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High Genetic Diversity on a Sample of Pre-Columbian Bone Remains From Guane Territories in Northwestern Colombia

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ABSTRACT Ancient DNA was recovered from 17 individuals found in a rock shelter in the district of "La Purnia" (Santander, Colombia). This region is the homeland of pre-Columbian Guane, whom spread over the "Río Suarez" to the "Río de Oro", and were surrounded to the west by the Central Andes, south and east by foothills of Eastern Andes, and north by the "Chicamocha" river canyon. Guanes established in a region that straddles the Andes and the northern Amazon basin, possibly making it an unavoidable conduit for people moving to and from South America. We amplified mtDNA hypervariable region I (HVI) segments from ancient bone remains, and the resulting sequences were compared with both ancient and modern mitochondrial haplogroups from American and non-American populations. Samples showed a distribution of 35% for haplogroup A, 41% for haplogroup B and 24% for haplogroup D. Nine haplotypes were found in 17 samples, indicating an unusually high genetic diversity on a single site ancient population. Among them, three haplotypes have not been previously found in America, two are shared in Asia, and one is a private haplotype. Despite geographical barriers that eventually isolated them, an important influence of gene flow from neighboring pre-Columbian communities, mainly Muiscas, could explain the high genetic polymorphism of this community before the Spanish conquest, and argues against Guanes as being a genetic isolate. Am J Phys Anthropol 146:637–649, 2011.

Interest in South American prehistory has increased in recent years, particularly due to recent archaeological discoveries that changed our understanding on how the continent was populated (Dixon, 1999; Dillehay, 2000). Archaeological evidence (Dixon, 1999; Dillehay, 2000) and molecular genetics (Bonatto and Salzano, 1997; Tarazona-Santos et al., 2001) indicate that South America was populated during the Pleistocene, but the number of migrations has not been clarified. Some researchers argue for a single migration (Moraga et al., 2000), while others support the hypothesis of two waves of migration toward South America (Greenberg et al., 1986; Wallace, 1992; Fox, 1996; Lalueza et al., 1997; Keyeux et al., 2002; Achilli et al., 2008), eventually including a more recent bi-directional gene flow between Siberia and the North American Arctic (Tamm et al., 2007). Fusselli et al. (2003) have proposed a two stage single migration that first went down the Andes and after, went up through the Amazon basin. In recent decades, population studies have focused on the molecular analysis of uniparental lineage markers, located in the mtDNA and Y chromosome, in order to elucidate human migration routes (Wallace et al., 1985; Schurr et al., 1990; Torroni et al., 1993a; Cavalli-Sforza et al., 1998).

These investigations have shown that Amerindian populations are characterized by 4 main mitochondrial haplogroups: A, B, C, and D (Schurr et al., 1990; Torroni et al., 1993a; Bandelt et al., 2003). A fifth haplogroup, named haplogroup X, is specific to North America and has not been reported in South America (Smith et al., 1999; Dornelles et al., 2005). Mitochondrial haplogroups previously defined on the basis of Restriction Fragment Length Polymorphisms (RFLP) (Torroni et al., 1993a) are associated with specific mutations located in the control region of the mtDNA genome (Schurr et al., 2004; Achilli et al., 2008). Haplogroup frequencies differ between populations and are often associated with cultural affiliation, language, or geographical location (O'Rourke et al., 2000; Bolnick and Smith, 2003).

Ancient DNA analysis (aDNA) is a useful tool allowing inferences of population evolutionary processes (Fehren-Schmitz et al., 2010) because most of the hypotheses on the American settlements have been made based on extant indigenous samples, providing only a projected evidence of a historical process that has taken place over

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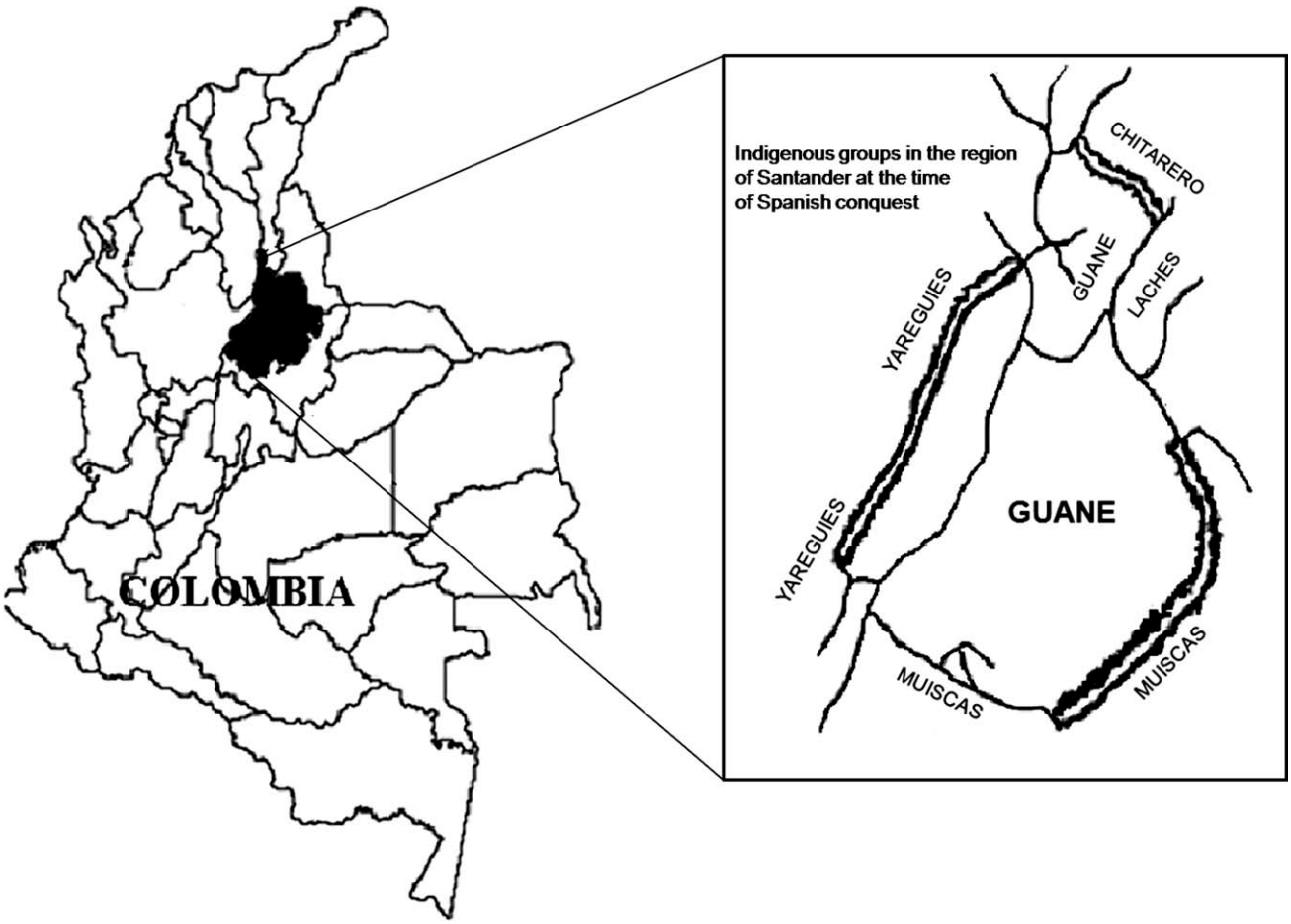

Fig. 1. Map of Colombia showing the location of Santander, and territory occupied by Guane communities at the time of the Spanish conquest (adapted from Morales, 1984).

long periods of time. Classical archeology has postulated that the peopling of Colombia shows at least three successive chronological phases in the pre-Columbian period, termed *Paleoindian* (hunter-gatherers), *Herrera* (agriculture and emerging ceramic), and *Agroceramic* (agriculture and developed pottery), based on the location or type of cultural activities deduced from funeral findings (Silva et al., 2008).

Few studies of ancient DNA (aDNA) have been carried out in Colombia. The first reports were published by Monsalve et al. (1996) after analyzing the mtDNA of six mummies, three of which were not specifically assigned to any particular group. The remaining three belonged to Tunebo, Lache, and Muisca pre-Columbian communities. These researchers found that three of the individuals belonged to haplogroup A, two to haplogroup C, and one individual showed characteristic nucleotide substitutions of haplogroup B, although his affiliation to this haplogroup is uncertain (Monsalve et al., 1996).

Molecular studies have also been carried out in ancient bone remains of ethnic groups belonging to different archaeological periods, mainly associated to the Agroceramic era (Guanes, Muiscas, Laches). By comparing available genetic data from the three archaeological periods, only haplogroups B and C have been found in Paleoindians (Fernández, 1999), as well as in individuals belonging to Herrera (Fernández, 1999; Monsalve et al., 1996; Silva et al., 2008) and Agroceramic periods (Monsalve et al., 1996; Sánchez, 2007; Fernández, 1999).

The Guane population, traditionally located in northeastern Colombia (Figs. 1 and 2) belongs to the Agroceramic period and has been of high anthropological interest. Bioanthropological studies in bone material from rock shelters excavated by W. Schotelius (Rodríguez, 1983) and others (Rodriguez, 1992; Correal and Flórez, 1992), characterized the physical aspect of the Guane as having a prominent and back convex shaped face, a very narrow nose, and graceful and slightly prominent cheekbones of average width, suggesting a Caucasoid appearance which clearly differentiated them from other neighboring populations (Simon, 1626; Correal and Flórez, 1992; Correal, 2004; Rodríguez, 2007). It has been argued that these distinct characteristics can be explained by geographical isolation and genetic drift processes suffered by this tribe (Rodriguez, 2007), even if they were integrated to the neighboring Muisca by cultural interchange (Langebaek, 1987).

The present study aims to analyze the genetic diversity of ancient Guane bone remains found in a rock shelter from La Mesa de los Santos, a known Guane pre-Columbian territory in Santander, northeastern Colombia. We sought to establish their possible biological relationship with other extant and extinct communities, and the extent of their genetic isolation. This last question is of marked interest, since archaeological data suggest a

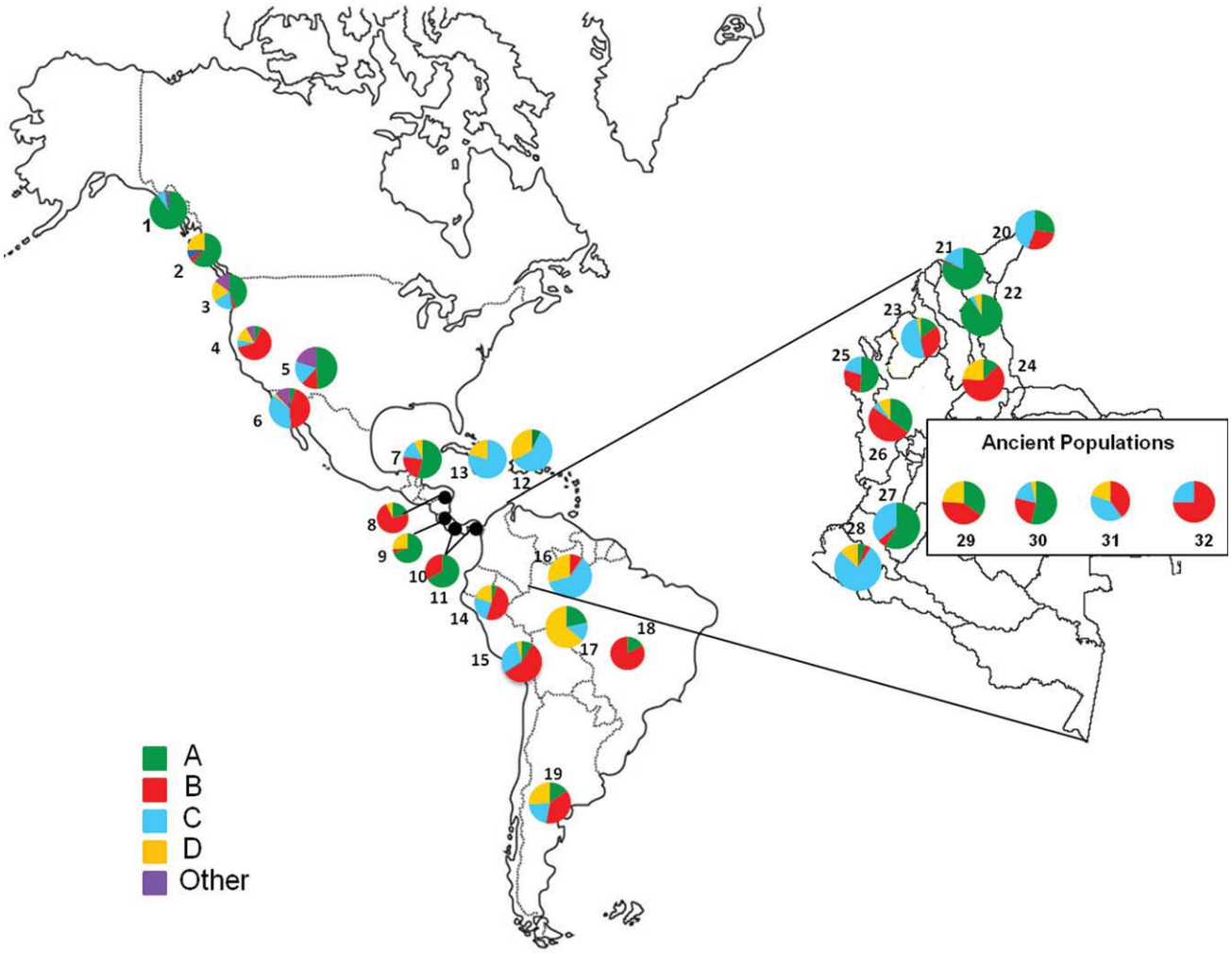

Fig. 2. Distribution of mtDNA haplogroups in Native American populations. Color keys for haplogroups A, B, C, and D is indicated in the lower left-hand corner. Numbers correspond to populations on Table 2. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

high degree of isolation, based on marked phenotypic differences with other groups.

MATERIALS AND METHODS

Population samples

Skeletal remains belonging to 17 individuals were found in the archaeological site located in "La Purnia", a district at "La Mesa de los Santos", Santander, Colombia (Fig. 1). These remains were found in 1988 by a group of students led by Professor Arturo Cifuentes from the Universidad Industrial de Santander (1990). The site includes two rock shelters reportedly used by Guane Amerindians as burial emplacements, near "El Salto del Duende" waterfall. Complex outfits and mortuary goods were found in the caves, including several rolled blankets in good condition along with a mummy, a loom, a baton stick, several ceramic pieces, necklaces, and scattered skeletons. Bones analyzed in this study had muscle tissue remains and skulls showed no evidence of deformation. Radiocarbon analysis of tissue samples from this archaeological site referred to as "Cueva del Duende" (Elf Cave) produced an estimated date of 1090 ± 70 AD (Beta analysis number 28746). Skeletal remains of individuals analyzed in this study have been assumed to belong to the same period, as there is only one pottery style associated to the studied bones and complex outfits related to identical mortuary practices in a sealed cave. Bone remains and mortuary goods have been preserved in the Laboratorio de Antropología Física at the Universidad Nacional de Colombia, in Bogotá.

Precautions taken to avoid contamination

To ensure the fidelity of the results, recommended criteria for aDNA by Cooper and Poinar (2000) and Gilbert et al. (2005), were followed to prevent contamination. Bone DNA extraction and amplification were performed in an laboratory for the exclusive analysis of aDNA at the Instituto de Genética Humana (IGH) in the Pontificia Universidad Javeriana in Bogotá, where separate areas were maintained for aDNA extraction and pre-PCR protocols. At the time of the referred experiments, access to aDNA facilities was restricted to the first four participants of this study, and only the first and fourth participants were present when aDNA was extracted and amplified. Pre-PCR facilities were regularly cleaned with 5% bleach for

15 min, and UV irradiated the night before each aDNA extraction. In order to ensure unidirectional restrictions, no post-PCR material was allowed to enter the pre-PCR rooms. Post-PCR experiments were performed at the common facilities of the IGH. Pipette filter tips, gowns, hats, masks, and gloves were used and were exclusive for each individual procedure. Only one specific sample was processed each day, to avoid cross contamination. Each sterile hood was cleaned with 5% sodium hypochlorite and subsequently with 70% ethyl alcohol. Finally, all material (pipettes, racks, plastic materials, gloves, gowns, hats, and masks) was irradiated with UV light for 1 hour. In order to identify and exclude any possible cross-contamination with aDNA, and any contamination with modern DNA, negative controls were used both in DNA extraction and amplification steps. In addition, multiple extractions and PCR amplifications were performed for each one of the samples. DNA from researchers involved in processing and handling of the samples was typed using the same techniques, in order to compare their DNA profile with that of the analyzed samples. Two samples were randomly selected for complete independent testing in another aDNA laboratory located on the Universidad de la Sabana in Chía (Cundinamarca), in order to confirm the reproducibility and specificity of the mitochondrial sequences obtained.

Ancient DNA extraction

Decontamination of the samples was conducted by completely removing the outer layer of bone using a DREMEL mototool® in a sterile hood, then by washing with sodium hypochlorite at 5%, for 10 min, followed by 70% ethyl alcohol, for 10 min, and then by exposing both sides of each bone under UV light for 10 min. We obtained, on average, 2 grams of pulverized bone from each individual using a DREMEL mototool® for every separate sample with a sterilized drilling head which was autoclaved for 30 min and then exposed under UV light for 30 min. The powdered samples were incubated at 56 °C for 48 hours in a solution containing 0.5M EDTA, 10% SDS, and proteinase-K (12.5mg/mL). DNA extraction was performed by the salting-out method (Silva et al., 2008) and then was purified using the DNA2000 kit (Corpogen). Preparation of every solution and reagent was performed in sterile conditions and strict precautions were taken to avoid modern DNA contamination. DNA samples were quantified on a NanoVue® (GE Healthcare, USA) following the manufacturer's instructions.

mtDNA analysis

Amplification of the HVI region of mtDNA was performed using L16209 and H16401 primers which were previously used and reported by our group (Silva et al., 2008). The amplification reaction had a final volume of 25 μL with 12.5 μL of a master mix (Green Master Mix, Promega), 0.5 μM of each primer and 5 μL of sample DNA. Amplification conditions were established as follows: initial denaturation at 94 °C for 5 min, followed by 28 cycles of denaturation at 94 °C for 45 seconds, annealing at 54 °C for 45 seconds, and extension at 72 °C for 45 seconds. A final extension at 72 °C for 7 minutes was included. Samples were reamplified with the same PCR conditions of the first amplification when the concentration of DNA was low, as determined when no DNA bands were visualized on 2% agarose gels, where PCR products were visualized after staining with ethidium bromide. Amplified products were purified and sequenced on an ABI 310 (Applied Biosystems) following the manufacturer's instructions. Sequences were aligned and compared with the human mtDNA Cambridge Reference Sequence (CRS) (Anderson et al., 1981) using SEQUENCHER 4.7 software.

Haplogroups B and D were confirmed by RFLP analysis, including *AluI* 5176 (to detect haplogroup D) and the mtDNA 9-pb deletion in the cytochrome oxidase II/ $tRNA^{Lys}$ region (to detect haplogroup B). Primer pairs used have been previously described by Torroni et al. (1993a). PCR was conducted on a final reaction volume of 25 μL with 12.5 μL master mix (Green Master Mix, Promega), 0.3 μM of each primer and 5 μL of sample DNA. Thermal conditions were established as follows: incubation at 94 °C for 5 min; 40 cycles at 94 °C for 45 sec; 52-62 °C for 45 sec, and 72 °C for 45 sec; final extension at 72 °C for 7 min. Each region was examined independently. PCR products were separated by electrophoresis in polyacrylamide gels and were detected by ultraviolet irradiation after staining with ethidium bromide. RFLP analyses were carried out by the first author in a third laboratory (Laboratorio de Identificación Humana, Instituto de Genética, Universidad Nacional de Colombia, Bogotá).

Data analysis

To elucidate biological relationships between pre-Columbian Guane and 28 contemporary Native American communities, and also to compare them to three previously reported pre-Columbian ancient populations, haplogroup data were obtained from published data (Schurr et al., 1990; Ward et al., 1991, 1993, 1996; Ginther et al., 1993; Shields et al., 1993; Torroni et al., 1993a,b; Santos et al., 1994; Batista et al., 1995; Kolman et al., 1995; Monsalve et al., 1996; Kittles et al., 1999; Lalueza-Fox et al., 2001, 2003; Keyeux et al.,2002; Melton et al., 2007; Lewis et al., 2007; Sanchez, 2007; Fernández, 1999; Silva, 2008). Haplogroup frequencies were compared and supported by a principal component analysis (PCA) with the MVSP 3.1 software. Intrapopulation variation was compared with the variation within populations on 19 American tribes (Fig. 2) based on two parameters: nucleotide diversity and haplotype diversity, using the method of Nei (1987). The spectrum of the molecular analysis in these samples covered the HVI region from nucleotide 16210 to nucleotide 16364. Interpopulation analysis on the same 19 populations was performed using the method of Tamura and Nei (1993), through a distance matrix between haplotypes using Arlequin 3.01 computer package. The results were visualized by a multidimensional scaling plot (MDS) using SAS 9.1 software. Median joining network analyses were conducted for haplogroups A and B using the NETWORK 4.0 software. A total of 320 mtDNA sequences extracted from published papers were used for haplogroup A, and 174 sequences for haplogroup B, on both Amerindian and Asian populations.

RESULTS

Evaluation of authenticity

No sign of contamination with modern or aDNA was observed in any blank extraction or any subsequent PCR. Only one out of nine haplotypes obtained in pre-

TABLE 1. Haplotypes found in the Guane population

		Human mtDNA position											RFLP		
HAPLOGROUP	**HAPLOTYPE**	1	1	1	1	1	1	1	1	1	1	1			*n*
		6	6	6	6	6	6	6	6	6	6	6			
		2	2	2	2	2	2	2	3	3	3	3	9-bp	*Alu I*	
		1	2	3	5	6	9	9	1	2	5	6			
		7	3	0	6	6	0	3	9	5	6	2			
	rCRS[a]	T	C	A	C	C	C	A	G	T	T	T			
A	1	–	T	–	T	–	T	–	A	–	–	C			1
	2	–	T	–	–	–	T	–	A	–	–	C			1
	3	–	T	–	–	T	T	–	A	–	–	C			1
	4	–	T	–	–	–	T	–	–	–	–	–			1
	5	–	T	–	–	–	T	–	A	–	C	C			2
B	6	C	–	–	–	–	–	–	–	–	–	–	+		5
	7	C	–	–	–	–	–	G	–	–	–	–	+		1
	8	C	–	G	–	–	–	–	–	–	–	–	NT		1
D	9	–	T	–	–	–	–	–	–	C	–	C		–	4
Total															**17**

[a] Cambridge Reference Sequence (Anderson et al., 1981; Andrews, 1999).

Columbian Guanes was identical to one of the mitochondrial DNA sequences obtained from the researchers directly involved in the analytical procedure. However, this sequence corresponds to the most common haplotype in contemporary Colombian mestizo populations, meaning that this particular haplotype can be proposed as an ancestral Amerindian marker. Nucleotide substitutions observed in two randomly chosen samples from which aDNA was extracted, amplified and sequenced independently in a different laboratory exactly matched those found on the same individuals in the IGH laboratory at the Pontificia Universidad Javeriana. Eleven out of seventeen (64.7%) samples were successfully extracted, amplified, and sequenced independently between two and four times, obtaining on each case reproducible results, corroborating the reliability of mtDNA sequences reported for each individual. Six samples were only amplified and sequenced once, showing clear sequences that allowed their haplogroup typing based on the reproducibility of the remaining samples which were considered an adequate control for both amplification and sequencing of aDNA. DNA concentrations obtained ranged between 7.7 and 86 ng/μl. These results indicate sufficient amounts of available DNA and ensure the possibility of reproducing the results of PCR amplification.

The authenticity of aDNA was further demonstrated by the impossibility to amplify the fragment of 278bp from aDNA with the primers used, unlike contemporary DNA samples, since Guane DNA samples showed clear signs of being partially degraded, such as smears on agarose gels in the low dilutions (data not shown). Amplification of the sequence corresponding to 229 bp fragment from ancient samples was successful, signifying that it was only possible to amplify the smallest DNA fragments.

Analysis of mtDNA

On 9 haplotypes identified among 17 sequences obtained from 17 individuals (Table 1), six sequences were classified as belonging to Haplogroup A based on the presence of C>T transitions in positions 16223 (C16223T) and 16290 (C16290T), and a G>A transition in position 16319 (G16319A), as proposed by Torroni et al. (1993a). Seven additional sequences were classified as haplogroup B based on the presence of a T>C transition found in position 16217 (T16217C); we were not able to confirm the presence of a complementary diagnostic haplogroup B polymorphism (T16189C), because it was located outside of the amplified region. B haplogroups detected by sequencing were confirmed by the presence of a diagnostic 9-pb deletion. No haplogroup C sequences were found in this pre-Columbian cohort. Finally, four sequences were classified as haplogroup D, based on the absence of diagnostic polymorphisms of other haplogroups. However, sequences corresponding to haplogroup D showed the frequent polymorphisms proposed by Torroni et al. (1993a) to define D-loop haplogroup classification (C16223T, T12325C and T16362C). RFLP analyses confirmed haplogroup D designations with the diagnostic absence of the *AluI* site at position 5176 (Table 1).

We found a high frequency of haplogroup B (41%), whereas lower frequencies were found for haplogroup A (35%) and haplogroup D (24%), with a total absence of haplogroup C. Haplogroup B was also found in high frequency in contemporary Amerindian populations such as North Yakima and Boruca in Central America; Guane-Butaregua, Waunana, and Embera in Colombia; and Xavante, Yungay, and Quechua in South America. Haplogroup C is found in low frequencies in North America, and is low to absent in Central America, but it is observed at high frequencies in extant populations of South America (Fig. 5). By contrast, haplogroup D has low frequencies in North America and Central America, and is frequently found in South America (Fig. 2, Table 2). A similar pattern of a predominant proportion of haplogroup B and unlike other extant Native American and Colombian populations, absence of haplogroup C was found in a contemporary population referred to by Keyeux et al. (2002) as Guane-Butaregua because they inhabit the same territories and are considered "Indians" by their neighbors.

It was noted that out of 9 different haplotypes found on these samples, five belong to haplogroup A, three to haplogroup B, and one to haplogroup D (Table 1). Haplotypes 2, 6, and 9 are the most common in America (Torroni et al., 1993a; Forster et al., 1996); haplotype 3 was found in the town of Bella Coola (British Columbia, Canada) and within a Maya population reported by Schurr et al. (1990); haplotype 4 in a sample from the Agroceramic period and in the Huetar from Costa Rica;

TABLE 2. Frequency distribution of mitochondrial DNA HVI haplogroups in America

Population[a]	*n*	A	B	C	D	Others
North America						
(1)[b]Haida[1]	41	0.90	0.00	0.07	0.00	0.02
(2) Bella Coola[1]	40	0.63	0.05	0.08	0.25	0.00
(3) Nuu-Chah-Nulth[2]	63	0.44	0.03	0.19	0.19	0.14
(4) Yakima[3]	42	0.07	0.64	0.07	0.14	0.07
(5) Cheyenne[4]	39	0.49	0.13	0.18	0.00	0.21
(6) Pima[4]	41	0.05	0.44	0.37	0.02	0.12
Central America						
(7) Maya[5]	27	0.52	0.22	0.15	0.07	0.00
(8) Boruca[6]	14	0.21	0.72	0.00	0.07	0.00
(9) Huetar[7]	27	0.70	0.04	0.00	0.26	0.00
(10) Ngöbe[9]	46	0.67	0.33	0.00	0.00	0.00
(11) Kuna[8]	63	0.71	0.29	0.00	0.00	0.00
(12) Tainos[10]	19	0.00	0.00	0.79	0.21	0.00
(13) Ciboney[11]	15	0.07	0.00	0.60	0.33	0.00
South America						
(14) Yungay[21]	20	0.05	0.50	0.25	0.20	0.00
(15) Quecua[21]	23	0.09	0.57	0.30	0.04	0.00
(16) Yanomami[20]	53	0.00	0.09	0.58	0.28	0.04
(17) Zoro[19]	29	0.21	0.00	0.14	0.62	0.03
(18) Xavante[19]	24	0.17	0.83	0.00	0.00	0.00
(19) Mapuche[18]	39	0.15	0.38	0.21	0.26	0.00
Colombia						
(20) Wayuu[12, 1]	30	0.27	0.27	0.43	0.00	0.03
(21) Arsario[12]	28	0.71	0.00	0.29	0.00	0.00
(21) Ijka[12]	31	0.90	0.03	0.06	0.00	0.00
(21) Kogi[12, 2]	21	0.81	0.00	0.19	0.00	0.00
(22) Chimila[13, 4]	35	0.89	0.00	0.03	0.06	0.03
(23) Zenu[13]	34	0.15	0.32	0.50	0.03	0.00
(24) Guane-Butaregua[13, 3]	33	0.12	0.64	0.00	0.24	0.00
(25) Tule-Cuna[13, 5]	30	0.50	0.27	0.20	0.00	0.03
(26) Embera (Cauca)[13]	21	0.33	0.48	0.05	0.09	0.00
(27) Paez[13]	31	0.58	0.07	0.36	0.00	0.00
(28) Guambiano[13]	23	0.04	0.04	0.78	0.13	0.00
Ancient populations						
(29) Guane[PS]	17	0.35	0.41	0.00	0.24	0.00
(30) Agroceramic[pool 14, 16, 17]	24	0.54	0.25	0.17	0.04	0.00
(31) Herrera[pool 14, 15, 16]	5	0.00	0.4	0.4	0.2	0.00
(32) Paleoindian[14]	4	0.00	0.75	0.25	0.00	0.00

[a] References: (1) Ward, 1993; (2) Ward, 1991; (3) Shields, 1993; (4) Kittles, 1999; (5) Schurr, 1990; (6) Torroni, 1993a; (7) Santos, 1994; (8) Batista, 1995; (9) Kolman, 1995; (10) Lalueza-Fox, 2001; (11) Lalueza-Fox, 2003; (12) Melton, 2007; (13) Keyeux, 2002; (14) Fernández, 1999; (15) Silva, 2008; (16) Monsalve, 1996; (17) Sanchez, 2007; (18) Ginther, 1993; (19) Ward, 1996; (20) Torroni, 1993b; (21) Lewis, 2007; (PS) Present study.
[b] Population code in Figure 2.

haplotype 7 in the town of Yungay (Northern Peru); haplotypes 1, 5, and 8 were not found in American populations included in the latter analysis (Table 3).

In order to look for haplotypic coincidences in the rest of the world, we expanded the number of sequences analyzed to include other contemporary populations such as China (Torroni et al., 1993a; Comas et al., 1998), Mongolia (Kolman et al., 1996), and Siberia (Torroni et al., 1993b) (Table 4). This new approach allowed us to find that haplotype 1 is shared with the Mongolian population, haplotype 4 is also present in China and Mongolia, and haplotype 5 is found in China and Siberia. Finally, haplotype 8 was not found in any of these populations and appears to be an exclusive pre-Columbian Guane haplotype.

TABLE 3. Guane haplotypes in America

	Haida	Bella Coola 1	Bella-Coola 2	Nuu-Chah-Nulth	Yakima	Cheyenne	Pima	Maya	Huetar	Ngöbe	Kuna	Tainos	Ciboney	Arsario	Kogi	Wayuu	Yungay	Quechua	Yanomami	Mapuche	Agroceramic	Herrera	Paleoindian
Haplotype 1																							
Haplotype 2	X	X		X	X	X			X	X	X		X	X	X	X				X	X		
Haplotype 3			X					X															
Haplotype 4									X												X		
Haplotype 5																							
Haplotype 6		X		X	X	X	X		X	X						X	X	X			X	X	X
Haplotype 7																	X						
Haplotype 8																							
Haplotype 9		X		X	X				X		X	X	X				X			X	X	X	

Shared haplotypes are marked with an X.

TABLE 4. Guane haplotypes in Asia.

	China	Mongolia	Siberia
Haplotype 1		X	
Haplotype 4	X	X	
Haplotype 5	X	X	X
Haplotype 8			

Shared haplotypes are marked with an X

Table 5 summarizes the values of diversity found in different extant and extinct Amerindian populations. Pre-Columbian Guane show a nucleotide diversity of 0.0231 in the analyzed fragment of 154 pb, which appears high when compared to other populations of North America such as Haida (0.0096) and Bella-Coola (0.0177) of Central America such as Ngöbe (0.0181), Kuna (0.0175), Taino (0.145), Ciboney (0.0174), and to populations in South America such as the Arsario (0.0164), Ijka (0.0067), Kogi (0.0142), Yanomami (0.017), Zoro (0.0187), and Xavante (0.0126). Nevertheless, pre-Columbian Guane showed similar values to other populations in North America, including Nuu-Chah-Nulth and Yakima, as well as Yungay and Quechua in South America, and also an ancient population from the Agroceramic period (Fernández, 1999; Silva et al., 2008; Monsalve, 1996). Gene diversity of the pre-Columbian Guane population was 0.875, which corresponds to what was found in the nucleotide diversity. This value is similar in populations of North America, such as the Nuu-Chah-Nulth, Cheyenne, and Pima, in populations of the Caribbean such as the Taino and Ciboney, and also in South American populations such as the Yungay, Quechua and Mapuche, or else from the Agroceramic period.

Median Joining Network (MJN) analysis of haplogroups A and B (Figs. 3 and 4) show a star shape that reflects a regular expansion of the population as suggested by others (Forster et al., 1996). Haplogroup A (Fig. 3) has a node where the most common Guane haplotype, termed GuaF18 (16223, 16290, 16319, and 16362), is shared by several groups of North, Central, and South America. This haplotype is ancestral to GuaF17 with a one-step mutation at position 16266. Three clear examples of homoplasy were observed in this MJN, which involved gene sequences belonging to Guane/China (16256/16356), Huetar/Boruca (16360/16319), and Guane/Muisca (16223/16290) populations. These homoplasies could probably be interpreted as a reversal phenomenon, either by mutational loss or gain.

MJN of haplogroup B (Fig. 4) has a master node with a common haplotype (16 217) with five of the analyzed sequences (GuaF13, GuaF22, GuaF20, GuaF7, and GuaF19). GuaF4 sequence has a mutation at position 16230 one step away from the ancestral node. GuaF3 sequence is shared by the sequence of Yungay with a one-step mutation at position 16293. A pre-Columbian Guane sequence (GUAN) analyzed by Fernández (1999), with a one-step mutation at position 16299 and the sequence found in a Pima Indian (Pima3) are homoplasic by reversion (mutational loss or gain), as well as the Mong77 (Mongolia) sequence with one step mutation at position 16261. The divergence of the population was estimated by performing a distance matrix with values of nucleotide diversity within and between populations, using the model of Tamura and Nei (1993) and multidimensional scaling of genetic distances (Fig. 6).

The genetic distances in Amerindian populations show geographic correlation ($r = 0.96$), especially in North and Central America. Central American groups especially Ngöbe, Huetar and Kuna, appear to be both geographically and genetically close to each other. Caribbean populations (Taino and Ciboneys) are closely related to the people of South America as they show high frequencies of haplogroups C and D, particularly to Yanomami and Zoro, but are isolated from other populations of North and Central America. Extant Amerindians from the "Sierra Nevada" of Santa Marta, Colombia (Ijka, Kogi, Arsario) are also found in one tightly related group, close to populations of North America (Haida, Bella Coola, Nuu-Chah-Nulth and Cheyenne). On the other hand, the pre-Columbian Guane population appears to be isolated from the preceding populations and can be only associated to samples from the Agroceramic period with similar values of nucleotide diversity.

TABLE 5. Genetic diversity in Amerindian populations

Population	n*	No. Haplotypes	K	h	P_w	π
North America						
Haida[1]	41	4	10	0.4402	1.48	0.0096
Bella Coola[1]	40	8	12	0.7833	2.74	0.0177
Nuu-Chah-Nulth[2]	63	22	17	0.9211	3.98	0.0258
Yakima[3]	42	16	20	0.8095	3.33	0.0216
Cheyenne[4]	39	20	24	0.9285	4.78	0.0311
Pima[4]	41	17	26	0.9024	4.73	0.0306
Central America						
Huetar[6]	27	6	7	0.6724	2.12	0.0137
Ngöbe[8]	46	4	7	0.5961	2.78	0.0181
Kuna[7]	63	5	8	0.5699	2.71	0.0175
Tainos[9]	19	9	9	0.883	2.24	0.0145
Ciboney[10]	15	9	8	0.9238	2.68	0.0174
South America						
Arsario[11]	28	2	6	0.4233	2.54	0.0164
Ijka[11]	31	3	8	0.1849	1.05	0.0067
Kogi[11]	21	3	7	0.538	2.20	0.0142
Wayuu[11]	30	5	13	0.7885	4.25	0.0276
Guane[PS]	17	9	11	0.8750	3.55	0.0231
Yungay[14]	20	14	18	0.9474	4.10	0.0266
Quechua[14]	23	15	17	0.9051	3.82	0.0248
Yanomami[15]	53	23	17	0.8367	2.73	0.017
Zoro[13]	29	6	12	0.6576	2.86	0.0187
Xavante[13]	24	3	6	0.6486	1.93	0.0126
Mapuche[12]	39	11	15	0.8934	4.27	0.0277
Agroceramic[16]	24	14	12	0.9275	3.43	0.0223

* n, size of the sample; k, number of different sequences; h, gene diversity; P_w, pairwise differences; π, nucleotide diversity.
[a]References: (1) Ward, 1993; (2) Ward, 1991; (3) Shields, 1993; (4) Kittles, 1999; (5) Schurr, 1990; (6) Santos, 1994; (7) Batista, 1995; (8) Kolman, 1995; (9) Lalueza-Fox, 2001; (10) Lalueza-Fox, 2003; (11) Melton, 2007; (12) Ginther, 1993; (13) Ward, 1996; (14) Lewis, 2007; (15) Torroni, 1993a; (16) Sanchez, 2007; (PS) Present study.

DISCUSSION

Mitochondrial haplogroups and haplotypes obtained, based on the sequences of the HVI region (16209-16401), in 17 pre-Columbian bone remains were analyzed and classified into haplogroups A, B, and D, based on the presence or absence of specific polymorphisms (Torroni et al., 1993a, Forster et al., 1996). These results show a predominance of haplogroup B (41%), and frequencies of 35% for haplogroup A, and 24% for haplogroup D. No sequences compatible with haplogroup C were found.

This pattern was similar to that reported by Keyeux et al. (2002) for a contemporary population from a village currently named Guane, near the region of Butaregua

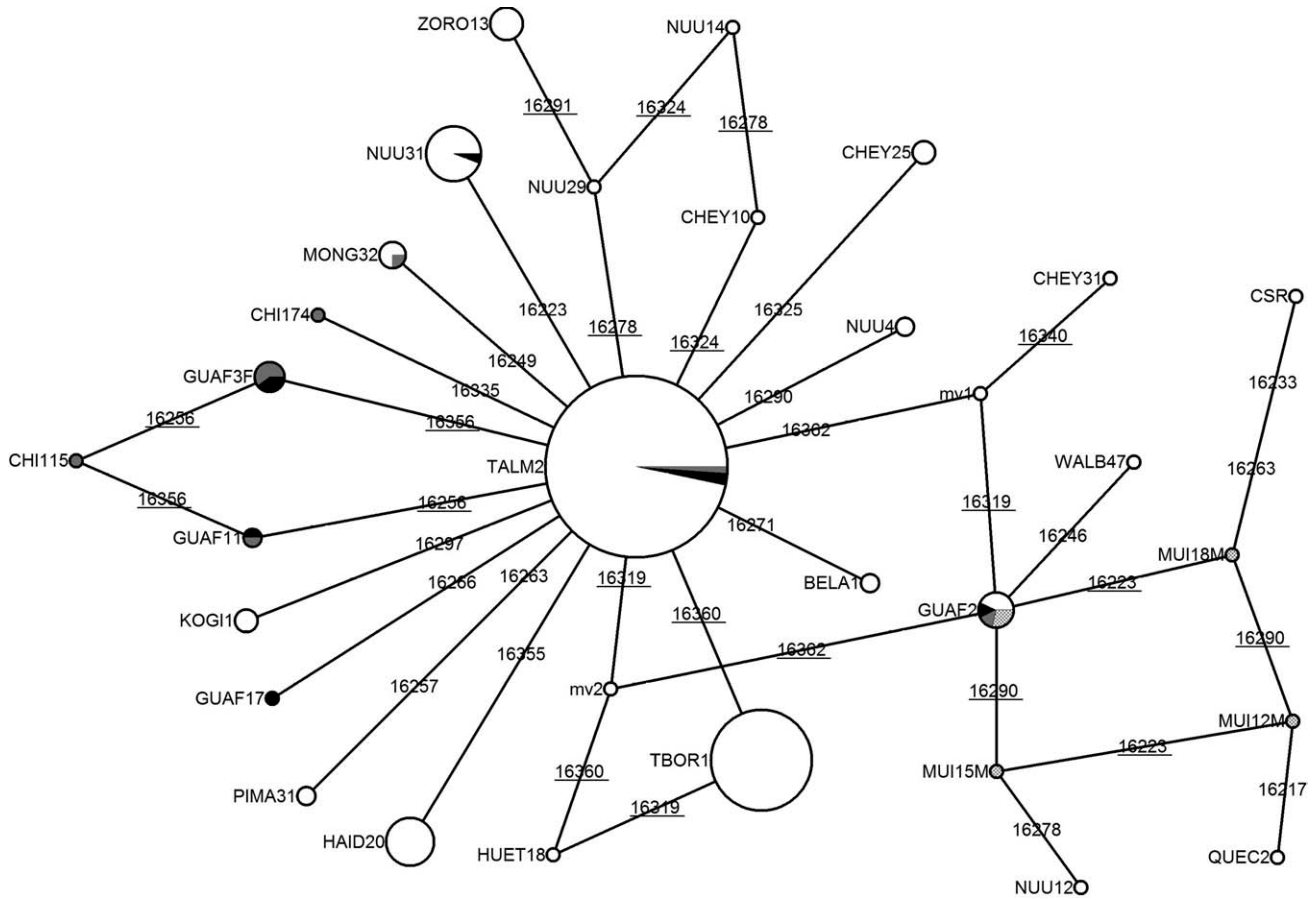

Fig. 3. Median Joining Network analysis on Haplogroup A, including 29 populations and 320 individuals. The central node corresponds to the most frequent haplotype (16223, 16290, 16319, 16362). The position of pre-Columbian Guane individuals in the network is indicated with black fillings; White filling on the circles represents contemporary native Amerindians; Gray filling represents Asian populations; Stripped filling represents Muisca population; mv1 and mv2 represent hypothetical nodes.

(Santander), with a high frequency of haplogroup B (64%), an equivalent frequency of haplogroup D (24%), 12% of haplogroup A, and complete absence of haplogroup C. When compared to other contemporary Colombian Amerindian populations, this group appears to be the only one that has a similar distribution of haplogroups as pre-Columbian Guane reported by us in the present work. Even if this population, analyzed by Keyeux et al. (2002) under the collective name of Guane-Butaregua, did not belong to an isolated Amerindian group, as samples were collected in the 1990's from farmers in this region, these individuals were reportedly considered by their neighbors and by themselves as being different from other local mestizos according to their cryptic language and particular cultural traditions (personal communications).Their cultural eccentricity has thus led other researchers to consider them a distinctive human extant isolate that could have arisen from the ancestral Guane (Keyeux et al., 2002). Our results support this hypothesis. The genetic findings of the present study show that current populations of rural areas of Santander (Guane-Butaregua) retain their indigenous ancestry as can be assumed by the equivalence of mtDNA marker frequencies in the pre-Columbian samples analyzed.

Some anthropologists have argued that the Guane was dramatically reduced in the eighteenth century, not so much as a consequence of violent actions, but rather to the admixture that led to the uprooting of traditions and identity (Morales, 1984). In addition to population fusion after the conquistadors invaded this region, mining operations in the neighboring Río de Oro valley, close to the town of Girón, forced the evacuation of villages (Morales, 1984). These two historical conditions, fusion and mining, notably influenced the disappearance of ethnic Guane in colonial times. This process ended up turning against the few surviving natives, as their members were progressively dispersed in the rural villages of Santander from the XVI century onwards. Other researchers argue that, even today, an Amerindian phenotype can be observed in many rural areas, particularly in nearby villages such as Simacota and Barichara, in which indigenous surnames also abound (Lucena, 1974). Guane traditions seem to persist only in periodic ant harvest and consumption (Morales, 1984).

From a genetic perspective, it has been suggested that haplogroup B may represent an independent migration toward South America, being found at low frequencies in Asia and North America (Torroni et al., 1992; Torroni et al., 1993a; Starikovskaya et al., 1998; Derenko et al., 2000). This haplogroup has been observed in high frequencies in populations including the three former Colombian archaeological periods (Paleoindian, Herrera, and Agroceramic), suggesting a historical continuity of

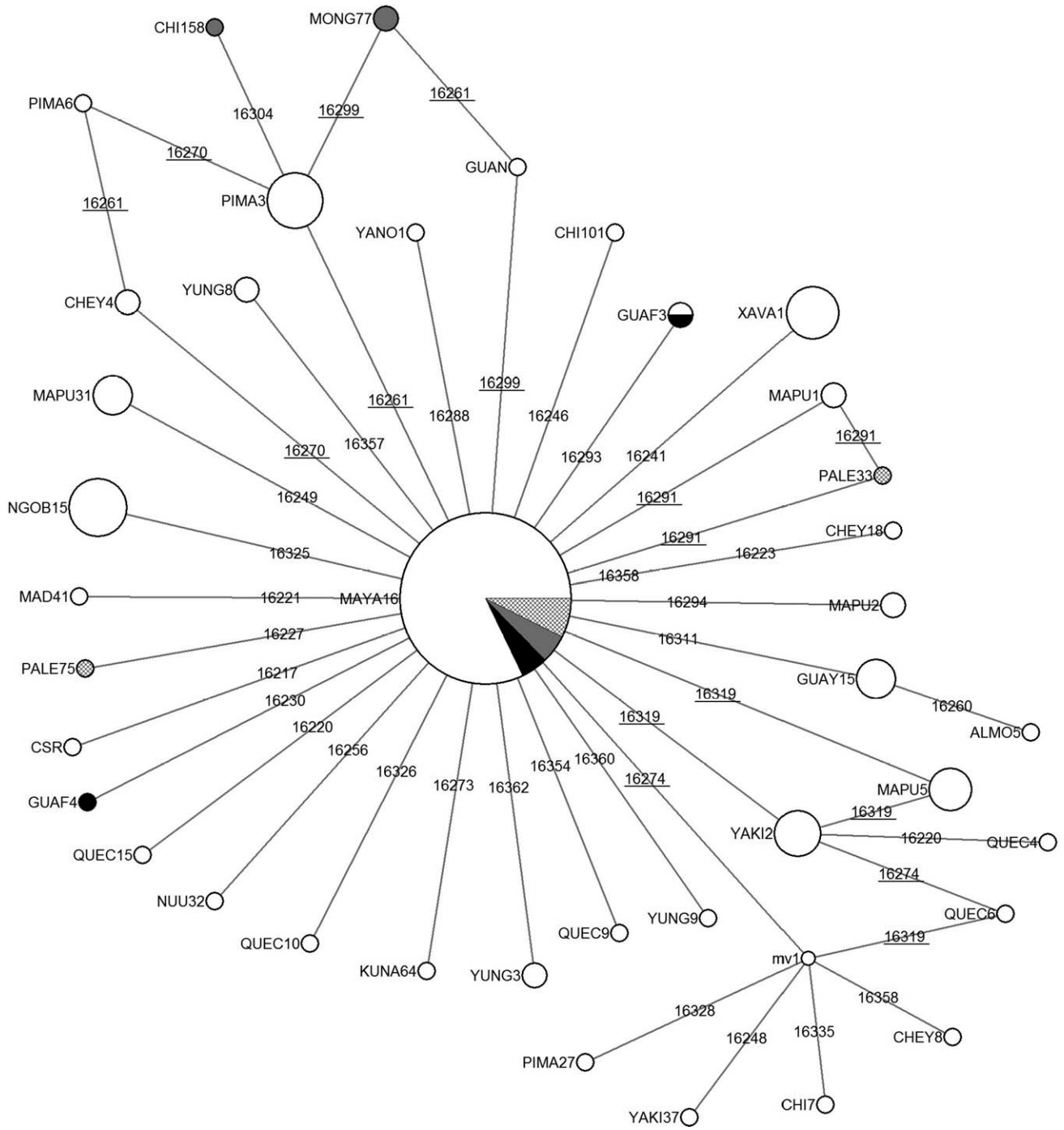

Fig. 4. Median Joining Network analysis on Haplogroup B, including 24 populations and 185 individuals. The central node corresponds to the most common haplotype (16 217). The position of pre-Columbian Guane individuals in the network is indicated with black fillings; White filling on the circles represents contemporary native Amerindians; Gray filling represents Asian populations; Striped filling represents Muisca population; mv1 represents an hypothetical node.

this haplogroup as can also be concluded based on the present data of a pre-Columbian Guane population (Fernández, 1999, Silva et al., 2008; Sánchez, 2007). This haplogroup is characteristic of other ancient samples in South America as those from Peru, Chile, and Argentina (Carnese et al., 2010, Kemp et al., 2009, Shinoda et al., 2006; Moraga et al., 2000 and 2005). As it has been suggested by Morales (1984) and others that post-Columbian Guane spoke a Chibchan dialect, the introduction of haplogroup A could be due to a recent gene flow originated in the neighboring Muisca, and the presence of haplogroups B and D would constitute a remnant from earlier populations. Three out of nine haplotypes reported in the present work among pre-Columbian Guane have not yet been reported in Amerindian populations. However, we found that two of these haplotypes are shared with contemporary individuals in Mongolia, China, and Siberia.

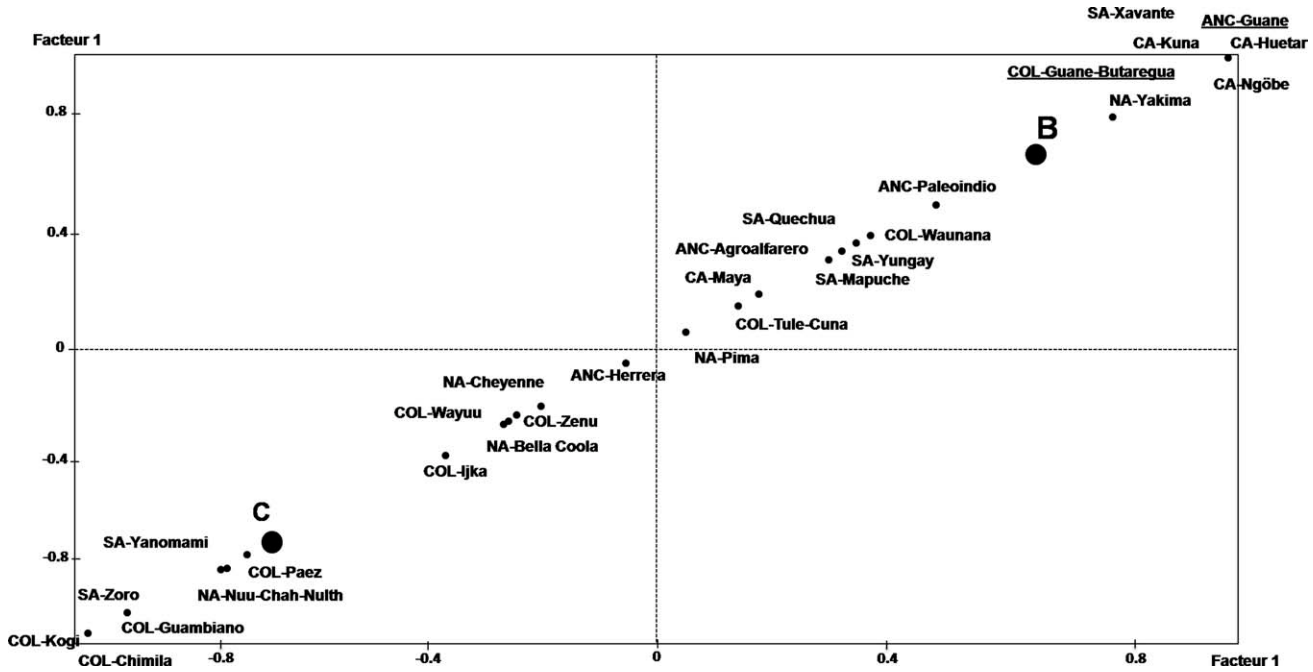

Fig. 5. Principal Component Analysis of distributions of haplogroups B and C in American populations. Abbreviated keynames for geographical distribution are: NA (North America), CA (Central America and Carribbean), SA (South America), COL (Colombia), ANC (Ancient Colombian).

As revealed by phylogenetic analyses performed on each one of the pre-Columbian Guane haplogroups, we found that haplotypes 1 and 5 are also associated with common ancestors in China (Torroni et al, 1993a; Comas et al., 1998), Mongolia (Kolman et al, 1996), and Siberia (Torroni et al. 1993b). These analyses support the hypothesis of an early geographic spread from Asia to South America. Interestingly, haplotype 8 was not found either in Asian or American Indian populations, constituting so far, a private haplotype of the Guane. According to Wells (2002), some Amerindian lineages have gone extinct in the last 15000 years and contemporary genetic diversities represent only a tiny fraction of what could have been found in Eurasia (Wells, 2002). These lineages could have disappeared through the bottleneck of the Spanish conquest. According to Schurr (1990), the genetic diversity of contemporary Native Americans has been further reduced with the arrival of European settlers and possibly many of the haplotypes that corresponded to Asian migratory waves have now disappeared (Schurr et al., 1990).

The high level of both nucleotidic and haplotypic diversity found in pre-Columbian Guane reported in this work and supported by pairwise differences as compared to other contemporary populations, indicates that there is no evidence of dramatic episodes of genetic drift in the genetic makeup of this population. According to genetic evidence obtained in neighboring indigenous groups, especially Muisca (Sanchez, 2007), some haplotypes such as haplotype 4 are also found in individuals in the Muisca from the Agroceramic period. According to the network analysis there is probably a homoplasy reversal with this haplotype involving 3 different individuals of the Muisca. This would indicate that there was a gene flow between Muisca and Guane people.

Previous anthropological assumptions on pre-Columbian Guane are contradicted by our results as the

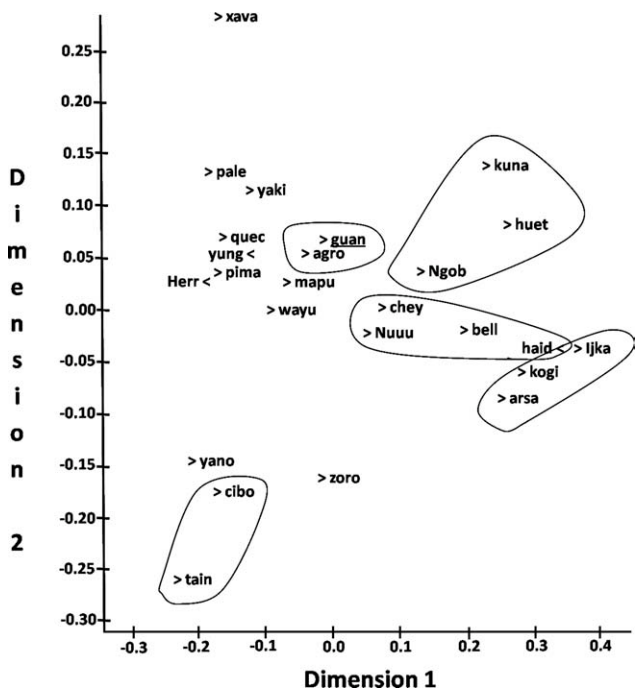

Fig. 6. Multidimensional scaling of genetic distances of 25 populations included on Table 5. Abbreviated keynames for populations indicated in parentheses are: Xava (Xavante), Pale (Paleoindians), Yaki (Yakima), Chec (Quechua), Yung (Yungay), Pima (Pima), Herr (Herrera), Guan (Guane), Agro (Agroceramic), Mapu (Mapuche), Wayu (Wayuu), Kuna (Kuna), Huet (Huetar), Ngob (Ngöbe), Chey (Cheyenne), Nuu (Nuu-Chah-Nulth), Bell (Bella Coola), Haid (Haida), Ijka (Ijka), Kogi (Kogi), Arsa (Arsario), Zoro (Zoro), Yano (Yanomami), Cibo (Ciboney), Tain (Tainos). Groupings refer to geographical vicinity of the corresponding populations.

natural borders of the Guane territories were considered to condition a traditional Guane geographic and genetic isolation (Rodríguez, 1999; Rodríguez, 2007). This hypothesis was based on peculiarities of Guane craniofacial bones which were reportedly homogeneous and distinguishable when compared to craniofacial bones of surrounding populations (Rodriguez, 1999; Correal, 2004). The genetic data presented in our study better align with the hypothesis of a genetic exchange of the Guane with neighboring Muisca populations, as supported by the discovery of the rare haplotype 4 shared by these two communities.

CONCLUSIONS

The frequencies of mitochondrial haplogroups reported in this study agree with the genetic continuity observed through the haplogroup B in earlier periods, and are also compatible with gene flow between different populations throughout Andean South America.

The finding of deeply rooted lineages from the first inhabitants of Asia in the Guane and Eurasian populations corroborates with the hypothesis of early American settlers migrating from Beringia in Siberia, extending southward from the region devoid of the North American ice sheet.

According to our data, the peopling of the Guane region in northwestern Colombia would have been originated by two pre-Columbian migration waves, with an initial introduction of haplogroup B in this region and afterwards an introduction of haplogroup A as a result of a later expansion of Chibchan speaking populations out of Central America.

The genetic composition of skeletal remains belonging to 17 individuals found in the same rock shelter reveals a high degree of diversity of this particular community, indicating that there is no evidence of dramatic episodes of genetic drift in the genetic makeup of this population before the Spanish conquest. It also strengthens the hypothesis that there was gene flow among the Andean people from the Agroceramic period.

In conclusion, Guane people appear to have been genetically heterogeneous, although an exceptional craniometric homogeneity has been reported by physical anthropologists. Despite the geographical barriers that surrounded them, they appear as having been influenced by gene flow from neighboring communities such as the Muisca with whom trading relationships have been well documented, thus allowing genetic interchange.

Furthermore, although the indigenous pre-Columbian Guane are considered non extant, our work shows a clear evidence of their genetic survival through maternal lineages.

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