Polymorphism of the leptin gene in buffalo breed groups from eastern Amazon

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Summary
The objective of this work was to characterize genetically some buffalo herds raised in Varzea (VA) and Terra-Firme (upland) (TF) ecosystems through polymorphism of the intron 2 of the leptin gene (*LEP*-1620). Two hundred seventy-nine animals from four distinct populations were evaluated using the PCR-RFLP method for LEP-1620 polymorphism (SNP) of the leptin gene with restriction enzyme *Bsa*AI. The animal samples were sorted into 4 groups, according to their breed and environmental origin: Mediterranean TF, Murrah TF, Mediterranean VA and Crossbreed VA. Two alleles (A and G) were detected and their frequencies were analyzed. Allele A frequency ranged from 0.395 (Mediterranean TF) to 0.850 (Murrah TF), with AA genotype ranged from 0.114 (Murrah TF) to 0.700 (Mediterranean TF). The observed and expected heterozygosities ranged from 0.268 (Mediterranean TF) to 0.562 (Murrah TF), and 0.255 (Mediterranean TF) to 0.478 (Murrah TF), respectively. The Hardy-Weinberg probabilities were greater than 0.05. The Crossbred herd in Varzea was the only population with significant inbreeding and the Shannon index ranged between 0.423 (Mediterranean TF) and 0.671 (Murrah TF).

Introduction

The buffaloes were introduced in Brazil during the nineteenth century from Asia, Italy and the Caribbean. They quickly adapted very well to Brazil's lands because of the similarity of the environmental conditions with their native place of origin (Zetouni *et al.* 2013). In Brazil, the region that stands out in the production of buffaloes is the North Region which has approximately 877 thousand animals, representing about 50% of the buffalo herd in this country (IBGE 2014). Although buffaloes show lower carcass yields compared to cattle, they present superior performance in low fertility soils, accelerated growth and ease of handling, since they can adapt well to wide climatic zones such as equatorial, tropical, subtropical, mediterranean and even higher latitudes, allowing their greater distribution in the world (Campanile *et al.* 2016). In addition, unlike cattle, these animals don't require living in farming-like fields to convert food (plants) into meat and milk. Since they can live and be very productive in natural landascapes like the lands of Marajo Island, their significant lower cost maintanace makes them animals of great economic value (Ramalho *et al.* 2013, Rodrigues *et al.* 2015).

The study of genetic variability has progressed in recent years with the advancement and application of molecular techniques. This enabled improving livestock productivity, since these innovations led to great amount of researches using several molecular markers associated to animal productivity traits to take place. No doubt this is good news, however, the

number of investigations of this sort in bufflaloes is still scarce. This development allowed the use of several molecular markers related to animal productivity. Therefore, the search for molecular markers that aid genetic breeding programs is of high importance. When polymorphisms are found to be associated with possible productive or reproductive traits, they contribute to improving the herd's qualities (Rodrigues *et al.* 2010, Marcondes and Righetti 2011, Zetouni *et al.* 2013, Barbosa *et al.* 2016).

Among the genes involved in food control and productivity, we can highlight the leptin hormone, which has a relationship with energy control, food consumption and body weight reduction. It also works as a chemical signal, communicating to the brain that the body has sufficient energy reserves to provide the beginning of puberty. In addition, it plays the role of maintenance of their entire reproductive life, regulating animal metabolism and the reproductive system (Kowalski *et al.* 2014, Pérez-Pérez *et al.* 2015).

Leptin is a protein hormone with 167 amino acids, but the active form does not present the first 21 amino acids and has a molecular weight of 16 kDa, and it is expressed by the obese (ob) gene, located on the fourth autosomal chromosome, in bovines, with three exons and two introns (Lara *et al.* 2011). Its expression is mainly in white adipose tissue, but it also can be expressed in brown adipose tissue, ovary, stomach and placenta as well. However, leptin is most active in the hypothalamus. Within hypothalamic cells, after leptin binds to its specific receptors, it is brought to the cell nucleus, where it then binds to DNA sequences that control the expression of appetite-enhancing neurotransmitter inhibitor genes, such as neuropeptide Y (NPY), and stimulate appetite-reducing genes, such as propofolomelanocotin (POMC), resulting in satiety. This hormone also acts as a signal to the hypothalamus that the animal is under food restriction due to decreased secretion of leptin, signaling the use of energy reserve, thus decreasing adipose tissue (Catunda *et al.* 2014).

Several studies of the leptin gene revealed the presence of single nucleotide polymorphisms (SNPs) that were used as molecular markers and associated with weaning weight (Souza *et al.* 2010), milk composition, duration and difficulty of parturition (Giblin *et al.* 2010), and its implication in meat tenderness (Lara *et al.* 2011), mainly in cattle. Although there are few works related to the leptin gene in buffalo species, one of the markers studied was *LEP*-1620 (A/G) SNP, located in intron 2 of the leptin gene. It is strongly related to milk production and characteristics such as fat and protein in buffaloes (Zetouni *et al.* 2013). Thus, it is possible that some of these SNPs found in this gene might be associated with productive and economic characteristics of the animals of zootechnical interest through the analysis of the candidate gene for increasing the productivity of these animals. In particular, it would be beneficial to know more about this marker because it is a potential tool to be used by ranchers for genetically improving the quality of raising and breeding buffaloes at eastern Amazon region (Kowalski *et al.* 2014). The objective of this work was to characterize some buffalo herds from Varzea and Terra-Firme (upland) systems from Eastern Brazilian Amazon, through polymorphism on intron 2 of leptin gene (*LEP*-1620).

Materials and methods

This study was submitted to the ethics committee for animal use and approved with protocol number 033/2015 of the Federal Rural University of Amazon. Fifty hairs per animal were collected with bulbs from 279 buffaloes: 164 of these animals came from two pupulations of the Varzea system (49 Mediterranean and 115 Crossbred), and 115 animals comimg from two populations of Terra Firme system (10 Mediterranean and 105 Murrah). The varzea system population, Mediterranean and Crossbred, were in extensive system with native pastures, no fences and practically no sanitary, zootechnical or reproductive control of the herds, while the two populations coming from Terra-firme system, the Mediterranean and the Murrah were in semi-extensive to extensive system with cultivated pastures and sanitary, zootechnical and reproductive control of herds.

After collection, the biomaterial was stored at 2 °C until DNA extraction. Forty hair bulbs were selected from each animal for DNA extraction. The phenolic method was used in this step, in 1.5 mL tubes, following the procedure described by Sambrook and colleagues (Sambrook *et al.* 1989). A 522 pb amplicon was obtained by polymerase chain reaction (PCR). The primers used were those described by Lien and colleagues (Lien *et al.* 1997) from the intron 2 region to the exon 3 of the buffalo leptin gene (F-5'GTC TGG AGG CAA AGG GCA GAC T 3' and R-5'CCA CCA CCT CTG TGG AGT AG 3'). PCR mixture was obtained to a final volume of 15 µl, by homogenically mixing the following: 1.5 µl 10x PCR Buffer, 0.6 µl 50 mM $MgCl_2$, 1.0 µl 1.25 mM of dNTP (Invitrogen, Fortaleza, CE, Brazil), 0.5 µl 10 µM of each primer (foward and reverse), 0.3 µl 5U Taq DNA polymerase (Ludwig Biotec, Alvorada, RS, Brazil), 3.0 µl Q-solution (Quiagen, Valencia, CA, USA), 1.0 µl 50-100 µM of genomic DNA and completed with 6.6 µl pure water. The temperature and time conditions were: initial denaturation at 94 °C for 5 min, followed

by 30 cycles with denaturation at 94 °C for 60 s, annealing at 60 °C for 60 s, extension at 72 °C for 60 s, and ending cycle with final extension at 72 °C for 10 min. The final PCR products were visualized on 1.5% agarose gel stained with Gelred (Biotium/USA). A 100 bp ladder was used as reference. For restriction fragment length polymorphism (RFLP) reaction, PCR products were subjected to (or digested by) the *B*saAI enzyme (New England Biolabs, Inc.) under the following conditions: 3 µl of PCR product, 0.5 µl of restriction enzyme, 1 µl of reaction buffer and 10.5 µl of distilled water. The 15 ul volume reaction was heated at 37 °C for 60 minutes. The RFLP products were submitted to eletrophorese on 1.5% agarose gel stained with Gelred (Biotium/USA) at 90 V for 30 min. All genotypes were determined by visually analyzing the lethgths revaled by UV transluminater gel photo (or prints).

The POPGENE software version 1.32 (Yeh *et al.* 1997) was used to obtain all the statistical data needed for the analytical part of this study, including: the allelic and genotypic frequencies, the diversity parameters as observed (ObsHe), expected heterozygosities (ExpHe), Hardy-Weinberg probabilities (HWP), inbreeding coefficient (Fis) and Shannon Index (SI). The software was also used to determine the genetic identities and genetic distances of Nei (Nei 1972, Nei 1978) among the populations studied, and it developed two dendrograms by the UPGMA method from the genetic distances of Nei (Nei 1972, Nei 1978). Another software, GENEPOP version 4.6, (Raymond and Rousset 1995) was used to estimate the genetic and genotypic differentiations between breed groups and the F statistics (Fst, Fis and Fit). The significant level was 5%.

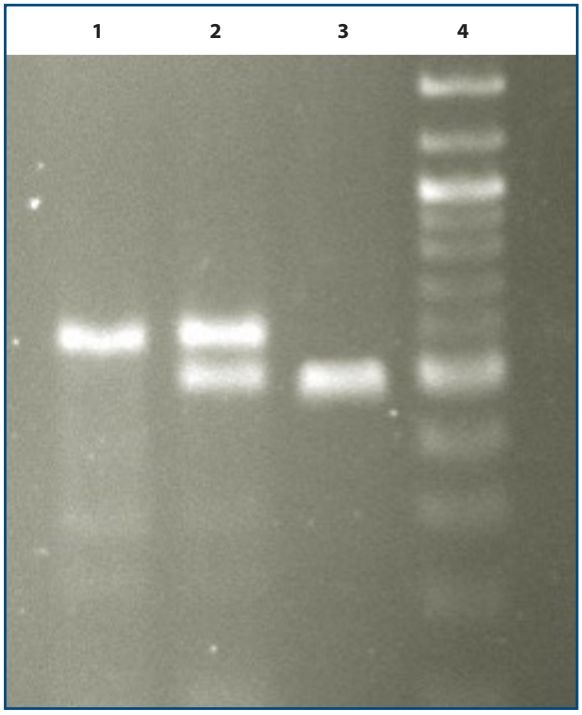

Figure 1. *Agarose gel (1.5%) demonstrating the genotype migration stands.* Lane 1 = AA, Lane 2 = AG, Lane 3 = GG and Lane 4 = DNA Marker (100bp). Fragment of 83 bp is not visible.

Results

We observed three digest stands (Figure 1): the homozygous AA (fragment of 522 bp), heterozygous AG (fragments of 522, 439 and 83 bp), and homozygous GG (fragments of 439 and 83 bp).

Table I shows the allelic and genotypic frequencies, and diversity parameters. The A allele was the most frequent in the Mediterranean breed (TF), Mediterranean breed (VA) and Crossbred (VA) populations, while the G allele was most frequent in the Murrah (TF) population.

The AA genotype was also the most frequent in the three populations where the A allele was the most frequent. However, the genotype heterozygous AG was the most frequent in the Murrah (TF) population. Observed and expected heterozygosities were lower in the Mediterranean (TF) population and higher in the Murrah (TF) population (Table I).

Both breed groups of the Terra-Firme and Varzea systems were within the Hardy-Weinberg equilibrium ($P > 0.05$), which indicates that the populations have imperceptible conditions of mutation, selection and migration (Table I). Only Crossbred population of Varzea presented inbreeding (Table I), being genetically similar. According to the Shannon

Table I. *Genetic diversity parameters of the LEP-1620 polymorphism in Amazon buffalo groups.*

Parameters	ME (TF)	MU (TF)	ME (VA)	CB(VA)	Total
Alleles*					
A	0.850	0.395	0.735	0.709	0.600
G	0.150	0.605	0.265	0.291	0.400
Genotypes*					
AA	0.700	0.114	0.510	0.513	0.369
AG	0.300	0.562	0.449	0.391	0.462
GG	0.000	0.324	0.041	0.096	0.169
Diversity					
HeOBS	0.268	0.562	0.449	0.391	0.462
HeEXP	0.255	0.478	0.390	0.413	0.480
HWP	0.660	0.080	0.320	0.540	0.520
Fis	-0.125	-0.171	-0.142	0.057	-0.108
SI	0.423	0.671	0.579	0.603	0.673

*Means frequencies; ME (TF) = Mediterranean Terra-firme; MU (TF) = Murrah Terra-firme; ME (VA) = Mediterranean Varzea; CB (VA) = Crossbred Varzea; HeOBS = Observed heterozygosities; HeEXP = Expected heterozygosities; HWP = Hardy-Weinberg probabilities; Fis = Inbreeding coefficient and Shannon indexes.

Table II. *Fst statistic among breed populations of buffaloes in Eastern Amazon.*

	ME (TF)	MU (TF)	ME (VA)	CB(VA)
ME (TF)	0.000			
MU (TF)	0.298	0.000		
ME (VA)	0.010	0.199	0.000	
CB (VA)	0.021	0.178	-0.006	0.000

ME (TF) = Mediterranean Terra-firme; MU (TF) = Murrah Terra-firme;
ME (VA) = Mediterranean Varzea; CB (VA) = Crossbred Varzea.

Table III. *Gene differentiations (below diagonal) and genotype differentiations (above diagonal) among breed populations of buffaloes in Eastern Amazon.*

	ME (TF)	MU (TF)	ME (VA)	CB(VA)
ME (TF)		HS	0.363	0.222
MU (TF)	HS		HS	HS
ME (VA)	0.397	HS		0.692
CB (VA)	0.211	HS	0.692	

HS = High significant; ME (TF) = Mediterranean Terra-firme;
MU (TF) = Murrah Terra-firme; ME (VA) = Mediterranean Varzea;
CB (VA) = Crossbred Varzea.

index, the highest value was found in Murrah (TF) with 0.671 (Table I), that is, it has greater diversity (Uramoto 2005, Silva *et al.* 2016) when compared to the lower value in Mediterranean (TF).

The F statistic for the leptin SNP for all buffalo populations was Fis = - 0.19, Fit = 0.34 and Fst = 0.13. Gene flow (Nm) estimated from Fst = 0.25 (1-Fst) / Fst was 1.69.

Table II shows the Fst values (Wright 1965) between populations. These values were considered high (Fst > 0.15) between the Mediterranean (TF) and Murrah (TF), 0.298, Mediterranean (VA) and Murrah (TF), 0.199, and Crossbred (VA) and Murrah (TF), 0.178.

In relation to the genic and genotype differentiations among the populations (Table III), the population of Crossbred (VA) and the population of Mediterranean (VA) are the groups with the lowest divergence.

Table IV shows the genetic identities and genetic distances of Nei among the studied populations. In this study, the populations of Mediterranean (TF) and Murrah (TF) were the most different, and the populations of Mediterranean (VA) and Crossbred (VA) were the most similar. To illustrate, Figure 2 represents two dendrograms, drawn from these genetic distances (Nei 1972, Nei 1978).

Discussion

The genetic polymorphism in discussion is on intron 2 of the leptina gene (Lien *et al.* 1997). The

Table IV. *Nei's genetic identity (above diagonal) and genetic distance (below diagonal) among breed populations of buffaloes in Eastern Amazon.*

	ME (TF)	MU (TF)	ME (VA)	CB(VA)
ME (TF)		0.688	0.991	0.982
MU (TF)	0.374		0.801	0.826
ME (VA)	0.009	0.222		1.002
CB (VA)	0.018	0.191	-0.002	

ME (TF) = Mediterranean Terra-firme; MU (TF) = Murrah Terra-firme;
ME (VA) = Mediterranean Varzea; CB (VA) = Crossbred Varzea.

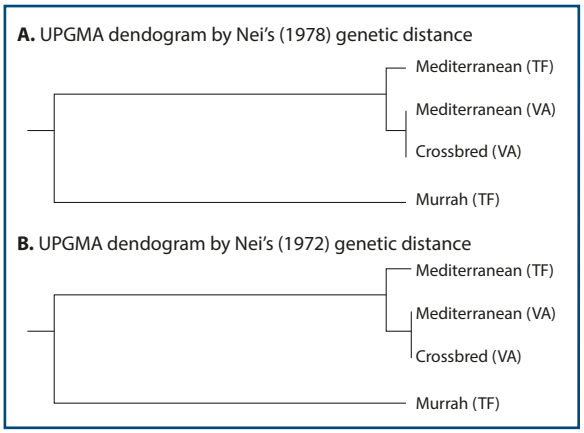

Figure 2. *Nei's dendrograms among breed populations of buffaloes in Eastern Amazon.*

results of the RFLP technique corroborate with those found by Lien and colleagues (Lien *et al.* 1997), who were the first to describe the substitution of a guanine (G) by an adenine (A) at the intron 2 in the leptin gene in bovine. Results were also corroborated with both Azari and colleagues (Azari *et al.* 2012), who studied the same gene when they described distributions of allelic and genotypic frequencies in three genetically different populations, including Holstein cows (*Bos taurus*), Mazandarani cattle (*Bos indicus*) and river buffaloes (*Bubalus bulalis*), and with Zetouni and colleagues (Zetouni *et al.* 2013a), who studied the existence of the same polymorphism, *LEP*-1620 (A or G), in buffaloes and their possible associations with milk, fat, protein and fat and protein percentages. In addition, the same authors found the same genotypes (AA, AG and GG) in all animals studied.

The values found for He were lower than those reported by Mishra and colleagues (Mishra *et al.* 2009), who found average heterozygosity of 0.572 for the Banni breed and 0.610 for the Murrah breed when they characterized 95 animals of these 2 breeds using 24 microsatellite markers, and by Marques and colleagues (Marques *et al.* 2011) who studied the genetic diversity of Brazilian buffaloes using twenty-five microsatellite

markers in five breeds (Carabao, Jafarabadi, Mediterranea and Murrah, plus the Baio type), with expected heterozygosity between 0.532 and 0.609. In the present work, we have observed less genetic variability when compared with what was presented by these authors.

Shannon's index is based on information theory (Ludwig *et al.* 1988) which provides a principle of degree of uncertainty, and it can predict which species an individual would randomly withdraw from the population. The higher the value of this index (Table I), the greater the degree of uncertainty, that is, the greater the genetic diversity (Uramoto 2005).

Fst is an index commonly used to measure distances between the studied populations, that is, the closer the values are to 1, the greater the differentiation between populations (Attia *et al.* 2014). These values were lower than those found by Marques and colleagues (Marques *et al.* 2011), with Fst = 0.1998 (0.1615-2,413). Fis is a parameter that expresses the occurrence of random mating in the population, that is, whether the population suffers inbreeding or not. In this study, the value of Fis was less than zero, and it shows the occurrence of mating between individuals that are not related. The Fit parameter expresses the difference of an individual's heterozygosity over (a or the) metapopulation. When both Fis and Fit values are close to zero, that means there is genetic variability in the population (Barros *et al.* 2011). That was the case with the results found in this study. Although the gene flow (Nm) above 1 means that the group does not have any significant genetic differentiation, when it is below 1, it is an evidence of genetic differentiation, and when it is greater than 4, this suggests a great amount of gene exchange (Wright 1965, Attia *et al.* 2014).

The highest value of Fst found, the one with the highest divergence (Wright 1965) when correlated, was observed in both the Mediterranean (TF) and Murrah (TF) breeds (Table III). Similar results were found by Albuquerque and colleagues (Albuquerque *et al.* 2006), who estimated the genetic variability with the use of RAPD markers among five groups of buffaloes raised in Brazil, two groups being conserved *in situ*, Carabao and Baio type, and three groups considered commercial breeds, Murrah, Jafarabadi and Mediterranean.

In this study, the population of Crossbred (VA) and the population of Mediterranean (VA) were the groups with the lowest divergence (Table III). This is explained by the fact that the two genetic groups have been submitted to crosses in the past (Albuquerque *et al.* 2006).

For Nei (Nei 1972, Nei 1978) the determination of the genetic distance allowed to verify the genetic variability among the populations. In this study, the genetic distance values reached revealed that the populations of Mediterranean (TF) and Murrah (TF) are the most different, and the populations of Mediterranean (VA) and Crossbred (VA) are the most similar (Table IV). These results confirmed a higher degree of kinship between the Crossbred and Mediterranean populations of the Varzea system, possibly due to long term interbreed between these two breeds (Figure 2).

Conclusions

The *LEP*-1620 molecular polymorphism suggested low genetic variations between the Mediterranean and Crossbred populations, which distinguish them from the Murrah breed. Both Mediterranean and Crossbred buffalo groups are preferred by milk production farmers in Eastern Amazon region. This suggests a possible selection of animals for milk production which present allele A at high frequency, specially in homozigose (AA).

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