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Application of AFLP Technique to the Study of Calving Interval Trait of Thai Swamp Buffalo (*Bubalus bubalis*)

Benchamart Moolmuang[a], Supajit Sraphet[a], Ancharlie Na-Chiangmai[b], Duncan R. Smith[a], Sakol Panyim[a,c] and Kanokporn Triwitayakorn[a*]

[a] Institute of Molecular Biology and Genetics, Mahidol University, Salaya, Nakhon Pathom 73170, Thailand.
[b] Department of Livestock Development, Phayathai Rd., Bangkok, 10400, Thailand.
[c] Department of Biochemistry, Faculty of Science, Mahidol University, Phayathai, Bangkok, 10400, Thailand.

* Corresponding author, E-mail: mbktw@mahidol.ac.th

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ABSTRACT: This study aimed to develop genetic markers specific for the calving interval (CI) trait of the domestic Thai buffalo (*Bubalus bubalis)* using the technique of Amplified Fragment Length Polymorphism (AFLP). Using two groups of buffalo with extreme CI phenotypes, several AFLP markers were identified. The DNA sequences of all identified polymorphic AFLP fragments were determined by DNA sequencing and analyzed with BLAST searches. Interestingly, the analysis showed that the sequence of the AFE7M24-2 marker has a high similarity with the prostaglandin f2α receptor gene, while the sequences of the AFE16M25-2 and AFE4M28-3 markers have high similarity with the follicle stimulating hormone receptor gene of sheep (*Ovis aries*).

KEYWORDS: AFLP, SCAR, Thai swamp buffalo, calving interval trait.

INTRODUCTION

The domestic Thai swamp buffalo (*Bubalus bubalis*) is a large ruminant that is traditionally kept as a draught animal working in rice fields and crop plantations. A recent survey has suggested that there are about 170 million swamp buffalo found globally[1]. However, while the overall number of buffalo world-wide is increasing at 1.3% annually, the number of animals in Thailand is decreasing dramatically. From 1990 to 2003, the number of buffalo in Thailand decreased by more than 50%, from 5 million to 1.8 million or an average decline of nearly 22% per year[2]. Therefore, the selection and improvement of phenotypic traits, such as the fertility performance of offspring production, as well as research and development programs may help farming systems to increase the productivity of the buffalo.

Fertility in animals is a complex trait that comprises several components and it is greatly affected by differences or changes in the environment, especially the nutritional status[3,4]. Studies that can improve reproduction of buffalo have been affected by several physiological factors including inherent problems such as the low number of primordial follicles in the buffalo ovary, poor fertility and seasonality of reproduction[5]. The fertility trait can be measured by calving interval (CI), calving rate, service per conception, age of the first calving and days to calving[6]. Female buffaloes that have either short or long calving intervals were selected in this study for the identification of genetic markers and genes involved in CI.

While the use of simple sequence repeat (SSR) or microsatellite markers in genetic analysis has several advantages over the other types of markers, including co-dominant inheritance (which allows discrimination between homo- and heterozygotes) and their ease of use, the significant disadvantage of microsatellites is that specific primers of known sequence are required. This means that microsatellites must be isolated and characterized for each individual species to be examined. These limitations have resulted in the development of alternative technologies which can be applied in the absence of specific sequence information. Given the lack of specific microsatellite markers for the swamp buffalo, an alternative approach was sought.

Recently, a combination of bulked segregant analysis and the Amplified Fragment Length Polymorphism (AFLP) technique has been proposed for the identification of trait specific markers in livestock species[7]. AFLP is a technique of DNA fingerprinting that involves PCR amplification of a subset of restriction fragments that are generated by the digestion of genomic DNA[8]. The advantages of AFLP over other marker systems are its suitability to simultaneously screen the whole genome and to provide a large number of markers that can be converted to a simple co-dominant locus specific marker, such as SCAR (Sequence Characterized Amplified Region) markers, that can be easily manipulated for rapid selection with one step PCR[9,10,11]. AFLP has been applied to several domesticated species including cattle[12,13], pigs[7,14] and chicken[15].

The development of markers related to calving interval, which reflects the fertility trait, will be useful for the identification of buffaloes with high fertility. This can be applied to the breeding program of swamp buffalo in

Thailand. However, there are a few reports that have described the genetics of the Thai swamp buffalo, including the studies of genetic diversity of the Asian water buffalo[16] and the Thai swamp buffalo[17], which analyzed the genetic diversity of this animal in Thailand. Moreover, the sequence of the polymorphic fragments can be used to identify the genes involved in the trait. The objective of this study was to develop AFLP markers for the identification of high and low fertility levels in buffalo using pooled DNA of the selected animals.

Materials and Methods

Experimental Animals

Two sets of 12 Thai swamp buffalo (n = 24) were used in this study. Samples were kindly provided by the Department of Livestock Development, Ministry of Agriculture and Cooperatives, Thailand. Animals in each set were classified into two groups, short and long CI, according to their records. Buffalo with CI of less than 500 days were classified into the short CI groups, whereas those with CI of greater than 500 days were classified into the long CI groups. The short and long CI groups of the first set (S1-pool and L1-pool), comprised seven buffalo each with mean CI of 392 and 812 days, while the short and long CI groups of the second set, (S2-pool and L2-pool), comprised five buffalo with mean CI of 401 and 701 days, respectively.

DNA Extraction and Preparation of High and Low Fertility Pools

Genomic DNA was extracted from blood samples with the QIAamp DNA blood kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. A total of 1μg of genomic DNA from individual animals of both short and long CI groups of the first and the second sets were pooled in order to obtain two sets of short CI-pooled (S1 and S2) and long CI-pooled (L1 and L2) DNA samples.

AFLP Analysis

AFLP analysis was performed as described by Vos et al[8]. A total of 500 ng of pooled genomic DNA was double digested with 10 U *Mse*I (New England BioLabs, Beverly, MA), followed by 12 U *Eco*RI (Promega, Madison, WI) in a final volume of 30 μl for 3 hr at 37 °C. Digested DNA samples were subsequently ligated to *Eco*RI and *Mse*I adapters. The ligation reactions were performed with 0.5 μM of *Eco*RI adapter, 5 μM of *Mse*I adapter, 1X ligation buffer, and 1 U T4 DNA ligase enzyme (USB Corporation, Cleveland, OH). The digested-ligated DNA samples were pre-amplified with a set of primers complementary to the *Eco*RI and *Mse*I adapters with one additional selective 3' nucleotide. The primer combination used in this pre-amplification was *Eco*RI+A and *Mse*I+C. The pre-amplification reaction was undertaken with 10 pM *Eco*RI+A primer, 10 pM *Mse*I+C primer, 200 μM dNTP (Promega), 1X PCR Buffer, 1.5 mM $MgCl_2$, and 1.5 U *Taq* polymerase (Promega). The pre-amplification PCR conditions consisted of 30 cycles of 94 °C for 45 sec, 56 °C for 45 sec, 72 °C for 1 min, and 1 cycle of 72 °C for 5 min. The pre-amplified products were diluted 50 fold in water and 1 μl was used for selective amplification with the *Eco*RI and *Mse*I primers containing an additional three selective nucleotides at the 3' end (*Eco*RI+ANN and *Mse*I+CNN). The PCR reaction was performed with 10 pM *Eco*RI+ANN, 10 pM *Mse*I+CNN, 200 μM dNTP (Promega), 1X PCR Buffer, 1.5 mM $MgCl_2$, and 1.5 U of *Taq* polymerase (Promega). The PCR profiles were as follows: 11 cycles of 94 °C for 30 sec, 65 °C (-0.7 °C/cycle) for 30 sec, 72 °C for 60 sec, and 25 cycles of 94 °C for 10 sec, 56 °C for 30 sec, 72 °C for 60 sec. The PCR products were mixed with 15 μl of denaturing solution (99% formamide, 0.05% xylene cyanol, and 0.04% bromophenol blue), denatured at 94°C for 5 min and kept on ice for 10 min before being analyzed by electrophoresis through 5% polyacrylamide sequencing gels run at constant voltage (1500 V) for 2 hr. DNA bands were visualized by the silver staining method described by Sambrook and Russel (2001). The AFLP fingerprints were then scored for polymorphisms which showed as the presence or absence of AFLP fragments in the S- or L-pools, but not both. The adapters and the primers used in the pre-amplification and final-amplification are shown in Table 1.

Table 1. Sequences of adapters and primers used in AFLP analysis.

Adapters and primers	Sequences
*Eco*RI adapters	5'-CTCGTAGACTGCGTACC-3' 3'-CTGACGCATGGTTAA-5'
*Mse*I adapters	5'-GACGATGAGTCCTGAG-3' 3'-TACTCAGGACTCAT -5'
*Eco*RI pre-amplification primer	5'-GACTGCGTACCAATTC**A**-3'
*Eco*RI final-amplification primer	5'-GACTGCGTACCAATTC**ANN**-3'
*Mse*I pre-amplification primer	5'-GATGAGTCCTGAGTAA**C**-3'
*Mse*I final-amplification primer	5'-GATGAGTCCTGAGTAA**CNN**-3'

N represents the selective nucleotides A, C, G, or T

Cloning of AFLP Fragments

The identified polymorphic AFLP fragments were cloned according to Meksem et al.[10]. The polymorphic fragments were excised from dried polyacrylamide gels, rinsed twice for 30 min with 50 μl distilled water at room temperature and incubated in 20 μl of distilled water at 37 °C overnight. The supernatants were transferred to new tubes and used as template for PCR reamplification using the same primer combination and PCR condition previously described for the AFLP reaction. The reaction was performed using 50 ng of each primer, 0.3 U of *Taq*

polymerase (Promega), 200 µM dNTP (Promega), 1X PCR buffer, and 1.5 mM $MgCl_2$. Cycle conditions were: 1 cycle at 94 ºC for 2 min, followed by 30 cycles of 94 ºC for 45 sec, 56 ºC for 45 sec, and 72 ºC for 1 min, and 1 cycle of 72ºC for 5 min. The reamplified products were analyzed by electrophoresis through 1.2% agarose gels, and the expected bands excised and purified from the gel as described by Sambrook and Russel[18]. The purified DNA fragments were cloned into the pGEM-T easy vector (Promega), as described in the manufacturer's protocol. Recombinant plasmids were used to transform *E. coli* strain DH5α using the heat shock method, as described by Sambrook and Russell (2001), and plated on LB plates containing 100 mg amplicilin, 20 µl X-Gal (20 mg/ml) and 4 µl IPTG (200 mg/ml). Plasmid DNA of 3-5 white colonies from each transformation were isolated using the alkaline lysis method[19] and sequenced.

DNA Sequencing and Analysis

Plasmid DNA of positive clones were sequenced in both directions with the DYEnamic ET Dye Terminator Cycle Sequencing Kit and analyzed on a MegaBACE 500 sequencer (Amersham Pharmacia Biotech Inc., Sunnyvale, CA). Sequencing of the 5'-end was performed following the manufacturer's protocol with the M13 forward primer. The AFLP sequences were subjected to BLASTN and BLASTX searches against the GenBank database.

Results and Discussion

The genomic DNA of short (S1 and S2) and long CI-pools (L1 and L2) were tested with 256 *Eco*RI and *Mse*I primer combinations of E-ANN and M-CNN. The AFLP analysis indicated that a total of 19 AFLP fragments are polymorphic. These polymorphisms were seen as the presence or absence of AFLP fragments when the S- and L-pools were compared in both sets of DNA samples. From the 19 AFLP fragments, a total of 6 markers were identified from the short CI-pools, while 13 markers were identified from the long CI-pools. These results showed the effectiveness of using AFLP coupled with bulked segregant analysis to screen for markers specific for a CI trait. The AFLP fingerprints showing the polymorphic profile are shown in Fig 1.

The 19 polymorphic AFLP bands identified from the two sets of pooled DNA samples were excised and purified from acrylamide gels. Seventeen were successfully reamplified and showed a single PCR product of the expected size when analyzed by electrophoresis through 1.2% agarose gels. These fragments were cloned and sequenced. The DNA sequences from 10 of the individual AFLP markers contained 2-3 different sequences, suggesting that these AFLP bands were composed of a number of different fragments, which have nearly identical fragment sizes but differ in sequences[10]. The AFLP sequences were analyzed by BLAST analysis.

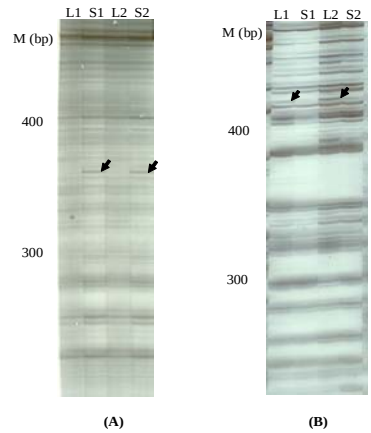

Fig 1. AFLP reactions derived from the first set of short calving interval pool (S1) and long calving interval pool (L1) and the second set of short calving interval pool (S2) and long calving interval pool (L2) analyzed by electrophoresis through 5% denaturing polyacrylamide gels. Panel A: The DNA fingerprint was generated with the E-ACG/M-CCT primer combination. Arrows indicate the polymorphic AFLP bands derived from the short calving interval pool. Panel B: The DNA fingerprint was generated with the E-ATC/M-CTC primer combination. Arrows indicate the polymorphic AFLP bands identified from the long calving interval pool.

Interestingly, the results of AFLP sequence analysis by BLAST showed that some of the sequences had similarity with known genes of other bovine species, such as *Bos taurus* (Table 2). The AFLP sequences of the AFE7M24-2 marker showed similarity with the prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) receptor gene. Prostaglandin $F_{2\alpha}$ has been shown previously to enhance fertility in cattle[20,21]. Prostaglandin $F_{2\alpha}$ may also exert a fertility effect by stimulating the release of luteinizing hormone (LH) upon decrease of the corpus luteum and reduced progesterone concentrations[20]. In addition, it has been reported that goats which were treated with prostaglandin, equine chorionic gonadotropin (eCG), and human chorionic gonadotropin (hCG) showed a significant increase in the number of follicles compared to those that received no treatment[22]. The sequences of the AFE16M25-2 and AFE4M28-3 markers both showed homology with the follicle stimulating hormone (FSH) receptor gene of sheep (*Ovis aries*). FSH is one of major hormones involved in follicle development[23,24] and directly affects fertility.

In this work, the AFLP technique proved to be an effective method for the development of trait specific genetic markers. The polymorphic markers found in this project as well as newly identified markers in the future will be useful for the study of Quatitative Trait Loci (QTL). However, to identify the QTL underlying the trait of interest, more markers and appropriate samples such as full-sib or half-sib progenies are required. Afterward, genetic linkage maps and phenotype will be integrated in order to identify the QTL underlying the

Table 2. AFLP sequence analysis against GenBank database.

AFLPsequences	Putative genes	GenBank accession no.	Species	Probability	Score
AFE6M18-1	- alpha-lactalbumin-like gene	AF327256	*Tragelaphus eurycerus*	254	1e-64
	- regakine-1 protein gene	BTA313203	*Bos taurus*	186	2e-44
	- interleukin 1, alpha (IL1A)	NM_174092	*Bos taurus*	125	6e-26
AFE7M24-2	- alpha-S1 casein gene and alpha-S1 casein-beta casein	AY154895	*Bos taurus*	180	1e-42
	- prostaglandin $F_{2\alpha}$ receptor gene	AY030405	*Ovis aries*	145	6e-32
AFE7M27-1	- kappa casein (CSN3) gene	AY380229	*Bos taurus*	113	1e-22
AFE12M29-1	- X-inactivation center region, Jpx and Xist genes.	AJ421481	*Bos taurus*	111	4e-22
AFE14M28-1	- interleukin 1, alpha (IL1A)	NM_174092	*Bos taurus*	406	1e-110
AFE16M25-2	- follicle stimulating hormone receptor gene	AF090438	*Ovis aries*	163	2e-37
	- lactoferrin gene	Z93399	*Bos taurus*	151	7e-34
	- steroid 21-hydroxylase gene,	AF163768	*Bubalus bubalis*	135	4e-29
AFE4M28-3	- sex-specific gonadal PISRT1 mRNA,	AF404302	*Capra hircus*	299	2e-78
	- follicle stimulating hormone receptor gene	AF090438	*Ovis aries*	315	3e-83

desired trait. The identified sequences can be further developed in the future to generate gene specific markers for the CI fertility trait in the Thai swamp buffalo. Moreover, the results of AFLP sequence analysis showed that the technique is not only useful as the initial step for the development of gene specific genetic markers, but also for screening of genes involved in the trait of interest.

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