Genetic characterization of Laotian native cattle using mtDNA haplotype and loci associated with economical traits, coat color, and a hereditary disorder

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ABSTRACT

To investigate genetic characteristics of the native cattle in Southeast Asian countries, we genotyped seven genes associated with economical traits, coat color, and hereditary disorder including SCD, SREBP1, EDG1, DGAT1, NCAPG, MC1R, and F11 genes in populations of Laotian native cattle by PCR or PCR-RFLP. The genotyping results indicate that only one genotype were observed in EDG1, DGAT1, NCAPG and F11 genes, suggesting that these genes are fixed to one allele in the populations of Laotian native cattle. On the other hand, two and three genotypes were observed in SCD and SREBP1 genes and the frequencies of the minor alleles of these genes were 0.09 and 0.20, respectively. For MC1R gene, two genotypes $E^+E^+$ and $E^+e$ were observed and frequencies of $E^+$, $E^-$, and e alleles were 0, 0.94, and 0.06, respectively. Our findings of the presence of these polymorphisms in zebu cattle are of particular interest regarding the origin of these polymorphisms. The sequence analysis of the D-loop region of mitochondrial DNA indicated that the Laotian native cattle possess three haplotypes that classified into a group of the zebu cattle haplotypes. The present findings will be informative for understanding the genetic characteristics of the native cattle of Southeast Asian countries.

Key words: Laos, mtDNA, Native cattle, Polymorphisms

INTRODUCTION

Various polymorphisms of genes associated with economical traits such as carcass weight, milk production, beef marbling, and fatty acid composition, coat color variations, and mutant alleles of genes responsible for hereditary disorders have been identified in cattle of different breeds, but little is known about these polymorphisms in native cattle of Southeast Asian countries. In particular, since most of these polymorphisms have been identified in the taurine cattle (Bos taurus), distribution of these polymorphisms in the zebu (Bos indicus) cattle including Southeast Asian native cattle will be informative for understanding the origin of these polymorphisms. Therefore, we investigated whether these polymorphisms are existing in native cattle of Laos that is one of the Southeast Asian countries, in this study.

The Laotian native cattle are indigenous “yellow Asian” cattle raised by traditional methods (Bouahom 2000). The coat colors of these cattle are mainly yellow to brown colors, but other various coat colors are also observed (Namikawa et al. 2000). Genotypes of blood protein types as well as mitochondrial DNA (mtDNA) and Y-chromosome haplotypes have indicated that the Laotian native cattle belong to zebu cattle (Nomura et al. 2000; Tanaka et al. 2000; Mannen et al. 2000).

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Accepted: June 13, 2017
In the present study, we genotyped the polymorphisms of stearoyl-CoA desaturase (SCD), sterol regulatory element-binding protein-1 (SREBP1), endothelial differentiation sphingolipid G-protein-coupled receptor 1 (EDG1), diacylglycerol o-acyltransferase 1 (DGAT1), non-SMC condensin I complex, subunit G (NCAPG), melanocortin 1 receptor (MC1R), and blood coagulation factor XI (F11) genes by PCR or PCR-RFLP assay. SCD and SREBP1 genes are associated with fatty acid composition and A allele of SCD and S allele of SREBP1 increase monounsaturated fatty acids contents (Taniguchi et al. 2004; Hoashi et al. 2007), EDG1 gene is associated with beef marbling and G allele of this gene increase beef marbling score (Yamada et al. 2008), DGAT1 gene is associated with milk composition and K allele of this gene increase fat content (Grisart et al. 2002), NCAPG gene is associated with carcass weight and G allele of this gene increase carcass weight (Eberlein et al. 2009), MC1R gene is involved in coat color determination and e, E+, and E0 alleles are associated with brown, dark brown, and black coat color, respectively (Klungland et al. 1995), and the mutant allele (-) of F11 is responsible for factor XI deficiency (Kunieda et al. 2005).

We also investigated haplotypes of D-loop region of mtDNA in the Laotian native cattle. Since mtDNA sequence show mode of maternal inheritance and their haplotypes are highly polymorphic, the polymorphisms of mtDNA have been extensively used for phylogenetic analysis of populations of livestock. Particularly, since the bovine mtDNA haplotypes are clearly classified into taurine type and zebu type groups (Achilli et al. 2009), we investigated the distribution of the mtDNA haplotypes that will be informative for genetic characterization of Laotian native cattle.

**MATERIALS AND METHODS**

Total 27 blood samples were collected from 17 and 10 animals of Laotian native cattle in Vientiane and Xiengkhuang provinces, respectively, during December 2014 to January 2015. White blood cells were isolated from these blood samples and genomic DNA was extracted from these cells by standard phenol-chloroform extraction method.

For genotyping, SREBP1 and F11 genes were amplified by PCR using the primer pairs listed in Table 1 and genotyped according to the length of the amplified fragments, as described by Hoashi et al. (2007) and Kunieda et al. (2005), respectively. SCD, EDG1, DGAT1, NCAPG, and MC1R genes were amplified using the primer pairs

| Table 1. Primer sequences, fragment lengths, annealing temperatures, and restriction enzymes for genotyping |
|----------|---------------------------------|--------|--------|-----------------|
| Gene     | Primer sequences (5' to 3') | Length (bp)* | Temp** | Restriction Enzyme |
| SCD      | F : GTGTCCGTGTTGTGTTCTCCTCCTGCC | 197 | 60 |  |
|          | R : AAATATCTCCTGGGGTGTATGGTCTTG | 156, 41 | NcoI |
| EDG1     | F : GTCTGACGCACACAGATCC | 378 | 62 |  |
|          | R : GAAAGACCTCAGGCGGCAGAT | 163, 215 | MscI |
| SREBP1   | F : CCACAACGCATCAAGAAGACGCTAC | 348, 432 | 65 |  |
|          | R : GCCCTTCCTGACCACCCAACTTAG |  |
| DGAT1    | F : GCACCATCCTCTTCTCAAG | 411 | 66 |  |
|          | R : GGAAGCGCTTTCGATG | 203, 208 |  |
| NCAPG    | F : ATTTAGAAACGACTCTGG | 129 | 51 |  |
|          | R : ATTTGATCTTCTTTATCAT | 66, 63 |  |
| F11      | F : TCACATCCTAATGCTGCTCTGC | 95, 110 | 60 |  |
|          | R : TCTACGATGTCCAGTCTCTCC |  |
| MC1R (E, E0) | F : AACCTGCACTCCCCATGTGACTCT | 154 | 65 |  |
|          | R : ACATGGTCACCTCGCTGCTGCTC | 96, 58 | MspI |
| MC1R (E, e) | F : ATCTGACGTCCGCTGCTCTGACT | 219 | 65 |  |
|          | R : GGCGTAAAAGAGATGGAGATGCTC | 138, 81 |  |
| mtDNA    | F : GCCCCATGCATATAAGCAGAG | 320 | 57 |  |
|          | R : CGAGATGCTCTATTTAAAGAG |  |

*Italicized letters indicate lengths of digested fragments.

**Annealing temperature.
listed in Table 1 and genotyped by PCR-RFLP methods using *Nco*I, *Msc*I, *Eae*I, *Tsp*_509I, *Msp*A11, and *Msp*I restriction enzymes, according to Taniguchi *et al.* (2004), Yamada *et al.* (2008), and Eberlein *et al.* (2009), respectively (Table 1). PCR reactions were performed in 10 µL reaction mixtures containing 20 ng of genomic DNA, 0.2 µM primers, 0.2 µmol/L dNTP, 5 × PCR buffer, and 0.5 U Go Taq DNA polymerase (Promega Corporation, WI, USA), for 35 to 40 cycles of denaturation at 94°C for 30-120 sec, annealing at the temperatures indicated in Table 1 for 30-60 sec, and extension at 72°C for 30-120 sec. After PCR amplification and restriction enzyme digestion, the PCR fragment or restriction fragments were electrophoresed in an agarose gel in TAE buffer, stained with ethidium bromide, and visualized using UV transilluminator.

To determine the haplotypes of mtDNA, a 320-bp fragment of the hypervariable D-loop region were amplified by using a pair of primer listed in Table 1 (Troy *et al.* 2001) and the amplified fragments were directly sequenced by using these primers. PCR reaction were carried out in 10 µL reaction mixtures containing 10 ng of genomic DNA, 0.2 µM primers, 0.2 µmol/L dNTP, 5 × PCR buffer, and 0.5 U Go Taq DNA polymerase (Promega Corporation, WI, USA), for 30 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec, and extension at 72°C for 90 sec (Beja-Pereira *et al.* 2006). The obtained sequences were aligned using MEGA7 (Kumar *et al.* 2016) and finally generated a data set in length of 240 bp, between 16023 and 16262 of reference mtDNA genome sequence V00654, following Chen *et al.* (2010).

**RESULTS AND DISCUSSION**

We genotyped the genes related to carcass weight, beef marbling, fatty acid composition, coat color variation, and hereditary disorders including *SCD, SREBP1, EDG1, DGAT1, NCAPG, MC1R,* and *F11* genes in the Laotian native cattle as the first step for the genetic characterization of native cattle of Southeast Asian countries. As results of the genotyping of the Laotian native cattle (Table 2), only one genotype of GG, KK, TT, and ++ were observed in *EDG1, DGAT1, NCAPG* and *F11* genes, respectively, suggesting that these genes are fixed to G, K, T and + alleles in the populations of Laotian native cattle. On the other hand, two genotypes of AV and VV were observed in *SCD* gene and three genotypes of SS, SL and LL were observed in *SREBP1* gene indicating that both A and V, and S and L alleles of these genes are present in the population of Laotian native cattle. The frequencies of the A allele of *SCD* gene and L allele of *SREBP1* gene were 0.09 and 0.20, respectively (Table 2). The observed distributions of the genotypes are not significantly different from those expected from Hardy-Weinberg Equilibrium Model. For *MC1R* gene, two genotypes E+e+ and e+e were observed but no E+Ed, E+Ed+, E+de and eed genotypes were observed, suggesting that the e allele, in addition to the wildtype E allele, are present in the population of Laotian native cattle at low frequency.

| Gene | Genotype distributions | allele frequencies | Chi-squarevalues for HWE* test |
|------|------------------------|--------------------|--------------------------------|
| *SCD* | A/A A/V V/V | A V | 0.09 0.91 0.3 |
| *EDG1* | G/G G/A A/A | G A | NA |
| *SREBP1* | S/S S/L L/L | S L | 0.80 0.20 0.02 |
| *DGAT1* | K/K K/A A/A | K A | NA |
| *NCAPG* | G/G G/T T/T | G T | NA |
| *F11* | +/- -/- ++/++ | +/- -/ - | NA |
| *MC1R* | E0/E0 E0/E+ E+/E+ E0/e+ e/e | E0 E+ e | 0 0.94 0.06 0.1 |

*HWE: Hardy-Weinberg Equilibrium
whereas the $E^{D}$ allele are not present the populations. The frequencies of $E^{P}$, $E^{+}$, and $e$ alleles were 0, 0.94, and 0.06, respectively.

The sequence analysis of the D-loop region of bovine mtDNA indicated that the 27 animals of the Laotian native cattle possess three haplotypes that belong to the I haplogroup of the bovine mtDNA (Table 3). The bovine mtDNA haplotypes have been clustered into taurine type (T*, T1, T2, T3, and T4) and zebu type (I1 and I2) haplogroups (Achilli et al. 2009). The 17 animals of Vientiane province possess haplotype I1, I1_51, and previously unidentified haplotype of the haplogroup I1 which is identical to Laos-F haplotype described in Mannen et al. (2000) (we tentatively designated this haplotype as I1_57), whereas the 9 animals of Xiengkhuang province possess haplotype I1 and I1_57. Our findings of the presence of I1 and I1_51 haplotypes and their relative frequencies in Laotian native cattle are in consistent with the previous report (Chen et al. 2010; Mannen et al. 2000).

The polymorphisms of the genes examined in the present study have been identified as the genes associated with particular traits of beef and dairy cattle in the taurine cattle including Japanese Black cattle. However distribution of these polymorphisms in the zebu cattle including Southeast Asian native cattle is remains unclear. Since remarkable differences in allelic frequencies of various genes between taurine cattle population and zebu cattle populations have been reported (Lin et al. 2010; Kaneda et al. 2011; Yonesaka et al. 2016), whether or not these polymorphisms observed in populations of taurine cattle are also observed in populations of zebu cattle is of particular interest. In the present study, we found that the polymorphisms of $SCD$, $SREBP1$, and $MC1R$ genes reported in taurine cattle were also observed in the population of Laotian native cattle. While the presence of $A$ allele of $SCD$ and $e$ allele of $MC1R$ in zebu cattle have been reported (Kaneda et al. 2011; Zhang et al. 2014), this is the first report for the presence of the $L$ allele of $SREBP1$ in zebu cattle. The present findings of the presence of the polymorphisms of these genes in both taurine and zebu cattle indicate that these polymorphisms are relatively old polymorphisms that are originated from common ancestor of both taurine and zebu cattle.

The polymorphisms of the $SCD$ and $SREBP1$ genes have been reported to be associated with fatty acid composition of beef meat (Taniguchi et al. 2004; Hoashi et al. 2007). The animals possessing the $A$ allele of $SCD$ and $L$ allele of $SREBP1$ showed increased mono-unsaturated fatty acid composition which results in lower melting points of meat fat. Therefore, our findings of the presence of the desired alleles of these genes in the populations of Laotian native cattle will be informative for the genetic improvement of meat quality of the populations by changing the fatty acid composition. In particular, since the frequencies of the desired alleles of these genes are relatively low in the populations of Laotian native cattle, the selection of the animals possessing the desired alleles of these genes might be effective for improving the average meat quality of the population.

In the present study, no $E^{D}$ allele of $MC1R$ was observed and the frequency of $e$ allele was very low in the populations of Laotian native cattle. The coat color of Southeast Asian native cattle is known to be highly variable, and we observed various coat colors including black, dark brown, yellow to brown, and grey to white coat colors in the populations of Laotian native cattle (Table 4) as previously reported (Namikawa et al. 2000). The contribution of the presence of the $e$ allele for these coat color variation is

| Table 3. Haplotypes of mtDNA D-loop region observed in Laotian native cattle |
|---------------------------------|-------------------|
| haplotype | base position |
|-----------|---------------|
| I1 (n=24) | T A T C A T - A C C C A C A C C C C - C A A G T T |
| I1_51 (n=1)| T A T C A T C A C C A C A C C C C - C A A G T T |
| I1_57 (n=2)| - A T C A T - A C C C A C A C C C C - C A A G T T |
| Reference sequence* | C G C T G C T G T T G T G T T T T T A T G - A C C |

*We use the bovine mtDNA sequence (accession number V00654) published by Anderson et al. (1982) as reference sequence.
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unclear, since no e/e genotype that could affect the coat color was observed in the 27 animal examined. However, the present finding of relatively low allelic variation of the MC1R gene indicate that MC1R is not major determinant of the coat color variation of Southeast Asian native cattle and other genes associated with cattle coat colors such as TYRP1 and ASIP genes (Mohanty et al. 2008) might be involved in the coat colors variation.

The present findings of the distributions of the genotypes of genes associated with economical traits, coat color, and a hereditary disorder and haplotypes of mtDNA will be informative for understanding the genetic characteristics of the native cattle of Southeast Asian countries.

REFERENCES

Achilli A, Bonfiglio S, Olivieri A, Malusà A, Pala M, Hooshiar Kashani B, Perego UA, Ajmone-Marsan P, Liotta L, Semino O, Bandelt HJ, Ferretti L, Torroni A. 2009. The multifaceted origin of taurine cattle reflected by the mitochondrial genome. PLoS One, 4: e5753.

Anderson S, De Bruijn MHL, Coulson AR, Eperon IC, Sanger F, Young IG. 1982. Complete sequence of bovine mitochondrial DNA conserved features of the mammalian mitochondrial genome. Journal of Molecular Biology, 156: 683-717.

Beja-Pereira A, Caramelli D, Lalueza-Fox C, Vernesi C, Ferrand N, Casoli A, Martini A. 2006. The origin of European cattle: evidence from modern and ancient DNA. Proceedings of the National Academy of Sciences of the United States of America, 103: 8113-8118.

Bouahom, B. 2000. Animal genetic resources in the Lao PDR: Current status and production systems. Report of the Society for Researches on Native Livestock, 18: 11-16.

Chen S, Lin BZ, Baig M, Mitra B, Lopes RJ, Santos AM, Magee DA, Azevedo M, Tarroso P, Sasazaki S, Ostrowski S, Mahgoub O, Chaudhuri TK, Zhang YP, Costa V, Royo LJ, Goyache F, Luikart G, Boivin N, Fuller DQ, Mannen H, Bradley DG, Beja-Pereira A. 2010. Zebu cattle are an exclusive legacy of the South Asia Neolithic. Molecular Biology and Evolution, 27: 1-6.

Eberlein A, Takasuga A, Setoguchi K, Pfuhl R, Flisikowski K, Fries R, Kloppe N, Fürbass R, Weikard R, Kühn C. 2009. Dissection of genetic factors modulating fetal growth in cattle indicates a substantial role of the non-SMC condensin I complex, subunit G (NCAPG) gene. Genetics, 183: 951-964.

Grisart B, Coppieters W, Farnir F, Karim L, Ford C, Berzi P, Cambisano N, Mni M, Reid S, Simon P, Spelman R, Georges M, Snell R. 2002. Positional candidate cloning of a QTL in dairy cattle: identification of a missense mutation in the bovine DGAT1 gene with major effect on milk yield and composition. Genome Research, 12: 222-231.

Hoashi S, Ashida N, Ohsaki H, Utsugi T, Sasazaki S, Taniguchi M, Oyama K, Mukai F, Mannen H. 2007. Genotype of bovine sterol regulatory element binding protein-1 (SREBP-1) is associated with fatty acid composition in Japanese Black cattle. Mammalian Genome, 18: 880-886.

Kaneda M, Lin BZ, Sasazaki S, Oyama K, Mannen H. 2011. Allele frequencies of gene polymorphisms related to economic traits in Bos taurus and Bos indicus cattle breeds. Animal Science Journal, 82: 717-721.

Klungland H, Vage DI, Gomez-Raya L, Adalsteinsson S, Lien S. 1995. The role of melanocyte-stimulating hormone (MSH) receptor in bovine coat color determination. Mammalian Genome, 6: 636-639.

Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Molecular Biology and Evolution, 33: 1870-1874.

Kunieda M, Tsuji T, Abbasi AR, Khalaj M, Ikeda M, Miyadera K, Ogawa H, Kunieda T. 2005. An insertion mutation of the bovine F11 gene is responsible for factor XI deficiency in Japanese Black cattle. Mammalian Genome, 16: 383-389.

Lin BZ, Sasazaki S, Mannen H. 2010. Genetic diversity and structure in Bos taurus and Bos indicus populations analyzed by SNP markers. Animal Science Journal, 81:

| Province      | Black | Dark Brown | Yellow - Brown | Gray - White | Total |
|---------------|-------|------------|---------------|--------------|-------|
| Vientiane     | 0     | 5          | 9             | 3            | 17    |
| Xiengkhuang   | 3     | 0          | 6             | 1            | 10    |
| Total         | 3     | 5          | 15            | 4            | 27    |

Table 4. Coat color distributions in Laotian native cattle
281-289.
Mannen H, Koumoto M, Tsuji S, Kurosawa Y, Nishibori M, Yamamoto Y, Okada Y, Kuroiwa A, Yamagata T, Namikawa T, Kiao K, Siksida P, Thongsay, Phet P, Bouahom B. 2000. Mitochondrial DNA variation and phylogenetic analysis of Laos native cattle. Report of the Society for Researches on Native Livestock, 18: 59-64.

Mohanty TR, Seo KS, Park KM, Choi TJ, Choe HS, Baik DH, Hwang IH. 2008. Molecular variation in pigmentation genes contributing to coat colour in native Korean Hanwoo cattle. Animal Genetics, 39: 550-553.

Namikawa T, Yamagata T, Mannen H, Kurosawa Y, Nishibori M, Yamamoto Y, Bouahom B, Vannasouk T, Seng Dara B, Keonouchanh S, Phouthavongs K, Novaha S, Phannavong B. 2000. Coat-color variations and body-measurements of the native cattle in Laos. Report of the Society for Researches on Native Livestock, 18: 37-43.

Nomura K, Takahashi Y, Amano T, Tanaka K, Yamagata T, Mannen H, Kurosawa Y, Nishibori M, Yamamoto Y, Namikawa T, Vannasouk T, Seng Dara B, Phouthavongs K, Novaha S, Phannavong B. 2000. Constitution of genes controlling blood protein types of Laos native cattle and their phylogenetic study. Report of the Society for Researches on Native Livestock, 18: 45-57.

Tanaka K, Okada Y, Kuroiwa A, Yamagata T, Namikawa T, Amano T, Mannen H, Kurosawa Y, Nozawa K, Nishibori M, Yamamoto Y, Nguyen HN, Phan XH, Trinh DT, Dang VB, Chau BL, Nguyen DM, Bouahom B, Vannasouk T, Seng Dara B, Keonouchanh S, Phouthavongs K, Novaha S, Phannavong B. 2000. An assay for paternal gene flow between the taurus-type and indicus-type cattle in Laos and Vietnam using variation in SRY gene. Report of the Society for Researches on Native Livestock, 18: 59-64.

Taniguchi M, Utsugi T, Oyama K, Mannen H, Kobayashi M, Tanabe Y, Ogino A, Tsuji S. 2004. Genotype of stearoyl-CoA desaturase is associated with fatty acid composition in Japanese Black cattle. Mammalian Genome, 14: 142-148.

Troy CS, MacHugh DE, Bailey JF, Magee DA, Loftus RT, Cunningham P, Bradley DG. 2001. Genetic evidence for Near-Eastern origins of European cattle. Nature, 410: 1088-1091.

Yamada T, Itoh M, Nishimura S, Taniguchi Y, Miyake T, Sasaki S, Yoshioka S, Fujita T, Shiga K, Morita M, Sasaki Y. 2008. Association of single nucleotide polymorphisms in the endothelial differentiation sphingolipid G-protein-coupled receptor 1 gene with marbling in Japanese Black beef cattle. Animal Genetics, 40: 209-216.

Yonesaka R, Sasazaki S, Yasue H, Niwata S, Inayoshi Y, Mukai F, Mannen H. 2016. Genetic structure and relationships of 16 Asian and European cattle populations using DigiTag2 assay. Animal Science Journal, 87: 190-196.

Zhang Y, Li Q, Ye S, Faruque MO, Yu Y, Sun D, Zhang S, Wang Y. 2014. New variants in the melanocortin 1 receptor gene (MC1R) in Asian cattle. Animal Genetics, 45: 609-610.