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ORIGINAL ARTICLE

Genotyping of the *kappa-casein* and *leptin* genes in Cuban water buffalo by PCR-RFLP

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ABSTRACT: Genetic analysis of loci affecting production and reproduction traits in livestock animals make possible the evaluation of animal breeding values using molecular markers. The aim of this study was to detect polymorphisms in the *leptin* and κ-*casein* genes of a Cuban water buffalo population to provide markers useful for associating studies. Genomic DNA from 68 unrelated Cuban buffaloes, uncontrolled crossbreds of river (Buffalypso) and swamp (Carabao) animals, was analyzed using PCR-RFLP. Fragments of *leptin* and ê-casein genes were amplified and digested with *Bsa* AI and *Hind* III/*Taq* I, respectively. All the amplified samples were monomorphic BB after digestion with *Hind* III/*Taq* I. Genotypic frequencies found in the *leptin* gene with *Bsa* AI were 0.47 (AA), 0.42 (AG) and 0.11 (GG). The population was in HWE (p=1.0000), possibly because there was not selection for this locus on it. The F_{IS} estimate (0.0212) showed no inbreeding in the population regarding this locus. He (0.436) and Ho (0.426) values were below 0.5 (50%), indicating low genetic variation in this locus in the population. PCR-RFLP detected the two genetic variants described for the *leptin* gene A1620G. However, a higher number of animals and SNPs in the *leptin* and/or other genes should be analyzed for a more accurate estimate of the genetic diversity in this population. DNA sequencing should be assayed by nucleotide sequence analysis to detect the new A and B variants of κ-casein reported in buffalo.

Key words: leptin, Casein, buffaloes, PCR-RFLP, genetic variants.

Genotipado por PCR-RFLP de los genes de la kappa-caseína y la leptina en búfalos de agua de Cuba

RESUMEN: El análisis genético de *loci*, que controla caracteres productivos y reproductivos, hace posible la evaluación del valor genético en el ganado con el uso de marcadores moleculares. El objetivo de este estudio fue detectar polimorfismos en los genes de la leptina y la κ-caseína en búfalos cubanos, para proveer marcadores útiles en estudios de asociación. Se amplificaron por PCR fragmentos de los genes de la leptina y la ê-caseína que se digirieron con *Bsa* AI y *Hind* III/*Taq* I, respectivamente. Todas las muestras amplificadas resultaron monomórficas BB tras la digestión con *Hind* III/*Taq* I. Las frecuencias genotípicas en el gen de la leptina fueron, 0.47 (AA), 0.42 (AG) y 0.11 (GG). La población se encontró en equilibrio de Hardy-Weinberg (p=1.0000), posiblemente, porque no está ocurriendo selección para dicho *locus*. El F_{IS} estimado (0.0212) mostró que no existe endogamia en la población cuando se considera este *locus*. Los valores de He (0.436) y Ho (0.426) estaban por debajo de 0,5 (50%), lo que indica que existe una baja variabilidad genética para este *locus* en la población. La PCR-RFLP permitió detectar las variantes genéticas descritas para A1620G en el gen de la leptina. Sin embargo, se deben analizar un mayor número de animales y SNPs, en este u otros genes, para estimar con mayor certeza la diversidad genética en esta población. Es necesario el uso de la secuenciación para detectar las nuevas variantes A y B de la κ-caseína que se han descrito en búfalos a través de esta técnica.

Palabras clave: Leptina, Caseína, búfalos, PCR-RFLP, variantes genéticas.

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INTRODUCTION

Identification of *loci* affecting production and reproduction traits in livestock animals would make possible the evaluation of animal breeding values using molecular markers. In genetic animal studies, much attention has been focused on the identification of single nucleotide polymorphism (SNP). These markers are extremely common throughout the genome and their identification is the first step in association studies (1).

Many studies in bovine have reported association between SNPs and traits of economic interest (2, 3, 4). Genetic polymorphism in milk proteins has raised great interest in the animal breeding and dairy industry, due to the relationship between milk proteins and milk production traits, composition, and quality (5, 6, 7). Kappa-casein (κ-*casein*, CSN3, K-Ca, kCn, CASK) is one of the most important and highly studied milk protein genes. In cattle 13 protein variants and one synonymous variant have been reported; however, the most frequent ones are A and B alleles (8). The variants of κ-*casein* affect casein content, protein content and cheese yield, as well as curd firmness.

The *leptin* gene is another important candidate gene that can be used to improve animal reproduction and production. Leptin and its receptor have been mapped in several species and a number of SNPs have been identified for further use in marker assisted selection programs (9). Polymorphism in the bovine *leptin* gene locus associated with genetic variation in energy balance, milk production, live weight, and fertility trait have been reported by many researchers (10, 11, 12).

Before the use of these genes for enhancing productivity and reproduction in farm animals, studies with different populations are required for a proper characterization of the robustness of their association with economically important traits across dairy/beef livestock (13). Effective genotyping of the κ-*casein* and *leptin* genes of buffaloes requires fast, efficient, and low cost methods, independent of age and sex. DNA based genotyping through polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) has made this evaluation possible as well as simple for a large number of animals (14). Therefore, the aim of the present study was to detect some SNPs, previously reported in cattle, in the *leptin* and κ-*casein* genes of a Cuban water buffalo population by PCR-RFLP to provide markers useful for association studies in this population.

MATERIAL AND METHODS

Genomic DNA isolation

Blood samples were collected from 68 unrelated Cuban buffaloes, uncontrolled crossbreds of river (Buffalypso) and swamp (Carabao) buffaloes belonging to a dairy herd at the Empresa Pecuaria Genética «El Cangre», in Mayabeque Province.

Genomic DNA was extracted using Promega Wizard® Genomic DNA purification kit (Promega, Madison, WI, USA) in accordance with the manufacturer's suggested protocol.

The quality and quantity of DNA (ng/µL) for each sample were analyzed in a spectrophotometer (Nanodrop ND1000, Thermo Scientific).

PCR amplification

A fragment of 522 bp located between intron 2 and exon 3 of the *leptin* gene was amplified by PCR using the primers 5´-GTC TGG AGG CAA AGG GCA GAG T-3´ and 5´-CCA CCA CCT CTG TGG AGT AG-3´ (15). The reaction mixture contained ~100 ng of genomic DNA, 0.2 mM of dNTPs, 2mM of $MgCl_2$, 0.4 µM of each primer, 1X of Taq Platinum DNA Polymerase Buffer, 0.75 U of Platinum®Taq DNA polymerase (Invitrogen™/ Life Technologies, Carlsbad, CA, USA), and nuclease free water (Promega, Madison, WI, USA) to make a final volume of 25 µl. Amplification conditions were as follow: 95°C for 5 min, 30 cycles at 94°C for 15sec, 65°C for 30 s and 72°C for 1 min, followed by a final extension step of 72°C for 5 min.

A 379 bp fragment from exon IV of κ-*casein* gene was amplified using the primers K-F: 5´-CACGTCACCCACACCCACATTTATC-3´ and K-R: 5´-TAATTAGCCCATTTCGCCTTCTCTGT-3´ (17). The PCR mixture was composed of ~100 ng of genomic DNA, 1XPCR master mix (Promega, Madison, WI, USA), 0,4 µM from each primer, 2 mM of $MgCl_2$, and nuclease free water (Promega, Madison, WI, USA) to make a final volume of 25 µl. The PCR reaction included pre-denaturation for 4 min at 95°C followed by 30 cycles at 94°C for 1 min, 56°C for 2min, 72°C for 1 min and a final extension of 7 min at 72°C.

The amplified products were stained with Blue Green Dye (LGC Biotecnologia, Cotia, SP, Brazil) and electrophoresed in agarose gel (2 %) for 45 min at 100 V in 0.5 X TBE buffer. The visualization was done under ultraviolet light and the gel was documented by Gel-Doc (Bio-Rad) equipment. The presence of the expected

bands and the quantity of PCR products were assessed by comparing with a 100 bp DNA ladder (Promega, Madison, WI, USA).

Genotyping using RFLP

The PCR products of the *leptin* gene amplification were digested with *Bsa* AI restriction enzyme to discriminate A or G allelic variants (15, 16). The restriction digestion was performed in a total volume of 15 µl containing 1X of enzyme buffers (ThermoScientific, Waltham, MA, USA) 0.1-0.5 µg of amplified DNA, 10 U *Bsa* AI (ThermoScientific, Waltham, MA, USA), and nuclease free water (Promega, Madison, WI, USA). The reaction mixture was incubated at 30°C for 3 h.

In the case of the κ-*casein* gene, the 379 bp PCR products were digested with *Hind* III or *Taq* I restriction enzymes to discriminate A or B allelic variants (14). The restriction digestions were performed in a total volume of 25 µl containing 1X of enzyme buffers (Promega, Madison, WI, USA), 0.1-0.5 µg of amplified DNA, 10 U *Hind* III or *Taq* I (Promega, Madison, WI, USA), and nuclease free water (Promega, Madison, WI, USA). The reaction mixture was incubated at 37°C (*Hind* III) or 65°C (*Taq* I) for 3 h.

The restriction products were stained with Blue Green Dye (LGC Biotecnologia, Cotia, SP, Brazil) and electrophoresed in agarose gel (3 %) for 45 min at 100 V in 0.5 X TBE buffer. The visualization was done under ultraviolet light and the gel was documented by Gel-Doc (Bio-Rad) equipment. The size of the bands was estimated with a 50 bp (*leptin* gene) or 100 bp DNA ladder (Promega, Madison, WI, USA).

Statistical Analysis

Direct counting was used to estimate genotype frequencies of gene genetic variants.

Deviations from Hardy-Weinberg Equilibrium (HWE) and Wright Fixation Index (F_{IS}, within population inbreeding estimate) were assed with GENEPOP v4.2 software (17) using exact tests that employ the Markov Chain method to estimate p-values (1000 dememorization steps, 100 batches and 1000 iterations). Allele frequencies and observed (Ho) and unbiased expected (He) heterozygosities were calculated using the Excell complement GenAlEx 6.5 (18).

RESULTS

All 68 samples were amplified using specific primers for the *leptin* gene. After digestion of PCR products with *Bsa* AI, an intact 522 bp fragment as AA genotype, 441 and 81 bp fragments as GG genotype, and 522, 441 and 81 bp fragments as GA genotype were observed (Figure 1). Genotypic frequencies were 0.47 for genotype AA, 0.42 for AG, and 0.11 for GG. The allelic frequencies found were 0.684 for the allele A and 0.316 for the allele G. The population was in HWE (p-value =1.0000). F_{IS} estimates were 0.0212. He value was 0.436, and Ho was equal to 0.426.

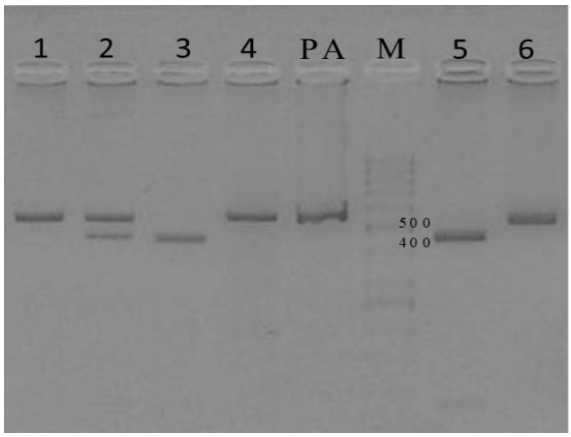

FIGURE 1. Pattern of bands corresponding to genotypes of leptin gene amplified with primers reported by Lien *et al.* (18) digested with *Bsa* AI. 1, 4, 6: Sample of animal showing AA genotype (Uncut 522 bp); 2: Sample of animal showing AG genotype (522 and 441 bp); 3, 5: Sample of animal showing GG genotype (441 bp); M: DNA Molecular weight marker and PA: Uncut amplified product. The 81 bp band was only visible in line 5./ *Patrón de bandas correspondientes a los genotipos del gen de la leptina amplificado con los cebadores descritos por Lien et al. (18) digerido con Bsa AI. 1,4,6: muestras de animales con genotipo AA (522 pb); 2: muestra de animal con genotipo AG (522 y 441 pb); 3,5: muestras de animales con genotipo GG (441 pb); M: patrón de peso molecular y PA: producto amplificado sin digerir. La banda de 81pb sólo se observó en la línea 5.*

Sixty-five samples were amplified with primers K-F and K-R for exon IV of κ-*casein*. After digestion of PCR products with *Hind* III, all animals were monomorphic, showing two fragments of 225 and 154bp corresponding to BB genotype (Figure 2). The 379bp amplified a fragment remained undigested by *Taq* I restriction enzyme in all animals, also showing a monomorphic BB pattern.

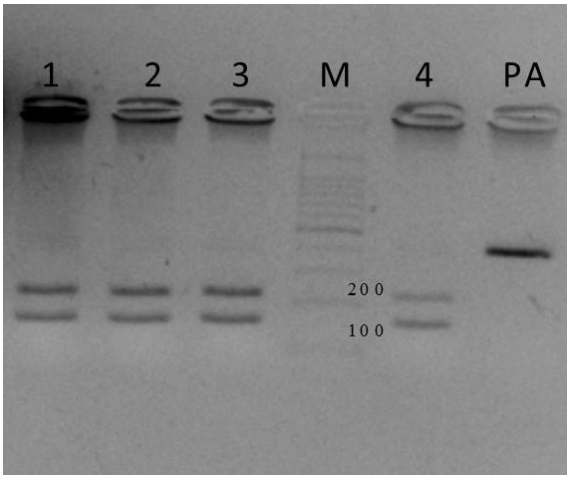

FIGURE 2. Pattern of bands (225 and 154 bp) corresponding to BB genotype of κ-*casein* gene (exon IV) amplified with primers reported by Mitra *et al*. (17) and digested with *Hind* III. 1-4: Sample of animals, M: DNA Molecular weight marker and PA: Uncut amplified product./ *Patrón de bandas (225 and 154 bp) correspondientes al genotipo BB del gen de la κ-caseina amplificado con los cebadores descritos por Mitra et al. (17) digerido con Hind III. 1-4: muestras de animales; M: patrón de peso molecular y PA: producto amplificado sin digerir.*

DISCUSSION

It has been demonstrated by interspecies comparisons that κ-*casein* possesses the highest degree of conservation among the casein genes (19). The *leptin* gene structure, intron/exon boundaries and amino acid sequence are highly conserved in mammalian species. Comparative study of intron 2 of the *leptin* gene between bovines, buffaloes and goats, revealed the sequence similarity of 95-98% and indicated that bovine were closer to buffaloes (10). The former explains that buffalo *leptin* and κ-*casein* genes were successfully amplified by PCR using primers designed for bovines. Failure of amplification in three samples in the κ-*casein* gene could be due to presence of PCR inhibitors in the DNA sample or because of the existence of mismatches in the union site of primers in the gene sequence.

Lien *et al*. (15) first described a guanine (G) to adenine (A) substitution in position 1620 in intron 2 of the *leptin* gene of Norwegian cattle identified by *Bsa* AI digestion. Chaudhary *et al*. (20) reported digestion of 522 bp PCR products with the *Bsa* AI restriction enzyme and revealed three genotypes in all the breeds of *Bos indicus, Bos taurus,* and Jersey cattle. Souza *et al*. (5) determined *leptin* A1620G to be associated with weaning weight (W) in three Nelore (*Bos indicus*) lines selected for growth. Significant effects (p=0.03) of this polymorphism were observed for W_{210}, with the demonstration of greater mean values for AA animals compared with AG and GG animals. There have been few studies on this SNP in water buffaloes.

The results of the present study in a Cuban buffalo population showed the presence of the three genotypes (AA, AG, and GG), with AA as the most frequent genotype. The genotype GG was the least frequent. In accordance with that fact, allele A showed the highest allele frequency. The population in HWE indicated that, possibly, there was not selection for this locus in the population. F_{IS} estimates showed that no inbreeding occurred in the population regarding this locus.

Heterozygosity value is the most accurate way to measure the genetic diversity of population (21) and to get an overview of the genetic variability (22). He and Ho values obtained in this study for the *leptin* gene were below 0.5 (50%). Javanmard *et al*. (23) suggest that heterozygosity values below 0.5 (50%) indicate low variation of a gene in the population. The number of samples, the number of alleles, and the allele frequencies influence heterozygosity values. A higher number of animals and SNPs in the *leptin* and/or other genes should be analyzed for more accurate estimate F_{IS} and genetic variation in this population.

Comparing with other researches made on water buffalo, Azari *et al.* (24) analyzed 53 Mazandarani native river buffaloes from Iran by PCR-RFLP in *leptin* gene using *Bsa* AI. They also observed three genotypes. The highest genotype frequency detected by the authors corresponded to AG (AB) (0.509) genotype, different from the results of the present study. They reported frequency of GG (BB) genotype as the lowest, in accordance with this work. The allelic frequencies that Azari *et al.* (24) reported for A and G (B) alleles were 0.61 and 0.35, respectively. The first one was higher, and the second lower than the allelic frequencies detected in the present study. The Mazandarani buffalo population was in HWE for the locus analyzed as was observed in the Cuban population. The Ho (0.509) was slightly superior to that observed in this study but it was still near to 0.5.

In another research, Zetouni *et al.* (16) studied a Brazilian buffalo population also by PCR-RFLP in the *leptin* gene using *Bsa* AI. The authors identified the three genotypes and reported the highest frequency for AG (0.54) genotype and the lowest for GG (0.16). The allele frequency they found for the A allele (0.57) was lower than that observed in this work and the allele

frequency for the G allele (0.43) was higher. The author informed that the population was in HWE for this locus as the population study in this research. The Ho (0.54) was higher than that obtained in the present work.

Kale *et al.* (25) reported that digestion of the 522 bp fragment of Murrah buffalo *leptin* gene with *Bsa* AI yielded an uncut fragment, which indicated the frequency of AA genotype as 1. The polymorphic restriction site was absent in the Murrah buffaloes studied exhibiting monomorphic pattern in the analyzed population.

The analyzed Cuban buffalos resulted from uncontrolled crossbred of Carabao and Bufallypso, which is a composite breed formed by Murrah, Surti, Jaffarabadi, Nelli and Bhadawari (26). Considering the results reported by Kale *et al.* (25), the contribution made by the Murrah Indian riverine breed to the formation of Buffalypso could explain why AA was the most frequent genotype in the studied Cuban population.

In exon IV of cattle κ-*casein* gene, alleles A and B were detected by differences at codons 136 and 148 (27). Allele A has 136Thr (ACC)/148Asp (GAT), where as allele B has 136Ile (ATC)/148Ala (GCT). All previous reports on genotyping of water buffalo κ-*casein* gene (exon IV) by PCR-RFLP analysis used restriction enzymes *Hind* III, *Hinf* I, and *Taq* I, which successfully identify cattle A and B genotypes. The results obtained in the analysis of Cuban buffalo κ-*casein* gene were in accordance with Mitra *et al*. (14), who reported monomorphism (BB) for this gene, showing similar band pattern of 225 bp and 154 bp by using restriction endonuclease *Hind* III in Murrah and Nili-Ravi buffalo breeds. In other researches using PCR-RFLP, Pipalia *et al.* (27), Riaz *et al.* (28), Abbasi *et al.* (29), Ren *et al*. (30), and Jaayid *et al.* (31) also found monomorphism (BB) for this gene in diverse buffalo breeds from different countries.

However, Sing *et al.* (32) found the two alleles A and B for κ-*casein* locus in Murrah and Bhadawari breeds by PCR-RFLP, but they reported monomorphism (BB) in Surti and Mehsana breeds of buffalo. Similarly, Patel *et al.* (33) found alleles A and B in Murrah, Surti, and Pandharpuri buffaloes. They observed BB and AB genotypes in these three breeds and monomorphism (BB) in Jaffarabadi breed. The authors found BB genotype frequency (0.968) very much higher than the AB genotype (0.032). In another work, Gouda *et al.* (34) reported detection of BB and AB genotypes in Egyptian buffalo, also with higher frequency of BB genotype (0.75). Lin *et al.* (35) also studied a sample from different breeds and crossbreds of buffalo in China by RFLP. They found the three κ-*casein* genotypes AA, AB, and BB, though the latter genotype occupied the largest proportion. The authors detected that most of the AB genotype and all of the genotype AA appeared in crossbred buffalo (Murrah + Nili-Ravi and river type + Chinese local swamp), while just several AB and none of AA genotype were found in pure river type buffalo (Murrah, Nili-Ravi).

In the present study, a population of uncontrolled crossbred animals (composite river breed + swamp) was analyzed. Taking into account the presence of alleles A and B for κ-*casein* locus in Murrah reported by Sing *et al.* (32) and Patel *et al.* (33), and the presence of AA and AB genotypes in crossbred buffalo (Murrah + Nili-Ravi and river type + Chinese local swamp) and AB genotype in pure Murrah, referred to by Lin *et al.* (35), it was expected that Cuban buffaloes showed a polymorphic pattern in κ-*casein* exon IV by PCR-RFLP. However, the results are not in agreement with those of all these authors and agree with those obtained by Otaviano *et al.* (36), who also found BB monomorphism in κ-*casein* exon IV when they studied Brazilian Murrah breed and its crossbreds by PCR-RFLP.

Buffalo κ-*casein* polymorphism has also been investigated using nucleotide sequence analysis, and two nucleotide variants at codons 135Thr (ACC)/Ile (ATC), and 136Thr (ACC/ACT) (silent mutation) have been reported (14, 37-39). Allele A has 135ThrACC/136ThrACC, whereas allele B has 135IleATC/136ThrACT. In comparing partial nucleotide sequences (from codon 130 to 149) of alleles A and B in cattle and buffalo, Nahas *et al.* (39) found that *Hind* III restriction site ''A^AGCTT'' was present in cattle allele B (148Ala) and in both buffalo alleles A and B, whereas *Hinf* I restriction site ''G^ANT'', contrary to *Hind* III, was present in cattle allele A (148Asp) and was missing in cattle allele B as well as in both buffalo alleles A and B. Since buffalo samples (both alleles A and B) followed cattle BB pattern, they were mistakenly assumed as BB monomorphic where in fact they would have been AA, BB or AB. *Taq* I restriction site T^CGA is present at codon 136 in cattle allele B (136Ile) but was absent in buffalo alleles A and B. These findings are in contrast with those results referred to by Sing *et al.* (32), Patel *et al.* (33), Gouda *et al.* (34) and Lin *et al.* (35).

PCR-RFLP technology has been extensively used in buffalo DNA analysis (14, 16, 20, 25, 31, 40-43). In this study, PCR-RFLP with *Bsa* AI was able to detect the two genetic variants described for the *leptin* gene A1620G. However, the monomorphism BB finding in κ-*casein* gen using *Hind* III and the absent of a *Taq* I restriction site, indicates the necessity of DNA sequencing, as a method with higher specificity and

accuracy (44), to detect the new A and B variants of κ-*casein* reported in buffalo by nucleotide sequence analysis (45, 47, 48). As it was referred by Caroli *et al.* (44), when funding is limited, researchers may use RFLP analysis for screening of the total sample set for polymorphism, after which, samples exhibiting different RFLP patterns may be subjected to DNA sequencing analysis.

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