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Genetic Variation of Cytochrome b Gene in Three Local Goat Breeds in Central Java of Indonesia

Research Article

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ABSTRACT

This study was conducted to elucidate the genetic diversity and phylogenic relationship of three local goat breeds in Central Java of Indonesia based on the cytochrome b (cyt b) gene sequence. Blood samples of 19, 15 and 12 heads of Kacang, Kejobong and Ettawa Grade breeds, respectively, were used as materials of the study. The multiple alignment of cyt b gene was conducted by comparing under study goat breeds and one deposited sequence in GeneBank (*Capra hircus*, accession number AB004072). Of the 16 sites of different nucleotide bases found, there were 12 sites that could be used as a marker to distinguish the three breeds of local goat in Indonesia with the *Capra hircus*. Genetic distance among local goats was quite close compared to genetic distance between those local goats to GenBank's *Capra hircus*. Phylogeny tree showed two main clusters, that was cluster A for *Capra hircus* and cluster B for three local goat breeds of Indonesia.

KEY WORDS genetic diversity, indigenous goat, mitochondrial DNA.

INTRODUCTION

Goat is used as the small household savings and can be used at any time to cover needs of the family. One of advantages in raising goat is faster business cycle compared to larger ruminants because goat has shorter reproductive cycle (Ball and Peters, 2004; Jainudeen *et al*. 2000). Central Java of Indonesia has local genetic resources for large and small ruminants. In goat, there are four local breeds, namely Kacang, Jawarandu, Ettawa and Kejobong. Phenotypic studies of the first three breeds of goat are many (Setiadi *et al.* 1995; Elieser *et al.* 2012; Kurnianto *et al.* 2013; Basbeth *et al.* 2015). However, genotypic diversity study, especially in the last breed, is lack (Lestari, 2017). Phenotypic study is usually conducted on qualitative and quantitative traits (Falconer and Mackey, 1997), but the study on genotypic study is based on analysis of blood plasma polymorphism (Elmaci *et al.* 2006; Mousavizadeh *et al*. 2009). In general, these studies are intended to obtain an overview of trait diversity in each of breed commodity.

Mitochondrial DNA (mtDNA) polymorphism has been applied to understand the phylogenetic relationship in many breed. It is well-known that the function of mtDNA has been a popular area of experiment in population genetic analysis (Zhao *et al.* 2013). Studies on mitochondrial DNA variability were conducted in Saudi Arabian goat breeds (Amer, 2014) and Indian sheep breeds (Sawaimul *et al.* 2014).

Molecular information can be obtained by conducting study on mtDNA fragment profile of cytochrome b (cyt b) gene. The cyt b is an essential part of the mtDNA, in which it plays role in estimating genetic diversity and phylogenetic relationship in some species, such as in cattle (Cai *et al.* 2007; Kim *et al.* 2013), sheep (Oner *et al.* 2013;

Sawaimul *et al.* 2014); pig (Yang *et al.* 2012) and duck (Sultana *et al.* 2016) . Because of its maternal inheritance and low recombination occurrence, cyt b gene has been used to study the genetic diversity and phylogenetic of either various species or breeds (Irwin *et al.* 1991; Pfeiffer *et al.* 2004; Barbar *et al.* 2014; Jiyanto *et al.* 2014). Analysis in more than two breeds of goat can give information about genetic distance among them. This study was conducted to elucidate the genetic diversity and phylogenic relationship of three local goat breeds in Central Java of Indonesia based on the cytochrome b (cyt b) gene sequence.

MATERIALS AND METHODS

Materials

Forty six heads of unrelated local goat in Central Java of Indonesia consisted of 19, 15 and 12 heads of Kacang, Kejobong and Ettawa Grade breed, respectively, were used as materials of this study. The blood samples of Kacang, Kejobong and Ettawa Grade goats were taken from Grobogan, Purbalingga and Kendal regencies, respectively.

Blood sample

Blood samples were collected from the jugular vein. Obtained blood samples were saved in ethylenediaminetetraacetic acid (EDTA) tube to avoid blood clotting and stored at -20 ˚C until DNA extraction.

DNA isolation

DNA extraction was conducted by using Genomic DNA Mini Kit from GeneAid with modification procedure of Sambrook *et al.* (1989). Blood sample of 400 µL was inserted into 1.5 mL of Eppendorf tubes. Red blood cell lysis buffer was added into the tube until it reached a volume of 1.5 mL, and then it was waited for 10 min. The samples were centrifuged at 3000 rpm for 5 min. Erythrocytes and leucocytes formed at the base of tube was taken, then transferred into another new tube. The 200 µL of GT Buffer solution (buffer wash) and 20 µL of Proteinase-K was added, then mixed. Samples were then incubated in a shaking water bath with a temperature of 47 ˚C for 16 h. Then, samples were removed and added to 200 mL of GB Buffer (digestion buffer), then mixed again and incubated for 20 min. Samples were transferred into the GD column tube (there was filter tool at the bottom of tube). The 200 mL of absolute ethanol was added to the tube, samples were centrifuged between 14000-16000 rpm for 2 min. The formed liquid at the bottom of tube was removed. At the filtered DNA, the W1 solution (substitute phenol to clean existed protein) was added about 400 µL. Samples were centrifuged at 14000-16000 rpm for 1 min, formed liquid at the bottom was discarded.

The 600 mL Wash Buffer Chloroform was added, then the samples were centrifuged by 14000-16000 rpm for 1 min and performed fluid was removed. Samples were centrifuged at 14000-16000 for 3 min in order to clean residual ethanol. The GD Colum was placed in 1.5 mL of eppendorf to remove ethanol. The 75 mL of Elution Buffer solution was added and incubated at room temperature. Sample was centrifuged by 14000-16000 rpm for 1 min. A solution of 75 mL of elution buffer was added again, and then the samples were centrifuged at a speed of 14000-16000rpm for 1 min. Pure DNA obtained was given and transferred to a new Eppendorf tube.

Polymerase chain reaction (PCR)

PCR composition consisted of 25 µL Kappa (PCR Mix), 1 µL primer forward 5'-TGGAATCTAACCATGACCAATG-3', 1 µL primer reverse 5'-GGCTATTCTCCTTTTCTGGTTT-3', 21 µL template DNA and 21 µL ddH2O. PCR amplification was performed with the following conditions: initial denaturation for 5 min at 94 ˚C, then the amplification reaction for 35 cycles consisting of the denaturation at 94 ˚C for 30 sec, annealing at a temperature of 48 ˚C for 45 sec, elongation at 72 ˚C for 60 sec, then terminated by the addition of elongation (extension) for 5 min at 72 ˚C and stored at 4 ˚C. Then PCR products test was conducted using 1% agarose gel electrophoresis.

DNA sequencing

Product of PCR amplification was purified and used as the template DNA for DNA sequencing reaction. Each PCR product was sequenced using the BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems Inc.) on an automated DNA sequencer (Applied Biosystems 373 DNA sequencer with XL upgrade computer system), following manufacturer's instructions.

Data analysis

The sequence results were analyzed using molecular evolutionary genetics analysis (MEGA) version 6.0 (Tamura *et al.* 2013). The multiple alignment of cyt b gene was conducted by comparing three breeds of goat to sequence of a reference breed in GeneBank, that was *Capra hircus* (AB004072). Genetic distance was analyzed with the Kimura 2 parameters model (Kimura, 1980). Phylogenetic tree was constructed by unweighted pair-group method with arithmetic mean (UPGMA) and made based on the cyt b gene sequences with bootstrap testing 1000x replication (Sneath and Sokal, 1973; Felsenstein, 1985). Nucleotide diversity (π) and haplotype diversity (Hd) were analyzed using DNA sequence polymorphism (DnaSP) version 5.0 (Rozas *et al.* 2003).

RESULTS AND DISCUSSION

The coding sequence of the cyt b gene in three breeds of local goat in Central Java were 1140 bp. The diversity of the cyt b gene in samples of Kacang, Ettawa Grade, Kejobong and *Capra hircus* (GeneBank D84201) are presented in Table 1.

Analysis showed there were 16 nucleotides' position that differ among 3 local breeds of goat compared to *C. hircus*. The different sites were found in nucleotide sequences of 49, 309, 396, 397, 441, 447, 450, 495, 572, 594, 643, 695, 714, 721, 852 and 1068. At sites of 309 and 1068, the nucleotide *C. hircus* was in the form of thymine (T), while the three local goats of Central Java were cytosine (C).

Table 1 Cytochrome b gene diversity based on nucleotide base in Kacang (K), Kejobong (KJ) and Ettawa Grade (PE) goats

Sample	Nucleotide base at i^{th} site															
	49	309	396	397	441	447	450	495	572	594	643	695	714	721	852	1068
C. hircus	G	T	C	C	C	C	C	A	G	C	A	C	T	C	C	T
K1	.	C	T	T	T	T	T	.	C	T	G	.	.	T	T	C
K2	.	C	T	T	T	T	T	.	C	T	G	.	.	T	T	C
K3	.	C	T	T	T	T	T	.	C	T	G	.	.	T	T	C
K4	A	C	T	T	T	T	T	.	C	T	G	.	.	T	T	C
K5	.	C	T	T	T	T	T	.	C	T	G	.	.	T	T	C
K6	.	C	T	T	T	T	T	.	C	T	G	.	.	T	T	C
K7	.	C	T	T	T	T	T	G	C	T	G	.	.	T	T	C
K8	.	C	T	T	T	T	T	.	C	T	G	.	.	T	T	C
K9	.	C	T	T	T	T	T	.	C	T	G	.	.	T	T	C
K10	.	C	T	T	T	T	T	.	C	T	G	.	.	T	T	C
K11	.	C	T	T	T	T	T	.	C	T	G	.	.	T	T	C
K12	.	C	T	T	T	T	T	.	C	T	G	.	.	T	T	C
K13	.	C	T	T	T	T	T	.	C	T	G	.	.	T	T	C
K14	.	C	T	T	T	T	T	G	C	T	G	.	.	T	T	C
K15	.	C	T	T	T	T	T	.	C	T	G	.	.	T	T	C
K16	.	C	T	T	T	T	T	G	C	T	G	.	.	T	T	C
K17	.	C	T	T	T	T	T	.	C	T	G	.	.	T	T	C
K18	.	C	T	T	T	T	T	.	C	T	G	.	.	T	T	C
K19	.	C	T	T	T	T	T	.	C	T	G	.	.	T	T	C
KJ1	A	C	T	T	T	T	T	.	C	T	G	.	.	T	T	C
KJ2	.	C	T	T	T	T	T	.	C	T	G	.	.	T	T	C
KJ3	.	C	T	T	T	T	T	.	C	T	G	.	.	T	T	C
KJ4	.	C	T	T	T	T	T	.	C	T	G	.	C	T	T	C
KJ5	.	C	T	T	T	T	T	G	C	T	G	.	.	T	T	C
KJ6	.	C	T	T	T	T	T	G	C	T	G	.	.	T	T	C
KJ7	.	C	T	T	T	T	T	.	C	T	G	.	.	T	T	C
KJ8	.	C	T	T	T	T	T	.	C	T	G	.	.	T	T	C
KJ9	A	C	T	T	T	T	T	.	C	T	G	.	.	T	T	C
KJ10	.	C	T	T	T	T	T	.	C	T	G	.	.	T	T	C
KJ11	.	C	T	T	T	T	T	.	C	T	G	.	.	T	T	C
KJ12	.	C	T	T	T	T	T	G	C	T	G	.	.	T	T	C
KJ13	.	C	T	T	T	T	T	G	C	T	G	.	.	T	T	C
KJ14	.	C	T	T	T	T	T	.	C	T	G	.	.	T	T	C
KJ15	.	C	T	T	T	T	T	.	C	T	G	.	.	T	T	C
PE1	.	C	T	T	T	T	T	.	C	T	G	.	.	T	T	C
PE2	.	C	T	T	T	T	T	.	C	T	G	.	.	T	T	C
PE3	.	C	T	T	T	T	T	.	C	T	G	.	.	T	T	C
PE4	.	C	T	T	T	T	T	.	C	T	G	.	.	T	T	C
PE5	.	C	T	T	T	T	T	.	C	T	G	.		T	T	C
PE6	A	C	T	T	T	T	T	.	C	T	G	.	.	T	T	C
PE7	.	C	T	T	T	T	T	.		T	G	T	.	T	T	C
PE8	.	C	T	T	T	T	T	.	C	T	G	.	.	T	T	C
PE9	.	C	T	T	T	T	T	G	C	T	G	.	.	T	T	C
PE10	.	C	T	T	T	T	T	G	C	T	G	.	.	T	T	C
PE11	A	C	T	T	T	T	T	.	C	T	G	.	.	T	T	C
PE12	A	C	T	T	T	T	T	.	C	T	G	.	.	T	T	C

The sites 396, 397, 441, 447, 450, 594, 721 and 852 of the nucleotide bases of *C. hircus* were cytosin (C), while the local goat was timine (T). At site572, nucleotide base of *C. hircus* was guanine (G), while the local goat was citosine (C). There were differences in nucleotide bases between local goat and *C. hircus* on site 49, 495, 695 and 714. It was stated by Pidancier *et al*. (2006) that *C. hircus* goat is a goat resulted from domestication of wild goat (*C. aegagrus*), in which the endemic area are Africa (South Africa), Slovenia, Europe (Switzerland), Asia (Malaysia). The similar statement on the origin of local goat in other area stated by Chen *et al.* (2006), in which the Chinese goats were obviously separated from wild goats and might come from *C. aegagrus.*

It can be seen in Table 2 that of the 16 sites of different nucleotide bases there are 12 sites that could be used as a marker to distinguish the local goat of Central Java with the ancestors, those were at site 309, 396, 397, 441, 447, 450, 572, 594, 643, 721, 852 and 1068. The genetic diversity of local goats in Central Java formed 5 haplotypes. Amer (2014) reported that the cyt b gene did not show any variation among studied Saudi Arabian goat breeds.

Each goat's breed in this research had different nucleotide diversity (π) value and haplotype diversity (Hd) value that shown in Table 3.

The Ettawa grade displayed the highest nucleotide diversity value (0.077±0.017) and haplotype diversity value (0.712±0.105).

According to Nei (1987), genetic diversity values ranged from 0.8 to 1 in the high category, while the value of 0.5 to 0.7 classified in the medium category and 0.1 to 0.4 classified in the low category. Smith and Chesser (1981) stated that the Hd value ≥ 0 < 0.5 is low haplotype diversity, while > 0.5 ≤ 1 belongs to the high haplotype diversity and more diverse genetic haplotype diversity, the level will be higher and vice versa.

Livestock domestication process has happened in thousands of years, even several million years ago. Goats that currently exist and appear in a variety of characteristics is the result of domestication. Occurrence of either genetically or phenotypic differences are resulted from the process of natural selection, as well as the evolution process in an area (FAO, 2011).

The genetic distance values among three breeds of local goat and between them and *C. hircus* are presented in Table 4. The low value of genetic distance among Kejobong, Kacang and Ettawa Grade goats indicated the low genetic relationships (0.0005-0.0007). The low value of genetic distance among Kejobong, Kacang and Ettawa Grade goats indicated strong genetic relationships between these breeds.

Table 2 Haplotype of Kacang (K), Kejobong (KJ) and Ettawa Grade (PE) goats

Haplotype	Sample code
Type 1	K4, Kj1, Kj9, PE6, PE11, PE12
Type 2	K7, K14, K16, Kj5, Kj6, Kj12, Kj13, PE9, PE10
Type 3	PE7
Type 4	Kj 4
More up	K1, K2, K3, K5, K6, K8K9, K10, K11, K12, K13, K15, K17, K18, K19, Kj2, Kj3, Kj7, Kj 8, Kj10, Kj11, Kj 14, Kj 15, PE1, PE2, PE3, PE4, PE5, PE8

Table 3 Genetic diversity of Kacang (K), Kejobong (KJ) and Ettawa Grade (PE) goats

No	Goat breed	Location	n	Genetic diversity		
				Hn	Hd (±SE)	π (±SE)
1	Kacang	Grobogan	19	3	0.368 (±0.125)	0.034 (±0.012)
2	Kejobong	Purbalingga	15	4	0.667 (±0.099)	0.007 (±0.015)
3	Ettawa Grade	Kendal	12	4	0.712 (±0.105)	0.077 (±0.017)

n: number of sample; Hn: number of haplotype; Hd: haplotype diversity; π: nucleotide diversity (%) and SE: standard error.

Table 4 Genetic distance estimates of evolutionary divergence over sequence pairs between groups

Breed	*Capra hircus**	Kacang	Kejobong
*Capra hircus**	-	-	-
Kacang	0.0108	-	-
Kejobong	0.0110	0.0005	-
Ettawa Grade	0.0111	0.0006	0.0007

* Breed was used as a comparison.
The number of base substitutions per site from averaging over all sequence pairs between groups are shown. Analyses were conducted using the Kimura 2-parameter model. The analysis involved 47 nucleotide sequences. Codon positions included were $1^{st} + 2^{nd} + 3^{rd}$ + noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 1140 positions in the final dataset. Evolutionary analyses were conducted in MEGA 6.

These breed are not isolated from each other, the breeder practice probably cross breeding.

Meanwhile, the genetic distance between local goats and *C. hircus* were further than that of within local goats (0.0108-0.0111). The each of local goat may be compared each other and to *C. hircus* obtained from genbank, because those goats are the same species.

The results of genetic distance estimation among Central Java local goats are parallel with statement of Machado *et al.* (2000), in which the analysis of the relationships among breeds are genetically far show large genetic distance, or *vice versa*.

Analysis of phylogeny can take advantage of sequence nucleotide and amino acid. Usually phylogeny analyze the change that occur in the evolution of organism (Dharmayanti, 2011). Evolution itself is a gradual change of organism leads to conformity with the time and place. Phylogeny tree of Kejobong, Kacang and Ettawa goats is presented in Figure 1. The result shows that 3 analyzed breeds of local goat in Central Java, all breeds had no typical cluster. The phylogeny analysis of within native animals tended to result in similarity (Odahara *et al.* 2006), in which less genetic variability of the Korean native goats was found compared to other Asian goat population.

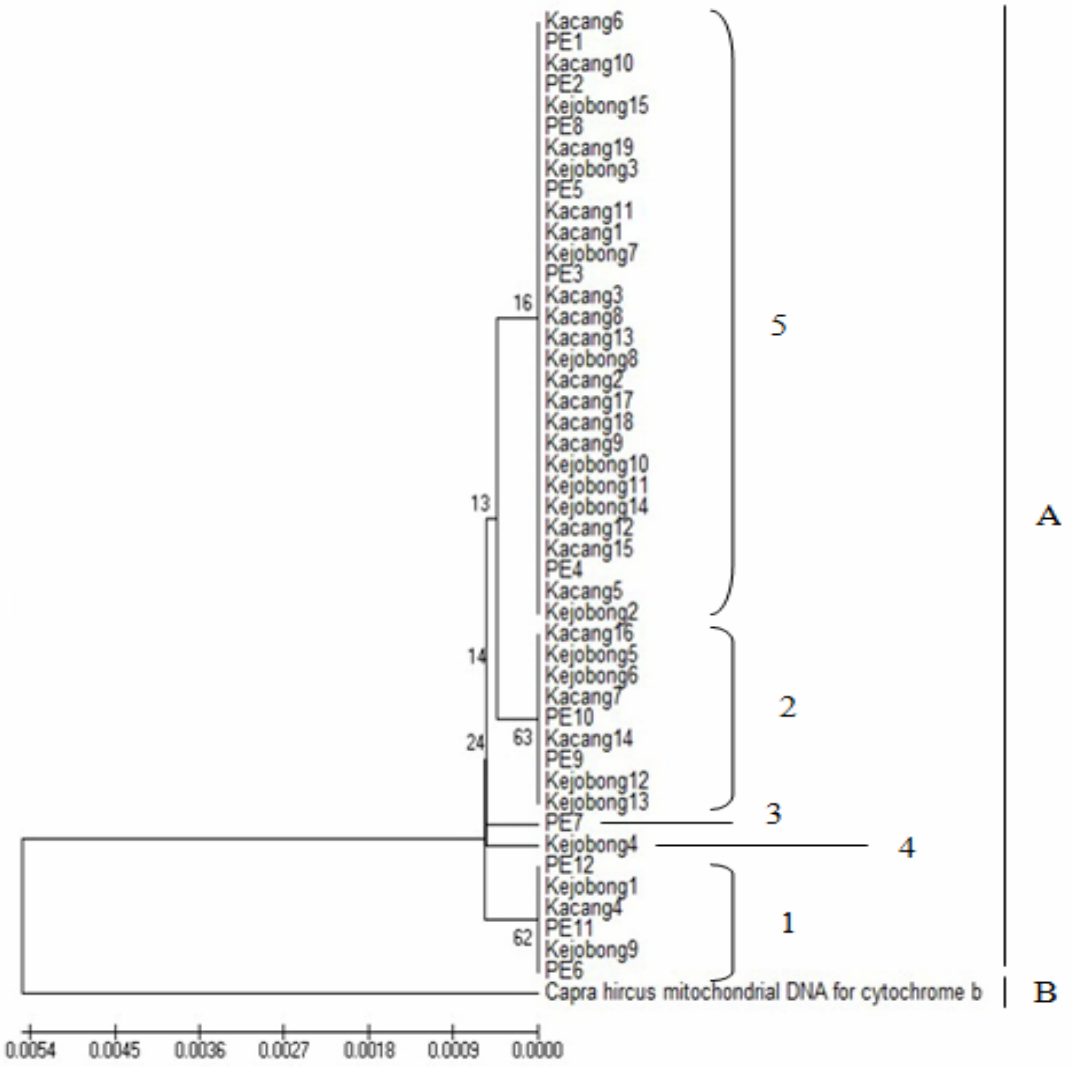

Figure 1 Phylogeny of three local goats and *C. hircus* based on *Kimura* 2-parameter model

Note: The evolutionary history was inferred using the UPGMA method. The optimal tree with the sum of branch length= 0.01297316 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. The analysis involved 47 nucleotide sequences. Codon positions included were 1^{st} + 2^{nd} + 3^{rd} + noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 1140 positions in the final dataset. Evolutionary analyses were conducted in MEGA 6.

Phylogeny tree showed two main clusters, that was cluster A for *C. hircus* and cluster B for three breeds of the local goat in Central Java. Cluster B consisted of 5 subgroups. The subgroup formed was not distinguish at each breed but follow the pattern formed by the haplotype number that caused by diversity in nucleotide sequence.

CONCLUSION

Of the 16 sites of different nucleotide bases there are 12 sites that could be used as a marker to distinguish the three breeds of local goat in Central Java, those are Kacang, Kejobong and Ettawa Grade, with the *C. hircus*. Genetic distance among local goat was quite close compared to genetic distance between those local goats to *C. hircus*.

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