2-delta) gene was found 1 SNP, rs41566363, with two genotypes, GG and CC, with average milk/day of 3.93 and 5.91 kg respectively (P<0.01). It consisted of Goszczynsk *et al.* (2018) studied in Brangus cattle; three genes were found in R5 of BOLADQA1 genes. They were TFAP2D, TFAP2B and PKHD1. The TFAP2D, TFAP2B genes were factors for transcription to form proteins during development. However, the association with the immune system was not clear. It also played a role in immunity due to regulating expression of T-cells (Hogan *et al*., 2004). It was the same reason as the BOLADQA1 gene.

Considered with allele and genotype frequency, the rs41566363 SNP was not suitable for development as a genetic marker for milk/day trait of swamp buffalo.

CONCLUSION

The relationship between SNP markers and milk/day trait of swamp buffalo was carried out by MassArray genotyping, 40 positions determined by 3 groups of genes, related hormones, energy balance and immune system. They detected 38 SNP, there were 10 polymorphic SNP with milk/day trait. The Leptin_5 (G3441A) was the most suitable to develop as MAS. The GHRL_2 (T380C) and BOLADQA1 (rs110525467) SNP were moderate. However, 3 SNP positions could be used as a genetic marker for selection and breeding. In other ways, the outside genetics can be introduced for the development of the milk/day trait of the swamp buffalo of this farm.

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