GENOTYPIC ANALYSIS OF *KAPPA-CASEIN*, *BETA-CASEIN*, AND *BETA-LACTOGLOBULIN* POLYMORPHISMS IN SIQUIJOR NATIVE CATTLE AND HOLSTEIN X SAHIWAL HYBRID CATTLE STOCKS IN THE PHILIPPINES

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ABSTRACT

The use of polymorphic gene markers is a widely used alternative to traditional methods of trait selection in livestock. This study aims to determine the polymorphism of three milk gene markers: κ-casein, β-casein, and β-lactoglobulin in the Siquijor native (SN, n=124) and Holstein x Sahiwal (HS, n=86) cattle. Genotyping was done primarily by PCR- RFLP analysis while PCR-sequencing was followed only for the β-casein of the SN herd. Results showed that for the β-lactoglobulin, the genotypic frequencies of AA, AB, and BB in the HS were 0.174, 0.464, and 0.362, whereas in the SN these were 0.025, 0.099, and 0.876 respectively. The distribution of κ-casein genotypes was 0.589, 0.286, and 0.125 for the AA, AB, and BB genotypes for the HS respectively, and 0.442, 0.425, and 0.133 for the SN. β-casein genotypes showed that the A1A1, A1A2, and A2A2 were 0.143, 0.755, and 0.102 respectively in the HS and 0.600, 0.320, and 0.080 in the SN. Our results show that the three genes are polymorphic in the SN and HS cattle. It is recommended to conduct an association study between these polymorphic variants and milk quality traits to confirm their usability as gene markers for future selection.

Keywords: *beta-casein, beta-lactoglobulin,* Dairy Cattle*, kappa-casein,* PCR-RFLP

INTRODUCTION

Conventional animal breeding relies on the selection of phenotypes that are advantageous to improve their progeny's performance. Technologies such as artificial insemination, multiple ovulations, and embryo transfer have aided animal breeding to make the selection process more efficient. However, conventional animal breeding has its limitations, particularly concerning the genotype and environmental (GE) interactions. This is limiting to the genetic improvement of farm animals as it does not consider all the sources of genetic variability in an efficient manner and is described to be expensive and time-consuming. Hence, molecular genetic techniques have been used as they provide researchers with a basis for traits at the DNA level which can help solve the problems found in conventional animal breeding (Salisu *et al.*, 2018). Molecular markers, a gene or DNA

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sequence with an associated trait and a known location are an example of these molecular genetic techniques (Al-Samarai and Al-Kazaz, 2015). Assessing genetic traits for animal breeding using this technique has been gaining interest in recent years as researchers utilized these tools to genetically identify traits among animals. A strategy known as marker-assisted selection (MAS) makes use of molecular markers to anticipate an animal's performance to select which individual has traits that can be bred to produce superior offspring. Thus, molecular markers can be an influential tool in the field of animal breeding as they can solve the limitations of conventional breeding techniques (Beuzen *et al.*, 2000).

The genes that express *κ-casein, β-casein*, and *β-lactoglobulin* proteins have been described to influence the overall milk quality and quantity of cattle and are utilized as molecular markers when evaluating their genetic potential. The gene expressing *κ-casein* is associated with controlling casein, a major protein found in cattle milk (Anggraeni *et al.*, 2017). Another protein, the *β-casein* influences the structure of casein specifically in the inner parts of the micelle (Glab and Boratynski, 2017). Whereas the gene for *β-lactoglobulin* has been known to affect the main proteins in whey (Botaro *et al.*, 2008). However, studies on the effects of polymorphisms of these genes on milk yield and quality have differing results which can be attributed to differences in breed, sample sizes, and environmental factors (Bangar *et al.*, 2021). Hence, it is important to identify the different polymorphisms of these molecular markers in various populations of cattle to get a better understanding of their general effects.

In this study, DNA samples extracted from Siquijor native and Holstein x Sahiwal hybrid cattle will be utilized to identify the various polymorphisms of *κ-casein*, *β-casein*, and *β-lactoglobulin* through PCR-RFLP and PCR-sequencing analysis to better understand the effect of the genes to these cattle breed. Specifically, this study will measure the frequency of the identified genotypes and alleles of the genes within the two herds of cattle and identify whether these populations are in Hardy-Weinberg equilibrium.

MATERIALS AND METHODS

The study was carried out between 2015 to 2022. Hair follicle samples were collected from a total of n=210 cattle from two farms in the Philippines. A total of n=86 commercial Holstein x Sahiwal hybrid cattle were sampled from Real Fresh Dairy Farms, Inc. in Bay, Laguna in Region IV-A (CALABARZON). Whereas n=124 Siquijor native cattle were sampled from the Ubay Stock Farm in Ubay, Bohol in Region 7 (Central Visayas). DNA extraction was performed using magnetic bead-based method (DNA IQ™ Promega Magnesphere, Promega Corp., USA). The sampling protocol was approved by the Animal Care and Use Committee of the University of the Philippines Los Baños (CAFS-2021-001).

PCR-RFLP for *κ-casein* analysis protocols were followed from the paper of Mitra *et al.* (1998). Each of the amplification reactions consisted of a 1x PCR buffer with 0.5 mM $MgCl_2$, 0.2 mM dNTP mixture, 0.2 μM primers (Forward: 5'-CACGTCACCCACACCCACATTTA-3'; Reverse: 5-'TAATTAGCCCATTTCGCCTTCTCTGT-3'), 0.2U *Taq* DNA polymerase (KAPA Biosystems), and 1 μl DNA template (5 ng/μl). The amplification procedure was then carried out on a thermocycler with the initial denaturation set at 95°C for 5 minutes, 30 cycles of denaturation (95°C for 30s), annealing (64°C for 30s), extension (72°C for 30s), and the final extension at (72°C at 10 minutes). The product was then subjected to RFLP analysis in a 3% agarose gel and digested using HinfI restriction enzyme.

Protocol for the PCR-RFLP analysis of the *β-lactoglobulin* was taken and

followed from Medrano and Aguilar-Cordova's (1990) paper. Each of the amplification reactions consisted of a 1x PCR buffer with 0.5 mM $MgCl_2$, 0.2 mM dNTP mixture, 0.2 μM primers (Forward: 5'- GTCCTTGTGCTGGACACCGACTACA -3'; Reverse: 5'-CAGGACACCGGCTCCCGGTATATGA-3'), 0.2U *Taq* DNA polymerase (KAPA Biosystems) and 1 μl DNA template (5 ng/μl). The amplification procedure was then carried out on a thermocycler with the initial denaturation set at 95°C for 5 minutes, 30 cycles of denaturation (95°C for 30s), annealing (66°C for 30s), extension (72°C for 30s), and the final extension at (72°C at 10 minutes). The PCR product was then subjected to RFLP analysis in a 3% agarose gel and digested by HaeIII restriction enzyme.

The PCR-RFLP protocol used to genotype the *β-casein* of individuals belonging to the Holstein x Sahiwal was taken from Miluchová *et al.* (2013). Each of the amplification reactions consisted of a 1x PCR buffer with 0.5 mM $MgCl_2$, 0.16 mM dNTP mixture, 0.4 μM primers (Forward: 5'- CCTTCTTTCCAGGATGAACTCCAGG-3'; Reverse: 5'-GAGTAAGAGGAGGGATGTTTTGTGGGAGGCTCT-3'), 0.04 U *Taq* DNA polymerase (Fermentas), and 1 μl DNA template (5 ng/μl). The amplification procedure was then carried out on a thermocycler with the initial denaturation set at 94°C for 5 minutes, 30 cycles of denaturation (94°C for 30s), annealing (58°C for 30s), extension (72°C for 30s), and the final extension at (71°C at 10 minutes). The PCR product was then subjected to RFLP analysis in a 3% agarose gel and digested by DdeI restriction enzyme.

Genotyping of the *β-casein* polymorphisms of Siquijor native cattle through PCR-RFLP produced no results. Hence, PCR-sequencing techniques were carried out to identify the genotypes of the herd. Each Siquijor native cattle was ranked based on the combined *κ-casein* and *β-lactoglobulin* genotypes that are favorable for cheese production which are the AB and BB genotypes. Only the top twenty-five Siquijor native cattle underwent further genotyping via PCR sequencing. Single Nucleotide Polymorphisms (SNPs) specifically for milk production were identified using PCR and Sanger sequencing. These target genes were the major milk structural protein caseins (CSN1S1, CSN2, CSN1S2, CSN3), whey, lactoferrin gene (anti-mastitis), acyl-coenzyme a: diacylglycerol acyltransferase 1 (DGAT1), and Booroola fecundity gene. However, only the milk structural protein caseins (CSN1S1, CSN2, CSN1S2, CSN3) were PCR amplifiable. Additionally, out of all these genes, only the CSN2 or the β-casein gene was able to cover the mutation site of interest. Hence, the top twenty-five individuals of the Siquijor native herd underwent genotyping of their *β-casein* gene. PCR products were sent to First Base Laboratories (Malaysia) for sequencing. The sequenced data was analyzed using NCBI, Chromas Lite, and ExPASy to locate and identify the mutation of interest.

Allele and genotypic data analysis were conducted using the collected genotype data from the *κ-casein, β-casein,* and *β-lactoglobulin* genes of the two herds. Samples were analyzed using RStudio and Microsoft Excel. The allele and genotypic frequencies were calculated for each of the genes in the two cattle herds based on the available data. A Chi-square analysis test was used to determine whether the genes within the two populations were in Hardy-Weinberg equilibrium.

RESULTS

The performed PCR-RFLP analysis on the *β-Lactoglobulin* gene resulted in a DNA with an amplicon size of 252 base pairs (bp). RFLP analysis in a 3% agarose gel and digestion by the HaeIII restriction enzyme generated three distinct genotypes: AA, AB, and

BB. Agarose gel electrophoresis patterns showed that the A allele had 2 fragments which were 99 and 148 bp in size whereas the B allele had 3 fragments which were 99 and two 74 bp. Table 1 shows the gene and genotype distribution, heterozygosity, and Chi-square test results within the herds. Among the two herds, the B allele showed a higher frequency with the Holstein x Sahiwal herd having a gene frequency of 0.59 and the Siquijor native herd having 0.93. As for the genotypic frequency, the AB genotype had the highest frequency (0.464) among the Holstein x Sahiwal herd and the BB genotype (0.876) among the Siquijor native. The AA genotype was the least occurring among the two populations as it had a genotype frequency of 0.174 and 0.024 in the Holstein x Sahiwal and Siquijor native, respectively.

Table 1. Gene and genotypic frequencies of *β-lactoglobulin* gene determined by PCR-RFLP.

Breed	**No. of Animal Tested**	**Heterozy-gosity**	**Chi-square test** (one degree of freedom)	**Gene Frequency**		**Genotype Frequency**		
				A	**B**	**AA**	**AB**	**BB**
Holstein x Sahiwal	69	0.48	$0.95^{n.s.}$	0.41	0.59	0.174	0.464	0.362
Siquijor Native	121	0.14	$0.0088^{n.s.}$	0.07	0.93	0.025	0.099	0.876

n.s.: Not significant

The PCR-RFLP analysis for the *κ-casein* gene resulted in a DNA with an amplicon size of 379 bp. RFLP analysis in a 3% agarose gel and digestion by the HinfI restriction enzyme generated three distinct genotypes: AA, AB, and BB. Agarose gel electrophoresis patterns showed that the A allele had 3 fragments which were 91, 132, and 156 bp in size whereas the B allele had 2 fragments which were at 91 and 288 bp. Table 2 shows the gene and genotype distribution, heterozygosity, and Chi-square test results within the herds. The A allele was the most common among the two herds with a frequency of 0.73 and 0.65 for the Holstein x Sahiwal and Siquijor native, respectively. The AA genotype had the highest genotypic frequency, particularly within the Holstein x Sahiwal group which comprised at least 0.589 of the population wherein the Siquijor native had an occurrence of 0.442 followed closely by the AB genotype (0.442). The BB genotype was the least occurring within the Holstein x Sahiwal (0.125) and Siquijor native (0.133) herd.

The PCR-RFLP analysis for the *β-casein* gene in the Holstein x Sahiwal herd resulted in a DNA fragment with an amplicon size of 121 bp. Through RFLP analysis, the product was placed in a 3% agarose gel and digested using DdeI restriction enzyme, resulting in three distinct genotypes: A1A1, A1A2, and A2A2 which were identified using the fragments that were generated. Table 3 shows the gene and genotype distribution, heterozygosity, and Chi-square test results within the herds. The A1A1 genotype had a 121 bp fragment, the A1A2 genotype had fragments that were 121 bp, 35 bp, and 86 bp, and the A2A2 genotype had two fragments which were at 35 and 86 bp. Allele frequency analysis of Holstein x Sahiwal

showed that the A1 allele occurred more with a frequency of 0.52 whereas the A2 allele had a frequency of 0.48. The genotypic analysis showed that the A1A2 was the most occurring genotype within the Holstein x Sahiwal hybrid herd which had a frequency of 0.755 and only about 0.143 and 0.102 of the herd had a homozygous A1 and A2 genotype, respectively.

Table 2. Gene and genotypic frequencies of *κ-casein* gene determined by PCR-RFLP.

Breed	**No. of Animal Tested**	**Heterozygosity**	**Chi-square test** (one degree of freedom)	**Gene Frequency**		**Genotype Frequency**		
				A	**B**	**AA**	**AB**	**BB**
Holstein x Sahiwal	56	0.39	$0.13^{n.s.}$	0.73	0.27	0.589	0.286	0.125
Siquijor Native	113	0.45	$0.81^{n.s.}$	0.65	0.35	0.442	0.425	0.133

n.s.: Not significant

Table 3. Gene and genotypic frequencies of *β-casein* gene determined by PCR-RFLP and PCR-sequencing.

Breed	**No. of Animal Tested**	**Heterozygosity**	**Chi-square test** (one degree of freedom)	**Gene Frequency**		**Genotype Frequency**		
				A	**B**	**AA**	**AB**	**BB**
Holstein x Sahiwal	49	0.49	$0.001^{n.s.}$	0.48	0.52	0.143	0.755	0.102
Siquijor Native	25	0.36	$0.82^{n.s.}$	0.76	0.24	0.600	0.320	0.080

n.s.: Not significant

The Siquijor native cattle were first ranked based on their *κ-casein* and *β-lactoglobulin* genotypes. The genotypes AB and BB for both genes were deemed as the desirable genotypes as they are described to impart better milk qualities for cheese production. These selected individuals were then put through PCR-sequencing for further genotypic analysis of their *β-casein* gene. The analyzed sequence data showed that the A1 allele was the majority as it was present in 0.76 of the sampled population whereas the A2 allele only accounted for at least 0.24 of the population. The genotypic analysis showed that most of the sampled population had the A1A1 genotype which had a frequency of 0.600 whereas the A1A2 and A2A2 genotypes had a frequency of 0.320 and 0.080 respectively.

DISCUSSION

The polymorphic variants of the three genes *β-lactoglobulin, κ-casein*, and *β-casein* have varying effects on a cow's milk quality and quantity depending on their breed. The *β-lactoglobulin* gene has been studied to affect the individual cow's overall milk quality and cheese yield. In this study, the AB genotype was measured to be the most frequently occurring genotype within the Holstein x Sahiwal herd whereas the BB genotype was the majority for the Siquijor native cattle herd. A study by Singh *et al.* (2014) conducted an analysis of the effects of *the β-lactoglobulin* genotype on the milk production of Indian Frieswal (Holstein-Sahiwal crossbreeds) and discovered that the AB and BB genotypes had a positive effect on the total milk yield and peak yield with these genotypes producing significantly more milk than their AA counterparts. Similar findings were also found in the paper by Badola *et al.* (2003) wherein the BB genotype produced the highest milk yield among the Holstein-Friesian cattle. However, Jersey and crossbred cattle for this study showed no significant differences in the genotypic effects of *β-lactoglobulin* on the milk yield, indicating the variation of effects of this gene depending on the cattle breed. In contrast, in the study by Zaglool *et al.* (2016), the Holstein-Friesian cattle showed significant milk and protein yield among individuals with the AA genotype whereas cows with the BB genotype were shown to have a higher fat percentage within their milk. Cases of different effects of the gene on the same breed have also been reported. In the same study by Badola *et al.* (2003) where the AA genotype showed a significant milk yield than other genotypes on Sahiwal cattle, another study showed that the effects of *β-lactoglobulin* were not significant on the same breed. Hence, it is important to further investigate the effects of this gene and its genotypes on a larger scale to come up with a conclusive statement about its effects on milk production (Mir *et al.*, 2014). This will be important for breeding programs of well-established cattle breed as well as understudied breeds like the Siquijor native cattle.

The second candidate gene, the *κ-casein* gene is said to be associated with traits such as total protein content, fat percentage, and milk production. The B allele of this gene is said to be desirable for cheese production (Sumaiya *et al.*, 2020). In this study, the AA genotype was measured to be the most occurring in the two cattle herds. Other literature such as the study of Singh *et al.* (2014) has found that the AB genotype in Holstein-Friesian x Sahiwal hybrids had a significant effect on the milk yield, peak yield, and yield at 300 days when compared to the AA genotype. However, in the study of Rachagani and Gupta (2008) the BB genotype in the Sahiwal breed showed more influence on the monthly and 305-day milk yield as well as the solids-non-fat, and protein yield. The B allele was stated to be the more desirable gene when it comes to cheese production by the study of Awad *et al.* (2016) wherein Holstein Friesian cattle with AB genotype had a significantly higher effect on milk yield and solids-non-fat when compared to the AA genotype. The paper of Volkandari *et al.* (2017) also supports this claim as they cited that this allele is favorable in increasing the yield of cheese and having superior renneting properties such as having shorter rennet coagulation time, a faster rate of curd firming when compared to other genotypes. Hence, it is recommended for the farms of the two cattle herd to increase the frequency of the B allele to take advantage of the benefits brought upon by this allele.

β-casein, the third candidate gene, and its polymorphisms are said to be associated with cheese yield, cheese quality, concentrations, and rennet quality with the A1 allele dubbed as the harmful allele as it is said to be a risk factor for various diseases (Massella *et*

al., 2017; Joshi *et al.*, 2021). In the study, PCR-RFLP and PCR-sequencing methods showed that the A1A2 genotype was the most frequent among the Holstein x Sahiwal cattle whereas the A1A1 genotype was the most frequent among the Siquijor native cattle herd. Among the two herds, the A2A2 was the least occurring genotype. According to several studies the A2 allele is considered as desirable as it imparts the positive effect of the other genotype without the risk factors associated with the A1 allele. In the study by Ganguly *et al.* (2013), positive relationships between the A2 variant with milk production traits in Holstein x Sahiwal hybrids have been observed particularly in its protein and milk yield. The study stated that individuals with the A1 variant are vulnerable to gastrointestinal proteolysis digestion that releases beta-casomorphin which is known for its potential risk to human cardiovascular diseases, type 1 diabetes, atherosclerosis, and sudden infant death syndrome. The study by Cuevas *et al.* (2021) found that *A2 β-casein* in Siquijor native and Holstein Friesian x Sahiwal cattle exhibited a higher scavenging activity which suggests that it is significant in maintaining antioxidant homeostasis within the human body. Hence, based on the genotypic and allelic frequency results of the herds in this study it is recommended for the farmers to increase the overall A2 allelic frequency of the two herds to produce healthier and better overall milk.

This was the first survey study of the *β-lactoglobulin, κ-casein,* and *β-casein* polymorphisms found in the Siquijor native cattle and Holstein x Sahiwal hybrid cattle herds from the Ubay stock farm and Real Fresh Dairy Farms respectively. Hence, this study will serve as a baseline for future research by cataloging variations of the genes that are currently present in both herds. Furthermore, this catalog may also aid in future breeding programs to heighten the frequency of the advantageous phenotype by having a record for each individual genotyped cattle. To summarize, various studies have repeatedly shown that the genotypes favorable for cheese production were the AB and BB for both the *β-lactoglobulin* and *κ-casein* genes and the A2 allele for the *β-casein*.

Overall, it is important to further investigate the actual association between the gene polymorphisms and the milk production traits of the two herds in the future. As stated earlier, the genotypic effects can largely vary depending on the breed and environment of the herd. Therefore, an in-depth study of the relationship between the gene polymorphisms to the performance of these specific herds can give a better understanding of the genotypic effects for future breeding programs.

CONCLUSION

Using PCR- RFLP and PCR sequencing analysis, we have shown that the three genes *κ-casein, β-lactoglobulin*, and *β-casein* were polymorphic in the Siquijor native and Holstein x Sahiwal cattle. It is recommended to further investigate the relationship between the genotypic variants and the actual measured performance traits of the same Holstein x Sahiwal and Siquijor native individuals. When associated with favorable traits is proven, it is suggested that farmers conduct selective breeding to increase the frequency of the beneficial genotypes and alleles within the herd.

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