Phylogenetic Tree of Kuantan Cattle by DNA Barcoding

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ABSTRAK

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Sapi kuantan merupakan breed sapi potong lokal Riau yang belum diketahui asal usulnya. Sapi kuantan banyak ditemukan di daerah Kabupaten Indargiri Hulu dan Kabupaten Kuantan Singingi. Berdasarkan tampilan fenotipe, sapi kuantan mirip dengan sapi pesisir (bangsa sapi potong lokal Sumatera Barat). Menurut beberapa peternak, asal-usul sapi kuantan merupakan sapi pesisir yang dibawa oleh perantau minang ke daerah ini. Tujuan penelitian ini untuk membuktikan apakah asal usul sapi kuantan merupakan sapi pesisir melalui analisis keragaman genetik menggunakan DNA barcode. DNA barcode yang digunakan adalah gene *Cytochrome oxidase sub unit I*, merupakan salah satu gene yang ditemukan pada DNA mitochondria. Isolasi DNA berhasil dilakukan pada 25 sampel darah sapi kuantan betina dewasa dan 18 sampel darah sapi pesisir betina dewasa. Amplifikasi ruas gene COI menggunakan metode *Polymerase Chain Reaction.* Primer forward yang digunakan dalam penelitian ini adalah F'5 TTCTCAACCAACCATAAAGATATTGG-3' dan primer reverse 5'-TAGACTTCGGGGTGTCCAAAGAATCA-3', mengapit ruas gene COI sapi kuantan dan sapi pesisir dari basa 5711 – 6420 (Genebank nomor akses NC_005971) sepanjang 710 bp. Hasil analisis sekuens menggunakan Program MEGA 5.2 menunjukkan bahwa ditemukan 6 titik polimorpik yang membentuk 7 haplotype pada sapi kuantan dan 9 titik polimorfik yang membentuk 12 haplotype pada sapi pesisir. Hasil analisis jarak genetik dan phon filogenetik menunjukkan bahwa sapi kuantan dan sapi pesisir berada pada kelompok yang sama dengan *Bos indicus*. Mutasi pada ruas gene COI sangat kecil dan belum dapat menjelaskan perbedaan diantara breed. Hasil penelitian ini juga menegaskan bahwa berdasarkan garis keturunan induk asal usul sapi kuatnan adalah dari *Bos indicus* sama seperti sapi pesisir.

Kata Kunci: Gene COI, Polimorfik, Sapi Kuantan, Jarak Genetik, Pohon Filogenetik

ABSTRACT

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Kuantan cattle is one of local beef cattle breed of Riau Province which its origin was unknown. Kuantan cattle are commonly found in Indragiri Hulu and Kuantan Singingi Regency. Based on phenotype characterizations, kuantan cattles are similar with pesisir cattle (West Sumatera beef cattle). Historically, kuantan cattle were pesisir cattle brought by "minang" immigrants (Immigrant from West Sumatera) to this region. The purpose of this study was to analyze the origin of the kuantan cattle through genetic diversity analysis using DNA barcode. DNA barcode used was Cytochrome oxidase subunit I gene which was found in the mtDNA. DNA isolation was done on 25 kuantan's blood samples and 18 pesisir blood samples. Amplification of COI gene segment used Polymerase Chain Reaction technique. The forward primer sequence used in this study was F'5 TTCTCAACCAACCATAAAGATATTGG-3' and the reverse primer sequence used was reverse 5'-TAGACTTCGGGGTGTCCAAAGAATCA-3. It squeezed kuantan and pesisir sequence 5711 - 6420 base (GeneBank accession number NC_005971) with length by 710 bp. Analysis result of sequence using MEGA 5.2 Program showed that there were 6 polymorphic sites establishing 7 haplotypes on kuantan cattle and 9 polymorphic sites establishing 12 haplotypes on pesisir cattle. Based on genetic distance and phylogeney tree, kuantan and pesisir cattle were in same group with *Bos indicus*. Mutation in the COI gene segment in this study was too small and was not able to distinguish the difference of those breeds. The result of neighbor joining analyze indicated that kuantan cattle origin was from *Bos indicus* just like pesisir cattle.

Key Words: COI Gene, Polymorphic, Kuantan Cattle, Genetic Distance, Phylogenetic Tree

INTRODUCTION

Kuantan cattle, one of local beef cattle breed of Riau Province which was registered as No.1052/Kpts/S.R.120/10/2014 by Ministry of Agriculture on 14 October 2014, has been designated as one of Indonesian native cattle breed. Morphologically, kuantan bull had body length by 103.78 cm; chest girth by 126.22 cm; and shoulder height by 99.28 cm, while kuantan cow had body length by 102.35 cm; chest girth

123.27 cm; and shoulder height 99.19 cm (Ministry of Agriculture Republic of Indonesia 2014). Kuantan cattle were found along the Kuantan River (from Kuantan Singingi Regency to Indragiri Hulu Regency). Total population of kuantan cattle in Indragiri Hulu Regency (5950 heads) was more than in Kuantan Singingi which were 2386 heads (Department of Animal Husbandry and Animal Health of Riau Province 2011). This kuantan cattle has been reared with extensive and semi intensive system for a long term.

According to Misrianti (2014), dominant color of kuantan cattle was white-brown with upward-curved horns and white legs. Phenotypically, kuantan cattle are similar with pesisir cattle and historically, kuantan cattle were pesisir cattle brought by "minang" immigrants (from West Sumatera) to this region. The development of every species under its particular natural ecosystem, environmental, and socio-economic conditions has led to each having its own specific genetic characteristics (Yang et al. 2013). Domestication of livestock species and a long history of migrations, selection and adaptation have created an enormous variety of breeds (Hailu & Getu 2015). The most domestic cattle in Indonesia belong to *Bos taurus* or *Bos indicus* while Indonesian Bali cattle was domesticated form banteng (*Bos javanicus*) (Kusdiantoro et al. 2009)

One of conventional methods applied in animal genetic resources assessment is morphological markers. Morphological markers, normally refer to external animal characteristics (animal's phenotype), which can be obtained by direct visual observation and measurement. Animal's phenotype is determined by its genetic, environment and interaction of both. Evolution of farm animal genetic resources through morphological markers based on subjective judgments and descriptions and the conclusions reached were often not completely accurate. Information on genetic diversity and origin of kuantan cattle has not been reported.

Determination of breed or line of livestock by Indonesian government was aimed to guarantee the utilization and conservation of animal genetic resources in a sustainable manner, as well as legal protection providing for breed or lines of the existing livestock. Management of animal genetic resources is essential for the achievement of global food security emphasizing global food production, improvement of productivity and food availability. People may use genetic resources to develop animal production to meet food requirement. However, sufficient genetic markers for evaluating population structure and other aspect of animal genetic resources require genetic diversity assessment. One method for population characterization was DNA barcoding (Yang et al. 2013; Hailu & Getu 2015). A DNA barcode was a short DNA sequence from a standardized region of the mitochondrial DNA gene, mitochondrial cytochrome c oxidase I (COI), used for species identification (Hebert et al. 2003).

COI gene was a gene that responsible for final step in phosphorylation before the establishment of adenosine triphosphate (ATP) (Sutrisno et al. 2013). COI gene had a conserve sequence of mitochondrial DNA on livestock (Mueller 2006) and might be used to analyze the origin of livestock (Wilson 2010) and was suitable for evolutionary studies (Lunt et al. 1996). The advantages of COI gene as a DNA barcode were (1) a relatively short length of the gene which was about 648 bp, (2) relatively stable and not prone to mutation, (3) variability was very low 1-2%, (4) much amount of copies was easily to be amplified than genes –gene nuclear DNA (Sutrisno et al. 2013).

Some research reports using DNA barcode on birds (Herbert et al. 2004), Indonesian local cattle (Febriana 2011), chicken (Gao et al. 2011), cetartiodactyla (Zein & Fitri 2012), bali cattle (Syed-Shabthar et al. 2013) and Indonesian local buffalo (Saputra et al. 2013) has been reported.

This study was aimed to obtain phylogenetic tree of kuantan cattle and to study whether pesisir cattle was the origin of the kuantan cattle based on DNA barcoding.

MATERIALS AND METHODS

Materials

Blood samples of more than two years old kuantan cow (n=25) from Kuantan Singingi (Riau Province) (Figure 1) and more than two years old pesisir cow (n=18) from central of superior cattle-forage breeding of Padang Mengatas, West Sumatera (Figure 2) were collected from jugular vein using 3 mL syringe. Those blood samples were preserved in EDTA and kept in room temperature from October to November 2015 for laboratory analysis.

DNA isolation

DNA isolation was done in Animal Molecular Genetic Laboratory, Bogor Agricultural University on November 2015. Genomic DNA was extracted using Phenol-chloroform Technique (Sambrook et al. 1989) and modified by buffer lysis cell use (250 µL 1 X STE, 40 µL SDS and 10 µL Proteinase-K). The DNA was purified by adding 40 µL 5M NaCl, 400 µL phenol chloroform and 400 µL CIAA (Chloroform Iso Amyl Alcohol) and precipitated using 40 µL 5M NaCl and 800 µL ethanol absolute. The precipitation was washed once by adding 800 µL of 70% ethanol and centrifuged at 12.000 rpm of speed for 5 minutes. Ethanol was discarded and evaporated. The precipitated DNA was dissolved in 100 µL of 80% TE (Elution buffer).

Figure 1. Kuantan cattle in Kuantan Singingi Regency, Riau Province

Figure 2. Pesisir cattle in central of superior cattle-forage breeding of Padang Mengatas, Lima Puluh Kota, West Sumatra

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5581 tctgaatttg caattcaacg tgtaaattca ccacagagct tggtaaaaag aggagtcaaa
5641 cctctatctt tagatttaca gtctaatgct ttgctcagcc attttaccca tgttcattaa
5701 ccgctgacta ttctcaacca accataaaga tattggtacc ctttacctac tatttggtgc
5761 ttgggccggt atagtaggaa cagctttaag ccttctaatt cgcgctgaat taggccaacc
5821 cggaactctg ctcggagacg accaaatcta caacgtagtt gtaaccgcac acgcatttgt
5881 aataatcttc tttatagtaa taccaatcat aattggaggg ttcggtaact gacttgttcc
5941 cctaataatt ggtgctcccg atatagcatt tccccgaata aataatataa gcttctgact
6001 tctccctccc tcattcctac tactcctcgc atcctctata gttgaagctg gggcaggaac
6061 aggctgaacc gtgtaccctc ccttagcagg caacctagcc catgcaggag cttcagttga
6121 tctaaccatt ttctctttac acttagcagg agtttcctca attttaggag ccatcaactt
6181 cattacaaca attatcaaca taaagccccc cgcaatgtca caataccaaa cccctctatt
6241 cgtatgatcc gtaataatta ccgccgtact actactactc tcgctccctg tattagcagc
6301 cggcatcaca atgctattaa cagaccggaa cctaaatаca actttcttcg acccggcagg
6361 aggaggagat cctattctat accaacactt attctgattc tttggacacc ccgaagtcta
6421 tattttaatc ttacctggat ttggaataat ctctcatatc gtaacctact actcaggaaa
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Figure 3. BICOI amplicon sequence, Genebank NC_005971. The primers sites were underlined and bold

Qualitative and quantitative test of DNA isolation

Qualitative test of DNA isolated was used on 1% agarose gel electrophoresis in 1XTAE solution. Electrophoresis was run at 100 volts for 35-40 minutes. Determination of concentration and purity level of the isolated DNA used spectrophotometry on 5 samples of the isolated DNA randomly.

DNA amplification and direct sequencing method

DNA was amplified using Polymerase Chain Reaction (PCR) technique carried out in Genetic and Breeding Laboratory, Faculty of Agricultural and Animal Science, Islamic State University of Sultan Syarif Kasim Riau on November 2015 - January 2016. Each PCR reaction was made with cocktail 50 ng (2-3 µL) DNA templates, 0.25 µM forward and reverse primer, 12.5 µL Dream Tag Green Master Mix from Thermo Scientific #K1081 and dH2O up to 25 µL. The forward primer sequence was F'5 TTCTCAACCAACCATAAAGATATTGG-3' and reverse primer sequence was reverse 5'-TAGACTTCGGGGTGTCCAAAGAATCA-3', in accordance with Febriani (2013). Position of forward and reverse primer in PCR product of COI gene was shown in Figure 3. Samples were initially denaturized at 93°C for 3 minutes and followed by 35-36 cycles of denaturation at 93°C for 30 second, annealing at 60°C for 30 second and extension at 72°C for 30 second. Final extension was at 72°C for 5 minutes. Master Cycler Personal 22331 Eppendorf was used for PCR amplification. PCR products were then separated on 1.5% agarose/1 x TAE, stained with 2.5 µL of ethidhium bromide (EtBr) and calibrated with 100 bp ladder marker. Electrophoresis chamber was run on 100

volt power supply for 35 minutes. Finally, the gel was visualized under Gel Doc. Forty three PCR product samples were then subjected to direct sequence (forward and reverse) analysis by dideoxy sequencing in ABI 3730 XL automated DNA sequencer at the First base laboratory Malaysia via commercial service on January 2016.

Data analysis

Results of sequences fragment of COI gene were analyzed with BioEdit (Hall 2011), MEGA version 5.2 software (Tamura et al. 2011). BLAST sequence was obtained from Gene Bank with accession number of KF 952276, KF 771228, KF 952284, KF 952285, HQ860420.1, HM 102290.1, KF771228.1, FJ 958333 and JX 218056.1. Method of neighboor-joining with kimura 2-parameter model was applied for reconstructing phylogeney tree.

RESULT AND DISCUSSION

Quality of DNA isolated and PCR amplification of COI gene

Qualitative method for identification, separation and purification of DNA fragments was by agarose gel electrophoresis (Fachtiyah, et al. 2011). DNA which might migrate on agarose gel or polyacrylamide gel was placed in a buffer solution which electrified in chamber. Negatively DNA will be moving positive direction. The success of DNA isolation characterized by a single band, bright and cleared in each well (Figure 4).

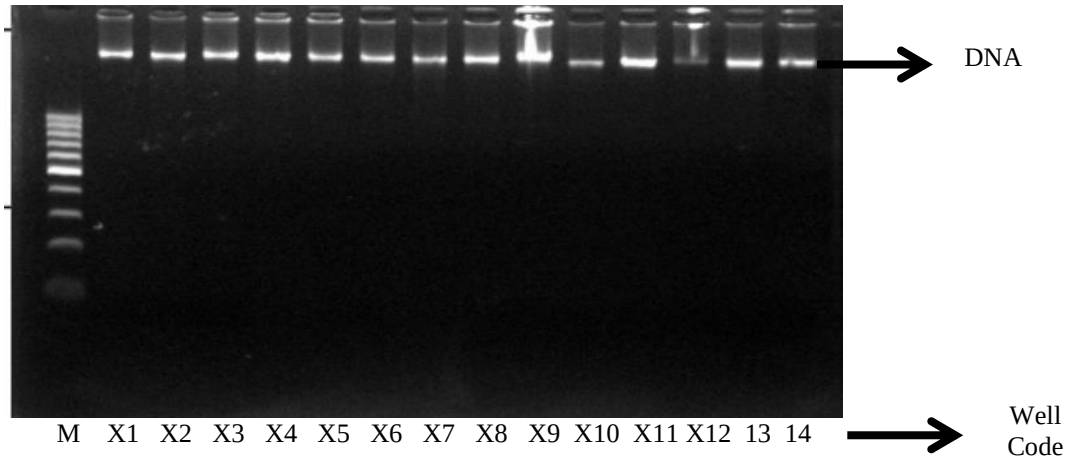

Figure 4. Qualitative test of DNA isolated

Table 1. Concentration and Purity Level of Isolated DNA

Sample Code	DNA Concentration (ng/µL)	Å 260	Å280	Purity Level
Blanco	0.80	0.017	0.021	0.8
Kuantan cattle 19	89.40	1.79	0.99	1.80
Kuantan cattle 23	77.55	1.55	0.86	1.80
Kuantan cattle 30	121.45	2.43	1.34	1.81
Kuantan cattle X7	44.20	0.88	0.51	1.75
Kuantan cattle X12	27.45	0.55	0.33	1.64
Pesisir cattle 4796	97.45	1.95	1.08	1.80
Pesisir cattle 4848	49.30	0.99	0.56	1.77
Pesisir cattle 4856	66.10	1.32	0.75	1.77
Pesisir cattle 4857	29.95	0.60	0.35	1.71
Pesisir cattle 4859	84.85	1.70	0.95	1.79

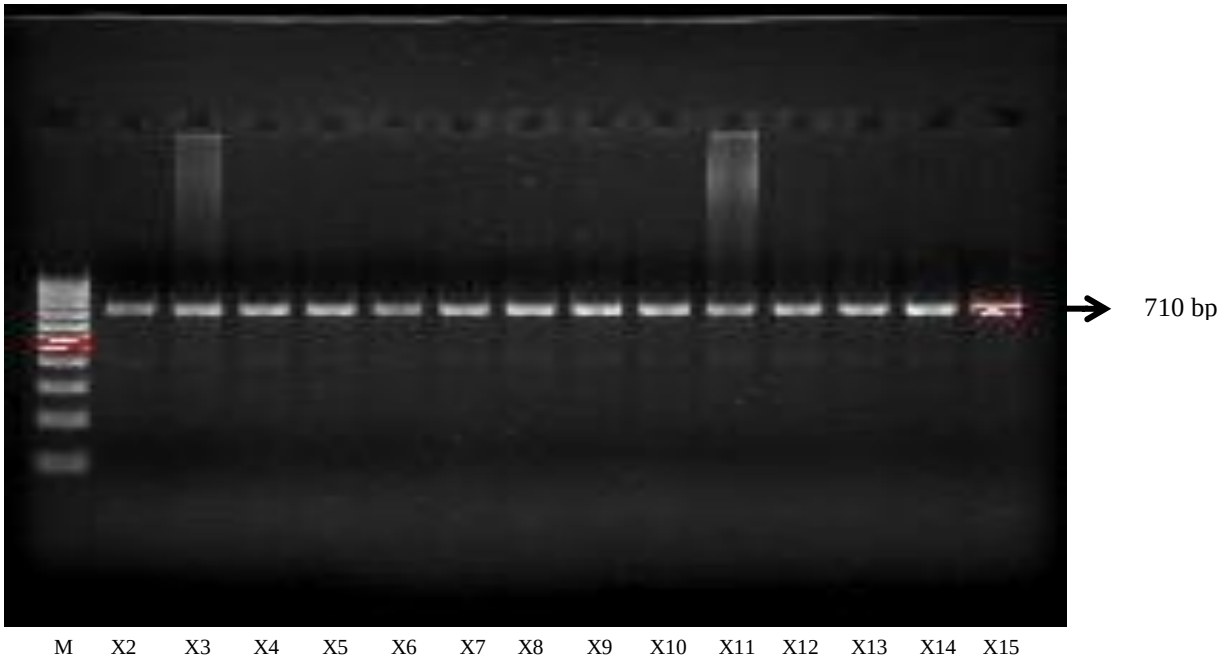

Figure 5. PCR amplification product of COI Gene (710 bp); M = DNA ladder 100 bp, X2, X3..Xn = samples codes

Concentration of isolated kuantan's DNA was 27.45-121.45 ng/µL with a purity level of 1.64 to 1.81 (Table 1). DNA concentration of pesisir cattle was 29.9597.45 ng/µL with purity level of 1.71 to 1.80. Good purity levels of DNA isolated were 1.8 to 2.0. If the value exceeds of 2.0, the solution tested was still contaminated by membrane protein or other compounds so that the levels of plasmid DNA obtained was not pure. If it was less than 1.8, it means that too much ddH_2O was taken while DNA taken was too slightly. The success of PCR process was determined by accurately and level of concentration of DNA used as a template. The range of concentrations of DNA used in the PCR process was 25-50 ng/mL.

Primer has amplified COI gene of kuantan cattle and pesisir cattle successfully. It was indicated by the appearance of a single band at 710 bp (Figure 5). Annealing temperature was 60^0C for 45 seconds.

Diversity of COI gene on Kuantan and Pesisir cattle

Alignment of 25 kuantan cattle sequences showed six point mutations (c.9, c.25, c.35, c.60, c.111, c.617) forming seven haplotypes (A, B, C, D, E, F and G) (Table 2). The A haplotype was dominant (76%, n=19) on Kuantan cattle. The B (n=X2), C (X5); D (15); E (19); F (X7) and G haplotype (X) of kuantan cattle were detected 4% separately. On pesisir cattle it showed nine point mutations (c.7, c.16, c.35, c.42, c.67, c.74, c.145, c.254 and c.659) forming twelve haplotypes (A, B, C, D, E, F, G, H, I, J, K and L) (Table 2). The A haplotype on kuantan cattle and pesisir cattle was exactly similar. The A haplotype on kuantan cattle was the highest proportion (76%) on population than the B and J haplotypes on pesisir cattle (16.67%).

Genetic distance and phylogeny tree of COI gene on Kuantan and Pesisir cattle

Results of the analysis of genetic distance showed that kuantan and pesisir cattle might be grouped into *Bos indicus* because it had a genetic distance of 0.000 in all haplotypes, except on K Haplotype on pesisir cattle (0.002). Genetic distance of kuantan cattle and *Bos taurus* was in the range of 0.10 to 0.12. This result explains that based on the DNA barcode, kuantan cattle is in the same cluster with *Bos indicus* as well as pesisir cattle, aceh cattle and PO cattle. Cattle breeds have been developed in different ways depending on regional climates, nutritional conditions and selection for different purposes. Genetic drift also contributed in the process of breed differentiation. In very closely related breeds, the number of mutations cannot explain the observed genetic variation even when highly mutable DNA sequences are used.

The result was in accordance with Mitchell et al. (2010) who said that the genetic diversity of COI gene in the Bovidae was between 0.0 -1.92% (average 0.245) and 9.77% in average between the species. Genetic distance in the Bos, Capra, and Ovis genus was 3.5%, 0.0%, 0.003% respectively. Genetic distance between javanicus - indicus and javanicus - taurus species was 0.066% and 0.014%, respectively (Zein and Fitri, 2012). In this study, genetic distance between kuantan

and *Bos indicus* cattle was 0.00 and between kuantan and *Bos Taurus* was among 0.10-0.12. Genetic distance in height of COI gene between the species was low. It showed that COI gene was effective for identification at the level of species, genus and family and was appropriate to be used as a DNA barcode. For small size (N=50) population and mutation rate of $\beta = 10^{-3}$, the difference between computed inbreeding value assumed that mutation was small (less than 7 % of true value) and mutations might be neglected during 200 generations (Laval et al. 2002).

Reconstruction of phylogeny tree of kuantan cattle used neighboor-joining method. The result showed that all samples of cattle kuantan were in the same cluster with the *Bos indicus* with gene bank access number of KF 952 285, KF 952 284, KF 771 228 and *Bos Taurus* with gene bank access numbers of JX 18056.1. It was vary from *Bos Taurus* access number of HQ 860420.1, HM 102290.1, KF 771228.1 and FJ 958 333 with the genetic distance of 0.001 (Figure 6). This research showed that based on the maternal line, the ancestor of kuantan cattle was *Bos indicus* as well as pesisir cattle.

Table 2. Type of haplotype of Kuantan and Pesisir cattle

Haplotype type	Point mutation														n (%)
	7	9	16	25	35	42	60	67	74	111	145	254	617	659	
Kuantan cattle															
A Haplotype	A	G	G	A	T	T	G	A	T	-	G	C	A	C	19(76%)
B Haplotype	A	G	G	-	-	T	G	A	T	-	G	C	A	C	1(4%)
C Haplotype	A	G	G	-	T	T	G	A	T	T	G	C	A	C	1(4%)
D Haplotype	A	R	G	A	T	T	G	A	T	-	G	C	A	C	1(4%)
E Haplotype	A	G	G	A	-	T	G	A	T	-	G	C	A	C	1(4%)
F Haplotype	A	G	G	-	-	T	-	A	T	-	G	C	A	C	1(4%)
G Haplotype	A	G	G	-	T	T	G	A	T	-	G	C	-	C	1(4%)
Pesisir Cattle															
A Haplotype	A	G	G	A	T	T	G	A	T	-	G	C	A	C	1(5.56%)
B Haplotype	C	G	G	A	T	T	G	-	T	-	G	C	A	C	3(16.67%)
C Haplotype	A	G	G	A	-	-	G	A	T	-	G	C	A	C	1(5.56%)
D Haplotype	A	G	R	A	T	T	G	A	T	-	G	C	A	Y	1(5.56%)
E Haplotype	A	G	R	A	T	T	G	A	T	-	G	C	A	C	1(5.56%)
F Haplotype	A	G	G	A	T	T	G	A	-	-	G	M	A	C	1(5.56%)
G Haplotype	A	G	G	A	-	-	G	A	T	-	G	M	A	C	1(5.56%)
H Haplotype	A	G	G	A	-	-	G	A	-	-	G	M	A	C	1(5.56%)
I Haplotype	A	G	G	A	-	-	G	A	T	-	G	C	A	C	2(11.11%)
J Haplotype	A	G	G	A	T	T	G	A	T	-	G	C	A	C	3(16.67%)
K Haplotype	A	G	G	A	T	T	G	A	T	-	T	C	A	C	1(5.56%)
L Haplotype_	-	G	G	A	T	T	G	A	T	-	G	C	A	C	2(11.11%)

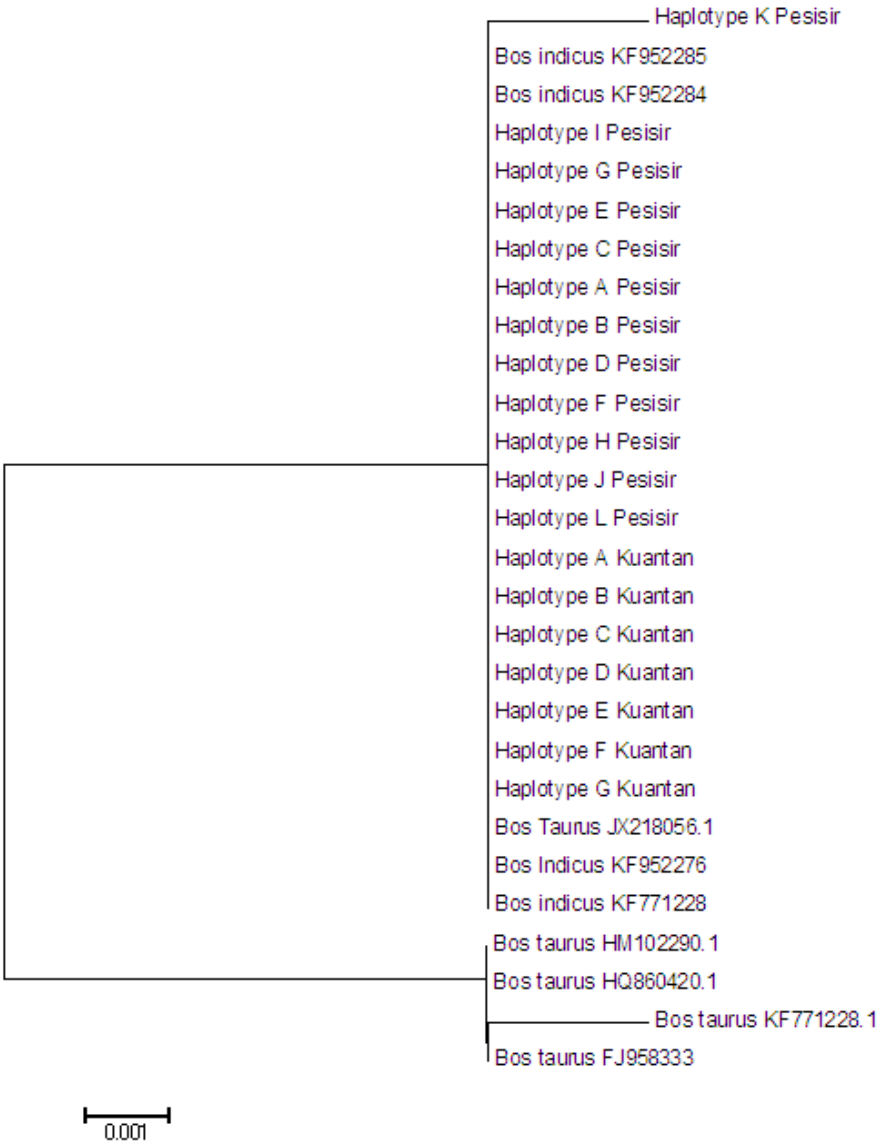

Figure 6. Phylogeny tree of Kuantan cattle by *Neighboor-Joining* Method

CONCLUSION

Results of sequence analysis using the program MEGA 5.2 showed six point mutations which established 7 haplotypes on kuantan cattle. Nine point mutations on pesisir cattle established 12 haplotypes. Based on genetic distance, kuantan and pesisir cattle were in same group with *Bos indicus*. Mutations in the COI gene segment of kuantan and pesisir cattle in this study were too small and the difference cannot be clearly explained. The results of neighbor joining analysis indicated that the origin of the kuantan cattle was *Bos indicus* based on maternal lineage.

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