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Genetic relationship of maternal lineages in Phetchaburi native cattle

Laosutthipong, C.* **and Chuawongboon, P.**

Faculty of Animal Sciences and Agricultural Technology, Silpakorn University, Phetchaburi IT campus, Sampraya, Chaam, Phetchaburi 76120, Thailand.

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Abstract Maternally inheritance can be described via mitochondrial DNA (mtDNA) analysis for evolution and genetic diversity study in animals and human, especially in cattle. The genetic relationship and cluster analysis of Phetchaburi native cattle in Thailand was investigated. All 7 semen samples collected from male native cattle breeders in sperm bank collection from Phetchaburi province. The mtDNA at D-loop region (467 bp.) was analyzed and the high variable region (288 bp.) was aligned with reference cattle sequences. The results revealed 26 polymorphic sites. The sequence identity of all samples ranged from 96.8 to 99.7% which most of them similar to *Bos taurus* (AY515627), except MT-1(MH746114) showed 99% identity to *Bos indicus* (HQ234738). All mtDNA sequences were classified into 3 haplotypes from 7 sperm samples and the haplotype diversity 0.524. The phylogenetic tree analysis indicated 6 breeders were grouped into *B. taurus* clade whereas only one breeder was located in *B. indicus* clade. This current results provide the genetic background of native breeder cattle in Phetchaburi province including genetic diversity and breeder grouping which may have implications for breeding management and conservation of Thai native cattle.

Keywords: genetic relationship, maternal lineages, native cattle

Introduction

Nowadays beef cattle are still an important economical animal in Thailand and their production situation have been considered for sufficiency and sustainability production to serve worldwide consumers. However, the efficiency of beef cattle production need to improve in various issues for example the shortage of beef cattle for all markets, lacking of motivation and knowledge of farmers, the strength in management and marketing of beef cooperatives including the ability to compete with overseas markets (Osothongs *et al.*, 2016). Genetic classification of beef cattle in Thailand can be classified into three groups: Thai Native cattle (61%), Brahman and Brahman crossbreds (35%), and *Bos taurus* (Charolais, Angus, and Simmental) crossbreds (4%) (Development, 2015). Thai-native cattle are *Bos indicus* traits which have Zebu

* **Coressponding Author**: Laosutthipong C.; **Email**: laosutthipong_c@silpakorn.edu

styles (fatty hump, large dewlap, and high temperature tolerant. They have divided into four varieties (Kho Khaolumpoon, Kho Isaan, Kho Lan, and Kho Chon which is categorized by phenotypic character, geographical regions, (Boonyanuwat *et al.*, 2005) and recently confirmed by genotypic analysis (Wangkumhang *et al.*, 2015).

From the 2013 annual database of animal farmers in Phetchaburi, most of beef cattle farming in Phetchaburi province are Thai-native cattle (73%). This information reported 10,243 farmers that culture 128,470 cattle which are Thai-native specie 93,962 cattle (Office, 2013). Small farmers in this province feed about 2-3 cattle in their house area during cultivation for many purposes such increasing income, using manure for fertilizer, and using agricultural waste as feed. Kho Lan in Phetchaburi province is used as rice post-harvest item and play role in the activity after farming by arrange the ear of rice in circular field, tied cows in a row (one by one) around the center, and stimulate cows for running during rice seed ground on land, or recently some people known as traditional play Kho Lan (racing cattle) competition. Interestingly, genetic characterization of Thai-native cattle in this region has no clearly information and need more investigation for genetic variation and cluster analysis for further local cattle husbandry management.

Genetic analysis in Thai-native cattle for genetic variation or clustering have been investigated by using various markers such as SNPs (Charoensook *et al*., 2012; Wangkumhang *et al.*, 2015), STR (Jirajaroenrat, 2008a) , and mitochondrial DNA (Jirajaroenrat, 2008b; Kornphan *et al.*, 2012; Sarataphan *et al*., 2017). The mitochondrial DNA (mtDNA) has been utilized for evolution and genetic variation study in human and animals in term of maternal inheritance (Avise *et al.*, 1987; Bradley *et al*., 1996; Nabholz *et al.*, 2008). Mitochondria is not only a power-house of cell (Shoubridge and Wai, 2007) but also genetic marker which has retain inside its DNA. In animal fertilization, sperm send only its DNA into egg and mitochondria in zygote come from egg cell only, so the mtDNA inherited as maternal lineage. The displacement loop (D-loop) region in mtDNA is non-coding part which control the replication and transcription and it has high evolution rate; so called hypervariable region (Brown *et al*., 1979). For this reason, D-loop in mtDNA has been used for evolution study, origin and migration study, and genetic variation in maternal lineage in cattle (Bradley *et al.*, 1996; Nabholz *et al.*, 2008; Troy *et al.*, 2001). The maternal lineage effected on milk yield and fat percentage were reported in the Holstein-Friesian female dairy cattle in German and USA (Boettcher *et al.*, 1996; Schutz *et al*., 1992; Schutz *et al.*, 1994). For beef cattle, the maternal inheritance using mtDNA has been studied in Japanese black cattle (Malau-

Aduli *et al*., 2004) , Brangus-Ibage cattle (Henkes *et al.*, 2005), and Australian cattle (Srirattana *et al.*, 2017).

In Thailand, the analysis of maternal inheritance in Thai-native cattle using mtDNA has also been described the genetic variation and cluster analysis (Jirajaroenrat, 2008b; Kornphan *et al.*, 2012; Sarataphan *et al.*, 2017). Sarataphan *et al*. reported that most of Thai-native cattle located in *Bos indicus* clade and Kho-Lan (racing cattle) was origin of the most Thai-native cattle (haplotype 22) (Sarataphan *et al.*, 2017). However, Kho Lan (racing cattle) that normally farming in Phetchaburi has not yet investigated the ancestry, genetic variation and clustering.

Artificial insemination (AI) of cattle in Thailand was performed since 1953 for the first improvement of dairy cattle farming and milk production (Aiumlamai, 2005). There are several advantages of AI for cattle such as herd genetic management, cost saving for breeding bull maintenance and the prevention of genital disease spreading (Foote, 2001). The successful of AI start from the efficiency of breeding bull semen collection and storage. Frozen in liquid nitrogen with appropriate preserved solution is now available methods for semen storage in semen bank (Baracaldo *et al*., 2007). The viability and motility of thawed sperms were not different from unfrozen semen during keeping in two years (Malik *et al.*, 2015) and the fertility deterioration with age has no effect from this method (Richardson *et al*., 2017).

Thai-native cattle semen banks are established in many locations in Thailand. The Livestock Semen Production Centre, Thailand provides the list of the native bull semen bank for example Bull Frozen Semen Production Station at Kasetsart University (Kamphaeng saen Campus), Doi Inthanon Royal Project's Livestock Semen Production Center, and North Eastern Deep Frozen Production and Research Centre (Paisan, 2018). For Thai-native cattle in Phetchaburi province, the semen collection and AI still less information and need more investigationt. Faculty of Animal Science and Agricultural technology, Silpakorn University has been collected breeding bull semen as sperm bank. Most of semen belong to Thai-native cows around Phetchaburi area for AI research. Anyway, the genetic history of these breeding cows are necessary for further management and AI application. Even through, the mtDNA from sperm was used as the genetic markers for cattle population study in the native Eastern European cattle in 2015 (Ilie *et al.*, 2015). This was the first study in Thai-native cattle that used sperm sample from semen to mtDNA sequence analysis for genetic variation study meanwhile the previous study which used blood and tissue sample for mtDNA study (Jirajaroenrat, 2008b; Kornphan *et al*., 2012; Sarataphan *et al.*, 2017; Sharma *et al*., 2015).

In this study, we analyzed the genetic information and cluster classification of Thai-native breeding cows in Phetchaburi province from semen bank by using mtDNA sequence from sperm. The main purpose was to identify the genetic diversity in maternal lineage of this breeding cattle for further breeding program management and Thai-native cattle conservation planning.

Materials and methods

Semen samples collection

Semen from seven Thai-native breeder bulls was collected in semen bank for artificial insemination at Faculty of Animal Science and Agricultural Technology, Silpakorn University. Cryo-sperm straws were kept in liquid nitrogen after semen collection using electro-ejaculation method. Bulls were treated in the individual farm at Phetchaburi province.

MtDNA Extraction

Semen 500 µl were thawed and moved from cryo-straw into 1.5-ml Eppendorf tube. The mtDNA was extracted using Genomic DNA mini kit (Tissue) (Geneaid Biotech Ltd., Taiwan) according to the manufacturer's protocol. Sperm samples were homogenized by grinding with Micropestle and added GT Buffer. After adding Proteinase K, sample mixtures were incubated at 60 ℃ for 30 min. Next, GBT Buffer and absolute ethanol were applied to lysate, followed by loaded in GD column. Then, column was centrifuged, discarded flow-through, applied with W1 and Wash Buffer, respectively. The extracted DNA was eluted in low salt Elution buffer and stored in -20 ℃ prior to use.

D-loop region PCR amplification and Sequencing

Total DNA template 10 ng/µl in each sample was used in PCR reaction. The D-loop region in the mtDNA was amplified using forward primer: mtBTD_F (5'-CCAATAACTCAACACAGAATTTGC -3') and reverse primer: mtBTD_R (5'-TAAGAGGAAAGAATGGACCGTTT -3') (Sarataphan *et al.*, 2017). The PCR reaction mixtures consist of 1x Ultra-pure Taq PCR master mix (1 U of Ultra-pure *Taq* polymerase, 2 mM $MgCl_2$ and 200 µM of each dNTPs) (Geneaid Biotech Ltd., Taiwan), 0.8 µM of each primer, and 2 µl of DNA template. The PCR cycle conditions were performed in the thermocycler

(Biometra® T-gradient Thermoblock Thermal Cycler, Germany) with the initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 1 min. After final extension at 72 ℃ for 7 min, the PCR products were cooled down to 20 °C.

The 467 bp. PCR products were determined on 1.5% agarose gel electrophoresis. The single DNA band was excised under UV-light and purified using the GenepHlow™ Gel/PCR Kit (Geneaid Biotech Ltd., Taiwan). Then, the purified PCR products were sent to DNA sequencing service which performed in ABI Prism 3730XL DNA sequencer (Biobasic Inc., Canada).

Data Analysis

All DNA sequences (467 bp of D-loop region in mtDNA) were edited using MEGA 7.0 software (Kumar *et al*., 2016) and aligned with sequences in GenBank database at website: http://www.ncbi.nlm.nih.gov/ using BLASTn tool for the percentage similarity determination.

All 7 full mtDNA sequences were submitted to GenBank database by using online BankIt program. Multiple sequence alignments were then analyzed by ClustalW and the pairwise sequence identity and nucleotide substitutions transition and transversion) was determined with published sequences of cattle from GenBank database running in BioEdit program (Hall, 1999). Reference cattle nucleotide sequences are Kho Khaolumpoon cattle (HM173342 and HM173351), Zebu cattle: Red Chittagong (DQ985396), Tharparkar (L27736), and Hariana (L27722), European cattle (Taurine): Angus (L27712), Friesian (L27718) Charolais (L27716), Africa cattle: White Fulani (L27720), N'Dama (L27730), Kenana (L27728), and Butana (L27714) and Thai-native cattle (Lampang breed) D-loop partial sequences (EU280245, EU280246, EU280247).

The edited 288 nucleotide of D-loop mtDNA was analyzed for hypervariable site, haplotype number, nucleotide diversity and phylogenetic tree construction. The phylogenetic trees were constructed using the UPGMA method (The evolutionary distances were computed using the p-distance method) and the Maximum Likelihood method based on the Kimura 2-parameter model in MEGA7 program (Kumar *et al.*, 2016).

Results

Genetic Variation in D-loop region

The mtDNA at D-loop region was amplified using PCR. The results showed 467 bp. PCR product after gel electrophoresis, ethidium bromide

staining and UV exposure (Figure 1). The nucleotide sequencing of purified PCR products were performed and each mtDNA at D-loop region sequence was edited using MEGA7 program. All seven mtDNA sequences (the partial D-loop region) were submitted to GenBank NCBI database using BankIt online submission program. The accession number for all sequences were assigned in MH746114 to MH746120.

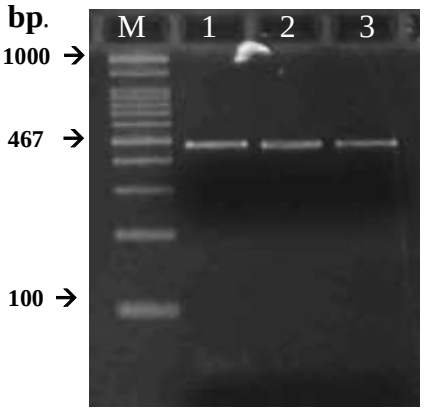

Figure 1. Example of mtDNA at D-loop region PCR products (467 bp), (Lane M = 100 bp DNA marker, Lane number 1, 2 and 3 = PCR products from three mtDNA templates 1, 2 and 3)

Nucleotide alignment using nucleotide BLAST program revealed that six samples (MH746115-MH746120) were 99% identity to *Bos taurus* (AY515627: Liping breed) and *B. indicus* (MF410539: DeHong Humped breed), whereas MH746114 was 99% identity to *B. indicus* (HQ234738: Tharparkar breed). The consensus sequence of all 7 samples were compared with all 15 reference sequences at position 16019 to 16303 of reference sequence V00654. The sequence identity of 467 bp.-length of mtDNA among seven samples from Thai-native cattle in Phetchaburi revealed 98.2-100% (Figure 2). From sequence identity matrix table, all of seven samples in this study were similar to Asia cattle such *B. indicus* (Zebu breed: HQ234738, DQ985396, L27736, L27722) and *B. taurus* (Liping breed: AY515627) (97.5%-100%) than European and Africa breed cattle (L27712, L27718, L27716, L27720, L27730, L27728, and L27714) (90.2%-92.3%).

Multiple sequence alignment of 22 sequences (MH746114 to MH746120 and 15 reference sequences) at position 16019 to 16303 (comparing with accession number V00654) of D-loop hypervariable region revealed 35 variable sites which have 30 parsimony informative sites and 5 singleton variable sites (Figure 3). When comparison of 288 nucleotides of this hypervariable site

among V00654 and seven Thai-native cattle sequence in this study, the variable sites were 26 regions. They were 24 base-transition (16 C<->T and 8 G<->A), one insertion (position 16203) and one deletion (position 16142). The number of haplotypes were classified as 13 haplotypes and haplotype diversity (H_d) were 0.8918. The haplotype distribution of Thai-native cattle in Phetchaburi showed the highest member in haplotypes 3 (MH746115, MH746116, MH746117, MH746119 and MH746120) which were the same haplotype to AY515627BT and HM173351BI (Kho Khaolumpoon). The haplotype 2 were MH746114 and HQ234738BI. Only sequence MH746118 was haplotype 4.

Genetic Relationship of Maternal Lineages of Phetchaburi Native Cattle

Phylogenetic analysis using cladistics method (Maximum Likelihood) and distance method (UPGMA) in Figure 4 and 5 revealed the relationship of maternal lineages in Thai-native cattle at Phetchaburi province. The 288 bp D-loop region of mtDNA in this study (GenBank accession number MH746114 to MH746120) were analyzed and grouped with mtDNA reference sequences from GenBank database. Both phylogenetic trees showed two major clades (clade 1 and 2). Clade 1 members were Asian cattle group while Clade 2 members were African and European cattle. All Thai-native cattle sequences in this study were grouped in Clade 1 which similar to Kho Khaolumpoon and Zebu strain.

Sequence Name	V00654BT	MH746114	MH746115	MH746116	MH746117	MH746118	MH746119	MH746120
V00654BT								
MH746114	90.5							
MH746115	91.2	98.5						
MH746116	91.2	98.5	100					
MH746117	91.2	98.5	100	100				
MH746118	91.6	98.2	99.6	99.6	99.6			
MH746119	91.2	98.5	100	100	100	99.6		
MH746120	91.2	98.5	100	100	100	99.6	100	
HQ234738BI	90.5	100	98.5	98.5	98.5	98.2	98.5	98.5
AY515627BT	91.2	98.5	100	100	100	99.6	100	100
HM173342BI	91.2	97.8	99.2	99.2	99.2	98.9	99.2	99.2
HM173351BI	91.2	98.5	100	100	100	99.6	100	100
DQ985396BI	90.5	97.8	99.2	99.2	99.2	98.9	99.2	99.2
L27736BI(Tharparkar)	90.2	97.5	98.9	98.9	98.9	98.5	98.9	98.9
L27722BI(Hariana)	90.5	97.8	98.5	98.5	98.5	98.2	98.5	98.5
L27712BT(Aberdeen Angus)	100	90.5	91.2	91.2	91.2	91.6	91.2	91.2
L27718BT(Friesian)	100	90.5	91.2	91.2	91.2	91.6	91.2	91.2
L27716BT (Charolais)	99.6	90.2	90.9	90.9	90.9	91.2	90.9	90.9
L27720BI(White Fulani)	98.9	90.2	90.9	90.9	90.9	91.2	90.9	90.9
L27730BT (N'Dama)	98.2	90.2	90.9	90.9	90.9	91.2	90.9	90.9
L27728BI (Kenana)	98.2	90.2	90.9	90.9	90.9	91.2	90.9	90.9
L27714BI(Butana)	98.5	91.2	91.9	91.9	91.9	92.3	91.9	91.9

Figure 2. Sequence Identity Matrix of seven sequences (MH746114 to MH746120) by comparing with 15 reference sequences (BT: *Bos taurus* and BI: *Bos indicus*). The D-loop mtDNA samples in this study was shaded in gray color and revealed the comparison percentage in this group

Figure 3. Multiple Sequence Alignment of D-loop Hypervariable Region. All 22 sequences (MH746114 to MH746120) and 15 references at position 16019 to 162307 of GenBank accession number V00654 were aligned using BioEdit program. The similar nucleotide base to V00654 were showed in dots (.) and dashes (-) in deletion

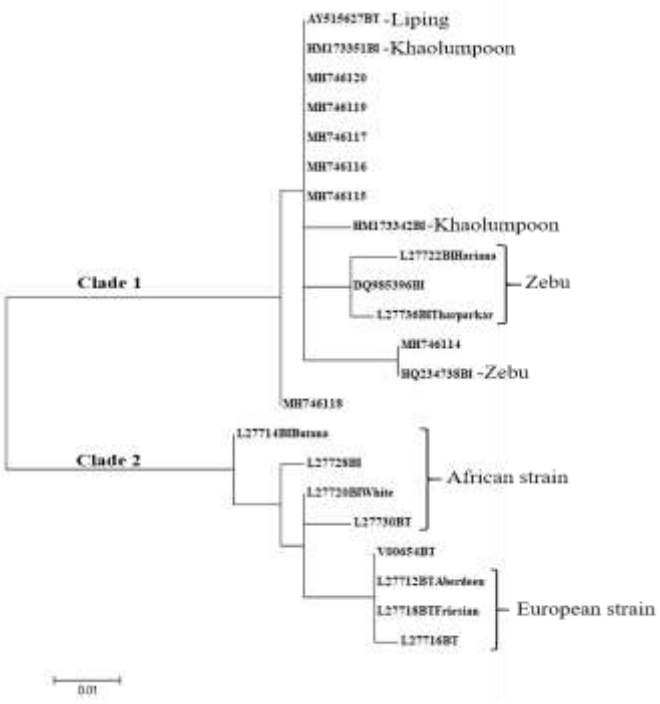

Figure 4. Molecular Phylogenetic analysis by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model in MEGA7 program. The 288 bp of mtDNA D-loop sequences from Thai-native cattle sperm (MH746114 to MH746120) were compared and grouped among 15 reference sequences

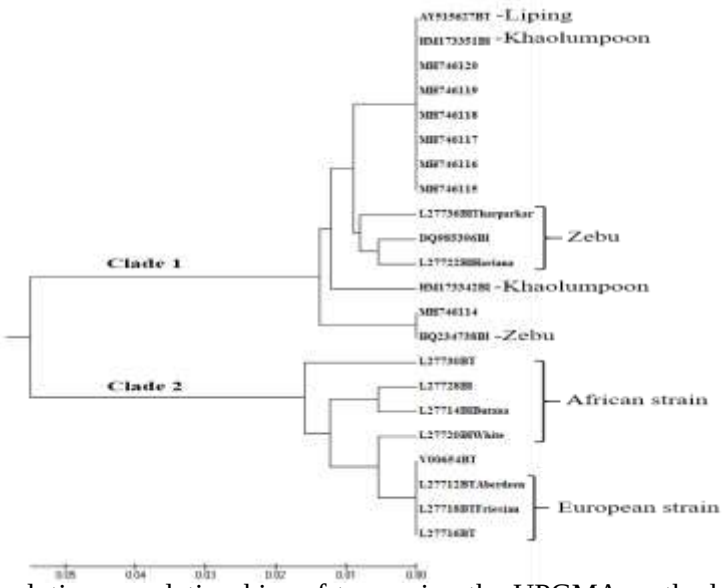

Figure 5. Evolutionary relationships of taxa using the UPGMA method, The 288 bp. D-loop region of mtDNA sequences from Thai-native cattle sperm (MH746114 to MH746120) were compared and grouped among 15 reference sequences using UPGMA method in MEGA7 program

Discussion

In this study, we used semen samples in sperm bank which were collected from seven Thai-native cattle in Phetchaburi province. The D-loop region on mtDNA from sperm were analyzed and identified genetic relationship among them and other cattle. The result from nucleotide BLAST and sequence identity matrix analysis showed that all seven samples in this study were similar to *B. indicus* (Zebu) in 99% (MF410539: DeHong Humped breed and HQ234738: Tharparkar breed). This result could be included in previously data that showed Thai-native cattle in various part in Thailand belong to *B. indicus* (Zebu) breed (Kornphan *et al.*, 2012; Sarataphan *et al.*, 2017; Wangkumhang *et al*., 2015).

From multiple sequence alignment of 22 sequences (MH746114 to MH746120 and 15 reference sequences) at position 16019 to 16303 of D-loop hypervariable region showed 35 variable sites (Figure 3) which were 24 base-transition (16 C<->T and 8 G<->A), one insertion (position 16203) and one deletion (position 16142). This high variable region data was nearly to previously analysis from Sarataphan, N. which report 50 variation sites (Sarataphan *et al*., 2017). This result indicated that Thai-native cattle had highly genetic variability. The number of haplotypes were classified as 13 haplotypes and haplotype diversity (Hd) were 0.8918, which mean that the high genetic variation.

Phylogenetic analysis revealed the relationship of maternal lineages in Thai-native cattle at Phetchaburi province because the 288 bp D-loop region of mtDNA in this study (GenBank accession number MH746114 to MH746120) were grouped in Clade 1 (Asian cattle group). All seven cattle in this province were closely related to other Asian cattle breed and clearly differentiate from African and European breed. This cattle data showed the same ancestral with other Thai-native cattle breed reports (Sarataphan *et al*., 2017; Wangkumhang *et al*., 2015).

In summary, the genetic relationship of maternal lineage successfully performed by using sperm mtDNA which was extracted from Thai-native cattle semen in Phetchaburi province. All of D-loop mtDNA sequences were submitted and deposited on GenBank NCBI database (Accession number: MH746114 to MH746120). These mtDNA nucleotide data were benefited to further genetic diversity of Thai-native cattle research in other regions.

All seven cattle in this study were grouped in Asian cattle breed and closely related to other Thai-native cattle such as Kho Khaolumpoon. Even though our information represents Thai-native cattle breed in Phetchaburi area, more genetic information data still require for fulfillment and better understanding in Thai-native cattle history. Therefore, our genetic information provides the knowledge in Thai-native cattle Phetchaburi province for further Thai-native breed management and conservation.

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