RFLP Marker Variation of Cytocrome *b* Gene and Genetic Relationship among Batur, Merino and Local Sheep Breeds

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Abstract. RFLP analysis of mitochondrial DNA cytochrome *b* gene was conducted to determine the diversity, status and close genetic relationships in a population of Batur sheep with the Merino and local sheep breeds (Garut, Thin Tail and Fat Tail). The research used genomic DNA of 27 samples of Batur, 15 Merino, 17 Garut, 15 Thin Tails and 15 Fat Tails sheep. The PCR process used two types of 25 nucleotides primers. The PCR products were checked by using 2% agarose gel electrophoresis. The PCR DNA fragment was digested by using Hae III at 37 ^{o}C and incubated for 10 hours. Similarities and differences of cytochrome b gene RFLP bands between individual samples of one and across populations, genetic distance, and close genetic relationship, were identified. The PCR process of the cytochrome b gene metochondrial DNA of the 45 samples of sheep yielded 359 bp band types. The digestion (cutting) of the PCR products of mitochondrial DNA cytochrome b gene by using Hae III resulted in RFLP band profiles of 128 up to 231 bp polymorphisms of cytochrome b gene. Although the Hae III restriction enzyme recognized only one restriction site, however, between samples of Batur, Merino, Garut, Thin Tail, and Fat Tails, there were monomorphism and polymorphism Hae III loci.

Key Words: RFLP, cytochrome b gene, genetic markers, genetic similarity, Batur sheep

Introduction

Batur Sheep has similar morphological features as well as a combination of local sheep and Merino. Batur sheep genetic status and geneology are unknown because they do not have a clear pedigree record. Study with a specific genetic marker can be used to know the genetic status and geneology of Batur sheep. Identifications by morphological markers are difficult to differentiate homozygous and heterozygous individual, therefore, the results are not accurate (Prayitno et al., 2011). Analysis by using Polymerase Chain Reaction Restriction Fragment Length Polymorphism (PCR-RFLP) technique found out that there were variability (polymorphism) at the few sequence restriction site of mitochondrial DNA cytocrome *b* gen between samples of Batur, Merino, Garut, Thin Tail and Fat Tails sheep. The specific restriction site can be used as a genetic marker for the identification of genetic status and to reveals (track) the geneology of individual in Batur, Merino, Garut, Thin Tail and Fat Tail sheep populations.

Molecular genetic has proven highly informative to investigate the relathionships between animal populations as well as to document the level of genetic variation resident within breed (Meadows et al., 2005). One lineage or clade of sheep is able to regenerate and to change the characteristics of the sheep, due to the process of adaptation to climatic conditions, feed, and resistance to disease. The lineages of sheep are moved from one to another area in order to improve the genetic quality (grading up) that can change the characteristics of sheep in an area. Offsprings from crosses of two or greater lineages can increase the genetic diversity of local lineage of sheep (Maijala, 1997). A separate approach has focused on maternal origins of both domestic sheep and their candidate progenitors via

certain analysis of the mithochondrial genome (Meadows et al., 2005). Mitochondria are organelle cells that contain DNA. In mammals, mitochondrial DNA is maternalist, therefore, it can be used to reveal (track) maternal ancestors who generate species, breed, lineage, clade or strains of individuals in the population. Inter-species of mammals have a sequence variety of cytochrome *b* gene, thus, it can be used to study the relationship closeness between the species of mammalian phylogeny. Sequence variety of the cytochrome *b* gene is useful to compare the studies between species of genus or family. Researches of polymorphisms of cytochrome *b* gene are widely used to study the classification and phylogenetic relationships between species (Mathee and Robinson, 1999). Study of the cytochrome *b* phylogeny is able to find out the differences between new species in one genus.

RFLP analysis can utilize the fragments of DNA from the results of mitochondrial DNA gene amplification of unknown nucleotide composition. Hienleder et al. (1998) identify and analyze the mitochondrial DNA of local sheep of five lineages from Europe, one from Africa and four from Asia to determine the phylogenetic relationship with the Mouflon (*Ovis musimon*), Urial (*Ovis vignel bochariensis*) and the Argali (*Ovis Ammon nigrimontena*). Meadow et al. (2005) identify and analyze the sequence of mitochondrial DNA in local sheep from India, Indonesia, Mongolia and Tibet as well as from Austria, Finland, Spain and Russia to reveal the gene flow between Asia and Europe clades of sheep. Jenifer et al. (2007) analyze mitochondrial DNA sequences to determine phylogenetic relationships closeness of local sheep in Turkey and Israel. Prayoga and Permana (2009) analyze the mitochondrial DNA to evaluate the genetic of Priangan sheep.

Materials and Methods

A research was conducted at the Laboratory of Biotechnology Study Center Gadjah Mada University. Approximately 3 ml blood sample was taken via jugular viena sheep population Batur, Merino, Garut, Thin Tail and Fat Tail of each are nine. Isolation of genomic DNA was phenol-chloroform methode was used with a protocol adopted from the method Sambrooke et al. (1989) modified. DNA pellets were washed twice with phenol-chloroform and presipitated using cold, absolute etahnol. After drying at room temperature and then dissolving in 100 mL TAE buffer, the DNA was checked by using 2% agarose gel electrophoresis. The concentration and purity of DNA was measured with a spectrophotometer at the wavelengths of 260 and 280 nm. The DNA then was stored at a temperature of -20 ^{0}C until ready to use for PCR-RFLP analysis. The amplification of mitochondrial-DNA cytochrome *b* gene used two types of primers, *Forward Cyt* b_1 5'-CCATCCAACATCTCAGCATGATGAA-3' (Ahmed and El-Mezawi, 2005), and *Riverse Cyt* b_2 5'-GCCCCTCAGAAT GATATTTGT CCTCA-3' (Ahmed and El-Mezawi, 2005). The PCR process used 25 µl final volume of 12.5 µl which consisted of Royal Mega Mix PCR Mix (Microzon, UK), 2 µl of two types primers (100 picogram/µl) and 1 µl of DNA sample (50 nanograms/µl) and 9,5 µl dH_2O. The PCR process of mitochondrial DNA cytochrome *b* gene was started with initial denaturation at 94 ^{0}C (2 min), rotated at 35 cycles with denaturation at 95 ^{0}C (30 sec), annealing at 53 ^{0}C (73 sec), initial polymerization at 72 ^{0}C (84 sec) and final polymerization at 72 ^{0}C (7 min). The PCR products were checked by electrophoresis with 2% agarose gel at a voltage of 5 volts cm^{-1}. The PCR products of the cytochrome *b* gene was digested with restriction endonuclease *Hae III* according to the modified standard procedure of Reis et al. (2001). A 200 µl-capacity tube was filled with 10 µl PCR products of the cytochrome *b* gene, 1 µl *Hae III* endonuclease enzyme (1 unit/µl), 1.5 µl buffer and 2.5 µl dH_2O. The mixture was incubated in a waterbath at a temperature of 37 ^{0}C for 10

hours. The products were digested (cut) using *Hae III* and then were separated by electrophoresis 2% agarose gel with a voltage of 5 volts cm^{-1}. After staining with ethdium bromide RFLP, the band profiles of mitochondrial DNA cytochrome *b* gene were observed in agarose gel and documented with a digital camera (5.0 Mpix M Zoom, Made in China). The size of RFLP DNA fragment bands of mitochondrial DNA cytochrome *b* gene was identified with DNA ladder from 100 to 3000 bp. This method was used to identify similarities and differences in the number, pattern and bands type between sheep samples for the determination of monomorphism and polymorphism of RFLP profile, in order to calculate the similarity of the bandsharing frequency of RFLP bands between the individuals in one and across populations. Bandsharing frequency of RFLP bands between the individuals across population was used to analyze the genetic similarity between individual samples and genetic distance across populations.

Genetic similarity between individual within population was estimated based bandsharing frequency by Lync and Maligan (1994) in Smith et al., (1996) usingthe formula:

$$B_{ab} = \frac{2B_{ab}}{(b_a + b_b)}$$

B_{ab} are everage bandd that similar a and b individual, whereas b_a and b_b the number of bands on a and b individual. 2 B_{ab} are number of bands that similar in a and b individual sample was compared.

Genetic similarity between individual cross population was estimated based bandsharing frequency by Lycnh (1990) using the formula:

$$B = 1 + B_{xy} - 0{,}5\,(B_xB_y)$$

Where, B_x and B_y are everage band that similar between individual within x and y populations. B_{xy} are everage band that similar between individual cross x and y population that compared

Bandsharing frequency were across population used to estimate genetic similarity and also calculated genetic distance between population. Genetic distance was estimated bya Lynch (1991) dalam Smith et al., (1996) using the formula:

$$D_{xy} = -\ln B_{xy} \left(\frac{B_{xy}}{(B_yB_y)^{0,5}} \right)$$

Where as D_{xy} are genetic distance, B_{xy} are average band that similar between individual cross x and y population, B_x and B_y are average band that similar between individual within x and y population compared. Genetic distance value were used to cluster analysis by Unweighted Pair Group Method Using Arihtmatic Average (UPGMA) for know closer genetic relationship between cross Batur, Merino, Garut, Thin Tail and Fat Tail shepp population.

Results and Discussion

The cytochrome *b* gene RFLP band profile of five breeds of sheep

Amplification of the mitochondrial DNA cytochrome *b* gene of Batur, Merino, Garut, Thin Tail and Fat Tails samples of sheep produced only 359 bp band (Figure 1). The PCR products band profile of mitochondrial-DNA cytochrome *b* gene from the sample populations of five breeds was monomorphism. Although aproximately 359 bp DNA fragments from all samples after the digestion with *Hae III* produced two fragment types only, however, between samples showed polymorphisms RFLP band profiles (Figure 2). The RFLP band profile of mitochondrial-DNA cytochrome *b* gene was generated from 45 samples, Batur were 141 and 218 bp (2 heads), 128 and 233 bp (7 heads), Merino 128 and 233 bp (9 heads), Garut 128

and 233 bp (2 heads), 138 and 221 bp (1 head), 241 and 218 bp (6 heads), Thin Tail 132 and 227 bp (1 head), 141 and 218 bp (8 heads), while the Fat Tails was 132 and 227 bp (9 heads). Based on the RFLP band profile of cytochrome *b* gene between samples, despite the *Hae III* restriction endonuclease enzyme recognized only one restriction site, however, there were monomorphism and polymorphism loci between the samples of Batur, Merino, Garut, Tail Thin and Fat Tails have *Hae III* sheep.

Referring the similarity of the RFLP band profile of mitochondrial-DNA cytochrome *b* gene between samples of Batur Merino, Garut, Thin Tail and Fat Tail sheep, it was assumed that the individual in Batur population was generated from a cross ancestors of female Merino and male Thin Tail, male and female ancestors of Merino or female Thin Tail and male Merino, whereas individuals in the population of Merino were generated from ancestors (parents) of female and male Merino breed. Individuals in Garut population were descended from crosses of male Merino to female Thin Tail, male Thin Tail to Merino and crossess between male Thin Tail or Merino with unidentified female parents. The individuals in the Thin Tail population were descended from crossbreedings between male Thin Tail and female Fat Tail, and between males of Thin Tails. Presumably most of the individuals in the population of Fat Tail were descendants of male and female Fat Tail's ancestors.

Stephen et al. (2009) state, increasing the genetic quality of clade, strains or breeds of local livestock requires knowledge of genetic differences or phenotypic characteristics between clade or breeds. One way to compare the native sheep in one area (ecotype) is to evaluate the spread (distribution) of the local livestock between regions. If the distribution is known, it can be used to manage local conservation of animal genetic material.

Merino sheep samples for this study came from farms in the Dandenong District, the state of Victoria, Australia, therefore, it was possible that the sheep was the offsprings of the breeding that have been done in a controlled selection system. Maijala (1997) states that the Booroola Merino breed that is also commonly called the Australian Merino, is a medium sheep type that carries the ovulation rate gene, *FecB*. However, there is also a Booroola Merino, that is classifies as a distinct breed. The emergence and formation of Booroola Merino breed was started in 1945 by the Commonwealth Scientific and Industrial Research Oganisation (CSIRO) in a farm called Booroola. At the initial period of formation, the breed was generated from prolific, female Merino breed. Until the year of 1958 the selections of male and female offsprings were still in progress. The selected offsprings of Booroola Merino as the products of the CSIRO breeding program then were bred in New Zealand, the United States, Canada, Algeria, Egypt, Israel, European countries, Central America, South America and South Africa.

According to Markens and Soemirat (1926) Thin Tail sheep of Java, Garut strain, is the result of crossbreeding of Priangan local sheep, Merino and Kaapstad of Africa, in about the year of 1865. The Merino was imported from Australia in 1860 and was maintained in Garut district to produce wool, meat and manure. Some male Merino sheep were given to the Regent (the Duke) of Limbangan (Garut) and some farmers in Garut district, and then were crossed with local sheep. The Cross breed of Garut sheep from Priangan areas were further distributed to the western part of Java island. In general, African sheep population has the characteristics of thin tail, fat tail, or contracted fat (Mason and Mule, 1960). Qualitative natures of Garut sheep were short ears (*rumpung*) to moderate (*ngadaun hiris*), long neck feathers, thin caudate, coat colors varies from black, black and white with gray, white with little black, white, gray and gray with white, brown or brown and white black with

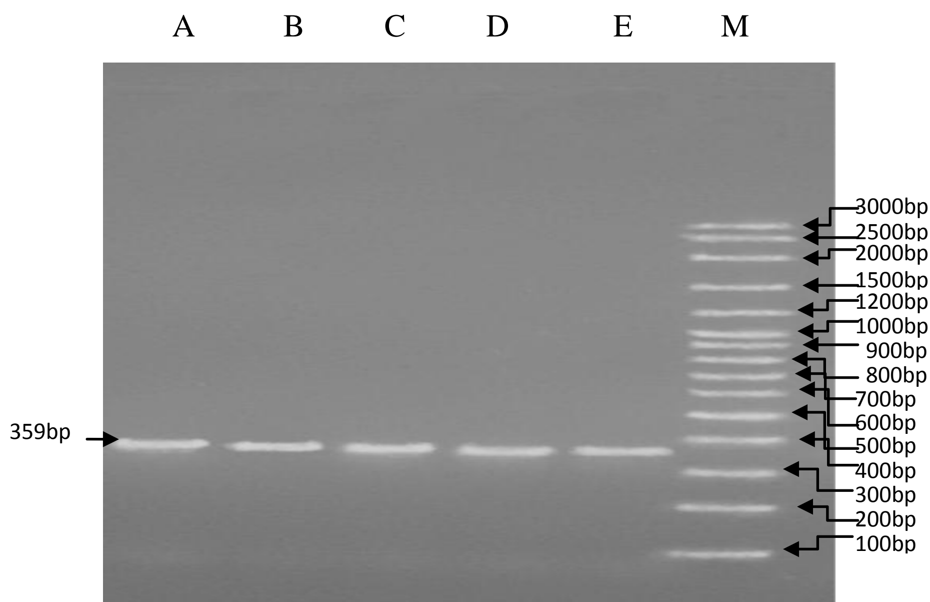

Figure 1. Band profiles of DNA fragments PCR product cytochrome *b* gene of mitochondrial DNA samples of sheep Batur (A), Merino (B), Garut (C), Thin Tail (D) and Fat Tail (E) and DNA Marker (M)

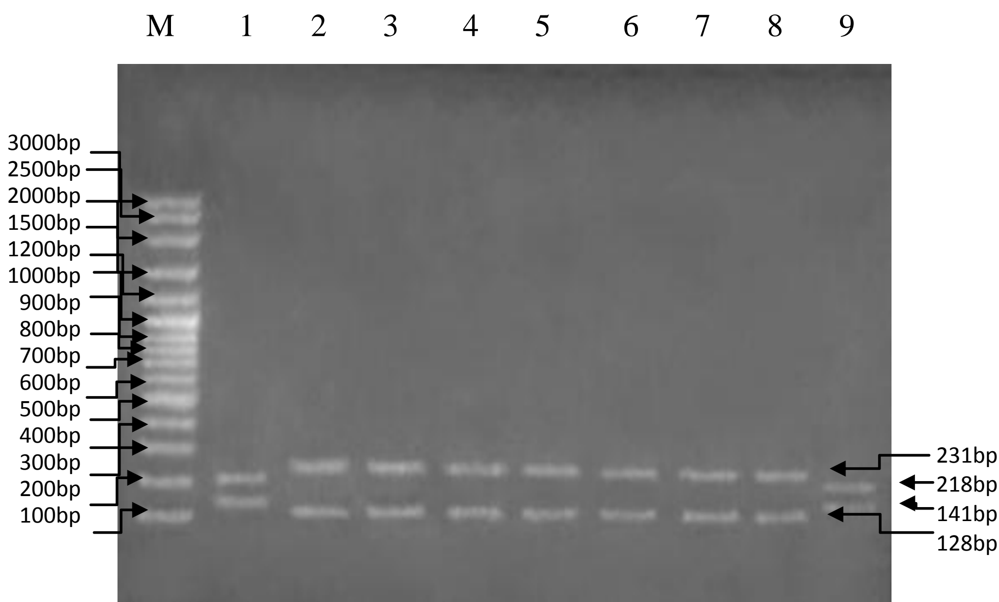

Figure 2. Sample of RFLP band profile of mitochondrial-DNA cytocrome *b* gene of Batur sheep digestion by restriction endonuclease *Hae III*

little white, black and white, gray, and white with black. Garut mitochondrial DNA amplification resulting in 1,447 bp DNA fragments. The results of DNA sequencing of PCR fragments of mitochondrial DNA indicated that there was a sequence variation of AT due to mitochondrial DNA mutations along the 75 base pairs, at position 15,790 up to 15,864 bp. The variation of DNA fragment size of mitochondrial-DNA PCR product indicated that Garut mitochondrial DNA deletion has occurred along the 75 base pair at position 15,790 up to 15,864 bp (Prayoga and Permana, 2009).

Uniform mitochondrial DNA was carried between individuals in the Fat Tail sheep population. It was expected because all the samples selected for this study had a common characteristic for the Fat Tailed sheep i.e long, thick tail, that are very easily distinguished among Batur, Merino, Garut and Thin Tail.

Genetic similarity between individuals one population Batur, Merino and Local sheep

The result of bandsahring freequency analysis of RFLP profile of mitochondrial-DNA cytochrome *b* gene between individuals within populations of Batur, Merino, Garut, Thin Tail, and Fat Tail sheep showed various values, ranged from 0, 54 to 1. The highest average value of similarity of the RFLP bandsharing frequency of mitochondrial-DNA cytochrome *b* gene was between individuals within populations of Merino or Fat Tail and the smallest was that of Garut sheep. Between individuals in the populations of Batur, Garut and Thin Tail, the value was lower than those of Merino and Fat Tail. Despite lower bandsharing frequency in Batur population than those in Merino, Fat Tails and Thin Tails, however, the value was higher than that of Garut. Bandsharing frequency of RFLP profile of cytochrome *b* gene between samples of Merino and Fat Tail suggested that all individuals in the two populations were descended from one breed female parent, Merino and Fat Tails, respectively. There were indications that the parents of individuals within populations of Batur, Garut and Thin Tail were from more than one strain or breed of female parent. There were indications that the female parent of individuals in the population of Batur and Garut were Merino and Thin Tail breed while the female parent of individuals in the population of Thin Tail were Thin Tail and Fat Tails breed. The results of a study by Meadows et al. (2005) reports that Thin Tail sheep of Java Garut strain showed 15 polymorphisms and haplotypes nucleotides with seven types of diversity level of 0.8. The average haplotype differences among individual of a population was 4.7%, with mitochondrial DNA nucleotide diversity of 2.11%. Tapio and Grigaliunaite (2002) analyzed the sequences in the control of mitochondrial DNA region of samples from various breeds; Thin Tail and Fat Tail of European origin, and they suggested that the genetic differences between populations was 27.84%.

Genetic similarity between individuals in cross-populations of Batur, Merino and Local sheep

The results of bandsahring freequency of RFLP profile of mitochondrial-DNA cytochrome *b* gene of between individuals across sheep populations of Batur, Merino, Garut, Thin Tail, and Fat Tail sheep showed various values. The average value of bandsharing frequency of mitochondrial-DNA cytochrome *b* gene was highest between individuals within populations of Batur and Merino and was smallest between individuals in Batur and Fat Tail, Thin Tail and Merino, Merino and Fat Tails, and also in Garut and Fat Tails. Inter-individuals across the populations of Batur and Garut, Batur and Thin Tail, Merino and Garut, Garut and Thin Tail, and also of Thin Tail and Fat Tail sheep, indicated lower bandsharing frequency of RFLP profile of cytochrome *b* gene than that of Batur and Merino. Bandsharing frequency of RFLP profile of cytocrom *b* gene across populations of Thin Tail and Garut was lower than that of Batur and Merino, but was higher than that of Batur and Thin Tail or Batur and Garut. These results illustrated that the majority of individuals in the population of Batur were regenerated from the female Merino breed and a small part was descendant of female Thin Tail. Whereas in Garut population, the majority of offsprings were generated from female Thin Tail, only smaller number of offsprings were generated from Merino. There were indications that some individual offsprings in Garut population were the descendants of female Merino, although it was of small proportions, in the range of 22 to 32%.

Tipio and Grigaliunaite (2002) analyzed the control region of mitochondria DNA from various breeds of Thin Tail and Fat Tails sheep of European origin and they indicated genetic differences between one breed of as much as 27.84%. The sequence of mitochondrial DNA samples from Asia origin found that there were 7 Thin Tail, Javanese, Garut strain sheep that carried mitochondrial DNA that was similar to haplotype B (sheep from Europe). If sheep are grouped according to geographic area of distribution in Asia or Europe, the largest sequence diversity was between breed of sheep, namely 81.5%, while the sequence diversity between the two geographic regions of distribution was only 2.7%. Sequence diversity between breed across geographic, distribution regions of Asia and Europe is 15.8% (Meadows et al., 2005).

Genetic distance among Batur, Merino, and local sheep populations

Analysis of genetic distance with frequency bandsharing RFLP profile of mitochondrial-DNA cytochrome *b* gene between individuals across the population samples of Batur, Merino, Garut, Thin Tail, and Fat Tail indicated various values of genetic distance. The smallest genetic distance between populations was that of Garut and Thin Tail, and the largest was between Batur and Fat Tail, Thin Tail with Merino, Merino with Fat Tails also Garut and Fat Tail. Batur populations showed the closest genetic distance with Merino, and Garut the loosest distance was with Thin Tail. Garut population had the closest genetic distance with Thin Tail and the loosest distance with Fat Tail. Batur population's genetic distance was closer to the Garut if compared toThin Tail, but Garut's was closer toThin Tail compared to Batur and Fat Tail. Merino population's genetic distance was the loosest to Thin Tail and Fat Tail. The closeness of genetic distance between Batur and Merino occurred because most samples of Batur sheep contained mitochondrial DNA of the female Merino breed, while the mitochondrial DNA from female Thin Tail was only found in smaller number of Batur sheep. There was no Mitochondrium from Fat Tail that was found in the population of Batur sheep, therefore, genetic distances was also very far away. Garut population genetic distance was closer to the Thin Tail if it was compared to the Batur and Merino, because most of the Garut samples (6 heads) contained mitochondrial DNA of female Thin Tail, while those that contained mitochondrial DNA from Merino were only two samples. Genetic distance of the Garut population was closer to the Thin Tail, Merino and Batur if compared with Fat Tail, because the samples of Garut and Batur indicated that they contained mitochondrial DNA from Thin Tail and Merino. In contrast, there was no indication of the existence of mitochondrial DNA from Fat Tail in the individual samples in the populations of Batur and Garut sheep.

Referring to similarities and differences in mitochondrial DNA content of individuals in the population Batur, Merino, Garut, Thin Tail and Fat Tail, it was suspected that individuals in the population of Batur was the result of crossbreeding of female Thin Tail with male Merino, or female and male parents of Merino. The genetic distance of the Garut population was closer to the Thin Tail if it was compared to the Merino, presumably most individuals in the population of Garut were regenerated from crossbreeding of female Thin Tail with male Merino, or female sheep that contained Merino's mitochondrial DNA that was mated with male Thin Tail, and crosses of female parents of unknown clade, breed or lineage with male Thin Tail or Merino.

The results of cluster analysis that used genetic distance values that were generated by unweighted Pair Group Method Arithmathic (UPGMA), obtained the smallest UPGMA value in the group of population Batur and Merino sheep and the largest of the group (Batur-Merino)-Garut-(Thin Tail-Fat Tail). Group Batur-

Table 1. Average value bandsharing frequency of the RFLP band cytochrome *b* gene mitochondrial DNA between individuals within population Batur, Merino and Local sheep the amplification results with two type of primer 25 nucleotides

	Sheep breeds				
	Batur	Merino	Garut	Thin tail	Fat tail
Bandsharing Frequency	0.57	1	0.54	0.66	1

Table 2. Average bandsharing frequency RFLP band cytochrome *b* gene of mitochondrial DNA between individuals across populations Batur, Merino and Local sheep

	Batur	Merino	Garut	Thin tail	Fat tail
Batur					
Merino	0.78				
Garut	0.32	0.22			
Thin tail	0.19	0.09	0.41		
Fat tail	0.07	0.08	0.10	0.11	

Table 3. Genetic distance based on average of bandsharing frequency value of RFLP band profile of mitochondrial-DNA cytochrome *b* gene across the population of Batur, Merino and Local sheep

	Batur	Merino	Garut	Ekor Tipis	Ekor Gemuk
Batur					
Merino	0,36				
Garut	0,56	1,23			
Ekor Tipis	1,50	3,44	0,16		
Ekor Gemuk	3,46	3,45	3,44	3,22	

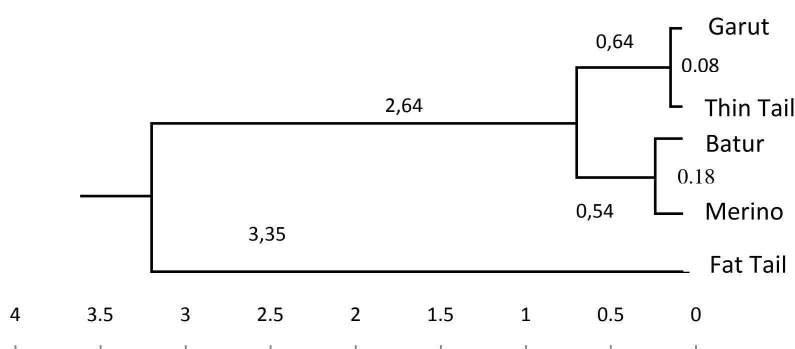

Figure 3. Phylogenetic tree genetic relationship of Batur with, Merino and Local sheep (Garut, Thin Tail and Fat Tail) based on RFLP profile cytocrom *b* gene mtDNA

Merino sheep population located (occupy) in the branch a cluster neighbor closest to the branch in dedogram of phylogeny tree. Thin Tail sheep populations occupying branches of a group adjacent with Fat Tails. Group of population Merino and Batur sheep forming branching adjacent to the Garut sheep (Figure 3). Referring to the pattern of branching in the dendogram phylogeny tree shows that the population of sheep Batur clade closely genetic relatedness (phylogenetic) to the population of Merino breed and way far apart genetic relatedness with a population of Fat Tails clads. The results of cluster analysis by UPGMA

indicated that the genetic relationships of most individuals of female parent in the Batur population was close to the Merino breed, whereas most individuals in the population Garut were descended from the females (ancestors) who had a close genetic relationship with the breeds of Thin Tail. The individuals in the populations of Batur and Garut were descended from the female Merino and Thin Tail, but there were few individuals in the population of Garut that were regenerated from African, Kaapstad sheep.

Evaluation of genetic diversity based on mitochondrial DNA may help to explain the differences in European and Asian local sheep. Phylogeny tree construction of many genes in mitochondrial DNA and the nucleus can support the hypothesis of two strains of female parent to be the ancestors of local sheep. It is further stated that the sequence of polymorphisms in the control region of mitochondrial DNA can be used as a basis to indicate that most species of sheep that are reared at farms are the offspring of two strains of females from Asia and Europe. The Urial mitochondrial DNA is very similar to samples of mitochondrial DNA haplotypes of native sheep of Europe, Africa and Central Asia. The results of phylogenetic analysis showed that Urial, Argali, and Mouflon are in a line with local sheep in Asia and Europe. The mitochondrial DNA sequence differences between that of Urial to that of Mouflon and local sheep were 5.59 and 6.97%, respectively. The shape of phylogeny tree showed that there was a branching in the European Mouflon, adjacent to the local sheep of Europe. The Asian local sheep formed a separate branch adjacent to Urial group. The differences of local sheep's haplotype mitochondrial DNA in Europe and Asia is estimated to have occurred from 375,000 to 750,000 years ago. The loss strains of sheep may happen because of genetic drift. (Hienleder et al. 1998).

Conclusions

Digestions (cuttings) of PCR products of mitochondrial-DNA cytochrome *b* gene of samples from populations of Batur, Garut and Thin Tail sheep by using *Hae III* restriction endonuclease enzyme generate polymorphism RFLP profiles, whereas RFLP profiles of samples from Merino and Fat Tail are monomorphisms. The genetic relationships of Batur sheep is closest to Merino and is far away apart to Fat Tail population. The individuals in the population of Batur sheep is suspected to be generated offsprings of cross breedings between female Thin Tail with male Merino sheep, and among the ancestor breeds of female and male Merino.

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