κ-CASEIN GENE POLYMORPHISMS IN RIVERINE AND SWAMP BUFFALO IN INDONESIA

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ABSTRACT

κ-casein is known as a gene that plays a role in controlling milk protein and also play a crucial role in the coagulation and curdling of milk. This study was aimed to identify polymorphisms of the κ-Casein gene of local buffaloes in Indonesia. A total number of 40 heads of riverine buffalo and 250 heads of swamp buffalo. This study used PCR-RFLP method, which amplification of the κ-Casein gene resulted an amplicon with length of 157 bp, located in exon 4. The amplified fragment were digested with EcoRV restriction enzyme, which cut the κ-Casein gene in exon 4 at nucleotides of GAT|ATC, revealed the presence of one polymorphism at the base position of 23 bp that occurs with a substitution of Ile (ATC) of the T genetic variant into Thr (ACC) of the C genetic variant. Genotyping κ-Casein gene in riverine buffalo produced two types of allele, namely C allele (157 bp) and T allele (136 and 21 bp). These two alleles resulted in three types of genotypes, namely CC, CT, and TT. Frequency of the C allele was dominant to T allele. κ-Casein gene in swamp buffalo was monomorphic with one allele, namely C allele. Heterozygosity value of riverine and swamp buffaloes were low. PIC value in riverine and swamp buffalo ranged 0.000-0.288. Fixation index of κ-Casein gene in riverine buffalo was low (Siborong-borong SBBC = -0.0036; Deli Serdang = -0.025), but in swamp buffalo was in fixation. This study showed that κ-Casein|EcoRV were polymorphic in riverine buffalo and monomorphic in swamp buffalo.

Keywords: Riverine buffalo, swamp buffalo, κ-Casein|EcoRV, PCR-RFLP, polymorphisms

Kata kunci: Kerbau sungai, kerbau rawa, gen κ-casein|EcoRV, PCR-RFLP, keragaman genetik

Kappa-casein (κ-casein) gene is known as a gene that plays a role in controlling milk protein and also play a crucial role in the coagulation and curdling of milk. This study was aimed to identify polymorphisms of the κ-Casein gene of local buffaloes in Indonesia. A total number of 40 heads of riverine buffalo and 250 heads of swamp buffalo. This study used PCR-RFLP method, which amplification of the κ-Casein gene resulted an amplicon with length of 157 bp, located in exon 4. The amplified fragment were digested with EcoRV restriction enzyme, which cut the κ-Casein gene in exon 4 at nucleotides of GAT|ATC, revealed the presence of one polymorphism at the base position of 23 bp that occurs with a substitution of Ile (ATC) of the T genetic variant into Thr (ACC) of the C genetic variant. Genotyping κ-Casein gene in riverine buffalo produced two types of allele, namely C allele (157 bp) and T allele (136 and 21 bp). These two alleles resulted in three types of genotypes, namely CC, CT, and TT. Frequency of the C allele was dominant to T allele. κ-Casein gene in swamp buffalo was monomorphic with one allele, namely C allele. Heterozygosity value of riverine and swamp buffaloes were low. PIC value in riverine and swamp buffalo ranged 0.000-0.288. Fixation index of κ-Casein gene in riverine buffalo was low (Siborong-borong SBBC = -0.0036; Deli Serdang = -0.025), but in swamp buffalo was in fixation. This study showed that κ-Casein|EcoRV were polymorphic in riverine buffalo and monomorphic in swamp buffalo. Keywords: Riverine buffalo, swamp buffalo, κ-Casein|EcoRV, PCR-RFLP, polymorphisms
INTRODUCTION

Beside as a meat producer and worker, buffalo also a producer of milk. In Indonesia, the production of buffalo milk is very slight. Contrast, in India buffalo is an important milch animal as more than 60% of the total milk produced is buffalo milk (Patel et al., 2007). Buffalo is considered to be a better converter of fibrous feeds into milk, and to be more resistant to disease and local climatic condition. In Indonesia, there were very little work has been carried out on buffalo genetics that could give an effective implementation on breeding program. Nowadays selection program has come to molecular approach that can give fast and efficient result. Selection on molecular markers is more reliable than other methods (Othman, 2005). Progress in molecular technology allows selection to be done at molecular level and have already uncovered a large number of genetic polymorphisms at the DNA level which are being used as genetic markers.

Milk and milk products is important for feeding because supply nutrients, energy, high quality protein, vitamins and mineral requirements. The composition of the milk of different species varies in the percentages of these constituents. All milks contain the same constituents, but in varying amounts (Olivario et al., 2005). Milk proteins are usually divided into two fractions. The first fraction is soluble fraction, named whey protein, constitutes the γ-lactalbumen and β-lactoglobulin. The second fraction is insoluble fraction, named whole casein, constitutes 4 different casein (alpha S1, alpha S2, Beta and kappa-caseins) (El-Rafey and Darwish, 1998). V aliant B of κ-Casein gene and local buffaloes.

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Information on polymorphisms of κ-Casein gene in buffalo in Indonesia is very limited. This study was aimed to identify the polymorphism of κ-Casein gene by using PCR-RFLP technique as a fast efficient and low cost method in Indonesia local buffaloes.

MATERIALS AND METHODS

Sources of Sample

Blood samples used totally were 290 buffaloes, that were collected from 40 riverine buffaloes from Siborong-borong SBBC (20 heads) and Deli Serdang (20 heads); as well as a number of 250 swamp buffaloes from Banten (30 heads), Semarang (30 heads), Mataram (30 heads), South Sulawesi (30 heads), Aceh (67 heads), Riau (25 heads), North Sumatera (24 heads), dan West Sumatera (14 heads). Blood samples were collected from the jugular vein using vacuum tubes containing with heparin. Blood samples were stored in absolute alcohol.

DNA extraction

Blood samples were collected from each buffalo in 10 mL non anticoagulan polypropylene tubes. Blood samples then were mixed with 96% ethanol. The process of DNA isolation used phenol-chloroform method (Sambrook et al., 1989) modified by Andreas et al., (2010) then were dissolved in TE buffer. Genomic DNA was stores at -20°C until amplification with polymerase chain reaction (PCR).
Polymerase Chain Reaction (PCR)

Amplification of Polymerase Chain Reaction (PCR) was carried out using specific primer by following from relevant references (Masina et al., 2007) GenBank Acc No. AM900443 which previously being modified for parts of exon 4. The primer design using PIRA technique which replace (cc) base become (ga) base in forward primer (Table 1). The PCR was performed in a final volume of 15 µL for each reaction containing 0.5 µL of genomic DNA, 5.85 µL destilled water, 0.3 µL of each primer, 0.05 µL taq phire, and 7.5 µL 2x PCR buffer. The reaction mixture was subjected to an initial 5 min of denaturation 95°C, followed by 35 cycles of denaturation 95°C for 20 sec, annealing 60°C for 30 sec, extention 72°C for 40 sec, and a final extension 5 min at 72°C. Electrophoresis used to check the PCR product.

Genotyping by PCR - RFLP

Visualization of amplification was analyzed on agarose gel 1.5% containing 2.5 µL EtBr (ethidium bromide), 0.5X TBE buffer (1 M Tris, 0.9 M Boric acid, 0.01 M EDTA PH 8.0) with a 100 bp ladder as a molecular weight marker for confirmation of the length of PCR product. For digestion by using enzyme and determination of RFLP, 5 µL of PCR products was added to 0.4 µL EcoRV enzyme, 1 µL distilled water, and 0.6 µL R buffer. The mixture was then incubated at 37°C for 16 hour. The digestion products were separated by horizontal electrophoresis (100 volts, 40 min) in 2% agarose gel in 0.5X TBE, 2.5 µL EtBr and 20 bp ladder as a molecular weight marker for confirmation of the length of PCR-RFLP product visualized on UV transiluminator.

Data Analysis

Genotype and Allele Frequencies

PCR-RFLP data were analyzed by calculating the frequencies of allele and genotype (Nei and Kumar, 2000). The genotype frequencies can be determined by calculating the ratio of specific genotypes in each population, were calculated by the following formula

\[ x_{ii} = \frac{n_{ii}}{N} \]

Allele frequency of an allele is the ratio of the overall alleles at a locus in a population. Allele frequency of κ-Casein|EcoRV were calculated by the following formula

\[ x_i = \frac{2n_{ii} + \sum n_{ij}}{2N} \]

Description:

\[ n_{ii} \] = Frequency of genotypes AiAi
\[ n_{ij} \] = Frequency of allele Ai
\[ N \] = Total sample

Heterozygosity

Heterozygosity was tested (Weir, 1996) by the following formulas

\[ H_o = \sum \frac{n_{ij}}{N} \]

\[ H_e = 1 - \sum x_i^2 \]

Description :

\[ H_o \] = Heterozygosity observation
\[ n_{ij} \] = Number of heterozygous animal
\[ N \] = Number of observed animal
\[ H_e \] = Heterozygosity expectation
\[ x_i \] = Frequency of allele
\[ q \] = Total allele

Polymorphic Informative Content (PIC)

The level of informative alleles were calculated using PIC value (Bostein et al., 1980)

Table 1. Forward and Reverse Primers for the Amplification of the κ-Casein Gene

| Primer Pair Name | Primer Sequence           |
|------------------|---------------------------|
| κ-Casein F       | 5’-GGTGAGCCTACAAGTGACAgTA-3’ |
| κ-Casein R       | 5’-TGCTCTGTTCAGTGACGTCCTAGAG-3’ |
by the following formula

\[ \text{PIC} = 1 - \sum_{i=1}^{n} p_i^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2p_i^2p_j^2 \]

Description:
- \( p_i \) = Frequency of allele-i
- \( n \) = Total allele

**Fixation Index**

Fixation index (Nei, 1987) in each source was obtained from equation

\[ F_{IS} = \frac{X_{kii} - X_{ki}^2}{X_{ki}(1 - X_{ki})} \]

Description:
- \( F_{IS} \) = Fixation Index
- \( X_{kii} \) = Frequency of homozygot genotype \( i \) in \( k \) population
- \( X_{ki} \) = Frequency of allele \( i \)

**RESULTS AND DISCUSSION**

**κ-Casein Gene Amplification**

Amplification of the κ-Casein gene resulted an amplicon with the length of 157 bp, which is located in partial exon 4. The amplification fragment of the κ-Casein gene was performed in the thermocycler machine with an annealing temperature of 60 °C. The amplification of κ-Casein gene fragment was carried on GeneAmp® PCR System 9700 (Applied Biosystem). Gene segment amplification products were visualized on 1.5% agarose gel as shown in Figure 1. Position of primers annealing on κ-Casein gene sequences shown in Figure 2.

**κ-Casein|EcoRV Gene Polymorphisms**

PCR-RFLP (restriction fragment length polymorphism) using EcoRV enzyme was used to genotype the Indonesian buffaloes. This enzyme recognized and cut at nucleotides of GAT|ATC sites. Forward primer that used in this research

![Figure 1. Visualization of κ-Casein Gene Amplification Results in 1.5% of Agarose Gel. M = Marker of 100 bp; No. 1-7 = Number of sample](image)

**Primer Forward**

12001 taccatcaat accattgtta gttgagcc tacaagtaca gataactcactg aagcaataga
dactcttag aaggttcttc aqaggttatt gagagtgtac ctgagacca

dactctcag gttacctca aagtcggtta aaaactctaa ggagacatca aagaagaca

**Primer Reverse**

![Figure 2. The Pattern Corresponding to the Sequence κ-Casein Gene in Buffalo (GeneBank Accession AM900443). Restriction Site at Position 12043](image)
was modified in order to get a restriction site by EcoRV enzyme. That technique known as PIRA (Primer Introduced Restriction Analysis), is widely used techniques in SNP detection. The method introduces an artificial restriction site into a PCR product by the use of a primer with a single-base mismatch close to its 3'end (Ke et al., 2001). Based on amplification results of the κ-Casein gene sequences, it was found one point of EcoRV restriction enzyme, it was in 12043 or on base position 21 bp of the PCR products. RFLP resulted in two fragments with the base length of 21 and 136 bp, it showed C allele. If there was a base change at position 12045, from the base T (Thimin) changing to C (Citosin), it caused EcoRV did not recognized and cut the fragment (bands) resulting T allele (Figure 3). Patel et al. (2007) reported his study in riverine buffalo that the restriction enzyme digestion analysis of κ-Casein indicates the presence of the two types of restriction pattern, two fragments of 266 and 84 bp for BB-genotype were observed while in the second pattern three fragments 266, 134/132, and 84 bp for AB-genotype were observed, but none of buffaloes indicated AA-genotype.

The diversity of κ-Casein in this study was founded in exon 4 region. Masina et al. (2007) reported in their research in water buffalo that the comparison of the sequences obtained from exons 1,2,3 and 5 did not reveal any polymorphism. As for the exon 4, the comparison of the obtained sequences confirmed the two single nucleotide polymorphisms already reported in literature at the fourth exon (T versus C responsible for amino acid substitution at position 135 and the silent mutation T versus C at codon 136).

Results from the PCR-RFLP of the κ-Casein|EcoRV gene in riverine buffalo segments were polymorphic. There were three genotypes, namely genotypes CC, CT, and TT derived from two alleles, namely C and T allele. Contrast, the κ-Casein|EcoRV gene in swamp buffalo was monomorphic because it just showed one allele (T) and all of the genotypes is CC. Nei and Kumar (2000) stated that an allele was polymorphic if the frequency of allele was equal or less than 0.99.

Three variant genotypes found in riverine buffalo of this study were CC, CT, and TT (Figure 3). Genotyping the κ-Casein gene, showed for the resulted 157 bp, identified for CC genotype; 136 and 21 bp for TT genotype; and 157, 136, and 21 bp for CT genotype. The observation for κ-Casein gene polymorphisms in exon 4 are similar to the findings of Riaz et al. (2008) who noted one allele B in Nili-Ravi breed of Pakistan. Raj et al. (2008), El-Rafey and Darwish (2007) and Otaviano et al., (2005) also found monomorphism (BB) for this gene in buffaloes. However, Patel et al. (2007) found two alleles A and B for κ-Casein locus in riverine buffalo, such as in Murrah, Surti, and Pandharpuri breeds of buffalo. The diversity of κ-Casein|EcoRV gene in buffalo (riverine buffalo and swamp buffalo) were indicated by the number of genotypes that appeared from each breeds.

Genotype and Allele Frequency of the κ-Casein|EcoRV Gene

Results of the κ-Casein|EcoRV gene analysis showed that the frequency of the CC genotype in all population of riverine and swamp buffaloes was higher than TT and CT genotype (Table 2). Frequency of CC genotype on riverine buffalo ranged 0.600 - 0.950. The highest frequency of

![Figure 3. Result of κ-Casein Gene Fragment Using PCR-RFLP Method with EcoRV Restriction Enzyme on 2% of Agarose Gel. M = Marker 20 bp; 1-16 = Number of samples; CC, CT, TT = Genotype](image-url)
CC, CT, and TT genotype was found in Deli Serdang (0.950), SBBC (0.350), and SBBC (0.050), respectively. Genotype CC was not found in Deli Serdang, but in that location showed same result as SBBC that the genotype CC (0.950) was higher than CT (0.050). The κ-Casein gene of swamp buffalo in all populations were monomorphic with one genotype, namely CC genotype.

The C and T alleles of riverine buffaloes in SBBC were 0.775 and 0.225; then 0.975 and 0.025 for Deli Serdang. The T and C alleles in swamp buffalo in all populations were 1.000 and 0.000, respectively. Patel et al. (2007) who reported in their research with κ-Casein gene of water buffalo that the frequency of BB genotype (0.968) was very higher than AB genotype (0.032) and AA genotype (0.00). This result indicating that most buffalo population has allele (B) for higher casein production.

Monomorphic allele in swamp buffalo was confirmed in several research in other countries, such as: India (Shende et al., 2009; Gangaraj et al., 2008), Mishr (Othman, 2005; Mahmoud et al., 2010; Dayem et al., 2008), Pakistan (Riaz et al., 2008), Brazil (Otaviano et al., 2005), Iran (Abassi et al., 2009), dan China (Ren et al., 2011).

Polymorphisms Degrees of the κ-Casein|EcoRV Gene

Heterozygosity value is the most accurate way to measure the genetic diversity of population (Nei and Kumar, 2000) and to get an overview of genetic variability (Marson et al., 2005). Heterozygosity values are influenced by the number of samples, the number of alleles and allele frequencies. The result of heterozygosity analysis and PIC value in riverine and swamp buffalo shown in Table 3.

Javanmard et al. (2005) suggest that heterozygosity values below 0.5 (50%) indicate low variation of a gene in the population. Table 3 showed that the heterozygosity of riverine and swamp buffalo were low. In riverine buffaloes, the observation heterozygosity values were lower than expected heterozygosity (Ho = 0.200 < He = 0.219) indicated that κ-Casein gene had a low level of heterozygosity. Swamp buffalo was not showed heterozygosity value because it was not showed allele variation. This might be caused by a lack attention on breeding program and there were no intensive selection based on the milk quality of

| Buffalo Breed  | Population | Genotype | Allele |
|----------------|------------|----------|--------|
| Riverine Buffalo | SBBC       | CC 0.600 (12) CT 0.350 (7) TT 0.050 (1) | C 0.775 T 0.225 |
|                | Deli Serdang | 0.950 (19) CT 0.050 (1) TT 0.000 (0) | C 0.975 T 0.025 |
|                | Sub Total   | 0.775 (31) CT 0.200 (8) TT 0.025 (1) | C 0.875 T 0.125 |
| Swamp Buffalo  | Banten      | 1.000 (30) CT 0.000 (0) TT 0.000 (0) | C 1.000 T 0.000 |
|                | North Sumatera | 1.000 (24) CT 0.000 (0) TT 0.000 (0) | C 1.000 T 0.000 |
|                | Semarang    | 1.000 (30) CT 0.000 (0) TT 0.000 (0) | C 1.000 T 0.000 |
|                | Mataram     | 1.000 (30) CT 0.000 (0) TT 0.000 (0) | C 1.000 T 0.000 |
|                | Aceh        | 1.000 (67) CT 0.000 (0) TT 0.000 (0) | C 1.000 T 0.000 |
|                | South Sulawesi | 1.000 (30) CT 0.000 (0) TT 0.000 (0) | C 1.000 T 0.000 |
|                | Riau        | 1.000 (25) CT 0.000 (0) TT 0.000 (0) | C 1.000 T 0.000 |
|                | West Sumatera | 1.000 (14) CT 0.000 (0) TT 0.000 (0) | C 1.000 T 0.000 |
|                | Sub Total   | 1.000 (250) CT 0.000 (0) TT 0.000 (0) | C 1.000 T 0.000 |

(...) = Number of samples; SBBC = Siborong-borong Buffalo Breeding Center
κ-Casein gene in both location. Hartl and Clark (1997) reported that expected heterozygosity value can be used as a way to estimate the breeding value (inbreeding) in a group of livestock. Generally, the expectation value of heterozygosity is a good indicator as a genetic identifier that can explain the genetic diversity in a population of domestic livestock (Moioli et al., 2004).

Besides heterozygosity value, polymorphism of a gene can be determined by calculating the PIC value. PIC value describe the values of corrected heterozygosity by the partially informative mating. PIC value have ranged from 0 – 1. PIC value equal null (PIC = 0) when only one allele is found in the genetic markers, whereas the PIC values equal one (PIC = 1) if there were an infinite number of alleles. If a gene has two alleles it will produce the maximum PIC value of 0.375 (Hildebrand et al., 1992).

Based on Tabel 3, PIC value equal null (PIC = 0) were found in all populations of swamp buffaloes because in those location κ-Casein|EcoRV gene were monomorphic found only one allele, namely C allele. PIC value in riverine buffaloes was higher in SBBC (PIC = 0.288) than Deli Serdang (PIC = 0.048).

**Fixation Index of κ-Casein|EcoRV gene**

Fixation index can be used to determine breeding pattern and selection in population. The value of fixation index could be positive or negative, it was influenced by selection, inbreeding, and assortative mating. The highest fixation index values in this study were low (Table 4). The κ-Casein gene was in fixation in swamp buffaloes in all populations due to a monomorphic occurrence. The fixation process could be caused by inbreeding in all populations swamp buffaloes were observed (Nei and Kumar, 2000)

**CONCLUSION**

κ-Casein|EcoRV gene in riverine buffalo in SBBC and Deli Serdang was polymorphic, with two alleles, namely C and T allele. κ-Casein|EcoRV gene in swamp buffalo was monomorphic with one allele, namely C allele. Heterozygosity value in riverine and swamp buffaloes were low (ranged from 0.000 – 0.350). Fixation Index values in riverine and swamp buffalo were low. The κ-Casein gene was in fixation in swamp buffaloes.
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Table 4. Fixation Index of κ-Casein Gene

| Population         | Allele | FISKI |
|--------------------|--------|-------|
| Riverine Buffalo   |        |       |
| SBBC               | T      | -0.0036 |
|                    | C      | -0.0036 |
| Deli Serdang      | T      | -0.0256 |
|                    | C      | -0.0256 |
| Swamp Buffalo      |        |       |
| Banten             | T      | 0.000 |
|                    | C      | 0.000 |
| North Sumatera     | T      | 0.000 |
|                    | C      | 0.000 |
| Semarang           | T      | 0.000 |
|                    | C      | 0.000 |
| Mataram            | T      | 0.000 |
|                    | C      | 0.000 |
| Aceh               | T      | 0.000 |
|                    | C      | 0.000 |
| South Sulawesi     | T      | 0.000 |
|                    | C      | 0.000 |
| Riau               | T      | 0.000 |
|                    | C      | 0.000 |
| West Sumatera      | T      | 0.000 |
|                    | C      | 0.000 |

SBBC = Siborong-borong Buffalo Breeding Center; FISki: Fixation Index
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