Reconstructing the Population History of Puerto Rico by Means of mtDNA Phylogeographic Analysis

Juan C. Martínez-Cruzado,[1*] Gladys Toro-Labrador,[1] Jorge Viera-Vera,[1] Michelle Y. Rivera-Vega,[1,2] Jennifer Startek,[1] Magda Latorre-Esteves,[1,3] Alicia Román-Colón,[1,4] Rebecca Rivera-Torres,[1,5] Iris Y. Navarro-Millán,[1,2] Enid Gómez-Sánchez,[1] Héctor Y. Caro-González,[1,6] and Patricia Valencia-Rivera[1,7]

[1]*Department of Biology, University of Puerto Rico at Mayagüez, Mayagüez, Puerto Rico 00680*
[2]*Escuela de Medicina, Universidad Autónoma de Guadalajara, Guadalajara, Jalisco CP45110, Mexico*
[3]*Department of Pathology, Harvard Medical School, Harvard University, Boston, Massachusetts 02115*
[4]*Medical Sciences Campus, University of Puerto Rico, San Juan, Puerto Rico 00936*
[5]*Department of Comparative Pathology, University of California at Davis, Davis, California 95616*
[6]*Department of Biological Sciences, Stanford University, Stanford, California 94305*
[7]*Department of Cell Biology, Harvard Medical School, Harvard University, Boston, Massachusetts 02115*

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ABSTRACT The haplogroup identities of 800 mtDNAs randomly and systematically selected to be representative of the population of Puerto Rico were determined by restriction fragment length polymorphism (RFLP), revealing maternal ancestries in this highly mixed population of 61.3% Amerindian, 27.2% sub-Saharan African, and 11.5% West Eurasian. West Eurasian frequencies were low in all 28 municipalities sampled, and displayed no geographic patterns. Thus, a statistically significant negative correlation was observed between the Amerindian and African frequencies of the municipalities. In addition, a statistically highly significant geographic pattern was observed for Amerindian and African mtDNAs. In a scenario in which Amerindian mtDNAs prevailed on either side of longitude 66°16′ West, Amerindian mtDNAs were more frequent west of longitude 66°16′ West than east of it, and the opposite was true for African mtDNAs. Haplogroup A had the highest frequency among Amerindian samples (52.4%), suggesting its predominance among the native Taínos. Principal component analysis showed that the sub-Saharan African fraction had a strong affinity to West Africans. In addition, the magnitudes of the Senegambian and Gulf of Guinea components in Puerto Rico were between those of Cape Verde and São Tomé. Furthermore, the West Eurasian component did not conform to European haplogroup frequencies. HVR-I sequences of haplogroup U samples revealed a strong North African influence among West Eurasian mtDNAs and a new sub-Saharan African clade. Am J Phys Anthropol 128: 131–155, 2005.

Recent technical advances have facilitated the discovery of genetic polymorphisms in the human population, many of which are useful as markers for prehistoric migrations that gave rise to continental and regional populations. Continental-population histories were reconstructed using Y-chromosome markers, which are paternally inherited (Hurles et al., 1998; Rosser et al., 2000; Bamshad et al., 2001; Hammer et al., 2001; Karafet et al., 2001; Kayser et al., 2001; Malaspina et al., 2001; Underhill et al., 2001; Bortolini et al., 2002, 2003; Cruciani et al., 2002; Lell et al., 2002; Pereira et al., 2002; Semino et al., 2002; Zerjal et al., 2002, 2003; Zegura et al., 2004), and mtDNA markers, which are inherited maternally (Merriwether and Ferrell, 1996; Comas et al., 1998; Starikovskaya et al., 1998; Richards et al., 2000, 2002; Forster et al., 2001; Kaestle and Smith, 2001; Malhi et al., 2001; Torroni et al., 2001a,b; Keyeux et al., 2002; Oota et al., 2002; Salas et al., 2002; Schurr and Wallace, 2002; Yao et al., 2002a,b; Kong et al., 2003), usually finding remarkable differences in sex migration histories.

In this study, we developed a hierarchical strategy that makes use of haplogroup-defining mtDNA restriction markers to identify maternal biological ancestries in a sample set randomly and systematically selected to be representative of the Puerto Rico population, a mixed Caribbean population of three principal components: Amerindian, sub-Saharan

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*Correspondence to: Dr. Juan C. Martínez-Cruzado, Department of Biology, University of Puerto Rico at Mayagüez, PO Box 9012, Mayagüez, PR 00681-9012. E-mail: jumartin@uprm.edu

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African, and West Eurasian. With some notable exceptions, most haplogroups are regarded as continent-specific. Thus, determining the haplogroup to which a mtDNA belongs usually identifies the mtDNA biological ancestry. The HVR-I sequence was used when biological ancestry could not be determined through restriction marker analysis.

The biological ancestries of a mixed people have implications in their population genetics and thus in public health. In terms of mtDNA ancestry, studies of European and North American populations related particular West Eurasian haplogroups to higher frequencies of some diseases such as Alzheimer's (Hutchin and Cortopassi, 1995), Leber hereditary optic neuropathy (Johns and Berman, 1991; Brown et al., 1997; Hofmann et al., 1997; Lamminen et al., 1997; Torroni et al., 1997; Howell et al., 2003), Wolfram syndrome and sudden infant death syndrome (Hoffman et al., 1997), and some conditions such as asthenozoospermia and nonasthenozoospermia (Ruiz-Pesini et al., 2000). Further, it was shown that the +10394 *Dde*I state plays a protective role against Parkinson's disease, and that its effect is stronger when it is combined with other polymorphisms that are specific to haplogroups J and K (van der Walt et al., 2003). Thus, the characterization of the mtDNA pool of any population may be instrumental in determining risk factors for various diseases and conditions.

In addition, biological ancestries imply human migration routes that shed light on the possible origins of introduced fauna and flora, including agricultural varieties. Moreover, biological ancestries play a fundamental role in population history which, as one of the main categories of cultural history, is essential to explain the social systems and behavioral guidelines that rule all aspects of social life. Population history considers population growth in relation to geographic regions, biological ancestries, and admixture, and thus plays a central role in the cultural development of a people (Fernández-Méndez, 1970).

It is estimated that from 60,000–600,000 Arawak-speaking Taíno Indians lived in Puerto Rico when it was discovered for the Europeans by Christopher Columbus in 1493 (Abbad, 1959; Fernández-Méndez, 1970). Traditional history tells us that they were decimated by war, hunger, disease, and emigration, such that they had totally disappeared by the end of the 16th century. The vast majority of Spanish settlers were single men, and mixing with Indian women commenced fully upon colonization in 1506. The Spanish Crown took measures to increment the number of "white" people on the island, including ordering "white" Christian female slaves to be sent to Puerto Rico in 1512. However, the 1530 census reported that only 57 of the 369 "white" men on the island were married to "white" women. Such "whites" were a minority. The census reported 335 "black" female slaves and 1,168 "black" male slaves, and a total of 1,148 Indians, both genders included (Brau, 1904). By this time, the base of the Puerto Rican economy was shifting from gold mine exploitation to sugar cultivation. African slaves became the cornerstone of the sugar industry.

Traditional history includes abundant evidence of the widely dispersed geographic origins of the sub-Saharan African peoples who were brought to the Americas, spanning Cape Verde on the northwest edge of sub-Saharan Africa to Mozambique and the island of Madagascar in the southeast. The arrival in Puerto Rico of people from various African regions can be confirmed by traditional festivals and other activities held in the names of African gods and by the use of words that can be found only in particular African regions. However, the lack of a classification system for slaves by tribe or even by geographic region during the Atlantic slave trade leaves doubts concerning the relative contribution of the different continental regions (Álvarez-Nazario, 1974).

Slaves were first brought to Puerto Rico in 1508 by its conquistador, Juan Ponce de León. These were residents of the Iberian Peninsula, many of North African, Senegambian, or Guinean origin (Álvarez-Nazario, 1974); others were Greek, Slavic, or Turkish (Thomas, 1997), and others Jewish (Díaz-Soler, 2000). The capture of sub-Saharan Africans with the goal of providing Spanish and Portuguese colonies in the Americas with a labor force, first in the search for gold and later in sugar plantations, started in 1518 (Díaz-Soler, 2000). Up to the beginning of the second half of the 16th century, almost all slaves originated in Senegambia and Guinea (Alegría, 1985). The island of São Tomé, with slaves acquired mainly from the Gulf of Guinea, was an important supplier thereafter. Throughout the 16th century and with the exception of one in the west coast, all 13 sugar mills in Puerto Rico were east of the La Plata River, which streams along longitude 66°16′ West (Gelpí-Baíz, 2000).

The Portuguese were the legal source of African slaves until 1640, at which time Spain suspended all contracts in retaliation for the revolution that removed their Spanish rulers. The resulting shortage of slave labor provoked the collapse of the sugar industry, starting a period of subsistence economy that lasted for a century and a half until the Crown suspended all taxes and source restrictions on the slave trade in 1789. The poor state of the economy hindered the importation of slaves, and the tax collected upon their sale made the illegal trade their main source. The illegal slave trade was circuitous in that the main slave sources were the Dutch colony of Curaçao and the English colony of Jamaica, in that order. Slaves brought from the Gold Coast (Ghana) were the most common in these colonies at the time. The illegal Puerto Rican harbors were located on the west and south coasts, where most of the island population lived (Álvarez-Nazario, 1974). The only legal harbor was far away in San Juan, the capital, and few legal immigrants made it to Puerto Rico during these times.

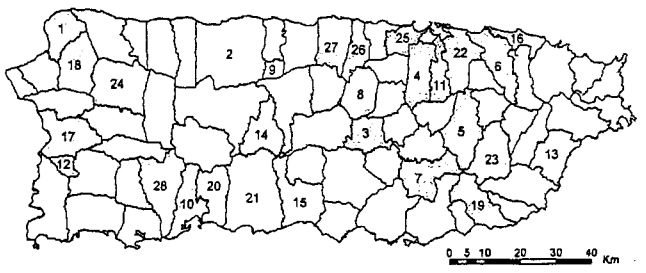
The importation of slaves increased dramatically as a consequence of the land and tax reforms of the last decades of the 18th century, and approximately two-thirds of all slaves ever brought to Puerto Rico arrived from that point in time until the abolition of slavery in 1873 (Álvarez-Nazario, 1974). By then, the African harbors most used by slave traders extended from the Gold Coast to Angola (Thomas, 1997).

This new wave of slaves found Puerto Rico mainly inhabited by *criollos*, Puerto Rico natives who were the product of centuries of admixture and generations living under a subsistence economy with little or no Spanish government intervention (Fernández-Méndez, 2000).

The Spanish Empire started to crumble at the beginning of the 19th century, and an 1815 royal decree permitted the settlement in Puerto Rico of foreign Catholics with their wealth and slaves. Thus, wealthy "white" refugees and other immigrants from Europe and the Americas made it to Puerto Rico in great numbers, stimulating the economy by developing the sugar industry in the coastal plains and the coffee and tobacco industries in the mountains. International treaties banned importation of slaves directly from Africa north of the Equator in 1817 and south of it in 1820. However, enforcement of the treaties was ineffective south of the Equator, where the Portuguese had bountiful slave factories. Thus, the illegal Angolan trade became substantial in the 19th century. Larger sources of Africans were probably the West Indies, because trade within the Caribbean was not banned and because escapees arriving to Puerto Rico were granted freedom. In this respect, migrations from the then Danish-ruled island of Saint Thomas, which acquired its slaves mainly from the Gold Coast to the Slave Coast in the Bight of Benin (Thomas, 1997), were a major source (Álvarez-Nazario, 1974).

Our results conform to most accounts of traditional history, but not at all with the extermination of the Taíno people as early as the 16th century, thus showing that population genetics has a lot to offer studies on Caribbean population history. It is important to note that neglected people rarely contribute to traditional history, and a great part of the cultural development of the Puerto Rican people occurred in the "darkness" of history, far away from the capital, as did the illegal trade that kept their subsistence economy alive.

In the interest of greater clarity, we often refer to Puerto Ricans carrying mtDNAs of Amerindian, African, or West Eurasian origin by such terms as Amerindians, Africans, and West Eurasians. However, it is important to keep in mind that we are referring to a thoroughly mixed population composed of people of a single culture and whose phenotypes do not predict individual mtDNA ancestries.



1- Aguadilla
2- Arecibo
3- Barranquitas
4- Bayamón
5- Caguas
6- Carolina
7- Cayey
9- Florida
10- Guayanilla
11- Guaynabo
12- Hormigueros
13- Humacao
14- Jayuya
15- Juana Díaz
16- Loíza
17- Mayagüez
18- Moca
19- Patillas
20- Peñuelas
21- Ponce
22- San Juan
23- San Lorenzo
24- San Sebastián
25- Toa Baja
26- Vega Alta
27- Vega Baja
28- Yauco

Fig. 1. Municipalities selected.

SUBJECTS, MATERIALS, AND METHODS

Subjects

A random sample of 872 housing units representative of the island of Puerto Rico was selected using a sampling frame developed by the Center for Applied Social Research (University of Puerto Rico at Mayagüez) for survey research in Puerto Rico, based on the 1990 Census of Population and Housing. Excluding the island municipalities of Vieques and Culebra from the sampling frame, 28 of the 76 municipalities in Puerto Rico were selected (Fig. 1), as per the following description. The eight most populated municipalities were selected with probability equal to one. Each was assigned a number of housing units proportional to its estimated population size, based on a total of 872 housing units for the entire island. To select the remaining 20 municipalities, the remainder of the island was divided into five geographical regions. Four municipalities from each region were selected at random with a probability proportional to estimated population size, while stratifying by estimated population size. They were assigned an equal number of housing units, proportional to the estimated population size of the geographic region they represented.

Thirty percent of the census tracts within each municipality were selected at random, with probability proportional to estimated population size. Having established an estimated number of housing units for each census tract based on the number of housing units for the municipality and the relative population sizes of the selected census tracts, census blocks were selected within them so that each would contribute an expected eight households to the sample. On the field, housing units were chosen by systematic sampling, with a preestablished random starting point for each block. This means that the actual number of housing units obtained from each block could be greater or smaller than initially expected, depending on how the number of housing units in it had changed since 1990. An adult was selected at random from each housing unit. Partici-

pation in the project was agreed to by appropriate informed consent.

DNA manipulation

Sample collection and DNA extraction were performed as in Martínez-Cruzado et al. (2001). Thereafter, a 200-μl aliquot from each 500-μl sample was purified, using the QIAamp DNA Mini Kit (Qiagen). To each aliquot, 36 μl of 60 mM Tris-HCl (pH 8.0), 60 mM Na_2EDTA (pH 8.0), 0.6 M NaCl, 0.24 mM DTT, and 12% SDS were added, followed by 250 μl of buffer AL and 250 μl of 100% ethanol. The aliquots were vortexed thoroughly, transferred to a spin column, and spun at 8,000 rpm for 1 min. The filter was washed by adding 300 μl of buffer AW1, spinning at 8,000 rpm for 1 min, adding 300 μl of buffer AW2, and spinning at 14,000 rpm for 5 min. DNA was eluted from the filter into two 100-μl aliquots. The eluate aliquots were kept at −80°C as backups until the end of the study.

Except for the cycling conditions (see below) and that 1.5 U of *Taq* DNA polymerase were used in each amplification reaction, the DNA amplification, restriction digestion, and agarose gel electrophoresis procedures were performed as in Martínez-Cruzado et al. (2001). The amplification reactions were usually subjected to one cycle of 2.5 min at 94°C, 32 cycles of 30 sec at 94°C, 1 min at 54°C, and 70 sec at 72°C, and one cycle of 10 min at 72°C. Primer annealing was achieved at 52°C to amplify the diagnostic site for macroparagroup L, and at 56°C to amplify the sites diagnostic for haplogroups G and L3d.

Haplogroup identification strategy and quality-control estimates

Studies involving high-resolution restriction analysis (Ballinger et al., 1992; Torroni et al., 1992, 1993a,b, 1994a–d, 1996, 1997; Chen et al., 1995, 2000), analyses of the complete sequence of mitochondrial chromosomes (Kong et al., 2003; Reidla et al., 2003), or complete (Herrnstadt et al., 2002) or partial (Silva et al., 2002) sequences of their coding region showed that all haplogroups are virtually monomorphic for the 10,394 *Dde*I and 10,397 *Alu*I sites, with the exception of haplogroup K. Thus, the a priori determination of the state of these sites quickly reduces the number of candidate haplogroups to which an unknown mtDNA may belong. Because these sites are close to each other, the 10394 *Dde*I/10397 *Alu*I motif (hereafter referred to as *the motif*) can be easily determined from a single amplicon.

Thus, each mtDNA sample was first tested for its motif. Depending on the result, each sample was then tested for the markers diagnostic for all haplogroups known to share its motif. The haplogroups, their motifs, their defining markers, and the primers used are shown in Table 1. Haplogroups that are defined by two or more markers invariably share at least one of them with some other haplogroup. Thus, tests on unshared haplogroup markers were performed only when the samples showed the shared ones. The two markers that define haplogroup L1b were tested on all (+/−) motif samples, as each by itself defines another (+/−) motif haplogroup. L is a macroparagroup, a large group of mtDNAs including several haplogroups and other paraphyletic mtDNAs (Chen et al., 1995; Salas et al., 2002). Among others, it includes haplogroup L2 (here further subdivided into L2a and L2* to pool subhaplogroups L2b, L2c, and L2d) and subhaplogroups L1b and L1c. All other L haplogroups and paraphyletic mtDNAs were included in paragroup L0 (Mishmar et al., 2003).

The testing of markers for all haplogroups within each motif group served as a quality-control measure, as it allowed us to detect false positives. In the few instances in which the mtDNA tested positive for no haplogroup-defining markers, its identity was determined by the sequence of its HVR-I and confirmed by restriction analysis. Thus, false negatives were also detected, and the likelihood of any error involving false haplogroup positives, false haplogroup negatives, or motif group misdiagnoses could be estimated experimentally. Such estimates were used to calculate the probabilities of any number of samples being misdiagnosed. Because all tests were performed independently, the likelihood that any two errors were committed in analyzing the same sample could be calculated based on the multiplicative rule of probability.

Amplicons to be sequenced were purified using the High Pure PCR Product Purification Kit (Roche Applied Science), as instructed by the manufacturer. Automated sequencing was performed at the New Jersey Medical School Molecular Resource Facility (University of Medicine and Dentistry of New Jersey), using an Applied Biosystems (ABI) model 3100 capillary sequencer after cycle sequencing with Dye Terminator mix version 2.0.

Biological ancestry determination and data analysis

Biological ancestries were inferred from haplogroup identity. Because only nine women of Asian ancestry were reported living in Puerto Rico in 1899 (Sanger et al., 1900), mtDNAs of haplogroups belonging to both the New World and Asia were assumed to be of Amerindian origin unless participant interviews revealed otherwise.

Data analysis was performed using the program SPSS 10.0.5 for Windows. To determine whether variation in participation rates or changes in population size occurring since 1990 in the sampled municipalities would lead to biased estimates of the parameters, we devised a weighting scheme. Through these weights, the number of samples provided by each municipality was adjusted so that it would be equal to the number expected by applying the original sampling proportions to the final sample

TABLE 1. Haplogroups with their motifs and defining markers, and primers used for their identification[1]

Motif[2]	Haplogroup	Defining markers	Primers[3]	Motif	Haplogroup	Defining markers	Primers
			L612 (22)				L4462 (21)
(−/−)	A	+663 *Hae*III	H742 (23)	(+/−)	I	−4529 *Hae*II	H4620 (19)
			L8216 (20)				L4160 (19)
(−/−)	B	9-bp del regV	H8296 (20)	(+/−)	J	+4216 *Nla*III	H4291 (21)
			L12237 (21)			+12308 *Hin*fI	L8921 (19)
(−/−)	F	−12406 *Hpa*I	H12485 (20)	(+/−)	K	−9052 *Hae*II	H9086 (21)
			L14711 (20)				L3517 (19)
(−/−)	HV	−14766 *Mse*I	H14885 (19)	(+/−)	L	+3592 *Hpa*I	H3667 (21)
		−14766 *Mse*I	L6958 (20)			+3592 *Hpa*I	As in L and L3e
(−/−)	H	−7025 *Alu*I	H7104 (19)	(+/−)	L1b	+2349 *Dpn*II	
		−14766 *Mse*I	L4462 (21)			+3592 *Hpa*I	L8921 (19)
(−/−)	V	−4577 *Nla*III	H4620 (19)	(+/−)	L1c	+9070 *Taq*I	H9086 (21)
			L4160 (19)			+3592 *Hpa*I	L16348 (21)
(−/−)	T	+4216 *Nla*III	H4291 (21)	(+/−)	L2*	+16389 *Hin*fI	H16401 (20)
			L12265 (21)			As in L2* plus	L13643 (19)
(−/−)	U	+12308 *Hin*fI	H12308[4] (23)	(+/−)	L2a	+13803 *Hae*III	H113809 (19)
		+12308 *Hin*fI	L8921 (19)				L9963 (21)
(−/−)	K	−9052 *Hae*II	H9086 (21)	(+/−)	L3b	+10084 *Taq*I	H10127 (19)
			L8216 (20)				L8558 (20)
(−/−)	W	+8249 *Ava*II	H8296 (20)	(+/−)	L3d	−8616 *Dpn*II	H8657 (20)
			L14390 (19)				L2272 (19)
(−/−)	X	+14465 *Acc*I	H14559 (19)	(+/−)	L3e	+2349 *Dpn*II	H2420 (19)
			L13233 (24)				L5121 (20)
(+/+)	C	+13262 *Alu*I	H13392 (24)	(+/+)	D	−5176 *Alu*I	H5229 (20)
			L7492 (20)				L4754 (22)
(+/+)	E	−7598 *Hha*I	H7641 (22)	(+/+)	G	+4831 *Hha*I	H4919 (19)

[1] L3* (+/−), M (+/+), N(−/−), (pre-HV)1 (−/−), and JT (−/−) have no defining marker.
[2] Refers to state of 10394 *Dde*I/10397 *Alu*I sites.
[3] Letters "L" and "H" denote extended mtDNA strand, light and heavy, respectively. Numbers indicate first nucleotide to be extended from primers, based on Cambridge reference sequence (CRS) (Anderson et al., 1981). Numbers in parentheses indicate primer length. When haplogroup is defined by more than one marker, primers shown belong to last marker used in procedure.
[4] H12308 is a mismatched primer with a G at position 12312, thus generating a *Hin*fI site when transition at position 12308 that characterizes haplogroup U is present.

size. The weights for municipality samples (W_m) were a function of the sampling proportion of the municipality (P_m), the final total obtained sample size (n), and the number of samples provided by the municipality (n_m), so that

$$W_m = (P_m \times n)/n_m.$$

A triangular graphic of ancestry distribution among municipalities was constructed using MATLAB. A projected plane representing a linear function of form w = f(X, Y, Z), in which plotted population dots were defined as the end of vectors with form w = Xi + Yj + Zk, where X, Y, and Z represented Amerindian, African, and West Eurasian frequencies, respectively, was produced. The sum of X, Y, and Z was equal to one. Their magnitudes were a function of the 30° and 60° angles. Vectors i, j, and k were their respective unit vectors in the positive directions of the coordinate axes x, y, and z.

To illustrate the geographic distribution of Amerindian mtDNA frequencies, municipalities were listed in order according to such frequencies and divided into 12 categories by creating a new category every time that the difference between two municipalities was 1.6% or more. Divisions were drawn halfway between the frequencies of such municipalities.

Principal component (PC) analyses were performed using the POPSTR program of Henry Harpending (University of Utah). They were based on population haplogroup frequencies, and included only populations with 17 samples or more. Sub-Saharan African mtDNAs were classified as follows. Macroparagroup L was divided into haplogroup L2 (further subdivided into L2a and L2*), subhaplogroups L1b and L1c, and paragroup L0 to pool all other haplogroups and paraphyletic mtDNAs within the macroparagroup. Paragroup L3A (Salas et al., 2002) was divided into L3b, L3d, L3e, L3f, L3g, and L3*. We designated U5b2 as a sub-Saharan African clade with the HVR-I sequence 16189-16192-16270-16320. Taken from one source were Shona (n = 17), Tongas (20), Shangaan (22), Chopi (27), Chwabo (20), Lomwe (20), Makonde (19), Makhwa (20), Ndau (19), Nyungwe (20), Nyanja (20), Ronga (21), Sena (21), and Tswa (19) from Mozambique (Salas et al., 2002), Brazil (65) (Alves-Silva et al., 2000), Bubi (45), São Tomé (49) (Mateu et al., 1997), Mandenka (118) (Graven et al., 1995), Serer (23), a group of other Senegalese tribes (48), a pool of Mauritanian and West Saharan tribes (24) (Rando et al., 1998), Tuareg (22), Yoruba (33), Hausa (20), Fulbe (60), Turkana (37), Somalia (27), Kikuyu (22) (Watson et al., 1997), Nubia (46) (Krings et al., 1999), Khwe (31) (Chen et al., 2000), and the southeastern islands of the Cape Verde Archipelago (169) (Brehm et al., 2002). From two sources were Biaka (34) and Mbuti (35) Pygmies (Chen et al., 1995; Watson et al.,

1997), Wolof (66) (Chen et al., 1995; Rando et al., 1998), and !Kung (62) (Watson et al., 1997; Chen et al., 2000). For West Eurasians, mtDNAs were classified as belonging to H, V, HV, (pre-HV)1, J, T, I, W, X, M, N, R, K, U*, U2, U5*, U5(a + b), U6, and U(others) to pool the remaining clades (U1, U3, U4, and U7). Populations were obtained from Rando et al. (1998) (23 Moroccan non-Berbers and 58 Moroccan Berbers), Brakez et al. (2001) (37 Moroccan Souss Valley inhabitants), and Richards et al. (2000). This last group of authors compiled data from several authors concerning 13 populations from North Africa and the Near East, as well as several populations from Europe. They classified the European populations into 10 geographic regions, and we observe those same classifications here. Amerindian populations were divided into 12 geographic regions and "Others." These were three from eastern North America (Mohawk (123) (Merriwether and Ferrell, 1996) and Ojibwa from Manitoulin Island (33) and northern Ontario (28) (Scozzari et al., 1997)), five from the Great Plains (Cheyenne/Arapaho (35), Sisseton/Wapheton Sioux (45), Turtle Mountain Chippewa (28) and Wisconsin Chippewa (62) (Malhi et al., 2001), and Siouan (34) (Lorenz and Smith, 1996)), six from the North American Southeast (Choctaw (27) (Lorenz and Smith, 1996), Creek (39) and Seminole (40) (Weiss and Smith, 2003), Oklahoma Muskoke (70) (Merriwether and Ferrell, 1996), and Oklahoma Red Cross Cherokee (19) and Stillwell Cherokee (37) (Malhi et al., 2001)), 15 from the North American Southwest (Akimal O'odham (43), Apache (38), Delta Yuman (23), Navajo (64), North Paiute/Shoshoni (94), Pai Yuman (27), River Yuman (22), Tauno O'odham (37), Zuni (26) (Malhi et al., 2003), California Penutian (17), Havasupai/Hualapai/Yavapai/Mojave (18), Jemez (36), Pima (37), Quechuan/Cocopa (23), and Washo (28) (Lorenz and Smith, 1996)), four from Mesoamerica (Maya (26), Mixtec (29), Nahua/Cora (32) (Lorenz and Smith, 1996), and North Central Mexico (199) (Green et al., 2000)), eight from eastern Central America (Bribri-Cabecar (24) (Torroni et al., 1993a), Emberá (Panamá) (44), Wounan (31) (Kolman and Bermingham, 1997), Guatuso (20), Teribe (20) (Torroni et al., 1994d), Huetar (27) (Santos et al., 1994), Kuna (63) (Batista et al., 1995), and Ngöbé (46) (Kolman et al., 1995)), 14 from western Colombia and Ecuador including the Andes (Cayapa (94) (Rickards et al., 1999), Chimila (34), Guambiano (23), Guane-Butaregua (33), Ijka-Arhuaco (40), Kogui (30), Paez (31), Tule-Cuna (29), Waunana (30), Yuco-Yukpa (88) (Keyeux et al., 2002), Emberá (Colombia) (41), Ingano (52), Wayuu (59), and Zenu (69) (Mesa et al., 2000; Keyeux et al., 2002)), nine from Colombia east of the Andes (Coreguaje (19), Curripaco (17), Guahibo-Sikuani (23), Guayabero (24), Huitoto (22), Murui-Muinane (18), Nukak (20), Piaroa (18) (Keyeux et al., 2002), and Tucano (71) (Mesa et al., 2000; Keyeux et al., 2002)), seven from the Amazon (Belén (Brazil) (81) (Batista dos Santos et al., 1999), Brazilian North (26) (Alves-Silva et al., 2000), Gaviao (27), Xavante (25), Zoró (30) (Ward et al., 1996), Ticuna (28) (Torroni et al., 1993a), and Yanomami (97) (Merriwether and Ferrell, 1996)), nine from the Peruvian, Bolivian, and Chilean highlands around Lake Titicaca (Atacameño (50) (Merriwether et al., 1995), Chimane (40), Ignaciano (21), Mosetén (19), Movima (22), Trinitario (33), Yuracaré (27) (Bert et al., 2001), Aymara (98), and Quechua (51) (Merriwether and Ferrell, 1996; Bert et al., 2001)), six from northern Argentina (Mataco from the provinces of Chaco (28), Formosa (44), and Salta (55), Pilaga (40), and Toba from the provinces of Chaco (28) and Formosa (26) (Demarchi et al., 2001)), and five from southern South America (Huilliche (89) (Merriwether and Ferrell, 1996), Mapuche-Argentina (50) (Bailliet et al., 1994), Mapuche-Chile (156) (Merriwether et al., 1995; Moraga et al., 2000), Pehuenche (204) (Merriwether and Ferrell, 1996; Moraga et al., 2000), and Yaghan (21) (Moraga et al., 2000)). Two "Other" populations were Bella Coola (36) (Lorenz and Smith, 1996) and Brazilian Southeast (33) (Alves-Silva et al., 2000).

Haplogroup diversity for the Amerindian mtDNAs was calculated using the method of Tajima (1989), $h = [1 - \Sigma x_i^2]n/(n - 1)$, where x_i is the frequency of each haplogroup and n is the sample size.

RESULTS

Response rate

All selected housing units were identified between August 6, 1999–March 19, 2000. Based on the 1990 Census of Population and Housing, a total of 872 housing units was selected. This translated into 1,067 because of housing growth through the decade. Eighty-one housing units were uninhabited. From the 986 remaining housing units, 876 selected individuals were contacted. Exactly 800 of these agreed to participate, for a response rate of 81.1% based on the 986 selected individuals. The sampling procedure results for each municipality and region are detailed in Table 2.

Haplogroup identification data quality

The haplogroup identification strategy described above allowed the detection of misdiagnoses of both motif and haplogroup-defining marker identities, and thus an estimation of the probability that any misdiagnoses may have gone undetected. The largest margin of error lies within the (+/−) motif group. Initially, all (+/−) samples were tested, among others, for the +3592 *Hpa*I and +2349 *Dpn*II markers but not for markers +9070 *Taq*I and +16389 *Hin*fI, which are necessary to discriminate L1c and L2, respectively, from all other mtDNAs within L (Table 1). Thus, the samples belonging to L1b (+3592 *Hpa*I/+2349 *Dpn*II) and L3e (−3592 *Hpa*I/+2349 *Dpn*II) were quickly identified, while the samples with the +3592 *Hpa*I/−2349 *Dpn*II profile had to be subjected to a second round of tests for

TABLE 2. Sampling procedure results categorized by region

Region	Municipality	Number of housing units	Uninhabited housing units	Inhabited housing units	Agreed to participate	Selected, not contacted	Declined to participate
Metro	Arecibo	33	3	30	26 (86.7%)	2 (6.7%)	2 (6.7%)
	Bayamón	60	7	53	43 (81.1%)	6 (11.3%)	4 (7.5%)
	Caguas	40	3	37	30 (81.1%)	7 (18.9%)	0
	Carolina	51	3	48	39 (81.3%)	4 (8.3%)	5 (10.4%)
	Guaynabo	23	2	21	16 (76.2%)	5 (23.8%)	0
	Mayagüez	33	3	30	26 (86.7%)	0	4 (13.3%)
	Ponce	37	3	34	27 (79.4%)	0	7 (20.6%)
	San Juan	118	5	113	78 (69.0%)	22 (19.5%)	13 (11.5%)
	Subtotal	395	29	366	285 (77.9%)	46 (12.6%)	35 (9.6%)
North	Florida	39	6	33	29 (87.9%)	4 (12.1%)	0
	Toa Baja	28	1	27	22 (81.5%)	1 (3.7%)	4 (14.8%)
	Vega Alta	50	3	47	38 (80.9%)	4 (8.5%)	5 (10.6%)
	Vega Baja	41	6	35	25 (71.4%)	7 (20.0%)	3 (8.6%)
	Subtotal	158	14	142	114 (80.3%)	16 (11.3%)	12 (8.5%)
East	Humacao	72	4	68	51 (75.0%)	11 (16.2%)	6 (8.8%)
	Loíza	46	1	45	37 (82.2%)	4 (8.9%)	4 (8.9%)
	Patillas	26	2	24	21 (87.5%)	1 (4.2%)	2 (8.3%)
	San Lorenzo	43	3	40	31 (77.5%)	7 (17.5%)	2 (5.0%)
	Subtotal	187	10	177	140 (79.1%)	23 (13.0%)	14 (7.9%)
South	Guayanilla	24	6	18	17 (94.4%)	0	1 (5.6%)
	Juana Díaz	23	1	22	19 (86.4%)	2 (9.1%)	1 (4.5%)
	Peñuelas	13	4	9	9 (100%)	0	0
	Yauco	27	2	25	22 (88.0%)	0	3 (12.0%)
	Subtotal	87	13	74	67 (90.5%)	2 (2.7%)	5 (6.8%)
West	Aguadilla	26	2	24	23 (95.8%)	1 (4.2%)	0
	Hormigueros	33	1	32	28 (87.5%)	2 (6.3%)	2 (6.3%)
	Moca	27	2	25	23 (92.0%)	0	2 (8.0%)
	San Sebastián	29	2	27	23 (85.2%)	1 (3.7%)	3 (11.1%)
	Subtotal	115	7	108	97 (89.8%)	4 (3.7%)	7 (6.5%)
Central	Barranquitas	38	2	36	30 (83.3%)	5 (13.9%)	1 (2.8%)
	Cayey	31	1	30	22 (73.3%)	7 (23.3%)	1 (3.3%)
	Corozal	29	1	28	23 (82.1%)	4 (14.3%)	1 (3.6%)
	Jayuya	27	2	25	22 (88.0%)	3 (12.0%)	0
	Subtotal	125	6	119	97 (81.5%)	19 (16.0%)	3 (2.5%)
Total	Total	1,067	81	986	800 (81.1%)	110 (11.2%)	76 (7.7%)

markers +9070 *Taq*I and +16389 *Hin*fI. HVR-I sequencing of those samples with no haplogroup-defining markers showed that the +3592 *Hpa*I motif of one of the 79 samples with the +3592 *Hpa*I/−2349 *Dpn*II profile initially went undetected. This gave us an experimental estimate of 1/79 for the frequency with which the +3592 *Hpa*I motif went undetected. Using such a frequency and a base of 49 L1b samples, we calculated a probability of 53.6% that none of the 38 samples identified as belonging to haplogroup L3e (−3592 *Hpa*I/+2349 *Dpn*II) may actually belong to L1b (+3592 *Hpa*I/+2349 *Dpn*II). Using bases of 50, 51, and 52 L1b samples, we calculated probabilities of 33.9%, 10.9%, and 2.4% that one, two, or three samples identified as L3e actually belong to L1b.

There are two other scenarios by which misdiagnoses could occur. One is the combination of a misdiagnosis of the sample motif group with a false positive for a haplogroup-defining marker. The other is the occurrence of both a false negative and a false positive for haplogroup-defining markers with the same sample. Based on the detection of 11 motif misdiagnoses (three samples misdiagnosed as (−/−), seven as (+/−), and one as (+/+)), six false positives (one each for the markers corresponding to A, D, HV, L, L3b, and J/T), 11 false negatives (three each for the markers of A and J/T, two for that of C, and one each for those of HV, L and L3b), the number of samples belonging to each motif group (377 (−/−), 233 (+/−), and 190 (+/+)), and the number of samples belonging to each haplogroup (Table 3), we estimate that the probability that no misdiagnoses were made under either of these two scenarios is 86.0%, and that the probability that two or more misdiagnoses were made is insignificant.

Haplogroup identities

Table 3 shows the distribution by municipality of all haplogroups found, their frequencies, and their biological origin. Only six of the 800 samples were confirmed, through HVR-I sequencing, as having 10394 *Dde*I/10397 *Alu*I motifs different from those corresponding to their haplogroups (Table 1). Specifically, we found two L3e, one L2*, one L2a, and one L1c sample to have (−/−) instead of (+/−) motifs. In addition, one haplogroup C sample had a (−/−) motif instead of the (+/+) expected.

On six occasions, samples were confirmed as having more than one haplogroup-defining marker. The −8616 *Dpn*II marker that characterizes haplogroup L3d was found in one L0 and one L1c sample. Furthermore, two L1b samples had the +4216 *Nla*III marker that characterizes haplogroups J and T. These were all regarded as belonging to macroparagroup L because of the known stability of the

TABLE 3. Haplogroup distribution by municipality

	Haplogroups																						
	Amerindian				Sub-Saharan African									West Eurasian									
Municipality	A	B	C	D	L0	L1b	L1c	L2*	L2a	L3*	L3b	L3d	L3e	U[1]	H	J	K	T	U	V	HV	R	Pre-HV	Totals
Aguadilla	6	4	7						1	1	1	2									1			23
Arecibo	10	1	8	1					3							1		1				1		26
Barranquitas	12		5	3		1		1				1	2		1	3			1					30
Bayamón	13	1	10	2	1	2	3[2]		1			1	2[3]	1	3	1			1		1			43
Caguas	11	1	5		1	1			1	1			4		4				1					30
Carolina	9	2	6	2		1	2		1	2	3	2	3		1	1	2		1				1	39
Cayey	7		4			2		3[3]	1				4	1										22
Corozal	7		9			3			1			1				1			1					23
Florida	9	1	9	1	1	1		1			2			1	2			1						29
Guayanilla	3		5	2	2		1							1	1[4]	1		1						17
Guaynabo	4	4	2			2	1		1				1			1								16
Hormigueros	11	2	8[3]		1	2	1								1	2								28
Humacao	15	1[5]	10	3		5	4[3]	3		1	1		1		4	1			1		1			51
Jayuya	8	3	4			2			1			1		1	1		1							22
Juana Diaz	11		6[6]			1	1																	19
Loiza	5		2		3	7	3	3	8[3]			5							1					37
Mayagüez	10	3	6					2	1				2				1		1					26
Moca	8	2	8[7]								1		1		1	2								23
Patillas	9	2	2							1	2	1	1		1	1			1					21
Peñuelas	3		2					1	1		1		1											9
Ponce	13	2	6	1		1			1						1	1	1							27
San Juan	16	3	14		2[2]	10[8]	2		4	3		2	6[3]	2	4		2	4	3	1				78
San Lorenzo	7	1	7	1		5		1	1		1	1	1		1	3			1					31
San Sebastián	10	2	3			2						2	1		1	1			1					23
Toa Baja	6	1	6		1	1			1			2	1		1	1			1					22
Vega Alta	13	2	9	1			3	1			2		3	2		1		1						38
Vega Baja	12	2	7						1		1				1				1					25
Yauco	8	2	4				1						4			1			2					22
Totals	256	42	174	17	12	49	22	16	29	9	15	21	38	9	29	23	7	8	18	1	3	1	1	800

[1] Corresponds to sub-Saharan African clade U5b2 (see text).
[2] Two samples (one each from Bayamón and San Juan) showed markers defining haplogroup L3d and macroparagroup L. These were regarded as belonging to macroparagroup L.
[3] Six samples (two belonging to haplogroup L3e and one each belonging to C, L1c, L2*, and L2a) contained (−/−) 10394 *Dde*I/10397 *Alu*I motifs instead of those corresponding to their respective haplogroups.
[4] One sample exhibited markers defining haplogroups H and B. Its HVR-I sequence showed that it belongs to haplogroup H.
[5] Humacao participant belonging to haplogroup B was of Chinese maternal ancestry.
[6] One Juana Diaz sample exhibited markers defining haplogroups C and E. Its HVR-I sequence showed that it belongs to haplogroup C.
[7] One Moca sample with (+/+) motif did not exhibit any haplogroup-defining marker, but its HVR-1 sequence revealed that it belongs to haplogroup C.
[8] Two San Juan samples exhibited marker defining haplogroups J and T in addition to those defining haplogroup L1b. They were regarded as belonging to haplogroup L1b.

TABLE 4. Haplogroup U HVR-I sequence types

Clade	Sequence type[1]	*n* (27)	Min reg cov[2]	Most likely origin
U6b	163, 172, 219, 311	2	081–526	Canary Islands
U6a	172, 189, 219, 278	1	161–526	North Africa
U5b2	189, 192, 270, 320	9	154–379	Sub-Saharan Africa
U5b*	189, 270	1	184–391	Europe
U5b*	189, 270, 311	1	184–394	Europe
U2[3]	189, 362	2	184–391	Europe or Near East
U*	192	1	154–394	Europe
U5*	224, 261, 270	1	154–391	North Africa
U5*	224, 270	7	154–382	North Africa
U5ala	256, 270	2	154–392	Europe

[1] Numbers represent positions at which transitions occur. They correspond to CRS (Anderson et al., 1981) minus 16000. Transitions at 16519 hypermutable site are ignored.
[2] Minimum region covered with confidence in all samples in each sequence type.
[3] Their belonging to haplogroup U2 is likely but not definite (see text).

L-defining +3592 *Hpa*I marker. The true haplogroup identities of the remaining two samples were determined from their HVR-I sequences. One haplogroup H sample had the 9-bp deletion between the $tRNA^{Lys}$ and COII genes that characterizes haplogroup B, and one haplogroup C sample had the −7598 *Hha*I mutation that characterizes Asian haplogroup E. Their respective HVR-I sequences were 16093-16362 and 16221-16223-16261-16298-16325-16327. Thus, the first lacked the transitions at positions 16189 and 16217 that characterize haplogroup B (Ginther et al., 1993; Horai et al., 1993), and the second possessed the haplogroup C-specific transitions at positions 16298 and 16327 as well as the Amerindian-specific transition at 16325 (Torroni et al., 1993b).

Samples that did not test positive for any haplogroup-defining marker were identified by sequencing their HVR-I as well as specific sites in their coding regions. Nine (+/−) mtDNAs were classified as L3* for having transitions at sites 10873 and 12705. The HVR-I sequences of two (−/−) mtDNAs not having transitions at 10873 or 12705 were 16288-16311 and 16126-16189-16362. The first (−/−) mtDNA had a transition at site 11719 but not at 16223, and was thus classified as belonging to R. The transitions at sites 16126 and 16362 showed that the second (−/−) mtDNA belonged to JT or (pre-HV)1 (Macaulay et al., 1999). The absence of a transition at 11719 showed that it belonged to (pre-HV)1 (Richards et al., 2000). Finally, the HVR-I sequence of one (+/+) sample that did not exhibit any haplogroup-defining marker was 16086-16183-16189-16223-16278-16298-16325-16327. Thus, it contained the 16223, 16298, 16325, and 16327 transitions specific for Native American haplogroup C, and transitions 16183, 16189, 16223, and 16278, which are found in most haplogroup X mtDNAs (Brown et al., 1998). However, it possessed the +10397 *Alu*I motif specific of macrohaplogroup M, to which haplogroup C but not haplogroup X belongs. This motif was shown to be very stable (Kivisild et al., 2002; Kong et al., 2003), and we thus regarded this mtDNA as belonging to haplogroup C. One haplogroup C mtDNA lacking the +13262 *Alu*I marker was previously described from the Amazonian Makiritare (Torroni et al., 1993a), and (+/+) mtDNAs lacking defining markers for haplogroups C and D seem to be common in Colombia (Keyeux et al., 2002; Rodas et al., 2003). No mtDNAs belonging to haplogroups E, F, G, I, M, N, JT, W, or X were found in our set of 800 samples.

Haplogroup U subdivisions

Among all haplogroups found here, U is the only one that was reported in significant numbers in more than one continental region (Torroni et al., 1996). It was thus necessary to study such mtDNAs in more detail to identify their biological origin. The HVR-I sequence of the 27 samples belonging to haplogroup U segregates them into 10 types (Table 4). Although haplogroup U is mostly regarded as a West Eurasian haplogroup, it is apparent that nine of these samples originate from sub-Saharan Africa. All share the same sequence type, which has not been found in Europe or the Near East despite the thousands of samples from these areas for which HVR-I was sequenced (Alves-Silva et al., 2000; Richards et al., 2000; Finnilä et al., 2001; Malyarchuk et al., 2002). However, it was found in one out of 60 Fulbe sequences (Watson et al., 1997), and in one of 38 and 23 Wolof and Serer sequences, respectively (Rando et al., 1998). We classify it as a member of clade U5b* because of its 16189, 16192, and 16270 motif (Richards et al., 2000). Its distinction is the addition of a transition at position 16320. We designate it as clade U5b2 to represent a sub-Saharan African clade with a transition at 16320 as its signature.

Eleven samples seem to originate from North Africa and the Canary Islands. Two samples sharing the same sequence exhibit the 16163 motif, which is diagnostic for the Native Canarian-specific clade U6b (Rando et al., 1999). Nine samples segregate into three North African sequence types. The most common type (16224-16270), comprising seven samples, may correspond to the 16093-16224-16270 type of apparently North African ancestry that was found in two Canarian Islands (Pinto et al., 1996; Rando et al., 1999), because our sequencing reactions did not

TABLE 5. L3 HVR-I sequence types*

Clade	Sequence type[1]	*n* (9)	Min reg cov[2]
L3g	15924, 114, 223, 293T, 311, 316, 355, 362, 399	1	15877–16400
L3*	086, 126, 179, 215, 223, 256A, 284, 311	2	15879–16395
L3*	129, 172, 174, 192, 218, 223, 256A, 311, 362	1	15860–16524
L3f1	129, 209, 223, 286, 292, 295, 311	1	15882–16395
L3*	129, 223, 256A, 311, 362	1	15860–16523
L3f1	145, 209, 223, 292, 295, 311	1	15882–16394
L3f1	209, 223, 274, 292, 311	1	15882–16384
L3*	223, 311, 362	1	16026–16400

[1] Numbers represent sites at which base substitutions occur. They correspond to CRS (Anderson et al., 1981) minus 16000 if only three digits are shown. Transversions relative to CRS are indicated by showing base identity after site number. Transitions at 16519 hypermutable site are ignored.
[2] Minimum region covered with confidence in all samples in each sequence type.

extend to the left of the 16154 site in these samples. No other mtDNAs have been found with the 16093-16224-16270 or the 16224-16270 sequence types elsewhere. Of the two remaining North African sequence types, one (16224-16261-16270) may have derived directly from the most common type, as it differs from it at only one site. The remaining one has been found mainly in North Africa, but also in the Near East and sub-Saharan Africa. Its highest frequency was reported in the Berber-speaking Mozabites of northern Algeria: 10 out of 85 samples (Côrte-Real et al., 1996). Other populations with lower frequencies are Moroccan Berbers and non-Berbers (Pinto et al., 1996; Rando et al., 1998), Egyptians (Krings et al., 1999), Syrians (Richards et al., 2000), and some East and West African tribes (Watson et al., 1997). It was also found in two of 54 samples from Portugal (Côrte-Real et al., 1996), but we believe its presence in the Iberian Peninsula is due to migrations related to the slave trade.

Two samples share the motif 16189-16362. They likely belong to the U2 clade, which is characterized by the 16051 motif (Kivisild et al., 1999; Macaulay et al., 1999), a site to which our sequencing reaction did not extend. However, most West Eurasian U2 mtDNAs, but not other haplogroup U clades, present substitutions at positions 16129 and 16362. Since these samples do not present motifs that would classify them under any other clade, but possess the 16362 transition, they likely belong to clade U2. Clade U2 is virtually absent in North Africa and is found in the Near East at somewhat higher frequencies than in Europe. However, the precise sequence type is found at a higher frequency in the Iberian Peninsula than in any Near Eastern population except the Kurdish (Richards et al., 2000). PC analysis does not assign the Puerto Rican West Eurasian population a decisively higher affinity to the Kurdish or the European Mediterranean Western Region population (see below). Thus, we can only conclude that these samples should originate either in the Iberian Peninsula or the Near East.

The remaining four sequence types, encompassing only five samples, are likely of European origin. One differs from the CRS only by a transition at position 16192. Although the exact sequence type has not been reported elsewhere, it is regarded as European in origin because of the instability of the 16192 site in haplogroup U mtDNAs and the fairly high frequency of the otherwise resulting sequence type (CRS) in the Iberian Peninsula. The remaining three European sequence types are either particular U5b* types common only throughout Europe or belong to subclade U5a1a, which evolved in Europe (Richards et al., 2000).

L3* subdivisions

The HVR-I sequences of the nine (+/−) samples for which no haplogroup-specific markers were found are shown in Table 5. They segregate into eight sequence types sharing the 16223-16311 motif. Three of the sequence types possess the 16209 transition diagnostic of the L3f clade and the 16292 transition of subclade L3f1 (Salas et al., 2002). Only these three sequences showed a 1-bp deletion in the 5-bp T-stretch that runs from 15940–15944 in the CRS. This deletion is not the result of errors in the CRS (Andrews et al., 1999); it may play a significant role in RNA translation efficiency, as it makes the T*C arm loop of the $tRNA^{Thr}$ only two nucleotides long, and may become a useful phylogenetic marker for group L3* clades or to further subdivide subclade L3f1.

Another sequence type contains the 16293T-16355-16362 motif of clade L3g. The four remaining sequence types encompass five samples and cannot be grouped into any L3* clade. These thus remain classified as L3*. However, four of these five samples seem to be not too distantly related, as they all share the 16256A transversion. Two of them also share transitions at positions 16129 and 16362.

Geographic distribution of mtDNAs by biological ancestry

Little change is observed when biological ancestry frequencies are corrected by sample weight. Frequencies and 95% confidence intervals of 61.0 ± 3.4% Amerindian, 27.5 ± 3.1% African, 11.4 ± 2.2% West Eurasian, and 0.1 ± 0.2% Asian (Table 3) are corrected to 61.3 ± 3.4% Amerindian, 27.2 ± 3.1%

TABLE 6. Weighted biological ancestry frequencies per municipality

Municipality	Ancestry			Total	Weight
	Amerindian	Sub-Saharan African	West Eurasian		
Aguadilla	22[1] (75.9)[2]	6 (20.7)	1 (3.4)	29	1.2873
Arecibo	18 (75.0)	3 (12.5)	3 (12.5)	24	0.8898
Barranquitas	15 (65.2)	4 (17.4)	4 (17.4)	23	0.7492
Bayamón	28 (60.9)	12 (26.1)	6 (13.0)	46	1.0658
Caguas	17 (56.7)	8 (26.7)	5 (16.7)	30	0.9791
Carolina	21 (48.8)	15 (34.9)	7 (16.3)	43	1.0980
Cayey	10 (50.0)	10 (50.0)	0	20	0.9183
Corozal	16 (69.6)	5 (21.7)	2 (8.7)	23	0.9817
Florida	19 (67.9)	6 (21.4)	3 (10.7)	28	0.9421
Guayanilla	8 (57.1)	3 (21.4)	3 (21.4)	14	0.8369
Guaynabo	12 (63.2)	6 (31.6)	1 (5.3)	19	1.2371
Hormigueros	21 (75.0)	4 (14.3)	3 (10.7)	28	1.0459
Humacao	14 (58.3)	7 (29.2)	3 (12.5)	24	0.4898
Jayuya	16 (69.6)	5 (21.7)	2 (8.7)	23	1.0544
Juana Diaz	15 (88.2)	2 (11.8)	0	17	0.8733
Loiza	5 (17.9)	22 (78.6)	1 (3.6)	28	0.7666
Mayagüez	19 (73.1)	5 (19.2)	2 (7.7)	26	0.9565
Moca	22 (78.6)	2 (7.1)	4 (14.3)	28	1.2396
Patillas	18 (62.1)	7 (24.1)	4 (13.8)	29	1.3563
Peñuelas	8 (57.1)	6 (42.9)	0	14	1.5450
Ponce	29 (80.6)	3 (8.3)	4 (11.1)	36	1.2969
San Juan	42 (42.0)	40 (40.0)	18 (18.0)	100	1.2878
San Lorenzo	13 (52.0)	8 (32.0)	4 (16.0)	25	0.8201
San Sebastián	17 (65.4)	6 (23.1)	3 (11.5)	26	1.1541
Toa Baja	17 (58.6)	8 (27.6)	4 (13.8)	29	1.3123
Vega Alta	18 (66.7)	8 (29.6)	1 (3.7)	27	0.7349
Vega Baja	19 (82.6)	2 (8.7)	2 (8.7)	23	0.8962
Yauco	10 (62.5)	4 (25.0)	2 (12.5)	16	0.7439
Total	489 (61.3)	217 (27.2)	92 (11.5)	798	—

[1] Numbers correspond to number of samples from each municipality times sample weight values rounded to integers.
[2] Percent frequencies are in parentheses.

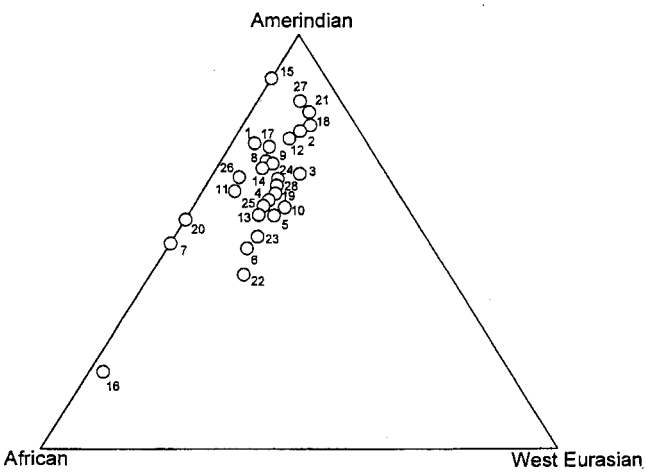


Fig. 2. Biological ancestry triangular graphic, showing biological ancestry frequencies of 28 municipalities. Municipalities are numbered as in Figure 1. Municipalities cluster close to vertex representing Amerindian frequencies equal to one, but with some degree of dispersion toward African vertex. Loíza dot lies apart from all other municipalities.

African, 11.5 ± 2.2% West Eurasian, and 0.0% Asian (Table 6).

Amerindian mtDNAs are the most common in all municipalities except Loíza, where African mtDNAs are more frequent, and Cayey, where the population is equally divided into African and Amerindian mtDNAs. Amerindian mtDNA frequencies are 50% or higher in all municipalities except Loíza, San Juan, and Carolina (Table 3).

In addition, West Eurasian frequencies are low in all municipalities (0–17.9%). Thus, in a triangular graph with axes representing biological ancestries, ancestry frequencies cluster close to the vertex where the Amerindian frequency equals one, and scatter next to the side defined by zero West Eurasian frequency, toward the vertex where African frequency equals one (Fig. 2). A negative Pearson correlation (−9.19) between African and Amerindian frequencies is observed that is significant at the 0.01 level (two-tailed test). That is, the biological ancestry frequency of municipalities can be virtually described by stating only their African or Amerindian frequencies.

Figure 3 divides the 28 sampled municipalities into 12 categories according to their Amerindian mtDNA frequencies, and divides Puerto Rico by longitude 66°16′ West, as 12 of the 13 sugar mills that worked throughout the 16th century were built east of it. It can be observed that the three municipalities with the lowest Amerindian frequencies are next to each other in San Juan and further east. Further, all 11 municipalities east of longitude 66°16′ West are among the 14 municipalities with the lowest Amerindian frequencies. There is a highly significant deviation from the null hypothesis that frequencies for all ancestries are the same east and west of longitude 66°16′ West (Pearson $\chi^2 = 43.70$, df = 2, $P \ll 0.001$). χ^2 tests also show highly significant

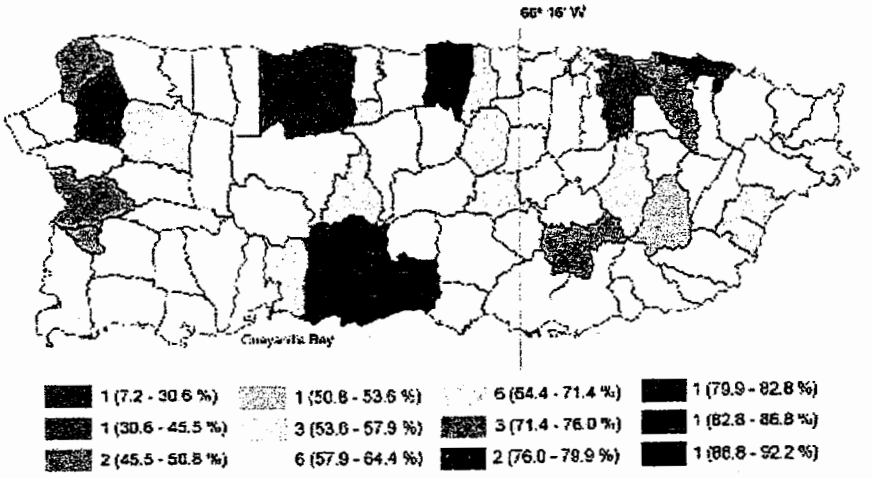


Fig. 3. Amerindian frequencies in 28 surveyed municipalities distributed in 12 categories. Shown next to each category shade indicator is number of municipalities in category and, in parentheses, Amerindian frequency range represented by category. Amerindian frequencies of single municipalities that are in first and last categories fall in middle of ranges represented by their categories. Fourteen municipalities with lowest Amerindian frequencies are 11 located east of longitude 66°16′ West and three surrounding Guayanilla Bay. See text for categorization methodology.

deviations from null hypotheses of equal frequencies on each side of longitude 66°16′ West for Amerindian (Pearson $\chi^2 = 41.72$, df = 1, $P \ll 0.001$) and African (Pearson $\chi^2 = 34.40$, df = 1, $P \ll 0.001$) mtDNAs. African mtDNAs are more frequent in the east than in the west; the reverse is true for Amerindian mtDNAs. No significant difference is found for West Eurasian mtDNAs.

Interestingly, the geographic distribution by biological ancestry does not fit expectations based on traditional history that place Amerindians fleeing to the mountains and African slaves working in sugar plantations on the coasts. The three municipalities with the highest Amerindian frequencies are coastal (Fig. 3), and χ^2 tests show that Amerindian frequencies in noncoastal municipalities are not significantly higher than those in coastal ones, and that African frequencies are not significantly higher in coastal than noncoastal municipalities.

Principal component analyses

To learn more about the origins of African mtDNAs in Puerto Rico, their weighted haplogroup frequencies (Table 7) were subjected to principal component (PC) analysis. Figure 4 plots the Puerto Rico African haplogroup frequencies with those of various contemporaneous African populations for the first two PCs (Fig. 4A), and the first and third PCs (Fig. 4B). The three PCs account for 28%, 23%, and 15% of the variation, respectively.

Little difference is found between the plots, which separately group the populations of West Africa and Mozambique, and scatter the East African populations in the lower side of PC2 and PC3. The Bubi from the island of Bioko are well-separated from all these groups. Three of four very old populations also separate well from these clusters. These are the Mbuti and Biaka Pygmies and the !Kung from South Africa. The South African Khwe fall within the Mozambique cluster. The ends of the West Africa cluster defined by PC1 are composed of populations located on the western coast to the left and populations located inland or close to the Gulf of Guinea to the right.

PC1 locates the population of Brazil outside of both the West African and Mozambican clusters. All other populations created by the slave trade are placed within the West African cluster. The southeastern Cape Verde Islands, which were uninhabited when discovered by the Portuguese and populated with slaves from the western coast of West Africa and inland thereafter (Thomas, 1997), fall in the middle of the West African cluster. To the right are located the Puerto Rico and São Tomé populations in that order, the latter lying at the edge of the West African cluster. Thus, a mainly West African origin is suggested for the African mtDNA fraction of Puerto Rico.

Figure 5 plots the Puerto Rico West Eurasian haplogroup frequency (Table 8) with those of European, Near Eastern, and Moroccan populations for the first two PCs (Fig. 5A), and for the first and third PCs (Fig. 5B). The three PCs account for 38%, 16%, and 13% of the variation, respectively. European and Moroccan clusters can be distinguished in the first plot, but fuse together in the second plot. The results are similar in many aspects to those obtained by Richards et al. (2002). The European Southeastern, Mediterranean East, and Mediterranean Central populations cluster a bit separately from the rest of the European populations, showing affinities to the Near Eastern populations, and the long-isolated Basques lie the farthest away from the Near Eastern populations. Another similarity is the scattering of Near Eastern populations, with the exception that the Kurdish, Armenian, and Turkish populations cluster together in both plots.

As Puerto Rico was a Spanish colony for four centuries, it is perhaps expected that its West Eurasian mtDNA population would lie close to the Mediterranean West population; it is noteworthy that on both plots the Mediterranean West population is the European population closest to Puerto Rico, but the Puerto Rico population lies outside the European

TABLE 7. Weighted data for sub-Saharan African haplogroups

	Sub-Saharan African haplogroups												
	L0	L1b	L1c	L2*	L2a	L3*	L3b	L3d	L3e	L3f	L3g	U5b2	Total
n	13	48	18	13	29	5	16	22	38	4	1	9	216
Frequency (%)	6.0	22.2	8.3	6.0	13.4	2.3	7.4	10.2	17.6	1.9	0.5	4.2	100.0

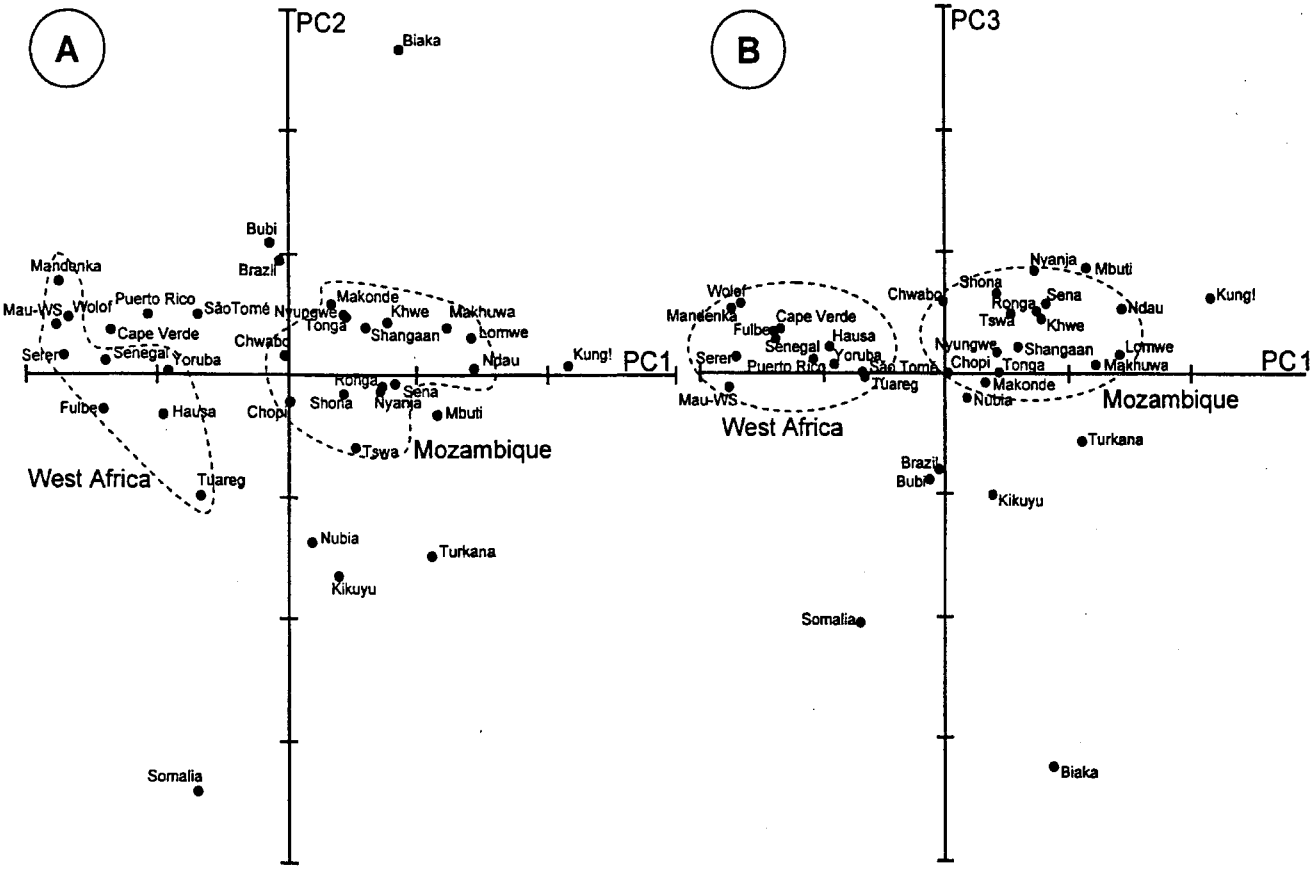


Fig. 4. PC analysis of sub-Saharan African populations based on mtDNA haplogroup frequencies. Crosslines indicate 0.1 units. **A:** PC1 vs. PC2. **B:** PC1 vs. PC3.

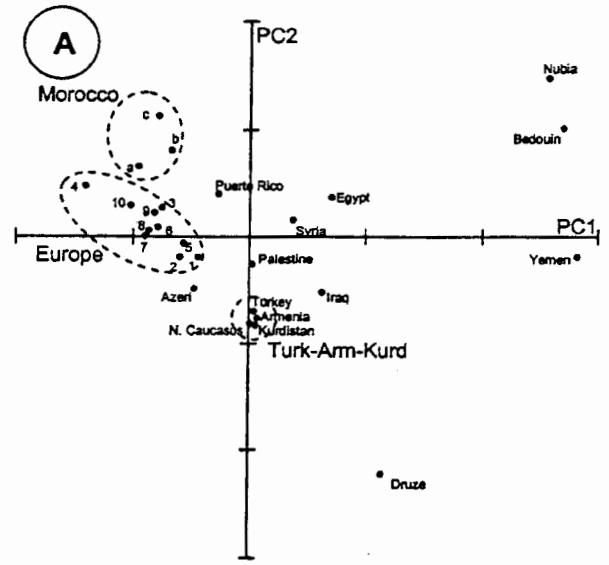
cluster. It lies closest to and about equidistant between the European and Moroccan clusters in the first plot, and closest to the Turkish-Armenian-Kurdish cluster in the second plot, suggesting that West Eurasian women who migrated to Puerto Rico had multiple origins.

The resolution power of the PC analysis of the Amerindian-weighted haplogroup frequencies (*n* for haplogroups A–D and X in Puerto Rico being 252, 42, 173, 14, and 0, respectively) is limited by the small number of haplogroups employed (Fig. 6). However, its frequent failure to group populations from particular geographic regions can often be explained by prehistoric demographic events. PC1 accounts for 38% of the variation, and although PC2 accounts for more variation (28%) than PC3 (20%), it is less effective at resolving most populations, as its resolution power lies on separating well Bella Coola from all other populations (Fig. 6, inset). The populations of five of the six North, Meso, and Central American regions, as well as those around Lake Titicaca and southern South America, generally lie closer to each other. Those from Colombia and the Amazon are widely dispersed. The Puerto Rico population is found in the bottom-left quadrant, next to the Western Colombian population of Paez.

DISCUSSION

Data quality and representativeness of the sample set

Our results can be deemed representative of the Puerto Rico population because we used a sampling frame developed for survey research in Puerto Rico that produced a sample set representative of the population based on the 1990 Census of Population and Housing, and obtained a response rate (81.1%) that complies with the standards and experience for survey research in the Island (Alegría et al., 2001;



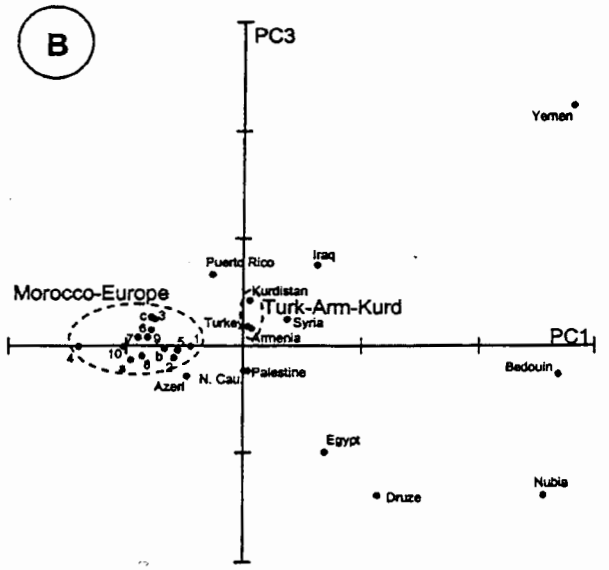


Fig. 5. PC analysis of West Eurasian populations based on mtDNA haplogroup frequencies. Crosslines indicate 0.1 units. **A:** PC1 vs. PC2. **B:** PC1 vs. PC3. Moroccan populations are designated as a, non-Berber Moroccans; b, Berbers; c, Souss Valley. Populations of European regions are designated as 1, Mediterranean East; 2, Mediterranean Central; 3, Mediterranean West; 4, Basque; 5, Southeast; 6, Alpine; 7, Northeast; 8, North Central; 9, Northwest; 10, Scandinavia.

Colón et al., 2001). Furthermore, no municipality had a particularly low response rate, San Juan (69.0%) having the lowest as the result of both the difficulty of contacting the selected people and a relatively high declination rate (Table 2). In addition, the biological ancestries among municipalities were not highly variable except for Loíza (Fig. 2), the response rate of which was good (82.2%). Thus, all significant variants that may exist in the population should have been adequately represented in the sample set. The very small differences observed between weighted and unweighted results add confidence to the assertion that the sample set is indeed representative.

TABLE 8. Weighted data for West Eurasian haplogroups

Haplogroup	*n*	Frequency (%)
H	29	31.5
HV	3	3.3
(Pre-HV)1	1	1.1
I	0	0.0
J	22	23.9
K	8	8.7
M	0	0.0
N	0	0.0
R	1	1.1
T	9	9.8
U*	1	1.1
U2	2	2.2
U5*	8	8.7
U5(a + b)	4	4.3
U6	3	3.3
U(others)[1]	0	0.0
V	1	1.1
W	0	0.0
X	0	0.0
Total	92	100.0

[1] Includes U1, U3, U4, and U7.

We estimated a rather high likelihood (33.9%) that one, and a fair one (10.9%) that two L1b samples may have been misdiagnosed as belonging to haplogroup L3e. Because both haplogroups are sub-Saharan African, the effects on our analyses of these possible misdiagnoses would be limited to the PCs (Fig. 5). In such cases, the Puerto Rico population would have plotted a little further to the left, as L1b is the haplogroup that contributes most significantly to the left side of PC1 (not shown). It would have plotted closer to the populations of Cape Verde and others found near the West Africa western coast, but still between Cape Verde and São Tomé. In addition, there is a fair possibility (14%) of other kinds of misdiagnoses occurring, but just once, and the effects of a single misdiagnosis in a set of 800 samples should be negligible.

Haplogroup specificity of the 10394 *Dde*I/10397 *Alu*I motif

Table 3 shows that five of the 211 African samples belonging to (+/−) haplogroups instead possessed (−/−) motifs. Chen et al. (1995) studied 96 Senegalese and 39 Biaka Pygmies belonging to (+/−) haplogroups, finding the (−/−) motif in five L1c Biaka Pygmies but none in the Senegalese. Another study on South African tribes found the (−/−) motif in one L3e and five L0 out of 31 Khwe samples and in two L0 of 43 !Kung, for a total of 13 (−/−) motifs in 209 samples (Chen et al., 2000). Our five (−/−) samples were found in two L3e and in one each of L1c, L2*, and L2a. It is thus clear that the (−/−) motif has arisen independently several times in Africa as well as in Europe (Finnilä et al., 2001; Herrnstadt et al., 2002), but it seems to be found in African tribes at

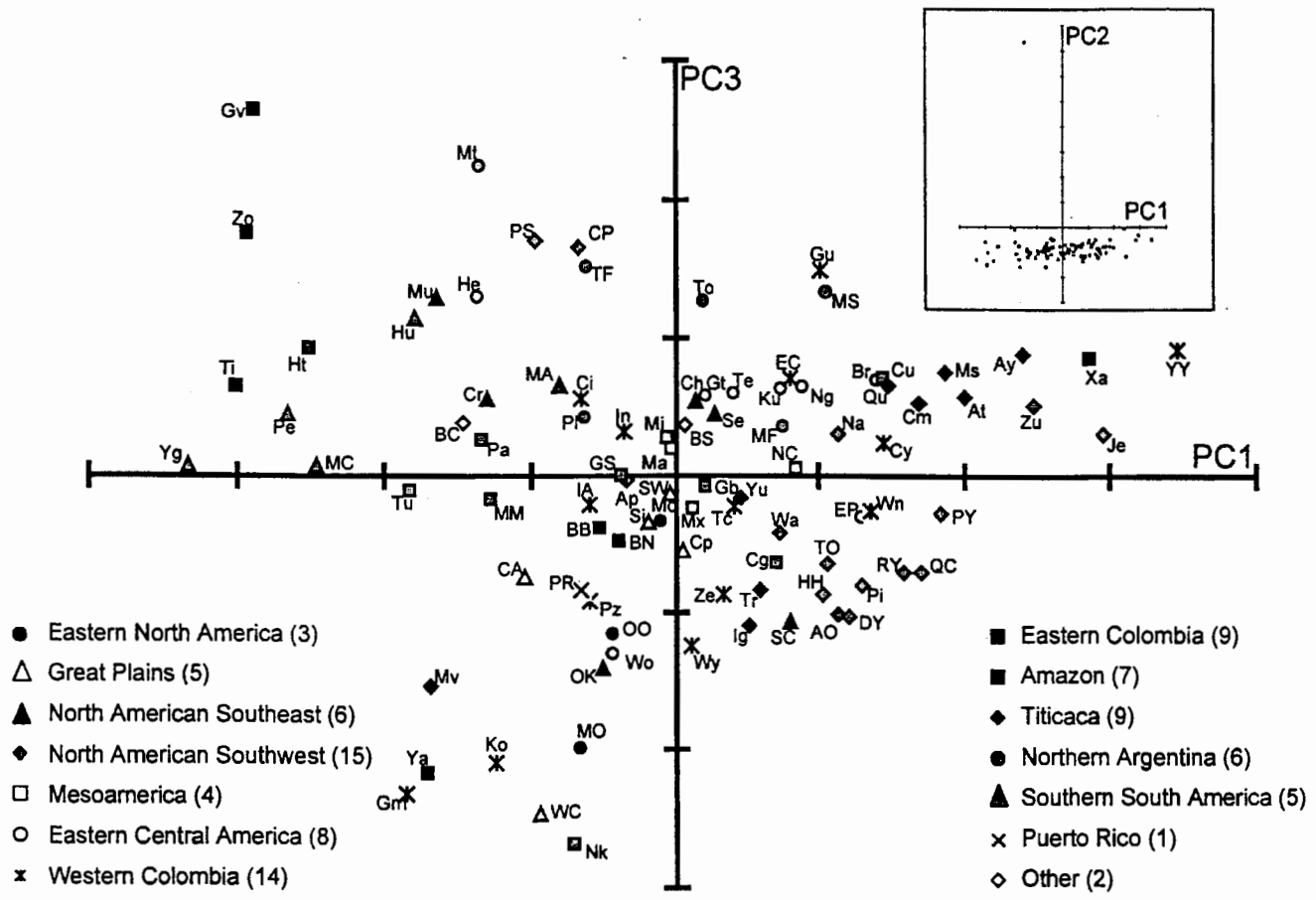


Fig. 6. PC analysis of Amerindian populations based on mtDNA haplogroup frequencies: PC1 vs. PC3. Inset: PC1 vs. PC2. Crosslines indicate 0.05 units. Number of populations from each geographic region is indicated in parentheses. Population codes are as follows. From eastern North America: Mo, Mohawk; MO, Manitoulin Island Ojibwa; OO, Northern Ontario Ojibwa; from Great Plains: CA, Cheyenne/Arapaho; Cp, Turtle Mountain Chippewa; Si, Siouan; SW, Sisseton/Wapheton Sioux; WC, Wisconsin Chippewa; from North American Southeast: Ch, Choctaw; Cr, Creek; Mu, Muskoke; OK, Oklahoma Red Cross; SC, Stillwell Cherokee; Se, Seminole; from North American Southwest: AO, Akimal O'odham; Ap, Apache; CP, California Penutian; DY, Delta Yuman; HH, Havasupai/Hualapai/Yavapai/Mojave; Je, Jemez; Na, Navajo; Pi, Pima; PS, North Paiute/Shoshoni; PY, Pai Yuman; QC, Quechuan/Cocopa; RY, River Yuman; TO, Tauno O'odham; Wa, Washo; Zu, Zuni; from Mesoamerica: Ma, Maya; Mi, Mixtec; Mx, North Central Mexico; NC, Nahua/Cora; from eastern Central America: Br, Bribri-Cabecar; EP, Emberá-Panamá; Gt, Guatuso; He, Huetar; Ku, Kuna; Ng, Ngöbé; Te, Teribe; Wo, Wounan; from western Colombia: Ci, Chimila; Cy, Cayapa; Gm, Guambiano; Gu, Guane-Butaregua; EC, Emberá-Colombia; IA, Ijka-Arhuaco; In, Ingano; Ko, Kogui; Pz, Paez; TC, Tule-Cuna; Wn, Waunana; Wy, Wayuu; YY, Yuco-Yukpa; Ze, Zenu; from eastern Colombia: Cg, Coreguaje; Cu, Curripaco; Gb, Guayabero; GS, Guahibo-Sikuani; Ht, Huitoto; MM, Murui-Muinane; Nk, Nukak; Pa, Piaroa; Tu, Tucano; from Amazon: BB, Belén-Brazil; BN, Brazilian North; Gv, Gaviao; Ti, Ticuna; Xa, Xavante; Ya, Yanomami; Zo, Zoró; from around Lake Titicaca: At, Atacameño; Ay, Aymara; Cm, Chimane; Ig, Ignaciano; Ms, Mosetén; Mv, Movima; Qu, Quechua; Tr, Trinitario; Yu, Yuracaré; from northern Argentina: MC, Mataco-Salta; MF, Mataco-Formosa; Mt, Mataco-Chaco; Pl, Pilaga; TF, Toba-Formosa; To, Toba-Chaco; from southern South America: Hu, Huilliche; MA, Mapuche-Argentina; MC, Mapuche-Chile; Pe, Pehuenche; Yg, Yaghan. PR, Puerto Rico. "Other" populations: BC, Bella Coola; BS, Brazilian Southeast.

uneven frequencies, being more prevalent in old tribes of South Africa and Central African Pygmies, and rare elsewhere. Our review of traditional history found no evidence of Pygmies being brought to the Americas, and suggests that none or very few members of South African tribes were brought to the Caribbean. Hence, our results may reflect what is found in more recent and widespread African tribes, most of which are related to Bantu expansions, and suggest that the (−/−) motif in (+/−) haplogroups is present at frequencies of 2–3% throughout most of sub-Saharan Africa.

The 10394 *Dde*I/10397 *Alu*I motif behaves more consistently among Amerindian mtDNAs, probably because these form a more recent group. Only one Amerindian mtDNA showed a motif inconsistent with its haplogroup identity. It belonged to the (+/+) haplogroup C, and probably suffered a back-mutation at position 10398, thus losing both restriction sites simultaneously. Because (with the exception of haplogroup U) all African haplogroups are (+/−), whereas none of the Amerindian haplogroups are, the few motif inconsistencies found here suggest that testing the 10394 *Dde*I/10397 *Alu*I motif is an

excellent starting point for sorting out African and Amerindian samples from mixed populations with high African and Amerindian affinities, such as those in the Caribbean.

Nonspecificity in haplogroup-defining markers

The 3592 *Hpa*I site that defines macroparagroup L has proven to be highly stable, being found strictly on mtDNAs belonging to haplogroups L1 or L2 in sub-Saharan Africa and elsewhere (Huoponen et al., 1997; Torroni et al., 1997). The story is not the same for the +4216 *Nla*III and −8616 *Dpn*II markers that define haplogroups J/T and L3d, respectively. Both (more often −8616 *Dpn*II than +4216 *Nla*III) have been found occasionally in mtDNAs belonging to other African and West Eurasian haplogroups (Finnilä et al., 2001; Herrnstadt et al., 2002). Thus, we deemed it unnecessary to determine the HVR-I sequence of the two L1b samples that exhibited the +4216 *Nla*III motif and of the single L0 and L1c samples that had the −8616 *Dpn*II motif (Table 3).

Other haplogroup-defining markers that were found to occur out of their expected haplogroups through HVR-I sequencing were the 9-bp deletion between the COII and $tRNA^{Lys}$ genes that defines haplogroup B and the −7598 *Hha*I marker of haplogroup E. The multiple independent emergences of the 9-bp deletion in Africa (Soodyall et al., 1996), India (Watkins et al., 1999; Clark et al., 2000), East Asia (Wrischnik et al., 1987; Yao et al., 2002a), Europe (Torroni et al., 1997), the New World (Torroni et al., 1993a), and Australia (Betty et al., 1996) are well-known. To our knowledge, this is the first time that it has been reported in a haplogroup H background. However, the −7598 *Hha*I motif that defines haplogroup E is much more stable. Hitherto, it had been found outside of a haplogroup E background only in Finnish belonging to haplogroup K (Finnilä et al., 2001), and in all reported Han Chinese belonging to haplogroup G (Yao et al., 2002a). It was found here in a haplogroup C background.

Designation of clade U5b2

Mitochondrial DNAs from northern sub-Saharan Africa with the (−/−) motif were first described by Chen et al. (1995). These turned out to belong to haplogroup U, as defined by the acquisition of a *Hin*fI site at position 12308 when using a mismatched primer (Torroni et al., 1996). Studies in northern sub-Saharan populations have often found mtDNAs whose HVR-I sequence circumscribe their identity to haplogroups H or U, suggesting the reintroduction to sub-Saharan Africa of West Eurasian mtDNAs through North Africa (Macaulay et al., 1999). Our results show that the sequence type 16189-16192-16270-16320 (Table 4) found in three African tribes but not in Eurasia belongs to haplogroup U. Its absence in all other haplogroup U sequences described to date strongly suggests that these mtDNAs have a very recent common origin in northern sub-Saharan Africa. Insofar as no coding region analysis other than at the 12308 site has been performed on these mtDNAs, we must depend on their HVR-I sequence for their classification. Haplogroup U was subdivided into eight clades plus haplogroup K, clade U5 being distinguished by its 16270 motif. U5 was further subdivided into U5a and U5b, and since only one clearly unrelated haplogroup U mtDNA possessing the 16320 has been reported (Malyarchuk et al., 2002), we depend on its transitions at sites 16189 and 16192, both unstable (Macaulay et al., 1999; Finnilä et al., 2000), to classify the African clade. The combination 16189 and 16192 was found in both U5a and U5b clades, but it was always accompanied by a transition at position 16256 within U5a, and by no substitution in particular within U5b (Richards et al., 2000). Thus, the parsimony rule guides us to classify sequence type 16189-16192-16270-16320 as a U5b clade. As clade U5b1 with the 16144 signature has already been described, we describe clade U5b2 with the 16320 signature. Coding-region analysis must be performed to confirm the assignment of these mtDNAs to subhaplogroup U5b.

L3 clades with no defining restriction markers

Within Africa, paragroup L3 is found at its highest frequencies in East Africa, where it originated and gave rise to those mtDNAs that migrated out of Africa (Quintana-Murci et al., 1999; Maca-Meyer et al., 2001). Salas et al. (2002) described haplogroups L3f and L3g, both virtually restricted to East Africa except subclade L3f1, which is common in West Africa and thus not surprisingly the only subclade of L3f found in Puerto Rico. Similarly, L3* is far more common in East than in West Africa, but the C-to-A transversion at position 16256 that appeared in four of the five Puerto Rican L3* mtDNAs (Table 5) has appeared just once in the continent, among the West African Tuareg (Watson et al., 1997). It may thus represent the signature of a small West African clade. Similarly, the 16114–16316 motif of the L3g sample places it in the L3g2 subclade, which might be predominantly of Central African origin (Bortolini et al., 2004; Salas et al., 2004a).

Amerindian mtDNA frequencies and traditional history

The combination of the high Amerindian mtDNA frequency found and the representativeness of the sample set leaves no doubts that the mtDNA pool of Puerto Ricans is predominantly Amerindian. Such preponderance extends throughout the island, with the notorious exception of Loíza (Fig. 2). Many of the slaves in the San Juan region emancipated upon the abolition of slavery in 1873 and not staying with their former masters were placed in Loíza. Because San Juan had an unusually high proportion of female domestic slaves (Negrón-Portillo and Mayo-

Santana, 1992), their transference to this lightly populated municipality increased its African maternal ancestry out of the range of all other municipalities.

Our results are in contrast with those based on genetic markers that estimate the contribution of Amerindians to the Puerto Rican gene pool at 18% (Hanis et al., 1991). This may be explained by the predominance of men among the Spaniards who arrived on the island. Population studies based on mitochondrial and Y-chromosome markers found strong sexual asymmetries in South America, mtDNAs usually being inherited from Amerindian women and Y-chromosomes from European men (Batista dos Santos et al., 1999; Alves-Silva et al., 2000; Carvajal-Carmona et al., 2000; Carvalho-Silva et al., 2001). Our preliminary results from Y-chromosome studies (unpublished) strongly suggest a similar scenario for Puerto Rico. The overall meaning of our mtDNA data is that the cumulative effect of female migrations to Puerto Rico for the last five centuries has been the reduction of the Amerindian mtDNA frequency from 100% to 61%, and (assuming equal reproductive rates) that about seven African women arrived in Puerto Rico for every three West Eurasians who did.

Remarkably, our mtDNA frequencies mimic those obtained from a study in which JC virus strains excreted by two groups of Puerto Rico residents were identified: 61% carried a JCV strain of Asian and Amerindian origin, 26% of African origin, and 13% European (Fernández-Cobo et al., 2001). The frequency distribution of the strains might be explained by fitness differences among viral strains. However, our results support an alternate explanation: that the viral transmission mechanism may favor the mother as vector. The combined identification of JCV strains and mtDNA ancestries should be undertaken to test for a possible correlation between them.

Traditional history provides abundant evidence that Amerindian slaves were brought to Puerto Rico during colonial times from Iberian possessions such as the islands of Margarita, Trinidad, Aruba, Bonaire, the Venezuelan coast (Fernández-Méndez, 1970), the Yucatán peninsula (Zavala, 1948), and Brazil (Tapia, 1945). However, the haplogroup diversity of Amerindian mtDNAs, calculated with weighted samples at 0.5934, is not high, as may have been expected if the Amerindian predominance in Puerto Rico was the consequence of post-Columbian migrations. Specifically, the Puerto Rico haplogroup diversity is higher than in 16 of 19 tribes in the American Southwest and Baja California (O'Rourke et al., 2000; Malhi et al., 2003), where recent population replacement and expansion events may have reduced the haplogroup diversity of the population (Kaestle and Smith, 2001). However, it is higher than in only eight of 29 tribes in the remainder of the contiguous United States and subarctic Canada (O'Rourke et al., 2000; Malhi et al., 2001; Weiss and Smith, 2003). Haplogroup diversities in South America are generally lower than in North America, but the haplogroup diversity in Puerto Rico is higher than in only 12 of 31 South American tribes outside Colombia (Demarchi et al., 2001). In Colombia, the Andes function as a barrier dividing tribes of haplogroup diversities higher to the east, and lower in the Andes and further west (Keyeux et al., 2002). Hence, the Puerto Rico haplogroup diversity is higher than that of only five of 10 tribes to the east, but of 11 of 15 tribes in the Andes and farther west. West of Colombia in eastern Central America, where recent population bottlenecks followed by population expansion events have drastically reduced haplogroup diversity, all five tribes examined have haplogroup diversities lower than Puerto Rico (Santos et al., 1994; Batista et al., 1995; Kolman et al., 1995; Kolman and Bermingham, 1997). In conclusion, the Puerto Rico haplogroup diversity is within the lower half of tribes in all regions of the subarctic New World, except for those regions where demographic histories were affected by strong and recent population expansion events. It is unlikely that the very large number of post-Columbian migrants needed to catapult the Amerindian frequency in Puerto Rico to 61% after the putative total extermination of the native population, arriving from various Spanish and Portuguese colonies, would by chance form a population of approximately median haplogroup diversity. More likely, most Amerindian mtDNAs in Puerto Rico have their origin in the native Taíno population.

The scarcity and geographic distribution of haplogroup D mtDNAs in Puerto Rico suggest that haplogroup D may have been imported during colonial times. Its geographic distribution is similar to that of sub-Saharan African mtDNAs, with a higher frequency on the eastern side of longitude 66°16′ West. It is known that, for centuries, one of the main Indian slave harbors in the Americas was located in Coro, northwestern Venezuela (Sued-Badillo, 1995). It provided plenty of Indian slaves to Aruba in the 19th century (Nooyen, 1965), where haplogroup D is predominant (Toro-Labrador et al., 2003), and it is possible that it provided slaves to Puerto Rico during that period. However, haplogroup D was found in six of 24 Taíno bone samples from the La Caleta archaeological site in the Dominican Republic (Lalueza-Fox et al., 2001), suggesting that genetic differences between Taínos on both islands were larger than the few cultural differences that have been acknowledged (Veloz-Maggiolo, 1991; Rouse, 1992).

Indian slave trade notwithstanding, the predominance of Amerindian mtDNAs in modern Puerto Rico coupled with the very large Atlantic slave trade strongly suggests that most of the Amerindian mtDNAs found are native, and that haplogroup A was predominant among the Puerto Rico Taínos. Thus, it is intriguing that ancient DNA studies on a total of 43 ancient remains from three archaeological sites in Cuba and one each in Puerto Rico and the

Dominican Republic found only one remain belonging to haplogroup A (Sánchez-Crespo, 1999; Lalueza-Fox et al., 2001, 2003). One explanation for this is that genetic diversity in Taínos may have existed mostly between and not within settlements. This could be explained by the matrilineal descent system of the Taínos, in which newborns would join their maternal clan (Keegan, 1997).

PC analysis was performed in an attempt to associate the Taínos to some continental region. The general picture that emerged in the PC1–PC3 plot (Fig. 6) was that of regional continuity affected frequently by population demographic events such as mass population movements, admixture, and genetic bottlenecks. The populations of five of the six North, Meso, and Central American regions showed some clustering, as did the populations around Lake Titicaca and farther south. All three eastern North American and four of the five Great Plains populations, which experienced population movement and admixture associated with the eastward migration of Algonquian-speaking groups (Malhi et al., 2001), fell in the bottom-left quadrant. Nine of 15 North American Southwest populations clustered in the bottom-right quadrant, the two recently arrived (after AD 1000) Na-Dene-speaking populations (Navajo and Apache) being among those scattered. In addition, the four Mesoamerican populations fell close to the axes' intersection. In a fashion similar to that of North American Southwest populations, five of the eight populations of eastern Central America were found in the upper-right quadrant, the two populations strongly influenced by recent migrations from Colombia (Emberá and Wounan) (Kolman and Bermingham, 1997) being among the three scattered. Not showing any cohesiveness were the populations of the North American Southeast, which may have experienced genetic bottlenecks produced by drastic population declines after contact (Weiss and Smith, 2003), and those from Colombia, the Amazon, and northern Argentina. Genetic isolation of small populations may have resulted from the extreme topography of the northern Andes. Larger genetic drift as a consequence of the smaller effective population sizes that may have resulted from restricted mobility in this topography, and later through the dense tropical forests of the Amazon, may have been translated into widely scattered populations in the PC plot for Colombia, the Amazon, and northern Argentina. By contrast, most of the populations in the highlands around Lake Titicaca and farther south that were found through control-region sequence analysis to have had large long-term effective population sizes (Fuselli et al., 2003) plotted close to each other. Five of the nine Lake Titicaca populations formed a cluster in the upper-right quadrant, and all five southern South American populations fell in the upper-left quadrant.

Our PC analysis failed to give an indication of the possible continental origins of the Taínos. Their sophisticated social and political organization, agroceramic culture, religious rituals, and Arawak language unmistakenly reveal the South American origin of their culture. However, the scattering of Colombian and Amazonian populations in the plot, coupled with the fact that the Taínos may have developed from the admixture of agroceramic South American cultures with nomads originating elsewhere (Veloz-Maggiolo, 1991; Rouse, 1992), further complicated the analysis. Clearly, expanded ancient DNA studies must be undertaken to better understand the prehistoric migrations that gave rise to the Taínos. Studies on modern populations, although affected by postcolonization migrations, may be useful in guiding and complementing ancient DNA studies.

The results presented here led us to reinterpret previous results. The 72.5% Amerindian mtDNA frequency found west of longitude 66°16′ West is very similar to the 69.6% reported by Martínez-Cruzado et al. (2001) for a group composed of 56 people living in communities known historically for their strong Indian background or volunteers having a mother or maternal grandmother with Indian-like phenotypic traits. Only four of these 56 people traced their maternal lineage to the east of longitude 66°16′ West, the remainder tracing it to the west. Thus, their relatively high Amerindian maternal ancestry can be explained simply by the geographical location of their maternal lineage west of longitude 66°16′ West. That is, if they share the location of their maternal lineages west of longitude 66°16′ West, Puerto Ricans with maternal ancestors with Indian-like phenotypic traits or living in communities known historically for their strong Indian background are not more likely to have an Amerindian mtDNA than those who do not. The significantly reduced Amerindian mtDNA frequency (52.6%) obtained from a random sample of 38 Puerto Ricans (Martínez-Cruzado et al., 2001) can be explained by the high number of participants who stemmed from east of longitude 66°16′ West. If only those tracing their maternal lineage west of longitude 66°16′ West are considered, the difference with the general population west of longitude 66°16′ West turns out to be insignificant (not shown).

Traditional history holds that Indians became extinct from disease, war, and slavery, leaving the whole island for the development of the Spanish colony. However, a new current of thought holds that although the number of Indians was lowered considerably by the conquest, and that their political, cultural, and religious systems disappeared in one or two generations, many survived and adapted to the conditions imposed by the colonial order, most of them living away from the colonial towns and plantations, but not in the hostile environment of the mountain chains (Sued-Badillo, 1995). The numbers of colonial settlers and the lands occupied by them were small enough to leave sufficient fertile cattle and crop land for Indians and mixed people of Indian ancestry whose presence was largely ignored

by the colonial government, and who developed into the so-called *criollos* of the late 18th and 19th centuries. Our overall results and the observed genetic bipartition defined by longitude 66°16′ West strongly support this view. Twelve of the 13 sugar-producing mills in Puerto Rico during the 16th century were located east of longitude 66°16′ West (Gelpí-Baíz, 2000). It is thus to be expected that most of the people related in any way to the colonial society established themselves in this region. However, and by no reason that could be related to any initiative from Spain, the majority of the population lived west of longitude 66°16′ West by 1776. Our work suggests that these people had a very high Amerindian ancestry. Furthermore, with the exception of San Juan and surrounding areas, the eastern side of Puerto Rico was virtually uninhabited (Abbad, 1959). Thus, land availability may have driven most of the 19th century immigrants to settle east of longitude 66°16′ West. Because most of the immigrants were of non-Amerindian origin, the result was a stronger reduction of the Amerindian frequency east than west of longitude 66°16′ West.

Sub-Saharan African and West Eurasian origins

It is apparent from a review of the literature on the Atlantic slave trade and Puerto Rico that the coast that extends from Ghana to Cameroon was the largest source of slaves to Puerto Rico, but that large numbers were also brought from the stretch extending from Senegambia to Sierra Leone, and from Congo and Angola, perhaps in that order, in addition to smaller contributions from other African regions.

Our PC analysis succeeds in distinguishing the West African populations from those of Mozambique. Furthermore, it plots the Brazilian population, which must have a very large Angolan component (Thomas, 1997), separate from both and next to the Bubi population that lives on the island of Bioko close to the Cameroon coast. This suggests that when sufficient data from Angola and Congo become available, PC analyses may be able to distinguish clusters corresponding to West, Southeast, and Central-West Africa.

A geographic gradient can be observed within the West Africa cluster at the left half of PC1 (Fig. 4), where the frequencies of L1b, L2*, and L3b (all more frequent in West Africa than in other African regions) are the main contributors, in that order. Those populations in or very close to Senegambia are found at the left edge of the cluster, and those in or closer to the Gulf of Guinea toward the right edge. The PC1 order of the populations created by the slave trade is consistent with historical accounts. The Cape Verde Islands, composed mainly of Senegambians but having also received people from other African regions, plot at the right edge of the Senegambian group. São Tomé, located in the Gulf of Guinea, and receiving mainly people from Ghana to northern Gabon (Tomás et al., 2002), lies at the right edge of the West African cluster. Brazil, with the largest Angolan and smallest Senegambian components (Salas et al., 2004b), lies to the right of the West African cluster. The Puerto Rico population lies between the Cape Verde and São Tomé populations, suggesting that it contains a Senegambian component smaller than that of Cape Verde but larger than that of São Tomé, and Gulf of Guinea, Congo, and Angolan components smaller than those of São Tomé but larger than those of Cape Verde.

The PC analysis of West Eurasian mtDNAs suggests that many of the West Eurasian women arriving to Puerto Rico were non-European in origin. The European populations cluster tightly, but the Puerto Rico population falls out of the European cluster, and is as close to it as to the Moroccan cluster in the first plot, and closer to the Turkish-Armenian-Kurdish cluster in the second (Fig. 5). In addition, 11 of the 18 haplogroup U West Eurasian mtDNAs found in Puerto Rico are likely not of European origin. This should not be surprising, as the vast majority of early colonizers were single men. This fact not only accounts for the disparity between West Eurasian Y-chromosome and mtDNA frequencies in Puerto Rico (unpublished results), but generated in the first decades of colonization a demand for white female slaves. Historical documents show that in 1512, the Spanish Crown encouraged the exportation of white female slaves to San Juan (Fernández-Méndez, 1970), and that in 1530 licenses were granted to export white Moorish slaves to the Americas, including to people with strong ties to Puerto Rico (Thomas, 1997). Raids of Arab towns produced many slaves for the Spaniards through the 16th century, and Moorish and Berbers (Álvarez-Nazario, 1974), as well as Slavs and Turks (Thomas, 1997), could still be found among slaves late in the 17th century. Moreover, in the 17th century, poor Canarian farmers moved with their families to Puerto Rico seeking incentives that were granted as part of a concerted effort to increase the white population of Puerto Rico (Fernández-Méndez, 1970). The location of the Puerto Rico population outside of the European cluster in the PC plot suggests that the combined input to Puerto Rico of all these sources was substantial, as compared to the European input.

It is noteworthy that the motif of sequence type 16224-16270, which represents eight samples and is by far the most frequent among our West Eurasian U mtDNAs (Table 4), has not been found elsewhere except for one sample each in the Canarian islands of Tenerife and Lanzarote that also possessed a transition at 16093, a site that was not sequenced in our samples. It is thus evident that the samples possessing the 16224 and 16270 motif are related to North Africa, probably to the Canary Islands, and maybe to the *guanches* (Canarian natives), because Lanzarote exhibits a strongly autochthonous genetic makeup (Rando et al., 1999). Furthermore, two other samples were identified as belonging to the Canarian-specific U6b clade, and one other to the North African-specific U6a. Thus, 11 of the 18 West

Eurasian U mtDNAs may be related to North Africans. Only the remaining seven samples could be of European origin. Combined with the PC analysis, this observation suggests that the European contribution to the West Eurasian mtDNA pool of Puerto Rico could be less than half. That North African and sub-Saharan African haplogroup U sequence types can be found at copy numbers of seven and nine, while those of likely European origin are never found at more than two, suggests that most of the European mtDNAs were introduced in Puerto Rico late in its history.

CONCLUSIONS

The hierarchical strategy employed here for RFLP mtDNA haplogroup identification was shown to be effective in the highly admixed population of Puerto Rico. The 10394 *Dde*I/10397 *Alu*I motif of each sample was determined first, followed by testing the haplogroup-defining markers of all haplogroups within the predetermined motif, and using HVR-I sequences to resolve inconclusive results. The effectiveness of the strategy depended largely on the stability of the motif, which was found to be inconsistent with the mtDNA haplogroup in only six of 800 samples. Because (with the exception of haplogroup U) all African haplogroups have (+/−) motifs, whereas none of the Amerindian haplogroups do, these results suggest that testing the 10394 *Dde*I/10397 *Alu*I motif is an excellent starting point for sorting out African and Amerindian samples from mixed populations with high African and Amerindian affinities, such as those in the Caribbean.

The findings here reported are consistent with most historical accounts, with the notable exception of the high frequency (weighted, 61.3%) of Amerindian mtDNAs found currently in Puerto Rico, among which haplogroups A (52.4%) and C (36.0%) are the most common. Their high frequency led us to the conclusion that most Amerindian mtDNAs in Puerto Rico should stem from the indigenous Taínos, and that haplogroups A and C were predominant among them. These results are in agreement with ancient DNA studies only in the high frequency of haplogroup C. Ancient DNA studies have so far found only one haplogroup A mtDNA among 15 Ciboney remains in Cuba (Lalueza-Fox et al., 2003), and none in the remains of 24 Taínos in the Dominican Republic (Lalueza-Fox et al., 2001) and four Pre-Taínos in Puerto Rico (Sánchez-Crespo, 1999). Furthermore, the low frequency of haplogroup D (2.9%) currently in Puerto Rico and its concentration in areas of higher sub-Saharan African ancestry suggest that it may have been absent in pre-Columbian Puerto Rico, but it was found in six of 24 Taíno remains in the Dominican Republic and in five of 15 Cuban Ciboney remains. Poor sampling and large genetic differences between archaeological sites could explain the virtual absence of haplogroup A in ancient samples. Substantial genetic differences between the islands, consisting of the absence in Puerto Rico of haplogroup D but its presence elsewhere, may account for the disparity in haplogroup D frequencies.

Because of their Arawakan culture, Taínos would be expected to be closely related to South American tribes. A haplogroup frequency-based principal component analysis shows some compartmentalization of regional populations, but not for Colombian or Amazonian populations, which tend to show lower effective population sizes, lower haplogroup diversities, and more susceptibility to genetic drift. Hence, these populations scatter widely throughout the plot, and the Puerto Rico population cannot be related to any particular region.

A principal component plot identifies West Africa as the main supplier of sub-Saharan African mtDNAs to Puerto Rico and to other populations created by the slave trade such as São Tomé and Cape Verde, but not Brazil. The main contributors to the first principal component of this plot are haplogroups considered West African-specific. This component generates a geographic gradient in which the westernmost populations of West Africa are placed at the left end of the plot, and populations farther to the east up to the Gulf of Guinea are placed further to the right. Along this component, Puerto Rico falls between Cape Verde to the left, and São Tomé to the right, suggesting a West African composition for Puerto Rico intermediate between these two populations. The strong West African composition of the sub-Saharan African fraction of Puerto Rico becomes evident by the relatively high frequency of a U5b HVR-I sequence type previously found hitherto only in West Africa, and defined here as clade U5b2.

For having been an European colony for 405 years, it would be expected that the West Eurasian fraction of Puerto Rico would show strong affinities with Europe. Principal component analysis shows that this is not the case. While European populations tend to cluster tightly, the Puerto Rico population remains separated from all populations, being approximately equally close to the European, Moroccan, and Turkish-Armenian-Kurdish clusters. HVR-I sequences of Puerto Rican haplogroup U mtDNAs show sequence types closely related to North Africa and the Canary Islands, as well as to Europe. North African and Canarian sequence types are not more varied than the European but have a higher average copy number, suggesting that their presence in Puerto Rico preceded that of most Europeans.

Within Puerto Rico, the expected difference in African mtDNA frequencies between coastal and noncoastal municipalities was found to be statistically insignificant. However, a highly statistically significant difference was found between the eastern and western sides of La Plata River, east of which most of the 16th century sugar mills were established. The weighted Amerindian frequency was higher in the western (72.3%) than in the eastern (50.0%) side. In addition, pockets of relatively low

Amerindian frequencies were found surrounding harbors.

As the area of the island of Puerto Rico is only 9,104 km^2, this work shows how much population structuring can occur within small regions, especially between metropolitan-cosmopolitan areas and nearby mountains which may hold old, traditional populations. This observation suggests that studies based on careful sampling in traditional populations, unaffected by frequent migrations common in metropolitan areas, may offer further information on the prehistoric migrations that gave rise to continental populations around the world.

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