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Mitochondrial DNA from Pre-Columbian Ciboneys From Cuba and the Prehistoric Colonization of the Caribbean

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ABSTRACT To assess the genetic affinities of extinct Ciboneys (also called Guanajuatabeys) from Cuba, 47 pre-Columbian skeletal samples belonging to this group were analyzed using ancient DNA techniques. At the time of European contact, the center and east of Cuba were occupied by agriculturalist Taino groups, while the west was mainly inhabited by Ciboneys, hunter-gatherers who have traditionally been considered a relic population descending from the initial colonization of the Caribbean. The mtDNA hypervariable region I (HVR-I) and haplogroup-specific markers were amplified and sequenced in 15 specimens using overlapping fragments; amplification from second extractions from the same sample, independent replication in different laboratories, and cloning of some PCR products support the authenticity of the sequences. Three of the five major mtDNA Amerindian lineages (A, C, and D) are present in the sample analyzed, in frequencies of 0.07, 0.60, and 0.33, respectively. Different phylogenetic analyses seem to suggest that the Caribbean most likely was populated from South America, although the data are still inconclusive, and Central American influences cannot be discarded. Our hypothesis is that the colonization of the Caribbean mainly took place in successive migration movements that emanated from the same area in South America, around the Lower Orinoco Valley: the first wave consisted of hunter-gatherer groups (ancestors of the Ciboneys), a subsequent wave of agriculturalists (ancestors of the Tainos), and a latter one of nomadic Carib warriors. However, further genetic studies are needed to confirm this scenario. Am J Phys Anthropol 121:97–108, 2003.

At the time of European contact, in the late 15th century, several different human groups inhabited the Caribbean islands. An agriculturalist group, known as the Tainos, occupied the west islands, including Hispaniola, Puerto Rico, central-eastern Cuba, Jamaica, and the Bahamas. The lesser Antilles, including the Windward Islands and Guadeloupe, were occupied by the Caribs (sometimes called the Island Caribs), nomadic warriors who raided the Taino settlements moving from the south at that time. Archaeological, linguistic, and genetic evidence suggests that both groups originated in South America, moving northwest along the Antilles arch, probably in successive migrations from some area close to the Orinocco River Valley or to the Guayanas (Rouse, 1986, 1992; Moreira de Lima, 1999). Both Tainos and Caribs spoke Arawakan languages, which belong to the Equatorial-Tucanoan family. In contrast, the so-called Mainland Caribs, a group that inhabited the mainland adjacent to the Windward Islands and maintained trading relations with the Island Caribs, spoke a Cariban language, from the Ge-Pano-Carib family. Moreover, linguists have identified words in the vocabulary of the Island Caribs which are not Arawakan, but typically Cariban (Rouse, 1986).

This was not quite the whole picture of the indigenous Caribbean peoples; there was yet another ethnic group, the Guanajuatabeys or Ciboneys, which mainly inhabited the western half of Cuba. They were hunter-gatherers who lived mainly on hunting, fishing, and mollusk gathering, lacking both pottery and agriculture, and who spoke an unknown language (Tabío and Rey, 1966; Dacal-Moure and Rivero de la Calle, 1984; Rouse, 1986). They became extinct very soon after European Contact, around 1520; the process of extinction was even faster than in

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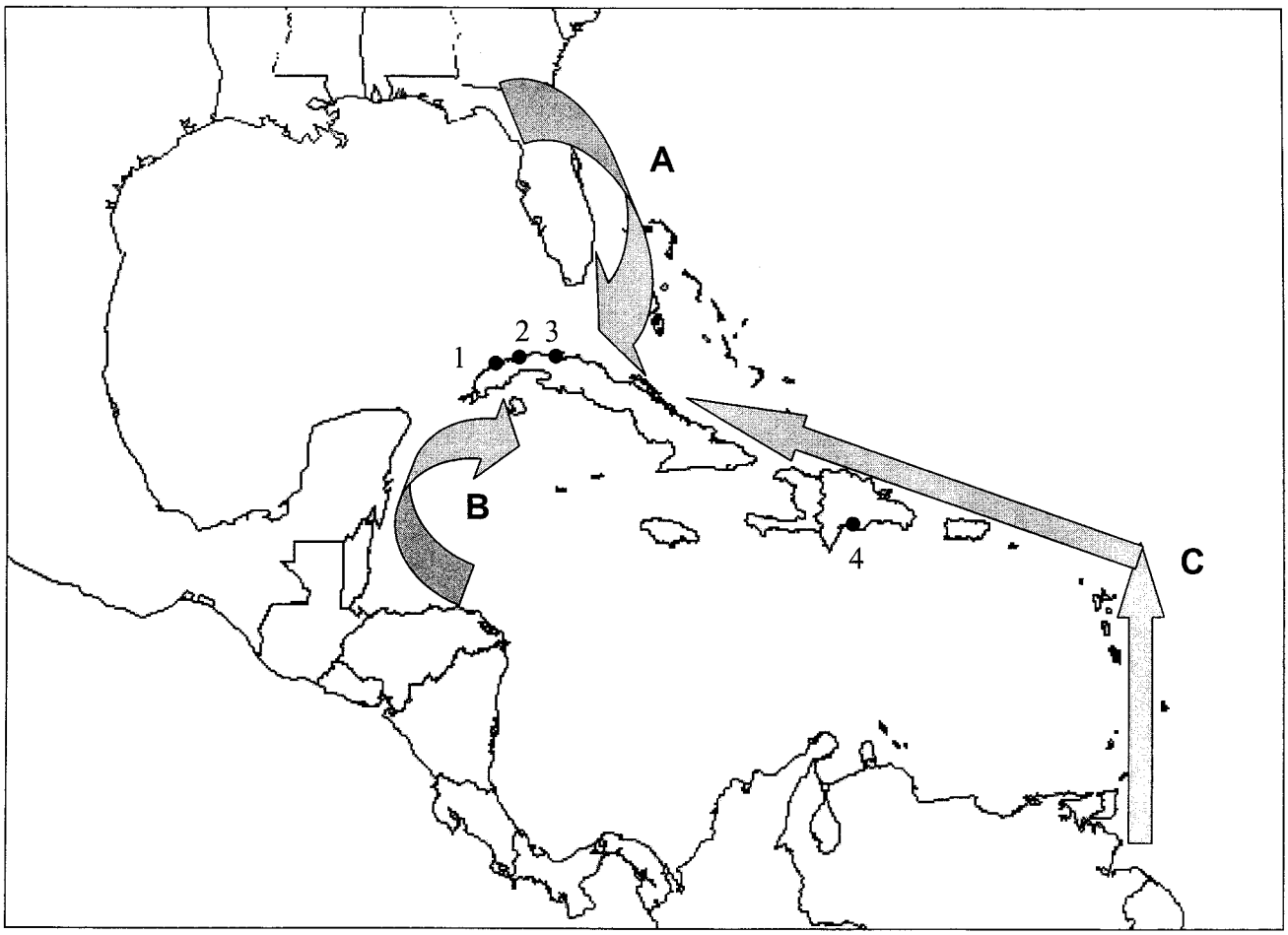

Fig. 1. Hypothetical migration routes in colonization of Caribbean and sites studied. A: Movements from North America. B: Movements from Central America. C: Movements from South America. Numbers refer to sites included in this study and in Lalueza-Fox et al. (2001): 1) Perico cave, 2) Mogote 3) Canimar, and 4) La Caleta.

the case of the Tainos, probably due to the lower population densities of these groups (Moreira de Lima, 1999). The Ciboneys have been traditionally considered a relic of an ancestral population, maybe descending from some of the first settlers of the Caribbean, a process that originated around 7,000 BP, associated with the spread of the Casimiroid lithic industry (MacNeish, 1982; Veloz Maggiolo, 1991; Rouse, 1992). The Casimiroid flints (which seem to have been woodworking tools) have traditionally been regarded as coming from Central America, maybe from Belize or the Yucatan Peninsula, where they may have originated around 9,500 BP (Callaghan, 1990). However, a contemporary Mesoindian culture, the Ostoiroid, appears to be the source of a migration that moved from northern South America to Hispaniola, Jamaica, and eastern Cuba, probably displacing or exterminating the Casimiroid foragers (Rouse, 1992). Therefore, while many scholars would agree with a peopling of the Caribbean in historic times (around 2,000 years before present) from South America in one or more migrations of Arawakan speakers, the origin and biological affinities of the Ciboneys remain a mystery (Fig. 1).

In this study, ancient DNA techniques were used to obtain genetic information from the extinct Ciboneys, taking advantage of the fact that the time span involved would allow DNA analysis. The fact that most Native Americans cluster in five major mitochondrial lineages (i.e., A, B, C, D, and X), determined by restriction-site gain or loss or by the presence of a 9-bp deletion, and are found to correlate with specific mtDNA control-region substitutions (e.g., Schurr et al., 1990; Torroni et al., 1992, 1993a,b; Horai et al., 1993), helps in the authentication of DNA data obtained from ancient Amerindian samples (Lalueza-Fox, 1996; Stone and Stoneking, 1998). The recovery of sequences from Taino remains from Hispaniola (Lalueza-Fox et al., 2001) allows us to further investigate the dynamics of the colonization of the Caribbean, a picture that can be enlarged with new genetic data from Ciboneys.

MATERIALS AND METHODS

Forty-seven samples from Ciboney culture sites were analyzed. The samples belong to three different sites: Perico I cave (N = 37), 25 km west of Bahia

Honda, in Pinar del Río (Cuba), Mogote La Cueva (N = 3), and Canimar (N = 7). Perico cave, one of the best studied preagriculturalist sites, has a radiocarbon dating of 1,990 ± 50 BP (unpublished data). The site was excavated in 1970 and 1997, and yielded the remains of at least 162 individuals (Travieso Ruíz et al., 1999). Mogote La Cueva is a site in Pinar del Río, and has been radiocarbon-dated at 1,620 BP (SI-424) (Tabío and Rey, 1966); Canimar is a rock shelter over the Canimar River (Matanzas), dated to 4,700 ± 70 BP (UBAR-171) (unpublished data). The specimens were chosen from a wider sample due to their good external preservation, taking in consideration a "fresh" aspect, lack of mineralization, absence of cracks or bone erosion, and completeness of the specimens. The skeletal material is hold at the Museo Antropológico Montané (Facultad de Biología, Universidad de La Habana, Havana, Cuba).

DNA extraction was undertaken following standard precautions to minimize risk of exogenous DNA contamination; the procedure was carried out in a dedicated ancient DNA room with positive air pressure, separated from the main laboratory and the common extraction room. Routine bleaching of surfaces, filter pipette tips, ultraviolet (UV) lights, sterile gloves, face masks, and aliquoted reagents were some of the precautions adopted during the extraction process.

The extraction method was similar to that published elsewhere (Lalueza-Fox et al., 2001). Teeth were immersed in bleach for 5 min, and after that immersed in 70% alcohol. A sample was taken with a drilling machine (Dremel) from the pulp chamber cavity and the surrounding dentine (approximately 0.2 g of powder, depending on the size of the tooth); bone samples were powdered in a coffee-grinding machine. The powder was washed overnight in10 ml 0.5 M EDTA to remove mineral salts; after centrifugation, the EDTA was removed and the remaining sample was incubated overnight at 37°C in a lysis solution with 8.5 ml of water, 1 ml 5% SDS, 0.5 ml 1 M Tris-HCl, pH 8.0, and 50 μl of 1 mg/ml proteinase K. After this procedure, the sample was extracted with phenol, phenol-chloroform, and chloroform; the supernatant was desalinized and concentrated with a Centricon-30 microconcentrator (Amicon) to a volume of 100 μl. The sample was finally incubated with 40 μl of silica suspension and centrifuged; the silica pellet was washed twice with 250 μl of 70% ethanol and left to dry for 20 min. DNA was eluted in 30 μl of sterile water. The procedure is a modification of that of Höss and Pääbo (1993).

PCR amplification was carried out with 1 μl of the template subjected to 40 cycles (1-min step at 94°C, 1-min step at 53–55°C, and 1-min step at 72°C) in a 25-μl volume containing 1 unit of Taq polymerase, 1 × reaction buffer (EcoGen), 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 2 mg/ml BSA, and 1 μM of each primer. Several negative controls (mock extraction and PCR controls without template) were added to each PCR reaction. Products were visualized on 0.8% low-melting-point agarose gels; bands were excised from the gels, melted at 65°C for 20 min, eluted in 150 μl of water, and subjected to another PCR of 30 cycles, with limiting primers and $MgCl_2$. The final PCR products were purified with microcolumns (Qiagen) and sequenced with an ABI 377A DNA sequencer (Applied Biosystems), following the supplier's instructions.

Four sets of overlapping primers (L16,055–H16,142, L16,131–H16,218, L16,209–H16,356, and L16,313–H16,401), published elsewhere (Handt et al., 1996; Stone and Stoneking, 1998), were used to amplify 354 bp of the mtDNA control region I, between positions 16,056–16,400 (Anderson et al., 1981). When possible, only the pairs L16,055–H16,218 and L16,209–H16401 were used. Six specimens (7160, C317, C319, 6620-1, 7146, and 2486) required the amplification of shorter fragments. When possible, two extracts were generated from the same sample; however, most of the teeth analyzed were too small to allow such a replication strategy.

To estimate the rate of Taq misincorporations due to template damage in our sample, one PCR product (16,209–16,401 or 16,055–16,218) from each of three different samples (C46, Cs/n and 7146) was cloned and sequenced. Twelve microliters of PCR product were treated with T4 polynucleotide kinase, purified, and ligated into a *Sma*I pUC18 plasmid vector, for 2 hr at 16°C, following the manufacturer's instructions (SureClone Ligation Kit-Pharmacia, Upssala, Sweden). Two microliters of the ligation product were transformed into 50 μl of competent cells, grown in 200 μl of LB medium for 1 hr and plated on IPTG/X-gal agar plates. White colonies grown after overnight incubation were transferred to 50-μl PCR reactions for 35 cycles; inserts that yielded the expected size in an electrophoresis were excised from the low-melting-point agarose gel, purified with silica, and sequenced as described previously.

One sample (6620-3) was sent to the Ancient Biomolecules Centre in Oxford for independent replication; the methodology used was as previously published (Cooper et al., 2001). The main differences were in the use of a high-fidelity enzyme (Platinum Taq Pfu Hi-fi, Gibco BRL), and that the control region was amplified twice as one 347-bp fragment (L16,055–H16,401) and one 147-bp fragment (L16,209–H16,356). The 147-bp fragment was cloned and sequenced.

To confirm the sequence haplogroup attribution, the mtDNA regions containing the haplogroup-specific marker were amplified and subjected to appropriate enzymatic digestion (for the A, C, and D haplogroups) or to electrophoresis with known size standards (for the B haplogroup) (Handt et al., 1996; Stone and Stoneking, 1998; Lalueza-Fox et al., 2001).

Statistical analysis

Intrapopulation mtDNA variation in the Ciboneys was measured with two parameters. Nucleotide diversity (π) and sequence diversity (D) parameters were computed (Nei, 1987).

To provide a phylogeographic context for testing the different hypotheses (Fig. 1) about the peopling of the Caribbean, data from North, Meso-, and South American groups with ethnic attribution and large sample sizes were compiled from the literature. The populations included are from North America: Athapascan (Shields et al., 1993), Bella Coola (Ward et al., 1993), Haida (Ward et al., 1993), Navajo (Torroni et al., 1993a,b), Nuu-Chah-Nulth (Ward et al., 1991), Oneota (Stone and Stoneking, 1998), Yakima (Shields et al., 1993), and Cherokee (Malhi et al., 2001); from Meso-America: Embera, Gavião (Ward et al., 1996), Huetar (Santos et al., 1994), Kuna (Batista et al., 1995), Ngöbé (Kolman et al., 1995), Quiché (Boles et al., 1995), and Taino (Lalueza-Fox et al., 2001); and from South America: Araucanians or Chileans (Horai et al., 1993), Cayapas (Rickards et al., 1999), Mapuches (Ginther et al., 1993), Xavante (Ward et al., 1996), Yanomami (Torroni et al., 1993a; Easton et al., 1996), Wounan (Kolman and Bermingham, 1997), and Zoro (Ward et al., 1996), as well as some individuals from related tribes (Wayampi, Kayapo, Arara, Katuena, Portujara, Awa-Guaja, and Tiriyo) grouped into the category "Amazonas" (Santos et al., 1996). Analyses including these samples were carried out considering sequences between positions 16,024–16,383.

A genetic distance matrix between populations was generated by intrapopulational correction of the interpopulational pairwise differences (intermatch-mismatch) with the Arlequin 2000 package (Schneider et al., 2000). Principal coordinates analysis was performed on the distance matrix with the NTSYS program, version 1.70 (Applied Biostatistics, Inc., Setauket, NY). An analysis of molecular variance (AMOVA) (Excoffier et al., 1992) was carried out using the Arlequin package, to estimate the most likely grouping of the Caribbean samples in the context of the three American subcontinents.

To unravel the phylogeny of the mitochondrial lineages, a reduced median network of the most common Caribbean haplogroup (C) was generated (Bandelt et al., 1995). Two hundred and twenty-nine C-haplogroup sequences from 24 Amerindian populations were included.

RESULTS

Two different sets of samples were analyzed in different periods in the laboratory in Barcelona: the first were 29 teeth samples from Perico site, while the second consisted of 18 bone samples from Perico, Mogote La Cueva, and Canimar. In the first set, only 7 of 29 samples extracted (24.1%) yielded positive amplifications and were subsequently sequenced (two of the amplified samples correspond to repeated samples from 2493 and 2486, extracted from two different teeth belonging to the same individual). In the second set, 10 of 18 samples (55.6%) extracted were amplified and sequenced. The general low amplification efficiency (38.3% for the whole sample) can be attributed both to the antiquity of the samples and the unfavorable climate, since the tropical conditions of the Caribbean do not help preserve DNA. Similar DNA deterioration was observed in ancient samples from India, were amino-acid analysis indicated that very little protein remained in the specimens (Kumar et al., 2000). However, a good amplification efficiency (70.4%) was observed in other samples from the Caribbean (Lalueza-Fox et al., 2001), although in this case the specimens were cortical bone samples and had more recent dates. Macroscopic selection prior to DNA analysis seems to be essential for obtainong high efficiency figures in warm areas.

Sequence authentication

Some of the main authentication criteria for ancient DNA, proposed by Cooper and Poinar (2000), and including the most stringent, i.e., independent replication, were followed. PCR products were cloned when clear heteroplasmies were observed in the direct sequencing, to unravel the possible heterogeneity in the sequences. Additional amplifications of small fragments were generated in some cases, including a sample (7146) in which multiple substitutions were observed in the clones; it has been observed that, due to the fragmentation inherent to ancient DNA, small fragments yield more reliable sequences (Krings et al., 1997).

The blanks showed no signs of contamination during the study; this and the fact that the sequences obtained are Amerindian, in a new laboratory where no modern Amerindian samples have been extracted and where all researchers are of European origin, support the authenticity of the sequences retrieved. The mtDNA of the researcher involved in the extraction and amplification of the samples in Barcelona (C.L.-F.) belongs to the European T haplogroup ([C]16,126, [T]16,294, [T]16,296, [C]16,304); it is interesting to note that this sequence has been found as a contaminant in one clone of the sample sent to Oxford. The mtDNA of the researcher in Oxford (M.T.P.G.) belongs to the V haplogroup (Table 1).

Sample 6620-3, which belonged to a haplogroup (A) different from the rest, was independently extracted, amplified, sequenced, and cloned in Oxford, and yielded the same sequence as in Barcelona. Also, two specimens duplicated in Barcelona from different teeth belonging to the same individual (2493 and 2486) produced the same sequences. Because substitutions due to template damage occur at random, it is expected that multiple amplifications of the same fragment will not show the same random damage substitutions. In three samples (2486, 7160, and C319), the [C]16,325 and [T]16,327 substitutions were observed in two different overlap-

TABLE 1. Mitochondrial sequences of Ciboneys and their haplogroup attributions[1]

Sample	Haplogroup	16103	16111	16126	16129	16223	16270	16290	16298	16311	16319	16325	16327	16362
Reference		A	C	T	G	C	C	C	T	T	G	T	C	T
6620–3*	A	·	T	·	A	T	·	**T**	·	·	**A**	·	·	C
2493	C	·	·	·	·	T	·	·	**C**	·	·	·	**T**	·
7152	C	·	·	·	·	T	·	·	**C**	·	·	·	**T**	·
2325–61	C	·	·	·	·	T	·	·	**C**	·	·	·	**T**	·
7145	C	·	·	·	·	T	·	·	**C**	·	·	C	**T**	·
C317	C	·	·	·	·	T	·	·	**C**	·	·	C	**T**	·
2486	C	·	·	C	·	T	·	·	**C**	C	·	C	**T**	·
c (s/n)	C	G	·	·	·	T	·	·	**C**	·	·	C	**T**	·
7160	C	·	·	·	·	T	T	·	**C**	·	·	C	**T**	·
c46	C	·	·	·	·	T	·	·	·	·	·	C	**T**	·
c47	D	·	·	·	·	T	·	·	·	C	·	**C**	T	**C**
C319	D	·	·	·	·	T	·	·	·	C	·	**C**	T	**C**
7146	D	·	T	·	·	T	·	·	·	·	·	·	·	**C**
7160a	D	·	·	·	·	T	·	·	·	·	·	**C**	·	**C**
6620-1	D	·	·	·	·	T	·	·	·	·	·	**C**	·	**C**

[1] Base positions refer to Cambridge reference sequence (Anderson et al., 1981).
* Corresponds to a sample independently replicated in Oxford (L16,209–H16,356 fragment). In bold, substitutions used to define each haplogroup.

ping fragments (16,209–16,356 and 16,313–16,401) generated from the original extracts, as well as the single [C]16,325 substitution in two other samples (C317 and 6620-1); the reproducibility of these substitutions supports the authenticity of the sequences.

Cloning results

The consensus sequences generated from the clones obtained for four samples display the substitutions found in the direct sequencing (Table 2). Samples Cs/n and C46 (Table 2a,c) display only singleton substitutions, that most likely correspond to cloning artifacts (Krings et al., 1997); sample 7146 (Table 2b) shows multiple substitutions at np 16,187[T] and np 16,194[G], as well as single substitutions at np 16,169 and np 16,126; however, the consensus sequence for these positions (C at np16,187, and A at np 16,194) was further established with the additional amplification and direct sequencing of the smaller 16,055–16,142 and 16,131–16,218 fragments, with unambiguous sequence results. In another sample, 6620-3 (Table 2d), 1 in 6 clones contained a different sequence of European origin that probably corresponded to a contaminant DNA from one of the researchers.

The error rates for samples Cs/n, 2230-6 (excluding the contaminant), and C46 are very similar to each other (2.60, 1.13, and 1.73 per kilobase, respectively), and are close to the lowest values described in ancient DNA studies (Cooper et al., 2001); the error rate for sample 7146 is slightly higher (3.05 per kilobase, considering each multiple substitution as a single event), which points to a higher degree of DNA degradation in this specimen.

Sequence sharing

The sequences obtained (Table 1) belong to the haplogroups A (n = 1), C (n = 9), and D (n = 5), as deduced from their diagnostic nucleotide substitutions (T in 16,290 and A in 16,319 for haplogroup A; C in 16,298 and T in 16,327 for haplogroup C; and C in 16,325 and C in 16,362 for haplogroup D), described as correlating with the enzymatic restriction haplogroup attribution (Torroni et al., 1992, 1993a,b). Most of the sequences belong to haplogroups C and D (60% and 33.3%, respectively), and one of them (6.7%) belongs to the A2 subgroup of haplogroup A. The C and D haplogroup attribution in these samples was confirmed by enzymatic digestion of the PCR product, including the diagnostic susbtitution. The amplification of the 9-bp deletion, diagnostic of the B haplogroup (Torroni et al., 1992, 1993a), confirmed the absence of this lineage in 19 Ciboney samples, including the 15 samples sequenced plus four additional samples that could not be sequenced.

Two sequences ([T]16,223, [C]16,325, [C]16,362 and [T]16,223, [C]16,298, [C]16,325, [T]16,327) were found in the Taino samples previously analyzed (Lalueza-Fox et al., 2001), and are also present in many Amerindian populations from both South and North America, since they represent the D and C haplogroup founding sequences. Other sequences found in the Ciboneys differ from previously described sequences by sites known to be hypervariable, such as C at np 16,311, C at np 16,362, C at np16,325, and C at nt 16,126 (Meyer et al., 1999).

The sequence of the A haplogroup ([T]16,111, [A]16,129, [T]16,223, [T]16,290, [A]16,319, [C]16,362) belongs to the A2 subhaplogroup, and has been mainly found in northern North American populations such as the Bella-Coola, the Nuu-Chah-Nulth, the Haida, and the Cheyenne-Arapaho (Ward et al., 1991, 1993; Malhi et al., 2001), although it has been described in Pehuenche aborigines from Chile (Moraga et al., 2000); moreover, the sequence is also

TABLE 2. DNA substitutions from cloned PCR products from four Ciboney samples[1]

a)							
	1	1	1	1	1	1	
	6	6	6	6	6	6	
	2	2	2	3	3	3	
	2	5	9	0	2	2	
	3	0	8	4	5	7	
Clone 1	T	G	C	·	C	T	
Clone 2	T	·	C	·	C	T	
Clone 3	T	·	C	·	C	T	
Clone 4	T	·	C	C	C	T	
b)							
	1	1	1	1	1		
	6	6	6	6	6		
	1	1	1	1	1		
	1	2	6	8	9		
	1	6	9	7	4		
Clone 1	T	·	·	T	·		
Clone 2	T	·	·	T	·		
Clone 3	T	·	T	T	·		
Clone 4	T	·	·	T	G		
Clone 5	T	·	·	T	G		
Clone 6	T	·	·	·	G		
Clone 7	T	C	·	·	·		
Clone 8	T	·	·	·	·		
Seq 1	T	·	–	–	–		
Seq 2	–	–	·	·	·		
c)							
	1	1	1	1			
	6	6	6	6			
	2	2	3	3			
	2	9	2	2			
	3	4	5	7			
Clone 1	T	G	C	T			
Clone 2	T	·	C	T			
Clone 3	T	·	C	T			
d)							
	1	1	1	1	1	1	1
	6	6	6	6	6	6	6
	2	2	2	2	2	3	3
	2	5	9	9	9	0	1
	3	2	0	4	6	4	9
Clone 1	T	G	T	·	·	·	A
Clone 2	T	·	T	·	·	·	A
Clone 3	T	·	T	·	·	·	A
Clone 4	T	·	T	·	·	·	A
Clone 4	T	·	T	·	·	·	A
Clone 6	·	·	·	T	T	C	·

[1] a, Cs/n, (L16,209-H16,401). b, 7146 (L16,055-H16,218). Seq 1, direct sequencing of 16,055–16,142 fragment; Seq 2, direct sequencing of the 16,131–16,218 fragment. c, C46 (L16,209–H16,401). d, 6620-3 (L16,209–H16,356). Changes are compared to reference sequence (Anderson et al., 1981). Clone number 6 from 6620-3 is a European DNA contaminant.

close to a sequence found in the Panamanian groups Kuna and Ngöbé that lacks the A in np 16,129, again a hypervariable position (Batista et al., 1995; Kolman et al., 1995).

Sequence and population diversity

Genetic diversity results for the Amerindian populations are shown in Table 3. The Ciboney nucleotide diversity is 0.0096 for the 354-bp fragment; this figure is one of the lowest of all Amerindian populations studied, although similar to the value found in other groups from North America, such as the Athapascan and the Haida; from Meso-America, such as the Tainos, the Kuna, and the Huetar; and from South America, such as the Xavante. In contrast, the sequence diversity is 0.9429, one of the highest described in Central and South American populations, although close to the values of other groups, such as Amazonas, Araucanians, Embera, Mapuche, Quiché, Nuu-Chah-Nulth, Tainos, and Wounan. The Ciboneys present a mean pairwise difference of 3.47, a low figure similar to that observed in other Amerindian populations, such as the Athapascan, Haida, Huetar, Kuna, Tainos, and Xavante. This figure, nonetheless, may be strongly affected by the haplogroup distribution.

Ciboney population relationships

The reduced median network of the C haplogroup (Fig. 2) shows a star-like shape that reflects a population expansion, as suggested by others (Forster et al., 1996); moreover, there is a clear geographic structure in the genealogy, with the central sequence found in North, Central, and South America, but with some branches only found in North America and many other found only in South America (the C haplogroup is almost absent in Central America). Taino sequences do not cluster in any exclusive North American branch; moreover, they are located in branches mainly shared with other Central or South American populations. Two Taino sequences ([C]16,126, [C]16,298, [C]16,325, [T]16,327 and [C]16,298, [C]16,325, [T]16,327, [G]16,254, [C]16,362) are related to an ancestral Amazonian sequence ([C]16,298, [C]16,325, [T]16,327) and to an ancestral Yanomami sequence ([C]16,298, [C]16,325, [T]16,327, [G]16,254), respectively; another Taino sequence ([C]16,189, [C]16,298, [C]16,325, [T]16,327, [C]16,362) is connected to the central sequence by another ([C]16,189, [C]16,298, [C]16,325, [T]16,327), so far only found in the Mapuche (Chile), and is related to another sequence ([C]16,183, [C]16,189, [C]16,298, [C]16,325, [T]16,327) only found in Mayas.

However, none of the Ciboney sequences are in branches exclusively found in either North or South America; for instance, one Ciboney sequence ([C]16,126, [T]16,223, [C]16,298, [C]16,311, [C]16,325, [T]16,327) is related to a Chilean/Taino ancestral sequence ([T]16,223, [C]16,298, [C]16,311, [C]16,325, [T]16,327), but was also found in one Oneota from North America (Stone and Stoneking, 1998). Another Ciboney sequence ([T]16,223, [C]16,298, [T]16,327) was also found in North American, South American, and even Asian populations, although it seems to be related to two other Taino sequences ([T]16,223, [C]16,298, [T]16,327, [C]16,362 and [T]16,223, [C]16,263, [C]16,298, [T]16,327), so far only found in the Caribbean; the remaining Ciboney sequences are related to the founding sequence by just one substitution and are exclusively found in the Caribbean, thus providing no phylogeographic information. In the network of the D haplogroup (not shown), the Caribbean sequences found also cluster in branches shared by North and South American populations. There is slight support for the origin of some Taino sequences from Central/South America, since in some cases these

TABLE 3. Diversity parameters in several Amerindian populations[1]

	n	*K*	D	Pw	π
North America					
Athapascan	21	12	0.9048	2.47	0.0069
Bella Coola	40	11	0.9038	5.02	0.0139
Haida	41	10	0.7085	2.49	0.0069
Navajo	17	10	0.8750	4.13	0.0115
Nuu-Chah-Nulth	63	28	0.9544	5.32	0.0148
Oneota	50	23	0.9061	5.07	0.0141
Yakima	42	20	0.8931	4.86	0.0135
Cherokee	30	13	0.8874	4.63	0.0129
Central America					
Embera	44	20	0.9419	5.87	0.0163
Huetar	27	7	0.7094	3.50	0.0097
Kuna	63	7	0.5919	3.30	0.0091
Ngöbé	47	8	0.7734	4.36	0.0121
Quiché	30	20	0.9747	6.06	0.0169
Wounan	31	15	0.9204	6.71	0.0186
South America					
Amazonas	92	41	0.9333	6.37	0.0177
Araucanians	45	24	0.9263	5.21	0.0145
Cayapas	120	13	0.7766	5.01	0.0139
Colombia	25	13	0.9333	6.50	0.0181
Gavião	27	7	0.8661	4.28	0.0119
Mapuche	39	13	0.9123	5.27	0.0153
Xavante	25	4	0.6767	3.00	0.0083
Yanomami	53	25	0.8389	4.08	0.0113
Zoro	30	9	0.7747	3.98	0.0111
Caribbean					
Ciboneys	15	10	0.9429	3.47	0.0096
Tainos	19	11	0.9181	2.86	0.0079

[1] *n*, sample size; *k*, number of different sequences; D, sequence diversity; Pw, mean pairwise difference; π, nucleotide diversity.

are in the tips of branches which are Central and/or South American-specific. However, the case for the Ciboney sequences is weak, since they are either in single branches or in branches found in both North and South America, and therefore without diagnostic power.

The results of the principal coordinate analysis on the mismatch-intermatch genetic distance matrix are given in Figure 3; the first two principal coordinates explain 65.6% of the total variance in the distance matrix (the first coordinate accounts for 43.8%, and the second for 21.8%); the first coordinate mainly separates South American from North American groups.

Overall, the population genetics of the Amerindians show geographical structure, especially noticeable in broad subcontinental entities, such as North, Meso-, and South America. It can be observed that the groups from Central America, especially the Chibcha-speakers (Ngöbé, Huetar, and Kuna), are quite close to each other. The South American populations (Amazonas, Araucanians, Cayapa, Gavião, Mapuche, Xavante, Yanomami, and Zoro), as well as the North Americans (Athapascan, Bella Coola, Haida, Navajo, Nuu-Chah-Nulth, Oneotas, and Yakima), are rather dispersed, and overlap with Central American populations. The two Caribbean samples tend to group together and with South American groups (specially the Yanomami), and are separated from the Meso- and North American populations. The Ciboneys are close to the Tainos, who are further separated from the mean variation.

To further investigate the genetic affinities of the Caribbean groups, several AMOVA analyses were generated, grouping the Ciboneys and the Tainos with North American, Central American, and South American groups, respectively. Including the Caribbean samples in the North America subcontinent, AMOVA showed that 2.61% of the genetic variation was among subcontinents ($P = 0.0303$), while 18.71% was within subcontinents ($P < 0.0001$); with the Caribbean samples in the Central American subcontinent, 2.10% of the genetic variation was among subcontinents ($P = 0.0675$), and 19.08% within subcontinents ($P < 0.0001$). When Caribbean samples were included in the South America subcontinent, the variation among subcontinents almost doubled (3.88%, with a probability significantly lower, $P = 0.0068$), while the variation within subcontinents decreased slightly (17.80%, with $P < 0.0001$). Thus, the subcontinent where the inclusion of the Caribbean groups adds less heterogeneity is clearly with South America.

In order to quantify the relative probabilities that the sample obtained could come from each of the three hypothetical source populations (North, Central, and South America), the probability of obtaining the observed frequencies for the five Amerindian mtDNA haplogroups (A, B, C, D, and X) from each subcontinent was estimated under three different multinomial distributions (Sokal and Rohlf, 1995). Each probability distribution was generated with the observed frequencies for the pooled samples for each subcontinental region (Lalueza-Fox et al.,

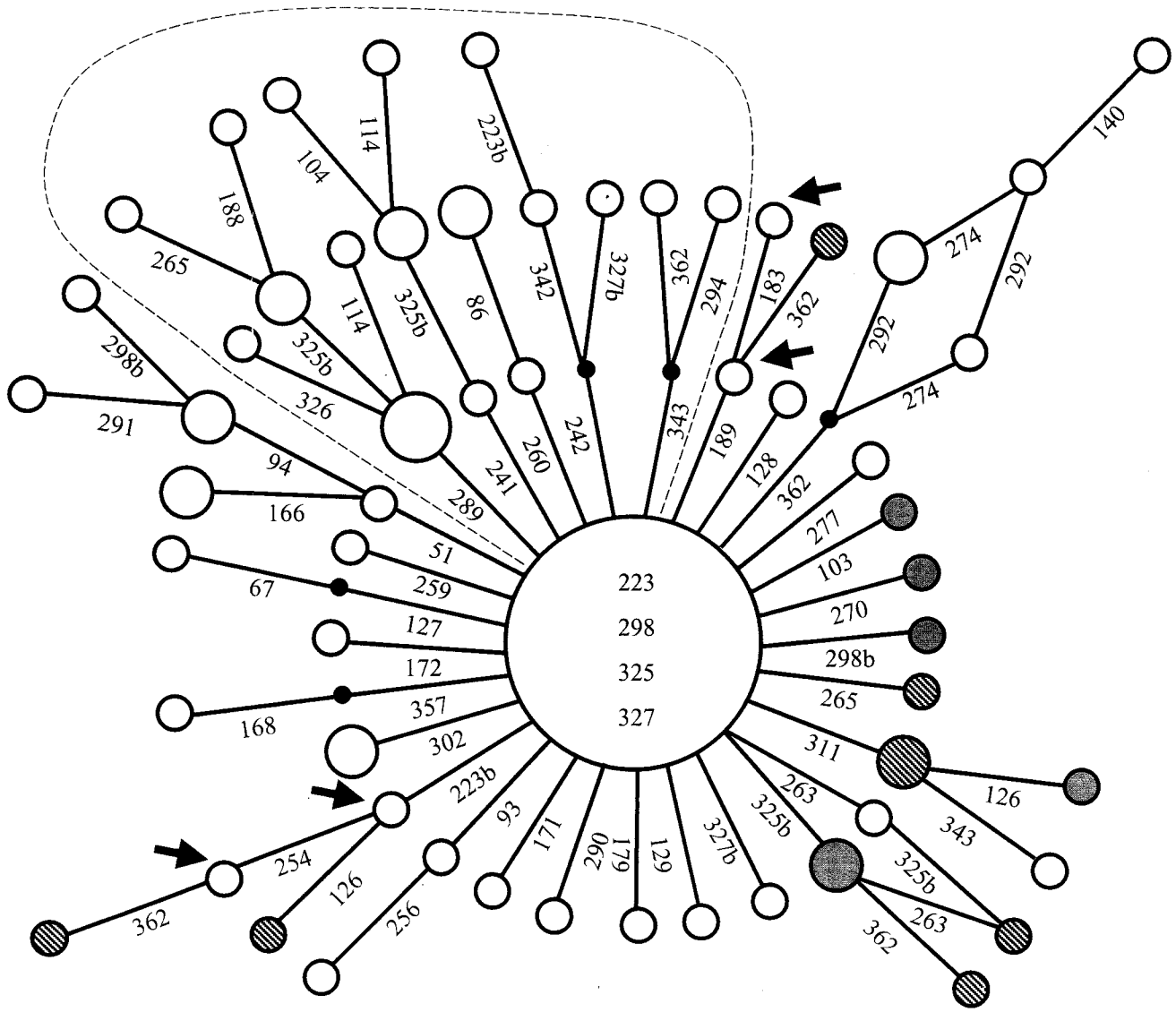

Fig. 2. Reduced median network of C Amerindian haplogroup, including 24 populations and 229 individuals. Nucleotide positions are as in Anderson et al. (1981); b indicates a reverse substitution; solid circles correspond to sequences not found; nodes represent single nucleotide change. Small circles, 1–5 individuals; medium circles, 6–10 individuals; large circles, more than 10 individuals; central node, 75 individuals. Grey circles correspond to Ciboney sequences, and hatched circles to Taino sequences (Lalueza-Fox et al., 2001). Dashed line encompasses lineages only found in North America. Arrows mark sequences related to some Caribbean sequences, that have only been found in Central and/or South American populations.

2001). The results show that the highest probability for the Ciboney haplogroup distribution is obtained when South America is the source population, being 130 times less probable when taking North America, and much less (on the order of 10^9) when considering Central America. This is probably attributable to the high frequency of the A haplogroup in Central America.

A different approach to recognize affinities in haplogroup distribution may be gained by comparing the Ciboney distribution with that of specific populations in the three main regions of the Americas close to the putative origin of the settlement of the Antilles (see Fig. 1). The populations chosen were the Seminoles (Florida) (Huoponen et al., 1997), the ancient Mayas from Xcaret (Quintana Roo, Mexico) (González-Oliver et al., 2001), and the Amazonian sample described in Materials and Methods. The likelihood that the Ciboneys and that each of the three populations comes from the same source (or general population) may simply be estimated as the P value from Fisher's exact test expanded to $2 \times n$ tables for each pair of populations (the Ciboneys being one in each case) and from the haplogroups found.

The Ciboney haplogroup distribution is significantly different from those of the three putative source populations, probably due to a founder effect; nonetheless, the results favor a closer relationship of the Ciboneys with the Amazonians than with the

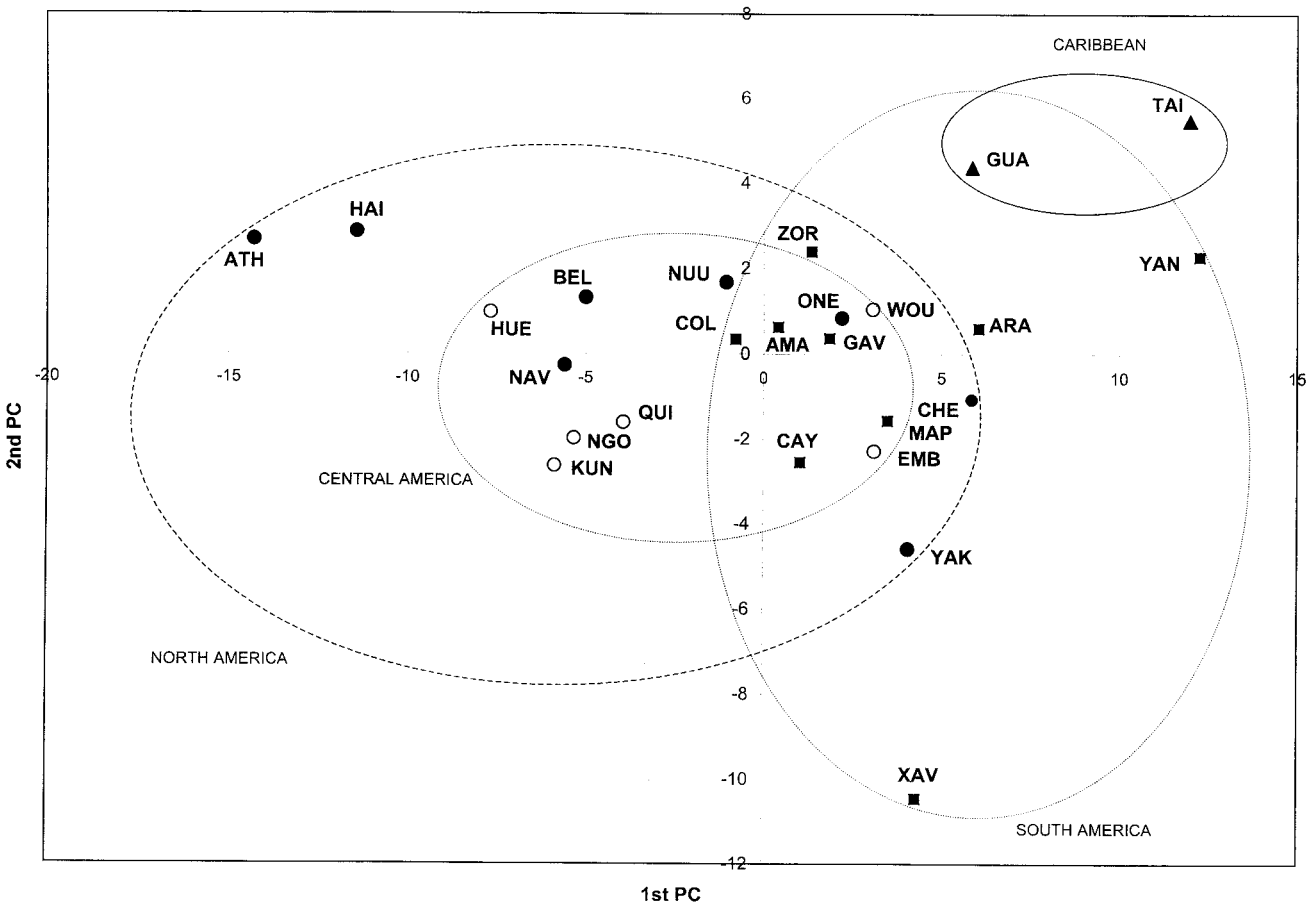

Fig. 3. First and second principal coordinates generated from intermatch-mismatch genetic distance among North American, Central American, South American, and Caribbean populations. First two principal coordinates account for 65.6% of variation in distance matrix. AMA, Amazonas; ARA, Araucanians; ATA, Atapascans; BEL, Bella Coola; CAY, Cayapa; CHE, Cherokee; COL, Colombia; EMB, Embera; GAV, Gavião; CIB, Ciboneys; HAI, Haida; HUE, Huetar; KUN, Kuna; MAP, Mapuche; QUI, Quiché; NAV, Navajo; NGO, Ngöbé; NUU, Nuu-Chah-Nulth; ONE, Oneota; TAI, Tainos; WOU, Wounan; YAK, Yakima; YAN, Yanomami; XAV, Xavante; ZOR, Zoro. ●, North American populations; ○, Central American populations; ■, South American populations; ▲, Caribbean populations.

other populations, since the P value is 10^3 times higher than for the comparison with the Seminoles and the Xcaret, yielding a dramatic difference (P value on the order of 3×10^{-6}).

DISCUSSION

From a geographical point of view, prehistoric migratory movements from South America into the Caribbean were more feasible than those from Central or North America to the Caribbean. All islands along the Caribbean chain can be seen from one another with three exceptions: the gaps are those between Tobago and the Windward Islands (at the beginning of the putative migration route), Cuba and Yucatan (210 km), and Cuba and Florida (180 km). Also, the strong Gulf stream only favored, as today, navigation from Cuba to North America, but not in the opposite direction (Moreira de Lima, 1999).

The putative origin of the Ciboneys has been debated using Caribbean archaeology, despite the problems of establishing connections between archaeological traditions and ethnic groups described by historic accounts, separated by hundreds or even thousands of years. Some scholars consider the Ciboneys to be direct descendants of people associated with the Casimiroid archaeological horizon (MacNeish, 1982; Veloz Maggiolo, 1991). The Casimiroid tradition, dated between ca. 4000–2000 BP, originated in Central America, and has been related to the first peopling of the Greater Antilles (MacNeish, 1982). Other scholars assume that the Ciboneys were in fact a relic population of pre-Arawakan speakers from South America (Rouse, 1986). In this sense, they could be descendants of immigrants associated with the preceramic Ostoiroid culture (ca. 4000 BP–AD 200), a tradition that moved into the Antilles from northern South America (Rouse, 1986). In this scenario, the Arawakan speakers (Tainos and Caribs) would have subsequently migrated along the chain of islands in successive waves from South America, pushing the remnants of the original migrants into peripheral areas such as western Cuba (Rouse, 1986).

The genetic data show that the American continent is rather homogenous, although a geographic structure in the mtDNA, especially in the haplogroup distribution and haplotype patterning, can be observed (e.g., Szathmary, 1993; Lalueza-Fox, 1996; Merriwether et al., 1995; Merriwether and Ferrell, 1996; Forster et al., 1996; Lorenz and Smith, 1996, 1997; Malhi et al., 2001, Salzano, 2002). The frequencies of the four major mtDNA lineages differ latitudinally and show a marked gradient from north to south; the general pattern along the continent is an increase of the C and D haplogroups to the south, parallel to a decrease in the A haplogroup frequency (Lalueza-Fox, 1996; Merriwether et al., 1995; Merriwether and Ferrell, 1996). Considering Amerindian samples published so far, the frequency of the A lineage is 52.6% in North America and 63.1% in Central America, while in South America it is only 11.8%. In contrast, the C haplogroup has a frequency of 15.3% in North America and 6.3% in Central America, but is as high as 20.2% in South America (Lalueza-Fox et al., 2001); similar figures can be observed for the D haplogroup. In North and Central American populations geographically close to the Caribbean, the haplogroup frequencies are quite different from those found in the Ciboneys and Tainos. In an ancient DNA analysis of 25 pre-Columbian Maya individuals from Xcaret (Quintana Roo, Mexico), roughly contemporary to the Ciboneys, 21 individuals (84%) were attributed to the A haplogroup, 2 (8%) to the C haplogroup, 1 (4%) to the B haplogroup, and 1 (4%) to "other" unspecified haplogroups, while the D haplogroup was absent (González-Oliver et al., 2001). In 37 Seminole individuals from Florida, 59.5% of the sequences belong to the A haplogroup, 21.6% to the B haplogroup, 8.1% to the C haplogroup, and 5.4% to the D haplogroup (there are two African sequences) (Huoponen et al., 1997). However, a recent genetic analysis of a modern sample from Puerto Rico (Martínez-Cruzado et al., 2001) revealed a significant proportion of A haplogroup sequences (56% of individuals with presumed Native American ancestry), which are absent in the Yanomami and quite uncommon in the Amazonian region; moreover, five samples carried both the diagnostic A haplogroup marker (663 *Hae*III) as well as the region V 9-bp deletion, a haplotype thus far only described in Maya, Boruca, and Mixtecs (Schurr et al., 1990; Torroni et al., 1993a). Martínez-Cruzado et al. (2001) suggested that there was some genetic contribution to the population of Puerto Rico from Central America, where the A haplogroup has a very high frequency.

Isolation and drift have shaped the genetic composition of the Amerindian populations to the point that there is considerable variation between adjacent tribes along the continent, especially in the haplogroup frequency distribution. Some groups show an absence of either one or another haplogroup; for instance, the Cayapas and the Ngöbé lack the D haplogroup, while the Gavião, Mataco, and Xavante lack the C haplogroup (Torroni et al., 1993a; Kolman et al., 1995; Ward et al., 1996; Rickards et al., 1999). A similar marked haplogroup variation seems to be present along the three neighboring Caribbean islands (Puerto Rico, Hispaniola, and Cuba) studied up to now, although factors such as recent admixture (in the case of Puerto Rico), small sample sizes, and the inclusion of different chronological periods may account for some of this mtDNA haplogroup heterogeneity.

Overall, the residual presence of the A lineage and the high frequencies of the C and D lineages in the sample studied seems to suggest that the place of origin of the Ciboneys from Cuba could be placed in South America, such as in the case of the Taino samples from Hispaniola (Lalueza-Fox et al., 2001). The absence of the B lineage in the Ciboneys may suggest that the Caribbean was peopled before the spreading of this lineage into the subcontinent, a fact that could be correlated with the absence of the B lineage in the southern end of South America (Lalueza-Fox, 1996). Alternatively, haplogroup B may have been lost in the founding event(s) of the Caribbean populations. The residual presence of the B haplogroup in Puerto Rico could be the result of a modern minor migration, according to Martínez-Cruzado et al. (2001).

It is recognized that haplogroup frequencies are variable between and within populations due to random sampling processes and/or genetic drift; moreover, lineage frequencies cannot be accurately estimated when low sample sizes, such as those generally obtained in ancient DNA studies, are used. Nevertheless, haplogroup frequencies may be indicative of the genetic relationships among populations (through the action of drift), and their composition may help estimate the likelihood of origin from putative source populations.

Phylogenetic analysis of the sequences suggests a closer relationship of some Taino sequences with South American rather than with North American populations, even if the information is not conclusive. In this sense, it is interesting to note that none of the Caribbean sequences cluster in any of the four haplogroup C branches exclusively found in North America (Fig. 2, dashed line). However, the existence of gene flow from North America cannot be rejected using the present data, since most of the sequences found in the Caribbean are widely distributed along the American continent, or (quite the reverse) are only Caribbean-specific. There is not enough phylogeographic structure in the mtDNA in the Americas to indisputably solve the problem.

The low nucleotide diversity and low mean pairwise differences of the Tainos suggest the existence of one or more founder effects in the colonization of the Caribbean (Lalueza-Fox et al., 2001). Given that the colonization waves may have emanated from the same source population, it is not possible to discern purely from genetic grounds whether the hunter-gatherer ancestors of the Ciboneys and the agricul-

turalist Tainos originated from one or two expansion movements. Since the mean pairwise difference of the Ciboneys is slightly higher (3.47) than that of the Tainos (2.96), two population expansions may have taken place. A population expansion from the same geographic source seems to be suggested by the C haplogroup network, where some sequences belonging to Tainos and Ciboneys tend to cluster in related branches. However, different origins for Ciboneys and Tainos cannot be rejected using the available data: while the latter seem to be related to South America, data from the former do not give unambiguous results in the phylogenetic analysis. A problem always present in evolutionary genetics is the availability of ancestral populations from which derived populations are generated; usually, extant populations have to be considered as parental to ancient populations. If two of the populations geographically close to Cuba (the Seminoles from Florida, and the contemporary Mayas from Xcaret, Quintana Roo) are taken as putative representatives of the Caribbean source populations, there is a clear discrepancy in the haplogroup distribution that makes a Ciboney origin unlikely from those or other closely related populations.

Our preliminary hypothesis is that the colonization of the Caribbean was mainly due to successive migration movements from mainland South America in different time periods, possibly related to different archaeological horizons and ethnic groups. An earlier expansion associated with the movement of hunter-gatherer groups into the Caribbean (ancestors of the Ciboneys) would have been followed by the migration of agriculturalist groups (ancestors of the Tainos) into the Caribbean. It is likely that there was yet a later movement associated with Carib infusions into the Taino settlements, that was still in process at the arrival of the Europeans and for which there is no genetic information at present. It is possible that all the Caribbean groups described at Contact time by Spanish accounts (Ciboneys, Tainos, and Caribs) were, from a genetic point of view, closely related. However, gene flow from Central or even North America cannot be ruled out, due to the small sample size available, the limited phylogenetic resolution of the sequences, and the lack of information about the regional distribution of the mtDNA haplogroups within some of the regions involved, specially the southeast of North America and the Orinoco River Valley, and for contemporary ancient populations. Additional analyses of more Caribbean samples from other islands, both modern and ancient, as well as those continental regions mentioned, are therefore needed to help obtain a more precise picture of the colonization of this area.

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