

Genetic Polymorphism of the Lactoferrin Gene in Dairy and Beef Cattles at National Artificial Insemination and Embryo Transfer Stations

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ABSTRAK

ANGGRAENI, A., G.E. MUMPUNIE, R. MISRIANTI dan C. SUMATRI. 2012. Polymorfisme genetik gen lactoferrin pada sapi perah dan potong di stasiun inseminasi butan dan embrio transfer nasional. *JITV* 17(4): 251-257.

Lactoferrin (LTF) adalah gen pengontrol komponen protein susu dan memiliki karakteristik sebagai antimikrobal. LTF pada susu berfungsi untuk mencegah diare, sedangkan pada sapi laktasi untuk mencegah mastitis pada ambing. Mempertimbangkan peran penting dari gen LTF, maka perlu dilakukan peningkatan kadar LTF dalam susu melalui seleksi pada taraf DNA. Polymorfisme genetik dari gen LTF diidentifikasi pada sapi perah dan potong dengan metoda *Polymerase Chain Reaction - Restriction Fragment Length Polymorphism* (PCR-RFLP), dengan enzim restriksi EcoRI. Genotyping dilakukan pada sapi perah Friesian Holstein (FH) total sejumlah 89 ekor, meliputi dari Balai Inseminasi Buatan Lembang (BIB Lembang) untuk 17 pejantan, Balai Besar IB Singosari (BBIB Singosari) untuk 32 pejantan, dan Balai embrio Transfer Cipelang (BET Cipelang) pada 40 dara. Genotyping dilakukan pula pada sapi potong dara berasal dari empat bangsa, meliputi Limousin (14 ekor), Angus (5 ekor), Simmental (13 ekor) dan Brahman (5 ekor) dari BET Cipelang. Gen LTF|EcoRI pada sapi perah dan potong pengamatan menghasilkan dua tipe alel, yaitu alel A dan B. Kedua jenis sapi tersebut menghasilkan hanya dua genotipe, yaitu genotipe AA dan AB, tanpa genotipe BB. Ini dapat menjadi hal yang baik karena genotipe AA dan AB dipertimbangkan berasosiasi dengan ketahanan pada mastitis. Nilai-nilai dari heterozygositas observasi (Ho) dari gen ini lebih tinggi dibandingkan heterozygositas ekspektasi (He). Disimpulkan bahwa gen LTF|EcoRI memiliki variasi yang baik pada sapi perah dan sapi potong dari ketiga balai bibit nasional tersebut.

Kata Kunci: Polymorfisme genetik, Friesian Holstein, gen Lactoferrin, PCR-RFLP

ABSTRACT

ANGGRAENI, A., G.E. MUMPUNIE, R. MISRIANTI and C. SUMATRI. 2012. Genetic polymorphism of the lactoferrin gene in dairy and beef cattles at national artificial insemination and embryo transfer stations. *JITV* 17(4): 251-257.

Lactoferrin (LTF) is a gene controlling milk protein components and having the characteristic as antimicrobial. LTF in milk has the function to prevent diarrhea, whereas its function in a cow is to prevent mastitis in udder(s). Considering the important roles of the LTF gene, improving LTF contain in milk through selection at the DNA level is necessary. Genetic polymorphism of the LTF gene was identified in dairy and beef cattles by Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR-RFLP) method using an EcoRI restriction enzyme. Genotyping was performed in Holstein Friesian (HF) dairy cattle for a total number of 89 heads, respectively from Lembang AI Station (Lembang AIS) by 17 bulls, Singosari Excellent AIS (Singosari EAIS) by 32 bulls, and Cipelang Embryo Transfer Station (Cipelang ETS) by 40 heifers. Genotyping was also conducted for four breeds of heifer beef cattle, providing Limousin (14 heads), Angus (5 heads), Simmental (13 heads) and Brahman (5 heads), from the Cipelang ETS. The LTF|EcoRI gene in the observed dairy and beef cattles resulted two types of alleles, i.e. A and B alleles. Either dairy and beef cattles had only two genotypes, i.e. AA and AB genotypes, without BB genotype. It could be a good thing as the AA and AB genotypes considerably be associated to mastitis resistance. The values of heterozygosity observation (Ho) of this gene were higher than those of heterozygosity expectation (He). It was concluded that a good variation in the LTF|EcoRI gene in either HF cattle or beef cattles from these three national breeding stations.

Key Words: Genetic Polymorphism, Holstein-Friesian, Lactoferrin Gene, PCR-RFLP

INTRODUCTION

National milk consumption has continually increased along with the increasing public awareness for the important need of drinking milk. Milk is rich in

nutrients, protein is one of them that is very useful for the body. High protein contents in milk will improve the quality of milk, not only as a functional food but also as a medical food. Milk protein in dairy cattle consists of two main components, namely casein and

whey protein. Whey protein contains lactoferrin, lactoperoxidase, lysozyme, and immunoglobulin, and others that are often referred to as anti-microbial proteins (ADLEROVA *et al.*, 2008).

Lactoferrin consists of a single polypeptide chain containing 703 amino acids folded into two globular lobes. These lobes, also called C – (carboxy) and N – (amino) terminal regions, are connected with a α -helix. Each lobe consists of two domains known as C1, C2, N1, and N2. The domains create one iron binding site on each lobe. Lactoferrin molecules contain varying numbers of sites for potential glycosylation, mostly on the surface of the molecule. The most common saccharide is mannose providing hexoses for around 3% and hexosamines for around 1%. Lactoferrin as a minor protein is naturally found in dairy cattle milk. Large quantities of lactoferrin are found in mammalian secretions, such as milk, tear, saliva, and seminal fluid; as well as in some white blood cells (ADLEROVA *et al.*, 2008). Lactoferrin in dairy cattle has a main function directly related to the immune system and has an important role to prevent and control mastitis (TENG, 2002). Lactoferrin binds iron (Fe) ions from microbes, so their growth can be inhibited (SACHACZUK *et al.*, 2005). In relation to this, an investigation on lactoferrin concentrations in the milk with different levels of the somatic cell count score were examined for the Chinese Holstein cows (198 heads). The result proved that milk lactoferrin concentration tended to be correlated with the somatic cell count score ($r = 0.375$). The finding suggested that milk lactoferrin could be useful indicator for intramammary infection in dairy cows (CHENG *et al.*, 2008).

Genetic polymorphism of the lactoferrin gene was found by Arnould *et al.* (2009) at exon 2, exon 11 and intron 8. Seyfert and Kuhn (1994) using the EcoRI restriction enzyme identified a point mutation at the intron 6 of this gene that consisted of two alleles, namely A and B, with the respective frequencies of 0.755 and 0.245. WOJDAK *et al.* (2006) also found a similar mutation at the intron 6, that resulted three genotypes successively AA (37.9%), BB (2.4%), and AB (59.7%). It was pointed out that lactoferrin level was significantly affected by a genetic factor, of which cows having AA genotype had a higher level of lactoferrin than those of cows with AB and BB genotypes. Therefore, cows with the AA genotypes were resistant to mastitis infection (CHANGHONG *et al.*, 2009). It was also confirmed that the lactoferrin gene was inherited in codominant and following a Mendel law (LEE *et al.*, 1997).

Due to the importance of the lactoferrin in inhibiting the growth of microbes, increasing lactoferrin in dairy cattle should be attempted. One of breeding method that can be done is through a selection process. Advances in molecular biotechnology enable selection has been done

until at DNA level, by finding the composition of nucleotide(s) of the lactoferrin gene suspected of affecting a high level of lactoferrin. The lactoferrin gene can be functioned as one of genetic markers of somatic cell concentration in milk, which is used as a marker of susceptibility or resistance to mastitis in dairy cows (WOJDAK *et al.*, 2006; JEMMALI *et al.*, 2011).

Study on genetic polymorphisms of the lactoferrin gene was therefore required for dairy cattles as breeding stocks to know their potency in producing offspring with the AA genotype that is expected of having resistance to mastitis. Investigation was also done for some beef cattle breeds as breeding stocks to get comparative information on genotype variants of this gene. This study therefore aimed to identify genetic polymorphism of the LTF|*EcoRI* gene by Polymerase Chain Reaction-Restriction Fragment Length Polymorphisms (PCR-RFLP) method in dairy and beef cattle at three National Cattle Breeding Stations.

MATERIALS AND METHODS

Blood Samples

Blood samples for DNA extractions were collected via jugular vein using a venoject needle in vacutainer tubes, then added by alcohol 70%. Blood samples were collected from Holstein Friesian (HF) dairy cattle for a total number of 89 heads. These HF cattle included bulls from Lembang Artificial Insemination Station (Lembang AIS) by 17 heads and Singosari Excellent AIS (Singosari EAIS) by 32 heads; as well as HF females from Cipelang Embryo Transfer Station (Cipelang ETS) by 40 heads. Blood samples were collected also from female beef cattle in Cipelang ETS for a total number of 37 heads. These beef cattle consisted of four beef cattle breeds providing for Limousin by 14 heads, Angus by 5 heads, Simmental by 13 heads, and Brahman by 5 heads. Blood collections were conducted during 2008 - 2009.

DNA extraction

DNA extraction was conducted during June to November 2010 at Molecular and Genetic Lab., Dept. of Science and Technology of Animal Production, Fac. of Animal Sciences, Bogor Agricultural University. DNA extraction was initially processed in a way that 200 μ l blood sample was added by 1000 μ l destillation water (DW), then centrifuged at a speed of 8000 rpm for 5 minutes. Formed supernatant was discarded, added by 10 μ l-proteinase K, 350 μ l 1xSTE (sodium tris-EDTA), and 40 μ l 10% SDS (sodium dodecyl sulfate), then incubated at 55 °C for 2 hours.

DNA molecules formed were purified by adding 40 µl 5M NaCl, 400 µl phenol solution and CIAA (chloroform iso amyl alcohol). DNA molecules were then separated from phenol by centrifugation at the speed of 12,000 rpm for 5 minutes. 40 µl of DNAs were added by 40 µl 5M NaCl and 800 µl absolute ethanol. DNA molecules then were left overnight at a temperature of -20°C.

DNA molecules centrifuged at 12,000 rpm for 5 min, available supernatants were then removed. The formed DNA precipitation was washed by adding 800 µl 70% ethanol, then centrifuged at 12,000 rpm for 5 minutes. The precipitated DNA molecules next were allowed to dry, then suspended in 100 µl 80% of TE buffer (tris EDTA).

DNA amplification

Primers used for amplification of the lactoferrin gene followed the study of WOJDAK *et al.* (2006) as following:

forward: 5'-GCC TCA TGA CAA CTC CCA CAC-3';

reverse: 3'-CAG GTT GAC ACA TCG GTT GAC-5'.

Reagent for the DNA amplification was generally performed using a DNA mixture consisting of 1 µl extracted DNA sample, 9.7 µl DW, 0.1 µl primer, 0.05 µl taq polymerase, 1.25 µl buffer, 0.15 µl dNTP and 0.25 µl MgCl. The mixture then was inserted into a PCR machine at a predenaturation temperature of 94°C for 5 min., 35 cycles (denaturation temp. of 94°C for 45 sec., annealing of 60°C for 45 sec. and elongation of 72°C for 1 min.) and for a final elongation of 72°C for 5 minutes.

Electrophoresis

Electrophoresis was carried out by using 5 µl PCR product on 1.5% agarose gel at 100 volts for 30 minutes. Gel was prepared by heating 0.45 g agarose, dissolved in 30 ml 0.5 xTBE solution, added by 2.5 µl EtBr during steaming process until a clear solution resulted. Liquid solution was poured into a printer and a comb was placed near the gel edge. The gel was then allowed to harden. After that, by removing the comb, 5µl PCR product was put in the formed wells of the gel, then mixed with loading dye (0.01% bromthymol blue, 0.01% Xylene cyanol and 50% glycerol).

The gel was placed into the electrophoresis gel tray already containing a buffer solution, then electricity was passed through. The DNA molecules with a negative charge, at neutral pH, moved to the positive one (anode). After electrophoresis was completed, the agarose gel was taken to determine the length(s) of the DNA band(s) using ultraviolet light, ie by drawing a

straight line between the band position of each DNA sample to the band position of the DNA marker.

Genotyping

Restriction Fragment Length Polymorphism (RFLP) was performed from which 5 µl PCR products were added by 1 µl DW, 0.3 µl EcoRI restriction enzymes and 0.7 µl buffer in a 0.5 ml tube. The mixture was incubated in an incubator at 37 °C for 16 hours. DNA samples were cut by the EcoRI enzymes, then electrophoresed on 2% agarose gel in a voltage of 100 volts for 30 minutes. After electrophoresis was completed, the agarose gel was taken to see the length(s) of the DNA band(s) using ultraviolet light, then compared with the marker. A similar migration position was considered as one type or one allele. Those fragmented DNAs were pictured for their patterns.

Frequencies of allele and genotype:

Genotype frequency was calculated by dividing the number of a particular genotype to overall samples, whereas allele frequency was the ratio between a particular allele to all alleles at the observed locus (NEI, 1987):

$$x_{ii} = \frac{n_{ii}}{N} \quad x_i = \frac{2n_{ii} + \sum n_{ij}}{2N}$$

Description:

x_{ii} = the ii^{th} genotype frequency

x_i = the i^{th} allele frequency

n_{ii} = individual number of the ii^{th} genotype

n_{ij} = individual number of the ij^{th} genotype

N = total number of individual samples.

Heterozygosity

Genetic diversity can be determined by comparing the value of observed heterozygosity (H_o) obtained from each population (Weir, 1996) to the value of expected heterozygosity (H_e) obtained based on allele frequencies (Nei and Kumar, 2000), as the following:

$$H_o = \sum_{i=j} \frac{n_{ij}}{N} \quad H_e = 1 - \sum_{i=1}^q x_i^2$$

Description:

H_o = observed heterozygosity (population)

H_e = value of expected heterozygosity

n_{ij} = number of heterozygous individuals

x_i = frequency of homozygous allele

N = number of individuals observed

q = number of allele

RESULTS AND DISCUSSION

Amplification of the LTF|*EcoRI* Gene

Amplification of the LTF gene by PCR method, following the work of Wojdak *et al.* (2006), from the results of this study was successful (100%). Amplification of 126 DNA samples from all of dairy and beef cattles resulted fragment lengths of 301 bp. The success of amplification of the LTF|*EcoRI* gene was determined by some factors, such as annealing temperature, Mg²⁺ concentration, primer concentrations, and targeted DNA concentration. An annealing position at nucleotide sequences of the intron 6 LTF|*EcoRI* gene, based on Gene Bank Access No. L19986.1, was shown in Figure 1.

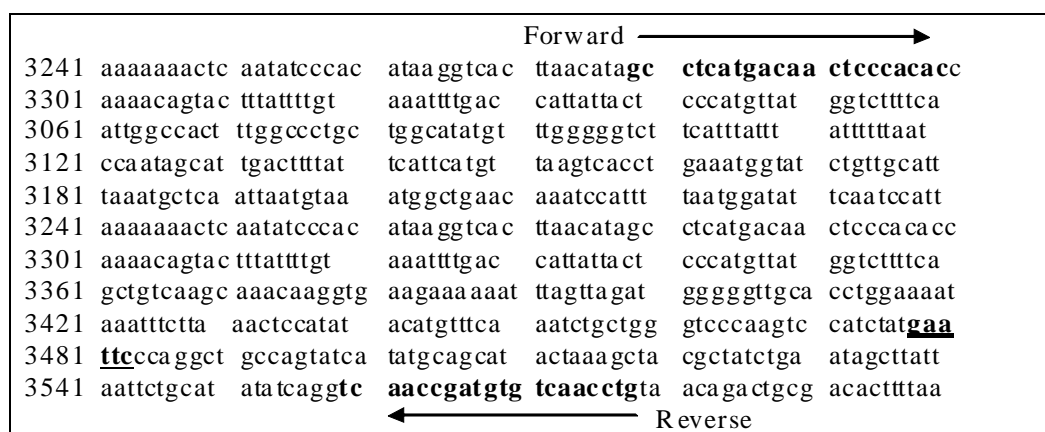
Polymorphism of the LTF|*EcoRI* gene

Detection of genetic polymorphism of the LTF gene by PCR-RFLP method using the *EcoRI* restriction enzyme cut the nucleotide sites at the 5'... G* AATTC ... 3'. The PCR products produced three types of DNA fragments, with the lengths of 100 bp, 201 bp, and 301 bp (Figure 2). Results from this study was similar to the study by Wojdak *et al.* (2006) that described for the achieving one DNA fragment with 301 bp length was identified as an A allele; while that for two DNA fragment lengths of 201 bp and 100 bp was identified as a B allele. Genotype variation of the LTF|*EcoRI* gene in HF and beef cattle observed was due to mutation, ie changes in a chemical structure of a gene leading to the changes in gene function. Mutation in the LTF gene studied was a kind of substitution mutation, ie changes

between purine-bases (AG) or between pyrimidine-bases (TC) (Figure 2).

Frequencies of genotype and allele of the LTF|*EcoRI* gene

Frequencies of genotypes and alleles of the LTF|*EcoRI* gene in HF cattle from Lembang AIS, Singosari EAIS and Cipelang ETS were listed in Table 1. The results in general showed that both HF dairy cattle and each beef cattle breed from each national breeding station produced only two types of genotypes, namely AA and AB genotypes with varying frequencies. Conversely none of BB genotype was found. By looking solely into dairy cattle, it showed that the highest frequency for the AA genotype was in HF cattle from the Cipelang ETS (0.72), whereas that for the AB genotype was in HF cattle from the Lembang AIS (0.76). For beef cattle, the highest frequency for the AA genotype was in Brahman cattle (0.60), while that for the AB genotype was in Angus (1.00). Further by considering both types of cattle, the frequencies of the two genotypes between HF dairy cattle and beef cattle were inversely proportional. In HF dairy cattle, the AA frequency was greater than the AB one (0.55 vs 0.45). Whilst in beef cattle, a higher frequency was found for the AB genotype than the AA genotype (0.62 vs 0.38). Based on these results, it could be expected that HF bulls and heifers from the two AISs and ETS would provide a higher probability of having the offsprings with the AA genotype (vs AB), but the opposite condition would be the case in beef cattle heifers from the ETS. However, the total frequencies of the AA and AB genotypes for the joining dairy and beef cattles were equal (0.50 vs 0.50).



Description:

Mutation occurrence at the cutting site of *EcoRI* restriction enzyme (GAATTC) at position 3419 (Intron 6)

A allele : 5'.....CATCTATG**A**TTCCAGGCT.....3'

B allele : 5'.....CATCTATG**A**TTCCAGGCT.....3'

Figure 1. Annealing position of the Primers (bold letters) at the sequences of the LTF| *EcoRI* gene (Gen Bank L19986.1 Access Number)

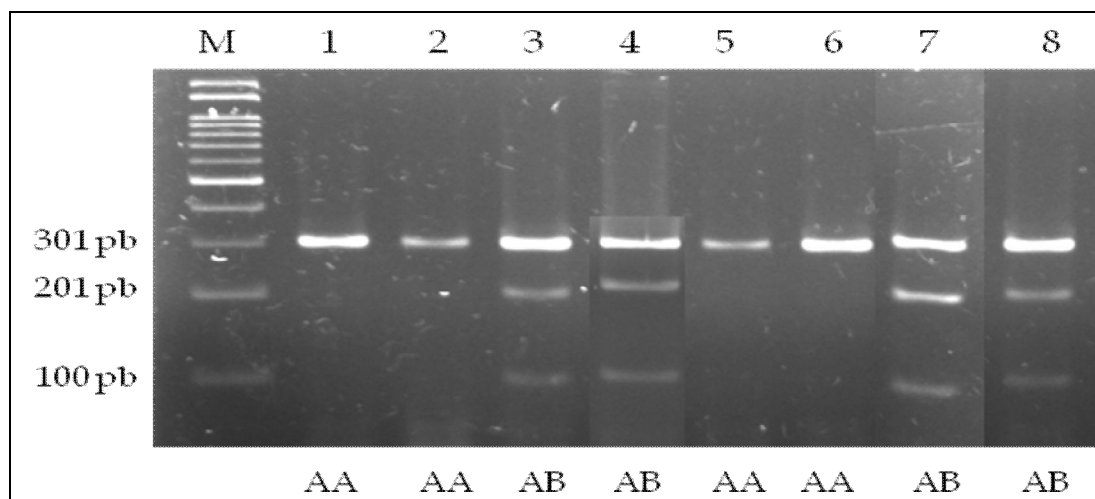


Figure 2. DNA band patterns at the intron 6 of the LTF gene cutting by the EcoRI restriction enzyme in HF cattle on 2% Agarose Gel. AA genotype for no. 1, 2, 5, and 6; AB genotype AB for no. 3, 4, 7 and 8, and M for marker

Total genotype frequencies of the LTF|*EcoRI* gene in HF dairy cattle from the two AISs and ETS resulted the frequencies of the AA and AB genotypes respectively 0.55 and 0.45, whilst for the BB genotype was 0. The same results were also found in all beef cattle observed. An absence of the BB genotype indicated that the HF bulls used at the National AISs and HF females at ETS carried more the AA and AB genotypes compared to the BB genotype. Previous studies such as by WOJDAK *et al.*, (2006), ADLEROVA *et al.* (2008), and Jemmali *et al.* (2011) noted a tendency of having a good resistance to mastitis for the AA and AB cows than the BB one. Based on that information, therefore it could be a good condition that both the observed HF bulls and heifers from these two national AISs and ETS, as well as the observed beef heifers from the ETS did not carry the BB genotype, that was associated with the mastitis susceptibility. The HF dairy bulls from the two AISs, through the AI mating of their frozen semen, as well as HF heifers and beef heifers from the ETS would produce offsprings with the AA and AB genotypes that are associated to mastitis resistance.

Frequency of an allele was a relative frequency of that allele in a population or number of an allele to the total number of alleles presented in a population (Nei and KUMAR, 2000). HF cattle from the joining three stations had the frequency of the A allele was higher than the B allele (0.77 vs. 0.23). Results from this study were not much different to some previous studies by finding a higher frequency of the A allele to the B allele, such as for HF dairy cattle in Poland (67,7% vs.

32,6%) (WOJDAK *et al.*, 2006) and HF dairy cattle in Iran (67,7% vs. 32,3%) (SHARIFZADEH and DOOSTI, 2011).

By considering HF dairy cattle, the A and B alleles had the the frequencies respectively in the ranges from 0.62 to 0.86 and from 0.14 to 0.38. For beef cattle, the frequencies of the A allele were from 0.50 to 0.80, while those of the B allele were from 0.21 to 0.50. An allele can be stated polymorphic if that allele has the frequency equal to or less than 0.99 (NEI, 1987). Based on this criteria, so the LTF|*EcoRI* gene owned by all of HF dairy cattle and four beef cattle breeds observed was polymorphic.

Estimation of heterozygosity value of the LTF|*EcoRI* gene

Genetic diversity can be measured accurately from heterozygosity value (H) (NEI, 1987). Estimation of the values of heterozygosity observation (H_o) and expectation (H_e) of the LTF|*EcoRI* gene were listed in Table 2. For both HF dairy and beef cattles from each station, the LTF|*EcoRI* gene had the values of $H_o > H_e$. TAMBASCO *et al.* (2003) stated that in the condition of the $H_o < H_e$, it might indicate a high degree of mating within the group taking place as the result of an intensive process of selection. The values of $H_o > H_e$ in this study could be HF bulls in AISs, HF heifers in ETS and beef heifers in ETS had genetic diversity of the LTF|*EcoRI* gene a fairly high level, as all of these cattle were imported from the origin countries.

Table 1. Frequencies of genotype and allele of the LTF|*EcoRI* gene in dairy and beef cattles

Breed/Cattle	Location	Genotype			Allele	
		AA	AB	BB	A	B
Dairy Cattle						
HF (17 hd.)	Lembang AIS	0.24 (4)	0.76 (13)	0.00 (0)	0.62	0.38
HF (32 hd.)	Singosari EAIS	0.50 (16)	0.50 (16)	0.00 (0)	0.75	0.25
HF (40 hd.)	Cipelang ETS	0.72 (29)	0.28 (11)	0.00 (0)	0.86	0.14
Sub total		0.55 (49)	0.45 (40)	0.00 (0)	0.77	0.23
Beef Cattle						
Limousin (14 hd.)	Cipelang ETS	0.57 (8)	0.43 (6)	0.00 (0)	0.79	0.21
Angus (5 hd.)	Cipelang ETS	0.00 (0)	1.00 (5)	0.00 (0)	0.50	0.50
Simmental (13 hd.)	Cipelang ETS	0.23 (3)	0.77 (10)	0.00 (0)	0.62	0.38
Brahman (5 hd.)	Cipelang ETS	0.60 (3)	0.40 (2)	0.00 (0)	0.80	0.20
Sub total		0.38 (14)	0.62 (23)	0.00 (0)	0.69	0.31
Total (126)		0.50 (63)	0.50 (63)	0.00 (0)	0.75	0.25

Description: (..) = number of cattle

Table 2. Estimation of the values of heterozygosity observations (Ho) and heterozygosity expectation (He) in the LTF|*EcoRI* gene

Breed	Location	He	Ho
Dairy cattle			
Holstein Friesian	Lembang AIS	0.47	0.76
Holstein Friesian	Singosari EAIS	0.38	0.50
Holstein Friesian	Cipelang ETS	0.24	0.28
Subtotal	-	0.35	0.45
Breed Cattle			
Limmousin	Cipelang ETS	0.34	0.43
Angus	Cipelang ETS	0.50	1.00
Simmental	Cipelang ETS	0.47	0.77
Brahman	Cipelang ETS	0.32	0.40
Subtotal	-	0.57	0.62
Total	-	0.38	0.50

According to JAVANMARD *et al.* (2005) if heterozygosity value was lesser than 0.5 (50%), so it indicated a low variation of a gene in that population. HF cattle from Lembang AIS had the highest heterozygosity value (0.76), while in beef cattle, the highest heterozygosity value was in Angus cattle (1.00). The heterozygosity value that was lower than 0.5 in HF cattle was from the Cipelang ETS (0.28), while in beef cattle was for those of Limmousine (0.43) and Brahman

(0.47). Nevertheless, based on the higher values of Ho to He for the observed dairy and beef cattles in each station, it could be stated that all of these cattles had a high level of heterozygosity. These higher values of the heterozygosity observation indicated a good variation in the LTF|*EcoRI* gene in either HF cattle or beef cattles from these three national breeding stations.

CONCLUSION

The LTF|*EcoRI* gene in HF dairy and beef cattle (Brahman, Angus, Simmental, and Limmousin) observed from the national AIS and ETS were polymorphic. Both HF dairy and beef cattle had only the AA and AB genotypes, without the BB genotype. It could be a good case as the AA and AB genotypes considerably be associated to mastitis resistance. As the values of Ho > He indicated a good variation in the LTF|*EcoRI* gene in either HF cattle or beef cattles from these three national breeding stations.

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