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Assessing the genetic diversity and ancestry of Hartón del Valle cattle using mitochondrial DNA¤

Diversidad genética y ancestría del ganado Hartón del Valle mediante ADN mitocondrial

Estimação da ancestralidade e da diversidade genética no gado Hartón del Valle utilizando DNA mitocondrial

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Summary

The Hartón del Valle (HV) cattle is a Criollo breed of Spanish descent that has adapted to the conditions of the Cauca valley (Colombia). This breed is currently listed as "vulnerable" because its population has dramatically declined in the last two years. Prompted by the uncertain future of this breed and its importance as a potential resource of valuable genes. Objective: this study addressed the diversity, genetic structure and ancestry of HV cattle using mitochondrial DNA (mtDNA). Methods: a 350-bp fragment was amplified and sequenced from 72 HV animals in 9 separate farms and from animals of Brahman and Holstein breeds. To analyze the breed's ancestry, the sequences were compared with 560 sequences available in the GenBank, representing 50 Bos taurus and Bos indicus breeds. Results: in accordance with the Spanish origin of the HV breed, there was a high representation of European mtDNA (91.7%) and a low representation of African (5.5%) and Middle Eastern mtDNA (2.7%). The average haplotype diversity was 0.65 ± 0.05. The farm with the oldest ancestry was the only population in which three mitochondrial lineages were observed; unfortunately, it was recently depopulated. Proximity was observed between HV and two Columbian breeds, the Romosinuano and Costeño con Cuernos. A comparative analysis with the sequences deposited in the GenBank from numerous breeds revealed the presence of 37 haplotypes, seven of which were unique to HV. Conclusions: the following Iberian breeds were found to be most closely related to HV: Tudanca, Rubia Gallega, Negra Serrana, Murciana, Pajuna, Avileña, Garvonesa and Mertolenga. Phylogenetic analysis confirmed the Iberian ancestry and some African influence on this Latin American Criollo breed.

Key words: *creole cattle, haplotypes, lineages, phylogeny.*

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Resumen

El Hartón del Valle (HV), es una raza adaptada a las condiciones del Valle del Cauca (Colombia) que está catalogada como "vulnerable" y cuya población ha descendido drásticamente en los últimos dos años. Objetivo: debido al futuro incierto y a la importancia como recurso potencial en la seguridad alimentaria regional se abordó el estudio de la diversidad, la estructura genética y la ancestría del HV, mediante ADN mitocondrial (ADNmt). Métodos: se amplificó y secuenció un fragmento de 350 pb en 72 animales HV, provenientes de nueve poblaciones y de controles de las razas Brahman y Holstein. Para analizar la ancestría las secuencias fueron comparadas con 560 secuencias de 50 razas Bos taurus y Bos indicus, depositadas en el GenBank. Resultados: de acuerdo con su origen español, se encontró una marcada influencia del ADNmt europeo (91.7%) y baja participación de taurinos de origen africano (5.5%) y del cercano Oriente (2.7%). La diversidad haplotípica promedio fue 0.65 ± 0.05. El hato más antiguo fue el único que mostró los tres linajes mitocondriales, sin embargo, este ha sido liquidado recientemente. Se observó proximidad entre el HV con las razas colombianas Romosinuano y Costeño con Cuernos. La comparación con las secuencias depositadas en el GenBank con diferentes razas reveló la presencia de 37 haplotipos, de los cuales siete fueron únicos en el HV. Conclusiones: las razas ibéricas más cercanas al HV fueron: Tudanca, Rubia Gallega, Negra Serrana, Murciana, Pajuna, Avileña, Garvonesa y Mertolenga. El árbol filogenético confirmó la ancestralidad ibérica y la influencia africana en las razas criollas de América Latina.

Palabras clave: *filogenia, ganado criollo, haplotipos, linajes.*

Resumo

O Hartón del Valle (HV) é uma* raça *adaptada* às condições *da região pertencente ao departamento do Valle del Cauca (Colombia). A raça está catalogada como vulnerável já que sua* população *tem sido reduzida drasticamente nos últimos dois anos. Objetivo: devido a incerteza no futuro da raça e a sua importancia como recurso potencial na* segurança *alimentaria regional, abordou-se o estudo da diversidade, a estrutura genética e a ancestralidade do gado HV, utilizando DNA mitocondrial (mtDNA). Métodos: para isto, se amplificou e sequenciou um fragmento de 350 pares de bases em 72 animais provenientes de nove* populações *e também de controles das* raças *Brahman e Holandesa. Para analizar a ancestralidade das sequências, estas foram comparadas com outras 560 de 50* raças *Bos taurus y Bos indicus depositadas no genebank. Resultados: de acordo com sua origem espanhola, foi encontrada uma marcada influência do DNA mitocondrial europeu (91.7%), e baixa* participação *de taurinos de origem africana (5.5%) e do Oriente Próximo (2.7%). A média da diversidade haplotípica foi de 0.65 ± 0.05. A população de gado HV mais antiga foi a única que apresentou as três linhagens mitocondriais encontradas; embora, esta população foi terminada recentemente. Observou-se também proximidade do gado HV com as* raças *colombianas da região Caribe, Romosinuano e Costeño con Cuernos. A comparação com as sequências depositadas no genebank com diferentes* raças *revelou a presença de 37 haplotipos, dos quais sete foram únicos no HV. Conclusões: as raças ibéricas mais aproximadas do HV foram: Tudanca, Rubia Gallega, Negra Serrana, Murciana, Pajuna, Avileña Garvonesa e Mertolenga. A árvore filogenética confirmou sua ancestralidade ibérica e a influência africana nas* raças *crioulas da América Latina.

Palavras chave*: filogenia, gado nativo, haplótipos, linhagens.*

Introduction

The origin of the Hartón del Valle (HV) cattle breed is similar to that of other Colombian Creole breeds. Iberian cattle brought by the Spanish during the colonial era underwent a long process of natural selection to adapt to local conditions, which is evidenced by the high reproductive rates, tolerance to parasite diseases and increased survival of these breeds (Casas and Valderrama, 1998).

In the last census of Creole cattle, the population consisted of 5,120 purebred animals (Martínez, 1999), and ten years later, estimates indicate that the population was reduced to 3,500 animals. Additionally, three of the four farms that provided breeding stock have closed down in recent years. The decrease in the number of Creole cattle has resulted from the conversion of cattle farms to sugar cane farms, the interbreeding of cattle with foreign breeds and the migration of cattle to other

provinces. In particular, the HV breed has never been included in the protection programs for Creole cattle, which were established by the Colombian government in the 1930s.

The analysis of mitochondrial DNA (mtDNA) sequences has been used extensively to study the origins of and the relationships among cattle breeds (Bruford *et al.,* 2003). Although the T3 haplogroup is predominant among American Creole cattle breeds, haplotypes of African origin have also been detected (Cymbron *et al.,* 1999; Miretti *et al.,* 2002; Beja-Pereira *et al.,* 2006; Egito, 2007; Magee *et al.,* 2002; Mirol *et al.,* 2003; Lirón *et al.,* 2006; Ginja *et al.,* 2009).

By studying the mtDNA control region in 110 samples from seven Colombian Creole cattle breeds, Carvajal-Carmona *et al.* (2003) found that 65% of the mitochondrial samples corresponded to European ancestry (T3), 26% to African ancestry (T1) and 9% to the T2 lineage. Using 21 samples from the HV breed, the percentages observed for the T1, T2 and T3 lineages were 38%, 29% and 33%, respectively (Carvajal-Carmona *et al.*, 2003).

The present study was motivated by concerns about the vulnerability and uncertain future of the HV breed, as well as the importance of the breed as a potential resource for regional food security. The aims of the study were to analyze the diversity, genetic structure and ancestry of the HV breed and its relationships with *Bos taurus* and *Bos indicus* breeds using mitochondrial DNA analysis.

Materials and methods

Sampling, amplification and sequencing of mtDNA

The mtDNA was obtained from blood samples, which were processed using the desalting method (Miller *et al.,* 1988). Because mtDNA haplotype diversity is low (≤ 0.03), 100 random samples were taken from unrelated animals in addition to 500 HV samples from the DNA bank at the National University of Colombia at Palmira. After analyzing the samples, however, only 72 sequences met the quality standards. These samples came from nine farms located in the Valle del Cauca: El Capricho (EC), Casa Rincón (CR), the National University of Colombia (UN), Gran Capricho (GC), Jamaica (JA), La Ondina (LO), Procampo (PR), San Rafael (SR) and Zanjón Hondo (ZH). The oldest herds and varied production systems (milk, meat and dual-purpose) were represented. As a reference, samples from unrelated Brahman (B; N = 6) and Holstein (H; N = 9) cattle were randomly selected from the DNA bank among 250 samples of these two breeds.

A 350-bp fragment located in the most highly variable region of the D-loop was amplified using the primers AN4 (5'-GGTAATGTACATAACATTAATG-3') and AN3 (5'-CGAGATGTCTTATTTAAGAGG-3'), as described by Cymbron *et al.* (1999). This sequence was selected to analyze the relationships between cattle breeds because it has been widely used in different studies of Iberian breeds and American Creole breeds (Bradley *et al.,* 1996; Beja-Pereira *et al.,* 2006). The amplification reactions were carried out using a 50 µl reaction volume with 50 ng of mtDNA, 0.6 units of Taq polymerase, 10X PCR buffer, 3.5 mM $MgCl_2$, 1.25 mM dNTPs and 20 µM forward and reverse primers. The PCR conditions were as follows: an initial denaturation for 3 minutes; 35 cycles of 94 °C for 20 seconds, 55 °C for 40 seconds and 72 °C for 40 seconds; and a final extension at 72 °C for 4 minutes. During the annealing step of the second amplification cycle, the reaction was paused at 55 °C for 3 minutes. The amplified product was visualized on a 1.5% agarose gel, which was stained with ethidium bromide and run at 70 volts for 30 minutes. The fragment size was compared to a 100-bp molecular weight marker. Both sides of the amplified DNA fragment were sequenced, and the editing and alignment were performed using Sequencer software (version 6.1.0, Gene Codes Corp.).

For the phylogenetic analysis of HV, the obtained sequences were compared with 560 mtDNA control region sequences deposited in GenBank, which represent 50 animals of the *Bos taurus* and *Bos indicus* breeds.

Statistical analysis

To infer the spatial distribution of haplotypes, a nested clade analysis was performed using TCS

software (version 1.1.3). This analysis represents the lowest possible number of steps (parsimonious method), which is interpreted as a distance measure, to accommodate a haplotype network (Clement *et al.,* 2000). The distribution of HV haplotypes and the relationships with the Colombian Creole, South American, European, Indian and African breeds were determined by constructing median-joining networks for the haplotypes (Bandelt *et al.,* 1999) using NETWORK software (version 4.1.0.8, Fluxus Technology Ltd., 2004).

Polymorphisms were identified by comparing the sequences to the reference sequence NC_001567 (2), which corresponds to the central haplotype (*Eucons*) of the European taurine cluster (T3) (Bandelt *et al.,* 1999). Variations from the consensus sequence or from another root haplotype (*Afcons*) were recorded. The *Afcons* haplotype is considered the root of the African taurine cluster (T1) and differs from *Eucons* by the C→T, T→C and T→C transitions at positions 16050, 16113 and 16255, respectively, which can be written as *Afcons* (Eucons050T-113C-255C). Distance matrices were generated according to the Kimura 2p model. Consensus networks were taken into account for the phylogenetic trees, which were constructed using the neighbor-joining method and Mega software (version 3.0) (Kumar *et al.,* 2005).

Results

Diversity of the mtDNA control region

Haplotypes and lineages: The analysis of the mtDNA hyper-variable region in 72 sequences from HV cattle and in 15 sequences from the reference breeds revealed 16 mitochondrial haplotypes defined by the polymorphisms at 26 sites (Table 1). In HV, 14 haplotypes were identified based on 24 polymorphic sites (18 transitions and six transversions). Haplotypes I to X corresponded to the T3 lineage, which is characteristic of European breeds; haplotype XI was the only representative of the T2 lineage, which is characteristic of Middle Eastern breeds; and haplotypes XII to XVI corresponded to the T1 lineage, which is characteristic of African breeds. Haplotype XV was unique to B, and haplotype IX was unique to H. All H individuals corresponded to the T3 lineage.

Zebu haplotypes were not observed in B, and 83% of the individuals belonged to the European lineage. The haplotype unique to B, haplotype XV, is derived from *Afcons* and has only been reported in Latin American Creole breeds; this haplotype was not observed in HV. In accordance with its European ancestry, the most common lineage in HV was the T3 lineage (91.7%), which was represented by nine haplotypes (Table 1, Figure 1). There were four T1 haplotypes (5.5%), indicating the contribution of African breeds to HV, and there was a single haplotype for the Near Eastern T2 lineage (2.7%). The most frequent haplotypes were I (57%), III (14%) and IV (11%) of the T3 lineage. The distribution of the haplotypes showed three levels of aggregation: the nine haplotypes of the T3 lineage, haplotype X of the T3 and T2 lineages and the four haplotypes of the African T1 lineage.

Among the nine farms, the LO estate had the only cattle population in which three lineages were observed, and there were seven total haplotypes in LO (Table 2). The UN and PR farms each had two lineages, and all other farms represented only the T3 lineage. Haplotype I was present in all of the sampled populations, and all of the JA individuals belonged to this group. Haplotypes III and IV were found in five and four populations, respectively, and haplotypes II, VI and VII were unique to CR, EC and ZH, respectively.

The average haplotype diversity was estimated using the Nei method (1987). In the nine HV herds, the diversity was 0.65 ± 0.05 (Table 3), which was lower than the diversity observed in B but greater than that in H. The GC, ZH and LO herds were notable for their high levels of haplotype diversity, UN was notable for its low diversity (0.26) and JA was notable for its uniform mtDNA.

Molecular analysis of variance did not reveal significant differences between the genetic groups (HV, H and B), and the fixation index (F_{ST}) was not significant because the heterogeneity of variances did not allow for the detection of differentiation. A low genetic structure was observed among the HV farms (F_{ST} = 0.035, p>0.06). Significant differences were observed between EC and ZH, between CR and ZH and between LO and ZH.

Table 1. mtDNA control region sequences and the number of haplotypes and lineages observed in 87 animals. The table shows the positions that differ with respect to the European consensus sequence (*Eucons*), as defined by Anderson *et al.* (1982) (in bold). Hartón del Valle (HV), Holstein (H), Brahman (B).

Haplotypes	16022	16050	16053	16055	16056	16057	16061	16066	16084	16087	16113	16115	16122	16127	16133	16135	16139	16165	16185	16195	16200	16234	16240	16247	16248	16255	Number of individuals/ haplotype			Lineage
	G	C	T	T	A	G	T	A	C	T	T	T	T	C	T	T	C	T	G	G	G	T	T	C	C	T	HV	H	B	
I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	41	6	3	
II	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	1				
III	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	10	1			
IV	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	8		2	T3	
V	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	2	1			
VI	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	1				
VII	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1				
VIII	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	1				
IX	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		1			
X	.	.	.	.	.	.	.	.	.	G	.	.	.	C	C	.	.	A	.	.	.	G	.	.	C		1				
Subtotal																											66	9	5		
XI	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	C		2			T2	
XII	.	T	.	.	.	.	G	T	.	C	.	.	.	.	.	T	.	.	.	.	.	.	.	.	C		1				
XIII	.	T	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	G	.	.	.	C		1			T1	
XIV	.	T	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	A	.	.	.	T	C		1				
XV	.	T	C	.	.	.	.	.	.	C	.	C	.	.	.	T	.	.	A	.	.	.	.	.	C				1		
XVI	A	T	.	C	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C		1				
Subtotal																											4		1		
Total																											72	9	6	87	

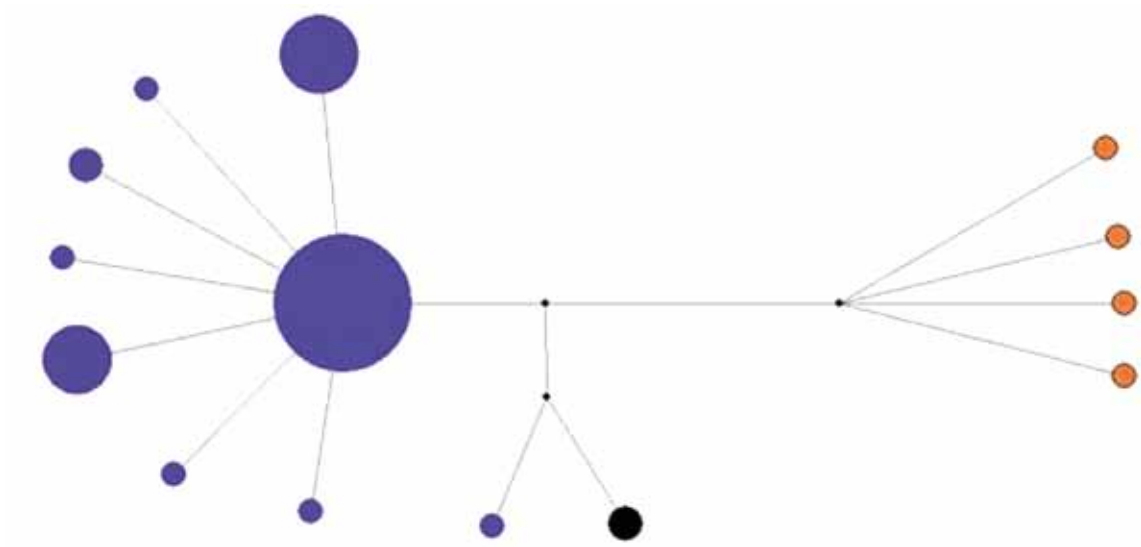

Figure 1. mtDNA haplotype network of 72 HV individuals constructed using the median-joining method (Bandelt *et al.,* 1999). The circles represent different haplotypes, and the areas of the circles are proportional to the frequency of each haplotype. The lineages are distinguished by color: T1 (orange), T2 (black) and T3 (violet). The lengths of the lines correspond to the number of mutational events separating haplotypes. The black circles represent extinct or unsampled haplotypes.

Table 2. Relative frequencies of haplotypes observed in HV, as shown for each farm and grouped according to lineage.

Lineage	Haplotype	Farms								
		EC	CR	UN	GC	JA	LO	PR	SR	ZH
T3	I	0.42	0.20	0.84	0.33	1.00	0.47	0.70	0.63	0.33
	II		0.20							
	III	0.33					0.20	0.10	0.13	0.33
	IV	0.08	0.60	0.07			0.06		0.25	
	V				0.33			0.10		
	VI	0.08								
	VII									0.33
	VIII	0.08								
	X				0.33					
T2	XI			0.07			0.06			
T1	XII							0.10		
	XIII						0.06			
	XIV						0.06			
	XVI						0.06			
	Num. Haplotypes	5	3	3	3	1	7	4	3	3

Table 3. Haplotype and nucleotide diversity in the nine HV populations and in the B and H samplings, as estimated according to the Nei method (1987) using Arlequin software. Number of samples (N).

Breed	Farm	N	Haplotype Diversity	Nucleotide Diversity
HV	EC	12	0.75 ± 0.09	0.04 ± 0.03
	CR	5	0.70 ± 0.21	0.03 ± 0.03
	UN	13	0.29 ± 0.15	0.04 ± 0.03
	GC	3	1.00 ± 0.27	0.18 ± 0.15
	JA	3	0.00 ± 0.00	0.00 ± 0.00
	LO	15	0.77 ± 0.10	0.11 ± 0.06
	PR	10	0.53 ± 0.18	0.07 ± 0.04
	SR	8	0.60 ± 0.16	0.03 ± 0.03
Subtotal	ZH	3	1.00 ± 0.27	0.05 ± 0.05
		72	0.65 ± 0.05	0.06 ± 0.04
B		6	0.73 ± 0.15	0.11 ± 0.08
H		9	0.58 ± 0.18	0.02 ± 0.02

Relationship of the Hartón del Valle breed with other Colombian Creole cattle breeds. The 72 HV sequences were aligned with 74 sequences of Colombian Creole cattle breeds (CCB) available in GenBank (Carvajal-Carmona *et al.,* 2003). The sequence comparisons revealed 19 haplotypes for the T3 lineage, 12 haplotypes for the T1 lineage and a single haplotype for the T2 lineage (Figure 2). Haplotypes V, VI, VII, VIII, X, XII and XIII identified in HV have not been reported in CCB. In accordance with the European ancestry of HV, most of the haplotypes corresponded to the T3 line. Of the 12 T1 haplotypes, four were observed in HV.

For the T2 lineage, represented by haplotype XI, there were two HV individuals and six CCB individuals. The phylogenetic trees of the seven CCB indicate that Blancorejinegro and Chino Santandereano are noticeably different from the other breeds. HV showed proximity to the Romosinuano breed and, to a lesser extent, to the Casanareño and San Martinero breeds (Figure 3, Table 4).

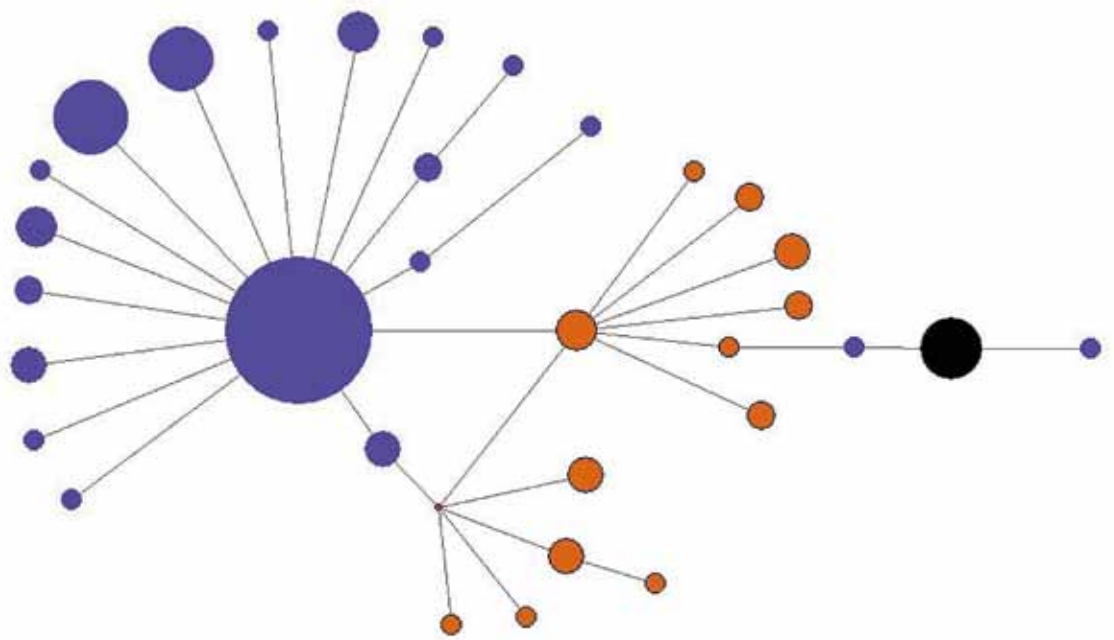

Figure 2. Haplotype network based on the comparison of 72 HV sequences and 74 sequences of Colombian Creole cattle breeds. The circles represent different haplotypes, and the areas of the circles are proportional to the frequency of each haplotype. The lineages are distinguished by color: T1 (orange), T2 (black) and T3 (violet).

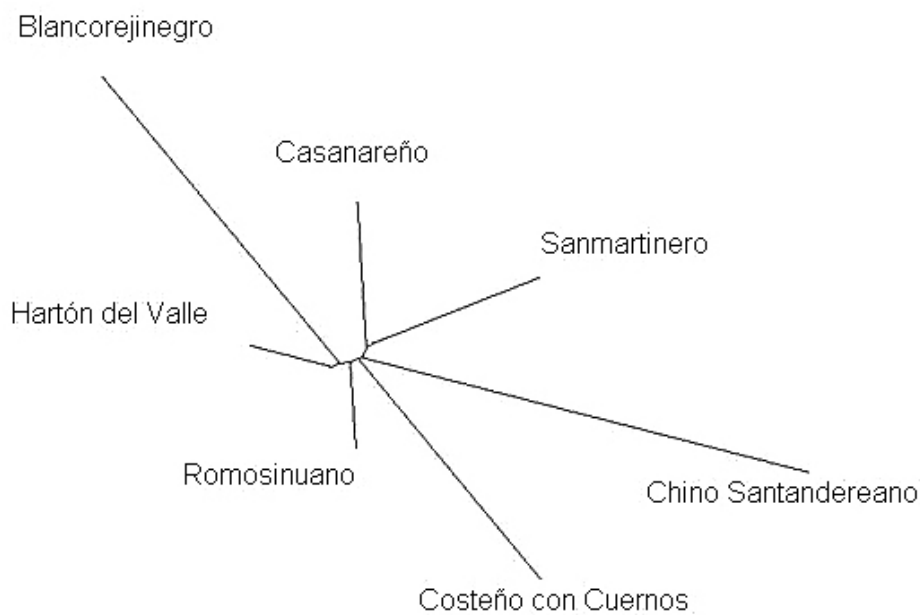

Figure 3. Phylogenetic tree of Colombian Creole cattle breeds inferred from a sequence within the D-loop. The framework was based on Kimura 2p distances, as grouped by the neighbor-joining method.

Table 4. Distance matrix for the Columbian Creole cattle breeds based on the Kimura 2p model and constructed using Mega software (version 3.0).

	SMT	ROS	CHS	BON	CAS	CCC	HV
SMT	*						
ROS	0.007	*					
CHS	0.013	0.010	*				
BON	0.014	0.010	0.017	*			
CAS	0.008	0.005	0.012	0.013	*		
CCC	0.010	0.007	0.014	0.014	0.009	*	
HV	0.008	0.004	0.012	0.012	0.007	0.009	*

San Martinero (SMT), Romosinuano (ROS), Chino Santandereano (CHS), Blancorejinegro (BON), Casanareño (CAS), Costeño con Cuernos (CCC), Hartón del Valle (HV).

The relationship of the Hartón del Valle breed with other breeds from around the world. Using TCS software, a haplotype network was constructed with 560 sequences representing 50 *Bos taurus* and *Bos indicus* breeds from various regions. After validating the network and excluding unrelated sequences from the information generated in this study, 353 sequences remained, which represented only the Iberian, African and Latin American Creole breeds. The network revealed the presence of 37 different haplotypes; nine of the haplotypes were observed in HV, and seven were unique (Figure 4). Fifty percent of the sequences (175/353) represented the central haplotype of the T3 cluster (*Eucons*). This group included 87 HV sequences, 29 sequences from Spanish breeds (Pajuna, Lidia, Rubia Gallega, Avileña, Mostrenca, Murciana, Monchina, Morucha, Negra Serrana, Retinta and Tudanca), 15 sequences from Portuguese breeds (Preta, Maronesa, Garvonesa and Mertolenga), 10 sequences from Argentine Creole breeds, 2 sequences from Bolivian Creole breeds, 6 sequences from Caribbean Creole breeds and 3 sequences from the African Kuri breed. Fifteen percent of the sequences represented the central haplotype of the African T1 cluster (*Afcons*), and the majority of these sequences (60%) came from animals of African cattle breeds (Egipcia, Kapsiki, N'Dama, Somba, White Fulani, Kuri and Kenana). A smaller number of the T1 sequences came from animals of Spanish, Portuguese, Caribbean Creole and Colombian Creole (one HV, two Romosinuano) breeds. The four HV sequences corresponding to the T1 lineage in this study were located in peripheral haplotypes. The unique haplotype representing the T2 lineage was present in five HV animals, two Blancorejinegro cattle and one Chino Santandereano individual. This T2 haplotype may have evolved in Colombia because it was not found in other breeds.

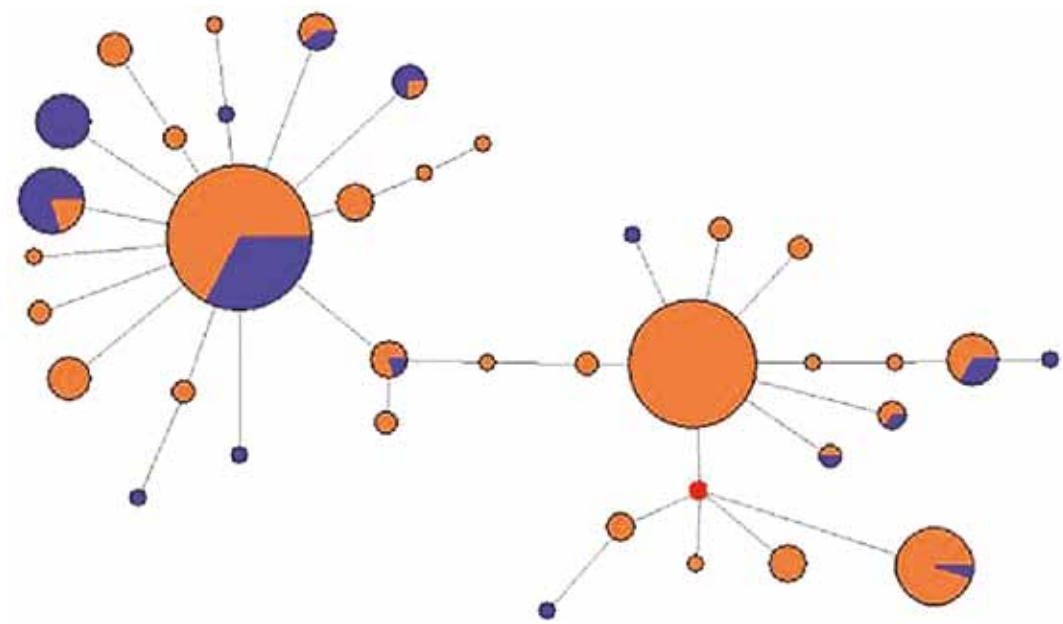

Figure 4. Haplotype network of 72 HV sequences (purple) compared with 353 sequences obtained from other parts of the world (orange). The circles represent different haplotypes, and the areas of the circles are proportional to the frequency of each haplotype. The lengths of the lines correspond to the number of mutation events that separate the haplotypes. The red dot is an extinct or unsampled haplotype.

The relationship between HV and the Iberian and African breeds is shown in Figure 5. HV was found to exhibit phylogenetic proximity with the Iberian breeds, namely, the Spanish breeds of Tudanca, Rubia Gallega, Negra Serrana, Murciana, Pajuna and Avileña, and the Portuguese breeds of Garvonesa and Mertolenga. The African breeds were present in a different cluster, with the Kuri breed being the most closely related to the Iberian breeds and HV. A phylogenetic tree was constructed using the sequences from Iberian, African and Latin American breeds (Figure 6). The observed relationships confirmed the Iberian ancestry of and African influence on the Latin American Creole breeds; of these, only the Guadeloupe Creole breed was distant from the others. With the exception of the Blancorejinegro and Chino Santandereano breeds, the Colombian breeds were grouped into a cluster containing Spanish and Portuguese breeds along with the Creole breeds of Argentina, Bolivia and the Caribbean.

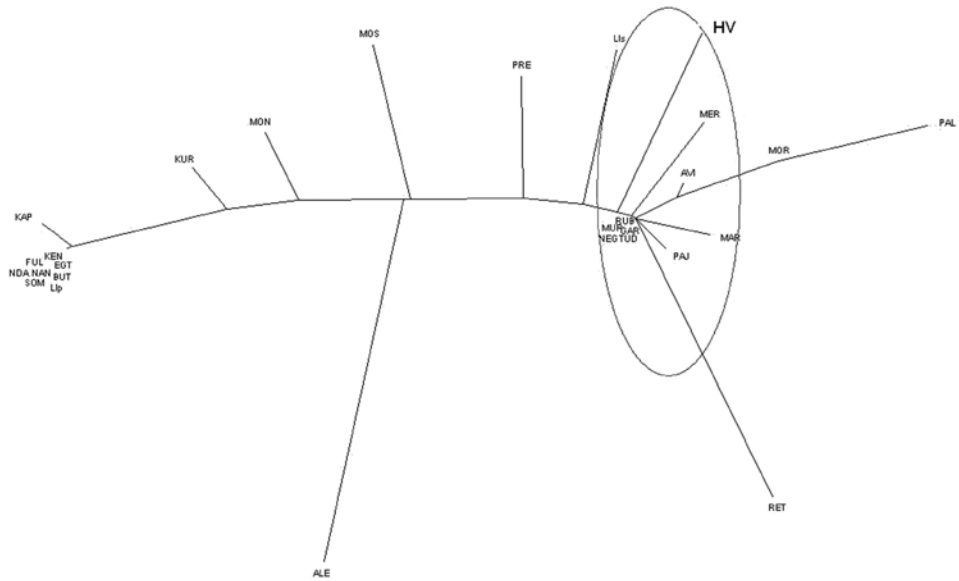

Figure 5. Phylogenetic tree relating the HV breed to Iberian and African breeds based on a sequence within the D-loop. The framework was based on Kimura 2p distances grouped by the neighbor-joining method and constructed using Mega software (version 3.0).

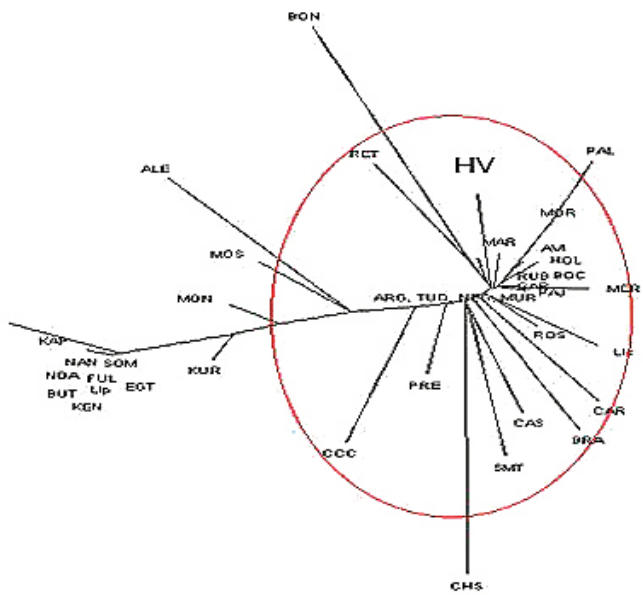

Figure 6. Phylogenetic tree relating HV to Iberian, African and Latin American breeds based on a sequence within the D-loop. The framework was based on Kimura 2p distances grouped by the neighbor-joining method and constructed using Mega software (version 3.0).

Discussion

Variation of the mtDNA control region in the Hartón del Valle breed

The findings of the present study reveal the highly diverse collection of mtDNA in the HV breed; more specifically, 14 haplotypes were identified representing three mitochondrial lineages. The haplotypes were defined based on the polymorphisms at 24 sites. Eighteen of these polymorphisms were transitions, and six were transversions, thereby corroborating the transitional bias previously described in cattle (Bradley *et al.,* 1996; Carvajal-Carmona *et al.,* 2003; Loftus *et al.,* 1994a). The number of haplotypes was greater than the numbers previously reported in HV (Carvajal-Carmona *et al.,* 2003) and in four Brazilian Creole breeds and one Argentine breed (Miretti *et al.,* 2002) and similar to the number identified in three Caribbean Creole breeds and the Nellore breed (Magee *et al.,* 2002).

Cattle were introduced to the Caribbean by the Spanish conquistadors in 1493, and by 1525, cattle populations had expanded to Central and South America. Cattle shipments from Portugal to Brazil have also been documented (Primo, 1992), along with the introduction of African cattle to the continent during the sixteenth and seventeenth centuries as a result of the slave trade. As previously reported by Carvajal-Carmona *et al.* (2003), HV sequences represent three of the four Old World cattle lineages, defined by Troy *et al.* (2001). The findings of the present study reveal a large proportion of European mtDNA (T3) and a low proportion of African mtDNA (T1) within the HV breed. The distribution of the lineages was 5.5%, 2.7% and 91.7% for the T1, T2 and T3 lineages, respectively. Several studies have shown that T3 is the most common haplogroup among American Creole breeds (Lirón *et al.,* 2006; Mirol *et al.,* 2003; Miretti *et al.,* 2002; Ginja *et al.,* 2009).

These findings contrast sharply with the distribution previously reported for HV (38%, 29% and 33% for T1, T2 and T3, respectively) by Carvajal-Carmona *et al.* (2003). The difference may reflect the increased sample size in the present study (72 versus 21 samples), along with the different sizes and varied production systems of the farms included in the study.

Haplotypes of African origin have been detected in Portuguese and Iberian breeds (Beja-Pereira *et al.,* 2006; Cymbron *et al.,* 1999; Miretti *et al.,* 2002), as well as American Creole breeds (Beja-Pereira *et al.,* 2006; Egito *et al.,* 2007; Lirón *et al.,* 2008; Miretti *et al.,* 2002; Mirol *et al.,* 2003). A high maternal contribution of African breeds has been observed in Brazilian Creole breeds (T3=44.4% and T1=40.7%), and Brazil is the only South American country in which cattle of Portuguese origin contributed to the Creole breeds (Egito *et al.*, 2007).

From 454 sequences of Creole (Colombia, Argentina, Brazil and West Indies), Iberian and African breeds, Lirón *et al.* (2006) found that the T3 haplogroup was the most common haplogroup among American Creole breeds (63.6%) followed by the African T1 haplogroup (32.4%) and the Middle Eastern T2 haplogroup (4%). Within the African lineage, two subclusters were identified: T1a, which has been reported in cattle in Brazil and the Lesser Antilles and with a distribution that does not include Africa, and T1, which is limited to the region of America colonized by the Spanish. In the present study, the majority of the HV samples corresponded to the European haplogroup (91.7%), and the percentage corresponding to the African lineage was significantly lower. A sample from a B individual corresponded the T1a haplotype, which may have originated in Brazil based on the findings that T1a is restricted to Brazil and the non-Spanish Caribbean Islands (Lirón *et al.,* 2008). Two HV individuals belonging to the same population were of the T2 lineage, which is associated with breeds from the Near East and has only been reported in Colombia.

Several explanations have been proposed for the presence of African haplotypes in American Creole cattle: 1) the introduction of the T1 lineage to America from West Africa through intermediate slave trade ports, 2) the entry of African cattle onto the Iberian Peninsula during the Arab invasion prior to the conquest of America and 3) the contribution

of Brazilian cattle with Portuguese ancestors that have African matrilineages (Lirón *et al.,* 2008; Magee *et al.,* 2002).

Haplotype diversity in HV (0.65 ± 0.05) was lower than that of breed B but greater than that of H. It is possible that the high diversity of HV has arisen since the time of the conquest and that a founding population of animals from different origins contributed to the breed. In support of this hypothesis, it has been reported that cattle arrived in the Valle del Cauca from the four cardinal directions (Finch, 1991). Cattle coming from the north and east derived from ancestral stock on the Caribbean coast, and those from the south originated in Ecuador. In 1540, cattle arrived in Cali via the trail through Dagua (further west); these cattle originated from Hispaniola and Nicaragua, and were introduced via the Panama Canal and the city of Buenaventura (Finch, 1991).

Among the nine farms, GC, ZH and LO had the highest haplotype diversity. LO was the only farm in which three mitochondrial lineages were observed, but the herd was liquidated in June 2009. The LO estate was established in 1890 and had the oldest tradition of cattle breeding, with founding animals that were descendents of the oldest herds in Roldanillo (Valle del Cauca). The high level of genetic diversity resulted from the use of a broad genetic base of pure livestock and the use of breeding bulls from other farms (Valderrama, 2008). The animals belonging to this herd were of great interest for the conservation of the genetic diversity of the breed, which was also observed using autosomal microsatellites and milk proteins (Álvarez, 2008).

Another herd with high diversity was GC, which, together with EC, belonged to Atogan Ltd., a cattle conglomerate dedicated to the production of milk and dairy products that was liquidated in 2008. The farms were established in 1955 with 40 HV cows, two HV bulls, two young HV-European mixed breeding bulls and 40 crossbred cows (Pardo Suizo, Zebu and Jersey). By eliminating the crossbreeds and by using the HV bulls, this conglomerate produced a purebred cattle herd. Later crosses were made using Holsteins and, to a lesser extent, German Anglers in order to improve milk production and certain characteristics of the breed.

By 1991, the farm had approximately 1,200 milking cows and, using artificial insemination, achieved an average production of 2,500 kg milk/day (Valderrama, 2008). The artificial insemination technique used in this population favored the sampling of a larger number of bulls over natural mating. Therefore, the high diversity of this population was a result of a multiracial founder population and the widespread use of bulls and crossing events. The ZH farm, with origins that are unknown, exhibited high haplotype diversity but not nucleotide diversity; this observation most likely reflects the population size (1,000 animals) because no animals from other farms have been introduced into this population. The UN herd represented two mitochondrial lineages but exhibited low haplotype diversity (0.29 ± 0.15). This population originated from a mixed Holstein herd, and in 1968, animals from three different farms (which are no longer in existence) were introduced into the herd.

In 2004, UN received animals on loan from the Ministry of Agriculture and Development of the Valle del Cauca, with animals that had been inbred for 20 years and later included individuals from four other herds (Valderrama, 2008). The results of the present study provide information critical for increasing the genetic variation of the UN conservation herd at the National University of Colombia. The university is the only state entity that maintains the HV breed, which was not included in the programs established in the 1930s by the Colombian government for the protection of Creole cattle breeds or in the National Development Plan for Creole Cattle Breeds developed by the Ministry of Agriculture and Rural Development in 2004.

Relationship of the Hartón del Valle breed with Colombian and Creole cattle breeds. A comparison of the seven Colombian Creole breeds studied by Carvajal-Carmona *et al.* (2003) with those in the present study revealed a high degree of proximity between the HV and the Romosinuano breed and a lesser degree of proximity with the Costeño con Cuernos, Casanareño and San Martinero breeds. This proximity was previously reported by Pinzón (1984), who attributed the origin of Romosinuano, HV and Chino Santandereano to the Costeño con Cuernos. The study also described how cattle

arrived at the Valle del Cauca from the four cardinal directions and how the cattle that came from the north and east were derived from ancestral stock developed on the Caribbean coast (Pinzón, 1991).

The Brahman breed was slightly more distant from HV, and it clustered with the Casanareño and San Martinero breeds (data not shown). Previous studies have revealed introgression with the Zebu breed in the Casanareño breed based on mtDNA analysis (Martínez *et al.,* 2006) and cytogenetic studies of the Y chromosome (Sánchez *et al.,* 2008).

Several studies have shown an early bifurcation in the bovine mtDNA between the two major taxa of domestic cattle, namely, *Bos indicus* and *Bos taurus,* indicating a divergence prior to domestication (Bradley *et al.,* 1996; Loftus *et al.,* 1994b). As in other reports investigating *Bos indicus* cattle in America, Zebu haplotypes were not observed in any of the Brahman samples analyzed. The American Zebu haplotypes were of taurine origin, indicating that they developed exclusively from female *Bos taurus* and male *Bos indicus* (Barrera *et al.,* 2006; Egito *et al.,* 2007; Meirelle *et al.,* 1999; Mirol *et al.,* 2003). In the United States, the origin of Brahman cattle dates back to the nineteenth century with contributions from the Guzerá, Nellore, Krishna Valley and Gyr breeds, which were imported from India and Brazil. In Colombia, Brahman cattle were crossed with Colombian Creole breeds and quickly spread throughout the country; this breed now constitutes more than 95% of the national Zebu herd (Pinzón 1991).

Relationship of the Hartón del Valle breed with other breeds worldwide

Although the identity of the ancestral founders of the American Creole breeds is uncertain, several reports have indicated the possible contribution of cattle from southern Spain. Some authors have claimed that ships came from Galicia in northern Spain and from the Canary Islands, a typical stopover for trips to America (Primo, 1992). Rouse (1977) discussed how the Spanish breeds Retinta, Berrenda, Cacereña and Andaluza Negra may have contributed to the development of American cattle. Casas and Valderrama (1998) suggested that the HV breed represents combinations of the following: Rubia Gallega and its descendants, Palmeña and Canaria; Asturiana de los Valles; and Menorquina, or Mahonesa. In the present study, HV was found to have high proximity with Iberian breeds and low proximity with African breeds. The closest Iberian breeds included the following: Tudanca, Rubia Gallega, Negra Serrana, Murciana, Pajuna and Avileña, as well as the Portuguese breeds Garvonesa and Mertolenga.

The study provides information regarding the conservation of the Hartón del Valle breed, which has a declining population. The gene pool of Hartón del Valle represents at least three sources: an African origin (5.5%), a Mediterranean European origin from the Middle East (2.7%) and a Mediterranean European origin (91.7%).

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