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Abstract

The Rama are a coastal population from Southern Nicaragua who in large part were able to resist, at least for a time, the cultural changes and social reorganization brought on by colonial and modern influences. Historical information leaves the Rama origins and biological relationships to nearby extinct and extant groups ambiguous. The objective of this study is to examine the internal genetic microdifferentiation based on the first hypervariable region of the mtDNA from a sample of approximately 20% of the population, and to expand the few available historical and anthropological data on the Rama by exploring the effects of cultural practices and historical events on genetic structure, providing an integrative perspective on the Rama genetic history. When considering differences in the spatial distribution and genetic diversity of the mtDNA haplotypes together with historical information on the Rama, a noteworthy pattern

emerges: first, haplotypes are differentially distributed among a central Rama community (Punta Águila) from the other five peripheral communities (AMOVA: $F_{ct} = 0.10$, $p < 0.001$) and their distribution is consistent with the historical relocation of this population after their split from Punta Gorda in the 18th century; second, differential genetic signatures found among central and peripheral Rama communities resemble two population histories: one of stability (haplogroup A2) and other of expansion (haplogroup B2), supporting the possibility that these patterns of genetic microdifferentiation between central and peripheral populations resulted from the 18th century unification in southern Nicaragua of the Rama and a group of Voto migrants from Costa Rica that later split and hived off to the Bay of Bluefields.

The scant bioanthropological research on contemporary indigenous groups from the Caribbean region of Southern Central America (SCA) has resulted in a limited understanding of intergroup relationships and genetic history. To date, most recent molecular research highlights the effects of migration on vast continental regions rather than assessing population dynamics of individual groups that occupy their own changing niches (Reich et al. 2012; Wang et al. 2007). In SCA, few studies have focused on the microevolutionary consequences of cultural practices or the recent effects of historical events such as migration and the selective forces that operate on the structure of small and isolated groups (Barrantes 1993; Thompson et al. 1992). Therefore, our primary goal in this investigation is to expand this knowledge by studying the population microdifferentiation and history of the Rama Amerindians from Central America (Figure 1) though their maternal genetic inheritance and exploring the forces of evolution impacting this population due to the influence of recent historical events.

Despite unresolved issues regarding the origins of the Rama, they have been recognized as a culturally (Conzemius 1930; Loveland 1975), linguistically (Constenla 2008; Craig 1990), and biologically unique indigenous population among other Caribbean populations in Nicaragua (D'Aloja 1939; De Stefano 1973; Schultz 1926). Recent studies in anthropological genetics and historical linguistics suggest that the Rama are related to other Chibchan speakers from SCA and northern South America (Constenla 2008; Melton et al. 2013), and were significantly impacted by paternal gene flow from Europeans (Melton et al. 2013). These investigations, however, have not integrated historical factors for better understanding the causes of the Rama's internal micro-differentiation that was detected by a previous study based on isonymy and genealogies (Baldi et al. 2014).

The present study examines the level of population micro-differentiation, based on the analysis of the first hypervariable segment of the mtDNA (HVS-I) of six Rama subpopulations in the southern Caribbean region of Nicaragua. Because the Rama maternal genetic inheritance was less impacted by non-indigenous migrants to the region after the 16th century in comparison with the Y-chromosome (Melton et al. 2013), mtDNA was used as an auxiliary method to explore the genetic structure of this population. While the sole use of this marker constrains the analysis of the entire diversity offered by other markers, when combined with ethnohistorical and demographic data mtDNA provides valuable insights into genetic history of a population.

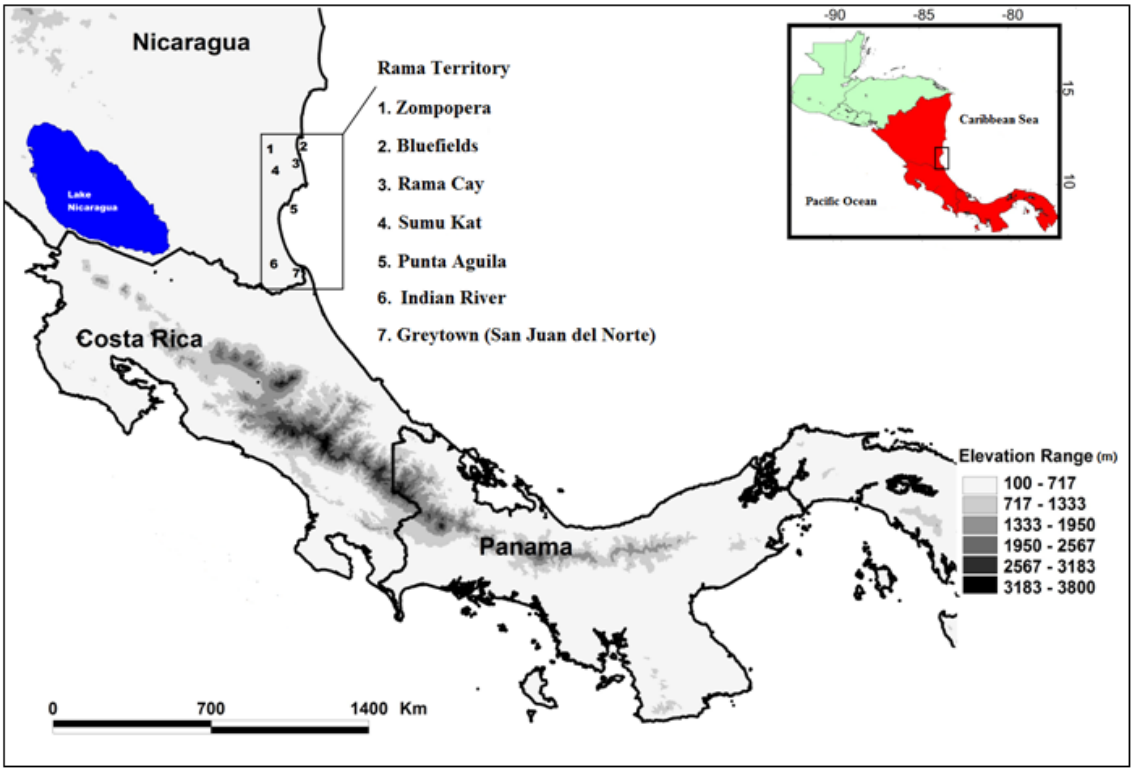

Figure 1. Seven Rama localities visited during fieldwork (2007 and 2009) in the southern Mosquitia region of Nicaragua (Baldi 2013).

Origin of the Rama

The pre-Columbian origin of the Rama remains uncertain, and available historical accounts are contradictory (Riverstone 2004; Smutko 1988). Some link the archeological evidence from the Caribbean coast with the Rama (Incer 1975; Riverstone 2004), others, with migrations from Mesoamerica or South America (Clark et al. 1984; Conzemius 1938; Magnus 1974; Stone 1972). Additional hypotheses state that the Rama are an amalgamation of a number of disparate groups from southern Nicaragua and northern Costa Rica or are the direct descendants of extinct Voto −

or Boto− , who inhabited the northern region of Costa Rica during the colonial period (Riverstone 2004). Contributing to the confusion, the name Rama has been used interchangeably with a number of names since at least the 1740s, when Don Diez Navarro visited the San Juan River and observed that whether or not some individuals can be distinguished as either "Caribs" (Rama) or "Moscos", they belong to the same nature (Romero 1995). The ambiguity of the chronicles contributes to difficulty faced by historians and anthropologists who wish to identify exact population names and localities.

In order to avoid confusion due to the interchangeable use of the names Voto and Rama in historical accounts, we refer here to the post-16th century population inhabiting the lowlands of northern Costa Rica and the San Juan River surroundings as the "Voto-Rama," to differentiate from the Voto of the foothills of the Cordillera Central, near the Poás and Barva volcanoes, and the lowlands of San Carlos in Costa Rica.

The history of migration of the Voto-Rama from Costa Rica to Nicaragua and its aggregation with the Rama from Punta Gorda is summarized in figure 2. During the 16th and 17th century, the San Juan River separating Costa Rica and Nicaragua functioned as a refuge for indigenous populations escaping European colonization in other regions of Costa Rica and likely from the Pacific region of Nicaragua. The San Juan River and its tributaries served as a home base for the Voto-Rama and a number of now extinct indigenous groups, such as Tises, Katapas, Abito, Pocosol, and Tori (Ibarra 2011; Solórzano 2000). In the 18th century the Voto-Rama migrated out from the San Juan River region to Punta Gorda in southern Nicaragua where a Rama faction also known as the "wild Caribs" resided (Romero 1995). This group is presumably today's Rama.

At the end of the 18th century and early in the 19th century a fraction of the Rama relocated to the Bay of Bluefields and Rama Cay while another Rama population remained in Punta Gorda. Oral traditions compiled by Moravian missionaries (Loveland 1975), as well as ethnographies (Conzemius 1927), indicate that these disparate groups were once a unified population, stating that a group moved to Rama Cay after being compensated by the Miskito kingdom for services rendered in an intertribal war against the Teribe from Costa Rica.

It is not clear if the Voto-Rama were completely assimilated by the Rama of Punta Gorda or if the group that split off Punta Gorda correspond with either of these two populations. What is assured is that the Rama that inhabit Rama Cay refer to the group at Punta Águila, near Punta Gorda, as the "real Rama" (Jerry Macrea, personal communication, 2009). In consequence, the isolation of these two groups gave rise to two dialectal variants, Rama Cay Creole, spoken mostly at Rama Cay, and a second variant that is spoken to the south, including in Punta Águila (Assandi 1983). In addition, myths of creation gathered from Rama Cay included an account of the migration from Punta Gorda to the Bay of Bluefields in the 18th century (Loveland 1975). These myths pinpointed Corn River, Snook Creek, Cane Creek, Monkey Point, and the Punta Gorda region as localities where Rama emerged before their migration north to the Bay of Bluefields (Loveland 1975; Schneider 1989).

In the late 20th century the overpopulation at the island of Rama Cay increased conflict and competition for land and marine resources with non-indigenous migrants, most of them from the Pacific coast of Nicaragua, induced the migration and re-colonization by the Rama from Rama Cay to southern Nicaragua such as San Juan del Norte (Greytown), Indian River and the Bay of Bluefields region. Aggregation of the Rama in communities is a recent phenomenon

resulting from the pressure for resources by foreign interests and Mestizo peasants (GTR-K 2007).

Based solely on ethnohistorical accounts, the hypotheses of origins of the Rama are difficult to test because of the existing discrepancy of locations, the complexity of population movements, the assimilation process, and the overlapping of cultures and names of the indigenous villages in the sixteenth century and later. However, the examination of additional mtDNA alongside available historical, archaeological, and linguistic information offer an alternative means of studying the effect of historical events on the genetic structure of the Rama. Future studies using full mitochondrial genomes and additional markers will led toward a more comprehensive analysis and interpretation of the population dynamics of the Rama.

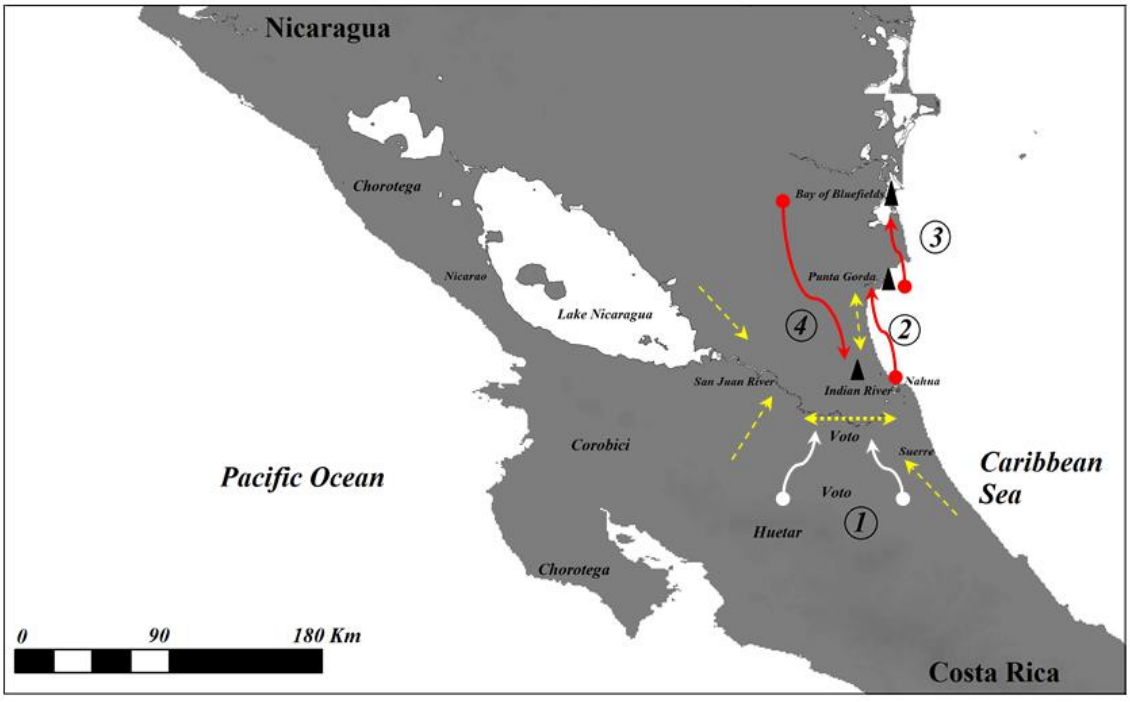

Figure 2. Migratory history of the Voto-Rama: (1) In the 16th and 17th century the San Juan River region functioned as a refuge for indigenous populations escaping European colonization

in other regions of Costa Rica and likely Nicaragua. Dashed and solid arrows indicate migrations of indigenous populations to the San Juan River region. The San Juan River and its tributaries was also a base for the Voto-Rama and a number of now extinct indigenous groups. (2) The Voto-Rama in the 18th century, migrated out from the San Juan River region to Punta Gorda where another Rama faction, known as "wild Caribs," resided. In the same century, sporadic migrations from Punta Gorda and Indian River protected them against the outbreak of diseases and slave raids. (3) A fraction of the Rama relocated in the Bay of Bluefields and Rama Cay (peripheral group) at the end of the 18th century and early in the 19th century while another fraction of the Rama remained in Punta Gorda (central group). The isolation of these two groups gives rise to dialectal variants, Rama Cay Creole and other Creole registers. (4) Overpopulation of Rama Cay and increased conflict and competition for land and marine resources induced migration and re-colonization in Southern Nicaragua and the Bay of Bluefields region in the late 20th century. Aggregation of the Rama in communities is a recent phenomenon resulting from the pressure for resources by foreign interests and Mestizo peasants.

MATERIALS AND METHODS

In order to investigate the genetic variation among six Rama subpopulations, we collected buccal swabs for DNA analysis and genealogical information of 190 participants from seven Rama communities along the southern Caribbean coast of Nicaragua during fieldwork in 2009. To these samples, we added seventy five additional samples collected by Melton et al (2013) [n = 265]. Details on geographical locations and general information on each Rama community visited during fieldwork are summarized in Baldi et al. (2014).

During fieldwork each participant signed a consent following the Helsinki protocol for the use of biological samples. The ethical approval of this research was endorsed by the

Institutional Review Board (IRB: 16735) at the University of Kansas, and accepted by the Rama community, and the GTR-K (Gobierno Regional Rama y Kriol) in the Autonomous region of Southern Nicaragua. Cells from swabs and mouth washes were collected in Cryotubes with 750 a μL of TE. The samples were then transported to the Laboratory of Biological Anthropology (LBA) of the University of Kansas for DNA extraction, analysis and storage.

DNA extraction

DNA extractions, RFLP (restriction fragment length polymorphism) for haplogroup identification, and PCRs for genetic sequencing were performed at the LBA. The DNA from mouth rinses obtained during fieldwork was extracted following the Chelex® protocol (Walsh et al. 1991), and the DNA from buccal swabs was extracted using the Evogen one® method (provided by Evogen Laboratories, Kansas City MO). Buccal swabs were centrifuged for four minutes at 10,000 rpm and then 50 μL of Evogen one® product were added to each tube. Tubes then were heated at 95 ^{o}C for two minutes after being gently vortexed. The supernatants containing the DNA, were transferred into of 5.0 mL collection tubes.

mtDNA HVS-I Sequencing and RFLP analyses

In order to characterize haplogroup variation, we first screened samples by RFLP analysis of the coding region. We tested first for haplogroups A2 and B2 which are the most frequent among the Rama (Melton et al. 2013). The remaining samples that did not test positive for these haplogroups were screened for haplogrups D1 and C1 and examined for African and Eurasian haplogroup variation based on maternal genealogical information and direct sequencing of the

HVS-I and HVS-II segment. RFLP screening for Amerindian haplogroups were performed using the following restrictions sites: +663 *Hae*III (hg. A2), +8,250 *Hae*III, (hg. B2), +13,259 *Hinc*II and +13,262 *Alu*I (hpl. C1), +5,176 *Alu*I (hpl. D1). Details on the primers, annealing temperatures, reagents, and endonuclease enzymes were previously described (Baldi 2013). In addition to these analyses, individuals were crosschecked with their respective HVS-I sequence and haplogroup assignation based on PhyloTree.org (Build 16) nomenclature (van Oven 2010).

In order to obtained greater genetic resolution on haplotype variation we analyzed 174 new mtDNA HVS-I samples from six Rama subpopulations (Sumu Kat: 15, Punta Águila: 21, Indian River: 10, Greytown: 41, Zompopera: 37, and Rama Cay: 80). These were added to 30 samples previously reported by Melton et al. (2013). Samples were characterized by the mtDNA HVS-I between the nucleotide position (np) 16,000 and 16,400 using the Big Dye® Terminator version 3.1 Cycle Sequencing Kit on an ABI 3130 Sequencer (Applied Biosystems, Foster City, CA) at the University of Kansas Natural History Sequencing Laboratory. Forward and reverse strands were sequenced for each sample in order to avoid phantom mutations, errors, and other artifacts. mtDNA chromatograms resulting from the previous analysis were edited using BioEdit (Hall 1999) and compared to the revised Cambridge Reference Sequence [rCRS] (Andrews et al. 1999). Variations in nucleotides deviating from the rCRS were recorded as DNA sequence variants.

ANALYTIC PROCEDURES

Genetic structure and diversity measurement

Genetic structure was approximated using the analysis of molecular variance [AMOVA] (Excoffier et al. 1992) and the *R*-matrix, a statistical technique that permits visualization of the relationships of populations by averaging genetic distances into a variance-covariance matrix (Harpending and Jenkins 1973). AMOVA was calculated based on geographical and kin affiliation criteria (Baldi et al. 2014) using pairwise genetic differences. The *R*-matrix analysis was performed in ANTANA (Harpending and Rogers 1984) and displayed in a principal component analysis (PCA) using HVS-I mtDNA sequence data. Tamura and Nei genetic distances (1993) were calculated on the mtDNA sequences and corrected for mutation rate heterogeneity using the γ-value of 0.26 (Meyer et al. 1999).

For detecting patterns of genetic discontinuity among the Rama subpopulations, Monmonier's algorithm (Monmonier 1973) and the interpolated genetic landscape (Miller et al. 2006) were applied. Monmonier's algorithm is a phylogeographic procedure that detects barriers of gene flow by identifying distances along a network of interconnected points (Dupanloup et al. 2002; Manni et al. 2004). We used the software Barrier v.2.2 (Manni et al. 2004) for detecting such genetic discontinuities. Results were plotted in a three dimensional geographical grid with x, y, and z axes using the software Alleles in Space (Miller 2005). In this representation, the z axis in the three dimensional grid represents the genetic differences between populations, whereas the x/y represents geographic coordinates. Valleys below the x/y plane represent genetic similarities, and the peaks above the x/y plane indicate genetic differences.

Phylogenetic and chronometric analyses

Median joining networks (MJ) of reduced reticulations (threshold = 1, ε = 0) were constructed to determine genetic relationships among haplotypes within the studied subpopulations using

mtDNA HVS-I genetic sequences and for two Native American haplogroups (A2 and B2). Networks were generated in the software NETWORK v.4.6.1.0 (Bandelt et al. 1999).

We approximated the time of the most recent common ancestor (MRCA) for haplogroups A2 and B2 using the mtDNA HVS-I (np 16,050 - 16,383) from the constructed MJ networks (Bandelt et al. 1999) with a rate of 1 mutation every 16,667 years (Soares et al. 2009). Mutational changes were counted from the network by means of the statistic ρ —rho—, which represents the average number of mutations between the root haplotype and individuals in the sample.

RESULTS

RFLP and Haplogroup Characterization

The RFLP analysis of the Rama sample revealed the presence of haplogroups (hgs) A2 and B2, which are representative of two of the four major clades present in America [A, B, C, and D] (Schurr et al. 1990; Wallace and Torroni 1992). Haplogroup B2, which accounted for 71% of the sample, was assigned by the presence of the +8,250 *HaeIII* marker identifying the 9bp deletion (-CCCCCTCTA-) at COII-tRNA^{lys}, and cross checked with genealogical information. Haplogroup A2, which accounted for 28% of the total sample, was assigned by the presence of the +663 *HaeIII* marker and cross checked with genealogical information. The remaining one percent of samples belong to haplogroups H2 and L3. A previous study found the same pattern of haplogroup variation among the Rama but in different proportions [A2: 8% and B2: 92%] (Melton et al. 2013).

An examination of additional mutational motifs in the HVS-I and II segment of an individual mtDNA, that was previously incorrectly assigned as C1 solely by the HVS-I (Baldi 2013), confirmed the presence of the European haplogroup H2a (H2a2a1d) through the transitions at np 16172C and 16263G . An additional sample corresponded to the African lineage L3 (L3h1b2). Both of these haplogroups are signatures of recent genetic admixture among the Rama's gene pool and were assigned through mutation assignments and crosschecked on phylotree.org (van Oven 2010; van Oven and Kayser 2009) and with the software Mitotool v. 1.1.2 (http://www.mitotool.org/).

Within the Rama population haplogroup B2 is most frequent, particularly among the communities of Sumu Kat, Rama Cay, Bluefields, Greytown, and Indian River. Haplogroup A2 is more frequent in Punta Águila and haplogroups A2 and B2 are equally represented in Zompopera. H2a and L3 lineages appear in Greytown close to the San Juan River between Costa Rica and Nicaragua (Table 1). From the 174 new sequences nine new haplotypes (hpl) [3, 4; 8, 9; 13-17] are added to the seven previously reported by Melton et al. (2013) among the Rama. Haplotypes 3 and 4 correspond to haplogroup A2, and haplotypes 8 and 9 and 13-15 to haplogroup B2. Haplotypes 13 and 14 correspond to haplogroups H2a and L3, respectively (Table 2).

Table 1. Haplogroup classification based on RFLP and HVS-I sequencing among seven Rama subpopulations Amerindians from Nicaragua.

	Haplogroups				
Subpopulations	A2	B2	H2	L3	Total ind.
Sumu Kat	3	28			31
Rama Cay	23	88			111
Bluefields	0	7			7
Punta Águila	13	9			22
Greytown	12	32	1*	1*	46
Indian River	4	6			10
Zompopera	19	19			38
Total	74	189	1	1	265
%	28	71	0.5	0.5	100

(*) HVS-II was additionally sequenced for this samples.

Table 2. Haplotype variation among six Rama subpopulations based on mtDNA HVS-I.

Hgs**	Haplotype (hpl)	16108	16111	16172	16174	16187	16189	16192	16217	16218	16223	16242	16250	16256	16258	16269	16270	16290	16291	16298	16311	16319	16325	16327	16334	16349	16360	16362	Rama Cay	Zompopera	Punta Águila	Greytown	Indian River	Sumu Kat	total
	CRS*	C	C	T	C	C	T	C	T	C	C	C	C	C	A	T	C	C	C	T	T	G	T	C	T	A	C	T							
A2	1	.	T	.	.	T	.	.	.	.	T	.	.	.	.	.	.	T	.	.	.	A	.	.	.	.	.	C	10	16	2	11	3	3	45
	2	.	T	.	.	.	C	.	.	.	T	.	.	.	.	.	.	T	.	.	.	A	.	.	.	.	.	C	5	1	9		1		16
	3	.	T	.	.	.	C	.	.	.	T	.	.	.	C	.	.		.	.	.	A	.	.	.	.	.	C		1	1	1			3
	4	.	T	.	.	.	C	.	.	.	T	.	.	.	C	.	.	T	.	.	.	A	.	.	.	.	.	C	1						1
	5	.	T	.	.	.	.	.	.	.	T	.	.	.	.	.	.	T	.	.	.	A	.	.	.	.	.	C	1		1				2
	6	.	T	.	.	.	.	.	.	.	T	.	.	.	.	.	.	T	.	.	.	A	.	.	.	.	T	C	1						1
B2	7	.	.	.	.	.	C	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	47	19	7	26	6	12	117
	8	A	.	.	.	.	C	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	5						5
	9	.	.	.	.	.	C	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	2						2
	10	.	.	.	.	.	C	.	C	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	2						2
	11	.	.	.	.	.	C	.	C	.	.	T	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	1						1
	12	.	.	.	.	.	C	.	C	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2					1	3
	13	.	.	.	.	.	C	.	C	.	T	.	.	.	.	C	.	.	.	.	.	.	.	.	C	.	.	.			1				1
	14	.	.	.	.	.	C	.	C	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.				1			1
	15	.	.	.	.	.	C	.	C	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	2						2
H2	16	.	.	C	.	.	.	.	.	.	T	.	.	.	.	.	.		.	C	C		C	T	.	.	.	.				1			1
L3	17	.	.	C	T	.	.	T	.	T	T	.	.	A	.	.	.	.	.	.	C	.	.		.	.	.	C				1			1
Totals																													79	37	21	41	10	16	204

(*) Revised Cambridge Reference Sequence (Andrews et al. 1999), (**) Nomenclature based on Phylotree.org (van Oven 2010; van Oven and Kayser 2009). Rama samples (n = 30) reported by Melton et al. (2013) are included in this table.

Genetic Diversity and Neutrality Tests

Haplotype diversity values (*h*), the number of variant sites (Θs), and the nucleotide diversity (Θ_π) were calculated to approximate the forces of evolution acting on each Rama locality (Table 3). Based on the magnitude of *h*, Punta Águila and Rama Cay are the most diverse communities compared to the other localities. The diversity estimator Θs shows a similar picture of diversity compared to the nearly uniform nucleotide diversity values across localities, except for Sumo Kat, that shows lower nucleotide diversity values ($\Theta_\pi = 2.74$). In addition, the number of polymorphic sites and haplotypes, as well as selective neutrality tests were calculated for the Rama as a whole. The Rama have 15 different haplotypes, low nucleotide diversity values ($\pi = 0.012$), and a moderate haplotype diversity value of 0.637, suggesting a relatively low genetic diversity shared among individuals.

Table 3. Molecular diversity indexes and neutrality tests among Rama subpopulations.

Statistics*	Rama	Sumu Kat	Punta Águila	Indian River	Greytown	Zompopera	Rama Cay	Mean± SD
Sample size	131	15	21	10	39	37	80	33.6 ± 25.51
Θ_s	4.22	2.46	3.05	2.82	2.36	2.15	10.90	3.96 ± 3.42
Θ_π	3.65	2.74	3.68	4.13	3.54	4.14	4.05	3.71 ± 0.53
Hapl. diversity *h* (SD)	0.64 ± 0.04	0.34 ± 0.12	0.72 ± 0.06	0.60 ± 0.13	0.48 ± 0.06	0.56 ± 0.04	0.65 ± 0.05	

(*) Two individuals that belong to the haplogroup H and L3 from Greytown were excluded from this analysis.

Haplotype Networks and Chronometry

In order to compare the sequence haplotype variation between Punta Águila, Zompopera, Rama Cay, Greytown, Indian River, and Sumu Kat a MJ network was constructed from mtDNA HVS-I sequences from np 16,050 to 16,383. Two separate networks for haplogroups A2 and B2 were generated showing large and small satellite nodes that represent cluster of haplotypes [I-VI] (Figure 3). Each node shows the frequency of individuals within a subpopulations in different colors.

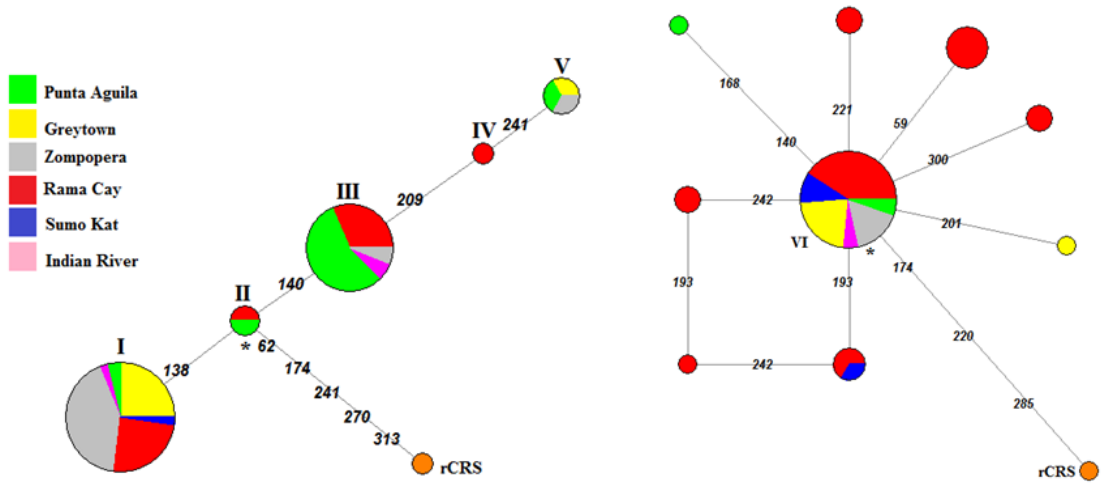

Figure 3. Network of haplotypes defined by sequence variants from the HVS-I (np 16,050-16,383) from six Rama subpopulations. Left, haplogroup A2 (node I = hpl. 1, node II = hpl. 5, node III = hpl. 2, node IV = hpl. 6, node V = hpl. 3); Right, haplogroup B2 (node VI = hpl. 7). Circles represent haplotypes that are proportional to their total frequency and are divided by

subpopulations. Mutations are shown on the linking branches of nodes and ancestral haplotypes are marked with an asterisk.

The general topology of the haplogroup A2 shows a stable population where genealogies are structured by nodes or groups of haplotypes of related lineages that coalesced in major nodes. Haplogroup A2 has five nodes that correspond with haplotypes 1-3, 5 and 6 from table 2. The haplotype 5 (basal node II) is the only haplotype shared between Rama Cay and Punta Águila. This node shares branches with two other major nodes: I (hpl. 1) and III (hpl. 2). Haplotype 1 is more frequent in Zompopera, Rama Cay, and Greytown, whereas haplotype 2 is more frequent in in Punta Águila. Two other low frequency haplotypes: hpl. 6 (node IV) and hpl. 4 (node V) diverged from node III.

In contrast, the topology of the B2 network shows a star-like phylogeny typical of an expanding population where a central node shares most of the haplotypes across subpopulations and low frequency haplotypes radiate from it. The most frequent haplotype of haplogroup B2 occurs in the ancestral node VI (hpl. 7). This haplotype is shared among other Central American populations (Kuna, Emberá, Zapatón-Huetar, Guatuso-Maleku, Guaymí, and Matambú-Chorotega (Melton et al. 2013) and it reaches its highest frequency at Rama Cay, Greytown, and Zompopera, and is less frequent in Punta Águila and Indian River, and moderately frequent in Sumu Kat. The star-like shape of this founder haplotype and associated nodes is indicative of population explosion and gain of genetic diversity. Satellite node 16,140 - 16,168 is present only in Punta Águila and corresponds with haplotype 13 from table 2.

In order to estimate the time of the merging of lineages to a most common ancestor for both haplogroups A2 and B2, the rho statistic (ρ) was calculated from the constructed phylogeny shown in the previous network. This statistic indicates the average number of mutations between

the root haplotypes 5 and 7 and descendent satellite nodes (Table 4). For haplogroup A2 nodes coalesced at 18,000 ± 15,033 YBP (ρ = 1.08 ± 0.90 [δ]). This time estimate falls within the range of other reported dates for haplogroup A2 (Achilli et al. 2008; Perego et al. 2012; Tamm et al. 2007). Contrary to this scenario, haplotypes that belong to the haplogroup B2 coalesced at 1,808 ± 785 YBP (ρ = 0.10 ± 0.04 [δ]); however, most of recent single mutations emerged around 300 YBP and are more frequent in Rama Cay. Only one satellite node of two mutational differences (hpl. 13) away from the basal haplotype (hpl. 7) is present in Punta Águila.

Table 4. Time estimates for haplogroups A2 and B2 based in HVS-I sequences.

Haplogroup	Haplotype	ρ ± δ	Years Before Present (YBP) ± SD
A2	5	(16111T, 16223T, 16290T, 16319A, 16362C)*	18,000 ± 15,033
	1	0.957 ± 0.957	15,957 ± 15957
	2	0.881 ± 0.881	14,845 ± 14,815
	3	1.80 ± 0.60	30,000 ± 10,000
	6	0.66 ± 0.33	11,111 ± 5,555
B2	7	(16111T, 16217C)*	1,808 ± 785
	8	0.004 ± 0.046	683 ± 683
	9	0.016 ± 0.016	280 ± 280
	10	0.016 ± 0.016	280 ± 280
	11	0.016 ± 0.084	282 ± 141
	12	0.025 ± 0.025	416 ± 416
	13	0.016 ± 0.016	282 ± 199
	14	0.008 ± 0.008	141 ± 141
	15	0.016 ± 0.016	280 ± 280

(*) Time estimates in years from central nodes 5 and 7 were calculated as one mutational event every 16,667 years. Only variable sites are shown within np 16,050 and 16,383.

Population Structure

In order to ascertain the relationship between the six Rama subpopulations a PCA plot of the *R*-matrix was constructed using HVS-I sequences (Figure 4). This plot retains 81% of the total genetic variation within the first and second dimension and separates the geographically peripheral populations of Rama Cay, Sumu Kat, Indian River, Greytown, and Zompopera from a central population, Punta Águila. This isolation is interpreted as a low rate of haplotype sharing between other peripheral Rama subpopulations and Punta Águila. Clusters are caused by differences in haplogroup frequencies. Rama Cay and Sumu Kat have the highest frequency of haplogroup B2 with respect to the lower cluster that includes Indian River and Greytown. Zompopera has equal frequencies of B2 and A2. In Punta Águila, A2 is predominant, for this reason it is an outlier in the diagram.

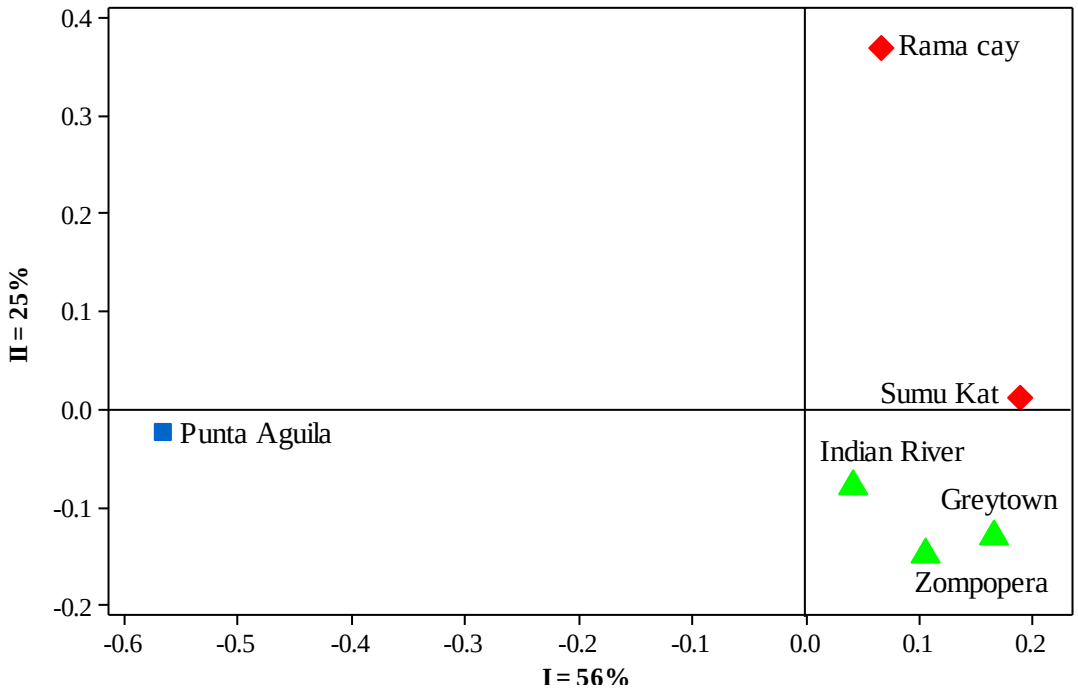

Figure 4. PCA of the *R*-matrix of six Rama subpopulations using mtDNA HVS-I sequences. In this plot the most divergent population is Punta Águila. The two axes (I and II) account for the 81% of the total variation in the plot.

Genetic Barriers and Phylogeographic Analysis

Monmonier's algorithm applied to a F_{st} distance matrix using HVS-I sequences of six Rama communities approximated the location of two genetic barriers indicating that the first barrier of gene flow isolated Punta Águila from the remaining communities between the Indio Maíz River to the south and the Bay of Bluefields to the north; the second, less robust barrier isolates Zompopera from Sumu Kat and Rama Cay. A possible geographic barrier between these communities is the Kukra River and surrounding forests. The resulting three dimensional diagram of these barriers depicts the genetic discontinuities between these Rama localities (Figure 5).

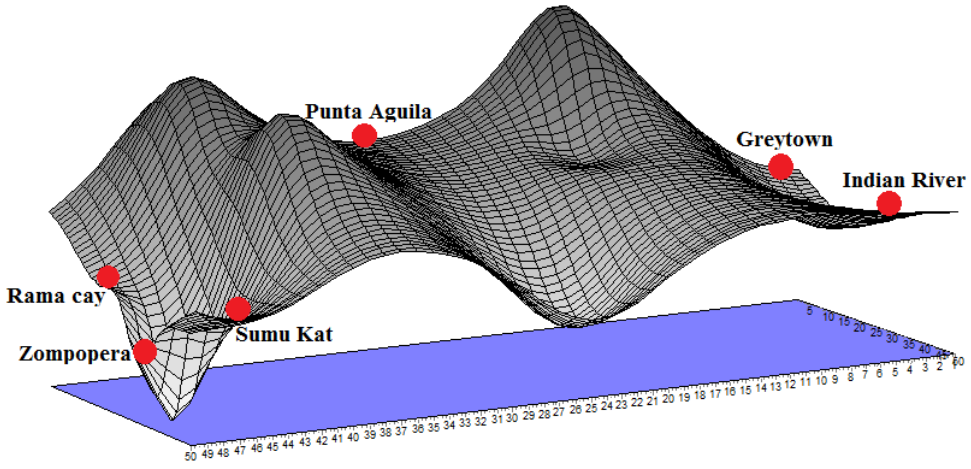

Figure 5. Interpolated genetic landscape connecting six Rama localities based on the method of Miller et al. (2006). The surface of this representation was based on genetic distances (z axes) and geographical locations of the communities defined by the x/y axes. In the genetic landscape

Punta Águila is the most isolated population followed by Zompopera. The second, weaker genetic barrier separates Zompopera from Sumo Kat and Rama Cay.

Three different hierarchical models were tested using AMOVA in order to investigate the presence of genetic substructuring within the Rama. Two models were based on geographic separation; the first one separated the east-west (Punta Águila and Zompopera) from the north-south boundaries that includes Indian River, Greytown, Rama Cay, and Sumu Kat. The second AMOVA divides northern (Sumu Kat, Rama Cay, and Zompopera), central (Punta Águila) and southern Rama localities (Indian River and Greytown). The third AMOVA tested two groups based on kinship relationships previously proposed by Baldi et al. (2014); Punta Águila differentiated from the remaining five localities of the Rama territory.

Table 5 presents the results of the first AMOVA based on the separation of eastern-western and northern-southern localities shows significant differentiation among these groupings (F_{ct} = 0.10183, $p < 0.001$) nonetheless the correlation among subpopulations within the Rama population as a whole was not significant (F_{sc}), although this component shows low values in the Geography II and Kinship groupings. The second AMOVA shows that 93.6% of the HVS-I variation is shared within northern and southern localities whereas 6% was attributed among communities and between groups, the remaining 0.5% of the variation is not significant when northern, central, and southern groups of communities were compared (F_{ct} = 0.0054). Zompopera is included in the northern group.

The AMOVA based on close kin affiliation between Punta Águila (central population) and the peripheral populations reveals that the highest (87% of the variation) is present within individual subpopulations, and 9.5% is among groups. The fixation index F_{ct} = 0.10183 ($p < 0.001$) accounts for the variation among central and peripheral groups.

Table 5. Analysis of molecular variance (AMOVA) of five Rama subpopulations.

Subdivision	Groups	Subpopulations	Source of variation	Percentage of variation	*F*-statistics	*p*-value
Geography I	East-West	Punta Aguila, Zompopera	Among groups	10.18	F_{ct}=0.10183	<0.001
	North-South	Indian River, Greytown, Rama Cay Sumu Kat	Among subpop. between groups	0.42	F_{sc}=0.00465	n.s.
			Within subpop.	89.40	F_{st}=0.10600	<0.05
Geography II	North	Sumo Kat, Rama Cay, Zompopera	Among groups	0.54	F_{ct}=0.00540	n.s.
	Central	Punta Aguila	Among subpop. between groups	5.87	F_{sc}=0.05900	<0.05
	South	Indian River, Greytown	Within subpop.	93.59	F_{st}=0.06408	<0.05
Kinship	Central	Punta Aguila	Among groups	10.12	F_{ct}=0.10123	<0.001
	Peripheral	**Sumo Kat, Rama Cay, Zompopera, Indian River, Greytown**	Among subpop. between groups	3.26	F_{sc}=0.03629	<0.05
			Within subpop.	86.62	F_{st}=0.13385	<0.05

DISCUSSION

Mitochondrial Diversity

Mitochondrial DNA haplogroups within the Rama belong to two (A2, B2) of the four major founding macro haplogroups (A, B, C, and D) in the Americas (Torroni et al. 1993; Wallace and Torroni 1992), as well as the African haplogroup L3 and the European H2. These results differ

somewhat from previously published results (Melton et al. 2013) due to the presence of two new haplogroups (H2 and L3). The percentages also changed due to the augmentation of the sample size (B2 = 71%, A2 = 28%, H2 = 0.5%, and L3 = 0.5%). Despite the new identification of two non-indigenous haplogroups in the Rama's gene pool, haplogroup B2 is still the most frequent.

Haplotype 7 of haplogroup B2 (16189C, 16217C) is the most common among the Rama and is shared with other SCA populations Kuna, Emberá, Zapatón-Huetar, Guatuso-Maleku, Guaymí, and Chorotega (Melton et al. 2013). The remaining B2 Rama haplotypes (3, 4; 8-15 in table 2) have not been detected among other SCA Chibchan populations. The time estimates of all variants in the B2 lineage were dated to historical times, around 1700 CE (current era), a date congruent with the relocation in the 18th century of a group of Voto-Rama Amerindians from the San Juan River refuge to Punta Gorda including Punta Águila, lands historically occupied by the Rama in Nicaragua (Incer and Perez-Valle 1999; Kemble 1884; Schnaider 1989). The recent population expansion shown in the network analysis of the haplogroup B2 suggests the possibility of gene flow between neighboring Voto-Rama demes around three hundred years ago might explain the high frequency of private haplotypes in today's Rama gene pool (see Ray et al. 2003), thus, the same variants should be expected in genetically related extant and extinct populations in northern Costa Rica.

The second most common variant (hpl. 1) of haplogroup A2 (16111T, 16187T, 16223T, 16290T, 16319A, 16362C) is shared with a number of Central American Chibchans (Maleku, Guaymí, and Ngöbé), Mesoamericans populations, and with non-Chibchan speakers from South America (Baldi 2013). According to the mtDNA molecular clock, haplogroup A2 most likely coalesced between 18,000 and 20,000 YBP. This estimation falls within the most accepted time

of colonization of Central America between 15,000 and 19,000 YBP based on mtDNA (Perego et al. 2012).

Haplogroup L3 is indicative of African mixture with the Rama and one individual in Greytown, belonging to the haplogroup H2, indicates recent European admixture. The gene flow between individuals of African and European ancestry was probably recent (Battistuzzi et al. 1986) and it is more frequent at Rama Cay, Punta Águila, and Greytown compared to any other Rama community according to admixture estimations obtained from previous surname analysis and a recent demographic survey (Baldi et al. 2014; GTR-K 2007).

According to the genetic diversity estimators (h and θ_s) applied only to native American haplogroup variation among the Rama, Rama Cay and Punta Águila are the most genetically diverse populations compared to the rest of the communities while values of nucleotide diversity are similar (average = 4.05) among localities. The relative values derived from the diversity index θ_s and h are consistent with the unbiased isonymy values (I_{ii}) calculated by Baldi et al. (2014) in which Rama Cay and Punta Águila are the more diverse subpopulations, Greytown and Zompopera are intermediate, and the less diverse and more isolated subpopulations were Indian River and Sumu Kat.

Genetic Substructure and history of the Rama

The network analysis for haplogroup A2 reveals a past population reduction (i.e. genetic bottleneck) in which the most ancestral node (II) is shared by only two communities, Punta Águila and Rama Cay, and is linked to other haplotypes of major frequency. Contrary to this, the star-like phylogeny of haplogroup B2 is characterized by a number of singletons that radiate from a large central node, indicating recent population expansion (Figure 4). Overall haplogroup

A2 is more frequent in Punta Águila compared to haplogroup B2, which is more common among the other five Rama subpopulations. The relationships of the haplotypes 2 and 3 of haplogroup A2 is depicted in the phylogenetic network where haplotype 2 (node III) is highly frequent in Punta Águila but is shared in low frequencies among Rama Cay, Indian River, and Zompopera; and haplotype 3 (node V) is shared between Punta Águila, Zompopera, and Greytown. This is an indicative of mitochondrial relationships between Punta Águila and other three communities. Haplogroup B2 shows a contrasting scenario of only one maternal relationship through the haplotype 7 between Punta Águila and other Rama communities.

The genetic differentiation of central and peripheral populations was tested using AMOVA and Monmonier's algorithm on mtDNA sequences. This analysis shows that 10% (F_{ct} = 0.10, $p < 0.001$) accounts for the genetic variation among peripheral and central groups; nevertheless, the correlation of Punta Águila and Zompopera and a group that includes the remaining northern and southern communities shows a similar F_{ct} value. This relationship is not surprising since Punta Águila and Zompopera shared A2 haplotypes as was previously described. In addition, analyses of the kin relationships between Rama subpopulations based on surname isonymy show affinities between Zompopera and Punta Águila (Baldi et al. 2014).

Congruent with AMOVA analyses, Monmonier's algorithm found a strong genetic barrier of gene flow that separates Punta Águila (central population) from the peripheral communities, and a less robust barrier of gene flow isolates Zompopera. Geographically, the strongest barrier is likely to be situated between the Bluefields Lagoon and Punta Gorda River. The reduced gene flow between central and peripheral groups was also verified through the PCA of the *R*-matrix.

Additional support of maternal genetic difference between Punta Águila and the peripheral Rama communities comes from the median networks and the PCA of the *R*-matrix. These analyses show that in Punta Águila A2 haplotypes are more frequent compared to peripheral communities where B2 is higher. Based on these analyses it can be proposed that affinal relationships based on kin might have deep historical roots that have persisted until the present. According to Baldi et al. (2014), marital practices, probably based on assortative mating, created consanguineal relationships and alliances that underlie the maternal genetic structure of the Rama and endured for generations, explaining the observed subdivision between central or peripheral communities. Affinal aggregation and vicinage is not random because it is based on generations of arranged marriages (explicit or not) with other known family groups (Loveland 1975).

The genetic structure of these central and peripheral groups suggests two evolutionary stories in concordance with their relative geographic isolation, migration, and kin structure. From the mitochondrial perspective, haplogroup A2 (hpl. 2) and B2 (hpl. 13) appear to be more related with the central population, Punta Águila, and are less diffused among peripheral Rama communities such as Zompopera. This situation leads to the proposal that said haplotypes were restricted to maternal lineages in the Punta Gorda region due to reduced genetic flow with other peripheral groups.

In synthesis, the peripheral group could represent a remnant population of the colonial Voto (Voto-Rama), whose descendants migrated first to Punta Gorda and then split at the end of the 18th century to the Bay of Bluefields, while a central group (including Punta Águila and surrounding communities) may have remained in the region of Punta Gorda for many generations.

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