Kappa casein (CSN3) gene polymorphism and its effect on cumulative milk yields of Holstein Friesian dairy cattle

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Abstract. Kappa casein (CSN3) is a standout amongst the most vital milk proteins in mammals that assumes a crucial part in milk quality and coagulation. This study aimed to determine genetic polymorphism of the Kappa casein gene (CSN3) and associate its genotype variants on various cumulative milk yields in Holstein Friesian (HF) dairy cattle. A number of 61 blood samples were collected from 2 Holstein Friesian populations, respectively, from IRIAP Breeding Station in Ciawi (61) and Lembang Artificial Insemination Center (Lembang AIC) (17). Real Time Polymerase Chain Reaction (RT-PCR) method was used to identify variant genotypes of the Kappa Casein gene. In population were detected all three genotypes GG, GT, and TT. The most frequent genotype was TT, with a frequency of 0.63. Results from the statistical association analysis between g.13975G>T CSN3 genotype and cumulative milk yield in standard lactation length were not significant.

1. Introduction
Milk production is a quantitative trait controlled by many genes, and its expression is an accumulation of genetic, environmental, and interaction influences. Selection can be accelerated through the use of genetic identifiers using molecular techniques. Selection based on genetic markers for certain traits will make the selection occur earlier, so unproductive livestock maintenance can be avoided. In addition, the application of genetic markers in livestock breeding programs can accelerate the improvement of the genetic quality of livestock. The productivity of dairy cows can be evaluated by measuring milk production during one lactation period. Milk production is usually relatively high after six weeks of lactation until it reaches maximum production, after which there is a gradual decrease in production until the end of lactation. In general, the length of the lactation period is 10 months (305 days) in cows that have a calf interval of 12 months. Milk production is generally controlled by external and internal factors. External factors are factors that come from outside the livestock body such as climate, amount and quality of feed, disease, and parasites [1], while internal factors are genetic factors, lactation period, milking frequency, age and body size of livestock, dry period, estrus cycle, pregnancy, ketosis and milk fever [2].

Selection of genetic superiority in cows can be done by identifying gene diversity related to the nature of milk production and quality. Genetic diversity can be used to estimate the presence or absence of selection in a population. Several studies have been conducted to determine the relationship between
genetic diversity and milk production in dairy cows. In dairy cows, it is known that major genes control the properties of milk proteins. By identifying genotypic polymorphisms from the milk protein gene family, consistent selection activities will provide a faster process in forming superior milk protein HF cattle strains. This is possible because the protein properties of dairy cows are controlled mainly by two gene families, namely casein and whey. Casein as the largest component of milk protein (78-82%) was controlled by only four casein genes, including s1-casein, -casein, s2-casein, and -casein. Many research reports indicate that certain milk protein variants can be associated with milk production [3,4].

The K-casein gene (CSN3) is located on chromosome 6q31 and is approximately 13 kb in length (Figure 1). The K-casein gene has 5 exons and 4 introns, and most of the coding sequences for the K-casein protein are located on exon 4 [5]. Although 9 variants have been found in the K-casein gene: A, B, C, E, F, G, H, I, and A1, the most common allele variants are A and B [6]. Variants A and B occur at amino acid positions 136 and 148 of the primary structure. At 136 amino acids, threonine (Thr) was replaced by isoleucine (Ile), while at 148 amino acids, aspartic acid (Asp) was replaced by alanine (Ala) [7]. When AA position 136 is Thr in the -CN gene, it is expressed as a CSN3*A allele, whereas when AA position 148 becomes Ala, it is identified as a CSN3*B allele.

Figure 1. Reconstruction of kappa casein (CSN3) gene structure in bovine chromosome 6

Genetic variants of the kappa casein (CSN3) gene have been of serious concern to the dairy industry. This is because the CSN3 gene has an important role in determining the protein content (casein) of fresh milk needed as a raw material for processing and processing milk into prime processed products, such as in the manufacture of cheese. There is a direct effect (BB genotype vs. AA genotype) on the CSN3 gene's superiority of milk protein content [8]. Parent genotype BB (vs. genotype AA) produced a milk protein content of more than 0.08% [9]. The effect of the -CN gene variant on the crude protein and casein content of milk has a genotype sequence BB > AB > AA, where the BB parent to AA has superior milk protein content between 0.1 – 0.2/100 g milk [10]. The protein content increased due to the addition of the level and amount of casein [11]. Meanwhile, BB genotype also gave better results for dairy products and processes, such as shortening rennet formation time and cheese smoothness [12]. The consistency of the effect of BB genotype on the Kappa-Casein gene (CSN3) on high milk protein was proven for domestic HF cattle in Indonesia [13]. The frequency of the CSN3 genotype was different in cows with different milk protein breeding values. BB and AB genotypes appeared in high frequency (0.64) in high protein NPs. In contrast, at low breeding values, the AA -CN genotype frequency was very high (0.80). In addition, the BB genotype produced a better milk yield than the AB and AA genotypes.

This research activity aimed to analyze the relationship between genotype diversity of the CSN3 (kappa casein) gene and milk production in HF cows.
2. Materials and methods

2.1. Animals
This research was conducted at the dairy cattle experimental station at IRIAP Ciawi–Bogor and the Animal Molecular Genetics Laboratory at Bogor Agricultural University. Blood collection for bulls and HF cows was carried out through the coccygeal vein. Sixty-one cows' blood samples were taken from the IRIAP Ciawi experimental station. Blood samples of HF bulls were obtained from active and inactive males in the Lembang Artificial Insemination Center (AIC) as many as 17 samples.

2.2. Milk yields
Milk production data were obtained from the recording of milk production data in 2013-2015 as many as 61 cows. Milk production data collection activities are carried out regularly and consistently. Daily milk production was obtained from morning and evening milking and recorded from the time the cow gave birth to the dry period.

Milk production data were analyzed to describe daily milk production during a 305-day lactation period. Daily milk production data is the sum of the daily test milk production obtained in each lactating cow's morning and evening milking. Milk production was analyzed to obtain: 1) daily test milk production, 2) Cumulative milk production, which was based on 15 daily interval observations.

Analysis of the relationship between the Kappa Casein genotype and milk production was carried out using the General Linear Model (GLM) analysis method with SAS Ver 9.1 software.

2.3. Milk yield classification
The grouping of milk production, namely high, medium, and low classifications based on the population mean value (8.32 ± 1.63), is combined with the difference between the maximum and minimum values to obtain a new standard deviation of 1.65. Thus, the population means and standard deviation are (8.32 ± 1.65) with the highest limit of 9.97 and the lowest being 6.67. On this basis, the classification is as follows: (1) high with protein content above 9.97 liters/head/day, (2) moderate with protein content 6.67−9.97 liters/head/day, and (3) low with protein content below 6.67 liters /heads /day.

2.4. Primer
The primer design to amplify SNPs g. 13975G>T CSN3 gene was performed using Primer Express software with amplicon recommendations ranging from 70-130 bp. Sequence primer consist of F: GAG GTT AAA CAG AAA GAT CAA TAA GAT A; R: GACCCAAAATCATGTAGACAGTGTGA; VIC: AACATTGGAGAGTCTAGGC; FAM: TTTGAGATTCTAGGCAAC.

2.5. Genotyping of SNPs g.13975G>T CSN3 using real time polymerase chain reaction (RT-PCR)
The amplification of CSN3 gene was carried out with the Applied Biosystem® 7500 Fast Real-Time PCR System. A total of 20 µl of the PCR reagent mixture was distributed into each PCR well which was filled with 5 µl of DNA sample, then spin down for 10 seconds and then put into the PCR machine. Components and composition of Real-Time PCR reagents consisted of 5 µl DNA, 1.5 µl Taqman GTXpress Master Mix (2x), 1.0 µl Custom Taqman SNP Genotyping Assays; 6.5 µl PCR grade water. DNA amplification was done with the pre-denaturation condition at 95°C for 20 min, 40 cycles consisting of denaturation at 95°C for 3 min, annealing primers at 60°C for 30 s and extension at 72°C for 5 min for 1 cycle and the final extention at 72°C for 5 min. The results of SNP genotyping can be seen with multicomponent plots and allelic discrimination plots with quality above 99%.

2.6. Data analysis
Genotype frequency represents the ratio of a genotype to the total population. Allele frequency is a ratio of an allele to the overall allele at a locus in the population. Mathematic models for genotype and allele frequencies [14] were as follows:
\[ x_{ii} = \left( \frac{n_{ii}}{N} \right) \times 100\% \\
\]
\[ x_i = \left( \frac{2n_{ii} + n_{ij}}{2N} \right) \]
\[ x_{ii} = \text{ii}\text{th genotype frequency} \\
\]
\[ x_i = \text{i}\text{th allele frequency} \\
\]
\[ n_{ii} = \text{number of individuals with ii genotype} \\
\]
\[ n_{ij} = \text{number of individuals with ij genotype} \\
\]
\[ N = \text{total number of individuals} \\
\]

\[ \text{Association of CSN3 gene genotype polymorphisms on 15-d cumulative milk yield was analyzed by the GLM (General Linear Model) procedure using the Statistical Analysis System (SAS) 9.4 software program [15].} \]

Mathematic model [16] was represented as follows:

\[ \gamma_{ij} = \mu + \alpha_i + \varepsilon_{ij} \]

\[ \gamma_{ij} = \text{a certain 15-d cumulative milk yield} \]
\[ \mu = \text{average} \]
\[ \alpha_i = \text{additive effect from i}\text{th genotype} \]
\[ \varepsilon_{ij} = \text{observed error} \]

3. Result and discussion

3.1. Genetic diversity of g. 13975G>T CSN3 gene within Holstein Friesian

Genotype and allele frequencies of The CSN3 of all observed HF cattle are presented in Table 1. The allele frequency in the CSN3 gene indicates that the T allele frequency is higher than the G allele in both the IRIAP and Lembang AIC populations. Allele frequency L of HF cattle observed from two locations ranged from 0.78 to 0.81. Higher L allele frequency in observed HF cattle caused a higher frequency of TT genotypes than GT and GG genotypes.

**Table 1. Frequency of alleles and genotypes of the CSN3 gene**

| Population      | n  | Alleles | Genotype |
|-----------------|----|---------|----------|
|                 |    | G       | T        | GG       | GT       | TT       |
| IRIAP           | 61 | 0.22    | 0.78     | 0.08     | 0.29     | 0.63     |
| Lembang-AIC     | 17 | 0.19    | 0.81     | 0.04     | 0.30     | 0.67     |

Note: G = Guanine, T = Thymine

3.2. HF cow’s milk yield at the IRIAP breeding station

Cumulative 15-day milk yield for 305 days of lactation in HF cows at IRIAP station is presented in Table 2. Milk production is the ability of an individual dairy cow to produce milk in a day. Based on Table 2, cumulative production data is obtained with a pattern that increases every 15 days of lactation. Cumulative milk production at 15 days of lactation was 130.65 liters. There was an increase in cumulative milk production of 15 days of lactation towards cumulative production of 30 days of lactation, which was 164 liters. In general, the increase in milk production ranged from 87.71-165.78 liters. Cumulative milk production of 305 days of lactation in HF cows at the IRIAP Station is 2546.44 liters or an average daily production of 8.34 liters/head/day. The milk production of HF dairy cows in their home country, namely Australia, reaches an average of 6,930 liters per lactation with an average lactation duration of 324 days or 21.3 liters/head/day [17]. After giving birth, milk production begins to increase and reaches peak production at 3 to 6 weeks, after which it decreases slowly until the end of lactation [18]. Meanwhile, milk production of dairy cows followed a regular pattern in each lactation [19]. Milk production will increase for 45 to 60 days after calving until it reaches production and decreases slowly until the end of lactation.
Table 2. 15- daily cumulative milk production in HF cows at the IRIAP Station

| Cumulative Milk Production (Day) | n  | Milk production (Liter) | Stddev | Minimum | Maximum |
|---------------------------------|----|-------------------------|--------|---------|---------|
| 15                              | 61 | 130.65                  | 24.85  | 58.80   | 198.30  |
| 30                              | 61 | 294.65                  | 41.37  | 138.70  | 393.30  |
| 45                              | 60 | 460.43                  | 64.01  | 239.60  | 607.40  |
| 60                              | 60 | 619.53                  | 88.46  | 336.80  | 829.80  |
| 75                              | 60 | 770.47                  | 108.34 | 446.60  | 1037.20 |
| 90                              | 59 | 919.27                  | 126.94 | 560.50  | 1242.90 |
| 105                             | 59 | 1062.40                 | 146.10 | 655.10  | 1450.00 |
| 120                             | 59 | 1198.81                 | 161.08 | 744.20  | 1611.40 |
| 135                             | 59 | 1328.07                 | 175.82 | 835.10  | 1773.80 |
| 150                             | 59 | 1475.58                 | 266.59 | 913.60  | 2873.30 |
| 165                             | 58 | 1596.37                 | 278.85 | 986.90  | 2986.90 |
| 180                             | 58 | 1685.80                 | 319.03 | 745.90  | 3087.80 |
| 195                             | 57 | 1792.60                 | 329.94 | 841.30  | 3175.70 |
| 210                             | 57 | 1885.93                 | 341.52 | 931.60  | 3260.80 |
| 225                             | 56 | 2011.30                 | 425.96 | 1012.20 | 3893.17 |
| 240                             | 56 | 2102.68                 | 434.54 | 1107.20 | 4004.17 |
| 255                             | 56 | 2224.54                 | 501.71 | 1220.30 | 4101.97 |
| 270                             | 55 | 2328.70                 | 496.70 | 1334.30 | 4192.77 |
| 285                             | 53 | 2429.47                 | 501.04 | 1440.30 | 4285.37 |
| 300                             | 53 | 2517.18                 | 504.82 | 1549.10 | 4366.57 |
| 305                             | 53 | 2546.44                 | 506.02 | 1585.37 | 4391.00 |

Stddev = Standard Deviation

Figure 2 shows a curve of the daily milk production of HF cows in Balitnak.

Figure 2. 15 daily cumulative milk production in HF cows at the IRIAP Station

In Figure 2, it can be seen that the daily milk production of HF cows at IRIAP Station for 305 days has an average of 8.34 liters/head/day. The minimum daily production is 5.21 liters/head/day, while the maximum daily production is 15.11 liters/head/day. The average daily production of HF cattle is lower than the report of [20], who reported that domestic dairy cows’ average milk production capacity could produce around 10 liters of milk/head/day. Meanwhile, the average milk production of dairy cows fed with rice straw and elephant grass were 10.87 liters/head/day and 11.11 liters/head/day, respectively [21]. It is estimated that environmental factors greatly affect cow’s milk production, including maintenance management and the quality of feed given to lactating cows.
3.3. Associations between the SNP c.13975G>T CSN3 gene polymorphism with cumulative milk yield (L) in HF Cows

The association of SNP diversity c.13975G>T of the CSN3 gene on cumulative milk production (liters) of HF cattle is shown in Table 3. The effect of GG, GT, and TT genotypes on milk production for 305 days showed no significant difference (P>0.05). The cumulative milk production for 305 days for GT genotype was higher than cows with GG and TT genotypes. This difference is clearly seen in the cumulative milk production of 305 days of lactation, with a range of differences in milk production of 285.19 and 108.97 liters compared to cows with GG and TT genotypes.

Table 3. Associations between the SNP g.13975G>T CSN3 gene polymorphism with cumulative milk yield (L) in HF Cows

| Cumulative milk yield | Genotype of g.13975G>T CSN3 Gene | SEM | Probability |
|-----------------------|----------------------------------|-----|-------------|
|                       | GG                               | GT  | TT          |
| 15                    | 106.70                           | 136.03 | 131.90     | 4.42 | 0.294 |
| 30                    | 297.85                           | 296.21 | 299.48     | 6.93 | 0.976 |
| 45                    | 481.75                           | 463.60 | 467.43     | 10.80 | 0.926 |
| 60                    | 639.45                           | 620.62 | 631.72     | 14.70 | 0.921 |
| 75                    | 778.35                           | 772.03 | 789.00     | 18.09 | 0.909 |
| 90                    | 892.85                           | 919.29 | 943.65     | 21.11 | 0.779 |
| 105                   | 1005.50                          | 1058.59 | 1096.15   | 23.83 | 0.571 |
| 120                   | 1121.55                          | 1190.95 | 1237.04   | 26.69 | 0.492 |
| 135                   | 1220.55                          | 1313.42 | 1371.92   | 29.35 | 0.369 |
| 150                   | 1320.15                          | 1434.94 | 1502.95   | 31.49 | 0.289 |
| 165                   | 1411.00                          | 1551.54 | 1629.31   | 33.52 | 0.214 |
| 180                   | 1486.15                          | 1658.21 | 1748.77   | 36.33 | 0.151 |
| 195                   | 1553.85                          | 1753.42 | 1859.79   | 39.27 | 0.107 |
| 210                   | 1643.75                          | 1837.96 | 1961.03   | 41.23 | 0.097 |
| 225                   | 1752.65                          | 2039.73 | 2057.05   | 75.40 | 0.629 |
| 240                   | 1867.05                          | 2129.03 | 2152.47   | 76.75 | 0.678 |
| 255                   | 1977.75                          | 2378.41 | 2248.67   | 96.73 | 0.587 |
| 270                   | 2091.90                          | 2463.14 | 2343.16   | 98.03 | 0.642 |
| 285                   | 2214.10                          | 2551.90 | 2428.62   | 99.37 | 0.684 |
| 300                   | 2325.70                          | 2635.96 | 2520.97   | 100.60 | 0.730 |
| 305                   | 2362.60                          | 2664.50 | 2551.50   | 70.28 | 0.743 |

Table 4 shows the genotype frequency of the c.13975G>T CSN3 gene based on the classification of high, medium, and low average daily milk production levels in HF cattle in IRIAP Station. Table 4 informed that HF cows with the TT genotype have a high frequency (0.67) in the classification of high milk production as many as 4 heads. The classification of moderate milk production was also dominated by the TT genotype (0.55). In the classification of low milk production, TT and GT genotypes have the same frequency (0.50).

Table 4. Genotype frequency of SNP g.13975G>T CSN3 gene based on the classification of high, medium and low milk production rates for HF cattle in IRIAP Station

| Classification of Milk Production | heads | c.13975G>T CSN3 | Genotypes Frequency |
|-----------------------------------|-------|----------------|---------------------|
|                                   |       | GG  | GT  | TT  | GG  | GT  | TT   |
| High                              | 6     | 0   | 2   | 4   | 0.00 | 0.33| 0.67 |
| Medium                            | 44    | 3   | 17  | 24  | 0.07 | 0.39| 0.55 |
| Low                               | 2     | 0   | 1   | 1   | 0.00 | 0.50| 0.50 |
| Total                             | 52    | 3   | 20  | 29  |    |     |      |

Note: Average daily milk production is high >9.97 liters/head/day; medium between 6.67-9.97 liters/head/day; low <6.67 liters/head/day.
The results obtained in this study are the same as those reported in Montbéliarde dairy cattle that the genotype for the CSN3 gene did not affect cumulative milk production [22]. A different report in Chinese Holstein cows showed that CSN3 gene with genotypes TT, TG, and GG produced significantly different milk production among the three, with the highest to lowest order being GG, GT, and TT [23]. The CSN3 gene polymorphism affects 305 days of cumulative milk production for Sahiwal cattle, where cows with BB genotype produced 305 days of milk production of 2284 kg, followed by cows with AA genotype with 1548 kg and finally cows with genotype AB with a production of 1336 kg [24]. Dairy cows with genotype BB have higher mean milk yields than AA and AB [25]. According to this finding, genotype AA was associated with higher milk production and fat content than BB. On the other hand, dairy cows with genotype BB had a higher milk protein content than cows with genotype AA and genotype AB [26].

4. Conclusion
Investigation of the relationship between variances CSN3 gene genotype in cumulative HF cow's milk production is carried out at IRIAP Station, West Java. The result showed that GT genotype cattle tend to have higher milk production than cows with TT and GG genotypes. However, statistical analysis proves that the GG, GT, and TT genotypes possessed by the CSN3 gene do not have a significant effect on all 15 daily cumulative milk production were observed. This result shows that examination of the CSN3 gene did not affect milk production of HF cows. This is because milk production is a quantitative trait controlled by the polygene. In addition, milk production is a quantitative trait that is also influenced by genetic and environmental factors. Several environmental factors may affect dairy products cows, including lactation (calving age), open days, dry days, calving season, and calving year [27].

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