ALLELIC AND GENOTYPIC FREQUENCY OF KAPPA CASEIN GENE IN DOUBLE PURPOSE CATTLE.

[FRECUENCIAS ALELICAS Y GENOTIPICAS DEL GEN KAPPA CASEINA EN BOVINOS DE DOBLE PROPOSITO]

N.G. Cortés-López[1], S. del Moral [1], José A. Rueda [2], C. Luna-Palomera [3], C.A. Meza-Herrera[4] and J. Abad-Zavaleta[1*]

*[1] Universidad del Papaloapan. Instituto de Biotecnología. Calle Circuito Central No. 200, Col. Parque Industrial. CP. 68301. Tuxtepec, Oaxaca, México. Tel. (287) 87 59 2 40, ext. 220.*E-mail: jabad@unpa.edu.mx.*
[2] Universidad del Papaloapan. Licenciatura en Zootecnia. Av. Ferrocarril S/N, Col. Ciudad Universitaria, C.P. 68400. Loma Bonita, Oaxaca, México. Tel. (281) 872-22-37, ext. 220.
[3]Universidad Juárez Autónoma de Tabasco. División Académica de Ciencias Agropecuarias. Av. Universidad S/N, Col. Magisterial, Villahermosa, Tabasco, México. Tel. (993) 35-8-15-85.
[4]Universidad Autónoma Chapingo. Unidad Regional Universitaria de Zonas Áridas. Bermejillo, Durango, México. Tel. (872) 77-6-01-60.
**Corresponding Author*

SUMMARY

In this work, allelic frequencies (A and B) of *CASκ* gene were determined as selection criteria on milk quality in double purpose cattle. Blood samples were taken at 200 bovine females and were placed in tubes containing EDTA. Genetic marker MB002 was amplified from extracted genetic material. RFLP testing was performed using restriction enzyme *Hinf I* for the diagnosis of A and B alleles on *CASκ*. Genotype frequencies obtained corresponded to 0.34, 0.01 and 0.65 for AA, BB and AB, respectively. Allelic frequency were 0.67 and 0.33 for A and B alleles, respectively. Also, there was a heterocigocity average of 0.6481. The study population is not in Hardy Weinberg equilibrium, the value of $\chi 2 = 14.8$ with 2 degrees of freedom ($P < 0.005$). Based on allelic frequency of *CASκ* B (0.34) observed in this study under theoretical assumptions, double-purpose cattle may be a feasible option to increase milk quality when BB sires are used for cross breeding. In this way, B alleles associated to higher milk quality can be enriched in few generations.

Keywords: double purpose bovine, allelic frequency, *CASκ* gene.

RESUMEN

En este trabajo, las frecuencias alélicas (A y B) del gen CASκ se determinaron como criterio de selección en la calidad de la leche en el ganado bovino de doble propósito. Se tomaron muestras sanguíneas de 200 hembras bovinas y se colocaron en tubos que contenían EDTA. Se amplifico el marcador MB002 a partir de material genético extraído. Los RFLP se realizaron con la enzima de restricción Hinf I para el diagnóstico de los alelos A y B en CASκ. Las frecuencias genotípicas obtenidas correspondían a 0.34, 0.01 y 0.65 para los alelos AA, BB y AB, respectivamente. Las frecuencias alélicas fueron de 0.67 y 0.33 para los alelos A y B, respectivamente. Además, se registró una heterocigosidad promedio de 0.6481. La población es estudio no se encuentra en equilibrio Hardy Weinberg, el valor ji cuadrada fue de $\chi 2 = 14.8$ con 2 grados de libertad ($P < 0.005$). Basado en la frecuencia alélica de CAS κ B (0.34) observada en este estudio, el ganado de doble propósito puede ser una opción viable para aumentar la calidad de la leche si se utilizan sementales con el genotipo BB para el cruce. De esta manera, los alelos B asociados a la calidad de la leche pueden mejorarse en pocas generaciones.

Palabras clave: Bovinos de doble propósito, frecuencias alélicas, gen *CASκ*

INTRODUCTION

Milk contains carbohydrates, lipids, proteins, essential amino acids, fatty acids, vitamins, inorganic elements and water. Milk proteins are divided in soluble and insoluble fractions. In the soluble fraction there are serum proteins: α-lactoalbumin (α-La) and β-lactoglobulin (β-Lg) while the insoluble fraction

contains caseins: α-s1 (αs1-Cn), α-s2 (s2-Cn), (β-Cn) and κ-casein (κ-CN). Casein function is to retain fatty acids in milk to form micelles and forming an emulsion (Thompson *et al*., 2009; Formaggioni *et al*., 1999). All caseins have a genetic polymorphism involving the replacement of one or two amino acids and occasionally deletion of a segment. The presence of certain genetic variants in milk has an important effect upon coagulation properties of milk (Corral *et al*., 2006; Gutiérrez *et al*., 2000; Fox *et al*., 2000; Formaggioni *et al*., 1999; Freyer *et al*., 1999).

In cheese production, k-CN is very important due to its involvement in stabilization and formation of micelles, preventing milk caseins precipitation. Caseins are encoded in a group of autosomal genes located on chromosome 6 at position 6q31-33 spanning 250 kb (Requena *et al*., 2007; Corral *et al*., 2006; Gutiérrez *et al*., 2000; Fox *et al*., 2000; Formaggioni *et al*., 1999; Freyer *et al*., 1999). κ-CN gene is comprised of 5 exons and 4 introns (Cervantes *et al*., 2007; Requena *et al*., 2007; López and Vasquez, 2004; Medrano and Cordova, 1990) (figure 1 and 2). Main allelic variants of κ-CN are A and B. These variants are present in all breeds with variable frequencies; however, other variants such as C, E, F o G have been also reported (Requena *et al*., 2007; Barroso *et al*., 1998; Horne and Muir, 1994).

Milk derived from animals with B variant has a higher proportion of κ-CN, smaller micelles, higher protein content, increased stability to heat and freezing, reduced coagulation time, a more consistent curd as well as increased cheese yield (5-10%) when compared to animals showing *CASκ* A (Requena *et al*., 2007; Barroso *et al*., 1998; López, 1998; Horne y Muir, 1994). These characteristics are important to dairy industry. High frequency of B allele is a common feature in most populations of dairy cattle (Osta, 1994; Zardowny and Kühnlein 1990) excepting Holstein cattle, where A allele is more common (Viana *et al*., 2001; Bonvillani *et al*., 2000).

Molecular marker MB002 (also known as JK5-JK3) has been used in several studies to identify allelic variants A and B (Veli *et al*., 1994). This molecular marker covers exon 4 and intron 4-5 with a size of 350bp (figure 1 and 2); Requena *et al*., 2007; López and Vásquez, 2004; Medrano and Córdova, 1990). Differences between A and B alleles can be defined using PCR-RFLP technique by restriction with *Hinf I* (Cervantes *et al*., 2007).

In tropical areas, double-purpose cattle is a term commonly used to describe crossbreeding of Zebu, Creole and European cattle in Mexico, double-purpose cattle has been used for both meat and milk production. At the Papaloapan region, crossbreeding of cattle has been done indiscriminately due to a lack of professional orientation. Occasionally, genetically superior sires have been used, however, most of the time only individuals with a desirable phenotype to the producer are used. Double-purpose cattle is a genetic group well adapted to harsh environmental conditions like high temperature and moisture, low quality of pastures, and high incidence of parasites commonly found in tropical and subtropical areas. Productivity of this system can be considered lower when compared to intensive systems specialized in milk and meat production. However, double-purpose genotypes have advantages with respect to specialized genetic groups, mainly when low-input production systems are considered. These marginal production systems under tropical and subtropical ecosystems are usually managed under low economic and biotic input schemes and with minimum technology (Ortega and Ward, 2005; Baez 2000). The objective of the present work was determine allelic frequencies A and B to *CASκ* in double purpose cattle as well as to discuss some implications to enhance possible strategies designed to promote genetic improvement for milk quality traits.

MATERIAL AND METHODS

Location and environmental conditions

This work was done in the Papaloapan region of Oaxaca This region is located northwest of the Oaxaca Mountains, at a north latitude of 18° 05' west long 96° 08', and 20 m altitude (OEIDRUS-OAXACA, 2005). Papaloapan region has a warm subtropical climate with an annual average temperature of 33 ° C and average annual rainfall ranging from 1,300 to 3,000 mm.

Samples and animals

The double-purpose cattle are crossbreeds of *Bos taurus, Bos indicus* and creole, intended for both meat and milk production. *Bos taurus* breeds admixed are American and European Brown Swiss, Holstein and Simental, *Bos indicus* breeds are Brahman, Gyr, Sardo Negro, Indobrasil, Nelore and Guzerat. However, admixture level is unknown given unsystematically and undocumented crossbreeding.

Blood samples were randomly taken from 200 females of different age, physical condition and physiological state, under lactation and representing 10% of total sampled population. Sampling was done in 25 different ranches.

Whole blood was placed in tubes containing EDTA (1.8 mg/ml final concentration) as anticoagulant.

Blood was ice transported and stored in a - 20 ° C freezer until processed.

Genotyping

DNA extractions were carried out using Blood DNA Extraction kit GF-1 (Vivantis) following manufacturer's recommendations. Polymerase chain reaction was carried out from a starting template of approximately 100 ng of genomic DNA in a 10 μL volume. Accu Prime Taq DNA polymerase System (Invitrogen) was used for amplification. Primers MB002F (ATCATTTATGGCCATTCCACCAAAG) and MB002R (GCCCATTTCGCCTTCTCTGTAACAGA) flanking MB002 molecular marker (fig. 1) were used at 10 pM. Reaction was conducted in a MAXYGENE termocycler (AXYGEN Scientific®). Temperature conditions were 94 °C for 5 min; 30 cycles of 94 °C for 1 min, 56.1 °C for 30 s and 72 °C for 30 s; final extension step for 10 min. PCR products were separated on 2% agarose gels stained with ethidium bromure 0.5% (v/v). Gel was examined with UV light via a photodocumentation system (INGENIUS SYNGENNE®).

PCR-RFLP (Restriction fragment length polymorphism) was conducted using amplification of MB002 molecular marker and restriction enzyme *Hinf I* (Fermentas®). Restriction digest was carried out in a final volume of 30 μL containing 10 μL of PCR product and 1 U of restriction enzyme on R buffer 10X for 2 h at 37°C (figure 2). Digested fragments were analyzed on 3% agarose gels. Band patterns were observed via photo documentation system (Ingenius SYNGENNE ®).

Statistical analysis

Allelic and genotypic frequencies were obtained by means of χ^2 test performed on statistical program SAS.

RESULTS

MB002 marker was PCR amplified from genomic DNA with oligonucleotides MB002F and MB002R, obtaining a 344bp fragment. From 200 samples tested, only 108 were PCR positive.

For those samples where marker MB002 was amplified, *Hinf I* was used (figure 2) to restrict PCR product and allow allele discrimination. Homozygous AA individuals rendered two distinct bands representing three restriction fragments, two fragments of similar length (131 and 132bp) and a smaller 81pb fragment. Homozygous BB individuals showed a clear two band (263 and 81bp) restriction pattern (figure 2 and 3). Heterozygous AB individuals (figure 3) showed a three fragment restriction pattern (263, 131/132 and 81bp).

Table 1 contains genotypic and allelic frequencies found and expected from the study population of dual purpose cattle. Genotype frequencies obtained corresponded to 0.34 for homozygous AA, 0.65 for heterozygous AB and 0.01 for homozygous BB. Allele frequencies of 0.67 for A allele and 0.34 for B allele were observed. Table 1 also shows average heterozygosity of 0.6481. The study population is not in Hardy Weinberg equilibrium ($P < 0.05$). A allele was more prevalent than B allele, showing a frequency of 0.67 and 0.34, respectively.

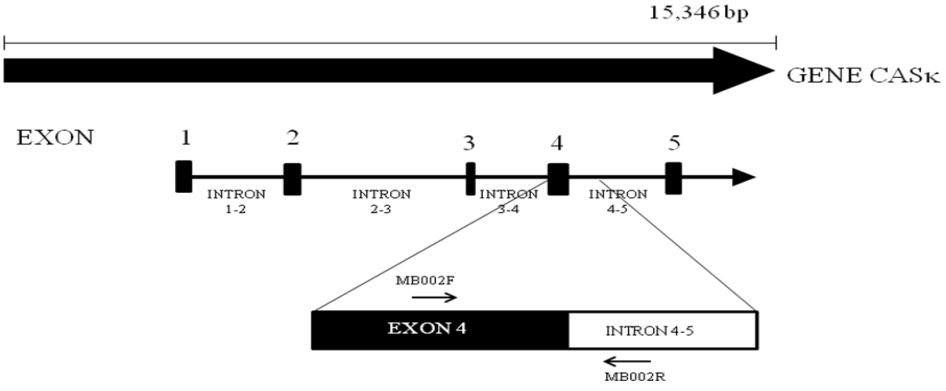

Figure 1. Exons and introns of *CASκ* gene from bovine.

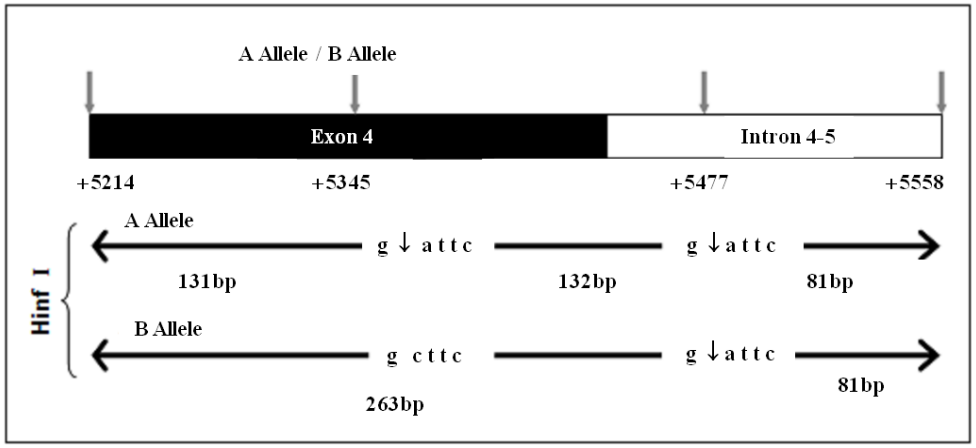

Figure 2. *Hinf I* restriction pattern of MB002 marker, using 344 bp PCR product illustrated in Fig 1 (adapted from Lopez and Vasquez, 2004).

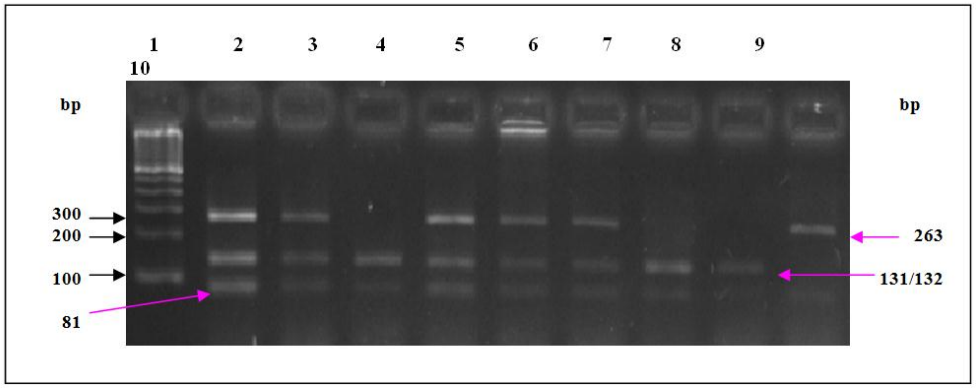

Figure 3. Representative agarose gel showing *CASκ* genotyping performed in this research in double purpose cattle. MWM: molecular weight marker, AA: homozygous A, BB: homozygous B, AB heterozygous.

Table 1. Genotypic and allelic frequencies observed and expected in double purpose cattle.

Genotype	Observed		Calculated		Allele	Allelic frequency
	Number of individuals	Genotypic frequency	Number of individuals	Genotypic frequency		
AA	37	0.34	48.00	0.44	A	0.67
AB	70	0.65	48.00	0.45	B	0.33
BB	1	0.01	12.00	0.11		
χ^2	14. 8	Degrees of freedom	2	(P < 0.005).		

DISCUSSION

From 200 samples tested, only 108 were PCR positive for marker MB002. As shown in figures 1 and 2, MB002 genetic marker is located in exon 4 and intron 4-5. Factors impeding amplification on all samples may be that intronic regions are poorly conserved among allelic variants (Cervantes *et al*., 2007; Requena *et al*., 2007; Lopez and Vasquez, 2004; Medrano and Cordova, 1990) and high variability in exon 4. This shows that genetic diversity of *CASκ* gene in exon IV has been underestimated in domestic livestock, and therefore it is necessary to devote more research efforts characterizing this genomic region (Chen *et al*., 2008). Because of exon and intron variability, it is possible that MB002F and MB002R primers here used have not found a complementary region and therefore marker amplification was not positive for those 92 samples. Therefore, it is possible that negative samples contain another allelic variant

than those expected in this work. We are currently looking for allelic variants in the negative samples.

Several studies evaluating genetic variability of *CASκ* gene in different breeds of dairy cattle have been done (Viana *et al*., 2001; Kemenes *et al*., 1999; Grosclaude, 1998; Van Eenennaam and Medrano, 1991, McLean *et al*., 1984). Holstein breed has been highlighted due to its high milk yield; although, it has been low ranked because of its decreased levels of total solids, fat and protein (Benavides, 2003). In contrast, Jersey breed depicts a decreased milk yield regarding to Holstein breed, nonetheless, quality of its milk is far superior compared to Holstein and other dairy breeds (Grosclaude, 1998; Van Eenennaam and Medrano, 1991, McLean *et al*., 1984). The main drawback of Jersey breed, however, is that its production is limited to certain environmental conditions and therefore it has not been commonly spread around the world. The latter has generated expectations about other alternatives in terms of milk production, considering both quality and volume, by other cattle breeds (Munizaga *et al*., 2004). Under such scenario, characterization of double-purpose cattle acquire relevance given its widespread use as genotypes that produce both milk and meat, mainly under tropical and subtropical regions, despite of adverse environmental conditions involving high temperature and high humidity. Studies on double-purpose cattle are scarce (Cervantes *et al*., 2007). The study here presented is the first of its kind in the Papaloapan region.

Several studies characterizing cattle breeds have shown that B allele has higher frequencies in *Bos taurus* with respect to *Bos indicus* (Golijow *et al*., 1999; Kemenes *et al*., 1999; Tambasco, 1998, Del Lama and Zago, 1996; Backer and Manwell, 1980). Likewise, in double-purpose cattle evaluated in this study, A allele was predominant over B allele (0.67 and 0.34, respectively), most likely due to high input from A allele races like Zebu, Holstein, Nelore and Guzerat, Gyr (0.74, 0.90, 0.91 y 0.92, 0.93, respectively).

Frequencies obtained for allelic variant B (0.34), surpassed those found in other breeds, such as Guernsey breed (Van Eenennaam and Medrano, 1991, Voelker *et al*., 1997), Shorthorn breed (Van Eenennaam and Medrano, 1991), Holstein (Viana *et al*., 2001), Zebu (Cervantes *et al*., 2007), Nelore, Guzerat, and Gyr (Kemenes *et al*., 1999) with values of 0.27, 0.11, 0.10, 0.26, 0.09, 0.08 and 0.07, respectively. In the Papaloapan region, commonly used breeds like Jersey, Brown Swiss and Normand have higher B alleles values (0.77, 0.57 y 0.56, respectively).

On the other hand, in several breeds (Holstein-Friesian Irish, Dutch-Friesian, Limousin, Montbeliarde, Charolais, Normande, Norwegian Red and Kerry) has been reported that A allele is dominant in all groups (dairy, beef and double purpose) with an allelic frequency of 0.89 in high-yielding dairy animals and beef animals 0.75. AB haplotype is comparatively rare in dairy cattle (0.11) compared with both beef and double purpose animals. BB haplotype, though rare overall (0.09), is higher in double-purpose animals (0.18) than dairy (0.056) animals (Keating *et al*., 2007).

Van Eenennaam and Medrano (1991) demonstrated that in mammary glands, allelic variant B has a higher level of expression with respect to allelic variant A and therefore, the proportion of κ-CN in milk depends on the animal genotype in the following order BB> AB> AA, reaching a protein difference of 3% in animals with allelic variant B against animals with allelic variant A. We found only one individual with BB genotype, because of high heterozygous frequency found.

Increasing BB frequency through targeted crossbreeding is an interesting strategy to increase cheese production. If a breed that shows an allelic frequency around 0.25 for kappa-casein B allele is crossed with selected BB stallions, in few generations BB frequency can be dramatically increased (Prinzenberg *et al*., 1996). Double-purpose cattle in the Papaloapan region can be subject of this strategy. It can be directed towards milk volume production given its high A allele frequency, or towards milk quality by increasing B allele frequency which is already high (Table 1).

Double purpose cattle is the main traditional alternative for milk production, considering an animal production model managed and operated under marginal conditions ,because its excellent adaptation to harsh environmental conditions in the tropics, where specialized breeds cannot develop to full potential.

As shown in table 1, genotype frequencies (AA, AB and BB) observed and expected under the equilibrium condition were not equal. Therefore, the population is not in H-W equilibrium and random mating for this *locus* did not occur in the last generations, according to χ^2 test. Heterozygosity measured (0.6481) suggests a highly hybrid poheterogeneity population regarding this locus, in the studied population, probably asa result of indiscriminate crossbreeding and lack of orientation toward a defined production goal. Finally, using PCR-RFLP -a simple and low cost tool- those animals with allelic variant B could be identified. Therefore, making genetic improvement programs through targeted crosses, with the goal of obtaining

animals with better milk quality, are feasible. This will contribute to economic development in the region. Moreover, the short time from collection of whole blood samples to genotype identification by PCR-RFLP offers great advantages in contrast to the time required to evaluate an animal by conventional methods. Recent reports indicate that improvements in this technique allow discriminating between 9 allelic variants in a basic molecular biology laboratory (Pacheco *et al*., 2011).

CONCLUSION

Through amplification and genotyping of marker MB002 is possible to identify allelic variants A and B of *CASκ* in double-purpose cattle from the Papaloapan region. In this study, only 108 out of 200 samples amplified marker MB002. Therefore, the remaining 92 animals should contain a different allelic variant than those studied in this work. In the population under study, A allele predominated over B allele, with a frequency of 0.67 over 0.34, respectively. High population heterogeneity makes feasible a genetic program to increase milk quality in double-purpose cattle. High frequency of heterozygotes AB is in contrast to almost absent frequency of homozygotes BB. This seems to be caused by indiscriminate crosses combined with minimal guidance to get a defined production goal. Present results will be used for genotypic and phenotypic indicators, in order to select those individuals with homozygous BB and heterozygous AB alleles to produce sires with BB genotype. Furthermore, using homozygous BB sires for crossbreeding will increase the frequency of B allele in the studied dual purpose cattle population and therefore it will improve milk quality, as well as cheese yield .

ACKNOWLEDGMENTS

This research was supported by a grant from PROMEP awarded to J.A-Z. (103.5/07/2740) and a student scholarship awarded. to N. G. C - L. We thank Lice Jennie Renn and PhD Julian Peña-Castro for their collaboration reviewing grammar of this manuscript.

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Submitted October 03, 2011 – Accepted November 07, 2011
Revised received November 22, 2011